In vitro evaluation of the enzymatic activity profile of non-*albicans* *Candida* species isolated from patients with oral candidiasis with or without diabetes

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Objective. To evaluate the expression of phospholipase (PL) and secreted aspartyl proteinase (SAP) by *Candida glabrata* and *C tropicalis* obtained from the denture biofilms of healthy participants (16 isolates), patients with oral candidiasis with diabetes (10 isolates), and patients with oral candidiasis without diabetes (25 isolates).
Study Design. After incubation, the supernatants and pellets of the isolates were used for the enzymatic assays and quantification of colony-forming units (CFU), respectively. Colorimetric tests were used with phosphatidylcholine as a substrate for PL and azocasein as a substrate for SAP, and the absorbances of the samples were measured. Enzymatic rates were calculated, and values were normalized by CFU. Results were analyzed with factorial analyses of variance ($\alpha = .05$).
Results. *C tropicalis* and *C glabrata* were proteolytic and phospholipolytic. The clinical sources of isolates had no significant effect on the enzymatic activities ($P > .05$). *C tropicalis* had significantly higher enzymatic activity for both PL and SAP ($P < .001$) than did *C glabrata*.
Conclusions. *C tropicalis* isolates produced significantly higher amounts of both enzymes than did the *C glabrata* isolates.
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Candida species (spp.) can be found as commensal inhabitants in the microbiota of human cavities.[1,2] However, these microorganisms can become opportunistic pathogens and cause infections ranging from simple mucocutaneous disorders, such as oral candidiasis,[3-5] to invasive diseases involving multiple organs.[6,7] Oral candidiasis is one of the most common candidal infections,[8] and although it is a local disease, it can spread through the bloodstream or upper gastrointestinal tract, leading to systemic infections with significant morbidity and mortality.[7] The occurrence of oral candidiasis has been related to several predisposing factors related to the host, including age and female gender, the use of dental prostheses, reduced salivary function, smoking habits, immunosuppressive diseases, and metabolic disorders, such as diabetes mellitus.[3-5,8]

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Patients with diabetes wearing removable dentures have shown higher incidence of *Candida* infection[9] and increased levels of *Candida* spp.[3,9-11] owing to the systemic consequences related to the disease. The higher concentration of salivary glucose levels observed in these individuals enhances the adhesion of *Candida* yeasts.[12] Their reduced salivary flow rate[13] also promotes the oral carriage of *Candida*. In addition, people with diabetes possess a deficient cellular innate immunity with a decreased activity of phagocytosis, intracellular killing, bactericidal activity, and chemotaxis,[14] rendering them even more predisposed to candidal and other infections. These factors may have a significant influence in the balance between the host and yeasts, favoring the transition of *Candida* isolates from commensal to pathogenic microorganisms.

Candida spp., the major etiologic factor for oral candidiasis (either with or without diabetes), have developed specific virulence attributes that give them the ability to disrupt the integrity of the host epithelial

Statement of Clinical Relevance

Although all *C tropicalis* and *C glabrata* isolates produced enzymes, *C tropicalis* secreted higher amounts of both phospholipase and proteinase. Inhibition of secretion of these enzymes might turn out to be particularly rewarding for oral candidiasis treatment and prevention.

barrier function, invade host tissues, and cause infections. In this context, the proteolytic and lipolytic exoenzymes seem to play an important role in *Candida* pathogenicity. Yeast adhesion to epithelial cell surfaces is recognized as an essential step in the process of candidal colonization and subsequent infection. However, once the first contact between host tissues and *Candida* has occurred, the enzymes enhance adherence and invasion by damaging or degrading cell membranes and extracellular proteins.[1,15-18] Among these enzymes, phospholipases (PLs) and secreted aspartyl proteinases (SAPs) can be pointed out as the most significant ones. The PLs, which are concentrated mostly in the tips of the hyphae, have an active role in the invasion of lesions in the host tissue, because this enzyme causes the rupture of the epithelial cell membrane and allows the hyphal tip to enter the cytoplasm.[16] SAP production is related to an increased ability of the microorganisms to colonize and penetrate host tissues and to evade the host's immune system by provoking the degradation of a large variety of host proteins.[1,15,17,18] The literature shows that enzymatic secretion from *Candida* spp. isolates may be determinant not only for the onset[15] and severity[19] of infections but also for commensal colonization of such yeasts.[1,17,18]

C albicans, the most prevalent yeast within the oral cavity, is a fundamental etiologic factor linked to the occurrence of oral candidiasis.[2,3-5,20] Nevertheless, the epidemiology of *Candida* infections has changed with the emergence of non-*albicans* spp., which have been increasingly described both in compromised and non-compromised hosts.[3-5,9-11,20] Multiple isolations of *Candida* spp. in structured biofilm communities were recorded in patients with oral candidiasis with or without diabetes, and the yeast associations most commonly observed were *C albicans* together with *C tropicalis*, *C glabrata*, or both.[3-5,9,20] In the context of enzyme secretion, these *Candida* spp. are known to possess PL and SAP genes[21,22] and to secrete these enzymes in vitro.[11,23-32] Thus, this may also be a virulent attribute to the pathogenicity of these non-*albicans Candida* spp. There are available data in the literature indicating that SAP[33] and PL[34] activities of *C albicans* were higher than those of the non-*albicans Candida* isolates. Nonetheless, studies comparing these non-*albicans Candida* spp. are sparse. The pathogenicity of *C tropicalis* and *C glabrata* should not be underestimated. A recent study found that a large percentage of oral non-*albicans* isolates, especially from *C glabrata*, was resistant or dose-dependent to azoles.[35] In addition, these 2 non-*albicans* spp. have the ability to cause fungemia in humans and are associated with higher mortality rates.[6,7] Thus, an evaluation of the enzymatic activity of these *Candida* spp. may have a therapeutic role in the future prevention and treatment of *Candida* infections.

There is a large variation in the expression of different extracellular hydrolytic enzymes among *Candida* spp. isolates, and this could be the result of factors such as the source of isolates, the high phenotypic variability of a certain isolate, or even variations in the methodology used.[10,11,23-34] Thus, it could be interesting to investigate whether there are some virulence factors, such as PL and SAP production, which activities could be enhanced in isolates from non-*albicans Candida* spp. obtained from patients with different systemic/oral conditions (with and without oral candidiasis and diabetes mellitus). To the authors' knowledge, there are only a few investigations that studied the expression of these 2 enzymes in oral non-*albicans Candida* isolates from diabetic individuals with or without oral candidiasis.[10,11] The characterization of the enzymatic activity profile of the strains responsible for a particular infection, including the less-studied *C glabrata* and *C tropicalis*, is essential to determine strategies for prevention, control, and treatment of this type of fungal infection and also as a possible target for developing novel therapeutic interventions. Thus, the central aim of the present study was to characterize the enzymatic profile of a Brazilian culture collection of 51 clinical isolates of non-*albicans Candida* spp. (*C glabrata* and *C tropicalis*) by assessing the expression of the extracellular hydrolytic enzymes PL and SAP. In addition, this study compared the rates of secretion of candidal histolytic enzymes of *C glabrata* and *C tropicalis* spp. isolated from healthy participants, patients with oral candidiasis with diabetes, and patients with oral candidiasis without diabetes.

MATERIAL AND METHODS

Clinical isolates of *Candida* spp.

In the present study, 51 clinical isolates of non-*albicans Candida* spp., obtained from the yeast culture collection of the Laboratory of Applied Microbiology, UNESP - Univ Estadual Paulista, Araraquara, São Paulo, Brazil, were used. In general, 31 isolates were identified as *C glabrata* and 20 as *C tropicalis*. These isolates were previously obtained from the biofilm of the fitting surfaces of the complete upper dentures of 210 edentulous patients with different systemic/oral conditions, in a previous investigation.[3] Sixteen clinical isolates were obtained from healthy participants who were not diabetic and had no clinical signs of oral candidiasis (13 *C glabrata* and 3 *C tropicalis*); 25 clinical isolates were obtained from patients with oral candidiasis without diabetes (14 *C glabrata* and 11 *C tropicalis*); and 10 clinical isolates were obtained from patients with oral candidiasis with diabetes (4 *C glabrata* and 6 *C tropicalis*).[3]

As described in our previous investigation,[3] all patients with type 2 diabetes were evaluated in relation to

their medical care of diabetes based on the recommendations of the Standards of Medical Care in Diabetes—2007.[36] Four clinical chemistry tests were performed to assess the degree of diabetic control before the onset of the study, and only patients fulfilling the recommended goals for the tests were selected: the fasting blood glucose level (90-130 mg/dL), the postprandial capillary plasma glucose (<180 mg/dL), the glycosylated hemoglobin level (<7%), and the serum lipids (low-density lipoprotein <100 mg/dL; triglycerides <150 mg/dL; and high-density lipoprotein >40 mg/dL). Serum creatinine (0.4-1.3 mg/dL) and urine tests (protein/glucose/nitrite free and leukocytes < 10 units/mm^3) were also carried out to evaluate the systemic health condition of the diabetic individuals.

The diagnosis of *Candida* infection was made by a specialist researcher,[3] who performed a comprehensive oral examination of the patients and classified their mucosal characteristics according to the criteria proposed by Newton: type I (initial stage of localized pinpoint hyperemia); type II (diffuse erythema confined to the denture-bearing surface); and type III (inflammatory papillary hyperplasia).[37] All procedures followed the criteria of Resolution 196/96 of the Brazilian Health Ministry, and the study was approved by the Ethics Committee of the Araraquara Dental School, UNESP - Univ Estadual Paulista. All demographic characteristics of the patients and *Candida* spp. prevalence are available in Table I.

The yeast identification procedures for *Candida* spp. included the presumptive identification on CHROMAgar *Candida*, the micromorphologic characteristics on corn meal agar with polysorbate 80 (Tween 80) for the production of hyphae and chlamydoconidia, and the assimilation of a variety of carbon and nitrogen sources using the ID32C yeast identification system (bioMérieux, Marcy-l'Etoile, France).[2-5,20] Two reference strains were used as controls: *C glabrata* ATCC 2001 and *C tropicalis* ATCC 4563. All isolates were maintained in yeast extract peptone dextrose (YEPD) medium (1% yeast extract, 2% Bacto peptone, 2% D-glucose, 2% agar) and frozen at −70°C until use.

Culture conditions

The microorganisms were subcultured onto Sabouraud Dextrose Agar (SDA) (Acumedia Manufactures Inc, Baltimore, MD, USA) plates supplemented with chloramphenicol (0.05 g/L) and incubated at 37°C for 24 to 48 hours. To prepare the yeast inocula, a loopful of the agar stock cultures was transferred to tubes containing 5 mL of Yeast Nitrogen Base broth (YNB) (Difco; Becton Dickinson, Sparks, MD, USA) supplemented with 100 mM glucose and incubated at 37°C overnight in an orbital shaker (75 rpm). After the incubation period, the tubes were centrifuged (5000 × *g* for 5 minutes) and the supernatants were transferred to sterile polypropylene microtubes (Eppendorf, Hamburg, Germany) and immediately used for the enzymatic activity assays. Cell pellets were reserved for quantification of cells by counting colony-forming units (CFU) and reporting of CFU per milliliter (CFU/mL).

Quantification of colony-forming units per milliliter

Cell pellets were harvested and washed twice with phosphate-buffered saline (PBS) (pH 7.2) at 5000 × *g* for 5 minutes. Serial decimal dilutions (in PBS) were made, and the number of *C glabrata* and *C tropicalis* was determined by pipetting replicate specimens (25 μL) of the suspensions on SDA medium supplemented with chloramphenicol. The plates were incubated during 48 hours at 37°C, and the values of CFU/mL were accounted.

Quantification of phospholipase secretion

Phospholipase production testing was performed according to the methodology proposed by Taniguchi et al.[38] with few modifications. Briefly, a solution was prepared from 100 mL of 50 mM Tris-HCl buffer (pH 7.5) plus 0.123 g of phosphatidylcholine (Sigma-Aldrich Brasil Ltda, São Paulo, São Paulo, Brazil), 250 μL of Triton X-100 (Sigma-Aldrich Brasil Ltda), 0.27 g of aluminum chloride (Sigma-Aldrich Brasil Ltda), and 0.124 g of bromothymol blue (Sigma-Aldrich Brasil Ltda). Phosphatidylcholine was dissolved under vigorous agitation at bench temperature (27°C) and filtered at 0.22 μM after 1 hour of agitation. Before use, this solution was stored for 1 week under refrigeration (5°C).[38] Immediately before the readings, the planktonic supernatants had their pHs corrected to 7.5 with 10 mM NaOH solution (Synth LabSynth Produtos para Laboratório Ltda, São Paulo, São Paulo, Brazil), and 500 μL of the supernatants was mixed with an equal volume of phosphatidylcholine solution in sterile polypropylene microtubes (Eppendorf). The microtubes were incubated for 1 hour at 35°C, after which the absorbance was determined by OD630 nm readings in 10-mm-path cuvettes in a spectrophotometer (Biospectro; Equipar Ltda, Curitiba, Paraná, Brasil). All tests were carried out in triplicate and at 3 independent moments.

A control assay, without the planktonic cells in the reaction mixture, was done and was used as the blank in all spectrophotometric measurements. The rates of absorbance shifts (ΔOD630 nm) for the repetitions were adjusted by the blank. One phospholipase unity (U) was arbitrarily established as the absorbance shift, by minute of reaction (60 minutes), multiplied by one

Table I. Demographic characteristics and frequency distribution (%) of *Candida* spp. of the healthy control participants and of patients with oral candidiasis with or without diabetes

Group	Demographic characteristics		Candida spp. prevalence		
	Mean age (y)	Gender (% female)	C albicans	C tropicalis	C glabrata
Healthy participants (n = 90)	65.5	72.3	64	2	14
Patients with oral candidiasis without diabetes (n = 80)	59.6	77.5	93	28	20
Patients with oral candidiasis with diabetes (n = 40)	62.4	87.5	100	20	10

Data from Sanitá et al.[3]

Table II. Factorial analyses of variance for the enzymatic activity data

Enzyme	Factor	Sum of squares	df	F	P
Phospholipase	(Intercept)	691.05	1	3945.10	<.001
	Candida spp.	1.86	1	10.60	.0022*
	Source of clinical isolate	0.61	2	1.75	.1847
	Candida spp. × source	0.32	2	0.92	.4067
	Residual	7.88	45		
Proteinase	(Intercept)	1075.73	1	5395.91	<.001
	Candida spp.	0.89	1	4.48	.0398*
	Source of clinical isolate	0.47	2	1.18	.3160
	Candida spp. × source	0.69	2	1.74	.1874
	Residual	8.97	45		

*Significant differences at $P < .05$.

thousand (U = ΔOD630 nm/min × 1000). The results of the specific phospholipase activity were expressed as a ratio between U and CFU (U/CFU).

Quantification of proteinase secretion

Proteinase production testing was performed according to the methodology proposed by Pande et al.[39] with few modifications. An azocasein solution (1%) was prepared using 1 g of azocasein (Sigma-Aldrich Brasil Ltda) plus 100 mL of 50 mM Tris-HCl buffer (pH 7.5). Then, 900 μL of the supernatant was mixed with 100 μL of the azocasein solution in sterile polypropylene microtubes, and the microtubes were incubated for 1 hour at 35°C. Thereafter, the reaction was stopped with 500 μL of 10% trichloroacetic acid (Sigma-Aldrich Brasil Ltda) during 10 minutes at room temperature. The stopped solution was centrifuged (5000 × *g* for 3 minutes), 500 μL of the supernatant was mixed with an equal volume of 0.5 M NaOH, and this mixture was incubated for 15 minutes at 35°C. The development of color was measured spectrophotometrically in 10-mm-path cuvettes at 440 nm. All tests were carried out in triplicate and at 3 independent moments.

A control assay, without the planktonic cells in the reaction mixture, was done and was used as the blank in all spectrophotometric measurements. The rates of absorbance shifts (ΔOD440 nm) for the repetitions were adjusted by the blank. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions, that gives rise to an increase of 0.001 unit of absorbance at 440 nm per minute of digestion. Thus, one proteinase unity was established as the absorbance shift, by minute of reaction (60 minutes), multiplied by one thousand (U = ΔOD440 nm/min × 1000). The results of the specific proteinase activity were expressed as a ratio between U and CFU (U/CFU).

Table III. Mean values of enzymatic activity for phospholipase (U/CFU × 10^{-7}) and proteinase (U/CFU × 10^{-7}) and SD of each *Candida* species from all clinical sources combined

Candida spp.	Phospholipase	Proteinase
C glabrata (n = 31)	3.59 (11.06)^{A}	0.064 (0.212)^{A}
C tropicalis (n = 20)	5.17 (4.99)^{B}	0.104 (0.116)^{B}

In columns, values with different uppercase letters were statistically different ($P < .05$).
U, enzyme unit; *CFU*, colony-forming unit.

Statistical analysis

There were 2 variables of interest in the present study, which were the phospholipase and proteinase activities (U-PL/CFU and U-SAP/CFU values, respectively). The factors analyzed were the *Candida* spp. (*C glabrata* and *C tropicalis*) and the clinical source of the isolates: healthy participants (no diabetes, no oral candidiasis), those with oral candidiasis without diabetes, and those with oral candidiasis with diabetes.

To obtain positive values of information and to achieve a normal distribution of the data, the negative values of the logarithmic function were used ($-\log_{10}$ [U/CFU]). Then, a 2 × 3 factorial analysis of variance (ANOVA) was applied, and a significance level of 5% was considered for all comparisons.

RESULTS

In general, all 51 clinical isolates evaluated here and the ATCC reference strains were PL and SAP positive. The

enzymatic activity values of *C glabrata* strains ranged from 0.28 to 61.69 U-PL/CFU $\times 10^{-7}$ and from 0.006 to 1.197 U-SAP/CFU $\times 10^{-7}$. The enzymatic activity values of *C tropicalis* strains ranged from 0.93 to 22.61 U-PL/CFU $\times 10^{-7}$ and from 0.015 to 0.457 U-SAP/CFU $\times 10^{-7}$. The enzymatic activity values of *C glabrata* ATCC strain were 1.03 U-PL/CFU $\times 10^{-7}$ and 0.052 U-SAP/CFU $\times 10^{-7}$. The values of *C tropicalis* ATCC strains were 5.32 U-PL/CFU $\times 10^{-7}$ and 0.089 U-SAP/CFU $\times 10^{-7}$.

Table II shows the results of the factorial ANOVAs. Although the 3 clinical sources of the isolates had no significant effect on the enzymatic activities ($P > .05$), the different *Candida* spp. showed a significant effect ($P < .001$) on the U values of both enzymes.

Because the 3 clinical sources of the isolates had no significant effect on the enzymatic activities for both enzymes, the mean values of U-PL and U-SAP activity of each *Candida* spp. were grouped. Table III shows the mean values of U-PL and U-SAP of each *Candida* spp. from all clinical sources combined. *C tropicalis* isolates had significantly higher enzymatic activity than those of *C glabrata*, for both PL and SAP.

DISCUSSION

The present study investigated the expression of SAP and PL by clinical isolates of 2 of the most common non-*albicans Candida* spp. (*C tropicalis* and *C glabrata*) obtained from the denture fitting surfaces of healthy participants, patients with oral candidiasis with diabetes, and patients with oral candidiasis without diabetes. In general, the data showed that all the 51 non-*albicans Candida* isolates evaluated were able to produce the 2 hydrolytic enzymes in vitro. These results are in agreement with other studies in which genetic sequences related to the SAP[22] and PL[21] genes were detected in some isolates of non-*albicans Candida*, such as *C tropicalis* and *C glabrata*. The specific roles of these 2 hydrolytic enzymes in *Candida* spp. colonization and infection processes are slightly different. In general, SAPs are capable of degrading numerous substrates that constitute host proteins in the oral cavity. Digestion of these nutrients may help *Candida* to acquire essential nitrogen for growth, to attach to and penetrate oral mucosa, or both.[17,18] SAPs are also able to break down portions of immunoglobulins, which are glycoproteins synthesized and secreted by plasma cells that function as antibodies. This mechanism plays a major role in disrupting humoral host defense.[1,15] Moreover, SAPs cause increased vascular permeability by activating one or more steps of the kallikrein-kinin system to release bradykinin, leading to inflammatory reactions[40] and clinical symptoms thereof. The main mechanism of action of PL is to target the membrane phospholipids and digest these components, leading to cell lysis. Because PLs are concentrated mostly in the tips of the hyphae, such host cell injury would be expected to facilitate the penetration of the infecting fungi.[16] In addition, PL has been found to be a potent inflammatory agent, inducing the accumulation of inflammatory cells and plasma proteins and release of various inflammatory mediators in vivo.[16] Given that the present study found that the same *Candida* strain was able to secrete both SAP and PL, it is probable that these enzymes execute their specific functions in concert, augmenting fungal virulence and the infection process.

The higher percentage of positive SAP/PL yeasts found in the present study is partially in agreement with previous in vitro studies. Several investigations found that some *C tropicalis* clinical isolates obtained from children with HIV,[24] patients with diabetes and denture stomatitis,[11] patients with denture stomatitis without diabetes,[27,28] elderly healthy individuals,[25] and hospitalized patients[26,30,31] were able to secrete both SAP and PL in vitro. In contrast, other studies found that only a few strains of *C tropicalis* were able to produce SAP, and none secreted PL.[23,29,32] Although *C glabrata* has been previously considered to be a non-enzyme-producing organism, all isolates evaluated in the present investigation were proteolytic and phospholipolytic. Some studies have found that some isolates of this *Candida* spp. were either able to secrete both enzymes[25,26,29,31] or to produce only one of them.[23,27,32] However, there have also been published reports that found no *C glabrata* enzyme-producing isolates.[11,33] One of the factors that could explain the discrepancy among studies is that SAP and PL expression can vary according to *Candida* spp. strain, and the site of isolation. In addition, as can be seen from the results in this study, the standard deviation of SAP and PL activities of *C tropicalis* and *C glabrata* isolates was large, reflecting the heterogeneity of isolates. Other authors also observed this intra- and interspecies variability,[30,31] and such findings highlight the complexity of infection caused by the different *Candida* spp. Differences among studies may also be attributed to the methods used. Obviously, the methods based on spectrophotometric comparison,[12,38,39] such as those used here, are considerably more sensitive than the methods based on the formation of an opaque halo of degradation in specific agar plates.[23,24,27-31] Although the agar plates assay is simple and fast, it is not especially accurate and may not be suitable for isolates with low levels of enzymatic activity.[16,30]

In the present study, the comparison of SAP and PL production from *C tropicalis* and *C glabrata* clinical isolates detected a significant difference between these 2 populations, regardless of their clinical source. In particular, *C tropicalis* isolates produced significantly

higher amounts of both enzymes than *C glabrata* isolates (see Table III), which may be the most interesting feature of this study. Furlaneto-Maia et al.[25] also found that *C tropicalis* produced more PL than *C glabrata*. They tested a limited number of isolates (6 and 5 of each species, respectively) and found only one strain of each species producing this enzyme. To the authors' knowledge, this study evaluated the enzymatic activities of a higher number of isolates of these 2 non-*albicans Candida* spp. when compared with previous investigations. The relationship of these 2 hydrolytic enzymes with other virulence factors is well documented. Lyon and Resende[41] found a positive correlation between higher SAP and PL production and greater adhesion to buccal cells and resistance to fluconazole of *C albicans* isolates. There is evidence indicating that biofilm formation is also positively associated with secretion of hydrolytic enzymes involved in proteolysis in patients with diabetes.[42] In addition, the production of PL has been associated with the presence of denture stomatitis,[32] and there is a trend with respect to disease severity and SAP expression.[19] These pieces of evidence and the aforementioned results could help explain some previous clinical findings in which the prevalence of *C tropicalis* increased in relation to the highest severity of denture stomatitis.[3] Recent studies conducted by our research group found that *C tropicalis* was considered the second or third most frequently isolated *Candida* spp. from patients with oral candidiasis.[3,5,20] In a recent epidemiologic study conducted in 11 Brazilian medical centers, *C tropicalis* was the second most frequent *Candida* spp., accounting for 24% to 25% of all candidemias.[6] Another investigation found that the most common *Candida* non-*albicans* spp. causing bloodstream infections in Latin America were *C tropicalis* or *C parapsilosis*, rather than *C glabrata*.[43] In fact, the literature contains substantial data indicating that *C glabrata* is found much more frequently in North America.[44] Besides the high prevalence in Brazil, the propensity of *C tropicalis* for dissemination and the high mortality associated with its infections[45] may be strongly related to the potential of virulence factors exhibited by this species, as observed in the present investigation. Recent literature reviews reported that *C tropicalis* can have similar or higher virulence than *C albicans*.[46,47] This could enhance the progression of infections caused by *C tropicalis* when compared with those caused by *C albicans* and other non-*albicans* spp.

In the present study, the analysis of SAP and PL production by *Candida* isolates from patients with oral candidiasis with or without diabetes and from healthy control participants did not find any significant differences among the 3 groups (see Table II). The same results were observed by Pinto et al.,[27] who found no significant differences in the expression of both enzymes between strains isolated from denture stomatitis and control individuals. Other authors also detected no significant differences in the in vitro expression of SAP among *Candida* isolates from patients with and without diabetes,[12] denture stomatitis,[28] and chronic erythematous candidiasis.[48] Contrasting results, however, have indicated that strains of *C albicans* from individuals with oral candidiasis were more proteolytic than commensal isolates.[19] More surprisingly, some authors found that enzyme secretion rates were higher in clinical isolates from healthy *Candida* carriers than those from individuals with oral candidiasis.[28] Considering that these exoenzymes are related to the occurrence and dissemination of *Candida* infections, it could be expected that their production should be higher in patients with the disease. However, the enzymatic secretion by some commensal microorganisms could be related to the fact that these enzymes are involved not only in the disease initiation but also in the colonization process.[1,17,18] Accordingly, Naglik et al.[1] found that several SAP genes are expressed in both symptomatic and asymptomatic *Candida* carriers.

The present work found that species other than *C albicans* had the ability to secrete both SAP and PL in vitro, with special attention being paid to the greater secretion rates of *C tropicalis* clinical isolates over the *C glabrata* ones. The pathogenicity of *Candida* spp. yeasts depends on these specific virulence mechanisms that give them the ability to colonize surfaces, to invade deeper host tissue, to evade host defenses, and to cause infections. Thus, the secretion of SAP and PL by the different *Candida* spp. could be considered a target for antifungal therapy, and, in this context, the use of inhibitors of enzymes secretion might turn out to be particularly rewarding for oral candidiasis treatment and prevention. Although in the present investigation a higher number of clinical isolates was tested compared with previous studies, it is necessary to consider that this is an initial evaluation that may guide further investigations using larger samples.

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