Human enamel colonization of *Candida albicans*

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ABSTRACT

Introduction: *Candida albicans* may be a commensal member of the oral microbiota, and may colonize the endodontic environment. Using an *in vitro* dentin infection model, the objective of this study was to evaluate the pattern of dentin colonization by *C. albicans* and the influence of thigmotropism on the colonization. **Methods:** An apparatus was designed being composed of two glass flasks connected by a silicone manifold. Internally, they were separated by a dental fragment protruding an acrylic disk. The upper and bottom flasks were filled with Sabouraud broth and *C. albicans* was inoculated in the upper flask. After 72 h at 37 °C, the device was aseptically dismounted and the dentinal fragment was prepared for scanning microscopy. **Results:** *Candida albicans* 1015 strain actively penetrated dentinal tubules and hyphae were the mainly growth form for the primary yeast invasion of human dentin. Yeast cells were observed in inner dentin layers. **Conclusions:** The direction of the hyphal tip was not influenced by the tubular nature of the dentin. In his view, only the pleomorphism has a significant role in the fungal colonization of human dentin.

Keywords: Infection. Periapical diseases. Dentin.

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Introduction

Under physiological conditions, the pulp tissue and the surrounding dentin are protected by enamel and cementum. Any factor that causes loss of these protective structures, such as caries, fracture, attrition, abrasion, scaling, and root planning, exposes dentin and eventually the pulp tissue to detrimental effects due to mechanical, chemical, and particularly microbial irritants.[25,26]

Exposed dentinal tubules are the main routes microorganisms have to the endodontic environment. The number of dentinal tubules per mm^2 of dentin ranges from 15,000 at the cementoenamel junction to 45,000 near the pulp.[5,11] Intratubular dentin deposition results in narrowed tubules as it is more advanced in the superficial dentin when compared to the dentin adjacent to the pulp and resulting in tubules with a uniform conic appearance. The largest depositions were observed around the pulp (approximately 2.5 µm diameter) and progressively decreased as they approached the cementoenamel junction (approximately 0.9 µm diameter).[3]

A reduced tubule diameter in the superficial dentin layers might hinder opportunistic yeasts of the genus Candida from penetrating the pulp environment. However, some studies have demonstrated the ability of these microorganisms to infect this dental tissue.[15,16,21,22,23,26,29] Furthermore, this ability has been suggested to be closely linked to the pleomorphic growth patterns that are most commonly exhibited by isolates of *C. albicans*.

Regarding the tubular nature of dentin and also of thigmotropism, the latter is defined as a directional response of a cell or tissue to topographic modifications of a surface[12] and is supposedly regarded as an important factor for the colonization of the dental pulp by Candida species. Nevertheless, this inference is based on models of oral and vaginal mucosal infections, where the thigmotropic response has already had a defined role. In the present study, we aimed to assess the pattern of dentin colonization by *C. albicans* and the influence of thigmotropism on the colonization using an *in vitro* dentin infection model.

Material and Methods

The apparatus used consisted of two glass flasks of equal volume (10 ml) and size (7 cm x 1 cm radius) connected by a hollow silicone ring (2.5 cm x 2.5 cm diameter). Flasks were connected to each end of the ring, and a small hole (0.5 mm) was created in the side of the ring to remove air bubbles and to allow us to insert the desired volume of microbiological culture medium. A plastic connector was attached to the hole in order to seal the system. This apparatus was made airtight using an acrylic disk, and all connections were sealed with rubber rings (0.7 mm thick) (Fig 1).

A dental fragment was added to the acrylic disc. Apices and crowns of recently extracted human permanent incisors were sectioned perpendicularly along the axis of the tooth using carboril disc with a profuse irrigation with distilled water. Two cylinders were

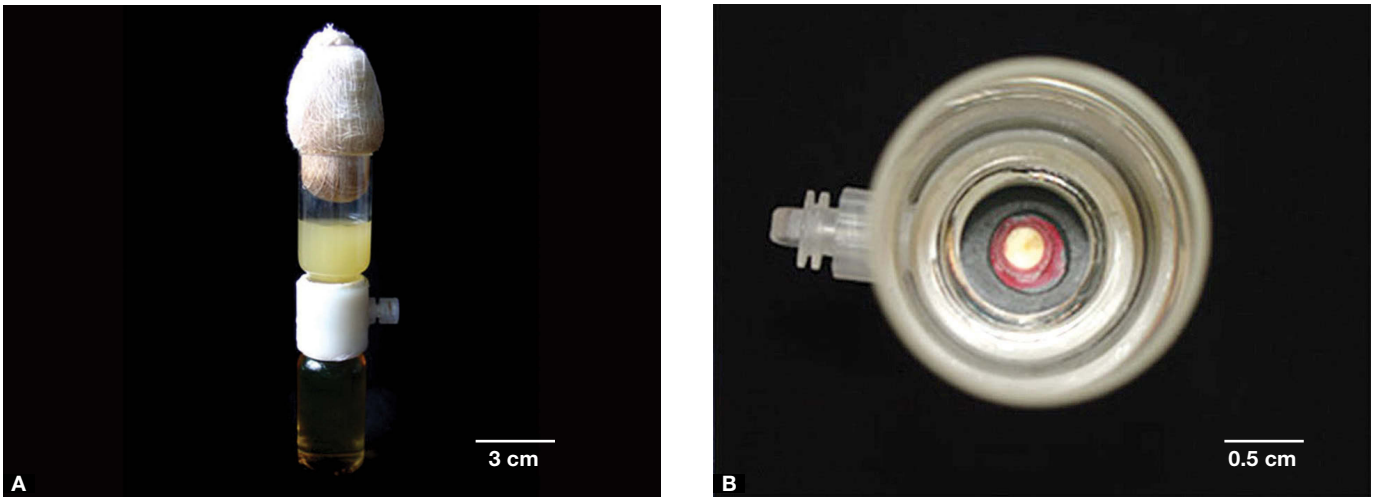

Figure 1. A) Side view of the apparatus for assessing dentinal colonization by *Candida albicans* 1015. **B**) Top view of an acrylic disc containing a dentinal fragment in its central portion.

made from each of the remaining root portions, and each cylinder was approximately 5 mm in diameter and 2 mm thick. Cementum was removed using a diamond bur. Then, dentinal cylinders were individually placed into the central hole that was previously made in the acrylic disc and fixed using thermopolymerizable acrylic resin (Fig 2). The smear layer was removed from some dentinal cylinders by immersing them in 17% EDTA and 5.25% NaOCl for 3 min.

Microorganisms and Culture Medium

After sterilizing the entire apparatus with gamma radiation, the top flask was filled with modified Sabouraud broth (2% glucose, 1% peptone, 0.5% yeast extract) containing 10^7 CFU/mL of the strain *C. albicans* 1015, which had been isolated from a necrotic root canal.[15] The density of the inoculum was standardized at an absorbance reading equivalent to 1.2 using spectrophotometry (O.D. 560 nm).

Sterile Sabouraud broth was placed into the bottom flask, which removed all of the air (Fig 1). The apparatus was incubated at 37°C until the culture medium in the lower flask was visibly cloudy, indicating microbial growth. Flasks without microbial inoculum were used as a negative control. Assays were persistently repeated, at least thirty times.

Scanning Electron Microscopy

As soon as microbial growth was observed in the bottom flask, the apparatus was dismantled under aseptic conditions. An optical microscope was used to analyze 10 µL aliquots of Sabouraud broth from the bottom portion. The acrylic discs containing dentinal fragments were washed three times with 0.1 M PBS and fixed for 1 h in a solution of 2.5% glutaraldehyde and 0.1 M PBS, pH 7.4. After the fixation period, acrylic discs were washed again in 0.1 M PBS and then sprayed with gold while under vacuum. Specimens were examined with a scanning electron microscope, model JEOL JSM-6360 LV (Tokyo, Japan), operating at an accelerating voltage of 15 kV.

Results

In experiments conducted on dentinal fragments both with and without the dentinal smear layer, the culture medium in the lower bottle was visually turbid after 72 h of incubation. Microscopic analysis of the aliquots from the culture medium in the lower compartment revealed growth of yeast cells morphologically identical to those previously inoculated in the top bottle.

In Figure 2, negative controls are seen with and without a smear layer.

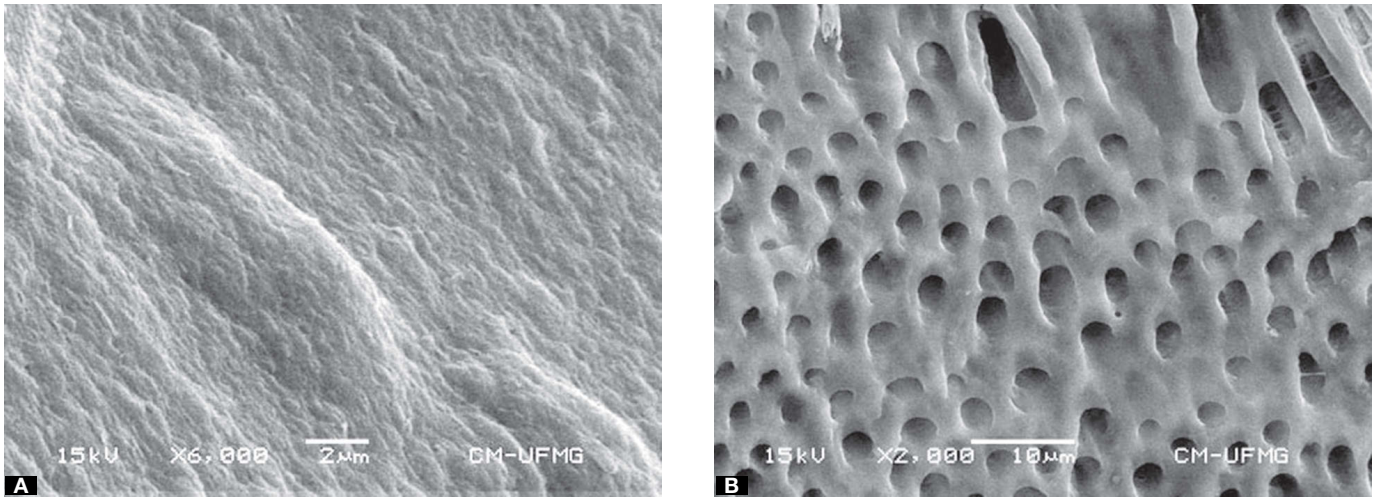

Figure 2. Dentinal fragments used as negative controls. **A**) Without smear layer. **B**) With smear layer.

Dentin colonization by *C. albicans* 1015 in the dental fragment with smear layer is shown in Figure 3. Yeast cells and hyphae were observed in the superficial layer of the fragment (Figs 3A, B and C). Hyphae showed branching and linearly extended into the substrate; however, the hyphae seemed randomly oriented and some

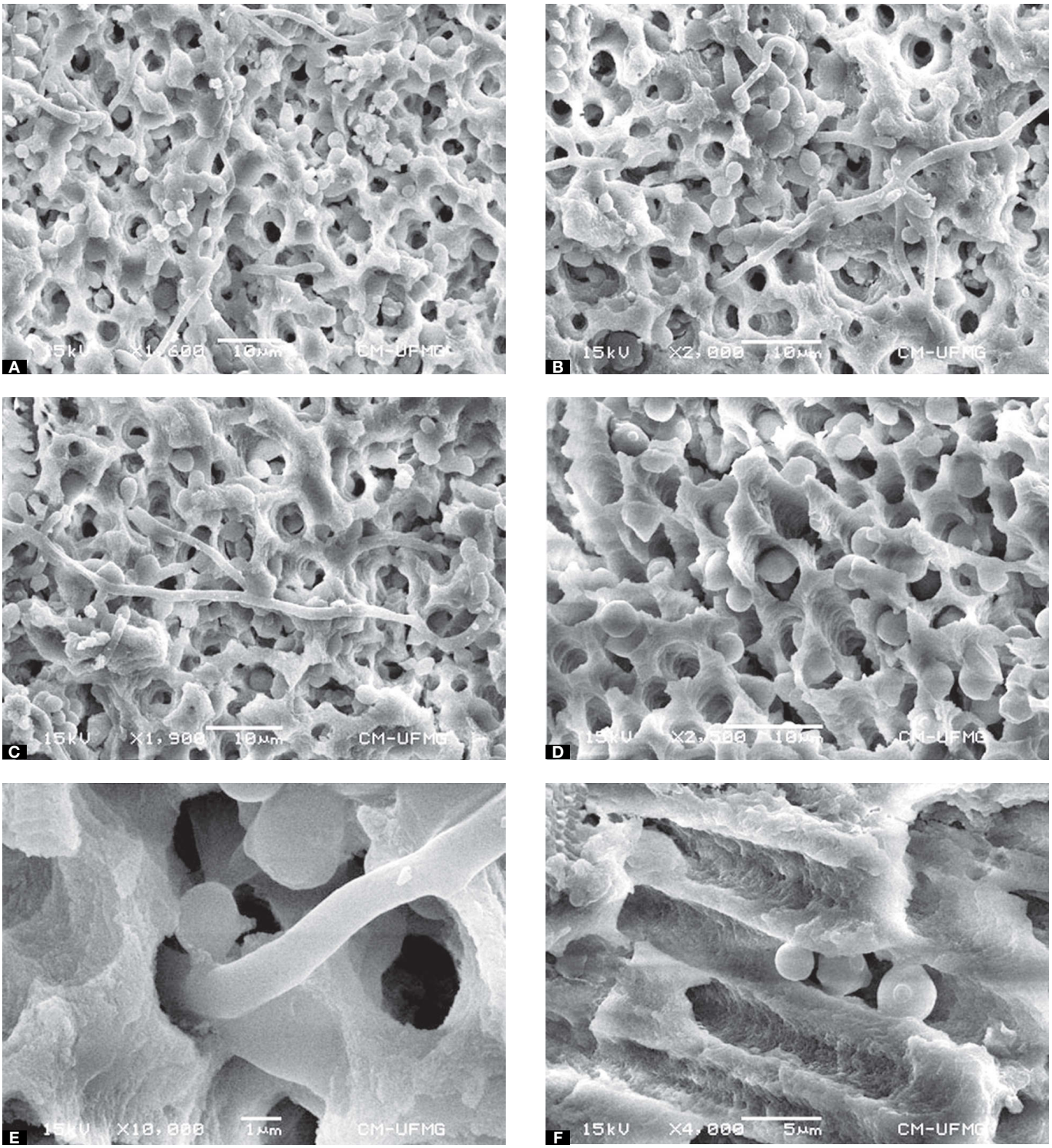

Figure 3. Electron micrograph showing colonization of dentinal tubules by C. *albicans* 1015 in dentin samples with the smear layer. **A**, **B**, **C**) Yeast cells and hyphae in the superficial dentin layer. (**D**) Yeast cells in dentinal tubules in the inner dentin layer. (**E**) Penetration of hyphae in dentinal tubules. (**F**) Yeasts attached to the dentinal tubule wall.

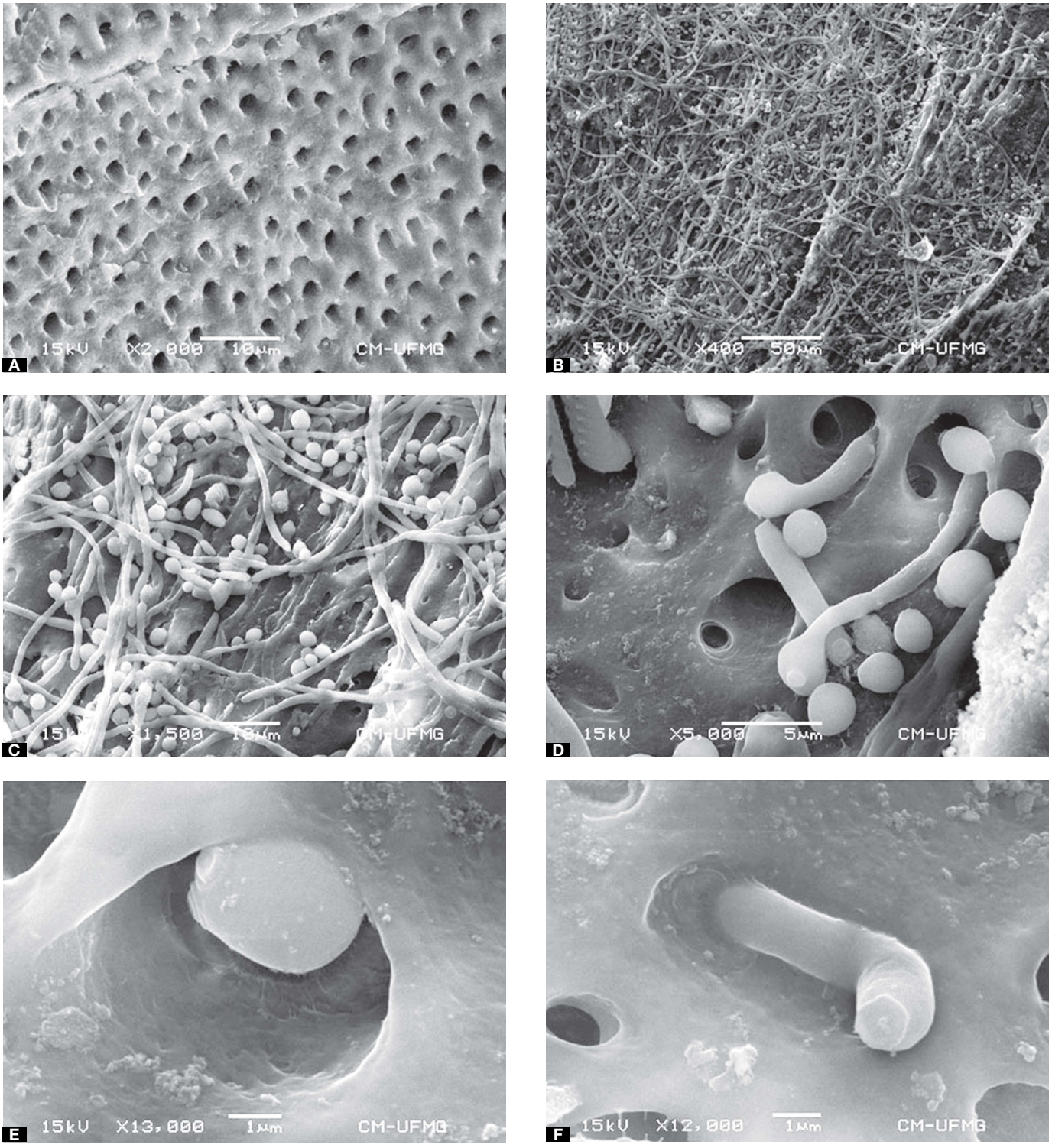

Figure 4. Electron micrograph showing C. *albicans* 1015 colonizing the dentinal tubules in dentin samples without the smear layer. **A**) Dentin sagittal section showing the absence of the smear layer in dentinal tubules (Negative control). **B**, **C**) Yeast cells and hyphae on the superficial dentin layer. **D**) Yeast cells and germ tubes in the inner dentin layer. **E**) Yeast adhered to the dentinal tubule wall. **F**) Hypha in a dentinal tubule.

headed toward the opening of the dentinal tubules. The predominant yeast growth was observed in the inner dentin layers (Figs 3D and F). Yeasts were present in various extensions of the dentinal tubules, and some cells exhibited budding, indicating growth potential.

Figure 4 shows dentinal colonization by *C. albicans* 1015 in the dental fragment that had been treated to remove the smear layer. After removal, the diameter of the dentinal tubules ranged from 1.27 µm to 5.50 µm. The superficial dentin layer was densely colonized by budding cells and hyphae (Figs 4B and C). Invasion of dentinal tubules by germ tube formation (Fig 4D) or by mycelium growth (Fig 4F) was evident in the inner most portions of the substrate. Yeast adherence to the dentinal wall by matrix secretion may be observed in the micrograph (Fig 4E).

Discussion

Colonization of dentinal tubules by microorganisms is considered a significant risk factor for early and persistent endodontic infection.[4] In this study, *C. albicans* 1015 was found to be able to invade dentinal tubules. Visualization of a large number of hyphae in the superficial dentin layers of dentinal fragments with and without the smear layer indicates that this invasion results from a morphological differentiation of the yeast cells that grow into mycelial form, thus entering the tubules. The presence of *C. albicans* in carious dentin fragments has been reported in clinical studies and confirms that these microorganisms are able to penetrate dentin *in vivo*.[2,9,10]

C. *albicans* cells usually have a spherical or oval cellular shape. Their blastospores may start forming hyphae that linearly extend and sometimes branch.[7,8] Pleomorphic growth patterns, described for this species, are related to their different morphologies, including germ tubes, blastospores, pseudohyphae, true hyphae, and chlamydospores.[14,19,30] All of these growth patterns, except chlamydospores, may assume other forms based on the following environmental conditions: pH, temperature, and nutrient supply.[6,19,28] Therefore, in addition to need to colonize the innermost dentin layers, the temperature of 37°C used in our study favored *C. albicans* 1015 pleomorphism on this substrate.

Multiple findings[18,20] suggest that the yeast-hypha transition is required for *C. albicans* virulence. However, some studies[6,19,27] highlight that the yeast morphology that is observed in both superficial and inner most layers of dentin fragments, with and without a smear layer, are critically important for microbial adhesion and rapid dissemination in various tissues and for biofilm formation, which are both processes indirectly related to virulence. The presence of the smear layer did not influence adhesion of *C. albicans* 1015 to the substrate in contrast to the other findings.[22]

In another study,[24] it was concluded that the presence of the smear layer enhanced the adhesion of *C. albicans* to human dentin due to a higher availability of Ca^{+2} ions and collagen. In the present study, removal of the smear layer did not affect microbial adhesion, suggesting that Ca^{+2} ions are less important for microbial colonization. Furthermore, the presence of germ tubes in the dentinal tubules in samples without a smear layer supports this conclusion because these structures are very substrate-adherent.[13,17]

The methodology used in the present study allowed us to determine whether dentinal tubules may be used as a route to colonize a sterile ecological niche, as it may occur *in vivo*. Although the penetration of dentinal tubules by *C. albicans* has been shown to be directly related to the polymorphism of this species, it seems that the growth direction of *C. albicans* hyphae was not influenced by the substrate topography. Hyphae were randomly oriented in both of the analyzed samples. Thus, yeast penetration of the dentinal tubules and colonization of the innermost dentin layers seems to be a natural consequence of the process and therefore is not due to active recognition of topographic changes, as it occurs during the thigmotropic response. Differentiated infection structures and structural modifications to the hyphae were not evident either.

As observed in the present study, *C. albicans* is able to invade dentinal tubules, like bacteria. However, the behavior of this yeast in this ecological niche should be further studied *in vivo*. *In vivo* and in the presence of other microbial groups and oral fluids, yeasts may show different morphological responses that may have varied impact on the pathogenesis of endodontic and periradicular infections.

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