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Research Article

Oral Dysbiosis Exacerbates *Candida parapsilosis* Sensu Stricto Biofilm Production via Up-Regulation of the *CPH2* Biofilm Master Gene

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ARTICLE INFO

Article history:
Received: 13 March, 2021
Accepted: 25 March, 2021
Published: 22 April, 2021

Keywords:
Candida parapsilosis sensu stricto
virulence
oral dysbiosis
oral eubiosis
CPH2 gene master

ABSTRACT

Introduction: *Candida parapsilosis* sensu stricto is the second to third most frequent cause of candidemia. Studies place this yeast as a frequent colonizer of niches of the oral cavity, predominantly in pathological conditions. We hypothesize that a buccal environment in dysbiosis enhances the virulence of *C. parapsilosis* sensu stricto.

Objective: To evaluate the phenotype and molecular level of the production of biofilm in oral isolates of *Candida parapsilosis* sensu stricto and correlate the results with the clinical origin (dysbiosis versus eubiosis).

Materials and Methods: The biofilm-forming ability was compared in 50 oral isolates of *Candida parapsilosis* sensu stricto obtained from patients with and without oral dysbiosis; by quantification of metabolic activity. The results were corroborated by confocal fluorescence microscopy, and correlated with the transcriptional activity of *CPH2*, by RT-qPCR. The data were analysed by Excel 2010, and InfoStat 2018, with a 95% confidence interval.

Results: The metabolic activity in biofilm was significantly higher in oral dysbiosis relative to control ($p = 0.0025$). Basal expression of *CPH2* increased 2.8 times more in oral dysbiosis related to the control condition and showed no significant differences with pathogenic isolates of this same yeast, derived from onychomycosis lesions.

Conclusion: The oral cavity in dysbiosis increases the virulence of *C. parapsilosis* sensu stricto due to possible changes in epigenetic marks. This finding suggests that the oral cavity in dysbiosis may be an alternative route to the skin in the epidemiology of nosocomial candidemia.

Introduction

Candida parapsilosis sensu stricto is a yeast that integrates together with *Candida orthopsilosis* and *Candida metapsilosis* a complex of three species, called the *Candida parapsilosis* complex or psilosis complex. Of the three fungal species, *Candida parapsilosis* sensu stricto stands out as the most isolated yeast in the different human ecological niches, both in health and disease conditions, however, there is still no global consensus on whether it is also the most virulent of the complex. In the last 20 years, this yeast has concentrated medical interest after being the second most isolated *Candida* species, after *Candida albicans*, in candidemia events especially in Southern Europe, China, Japan, and Latin America. The latter is exposed as the epicenter of candidemia due to *Candida parapsilosis* sensu stricto, reaching *Candida albicans* in some regions in the proportion of positive blood cultures [1-9].

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http://dx.doi.org/10.31487/j.DOBCR.2021.01.07

Studies dating from the 1990s place *Candida parapsilosis* sensu stricto as the second most isolated yeast, after *Candida albicans*, in niches of the oral cavity. However, in 2017 a study was published in which a shift in oral dominance of Candida albicans was reported, with a greater recovery of non-*Candida albicans* (NAC) species such as *Candida quercitrusa and Candida parapsilosis* sensu stricto. In 2017, our research group demonstrated through a retrospective, cross-sectional and comparative study a greater recovery of *Candida parapsilosis* sensu stricto, compared to the other species of the complex, from oral cavity niches under the condition of gingivo-periodontal disease; with higher biofilm production when the isolates came from a dysbiotic environment [10-15]. Based on this antecedent, we wonder if an oral environment under ecological imbalance exacerbates the virulence of *Candida parapsilosis* senu stricto, via up-regulation of the transcriptional activity of key genes for biofilm formation. To answer the research question, we set out to measure and compare *in vitro* biofilm production in a collection of oral isolates of this *Candida* species, recovered under oral health conditions (eubiosis) and periodontal disease (dysbiosis) and correlate these results with the level of gene expression of key biofilm regulators described in this fungal model.

The present study acquires relevance from the moment in which a greater frequency of recovery of *Candida* species was reported, especially NAC species from subgingival niches with periodontal pathology, depending on the severity of the disease and the presence of prosthetic devices such as implants. Additionally, a longitudinal and prospective study, published in 2006, located the mouth under a state of poor hygiene as a key risk factor for the development of a superficial or invasive intra-oral or extra-oral nosocomial fungal infection [16-20].

Materials and Methods

Through a basic, retrospective, cross-sectional and comparative research study, we evaluated the virulence of this fungal model expressed through its ability to produce biofilm *in vitro*. This phenotype was studied both at the phenotype level and at the molecular level, in the latter case measuring the basal transcriptional activity of the *CPH2* biofilm master regulatory gene. The isolates came from oral cavity, under different clinical conditions: eubiosis (oral health) and dysbiosis (gingivo-periodontal disease).

Biofilm growth was induced in RPMI 1640 medium, and the results obtained were corroborated with the structure and topography of the biofilm formed by selected strains, according to the phenotype detected, by fluorescence confocal microscopy. The results obtained from the phenotype study were correlated with the molecular virulence study, in which we analysed the baseline transcriptional activity of the *CPH2* gene, defined in a previous study as a specific biofilm regulator for this fungal model [21].

I Strain, Isolates and Media

Virulence in this fungal model was studied through the biofilm-forming capacity *in vitro*, since studies have proposed this attribute as one of the most important in the pathogenesis of this yeast [3]. Biofilm production was analysed in a collection of 50 oral isolates, of which 25 isolates came from patients with a diagnosis of oral health, and 25 isolates came from patients with gingivo-periodontal disease; all of them recovered in a previous study and categorized according to the penultimate classification of periodontal diseases published in 1999, by a dentist calibrated for this purpose [22].

The isolates used in this study were identified in a previous study as *C. parapsilosis* sensu stricto, by conventional phenotypic and molecular methods (PCR and Sanger sequencing plus bioinformatic analysis). For the biofilm assay, we used the ATCC 10231 strain of *C. albicans*, defined by the CLSI (Clinical and Laboratories Standard Institute) as non-pathogenic, as a negative running control [14, 23]. For the reconstitution of the stored and cryopreserved isolates, a preliminary BHI broth (Merk) supplemented with antibiotic was used, incubated for 48 to 72 hours at 37ºC; the cultures obtained were amplified on Saburaud dextrose agar (SDA) medium supplemented with chloramphenicol (Becton Dickinson), incubated for 24 hours at 28ºC [24]. With the growth obtained in SDA, fungal suspensions were prepared in sterile physiological solution, at a concentration of $1x10^7$ cells/ml [25].

II Quantification *in vitro* Biofilm Production

In vitro biofilm production was studied in a collection of 50 oral isolates of *Candida parapsilosis* sensu stricto, obtained under two different clinical conditions (eubiosis versus oral dysbiosis). This phenotype was estimated by evaluating the metabolic activity present in the biofilm, which was induced in 96-well polystyrene microtiter plates in RPMI 1640 medium 1X (supplemented with L-glutamine), described by Treviño Rangel *et al.*, in 2015; and the absorbance of the soluble chromogenic product 'formazan', generated after metabolic reduction of the tetrazolium salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenil]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) by mitochondrial dehydrogenases, which are only active in viable fungal cells, was measured by mass spectrometry at a wavelength of 490 nm; and the density of formazan was considered proportional to the viability of the biofilm [25, 26]. Each sample was analysed in 4 replicas and in two independent experiments. The quantity of biofilm formed by an isolate was classified according to the guidelines by Sánchez *et al.*, and Treviño Rangel *et al.* [26, 27].

III Evaluation of Biofilm with Confocal Laser Scanning Microscope (CLSM)

In order to obtain a three-dimensional reconstruction and evaluate the architecture of the biofilm in this *Candida* species, the biofilm formed by a representative isolate of each phenotype (high and low biofilm forming strains, according to XTT test results) was examined by CLSM [28]. Biofilms grown on thermanox supports were stained with acridine orange and visualized by an Olympus FV100 scanning laser confocal microscope. For the visualization of the images, the IMAGE J software was used with which the three-dimensional (3D) reconstruction and the export of each captured image to TIFF format was performed.

IV Measurement of Gene Expression by RT-qPCR Applied to the *CPH2* Master Gene Depending on the Clinical Origin

CPH2 transcriptional activity was determined by quantitative real time reverse transcription PCR (RT-qPCR) in 3 oral isolates randomly selected from each clinical group. The expression of the target gene was relativized to the gamma tubulin gene 4 (*TUB4*), and the method used

was the comparative "Cq algorithm" (ΔCq), and expression relative to a calibrator or reference sample by calculating the parameter 2ΔΔCq; for which we follow the Bustin´s MIQE guidelines published in 2009 [29]. The ATCC 22019 strain of *Candida parapsilosis* sensu stricto, defined as non-pathogenic by CLSI, was used as a reference sample (basal expression) in the analysis of the differential expression of the *CPH2* gene in each of the included samples [30]. Three biological replicates were tested for each clinical condition, and in turn each sample was tested in triplicate, in three independent experiments.

For the gene expression assay, we used the Qiagen RNA extraction kit (Rnasy mini kit), for which we followed the manufacturer's instructions; the solutions obtained were stored at -70°C and were reverse transcribed to cDNA using the iScript reverse-transcriptase from Biorad. The cDNA solutions were stored at -20°C, according to the manufacturer's instructions.

V Statistical Analysis

We used the Microsoft Excel 2010 program to generate the database, and the statistical analysis was performed with the statistical package of the Universidad Nacional de Córdova (InfoStat version 2018). For the descriptive statistics, we used measures of central tendency as mean and standard deviation. We run the inferential statistics with Student's t test for two independent samples and two-factor ANOVA with K = 1 observation, after evaluation of assumptions of normality and equality of variances. The detection of extreme data significantly far from the mean was assessed by the Grubss test.

Results

I Measurement of Biofilm-Producing Ability *in vitro* in a Collection of Oral Isolates of *Candida parapsilosis* Sensu Stricto

The *in vitro* biofilm formation estimated by colorimetric method with tetrazolium salt XTT, recognized 100% of the isolates as biofilm producers, with 84% of the isolates demonstrating a "high producer" phenotype; and the difference between the recognized phenotypes (high and low biofilm forming strains) in the collection of isolates was statistically significant [P <0.0001] (Figure 1).

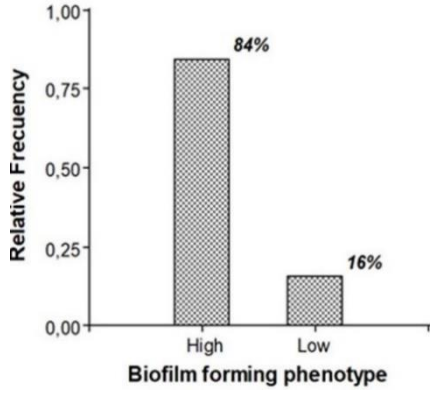

Figure 1: Frequency distribution for biofilm forming ability in RPMI 1640 culture medium and estimated by XTT method.
Student's test showed a significant difference (P <0.0001) between high and low phenotypes detected by this method.
RF: Relative Frequency.

The morphological and architectural study of the biofilm by CLSM, corroborated the results evidenced by the XTT colorimetric test. Indeed, those isolates that demonstrated high biofilm producing ability *in vitro* were able to generate dense and highly organized biofilm structures on thermanox supports, with a two-dimensional spider web-like structure, and with an architecture based on series of peaks and valleys, whose signal intensity, height and area it differed dramatically from the structure generated by the isolates categorized according to the XTT assay as "low biofilm formers" (Figures 2-4).

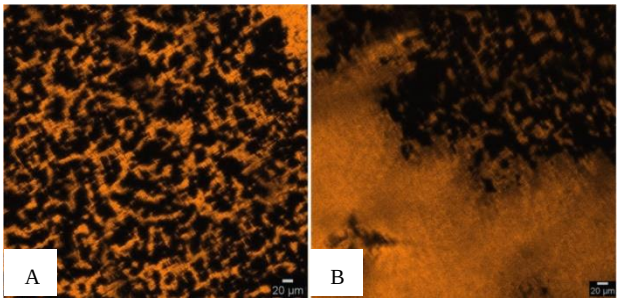

Figure 2: Biofilm architecture in two dimensions, generated by high biofilm forming strains, according to XTT colorimetric assay.
(A) Image obtained with a 20X objective lens showing a structure formed by interconnected cords of cells and extracellular matrix forming a network with a "spider web" pattern. **(B)** Image obtained with the same magnification which shows an area with abundant density of extracellular matrix in thick and dispersed aggregates.

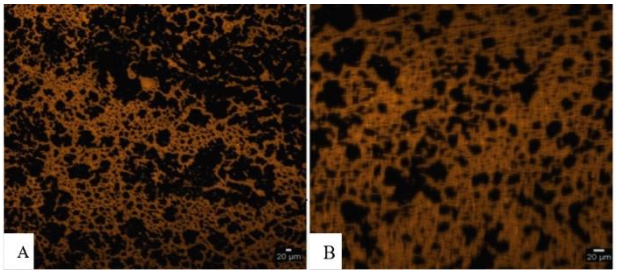

Figure 3: Biofilm architecture in two dimensions, generated by "low biofilm producing" strains, according to XTT colorimetric assay.
(A) Image obtained at 10X demonstrates a reticular topology with an "alveolar bone" pattern (the bar represents the scale). (**B)** The same image obtained at 20X shows the same structure observed at 10X for a "low biofilm producer" strain according to the XTT colorimetric assay.

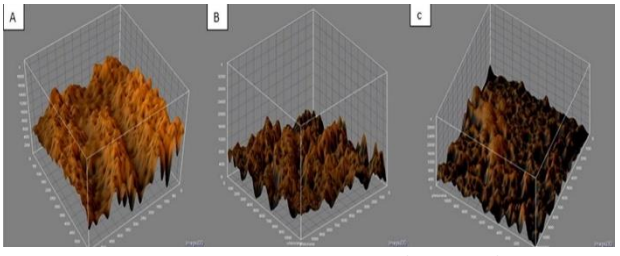

Figure 4: Reconstruction in three dimensions for the biofilm generated by strains defined according to XTT test as "high **(A)** and low biofilm formers **(B)** & **(C)**".
In the two categories of biofilm (A= high biofilm former/ B and C= low biofilm former) a three-dimensional structure based on a series of peaks and valleys is recognized, whose height and base vary according to the biofilm-forming capacity. The high producing strain (A) forms conical structures over 100um in height, while the low producing strains (B and C) form conical structures less than 100um in height.

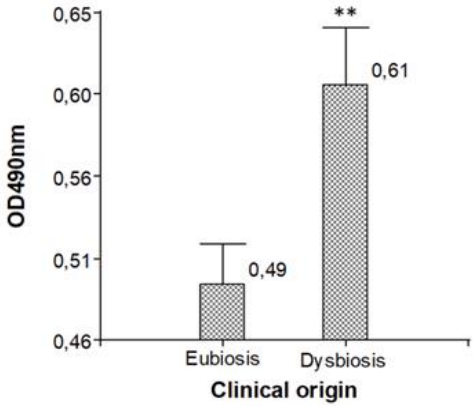

Figure 5: Comparison of average absorbance of the formazan product after reduction of XTT by viable fungal cells grown in RPMI 1640 medium, according to clinical provenance of isolates.

Data are expressed as mean and standard deviation and are representative of four technical replicas and two independent experiments. The difference between means was determined by Student's right-tail test for two independent samples; (**): $P < 0.01$.

II Biofilm-Forming Ability of Oral Isolates of *Candida parapsilosis* Sensu Stricto Depending on its Clinical Origin

When the metabolic activity in biofilm (OD490nm) was related to the clinical origin (eubiosis and oral dysbiosis), we obtained significantly higher biofilm production by the isolates recovered in the condition of oral dysbiosis (Figure 5). Because the difference found was very significant, we asked ourselves if there is an association between the biofilm-forming phenotype with a certain clinical oral condition. To answer this question, we related the variable OD490nm based on the clinical origin partitioned by the biofilm forming phenotype, and the statistical analysis was carried out with two-way ANOVA and K = 1 observation, with a subsequent Bonferroni test. Figure 6 demonstrates the existence of a statistically significant association between a high biofilm forming phenotype, and the clinical condition of dysbiosis or ecological imbalance in this fungal species.

III Relative Expression of the *CPH2* Biofilm Master Gene in Oral Isolates of *C. parapsilosis* Sensu Stricto, by RT-qPCR

The results broken down in (Table 1) indicate that the expression of the target gene *CPH2* is frequently higher in the situation of oral dysbiosis. The 2ΔΔCq parameter showed that basal expression of *CPH2* is 2.8 times higher in the oral dysbiosis condition compared to the calibrator or control condition (oral eubiosis) (Table 2). When we analysed the differential expression of the target gene *CPH2* in each of the isolates of *Candida parapsilosis* sensu stricto, relative to the calibrator strain ATCC, we obtained that of the three strains derived from the oral dysbiosis condition, two strains demonstrated for this regulator a basal expression level significantly higher. Whereas that, of the three strains of this *Candida* species derived from the oral eubiosis condition, only one showed a baseline expression level for *CPH2* significantly higher than that demonstrated by the ATCC strain or calibrator (Figure 7).

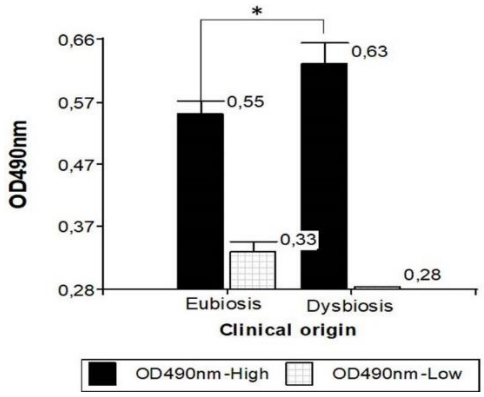

Figure 6: Metabolic activity in biofilm, relative to the clinical origin of oral isolates, and biofilm-forming ability.

ANOVA two factors with subsequent Bonferroni test for pairwise comparisons; (*): $P < 0,05$.

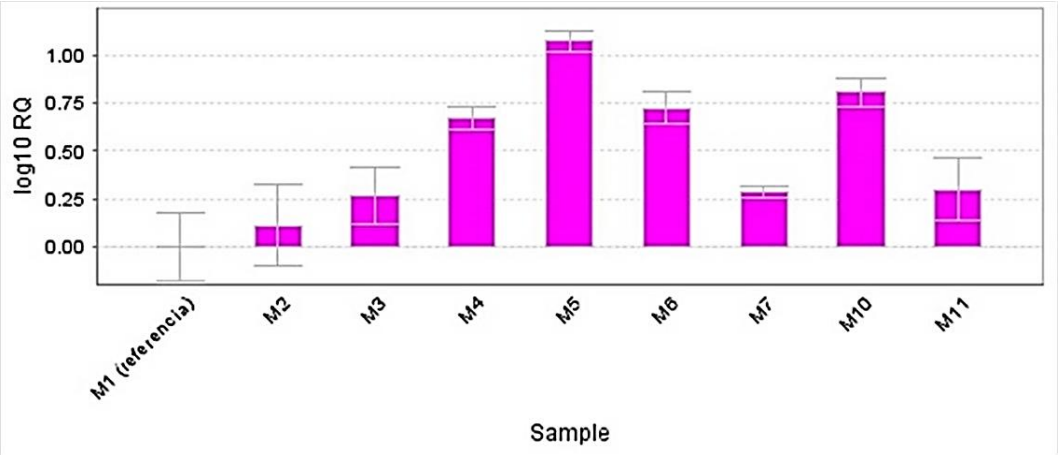

Figure 7: Differential expression of the target *CPH2* gene in each of the analysed samples (n = 8), relative to the calibrator (sample 1 equivalent to strain ATCC 22019). Figure provided by the StepOne-Plus software.

Samples M2, M3 and M4 represent the control condition (eubiosis). Samples M5–M7 represent the study or problem condition (dysbiosis). The results are representative of three technical replications for each sample analysed, in a single experiment; and are expressed as mean and standard deviation of the quantification ratio (RQ), a value that is equivalent to the differential expression of the *CPH2* gene relative to the reference sample M1 (baseline expression). This trend was observed in the three experiments performed independently.

Table 1: Proportion of clinical isolates of *Candida parapsilosis* sensu stricto that demonstrated by RT-qPCR a basal expression level of *CPH2* increased by more than 3 units with respect to the calibrator (*C. parapsilosis* sensu stricto strain ATCC 22019).

Clinical condition	Number of isolates	Number of isolates with *CPH2* expression increased by more than 3-fold with respect to the calibrator gene (%)
Oral eubiosis (commensal isolates)	3	1 (33.3)
Oral dysbiosis (commensal isolates)	3	2 (66.6)
Onychomycosis (pathogenic isolates)	2	1 (50)

Values are representation of a single experiment.

Table 2: Average and standard deviation for ΔCq in both clinical conditions (eubiosis and dysbiosis) in three independent experiments.

Experiment	Eubiosis ΔCq(μ)	Dybiosis ΔCq(μ)	ΔΔCq	$2^{-\Delta\Delta Cq}$
1º	0.14	-1.33	-1.47	2.77
2º	-2.52	-4.32	-1.8	3.46
3º	0.9	-0.24	-1.14	2.2
Average between experiments	-0.49	-1.96	-1.47	**2.81**
Standard deviation between experiments	±1.80	±2.11	±0.33	±0.63

The average 2-ΔΔCq value indicates that in dysbiosis the *CPH2* expression is 2.8 times different relative to the calibrator (eubiosis condition), [95% CI: 2.1-3.5]. The average values of each experiment are representative of 3 technical replicates per test.
DS: Standard Deviation.

When we related the baseline expression of this gene relative to the clinical origin of the isolates (eubiosis, oral dysbiosis), although the transcriptional activity of *CPH2* showed a tendency to be higher in the oral dysbiosis group, we did not obtain significant differences. However, the basal expression of *CPH2* demonstrated by the buccal strains of *C. parapsilosis* sensu stricto was similar, without significant differences with the level of expression evidenced by three pathogenic strains of this same *Candida* species derived from onychomycosis lesions (Figure 8). This result places the oral cavity as a complex niche, capable of bringing the basal expression of biofilm master genes in commensal strains of *C. parapsilosis*, to levels comparable to those exhibited by cutaneous strains of this same *Candida* species, obtained in context pathogen.

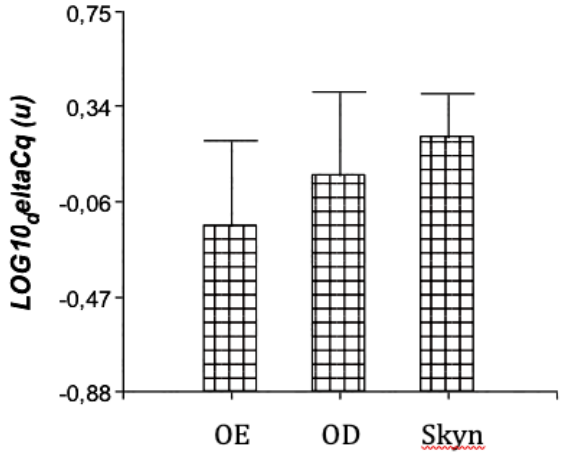

Figure 8: Expression of the *CPH2* gene relative to the normalizer gene (*TUB4*) based on the clinical origin of the isolates.
The values are representative of three technical replications in three independent experiments and are expressed as mean and standard deviation (error bars).
OE: Buccal Eubiosis; OB: Oral Dysbiosis.

Discussion and Conclusion

There are no reports, in this fungal model, regarding the effect of the environment on the transcriptional activity of *CPH2*; the literature only presents evidence, for the moment, with the master biofilm regulator *BCR1*. Indeed, our research group demonstrated in 2020 using the RT-PCR approach that the basal transcriptional activity of *BCR1* increased significantly when *Candida parapsilosis* sensu stricto came from oral niches under a situation of oral dysbiosis [31]. In agreement with our results for the *BCR1* target in this fungal model, a recent study demonstrated the ability of this biofilm regulator gene to respond to environmental signals, such as a high manganese level, resulting in an exacerbated biofilm phenotype [32].

Periodontal or gingivo-periodontal disease (PGD) comprises a set of inflammatory conditions that affect the gingiva and the tooth insertion apparatus (periodontal ligament, alveolar bone and root cement), with different stages or phases and varying degrees of progression or severity; polymicrobial in nature, and multifactorial etiopathogenesis, which can lead to tooth loss and contribute to systemic inflammation [33, 34]. This condition is characterized by a dysbiosis of the subgingival environment, with a predominance of periodontopathogens such as *Porphyromonas gingivalis*, which would contribute to increasing the virulence of the entire microbial community [35]. Additionally, an alteration in the REDOX balance has been reported in this pathology; based on our results and other findings, we postulate that probably the variations in the subgingival environment with an increase in oxidant species and proinflammatory cytokines influence the epigenetics of *Candida parapsilosis*, translating into an increased transcriptional activity of global biofilm regulatory genes [36, 37].

Regarding the differential expression obtained for the *CPH2* target depending on the clinical origin of the isolates, similar findings have been described in the *C. albicans* and *Staphylococcus aureus* models, in which a higher level of virulence has been demonstrated both at the level

of phenotype and at the molecular level depending on the clinical origin of the strains [38, 39]. This finding acquires high clinical relevance, since it places the oral cavity under pathological conditions as an alternative route to the skin in the epidemiology of nosocomial candidemia. Further, *C. parapsilosis* sensu stricto could favour the progression of periodontal disease, especially in the clinical form of peri-implantitis; indeed, antecedents show an increase in the frequency of recovery of *C. parapsilosis* at the level of subgingival niches of patients with periodontal disease as a function of the severity of the disease and the wearing of prosthetic devices [16-19]. Furthermore, studies carried out in the *Candida albicans* model reveal the ability of yeasts with a hyphal and/or pseudo-hyphal growth pattern to promote the growth and survival of key periodontopathogens such as *Porphyromonas gingivalis* [40, 41].

Ethical Approval

This study was approved by the Ethics Committee of the School of Dentistry of the University of Buenos Aires, with resolution number 012/2016CETICAFOUBA.

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