

Oral dysbiosis exacerbates the virulence of *Candida parapsilosis* sensu stricto via up-regulation of the CPH2 biofilm master gene

Rodríguez María Lourdes ^{1,4,*}, Rosa Alcira Cristina ², Nastri María Lorena ³ and Jewtuchowicz Virginia Martha ⁴

¹ Department of Microbiology and Diagnostic Clinic, Faculty of Dentistry, University of Cuenca, Ecuador.

² Microbiology Department, Faculty of Dentistry, University of Buenos Aires, Argentina.

³ Endodontics Department, Faculty of Dentistry, University of Buenos Aires, Argentina.

⁴ Research Institute in Microbiology, Parasitology and Immunology (IMPAM), Faculty of Medicine, University of Buenos Aires, Argentina.

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Abstract

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 at the phenotype and molecular level the production of biofilm in oral isolates of *C. parapsilosis* sensu stricto and correlate the results with the clinical origin (dysbiosis versus eubiosis).

Material and methods: The biofilm-forming ability was compared in 50 oral isolates of *C. parapsilosis* sensu stricto obtained from patients with and without oral dysbiosis; by quantification of biofilm biomass and metabolic activity. The results were corroborated by optical and confocal fluorescence microscopy, and correlated with the transcriptional activity of *CPH2*, by RT-qPCR. The data were analyzed 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.

Keywords: *Candida parapsilosis* sensu stricto; Virulence; Oral dysbiosis; Oral eubiosis; *CPH2* gene master.

1. Introduction

Nosocomial blood infections have *Candida* spp. as the fourth most common causal agent [1], with *Candida parapsilosis* sensu stricto behaving as the second to third most isolated *Candida* species in invasive infections acquired in the ICU globally [2, 3], with the highest concentration in the South from Europe, some regions of Asia, and Latin America [4, 5]. Epidemiological surveillance studies show that the recovery of *C. parapsilosis* sensu stricto in candidemia events equals, and even exceeds *Candida albicans* in regions such as Serbia, Puerto Rico, Venezuela and Brazil [6-9].

* Corresponding author: Rodríguez ML. Phone: 593 0988820262, Email: maria.rodriquezc84@ucuenca.edu.ec
University of Cuenca, School of Dentistry, Chair of Microbiology and Diagnostic Clinic.

C. parapsilosis sensu stricto behaves as a frequent colonizer of the oral cavity [10-13], predominating in pathological conditions. Regarding this, our research group previously demonstrated that the probability of recovering this yeast in oral cavity sites is almost four times higher in subjects with periodontal disease [14]; and their biofilm-producing ability was shown to be significantly different depending on the clinical origin of the isolates, in a pilot study published in 2017 [15]. Given this background, we hypothesize that the virulence of *C. parapsilosis* sensu stricto is influenced by the ecological niche from which the isolates come, via epigenetic regulation. To validate this hypothesis, we set out to study the production of biofilm *in vitro* at a phenotype and molecular level in a collection of clinical isolates of *C. parapsilosis* sensu stricto from different oral clinical conditions.

The proposed methodology and the tests carried out allowed us to establish the role of the ecological niche in the virulence of this *Candida* species. The oral cavity under dysbiosis, was shown to have a dramatic impact on the virulence of *C. parapsilosis* sensu stricto, via up-regulation of *CPH2*

2. Material and methods

In order to achieve the proposed objective and validate the established hypothesis, we designed a basic research study, retrospective, cross-sectional and comparative. In it, the ability to produce biofilm was collated by colorimetric methods in a collection of clinical isolates of *C. parapsilosis* sensu stricto, defined as such in a previous study by both molecular and conventional phenotypic methods. The isolates came from oral cavity, under different clinical conditions: eubiosis (oral health) and dysbiosis (gingivo-periodontal disease). The results of the biofilm study were corroborated by light microscopy and confocal fluorescence microscope. Phenotype results were also correlated with the expression level of the global biofilm regulator *CPH2*, defined as such by Holland and collaborators [16].

The methodological design of this study was approved by the Ethics Committee of the Faculty of Dentistry of the University of Buenos Aires (Resolution number 012 / 2016CETICAFUUBA).

2.1. Strain, isolates and media

To study the potential impact of a dysbiotic environment on the virulence of *C. parapsilosis* sensu stricto, we used 50 isolates of *C. parapsilosis* sensu stricto (defined by phenotypic and molecular studies) from patients diagnosed with gingival-periodontal disease (GPD). GPD is a model par excellence for chronic inflammatory disease and is characterized by an association of alterations in oral pH, REDOX imbalance, oxidative stress, and changes in diversity and composition of the microbiota towards a polarization of the red and orange complex [17-19]. These attributes make GPD a good model for studying the impact of the oral microenvironment on the phenotype of a given microbial model.

For the experimental phase, we used a strain of *Candida albicans* ATCC 10231 as positive control for the run, because it has been declared a pathogen according to the criteria of the CLSI (Clinical and Laboratories Standard Institute) [20].

The clinical isolates used in this study are stored and cryopreserved in the Mycology center of the Institute for Research in Microbiology and Parasitology (IMPAM), Faculty of Medicine, University of Buenos Aires. The clinical categorization of these was carried out in a previous research study. The preserved isolates were reconstituted in BHI (brain-heart infusion) broth (Merck) at 37 °C. The growths obtained in BHI broth were plated in Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (Becton Dickinson), incubated at 28 °C. [21]; with cultures obtained in SDA, suspensions were prepared at 1×10^7 cells/ml in saline solution.

2.2. Quantification *in vitro* biofilm production

We measured the biofilm-forming ability in 96-well polystyrene microtiter plates (catalog number 167008 from Nunc or from Techno Plastic Products AG) by total biofilm biomass quantification assay with violet crystal (VC) in RPMI 1640 medium 1X (supplemented with L-glutamine), described by Treviño Rangel et al., in 2015 [22]. Each sample was analyzed in 4 replicas and in two independent experiments. To increase the confidence level of the results, we used an alternative method for quantification of biofilm *in vitro* based on measuring metabolic activity in each well by enzymatic reduction of the tetrazolium salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) by mitochondrial dehydrogenases, which are only active in viable fungal cells. Only viable cells will be able to reduce XTT salt in a chromogenic product (formazan) whose optical density is measured at 490 nm [23]. The quantity of biofilm formed by an isolate was classified according to the guidelines by Sánchez et al. [24], and Treviño Rangel et al. [22]

2.3. Evaluation of biofilm with optical microscopy (OM)

Biofilm morphology and topology were examined under optical microscope (Olympus), following the protocol described by Oggioni and collaborators [25], in 2006. Images were obtained with a SAMSUNG Galaxy J7 and exported to TIFF format.

2.4. 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, low and null biofilm forming according to VC test results) was examined by CLSM [26].

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.

2.5. Measurement of gene expression by RT-qPCR applied to the *CPH2* master gene depending on the clinical origin of the isolates.

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 $2^{\Delta\Delta Cq}$; for which we follow the Bustin’s MIQE guidelines published in 2009 [27]. The ATCC 22019 strain of *C. parapsilosis sensu stricto*, defined as non-pathogenic by CLSI [28], 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. Three biological replicates were tested for each clinical condition, and in turn each sample was tested in triplicate, in three independent experiments.

The RNA used in this study was obtained by means of a column purification system (Qiagen). The extracts obtained were analyzed for yield, purity, and integrity; and were stored at -70 °C with Promega RNase inhibitor. Subsequently, a microgram of RNA was restricted to cDNA, for which the iScript retrotranscriptase from Biorad was used. The cDNA matrices were stored at -20 °C.

2.6. Statistical analysis

Data were processed and analyzed in Microsoft Excel 2010 and the InfoStat 2018 statistic package. Difference between means was evaluated by right-tailed Student’s *t*-test for two independent samples after verifying the assumptions of normality, independence and homogeneity of variances, with a 95 % confidence interval, considering as significant a *P* value lower than error alpha ($\alpha= 0.05$). To determine whether there is significant association between biofilm-forming phenotype and clinical source of isolates, ANOVA with two fixed factors was used and *K*=1 observation, and Bonferroni’s test for pairwise comparisons. ANOVA validity was tested with Shapiro Wilks’ test, Q-Q plot test and Levene’s test on residuals. Presence or absence of outliers was determined by Grubb’s test for outliers (data not revealed). The correlation between variables was analyzed by Spearman test and scatter plot.

Sample size was calculated using statistical software from the InfoStat 2018 package.

3. Results

3.1. Measurement of biofilm-producing ability in vitro in a collection of oral isolates of *C. parapsilosis sensu stricto*

The phenotype "high biofilm forming" predominated in the collection of oral isolates of this *Candida* species evaluated, by the two biofilm reading methods used (VC and XTT). However, the VC colorimetric method recognized 12% of isolates as "null biofilm forming" (Fig. 1), while the XTT colorimetric method did not recognize isolates incapable of forming biofilm in vitro (Fig. 2). To resolve this discrepancy, we studied the morphology and architecture of the biofilm generated by a group of oral isolates of *C. parapsilosis sensu stricto* randomly selected from each phenotype recognized by the VC reading method.

The morphological and architectural study of the biofilm, both by OM and CLSM, demonstrated a biofilm-producing ability in the group of isolates categorized by VC as "null biofilm forming". The biofilm formed by this last group of

isolations showed morphological, topological and architectural similarity with the biofilm formed by the strains categorized as "low biofilm forming" according to the VC colorimetric test (Figs. 3, 4, 5, and 6). The morphological and structural characterization of the biofilm by OM and CLSM confirms the phenotypic characterization established according to the results of the colorimetric test based on tetrazolium salts (XTT).

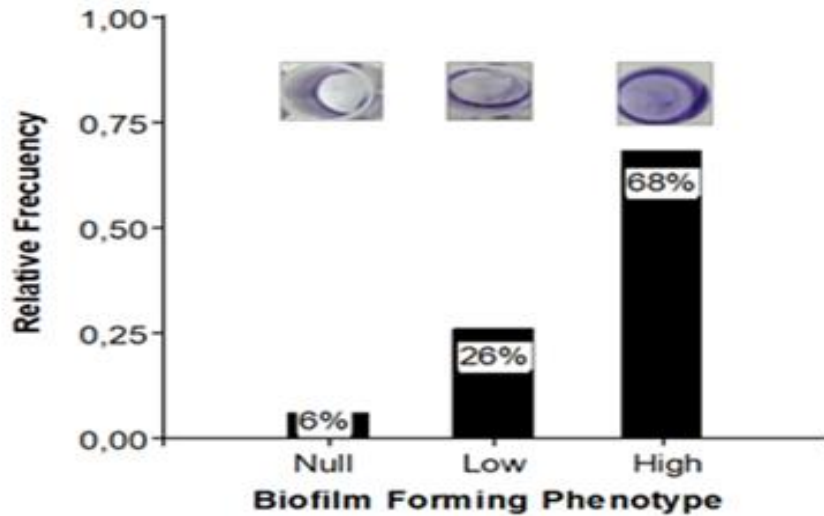


Figure 1 Frequency distribution for biofilm forming ability in RPMI 1640 culture medium and estimated by violet crystal method.

Note: 68% of the isolates formed high levels of biofilm in RPMI medium (RF: relative frequency). The difference between the three groups was determined by ANOVA one factor.

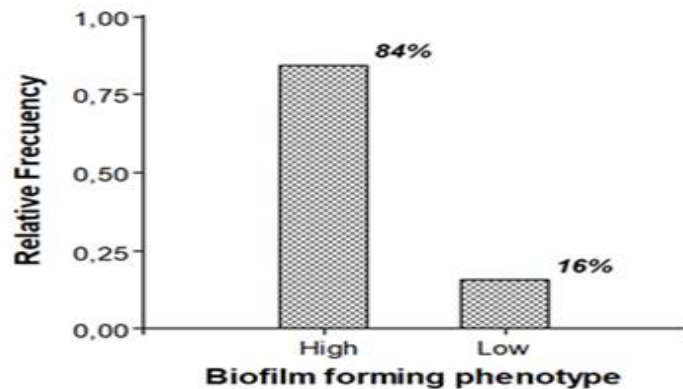


Figure 2 Frequency distribution for biofilm-forming ability in RPMI 1640 culture medium and estimated by XTT method.

Note Student's test showed a significant difference ($P < 0.0001$) between high and low formator phenotypes detected by this method (RF = relative frequency).

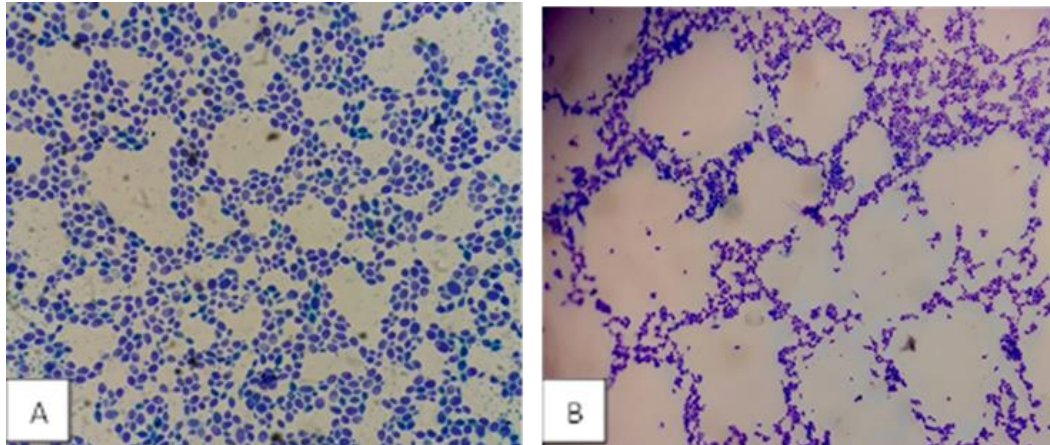


Figure 3 Biofilm topology formed by isolates with low and zero biofilm-producing capacity, according to the violet crystal test, seen by OM.

Note: **A**= Isolation “low biofilm forming” defined by VC method. The topography is reticular, the ECM is scarce and it is arranged as a mesh or scaffold that supports the cells, which are mainly ovoid yeasts and blastospores (40X). **B**= Isolation defined by VC method as “null biofilm forming”, however, it shows to form an intelligently organized structure of cells, which are arranged by drawing a network. ECM is very rare, and the cell morphology is exclusively yeast (10X).

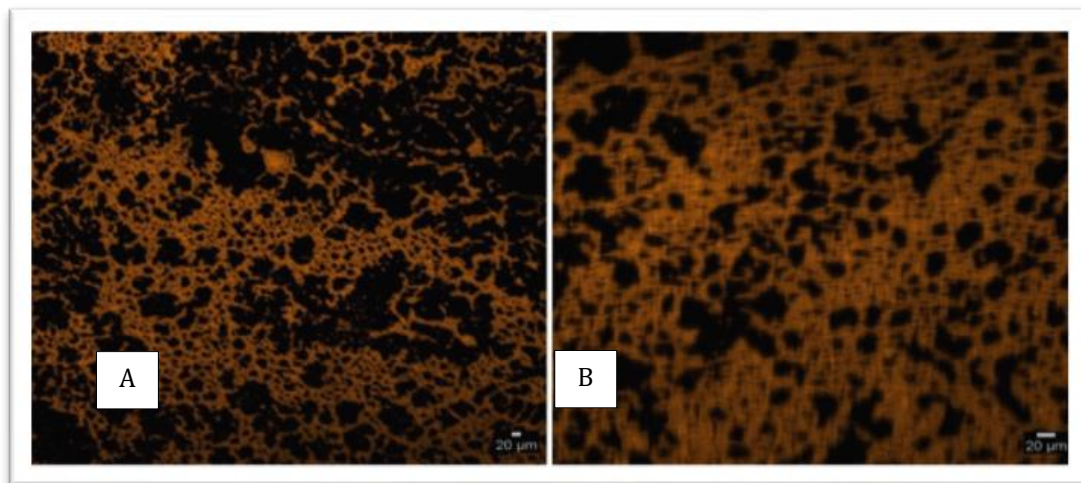


Figure 4 Biofilm architecture in two dimensions generated by “low biofilm-forming” strain, according to VC colorimetric assay.

Note: **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 strain with a “low biofilm-forming phenotype, according to the VC colorimetric assay.

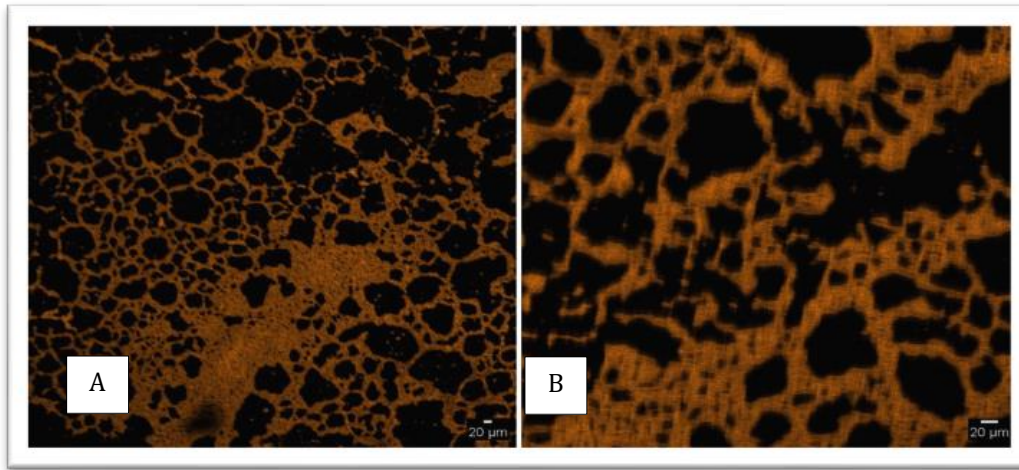


Figure 5 Biofilm architecture in two dimensions, generated by a strain with a “null biofilm-forming phenotype”, according to VC colorimetric assay.

Note: 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 strain with a “null biofilm-forming phenotype”, according to the VC colorimetric assay. The topology demonstrated by this strain is highly similar to the topology generated by the low biofilm-forming strain.

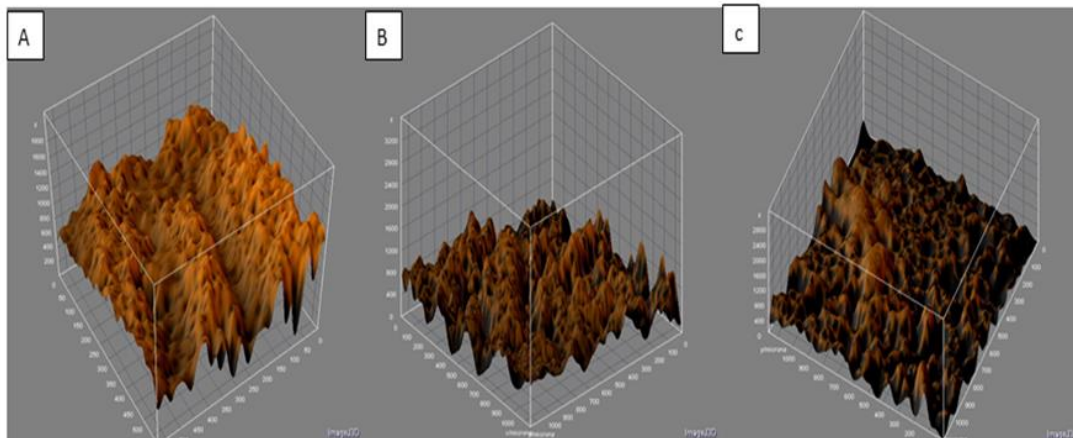


Figure 6 Reconstruction in three dimensions for the biofilm generated by strains defined according to VC test as “high (A), low (B) and null biofilm-forming (C)”.

Note: In the three categories of biofilm (A, B and C) a three-dimensional structure based on a series of peaks and valleys is recognized, whose height and base vary according to the biofilm-forming ability. The “high biofilm-forming strain” (A) forms conical structures over 100 µm in height, while the “low and null biofilm forming” strains (B and C) form conical structures less than 100 µm in height. The biofilm architecture of “low (B) and null biofilm forming” (C) strains does not reveal significant differences; indeed, the intensity of the fluorescence signal is comparable, which indicates similarity in cell density and extracellular matrix.

3.2. 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 (Fig. 7). 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 8 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 specie.

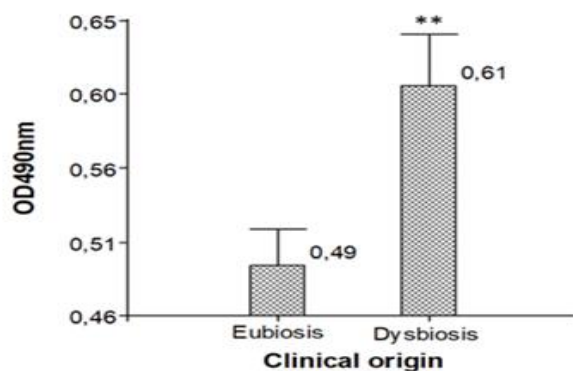


Figure 7 Comparison of average absorbance of the formazan product after reduction of XTT by viable fungal cells grown in RPMI 1640 medium, according to clinical provenance of isolates.

Note: 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; (**): $P < 0.01$.

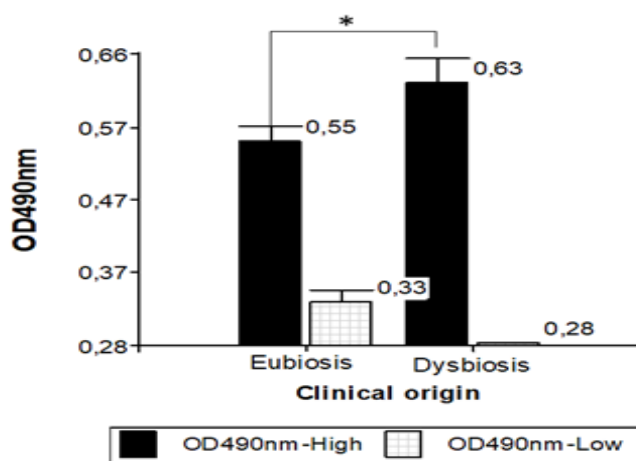


Figure 8 Metabolic activity in biofilm, relative to the clinical origin of oral isolates, and biofilm-forming ability.

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

3.3. 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 $2\Delta\Delta Cq$ parameter showed that the 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 related the baseline expression of this gene relative to the clinical origin of the isolates (eubiosis and 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 (Fig. 9). 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.

Table 1 Proportion of clinical isolates of *C. parapsilosis* sensu stricto that demonstrated by RT-qPCR a basal expression level of *CPH2* gene 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 <i>CPH2</i> expression increased by more than 3 fold with respect to the calibrator (%)
Oral eubiosis (commensal isolates)	3	1 (33.3)
Oral dysbiosis (commensal isolates)	3	2 (66.6)
Onychomycosis (pathogenic isolates)	2	1 (50)

Note: Values are representation of a single experiment.

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

Experiment	Oral eubiosis $\Delta Cq(\mu)$	Oral dybiosis $\Delta Cq(\mu)$	$\Delta\Delta Cq$	$2 - \Delta\Delta Cq$
1 ^o	0.14	-1.33	-1.47	2.77
2 ^o	-2.52	-4.32	-1.8	3.46
3 ^o	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

Note: The average $2 - \Delta\Delta Cq$ value indicates that in oral dysbiosis the *CPH2* gene 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].

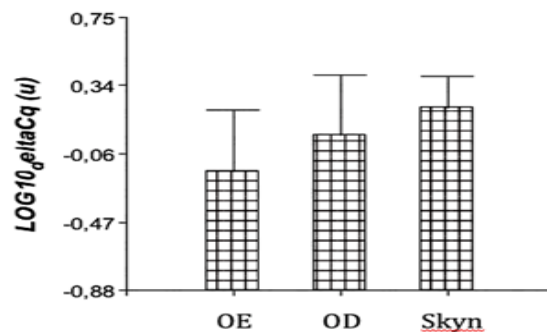


Figure 9 Expression of the *CPH2* gene relative to the normalizer (*TUB4*) based on the clinical origin of the isolates.

Note: 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].

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 *C. parapsilosis* sensu stricto came from oral niches under a situation of oral dysbiosis [29]. Further, Shafeeq and his group [30] published in 2019 a study in which they demonstrate the property of *BCR1* to

respond to high levels of manganese, which led to the formation of biofilm in strains previously defined as non-forming and low biofilm-forming. This finding demonstrates the ability of *BCR1* to respond to signals from the environment. In agreement with the findings of Shafeeq et al. [30], and under our experimental conditions, the basal transcriptional activity of *BCR1* was shown to be higher in those isolates that recovered under conditions of gingivo- periodontal disease (dysbiosis). This condition is characterized by the imbalance of the colonizing microbiota, with a periodontopathogens predominance of the red and orange complex [31], in addition to the REDOX imbalance [32, 33]. Currently, it is not known whether the REDOX imbalance is a cause or consequence of gingivo-periodontal disease. The results of this study lead us to hypothesize that fungal cells can respond to high levels of pro-oxidant species by up-regulating biofilm regulatory genes, such as *BCR1* and *CPH2*, to adapt to such a stressful situation. Indeed, it has been shown that free radicals can directly or indirectly alter various cellular and physiological mechanisms by acting on lipids, DNA, and proteins [32].

We postulate that, these differences in the basal expression of *BCR1* and *CPH2* are due to changes in the epigenetic marks that control the transcriptional activity of this gene, which would change when faced with signals from the environment, which may be physical, chemical or mechanical. All these stimuli constantly act in the oral cavity, which exhibits it as a complex niche. Indeed, similar findings have been reported in the *C. albicans* [34] and *Staphylococcus aureus* [35] models.

It would be necessary to validate this hypothesis with approaches that analyze the epigenetic profile in clinical isolates of *C. parapsilosis sensu stricto* obtained from different niches.

Our results suggest that the oral cavity under pathological conditions may function as an alternative route to the skin in the epidemiology of nosocomial candidemia. Further, *C. parapsilosis sensu stricto* could favor 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 [36-39]. Furthermore, studies carried out in the *C. 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].

4. Conclusion

From our results we can infer that the oral cavity under a situation of ecological imbalance, as occurs during periodontal disease, can exacerbate the virulence of *C. parapsilosis sensu stricto*, reflecting in a greater capacity to produce biofilm. It is likely that this effect results from epigenetic modifications, which should be studied in a later study.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors have no conflicts of interest.

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