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Prevalence of Yeast Other than *Candida albicans* in Denture Wearers

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Abstract

Purpose: The isolation of yeast species other than *Candida albicans* from the oral mucosa has been increasing in frequency, suggesting that those may constitute emerging potential oral colonizers. The purpose of this work was to determine whether yeast species other than *C. albicans* are associated with factors related to wearing of dental prostheses.
Materials and Methods: tRNA-PCR fingerprinting and sequencing of the 26S rDNA D1/D2 domain were used to identify all yeasts isolated from CHROMagar™ Candida cultures of oral swabs collected from 178 patients.
Results: Besides *C. albicans*, 13 other species were identified, corresponding to 34% of the yeast isolates. The majority of the non-*C. albicans* species were not detected as single colonizers but rather in co-colonization with one or two other yeasts, often with *C. albicans*. No significant associations were found with non-*C. albicans* species. On the contrary, the best-fitted logistic regression model predicts that either wearing a denture (adjusted odds = 4.6) or insufficient oral hygiene (adjusted odds = 2.3) are risks for colonization by yeast, in general.
Conclusions: The colonization with non-*C. albicans* species and co-colonization were not independently associated with any of the analyzed host-related factors. In particular, neither wearing a removable denture nor being elderly were significant predictors.

Candida albicans is a known microorganism of the oral microbiome, also present in healthy individuals.[1] Recently, much attention has been paid to more unusual yeasts, non-*C. albicans* species, as potential oral colonizers. This increased attention to non-*C. albicans* species is probably due to the growing population of individuals with immunocompromising conditions, such as HIV infection, *diabetes mellitus*, or immunosuppressive therapies. This population seems to be more prone to colonization or infection by these atypical species,[2-6] generally after antifungal treatments.[7] Non-*C. albicans* are ubiquitous species that may naturally come into contact with the mouth and establish themselves as colonizers. The problem stems from the fact that oral colonization is a primary stage necessary for the onset of candidiasis, a superficial yeast infection common in moist areas of the body, which may further develop and become systemic in immunocompromised hosts.[8-9] Furthermore, some of these yeasts, particularly *C. glabrata* and non-*Candida* genera, are often resistant to therapeutic antifungal agents, which may be responsible for higher rates of mortality in those cases of candidemia.[9] Additionally, more discriminative methods of identification are currently employed, bringing to light non-*C. albicans* spp. that might otherwise have gone unnoticed. Due to the above-mentioned reasons, reports of candidemia cases attributed to non-*C. albicans* have increased.[10-12]

Although oral colonization is a rather common occurrence, a number of factors may predispose one to it, including that of wearing dental prostheses, denture stomatitis, insufficient oral hygiene, or old age; however, the relationship between those factors and colonization remains uncertain, in particular, those that concern colonization by non-*C. albicans* species. In fact, the various factors that may contribute to creating the advantageous conditions for colonization in the host are usually evaluated individually, which tends to blur the conclusions. Moreover, most studies have focused largely on the relationship of those host factors either with *C. albicans* or with the totality of yeast (considering all genera and species) colonization. In this study, we aim to determine whether the usual risk factors, such as wearing denture, the type of prosthesis, old age, gender, or oral hygiene frequency[13-17] are predictors for non-*C. albicans* colonization and the weight of

each predictor's contribution by using a logistic regression approach.

Materials and methods

Population

A total of 178 patients, (mean age: 57 years, range: 26 to 84 years) who attended dental appointments at the University Dental Clinic at Instituto Superior de Ciências da Saúde Egas Moniz (ISCSEM), Caparica, Portugal, were enrolled in this prospective, observational study. Information on each patient such as age, gender, oral hygiene frequency (times per day), tobacco and alcohol consumption habits, intake of antifungal or antibiotic agents, and the existence of immunosuppressive disease were recorded prior to oral examination by a clinician to evaluate the dental prosthesis, prosthesis base material, and mucosal lesions. Eligibility criteria excluded any patients under 18, or taking antifungal or antibiotic agents over the past 6 months, or suffering from *diabetes mellitus*, or with HIV infection, or declaring some other immunosuppressive disease. The protocol was approved by the Ethics Committee of the ISCSEM, and all participants signed the informed consent form before being included.

Sample collection and microbiological analysis

Samples were collected by swabbing the oral mucosa under the prosthesis and palate with sterile cotton swabs that were immediately processed. In the case of nondenture wearers only the palate was sampled. Primary isolation was made on the selective, differential CHROMagar™ Candida medium (CHROMagar, Paris, France) incubated aerobically at 30°C for 48 hours, followed by a presumptive identification based on the morphology and color of the colonies. Pure cultures were achieved by streaking representative colonies (each color and/or different morphology) of each sample on Sabouraud dextrose agar with chloramphenicol (Biomérieux, Marcy l'Etoile, France) to prevent bacterial growth and were stored frozen at −80°C in 15% (v/v) sterile glycerol. Molecular methods were used for further identification, as described in the following sections.

tRNA-PCR fingerprinting

Total DNA was isolated as previously reported[18] using glass beads for cell disruption. The supernatant containing genomic DNA was diluted in autoclaved double-distilled water (1:250), and 5 μl were used directly in the PCR. tRNA-PCR fingerprinting of genomic DNA employed a single primer T3B (5′-AGGTCGGTTCGAATCC-3′), following Thanos et al's protocol,[19] without the final step for product condensation. Amplification was performed in a final volume of 25 μl with 1U of *Taq* polymerase (GE Healthcare, Waukesha, WI) in an MJMini™ Personal Thermal Cycler (Bio-Rad, Hercules, CA),and gel electrophoresis images were acquired with the GelDoc XR System software (Bio-Rad). DNA banding patterns from the isolates and those from type strains were grouped by similarity in a dendrogram using Pearson's correlation coefficient and the UPGMA clustering method implemented in the GelCompar 4.1. software.[20]

Sequence of the D1/D2 domain of the 26S ribosomal DNA

The primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and LR6 (5′-CGCCAGTTCTGCTTACC-3′) were used in a PCR to amplify a fragment encompassing the D1/D2 26S rDNA domain. PCR products were then purified with the NucleSpin® Extract II kit (Macherey-Nagel, Düren, Germany) and used in the cycle sequencing reaction with the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′). To identify the selected isolates, the sequences obtained were aligned, visually corrected, and compared with all gene fungal sequences available at the GenBank database. Alignment and phylogenetic relationship analyses were conducted using *MEGA* version 5,[21] using the maximum parsimony method.

Reference strains

Nine type strains of clinical relevance were used as references: *Candida albicans* (PYCC 3436, ATCC 18804, CBS 562), *Candida tropicalis* (PYCC 3097, ATCC 750, CBS 94), *Candida glabrata* (PYCC 2418, ATCC 2001, CBS 138), *Candida parapsilosis* (PYCC 2545, ATCC 22019, CBS 604), *Candida krusei* (PYCC 3341, ATCC 6258, CBS 573), *Candida lusitaniae* (PYCC 2705, ATCC 34449, CBS 4413), *Candida guilliermondii* (PYCC 2730, ATCC 6260, CBS566), *Candida stellata* (PYCC 3044, ATCC 10667, CBS 843), supplied by the Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal; and *Candida dubliniensis* CBS 7987, supplied by the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Antifungal susceptibility testing

Susceptibility to the two clinically used antifungal agents fluconazole (25 μg disks from Oxoid, Spain) and voriconazole (1 μg disks from Oxoid, Spain) was tested for all isolates by a system of disk diffusion in Muëller-Hinton agar supplemented with 2% (w/v) glucose and 0.5 mg/ml of methylene blue, for 18 to 24 hours at 30°C. Classification was made according to Pfaller et al[22] (i.e., susceptibility for zone diameters $\geq$19 mm to fluconazole and $\geq$17 mm to voriconazole, susceptibility dose-dependent within 15 to 18 mm to fluconazole and within 14 to 16 mm to voriconazole, and resistant for $\leq$14 mm to fluconazole and $\leq$13 mm to voriconazole).

Statistical analysis

The Mantel-Haenszel chi-square test was used to evaluate linear association between non-*C. albicans*, multiple species or yeast colonization and the variables gender, wearing and type of removable dentures, oral mucosal lesions (denture stomatitis), oral hygiene frequency, and tobacco or alcohol consumption habits ($p < 0.05$ significant). Subsequently, a full logistic regression analysis was done with age, use, and type of removable dental prosthesis, to identify a possible relationship with the colonization by non-*C. albicans* yeasts. For yeast colonization, the independent variables (determined by Mantel-Haenszel chi-square test) age, denture wearing, and hygiene frequency were entered into a backward stepwise logistic

regression analysis using the likelihood ratio method to assess its relationship with yeast colonization. All statistical analyses were performed with IBM SPSS Statistics software version 19.0 (SPSS Inc., Chicago, IL).

Results

Colonization by Candida spp.

Identification of the yeast isolates was done by tRNA fingerprinting, a PCR approach that employs an unspecific primer, T3B, and expeditiously provides a reliable identification, to the species level, of a large number of isolates. This technique was previously used to identify clinical *Candida spp*.[19,23] The identification was accomplished through comparison of the DNA profiles obtained with the T3B primer in a dendrogram (Fig 1). Most of the DNA profiles could be clearly discriminated and grouped together with one of the type strains of *Candida* included in the study as references. Those strains whose DNA profiles were not sufficiently discriminative were identified by comparing the sequence of a D1/D2 region of the larger rDNA subunit with homologous sequences from type strains retrieved from GenBank.

The most frequently isolated non-*C. albicans* species were *C. glabrata* followed by *C. tropicalis, C. parapsilosis*, and *C. lusitaniae* (Table 1); however, nine additional species were collected so that, on the whole, the non-*C. albicans* yeasts corresponded to 34% of the yeast isolates, (i.e., the remaining 66% were *C. albicans*). Seven isolates did not belong to the *Candida* genus nor are commonly detected in oral samples. The majority of the non-*C. albicans* isolates, 67%, were not detected as single colonizers but in co-colonization with one or two other yeast species, often with *C. albicans* (Table 2). The presence of more than one species in each oral swab sample was uncovered because primary isolation was done on CHROMagar™ candida medium which distinguishes *C. albicans*, *C. tropicalis*, and *C. krusei* strains by colony color and morphology. Non-*C. albicans* had diverse white, pink, and purple shades allowing for an easy differentiation of strains present simultaneously in the same oral sample.

The medical relevance of non-*C. albicans* strains relies mostly on their higher resistance to the traditional antifungals used for infection treatment. In fact, 50% of *C. glabrata* and 25% of *C. parapsilosis,* the most prevalent non-*C. albicans* isolates, were resistant or susceptible-dose dependent to fluconazole. Similarly, for voriconazol, resistance or susceptibility-dose dependent were observed for 38% of *C. glabrata* and 25% of *C. parapsilosis* isolates, whereas for C. *albicans* this phenotype was observed for only 5% of the isolates. The *Rhodotorula mucilaginosa* isolate was also resistant to both therapeutic agents. All the other isolates were susceptible to both antifungals.

Colonization with non-*C. albicans* and co-colonization were not significantly correlated with the variables studied

The colonization's distribution through the different participant groups is given in Table 3. Seventy-two patients (27 male, 45 female), corresponding to 40% of the population, were colonized by yeasts; 33.3% from those 72 harbored non-*C. albicans*, and 19.4% harbored multiple species. Among multiple species carriers, three harbored simultaneously three or four species. Patients wearing complete dentures appear to have a greater tendency (25%) to carry yeasts other than *C. albicans* than those with a partial prosthesis (12%) or with no denture (8%); however, this effect was not specific for non-C. *albicans* because the same increase in the frequency of isolation occurs for *C. albicans*. About 72% (101 from a total of 140) of the patients with dentures wore acrylic resin-based dentures and showed a considerable higher frequency of non-*C. albicans* (19%) than their metal counterparts (5%). Nevertheless, the full logistic regression model did not identify any co-variable as a predictor for colonization with non-*C. albicans*. The same lack of association was noted for co-colonization when the same variables were evaluated, but five out of the six pairs of *C. albicans*/*C. glabrata* were isolated in participants wearing dentures and with oral mucosal lesions.

Wearing dentures and poor oral hygiene predispose to the colonization by yeasts

When yeast colonization was evaluated, considering all species instead of only the non-*C. albicans* ones, a significant association was found with three of the possible risk factors, namely, denture wearers, especially those with complete dentures, and individuals with poor oral hygiene and lesions on the oral mucosa (Table 4). Not surprisingly, nearly all of the patients clinically diagnosed with oral mucosal lesions wear dentures, with a prevalence of about 60% within this group. Wearing dentures and suffering from mucosal lesion were strongly associated with each other ($\chi^2_{\text{MH}} = 36.95$, $p < 0.001$), with the odds of developing mucosal lesion increasing almost 56-fold in those subjects. Thereby, suffering from mucosal lesion is a dependent variable and was not included in the backward stepwise logistic regression analysis to model the risk of being colonized by yeast. The model obtained was a function of denture wearing and hygiene frequency even if with different weights (Table 4). This means, for example, that an individual who wears a denture, and in addition performs oral hygiene less than twice a day has a 63% probability of being colonized with yeasts; this probability decreases to 42% upon improvement of oral hygiene. Contrary to that, age, gender, and tobacco and alcohol consumption habits by themselves did not significantly influence the colonization by yeast strains.

Discussion

Yeasts other than *C. albicans* may only be incidentally present

The prevalence of the less common yeasts, other than *C. albicans,* in the oral cavity was observed in 33.3% of the colonized patients, corresponding to 13.5% of the whole population. This frequency is within the range of 9% to 20% previously reported in studies for noninstitutionalized patients,[14,24-25] including in Portugal, the same geographic region.[26] Hospitalized or immunosuppressed patients have a higher prevalence of non-*C. albicans*.[9,27-29] However, that increase is not specific for

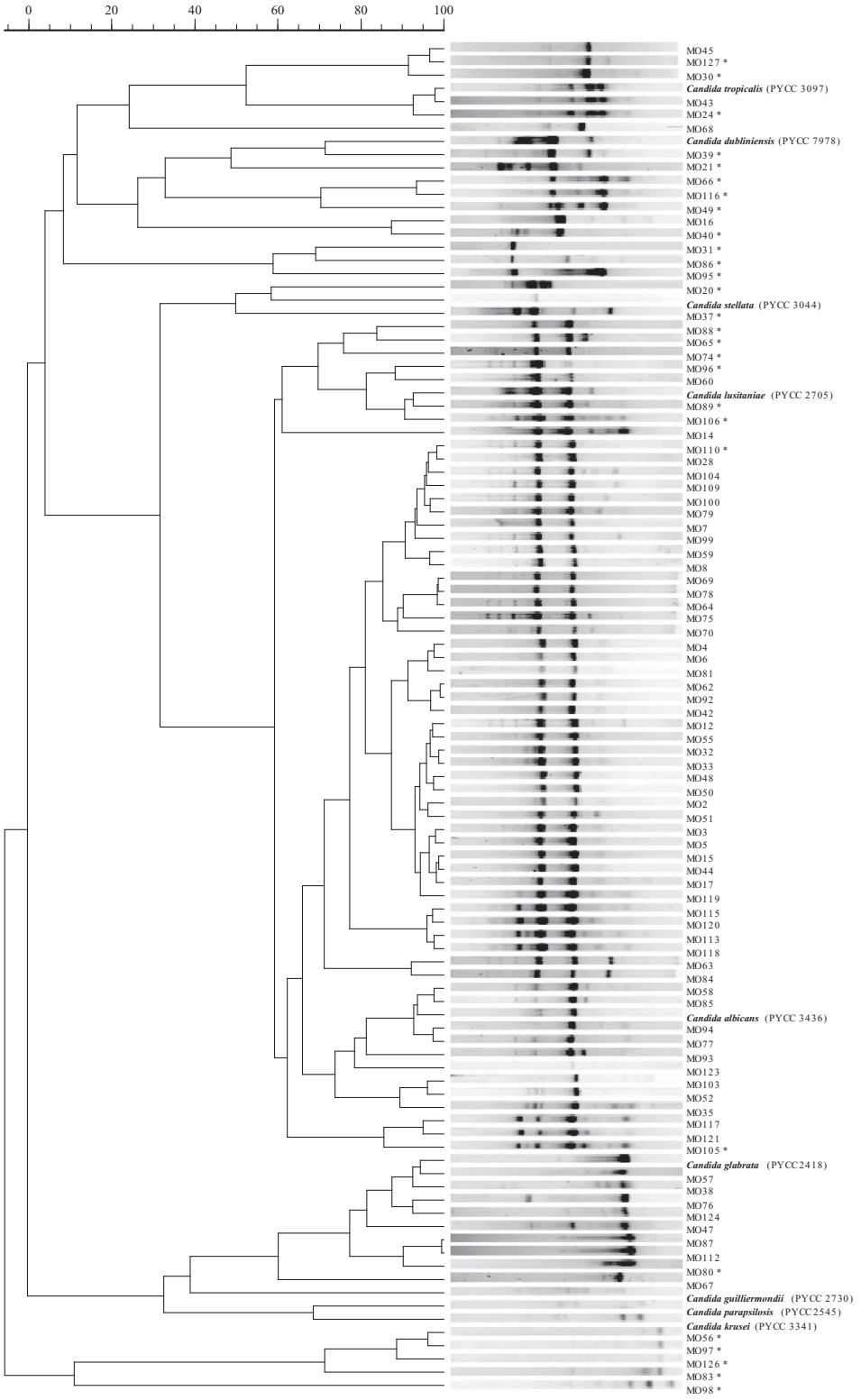

Figure 1 Dendrogram showing the cluster analysis of the genotypes of the oral yeast isolates and type strains (identified in the last column) obtained by DNA fingerprinting with the primer T3B. The dendrogram was constructed using UPGMA clustering method based on Pearson's correlation coefficient. The scale bar indicates the similarity index. Co-phenetic correlation coefficient was 0.938. The asterisk on the isolate code indicates those who were sequenced to confirmation.

Table 1 Number of yeast species isolated from the oral mucosa of the studied population

Organism	No. of isolates (%)[a]
C albicans	59 (66)
C. glabrata	8 (9.0)
C. tropicalis	4 (4.5)
C. parapsilosis	4 (4.5)
Clavispora lusitaniae	4 (4.5)
Saccharomyces cerevisiae	2
C. dubliniensis	1
C. guillermondi	1
C. rugosa	1
Rhodotorula mucilaginosa	1
Pichia norvegensis	1
Torulaspora delbrueckii	1
Debaryomyces hansenii	1
Trichosporon cutaneum	1

[a]Total of 89 yeast isolates.

Table 2 Co-colonization among colonized participants

Organisms	No. of participants
C. albicans + *C. glabrata*	3
C. albicans + *C. glabrata* + other	3
C. albicans + *C. tropicalis*	1
C. albicans + *S. cerevisiae*	1
C. albicans + *D. hansenii*	1
C. albicans + *P. norvegensis*	1
C. albicans + *T. cutaneum*	1
C. glabrata + *C. parapsilosis*	1
C. parapsilosis + *C. rugosa*	1
C. lusitanea + *C. lusitanea*[a]	1

[a]Different colors on CHROMagar™ Candida medium.

Table 3 Association of oral yeast carriage with different participant groups

	No. of participants (%)			
Variables	n	Yeast	Non-*C. albicans*	Multiple species[a]
Gender				
Male	62	27(44)	9(15)	4(6)
Female	116	45(39)	15(13)	10(8)
No prosthesis	38	7(18)	3(8)	2(5)
With prosthesis	140	65(46)	21(15)	12(9)
Partial prosthesis	104	40(38)	12(12)	6(6)
Complete prosthesis	36	25(69)	9(25)	6(17)
Prosthesis base material				
Acrylic	101	48(48)	19(19)	12(12)
Metal	39	17(44)	2(5)	0
Presence of mucosal lesion	85	44(52)	14(16)	8(9)
Hygiene frequency (Once a day)	39	22(56)	6(15)	4(10)

[a]Carriers of 2 to 4 different species.

non-*C. albicans* because higher prevalence of colonization with yeast, in general, and co-colonization are also observed. Co-colonization is also commonly reported and has been found in similar proportions to non-*C. albicans* (9.3% to 24%).[16,26,29-30] This is in agreement with our finding that the majority of the non-*C. albicans* species were cohabitants, frequently with *C. albicans*. A hypothesis is that a former colonization by *C. albicans* can facilitate the fixation of species less adapted to adhere to the oral mucosa or other oral surfaces.[31] Only 13 out of the 72 yeast carriers harbor solely non-*C. albicans* species, suggesting a lower propensity of these yeasts to colonize the oral cavity.[32-35] *C. glabrata* seems to be an exception, and although less pathogenic than *C. albicans*,[36] it showed a superior adherence capability to the materials of the prosthesis,[37] promoting its oral colonization.[38] Although not statistically significant, our finding that patients with acrylic-based prostheses had a higher frequency of non-*C. albicans spp*. concurs with that idea. Furthermore, most of the pairs found were *C. albicans* with *C. glabrata* and were isolated from participants wearing dentures. This was also observed by Coco et al, who proposed that their synergistic association may potentiate their pathogenicity to explain their presence in patients with extensive oral inflammation.[39] An in vitro human oral epithelium model also evidenced an increased tissue invasion and damage after infection with a mixed population of *C. glabrata* and *C. albicans*.[35] However, this synergy might be strain dependent.[36]

Age, gender, removable dental prosthesis, type of prosthesis, oral mucosal lesions, oral hygiene frequency, and tobacco and alcohol drinking habits are among the most consistently mentioned host aspects eventually predisposing for colonization. Yet none was a significant predictor of colonization with non-*C. albicans* or with multiple species simultaneously. Age, in particular, has been a matter of great debate because various intrinsic factors change in older patients, decreasing the protective function of saliva and could be related to an increase in colonization by non-*C. albicans;* however, we did not find any relation between colonization by non-*C. albicans* and age.

The oral colonization by these less frequent species can be transient and due to casual environmental exposure (i.e., food, water, soil, leaves, fingers, and hair contact). In fact, *Trichosporum cutaneum* is an opportunistic dimorphic yeast, a minor component of normal hair skin flora and already identified in oral samples.[1,40] Similarly, *Saccharomyces*, *Rhodotorula, Pichia*, *Debaryomyces*, and several *Candida* non-*albicans* are genera normally associated with those commonplace environmental sources that easily come into contact with the mouth. It would be interesting to follow these participants to see if the pattern and number of non-*C. albicans* colonization is stable over time. If not, the presence of these yeasts has questionable clinical significance. The findings of a systematic review concerning several studies on candidemia support the idea of fortuitousness. Species distribution was essentially found to be dependent on geography, with the highest frequency of non-*C. albicans spp* observed in South European, South American, and Asian countries.[10] Although the above-mentioned genera may be incidentally present, they are among the most frequent non-*Candida* yeasts isolated as pathogens in candidemia[11] and so, it might be of concern if those species are detected in the oral

Table 4 Evaluation of denture-related variables as risk factor for colonization with yeast species

	Non-*C. albicans*			Yeasts						
	χ^2 test			χ^2 test			Logistic regression			
Denture-related variables	*Odds*	χ^2_{MH}	*p*	*Odds*	χ^2_{MH}	*p*	*Adjusted odds*	B	95% CI	*p*
Age	–	–	–	–	–	–				0.190
Gender	0.875	0.004	0.949	0.822	0.206	0.650				
Wearing prosthesis	2.058	0.752	0.386	3.831	8.557	0.003	4.56	1.518	1.8-11.8	0.002
Complete prosthesis	2.558	2.798	0.094	3.636	9.048	0.003				
Type of prosthesis*	4.292	3.106	0.078	1.172	0.052	0.819				
Mucosal lesion	1.637	0.798	0.372	2.494	7.728	0.005				
Hygiene frequency	1.333	0.077	0.782	2.381	4.685	0.030	2.33	0.848	1.1- 5.0	0.029

*Acrylic prosthesis.

cavity of immunocompromised patients, even if temporarily, because there is a risk of the onset of a serious yeast infection. This relationship between colonization and candidemia was observed in elderly patients[9] and in individuals who received organ transplants.[8,41] Moreover, some of these species, an example of which is *C. glabrata*, exhibit intrinsically or acquired lower sensitivity to some antifungal agents[12,42] as our results also confirm.

Denture wearing and poor oral hygiene are predictors of yeast colonization

Evaluating the data presented in this work by a logistic regression approach supported the conclusion that, with the exception of the use of a denture and the lack of adequate oral hygiene, none of the other variables appeared to be relevant to predispose healthy participants to yeast colonization. Oral hygiene is not an easily measured variable, and possibly, that is why it has been alternately referred to as contributing[17,43] or not[15] to the presence of yeasts or even to oral candidiasis. The odds of being colonized increased 2.3-fold in participants who stated they performed oral hygiene only once a day. Although that was not accurately evaluated, oral hygiene can be a somewhat underestimated true risk factor because patients tend to deliberately hide some lack of hygiene.

Altogether, the results highlight the emphasis that dentists should give to the education of patients with prostheses, which may at least reduce their probability of developing oral candidiasis, an uncomfortable and often painful infection. In addition to predispose to colonization, wearing a denture often causes denture stomatitis[13] as we also observed among our population. The local inflammation caused by the friction of the denture[44] may contribute to the invasion of the epithelium by yeasts,[45] thereby triggering candidiasis. The situation worsens if patients wear complete or unsuitable dentures and/or do not remove them during the night.[13,17] Besides harboring yeast, the prosthesis is also a reservoir of a high number of bacteria genera, including pathogens, which may represent an additional health risk for both the denture wearer and the community.[17,46]

Conclusion

Non-*C. albicans* yeasts are not usually considered regular human flora, and apparently their occurrence in the mouth may be incidental. This is suggested by the fact that they are not associated with any factors included in this study, a situation which differs considerably from *C. albicans*; however, our results also suggested that an additional surface, as in the case of an acrylic prosthesis, may contribute to the appearance of non-*C. albicans* yeasts, in particular *C. glabrata*.

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