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DEVELOPMENT OF *CANDIDA*-ASSOCIATED DENTURE STOMATITIS: NEW INSIGHTS

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ABSTRACT

Despite therapeutic progress, opportunistic oral fungal infectious diseases have increased in prevalence, especially in denture wearers. The combination of entrapment of yeast cells in irregularities in denture-base and denture-relining materials, poor oral hygiene and several systemic factors is the most probable cause for the onset of this infectious disease. Hence colonization and growth on prostheses by *Candida* species are of clinical importance. The purpose of this review is to critically discuss several key factors controlling the adhesion of *Candida* species which are relevant to denture-associated stomatitis. Although there is some consensus on the role of surface properties, studies on several other factors, as the use of denture liners, salivary properties and yeast-bacterial interactions, have shown contradictory findings. A comprehensive fundamental understanding is hampered by conflicting findings due to the large variations in experimental protocols, while other factors have never been thoroughly studied. Surface free energy and surface roughness control the initial adherence, but temporal changes have not been reported. Neither have *in vivo* studies shown if the substratum type is critical in dictating biofilm accumulation during longer periods in the oral environment. The contribution of saliva is unclear due to factors like variations in its collection and handling. Initial findings have disclosed that also bacteria are crucial for the successful establishment of *Candida* in biofilms, but the clinical significance of this observation is yet to be confirmed. In conclusion, there is a need to standardize experimental procedures, to bridge the gap between laboratory and *in vivo* methodologies and findings and – in general – to thoroughly investigate the factors that modulate the initial attachment and subsequent colonization of denture-base materials and the oral mucosa of patients subjected to *Candida* infections. Information on how these factors can be controlled is required and this may help to prevent the disease. The societal impact of such information is significant given the magnitude of the candidosis problem worldwide.

Key words: *Candida albicans*. Biofilm. Denture. Saliva. Bacteria.

INTRODUCTION

Candida infections receive increasing attention, presumably due to the increased prevalence worldwide. Numerous studies have shown that several *Candida* species possess a multitude of virulence mechanisms leading to successful colonization and infection of the host when suitable conditions occur. The recognition that *Candida* is an important pathogen has led to many laboratory studies evaluating these virulence attributes in an attempt to clarify the pathogenesis of the disease. The progress made in understanding some of these features, such as the mechanisms that result in adherence to surfaces[79], cell surface hydrophobicity[32], and saliva[13] is very impressive though yet in many aspects inconclusive. Knowledge about how the adherence and biofilm formation process takes place and how to avoid or at least diminish *Candida* colonization are mandatory in clinical practice. This review aims to critically discuss several key factors controlling the adhesion of *Candida* species which are relevant to denture-associated stomatitis, to highlight areas of current controversy and to suggest future research.

Role of surface properties on *Candida* colonization

Fungi normally live as innocuous commensals and

colonize various habitats in humans, notably skin and mucosa[63,88]. Commensal existence of oral *Candida* species varies from 20% to 50% in a healthy dentulous population[79,88]. As growth on surfaces is a natural part of the *Candida* lifestyle[51], one can expect that *Candida* colonizes denture.

There is a large body of evidence indicating that *Candida* is able to adhere to acrylic resin dentures. This is the first step that may lead to the development of the infectious process and that may ultimately result in varying degrees of denture stomatitis of the adjacent mucosa[13,15,84]. *Candida* adheres directly or via a layer of denture plaque to denture base (polymethylmethacrylate – PMMA)[7,23,86]. Without this adherence, micro-organisms would be removed from the oral cavity when saliva or food is being swallowed.

It is well-known that innumerable factors are involved in the adhesion of *Candida* to the acrylic resin base, though contradictory results have been reported from *in vitro* studies[68,78,93]. Substrate surface properties, as surface charge, surface free energy, hydrophobicity, and roughness have all been reported to influence the initial adhesion of micro-organisms[8,104]. Microbial adhesion on biomaterial surfaces depends on the surface structure and composition of biomaterials, and on the physicochemical properties of the microbial cell surface, again its surface charge and hydrophobicity[4,11]. Components of the resilient denture liners and acrylic resin may reduce the adhesion and inhibit the growth of *Candida*[45,105,108].

(a) Surface free energy and surface roughness

Surface free energy is one of the main factors related to the development of denture related candidosis[67]. It is defined as the interaction between the forces of cohesion and adhesion and predicts whether or not wetting occurs[113]. A linear relationship between contact angle measurements on various types of substratum and *Candida albicans* adherence has been demonstrated, i.e. the higher the surface free energy, the higher will be the adhesion of micro-organisms and alternatively, the more hydrophobic the surface, the less cell adherence is expected[33,45,67].

Although the cited reports have found correlations between surface free energy and microbial' adhesion[12], other factors should also be considered, such as cell surface factors, diet, salivary composition and secretion rates, and antibody titers, which are all controlling factors in plaque formation[9] and could therefore influence yeast attachment. These many confounding factors might explain why recent studies have failed to show a direct correlation between surface free energy values and the adhesion of *Candida* species[68,78,93,110].

Higher adherence of particular *Candida* species, e.g. *C. tropicalis*, *C. glabrata* and *C.dubliniensis*, when compared with *C. albicans*, might be attributed to their relative surface free energy values, since hydrophobic micro-organisms seem to be more adherent to acrylic surfaces. While there are no studies regarding hydrophobicity of *C. tropicalis* and *C. dubliniensis*, Luo and Samaranayake[55] (2002) stated that *C. glabrata* is more hydrophobic than *C. albicans*.

Commonly used biomaterials exhibit significant differences in surface free energy. Heat-polymerized acrylic resin was reported to be more wettable than microwave-polymerized acrylic resin, due to acid-base interactions[68,94].

Surface roughness is calculated as the arithmetic average deviation of the surface valleys and peaks of a given surface[1]. It directly influences micro-organisms initial adherence to surfaces, biofilm development, and *Candida* species colonization. Materials with the roughest surface usually exhibit higher yeast counts[70,78,83,105]. This happens because surfaces may serve as a reservoir, with surface irregularities providing an increased chance of micro-organism retention and protection from shear forces, even during denture cleaning. In addition, these irregularities sometimes allow the entrapped microbial cells time to attach irreversibly to a surface[98].

Quirynen, et al.[79] (1990) postulated a threshold roughness value (0.2 μm) below which no effect on the adhesion should be expected. Smooth and highly polished surfaces are of utmost importance not only for patient's comfort but also for denture/restoration longevity, good aesthetical results, oral hygiene and low plaque retention[101].

The presence of saliva is known to change this scenario. The nature of the substratum may influence the formation and the composition of the salivary pellicle, which layer may then become more relevant than the surface properties of the dental material itself[30]. It has been shown that saliva immersion decreases the surface roughness[83] and surface free energy[94] of acrylic resins. This might explain the general decrease of *Candida* species in those studies where specimens were coated with saliva. Saliva, its components and properties on *Candida* adherence and colonization is thoroughly discussed in the following paragraph *Role of the salivary properties on Candida colonization.*

The available studies on surface properties raise questions regarding the role of surface free energy and surface roughness. There is general agreement that the hydrophobicity of the cell surface and substratum is an important predictor in the adhesion process, i.e. surface free energy indicates the ease with which saliva spreads over a surface[67,94]. There is also consensus on the role of surface roughness and the initial adherence process, i.e. surface roughness is positively correlated with the rate of bacterial/fungal colonization of biomaterials. If such rougher surfaces become exposed to the oral environment, they may be more susceptible to micro-organisms adhesion and biofilm formation and lead to infections. However, no studies on the application of certain treatments on different substratum types have been reported (i.e. application of different treatments diminishes the number of yeasts but may lead to detrimental changes of the substratum). *In vivo* studies may lead to different outcomes when compared with *in vitro* studies.

(b) Denture liners surface and characteristics

New materials have been developed in order to reduce and redistribute occlusal forces from dentures that might damage the underlying mucosal tissues[60,97]. In recent years,

the use of denture liners, either hard or soft, has increased.

Liners are needed in many clinical situations in which patients have thin, sharp, or badly resorbed residual alveolar ridges or chronic tissue irritation from dentures[57,60]. Even though these materials exhibit excellent tissue tolerance, one of the problems is the colonization of *Candida* spp. on and within the material. Fungal growth is known to destroy the surface properties of the liner and this may lead to irritation of the oral tissues. This is due to a combination of increased surface roughness and high concentrations of exotoxins and metabolic products produced by the fungal colonies[57]. This observation is the rationale why attempts have been undertaken to incorporate antifungal agents or antiseptics in these materials.

Unfortunately, conflicting adherence/colonization results are reported on these lining materials. Some in vitro studies reported significant inhibitory effects on *C. albicans*[21,112]. More recent studies, however, showed only limited antifungal properties and no significant reduction on *Candida* adherence and colonization[17,21,24,31,49,50,53,58,75,78].

As can be seen in Figure 1A and B and as was also reported previously[105], denture liners, especially the soft ones, introduce a higher surface roughness. The porous surface texture of the material will entrap yeast cells (Figure 2A and B), leading to an increased (re)colonization in spite of the antifungals. Concomitantly, the nutrient-rich environment of the oral cavity might overrule any inhibitory effect induced by antifungals released from the denture liners[31].

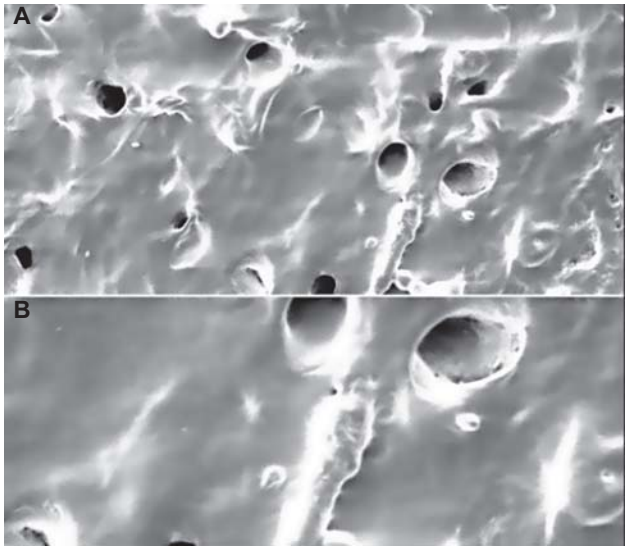

FIGURE 1A and B- Scanning electron microscopy of a soft denture reliner showing the extents of defect; it is notable to observe that the material not only exhibits porosities, but also show surface irregularities, which may turn into adhesion sites (A: x 40; B: x 100). Sample analyzed was prepared according to the manufacturer's directions (CoeSoft, GC America, Alsip IL, USA). It was subsequently mounted on a stub, air-dried, sputtercoated with gold (Balzers Union MED 010 evaporator), and examined with a Zeiss (Thornwood, NY) DSM940A scanning electron microscope at an accelerating voltage of 20.0 kV for surface characterization

Even though some *in vitro* studies have shown limited inhibitory effects, a reasonable explanation on why lining materials do not keep their antifungal characteristics could be the constant bathing in saliva in the mouth. Saliva extracts the antifungal ingredients, possibly even within a short time after the denture is placed in the oral environment, or dilutes the concentration near the denture surface to below fungicidal concentrations. Moreover, the antifungal included might not be effective against the particular *Candida* species (or mixture of micro-organisms, see below) that is causing the infection. Judging the literature the need emerges to systematically evaluate liners against various *Candida* species in relevant assays, e.g. involving various *Candida* and bacterial mixtures and saliva.

Role of salivary properties on *Candida* colonization

The role of human saliva in the *Candida* adhesion process is still controversial[68,73]. Saliva shows a physical cleaning effect and innate defence molecules, including lysozyme, histatin, lactoferrin, calprotectin and IgA[20,96], interact with *Candida* species, thereby decreasing

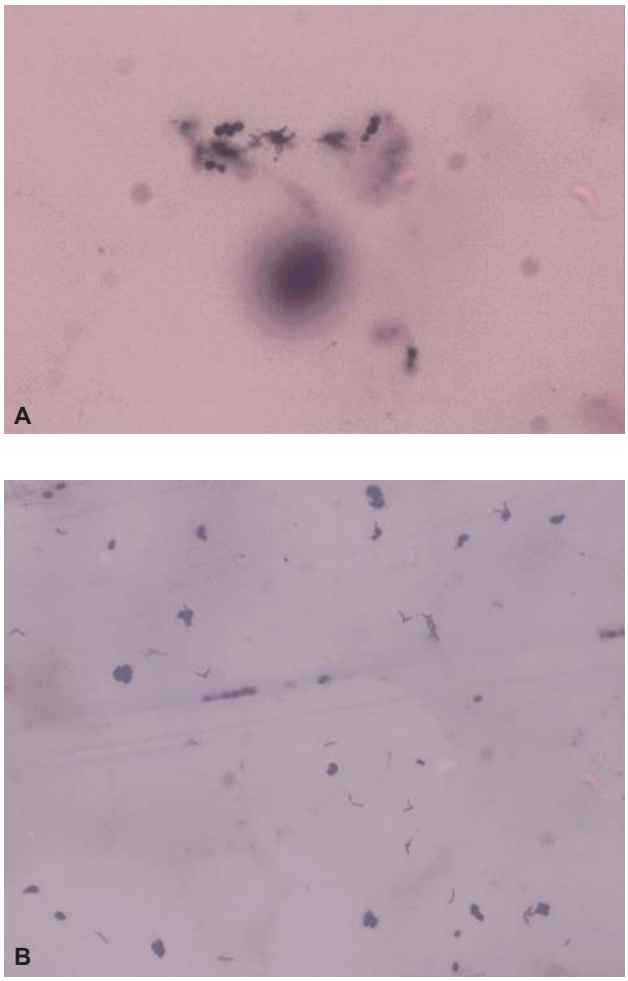

FIGURE 2A and B- Adherence of *Candida albicans* and bacteria on a soft denture liner coated with saliva
A – Note that bacteria and fungi are united. B – The sample was not coated with saliva; note that bacteria and fungi do not seem connected when compared to the coated sample

adherence to and colonization of oral surfaces. Other components in whole saliva, including mucins[20,25], statherin[42] and proline-rich-proteins[13,96] have been reported to adsorb to *C. albicans*, thereby facilitating adherence to saliva-coated acrylic resins[2].

However, studies regarding the influence of whole saliva on *Candida* adherence are mutuality contradictory and no consensus can be found in the literature (Table 1). Several investigators reported that a saliva coating reduces the adherence of *C. albicans* in acrylic resin based materials[6,59,65,66,68,72,78,86,110] Others showed increased adherence rates with saliva coating[23,65,71,102]. Three other research groups found no effect at all of a saliva coating[41,72,97]. A dynamic effect, depending on the morphological phase of *C. albicans* was also found[84,91], where initially adherence was increased, but subsequently decreased after 24 hours.

Several reasons might explain these divergent results. The most important are probably differences in the use of stimulated versus unstimulated saliva, resulting in different protein composition and viscosity, hence protection[103]. Furthermore, different incubation periods, use of filtered or whole saliva, different saliva temperatures when performing the study, and the presence or absence of nutrients in the different studies may have interfered with cell viability and adherence capacity[20,41,83,86]. Obviously inter-individual variations in the composition of saliva affect the outcome of three component adherence system studies of substratum, saliva and yeast[19,25,68,73,78].

In the oral cavity a denture is coated with a salivary pellicle, which provides receptor sites for the adherence of micro-organism[28]. Again surface roughness and surface free energy are confounding factors in the coating. Although surface characteristics are important in determining the final composition of an acquired pellicle and hence can dictate

TABLE 1- The effect of saliva on *Candida* species adherence/biofilm formation on acrylic surfaces, according to published data

Authors	Saliva Collection	Saliva Type	*Candida* Species	Effect on *Candida* spp.
Samaranayake, et al.[86], 1980	Unstimulated	Whole	*C. albicans*	Reduction
	Stimulated	Parotid	*C. albicans*	No effect
MacCourtie, et al.[61], 1986	Unstimulated	Whole	*C. albicans*	Reduction
Nikawa, et al.[72], 1992	Unstimulated	Whole	*C. albicans*	No effect
Vasilas, et al.[102], 1992	Stimulated	Whole	*C. albicans*	Increase
		Parotid	*C. albicans*	Increase
		Submandibular-Sublingual	*C. albicans*	Increased/reduced[1]
Edgerton, et al.[23], 1993	Stimulated	Submandibular-Sublingual	*C. albicans*	Increase
		Mucin-free	*C. albicans*	No effect
Nikawa, et al.[71], 1993	Unstimulated	Whole	*C. albicans*	Increase
Waters, et al.[110], 1997	Unstimulated	Whole	*C. albicans*	Reduction
Radford, et al.[81], 1999			*C. albicans*	
Millsap, et al.[65], 1999	Stimulated	Whole	*C. albicans*	Reduction/Increase[2]
San Millán, et al.[91], 2000	Unstimulated	Whole	*C. albicans*	Increased/reduction[3]
Millsap, et al.[66], 2001	Stimulated	Whole	*C. albicans*	Reduction
			C. krusei	Reduction
			C. tropicalis	Reduction
Ramage, et al.[85], 2001	Stimulated	Whole	*C. dubliniensis*	Increase
Maza, et al.[59], 2002	Unstimulated	Whole	*C. albicans*	Reduction
Bosch, et al.[6], 2003	Unstimulated	Whole	*C. albicans*	Reduction
Jin, et al.[41], 2004	Unstimulated	Whole	*C. albicans*	No effect
Ramage, et al.[84], 2004	Stimulated	Whole	*C. albicans*	Increase[4]
Moura, et al.[68], 2006	Stimulated	Whole	*C. albicans*	Reduction
			C. glabrata	No effect
			C. dubliniensis	Reduction/no effect[5]
			C. tropicalis	Reduction
Pereira-Cenci, et al.[78], 2007	Stimulated	Whole	*C. albicans*	Reduction
			C. glabrata	Reduction
Tari, et al.[97], 2007	Stimulated	Whole	*C. albicans*	No effect

[1]dependent upon the donor; [2]dependent upon the co-existence with other bacteria; [3]dependent on *Candida* morphological phase; [4]but decreased over time. [5]dependent upon the substratum

colonization of *Candida* species, there are only few studies where the effects of different types of acrylic resins on this process are compared[67,83].

Studies dealing with the effect of saliva on adherence of *Candida* species, other than *C. albicans*, to acrylic resins *in vitro* and *in vivo*, indicate variable adherence levels[66,68,78]. *C. dubliniensis* counts have been shown to decrease[25], increase[85] or show no effect[68] in the presence of saliva, while *C. glabrata* counts were not influenced by saliva in one study[68] but decreased in another report[78].

Thus there is contradicting evidence with regard to the relationship *in vitro* between saliva and *Candida* adhesion. In general it may be concluded that low molecular weight proteins are related to the adherence levels of *Candida*[10]. This is in agreement with clinical studies[20,74,80,96], where patients with low or impaired salivary flow and/or composition presented higher *Candida* species counts when compared with saliva from patients with normal salivary flow. Collectively this confirms the regulating role of saliva in inhibiting *Candida* species adherence.

Candida species' shift

The *Candida* species most often reported to be associated with oral mucosal lesions is *Candida albicans*. But *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. dubliniensis* have also been isolated from diseased tissues[18,56,89,90]. Recently a shift in disease-associated *Candida* species from *Candida albicans* towards these non-*albicans* species was observed[48,87,107]. While *C. albicans* is still by far the predominant isolate under inflammatory conditions[34], *C. glabrata* emerges as the second most prevalent species, frequently isolated from acrylic denture surfaces and the palatal mucosa[89]. *Candida glabrata* used to be considered a non-pathogenic *Candida* species, but the increased use of immunosuppressive drugs, as a cure of the immunosuppressive syndrome, have now led to increasing *C. glabrata* infections with high mortality rates[47]. The explanation for this trend towards morbidity due to ''less pathogenic'' yeasts remains to be established, but it has already been suggested that the increased worldwide use of antifungals has contributed to this phenomenon[92,95]. Besides the shift from *C. albicans* to *C. glabrata*, there is increasing evidence that more than one *Candida* species may simultaneously colonize mucosal habitats, as reported for the oral mucosa[22], tongue and palate[92], both in healthy and diseased subjects.

Bacteria and *Candida* interactions

Microbial cell to cell communication plays an important role in the colonization process. Micro-organisms present in the oral environment interact with each other in many ways, such as by using each other's metabolic end-products, or by communicating more directly through signalling molecules[5]. Understanding the complex interactions between surfaces, saliva, eukaryotic and prokaryotic micro-organisms during infections is crucial in developing prevention and treatment strategies. In studies on *Candida* biofilm formation and *Candida* susceptibility, the characteristics of the oral environment in which the biofilms are naturally formed should be mimicked as closely as feasible[52].

The multicellular lifestyle of bacterial and yeast biofilms[44,69] is induced by environmental stress and/or restricted nutrient supplies[76]. These cooperation lead to adaptation to natural stress responses and result in a balanced microflora[62,64,76,77]. In addition to various forms of metabolic dependence micro-organisms may co-aggregate, with two or more genetically distinct strains interacting through specific cell to cell recognition[38]. Such co-aggregation has been observed between *C. albicans* and several other oral micro-organisms[36,37,39] and is an important factor in the microbial colonization and progression of infections in the oral cavity.

Bacteria and yeasts also interact via quorum sensing (QS). Quorum sensing is a polymicrobial coordination within a microbial community, based on excreted small molecules triggering a genetic response when present in sufficiently high concentrations. QS occurs both in single species bacterial communities and in complex mixed bacterial-yeast communities[16,43]. A recent study[35] showed that *Candida* hyphal formation can be modulated by Gram negative bacterial quorum sensing molecules. Particularly in the multispecies biofilm communities QS molecules may accumulate to high concentrations and hence are important in controlling physiology and homeostasis[46].

Although studies on biofilm development and species interactions have, so far, focused largely on bacterial species it has become clear that synergistic interactions among micro-organisms increase the efficiency of the impropagation[29,54]. Oral biofilm are not random mixtures of micro-organisms; but organized structures though varying in space and time while modulating adherence and metabolic properties[99]. Immediately after brushing or prophylaxis, the surface will be recoated with salivary pellicle and the first pioneer bacteria will colonize. These "early colonizers" are followed by the "late colonizers", if the conditions of/in the biofilm become amenable for other species to survive[40].

Although there is variability in composition of an oral biofilm community depending on patient dependent characteristics, the mere presence of a specific micro-organism does not induce pathology. Typically this depends on a complex of micro-organisms-host interactions that modulate the host's response leading to inflammation. Depending on the local conditions, bacteria may provide fungi with compounds that activate virulence determinants of fungi[109]. This is not only important for *Candida* infections but also why *Candida* may be responsible for non-*Candida* infections induced by the patient's indigenous microflora[27].

Several researchers have studied interactions among *Candida* and bacteria in an attempt to determine how oral bacteria may modulate *Candida* adherence and colonization. The influence of *Streptococcus salivarius* has been reported to decrease *Candida* adherence[86], while cooperation between several *Streptococci* and *Candida albicans* has also been reported[7,106]. Other research groups assessed *in vivo* biofilms, with various plaque collection methods

generally destructive to the biofilm structure[14,26,82,84,111]. In contrast, the new confocal scanning laser microscopy using molecular biological staining techniques may elucidate unsolved issues or even identify artefacts arising from traditional methodologies. A recent study using acrylic resin samples of denture wearers *in vivo* has shown that different subjects present different biofilm formation rates, architecture and densities[3]. Unfortunately, the only substratum tested was acrylic resin and there was no attempt to characterize the surface properties, which might have resulted in a better understanding of the process. Clearly, understanding the biofilm behaviour of *Candida* species under various environmental conditions is the key to the development of effective preventive measures for *Candida* infections[100]. Further studies are needed to establish whether or not these interactions are strain-specific and on which other parameters they depend. As a result it may be possible to identify the stages when *C. albicans* and other emerging pathogenic species can be targeted in treatment and prevention.

Future research and final remarks

From the literature the picture emerges that many factors determine *Candida* harbouring biofilms. These factors include surface properties, micro-organisms interactions, biofilm architecture, and saliva. Obviously it is tempting to study the individual parameters in simple mechanistic studies. However, the level of contradictions in the pertaining literature should be interpreted by assuming multiple interactions between the various factors. A meaningful study of *Candida* biofilms thus only seems possible when the various factors are studied in a comprehensive experimental design.

As recent studies are pointing to the role of multi-species biofilms on the onset of the disease, studies that may explain how such biofilms interact with surfaces and how to prevent their growth are important. Fungal adhesion may be greater in materials presenting higher surface roughness. Consequently, the rehabilitation material chosen in clinical situations has to be carefully considered. When the oral cavity is re-colonized after antimycotic treatment withdrawal in patients with oral candidiasis, the yeasts may be harboured in more remote sites of the material.

While the initial adhesion of *Candida* species is influenced by surface roughness, and may be influenced by the materials' surface free energy (question still under discussion), these characteristics should be evaluated in *in vivo*-like conditions. Indeed, the presence of a rehabilitation material that could favour health and avoid the oral cavity re-colonization is mandatory. Therefore, studies that could explore the factors related to initial re-colonization by *Candida* in different materials are of utmost importance. The relationship of denture base materials and their effect on fungal growth requires further investigation through epidemiologic, clinical, and basic research. These new studies may include surface characteristics, but other important matters discussed on this review are fundamental to facilitate treatment protocols. New research should be on multispecies biofilm, as close as possible to the *in vivo* situation. Furthermore, other emerging fungal pathogens, such as *Candida glabrata*, should be under investigation, as the results found for one *Candida* species (mainly *Candida albicans*) may not generally hold, again in experimental setups where other organisms and saliva are present.

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