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The Oral Cavity: A Reservoir that Favors Colonization and Selection of *Candida parapsilosis* sensu stricto Strains with High Pathogen Potential Under Conditions of Gingival-periodontal Disease

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Abstract

The *Candida parapsilosis* complex comprises three genetically related species: *Candida parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis*. During the last decade, *Candida parapsilosis* sensu stricto has become the second most commonly isolated yeast from patients with blood stream invasive fungal infections, following *Candida albicans.* Despite its clinical significance, little is known about its distribution and behavior in oral cavity niches. Given this background, we intended to perform a molecular characterization of 38 isolates of the *Candida parapsilosis* complex collected from oral cavities of immunocompetent patients with and without gingival-periodontal disease, with the aim of determining its distribution and establishing a correlation with clinical data. Besides, we assessed the virulence of the strains by examining the in vitro biofilm-forming capacity in two different culture media: YPD and RPMI 1640. Being more frequently isolated from oral cavities with pathological conditions, 89.5% of the strains were reconfirmed to be *Candida parapsilosis* sensu stricto. More than 70% of the *Candida parapsilosis* sensu stricto strains showed biofilm-forming capacity in both culture media, reflecting a statistically significant association between biofilm-forming capacity and the clinical conditions of the subjects from whom the strains were isolated. Among the three complex species, *Candida parapsilosis* sensu stricto is a normal commensal in the oral cavity, being more frequently recovered in inflammatory conditions. Gingival-periodontal disease may be a factor triggering overgrowth and virulence of *Candida parapsilosis* sensu stricto commensal strains.

Keywords: *Candida parapsilosis* complex, *Candida parapsilosis* sensu stricto, Oral colonization, Biofilm, Gingival-periodontal disease.

Introduction

Within the healthy population, 40-60 % of subjects carry species of the genus *Candida* in their saliva and oral mucosa[1-4]; certain local and systemic conditions may turn this ecological niche into a site likely to trigger yeast overgrowth [3,5], *Candida albicans* being the most frequently recovered species from immunocompetent and immunocompromised subjects [1,5-9]. Other species less frequently isolated, which have been recovered from periodontal environments and the oral mucosa, are the following: *C. parapsilosis*, *C. dubliniensis*, *C. tropicalis* and *C. guilliermondi*, among others [9-14]. However, over the last ten years, some studies have found an increasein the non-*albicans Candida* (NAC) recovery frequency in oral mucosa: from 10 % in 1996 [1] to 15.0% [7] in 2010, 15.4 % in 2011 [8], and 25.0% in 2017 [14] in the case of *C. parapsilosis* at the level of healthy

subjects. Among the local and/or systemic factors associated with the higher distribution of NAC species in the oral cavity, we could mention the use of prosthetic devices [11]; being elderly, [15] elderly with low body mass index[16]; being an oncological patient undergoing chemotherapy or radiotherapy [17,18]; being a diabetic patient with poor metabolic control [19], being a man using anabolic androgenic steroids [20] or a woman using oral contraceptives [21].

Of all the NAC species, during the last few years, *Candida parapsilosis* has become an emerging nosocomial pathogen [22], stimulating interest among the medical community. *Candida parapsilosis* constitutes a complex comprising three species (*Candida parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis*) which are phenotypically indistinguishable but genetically heterogeneous, called *Candida parapsilosis* complex. According to literature, *C. parapsilosis* sensu stricto is the most prevalent species in different human ecological niches, either in healthy or diseased subjects; it is also the most pathogenic species and the one most commonly recovered from adults and preterm neonates suffering from an invasive infection [23-25].

Reports have indicated that *C. parapsilosis* and *C. dubliniensis* are the *Candida* species most frequently detected in oral niches after *C. albicans* among immunocompetent subjects [7,8,11,12,14,26]. Nevertheless, the epidemiology and behavior of the *C. parapsilosis* complex given different conditions of the oral cavity are still unknown. For that reason, the aim of this study was to evaluate the distribution and virulence of a collection of *C. parapsilosis* complex oral isolates from immunocompetent patients with or without a diagnosis of gingival-periodontal disease, in order to find which of the complex species is more prevalent in the oral cavity and determine whether oral inflammatory conditions affect the colonization and virulence of any of these three species.

Various methods adopted to assess *Candida* biofilm formation have been reported to date [27]. In this study, we examined the biofilm-forming capacity of a subgroup of 28 clinical isolates identified as *C. parapsilosis* sensu stricto through molecular techniques, applying the crystal violet staining assay. This method is considered to be the most effective to determine biofilm biomass formation given that it includes metabolically active and inactive cells, and the extracellular matrix component [27,28].

Materials and methods

In order to accomplish the stated objectives, a retrospective, cross-sectional, comparative study was designed. A collection of 38 oral isolates of the *C. parapsilosis* complex, defined through conventional phenotypic methods (CHROMagar*Candida* differential chromogenic medium [Becton-Dickinson] and micromorphology), from immunocompetent adult patients (aged 18-65) was analyzed; these subjects were non-smokers who had not used antimicrobials six months before sampling, with or without adiagnosis of gingival-periodontal disease, adopting the criteria established during the last American Academy of Periodontology meeting for the diagnosis of periodontal disease and conditions, published in the Annals of Periodontology (1999) [29]. Samples were collected in a previous research study by a dentist screened for that purpose. The isolates were stored in glycerol at -20°C, at the Microbiology, Parasitology and Immunology Institute (IMPaM) Mycology Center, School of Medicine, University of Buenos Aires (UBA). The present research was approved by the ethics committee of the Faculty of Odontology UBA, with **Nº 012 / 2016CETICAFOUBA**. The resolution was issued on November 7, 2016 with file number: 0048223/2016.

Strains and media

Besides the 38 isolates of the *C. parapsilosis* complex from different areas of the oral cavity (cheek, tongue, palate) and subgingival niches, the ATCC strains *Candida parapsilosis* sensu stricto (**ATCC® 22019™**), *Candida orthopsilosis (***ATCC® 96139™**) and *Candida metapsilosis* (**ATCC® 96144™**) were used as positive controlsfor each PCR assay. The stored strains were reactivated with Brain-Heart-Infusion (BHI) Broth (Merck) at 37°C. The biofilm assay was conducted in two culture media: YPD supplemented with chloramphenicol and RPMI 1640 supplemented with L-glutamine (Life Technologies).

Species identification

Cryopreserved isolates were cultivated in Sabouraud agar for 24 hours at 28°C and then in YPD broth for 18 hours at 37°C. DNA extraction was performed with the Qiagen column kit (QIAamp DNA Blood Mini Kit). A PCR assay optimization was performed with specific primers derived from sequences of the ITS1-5.8SrRNA-ITS2 region (Table 1), according to a study conducted by Asadzadeh *et al* [30]. The results were validated using Sanger sequencing, for which we employed an end-point

Table 1: Primers that were used for the rapid identification at the species level of the *C. parapsilosis* complex Source: Asadzadeh M, *et al* [30]

Primer	Target gene	Direction	Species specificity	Sequence	Amplification size
CPAF	ITS 1	Forward	*C. parapsilosis*	TTTGCTTTGGTAGGCCTTCTA	379pb
CPAR	ITS 2	Reverse		GAGGTCGAATTTGGAAGAAGT	
CORF	ITS 1	Forward	*C. orthopsilosis*	TTTGGTGGCCCACGGCCT	367pb
CORR	ITS 2	Reverse		TGAGGTCGAATTTGGAAGAATT	
CMEF	ITS 1	Forward	*C. methapsilosis*	TTTGGTGGGCCCACGGCT	374pb
CMER	ITS 2	Reverse		GAGGTCGAATTTGGAAGAATGT	

PCR with ITS 1 and ITS 4 panfungal primers to amplify and sequence the ITS 1-ITS 4 region of the 28S ribosomal RNA gene, as described by White *et al* [21]. Amplified fragments were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the ABI Prism 3730XL DNA Analyzer (Applied Biosystems, Bs.As.-Argentina), with primers ITS 1 and ITS 4. The sequences found were analyzed with the BLAST (Basic Local Alignment Search Tool) sequence comparison algorithm (http://www.ncbi.nlm.nih.gov/BLAST). For the phylogenetic analysis, we employed BioEdit, a sequence alignment editor, and MEGA 06, a multiple sequence alignment program, as well as a neighbor joining algorithm. The phylogenetic tree was built with sequences from ATCC *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* reference strains, as well as with sequences chosen randomly from those resulting positive for the PCR assay with specific primers.

In vitro virulence analysis

We studied the biofilm-forming capacity of 28 strains by means of biomass biofilm quantification through crystal violet staining; for this purpose, strains were grown on two culture media: YPD (supplemented with antibiotic) and RPMI 1640 (supplemented with L-glutamine) using 96-well microtiter plates (Nunc™ or Techno Plastic Products AG ninety six-well polysterenemicrotiter plates, catalog number 167008). Each strain was analyzed in 8 replicates, and in two independent experiments.

The protocol used to measure biofilm biomass with crystal violet staining in RPMI and YPD media was the one described by Treviño R, *et al.*[31] and Durán E, *et al.* [32].

The amount of biofilm produced by an isolate was categorized according to the classification found in Sanchez L. *et al.* [6] and Treviño R, *et al.* [31]: high producers (≥0.41), low producers (0.11-0.40) and non-producers (≤0.10); considering that Sanchez L *et al.* breaks down the high biofilm producersinto strong and moderate biofilm producers (Table 2).

Statistical analysis

All the data gathered was examined and studied by means of Microsoft Excel 2010 and the InfoStat 2016 statistical software. Mode, mean and standard deviation were used for statistical calculations. A 95 % confidence interval was applied, considering a p value lower than alpha error (alpha= <0.05) significant. Two tests were done to establish the association among variables: the Fisher Exact Test for qualitative variables, and the Mann-Whitney U test (two-sided) for quantitative variables. Strength of association was measured using prevalence ratio (PR).

Table 2: Based clasification of *Candida* spp. biofilm formation

Group	Biofilmformation	OD 595nm	CFU $Log_{10\ cells/ml}$
I	Not producer	≤0.10	$<0.1x10^8$
II	Weak producer	0.11-040	$0.1-0.75x10^8$
III	Moderate producer	0.41-0.74	$0.76-2x10^8$
IV	Strong producer	≥0.75	$>2x10^8$

Source: Sánchez L, *et al* [6]

Table 3: Distribution of species of the *Candida parapsilosis* complex in the analyzed sample

Species	AF	RF	PF	$CI_{95\%}$
***C. parapsilosis* sensu stricto.**	34	0.895	89.5%	88.4-89.7
C. orthopsilosis	0	0	0	0
C. metapsilosis	0	0	0	0
Otras	4	0.105	10.5%	9.1-12.9
Total	38	1	100%	

Mode: *Candida parapsilosis* sensu stricto

Note: AF (absolute frequency); RF (relative frequency); PF (percentage frequency); CI95 (95% confidence interval). Others: *Candida* species different from the *C. parapsilosis* complex

Table 4: Conditional probabilities according to the presence or absence of GPD

Oral condition	Species		Total N(%)
	C. parapsilosis sensu stricto N(%)	Otras N(%)	
GPD	27 (79)	0	**27(71)**
Whithout GPD	7 (21)	4 (100)	**11(29)**
Total N(%)	34 (100)	4 (100)	38(100)

Note: GPD: gingival-periodontal disease; Fisher`s test: 0.36/p=0.0045; PR: 3.9 (IC95%: 3.1 – 4.7)

Results

Out of the 38 isolates of the *C. parapsilosis*complex from the mouth (oral mucosa and subgingival niches), 34 (89.5 %) were reconfirmed to be *C. parapsilosis* sensu stricto (Figure 1), whereas, 4 were not identified as any of the three species of the complex. None of the isolates were identified as *C. orthopsilosis* or *C. metapsilosis,* the two less commonly recovered strains of the complex.

PCR results were validated through the phylogenetic analysis of sequences obtained by means of Sanger automated sequencing and analyzed with BioEdit and MEGA 06 programs (Figure 2).

The BLAST analysis of the 5 strains that were negative according to PCR for the three strains in the complex determined the following identification:

Cp36A: 100% identical to *C. albicans*

Cp53.1: 100% identical to *C. albicans*

Cp78/2.2: 100% identical to *C. pararugosa*

Cp78PA: 100% identical to *Kluyveromyces marxianus*

Cp381: 100% identical to *C. albicans*

When comparing the species recovered with the oral condition of patients at the moment of sample collection (according to medical records), we found that the recovery frequency of *Candida parapsilosis* sensu stricto was different for pathological and healthy oral niches. The strength of association resulting from prevalence ratio was 3.9, which means that the probability of recovering *Candida parapsilosis* sensu stricto in gingival-periodontal conditions is four times higher than in healthy ones.

In vitro biofilm-forming capacity study

Figure 3 shows that out of all the strains studied (N=28) in RPMI medium, 92 % (26 strains) had biofilm-forming capacity. Only two strains did not form in vitro biofilm in this culture medium. Phenotypic assessment showed that 71 % of the *C. parapsilosis* sensu stricto strains were high biofilm producers.

Figure 4 shows that, in YPD medium, 24 out of the 28 strains analyzed formed in vitro biofilm, while 4 strains had no biofilm-forming capacity in this medium. Phenotypic assessment showed that 79 % of the *C. parapsilosis* sensu stricto strains were high biofilm producers. These results are similar to the ones found in the RPMI medium.

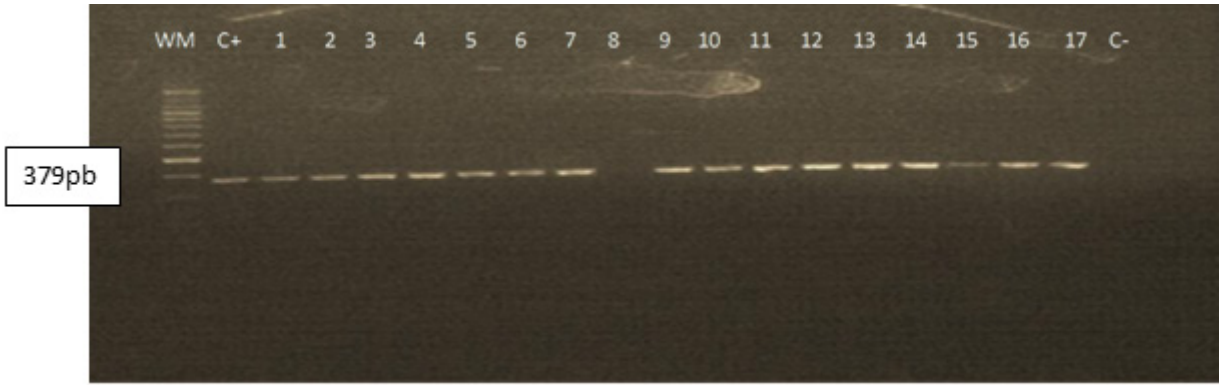

Figure 1: Electrophoretic run of PCR product from 17 samples of various oral cavity niches: A pattern of 379bp banding is observed (Note: **WM:** marcador de peso; **C+:** positive control; **C-:** negative control)

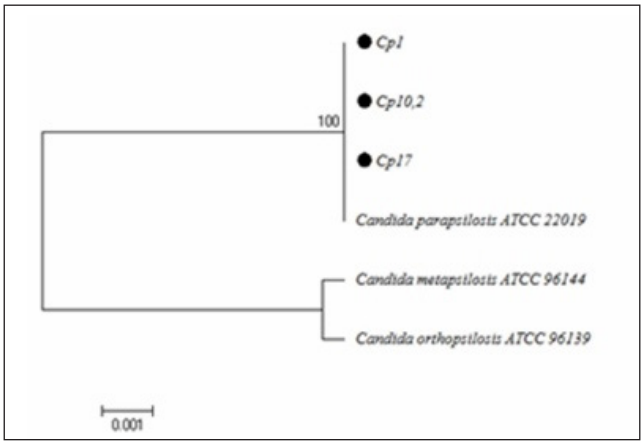

Figure 2: Phylogram constructed with reference strains and 3 unknown strains

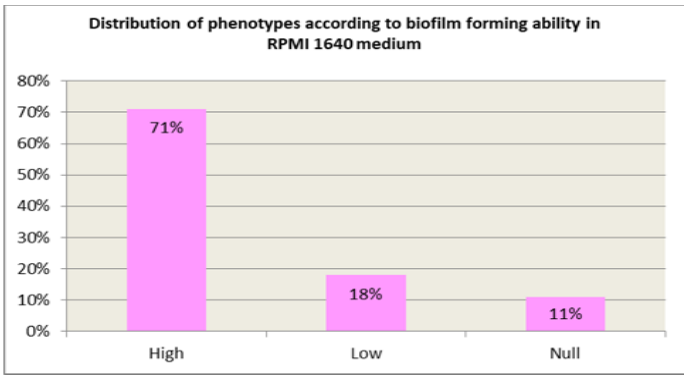

Figure 3: Biofilm forming ability measured in strains grown in RPMI medium

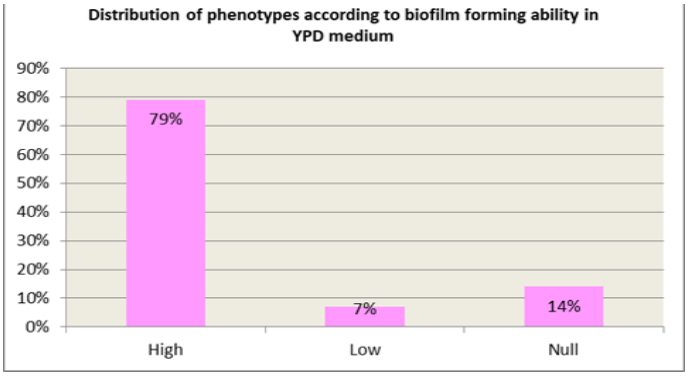

Figure 4: Biofilm forming ability measured in strains grown in YPD medium

Figure 5 shows the mean value and the standard deviation of absorbances found in the total number of *C. parapsilosis* sensu stricto strains, according to the oral condition they were isolated from: with and without gingival-periodontal disease, compared in two culture media, YPD and RPMI. Firstly, the chart shows that in both YPD and RPMI media, strains form more biofilm biomass in GPD condition (averageAbs YPD: 1.20 ±0.90 and averageAbs RPMI: 0.60 ±0.48); regarding the oral condition without GPD (averageAbs YPD: 0.46 ±0.53 and averageAbs RPMI: 0.36 ±0.37). The significance test (Mann-Whitney U test) demonstrated that, according to the comparison of the medians in GPD condition vs. without GPD, *C. parapsilosis* sensu stricto strains from an inflammatory environment are likely to be more virulent than those isolated in non-inflammatory oral conditions (YPD: p=0.02/ RPMI: p=0.04). However, with YPD medium, the absorbance levels were higher than in RPMI medium (1.02 ±0.88; 0.60 ±0.45), which means that YPD promotes more biofilm formation as opposed to RPMI 1640 at 24 hours after growth.

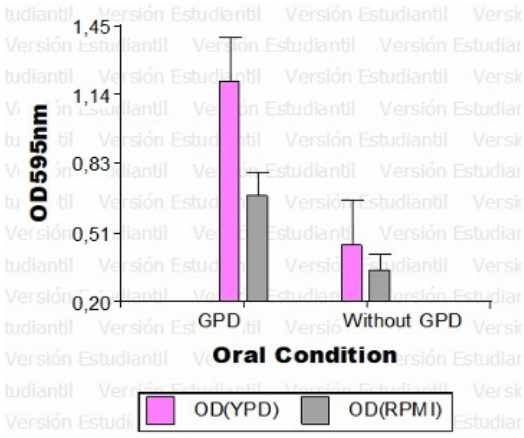

Figure 5: Average of abosorvancias of solutions crystal violet (Abs CV) obtained from biofilm of 24 hours of strains *Candida parapsilosis* sensu stricto in means YPD vs RPMI, analyzed according to buccal condition in which they were isolated
[**Test Mann Whitney (YPD) U:** 59.50/p=0.02; **Test Mann Withney (RPMI)U:** 64.50/p=0.04]

Discussion

Currently, *Candida parapsilosis*is considered as a nosocomial emerging pathogen [33-36], and its growing resistance to azoles and echinocandins [37-39] has put it under the spotlight in the last few years. It is a complex of 3 phenotypically indistinguishable species, but, due to their genetic heterogeneity, it is possible to differentiate them only with molecular techniques [40].

In this complex, *C. parapsilosis* sensu stricto is the species most frequently recovered from clinical isolates of immunocompetent and immunocompromised patients, both in normal and pathological conditions [7,41-47]. This knowledge has been supported by many studies around the world. However, there are few studies reporting the prevalence and distribution of the species in this complex under healthy and pathological conditions in the mouth. So far, *C. parapsilosis* sensu stricto is known to be the most prevalent species in oral cavity niches under immunocompetent conditions, regardless of the geographical region. This has been reported by studies from the USA, [7,48] Portugal, [42] Turkey [45] and China, [40] although with a reduced sample size. On the other hand, only one study made in Brazil analyzed the distribution of the species in this complex inside the mouths of immunocompromised HIV-positive patients. *C. metapsilosis* was the most frequently isolated species followed by *C. parapsilosis* sensu stricto, although the difference was not statistically significant, and the sample used was also very slender [49].

In the present study, only *C. parapsilosis* sensu stricto was isolated, showing a prevalence of 89.5 % in all the oral cavity samples. This study determined that *C. parapsilsosis* sensu stricto is more likely to be recovered from oral cavities under pathological conditions, which agrees with a Chilean study published in (2008) [10], where a lower yeast prevalence is observed, evidenced in a lower count of colony-forming units (CFU/ml) of *Candida* species among periodontally healthy subjects when compared to individuals with periodontal disease; the difference being statistically significant. Similar results were reported by Canabarro A *et al* in (2013) [50], since the results of their investigation showed that patients with severe chronic periodontitis are significantly more colonized, at the level of subgingival niches, by several species of *Candida* especially *Candida albicans* with respect to subjects periodontally healthy. However, contrary to our results and those of Urzua B *et al*, and Canabarro A *et al*, Laurenço A *et al* (2017) [14] did not find significant differences in the frequency of recovery of *Candida parapsilosis* complex between immunocompetent subjects with periodontal disease vs healthy. Results similar to those of Laurenco *et al* in Brazil are reported by Peters B *et* al [48]in this same year in USA, demonstrating that subjects with periodontal disease, although, are more colonized and show a higher relative abundance of *Candida* species with respect to periodontally healthy subjects, however the differences were not significant either. The explanation for this lack of agreement may be due to several factors including: a) diversity in study design; b) very small sample sizes; c) the different sensitivity of the microbial sampling techniques, since samples based on saliva collection fail to detect all the fungal and

bacterial species present in subgingiva.

Among clinical *Candida* strains, biofilm formation is variable and depends on the *Candida* species [6,51]. This fact has already been demonstrated in several studies; it has also been reported that biofilm formation by *Candida parapsilosis* is a key virulence factor in this species, highly dependent on the strain [51,52], an unproven characteristic in other NAC species such as *C. glabrata* and *C. tropicalis* [51]. In our case, from all the clinical *C. parapsilosis* sensu stricto isolates sorted according to biofilm biomass, concurrent results with those from Silva *et al.* [48] and Pannanusorn *et al.* [52] were found, since we identified strains with different biofilm-forming capacity (high, low and nonexistent), where high biofilm-forming capacity strains predominated in both RPMI and YPD media (71 % and 79 %, respectively). This result also agrees with the findings from Silva *et al*., given that in the clinical *C. parapsilosis* sensu stricto isolates analyzed by them regarding biofilm-forming capacity, oral isolates were found to be the most common biofilm producers with respect to strains from vaginal and urinary tracts. This characteristic was not observed in other NAC species such as *C. glabrata* y *C. tropicalis* [51].

Our hypothesis is that an oral inflammatory environment caused by overgrowth of periodontal pathogens during gingival-periodontal disease triggers and/or aggravates the virulence of *C. parapsilosis* sensu stricto commensal strains due to inheritable epigenetic changes. This is because the DNA sequence variability between this species strains has proven to be minimal even in isolates from different geographical regions [53]. With respect to this, we found that isolates derived from oral niches with GPD showed a significantly higher biofilm biomass compared to strains isolated in healthy oral conditions. This result was confirmed in two different culture media, RPMI 1640 and YPD, and agrees with reports by Hasan F. *et al*. (2009) [54] and Jain N. *et al*. (2007) [55], who also suggested that the origin and selection of isolates may be an influential factor in this *Candida* species biofilm formation. Furthermore, conversion of *Candida* species from commensalism to parasitism and their overgrowth are usually associated with changes in the intraoral environment such as unhygienic prostheses and xerostomia, or systemic factors such as type 2 diabetes (DM2) and immunodeficiencies. In any case, there are no published data so far about the effect of oral environment conditions on *Candida parapsilosis* pathogenicity. This is the first study to analyze the distribution and behavior of *C. parapsilosis* sensu stricto oral strains regarding the clinical conditions under which they were isolated.

In the biofilm biomass measurement assay, higher absorbance levels were reached with YPD medium as opposed to RPMI medium. This result agrees with those of Tan Y. *et al.*, (2016) [56]. This study showed that YPD and BHI culture media produce larger biofilm quantities and trigger stronger metabolic activity with respect to RPMI, concluding that culture conditions (medium composition, inoculum size, incubation period) affect in vitro biofilm formation.

Our study findings are highly relevant, since *C. parapsilosis* sensu stricto is the most prevalent species of the complex in different ecological niches, either in states of immunocompetence or deterioration of the immune system. On the other hand, there is enough evidence to indicate that this is the second or third most frequently isolated yeast after *Candida albicans* in blood-cultures from candidemia patients in Latin America [57], Asia [58] and Europe [59]. This is an important antecedent given that *C. albicans* and, on a smaller scale, other *Candida* species are reported to be frequently found in adults and children's oral cavities, serving as reservoir for inoculation and infection of any part of the body [60,61]. These infections are difficult to treat with antifungals, which means a high mortality rate [62,63]. Consequently, if the oral cavity in pathological conditions is a reservoir triggering *Candida parapsilosis* strains colonization and pathogenicity, such condition is an important risk factor for candidemia or invasive infections by this yeast. In fact, Fanello *et al*. (2006) [61], in a longitudinal study, showed a significant association between density of the species of the genus *Candida* in the oral cavity and the onset of a nosocomial fungal infection, which may be inside or outside the mouth. Moreover, such condition (GPD) may cause strains from this species to be more active in the tissue injury observed in periodontal disease. Concerning this, a study published in RevistaIberoamericana de Micología (Iberoamerican Journal of Mycology) by Rubio *et al*. (2012) [64] found *Candida* presence in 35% of samples of supragingival and subgingival plaque, biopsies from periodontal bags and soft tissues, which also shows the presence of filaments invading the connective tissue with relation to anaerobic microorganisms (Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans) in patients with periodontitis [48]. The above mentioned facts explain the need for this study.

Conclusions

- *C. parapsilosis* sensu stricto is probably the species in the complex which most frequently colonizes oral cavity niches of immunocompetent patients, its recovery being more common in oral inflammatory conditions.
- *C. orthopsilosis* and *C. metapsilosis* seem to be rare colonizing species in oral cavity niches of immunocompetent patients, regardless of their clinical condition.
- Biofilm-forming capacity of the *C. parapsilosis* sensu stricto species depends on the strain. However, it is likely that the virulence of this *Candida* species is exacerbated in oral dysbiosis due to heritable epigenetic changes.
- YPD medium is more effective and less expensive than RPMI 1640 to stimulate biofilm growth at 24 hours.

In any case, our results have to be corroborated with a prospective study in a larger number of clinical isolates.

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