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Virulence factors in *Candida albicans* and *Candida parapsilopsis* species and minimal inhibitory concentration for fluconazole

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Abstract

This study pursued the analysis of pathogenity and virulence factors of two species belonging to the genus Candida: Candida albicans and Candida parapsilosis. Analyses done in this direction were determining adherence capabilities to the Hela-2 cellular line, hydrolytic enzyme production capacities (proteinases, phospholipases, hemolytic factor) and fluconazole susceptibility.

Results obtained showed a higher expression of virulence factors in Candida albicans than in Candida parapsilosis. Candida albicans showed increased adherence capabilities to the Hela cellular line, increased hydrolytic enzymes synthesis, and some of the strains may even develop resistance to fluconazole. Candida parapsilosis has lower adherence capabilities than Candida albicans and hydrolytic enzyme production is less or none.

Keywords*:* Pathogenity, *Candida albicans*, Adherence

Introduction

Infections caused by species of the genus *Candida* have lately taken a high place in the hierarchy of lethal infections. Due to this fact, but also because the antifungal substances involved in the therapy of these infections have a high degree of toxicity for the human body, knowledge of the virulence and pathogenity factors is important in developing strategies against these microorganisms.

Species of the genus *Candida* are commensal microorganisms found in teguments, digestive tractus, urogenital tractus. Certain circumstances such as immunosuppression (as a result of drugs and/or alcohol consumption, but also as a result of chemotherapy, viral infections,) prolonged administering of broad-spectrum antibiotics, pH modifications of the vaginal mucosa (with pregnant women), the use of implants made from different biomaterials determine in some cases the switch from commensalisms to parasitism (1,6). Among the infections caused by these microorganisms are: angular cheilitis, periodontitis, osteitis, faringitis, vaginitis, (10).

The pathogenity of these microorganisms is due to the presence of virulence factors such as: cellular dimorphism, adherence capabilities to cellular and inert substrate, the secretion of hydrolytic enzimes and resistance to antifungal chemicals (4,5, 8,11).

The purpose of this study is to show the virulence factors of *C. albicans* and *C.*

parapsilosis species and to determine the minimum inhibitory concentration for fluconazole, an antifungal that's frequently used in yeast infections.

Materials and methods

Microorganisms: 8 pathogenic yeast strains belonging to the genus *Candida* isolated from patients with different illnesses were used. 6 of these strains – *C. albicans* CMGB 322, 325, 236, 327, 328 and 329 and 2 *C. parapsilopsis* CMGB323 and CMGB324 strains. These strains were isolated from vaginal infection (CMGB322), lung infections (CMGB323 and 324) and urinary infections (CMGB325, 326, 327 and 328). The strains identified were included into the microorganism collection of MICROGEN research center. The reference strains were *C. albicans* ATCC 10231 and *C. krusei* CMGB 221.

Yeast filamentation, adherence and invasiveness capabilities to cellular substrate

The HeLa-2 human tumor cell line was used in order to determine the adherence of pathogen yeasts and their pathogenic potential. The line was obtained by cultivating cells in Eagle MEM (EMEM-Gibco) medium, with added 10% bovine fetal serum, 1% glutamine, penicillin-streptomycin solution 1%, fungizone solution 1%, ITS 1%. 1 ml yeast cellular suspension (that had been cultivated for 18 hours in liquid YPG at 37°C) was added to the HeLa plates and incubated for 2 hours at 37°C. After the incubation period, the cellular monostrate was washed with a saline buffer solution pH 7.4 and fixed for 5 minutes in methanol. After this step, the plates were colored with Giemsa solution for 20 minutes and then washed with tap-water and dried. Examination was done at a microscope with an immersion objective (2).

Proteinase synthesis

Culture medium with the following composition was used to determine proteinase synthesis (%):YNB1,17, agar1,5, (V fraction) 0,2, and glucose0,2. The strains were grown in liquid YPG – Yeast Peptone Glucose – containing (%): yeast extract 1, glucose 2, peptone 1, for 20 hours at 37°C. At the end of the incubation period, 1,5 ml of culture was centrifuged for 6 minutes at 5000 RPM and the sediment washed twice with sterile distilled water and resuspended in sterile distilled water to a cellular density of 10^8 cells/ml. 10 µl of suspension were seeded into Petri dishes containing culture medium supplemented with BSA and incubated at 28°C for 7 days.

At the end of the incubation time colony diameter and total colony and halo areas were measured.

This is a semi-quantitative activity dosage method and is expressed by the proteinase synthesis index (Pz), calculated according to formula (Luo):

$$Pz = \frac{colony\ diameter}{total\ diameter\ (colony + halo)}$$

Phospholipase synthesis

Phospholipase synthesis was done by using a minimal growth medium containing (%)peptone 0,48, dextrose 4, sodium chloride 7,3, calcium chloride 0,06 and agar-agar 2 supplemented with 8% egg yolk. 10 µl of suspension were seeded into Petri dishes containing this medium and incubation was done at 37°C for 48 hours. Phospholipase actitivity (Pz) was determined like proteinase synthesis.

Hemolytic factor synthesis

In order to determine hemolytic factor synthesis abilities the strains were grown on Saboraud (Promega) medium supplemented with 3% glucose and 7% rabbit's blood. 10 µl of suspension were seeded on the plate and incubation conditions were 37°C for 48 hours. The haemolysis index (Hi) was done with the same method as Pz index.

Fluconazole minimum inhibitory concentration

In order to determine the sensibility to fluconazole, the strains were grown on liquid YPG medium for 20 hours. At the end of the incubation time cells were sedimented, washed twice with sterile distilled water and then diluted in sterile distilled water to 0,5 McFarland density. This suspension was used to evenly seed on Saboraud medium plates using a sterile swab. The fluconazole-impregnated strip was placed in the middle of the plate.

Plate incubation was done for 24 hours at 37°C and at the end of the incubation time the minimum inhibitory concentration.

Results and discussions

A set of 8 strains of pathogenic yeasts belonging to the Microorganism Collection of The Center for Research, Training and Consulting – MICROGEN and identified through polypahsic taxonomy methods (data not shown) was used for this study. After polyphasic identification 6 of these strains (CMGB 322, 325, 236, 327, 328 and 329) were identified as being *C. albicans* and two strains (CMGB323 and CMGB324) were identified as belonging to the species *C. parapsilopsis*.

Yeast filamentation and adherence capability to cellular substrate (HeLa-2 line)

Adherence is a concept of fundamental importance due to the fact that adherence mechanisms are the first stage necessary for most physiological and pathological processes (1, 8). It is noted that in the cases of the studied microorganisms, the *C. albicans* strains CMGB 322, 325, 326 327 , 328 and *C. albicans* CMGB 329 have high adherence both to the HeLa-2 cellular line and to the inert substrate (adherence plate) and develop filamentation capacity due to the bovine fetal serum in the growth medium. Unlike these, the *C. parapsilosis* strains CMGB 323 and CMGB 324 adhere to the cellular substrate but with a far lower frequency than the strains identified as being *C. albicans* and do not form filaments.

Proteinase synthesis

In a first stage the strains were tested for proteinase synthesis activity. It is known that, in order to penetrate and invade the host organism during the infectious process, microorganisms must present certain abilities, among which is synthesis of proteinases, enzymes involved in damaging tissue (11). For *C. albicans* these enzymes are coded by 10 SAP genes (secreted aspartil proteinases). The other species of the genus *C.* posses only a part of these genes, which is correlated with low pathogenity and virulence compared to *C. albicans (12). C. albicans* strains manifested a lysis halo associated with proteinase production whereas *C. parapsilosis* strains did not present a halo (Figure 2).

Protease activity quantification can be achieved (Figure 3) by calculating the Pz index.

Pz index values obtained were between 0.4 and 1. Strains with Pz index less than 0,7 were classified as high protease activity and those with Pz index higher than 0,7 were classified as low protease activity.

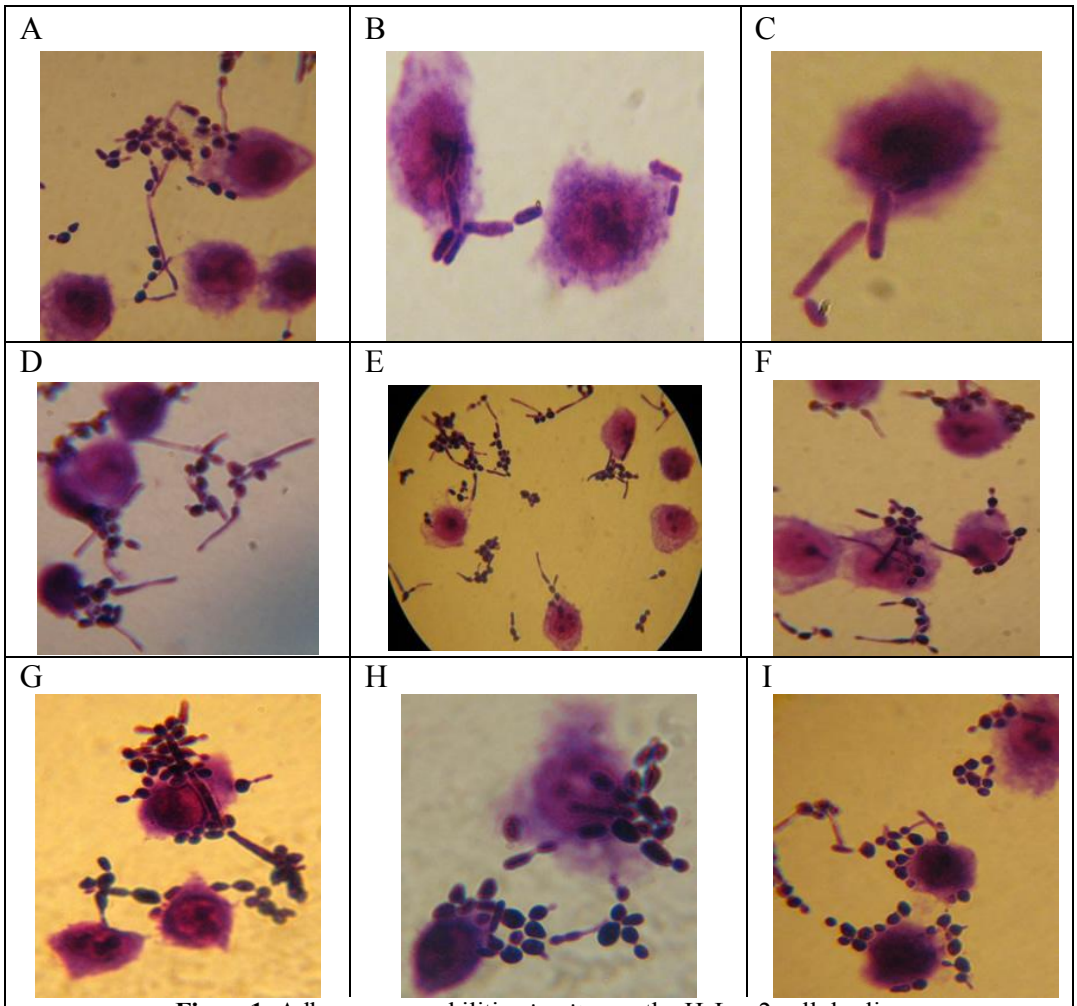


Figure1. Adherence capabilities *in vitro* on the HeLa -2 cellular line
A- *C. albicans* CMGB 322, **B-** *C. parapsilosis* CMGB 323, **C-** *C. parapsilosis* CMGB 324, **D-** *C. albicans* CMGB 325, **E-** *C. albicans* CMGB 326, **F-** *C. albicans* CMGB 327, **G-** *C. albicans* CMGB 328, **H-***C. albicans* ATCC 10231, **I-** *C. albicans* CMGB 328, **J-** *C. albicans* CMGB 329

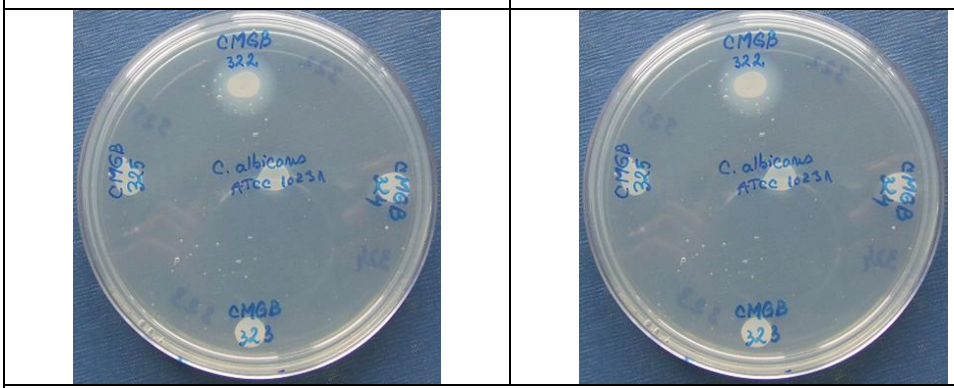


Figure 2 Showing aspartyl-proteinase activity
A- *C. albicans* CMGB 322, *C. parapsilosis* 323, *C. parapsilosis* 324, *C. albicans* 325, *C. albicans* ATCC 10231
B- *C. albicans* CMGB 326, *C. albicans* 327, *C. albicans* 328, *C. albicans* 329, *C. albicans* ATCC 10231

Phospholipase synthesis

Phospholipase synthesis was highlighted by growing the strains on a culture medium supplemented with egg yolk and observing the build-up of white precipitate under the colonies. After 24 hours incubation all the strains grew well, except for *C. parapsilosis* CMGB 323, *C. parapsilosis* 324 and *C. albicans* CMGB 328 that manifested slower development. *C. albicans* ATCC 10231 (reference strain), *C. albicans* CMGB 325, *C. albicans* 326, *C. albicans* 327, and *C. albicans* 329 strains manifested build-up of precipitate under the colony. The precipitate that forms and accumulates under the colony is a complex between calcium chloride (from the culture medium) and the fatty acids released into the medium by the action of phospholipases on egg yolk (Figure 4) (3, 9).

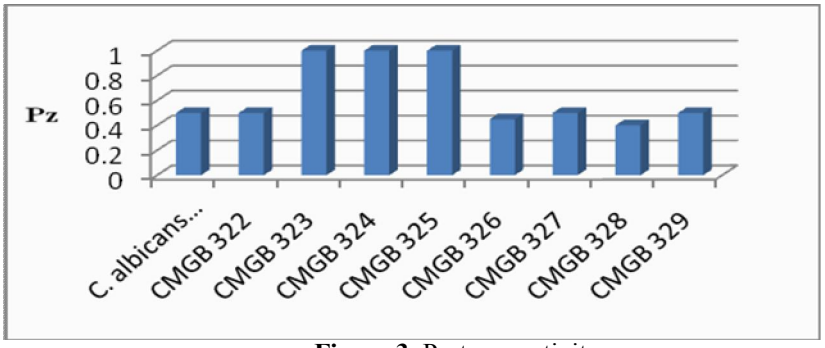


Figure 3. Protease activity

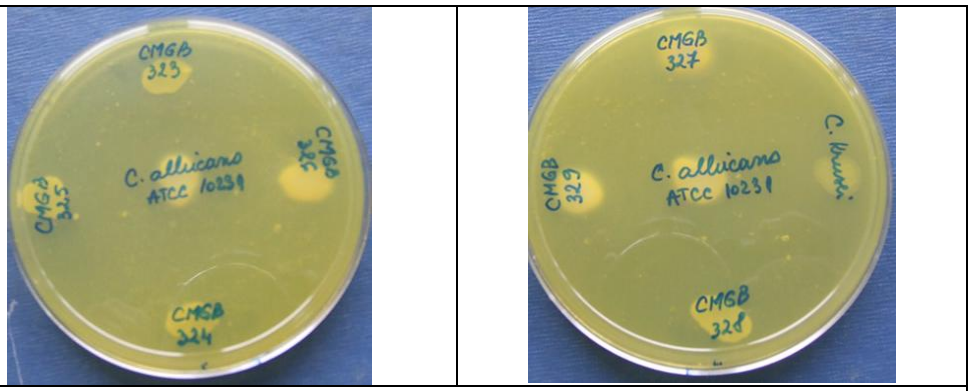


Figure 4. Phospholipase activity (after 24 hours incubation time)

After 48 hours, an increase in phospholipase activity was observed for most of the strains except *C. krusei* CMGB 221 (reference strain), *C. parapsilosis* CMGB 323 and *C. albicans* CMGB 328 (Figure 5).

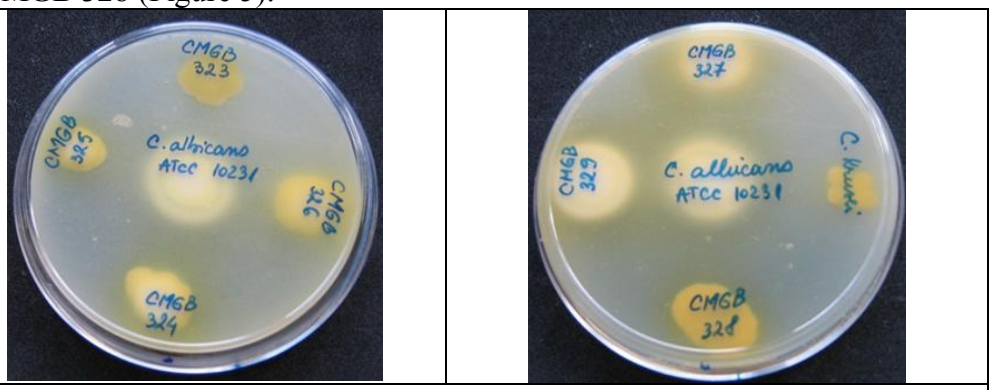


Figure 5. Phospholipase activity (after 48 hours incubation time)

After analysis, isolates were classified according to Pz index value into strains with low phospholipase activity (Pz higher than 0,7 - strains *C. krusei* and *C. parapsilosis* CMGB 323), with medium phospholipase activity (Pz very close to 0,7 - *C. parapsilosis* CMGB 324 and *C. albicans* CMGB 325) and strains with high phospholipase activity (Pz less than 0,7 - strains *C. albicans* CMGB 326, *C. albicans* CMGB 327, *C. albicans* CMGB 328, *C. albicans* CMGB 329 and *C. albicans* ATCC 10231 (Figure 6).

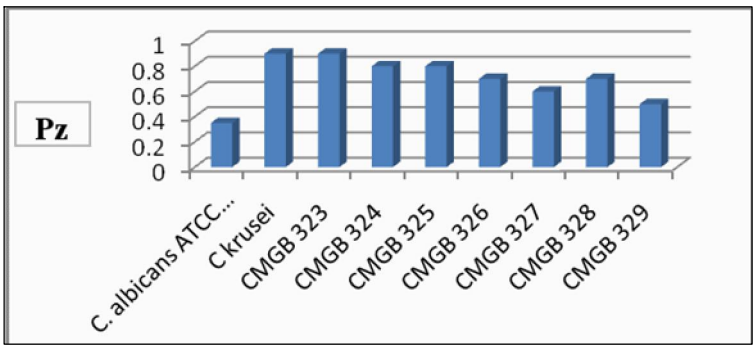
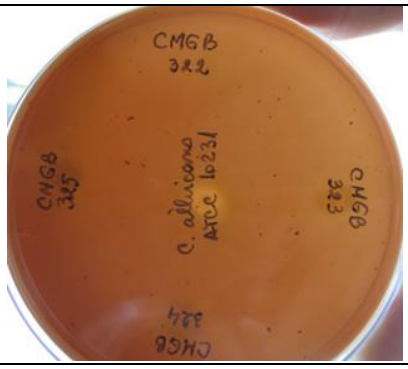
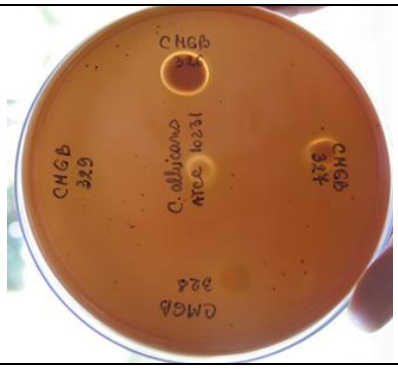


Figure 6. Phospholipase activity

Hemolytic factor synthesis

The ability of pathogenic yeasts to retain iron was shown to represent an essential step in both survival and infectious process. *C. albicans* has the ability to grow on minimal growth medium supplemented with rabbit's blood which is shown by the presence of a clear halo around the culture spot tsang, Linares.

C. albicans CMGB 326, *C. albicans* 327 and *C. albicans* ATCC 10231 (reference strain) showed strong hemolytic activity (Hi between 0,4 – 0,6) both after 24 and 48 hours of incubation, while *C. albicans* CMGB 322, *C. parapsilosis* 324 and *C. albicans* CMGB 325 showed only weak hemolytic activity (Hi between 0,9 – 1,0) after 48 hours (Figure 7 and Figure 8).

A	A
	

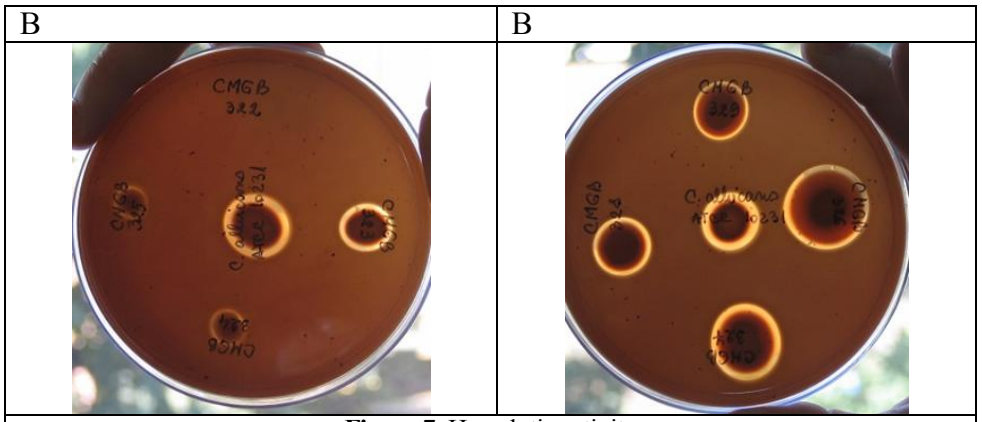


Figure 7. Hemolytic activity
A-(24h incubation) *C. albicans* CMGB 322, *C. albicans* CMGB 325, *C. albicans* CMGB 326, *C. albicans* CMGB 327, *C. albicans* CMGB 328, *C. parapsilosis* CMGB 323, *C. parapsilosis* 324
B-**(48h incubation)** *C. albicans* CMGB 322, *C. albicans* CMGB 325, *C. albicans* CMGB 326, *C. albicans* CMGB 327, *C. albicans* CMGB 328, *C. parapsilosis* CMGB 323, *C. parapsilosis* 324

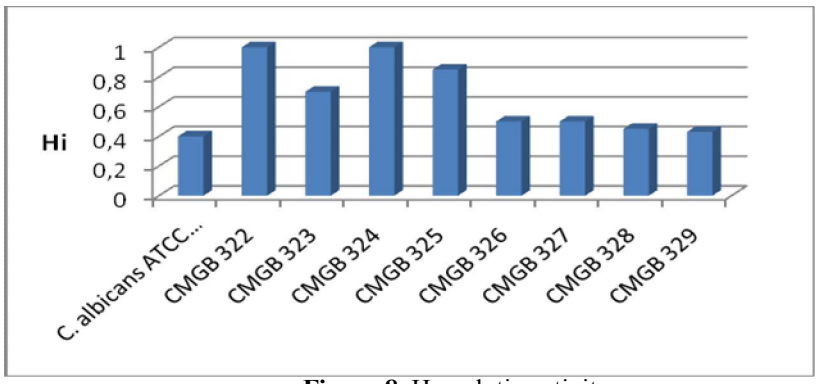


Figure 8. Hemolytic activity

Fluconazole minimum inhibitory concentration (MIC)

Fluconazole is one of the widest used antifungals for the treatment of infections caused by pathogenic yeasts due to high antimicrobial activity and low toxicity for the human body (7). The tests were performed on 2 *C. albicans* strains (CMGB 325 and CMGB 329) and 2 *C. parapsilosis* strains (CMGB 323 and CMGB 324). Testing revealed that *C. parapsilosis* strains and *C. albicans* strain CMGB 325 manifested sensibility to fluconazole at the folowing minimum inhibitory concentrations: 1,5µg/ml *(C. parapsilosis* CMGB 323), 1.0µg/ml (*C. parapsilosis* CMGB 324) and 0,50µg/ml *(C. albicans* CMGB 325). *C. albicans* CMGB 329 was the only strain resistant to fluconazole (Figure 9).

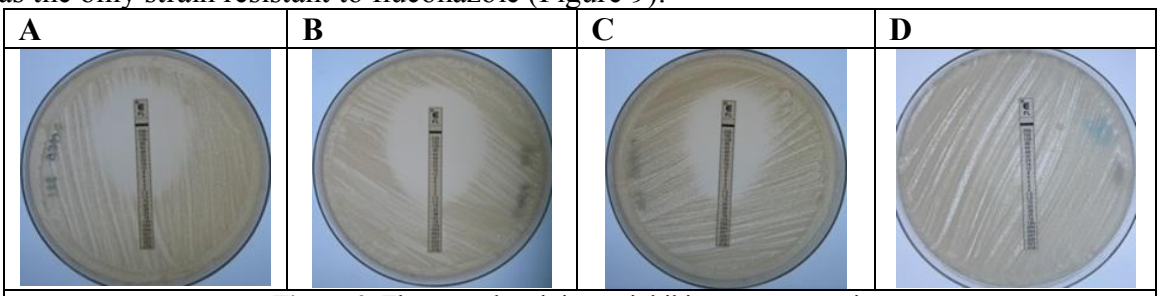


Figure 9. Fluconazole minimum inhibitory concentration
A- *C. parapsilosis* CMGB 323, **B-** *C. parapsilosis* CMGB 324, **C-** *C. albicans* CMGB 325, **D-** *C. albicans* CMGB 329

Comparative evaluation of the tested strains' virulence

The analysis of virulence traits (table 1) reveals that:

- Enzymatic activity level is species dependent.
- The lowest activity was observed for *C. parapsilopsis* CMGB 324.
- *C. albicans* strain CMGB 329 shows maximum enzymatic activity also correlated with fluconazole resistance.

Table 1. Virulence traits of yeast strains

Strain		**Protease activity (Pz)**	**Phospholipase activity (Pz)**	**Hemolytic activity (Hi)**	**MIC (µg/ml)**
*C. albican*s	CMGB 322	0,5	-	1	-
	CMGB 325	1	0,8	0,85	0,50
	CMGB 326	0,45	0,7	0,5	-
	CMGB 327	0,5	0,6	0,5	-
	CMGB 328	0,4	0,7	0,45	-
	CMGB 329	0,5	0,5	0,43	R
	C. albicans ATCC 10231	0,5	0,35	0,4	-
C. parapsilosis	CMGB 323	1	0,9	0,7	1,5
	CMGB 324	1	0,8	1	1

Conclusions

Virulence factors from two *Candida* species: *C. albicans* and *C. parapsilosis,* were analyzed in this study and the following was concluded:

1. *C. albicans* strains show adherence to cellular lines, increased hydrolytic enzyme secretion ability and some are resistant to fluconazole, while *C. parapsilopsis* strains have lower cellular line adherence and the absence of hydrolytic enzyme secretion or only weak secretion and are sensible to fluconazole;
2. *C. albicans* strain CMGB 329 stood out as having high enzymatic activity (protease, phospholipase and hemolytic) correlated with fluconazole resistance.

References

1. CALDERONE R. A., BRAUN P. Adherence and Receptor Relationships of *Candida albicans*. Microbiological Reviews, 1991, **55** (1), p: 1-20.
2. CHIFIRIUC M. C., LARION C., IORDACHE C., LIXANDRU M., DRACEA O., BLEOTU C., BUCUR M, ISRAIL A. M. Influence of soluble mediators upon the expression of different physiological and virulence hallmarks of bacteria. Roumanian Biotechnological Letters.2008, **13** (2), p: 3631-3642
3. GHANNOUM M. A. Potential Role of Phospholipases in Virulence and Fungal Pathogenesis, Clinical Microbiology Reviews, 2000, **13** (1), p: 122-143
4. HUBE B., NAGLIK J. *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiology, 2001, p: 1997-2005.
5. LINARES C. E. B., LORETO E. S., SILVEIRA C. P., POZZATTI P., SCHEID L. A., SANTURIO L. M., ALVES S. H. Enzymatic and hemolytic activities of *Candida dubliniensis* strains. Rev. Inst. Med. Trop. S. Paulo. 2007, **49** (4), p: 203-206
6. NAGLIK R. J., CHALLACOMBE S. J., HUBE B. *Candida albicans Secreted Aspartyl Proteinase in Virulence and Pathogenesis*, Microbiology and Molecular Biology Reviews, 2003, p: 400-428,
7. PFALLER M.A.,DIEKEMA D.J.,MESSERS.A.,BOYKEN L.,HOLLIS R.J.,. Activities of fluconazole and voriconazole against 1,568 recent clinical isolates of *Candida* species determined by broth microdilution

disk diffusion, and etest methods:report from the ARTEMIS global antifungal susceptibility program, 2001. Journal of Clinical Microbiology, 2003, **41(**4), p:1440-1446.

8. SENET J. M. *Candida* adherence phenomena, from commensalism to pathogenicity. Internatl Microbiol., 1998, **1**, p: 117-122.
9. TSANG C. S. P., CHU F. C. S., LEUNG W. K., JIN L. J., SAMARANAYAKE L. P, SIU S. C. Phospholipase, proteinase and hemolytic activities of *Candida albicans* isolated from oral cavities of patients with type 2 diabetes mellitus. Microbiol., 2007, **56**, p: 1393-1398
10. WEBB B. C., THOMAS C. J., WILLCOX M. D. P., HARTY D. W. S., KNOX K. W. *Candida*- associated denture stomatitis. Aetiology and management: A review. Part2. Oral diseases caused by *Candida* species. Australian Dental Journal 1998, **43**, p: 160-166
11. WU T., WRIGHT K., HURST S. F., MORRISON C. J. Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in subinhibitory concentrations of fluconazole. Antimicrobial Agents and Chemotherapy, 2000, **44** (5), p: 1200-1208.
12. ZAUGG C.,VON- ZEPELIN M., REICHARD U., SANGLARG D., MONOD M. Secreted Aspartic proteinase Family of *Candida tropicalis*, Infection and Immunity, 2000, **69** (1), p: 405-412