



UNESP - Universidade Estadual Paulista

**“Júlio de Mesquita Filho”**

Faculdade de Odontologia de Araraquara



**BEATRIZ HELENA DIAS PANARIELLO**

**INFLUÊNCIA DE ALTERAÇÕES GENÉTICAS, DO FLUCONAZOL E DE ENZIMAS HIDROLÍTICAS NA MATRIZ EXTRACELULAR DE BIOFILMES DE *Candida* SUSCEPTÍVEL E RESISTENTE A FLUCONAZOL**

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Tese apresentada ao Curso de Pós-Graduação em Reabilitação Oral- Área de Prótese, da Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista “Júlio de Mesquita Filho”, para obtenção do título de Doutora em Reabilitação Oral.

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**Co-orientadora:** Prof<sup>ª</sup>. Dr<sup>ª</sup>. Marlise Inêz Klein

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Panariello BHD. Influência de alterações genéticas, do fluconazol e de enzimas hidrolíticas na matriz extracelular de biofilmes de *Candida* susceptível e resistente a fluconazol [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

## RESUMO

Biofilmes formados por *Candida* estão relacionados a infecções bucais, como a candidíase. Embora a resistência do biofilme seja multifatorial, a proteção exercida por sua matriz extracelular (MEC) é importante para os altos níveis de resistência a drogas antifúngicas. O conhecimento dos princípios estruturais da MEC possibilita maior compreensão de como atuar para desorganizá-la e melhorar a difusão de agentes antifúngicos para que atinjam mais eficientemente o biofilme, além de, futuramente, possibilitar o desenvolvimento de terapias mais eficazes para o controle da formação de biofilmes. Sendo assim, os objetivos principais deste estudo foram: (1) verificar a influência da inativação de genes envolvidos na filamentação (EFG1 e TEC1) em características estruturais dos biofilmes e na produção de componentes da MEC; (2) verificar a influência do fluconazol (FLZ) na MEC de biofilmes de *Candida albicans* ATCC 90028 (susceptível a fluconazol- CaS), *C. albicans* ATCC 96901 (resistente a fluconazol- CaR), *Candida glabrata* ATCC 2001 (susceptível a fluconazol- CgS) e *C. glabrata* ATCC 200918 (resistente a fluconazol- CgR) e (3) estudar a ação de enzimas hidrolíticas (DNase, Dextranase e  $\beta$ -glucanase individualmente ou em diferentes combinações) sobre a MEC de biofilmes de CaS e CaR. Biofilmes maduros (48 horas) foram analisados através de contagem de unidades formadoras de colônia (ufc/mL), peso seco total, peso seco insolúvel e proteínas insolúveis. Os componentes da MEC- polissacarídeos solúveis em álcali (ASPs), polissacarídeos solúveis em água (WSPs), DNA extracelular (eDNA) e proteínas solúveis foram quantificados. No estudo 1, foi observado que o conteúdo de ASPs é significativamente maior em cepa parental de *C. albicans* em comparação com as cepas mutantes  $\Delta/\Delta$  efg1 e  $\Delta/\Delta$  tec1, o que indica que a produção de ASPs pode estar relacionada à morfologia celular filamentosa em *C. albicans*. No estudo 2, observou-se que as biomassas totais e WSPs foram significativamente reduzidos pelo FLZ na MEC de CaS, CaR, CgS e CgR, mas as quantidades de eDNA e proteínas não foram influenciadas pela presença de FLZ nem pelo tipo de cepa. O FLZ interferiu na morfologia celular e na estrutura dos biofilmes, reduzindo a formação de hifas nos biofilmes de CaS e CaR e diminuindo o número de células nos biofilmes de CgS e CgR. No estudo 3, observou-se que exposição de biofilmes maduros à DNase por 5 minutos reduziu o conteúdo de eDNA, polissacarídeos e proteínas solúveis da MEC de CaS e CaR, sendo um promissor adjuvante para terapias antibiofilme. A redução de polissacarídeos extracelulares e

do conteúdo de proteínas pela DNase indicam que esses componentes estão interligados ao eDNA na MEC de CaS e CaR. Portanto, células filamentosas têm tendência de produzirem mais exopolissacarídeos, e estes componentes estão interligados ao eDNA e a proteínas solúveis na MEC de biofilmes de *C. albicans*. Para reduzir componentes da matriz e desorganizar a estrutura formada por eDNA-exopolissacarídeos-proteínas, a aplicação de enzima DNase por 5 min em biofilmes maduros de *C. albicans* se mostrou eficaz.

Palavras chave: Candida. Biofilmes. Matriz extracelular. Resistência a medicamentos. Fluconazol. Mutação.

Panariello BHD. Influence of genetic alterations, fluconazole and hydrolytic enzymes on the extracellular matrix of fluconazole-susceptible and -resistant *Candida* biofilms [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

## ABSTRACT

Biofilms formed by *Candida* are related to bucal infections, such as candidiasis. Although the biofilm resistance is multifactorial, the protection exerted by its extracellular matrix (ECM) is essential for its high levels of resistance to antifungals. The knowledge of the structural principles of the ECM permits a better understanding of how to disorganize the ECM and improve the diffusion of antifungal drugs to reach the biofilm. Moreover, the study of the ECM may enable the development of more effective therapies to control biofilm formation. Thus, the main objectives of this study were: (1) to verify the influence of the inactivation of genes involved in filamentation and structural characteristics of the biofilms (EFG1 and TEC1) on the production of ECM components; (2) to verify the influence of fluconazole (FLZ) on the biofilms' ECM of *Candida albicans* ATCC 90028 (fluconazole-susceptible: CaS), *C. albicans* ATCC 96901 (fluconazole-resistant: CaR), *Candida glabrata* ATCC 2001 (fluconazole-susceptible: CgS) and *C. glabrata* ATCC 200918 (fluconazole-resistant: CgR) and (3) to study the action of hydrolytic enzymes (DNase, Dextranase and  $\beta$ -glucanase individually or in different combinations) on the ECM of CaS and CaR biofilms. Mature biofilms (48 hours) were analyzed by colony counting forming units (cfu/mL), total dry weight, insoluble dry-weight and total proteins. ECM components- alkali-soluble polysaccharides (ASPs), water-soluble polysaccharides (WSPs), extracellular DNA (eDNA) and soluble proteins- were quantified. In study 1, it was observed that ASPs content is significantly higher in *C. albicans* parental strain compared to the mutant strains  $\Delta/\Delta$  *efg1* and  $\Delta/\Delta$  *tec1*, indicating that the production of ASPs may be related to the filamentous cell morphology in *C. albicans*. In study 2, it was observed that the total biomasses and WSPs were significantly reduced by FLZ in the ECM of CaS, CaR, CgS and CgR, but the amounts of eDNA and proteins were not influenced by the presence of FLZ nor by type of strain. FLZ interfered on the cellular morphology and structure of biofilms, reducing hyphae formation in CaS and CaR biofilms and reducing the number of cells in CgS and CgR biofilms. The study 3 demonstrated that exposure of mature biofilms to DNase for 5 minutes reduced the eDNA, polysaccharides and soluble proteins of the ECM of CaS and CaR, being a promising adjuvant for antibiofilm therapies. The reduction of extracellular polysaccharides and soluble proteins by DNase indicates that these components are intertwined to eDNA in the ECM of CaS and CaR. Therefore, filamentous cells tend to produce more

exopolysaccharides, and these components are intertwined to eDNA and soluble proteins in the ECM of *C. albicans* biofilms. To reduce matrix components and disrupt the structure formed by eDNA-exopolysaccharide-proteins, 5 min exposure of mature biofilms to DNase showed to be effective.

Keywords: Candida. Biofilms. Extracellular matrix. Drug resistance. Fluconazole. Mutation.

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## 1 INTRODUÇÃO

Os microrganismos do gênero *Candida* são fungos comensais da cavidade oral de indivíduos saudáveis que podem se tornar agentes patogênicos oportunistas em algumas situações, por exemplo, quando há alterações no sistema imunológico, disfunção metabólica ou em população de idade avançada (Abaci et al.<sup>1</sup>, 2010; Dagistan et al.<sup>7</sup>, 2009; Li et al.<sup>13</sup>, 2007; Luo, Samaranayake<sup>15</sup>, 2002; Pfaller, Diekema<sup>31</sup>, 2007; Samaranayake, Samaranayake<sup>38</sup>, 2001). Além disso, o uso crescente de antibióticos de amplo espectro, quimioterapias citotóxicas e transplantes também intensificam o risco de infecções por esses fungos oportunistas (Pfaller, Diekema<sup>31</sup>, 2007). Essas infecções são conhecidas como candidíase. A *Candida albicans* é a espécie mais prevalente associada a candidíase, seguida pela *Candida glabrata*, que é a espécie não-*albicans* mais prevalente que tem sido associada ao desenvolvimento de infecções orais (Abaci et al.<sup>1</sup>, 2010; Dagistan et al.<sup>7</sup>, 2009; Li et al.<sup>13</sup>, 2007; Luo, Samaranayake<sup>15</sup>, 2002; Samaranayake, Samaranayake<sup>38</sup>, 2001). A crescente importância de *C. glabrata* como um oportunista em indivíduos imunocomprometidos tem sido relatada (Bennet et al.<sup>3</sup>, 2001; Pfaller, Diekema<sup>31</sup>, 2007), principalmente devido a sua habilidade inata para adquirir resistência antifúngica (Mann et al.<sup>17</sup> 2009; Tsai et al.<sup>44</sup>, 2010).

Infecções causadas por *Candida* estão frequentemente associadas à formação de biofilmes (Nobile, Mitchell<sup>26</sup>, 2007). O crescimento de biofilme se inicia quando células planctônicas aderem a um determinado substrato. Em seguida, ocorre proliferação de células de levedura na superfície do substrato e o início do desenvolvimento de hifas. O passo final para o desenvolvimento do biofilme é o estágio de maturação, no qual o crescimento em forma de levedura é reprimido, o crescimento de hifas se eleva e matriz extracelular (MEC) encobre o biofilme (Blankenship, Mitchell<sup>5</sup>, 2006). A via de desenvolvimento de hifas é crítica para que haja formação significativa de biomassa de biofilme (Ramage et al.<sup>35</sup>, 2002). Mutantes com defeitos no fator de transcrição EFG1 (enhanced filamentous growth), o principal ativador do desenvolvimento de hifas, não foram capazes de formar nem mesmo uma monocamada de células sobre superfícies de poliestireno (Ramage et al.<sup>35</sup>, 2002). Além de EFG1, o fator de transcrição TEC1 também é necessário para a formação de hifas (Schweizer et al.<sup>40</sup>, 2000). Foi observado que biofilmes produzidos por cepa mutante com ausência do fator de transcrição TEC1 ( $\Delta/\Delta$  *tec1*) eram rudimentares, possuindo menos de 20  $\mu$ m de espessura (Ramage et al.<sup>35</sup>, 2002). Cepas mutantes com defeitos em genes de filamentação são menos virulentas do que suas cepas parentais e apresentam menores níveis de infectividade de células endoteliais e catéteres (Lewis et al.<sup>12</sup>, 2002; Lo et al.<sup>14</sup>, 1997).

Algumas espécies de *Candida* possuem resistência intrínseca a drogas antifúngicas, especialmente ao fluconazol (Pfaller, Diekma<sup>31</sup>, 2007; Tsai et al.<sup>44</sup>, 2010). Em contraste, a resistência pode ser desenvolvida pelo microrganismo após longos períodos de exposição a antifúngicos (Shapiro et al.<sup>41</sup>, 2011). Dessa forma, uma grande preocupação com os biofilmes de *Candida* é que suas células podem ter uma susceptibilidade reduzida contra azóis e polienos, devido ao desenvolvimento de resistência (Pfaller et al.<sup>30</sup>, 2002). A resistência dos biofilmes de *Candida* é multifatorial e está associada ao estado fisiológico das células, à ativação de bombas de efluxo de drogas e ao efeito protetor dos  $\beta$ -glucanos presentes na MEC de *C. albicans*, que se ligam ao fluconazol e à anfotericina B (Nett et al.<sup>24</sup>, 2007, Nett et al.<sup>25</sup>, 2010), dificultando a penetração desses fármacos nos biofilmes (Vediyappan et al.<sup>45</sup>, 2010).

Foi demonstrado que a MEC de biofilme de *C. albicans* possui grandes quantidades de  $\beta$ -1,6 glucanos e  $\alpha$ -mananas, que interagem para formar um complexo manano-glucano (MGCx) (Mitchell et al.<sup>20</sup>, 2015; Zarnowski et al.<sup>47</sup>, 2014). Esta interação de exopolissacarídeos foi considerada crucial para a proteção do biofilme contra tratamento medicamentoso (Mitchell et al.<sup>21</sup>, 2016). Além disso, demonstrou-se que o DNA extracelular (eDNA) contribui para a integridade estrutural do biofilme de *C. albicans* (Martins et al.<sup>18</sup>, 2012; Rajendran et al.<sup>34</sup>, 2014). Esforços para hidrolisar polissacarídeos e ácidos nucleicos da MEC têm sido eficazes na sensibilização de biofilmes de *Candida* e *Aspergillus* (Martins et al.<sup>18</sup>, 2012; Mitchell et al.<sup>20</sup>, 2015; Nett et al.<sup>24</sup>, 2007; Rajendran et al.<sup>33</sup>, 2013). O acúmulo de  $\alpha$ -mananos foi bloqueado com  $\alpha$ -manosidase, uma enzima que catalisa a hidrólise de resíduos terminais não redutores de  $\alpha$ -D-manose em  $\alpha$ -D-manosídeos, aumentando a atividade do fluconazol contra os biofilmes de *C. albicans* (Mitchell et al.<sup>20</sup>, 2015). Além disso, biofilmes de 24 horas desafiados com RPMI contendo diferentes concentrações de antifúngicos isolados ou em combinação com DNase mostraram que a adição de DNase aumentou a susceptibilidade das células de *C. albicans* à anfotericina B (Martins et al.<sup>18</sup>, 2012). Ademais, demonstrou-se que a combinação de biofilmes com DNase associada a anfotericina B e caspofungina melhorou significativamente a susceptibilidade antifúngica em biofilme de *Aspergillus fumigatus* (Rajendran et al.<sup>33</sup>, 2013).

A matriz extracelular é considerada um dos maiores desafios no controle do biofilme oral (Panariello et al.<sup>29</sup>, 2017). Sendo assim, o conhecimento dos princípios estruturais da matriz extracelular possibilita maior compreensão de como atuar para desorganizá-la e melhorar a difusão de agentes antifúngicos através do biofilme, a fim de que atinjam mais eficientemente as células de *Candida*. Além disso, possibilita que, futuramente, sejam desenvolvidas terapias mais eficazes para o controle da formação e patogenicidade de biofilmes de *Candida*. Portanto, os objetivos principais deste estudo foram: (1) caracterizar a matriz extracelular de biofilmes



de cepas mutantes ( $\Delta/\Delta$  *efg1* e  $\Delta/\Delta$  *tec1*) e cepa parental (wild-type-WT) de *C. albicans* para verificar a influência da inativação de genes envolvidos na filamentação e em características estruturais dos biofilmes na produção de componentes da MEC; (2) caracterizar a MEC de biofilmes de *C. albicans* e *C. glabrata* susceptíveis e resistentes ao fluconazol na presença e na ausência desta droga para verificar sua influência na MEC dos biofilmes destas cepas e (3) estudar a ação de enzimas hidrolíticas (DNase, Dextranase e  $\beta$ -glucanase individualmente ou em diferentes combinações) sobre a MEC de biofilmes de *C. albicans* susceptível e resistente a fluconazol.

## 2 PROPOSIÇÃO

1. Verificar a influência da inativação de genes envolvidos na filamentação e em características estruturais dos biofilmes (TEC1 e EFG1) na produção de componentes da MEC em *C. albicans*.
2. Verificar a influência do fluconazol na MEC de biofilmes de cepas de *C. albicans* e *C. glabrata* susceptíveis e resistentes a fluconazol.
3. Analisar a ação de enzimas hidrolíticas (DNase, Dextranase e  $\beta$ -glucanase individualmente ou em diferentes combinações) sobre a matriz extracelular de biofilmes de *C. albicans* susceptível e resistente ao fluconazol.

### 3 PUBLICAÇÕES

#### 3.1 Publicação 1

##### **Inactivation of genes TEC1 and EFG1 in *Candida albicans* influences extracellular matrix composition and biofilm morphology**

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**ABSTRACT**

**Background:** Infections caused by *Candida* spp. have been associated with formation of a biofilm, i.e. a complex microstructure of cells adhering to a surface and embedded within an extracellular matrix (ECM).

**Methods:** The ECMs of a wild-type (WT, SN425) and two *Candida albicans* mutant strains,  $\Delta/\Delta$  *tec1* (CJN2330) and  $\Delta/\Delta$  *efg1* (CJN2302), were evaluated. Colony-forming units (cfu), total biomass (mg), water-soluble polysaccharides (WSPs), alkali-soluble polysaccharides (ASPs), proteins (insoluble part of biofilms and matrix proteins), and extracellular DNA (eDNA) were quantified. Variable-pressure scanning electron microscopy and confocal scanning laser microscopy were performed. The biovolume ( $\mu\text{m}^3/\mu\text{m}^2$ ) and maximum thickness ( $\mu\text{m}$ ) of the biofilms were quantified using COMSTAT2.

**Results:** ASP content was highest in WT (mean  $\pm$  SD:  $74.5 \pm 22.0$   $\mu\text{g}$ ), followed by  $\Delta/\Delta$  *tec1* ( $44.0 \pm 24.1$   $\mu\text{g}$ ) and  $\Delta/\Delta$  *efg1* ( $14.7 \pm 5.0$   $\mu\text{g}$ ). The protein correlated with ASPs ( $r = 0.666$ ) and with matrix proteins ( $r = 0.670$ ) in the WT strain. The population in  $\Delta/\Delta$  *efg1* correlated with the protein ( $r = 0.734$ ) and its biofilms exhibited the lowest biomass and biovolume, and maximum thickness. In  $\Delta/\Delta$  *tec1*, ASP correlated with eDNA ( $r = 0.678$ ).

**Conclusion:** ASP production may be linked to *C. albicans* cell filamentous morphology.

**KEYWORDS:** *Candida albicans*, biofilm, EFG1, TEC1, extracellular matrix

## Introduction

The microorganisms of the genus *Candida* are opportunistic fungi that are present in the oral cavity. When there is an imbalance in the immune system of the host, the oral microbiota is altered, and these microorganisms may invade the oral tissues [1,2]. Clinical manifestations of infections caused by *Candida* spp. can be superficial, such as oropharyngeal candidiasis (OPC) and/or systemic (e.g. candidemia). The OPC is an indicator of the development of AIDS and depending on the stage of the infection by HIV, about 90% of the patients show OPC [3,4]. *Candida albicans* is the most prevalent specie related to this infection [5-8].

Infections caused by *Candida* spp. are often associated with biofilm formation. Biofilm is a complex microstructure of cells adhered to a surface and embedded within an extracellular matrix (ECM) made up of secreted microbial and host-derived substances (i.e. saliva components) and cells lysis [9]. The ECM contributes to the biofilm build up, preservation of the biofilms' architecture and maintenance of stable interactions between cell-cell and cell-surface and the environment [10]. Among the substances found in the ECM are polysaccharides, proteins, and nucleic acids, all of which play a major role in biofilms [9]. Three classes of conventional antifungals are used for treating infections caused by *Candida*: azoles (e.g. fluconazole), polyenes (e.g. amphotericin B) and echinocandins (e.g. caspofungin) [11]. However, antifungal drug resistance can arise from all drug classes and includes acquired resistance in susceptible strains and selection of innately less susceptible species [12].

Antifungals resistance in *Candida* biofilms is multifactorial and is associated with the physiological state of the cells, the activation of drug efflux pumps and the protective effect of the ECM performed by  $\beta$ -glucans (an alkali-soluble exopolysaccharide or ASP), which bind to fluconazole [13] and amphotericin B, preventing the penetration of drugs into the biofilm [14]. In addition to the protective effects of ECM performed by  $\beta$ -glucan, it has been shown that the extracellular DNA (eDNA) is another key component, contributing to the structural integrity of

*C. albicans* biofilm [15]. The ECM of *C. albicans* strain K1 was tested using *in vitro* and animal models, and the ECM composition was 55% protein, 25% carbohydrate, 15% lipid, and 5% nucleic acid, while  $\beta$ -1,3-glucan comprised only a small portion of the total matrix [16].

The “hyphal development pathway is critical for formation of significant biofilm mass” [17]. Mutants defective in the enhanced filamentous growth transcriptional factor (EFG1), a major activator of hyphal development, presented impaired formation of monolayer of cells on polystyrene surfaces [17]. This defect in biofilm development may be because of altered surface-protein composition and adherence properties of the EFG1 null mutant ( $\Delta/\Delta$  efg1) [18]. In addition, the lack of functioning EFG1 in *C. albicans* strains yielded only pseudohyphae on solid media and without growth in liquid media [19]. Tec1p is a TEA/ATTS transcription factor and it is required for hyphal formation [20]. Biofilm produced by the tec1 null mutant ( $\Delta/\Delta$  tec1) strain was rudimentary with less than 20  $\mu$ m deep and composed exclusively of yeast cells [17], while its parental strain formed a biofilm 250–450  $\mu$ m deep that included many hyphal filaments [17]. Therefore, mutant strains defective in the filamentation genes EFG1 and TEC1 are considered less virulent than their wild-type counterparts, because they present decreased levels of infectivity of endothelial cells and plasma-coated catheters [21, 22].

The study of *C. albicans* mutants with defective capability of forming biofilms is needed to evaluate the differences in the ECM components and structure when there is deficient formation of biofilm, facilitating the understanding of which components are related to a regular biofilm formation. Knowing the assembly principles of the matrix helps on deciding in which component or components new therapies should focus at to design effective treatments to control fungal biofilm formation and pathogenesis. Therefore, the aim of this study was to characterize the ECM of wild-type and mutant ( $\Delta/\Delta$  efg1 and  $\Delta/\Delta$  tec1) *C. albicans* strains.

## **Materials and methods**

### ***Biofilm formation and processing***

The microorganisms used for this experiment were *C. albicans* SN425 (wild-type strain-WT), *C. albicans* CJN2302 and *C. albicans* CJN2330, the last two ones are mutant strains with deficient biofilm formation ability,  $\Delta/\Delta\text{tec1}$  and  $\Delta/\Delta\text{efg1}$  [23]. TEC1 is primarily an activator of its biofilm-relevant direct target genes and EFG1 is both activator and repressor [23]. The microorganisms stored at  $-80^{\circ}\text{C}$  were seeded onto Petri dishes with SDA (Sabourand dextrose agar) culture medium supplemented with chloramphenicol and incubated at  $37^{\circ}\text{C}$  for 48 h. Next, starter cultures containing about 5 colonies were grown using YNB medium (Yeast Nitrogen Base- DIFCO, Detroit, Michigan, USA) supplemented with 100 mM of glucose, and incubated at  $37^{\circ}\text{C}$ . After 16 h of incubation, the starter cultures were diluted with fresh YNB medium supplemented with 100 mM glucose (1:20 dilution). These inoculum cultures were incubated at  $37^{\circ}\text{C}$  until the three strains reached the mid-log growth phase (8 hours, Fig. S1). Then, the  $\text{OD}_{540\text{nm}}$  of the inoculums was adjusted to reach  $10^7$  cells  $\text{mL}^{-1}$ . Next, 1 mL of the inoculum of each strain was added to the wells of a 24-well polystyrene plate (Techno Plastic Products- TPP, Trasadingen, Switzerland). The culture plate was incubated at  $37^{\circ}\text{C}$  for cell adhesion to the substrate. After 90 min, the wells were washed twice with sterile 0.89% NaCl solution to remove non-adhered cells. Next, 1 mL of RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, Missouri, USA) at pH 7 was added to each well.

After 24 hours of biofilm formation, the culture medium was removed by aspiration and fresh RPMI buffered with MOPS (1 mL, pH 7.0) was added to each well. After 48 hours of biofilm formation, the wells were washed twice with 0.89% sterile NaCl solution. Biofilms were processed following the flowchart in Fig. 1. Briefly, biofilms were removed by scraping each well with a pipette tip and 2 mL of sterile 0.89% NaCl. From the biofilm suspension, 0.1 mL was used for the 10-fold serial dilution and culture on SDA plates for recovery of colony forming units (CFU) and 0.1 mL was used for dry weight (biomass) determination [24]. The

remainder of the volume was vortexed vigorously at high speed for 1 min for all samples during processing for mechanical disruption of the ECM and centrifuged at 5500 xg for 10 min (4°C). The supernatant was stored in another tube and the precipitate with the cells and the insoluble components of the ECM was washed twice with sterile milli-Q water (5500xg /10 min/ 4°C). From the stored supernatant, it was separated 1 mL for the quantification of water-soluble polysaccharides (WSP) [25], 0.650 mL for eDNA analysis [26] and 0.150 mL for protein tests [27]. The precipitate was re-suspended in water then 0.05 mL was separated for the quantification of protein [27] and 0.95 mL was separated for the determination alkali-soluble polysaccharide ASP [25].

### ***Protein quantification***

Proteins in the ECM (soluble or supernatant) and in the insoluble part of biofilms were quantified. The proteins from the insoluble part were extracted by boiling at 100°C for 1 h at 1000 rpm. Bovine serum albumin solution (P5369, Sigma-Aldrich, St. Louis, MO, USA) was prepared in saline buffer and the following concentrations were used as standard curve: 0 mg/mL, 0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL e 1.4 mg/mL. In 96 well plates, 200 µL of Bradford Reagent (B6916, Sigma-Aldrich, St. Louis, MO, USA) was mixed with 5 µL of each curve point and biofilm samples. The reaction was carried out during 30 min, and the absorbance at 595 nm was determined in a spectrophotometer.

### ***Water-soluble polysaccharides (WSP) analysis***

An aliquot of 1 mL per sample of the homogenized supernatant was transferred to sterile centrifuge tubes to which 2.5 volumes of 95% ethanol were added. The WSP were precipitated for 18 h at -20 °C and centrifuged at 9500 xg for 20 min at 4°C. After centrifugation, the supernatants were discarded. The samples were washed three times with ice-cold 75% ethanol



and the pellets were air-dried. Each pellet was resuspended with 1 ml of water, and total carbohydrates were quantified using the phenol-sulfuric acid method [25]. Glucose was used for standard curve (0, 2.5, 5, 10, 15, 20 and 25  $\mu\text{L}$  glucose per tube). The method consists of adding 200  $\mu\text{L}$  of 5% phenol to a glass tube containing 200  $\mu\text{L}$  of the sample or standard curve point (triplicate per sample). After carefully mixing, one mL of sulfuric acid was added to each tube under agitation. After 20 min of reaction, samples were measured using a spectrophotometer (490 nm).

#### ***Alkali-soluble polysaccharides (ASP) analysis***

Aliquots of 0.95 mL of each biofilm suspension were centrifuged (13000  $\times g$ /10 min /4  $^{\circ}\text{C}$ ). The supernatant of each tube was carefully removed and discarded. The pellets were then dried in a desiccator for one week. The pellets were weighed and 300  $\mu\text{L}$  of 1N NaOH per 1 mg of the dry-weight were added. The pellets with 1N NaOH were incubated for 2 h at 37 $^{\circ}\text{C}$  and then were centrifuged at 13000  $\times g$  for 10 min. The supernatants were cautiously collected with a pipette and transferred to new microcentrifuge *tubes*, preserving just the pellet. Once more, the same previous volume of 1N NaOH was added to the tubes containing the pellets, and the same steps as above were repeated for the extraction of ASP. After incubation, samples were centrifuged (13000  $\times g$  /10 min) and the supernatants were carefully collected and added to the previously collected supernatant. For the third extraction, the same steps above were repeated, but this time, the samples were not incubated for 2 h before centrifugation. After three extractions, three volumes of cold 95% ethanol were added to each sample. The samples were then stocked at -20  $^{\circ}\text{C}$  for 18 h for precipitation of ASP. After precipitation of ASP, the tubes were centrifuged (9500  $\times g$ /20 min /4  $^{\circ}\text{C}$ ), and the supernatants were discarded. Each resulting pellet was washed three times with ice-cold 75% ethanol and air-dried, following the procedures performed for WSP samples. The pellets were resuspended in the same total volume of the

original extraction with 1N NaOH. Finally, the samples were ready for quantification of total carbohydrates using the phenol-sulfuric acid method as described for WSP analysis.

### ***eDNA analysis***

Aliquots of 0.65 mL of the supernatant of biofilms suspensions were mixed with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) for eDNA extraction. The aqueous phase of each sample was mixed with 3 volumes of isopropanol and 1/10 volume of 3M sodium acetate (pH 5.2) and stored at -20° C for 18 h. The eDNA precipitated with isopropanol was collected by centrifugation (13000 xg/20 min/4 °C) and washed 3 times with ice-cold 70% ethanol, air dried, and then dissolved in 10 µL of TE buffer (Tris HCl/1 mM EDTA, pH 8.0). The amount of eDNA was determined using a spectrophotometer with light length of 260 nm.

### ***Variable pressure scanning electron microscopy protocol (VPSEM)***

After 48 h of biofilm formation, the samples were transferred directly to the VPSEM [Zeiss EVO 50 (Carl Zeiss Microscopy, LLC, Thornwood, NY) chamber and imaged at 100 Pa. VPSEM images were captured at a working distance of 6.5 and 7.0 mm and field widths of 10 µm and 20 µm [28].

### ***Confocal scanning laser microscopy (CSLM)***

The biofilm morphology was determined by CSLM. Leica TCS SP5 microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a HCX APOL U-V-I 40X/0.8-numerical-aperture water immersion objective was used. The biofilms were stained with a live/dead viability kit (Molecular Probes, Invitrogen, Eugene, Oregon, USA). The stain was prepared by diluting 1.5 µL of SYTO 9 and 1.5 µL of propidium iodide in 1.0 mL of sterile 1% phosphate buffered solution (pH 7.4) [29]. The plates were incubated at room temperature in the dark for 15 min and examined under a CSLM. The biovolume ( $\mu\text{m}^3/\mu\text{m}^2$ ) and maximum thickness ( $\mu$ )

[30] of the biofilms were quantified using COMSTAT2- <http://www.comstat.dk>; and the images were rendered in the Amira software (Mercury Computer Systems Inc., Chelmsford, MA).

### ***Statistical analyses***

All the experiments were repeated on three separated occasions, with four replicates (n=12). Data was analyzed by one-way ANOVA with Tukey post-hoc test ( $\alpha = 0.05$ ). A Pearson's correlation test ( $r$ ) was applied to check correlations between the different ECM components. Correlation was considered significant at the 0.05 level (2-tailed). All the tests were performed using IBM SPSS Statistics 19.

### **Results**

The quantitative data of population, dry weight, protein and extracellular matrix components are displayed in Table 1. The population data demonstrated significant differences between WT and  $\Delta/\Delta$  *efg1* and  $\Delta/\Delta$  *tec1* and  $\Delta/\Delta$  *efg1* ( $p < 0.001$ ) while no statistical differences were observed between WT and  $\Delta/\Delta$  *tec1* strains. The dry weight (total biomass) of the WT strain is higher than the mutant strains ( $p < 0.005$ ). The proteins are significantly higher ( $p < 0.05$ ) in the  $\Delta/\Delta$  *efg1* mutant strain. The ASP data showed significant differences for all the strains ( $p = 0.000$ ), being the WT the strain that possess the higher amount of this ECM component ( $74.5 \pm 22.0 \mu\text{g}$ ), followed by  $\Delta/\Delta$  *tec1* ( $44.0 \pm 24.1 \mu\text{g}$ ) and  $\Delta/\Delta$  *efg1* ( $14.7 \pm 5.0 \mu\text{g}$ ). However, the other parameters, eDNA, WSP and matrix proteins, showed no significant differences among the strains ( $p > 0.05$ ; Table 1).

Pearson's Correlation was applied to compare the ECM components between each other. This analysis showed a significant correlation ( $p < 0.05$ ) between the ASP and protein content in the WT strain ( $r = 0.666$ ) as well as for protein and matrix protein ( $r = 0.670$ ) (Table 2). The population of  $\Delta/\Delta$  *efg1* significantly correlated to the protein content of its ECM ( $r = 0.734$ ) (Table 3). The  $\Delta/\Delta$  *tec1* mutant strain showed a significant correlation between the ASP content and the eDNA in its ECM ( $r = 0.678$ ) (Table 4).

The WT strain present typical thick biofilm architecture in visual appearance (a) with the presence of abundant ECM (b and c), as observed by VPSEM. In contrast,  $\Delta/\Delta$  *efg1* mutant shows sparse thin biofilm growth patterns (d) and morphologically distinct ECM (e, f) in comparison to the WT (b, c) and  $\Delta/\Delta$  *tec1* strains (h, i). The  $\Delta/\Delta$  *tec1* mutant present defects in visual appearance (g) compared to the WT strain (a), however, its ECM is morphologically similar to WT (h, i).

CSLM representative images of the 48 h biofilms of *C. albicans* strains are shown in Fig. 3. Multidimensional imaging of live (green) cells can be observed at different depths of the biofilms. In addition, negligible amount of dead (red stained) cells were observed (not depicted in Fig. 3). The images show that the biofilm formed by the WT strain (Fig. 3a-1) has an elevated number of hyphae, contrary to the biofilm formed by  $\Delta/\Delta$  *efg1* mutant strain, that did not exhibit hyphae (Fig. 3a-2). The biofilm formed by  $\Delta/\Delta$  *tec1* mutant strain (Fig. 3a-3) exhibited hyphae, but in a smaller quantity than the WT strain (Fig. 3a-1). The orthogonal view of biofilms showed that the mutant strains biofilms of  $\Delta/\Delta$  *efg1* (Fig. 3a-2) and  $\Delta/\Delta$  *tec1* (Fig. 3a-3) are thinner than the WT (Fig. 3a-1), specially the  $\Delta/\Delta$  *efg1*, which possesses the thinner structure confirmed by the biovolume (Fig. 3b) and maximum thickness of biofilms (Fig. 3c). The profiles of the distribution of *C. albicans* WT and mutant strains in the 48 h-old biofilms are shown in Fig. 3d, where it can be observed that the biofilm profile of the WT and  $\Delta/\Delta$  *tec1* strains are similar, while the  $\Delta/\Delta$  *efg1* mutant showed a very low percentage of coverage.

## Discussion

The biofilm architecture contributes to the maintenance of stable interactions between cell-cell, cell-surface, and the environment [31]. Furthermore, it protects against phagocytic cells and works as a scaffold for preserving biofilm integrity by limiting the diffusion of noxious substances into the biofilm [32]. Although biofilm resistance is multifactorial [33], the protection exerted by the ECM is a key supporter to the high levels of antifungal drug resistance

displayed by *C. albicans* biofilms [13, 14, 34]. It has been demonstrated that transcriptional regulatory genes in *C. albicans*, including TEC1 and EFG1, regulate biofilm formation [23, 35, 36]. Thus, understanding how these transcriptional regulatory genes influence the ECM composition is paramount to better prevent or impair biofilm formation.

The present study demonstrated that ASP are major components of the ECM of the WT strain, and that these components are significantly reduced in the mutant strains with biofilm formation deficiency ( $\Delta/\Delta$  efg1 and  $\Delta/\Delta$  tec1), being  $\Delta/\Delta$  efg1 the strain that possess the smaller quantities of these components. A significant correlation ( $p < 0.05$ ) between the protein and the ASP content in the ECM of the WT strain ( $r = 0.666$ ) was present, so the production of ASP in this strain might be influenced by the proteins and vice-versa (Table 2). It appears that filamentous cells build-up ECM richer in ASP than non-filamentous cells. Early studies showed a correlation between ECM and levels of resistance against fluconazole and amphotericin B [37, 38]. An ECM component with a role in antifungal drug resistance is  $\beta$ -1,3-glucan (an ASP) which acts through a mechanism of drug sequestration.

Delivery of  $\beta$ -1,3-glucan to the ECM is controlled by a glucan-modifying pathway composed of Bgl2p and Phr1p (glycosyltransferases) and Xog1p (glucanase) [39]. It has been shown that the deletion of any of the genes encoding these proteins resulted in at least 10-fold reduction in matrix  $\beta$ -1,3-glucan content and formed more vulnerable biofilms, that were easily disrupted [39]. Moreover, biofilms formed by these mutants showed less ability to sequester fluconazole and higher susceptibility to this drug [39]. In addition to drug sequestration, it has been demonstrated that the production of  $\beta$ -1,3-glucan by *C. albicans* biofilms hinders the production of reactive oxygen species by neutrophils and protects the cells in biofilm from neutrophil killing [40]. The  $\beta$ -1,3-glucan binds to fluconazole preventing this drug from reaching its cellular targets [13, 39, 41-42]. A similar effect was detected for amphotericin B [18] and for other classes of antifungal agents as well [41]. It has been demonstrated that EFG1

intermediates tolerance of *C. albicans* to azoles (i.e. fluconazole, ketoconazole, itraconazole) and polyenes, including amphotericin B [43]. Moreover, a mutant  $\Delta/\Delta$  *efg1* *C. albicans* strain showed higher susceptibility to these drugs, including miconazole and caspofungin [43, 44]. Thus, the ASP of the ECM has a potential role in the tolerance to antifungals and biofilm structure, since it is much more accumulated in the ECM of the WT strain in contrast to the more susceptible mutant  $\Delta/\Delta$  *efg1*.

Another ECM component with a role in antifungal drug resistance is eDNA. This component is a key matrix component of fungal and bacterial biofilms that enables adhesion to distinct surfaces and binds with other biopolymers, giving biofilms structural integrity and stability [34, 45, 46]. The addition of DNase increases the susceptibility of mature *C. albicans* biofilms against some antifungal agents [47]. The present study found a significant correlation between the eDNA and ASP content in the ECM of  $\Delta/\Delta$  *tec1* mutant strain ( $r=0.678$ ) (Table 4), indicating that the production of these components happens together on each other in this strain. Although the precise mechanism by which eDNA is released and contributes to drug resistance remains unclear [34], it has been suggested that eDNA may be released during hyphal growth [48]. However, the present study shows that independent of the presence of hyphae all the biofilms produced similar quantities of eDNA, contrary to ASP, where filamentous cells produces more ASP than non-filamentous cells. Therefore, the mutant strain  $\Delta/\Delta$  *efg1* that does not form hyphae can produce eDNA and this indicates that the eDNA production may not be necessarily related to hyphal growth.

The  $\Delta/\Delta$  *efg1* population ( $\text{Log}_{10}$  CFU  $\text{mL}^{-1}$ ) is higher than WT and  $\Delta/\Delta$  *tec1* cells. The smaller size and unicellularity can be considered reasons to explain differences in fungal population observed between mutant  $\Delta/\Delta$  *efg1* and WT strains [49]. Moreover, cells lacking EFG1 genes showed increased colonization of the gastrointestinal tract of mice [50]. As the  $\Delta/\Delta$  *efg1* mutant strain did not form hyphae, the smaller size of its cells might have influenced

in the higher number of viable cells for colony counting. In contrast, the strain  $\Delta/\Delta$  *tec1* presented similar population to WT strain, which are statistically smaller than the  $\Delta/\Delta$  *efg1* mutant strain. This might have happened probably because these strains present hyphae. However, the differences in population between all the strains is less than 1 log, meaning that this result may not be biologically significant. On the other hand, the biomass of the mutant strains ( $\Delta/\Delta$  *efg1* and  $\Delta/\Delta$  *tec1*) is lower than the biomass of the WT strain, confirming that these mutants have defective capability of forming biofilm. In addition, the biovolume of the mutant strains is also lower than the WT one (Fig. 3).

The quantitative analysis of proteins showed the mutant strain  $\Delta/\Delta$  *efg1* possesses higher contents of proteins when compared to the WT and  $\Delta/\Delta$  *tec1* mutant strain, which makes sense because  $\text{Log}_{10}$  (CFU mL<sup>-1</sup>) significantly influenced the protein in the biofilm of this strain ( $r=0.734$ ) (Table 3). Pearson's correlation test applied to the current data showed a significant ( $p<0.005$ ) correlation between the protein and the matrix proteins ( $r= 0.670$ ) in the WT strain (Table 2). Proteins encompass a large portion of the biomass in many microbial biofilms [31, 51, 52], having been shown to comprise more than half of the *C. albicans* ECM by weight [16]. A comparison of the matrix proteome of *C. albicans* and total matrix proteins identified several similarities between them, including a large amount of proteins involved in carbohydrate and amino acid metabolism [53, 54]. The known function of most proteins is related to metabolism [16], suggesting that the ECM might “function as an external digestive structure that disrupts extracellular biopolymers as an energy source” [16]. Thus, the higher amounts of protein related to the higher population values observed in the  $\Delta/\Delta$ *efg1* might represent an extra effort of these strain to obtain energy to arrange as a biofilm.

Evaluation of intact biofilm architecture via VPSEM and CSLM provide valuable information of cells and ECM spatial organization. VPSEM preserves the ECM since it does not require sample dehydration process and high chamber vacuum [28]. The images show that

while WT strain present typical bulky biofilm architecture encased by extracellular matrix material (Fig. 2b and Fig. 2c), the  $\Delta/\Delta$  *efg1* mutant strain is morphologically distinct from the WT (Fig. 2e and Fig. 2f). On the other hand,  $\Delta/\Delta$  *tec1* mutant formed a biofilm with ECM comparable to the WT (Fig. 2h and Fig. 2i). Thus, the similarity between the ECM produced by the WT and  $\Delta/\Delta$  *tec1* mutant strain can be related to the growth of pseudo hyphae and hyphae, which goes along with the production of ECM [55].

Moreover, the CLSM images corroborate the results obtained with VPSEM, showing that WT reference strain formed a thick and bulky biofilm on the surface of the polystyrene plate (Fig. 3a-1). However, the mutant strains tested showed defects in biofilm formation, especially  $\Delta/\Delta$  *efg1*, which is severely defective (Fig. 3a-2), while the  $\Delta/\Delta$  *tec1* mutant had less pronounced defects (Fig. 3a-3). These results agree with a study that analyzed the same strains [23]. The measurements of the biovolume and maximum biofilm thickness obtained by COMSTAT2 confirms these results, showing that the  $\Delta/\Delta$  *efg1* mutant strain has the lowest biovolume and maximum thickness ( $p > 0.05$ ) compared to the other studied strains. The biofilm profile of this strain is markedly different from the other studied strains, showing a small percentage of coverage area (Fig. 3d). In addition, previous data shows that  $\Delta/\Delta$  *efg1* mutant strains have impaired hyphae growth under many conditions [19, 21]. Contrary to a previous report that found that biofilm produced by the *tec1* null mutant ( $\Delta/\Delta$  *tec1*) strain was composed exclusively of yeast cells [17], in the present study it was observed that  $\Delta/\Delta$  *tec1* is a defective mutant that exhibits hyphae; however, its biofilm displayed defective visual appearance and smaller quantity of hyphae when compared to the WT strain (Fig. 3a-3). Despite of the visual and microscopically differences between WT and  $\Delta/\Delta$  *tec1* strains, they have a comparable biovolume and maximum biofilm thickness ( $p > 0.05$ ); in addition, the biofilm percentage coverage profile of these strains is also similar (Fig. 3d).



This study characterized the ECM of *C. albicans* WT and mutant strains derived from it. Despite that the  $\Delta/\Delta$  *efg1* mutant strain present severe biofilm defects, its cells grow more in numbers than the WT and  $\Delta/\Delta$  *tec1* mutant strains and it matches the higher quantity of proteins in biofilm. The amounts of eDNA, WSP and matrix soluble proteins are similar between the strains, but the eDNA correlates with the ASP content in the ECM of the  $\Delta/\Delta$  *tec1* strain. On the other hand, the ASP content is significantly higher in the WT strain in comparison to the mutant strains, which indicates that ASP production may be linked to *C. albicans* cell filamentous morphology.

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Table 1. Population and biochemical composition of *C. albicans* biofilms. Mean and standard deviations of Log<sub>10</sub> (CFU mL<sup>-1</sup>), eDNA (μg), WSP (μg), ASP (μg), soluble (μg) and total protein (μg) for *C. albicans* SN 425 (wild-type), *C. albicans* CJN 2302 (Δ/Δ *efg1*) and *C. albicans* CJN 2330 (Δ/Δ *tec1*).

Strains	Biofilm components				ECM components									
	CFU mL <sup>-1</sup> Log <sub>10</sub>		Dry weight (mg)	Protein (μg)	ASP (μg)	WSP (μg)	eDNA (μg)	Matrix protein (μg)						
<i>C. albicans</i> SN 425 (WT)	6.89 (0.07)	A	24.5 (3.7)	A	47.8 (1.5)	A	74.5 (22.0)	A	47.7 (13.4)	A	20.1 (7.1)	A	13.8 (9.8)	A
<i>C. albicans</i> CJN 2302 (Δ/Δ <i>efg1</i> )	7.16 (0.17)	B	18.4 (2.8)	B	50.3 (2.3)	B	14.7 (5.0)	B	52.2 (7.8)	A	27.4 (7.6)	A	20.4 (11.3)	A
<i>C. albicans</i> CJN 2330 (Δ/Δ <i>tec1</i> )	6.83 (0.18)	A	18.5 (3.0)	B	46.5 (1.4)	A	44.0 (24.1)	C	46.1 (11.3)	A	34.2 (14.4)	A	17.4 (4.3)	A

Comparisons by one-way ANOVA and Tukey post-hoc test: means followed by the same letter in column are not significantly different from each other.



Table 2. Pearson correlation of  $\text{Log}_{10}(\text{CFU mL}^{-1})$  and ECM components for the WT strain (SN 425).

WT strain (SN 425)		$\text{Log}_{10}$	ASP	WSP	Total Protein	Matrix Protein	eDNA
$\text{Log}_{10}$	Pearson Correlation	1	.372	.262	.173	.017	-.167
	Sig. (2-tailed)		.234	.411	.590	.957	.605
	N		12	12	12	12	12
ASP	Pearson Correlation		1	.184	<b>.666*</b>	.361	.273
	Sig. (2-tailed)			.567	<b>.018</b>	.249	.391
	N			12	<b>12</b>	12	12
WSP	Pearson Correlation			1	.065	.017	-.326
	Sig. (2-tailed)				.841	.959	.301
	N				12	12	12
Protein	Pearson Correlation				1	<b>.670*</b>	.376
	Sig. (2-tailed)					<b>.017</b>	.229
	N					<b>12</b>	12
Matrix Protein	Pearson Correlation					1	-.032
	Sig. (2-tailed)						.921
	N						12
eDNA	Pearson Correlation						1
	Sig. (2-tailed)						
	N						

\* Correlation is significant at the 0.05 level (2-tailed)

Table 3. Pearson correlation of  $\text{Log}_{10}(\text{CFU mL}^{-1})$  and ECM components for the  $\Delta/\Delta$  efg1 mutant strain (CJN 2302)

$\Delta/\Delta$ efg1		$\text{Log}_{10}$	ASP	WSP	Total Protein	Matrix Protein	eDNA
$\text{Log}_{10}$	Pearson Correlation	1	.246	.057	<b>.734*</b>	-.286	-.315
	Sig. (2-tailed)		.442	.861	<b>.010</b>	.367	.318
	N		12	12	<b>12</b>	12	12
ASP	Pearson Correlation		1	.477	-.133	.252	.290
	Sig. (2-tailed)			.117	.696	.429	.361
	N			12	12	12	12
WSP	Pearson Correlation			1	.236	-.160	.080
	Sig. (2-tailed)				.485	.619	.805
	N				12	12	12
Protein	Pearson Correlation				1	-.205	.538
	Sig. (2-tailed)					.545	.071
	N					12	12
Matrix Protein	Pearson Correlation					1	-.247
	Sig. (2-tailed)						.463
	N						12
eDNA	Pearson Correlation						1
	Sig. (2-tailed)						
	N						

\* Correlation is significant at the 0.05 level (2-tailed).

Table 4. Pearson correlation of  $\text{Log}_{10}(\text{CFU mL}^{-1})$  and ECM components for the  $\Delta/\Delta$  *tec1* mutant strain (CJN 2330).

$\Delta/\Delta$ <i>tec1</i>		$\text{Log}_{10}$	ASP	WSP	Total Protein	Matrix Protein	eDNA
$\text{Log}_{10}$	Pearson Correlation	1	-.080	.171	-.155	-.171	-.194
	Sig. (2-tailed)		.804	.596	.630	.595	.567
	N		12	12	12	12	12
ASP	Pearson Correlation		1	.285	.106	.190	<b>.678*</b>
	Sig. (2-tailed)			.369	.743	.554	<b>.022</b>
	N			12	12	12	<b>12</b>
WSP	Pearson Correlation			1	-.002	-.535	.219
	Sig. (2-tailed)				.995	.073	.518
	N				12	12	12
Protein	Pearson Correlation				1	.000	.098
	Sig. (2-tailed)					1.000	.775
	N					12	12
Matrix Protein	Pearson Correlation					1	.322
	Sig. (2-tailed)						.334
	N						12
eDNA	Pearson Correlation						1
	N						

\* Correlation is significant at the 0.05 level (2-tailed).

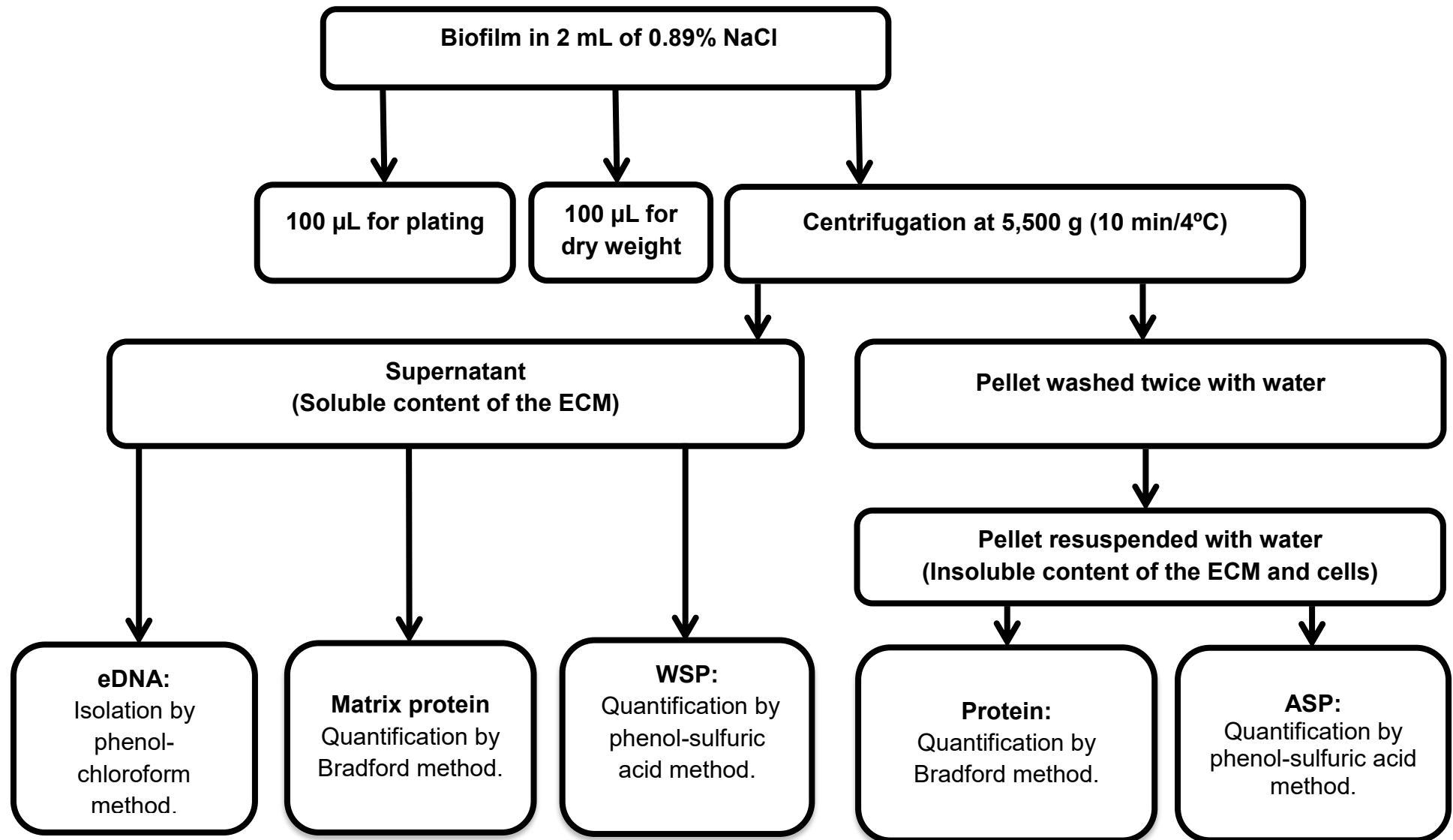
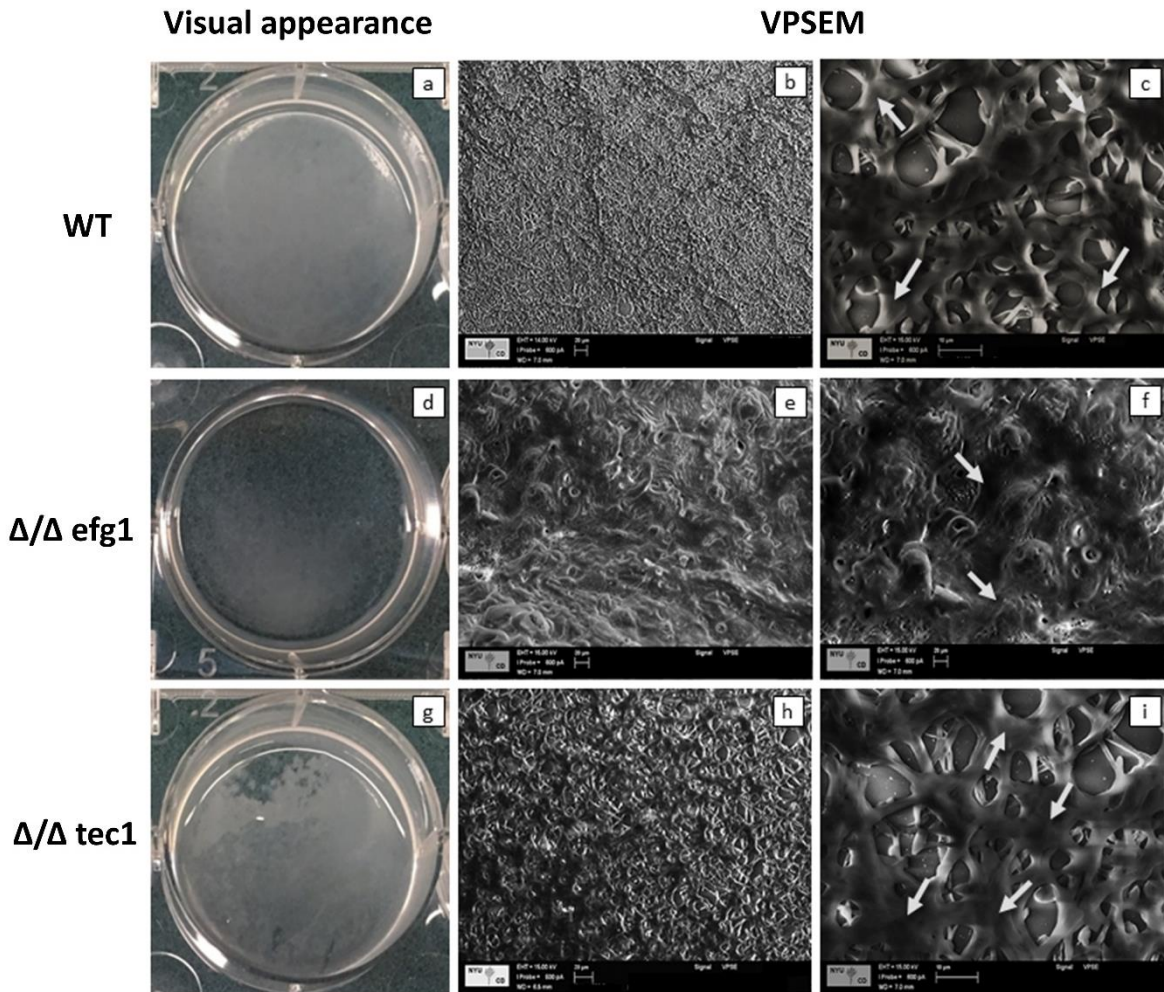
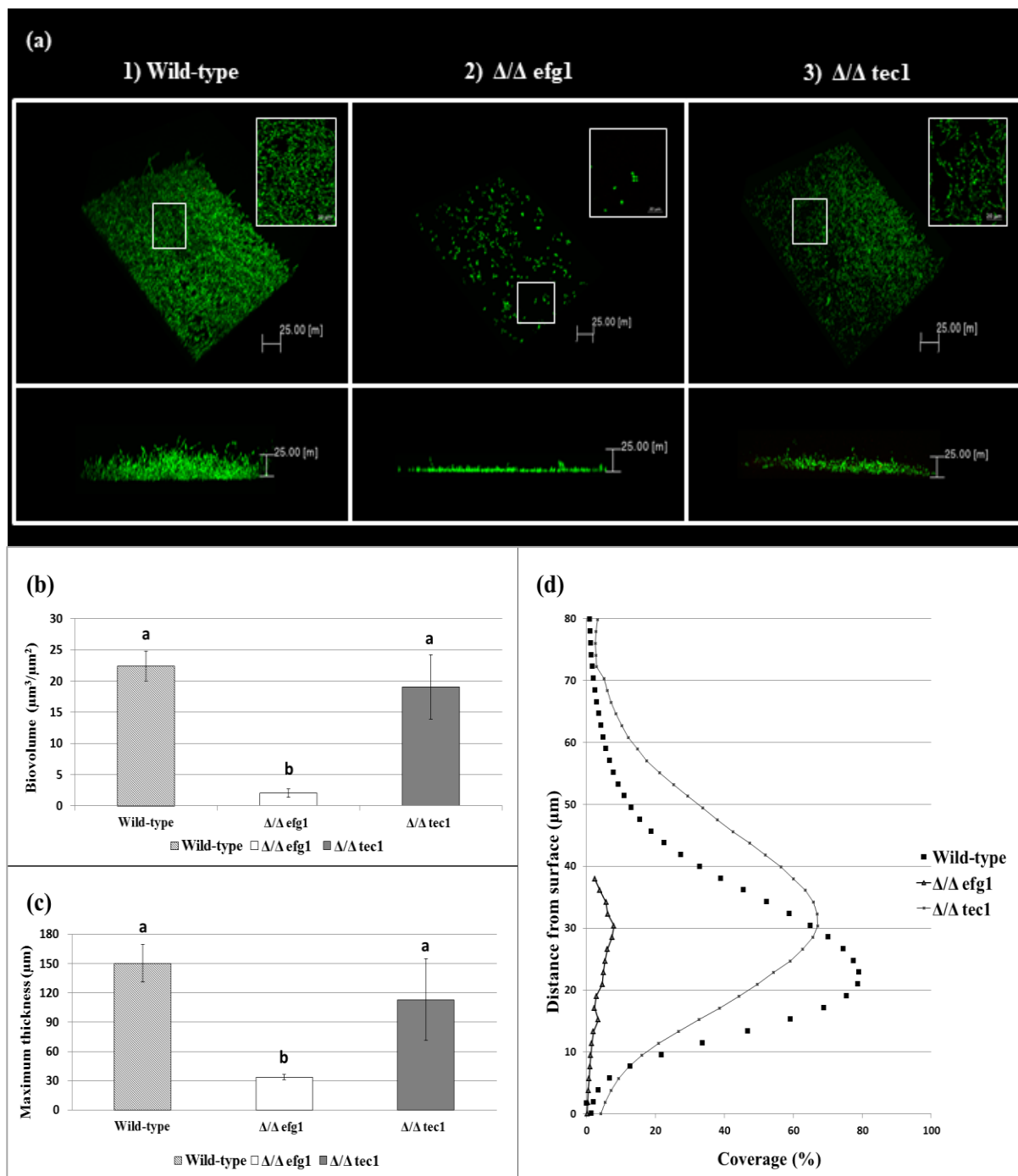
**Fig. 1:** Biofilm characterization flowchart.

Figure 2. Visual macroscopic appearance and overall *C. albicans* biofilm structure by VPSEM.



The WT strain present typical thick biofilm architecture in visual appearance (a) with the presence of abundant ECM (b and c), exemplified by the arrows (c). The  $\Delta/\Delta$  *efg1* mutant shows sparse thin biofilm growth patterns (d) and an ECM (e, f) morphologically distinct from the WT (b, c) and  $\Delta/\Delta$  *tec1* strains (h, i). The  $\Delta/\Delta$  *tec1* mutant present defects in visual appearance (g) compared to the WT strain (a), however, its ECM is morphologically similar to the WT (h, i, see arrows).

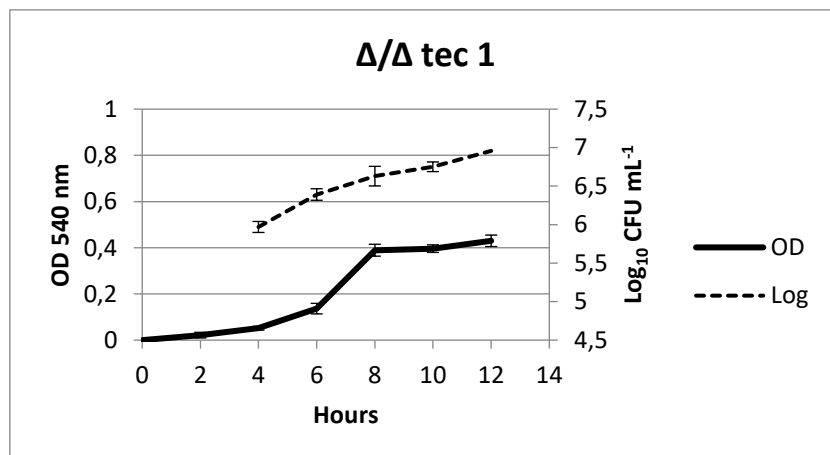
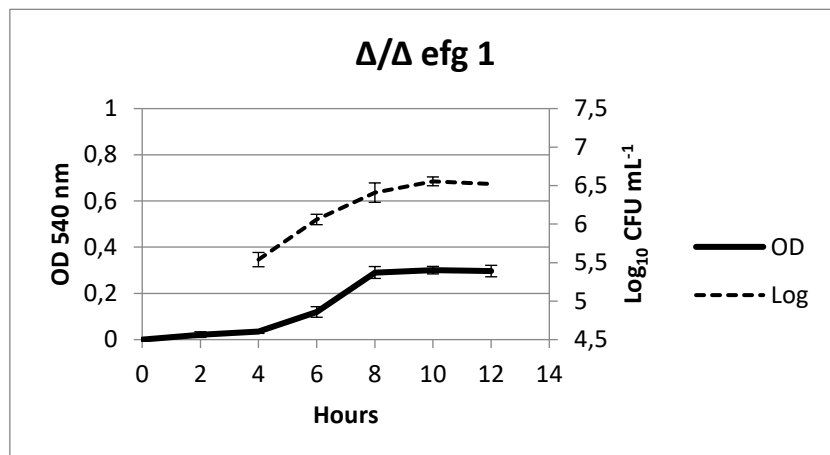
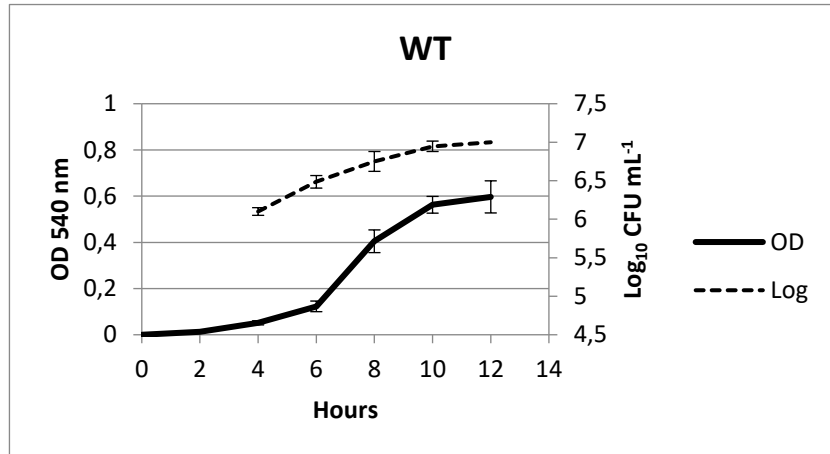
Figure 3. *C. albicans* biofilms structures and corresponding quantitative data from CLSM assays.



(a) Representative three-dimensional and orthogonal images of the structural organization of the 48h biofilms of *C. albicans* wild-type (1) and mutant strains-  $\Delta/\Delta$  *efg1* (2) and  $\Delta/\Delta$  *tec1* (3) (green color denotes labeling with SYTO9 for live yeast cells). Mean and SD of biovolume (b) and average biofilm thickness (c) of *C. albicans* wild-type and mutant strains ( $\Delta/\Delta$  *efg1* and  $\Delta/\Delta$  *tec1*) determined by COMSTAT2 analysis. The average biovolume and biofilm thickness were calculated from five independent samples from each strain. Values followed by the same letter are not significantly different ( $P > 0.05$ ), as determined by an analysis of variance for all pairs using Tukey's test. The profile of the distribution of *C. albicans* WT and mutant strains ( $\Delta/\Delta$  *efg1* and  $\Delta/\Delta$  *tec1*) in the biofilms is represented in (d).

### Supplemental material

**Figure S1:** Growth curves of *C. albicans* strains SN 425 (WT), CJN 2302 ( $\Delta/\Delta$  efg1) and CJN 2330 ( $\Delta/\Delta$  tec1). Planktonic cultures were performed in YNB medium supplemented with 100 mM of glucose and incubated at 37° C. The optical density (OD at 540 nm) and the population ( $\text{Log}_{10}$  CFU  $\text{mL}^{-1}$ ) were determined over time.



### 3.2 Publicação 2

**Fluconazole impacts the extracellular matrix of fluconazole-susceptible and resistant *Candida albicans* and *Candida glabrata* biofilms.**

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## ABSTRACT

**Background:** Fluconazole (FLZ) is a drug commonly used for the treatment of *Candida* infections. However,  $\beta$ -glucans in the extracellular matrices (ECM) hinder FLZ penetration into *Candida* biofilms, while extracellular DNA (eDNA) collaborates with biofilm's architecture and resistance.

**Methods:** This study characterized biofilms of fluconazole-sensitive (S) and -resistant (R) *Candida albicans* and *Candida glabrata* in the presence or absence of FLZ focusing the ECM traits. Biofilms of *C. albicans* ATCC 90028 (CaS), *C. albicans* ATCC 96901 (CaR), *C. glabrata* ATCC 2001 (CgS) and *C. glabrata* ATCC 200918 (CgR) were grown with RPMI medium with or without biofilm FLZ sub-minimum inhibitory concentrations (MIC) per strain (37°C/48 h). Biofilms were assessed by CFU/mL, biomass and ECM components (alkali-soluble polysaccharides-ASP, water-soluble polysaccharides-WSP, eDNA and proteins). Scanning Electron Microscopy (SEM) was also performed. Data were analyzed by two-way ANOVA tests ( $p \leq 0.05$ ).

**Results:** In biofilms, FLZ reduced the CFU/mL of all strains ( $p=0.000$ ), except for CaS ( $p=0.937$ ). However, the ASP amounts in CaS were significantly reduced by FLZ ( $p=0.034$ ), while the drug had no effect on the ASP amounts of the other strains ( $p>0.05$ ). Total biomasses and WSP were significantly reduced by FLZ in the ECM of all microorganisms ( $p=0.000$ ), but eDNA and proteins amounts were not influenced by the presence of FLZ nor by the type of the strain ( $p>0.05$ ). FLZ affected the cell morphology and biofilm structure by hindering hyphae formation in CaS and CaR biofilms, by decreasing the number of cells in CgS and CgR biofilms and by yielding sparsely spaced cell agglomerates on the substrate.

**Conclusion:** FLZ hindered the accumulation of WSPs and reduced the biomasses of the biofilms by decreasing hyphae prolongations and the number of cells.

**KEYWORDS:** Biofilm, *Candida albicans*, *Candida glabrata*, Extracellular matrix, Fluconazole, Fluconazole-resistant.

## Introduction

*Candida* spp. are commensal fungi of the oral cavity of healthy individuals that can become opportunistic pathogens in some situations, for example, when there are changes in immune system, metabolic dysfunction or in high-age population [1-7]. Moreover, growing usage of broad-spectrum antibiotics, cytotoxic chemotherapies and transplantation also intensifies the risk for infections by these opportunistic fungi [7]. These infections are known as candidiasis. *Candida albicans* is the main specie associated to this disease, while *Candida glabrata* is the most predominant non-albicans specie that has been linked to the development of oral infections [1-6]. *C. glabrata* is considered a pathobiont pathogen [7-8], mainly due to its innate resistance to azoles [9-10].

*Candida* infections are frequently related to the establishment of biofilms. Biofilms are composed by microbial cells that are attached to a substrate and surrounded by an extracellular matrix (ECM) [11]. The ECM is composed of secreted microbial and host-derived substances and cells lysis [11], and collaborates to the conservation of the biofilms architecture and to the preservation of stable interactions between cell-cell, cell-surface, and the environment [12]. Although polysaccharides and protein are the most widely studied substances in biofilms ECMs, other molecules, such as nucleic acids, are important to their function [11].

Treatments for oral infections caused by *Candida* use topical [13] and systemic [14] antifungal medication, such as Fluconazole (FLZ). Systemic antifungal medication is usually prescribed for individuals with compromised overall health and in the episodes of recurrent infections [14]. An important aspect to be considered in therapy with topical or systemic antifungal refers to the resistance that *Candida* species can present to these drugs. Resistance to antifungal agents can be defined as the persistence or progression of an infection after application of antimicrobial treatment [15-16]. Some studies have observed the emergence of resistant microorganisms during long-term or prophylactic treatment [17-18]. Intrinsic or

primary resistance occurs when a microorganism has low susceptibility to a medication, prior to its exposure to the agent. Some *Candida* species possess intrinsic resistance to antifungal drugs, especially to fluconazole [7, 10]. In contrast, the secondary resistance is one that can be developed by the microorganism after long periods of exposure to antifungal drugs [15]. Thus, a major concern with *Candida* spp. biofilms is that their cells may have reduced susceptibility against azoles and polyenes, due to development of resistance [19-20].

The resistance of *Candida* biofilms is multifactorial and involves the stimulation of drug efflux pumps, the cells physiological state and the protection exerted by the ECM via  $\beta$ -glucans, that bind to FLZ and amphotericin B [21], avoiding the diffusion of these antifungals through biofilms [22]. *C. albicans* biofilms that grew under constant flow produced a higher quantity of ECM than those grown statically and presented higher resistance to amphotericin B, showing that the matrix can expressively influence on drug resistance in *Candida* [23]. In the same study, the authors detected that the ECM of *C. albicans* GDH 2346 (NCYC 1467) had carbohydrate, protein, hexosamine, phosphorus and uronic acid. Nevertheless, the main constituent of the ECM *C. albicans* was glucose (32%) [23]. Besides  $\beta$ -glucan, it has been revealed that the extracellular DNA (eDNA) is also a relevant constituent of the ECM that guarantees the structure integrity of *C. albicans* biofilm [24]. Furthermore, nuclear magnetic resonance (NMR) investigation verified interaction of ECM aggregate with fluconazole, demonstrating a role in drug resistance [25].

The difficulties in determining the key ECM components have been a challenge to the understanding of how the ECM interferes in *Candida* drug resistance [26]. Thus, better knowledge of the assemblage and functional properties of the matrix and FLZ influence on it will enable the design of more effective therapies to control *Candida* pathogenesis and biofilm development Here we characterized the biofilms of fluconazole-susceptible and -resistant *C.*

*albicans* and *C. glabrata* strains in the presence or absence FLZ to evaluate the drug interference in the ECM of these microorganisms.

## **Material and Methods**

### ***Microorganisms***

Four American Type Culture Collection (ATCC) *Candida* species were used in this study. *C. albicans* ATCC 90028 (fluconazole-susceptible; CaS) was originally isolated from blood in Iowa, USA. *C. albicans* ATCC 96901 (fluconazole-resistant; CaR) was isolated from the mouth of an HIV-positive patient in Omaha, NE, USA. *C. glabrata* ATCC 2001 (fluconazole-susceptible; CgS) was isolated from feces in Wayne, PA, USA and *C. glabrata* ATCC 200918 (fluconazole-resistant; CgR) was isolated from human tongue in Santa Rosa, CA, USA.

### ***Growth curves***

To ensure the reproducibility of the biofilms model, growth curves were constructed based on optical density (OD) at 540 nm wavelength. Growth curves were performed on three different occasions with three replicates. The number of colony forming units (CFU) were determined at four-time points (Figure 1).

The microorganisms kept at -80°C were seeded onto Petri dishes with SDA (Sabourand dextrose agar- DIFCO, Detroit, Michigan, USA) culture medium supplemented with chloramphenicol (50 mg/L) [27] and incubated at 37°C for 48 h. Then, 5 colonies of each microorganism were added separately to tubes containing YNB medium (Yeast Nitrogen Base- DIFCO, Detroit, Michigan, USA) supplemented with 100 mM of glucose [27] and these pre-inoculums were incubated at 37°C for 16 h. Afterwards, the pre-inoculums were diluted with fresh YNB medium plus 100 mM glucose (1:20 dilution for *C. albicans* strains and 1:10 for *C. glabrata* strains) to form the inoculums, and the OD<sub>540 nm</sub> and CFU were determined every 2 h

in a total of 16h for each strain. Biofilms formation was performed with cells at mid-log growth phase of each microorganism (Figure 1).

### ***Susceptibility testing and fluconazole concentrations for biofilms formation***

The CLSI M27-A3 broth microdilution susceptibility method [28] was performed to examine the minimal inhibitory concentrations (MIC) of FLZ against planktonic fluconazole susceptible and –resistant *C. albicans* and *C. glabrata* strains. Fluconazole powder (F8929, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile ultrapure water [29]. As a negative control, a 100  $\mu$ L of 2 $\times$  concentrated RPMI 1640 buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic (MOPS) plus a 100  $\mu$ L of sterile ultrapure water was used (no cells and antifungal agent). As a positive control, only cell suspensions were tested without the antifungal agent. Serial two-fold dilutions of fluconazole (range: 0.125 to 512  $\mu$ g/ml) in RPMI 1640 medium buffered to pH 7.0 with 0.165 MOPS buffer were inoculated in 96-well plates with each microorganism suspension adjusted to achieve a final inoculum concentration of  $0.5 \times 10^3$  to  $2.5 \times 10^3$  cells/mL based on the growth curves (Figure 1). The plates were incubated at 37°C and observed for the presence or absence of growth at 24 h. In addition to visual endpoint readings, the optical density of each plate well was measured at 562 nm after 24 h of incubation. MICs in spectrophotometer were based on the density of the growth control and were considered the lowest drug concentrations that resulted in minimum 90% decrease in growth related to the drug-free growth control [30-31]. As cells in biofilms are more resistant to drugs [32], the concentrations of 5X and 10X were tested in biofilms [33]. The 5X MIC concentration was chosen because 10X MIC almost completely inhibited the formation of biofilm (data not shown), and this was not the goal of the study since the inhibition of biofilm does not allow the evaluation of FLZ effects on biofilms' ECMs. Thus, 5X MIC concentrations were used for biofilm formation.

### ***Biofilm formation and processing***

Biofilms formation and processing for Log<sub>10</sub> CFU/mL, total biomass, insoluble biomass, proteins (from the insoluble part of the biofilm and from the supernatant), ASP, WSP and eDNA were performed according to the methodology described by Panariello et al. (2017) [34]. Briefly, biofilms of CaS, CaR, CgS and CgR were formed for 48 h in RPMI buffered with MOPS (pH 7.0). Biofilms (48 h) were washed twice with 0.89% NaCl and detached by individually scratching the wells with a pipette tip and 2 mL 0.89% NaCl. Then, biofilms were separated for Log<sub>10</sub> (CFU/mL) determination (0.1 mL) and for total biomass determination [35] (0.1 mL). After that, biofilms were dynamically vortexed, centrifuged (5,500 x g/10 min/4°C) and the supernatant containing the soluble part of the matrix was separated from the *pellet*, which contains the insoluble part of the matrix. The supernatant was divided for the quantification of WSPs (1 mL) [36], eDNA (0.650 mL) [37] and matrix protein in the soluble portion (0.150 mL) [38]. The *pellet* was washed twice and resuspended in sterile ultrapure water, then, it was separated for the quantification of insoluble biomass (0.8 mL), protein from the insoluble portion (0.05 mL) [38] and ASPs (0.95 mL) [36].

### ***Scanning electron microscopy (SEM)***

Biofilms were grown over polystyrene pieces obtained from the bottom of 24-well plates [39]. Before its use, they were sterilized in a microwave for 3 min at 650 W [40] and dried at flow chamber with UV light for 30 minutes. After 48 h of biofilms growth, the media was removed, and the wells were washed twice with 1 mL of sterile 0.89% NaCl. Next, the biofilm samples were fixed with 2.5% glutaraldehyde (60 min/ room temperature), washed twice with sterile 0.89% NaCl, and dehydrated. The dehydration process was performed with series of washes with ethanol 70% and 90% for 60 min each, followed by 5 washes of 30 min with absolute ethanol. The samples were stored in a desiccator with silica for 7 days to guarantee moisture-free samples. Then, the samples were fixed in aluminum stubs, sputter coated with gold, and

observed with a JEOL JSM-6610LV Scanning Electron Microscope, using magnifications of 160, 600 and 1200.

### ***Statistical analyses***

Normal distribution of data was verified by Shapiro-Wilk test and homogeneity of variance was checked by Levene test ( $\alpha = 0.05$ ). The quantitative data of CFU/mL, biomass, proteins from the insoluble portion and ECM components were statistically analyzed by two-way analysis of variance (ANOVA) considering the presence or absence of FLZ and the different strains (CaS, CaR, CgS, CgR). When the postulation of normality was not encountered, data were ranked and non-parametric analysis (ANOVA on ranks) was applied ( $\alpha=0.05$ ). For multiple comparisons, Tukey *post hoc* test was applied for homoscedastic data and Games-Howell *post hoc* test for heteroscedastic data ( $\alpha=0.05$ ). Analyses were done in the software SPSS (IBM® SPSS® Statistics, version 20, Chicago, IL, USA).

## **Results**

### ***Susceptibility testing and fluconazole concentrations for biofilms formation***

The MIC was performed in duplicate at three different occasions. The MIC<sub>90</sub> for planktonic cells were: CaS= 16  $\mu\text{g/mL}$ ; CaR= 256  $\mu\text{g/mL}$ ; CgS= 8  $\mu\text{g/mL}$  and CgR= 256  $\mu\text{g/mL}$ . The MIC<sub>90</sub> concentration found for planktonic cells of CaS; however, is not typical for sensitive strains. As cited before, cells in biofilms are more resistant than cells in planktonic cultures, thus, for biofilm formation, 5X MIC was applied: CaS= 80  $\mu\text{g/mL}$ ; CaR= 1280  $\mu\text{g/mL}$ ; CgS= 40  $\mu\text{g/mL}$  and CgR= 1280  $\mu\text{g/mL}$ .

### ***Biofilm and ECM characterization***

Log<sub>10</sub> (CFU/mL) presented normal distribution and homoscedasticity (Levene test:  $p=0.110$ ), thus it was analyzed by two-way ANOVA with “FLZ” and “strains” as main effects. There was a significant interaction between the factors “FLZ” and “strains” ( $p=0.000$ ), so one-way ANOVA followed by Tukey *post hoc* test was performed. FLZ

significantly reduced ( $p=0.000$ ) the CFU/mL of CaR (reduction of 0.88 logs), CgS (reduction of 0.71 logs) and CgR (reduction of 0.70 logs). On the other hand, the statistics pointed that the reduction of 0.67 logs caused by FLZ on CaS biofilms was not statistically significantly ( $p=0.937$ ). Moreover, CaS presented the lowest CFU/mL of all the strains in the absence of FLZ ( $p=0.000$ ). Mean and standard deviations of  $\text{Log}_{10}$  (CFU/mL) are represented in Figure 2.

The total biomass data did not show normal distribution; therefore, data were ranked. The non-parametric analysis showed that the factor “FLZ” caused significant interference in the results ( $p=0.000$ ), reducing the biomasses of all the strains (Figure 3).

The insoluble biomass data did not show normal distribution; thus, data were ranked, and non-parametric analysis was performed. An interaction between the factors “FLZ” and “strains” was observed ( $p=0.000$ ). Levene’s test showed that data were heteroscedastic ( $p=0.06$ ), so one-way ANOVA on ranks with Welch correction and Games-Howell *post hoc* test was applied. The insoluble biomass of CaS ( $p=0.038$ ), CaR ( $p=0.000$ ) and CgR ( $p=0.000$ ) were significantly reduced by the presence of FLZ. On the other hand, CgS showed a higher insoluble biomass in the presence of FLZ when compared to CgS in the absence of FLZ ( $p=0.000$ ). Mean and standard deviations of insoluble biomasses are represented in Figure 4.

Protein data from the insoluble portion met the criteria of normality, therefore two-way ANOVA was performed, showing that there were no interactions of factors “FLZ” and “strain” in the results obtained ( $p>0.224$ ). Thus, no *post hoc* tests were performed. Consequently, protein amounts from the insoluble portion were not affected by FLZ and the quantities of this component are not influenced by the type of the strain (Figure 5).

For WSP data the assumption of normality was not found, thus data were ranked. The non-parametric analysis showed that the only factor that influenced the quantity of WSP was “FLZ” ( $p=0.000$ ), reducing this component in the ECM of all the evaluated strains (Figure 6).



ASP data did not meet the assumptions of normality; therefore, data were ranked. Levene's test pointed to the homogeneity of variances ( $p=0.294$ ). As the non-parametric test showed that there was a significant interaction between the factors "FLZ" and "strains" ( $p=0.002$ ), data were submitted to one-way ANOVA on ranks with Tukey *post hoc* test. Statistics demonstrated that ASP amount was significantly reduced by FLZ on CaS ( $p=0.034$ ). However, for the other strains, FLZ did not alter ASP amounts ( $p>0.200$ ). Moreover, CgS produces significantly smaller amounts of ASP than the other evaluated strains ( $p=0.008$ ). Mean and standard deviations of ASP are represented in Figure 7.

eDNA data did not show normal distribution, so data were ranked. The non-parametric analysis revealed that there were no interactions of any factors in eDNA data ( $p>0.006$ ), thus, no *post hoc* tests were performed. Therefore, eDNA amounts were not affected by FLZ and the type of the strain does not interfere with the amounts of eDNA (Figure 8).

Matrix protein data did not meet the assumptions of normality and data were ranked. The non-parametric analysis showed that the "strain" was the only factor that interacted with the results obtained ( $p=0.000$ ). Levene's test showed that matrix protein data are homoscedastic ( $p=0.176$ ), therefore one-way ANOVA on ranks with Tukey *post hoc* test was applied for multiple comparisons. However, the analysis showed no statistical differences in the amount of matrix proteins between the strains ( $p>0.05$ ) (Figure 9).

### ***Biofilm structure***

SEM was performed to examine the overall biofilm structure of different microorganisms in the presence and in the absence of FLZ. Especial attention was given to the cells morphology and spatial organization on substrate, as well as on ECM covering and/or linking cells in these biofilms. Figure 10 shows the SEM images of CaS (Figure 10 A, B, C) and CaS+FLZ (Figure 10 D, E, F), demonstrating that FLZ reduced the size of hyphae. A closer

look at the structures shows that without FLZ cells are embedded in the ECM, as shown by the arrow (Figure 10 C).

Figure 11 shows the SEM images of biofilms formed by CaR (Figure 11 A, B and C) and CaR+FLZ (Figure 11 D, E and F). The CaR biofilm possesses large amounts of yeast cells and elongated hyphae structures (Figure 11 A, B, C). Figure 11 C shows the union of cells within biofilms through their extracellular matrices, as exemplified by the arrow. FLZ reduced the size and number of hyphae, remaining mostly cells with yeast morphology (Figure 11 C, D, E).

Figure 12 shows the SEM images of biofilms formed by CgS (Figure 12 A and B) and CgS+FLZ (Figure 12 C and D). CgS biofilm is composed by yeast cells (Figure 12 A) linked by ECM (Figure 12 B). The presence of FLZ reduced the number of cells (Figure 12 C) and the ECM (Figure 12 D).

Figure 13 shows the SEM images of biofilms formed by CgR (Figure 13 A and B) and CgR+FLZ (Figure 13 C and D). CgR biofilm presents a high quantity yeast cells (Figure 13A) and clusters interconnected by ECM (Figure 13 B). The presence of FLZ reduced the number of cells (Figure 13 C) and the ECM (Figure 13 D).

## **Discussion**

Fluconazole is often a chosen treatment for *Candida* infections because of its low cost and availability for oral administration [41]. This drug prevents the biosynthesis of ergosterol through the cytochrome P450 enzyme 14- $\alpha$  demethylase, which catalyzes the conversion of lanosterol to ergosterol [42]. The reduction of ergosterol changes the fluidity of the membrane and the activity of numerous membrane-bound enzymes, hindering the fungal growth and replication [42]. However, fluconazole-resistance is rising in *Candida* species [41, 44-45], requiring a better understanding of the action of this drug in *Candida* biofilms. Due to the mechanism of action of FLZ, variations in the architecture of the ECM may happen during

*Candida* biofilm formation in the presence of this medication [33, 46]. Here we characterized the biofilms formed by fluconazole-susceptible and -resistant *C. albicans* and *C. glabrata* strains in the presence or absence FLZ to evaluate the drug interference in the ECM of these microorganisms.

Surprisingly, no significant reduction in CaS counts was observed after exposure to 5x MIC doses of FLZ (Figure 2), and the images showed that FLZ reduced the size of hyphae in strain (Figures 10 C, D, E). However, the growth of CaR biofilms in the presence or absence of FLZ showed that the drug significantly reduced its population ( $\text{Log}_{10}$  CFU/mL) (Figure 2). In addition, SEM images showed that FLZ reduced hyphae formation in CaR (Figures 11 D, E, F). This result corroborates to a previous study that observed that FLZ has a direct inhibitory effect on hyphal formation [47]. Because FLZ interferes with the ergosterol pathway and ergosterol is necessary for hyphae formation, the presence of FLZ inhibits the transition from yeast to hypha [47], even in a culture media that promotes *C. albicans* filamentous morphology. Thus, taking into consideration the hyphae is the invasive form of *C. albicans* [48], the reduction of its size in both CaS and CaR is an important result to the reduction of the virulence of this strain. Likely to what happened to *C. albicans* strains, FLZ significantly reduced the CFU/mL in CgS and CgR (Figure 2). These results were confirmed by the SEM images of CgS (Figures 12 D and E) and CgR (Figures 13 D and E), which showed fewer cells and cell agglomerates are more sparsely distributed on the substrate. It has been reported that *C. glabrata* has intrinsic decreased susceptibility to FLZ and another classes of azoles antifungals [41, 49-50], nevertheless, the results of the present study showed that biofilm formation with 40  $\mu\text{g/mL}$  of FLZ for CgS and 1280  $\mu\text{g/mL}$  of FLZ for CgR can reduce the cell viability of these microorganisms. Therefore, the log reduction values for all strains is very close (CaS: reduction of 0.67 logs; CaR: reduction of 0.88 logs; CgS: reduction of 0.71 logs and CgR: reduction of 0.70 logs).

An important finding of this study was that FLZ acted on the ECM of all the microorganisms by reducing the production of WSPs (Figure 6). *Candida* biofilm matrix has  $\alpha$ -1,2 branched and  $\alpha$ -1,6 mannans (WSPs) associated with  $\beta$ -1,6 glucans (ASPs), constituting a mannan-glucan complex (MGCx) [25]. Considering that interactions of ECM polysaccharides are required for biofilm antifungal resistance [51], the reduction on WSP amounts in the ECM of the biofilms in the presence of FLZ might alter the constitution of the MGCx, leading to a higher susceptibility of the biofilms to antifungal therapy. Another noteworthy remark of this study is that FLZ caused an important antibiofilm activity by causing a significant reduction of the total biomasses of all the microorganisms studied. Cells and ECM components compose the biomasses, thus, the significant decreasing of WSP as well as the reduction of the size of hyphae in CaS (Figure 10 D, E, F) and CaR (Figure 11 D, E, F) and the reduction of cells in CgS (Figure 12 C, D) and CgR (Figure 13 C, D) might be responsible for the significant reduction of the biomasses.

In addition, FLZ significantly reduced the ASPs content only in the ECM of CaS strain biofilm (Figure 7). The ASPs such as  $\beta$ -1,3 glucans, sequester antifungals preventing them from diffuse through the ECMs of *C. albicans* [21, 51-54] and non-albicans biofilms [55]. It has been demonstrated the role of  $\beta$ -1,3 glucan in antifungal resistance as it binds to FLZ avoiding this drug to reach its targets [21, 51-55]. Thus, ASPs significant reduction in the ECM of CaS biofilms demonstrates that the biofilms of this specific strain get more vulnerable when FLZ is present. Moreover, although ASPs can bind to FLZ, when 80  $\mu$ g/mL of FLZ is present during CaS biofilm formation the drug can evade this effect and cause ASPs reduction.

The insoluble content of the biofilm (i.e. ASP and cells, which contains the proteins) composes the insoluble biomass. The results showed that in the presence of FLZ there was a significant reduction of the insoluble biomass of CaS, CaR and CgR (Figure 4) probably caused by the reduction of hyphae and blastopores, and by the numerical reduction of the ASP content.

However, CgS showed a significant increase in the insoluble biomass in the presence of FLZ. Variations in the ECM might happen when ions are available [56]. Possibly ions from FLZ ( $C_{13}H_{12}F_2N_6O$ ) might have interacted with carboxylic acid groups of the matrix to produce grids of macromolecules with higher viscosity resulting in a stickier polysaccharide matrix [56].

FLZ caused no effects on eDNA, proteins (from the insoluble part of the biofilm) and matrix proteins amounts in all the strains evaluated. Interestingly, the amounts of these components are also not different among the different types of strains evaluated. The production of eDNA has been associated to hyphal growth [57]. Nevertheless, the present study demonstrated that even in the absence of hyphae (e.g. in *C. glabrata* biofilms), all biofilms released comparable amounts of eDNA both in the presence or absence of FLZ. This result corroborates to a previous finding that observed that a strain defective in hyphae formation ( $\Delta/\Delta$  *efg1*) was capable to produce eDNA similarly to its parental strains that formed hyphae [34]. In *C. albicans*, eDNA contributes to the preservation and stability of mature biofilms, but not to their establishment [23, 24], while the recognized role of the majority of proteins is linked to metabolism [25]. In addition, eDNA performs as a regulator of biofilm cell antifungal resistance against echinocandins and amphotericin B but does not seem to significantly contribute to FLZ resistance [58]. This finding corroborates with the results found here, since FLZ did not affect the production of eDNA, as well it did not affect the production of proteins.

The present study showed that WSPs and biomasses are directly affected by the presence of FLZ during biofilm formation, while eDNA and proteins remains similar independent of the strain. Thus, FLZ hindered the accumulation of WSPs and reduced the biomasses by acting on hyphae and blastopores. The reduction of WSP and total biomasses may be related to biofilms susceptibility to therapeutics and virulence, making those growing in FLZ more susceptible and potentially less virulent. However, as FLZ is fungistatic, there is a chance for the development of acquired resistance during the treatment with this antifungal [45]. Moreover, the drug in 5X

MIC concentrations during biofilm formation had no effect on eDNA and proteins. Therefore, it is important to consider alternative ways to disorganize the ECMs to get a better diffusion of the drug through the biofilms to reach the cells.

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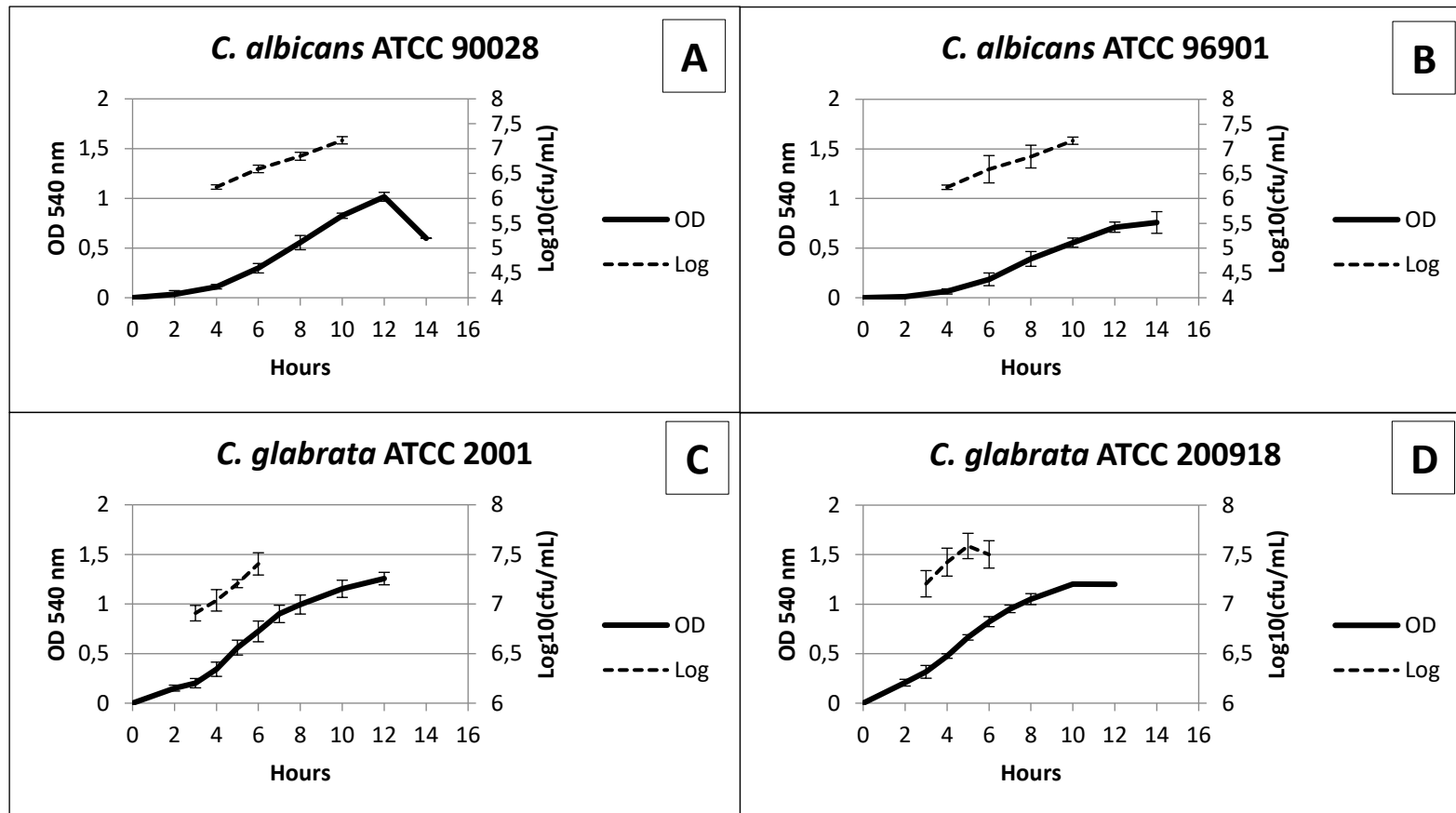
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**Figure 1.** Growth curves of the microorganisms (A) *C. albicans* ATCC 90028 (CaS); (B) *C. albicans* ATCC 96901 (CaR), (C) *C. glabrata* ATCC 2001 (CgS) and (D) *C. glabrata* ATCC 200918 (CgR). In the mid-log phase, the mean  $\pm$  standard-deviation of OD<sub>540nm</sub> for CaS is  $0.55 \pm 0.08$  and the Log<sub>10</sub> CFU/mL is  $6.8 \pm 0.1$ . For CaR the OD<sub>540nm</sub> is  $0.39 \pm 0.08$  and the Log<sub>10</sub> CFU/mL is  $6.8 \pm 0.3$ . For CgS the OD<sub>540nm</sub> is  $0.70 \pm 0.08$  and the Log<sub>10</sub> CFU/mL is  $7.4 \pm 0.1$ . For CgR the OD<sub>540nm</sub> is  $0.65 \pm 0.09$  and the Log<sub>10</sub> CFU/mL is  $7.6 \pm 0.2$

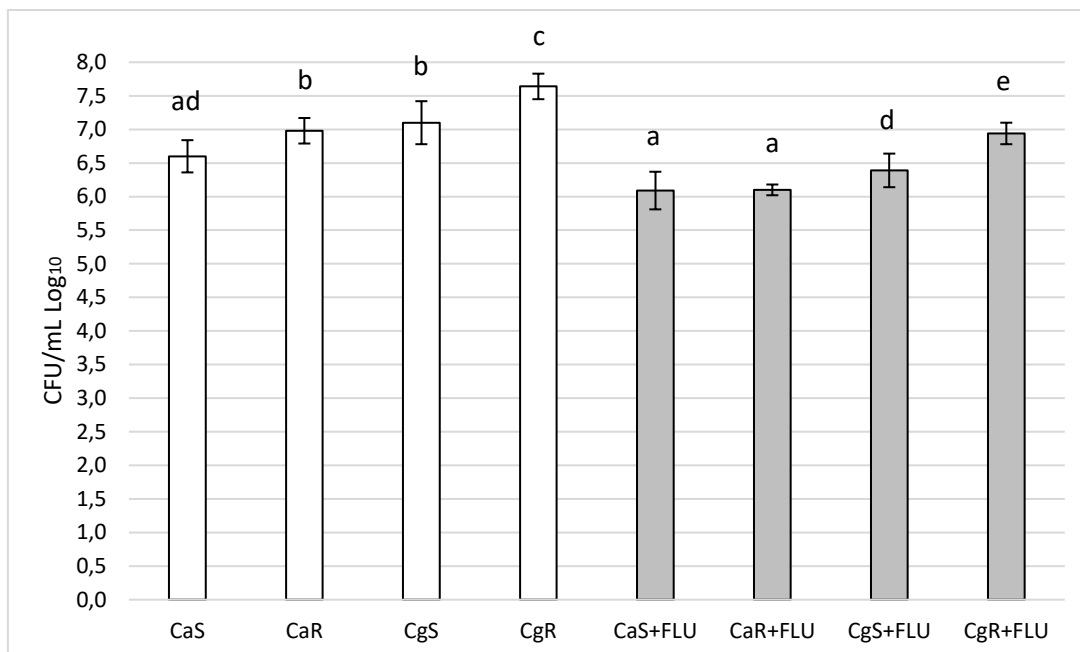


Figure 2: Mean values and standard deviations of Log<sub>10</sub>(CFU/mL) of CaS, CaR, CgS and CgR in the absence (white bars) or in the presence (grey bars) of FLZ. Bar charts were applied for normal data submitted to parametric analysis (ANOVA). Equal letters designate statistical correspondence ( $p < 0.05$ ).

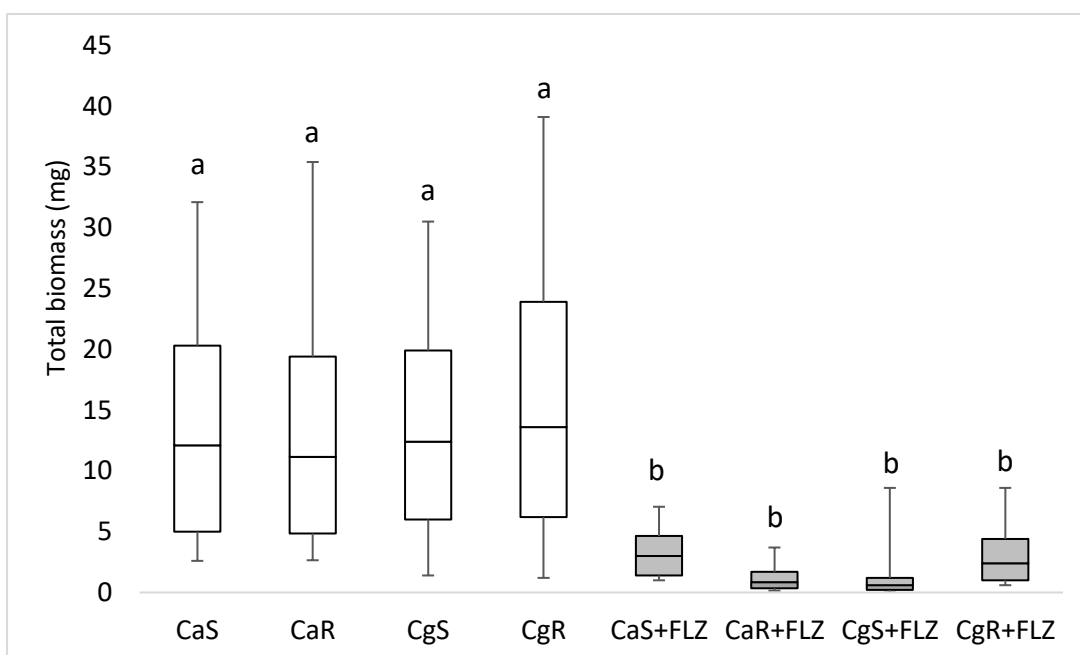


Figure 3: Box-plot of total biomass (mg) in CaS, CaR, CgS and CgR in the absence (white boxes) or in the presence (grey boxes) of FLZ. The box-plot shows the median (dash), the first and third quartiles (outer edges of box), and the highest and maximum values (error bars). A non-parametric analysis (ANOVA on rank) was applied. Equal letters designate statistical correspondence ( $p < 0.05$ ).

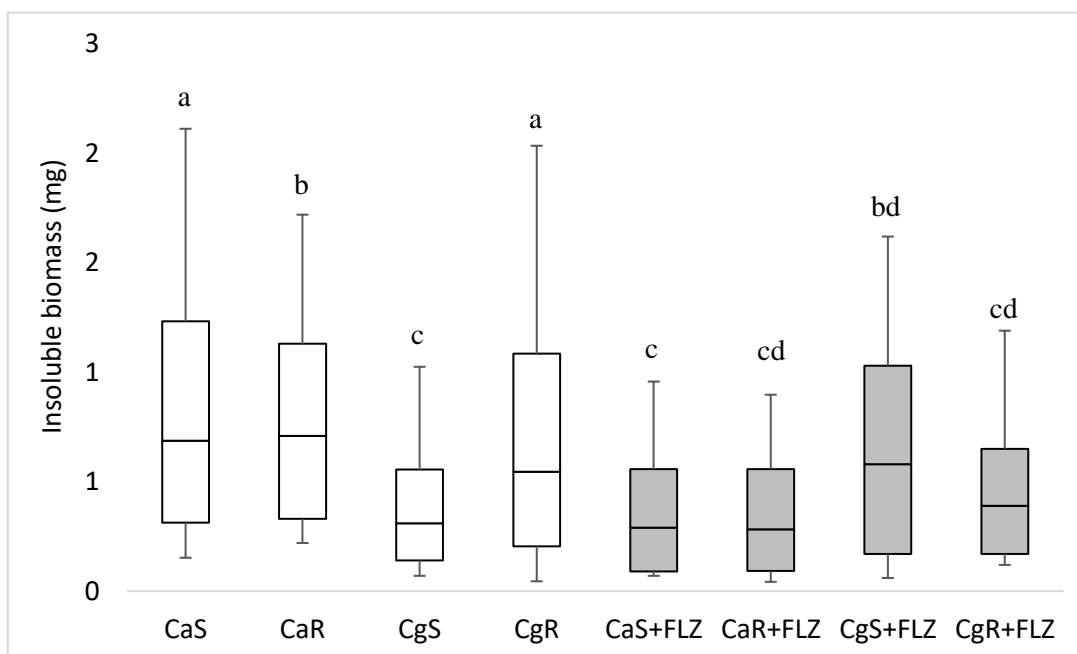


Figure 4: Box-plot of insoluble biomass (mg) in CaS, CaR, CgS and CgR in the absence (white boxes) or in the presence (grey boxes) of FLZ. The box-plot shows the median (dash), the first and third quartiles (outer edges of box), and the highest and maximum values (error bars). A non-parametric analysis (ANOVA on rank) was applied. Equal letters designate statistical correspondence ( $p < 0.05$ ).

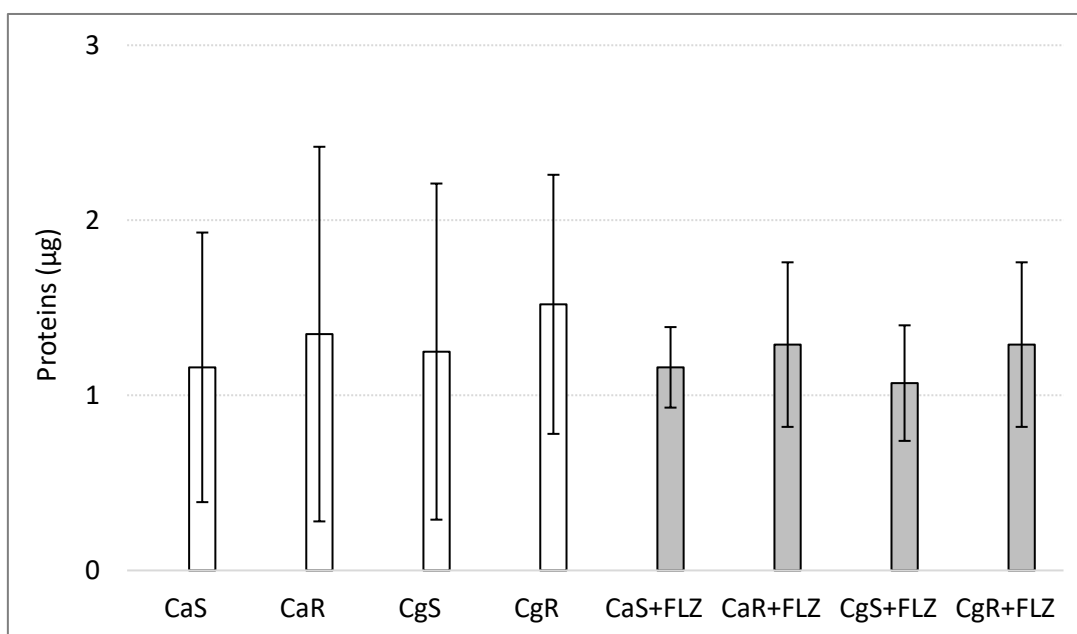


Figure 5: Mean values and standard deviations of proteins from the insoluble portion ( $\mu\text{g}$ ) in CaS, CaR, CgS and CgR in the absence (white bars) or in the presence (grey bars) of FLZ. Bar charts were applied for normal data submitted to parametric analysis (ANOVA). There was no statistical difference between all conditions depicted ( $p > 0.05$ ).



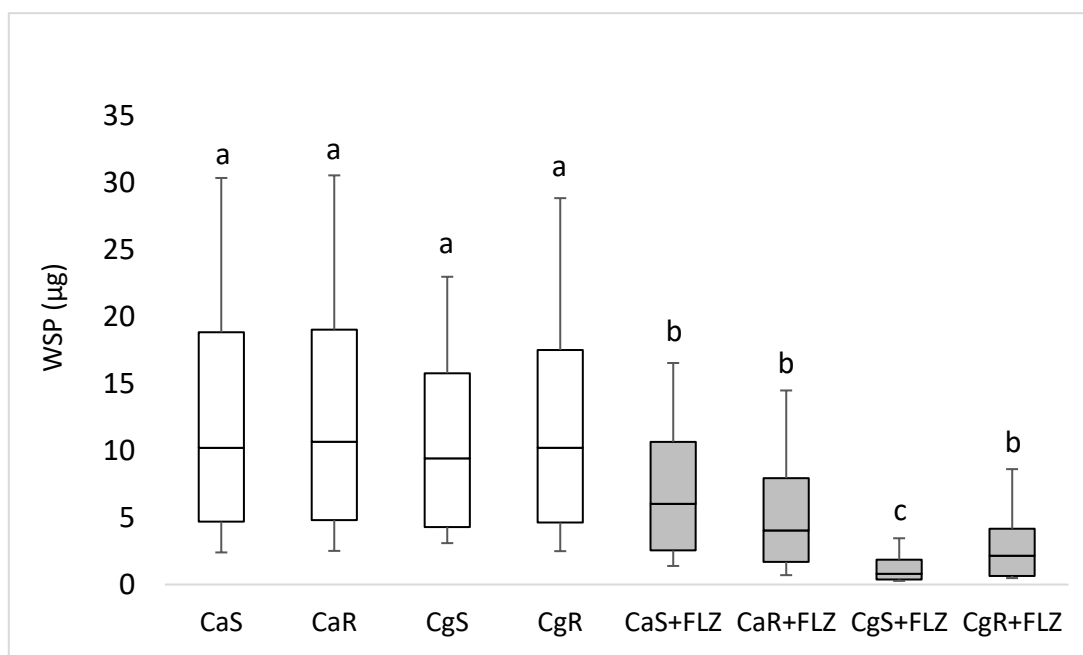


Figure 6: Box-plot of WSP amounts ( $\mu\text{g}$ ) in CaS, CaR, CgS and CgR in the absence (white boxes) or in the presence (grey boxes) of FLZ. The box-plot shows the median (dash), the first and third quartiles (outer edges of box), and the highest and maximum values (error bars). A non-parametric analysis (ANOVA on rank) was applied. Equal letters designate statistical correspondence ( $p > 0.05$ ).

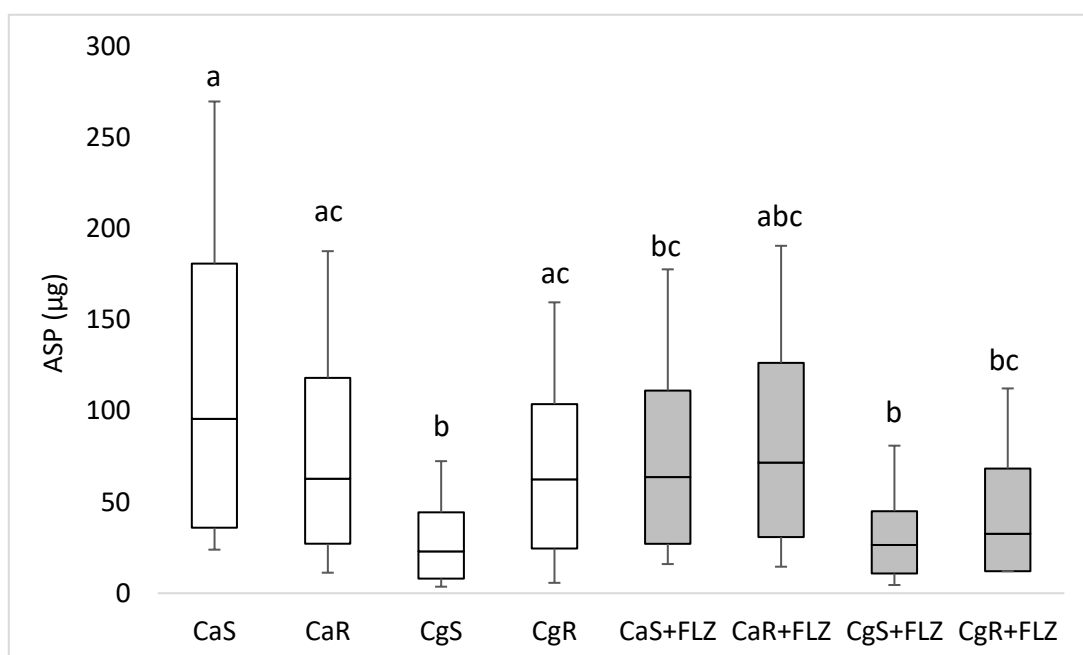


Figure 7: Box-plot of ASP amounts ( $\mu\text{g}$ ) in CaS, CaR, CgS and CgR in the absence (white boxes) or in the presence (grey boxes) of FLZ. The box-plot shows the median (dash), the first and third quartiles (outer edges of box), and the highest and maximum values (error bars). A non-parametric analysis (ANOVA on rank) was applied. Equal letters designate statistical correspondence ( $p < 0.05$ ).

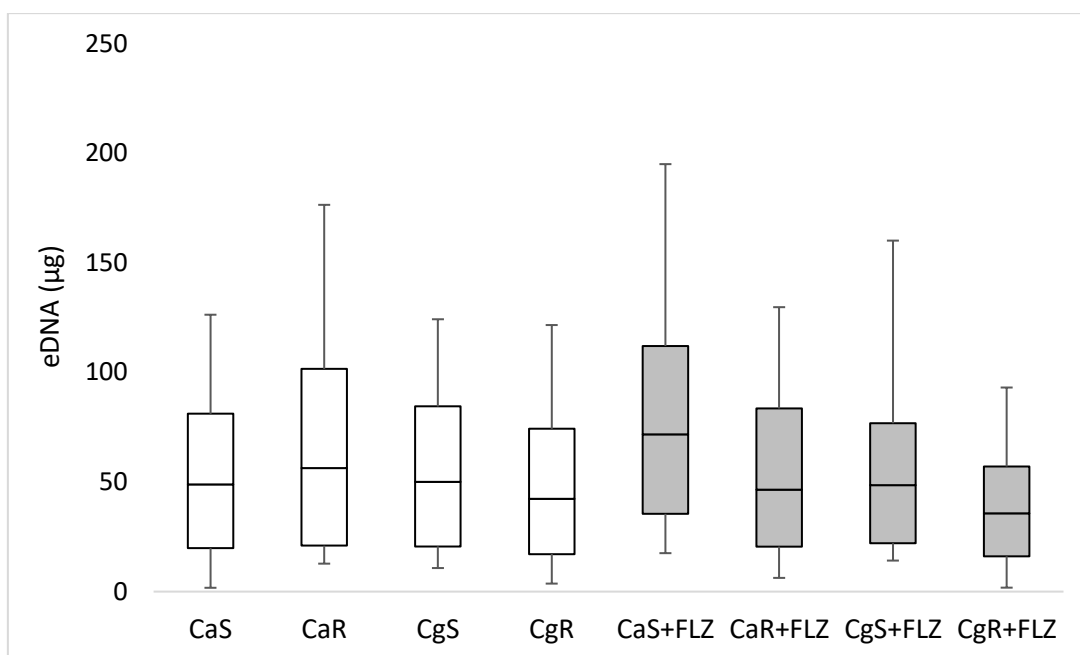


Figure 8: Box-plot of eDNA amounts ( $\mu\text{g}$ ) in CaS, CaR, CgS and CgR in the absence (white boxes) or in the presence (grey boxes) of FLZ. The box-plot shows the median (dash), the first and third quartiles (outer edges of box), and the highest and maximum values (error bars). A non-parametric analysis (ANOVA on rank) was applied. There was no statistical difference between all conditions depicted ( $p>0.05$ ).

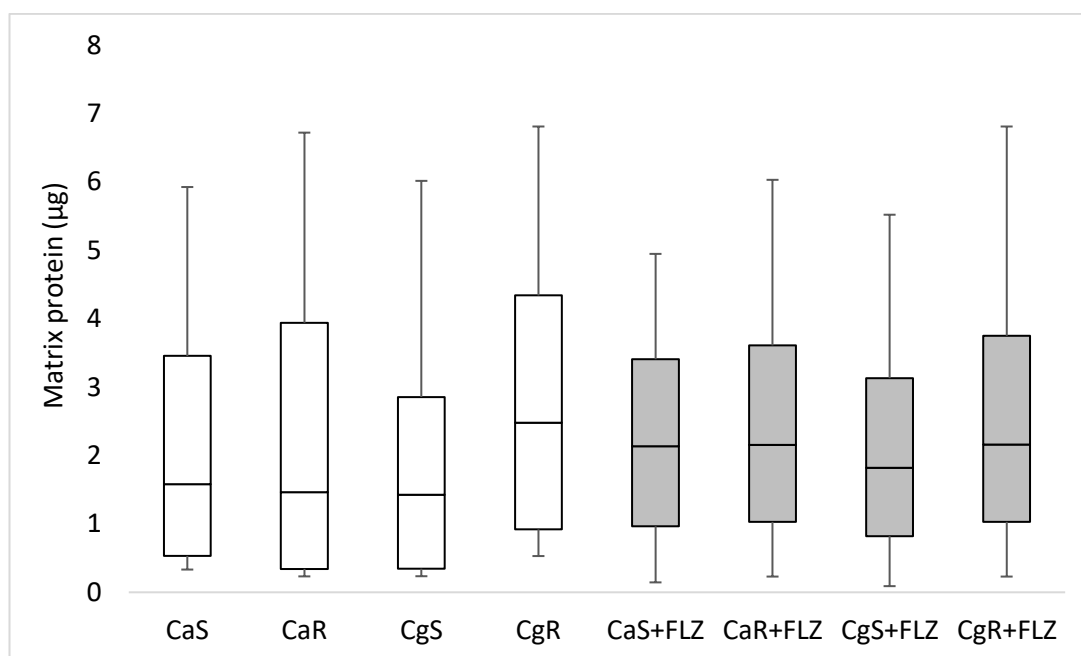
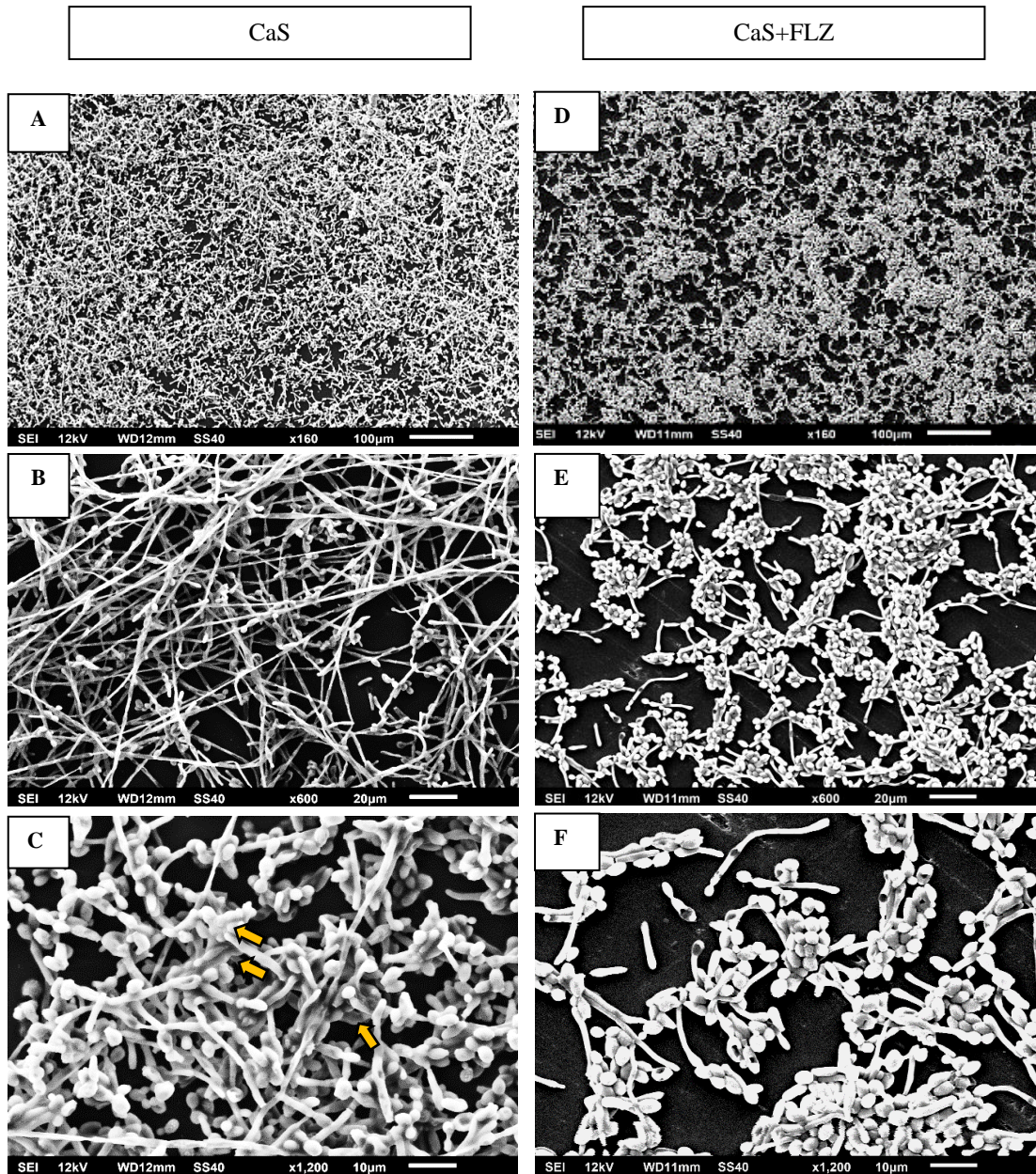
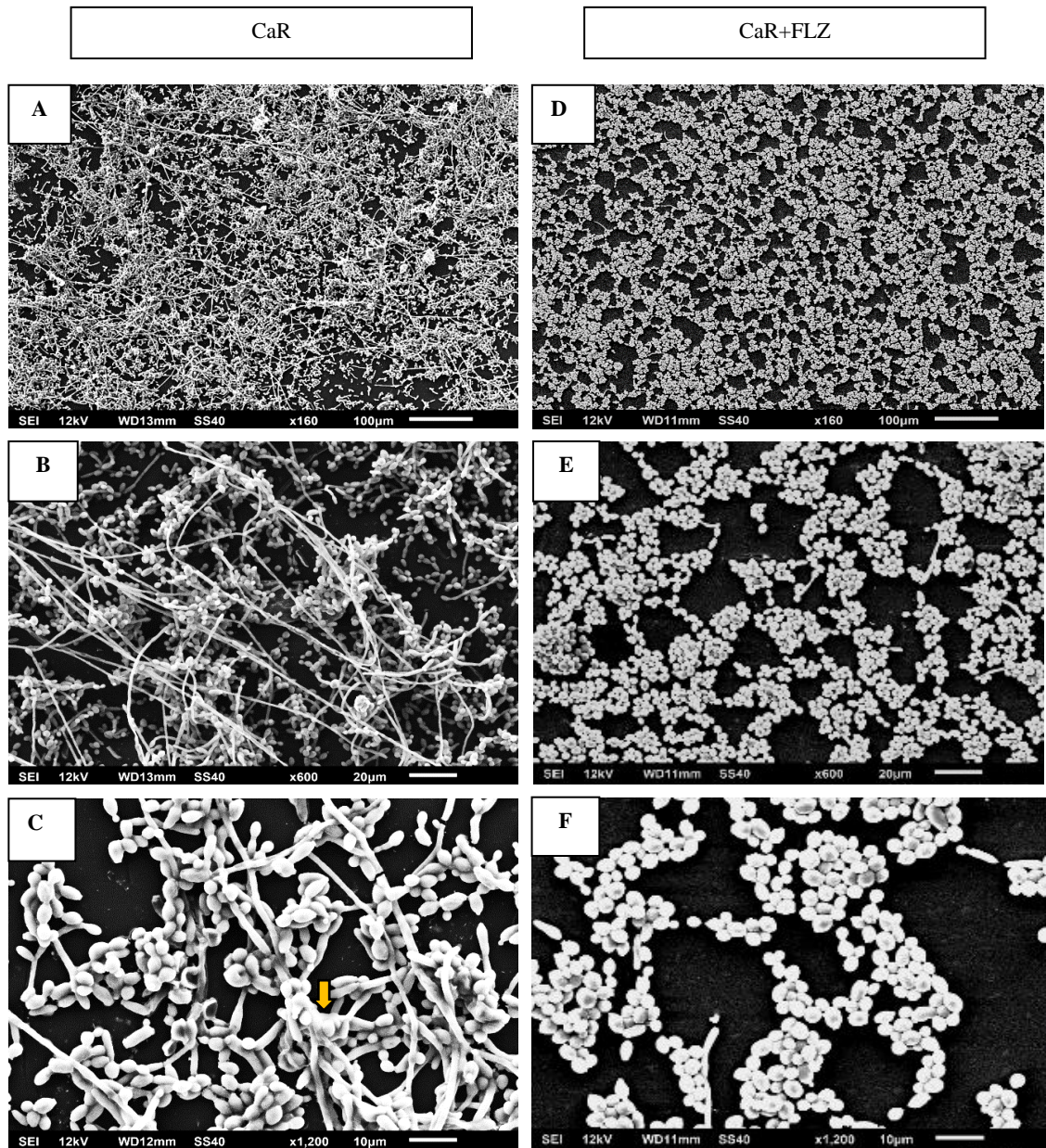


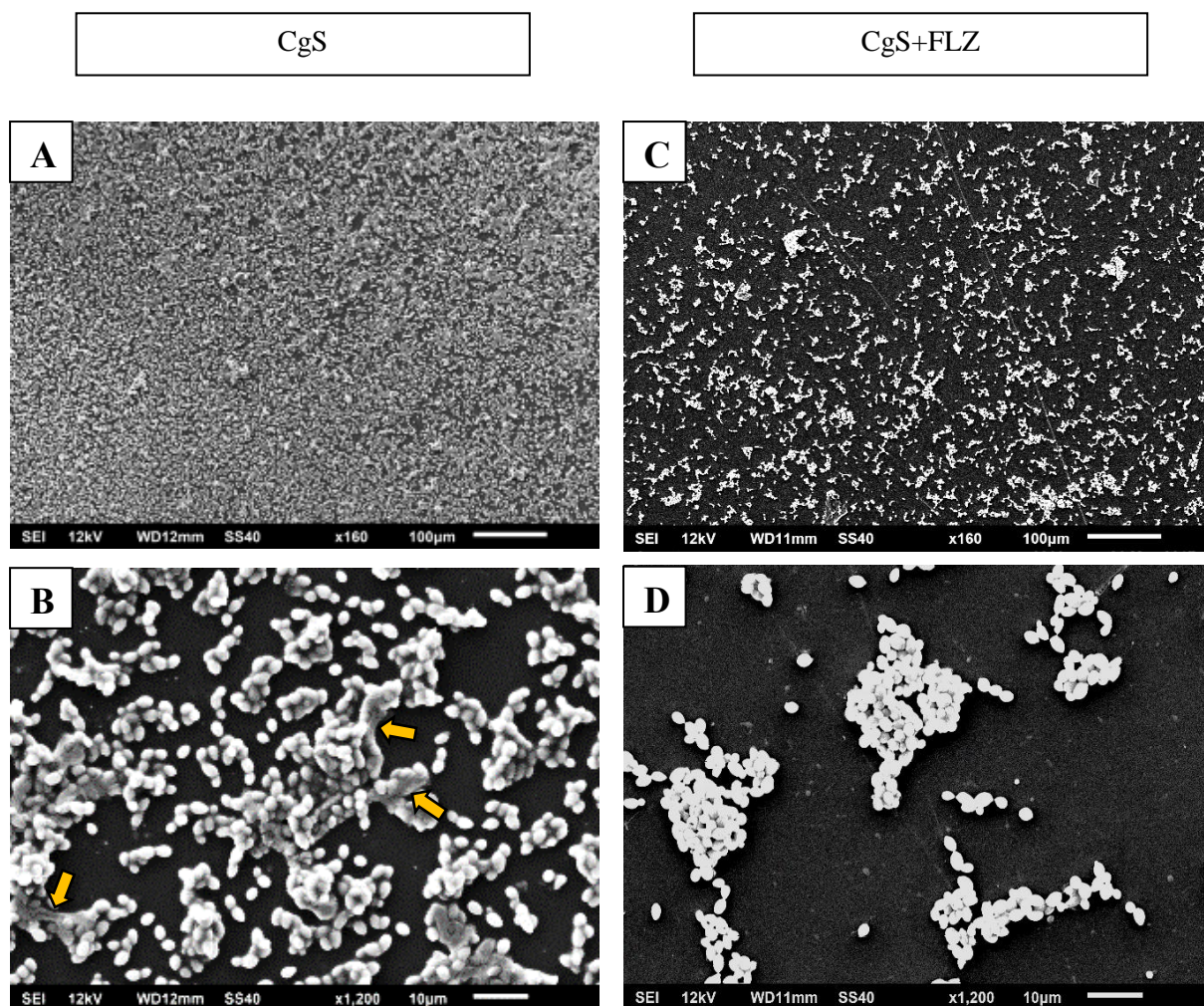
Figure 9: Box-plot of matrix protein amounts ( $\mu\text{g}$ ) in CaS, CaR, CgS and CgR in the absence (white boxes) or in the presence (grey boxes) of FLZ. The box-plot shows the median (dash), the first and third quartiles (outer edges of box), and the highest and maximum values (error bars). A non-parametric analysis (ANOVA on rank) was applied. There was no statistical difference between all conditions depicted ( $p>0.05$ ).



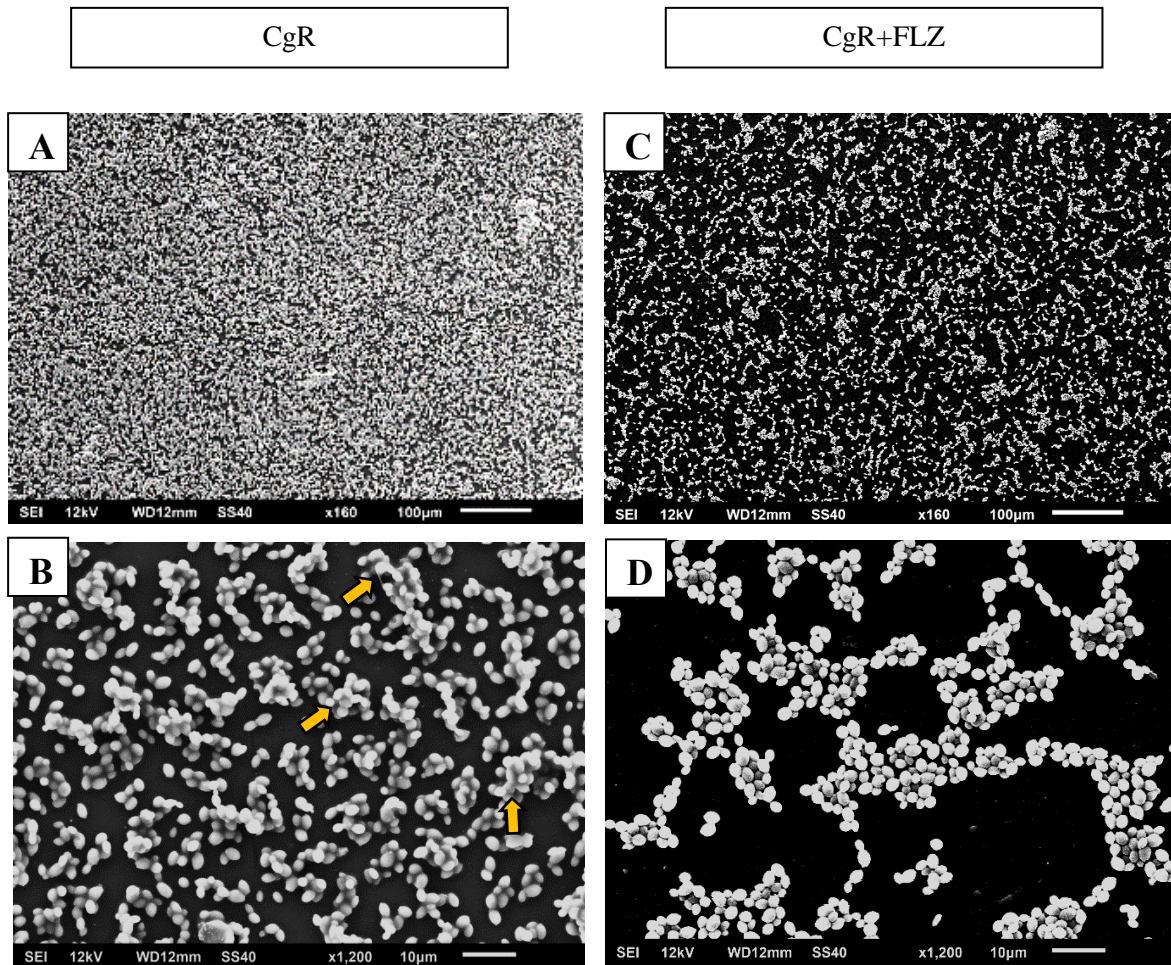
**Figure 10.** SEM images of CaS (A, B, C) and CaS+FLZ (D, E, F). FLZ (80  $\mu\text{g/mL}$ ) reduced the size of the hyphae (D, E, F). A closer look at the cells structures shows that without FLZ it is possible to see the cells embedded in the ECM, as shown by the arrow (C).



**Figure 11.** SEM images of CaR (A, B, C) and CaR+FLZ (D, E, F). The CaR biofilm possesses large amounts of yeast cells and elongated hyphae structures (B, C). Image C shows the union of cells within biofilms through their extracellular matrices, as exemplified by the arrow. FLZ (1280  $\mu\text{g}/\text{mL}$ ) reduced the number and size of hyphae, remaining mostly yeast cells (D, E, F).



**Figure 12.** SEM images of CgS (A, B) and CgS+FLZ (C, D). CgS biofilm is composed by yeast cells (A) linked by ECM (B). The presence of FLZ (40 µg/mL) reduced the number of cells (C) and the ECM (D).



**Figure 13.** SEM images of CgR (A, B) and CgR+FLZ (C, D). CgR biofilm presents a high quantity yeast cells (A) and clusters interconnected by ECM, exemplified by arrows (B). The presence of FLZ (1280 µg/mL) reduced the number of cells (C) and the ECM (D).

### 3.3 Publicação 3

**Intertwined eDNA, polysaccharides and proteins in the extracellular matrix of fluconazole-susceptible and -resistant *Candida albicans* are reduced by DNase.**

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## ABSTRACT

**Background:** The mature biofilm is enclosed by an extracellular matrix (ECM) that protects it against antimicrobials. *Candida albicans* ECM is composed by polysaccharides, proteins and extracellular DNA (eDNA). Therefore, the aim of the present study was to analyze how the ECM of fluconazole- susceptible and -resistant *C. albicans* strains responds to challenges with a panel of hydrolytic enzymes.

**Methods:** Biofilms of fluconazole-susceptible (ATCC 90028) and -resistant (ATCC 96901) *C. albicans* were grown in RPMI medium at 37° C for 48 h. Next, the hydrolytic enzymes  $\beta$ -glucanase, Dextranase, and DNase were applied alone and in combination to biofilms for 5 min. Biofilms were evaluated by colony forming units (CFU), total biomass, insoluble dry-weight and ECM components (water- soluble polysaccharides—WSP, alkali-soluble polysaccharide—ASP, proteins and eDNA).

**Results:** The treatment with DNase produced the most satisfactory outcomes, reducing eDNA, ASP, and soluble proteins in biofilms by both strains, and WSP in biofilm by the fluconazole-resistant strain. The reduction of extracellular polysaccharides and protein content by DNase indicates that these ECM components may be intertwined with eDNA.

**Conclusion:** DNase may be a promising adjuvant for antibiofilm therapies against both fluconazole-susceptible and -resistant *C. albicans*.

**KEYWORDS:** *Candida albicans*, biofilm, extracellular matrix, hydrolytic enzymes



## Introduction

*Candida albicans* asymptotically colonizes the oral cavity, gastrointestinal and reproductive tracts, and skin of most humans [1]. Nevertheless, changes in the host microbiota, variations in the host immune response or alterations in the local milieu may promote overgrowth of *C. albicans* and cause infections [1]. *Candida* infections are particularly serious in immunocompromised individuals and healthy people with implanted medical devices [2]. Most of infections caused by *Candida* are associated with biofilm formation [3]. Biofilm growth begins when planktonic cells adhere to a surface followed by proliferation of the yeast cells across the substrate surface. Then, the hyphal development starts, as well as production of and assembly of an extracellular matrix (ECM) [4]. The final step of biofilm development is the maturation stage, in which yeast-like growth seems to be repressed, hyphal growth is elevated, and the ECM encases the microbial cells within the biofilm [4]. The ECM is considered essential for the existence of the biofilm lifestyle [5].

Biochemical analyses have revealed the general composition of the *C. albicans* biofilm ECM, which includes polysaccharides, proteins, lipids, and nucleic acids [6]. The ECM mediates adhesive and cohesive interactions, providing mechanical stability to the biofilms [6] and acting as a digestive system that provides a nutrient source for the conglomerate of cells [7]. In addition, the ECM is a physical barrier that protects biofilms cells from the attack by the immune system and from antifungals [3], contributing to drug resistance. Regarding antifungal resistance, the  $\beta$ -glucans present in the ECM of *C. albicans* bind to fluconazole and amphotericin B [8], hindering the penetration of these drugs into biofilms, reducing their effect [9].

The resistance of *Candida* in biofilms is multifactorial and is associated to the physiological state of the cells, the activation of drug efflux pumps and the protective effect of the ECM performed by  $\beta$ -glucans [8]. Some *Candida* species possess intrinsic resistance to

antifungal drugs, especially to fluconazole [10, 11]. In contrast, the resistance can be developed by the microorganism after long periods of exposure to antifungal drugs [12]. Hence, a major concern with *Candida* biofilms is that their cells may have reduced susceptibility against azoles and polyenes, due to development of resistance [10,13]. It has been shown that the *C. albicans* biofilm ECM has larger quantities of  $\beta$ -1,6 glucan and  $\alpha$ -mannan, which interact to form a mannan-glucan complex (MGCx) [7, 14]. This polysaccharide interaction was found to be crucial for protection of the biofilm from drug treatment with fluconazole and amphotericin-B [8, 9].

In addition to the protective effect of the biofilm performed by  $\beta$ -1,6 glucan and  $\alpha$ -mannan, it has been shown that the extracellular DNA (eDNA) contributes to the structural integrity of *C. albicans* biofilm [15]. Efforts to hydrolyze these polysaccharide and nucleic acid ECM components have been efficacious in sensitizing both *Candida* and *Aspergillus* biofilms to available antifungals. Mannan accumulation was blocked with  $\alpha$ -mannosidase—an enzyme that catalyzes the hydrolysis of terminal mannosides—enhancing the activity of fluconazole against susceptible *C. albicans* biofilms [14]. It has been demonstrated that 24-hours exposure to the enzyme  $\beta$ -1,3 glucanase at 2.5 units/ml essentially eliminated the biofilm process in a rat catheter model [8]. Furthermore, 24h-old biofilms challenged with RPMI containing different concentrations of antifungals alone or in combination with DNase 24-h showed that the addition of DNase enhanced the susceptibility of *C. albicans* cells to amphotericin B [15]. Moreover, it was shown that the combination of biofilms with DNase plus amphotericin B and caspofungin significantly improved antifungal susceptibility in *Aspergillus fumigatus* biofilm [16].

It has been demonstrated that the use of enzymes helps biofilm penetration by antimicrobials through the ECM, representing a promising approach for the therapy of biofilm-related diseases [17]. Therefore, the goal of the present study was to target ECM components of fluconazole-susceptible and -resistant *C. albicans* biofilms using the hydrolytic enzymes  $\beta$ -

glucanase, dextranase and DNase individually or in different combinations to evaluate the influence of the enzymatic treatment on the ECM.

## **MATERIALS AND METHODS**

### ***Biofilm formation***

The microorganisms used for this experiment were *C. albicans* ATCC 90020 (fluconazole-susceptible; CaS) and *C. albicans* ATCC 96901 (fluconazole-resistant; CaR). The microorganisms stored at -80°C were seeded onto Petri dishes with SDA (Sabourand dextrose agar) culture medium supplemented with chloramphenicol and incubated at 37°C for 48 h. Next, starter cultures containing about 5 colonies were grown using YNB medium (Yeast Nitrogen Base- DIFCO, Detroit, Michigan, USA) supplemented with 100 mM of glucose, and incubated at 37°C. After 16 h of incubation, the starter cultures were diluted with fresh YNB medium supplemented with 100 mM glucose (1:20 dilution). These inoculum cultures were incubated at 37°C until the two strains reached the mid-log growth phase [18]. Then, the OD<sub>540nm</sub> of the inoculums was adjusted to reach 10<sup>7</sup> CFU/mL. Next, 1 mL of the inoculum of each strain was added to the wells of a 24-well polystyrene plate (Techno Plastic Products-TPP, Trasadingen, Switzerland). The culture plate was incubated at 37°C under stirring (75 rpm) for cell adhesion to the substrate. After 90 min, the wells were washed twice with sterile 0.89% NaCl solution to remove non-adhered cells. Subsequently, one mL of RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, Missouri, USA) at pH 7 was added to each well.

After 24 hours of biofilm formation, the culture medium was removed by aspiration and fresh RPMI buffered with MOPS (1 mL, pH 7.0) was added to each well, followed by another incubation during 24h. After 48 hours of biofilm formation, the wells were washed twice with 0.89% sterile NaCl solution and the incubation of enzymes in the biofilms was performed.

### *Application of enzymes application in the biofilms*

Stock solutions of  $\beta$ -(1 $\rightarrow$ 3)-D-Glucanase or  $\beta$  from *Helix pomatia* (67138, Sigma-Aldrich, St. Louis, MO, USA), dextranase or Dex from *Penicillium* sp. (D5884, Sigma-Aldrich) and DNase I from bovine pancreas (AMPD1, Sigma-Aldrich) were freshly prepared before each experiment in 0.1 M sodium acetate buffer (pH 5.5). To disorganize the ECM, but not to destroy it, the concentration of the enzymes was chosen based on the ASP, WSP and DNase concentrations from matrix examination experiments described previously [18]. Additionally, it was performed preliminary tests using the concentrations cited ahead and distinct incubation times. The shortest time that indicated hydrolysis of ECM components was 5 minutes, hence, it was chosen for the study (data not shown). Thus, the final concentrations of the working solutions were 2 units/mL of  $\beta$ -glucanase; 1 unit/mL of Dextranase and 20  $\mu$ units/mL of DNase. The enzymes combinations ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and  $\beta$ +Dex+DNase) were used in the same final concentrations mentioned above for each enzyme. The control solutions were 0.89% NaCl and 0.1 M sodium acetate buffer (pH 5.5).

After 48 h of biofilm formation, the culture medium was removed, the biofilms were washed twice with 0.89% NaCl solution and 250  $\mu$ L of working enzyme solutions and control solutions were added to the biofilms. Plates were incubated at 37 $^{\circ}$  C for 5 minutes. After this period, for the groups which DNase was applied, 20 units/mL of stop solution from the DNase I kit (AMPD1, Sigma-Aldrich) was applied to the biofilms. All biofilms, including those of the control groups, were washed twice with 0.89% NaCl solution. Then, the biofilms were removed by scraping each well with a pipette tip and 2 mL of sterile 0.89% NaCl. At this point, biofilms were vortexed and processed according to the flowchart represented in Figure S1 from previous manuscript [18]. From the biofilm suspension, 0.1 mL was applied for 10-fold serial dilution and plating on SDA plates for CFU and 0.1 mL for the total biomass (total dry-weight) [19], which comprehends cells and all ECM components. The remaining volume (1.8 mL) was

centrifuged at 5500 xg for 10 min (4°C). The supernatant containing the soluble part of the ECM was stored in another tube and the precipitate with the cells and the insoluble components of the ECM was washed twice with 1.8 mL of sterile milli-Q water (5500xg /10 min/ 4°C). The supernatant (1.8 mL) was separated for quantifying water-soluble polysaccharides (WSP) [20], eDNA [21] and soluble proteins [22]. The pellet was resuspended in 1.8 mL of water, of which 0.8 mL was used to calculate the insoluble dry-weight (which includes cells and insoluble ECM components that were not removed during biofilm suspension washes), 0.05 mL for the quantification of total proteins [22] and 0.95 mL for the determination of water-insoluble polysaccharides (ASP—alkali soluble polysaccharide) [20].

### ***Statistical analyses***

All experiments were repeated on four distinct occasions in duplicate (n=8). The normality of the variables was verified by the Shapiro-Wilk test and the homogeneity of variances was verified by the Levene test ( $\alpha=0.05$ ). After verifying that the variables presented a normal distribution, data of biofilm components [ $\text{Log}_{10}$  (UFC / mL), total biomass, insoluble biomass and proteins] and ECM's components [ASPs, WSPs, eDNA and soluble proteins] were analyzed by one-way ANOVA with Tukey *post-hoc* test for homoscedastic data and Games-Howell *post-hoc* test for heteroscedastic data, considering a significance level of 5%. Analyses were performed using SPSS software (IBM® SPSS® Statistics, version 20, Chicago, IL, USA).

### ***Results***

$\text{Log}_{10}$  (CFU/mL) data of CaS and CaR are plotted in Figure 1. For CaS, Levene's test demonstrated that  $\text{Log}_{10}$  (CFU/mL) data is heteroscedastic ( $p=0.000$ ), so the Games Howell *post-hoc* test was applied. Analysis showed that there were no statistical differences in  $\text{Log}_{10}$  (CFU/mL) values between the groups treated or not with enzymes individually or in combinations ( $p>0.05$ ). For CaR,  $\text{Log}_{10}$  (CFU/mL) data is also heteroscedastic ( $p=0.000$ ). As

it was observed for CaS, statistical tests showed that there were no differences in  $\text{Log}_{10}$  (CFU/mL) values between the groups ( $p > 0.05$ ). So, the enzymes individually or in combination does not interfere in cell viability.

The total biomass (mg) data of CaS and CaR are represented in Figure 2. According to Levene's test, the total biomass data of CaS is heteroscedastic ( $p = 0.006$ ), so Games Howell *post-hoc* test was applied. The test showed that there were no statistically significant differences of total biomass between the studied groups, that is, the presence of enzymes individually or in combinations does not reduce the total biomass in CaS. In CaR, total biomass data is also heteroscedastic ( $p=0.001$ ). Statistical analysis showed that CaR+ $\beta$ +Dex had significantly lower total biomass than CaR+ Buffer ( $p=0.014$ ) and CaR+ $\beta$  ( $p=0.018$ ).

Data of insoluble biomass (mg) of CaS and CaR are shown in Figure 3. Data of insoluble biomass for CaS is heteroscedastic ( $p=0.000$ ). Games Howell's *post-hoc* test demonstrated that there were no significant statistical differences in the insoluble biomass of the studied groups ( $p > 0.05$ ), that is, the presence of the enzymes individually or in combinations did not alter the insoluble biomass in CaS. On the other hand, data of insoluble biomass for CaR were homocedastic ( $p=0.058$ ), so Tukey's *post-hoc* test was applied. Statistical analysis showed that the group CaR+ $\beta$ +Dex presented significantly higher insoluble biomass than CaR ( $p=0.008$ ), CaR+Buffer ( $p=0.029$ ), CaR+Dex ( $p=0.000$ ) and CaR+DNase ( $p=0.000$ ). Moreover, CaR+ $\beta$  presented significantly higher insoluble biomass than CaR+DNase ( $p=0.046$ ), whereas CaR+Dex presented significantly lower insoluble biomass than CaR+ $\beta$ +Dex ( $p=0.000$ ), CaR+ $\beta$ +DNase ( $p=0.005$ ) and CaR+All enzymes ( $p=0.005$ ).

Proteins ( $\mu\text{g}$ ) data are shown in Figure 4. Regarding CaS protein amounts, it was observed by Levene's test that data were heteroscedastic ( $p=0.000$ ). After application of Games Howell's *post-hoc* test, it was observed that there were no statistical differences of protein amounts between the groups ( $p > 0.05$ ), that is, the presence of enzymes does not alter the amount

of total proteins in CaS. In CaR, protein data were also heterocedastic ( $p=0.028$ ), so, Games Howell's *post-hoc* test was applied. It was observed that like happened with CaS, no statistical differences in protein amounts was observed between the groups ( $p>0.05$ ).

ASPs ( $\mu\text{g}$ ) data for CaS and Car are depicted in Figure 5. For CaS, ASPs data is homoscedastic according to the Levene's test ( $p=0.229$ ). Tukey's *post-hoc* test showed that the group CaS+All enzymes presented significantly lower amounts of ASPs in its ECM than those presented by the groups CaS+ $\beta$  ( $p=0.025$ ) and CaS+Dex ( $p=0.003$ ). That is, the presence of DNase in combination with the other enzymes is essential to cause the reduction of ASPs in this strain. ASPs data of CaR also presented homoscedasticity ( $p=0.180$ ). Tukey's *post-hoc* tests demonstrated that the presence of dextranase (CaR + Dex) significantly reduced the ASPs in comparison to the control without enzymes CaR ( $p=0.026$ ). The same was observed with the combination of all enzymes (CaR+All enzymes) compared to the control without enzymes CaR ( $p=0.040$ ). In addition, CaR+ $\beta$ +DNase showed significantly higher amounts of ASPs than CaR+ $\beta$  ( $p=0.008$ ), CaR+Dex ( $p=0.002$ ) and CaR+All enzymes ( $p=0.003$ ).

WSPs ( $\mu\text{g}$ ) data for CaS and CaR are plotted in Figure 6. Regarding WSPs, Levene's test showed that data for CaS were heteroscedastic ( $p=0.001$ ). Games Howell's *post-hoc* test did not find statistical differences between WSP content in enzyme-treated and non-enzyme treated groups ( $p>0.05$ ), i.e. the presence of enzymes individually or in combination does not alter the WSP content from the ECM of CaS. For the analysis of WSPs data in CaR, Games Howell *post-hoc* test was also applied, since Levene's test pointed to heteroscedasticity of the variances ( $p=0.020$ ). It was observed that CaR possesses significantly higher quantity of WSPs than CaR+Dex ( $p=0.009$ ), CaR+DNase ( $p=0.010$ ) and CaR+ $\beta$ +DNase ( $p=0.023$ ). The same was observed for CaR+Buffer, which has statistically higher WSPs than CaR+Dex ( $p=0.002$ ), CaR+DNase ( $p=0.002$ ) and CaR+ $\beta$ + DNase ( $p=0.004$ ). The presence of all enzymes (CaR+All

enzymes) resulted in higher amounts of WSPs than CaR+Dex ( $p=0.004$ ), CaR+DNase ( $p=0.003$ ) and CaR+ $\beta$ +DNase ( $p=0.006$ ).

The eDNA ( $\mu\text{g}$ ) data are represented in Figure 7. eDNA data of CaS were assessed through Games Howell *post-hoc* test after verification of heteroscedasticity by the Levene test ( $p=0.000$ ). It was observed that the eDNA content was significantly reduced by DNase (CaS+DNase) when compared to the control groups CaS ( $p=0.020$ ) and CaS+Buffer ( $p=0.015$ ). In CaR, the Levene test also pointed to heteroscedasticity of variances ( $p=0.001$ ) and Games Howell's *post-hoc* test was applied. Statistical analysis showed that the presence of the enzyme DNase (CaR+DNase) significantly reduced the amount of eDNA in comparison to the control CaR ( $p=0.002$ ). In addition, CaR+ $\beta$ +Dex ( $p=0.001$ ), CaR+ $\beta$ +DNase ( $p=0.001$ ) and CaR+Dex+DNase ( $p=0.002$ ) also showed significantly lower amounts of eDNA compared to the control CaR. In addition, the enzyme DNase (CaR + DNase) caused a significant reduction of eDNA in comparison to CaR+Buffer group ( $p=0.044$ ). Furthermore, the groups CaR+ $\beta$ +Dex ( $p=0.042$ ) and CaR+ $\beta$ +DNase ( $p=0.045$ ) also presented significantly lower amounts of eDNA than the group CaR+Buffer. On the other hand, the amount of eDNA in the group CaR+All enzymes is significantly higher than the amount of eDNA presented by the other studied groups ( $p < 0.001$ ), with the exception of the group CaR+ $\beta$  ( $p=0.057$ ).

The soluble proteins ( $\mu\text{g}$ ) data for CaS and CaR are represented in Figure 8. Regarding soluble protein data, the Levene test pointed to heteroscedasticity of CaS variances ( $p=0.000$ ). After application of Games Howell *post-hoc* test, it was observed that all enzymes studied, individually or in combination, significantly reduced the amount of soluble proteins compared to the control group CaS [CaS+ $\beta$  ( $p=0.004$ ); CaS+Dex ( $p=0.031$ ); CaS+DNase ( $p=0.001$ ); CaS+ $\beta$ +Dex ( $p=0.002$ ); CaS+ $\beta$ +DNase ( $p=0.002$ ); CaS+Dex+DNase ( $p=0.007$ ) and CaS+All enzymes ( $p=0.002$ )]. Likewise, CaR presented heteroscedastic data ( $p=0.000$ ). Statistical analysis of CaR soluble proteins data showed that there was a significant reduction in the



amount of soluble proteins in all groups treated with enzymes (individually or in combinations) when compared to the control groups CaR ( $p < 0.002$ ) and CaR+Buffer ( $p < 0.004$ ).

### ***Discussion***

Based on the importance of the ECM for the protection of *Candida* cells in the biofilm [6], the present study focused on finding alternatives to open ways through the ECM that may improve the action of antifungal therapies. The intention was to disorganize the ECM using hydrolytic enzymes, but not to destroy the whole ECM, once this can provoke the dispersal of the cells to colonize another site.

The fluconazole susceptibility and resistance of the strains used in this study was confirmed before, being CaR ( $MIC_{90} = 256 \mu\text{g/mL}$ ) 16 times more resistant than the CaS ( $MIC_{90} = 16 \mu\text{g/mL}$ ) [18]. In addition, the present study confirms previous results [18] that demonstrated that ECM (ASP, WSP, e DNA and proteins) of both strains was similar.

The present study observed that the treatment during a short exposure time with the enzymes alone or in combination had no effect on population and total biomass (total dry-weight) of fluconazole-susceptible and -resistant strains. The same was observed for the insoluble dry-weight in CaS biofilm. However, the presence of  $\beta$ -glucanase alone and in combination with Dextranase and DNase significantly increased the insoluble dry-weight of CaR. It has been recognized that changes in the ECM may occur when ions are present [23]. Ions from the sodium acetate buffer ( $\text{C}_2\text{H}_3\text{NaO}_2$ ) and ions present in the matrix, such as  $\text{Ca}^{2+}$  and  $\text{K}^+$ , may specifically interact with exposed carboxylic acid groups on the polysaccharides matrix to produce nets of macromolecules with increased viscosity [23]. It is possible that the enzyme  $\beta$ -glucanase caused the exposure of carboxylic acid groups of polysaccharides molecules in the ECM of fluconazole-resistant strain, permitting the interaction of these groups with ions and resulting in a more viscous polysaccharide matrix that enhanced the insoluble

dry-weight. The composition of the buffer used during biofilms incubation with enzymes may have help in this process.

On the other hand, none of the enzymes or combinations of them reduced the total protein amount in fluconazole-susceptible and -resistant *C. albicans* biofilms. The total protein content includes proteins from the ECM that were not removed during the biofilm washes and proteins from the cells per se. Thus, as none of the enzymes or combinations of them acted on the population of fluconazole-susceptible and -resistant strains, the unaltered quantity of total protein is related to the unchanged number of cells.

Overall, the quantities of ECM components are pretty much similar between fluconazole-susceptible and -resistant strains biofilms. However, the response to short time exposure to distinct hydrolytic enzymes and combinations of these enzymes yielded distinct patterns of ECM components amounts, indicating possible structural differences in the linkages of ECM building blocks that may contribute to *C. albicans* strains innate resistance to conventional antifungal drugs.

The combination of all enzymes demonstrated reduction of ASP in the ECM of both strains. Moreover, the enzymes  $\beta$ -glucanase, Dextranase, DNase and the combination Dextranase and DNase also reduced significantly the ASP content in fluconazole-resistant strain. The polysaccharides in *C. albicans* ECM ( $\alpha$ -mannans,  $\beta$ -1,6 glucan and  $\beta$ -1,3 glucan) are physically associated in the mannan-glucan complex (MGCx) [7,14]. Taking into consideration that the enzyme  $\beta$ -glucanase was chosen for hydrolysis of  $\beta$ -1,3-glucan and the enzyme Dextranase was selected for hydrolysis of  $\alpha$ -(1,6)-glucosidic linkages in dextran, the results of the present study confirms that the ECM polysaccharides are interconnected, since  $\beta$ -glucanase and Dextranase reduced the ASP content in fluconazole-resistant strain. On the other hand, DNase was chosen for hydrolysis of the eDNA content from the ECMs of both strains, but, surprisingly, the presence of DNase alone and in combination with the other tested enzymes

had a significant effect on reducing ASP, mainly in the ECM of CaR. Moreover, in CaS, the enzyme DNase alone and in combination with all enzymes numerically reduced the ASP amounts compared to the controls and significantly reduced this component compared to the treatment with Dextranase alone. Therefore, our data indicate that eDNA is linked to and/or enclosed by components of the MGCx complex. Thus, DNase hydrolysis of eDNA breaks down the organization of complexes of eDNA-alkali soluble exopolysaccharides, consequently decreasing the amount of the polysaccharides in which eDNA molecules are attached to.

It was observed that Dextranase, DNase and the combination of all enzymes significantly reduced the WSP in the ECM of CaR, following the same tendencies observed for ASP in this strain. However, none of the enzymes or combinations of them altered the WSP content in the ECM of CaS. It has been described that the secretion of  $\beta$ -1,6-glucan may be only possible because of its tight physical interaction with water-soluble mannans [7]. Apparently, although the ASP and WSP amounts of CaS and CaR are similar, the physical interaction between the polysaccharides in the MCGx complex in the ECM of each strain may be different. This is, CaS may present stronger attachment/linkages between the polysaccharides that did not allowed the hydrolysis of WSP by the enzymes. Thus, for fluconazole-susceptible strain, DNase or the combination of all enzymes is required for a better reduction of ASP in the MGCx complex, however, more time of action and/or a higher concentration of the enzymes is needed for reducing WSP.

Considering the importance of eDNA to the ECM of *C. albicans*, contributing to biofilm structural integrity and resistance against antifungals [14-16], targeting eDNA via DNase demonstrated a positive result in the present study, as this enzyme significantly reduced the eDNA amounts in biofilms of both fluconazole-susceptible and -resistant strains. Moreover, the combination of DNase with  $\beta$ -glucanase and Dextranase were also effective in reducing the eDNA amounts in CaR. In addition, the combination of  $\beta$ -glucanase with Dextranase

significantly reduced the eDNA content in CaR too. This result reinforces the possibility that eDNA is linked to and/or surrounded by the components of the MGCx complex in the ECM of *C. albicans* strains. Unexpectedly, the combination of all enzymes increased the eDNA content in the ECM of CaR biofilm. It is possible that the combination of all enzymes caused an elevated disorganization of the ECM, liberating the eDNA content that was encased in deeper layers of ECM. Thus, the treatment of CaR with all enzymes together ( $\beta$ -glucanase+Dextranase+DNase) should be avoided.

Proteins in the ECM may operate as an exterior digestive structure that disrupts extracellular biopolymers as an energy source [7]. The present study showed that the proteins are the less abundant components of the ECM of both fluconazole susceptible and -resistant *C. albicans* strains, contradicting a previous study that showed that the proteins were the most abundant component, representing 55% of the ECM of a *C. albicans* strain clinically isolated from a systemic biofilm *Candida* infection (strain K1) [7]. However, most of proteins identified in the cited study was from the host, as it was obtained from an animal central venous catheter biofilm model. In the present study, it was observed that the soluble protein (from the ECM) content were significantly reduced by the treatment with the enzymes alone and with the combinations of them both in *C. albicans* strains used. It has been demonstrated that proteins adhere to polysaccharides in the ECM [23, 24]. Whereas proteases would certainly affect those proteins which interact with polysaccharides within biofilms, it has been demonstrated that polysaccharases and polysaccharide lyases have a much greater effect [23, 25, 26]. The results of the present study support these previous observations, as soluble proteins were reduced by  $\beta$ -glucanase, Dextranase and DNase. Moreover, the present study demonstrates that the ECM components (polysaccharides, eDNA and proteins) are interconnected in the ECM of fluconazole-susceptible and -resistant strains.

In general, DNase treatment produced satisfactory results decreasing ASP, eDNA and soluble proteins in both fluconazole-susceptible and -resistant *C. albicans* biofilms and reducing WSP in fluconazole-resistant strain. Thus, DNase treatment reduced most ECM components that can hinder antifungal drug penetration into biofilms. The reduction of biofilm ECM components is very relevant, since it can open pathways in the ECM that could facilitate the diffusion of antifungal agents into the biofilm, overcoming the resistance derived from ECM components. Moreover, the reduction of extracellular polysaccharides and protein content by DNase indicate that these ECM components are intertwined with eDNA. Therefore, the exposure of 48h-old biofilms for 5 min with DNase reduces eDNA, polysaccharides and soluble proteins of fluconazole-susceptible and -resistant *C. albicans* ECMs, being a promising adjuvant to antibiofilm therapies.

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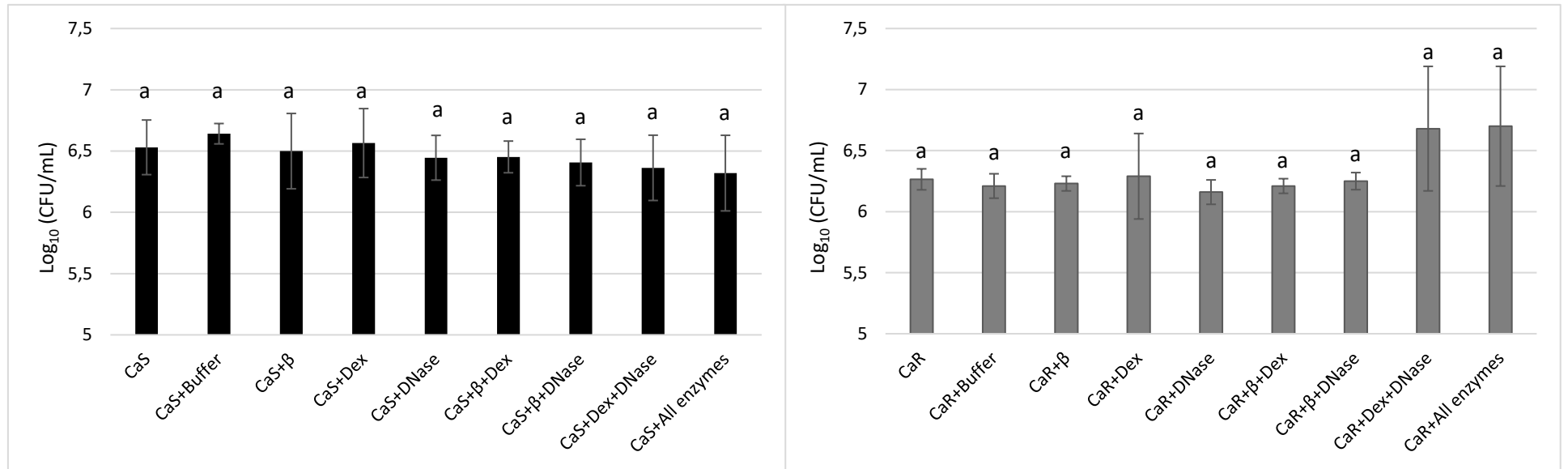


Figure 1: Data shown are means and standard deviations of Log<sub>10</sub> (CFU/mL) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to β-glucanase (β), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37° C for 5 min with 2 units/mL of β-glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes (β+Dex, β+DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence (p>0.05).

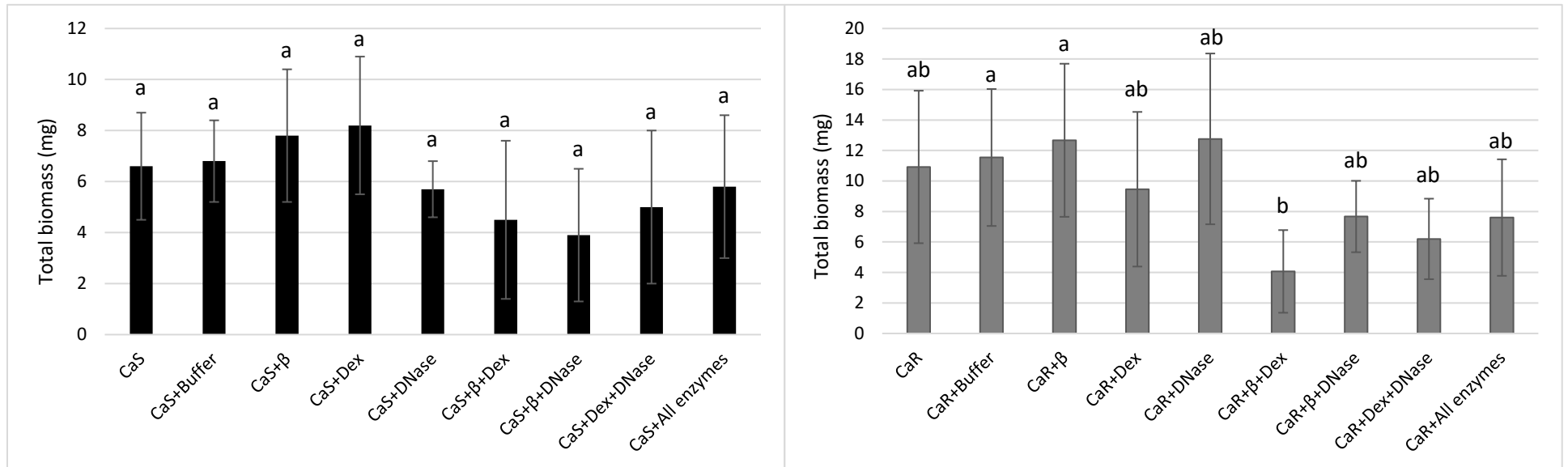


Figure 2: Data shown are means and standard deviations of total biomass (mg) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37° C for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).

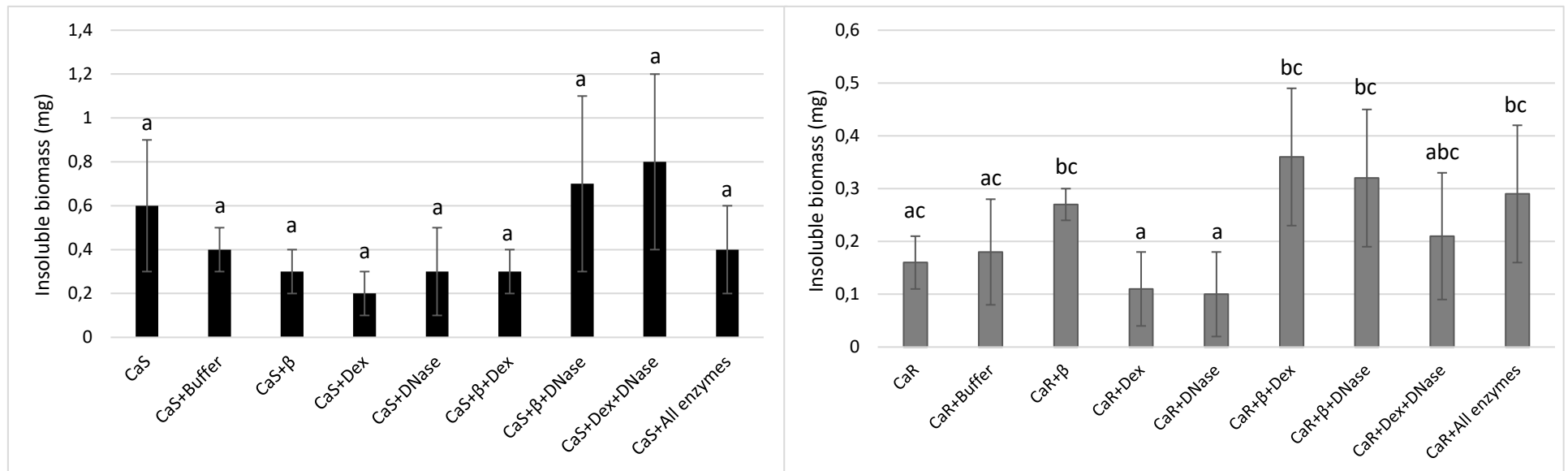


Figure 3: Means and standard deviations of insoluble biomass (mg) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37° C for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).

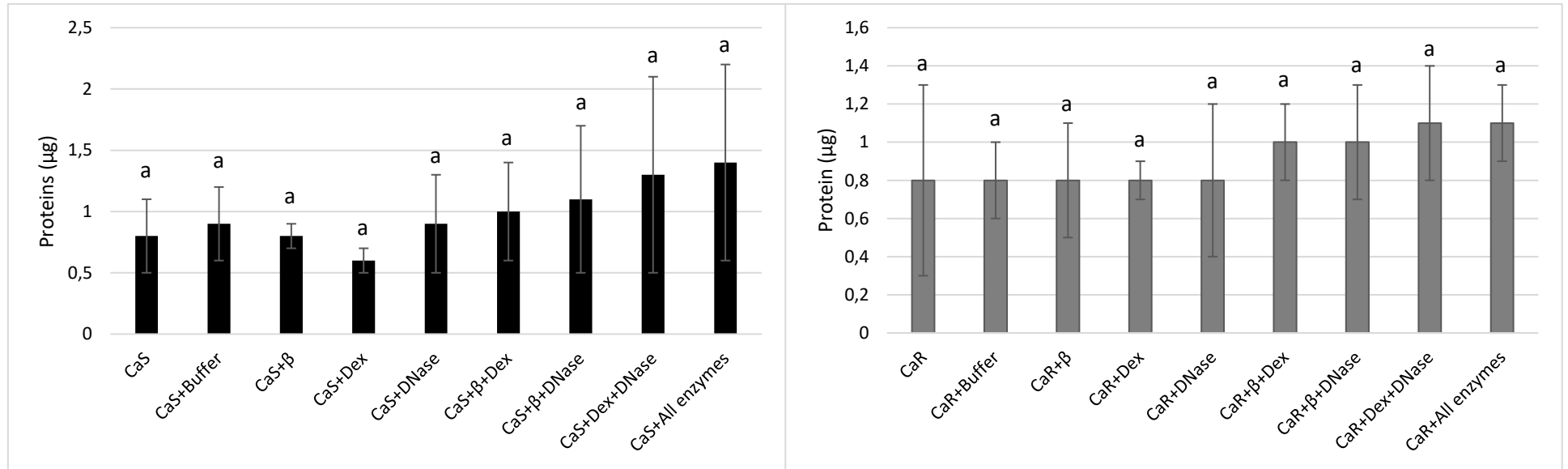


Figure 4: Means and standard deviations of proteins ( $\mu\text{g}$ ) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at  $37^\circ\text{C}$  for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).

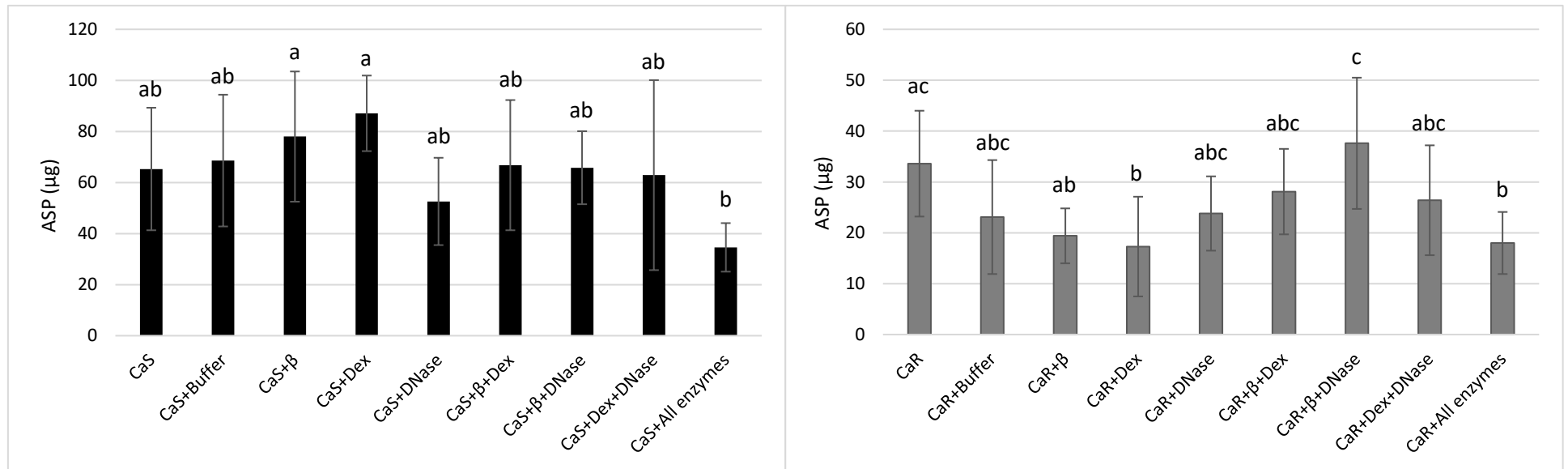


Figure 5: Means and standard deviations of ASP ( $\mu\text{g}$ ) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37° C for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).

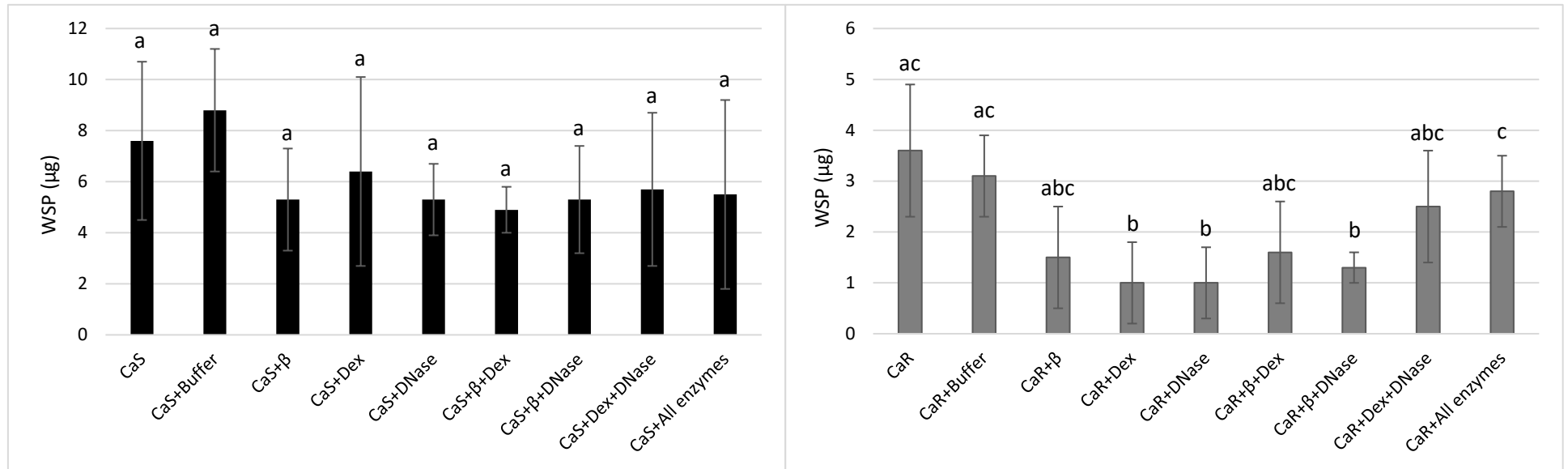


Figure 6: Means and standard deviations of WSP ( $\mu\text{g}$ ) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37° C for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).

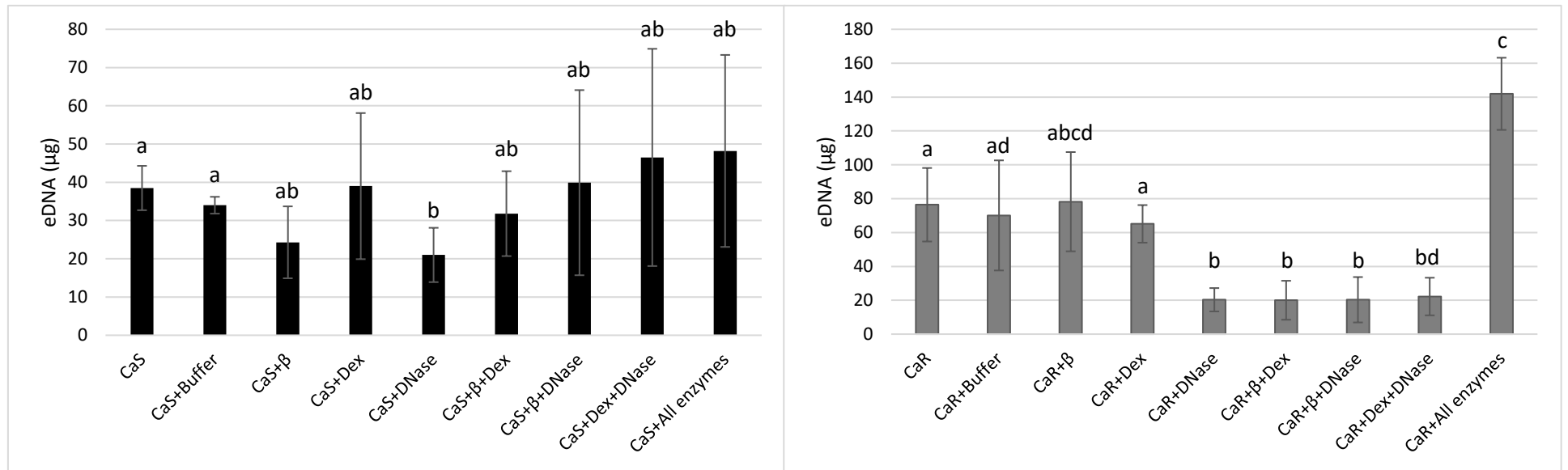


Figure 7: Means and standard deviations of eDNA ( $\mu\text{g}$ ) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at 37° C for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).

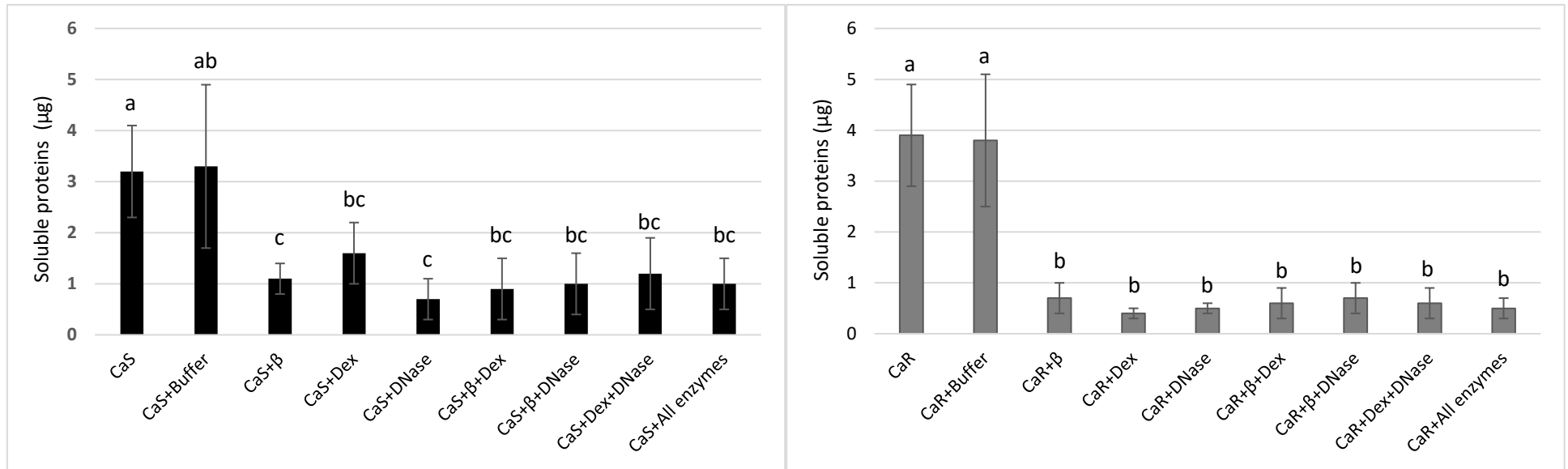


Figure 8: Means and standard deviations of soluble proteins ( $\mu\text{g}$ ) of fluconazole-susceptible (black bars) and -resistant *C. albicans* (grey bars) strains after exposure to  $\beta$ -glucanase ( $\beta$ ), Dextranase (Dex), DNase and the combination of these enzymes. 48 h-old biofilms were incubated at  $37^\circ\text{C}$  for 5 min with 2 units/mL of  $\beta$ -glucanase, 1 unit/mL of Dextranase, 20 units/mL of DNase and with the combination of these enzymes ( $\beta$ +Dex,  $\beta$ +DNase, Dex+DNase and All enzymes) following the same individual concentrations of each enzyme. Equal letters mean statistical correspondence ( $p > 0.05$ ).



## 4 DISCUSSÃO

A arquitetura do biofilme contribui para a manutenção de interações estáveis entre células, superfície celular e ambiente<sup>8</sup>. Além disso, protege contra células fagocíticas e preserva a integridade do biofilme, limitando a difusão de substâncias nocivas<sup>5</sup>. Embora a resistência do biofilme seja multifatorial<sup>36</sup>, a proteção exercida pela matriz extracelular (MEC) é fundamental para os altos níveis de resistência a drogas antifúngicas em biofilmes de *C. albicans*<sup>18, 20, 24, 25, 34, 45</sup>. A via de desenvolvimento de hifas é crítica para que haja formação significativa de biomassa de biofilme<sup>35</sup>. Cepas mutantes com defeitos em genes de filamentação são menos virulentas que as suas cepas parentais<sup>12, 14</sup>.

No estudo 1, foi realizada a análise das matrizes extracelulares de cepa parental (WT) e de cepas mutantes ( $\Delta/\Delta$  *efg1* e  $\Delta/\Delta$  *tec1*) de *C. albicans* com deficiência na formação de hifas e/ou biofilmes. Imagens obtidas por fotografias, microscopia eletrônica de varredura por pressão variável (VPSEM) e microscopia de varredura confocal a laser (CSLM) mostraram que a cepa WT apresenta uma arquitetura típica de biofilme volumoso de *C. albicans*, consistindo predominantemente de hifas combinadas com células em formato de levedura e encobertas por MEC. Por outro lado, a cepa mutante  $\Delta/\Delta$  *efg1* mostrou padrão de crescimento esparso, biofilme menos espesso e ausência de hifas. A cepa mutante  $\Delta/\Delta$  *tec1* formou hifas verdadeiras, mas a quantidade de hifas nesta cepa é menor em comparação com a cepa parental (WT). As medidas de biovolume e de espessura máxima dos biofilmes obtidas através do *software* COMSTAT2 confirmam esses resultados, mostrando que a cepa mutante  $\Delta/\Delta$  *efg1* tem menor biovolume ( $\mu\text{m}^3/\mu\text{m}^2$ ) e menor espessura ( $\mu\text{m}$ ) em relação às demais cepas estudadas. O perfil de biofilme da cepa mutante  $\Delta/\Delta$  *efg1* é notavelmente diferente do perfil de biofilme das outras cepas estudadas, mostrando uma pequena porcentagem de área de cobertura. Apesar das diferenças visuais e microscópicas entre as cepas WT e  $\Delta/\Delta$  *tec1*, estas possuem biovolume e espessuras similares de biofilme.

A matriz do biofilme de *Candida* possui  $\alpha$ -mananos, que são polissacarídeos solúveis em água (WSPs), associados a  $\beta$ -glucanos, que são polissacarídeos solúveis em álcali (ASPs), constituindo o complexo manano-glucano (MGCx)<sup>47</sup>. A análise das matrizes extracelulares da cepa WT e das cepas mutantes ( $\Delta/\Delta$  *efg1* e  $\Delta/\Delta$  *tec1*) mostrou que os ASPs são os componentes mais abundantes da MEC do biofilme da cepa WT, além de mostrar a tendência que células filamentosas (WT e  $\Delta/\Delta$  *tec1*) possuem em

produzirem maior quantidade de ASPs do que a cepa  $\Delta/\Delta$  *efg1*, que não produziu hifas. Foi demonstrado que o fator de transcrição EFG1 interfere na tolerância de *C. albicans* a azóis e polienos<sup>32</sup>. Além disso, foi demonstrado que uma cepa de *C. albicans* com ausência do fator de transcrição EFG1 ( $\Delta/\Delta$  *efg1*) apresentou maior susceptibilidade a estes fármacos, incluindo miconazol e caspofungina<sup>4, 32</sup>. Dessa forma, o estudo 1 confirmou que os ASPs da MEC têm papel na tolerância aos antifúngicos, uma vez que se encontram mais acumulados na MEC de cepa parental do que nas cepas mutantes ( $\Delta/\Delta$  *efg1*,  $\Delta/\Delta$  *tec1*). Por outro lado, a quantidade de WSPs na MEC de cepas mutantes e na cepa parental é similar.

O DNA extracelular (eDNA) também é um componente fundamental da MEC de *Candida*. O eDNA permite a adesão a superfícies distintas e se liga a outros biopolímeros, proporcionando integridade e estabilidade estrutural aos biofilmes<sup>27, 39</sup>. Além disso, o eDNA está relacionado à maior resistência do biofilme a antifúngicos<sup>18</sup>. Embora o mecanismo preciso pelo qual o eDNA é liberado e contribua para a resistência aos fármacos permaneça incerto<sup>9</sup>, foi sugerido que o eDNA é liberado durante o crescimento das hifas<sup>34</sup>. No entanto, o estudo 1 mostrou que, independentemente da presença de hifas, todos os biofilmes produziram quantidades similares de eDNA, contrariamente aos ASPs, que foram produzidos em maior quantidade por células filamentosas. Portanto, como a cepa mutante  $\Delta/\Delta$  *efg1*, que não produziu hifas, foi capaz de liberar eDNA em sua MEC, isso indica que a produção de eDNA pode não estar necessariamente relacionada ao crescimento das hifas.

A função conhecida da maioria das proteínas está relacionada ao metabolismo<sup>47</sup>. A análise quantitativa das proteínas totais no estudo 1 mostrou que a cepa mutante  $\Delta/\Delta$  *efg1* possui maior número de proteínas quando comparada à cepa parental e à cepa mutante  $\Delta/\Delta$  *tec1*, e que essas quantidades de proteínas estavam relacionadas aos valores populacionais (ufc/mL) mais altos observados em  $\Delta/\Delta$  *efg1*. Estes resultados podem representar um esforço extra da cepa mutante  $\Delta/\Delta$  *efg1* para obter energia e se organizar como biofilme. Portanto, o estudo 1 mostrou que as quantidades de eDNA, WSPs e proteínas solúveis na MEC dos biofilmes da cepa WT e das cepas mutantes ( $\Delta/\Delta$  *efg1* e  $\Delta/\Delta$  *tec1*) são semelhantes. Por outro lado, o conteúdo de ASPs é significativamente maior na WT em comparação com as cepas mutantes, o que indica que a produção de ASPs pode estar ligada à morfologia celular filamentosa em *C. albicans*. Devido ao papel de proteção do biofilme exercido por este componente da MEC, os ASPs devem ser alvo para novas estratégias de controle de biofilmes.

O fluconazol é um fármaco comumente aplicado para o tratamento de infecções fúngicas. Este fármaco previne a biossíntese de ergosterol através da inibição da enzima 14- $\alpha$  desmetilase, pertencente à família do citocromo P450, que catalisa a conversão de lanosterol em ergosterol<sup>23</sup>. A redução do ergosterol altera a fluidez da membrana e a atividade de numerosas enzimas ligadas a ela, dificultando o crescimento e a replicação fúngica<sup>16</sup>. No entanto, a resistência ao fluconazol está aumentando em espécies de *Candida*<sup>22, 46</sup>, exigindo uma melhor compreensão sobre a ação desse fármaco nos biofilmes deste fungo. Sendo assim, no estudo 2, avaliou-se a influência da resistência ao fluconazol na MEC de biofilmes de *C. albicans* e *C. glabrata* susceptíveis e resistentes a fluconazol quando seus biofilmes foram formados na presença ou ausência desta droga.

Surpreendentemente, nenhuma redução significativa na contagem de CaS foi observada após a exposição a doses de 5x CIM de FLZ. No entanto, os valores de redução em Log<sub>10</sub> para todas as cepas são muito próximos (CaS: redução de 0,67 Log<sub>10</sub>, CaR: redução de 0,88 Log<sub>10</sub>, CgS: redução de 0,71 Log<sub>10</sub> e CgR: redução de 0,70 Log<sub>10</sub>). Por outro lado, as imagens do estudo 2 mostraram que o FLZ reduziu o tamanho das hifas em CaS. Levando-se em consideração que as hifas são a forma invasiva de *C. albicans*<sup>14</sup>, a redução de seu tamanho é um resultado importante para a redução da virulência dessa cepa. O crescimento de biofilmes de CaR na presença ou ausência de FLZ mostrou que o medicamento reduziu significativamente a contagem de colônias viáveis em Log<sub>10</sub>. Além disso, as imagens obtidas através de microscopia eletrônica de varredura (MEV) mostraram que o FLZ reduziu a formação de hifas em CaR. Este resultado corrobora com um estudo anterior que observou que o FLZ tem efeito inibitório direto na formação de hifas<sup>11</sup>. Como o FLZ interfere na via do ergosterol e o ergosterol é necessário para a formação de hifas, a presença de FLZ inibe a transição de leveduras para hifa<sup>11</sup>. Da mesma forma, FLZ reduziu significativamente o Log<sub>10</sub> em CgS e CgR. Estes resultados foram confirmados pelas imagens obtidas através de MEV dos biofilmes de CgS e CgR. Foi relatado que *C. glabrata* tem susceptibilidade intrínseca diminuída ao FLZ e outras classes de azóis antifúngicos<sup>28, 31, 46</sup>, no entanto, os resultados do estudo 2 mostraram que a formação de biofilmes com 40  $\mu\text{g/mL}$  de FLZ em CgS e 1280  $\mu\text{g/mL}$  de FLZ em CgR podem reduzir a viabilidade celular destes microrganismos.

Na MEC, o FLZ atuou reduzindo significativamente o conteúdo de ASPs em CaS, o que não aconteceu com as outras cepas. Os ASPs, como os  $\beta$ -1,3-glucanos, sequestram os antifúngicos, impedindo-os de se difundirem através da MEC de *Candida*<sup>20, 24, 25</sup>. O papel dos  $\beta$ -1,3-glucanos na resistência antifúngica tem sido demonstrado, ou seja, foi

verificado que os  $\beta$ -1,3-glucanos se ligam à FLZ evitando que a droga atinja seus alvos no biofilme<sup>20, 24, 25</sup>. Assim, a redução significativa de ASPs na MEC de CaS demonstra que os biofilmes dessa cepa específica tornam-se mais vulneráveis quando o FLZ está presente. Além disso, embora os ASPs possam se ligar à FLZ, quando 80  $\mu$ g/mL de FLZ estão presentes durante a formação do biofilme de CaS, a droga pode suprimir esse efeito e causar a redução de ASPs.

Um achado importante do estudo 2 foi que o FLZ atuou na MEC de todos os microrganismos reduzindo a produção de WSPs. A matriz do biofilme de *Candida* possui  $\alpha$ -mannanos (WSPs) associados a  $\beta$ -glucanos (ASPs), constituindo o complexo manano-glucano (MGCx)<sup>47</sup>. Considerando que as interações dos polissacarídeos na MEC são necessárias para a resistência antifúngica do biofilme<sup>20</sup>, a redução da quantidade de WSPs na MEC dos biofilmes em presença do FLZ pode alterar a constituição do MGCx, levando a uma maior susceptibilidade dos biofilmes à terapia antifúngica. Outra observação relevante do estudo 2 é que o FLZ causou uma importante atividade antibiofilme ao reduzir significativamente as biomassas totais de todos os microrganismos estudados. Células e componentes da MEC compõem as biomassas, assim, a diminuição significativa dos WSPs, bem como a redução do tamanho das hifas em CaS e CaR e a redução de células de CgS e CgR podem ser responsáveis pela redução significativa das biomassas. O conteúdo insolúvel do biofilme (por exemplo, ASPs e células, que contém as proteínas) compõem a biomassa insolúvel. Os resultados mostraram que na presença de FLZ houve redução significativa da biomassa insolúvel de CaS, CaR e CgR, provavelmente causada pela redução do tamanho das hifas e da quantidade de células, além da redução de ASPs. No entanto, CgS mostrou um aumento significativo na biomassa insolúvel na presença de FLZ. Variações na MEC podem ocorrer quando íons estão disponíveis<sup>43</sup>. Possivelmente, os íons do FLZ ( $C_{13}H_{12}F_2N_6O$ ) podem ter interagido com grupos de ácido carboxílico da matriz para produzir grades de macromoléculas com maior viscosidade, resultando em uma matriz de polissacarídeo mais pegajosa<sup>43</sup>.

O FLZ não causou efeitos em eDNA, proteínas (da parte insolúvel do biofilme) e proteínas da matriz (solúveis) em todas as cepas avaliadas. Curiosamente, as quantidades desses componentes também não são estatisticamente diferentes entre os diferentes tipos de cepas avaliadas. A produção de eDNA tem sido associada ao crescimento das hifas<sup>34</sup>. No entanto, o estudo 2 mostra que, mesmo na ausência de hifas (por exemplo, em biofilmes de *C. glabrata*), todos os biofilmes produziram quantidades comparáveis de eDNA na presença ou ausência de FLZ, corroborando com os resultados do estudo 1. Em

*C. albicans*, o eDNA contribui para a preservação e estabilidade dos biofilmes maduros, mas não para o seu estabelecimento<sup>18</sup>; enquanto o papel reconhecido da maioria das proteínas está ligado ao metabolismo<sup>47</sup>. Além disso, o eDNA atua como um regulador da resistência antifúngica das células do biofilme contra equinocandinas e anfotericina B, mas não parece contribuir significativamente para a resistência à FLZ<sup>18</sup>. Esse achado corrobora com os resultados do estudo 2, uma vez que o FLZ não afetou a produção de eDNA, além de não afetar a produção de proteínas.

O estudo 2 mostrou que os WSPs e as biomassas são diretamente afetados pela presença de FLZ durante formação de biofilmes, enquanto o eDNA e as proteínas permanecem estáveis, independentemente do tipo de cepa. Assim, FLZ impediu o acúmulo de WSPs e reduziu as biomassas, atuando em hifas e reduzindo a quantidade de células de *C. glabrata* susceptível e resistente à fluconazol. A redução de WSPs e das biomassas totais pode estar relacionada à susceptibilidade dos biofilmes à terapêutica e à virulência, tornando os biofilmes que crescem na presença de FLZ mais susceptíveis e potencialmente menos virulentos. No entanto, como FLZ é fungistático, há uma chance para o desenvolvimento de resistência adquirida durante o tratamento com este antifúngico. Além disso, o FLZ teve efeitos mínimos na redução de Log<sub>10</sub>, bem como não teve efeito sobre eDNA e proteínas. Portanto, é importante considerar alternativas para desorganizar as MECs à fim de se obter uma melhor difusão da droga através dos biofilmes. Sendo assim, o estudo 3 se concentrou em encontrar alternativas para abrir caminhos através da MEC que possam melhorar a ação das terapias antifúngicas. A intenção era desorganizar a MEC usando enzimas hidrolíticas, mas não destruí-la, uma vez que isso poderia provocar a dispersão das células para colonizarem outros locais.

No estudo 3, observou-se que o tratamento durante um curto período de exposição com as enzimas isoladas ou em combinação não teve efeito sobre o Log<sub>10</sub> de cepas de *C. albicans* susceptíveis e resistentes ao fluconazol, e o mesmo foi observado com relação à biomassa total de CaS. No entanto, a combinação de  $\beta$ +Dex causou redução significativa da biomassa total em relação ao controle CaR+Tampão, o que pode ter ocorrido devido a redução de polissacarídeos e proteínas, comentadas à frente. Por outro lado, a presença de  $\beta$ -glucanase individualmente e em combinação com Dextranase e DNase (CaR+ $\beta$ , CaR+ $\beta$ +Dex, CaR+ $\beta$ +DNase e CaR+Todas as enzimas) aumentou significativamente a biomassa insolúvel de CaR. Foi reconhecido que mudanças na MEC podem ocorrer quando os íons estão presentes<sup>43</sup>. Os íons do tampão de acetato de sódio (C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub>) e os íons presentes na matriz, como Ca<sup>2+</sup> e K<sup>+</sup>, podem interagir com grupos

de ácido carboxílico expostos na matriz de polissacarídeos para produzir macromoléculas de alta viscosidade<sup>43</sup>. É possível que a enzima  $\beta$ -glucanase tenha causado a exposição de grupos de ácidos carboxílicos de moléculas de polissacarídeos na MEC da cepa resistente ao fluconazol, permitindo a interação desses grupos com íons e resultando em uma matriz de polissacarídeos mais viscosa, que aumentou a biomassa insolúvel. A composição do tampão utilizado durante a incubação dos biofilmes com as enzimas pode ter ajudado neste processo. Por outro lado, nenhuma das enzimas ou combinações delas reduziu a quantidade total de proteínas (proteínas insolúveis) em biofilmes de *C. albicans* susceptíveis e resistentes ao fluconazol. O teor de proteínas totais inclui proteínas da MEC que não foram removidas durante as lavagens de biofilme, incluindo também as proteínas das células. Assim, como nenhuma das enzimas ou combinações delas agiu sobre a população de cepas susceptíveis e resistentes ao fluconazol, a quantidade inalterada de proteína total pode estar relacionada ao número inalterado de células.

A combinação de todas as enzimas (CaS+Todas as enzimas) reduziu significativamente a quantidade de ASPs na MEC de CaS quando em comparação com CaS+ $\beta$  e CaS+Dex, demonstrando importante ação da DNase para redução de ASPs na MEC desta cepa. Além disso, a enzima dextranase (CaR+Dex) e a combinação de todas as enzimas (CaR+Todas as enzimas) também reduziram significativamente o conteúdo de ASPs em CaR em comparação ao controle sem enzimas. Levando-se em consideração que a enzima  $\beta$ -glucanase foi escolhida para hidrólise de  $\beta$ -1,3-glucano e a enzima dextranase foi selecionada para hidrólise de ligações  $\alpha$ -(1,6)-glicosídicas em dextrano, os resultados do presente estudo confirmam que os polissacarídeos da MEC estão interligados, uma vez que a  $\beta$ -glucanase e a dextranase reduziram o teor de ASPs em *C. albicans* susceptível e resistente ao fluconazol. Por outro lado, a DNase foi escolhida para a hidrólise do conteúdo de eDNA das MECs de ambas as cepas, mas, surpreendentemente, a presença de DNase em combinação com outras enzimas testadas teve um efeito significativo na redução de ASPs tanto em CaS quanto em CaR. Além disso, observou-se que CaR+Dex, CaR+DNase e CaR+ $\beta$ +DNase reduziram significativamente a quantidade de WSPs na MEC de CaR, ou seja, como esperado,  $\beta$ -glucanase não teve efeito de redução de WSPs em CaR, porém, a ação da DNase mostra que o eDNA está ligado e/ou englobado por exopolissacarídeos do complexo MGCx, fazendo com que a ação da DNase sobre o eDNA quebre a organização dos complexos formados por exopolissacarídeos-eDNA, causando redução de ASPs e WSPs nos quais as moléculas de eDNA estão ligadas. Porém, nenhuma das enzimas ou combinações delas

alterou o conteúdo de WSPs na MEC de CaS. Foi descrito que a secreção de  $\beta$ -1,6-glucanos é possível devido à sua interação física com mananos solúveis em água<sup>47</sup>. Aparentemente, a interação física entre os polissacarídeos do complexo MCGx na MEC das cepas CaS e CaR pode ser diferente.

Considerando-se a importância do eDNA para a MEC de *C. albicans*, contribuindo para a integridade estrutural do biofilme e resistência contra os antifúngicos<sup>18, 20, 34</sup>, a redução do eDNA via DNase no estudo 3 demonstrou um resultado positivo, uma vez que esta enzima reduziu significativamente a quantidade de eDNA nos biofilmes de ambas as cepas. Além disso, a combinação de DNase com  $\beta$ -glucanase e dextranase também foi eficaz na redução da quantidade de eDNA em CaR. Ainda, a combinação de  $\beta$ -glucanase com dextranase reduziu significativamente o conteúdo de eDNA em CaS. Este resultado reforça a possibilidade de que o eDNA esteja ligado e/ou englobado pelos componentes do complexo MGCx nas MECs das cepas de *C. albicans*. Inesperadamente, a combinação de todas as enzimas aumentou o conteúdo de eDNA na MEC do biofilme CaR. É possível que a combinação de todas as enzimas tenha causado uma desorganização elevada da MEC, liberando o conteúdo de eDNA que foi encapsulado em suas camadas mais profundas. Assim, o tratamento de CaR com todas as enzimas em conjunto ( $\beta$ -glucanase + Dextranase + DNase) deve ser evitado.

O teor de proteína solúvel foi significativamente reduzido pelo tratamento com as enzimas individualmente e em combinações em CaS e CaR. Foi demonstrado que as proteínas aderem aos polissacarídeos na MEC<sup>2, 43</sup>. Considerando-se que as proteases certamente afetariam as proteínas que interagem com polissacarídeos dentro dos biofilmes, demonstrou-se que as polissacarases e as polissacarídeos liases têm um efeito muito maior<sup>10, 42, 43</sup>. Os resultados do presente estudo apoiam essas observações anteriores, uma vez que as proteínas solúveis foram reduzidas pela  $\beta$ -glucanase, dextranase e, também, pela DNase. Além disso, o estudo 3 demonstrou que exopolissacarídeos, eDNA e proteínas estão interligados na MEC das cepas de *C. albicans* susceptível e resistente ao fluconazol estudadas.

Em geral, o tratamento com DNase produziu resultados satisfatórios que diminuiram o conteúdo de ASPs, eDNA e proteínas solúveis em biofilmes de *C. albicans* susceptível e resistente ao fluconazol, além de reduzir WSPs na cepa resistente ao fluconazol. Assim, o tratamento com DNase reduziu a maioria dos componentes da MEC que podem dificultar a penetração de antifúngicos em biofilmes de *Candida*. A redução dos componentes da MEC do biofilme é muito relevante, uma vez que pode abrir

caminhos na MEC que poderiam facilitar a difusão de antifúngicos no biofilme, superando a resistência derivada dos componentes da MEC. Além disso, a redução de exopolissacarídeos e de proteínas pela DNase indicam que esses componentes estão entrelaçados com o eDNA na MEC de *C. albicans*. Portanto, a exposição de biofilmes de 48h por 5 min à DNase reduz eDNA, exopolissacarídeos e proteínas solúveis da MEC de *C. albicans* susceptível e resistente a fluconazol, indicando que a DNase pode ser um promissor adjuvante para terapias antibiofilme.

De modo geral, os estudos mostraram a importância da MEC na formação e estabelecimento de biofilmes de *Candida*, e que a presença de ASPs está relacionada à morfologia celular filamentosa em *C. albicans*. Além disso, os estudos demonstraram que o FLZ tem interfere na MEC de cepas de *C. albicans* e *C. glabrata* susceptíveis e resistentes ao fluconazol. No intuito de melhorar a ação de terapias antibiofilme, o uso de enzimas hidrolíticas pode ser uma alternativa promissora, sendo assim, enzimas hidrolíticas que potencialmente teriam ação de desorganizar a MEC foram estudadas. Verificou-se que os componentes da MEC estão interligados na matriz de cepas de *C. albicans* susceptível e resistente a fluconazol, e que a aplicação da enzima hidrolítica DNase em biofilmes maduros de 48 h por apenas 5 min foi capaz de desorganizar a MEC destas cepas. Sendo assim, os estudos aqui apresentados mostraram a importância do estudo da MEC de biofilmes de *Candida* para entender o estabelecimento, virulência e resistência ao FLZ nestes biofilmes. Além disso, os estudos aqui presentes demonstraram o potencial da enzima DNase em desorganizar a MEC de biofilmes de *C. albicans*, com o intuito de, futuramente, melhorar a penetração de agentes terapêuticos e facilitar o tratamento das infecções causadas por biofilmes de *Candida*.



## 5 CONCLUSÃO

1. O conteúdo de ASPs é significativamente maior em cepa parental de *C. albicans* (SN425) em comparação com as cepas mutantes com deficiência na formação de hifas e/ou biofilmes,  $\Delta/\Delta$  *efg1* (CJN 2302) e  $\Delta/\Delta$  *tec1* (CJN 2330), indicando que a produção de ASPs pode estar relacionada à morfologia celular filamentosa.
2. WSPs e as biomassas são diretamente afetados pela presença de FLZ durante formação de biofilmes, enquanto eDNA e proteínas permanecem estáveis nas cepas *C. albicans* ATCC 90028 (susceptível à fluconazol), *C. albicans* ATCC 96901 (resistente a fluconazol), *C. glabrata* ATCC 2001 (susceptível à fluconazol) e *C. glabrata* ATCC 200918 (resistente a fluconazol).
3. FLZ impediu o acúmulo de WSPs e reduziu as biomassas, atuando em hifas em *C. albicans* ATCC 90028 e *C. albicans* ATCC 96901, e reduzindo a quantidade de células em *C. glabrata* ATCC 2001 e *C. glabrata* ATCC 200918.
4. A exposição de biofilmes de 48 horas a DNase reduziu eDNA, polissacarídeos e proteínas solúveis das matrizes extracelulares de *C. albicans* ATCC 90028 e *C. albicans* ATCC 96901.
5. A redução de polissacarídeos extracelulares e de proteínas solúveis pela DNase sugere que estes componentes estejam interligados ao eDNA nas matrizes extracelulares de *C. albicans* ATCC 90028 e *C. albicans* ATCC 96901.

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\* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-marco-2015.pdf>

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