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BIOATIVIDADE, BIOESTRUTURA E MORFOLOGIA DE BIOFILMES DE *Candida* spp. DESENVOLVIDOS NA PRESENÇA DE FLUCONAZOL

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Doutor em Clínica Odontológica – Área de Prótese Dental.

Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

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RESUMO

O biofilme de *Candida* spp. formado na superfície de próteses removíveis, é o principal fator etiológico da candidose, sendo a *C. albicans* e a *C. glabrata* as espécies mais prevalentes nesta condição. O antifúngico fluconazol (FLZ) é frequentemente utilizado no tratamento da candidose, porém o sucesso tem sido limitado devido a resistência desenvolvida pela *Candida* a esse medicamento. Considerando a importância da estrutura e morfologia do biofilme de *Candida* na candidose, o objetivo neste estudo foi avaliar o efeito de FLZ na bioatividade, bioestrutura e morfologia celular de biofilmes de *Candida* spp. desenvolvidos na presença deste antifúngico. Espécimes (10 mm x 2 mm) foram confeccionados utilizando resina de poli(metilmetacrilato) (PMMA), polimerizada por banho de água quente. Películas de saliva foram formadas na superfície da PMMA, e biofilmes de um isolado referência e dois isolados clínicos de *C. albicans* (ATCC 90028, P01, P34) e *C. glabrata* (ATCC 2001, P11, P31) foram desenvolvidos por 48h. Dois grupos foram formados: controle e experimental. FLZ a 2,56 µg/mL, concentração biodisponivel na saliva, foi adicionado ao meio de cultura do grupo experimental. Os meios de cultura do grupo controle e experimental foram trocados a cada 24 h. As bioatividades dos biofilmes foram avaliadas utilizando análise colorimétrica de redução por XTT. A bioestrutura analisada através do Microscópio Confocal à Laser e a morfologia celular avaliada utilizando o Microscópio Eletrônico de Transmissão. Os dados foram analisados pelo Test t de Student com nível de significância de 5%. A presença do FLZ reduziu a bioatividade de todos os biofilmes de *C. albicans* ($p<0.001$), porém não alterou a estrutura e morfologia da *C. albicans* P34. Quanto à bioatividade e bioestrutura dos biofilmes de *C. glabrata*, não foram encontradas diferenças estatisticamente significantes entre os grupos controle e experimental. Pode-se concluir que as alterações da bioatividade, bioestrutura e morfologia celular, como resposta ao tratamento com FLZ, na concentração biodisponível na saliva, depende da cepa de *Candida* spp. avaliada.

Palavras-chave: Biofilme, *Candida albicans*, *Candida glabrata* e fluconazol.

ABSTRACT

Candida spp. biofilm formed on removable denture surfaces is considered the main etiologic factor of candidosis, being the *C. albicans* and *C. glabrata* the species most frequently found in this condition. The antifungic fluconazol (FLZ) is commonly used in the treatment of candidosis, however its success is limited due to the resistance developed by Candida to this medicament. Considering the importance of the structure and morphology of *Candida* biofilms in the candidosis, the aim of this study was to evaluate the effect of FLZ on the bioactivity, biostructure and morphology of *Candida* spp. biofilms formed in the presence of this antifungal agent. Specimens (10 mm x 2 mm) were fabricated using water bath poly(methylmethacrylate) resin (PMMA). Salivary pellicles were formed on the PMMA surface, and biofilms of a reference strain and two clinical isolates of *C. albicans* (ATCC 90028, P01, P34) and *C. glabrata* (ATCC 2001, P11, P31) were developed for a period of 48h. Two groups were formed: control and experimental. FLZ at 2.56 µg/mL, concentration bioavailable in saliva, was added to the medium of the experimental group. The culture mediums of the control and experimental groups were changed at 24 hours. The bioactivities of the biofilms were evaluated with XTT reduction colorimetric assay. The biostructure was analyzed by the Confocal Scanning Laser Microscopy and the cell morphology analyzed by the Transmission Electron Microscopy. The data were analyzed by Student's t-test, with significance level set at 5%. The presence of FLZ decreased the bioactivity of all *C. albicans* biofilms ($p<0.001$), it did not change the structure and morphology of P34. As regards *C. glabrata* biofilms bioactivity and biostructure, no statistically significant differences were found between control and experimental groups for biofilms of all strains. It could be concluded that the alterations in bioactivity, biostructure and cell morphology in response to the treatment with fluconazole, bioavailable concentration present in saliva, depends on the *Candida* spp. strain

Keywords: Biofilm, *Candida albicans*, *Candida glabrata* and fluconazole.

SUMÁRIO

INTRODUÇÃO

A cavidade bucal oferece uma variedade de nichos à colonização microbiana, permitindo a sobrevivência de bactérias, vírus e fungos (Socransky & Haffajee, 1994; Sweeney *et al*., 1998; Jabra-Rizk *et al*., 2001). Dentre os fungos destaca-se as *Candida* spp., presentes na cavidade oral na forma comensal em cerca de 20% a 50% da população dentada saudável (Ellepola & Samaranayake, 2000; Perezous *et al*., 2005). Contudo, sob determinadas condições, a *Candida* comporta-se como patógeno oportunista, causando infecções que vão desde lesões nas mucosas até disseminações sistêmicas graves e invasivas (de Repentigny *et al*., 2000; Leung *et al*., 2000). Várias são as condições predisponentes ao desenvolvimento da candidose, com destaque para os tratamentos imunossupressores, síndrome da imunodeficiência adquirida, uso indiscriminado de antibióticos e próteses mal adaptadas e/ou precariamente higienizadas (Wilkieson *et al*., 1991; Cheng *et al.,* 2005; Nucci & Marr, 2005).

C. albicans é considerada o principal agente etiológico da candidose oral, devido à sua capacidade de aderir e colonizar a base das próteses e a mucosa subjacente (Cannon & Chaffin, 1999; Akpan & Morgan, 2002; Barbeau *et al*., 2003). Entretanto, *C. glabrata* é também frequentemente isolada das superfícies de próteses e da mucosa oral (Pereira-Cenci *et al*., 2008), representando o segundo patógeno mais prevalente nessas infecções (Lachke *et al*., 2002).

Visando o restabelecimento da saúde do paciente com candidose, tem-se preconizado a utilização de fármacos, dentre os quais se destaca o uso do fluconazol (FLZ), fungistático pertencente ao grupo dos azóis (Spellberg *et al*., 2006), devido a sua alta biodisponibilidade e a facilidade de administração via oral ou intravenosa. Adicionalmente, destaca-se o baixo custo deste medicamente, uma vez que sua patente é de domínio público (Spellberg *et al,.* 2006). O FLZ limita a biossíntese do ergosterol, componente essencial para manutenção da integridade da membrana plasmática. (Niimi *et al*., 2010; Ellepola & Samaranayake, 2000; Cannon *et al*., 2009).

No entanto, algumas dificuldades têm sido reladas em relação ao tratamento com FLZ, tais como a resistência adquirida pelos microrganismos aos componentes azólicos, (Chandra *et al*., 2001; Mann *et al*., 2009), o aumento da prevalência de espécies *não-albicans* nas infecções (Bagg, *et al*., 2003; Li *et al*., 2007) e a complexa estrutura do biofilme de *Candida* que dificulta a penetração dos agentes antifúngicos (Baillie & Douglas 1999).

A resistência adquirida aos antifúngicos tem sido um dos principais problemas nas infecções por *Candida*, pois permite o desenvolvimento de novas linhagens resistentes (Mann *et al*., 2009). Em alguns casos, a terapia com antifúngicos da classe dos azóis reduz a condição inflamatória inicial, porém os aspectos clínicos são recorrentes após a supressão do tratamento (Chandra *et al*., 2001; Mann *et al*., 2009). Os insucessos clínicos geralmente correlacionam-se com a sensibilidade diminuída dos isolados ao FLZ (Dupont & Drouhet, 1988; Cameron *et al*., 1993; Odds, 1993) e estão frequentemente relacionados a tratamentos anteriores com esta droga (Garcia-Hermoso *et al*., 1995).

Também tem sido observado o aumento da prevalência de infecções causadas por *Candida não-albicans,* (Bagg *et al*. 2003; Li *et al*., 2007), especialmente as ocasionadas pela *C. glabrata*, que são mais difíceis de serem tratadas e estão fortemente associadas às infecções sistêmicas generalizadas com alta taxa de mortalidade (Li *et al*., 2007; Pfaller & Diekema, 2007). Esse aumento na prevalência ainda não está completamente elucidado, mas pode estar relacionado com os repetitivos tratamentos antifúngicos, os quais têm maior efeito sobre as espécies de *C. albicans*, favorecendo o desenvolvimento das espécies *não-albicans* (Procop & Roberts, 2004).

Outro fator que dificulta a terapêutica antifúngica é o fato que as *Candida* spp. apresentam a capacidade de se aderir às superfícies de próteses, e de formar comunidades microbianas conhecidas como biofilmes. Os biofilmes de *Candida* são constituídos por camadas de células envoltas por uma matriz extracelular polimérica. A matriz extracelular dificulta a penetração dos agentes

antimicrobianos nas camadas mais basais dos biofilmes, limitando a ação dos agentes antifúngicos (Baillie & Douglas, 1999).

Todos esses fatores resultam em uma menor susceptibilidade dos biofilmes de *Candida* ao tratamento antifúngico. A susceptibilidade ao FLZ vem sendo avaliada através da análise colorimétrica por redução de XTT (Kuhn et al., 2003), que é uma substância incolor que quando metabolizada pelas células microbianas é convertido em um produto com coloração amarronzado denominado formazan. Este promove uma alteração na coloração dos meios em maior ou menor intensidade possibilitando a mensuração da susceptibilidade antifúngica de biofilmes sem a disruptura estrutural do mesmo (Kuhn *et al*., 2003).

Apesar de alguns estudos avaliarem o efeito do FLZ nos biofilmes de *Candida*, utilizando o XTT, esses estudos primeiramente formaram biofilmes, quando estes estavam maduros eram submetidos à aplicação única do FLZ (Konopka *et al*., 2010; Chandra *et al*., 2001). Nenhum estudo foi encontrado que simulasse a condição clínica na qual os biofilmes de *Candida* spp. são capazes de se desenvolver sobre as superfícies protéticas na presença do FLZ, condição que favorece o desenvolvimento da resistência adquirida dos biofilmes de *Candida* spp. ao FLZ.

Diante disto, o presente estudo teve o objetivo de avaliar se biofilmes de *Candida* spp. desenvolvidos na constante presença do FLZ apresentam alterações na sua bioatividade, bioestrutura e morfologia celular.

Bioactivity, biostructure and morphology of C*andida* spp. biofilms grown in the presence of Fluconazole

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of Fluconazole (FLZ) on the bioactivity, biostructure and morphology of *Candida* spp. biofilms formed in the presence of this antifungal agent. Materials and methods: Specimens were fabricated using water bath poly(methylmethacrylate) resin (PMMA). Salivary pellicles were formed on the PMMA surface, and biofilms of a reference strain and two clinical isolates of *Candida albicans* (ATCC 90028, P01, P34) and *Candida glabrata* (ATCC 2001, P11, P31) were developed for a period of 48 h. Two groups were formed: control and experimental. FLZ at 2.56 µg/mL, concentration bioavailable in saliva, was added to the medium of the experimental group. The culture mediums of the control and experimental groups were changed at 24 h. The bioactivities of the biofilms were evaluated with XTT reduction colorimetric assay. The biostructure was analyzed by Confocal Scanning Laser Microscopy and the morphology by the Transmission Electron Microscopy. The data were analyzed by the Student's t-test, with significance level set at 5%. Results: The presence of FLZ decreased the bioactivity of all *C. albicans* biofilms ($p<0.001$), however, it did not change the structure and morphology of P34. As regards *C. glabrata* biofilm bioactivity and biostructure, no statistically significant differences were found between control and experimental groups for biofilms of all strains. Conclusion: The alterations in bioactivity, biostructure and cell morphology in response to the treatment with fluconazole, at the bioavailable concentration present in saliva, depend on the *Candida* spp. strain.

Keywords: Biofilms, *Candida*, *Candida albicans*, *Candida glabrata* and Fluconazole

Introduction

Candida species are commensal microorganisms with a presence that ranges from 20% to 50% of the microorganisms in the oral cavity of healthy dentate population.[1] However, under predisposing conditions, *Candida* spp. can behave as an opportunistic pathogen causing a variety of infections ranging from mucosal lesions to severe systemic dissemination.[2,3] Among these infections, *Candida*-associated denture stomatitis is a common disease, observed in approximately 11% to 67% of denture wearers,[4] *C. albicans* being the predominant isolate in these conditions.[5] However, *C. glabrata* has frequently been isolated from acrylic surface and the palatal mucosa,[5] and represents the second most prevalent fungal pathogen in the oral cavity.[6]

Fluconazole (FLZ) has been the preferred antifungal agent for the treatment of mucosal and systemic *Candida* spp. infections.[7] The wide use of FLZ to treat *Candida* infections can be attributed to its high bioavailability, reduced cost and the possibility of being administrated orally or intravenously.[7] However, acquisition of resistance to azole compounds has been recorded with several organisms, in particular *C. albicans*.[8] Acquired resistance to antifungal agents has been one of the major problems, as the treatment can lead to selection of microorganisms, favoring infections caused by *Candida non-albicans*.[9,10] In particular infections caused by *C. glabrata*, which is naturally more resistant to antifungal treatment and strongly associated with generalized systemic infections with high mortality rates.[10,11]

Although some studies have been conducted evaluating the effect of FLZ on *Candida* biofilms or as planktonic cells[12-15] these studies were conducted using FLZ after biofilm growth[13,14] measuring the bioactivity of biofilms. However, no study was found, which simulated the clinical condition in which *Candida* biofilms were allowed to grow on the denture surfaces while the patients were undergoing FLZ therapy, a condition that could lead to *Candida* spp. developing resistance to FLZ.

Thus, the aim of the present study was to evaluate whether *Candida* spp. biofilms grown while they were under constant administration of FLZ could have their normal bioactivity, cellular morphology and biostucture altered.

2. Materials and Methods

2.1. Experimental design

This in vitro study had a randomized and blinded design. Specimens of poly(methylmethacrylate) resin were fabricated according to the manufacturer's instructions. After this, the surface roughness was measured and the specimens were randomly divided into 12 groups for biofilm assays. Biofilms of one reference strain and two clinical isolates of *C. albicans* (ATCC 90028, P01, P34) and *C. glabrata* (ATCC 2001, P11, P31) were allowed to develop on specimen surfaces. Two groups were formed: control and experimental. FLZ at 2.56 µg/mL, concentration bioavailable in saliva[16], was added to the medium of the experimental group. The biofilms were developed for 48h, and the bioactivity was

evaluated using XTT. Confocal Scanning Laser Microscopy (CLSM) and Transmission Electron Microscopy (TEM) were used for biostructure and morphology analyses, respectively.

2.2. Preparation of specimens

Specimens were fabricated using acrylic resin polymerized by hot water bath (QC-20 PMMA - Dentsply Ltd., Weybridge, England), according to the manufacturer's instructions, at room temperature (25 ± 1ºC) and 50 ± 5% (relative humidity) under aseptic conditions, using a metal matrix (10 mm in diameter and 2 mm in thick). The specimens were immersed in distilled water at 37 ºC for 48h for residual monomer release.[17] Specimens were ground using progressively smoother aluminum oxide papers (320, 400, and 600 - grit) in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil). The specimens were disinfected with 70% alcohol, and washed twice with sterile distilled water, and then ultrasonicated for 20 min to remove any contaminates and residues from the surface.

2.3. Surface Roughness Measurements

Surface roughness of the acrylic resin specimens was measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd, Kosaka, Japan) accurate to 0.01 µm with total measurement length of 3.2 mm and 0.5 mm/s. Three readings were made for each specimen, and a mean value was calculated.[18] Surface roughness was standardized at 0.31 ± 0.02 µm.

2.4. Biofilm assay

Biofilm assays were performed using two reference strains: *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 and two clinical isolates of each strain (P01 and P34) and (P11 and P31), respectively. The clinical isolates used were from denture wearers without candidosis. Before the experimental procedures, the identity of all isolates was reconfirmed by CHROMagar® *Candida* test (Difco Laboratories, Detroit, MI, USA), and carbohydrate assimilation test using Vitek-2 identification system (bioMérieux, Marcy l'Etoile, France).[19]

Prior to each experiment, each *Candida* strain was grown aerobically on Sabouraud Dextrose Agar at 37°C for 18 h. Then it was inoculated in Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 50 mM glucose and incubated aerobically at 37 °C overnight in an orbital shaker (model NT 151; Kline Shaker; Nova Tecnica Laboratory, Sao Paulo, Brazil). When cells were in the exponential growth phase, they were harvested and washed twice with 25 mL of phosphate buffered saline (PBS; pH 7.2). After, yeast cells were resuspended in YNB supplemented with 100 mM glucose and the suspensions were optically adjusted to density of 10^7 cells/mL.

Biofilms were formed on saliva-coated acrylic resin. The saliva was collected during masticatory stimulation with Parafilm M (American Can Co., Greenwich, CT, USA) in an ice-chilled polypropylene tube and clarified by centrifugation at 10 000 g for 5 min at 4°C. The volunteers provided written informed consent previously approved by the Ethics Committee of Piracicaba Dental School (042/2008). For every experiment the saliva sample was collected at the same time of day and the

volume limited to 50 mL per collection period, in order to make allowance for the circadian rhythm in saliva composition.[20] The supernatant was filtered through a 0.22 µm membrane filter (Corning, NY, USA) and immediately used.[21]

Under aseptic conditions, each specimen was placed inside a well of a pre-sterilized flat bottomed 24-well tissue culture plate and 1 mL of saliva was added. The plate was incubated for 60 min at 37 ^{o}C in an orbital shaker.[22]

Saliva coated specimens were transferred to another pre-sterilized flat bottomed 24-well tissue culture plate, and 2 mL of standard yeast cell suspensions (10^{7}cells/mL) were added to each well and incubated under agitation at 37 ^{0}C for 1.5 h (adhesion phase) in an orbital shaker. After the adhesion phase, the cell suspensions were aspirated and each specimen was gently washed twice with PBS. Afterwards, 2 mL of YNB medium with 100 mM glucose was added to the control group and a mixture of YNB with 100 mM glucose + FLZ (Sigma-Aldrich Corp, St. Louis., MO, USA) at 2.56 µg/ml concentration was added to the experimental groups.[16]

The plates were incubated under agitation at 37 ^{o}C for 48 h in an orbital shaker. After the first 24 h of incubation, the medium was aspirated and biofilms were washed twice with PBS followed by addition of 2 mL of medium (control group) or medium with FLZ (experimental group). Then the biofilms were returned to orbital shaker for a further 24 h to be analyzed.

*2.4.1. B*ioactivity *analysis*

Biofilm bioactivity was performed by XTT reduction assay as previously described.[23] XTT solution was prepared by dissolving the XTT salt (Sigma-Aldrich Corp, St. Louis., MO, USA) in PBS containing 200 mM of glucose. XTT final concentration was 1mg/mL.[23] The solution was filter-sterilized and stored frozen -70^{o}C until use. Menadione (Sigma-Aldrich Corp, St. Louis., MO, USA) solution at a 0.4 mM concentration in acetone was prepared immediately before each assay. For each assay, XTT solution was thawed on ice and mixed with Menadione solution to a 20:1 volume ratio.

Specimens with biofilm were gently placed inside another pre-sterilized flat bottomed 24-well tissue culture plate and 2 mL of the XTT solution (PBS+ 200mM glucose-XTT-Menadione) were added to each well. The plates were covered with aluminum foil and incubated in the dark under agitation at 37 ^{o}C for 3 h.[23] Thereafter, the solution was centrifuged and 500 mL were transferred to spectrophotometer cuvettes. The bioactivity assay was performed using a spectrophotometer (Beckman Coulter, Indianapolis, IN, USA) and the readings were made at 490 nm. The bioactivity assays were performed in triplicate in three independent experiments on different days.

2.4.2. Biostructure analysis

The specimens with biofilms were gently placed inside pre-sterilized flat bottomed 24-well tissue culture plates and stained using Molecular Probe's Live/Dead *BacLight* viability kits) (Invitrogen-Molecular Probes, Eugene, OR, USA). A kit

comprising SYTO-9 and propidium iodide (PI) was used. STYO-9 is a green fluorescent nucleic acid stain, generally labeling both live and dead microorganisms. PI, in contrast, is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes, thus only the dead microbes are visualized. Biofilms were incubated with SYTO-9 and PI at 30^{o}C for 20 min in the dark before CSLM analyses. The images of stained biofilms were captured using a CSLM system (Leica Microsystems CMS, Mannheim, German).

A series of images were obtained for each position at 1 µm intervals in the z-axis to obtain a three dimensional view of the biofilms (from substratum to the top of the biofilms). Five representative randomly selected positions from each corner and the middle of the specimens were examined for each specimen, in two independent experiments on different days.

COMSTAT analysis is a software program for quantification of three-dimensional biofilm structures. It analyses stacks of images acquired with CLSM. Z -series images of 48h biofilms were collected by CLSM. The z-slices of images were exported to COMSTAT software and analyzed. The parameters analyzed include total biomass, average thickness and black spaces.

2.4.3. Cell morphology analysis

The specimens were placed inside a polypropylene tube containing 3 mL of sterilized PBS. Adherent micro-organisms were removed from the specimens by sonication at 7 W for 30 s.[24] Once disaggregated, the cells were centrifuged (3000 rpm), and the pellet was fixed, dehydrated and included for TEM analysis.

2.5. *Statistical analysis*

Statistical analysis was performed with SAS 9.0 software (SAS Institute Inc., Cary NC, USA). The variables were bioactivity and biostructure (biomass, thickness and black spaces). The assumptions of equality of variances and normal distribution of errors were tested for each variable, and when violated, the data were transformed as suggested by the software.[25] As mean values were not normally distributed, the bioactivity data were transformed by exponentiation ($y^{2.3}$). The comparison between control and experimental group, for each strain, was performed using the Student's t-test. The significant level was fixed at 5%.

Results

The bioactivity and biostructure of *C. glabrata* biofilms were not altered by the presence of FLZ ($p>0.05$) (Table 1-2, Fig. 3). In contrast, a significant reduction in biofilm bioactivity was found for *C. albicans* biofilms ($p<0.001$) developed in the presence of FLZ. The bioactivity decreased 75% for ATCC 90028 and P01, and 60% for P34 (Table 1).

Table 1- Bioactivity of *Candida spp* biofilms (means ± s.d).

	Bioactivity	
C. albicans	**Control**	**Experimental**
ATCC 90028	3.9±0.1 A	1.0±0.3 B
P01	3.5±0.3 A	0.9±0.3 B
P34	3.6±0.2 A	1.4±0.4 B
C. glabrata		
ATCC 2001	3.9±0.2 A	3.9±0.2 A
P11	3.8±0.2 A	3.8±0.1 A
P31	3.9±0.3 A	3.9±0.1 A

Distinct letters represent statistically significant differences between control and experimental groups within each *Candida* strain (t-test; $p<0.05$).

The biostructure of *C. albicans* ATCC 90028 ($p<0.001$) and P01 biofilms ($p<0.05$), was affected by FLZ, increasing their thickness, biomass and the distances between cells ("black spaces"), Table 2. These changes can be observed in CSLM images (Fig 1 and 2), in which live cells are shown in green and dead cells shown in red and are embedded within a matrix of polysaccharides, represented as "black spaces" as no stain for polysaccharides was used. *C. albicans* P34 and *C. glabrata* strains showed no alteration in the biostructure of the biofilms, developed in the presence of FLZ (Table 2 and Fig. 3).

Table 2- Biomass ($\mu m^3/\mu m^2$), average thickness (µm), average of black spaces (µm) of *Candida* spp. biofilms (means ± s.d.).

	Biomass		**Average Thickness**		**Black spaces**	
C. albicans	**Control**	**Experimental**	**Control**	**Experimental**	**Control**	**Experimental**
ATCC 90028	13.9±1.7 A	22.5±1.5 B	12.9±1.7 A	21.5±1.5 B	7.4±0.9 A	11.7±0.8 B
P01	16.4±3.7 A	19.9±3.3 B	15.4±3.7 A	18.90±3.3 B	8.7±1.8 A	10.4±1.6 B
P34	19.3±4.9 A	18.3±4.6 A	18.3±4.9 A	17.3±4.6 A	10.1±2.5 A	9.6±2.3 A
C. glabrata						
ATCC 2001	14.2±4.6 A	15.7±3.5 A	13.2±4.6 A	14.7±3.5 A	7.6±2.3 A	8.3±1.7 A
P11	16.8±6.0 A	15.4±2.5 A	15.8±6.0 A	14.4±2.5 A	8.9±3.0 A	8.2±1.2 A
P31	13.3±3.6 A	14.8±2.4 A	12.3±3.6 A	13.8±2.4 A	7.1±1.8 A	7.9±1.2 A

Distinct letters represent statistically significant differences between control and experimental groups within each *Candida* strain. (t-test; $p<0.05$).

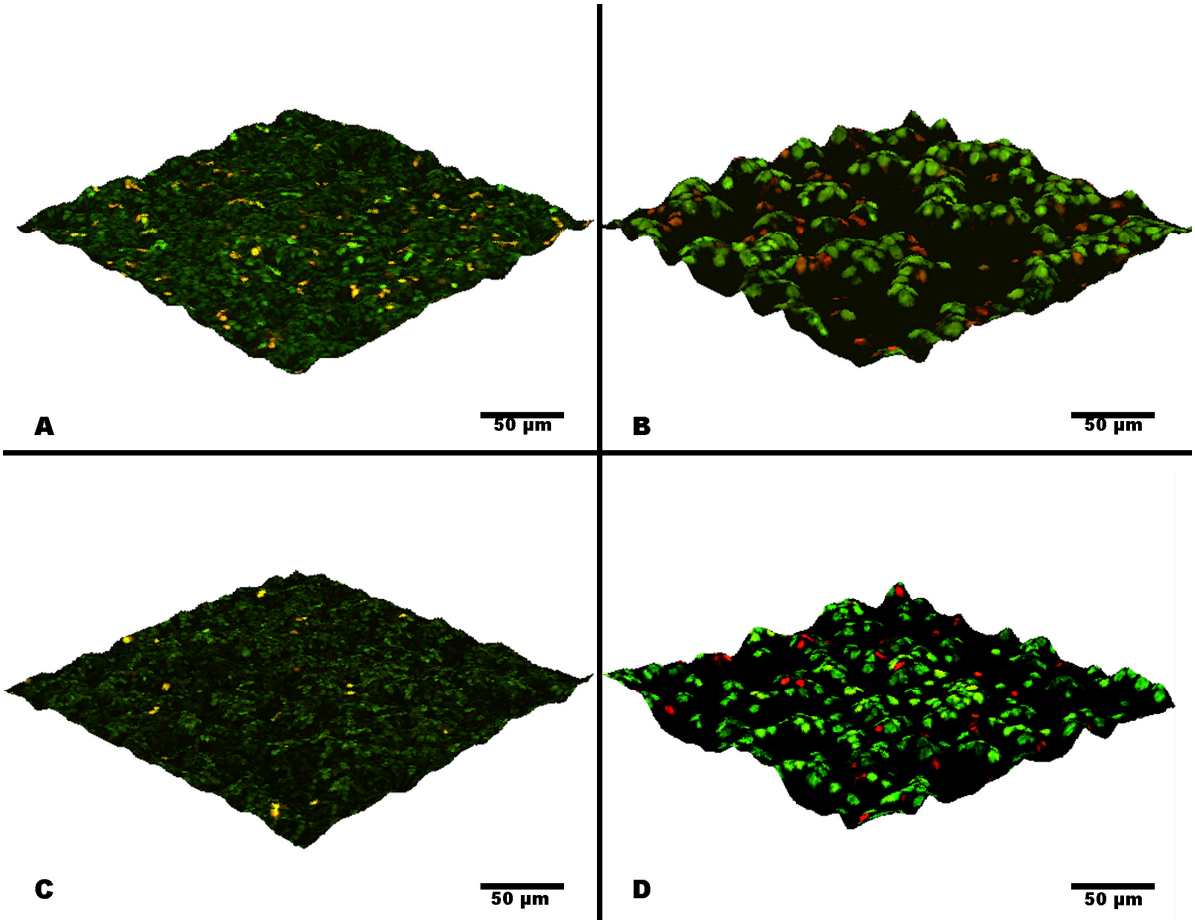

Figure 1 – CLSM 3D images showing observations of structure of *C. albicans* ATCC 90028 - (A) control group, (B) experimental group and *C. albicans* P01 - (C) control group, (D) experimental group.

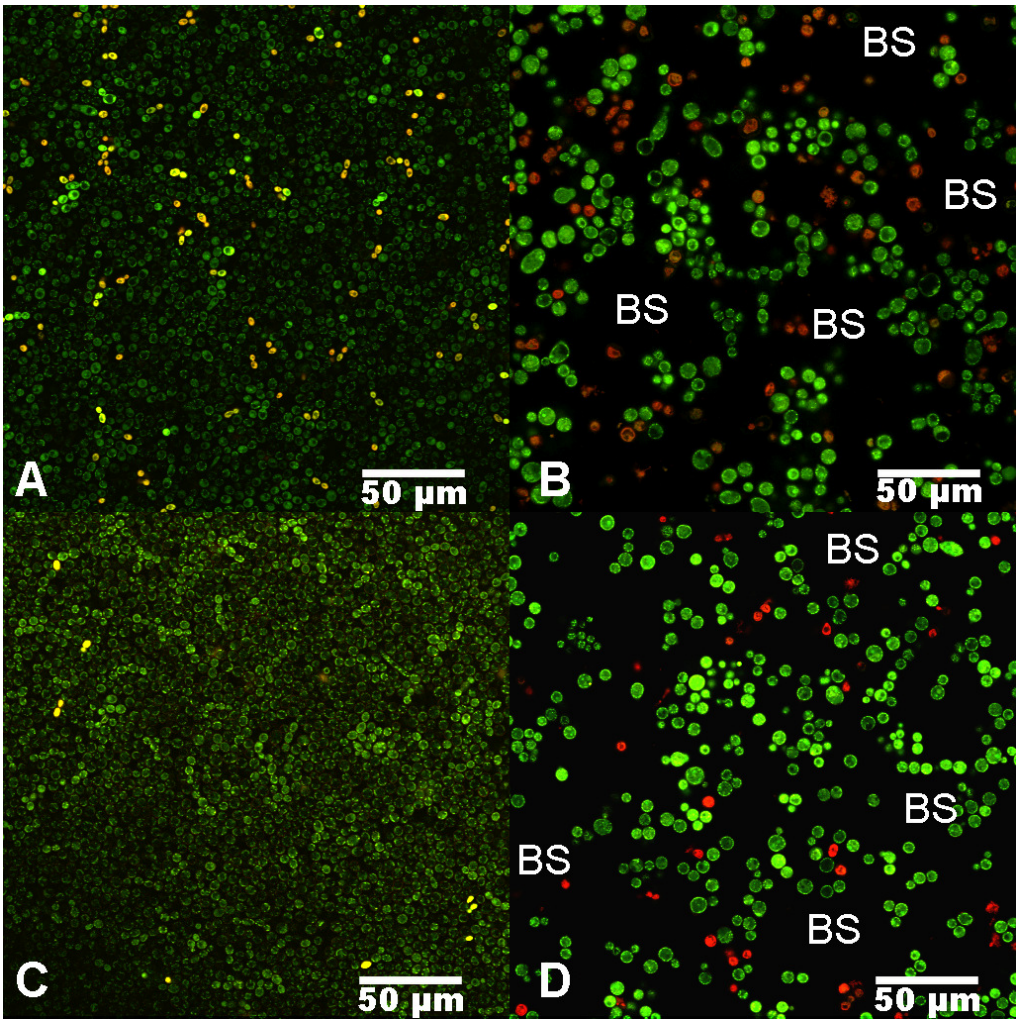

Figure 2 – CLSM images showing observations of *C. albicans* ATCC 90028 - (A) control group, (B) experimental group and *C albicans* P01 - (C) control group, (D) experimental group. Live cells appear green while dead cells appear red and black spaces (BS).

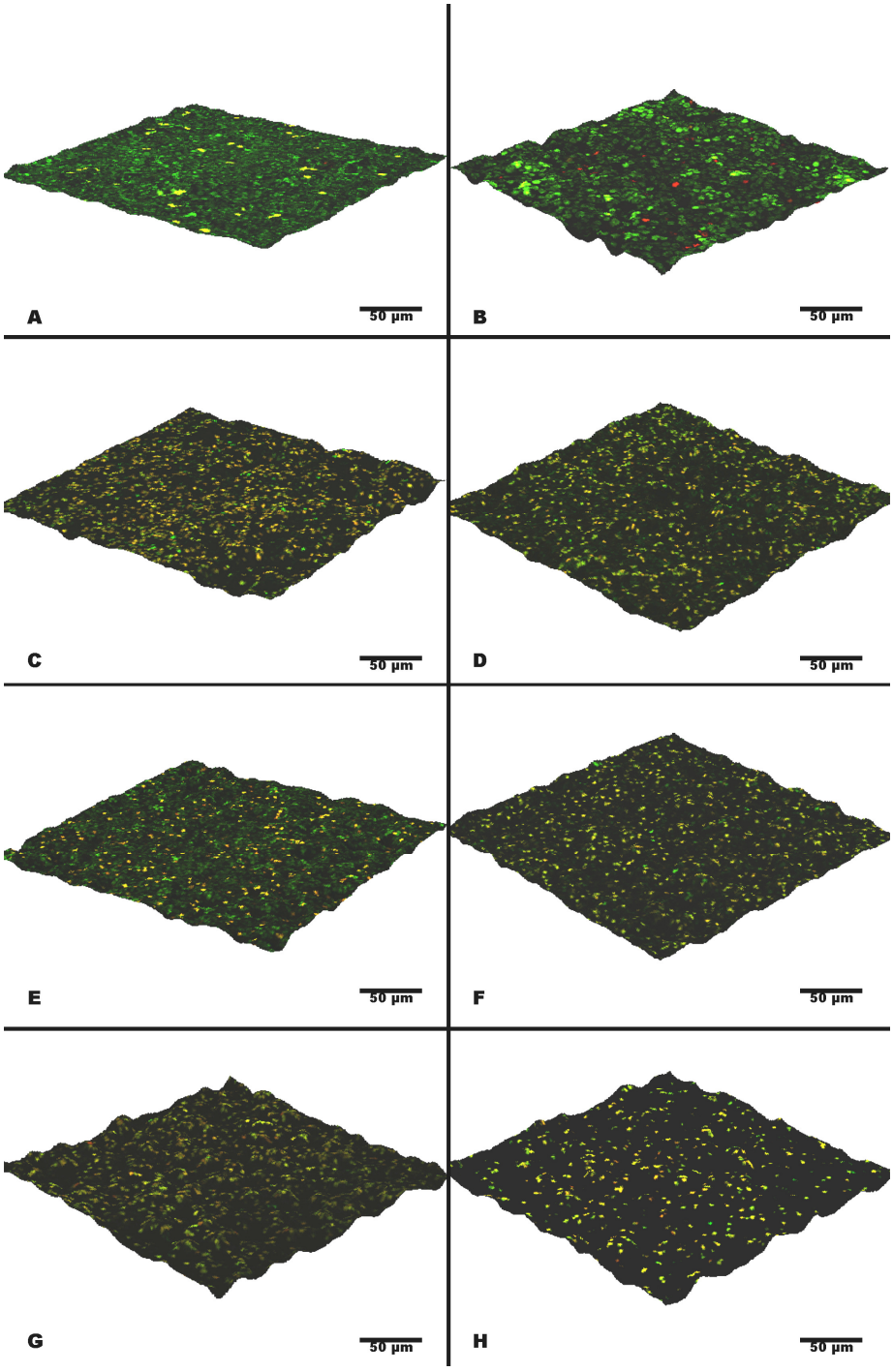

Figure 3 – CLSM 3D images of structure of *C. albicans* P34 - *(*A) control group, (B) experimental group; and *C. glabrata* strains*:* ATCC 2001 – (C) control, (D) experimental group; P11 - (E) control group, (F) experimental group; P31- (G) control group, (H) experimental group.

The effect of FLZ resulted in alterations in the morphology of some cells of *C. albicans* ATCC 90028 and P01. These changes were an increase in the amount of vacuoles and nuclei with deformed shape (Fig. 4). As in the CLSM images, the TEM showed an increase in the cell volume of *C. albicans* ATCC 90028 and P01 developed in the presence of FLZ (Fig 4).

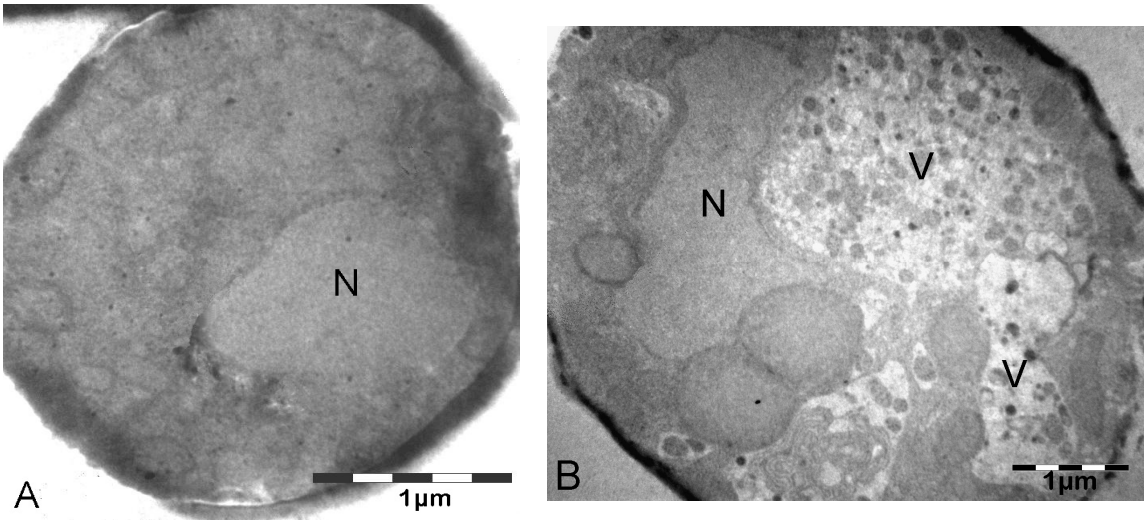

Figure 4 - TEM images showing visualization of morphology of *C. albicans* ATCC 90028*:* (A) control group, (B) experimental group. It is possible to visualize the nucleus (N) and vacuoles (V).

Discussion

Although, the effect of FLZ on *Candida* biofilms has been extensively investigated in literature,[12,14,15,26,27] there is little information about biofilms developed in the constant presence of FLZ.[13,14] The present biofilm growth model simulated *in vivo* conditions in which patients wearing dentures and presenting stomatitis are under

FLZ therapy regimen. Despite the FLZ treatment, in some cases, biofilms continued to develop over their dentures. Thus, understanding the behavior of *Candida* spp. biofilm growth under FLZ therapy, may be the key to the development of protective approaches to *Candida*-related diseases.

The bioactivity of *C. glabrata* was not altered by the presence of FLZ. These findings are in contrast with those from Konopka, 2010[14] who used FLZ in the same or higher concentration as used in the present study, and showed that *C. glabrata* biofilms were more sensitive to FLZ than *C. albicans* biofilms. Nevertheless, the present study corroborates other reports, which have demonstrated that *C. glabrata* is naturally more resistant to treatment with FLZ than *C. albicans.*[10,26,28] The resistance to FLZ acquired by *Candida*, especially *C. glabrata*, has been reported as being involved with the efflux pumps. These pumps are constituted by proteins in the cell membrane that pump the drug out of the cells, reducing the intracellular drug concentration, to a level at which FLZ has no effect on the cell.[10,29]

The present study showed that the *C. albicans* biofilms developed in the presence of FLZ, at a bioavailable concentration in saliva (2.56 µg/mL), reduced the metabolic activity by 60% for P34 and 75% for ATCC 90028 and P01. The results of this study differ from the findings of Kanopka *et al*., 2010,[14] who did not find significant reduction in the metabolic activity of *C.albicans* biofilms developed for 48h and then treated with FLZ at concentrations ≤ 3.0 µg/mL for another 24h. A previous study, conducted by Chandra *et al*., 2001,[13] showed that when using concentrations lower than 64 µg/mL, the *C. albicans* biofilms did not reach a

reduction of 50% in metabolic activity. The factor that may have influenced the lower bioactivity in the experimental group of the present study was the fact the the biofilms were grown in the constant presence of FLZ, while Chandra *et al,* 2001[13] and Kanopka *et al.,* 2010[14] first grew the biofilms, and afterwards incubated these biofilms with FLZ. Moreover, the differences found between the studies may be related to the different strains and to the fact that in the present study the experimental group was exposed to a new dose of the drug every 24h, considering that the half-life of FLZ ranges from 27- 37 h.[28]

The bioactivity of the *C. albicans* biofilms in the presence of FLZ was reduced, but it was important that the biofilm biostructure was also evaluated. This is because, a study showed that it co uld not be assumed that there was a direct relationship between the number of cells and XTT assay, as this ratio is not always constant among the *Candida* species, including *C. albicans.*[30] For this reason the present study used CSLM as an auxiliary method of analysis to assist the XTT assay, considering that CSLM allows biofilms to be evaluated with their structures preserved and in three dimensional format. Additionally, COMSTAT software was used, which evaluates the biofilm biostructures numerically.[31,32]

As regards biostructure, FLZ did not alter the thickness, biomass distance, and black spaces of *C. glabrata* and *C. albicans* P34 biofilms. As mentioned, the *C. glabrata* is naturally more resistant to treatment with FLZ.[10,26,28] Nevertheless, the fact that the biostructure of *C. albicans* P34 was not changed, although the metabolic activity was reduced by 60%, could be related to ability of *Candida* to reduce its metabolic activity as a protective mechanism in adverse situations,[10,29]

which, in the this present study was the presence of FLZ. However, *C. albicans* ATCC 90028 and P01 were sensitive to treatment with FLZ, once since they reduced their metabolic activity and in the presence of this antifungal agent their thickness, biomass, and black spaces increased. The increase in the average thickness of biofilms may be related to the increase the black spaces, as these spaces may be occupied by the polysaccharide matrix. The increase in biomass could be associated with the increase in cell volume. In the TEM images were it was observed that the cells developed in the presence of FLZ presented a deformed nucleus and a significant increase in the number of vacuoles. These vacuoles could be correlated to the FLZ action, which inhibits the ergosterol biosynthesis, a component of the plasmatic membrane. With this inhibition, toxic substances that are ergosterol precursors accumulate in the cell, [26,29] probably in these vacuoles.

The results showed that the structure of *C. albicans* ATCC 90028 and P01 were altered by FLZ, but this drug was not able to prevent the development of these biofilms. Could these structural alterations be related to a response to the exposure to FLZ resulting in an increase of the virulence of these biofilms? Further studies should be conducted to evaluate this hypothesis considering that the increase in the enzymatic activity is able to lysate the host cells, inducing the clinical aspects of candidosis.[33]

Conclusion

The alterations in bioactivity, biostructure and cell morphology as a response to the treatment with fluconazole, at the concentration bioavailable in saliva, depends on the *Candida* spp. strain evaluated.

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CONCLUSÃO

Dentro das limitações deste estudo, pode-se concluir que as alterações na bioatividade, bioestrura e morfologia celular, como resposta ao tratamento com FLZ, na concentração biodisponível na saliva, depende da cepa de *Candida* spp. avaliada.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Commitee of Medical Journal Editors - Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline

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Anexo 1 – Certificado de Aprovação do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Formação e desenvolvimento de biofilme de Candida spp. sobre a superfície de resina a base de poli(metilmetacrilato) na presença de saliva, plasma e agente antifúngico: efeitos sobre fatores de virulência, morfologia e quantificação do biofilme"**, protocolo nº 042/2008, dos pesquisadores Altair Antoninha Del Bel Cury, Priscila Nogueira Gomes, Wander José da Silva e William Custodio, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 04/02/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Formation and development of biofilm of Candida spp. on the surface of poly(methylmetacilate) containing resin in the presence of saliva, plasma and antifungic agent: effects on virulence factors, morphology and quantification of biofilm"**, register number 042/2008, of Altair Antoninha Del Bel Cury, Priscila Nogueira Gomes, Wander José da Silva and William Custodio, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 02/04/2009.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

Anexo 2 – Figuras

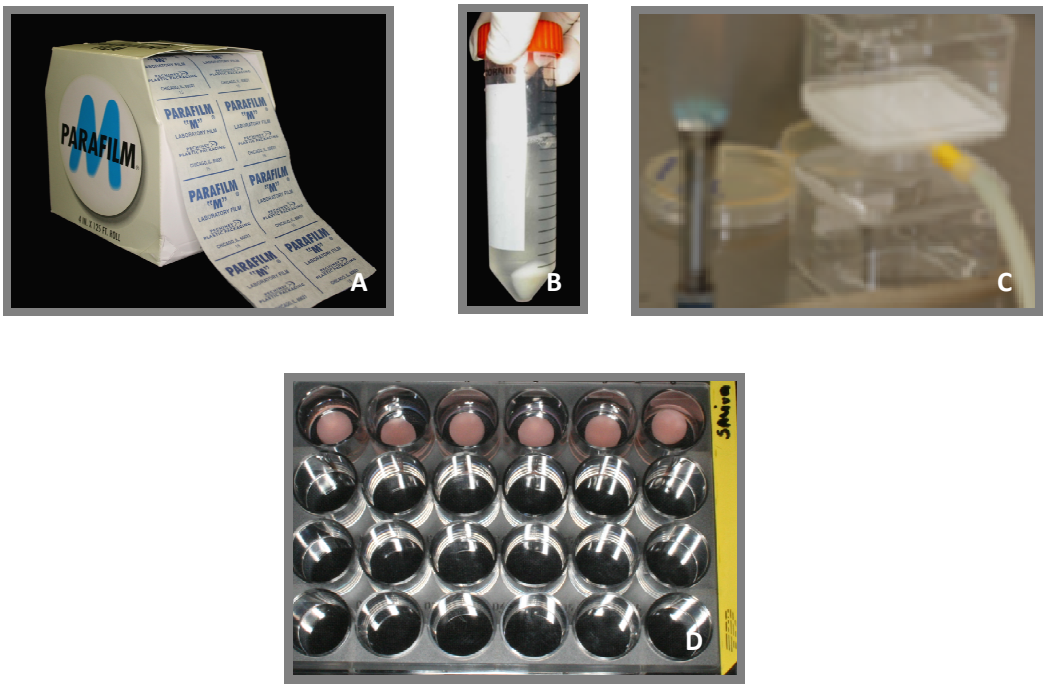

Figura 2. Parafilme utilizado para coleta de saliva estimulada (A), saliva após centrifugação (B), esterilização do sobrenadante da saliva em filtro de membrana (C) e espécimes posicionados em placa de 24 poços de cultura de tecido com saliva para formação da película adquirida (D).

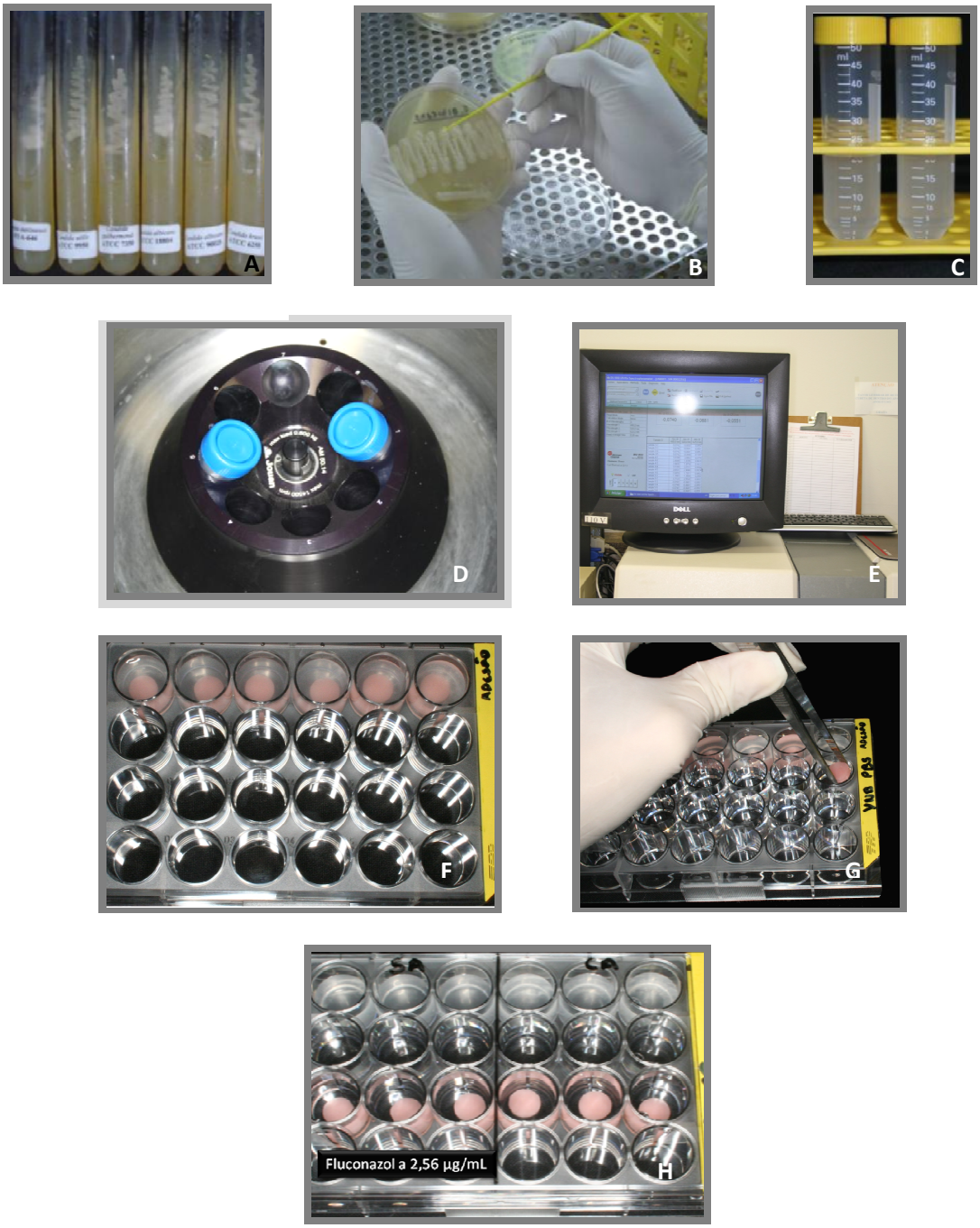

Figura 2. Cultura original das cepas de *Candida* (A), reativação em Agar Sabouraud (B), inóculo em YNB (C), centrifugação do inóculo (D), especrofotometro utilizado para ajuste da concentração de células no inóculo (E), adição do inóculo sobre os espécimes com película adquirida já formada (F), lavagem dos espécimes em PBS para remoção de células não aderidas (G) e espécimes em meio de cultura YNB, grupo controle e grupo experimental (H).

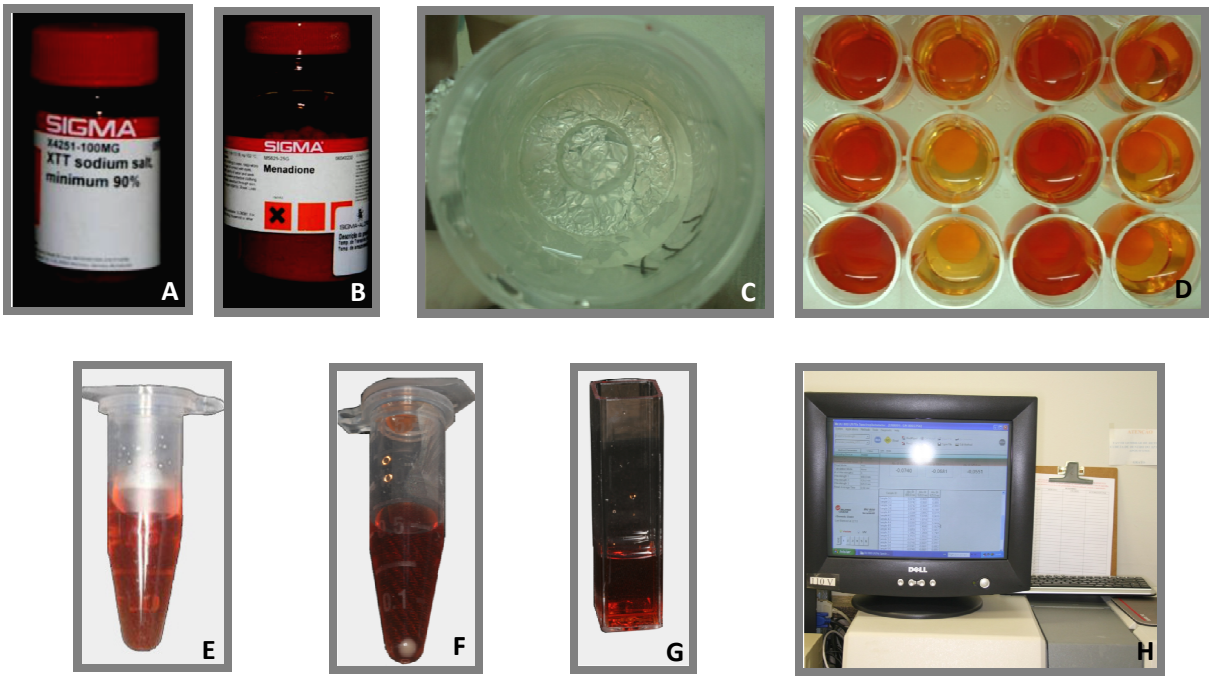

Figura 3. Sal de XTT (A), Menadiona (B), solução incolor de XTT antes da oxidação (c). Solução de XTT após a oxidação (D), transferida para eppendorf (E), após centrifugação (F) e transferida para cubeta de espectrofotômetro (G) para leitura da alteração colorimétrica em espectrofotômetro (H).

Figura 4. Corante “Molecular Probe’s Live/Dead *BacLight* viability kits” (A), Microscópio Confocal a Laser (B).

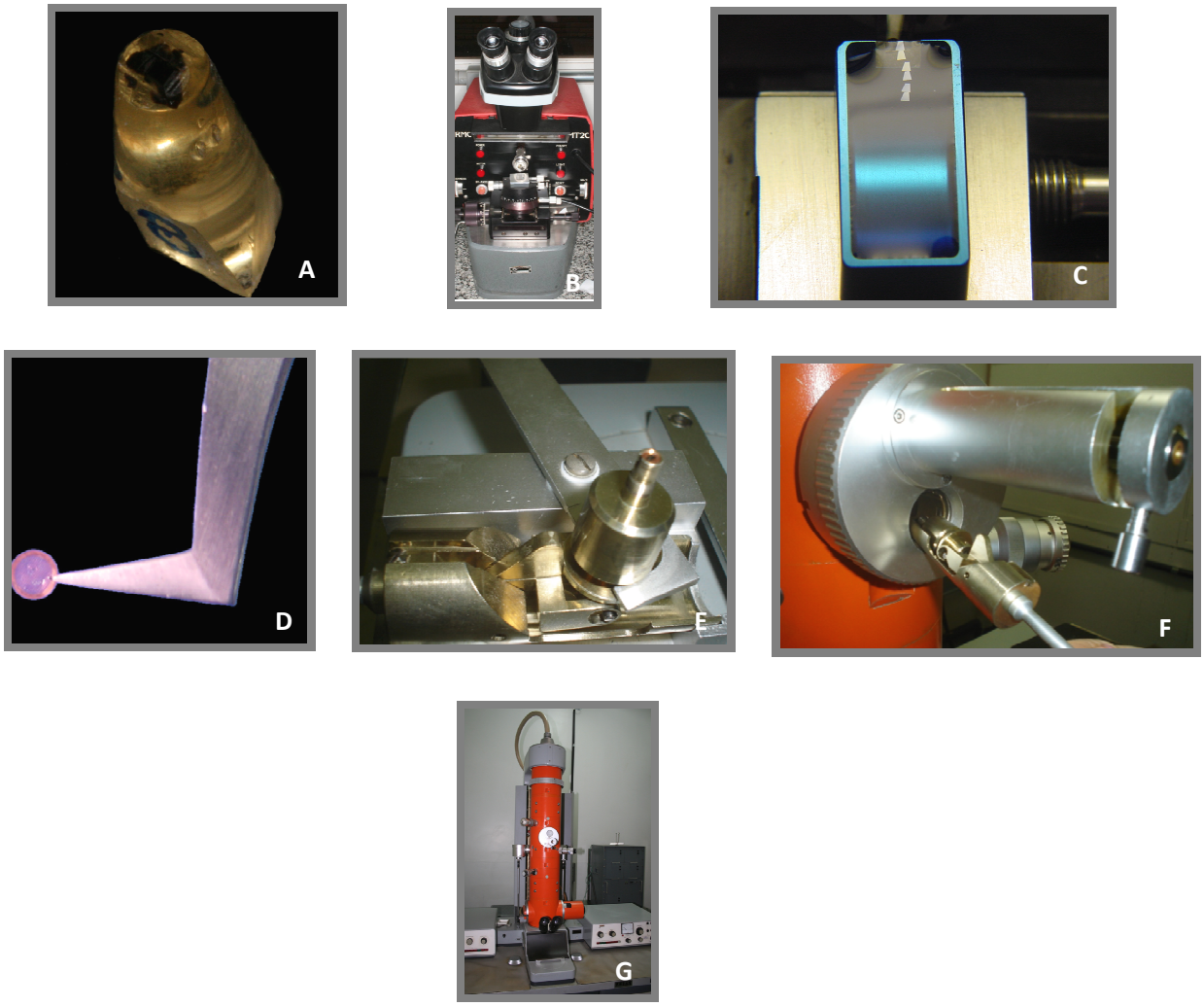

Figura 5. Pellet incluído em resina Dr. Spurr (A), ultrmicrótomo MT2C (B), navalha de diamante realizando cortes de 70 – 90 nm de espessura do pellet incluído resina (C), cortes coletados em grades de cobre (D), grade de cobre posicionada no suporte do MET (E), suporte sendo posicionado no MET (F) e Microscópio Eletrônico de Transmissão EM-900 (G).

Anexo 3 – Comprovante de submissão do artigo

Elsevier Editorial System(tm) for Archives of Oral Biology
Manuscript Draft

Manuscript Number: AOB-D-10-00524

Title: Bioactivity, biostructure and morphology of Candida spp. biofilms grown in the presence of Fluconazole

Article Type: Original Paper

Keywords: Biofilms, Candida, Candida albicans, Candida glabrata and Fluconazole

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Abstract: Objective: The aim of this study was to evaluate the effect of Fluconazole (FLZ) on the bioactivity, biostructure and morphology of Candida spp. biofilms formed in the presence of this antifungal agent.
Materials and methods: Specimens were fabricated using water bath poly(methylmethacrylate) resin (PMMA). Salivary pellicles were formed on the PMMA surface, and biofilms of a reference strain and two clinical isolates of Candida albicans (ATCC 90028, P01, P34) and Candida glabrata (ATCC 2001, P11 and P31) were developed for a period of 48 h. Two groups were formed: control and experimental. FLZ at 2.56 μg/mL, concentration bioavailable in saliva, was added to the medium of the experimental group. The culture mediums of the control and experimental groups were changed at 24 h. The bioactivities of the biofilms were evaluated with XTT reduction colorimetric assay. The biostructure was analyzed by Confocal Scanning Laser Microscopy and the morphology by the Transmission Electron Microscopy. The data were analyzed by the independent sample Student's t-test, with significance level set at 5%.
Results: The presence of FLZ decreased the bioactivity of all C. albicans biofilms ($p<0.001$), however, it did not change the structure and morphology of P34. As regards C. glabrata biofilm bioactivity and biostructure, no statistically significant differences were found between control and experimental groups for biofilms of all strains.
Conclusion: The alterations in bioactivity, biostructure and cell morphology in response to the treatment with fluconazole, at the bioavailable concentration present in saliva, depends on the Candida spp. strain.

Anexo 4 – Análise Estatística

Obs	tipo	especie	grupo	biomassa	espessura	bspace	xtt	trans
1	ATCC	atcc_alb	com_ant	20.0000	19	10.4998	0.7630	0.5368
2	ATCC	atcc_alb	com_ant	21.0000	20	10.9998	1.1326	1.3316
3	ATCC	atcc_alb	com_ant	24.0000	23	12.4998	1.2179	1.5736
4	ATCC	atcc_alb	com_ant	21.9999	21	11.4998	0.7977	0.5946
5	ATCC	atcc_alb	com_ant	21.9999	21	11.4997	0.6596	0.3840
6	ATCC	atcc_alb	com_ant	22.0000	21	11.4998	0.8151	0.6249
7	ATCC	atcc_alb	com_ant	23.0000	22	11.9998	1.0907	1.2210
8	ATCC	atcc_alb	com_ant	22.0000	21	11.4998	1.4660	2.4105
9	ATCC	atcc_alb	com_ant	25.0000	24	12.9998	0.8159	0.6263
10	ATCC	atcc_alb	com_ant	24.0000	23	12.4998	.	.
11	ATCC	atcc_alb	sem_ant	15.0000	14	7.9999	3.9818	23.9984
12	ATCC	atcc_alb	sem_ant	13.0000	12	6.9999	3.8568	22.3009
13	ATCC	atcc_alb	sem_ant	13.0000	12	6.9999	3.9818	23.9984
14	ATCC	atcc_alb	sem_ant	13.0000	12	6.9999	3.8716	22.4982
15	ATCC	atcc_alb	sem_ant	13.0000	12	6.9999	3.6955	20.2138
16	ATCC	atcc_alb	sem_ant	18.0000	17	9.4999	3.8716	22.4982
17	ATCC	atcc_alb	sem_ant	14.0000	13	7.4999	3.8591	22.3315
18	ATCC	atcc_alb	sem_ant	15.0000	14	7.9999	3.8591	22.3315
19	ATCC	atcc_alb	sem_ant	13.0000	12	6.9999	4.1602	26.5438
20	ATCC	atcc_alb	sem_ant	12.0000	11	6.4999	.	.
21	ATCC	atcc_gla	com_ant	21.0000	20	10.9998	3.7642	21.0886
22	ATCC	atcc_gla	com_ant	19.0000	18	9.9999	3.7642	21.0886
23	ATCC	atcc_gla	com_ant	18.0000	17	9.4999	3.8611	22.3581
24	ATCC	atcc_gla	com_ant	18.0000	17	9.4999	4.0131	24.4345
25	ATCC	atcc_gla	com_ant	18.0000	17	9.4999	4.1892	26.9713
26	ATCC	atcc_gla	com_ant	13.0000	12	6.9999	4.4902	31.6381
27	ATCC	atcc_gla	com_ant	12.0000	11	6.4999	3.7663	21.1156
28	ATCC	atcc_gla	com_ant	14.0000	13	7.4999	3.8632	22.3861
29	ATCC	atcc_gla	com_ant	13.0000	12	6.9999	3.8632	22.3861
30	ATCC	atcc_gla	com_ant	11.0000	10	5.9999	.	.
31	ATCC	atcc_gla	sem_ant	18.0000	17	9.4999	3.8611	22.3581
32	ATCC	atcc_gla	sem_ant	18.0000	17	9.4999	3.7642	21.0886
33	ATCC	atcc_gla	sem_ant	21.0000	20	10.9998	3.7642	21.0886
34	ATCC	atcc_gla	sem_ant	16.0000	15	8.4999	4.0131	24.4345
35	ATCC	atcc_gla	sem_ant	18.0000	17	9.4996	4.1892	26.9713
36	ATCC	atcc_gla	sem_ant	13.0000	12	6.9999	4.1892	26.9713
37	ATCC	atcc_gla	sem_ant	10.0000	9	5.4999	3.8632	22.3861
38	ATCC	atcc_gla	sem_ant	8.0000	7	4.4999	3.8632	22.3861
39	ATCC	atcc_gla	sem_ant	9.0000	8	4.9999	3.7663	21.1156
40	ATCC	atcc_gla	sem_ant	11.0000	10	5.9999	.	.
41	Isolado	P01	com_ant	20.9960	20	10.9954	0.6872	0.4220
42	Isolado	P01	com_ant	17.9955	17	9.4917	1.2393	1.6380
43	Isolado	P01	com_ant	22.9881	22	11.9809	0.9313	0.8490
44	Isolado	P01	com_ant	19.9743	19	10.4606	0.6853	0.4193
45	Isolado	P01	com_ant	23.9635	23	12.4362	0.3295	0.0778
46	Isolado	P01	com_ant	22.0000	21	11.4998	0.7066	0.4499
47	Isolado	P01	com_ant	23.0000	22	11.9998	1.4536	2.3639
48	Isolado	P01	com_ant	17.0000	16	8.9999	0.8769	0.7392
49	Isolado	P01	com_ant	17.0000	16	8.9999	1.0278	1.0651
50	Isolado	P01	com_ant	14.0000	13	7.4999	.	.
51	Isolado	P01	sem_ant	26.0000	25	13.4998	3.3558	16.1931
52	Isolado	P01	sem_ant	15.9998	15	8.4999	3.7563	20.9869
53	Isolado	P01	sem_ant	17.0000	16	8.9999	3.1475	13.9740
54	Isolado	P01	sem_ant	16.0000	15	8.4999	3.5651	18.6108
55	Isolado	P01	sem_ant	16.0000	15	8.4999	3.3890	16.5640
56	Isolado	P01	sem_ant	14.0000	13	7.4999	3.1672	14.1760
57	Isolado	P01	sem_ant	12.0000	11	6.4999	3.6772	19.9843
58	Isolado	P01	sem_ant	16.0000	15	8.4999	3.9782	23.9485
59	Isolado	P01	sem_ant	16.0000	15	8.4999	3.5522	18.4563
60	Isolado	P01	sem_ant	15.0000	14	7.9999	.	.
61	Isolado	P11	com_ant	17.0000	16	8.9999	3.8295	21.9395
62	Isolado	P11	com_ant	14.0000	13	7.4999	4.1305	26.1100
63	Isolado	P11	com_ant	15.0000	14	7.9999	3.9545	23.6217
64	Isolado	P11	com_ant	17.0000	16	8.9999	3.8392	22.0675

65	Isolado	P11	com_ant	16.0000	15	8.4999	3.7423	20.8074
66	Isolado	P11	com_ant	13.0000	12	6.9999	3.6631	19.8085
67	Isolado	P11	com_ant	20.0000	19	10.4998	3.7368	20.7372
68	Isolado	P11	com_ant	13.0000	12	6.9999	3.8337	21.9949
69	Isolado	P11	com_ant	12.0000	11	6.4999	3.8337	21.9949
70	Isolado	P11	com_ant	17.0000	16	8.9999	.	.
71	Isolado	P11	sem_ant	16.0000	15	8.4999	3.9545	23.6217
72	Isolado	P11	sem_ant	14.0000	13	7.4999	4.1305	26.1100
73	Isolado	P11	sem_ant	12.0000	11	6.4999	3.7326	20.6836
74	Isolado	P11	sem_ant	10.0000	9	5.4999	3.7423	20.8074
75	Isolado	P11	sem_ant	7.0000	6	3.9999	3.6631	19.8085
76	Isolado	P11	sem_ant	24.0000	23	12.4998	3.6631	19.8085
77	Isolado	P11	sem_ant	24.0000	23	12.4998	3.7368	20.7372
78	Isolado	P11	sem_ant	20.0000	19	10.4998	3.8337	21.9949
79	Isolado	P11	sem_ant	22.0000	21	11.4998	3.5907	18.9196
80	Isolado	P11	sem_ant	19.0000	18	9.9999	.	.
81	Isolado	P31	com_ant	11.0000	10	5.9999	3.9545	23.6217
82	Isolado	P31	com_ant	12.0000	11	6.4999	3.9545	23.6217
83	Isolado	P31	com_ant	15.0000	14	7.9999	3.9545	23.6217
84	Isolado	P31	com_ant	12.0000	11	6.4999	3.9642	23.7552
85	Isolado	P31	com_ant	17.0000	16	8.9999	3.9642	23.7552
86	Isolado	P31	com_ant	17.0000	16	8.9999	4.1403	26.2527
87	Isolado	P31	com_ant	14.0000	13	7.4999	3.7368	20.7372
88	Isolado	P31	com_ant	17.0000	16	8.9999	3.7368	20.7372
89	Isolado	P31	com_ant	17.0000	16	8.9999	3.8337	21.9949
90	Isolado	P31	com_ant	16.0000	15	8.4999	.	.
91	Isolado	P31	sem_ant	10.0000	9	5.4999	3.9545	23.6217
92	Isolado	P31	sem_ant	9.0000	8	4.9999	4.4316	30.6965
93	Isolado	P31	sem_ant	14.0000	13	7.4999	3.8295	21.9395
94	Isolado	P31	sem_ant	11.0000	10	5.9999	3.7423	20.8074
95	Isolado	P31	sem_ant	9.0000	8	4.9999	4.1403	26.2527
96	Isolado	P31	sem_ant	14.0000	13	7.4999	3.5962	18.9863
97	Isolado	P31	sem_ant	14.0000	13	7.4999	3.7368	20.7372
98	Isolado	P31	sem_ant	20.0000	19	10.4998	3.7368	20.7372
99	Isolado	P31	sem_ant	16.0000	15	8.4999	3.8337	21.9949
100	Isolado	P31	sem_ant	16.0000	15	8.4999	.	.
101	Isolado	P34	com_ant	22.9999	22	11.9996	1.7725	3.7303
102	Isolado	P34	com_ant	16.9998	16	8.9995	1.4990	2.5371
103	Isolado	P34	com_ant	15.0000	14	7.9999	1.4367	2.3011
104	Isolado	P34	com_ant	14.0000	13	7.4999	1.6500	3.1638
105	Isolado	P34	com_ant	11.0000	10	5.9999	0.6356	0.3526
106	Isolado	P34	com_ant	24.0000	23	12.4998	1.1362	1.3414
107	Isolado	P34	com_ant	23.0000	22	11.9998	1.7851	3.7916
108	Isolado	P34	com_ant	23.0000	22	11.9998	1.5431	2.7121
109	Isolado	P34	com_ant	18.0000	17	9.4999	1.1795	1.4619
110	Isolado	P34	com_ant	15.0000	14	7.9999	.	.
111	Isolado	P34	sem_ant	13.0000	12	6.9999	3.9926	24.1484
112	Isolado	P34	sem_ant	14.9999	14	7.9998	3.3236	15.8380
113	Isolado	P34	sem_ant	18.9999	18	9.9998	3.8677	22.4461
114	Isolado	P34	sem_ant	18.0000	17	9.4999	3.6231	19.3146
115	Isolado	P34	sem_ant	12.9999	12	6.9999	3.2921	15.4949
116	Isolado	P34	sem_ant	18.0000	17	9.4999	3.5139	18.0018
117	Isolado	P34	sem_ant	23.0000	22	11.9998	3.7563	20.9869
118	Isolado	P34	sem_ant	28.0000	27	14.4998	3.6102	19.1568
119	Isolado	P34	sem_ant	23.0000	22	11.9998	3.5011	17.8513
120	Isolado	P34	sem_ant	23.0000	22	11.9998	.	.

tipo=ATCC especie=atcc_alb grupo=com_ant

Variable	Mean	Std Dev	N	Variation
biomassa	22.5	1.5	10	6.7
espessura	21.5	1.5	10	7.0
bspace	11.7	0.8	10	6.4
xtt	1.0	0.3	9	27.3
xtt_trans	1.0	0.7	9	64.0

tipo=ATCC especie=atcc_alb grupo=sem_ant

Variable	Mean	Std Dev	N	Variation
biomassa	13.9	1.7	10	12.4
espessura	12.9	1.7	10	13.4
bspace	7.4	0.9	10	11.6
xtt	3.9	0.1	9	3.3
xtt_trans	23.0	1.7	9	7.6

tipo=ATCC especie=atcc_gla grupo=com_ant

Variable	Mean	Std Dev	N	Variation
biomassa	15.7	3.5	10	22.1
espessura	14.7	3.5	10	23.6
bspace	8.3	1.7	10	20.8
xtt	3.9	0.2	9	6.2
xtt_trans	23.7	3.5	9	14.9

tipo=ATCC especie=atcc_gla grupo=sem_ant

Variable	Mean	Std Dev	N	Variation
biomassa	14.2	4.6	10	32.2
espessura	13.2	4.6	10	34.6
bspace	7.6	2.3	10	30.0
xtt	3.9	0.2	9	4.4
xtt_trans	23.2	2.4	9	10.3

tipo=Isolado especie=PO1 grupo=com_ant

Variable	Mean	Std Dev	N	Variation
biomassa	19.9	3.3	10	16.5
espessura	18.9	3.3	10	17.4
bspace	10.4	1.6	10	15.6
xtt	0.9	0.3	9	37.8
xtt_trans	0.9	0.7	9	80.1

tipo=Isolado especie=PO1 grupo=sem_ant

Variable	Mean	Std Dev	N	Variation
biomassa	16.4	3.7	10	22.3
espessura	15.4	3.7	10	23.8
bspace	8.7	1.8	10	21.0
xtt	3.5	0.3	9	7.8
xtt_trans	18.1	3.3	9	18.0

tipo=Isolado especie=P11 grupo=com_ant

Variable	Mean	Std Dev	N	Variation
biomassa	15.4	2.5	10	16.0
espessura	14.4	2.5	10	17.1
bspace	8.2	1.2	10	15.0
xtt	3.8	0.1	9	3.6
xtt_trans	22.1	1.8	9	8.3

tipo=Isolado especie=P11 grupo=sem_ant

Variable	Mean	Std Dev	N	Variation
biomassa	16.8	6.0	10	35.5
espessura	15.8	6.0	10	37.7
bspace	8.9	3.0	10	33.5
xtt	3.8	0.2	9	4.4
xtt_trans	21.4	2.2	9	10.5

tipo=Isolado especie=P31 grupo=com_ant

Variable	Mean	Std Dev	N	Variation
biomassa	14.8	2.4	10	16.2
espessura	13.8	2.4	10	17.4
bspace	7.9	1.2	10	15.2
xtt	3.9	0.1	9	3.3
xtt_trans	23.1	1.7	9	7.5

tipo=Isolado especie=P31 grupo=sem_ant

Variable	Mean	Std Dev	N	Variation
biomassa	13.3	3.6	10	26.8
espessura	12.3	3.6	10	28.9
bspace	7.1	1.8	10	24.9
xtt	3.9	0.3	9	6.6
xtt_trans	22.9	3.6	9	15.7

tipo=Isolado especie=P34 grupo=com_ant

Variable	Mean	Std Dev	N	Variation
biomassa	18.3	4.6	10	25.4
espessura	17.3	4.6	10	26.8
bspace	9.6	2.3	10	24.1
xtt	1.4	0.4	9	26.2
xtt_trans	2.4	1.2	9	48.4

tipo=Isolado especie=P34 grupo=sem_ant

Variable	Mean	Std Dev	N	Variation
biomassa	19.3	4.9	10	25.5
espessura	18.3	4.9	10	26.9
bspace	10.1	2.5	10	24.3
xtt	3.6	0.2	9	6.5
xtt_trans	19.2	2.9	9	15.0

T-TEST FOR THE MEANS OF BIOMASSA WITHIN GRUPO

---------- ESPECIE = P01 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	19.89174	3.275	1.0357
sem_ant	10	16.39998	3.6576	1.1566

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	2.249	18	0.0373
Not Equal	2.249	17.78	0.0374

---------- ESPECIE = P11 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	15.4	2.4585	0.7775
sem_ant	10	16.8	5.9591	1.8844

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-0.687	18	0.5010
Not Equal	-0.687	11.98	0.5053

---------- ESPECIE = P31 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	14.8	2.3944	0.7572
sem_ant	10	13.3	3.5606	1.126

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	1.105	18	0.2835
Not Equal	1.105	15.76	0.2855

---------- ESPECIE = P34 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	18.29997	4.644	1.4686
sem_ant	10	19.29997	4.9228	1.5567

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-0.467	18	0.6459
Not Equal	-0.467	17.94	0.6459

---------- ESPECIE = atcc_alb ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	22.49998	1.5092	0.4773
sem_ant	10	13.9	1.7288	0.5467

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	11.850	18	<.0001
Not Equal	11.850	17.68	<.0001

---------- ESPECIE = atcc_gla ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	15.7	3.4657	1.096
sem_ant	10	14.2	4.5656	1.444

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	0.828	18	0.4188
Not Equal	0.828	16.79	0.4195

T-TEST FOR THE MEANS OF ESPESSURA WITHIN GRUPO

---------- ESPECIE = PO1 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	18.9	3.2813	1.0376
sem_ant	10	15.4	3.6576	1.1566

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	2.252	18	0.0370
Not Equal	2.252	17.79	0.0372

---------- ESPECIE = P11 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	14.4	2.4585	0.7775
sem_ant	10	15.8	5.9591	1.8844

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-0.687	18	0.5010
Not Equal	-0.687	11.98	0.5053

---------- ESPECIE = P31 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	13.8	2.3944	0.7572
sem_ant	10	12.3	3.5606	1.126

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	1.105	18	0.2835
Not Equal	1.105	15.76	0.2855

---------- ESPECIE = P34 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	17.3	4.644	1.4686
sem_ant	10	18.3	4.9227	1.5567

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-0.467	18	0.6459
Not Equal	-0.467	17.94	0.6459

---------- ESPECIE = atcc_alb ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	21.5	1.5092	0.4773
sem_ant	10	12.9	1.7288	0.5467

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	11.850	18	<.0001
Not Equal	11.850	17.68	<.0001

---------- ESPECIE = atcc_gla ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	14.7	3.4657	1.096
sem_ant	10	13.2	4.5656	1.4438

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	0.828	18	0.4188
Not Equal	0.828	16.79	0.4195

T-TEST FOR THE MEANS OF BSPACE WITHIN GRUPO

---------- ESPECIE = PO1 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	10.43641	1.6301	0.5155
sem_ant	10	8.699868	1.8288	0.5783

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	2.242	18	0.0378
Not Equal	2.242	17.77	0.0380

---------- ESPECIE = P11 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	8.19987	1.2292	0.3887
sem_ant	10	8.899857	2.9795	0.9422

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-0.687	18	0.5010
Not Equal	-0.687	11.98	0.5053

---------- ESPECIE = P31 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	7.899879	1.1972	0.3786
sem_ant	10	7.149888	1.7803	0.563

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	1.105	18	0.2835
Not Equal	1.105	15.76	0.2855

---------- ESPECIE = P34 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	9.649796	2.3219	0.7343
sem_ant	10	10.14983	2.4613	0.7783

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-0.467	18	0.6459
Not Equal	-0.467	17.94	0.6459

---------- ESPECIE = atcc_alb ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	11.74979	0.7546	0.2386
sem_ant	10	7.449886	0.8644	0.2734

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	11.850	18	<.0001
Not Equal	11.850	17.68	<.0001

---------- ESPECIE = atcc_gla ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	10	8.349871	1.7328	0.548
sem_ant	10	7.599853	2.2827	0.7219

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	0.828	18	0.4187
Not Equal	0.828	16.79	0.4195

T-TEST FOR THE MEANS OF XTT_TRANS WITHIN GRUPO

---------- ESPECIE = P01 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	9	0.891572	0.7142	0.2381
sem_ant	9	18.09933	3.2576	1.0859

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-15.479	16	<.0001
Not Equal	-15.479	8.77	<.0001

---------- ESPECIE = P11 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	9	22.12017	1.8446	0.6149
sem_ant	9	21.38793	2.2359	0.7453

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	0.758	16	0.4595
Not Equal	0.758	15.44	0.4599

---------- ESPECIE = P31 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	9	23.12191	1.7319	0.5773
sem_ant	9	22.86371	3.5939	1.198

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	0.194	16	0.8485
Not Equal	0.194	11.53	0.8494

---------- ESPECIE = P34 ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	9	2.37689	1.1513	0.3838
sem_ant	9	19.24875	2.8831	0.961

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-16.304	16	<.0001
Not Equal	-16.304	10.49	<.0001

---------- ESPECIE = atcc_alb ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	9	1.033703	0.662	0.2207
sem_ant	9	22.96831	1.739	0.5797

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	-35.365	16	<.0001
Not Equal	-35.365	10.27	<.0001

---------- ESPECIE = atcc_gla ----------

Sample Statistics

Group	N	Mean	Std. Dev.	Std. Error
com_ant	9	23.71856	3.5324	1.1775
sem_ant	9	23.20002	2.3786	0.7929

Hypothesis Test

Null hypothesis: Mean 1 - Mean 2 = 0
Alternative: Mean 1 - Mean 2 ^= 0

If Variances Are	t statistic	Df	Pr > t
Equal	0.365	16	0.7197
Not Equal	0.365	14.02	0.7203