(RESEARCH ARTICLE)

The oral cavity promotes virulence of *Candida parapsilosis* sensu stricto via up-regulation of *BCR1*

Rodríguez María Lourdes [1, 2, *], Alcaraz Eliana Sabrina [3], Rosa Alcira Cristina [4] and Jewtuchowicz Virginia Marta [1]

[1] *Instituto de Investigaciones en Microbiología y Parasitología (IMPaM), Facultad de Medicina, Universidad de Buenos Aires. Buenos Aires-Argentina.*
[2] *Cátedra de Semiología y Clínica de Diagnóstico, Universidad de Cuenca. Cuenca-Ecuador*
[3] *Instituto de Investigaciones en Bacteriología y Virología Molecular (IBaViM). Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Buenos Aires-Argentina.*
[4] *Departamento de Microbiología, Facultad de Odontología, Universidad de Buenos Aires. Buenos Aires-Argentina*

Abstract

Candida parapsilosis sensu stricto behaves as a frequent colonizer of niches of the oral cavity, predominantly in pathological conditions. Studies have suggested the influence of the ecological niche in the virulence of *C. parapsilosis*. Given this background, we hypothesize that the conditions of the niche affect the virulence of *C. parapsilosis* sensu stricto due to inheritable epigenetic changes. Measure and compare the virulence, at the phenotype and molecular level, of clinical isolates of *C. parapsilosis* sensu stricto from different niches and clinical conditions. Biofilm-forming ability was compared in 20 clinical isolates of *C. parapsilosis* sensu stricto obtained from blood (candidemia), skin (onychomycosis), and oral cavity (eubiosis and dysbiosis); by quantification of biofilm biomass, and metabolic activity. The results were corroborated by optical microscopy and correlated with the basal expression of the global biofilm regulator BCR1 by RT-PCR. Biofilm production and baseline BCR1 expression were significantly different depending on the ecological niche and the clinical origin of the isolates. The oral cavity exerts a preponderant role in the modulation of the virulence of this yeast via regulation of BCR1. The biofilm-forming ability in *C. parapsilosis* sensu stricto is dependent on the strain, but can be modulated by environmental conditions or ecological niche via epigenetic regulation of global biofilm regulators such as BCR1.

Keywords: *Candida parapsilosis* sensu stricto; Virulence; BCR1; Ecological niche

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].

Candida 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,

* Corresponding author: Rodríguez María Lourdes E-mail address: maria.rodriguezc84@ucuenca.edu.ec/ Phone: 549-1168186883

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 niches and 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. Of all the niches evaluated, the oral cavity, and especially the oral cavity under dysbiosis, was shown to have a dramatic impact on the virulence of *Candida parapsilosis* sensu stricto, via up-regulation of *BCR1*.

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 *Candida parapsilosis* sensu stricto, defined as such in a previous study by both molecular and conventional phenotypic methods. The isolates came from different niches: blood, skin and oral cavity, under different clinical conditions. The blood isolates were derived from subjects diagnosed with candidemia. While, the skin isolates recovered in onychomycosis lesions; and the isolations of the oral cavity were obtained under conditions of eubiosis (oral health) and dysbiosis (gingivo-periodontal disease). The results of the biofilm study were corroborated by light microscopy. Phenotype results were also correlated with the expression level of the global biofilm regulator *BCR1*, 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 / 2016CETICAFOUBA).

2.1. Strain, isolates and media

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. There were a total of 20 *Candida parapsilosis* sensu stricto isolates from each ecological niche: blood, skin and oral cavity. To study the potential impact of a dysbiotic environment on the virulence of *Candida parapsilosis* sensu stricto, we used 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 preserved isolates were reconstituted in BHI (brain-heart infusion) broth (Merck) at 37 ºC. The growths obtained in BHI broth were plated in Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (Becton Dickinson), incubated at 28 ºC. [21]. With cultures obtained in SDA, suspensions were prepared at $1x10^7$ 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 crystal violet (**CV**) 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-sulfophenil]-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], as: high producers (≥0.41), low producers (0.11-0.40), and null producers (≤0.10). Sánchez et al. break down high producers into strong and moderate biofilm producers (Table 1).

2.3. Evaluation of biofilm with optical microscopy (OM)

Biofilm morphology and topology were examined under optical microscope (Olympus), following the protocol described for Oggioni and collaborators [25], in 2006.

Images were obtained with a SAMSUNG Galaxy J7 and exported to TIFF format.

2.4. Measurement of gene expression by RT-PCR applied to the *BCR1* master gene depending on the clinical origin.

BCR1 transcriptional activity was determined by reverse transcription PCR (RT-PCR) in 4 oral isolates randomly selected from each clinical group. The expression of the target gene was relativized to the Actin 1 gene (*ACT1*).

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 ºC 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 ºC.

PCR reactions were carried out in laminar flow; and the Hiaway Taq polymerase and dNTPs from Invitrogen were used. The cycling conditions corresponded to a first stage of denaturation at 95 ºC for 5 minutes, followed by 40 cycles, each based on 3 stages of: 1) denaturation at 95 ºC for 1 minute, 2) hybridization at 55 ºC for 30 seconds, and 3) Extension of the enzyme at 72 ºC for 1 minute. Followed by a final stage of extension at 72 ºC for 5 minutes. Both genes, both internal control and target, were amplified under the same conditions regarding reagent concentration, sample and thermocycling parameters. Table 2 shows the composition and volumes of this retro-transcription PCR.

The obtained amplification products were developed by electrophoretic running on 2% agarose gel, stained with ethidium bromide at a concentration of 0.15 mg per gram of agarose. The electrophoretic run was performed at 82 volts, for one hour and twenty minutes. A 100 bp molecular weight marker (Embiotec) was used; and the volume sown was that suggested by the manufacturer (5 μl). The samples were seeded by clinical group, in a volume of 10 μl each.

The quantitative analysis of the amplicons evidenced as bands in the electrophoretic run was carried out by the IMAGE J program.

2.5. 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 validness 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 and discussion

3.1. Study of virulence to level of phenotype

3.1.1. Measurement of biofilm-producing ability in vitro in a collection of clinical isolates of Candida parapsilosis sensu stricto

Figure 1 shows the variable biomass level of biofilm expressed through the optical density of the crystal violet solutions measured at 595 nm (OD595nm), depending on the clinical origin of the isolates. Statistical analysis showed that invasive isolates (blood) formed significantly more biofilm biomass compared to those from skin and oral cavity. It is noteworthy that the skin isolates did not show a significant difference with respect to the mouth isolates in the biofilm-producing ability. A similar behavior was observed with the alternative method based on tetrazolium salts. But also, we did not obtain a significant difference regarding the metabolic activity in biofilm (OD490nm) between the blood and oral cavity isolates (Fig. 2).

Given the dramatic result of the colorimetric study based on metabolic activity, showing a similar level of virulence between invasive and oral isolates of *C. parapsilosis* sensu stricto, we expanded the sample size for oral isolates and compared their biofilm-forming capacity in vitro according to origin clinical (eubiosis versus dysbiosis), in two different nutritional conditions (YPD 2X broth and RPMI 1640, supplemented with 1X L-glutamine). Biofilm production was estimated by both CV and XTT. Both development methods showed a predominance of the high biofilm-forming phenotype in the collection of oral isolates (n = 25 for eubiosis and n = 25 for dysbiosis), with a significant difference between phenotypes (Figs. 3-5). Therefore, the observations they are not a product of chance.
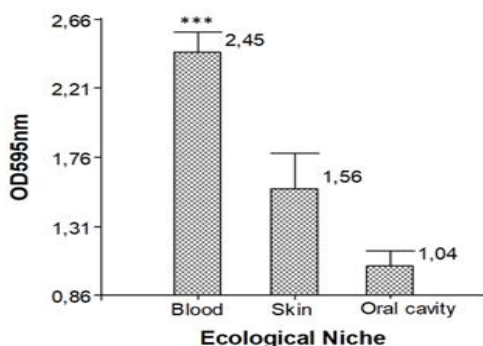

Figure 1 Biofilm biomass level depending on the type of niche. (n = 20) The data are representative of four technical replicates by isolation, and two independent experiments. Statistical analysis was performed by ANOVA 1 factor, with subsequent Bonferroni test.; [(*): $P < 0.0001$].
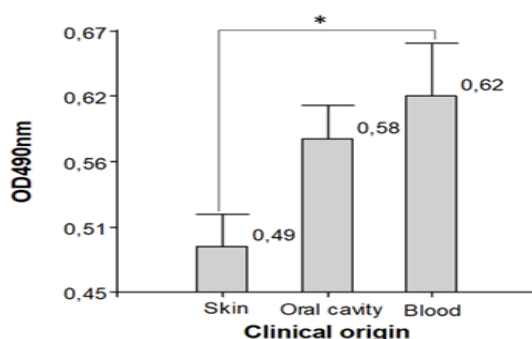

Figure 2 Metabolic activity in biofilm depending on the type of niche (n = 20). The data are representative of four technical replicates by isolation, and two independent experiments. Statistical analysis was performed by ANOVA 1 factor, with subsequent Bonferroni test; [(*): $P < 0.05$].
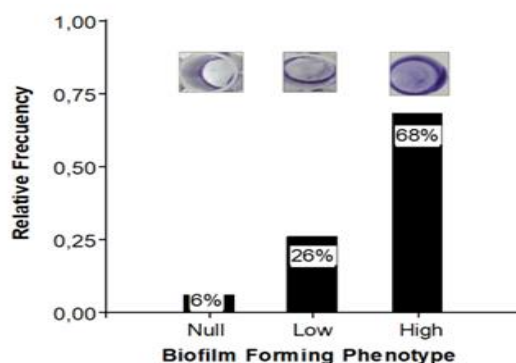

Figure 3 Frequency distribution for biofilm forming ability in RPMI 1640 culture medium and estimated by violet crystal method.

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 1 factor.
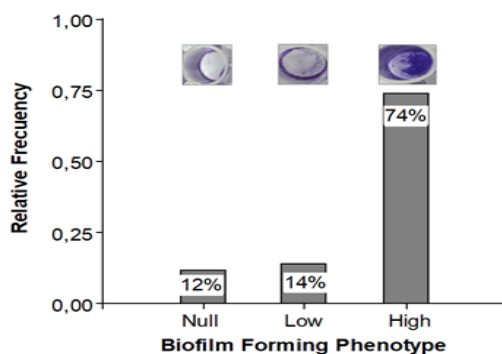

Figure 4 Frequency distribution for biofilm forming ability in YPD broth and estimated by violet crystal method.

Note: 88% of the isolates formed biofilm in this culture medium. (RF: relative frequency). The difference between the three groups was determined by ANOVA 1 factor.
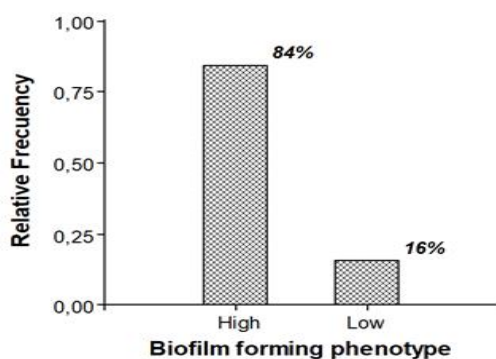

Figure 5 Frequency distribution for biofilm-forming ability in RPMI medium and estimated by XTT method.

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

3.2. Morphological and architectural characterization of the biofilm formed by oral isolates of *Candida parapsilosis* sensu stricto.

The study of the biofilm by light microscopy revealed two different topographies for the high forming strains: 1) Compact - type spider web, characterized by presenting abundant extracellular matrix (ECM) blurred between the cells (blue). The cellular component is dense, formed predominantly by spherical yeasts (lilac) (Fig. 6-A and B); 2) Lax - type serpentine, characterized by presenting less ECM, which accumulates between and on the cells in coarse aggregates with a serpentine appearance. The cellular component is made up of elliptical yeasts and pseudohyphae (Fig. 7-A and B).
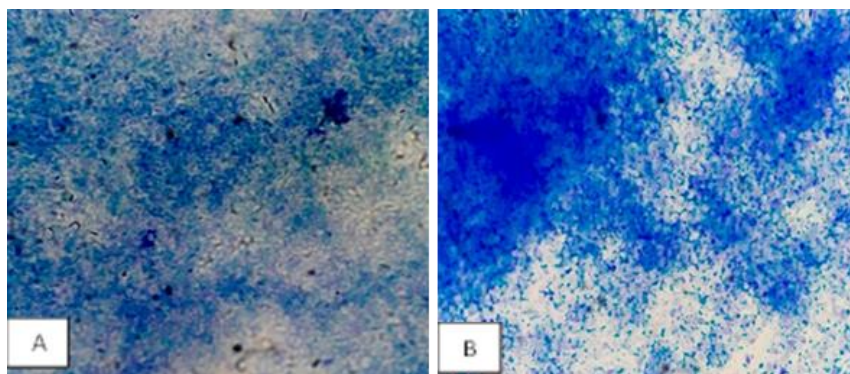
Figure 6 Isolation biofilm "*high former*" of *C. parapsilosis* sensu stricto seen by M.O. Topography in "*spider web*"

Note: **A**= Image obtained with 40X objective lens; **B** = Image obtained with 100X objective lens. Note the abundance of the ECM. The cellular component is predominantly spherical yeast.
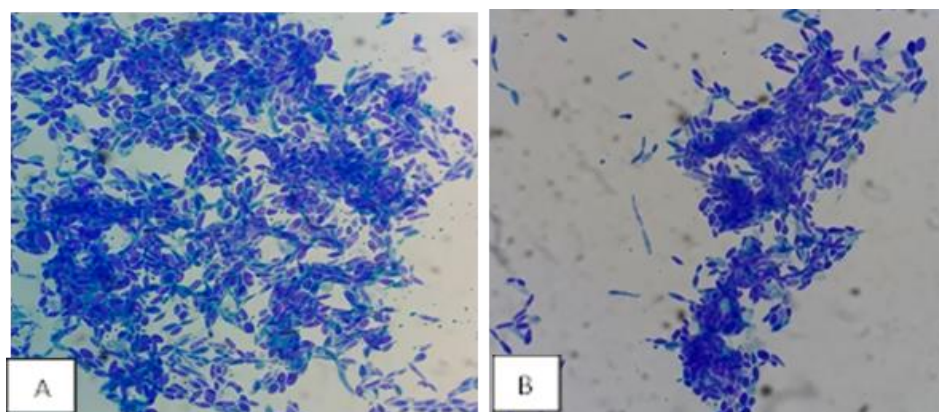

Figure 7 Isolation biofilm "*high former*" of *C. parapsilosis* sensu stricto seen por M.O. Topografhy in "*serpentine type*".

Note: A and B= Microphotographs with 400X magnification. Note the predominance of the cellular component over the ECM. The prevailing cell morphology is filamentous.
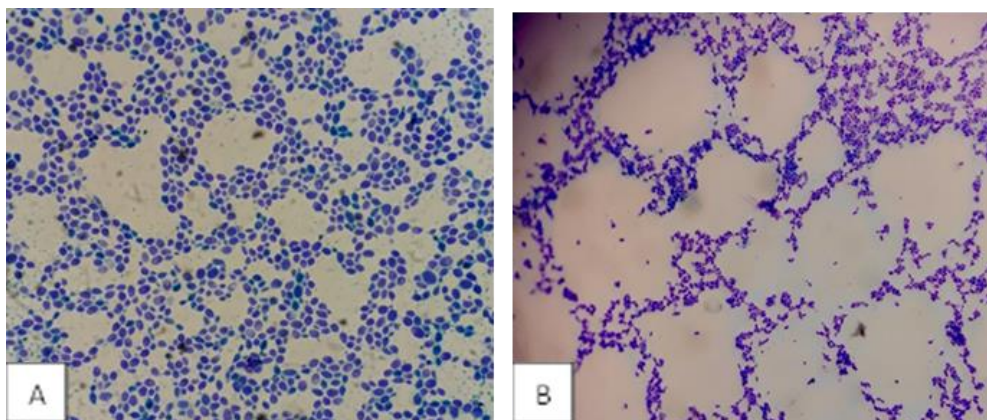

Figure 8 Biofilm formed by "*low and null forming*" isolates of *C. parapsilosis* sensu stricto, seen by MO.

Note: **A**= Isolation "*low biofilm former*" defined by CV. 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 CV as "*null formator*", 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).

The three strains characterized by CV as low biofilm formers evidenced the study by MO to generate highly organized structures, based on interconnected cell cords that draw a network. The ECM is scarce (Fig 8-A). This same structure was observed in the strains defined by CV as "forming nulls" (Fig. 8-B). This means that those strains categorized by the CV colorimetric study as null forming are actually biofilm forming strains. They were defined in the alternative colorimetric study (XTT) as low biofilm-forming strains.

The morphological and structural characterization of the biofilm by MO confirms the phenotypic characterization established according to the results of the colorimetric test based on tetrazolium salts (XTT).

3.2.1. Biofilm-forming hability 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. 9). 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 10 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.
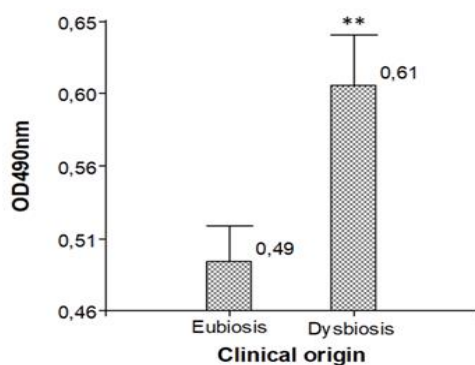

Figure 9 Comparison of average absorbance of the formazan product after reduction of XTT by viable fungal cells grown in RPMI 1640 medium, according to clinical provenance of isolates.

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; (**): $P < 0.01$.
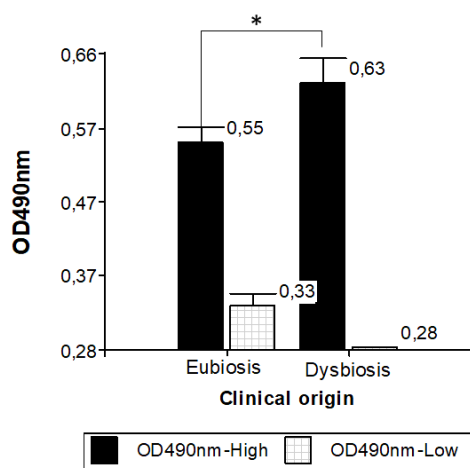
Figure 10 Metabolic activity in biofilm, relative to the clinical origin of oral isolates, and biofilm-forming hability.

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

3.3. Study of virulence to level molecular

Figure 11 shows the result of the electrophoretic run for the expression products of the *BCR1* and *ACT1* genes. The latter used as a standardizer.

Subjective analysis allows establishing over-expression of *BCR1* in the group of matrices from oral dysbiosis condition. At the same time, the quantitative study of the PCR products by IMAGE J showed significantly higher basal expression of the *BCR1* regulator in the condition of oral dysbiosis compared to the control group (eubiosis) (Fig. 12). Given this result, we wonder if the basal transcriptional activity of *BCR1* correlates with the biofilm-forming capacity estimated through the metabolic activity present in the biofilm. To answer this question, we measured the level of correlation between both quantitative variables (fold change and OD490nm) by Pearson's test, obtaining a coefficient of 0.89, plus a value of $P = 0.008$, therefore, the correlation is significantly linear and is also positive (Fig. 13).
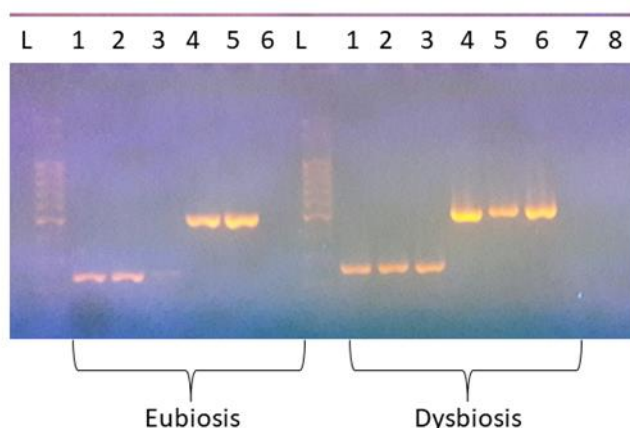

Figure 11 Differential expression of the target *BCR1* gene according to clinical origin (eubiosis versus dysbiosis) in oral isolates of *C. parapsilosis* sensu stricto.

Legend: L= ladder (100bp molecular weight marker); Columns 1,2,3 = samples processed with Act1 primers; Columns 4,5, and 6 = samples processed with Bcr1 primers; Columns 7 and 8 = negative controls of Act1 and Bcr1 respectively. 490 bp product = *BCR1* transcript; 245bp product = *ACT1* transcript.
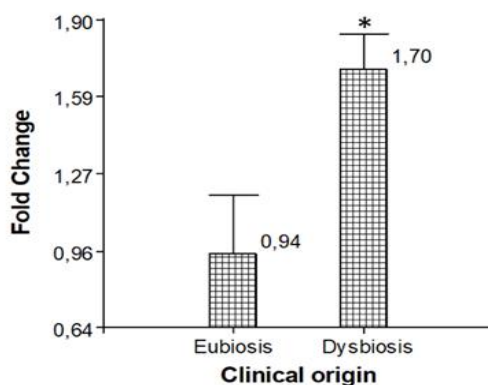

Figure 12 *BCR1* fold change in oral isolates of *C. parapsilosis* sensu stricto, depending on the clinical origin. Note: Results are representative of 3 biological replicates, and two independent experiments; [(*): $P < 0.05$].
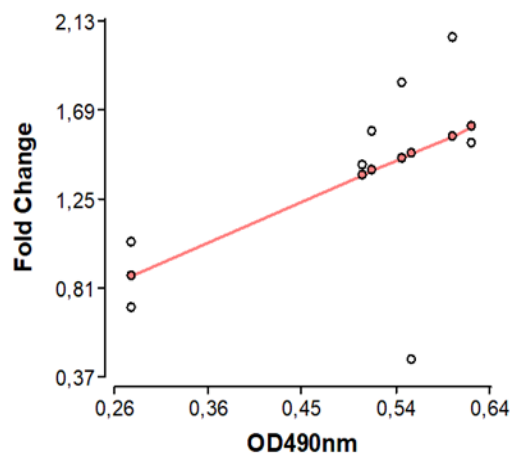
Figure 13 Scatter diagram showing linear and positive correlation between the fold change quantitative variables, and OD490nm. Note: ANOVA analysis showed that the model is statistically significant ($P = 0.02$).

The results disaggregated so far indicate that *C. parapsilosis* sensu stricto cells that colonize niches of the oral cavity, more frequently overexpress the *BCR1* regulator in dysbiosis condition, and this expression correlates with their metabolic activity in biofilm measured in vitro (Fig. 13). This panorama led us to wonder if *C. parapsilosis* sensu stricto cells isolated from skin and nails, in the context of pathogens (onychomycosis), show a different behavior regarding the transcriptional activity of *BCR1*, compared to oral colonizers of this same species. To answer this question, we randomly chose the cDNA of 3 skin isolates of *C. parapsilosis* sensu stricto obtained from onychomycosis. This cDNA was subjected to RT-PCR for *BCR1*; and we carried out a Student's t-test for two independent samples (skin versus oral cavity), with a two-tailed test, and an alpha error of 0.05. Figure 14 shows a clear difference in the transcriptional activity of *BCR1*, its expression being significantly more important in the isolates of *C. parapsilosis* derived from the oral cavity compared to those of the skin. This finding indicates that the oral cavity exerts an important impact as a complex niche, modulating the expression of a global biofilm regulator, such as *BCR1*.
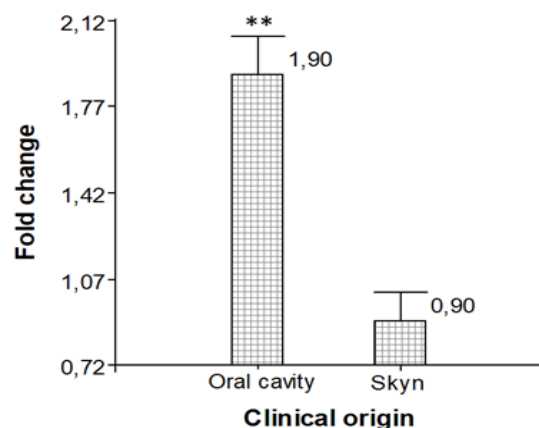
Figure 14 *BCR1* fold change in clinical isolates of *C. parapsilosis* sensu stricto, depending on the ecological niche.

Note: The results are representative of 3 biological replicates, and two independent experiments; [(**): $P < 0.001$, with Satterwhite correction].

4. Discussion

Our research group established, through a basic, cross-sectional and retrospective research study, that of the three species of the psilosis complex, *C. parapsilosis* sensu stricto behaves as a habitual colonizer in niches of the oral cavity, especially in conditions of gingivo-periodontal disease [14]. Given this background, we wonder if the virulence of *C.*

parapsilosis sensu stricto is different depending on the clinical origin of the isolates. Our study question is based on the findings of Silva et al. [26], Tavanti et al. [27], Sánchez et al. [24], as well as Pimentel de Barros and collaborators [28], who suggested the influence of clinical origin on the biofilm-producing capacity in Candida species.

Among clinical Candida isolates, biofilm formation is variable and depends on the Candida species in question [24]. This knowledge has already been demonstrated in several works, as it has also been established that the biofilm formation by *Candida parapsilosis* is a key virulence factor in this species, being highly dependent on the strain [22, 29]. Under our experimental conditions, the biofilm-forming capacity in *C. parapsilosis* sensu stricto proved to be variable (Figs. 3 and 4), showing a relationship with the clinical origin of the isolates (Figs. 9 and 10). Of the niches tested, the oral cavity proved to have a significant impact on the virulence of *C. parapsilosis*, both at the phenotypic and molecular level, significantly increasing the basal expression of the global biofilm regulator *BCR1*, which translated into greater biofilm formation in vitro when was compared with the skin isolates. Our results are compatible with those of Silva et al. [26], Tavanti et al. [27], and Silva Días et al. [30]. These groups reported that the biofilm formation and the rate of adhesion are higher in oral and muco-cutaneous strains of *C. parapsilosis.*

Given the dramatic in vitro behavior of the oral isolates of *C. parapsilosis*, we hypothesized that an oral environment in dysbiosis, associated with an overgrowth of pathobionts, promotes and / or exacerbates the virulence of commensal strains of *C. parapsilosis* sensu stricto due inheritable epigenetic changes. This hypothesis is supported by the findings of Tavanti et al. They reported that the variability in the DNA sequence between isolates of this species with different clinical and geographical origins is minimal, and is not related to the wide phenotypic variability detected [27]. The research led us to establish that those isolates derived from oral niches under conditions of gingivio-periodontal disease (dysbiosis), formed a greater biomass of biofilm with respect to isolates obtained under the condition of eubiosis, although the difference was not significant by CV test. However, the alternative method used to measure metabolic activity in biofilm, by reduction of tetrazolium salts, allowed to observe statistically significant differences in the biofilm-forming ability between oral isolates of this Candida species regarding its clinical origin (dysbiosis versus eubiosis). Additionally, the XTT assay allowed us to detect an association between the biofilm-forming phenotype and the clinical origin of the oral isolates. Statistical analysis indicated a significant association between these two categorical variables, suggesting that the biofilm-forming ability in this fungal model would also be modulated by the conditions of the ecological niche, or the environment. These results were compatible with the reports of Hasan et al. [31], and Jain et al. [32], who also suggested that the origin and selection of isolates may be a factor influencing biofilm formation in Candida species. Furthermore, the conversion of Candida species from commensalism to parasitism and overgrowth are usually associated with changes in the intraoral environment, such as: use of unsanitary prostheses and xerostomia, or systemic factors such as diabetes mellitus with poor metabolic control, and immunodeficiencies. In any case, to date, there are no published data regarding the impact of conditions in the oral environment on the pathogenicity of this yeast model. This is the first study that analyzes the behavior of a large collection oral isolates of *C. parapsilosis* sensu stricto based on the clinical conditions in which they were obtained.

Colorimetric and morphological studies of the biofilm showed that both the biofilm-producing capacity and its topography are highly strain-dependent attributes in this fungal species. Properties that have not been evidenced in other NAC species such as *Candida tropicalis* and *Candida glabrata* [24]. Among the high biofilm-forming strains, two well-differentiated structures were recognized by OM. One of them based on spider web pattern, with high cell density and ECM; and another looser one with less ECM, which was distributed among the cells as serpentine arrangements. In this serpentine variant, the pseudohypha was the main cell morphology. These findings are similar to the biofilm morphologies described by Panannusorn et al., in this fungal model [29]. Among the low biofilm-forming strains, we detected a single topography, based on more or less dense cell cords interconnected and supported by a thin ECM. The organization of cells in this group of strains drew a lattice structure. This result is compatible with the findings of Brilhante and collaborators [33].

A study that determined a before and after on the transcriptional regulation of biofilm in *C. parapsilosis* sensu stricto was that of Holland et al. [16], published in 2014. In it, the network of genes that control biofilm production in this yeast was established. Some of these genes showed shared functions with biofilm regulatory genes present in *C. albicans*, such as *BCR1*. In the same year, new evidence appeared that postulated the biofilm-producing capacity as a highly dependent quality of the strain in this Candida species, and at the same time dependent and independent of *BCR1*. Regarding the latter, Pannanusorn and his group [29] observed that only strains with low biofilm-producing capacity overexpress *BCR1* during biofilm growth, the opposite occurring in high-producing strains. In the latter group, *BCR1* would act as a virulence modulator, controlling both biofilm production and filamentation. These findings would place this gene as an important regulator of virulence in this Candida species. However, our results do not correspond to those of Pannanusorn and his group [29]. In our collection of isolates, and under our experimental conditions, the basal transcriptional activity of *BCR1* showed a significant and linear correlation with the ability to produce biofilm. This

finding suggests that *C. parapsilosis* sensu stricto during biofilm growth, would express more or less Bcr1 depending on its formative capacity. This result is compatible with the study by NiKoomanesh et al., who also demonstrated a positive relationship between the expression of the *BCR1* gene and the formation of biofilm in isolates of *Candida albicans* [34]. Either way, both our results and those of Pannanusorn et al. [29], locate *BCR1* as a key regulator of biofilm production in this fungal model.

The molecular study of the isolates allowed the detection of significant differences in the basal expression of *BCR1* relative to the clinical origin. We postulate that this difference in the basal expression of *BCR1* is 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. 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. An antecedent that supports this hypothesis is the work of Treviño Rangel and his group [22], published in 2015. They did not find variations in the *BCR1* sequence that could explain the phenotypic variability observed in *C. parapsilosis* sensu stricto strains. They also confirmed Pannanusorn's findings [29] regarding the biofilm-forming ability in this yeast (strain dependent property). Additionally, Tavanti et al. [27] reported in 2010 that the biofilm-producing capacity in *C. parapsilosis* sensu stricto varied from strain to strain, observing an association with the clinical origin. Furthermore, this group did not detect significant genetic variability that could explain the phenotypic heterogeneity observed in this fungal species. Until now, there are no published studies evaluating the differential expression of *BCR1* between isolates of *C. parapsilosis* from different niches, or of different clinical origin. Only in the *C. albicans* model was a study published in 2017, with a somewhat similar method, in which Pimentel de Barros et al. [28] compared the variables: a) Biofilm formation, b) Expression of virulence genes in biofilm (among them *BCR1*) at different times (0, 12, 24, 48 hours), and c) Pathogenicity in an invertebrate model (Galleria mellonella), between two strains of *C. albicans*. One of them corresponded to an isolation of the oral cavity in an HIV positive patient (Ca60 strain), and the other corresponded to an ATCC 18804 strain, isolated from a human skin lesion (According to American Type Culture Collection (ATCC): www.atcc.org. Last accessed on 29/03/2020]. In general, the study reported that both strains differed in: the amount of biofilm formed, the pathogenicity, as well as in the transcriptional profile of virulence genes. The strain derived from the oral cavity (Ca60) proved to be more pathogenic, and showed significantly elevated levels of the *HWP1*, *ALS3*, *SAP5*, *PLB2*, and *LIP9* genes, compared to the ATCC strain. In relation to the particular expression of *BCR1*, the study by Pimentel de Barros et al. [28] showed a much higher level of expression for this gene in the Ca60 strain, compared to the reference strain (ATCC 18804), at all biofilm times studied, including basal or zero hour expression of the biofilm.

Taken together, our findings suggest that the oral cavity, unlike the skin, functions as a complex niche, in which physical, chemical, and mechanical stimuli, such as masticatory forces, act. With respect to the latter, recent evidence, resulting from studies in human cells, suggests that eukaryotic cells can perceive tension stimuli and transmit them to the nucleus through cytoskeletal physical junctions (perinuclear stress fibers), and a cascade of biochemical pathways (pathways of Calcium and Rho-Rock) conditioning changes in gene expression profiles mediated by epigenetic modifications [35, 36]. Therefore, based on these antecedents, we postulate that the mechanical forces acting on the oral cavity, depending on its orientation; In addition to the shape and area of the different substrates or niches that we find in the oral cavity, they regulate in different ways the physical properties of the cytoskeleton, altering the properties of the nucleus and the gene expression programs in the fungal cells that colonize this ecological niche.

On the other hand, Shafeeq and his group [37] 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. [37], 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. This condition is characterized by the imbalance of the colonizing microbiota, with a periodonto-pathogen predominance of the red and orange complex [38], in addition to the REDOX imbalance [39, 40]. 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*, 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 [39].

5. Conclusion

The biofilm-forming capacity and structure is strain dependent in the *Candida parapsilosis* sensu stricto species. However, their ability to produce biofilm is affected by the clinical origin of the isolates and / or the conditions of the ecological niche via up-regulation of *BCR1*. We postulate that the transcriptional activity of this gene is regulated by

environment signals (physical, chemical and mechanical), which modify the epigenetic marks that regulate the expression of this gene. The oral cavity in a dysbiosis condition promotes the virulence of this Candida species. This finding places the oral cavity as an alternative route to the skin in the epidemiology of nosocomial candidemia. Biofilm production can be estimated by different indirect methods. In our experience, the quantification of the metabolic activity present in the biofilm proved to be a more robust method when correlating with the morphological analysis (OM) of said structure.

Compliance with ethical standards

Disclosure of conflict of interest

The authors have no conflicts of interest.

References

[1] Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP and Edmond MB. (2004). Nosocomial blood-stream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical Infectious Disease, 39(3), 309-17.

[2] Pfaller MA, Moet GJ, Messer SA, Jones RN and Castanheira M. (2011). Candida bloodstream infections: comparison of species distributions and antifun- gal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008–2009. Antimicrobial Agents Chemotherapy, 55(2), 561–6.

[3] Tóth R, Nosek J, Mora-Montes HM, Gabaldon T, Bliss JM, Nosanchuk JD, Turner SA, Butler G, Vágvölgyi C and Gácser A. (2019). *Candida parapsilosis*: from genes to the bedside. Clinical Microbiology Reviews, 32, e00111- 18.

[4] Quindos G. (2014). Epidemiology of candidaemia and invasive candidi- asis. A changing face. Revista Iberoamericana de Micología, 31(1), 42-8.

[5] Guinea J. (2014). Global trends in the distribution of Candida species causing candidemia. Clinical Microbiology and Infection, 20(6), 5-10.

[6] Arsic´ Arsenijevic´ V, Otaševic´ S, Janic´ D, Minic´ P, Matijaševic´ J, Medic´ D, Savic´ I, Delic´ S, Nestorovic´ Laban S, Vasiljevic´ Z and Hadnadjev M. (2018). Candida bloodstream infections in Serbia: first multicentre report of a national prospective observational survey in intensive care units. My- coses, 61(2), 70-78.

[7] Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ and Chiller T. (2012). Species identification and antifungal susceptibility testing of Candida blood- stream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. Journal of Clinical Microbiology, 50(11), 3435-42.

[8] Conde-Rosa A., Amador R, Perez-Torres D, Colon E, Sanchez-Rivera C, Nieves-Plaza M, Gonzalez-Ramos M and Bertran-Pasarell J. (2010). Candidemia distribution, associated risk factors, and attributed mortality at a university-based medical center. Puerto Rico Health Science Journal, 29(1), 26-29.

[9] da Matta D, Remondi A and Lopez Colombo A. (2017). Revisiting Species Distribution and Antifungal Susceptibility of Candida Bloodstream Isolates from Latin American Medical Centers. Journal of Fungi, 3(2), 24.

[10] Kleinegger CL, Lockhart SR, Vargas K and Soll DR. (1996). Frequency, intensity, species, and strains of oral Candida vary as a function of host age. Journal of Clinical Microbiology, 34 (9), 2246-54.

[11] Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A and Gillevet PM. (2010). Characterization of the Oral Fungal Microbiome (Mycobiome) in Healthy Individuals. PLoS Pathogens, 6(1), e1000713.

[12] Yang Y, Leaw S, Wang A, Chen HT, Cheng WT and LO HJ. (2011). Characterization of yeasts colonizing in healthy individuals. Medical Mycology January, 49, 103–106.

[13] Peters B, Wu J, Hayes R and Ahn J. (2017). The oral fungal mycobiome: characteristics and relation to periodontitis in a pilot study. BMC Microbiology, 17(157), 2-11.

[14] Rodríguez L and Jewtuchowicz V. (2016). Molecular characterization of *Candida parapsilosis* species complex in niches of the oral cavity in a cohort of patients from Argentina with different oral and dental clinical manifestations. Journal of Dental Science and Therapy, 1(1), 18-25.

[15] Rodríguez L, Rosa A, Rodríguez J, Nastri L and Jewtuchowicz V. (2018). 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. Journal of Dental Science and Therapy, 2(1), 1-9.

[16] Holland LM, Schröder MS, Turner SA, Taff H, Andes D, Grózer Z, Gácser A, Ames L, Haynes K, Higgins DG and Butler G . (2014). Comparative phenotypic analysis of the major fungal pathogens *Candida parapsilosis* and Candida albicans. PLoS Pathogens, 10, e1004365.

[17] Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN and Battino M. (2009). Metabolic syndrome and periodontitis: is oxidative stress a common link? Journal of Dental Research, 88(6), 503-518.

[18] D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J and Donos N. Oxidative stress, systemic inflammation, and severe periodontitis. Journal of Dental Research, 89(11), 1241-6.

[19] Monzón J, Acuña M and Cuzziol F. (2015). Salivary as an indicator of changes in periodontal tissues. Revista de la Facultad de Odontología FOUNNE, 8(1), 7-20.

[20] Susewind S, Lang R and Hahnel S. (2015). Biofilm formation and Candida albicans morphology on the surface of denture base materials. Mycoses, 58(12), 719-27.

[21] Durán EL, Mujica MT, Jewtuchowicz VM, Finquelievich JL, Pinoni MV and Iovannitti CA. (2007). Examination of the genetic variability among biofilm-forming Candida albicans clinical isolates. Revista Iberoamericana de Micología, 24(4), 268-71.

[22] Treviño-Rangel RJ, Rodríguez-Sánchez IP, Rosas-Taraco AG, Hernández-Belloa R, González JG and González GM. (2015). Biopelícula formation and genetic variability of BCR1 gene in the *Candida parapsilosis* complex. Revista Iberoamericana de Micología, 32(3), 180-4.

[23] Peeters E, Nelis HJ and Coenye T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of Microbiological Methods, 72(2), 157-65.

[24] Sánchez-Vargas LO, Estrada-Barraza D, Pozos-Guillén AJ and Rivas-Cáceres R. (2013). Biopelícula formation by oral clinical isolates of Candida species. Archives of Oral Biology, 58(10), 1318-26.

[25] Oggioni MR, Trappetti C, Kadioglu A, Cassone M , Iannelli F, Ricci S, Andrew PW and Pozzi G . (2006). Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Molecular Microbiology, 61(5), 1196–1210.

[26] Silva S, Henriques M, Martins A, Oliveira R, Williams D and Azeredo J. (2009). Biofilms of non-Candida albicans Candida species: quantification, structure and matrix composition. Medical Mycology, 47(7), 681-89.

[27] Tavanti A, Hensgens LA, Mogavero S, Majoros L, Senesi S and Campa M. (2010). Genotypic and phenotypic properties of *Candida parapsilosis* sensu strictu strains isolated from different geographic regions and body sites. BMC Microbiology, 10(203), 1-11.

[28] de Barros PP, Rossoni RD, De Camargo Ribeiro F, Campos Junqueira J and Cardoso Jorge AO. (2017). Temporal Profile of Biofilm Formation, Gene Expression and Virulence Analysis in Candida albicans Strains. Mycopathologia, 182, 285–295.

[29] Pannanusorn S, Ramírez-Zavala B, Lünsdorf H, Agerberth B, Morschhäuser J and Römling U. (2014). Characterization of Biofilm Formation and the Role of BCR1 in Clinical Isolates of *Candida parapsilosis*. Eukaryotic Cell, 13(4), 438-51.

[30] Silva-Dias A, Miranda IM, Branco J, Monteiro-Soares M, Pina-Vaz C and Rodrigues AG. (2015). Adhesion, biofilm formation, cell surface hydrophobicity and antifungal planktonic susceptibility: relationship among Candida spp. Frontiers Microbiology, 6, 205.

[31] Hasan F, Xess I, Wang X, Jain N and Fries BC. (2009). Biofilm formation in clinical Candida isolates and its association with virulence. Microbes and Infection, 11(8-9), 753-761.

[32] Jain N, Kohli R, Cook E, Gialanella P, Chang T and Fries BC. (2007). Biofilm formation by and antifungal susceptibility of Candida isolates from urine. Applied Environmental Microbiology, 73(6), 1697-1703.

[33] Brilhante RS, Sales JA, Queiroz da Silva ML, de Oliveira JS, Pereira LA, Pereira-Neto WA, Cordeiro R, Costa JJ, Castelo-Branco D, Marcos and Gadelha F. (2018). Antifungal susceptibility and virulence of *Candida parapsilosis* species complex: an overview of their pathogenic potential. Journal of Medical Microbiology, 67(7), 903-14.

[34] Nikoomanesh F, Roudbarmohammadi S, Roudbary M, Bayat M, Heidari G [2016]. Investigation of *bcr1* gene expression in *Candida albicans* isolates by RT-PCR technique and its impact on biofilm formation. Infect Epidemiol Med, 2(1):22-4.

[35] Alisafaei F, Jokhun S, Shivashankary GV and Shenoyn VB. (2019). Regulation of nuclear architecture, mechanics, and nucleocytoplasmic shuttling of epigenetic factors by cell geometric constraints. PNAS, 116(27), 13200–209.

[36] Wang N and Stamenovic D. (2000). Contribution of intermediate filaments to cell stiffness, stiffening and growth. Am. J. Physiol. Cell Physiol, 279, 188-194.

[37] Shafeeq S, Pannanusorn S, Elsharabasy Y, Ramírez-Zavala B, Morschhäuser J and Römling U. (2019). Impact of manganese on biofilm formation and cell morphology of *Candida parapsilosis* clinical isolates with different biofilm forming abilities. FEMS Yeast Research, 19(6), foz057.

[38] Tomás I, Camelo-Castillo A, Balsa-Castro C, Castellano A, Novoa L and Mira A. (2016). Nuevo modelo de patogenia de la periodontitis crónica: de la enfermedad infecciosa a la disbiosis polimicrobiana. RCOE, 21(3), 131-145.

[39] Lourido Pérez H, Martínez Sánchez G, Fleitas Vigoa D and Fernández Becerra J. (2009). Ambiente redox salival: Comparación entre pacientes con enfermedad periodontal inflamatoria y pacientes periodontalmente sanos. Revista de Ciencias Médicas, 13(2).

[40] Sculley D and Langley-Evans S. (2002). Salivary antioxidants and periodontal disease status. Proccedings of the Nutrition Society, 66(1), 137-143.

How to cite this article

Rodríguez ML, Alcaraz ES, Rosa AC and Jewtuchowicz VM. (2020). The oral cavity promotes virulence of *Candida parapsilosis* sensu stricto via up-regulation of *BCR1*. World Journal of Advanced Research and Reviews, 6(1), 120-133.