PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM ODONTOLOGIA

ANNE CAROLINE MORAIS CALDEIRÃO

EFEITO DE NANOCARREADORES DE DROGAS ANTIFÚNGICAS SOBRE ESPÉCIES DE CANDIDA EM BIOFILMES MICROCOSMOS

Presidente Prudente - SP
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Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Odontologia – Área de concentração: Clínica Odontológica

Orientador:
Prof. Dr. Douglas Roberto Monteiro

Presidente Prudente - SP
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Presidente Prudente, 19 de março de 2021.

BANCA EXAMINADORA

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Presidente Prudente - SP

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Prof. Dr. Juliano Pelim Pessan
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DEDICATÓRIA

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“Em algum lugar, algo incrível está esperando para ser descoberto”.

(Carl Sagan)

RESUMO

Efeito de nanocarreadores de drogas antifúngicas sobre espécies de Candida em biofilmes microcosmos

A resistência das espécies de *Candida* às terapias convencionais tem motivado o desenvolvimento de nanocarreadores antifúngicos baseados em nanopartículas de óxido de ferro (NPsOF) revestidas com quitosana (QTS). Este estudo avaliou os efeitos de NPsOF-QTS como carreadores de miconazol (MCZ) ou fluconazol (FLZ) sobre biofilmes microcosmos salivares. *Pool* de saliva de dois voluntários saudáveis suplementada com *Candida albicans* e *Candida glabrata* foi o inóculo para a formação de biofilmes. Os biofilmes foram formados por 96 horas sobre discos de vidro no *Amsterdam Active Attachment Model* e tratados por 24 horas com os nanocarreadores contendo diferentes concentrações de cada antifúngico (78 e 156 µg/mL). MCZ ou FLZ (156 µg/mL) e biofilmes não tratados foram considerados como controles. Os efeitos antibiofilme foram avaliados pela enumeração das unidades formadoras de colônias (UFCs), composição da matriz extracelular, produção de ácido lático, além da análise da estrutura e das células vivas/mortas do biofilme por microscopia confocal de varredura a laser (MCVL). Os dados foram analisados por ANOVA a um critério e teste de Fisher LSD ($\alpha = 0,05$). NPsOF-QTS carreando MCZ ou FLZ foram os tratamentos mais eficazes na redução de UFCs em comparação com qualquer agente antifúngico sozinho para *C. albicans* e com MCZ para *C. glabrata*. Reduções significativas em estreptococos do grupo mutans e *Lactobacillus* spp. foram encontradas, principalmente para o nanocarreador de MCZ. Os antifúngicos e seus nanocarreadores também mostraram proporções significativamente maiores de células mortas em comparação ao biofilme não tratado por MCVL ($p < 0,001$), promoveram reduções significativas na produção de ácido lático e aumentaram significativamente alguns componentes da matriz extracelular. Esses resultados reforçam o uso de nanocarreadores como alternativas eficazes para combater infecções fúngicas orais.

Palavras-chave: Antifúngicos; Biofilmes; *Candida*; Nanocarreadores; Nanopartículas de óxido de ferro.

ABSTRACT

Effect of nanocarriers of antifungal drugs on Candida species in microcosm biofilms

Resistance of *Candida* species to conventional therapies has motivated the development of antifungal nanocarriers based on iron oxide nanoparticles (IONPs) coated with chitosan (CS). This study evaluated the effects of IONPs-CS as carriers of miconazole (MCZ) or fluconazole (FLZ) on microcosm biofilms. Pooled saliva from two healthy volunteers supplemented with *Candida albicans* and *Candida glabrata* was the inoculum for biofilm formation. Biofilms were formed for 96 h on coverslips using the Amsterdam Active Attachment model, followed by 24 h treatment with nanocarriers containing different concentrations of each antifungal (78 and 156 μg/mL). MCZ or FLZ (156 μg/mL), and untreated biofilms were considered as controls. Anti-biofilm effects were evaluated by enumeration of colony-forming units (CFUs), composition of the extracellular matrix, lactic acid production, and structure and live/dead biofilm cells (confocal laser scanning microscopy - CLSM). Data were analyzed by 1-way ANOVA and Fisher LSD's test ($\alpha = 0.05$). IONPs-CS carrying MCZ or FLZ were the most effective treatments in reducing CFUs compared to either antifungal agent alone for *C. albicans* and MCZ for *C. glabrata*. Significant reductions in mutans streptococci and *Lactobacillus* spp. were shown, though mainly for the MCZ nanocarrier. Antifungals and their nanocarriers also showed significantly higher proportions of dead cells compared to untreated biofilm by CLSM ($p < 0.001$), and promoted significant reductions in lactic acid, while simultaneously showing increases in some components of the extracellular matrix. These findings reinforce the use of nanocarriers as effective alternatives to fight oral fungal infections.

Keywords: Antifungals; Biofilms; *Candida*; Nanocarriers; Iron oxide nanoparticles.

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

1 Introduction

Polymicrobial biofilms are communities comprising multiple species of microorganisms, including bacteria and fungi, attached to a surface and organized within an extracellular polymeric matrix[1]. In the human body, the presence of structured microbial consortia in biofilms is often observed, modulating the states of health and disease[1]. The oral cavity is considered one of the most favorable environments for polymicrobial biofilm formation due to its complex features and presence of various retentive niches[1,2], including mucous surfaces, lingual dorsum, tooth hard surfaces, and sub- or supra-gingival compartments[2]. It is thought that the oral microbiome has around 700 different species colonizing this environment[3].

Candida species are important contributors for the oral microbiome and may establish a commensal relationship with other microbial species, mainly in healthy individuals[4]. In general, *Candida* yeasts have high capacity to form biofilms[5] and to induce infections when there are local or systemic disorders, particularly in the immunocompromised[4,5]. Accordingly, a homeostatic imbalance occurs, followed by yeast cells proliferation, establishing a pathological condition[4,5]. Fungal infections affect around one billion people[6], and account for an annual mortality rate of approximately 1.7 million individuals worldwide[6,7]. In such infectious processes, *Candida albicans* stands out as one of the main etiological agents[8-10], which is present in about 95% of candidiasis clinical cases[11]. Nonetheless, *Candida glabrata* has been recognized as an important candidiasis-related pathogen in recent years, mainly due to its resistance to antifungal treatments[8] and high prevalence in systemic infections[12].

Among the drugs prescribed to manage candidiasis, miconazole (MCZ) and fluconazole (FLZ) have been frequently used as topical and systemic antifungals,

respectively[13-15]. Despite the favorable pharmacological properties of these antifungal agents, limitations related to their use have been reported in recent years, encompassing the reduction of antifungal efficacy ought to microbial resistance[16-19], which hinders the action of drugs and makes them less bioavailable[17,19]. Consequently, administration of higher doses and/or increased frequency are required[19], which may intensify side effects such as local burning sensation, nausea, vomiting, gastrointestinal disturbances and hepatotoxicity[11,19]. Another clinical challenge found in the treatment of *Candida* infections refers to the lower availability in the market of antifungals[4,5] compared to antibacterials[5].

Strategies for circumventing the limitations reported above include the study of new alternatives to control fungal infections. In this sense, advances in nanotechnology-based therapies have enabled combining drugs with nanoparticles for improving therapeutic performance of the compound carried, as well as for reducing its side effects[20,21]. Among the numerous nanometric materials available, iron oxide nanoparticles (IONPs) have shown wide application in the biomedical field, including drug delivery, due to factors associated to synthesis process, biocompatibility, chemical stability and surface modification capacity[22-24]. As for the latter, chitosan (CS) is a biopolymer with antimicrobial activity successfully used to coat IONPs[21,25,26]. This polymer may establish electrostatic interactions or hydrogen bonds with IONPs[27], favoring the stabilization of nanoparticles under physiological conditions, in addition to allowing the anchoring of drugs[28].

Recently, nanocarriers of MCZ or FLZ assembled from CS-coated IONPs showed similar or superior effects on planktonic cells and biofilms of *C. albicans* and *C. glabrata* compared to those found for each antifungal alone[21,22]. Moreover, we have shown promising effects within orally relevant interkingdom biofilm models,

though these were limited to only *C. albicans* and a small panel of preselected oral pathogens[29]. However, these data suggested that targeting the yeast had the potential to destabilise the bacterial components of interkingdom consortia. Therefore, despite these favorable results, the effects of these nanocarriers on *Candida* species in complex polymicrobial interkingdom biofilms remain unknown. We hypothesized that antifungal containing CS-coated IONPs would exert a direct and indirect antimicrobial effect on complex biofilms. Therefore, the aim of the present study was to evaluate the effect of nanocarriers of MCZ or FLZ on undefined microcosm biofilms formed from human saliva supplemented with *C. albicans* and *C. glabrata*.

2 Materials and methods

2.1 Assembly and characterization of the nanocarriers

IONPs-CS-MCZ and IONPs-CS-FLZ nanocarriers were obtained by mixing each antifungal with a known concentration of CS-coated IONPs, as previously detailed[21,22]. For characterization, the physico-chemical tests of X-ray diffraction, Fourier-transform infrared spectroscopy, thermogravimetric analysis, transmission electron microscopy and dynamic light scattering all showed that these antifungal agents were effectively immobilized in the IONPs-CS compound[21,22]. Furthermore, the crystalline structure of the IONPs was not affected after nanocarrier formation, which displayed diameters ≤ 317 nm[21,22].

2.2 Effects of the nanocarriers on salivary microcosm biofilms

2.2.1 Candida *strains and growth conditions*

Two standard strains tested in the present study were purchased from the American Type Culture Collection (ATCC): *C. albicans* (ATCC 10231) and *C. glabrata* (ATCC 90030). Stock cultures were propagated on Sabouraud dextrose agar (Difco, Le Pont de Claix, France) at 37 ºC for 48 h. Colonies of each species derived were individually inserted in 30 mL of Sabourand dextrose broth (Difco) and incubated overnight at 37 ºC in an orbital shaker (120 rpm). The yeast cells were then centrifuged (8000 rpm, 5 min), washed with phosphate buffered saline (PBS; 0.1 M, pH 7.0) and adjusted in a Neubauer chamber to 1×10^7 cells/mL in human saliva.

2.2.2 Collection of human saliva

This study was approved by the local Ethics Committee (CAAE: 22111419.3.0000.5515). Two healthy volunteers (non-smokers) who did not use either oral antimicrobial mouth rinses (over the last 30 days) or systemic antibiotics (over the last 180 days) were selected[30]. Donors also refrained from brushing their teeth on the night before and day of collection, and refrained from drinking alcohol in this period. Saliva collection was performed in the morning, at least two hours after eating and/or drinking[30]. Saliva production was stimulated by chewing flexible film (Parafilm® M, Sigma-Aldrich, St. Louis, MO, USA), and the saliva pool from the two donors/volunteers was stored in polypropylene tubes (on ice)[31,32]. The final saliva sample was diluted (1:1) with 60% sterile glycerol and stored at -80 ºC until use[32].

2.2.3 Microcosm biofilm formation and treatment with nanocarriers

Microcosm biofilms were formed on glass discs (coverslips, 12 mm in diameter; Menzel, Braunschweig, Germany) vertically positioned in the Amsterdam Active Attachment model (AAA), as described in detail by Exterkate *et al*.[32] Briefly, saliva

pool was diluted (50-fold) in McBain medium[33], whose composition for 1 L of deionized water consisted of 2.5 g mucin (Sigma-Aldrich), 2 g Bacto peptone (Difco), 2 g Trypticase peptone (BBL), 1 g yeast extract (Sigma-Aldrich), 0.35 g NaCl (Sigma-Aldrich), 0.2 g KCl (Sigma-Aldrich), 0.2 g $CaCl_2$ (Sigma-Aldrich), 0.1 g cysteine hydrochloride (Sigma-Aldrich), 0.001 g hemin (Sigma-Aldrich), and 0.0002 g vitamin K1 (Sigma-Aldrich)[33], supplemented with 0.2% sucrose (Sigma-Aldrich) v/v and 50 mmol PIPES (Sigma-Aldrich), at pH 7.0[32]. *Candida albicans* and *C. glabrata* were added at a final concentration of 1×10^7 cells/mL to the constituted by human saliva. This supplementation was performed to mimic a microcosm of oral fungal infections, as well as to ensure the presence of *Candida* species in the polymicrobial biofilm. The inoculum was pipetted (1.5 mL) into each well of a 24-well plate (Falcon®; Corning Incorporated - Life Sciences, New York, USA). The plate was closed with the AAA-model lid (containing the coverslips), which was then anaerobically incubated at 37 ºC (Anaerobac; Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil). After 8 h of incubation, the culture medium was replenished by adding 1.5 mL of pure McBain medium in a fresh 24-well plate. This was closed with the same AAA-model lid containing the coverslips with adhered cells. Microcosm biofilms were formed for 96 h, with daily replenishment of the culture medium.

After biofilm formation, the nanocarriers were diluted in McBain medium to reach final concentrations of MCZ and FLZ of 78 and 156 µg/mL, generating two nanocarriers for each antifungal drug: IONPs-CS-MCZ78, IONPs-CS-MCZ156, IONPs-CS-FLZ78 and IONPs-CS-FLZ156. These concentrations were based on the values of minimum inhibitory concentration (MIC) previously published[21], which are equivalent to 50- and 100-fold the MIC of IONPs-CS-MCZ for *C. glabrata*. For the treatment, the lid of the AAA-model containing 96-h-old biofilms was transferred to a

fresh 24-well plate containing 1.5 mL of each nanocarrier suspension, and this was incubated for 24 h. MCZ and FLZ alone, both at 156 µg/mL, were tested as positive controls, while biofilm exposed to pure McBain medium was considered as negative control (NC).

2.2.4 Quantification of cultivable cells

Coverslips with treated biofilms were washed three times with PBS (by transferring the AAA-model lid to 24-well plates containing fresh solutions) to remove weakly adhered cells, and transferred to 5 mL sterile tubes containing 1 mL of PBS. The tubes were placed in an ultrasonic bath for 2 min (55 W; Ultronique, São Paulo, Brazil) and vortexed (1 min). The resulting microbial suspensions were then serially diluted in PBS and plated in the following culture media: (i) Trypticase soy agar (TSA; Difco) with glucose (2 g/L), 5% fresh sheep blood, hemin (10 mL of 0.05% stock solution per liter of medium) and menadione (200 µL of 0.5% stock solution per liter of medium) to count total aerobic and anaerobic microorganisms[32]; (ii) Mitis salivarius agar (MSA; Difco) supplemented with bacitracin (3.3 mg/L), potassium tellurite (1%) and sucrose (15%) for mutans streptococci counts[34]; (iii) Rogosa agar (RA; Difco) supplemented with acetic acid (0.132%) to quantifiy *Lactobacillus* spp.[35]; (iv) CHROMagar *Candida* (Difco) to count *C. albicans* and *C. glabrata*.

TSA plates for total aerobes and CHROMagar *Candida* were aerobically incubated, while TSA plates for total anaerobes were incubated in anaerobiosis. MSA and RA plates were incubated under microaerophilic conditions (5% CO_2; Microaerobac, Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil). The number of colony-forming units (Log_{10} CFU/mL) was counted after 48-72 h of incubation at 37 ºC.

2.2.5 Composition of the extracellular matrix of microcosm biofilms

Coverslips containing the resulting biofilms after treatment were inserted into polypropylene tubes with 2 mL of PBS and vortexed (1 min) to detach biofilms. Afterwards, the tubes were placed in an ultrasonic bath for 2 min (55 W; Ultronique), vortexed for 1 additional min and centrifuged (3000 × *g*, 10 min). The supernatant was then filtered through a syringe filter (0.22 µm) to separate the liquid phase of the matrix from the cell pellet[36]. The tubes containing the cell pellets were dried until a constant dry weight was attained, and the difference between this weight and that from the empty tube was considered to be the final dry weight of the biofilm.

The bicinconinic acid method (Kit BCA, Sigma-Aldrich) was used to determine proteins from extracellular matrix, as previously detailed[36]. Briefly, 200 µL of the BCA kit reagent mixture were added to 25 µL of the liquid phase of the extracellular matrix in a 96-well plate (Kasvi, São José dos Pinhais, Brazil). After 30 min of incubation at 37 ºC, the absorbance was read at 562 nm, and the standard curve was constructed from known concentrations of bovine serum albumin. In turn, the quantification of carbohydrates was based on the method proposed by Dubois *et al*.[37], using different concentrations of glucose as standard. A volume of 500 µL of the liquid phase of the extracellular matrix was added to the mixture of phenol with sulfuric acid in glass tubes, which remained at rest for 15 min[36]. Next, the absorbance of the solution was read at 490 nm. For DNA quantification, the absorbance of the liquid phase of the matrix (2 µL) was read on a Nanodrop spectrophotometer (Eon Microplate Spectrophotometer; Bio Tek, Winooski, USA) at 260-280 nm[36]. All data obtained from the matrix components were represented according to the dry weight of the biofilms (mg/g dry weight).

2.2.6 Lactic acid production assay

The wells of a new 24-well plate were filled with 1.5 mL of buffered peptone water (BPW) with 0.2% glucose[32], and the plate was closed with the AAA-model lid after the biofilm treatment period with the nanocarriers. The plate was incubated for 3 h at 37 ºC in anaerobiosis, and the lactate concentration in the BPW solution was enzymatically determined (Lactate Dehydrogenase; Sigma-Aldrich) by reading the absorbance at 340 nm, using sodium L-lactate (Sigma-Aldrich) as a standard, ranging from 0 to 10 mM[38]. The values obtained in absorbance/cm^2 were converted into mM in a Microsoft Excel software spreadsheet (Version 2010, Microsoft Corp., Redmond, Wash., USA) to determine the analytical parameters.

2.2.7 Structural analysis of biofilms

After 24 h of treatment of the 96-h biofilms with the different compounds, the structural analysis was performed by confocal laser scanning microscopy (CLSM). Coverslips containing biofilms were washed with PBS, stained with SYTO9 green fluorescent dye and propidium iodide using the FilmTracer™ Live/Dead™ Biofilm Viability Kit (Invitrogen, Life Technologies Corporation, Eugene, Oregon, USA), and observed under a confocal microscope (Nikon C2/C2si, Tokyo, Japan), as previously described[21]. Three images from each group were obtained and processed in the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), and the percentages of dead cells were determined by dividing the intensity of red fluorescence (dead cells) by the intensity of green-red fluorescence (total cells).

2.3 Statistical analysis

All biofilm experiments were performed in triplicate, on three different occasions. For the statistical analysis of extracellular matrix components, the results were transformed into a cubic root. All biofilm data showed normal distribution (Shapiro-Wilk test) and were analyzed by 1-way ANOVA and Fisher LSD's *post hoc* test ($\alpha = 0.05$), using the SigmaPlot software (version 12.0; Systat Software Inc., San Jose, USA).

3 Results

3.1 Quantification of cultivable cells

For total anaerobes and aerobes, MCZ and IONPs-CS-MCZ156 were the only treatments that significantly reduced the number of CFUs compared to NC (Fig. 1A and B). IONPs-CS-MCZ156 was the most effective treatment, which was significantly better than free MCZ, leading to reductions of 5.65- ($p = 0.046$) and 4.43-$\log_{10}$ ($p = 0.037$) for total anaerobes and aerobes compared to NC, respectively.

All compounds significantly decreased the number of CFUs of mutans streptococci compared to NC (Fig. 1C), except IONPs-CS-FLZ78. Comparing the effects among each antifungal with their respective nanocarriers, IONPs-CS-MCZ156 was more effective (reduction of 5.38-$\log_{10}$; $p < 0.001$) than MCZ (reduction of 3.19-$\log_{10}$; $p < 0.001$) in reducing CFUs compared to NC (Fig. 1C). On the other hand, FLZ did not statistically differ from IONPs-CS-FLZ78 and IONPs-CS-FLZ156. Regarding the quantification of *Lactobacillus* spp., only IONPs-CS-MCZ156 promoted a significant decrease in CFU number compared to NC (3.47-$\log_{10}$, $p = 0.009$; Fig. 1D).

MCZ, IONPs-CS-MCZ78 and IONPs-CS-MCZ156 significantly reduced CFU numbers of *C. albicans* and *C. glabrata* compared to NC (Fig. 1E and F). A dose-

dependent effect was noted for the nanocarrier of MCZ, with higher reductions promoted by IONPs-CS-MCZ156 compared to IONPs-CS-MCZ78. In addition, IONPs-CS-MCZ156 was the most effective treatment, achieving reductions of 3.6- ($p < 0.001$) and 5.33-$\log_{10}$ ($p < 0.001$) compared to the NCs, respectively for *C. albicans* and *C. glabrata* (Fig. 1E and F). As for FLZ and its nanocarriers, IONPs-CS-FLZ156 was more effective in reducing the number of *C. albicans* cells than FLZ alone, while for *C. glabrata* these compounds behaved similarly (Fig. 1E and F).

3.2 Quantification of extracellular matrix components

IONPs-CS-MCZ156 promoted a 5.74-fold increase ($p = 0.014$) in protein content compared to NC (Table 1). On the other hand, no significant differences among NC, FLZ and IONPs-CS-FLZ156 were observed regarding this parameter. As for carbohydrate content, MCZ and IONPs-CS-MCZ156 did not differ from one another, but promoted increases of 18.86- ($p = 0.041$) and 29.44-fold ($p = 0.006$) compared to NC, respectively. Treatments with FLZ and IONPs-CS-FLZ156 did not differ from one another, but promoted increases of 14.17- ($p = 0.017$) and 14.81-fold ($p = 0.015$) in carbohydrate content compared to NC, respectively. For DNA content, treatments with MCZ, IONPs-CS-MCZ156 and FLZ resulted in increases of 4.81- ($p < 0.05$), 3.21- ($p < 0.05$) and 4.17-fold ($p = 0.014$) in comparison to NC, respectively (Table 1).

3.3 Quantification of lactic acid

MCZ, IONPs-CS-MCZ78 and IONPs-CS-MCZ156 did not significantly differ from one another, but led to significant reductions (ranging from 91.5 to 93.2%; $p < 0.001$) in acid production compared to NC (Fig. 2). The same trend was found for FLZ and its

nanocarriers, with significant reductions ($p < 0.001$) in lactic acid production of 89.7%, 90.8% and 91.9% compared to NC, respectively for FLZ, IONPs-CS-FLZ78 and IONPs-CS-FLZ156 (Fig. 2).

3.4 Structural analysis of biofilms

CLSM images showed biofilms composed by clusters of microbial cells partially covering the surface of the coverslips, regardless of the group evaluated (Fig. 3a-e). Treatments with MCZ, IONPs-CS-MCZ156, FLZ and IONPs-CS-FLZ156 resulted in biofilms with significantly higher proportions of dead cells compared to the NC group ($p < 0.001$; Fig. 3f). In turn, IONPs-CS-MCZ156 and IONPs-CS-FLZ156 led to similar reductions in cell viability compared to MCZ and FLZ, respectively (Fig. 3f).

4 Discussion

Favorable antifungal effects of MCZ and FLZ nanocarriers have been previously reported in studies performed with mono- or dual-species biofilms of *C. albicans* and *C. glabrata*[21,22]. Moreover, we were able to show a positive effect of this chemistry within controlled interkingdom biofilms[29]. Nonetheless, to better mimic the context of the oral microbiome of patients with oral candidiasis, in which *Candida* species are increasing in number and coexisting with other microbial species, the effects of the aforementioned nanocarriers were evaluated on *C. albicans* and *C. glabrata* in complex ‘real world’ microcosm biofilms. Interestingly, the results of the present study showed that the nanocarriers maintained their effectiveness on *Candida* species, even when they are present and integrated within polymicrobial biofilms with complex architecture.

In general, IONPs-CS-MCZ156 and IONPs-CS-FLZ156 were the most effective treatments in reducing the number of *Candida*, overcoming the effect promoted by each antifungal tested in its free form for *C. albicans* (both nanocarriers) and *C. glabrata* (MCZ nanocarrier) (Fig. 1E and F). These findings may be associated with a cooperative action among the three compounds that generate the nanocarriers, so that IONPs functioned as carriers, favoring the penetration of antifungal drugs into the deeper layers of microcosm biofilms. This assumption was previously confirmed, considering that Fe atoms were visible by energy dispersive spectroscopy for elemental mapping in the deeper layers of biofilms treated with nanocarriers based on IONPs-CS[22,26]. In addition, IONPs are able to depolarize the microbial membrane, to induce the production of reactive oxygen species and to generate oxidative stress that disturbs cellular homeostasis[24,29], thus contributing to the observed anti-biofilm effects. In turn, the CS coating stabilizes IONPs and facilitates their penetration through the different layers of biofilms, since the positive charge of CS has an electrostatic interaction with the negative charge of the microbial membranes[24,29]. Due to its mucoadhesive property, CS may also contribute to the retention of the nanocarrier in the target cells, in addition to improving the pharmacokinetics and biodistribution of the carried drugs, favoring cell death[24,29]. On the other hand, MCZ and FLZ act on lanosterol 14-α-sterol demethylase, which participates in ergosterol formation (component of the fungal cell wall)[11,39]. MCZ was also shown to promote oxidative stress due to increases in the production of reactive oxygen species[40].

For *C. glabrata* CFUs, however, IONPs-CS-FLZ156 was not able to overcome the reducing effect generated after treatment with free FLZ (Fig. 1F), demonstrating that the effect of this nanocarrier is primarily dependent on the presence of FLZ.

Candida glabrata has a low intrinsic sensitivity to FLZ[11,41] due to its capacity for specific mutations in the CDR1, CDR2 and MDR1 genes, which are characteristic of azole resistance and are encoded for the action of efflux pumps on the cytoplasmic membrane[39]. Another factor to be highlighted refers to the routes of administration of the different antifungals. FLZ seems to be more relevant for systemic candidiasis (moderate to severe), demanding the use of higher doses to achieve greater effectiveness against *Candida* compared to MCZ, which requires lower doses due to its topical use[11,42].

It was previously demonstrated that FLZ and IONPs-CS-FLZ did not differ from each other, but promoted significant reductions in the number of CFUs of single biofilms of *Candida* species at 1250 μg/mL[22], which corresponds to ~ eight-fold increase over the concentration tested in the present study (156 μg/mL). These findings are extremely relevant from a clinical point of view, as they indicate a superior effect of IONPs-CS-FLZ on *Candida* in polymicrobial consortia, as normally occurs in the oral cavity under pathological conditions. In addition, these results may be indicative of a reduction in the cytotoxic potential of FLZ, since it would make possible the use of lower and more effective doses to combat fungal infections. In contrast, although IONPs-CS-MCZ78 was more effective than free MCZ on *C. albicans* and *C. glabrata* forming mono- or dual-species biofilms[21], this trend was only observed in the present study when the nanocarrier had twice the concentration of MCZ (156 μg/mL). In fact, IONPs-CS-MCZ156 reduced the number of *C. albicans* CFUs by 3.6-$\log_{10}$, and completely eradicated *C. glabrata* cells from the microcosm biofilm (Fig. 1). Taken together, these findings indicate that the antibiofilm effect is dependent on the concentration of drug carried and that *Candida* species may be more tolerant to IONPs-CS-MCZ when present in polymicrobial biofilms. This

suggestion is corroborated by a recent study showing that the reduction in *Candida* promoted by IONPs-CS-MCZ was accompanied by greater reductions in bacterial cells in three models of pathogenic polymicrobial oral biofilms (gingivitis, denture and caries)[29]. These reductions reflected an increase in the percentage of *C. albicans* in the final composition of the three biofilm models, suggesting that the fungal cells were protected from the action of IONPs-CS-MCZ by bacterial cells[29]. Indeed, Kean and colleagues reported that in dual-species biofilms of *C. albicans* and *Staphylococcus aureus* treated with MCZ, that sensitivity is significantly reduced, supporting the notion of synergistic tolerance in interkingdom biofilms[43].

All compounds evaluated in the present study were also able to significantly reduce the number of CFUs of mutans streptococci compared to NC, except IONPs-CS-FLZ78 (Fig. 1C). These findings may be explained by the interactions established among microorganisms within biofilms. In this context, a symbiotic mechanism between *Streptococcus mutans* and *C. albicans* has been reported in several studies[1,44-46], showing that glycosyltransferases (Gtfs) produced and secreted by mutans streptococci promote the breakdown of glucose in monosaccharides, which are metabolized by *Candida* species. This facilitates *Candida* growth and contributes to the production of acids, creating a low-pH environment that helps in the maintenance and survival of *S. mutans*[44-46]. In addition, Gtfs may bind to *Candida* cell surfaces and convert sucrose into glucans[44]. These extracellular polymers, in conjunction with the larger surface area of fungal cells (yeasts and hyphae), create propitious conditions for *S. mutans* adherence[46,47]. Specific cell wall receptors (Als3p adhesin) also favor the adherence of other microorganisms to the hyphae of *C. albicans*, including *C. glabrata*[4], *Lactobacillus* spp. and other aerobic and anaerobic bacteria. Consequently, the reductions found for *Candida* species directly influenced

the survival conditions of mutans streptococci, impairing their adherence and permanence in the biofilm.

For *Lactobacillus* spp. (Fig. 1D), and total anaerobes and aerobes (Fig. 1A and B), IONPs-CS-MCZ156 was the most effective treatment in reducing CFUs. These results remain consistent with those previously discussed and reinforce the role of positive interactions between *Candida* and other microorganisms in the colonization, survival and susceptibility of microcosm biofilms to the compounds tested[48]. Furthermore, although MCZ is a typically antifungal drug, the findings of the present study highlight its antibacterial potential. Probably, free MCZ or conjugated to the core-shell system (IONPs-CS) inhibited bacterial flavohemoglobins, which are responsible for nitric oxide metabolism, resulting in microbial cell death[49,50]. CLSM analysis corroborate these findings, considering that MCZ and IONPs-CS-MCZ156 behaved similarly and presented significantly higher percentages of dead cells than NC control (Fig. 3).

On the other hand, FLZ, IONPs-CS-FLZ78 and IONPs-CS-FLZ156 were not able to significantly reduce the number of total anaerobes (Fig. 1A), total aerobes (Fig. 1B) and *Lactobacillus* spp. (Fig. 1D) compared to NC. A previous study also demonstrated that FLZ was unable to lead to bacterial death[51], corroborating the findings of the present study. Glucans produced by *S. mutans* Gtfs may have sequestered FLZ, limiting its penetration into the biofilm and reducing its effectiveness on bacteria[45,51]. Although the CFU reductions found for *Candida* and *Streptococcus* species after treatment with FLZ and its nanocarriers are in line with CLSM results (Fig. 3), such reductions were not reflected in changes in the total microbial load (Fig. 1). These discrepancies may be justified by the limitation of the CLSM analysis, which did not represent the entire sample, unlike the CFU analysis.

Furthermore, treatments with FLZ and IONPs-CS-FLZ might have favored the development of species that compete for nutrients and binding sites with *Candida* and *Streptococcus*, keeping the total microbial load stable.

In the study reported here, high percentages of lactic acid reduction were found after biofilm treatment with all compounds (Fig. 2). The breakdown of glucose precedes the production of lactic acid by bacteria, mainly *Lactobacillus* spp., besides *Streptococcus*, *Enterococcus* and other microbial genera, and plays important roles in the survival and maintenance of these species[52,53]. *Streptococcus mutans*, *S. oralis*, *S. mitis* and *S. gordonii* are all primary colonizers that offer adhesion sites for fungal colonization, in addition to being considered sources of carbon and lactic acid for *Candida*[4]. In turn, *Candida* species in human saliva also contribute to biofilm pH reduction by producing various acids (pyruvate, lactate and acetate)[54], which favor the activation of acid proteolytic enzymes that damage host tissues. Therefore, the microbial reductions found in the present study directly reflected in decreases in the production of lactic acid by microcosm biofilms. In clinical terms, these results are favorable and highlight that both nanocarriers are capable of affecting an important microbial virulence factor associated with oral candidiasis (acid production).

Regarding the extracellular matrix, there was an overall trend of increases in the values of proteins, carbohydrates and DNA after treatment with the different compounds, with significant differences between NC and IONPs-CS-MCZ156 (for all components) and between NC and IONPs-CS-FLZ156 (for carbohydrates) (Table 1). These increases seem to be related to CFU results, considering that intracellular constituents of dead cells may have been incorporated into the extracellular matrix. Moreover, a higher production of matrix by the remaining biofilm cells may explain these findings, representing an attempt of cellular self-protection against aggression

caused by treatments. A previous study demonstrated that FLZ and IONPs-CS-FLZ significantly increased the components of the extracellular matrix of single biofilms of *C. albicans* and *C. glabrata*[22]. In contrast, MCZ alone or conjugated with the nanocarrier acted at the cellular level, without affecting the matrix of mono- or dual-species *Candida* biofilms[21]. These previous results compared to those obtained here emphasize that the nanocarriers' effects on the extracellular matrix are directly influenced by the type of biofilm analyzed.

In conclusion, IONPs-CS-MCZ and IONPs-CS-FLZ were effective in reducing *Candida* species in salivary microcosm biofilms, surpassing the effects promoted by antifungals in their free form for some of the variables analyzed. Furthermore, significant reductions in the number of mutans streptococci and *Lactobacillus* spp. were found, mainly for IONPs-CS-MCZ. The nanocarriers also promoted significant reductions in the production of lactic acid, and increases in some components of the extracellular matrix of microcosm biofilms. Thus, the study's hypothesis was partially accepted. Future studies evaluating the effects of nanocarriers on microbial ecology (by next generation sequencing) and proteomic profile of the microcosm biofilm, as well as their cytotoxic effects using models of reconstituted human epithelium may contribute to improving the development of antifungal nanocarriers with high sensitivity and selectivity.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1. Mean values (standard deviation) of protein, carbohydrate and DNA contents extracted from the extracellular matrix of salivary microcosm biofilms treated with miconazole (MCZ) and fluconazole (FLZ), alone or forming nanocarriers

Matrix components (mg/g of biofilm dry weight)	Compounds					
	NC	MCZ	IONPs-CS-MCZ156	NC	FLZ	IONPs-CS-FLZ156
Proteins	20.56 (2.38)[a]	72.16 (41.62)[ab]	118.10 (44.23)[b]	20.56 (2.38)[a]	65.14 (37.77)[a]	57.76 (44.73)[a]
Carbohydrates	35.33 (11.49)[a]	666.36 (494.17)[b]	1040.32 (145.18)[b]	35.33 (11.49)[a]	500.68 (351.48)[b]	523.30 (320.66)[b]
DNA	6.75 (0.94)[a]	32.50 (11.36)[b]	21.69 (14.78)[b]	6.75 (0.94)[a]	28.21 (13.95)[b]	13.18 (5.53)[ab]

Note: for each component of the extracellular matrix, different lowercase letters represent significant differences among the groups (one-way ANOVA and Fisher LSD's test; $p < 0.05$). Statistical comparisons were performed separately for each antifungal, its respective nanocarrier and negative control (NC). Chitosan (CS)-coated iron oxide nanoparticles (IONPs) carrying MCZ (IONPs-CS-MCZ156) or FLZ (IONPs-CS-FLZ156), both at 156 µg/mL.

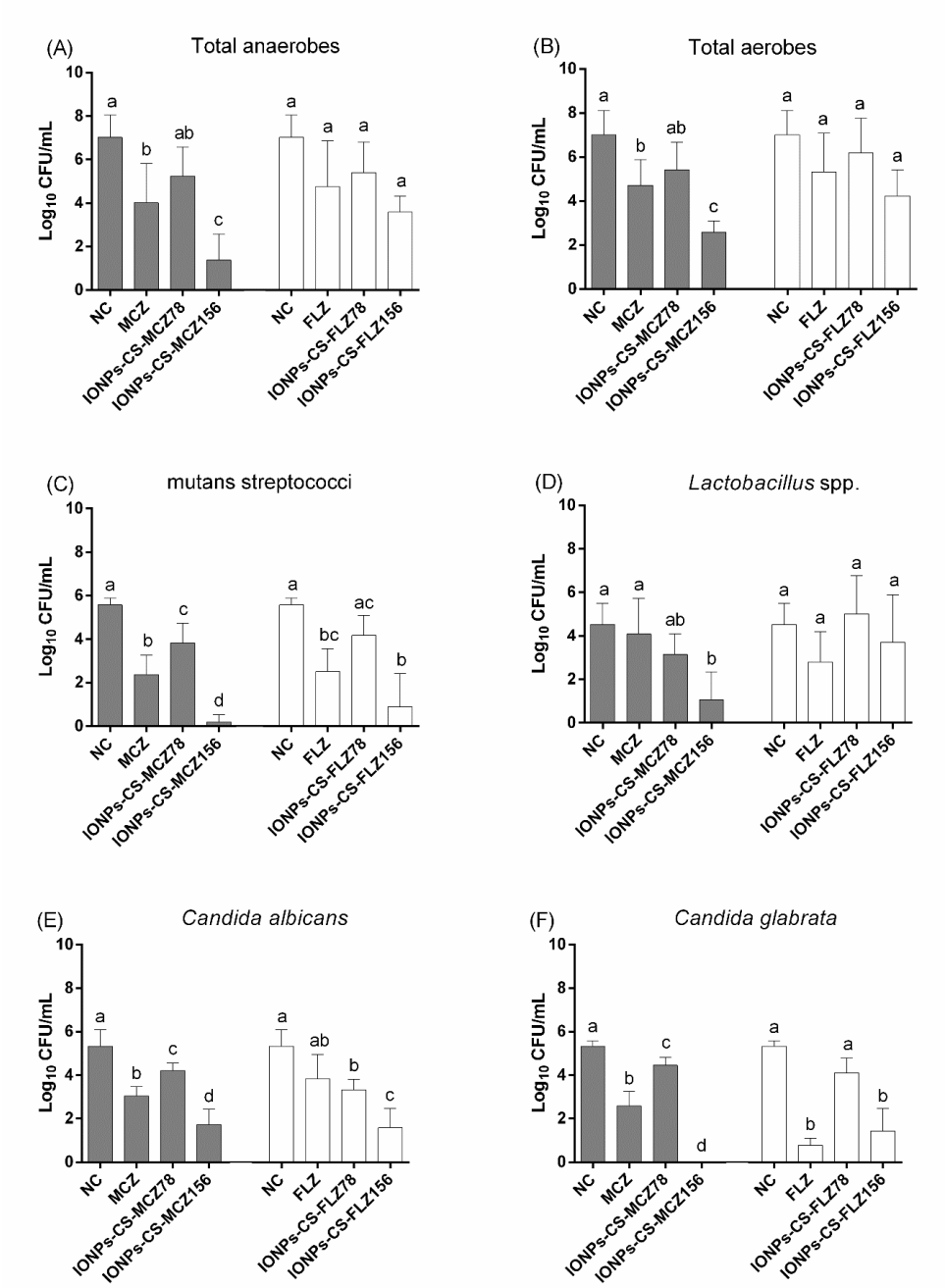

Figure 1. Quantification of colony-forming units (Log_{10} CFU/mL) of total anaerobes (A), total aerobes (B), mutans streptococci (C), *Lactobacillus* spp. (D), *Candida albicans* (E) and *Candida glabrata* (F) from microcosm biofilms formed for 96 h and treated with different compounds. Biofilms were treated during 24 h with miconazole at 156 µg/mL (MCZ), chitosan (CS)-coated iron oxide nanoparticles (IONPs) carrying MCZ at 78 (IONPs-CS-MCZ78) and 156 µg/mL (IONPs-CS-MCZ156), fluconazole at 156 µg/mL (FLZ) and FLZ-containing nanocarrier at 78 (IONPs-CS-FLZ78) and 156 µg/mL (IONPs-CS-FLZ156). Negative control (NC) represents biofilm formed for 120 h with pure culture medium. Error bars depict standard deviations of the means. Different lowercase letters represent significant differences among the groups (one-way ANOVA and Fisher LSD's test; $p < 0.05$). Comparisons were performed separately for each antifungal, its respective nanocarrier and NC.

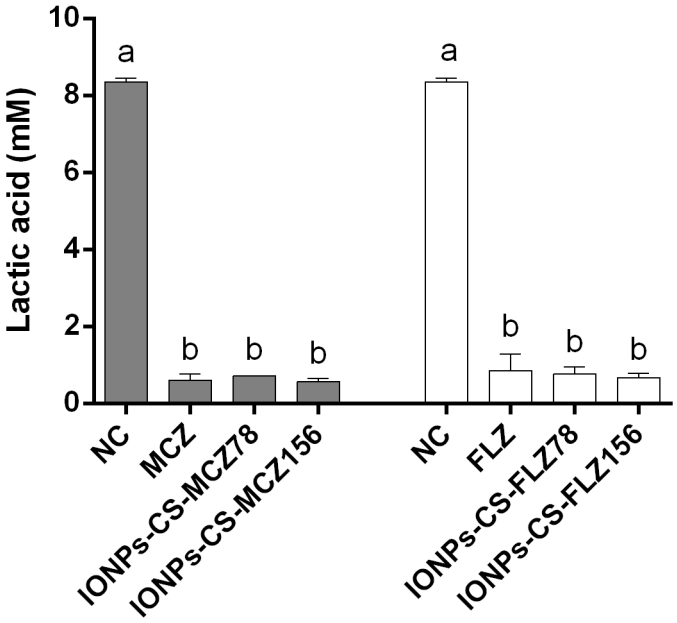

Figure 2. Mean values (standard deviation) of lactic acid concentration from microcosm biofilms formed for 96 h and treated with different compounds. Biofilms were treated during 24 h with miconazole at 156 µg/mL (MCZ), chitosan (CS)-coated iron oxide nanoparticles (IONPs) carrying MCZ at 78 (IONPs-CS-MCZ78) and 156 µg/mL (IONPs-CS- MCZ156), fluconazole at 156 µg/mL (FLZ) and FLZ-containing nanocarrier at 78 (IONPs-CS-FLZ78) and 156 µg/mL (IONPs-CS-FLZ156). Negative control (NC) represents the biofilm formed for 120 h with pure culture medium. Error bars depict standard deviations of the means. Different lowercase letters represent significant differences among the groups (one-way ANOVA and Fisher LSD's test; $p < 0.05$). Comparisons were performed separately for each antifungal, its respective nanocarrier and NC.

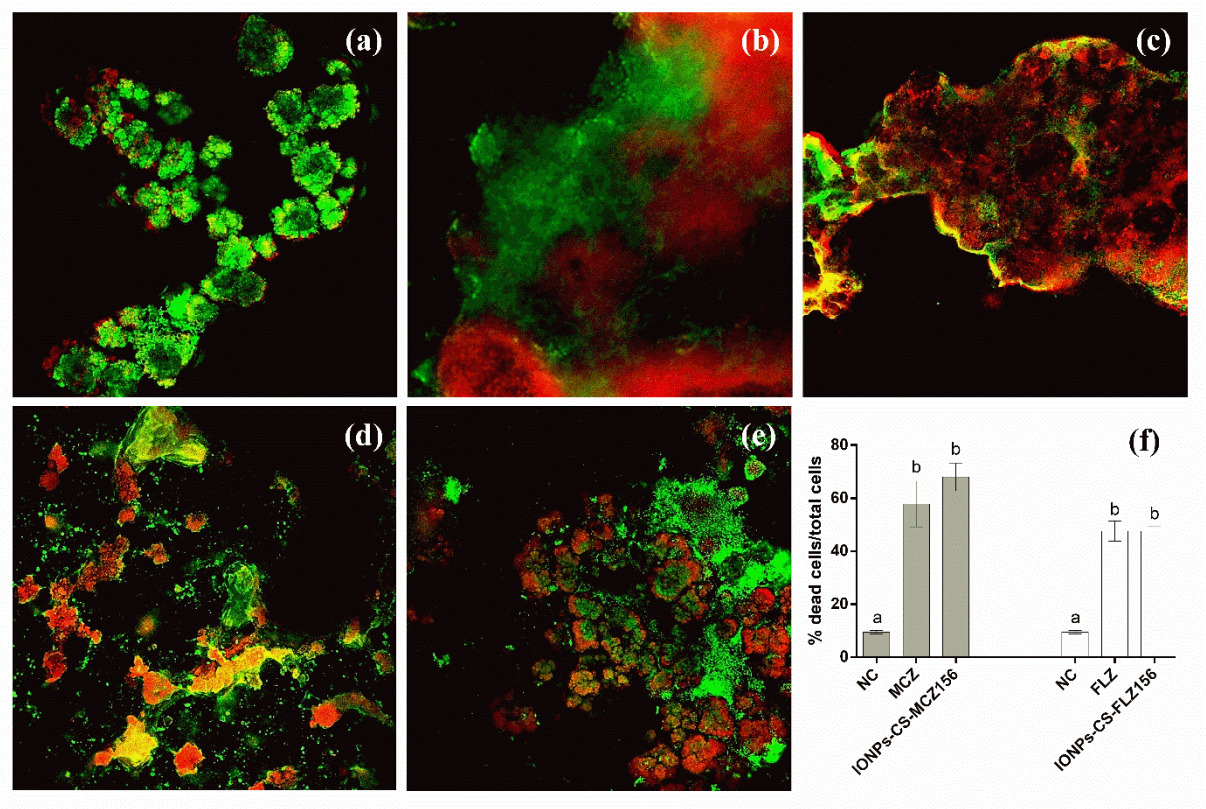

Figure 3. Confocal laser scanning microscopy images of 96-h microcosm biofilms treated during 24 h with miconazole (MCZ) at 156 µg/mL (b), chitosan (CS)-coated iron oxide nanoparticles (IONPs) carrying MCZ at 156 µg/mL (c), fluconazole (FLZ) at 156 µg/mL (d) and FLZ-containing nanocarrier at 156 µg/mL (e). Negative control (a) represents the biofilm formed for 120 h with pure culture medium. Red and green fluorescence indicate dead and living cells, respectively. Magnification: 20x. The image (f) represents the percentage of dead cells in relation to the total cells, and different lowercase letters represent significant differences among the groups (one-way ANOVA and Fisher LSD's test; $p < 0.05$). Comparisons were performed separately for each antifungal, its respective nanocarrier and NC negative control.

ANEXO A

Manuscript Preparation - Medical Mycology

Maximize the impact of your research by reviewing the Author Resource Guide and SEO made simple

Submission

All submissions must be uploaded as Word files to the *Medical Mycology* ScholarOne Manuscripts site. Users who have not previously submitted work through this site must create an account from the link on the login page. Assistance with this and all other areas of the site is available in the User Guide, which is accessed via the 'Get Help Now' button at the top right corner of all ScholarOne Manuscripts web pages. For additional help, please contact mm.editorialoffice@oup.com.

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Article types

Papers may be submitted in the following categories.

Original Papers

Manuscripts must have: (a) a Cover-Page that includes the full title and a short title, name(s) and affiliation(s) of all author(s), the name, telephone number and email address of the designated corresponding author, and a list of up to five keywords; (b) a separate page for an Abstract of up to 250 words prepared without sub-headers; (c) manuscript text consisting of Introduction, Materials and Methods, Results, Discussion and References; (d) References must be cited sequentially in the order in which they first appear in the text, in superscript numerals, and included in the same numerical order in the Reference section; (e) each Table should be submitted as a separate page and should have footnoted descriptions of all abbreviations contained in the Table (see section below on Tables for more detailed information); (f) each Figure should be submitted as a separate file (page), with appropriate figure legends allowing a reader to understand their contents without reference to the text (see section below on Figures for more detailed information). Manuscripts MUST be in English, double-spaced, in no less than size 12 point, line numbering should be turned on in Microsoft Word and lines numbered consecutively, and up to 5000 words excluding cover page, abstract, references, and tables/figures.

Reviews

For this article type, authors must first electronically submit an outline of their proposed article for evaluation by the journal. The outline should be no more than two, double-spaced pages in 12 point, in which the authors describe the objectives and contents of the report. The outline must be submitted to Reviews Editor Malcolm Richardson at Malcolm.Richardson@manchester.ac.uk. Once the proposal has been evaluated, the authors will be informed of the results of the Review Editor's initial

consideration of their proposal. Reviews are NOT restricted by the formatting or length requirements of Original Papers.

Case Reports

Medical Mycology no longer publishes Case Reports. Such articles should be submitted to ISHAM's other journal, Medical Mycology Case Reports. *Medical Mycology* Case Reports.

Brief Reports

These concisely written manuscripts are to provide an opportunity for the presentation of innovative well-defined studies that do not warrant a full-length original paper. These manuscripts are not for the publication of preliminary data.

The manuscript style is as follows: Cover page with title, authors, afiliations, corresponding author and 5 key words. An abstract of no more than 100 words. The text should be no more than 1200 words with no headers or subheadings included, but must contain a brief introduction, methods, results and conclusions. Limit references to those that are absolutely necessary and no more than 20. No more than 2 tables or figures are allowed (e.g., 2 figures, 2 tables, or 1 figure and 1 table).

Letters to the Editor

Letters are to allow readers the opportunity to discuss issues related to original articles, short communications, and reviews previously published in *Medical Mycology*. Letters are NOT to be used for the presentation of the authors' preliminary data from their own investigations. Letters should be no longer than 750 words, excluding references.

Manuscript format and structure

Please refer to a recent issue of *Medical Mycology* for guidance on style and layout of articles. Also refer to the Article Types section above for guidance on relevant information for each article type.

Language Editing

Language editing, especially if your first language is not English, to ensure that the academic content of your paper is fully understood by journal editors and reviewers is optional. Language editing does not guarantee that your manuscript will be accepted for publication. Further information on this service is available on the language services page. Several specialist language editing companies offer similar services and any of them can be used. Authors are liable for all costs associated with such services.

File Formats

All manuscripts must be submitted as Microsoft Word documents (.doc,.docx, .rtf, .pdf files).

Manuscript Preparation

Manuscripts MUST be in English, double-spaced, in no less than size 12 point, organized as appropriate for the manuscript type, have line numbering enabled and lines numbered consecutively, and have one inch margins all around. Statistics and measurements should be given in numerals when followed by a unit, e.g., mg, ml, etc. In contrast, numerals employed in other circumstance, e.g., two patients, should be spelled out if less than ten (10). ALL abbreviations, even those commonly used in medical mycology, e.g., Sabouraud glucose agar (SGA), must be defined when first used in the abstract and text. Please NOTE that the inability of authors to clearly present the contents of their submissions in English may constitute grounds for the immediate rejection of the papers or their return to authors so that they can correct their English usage prior to assignment to an associate editor.

References

Authors are responsible for the accuracy and completeness of their references and for correct text citation. In preparing your citations please follow the AMA Manual of Style, 11th Edition.

Some key style points that Medical Mycology articles must follow:

- Number references in the order they appear in the text; do not alphabetize.
- In text, tables, and legends, identify references with superscript numerals.
- When listing references, follow AMA style and abbreviate names of journals according to the journals listed in PubMed. The abbreviation should be in italic font and a period inserted after the □nal abbreviation, e.g., J Clin Microbiol.
- List all authors up to 6, but if more than 6, list the □rst 3 by name and the rest by “et al.”

Examples of reference style:

Journal article

Author AB, Author CD, Author EF. Title of paper. *J Title Abbrev*. 1995; 00: 000–000.

Author AB, Author CD, Author EF, et al. [if more than six authors] Title of paper. *J Title Abbrev* . 1995; 00: 000–000.

- If the title is in a language other than English, the authors should provide the English translation. The name of the original language should be included within a bracket, e.g., [in Spanish], after pagination at the end of the citation. For example, Author AB, Author CD, Author EF, et al. [if more than six authors] Title of paper. J Title Abbrev 1995; 00: 000–000 [in Spanish].

Book chapter

Author AB, Author CD, Author EF, et al. [if more than six] Chapter title. In: Editor AB, Editor CD, eds. Book Title With Initial Uppercase Letters (as indicated here and in italic font), 5th edn. Place: Publisher, 1995: 000–000.

Book

Author AB, Author CD (eds). Book Title With Initial Uppercase Letters (as indicated here and in italic font), 5th edn. Place: Publisher, 1995.

Thesis

Author AB. Paper title with lowercase initials to all words, except the □rst word. PhD thesis, University of X, 1995.

Conference proceedings/Supplements

Author AB. Paper title. In: Editor AB, ed. Proceedings Title, place, date. Place: publisher, 1995: 000–000.

Meeting abstracts

Abstracts of works presented at scientific meetings should be cited in brackets within the text of the manuscripts rather than in the Reference sections. The citations should be formatted as:

Author AB. Abstract title. Name of meeting, its location, the date and abstract number.

Personal communications, Unpublished results etc.

References to personal communications, unpublished results and papers submitted for publication (but not yet accepted) should only appear in brackets in the text, and in the following form: [A. B. Author, unpublished results] or [C. D. Author, personal communication].

Examples:

Staab JF, Neofytos D, Rhee P et al Target enzyme mutations confer di□erential echinocandin susceptibilities in *Candida kefyr*. *Antimicrob Agents Chemother*. 2014; 58: 5421–5427.
Pfaller MA, Diekema DJ. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2010. *J Clin Microbiol*. 2012; 50: 2846–2856.

Lay summary

Authors of all article types are encouraged to submit a lay summary as part of the article, in addition to the main text abstract. The lay summary should clearly summarize the focus and findings of the article for non-expert readers and will be published as part of the article online. The lay summary should be submitted for peer review as part of the main manuscript file, under the heading 'Lay summary,' before the article's main text. The lay summary should be no longer than 300 characters. As with a main abstract, avoid citations and define any abbreviations.

Tables

Tables should present only essential data, must be numbered sequentially in the order in which they are first referenced in the text, and all MUST be referred to in the text. Each table should be supplied as a separate page at the end of the main document and not imbedded within the body of the text. The table header should be brief and self-explanatory. If it contains genera and species names, these must be spelled out in full, e.g., *Candida albicans,* not *C. albicans.* Column headings should be brief and include units in parentheses where applicable. Only horizontal rule lines should be used and then only for headers and footers. Tables should be considered as "stand alone" documents through which data are summarized for presentation to readers. As such, all abbreviations employed in the tables MUST be de□ned footnotes included directly below the tables.

Figures/Images

Images should be submitted as TIF or EPS files. Images should be of a suficient resolution, i.e. a minimum of: 300 dpi for halftones/color, 600 dpi for combination halftones and 1200 dpi for line art. Illustrations should be submitted as individual □les, separate from the manuscript text file. An image that will be column-width should be 3.25 inches (8.13 cm) wide and an image that will be the full width of the page should be 6.75 inches (16.88 cm) wide.

NO part of any image may be enhanced, removed, relocated, introduced or manipulated in any manner after the capture of the original image unless the authors provide the editor with a clear and accurate description of the nature of the image manipulation.

Figures, like tables, should be considered as "stand alone" components of the manuscript. As such, authors by should provide legends at the end of the text, following the Reference section, which clearly describe the significant features of the illustrated objects. In addition, authors may employ, as warranted, arrows or similar indications.

Supplementary Files

Supplementary information, including extensive data sets, large figures, audio or video material, can be made available. Please do not include supplementary information within the main manuscript □le but upload the material as separate file(s), labeled as "Supplemental Digital Content". Please include a sequential number (S1, S2, etc.) for each file and a brief description of the content at the appropriate position in the text. Please note that supplementary files are not copy-edited and will be presented as submitted.

Nomenclature

Proposals of new fungal taxa MUST conform to the requirements outlined in the International Code of Nomenclature for Algae, Fungi, and Plants. The proposed taxon must be supported by deposition of cultures and/or the unique nucleotide and/or amino acid sequences in appropriate collections or databases, along with the new binomial. The identifier assigned to the taxon by these recognized repositories, e.g., Mycobank, must be included in the text, tables and figures. In addition, binomials should appear in italics, must be spelled out in full when first used in the abstract, text, figures/tables. Thereafter, a generic name may be abbreviated to the first initial only, e.g., *Candida albicans* when first cited and *C. albicans* in the remainder of the abstract and text, except at the beginning of a sentence where the genus should be spelled out in entirety. Species names may NEVER be used without the generic full name or abbreviation combined with the full name of the species. For example, it must be *Candida albicans* or *C. albicans*, but never *albicans*, or *C. neoformans var. grubii* but never *var. grubii*. The use of abbreviations of generic and species names, e.g., (Ca) for C. albicans, in the text is NOT permitted.

Nucleotide and/or Amino Acid Sequence Data

DNA, RNA, and/or amino acid sequence data must be deposited in a freely accessible database. Access to the sequence data must be made available to the editors and reviewers upon submission of the manuscript and must be available to everyone upon publication of the manuscript. The GenBank (Nucleotide- NCBI); or European Nucleotide Archive (European Nucleotide Archive EMBL-EBI or Bioinformation and DDBJ Center) accession numbers should be included in the Materials and Methods Section of the manuscript. However, in a figure of a phylogenetic tree, the numbers should be shown along with species names (e.g., *Malassezia globosa* AB099880) (No descriptions are needed in the Materials and Methods Section in this case).

High-Throughput Functional Genomics Data, Microarray, Next-Generation Sequencing Data

For papers that contain high throughput functional genomics, whole genome and microarray data must be deposited in the appropriate public database (e.g. GEO, ArrayExpress or CIBEX) Microarray data should comply with the Minimum Information About a Microarray Experiment (MIAME) standards, except in cases where a microarray is used to identify one, or more, genes, for primary genetic screening. Submission of microarray data to a central repository must be completed before submission of a manuscript, and accession numbers for the data must be included in the original submission of manuscripts in order to be available for the reviewing process.

Phylogenetic Data

Phylogenetic data (e.g., trees of species, trees of populations, trees of genes) representing the fungal taxa that are presented in a relevant manuscript should be freely accessible to researchers through a public phylogenetic information repository (e.g. TreeBASE).

Data on New Macromolecule Structures

It is mandatory to deposit new macromolecule structures in the Protein Data Bank (PDBj or RCSB PDB), and the accession numbers must be included in the manuscript prior to acceptance. However, inclusion of the coordinates in the original submission of the manuscript is encouraged in order to facilitate and expedite the reviewing process.

Strain Sharing

Authors reporting new taxa must deposit the type strain, but preferably also other isolates in a publicly accessible culture collection. Authors of papers on known taxa describing novel properties (e.g., pathogenicity to humans and animals, genetic resistance to antifungal agents, identification of new bioactive metabolites) must deposit the strain(s) in publicly accessible culture collections and provide accession number(s) in the manuscript. Authors of first reported fungal strains in a certain geographical location or a new habitat must also submit these strains in publically accessible culture collections and accession number(s) should be provided in the manuscript. These strains should be available in good faith to the scientific community through registered culture collections and their supply must conform to current national and international laws and regulations.

To ensure strain authenticity authors should indicate laboratory strain designations and donor sources as well asoriginal culture collection identification numbers.

Medical Mycology encourages the exchange of organisms and genetic constructs among researchers. Thus, strains andgenetic constructs such as plasmids that are not deposited in a publicly accessible culture collection should be made available by the authors on request for scientific purposes.

Acknowledgments

The work of other individuals who assisted in the studies or in the preparation of the submission must be specifically indicated in the Acknowledgments section of the paper. In addition, all sources of grant funding must be given and grant numbers given.

Funding

Details of all funding sources for the work in question also should be given in the Acknowledgments section. The following rules should be followed:

- The sentence should begin: “This work was supported by …”
- The full official funding agency name should be given, i.e. “the National Cancer Institute at the National Institutes of Health” or simply “National Institutes of Health” not “NCI” (one of the many sub-institutions) or “NCI at NIH.”
- Grant numbers should be complete and accurate and provided in brackets as follows: “[grant number ABX CDXXXXXX]”
- Multiple grant numbers should be separated by a comma as follows: “[grant numbers ABX CDXXXXXX, EFX GHXXXXXX]”
- Agencies should be separated by a semi-colon (plus “and” before the last funding agency)
- Where individuals need to be specified for certain sources of funding the following text should be added after the relevant agency or grant number “to [author initials].”
- An example is given here: “This work was supported by the National Institutes of Health [P50 CA098252 and CA118790 to R.B.S.R.] and the Alcohol & Education Research Council [HFY GR667789].”

Crossref Funding Data Registry

In order to meet your funding requirements authors are required to name their funding sources, or state if there are none, during the submission process. Please follow this link for more information about the CHORUS initiative.

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ANEXO B

UNOESTE - UNIVERSIDADE DO OESTE PAULISTA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito de nanocarreadores de drogas antifúngicas sobre espécies de Candida em biofilmes microcosmos

Pesquisador: Douglas Roberto Monteiro

Área Temática:

Versão: 1

CAAE: 22111419.3.0000.5515

Instituição Proponente: ASSOCIACAO PRUDENTINA DE EDUCACAO E CULTURA APEC

Patrocinador Principal: UNOESTE - Universidade do Oeste Paulista

DADOS DO PARECER

Número do Parecer: 3.631.766

Apresentação do Projeto:

O projeto foi redigido de forma clara, permitindo a adequada compreensão do estudo, seus objetivos e material e métodos utilizados para atendê-los. Para tanto, saliva proveniente de um doador sadio será usada como inóculo para a formação de biofilmes microcosmos, os quais serão formados durante 48 horas em discos de vidro no modelo de Adesão Ativa de Amsterdam. Após, os biofilmes serão tratados durante 24 horas com diferentes concentrações dos nanosistemas. O efeito antibiofilme dos nanosistemas será avaliado por meio da contagem do número de células cultiváveis, composição da matriz extracelular (proteínas, carboidratos e ácidos nucleicos) e análise da estrutura dos biofilmes. Controles apropriados serão incluídos em todas as análises.

Hipótese: A hipótese a ser testada no presente estudo é a de que os nanocarreadores de MCZ e FLZ apresentam efeito antimicrobiano sobre C. albicans e C. glabrata em biofilmes microcosmos, e que este efeito é superior àquele encontrado para as drogas utilizadas sozinhas (sem core-shell). Os resultados obtidos poderão contribuir para o desenvolvimento de novos nanobiomateriais para usoodontológico.

Objetivo da Pesquisa:

O presente trabalho tem por objetivo sintetizar dois novos nanosistemas carreadores de drogas (DRO) antifúngicas (miconazol e fluconazol) com base em nanopartículas magnéticas de óxido de

Endereço: Rodovia Raposo Tavares, Km 572
Bairro: Bairro Limoeiro **CEP:** 19.067-175
UF: SP **Município:** PRESIDENTE PRUDENTE
Telefone: (18)3229-2079 **Fax:** (18)3229-2080 **E-mail:** cep@unoeste.br

ferro (NPMs) e quitosana (QTS), bem como avaliar o efeito antimicrobiano destes nanosistemas frente aos microrganismos Candida albicans e Candida glabrata em biofilmes microcosmos.

Avaliação dos Riscos e Benefícios:

Segundo os pesquisadores, "Os riscos são mínimos, sendo os mesmos que ocorrem em exame de rotina da boca" e "O benefício será para o desenvolvimento de um nanosistema que poderá auxiliar no tratamento de infecções virais e fúngicas da cavidade bucal".

Comentários e Considerações sobre a Pesquisa:

O tema é relevante e importante. O pesquisador responsável tem adequado conhecimento na área. Foram estabelecidos critérios de inclusão e exclusão do estudo.

Considerações sobre os Termos de apresentação obrigatória:

1. Folha de rosto: presente e correta.
2. TCLE: os pesquisadores pedem dispensa do TCLE com a seguinte justificativa "A coleta das amostras de saliva serão da própria pesquisadora (Anne Caroline Morais Caldeiro), a qual já faz parte da equipe de trabalho. Sendo assim, acreditamos que no seja necessário o termo de consentimento, visto que a coleta será realizada por ela mesma".
3. Termo de assentimento: não se aplica.
4. Autorização do responsável pelo local a ser desenvolvida a pesquisa: presente e correta.
5. Autorização de contato com os participantes da pesquisa: não se aplica.
6. Autorização para utilização de prontuários e documentos / base de dados eletrônicos: não se aplica.
7. Termo de compromisso: presente e correto.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não foram observados óbices éticos.

Considerações Finais a critério do CEP:

Em reunião realizada no dia 08/10/2019, o Comitê de Ética em Pesquisa da Universidade do Oeste Paulista (CEP-UNOESTE), concordância com o parecerista, considerou o projeto APROVADO.

Solicitamos que sejam encaminhados ao CEP:

1. Relatórios anuais, sendo o primeiro previsto para 30/05/2021-.

UNOESTE - UNIVERSIDADE DO OESTE PAULISTA

2. Comunicar toda e qualquer alteração do Projeto e Termo de Consentimento Livre e Esclarecido. Nestas circunstâncias a inclusão de participantes deve ser temporariamente interrompida até a aprovação do Comitê de Ética em Pesquisa.
3. Comunicar imediatamente ao Comitê qualquer Evento Adverso Grave ocorrido durante o desenvolvimento do estudo.
4. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 (cinco) anos, após conclusão da pesquisa, para possível auditoria dos órgãos competentes.
5. Este projeto está cadastrado na CPDI-UNOESTE sob o número 5559.

Obs.: O PROJETO SÓ PODE SER INICIADO (EXECUTADO) QUANDO RECEBER O PARECER FINAL APROVADO TANTO NO CEP QUANTO NO COMITÊ ASSESSOR INSTITUCIONAL DE PESQUISA (CAPI).

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1390645.pdf	26/09/2019 09:26:02		Aceito
Declaração de Instituição e Infraestrutura	Documento57.pdf	26/09/2019 09:25:42	Anne Caroline Morais Caldeirão	Aceito
Declaração de Pesquisadores	termodecompromisso.pdf	26/09/2019 09:25:19	Anne Caroline Morais Caldeirão	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	solicitacaodedispensa.pdf	26/09/2019 00:02:06	Anne Caroline Morais Caldeirão	Aceito
Folha de Rosto	folhaderostoAnne.pdf	19/09/2019 14:52:47	Anne Caroline Morais Caldeirão	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_de_pesquisa.pdf	20/08/2019 19:16:59	Anne Caroline Morais Caldeirão	Aceito
Cronograma	Cronograma.docx	05/07/2019 09:25:55	Anne Caroline Morais Caldeirão	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:

Endereço: Rodovia Raposo Tavares, Km 572
Bairro: Bairro Limoeiro **CEP:** 19.067-175
UF: SP **Município:** PRESIDENTE PRUDENTE
Telefone: (18)3229-2079 **Fax:** (18)3229-2080 **E-mail:** cep@unoeste.br

UNOESTE - UNIVERSIDADE
DO OESTE PAULISTA

Continuação do Parecer: 3.631.766

Não

PRESIDENTE PRUDENTE, 09 de Outubro de 2019

Assinado por:
Aline Duarte Ferreira
(Coordenador(a))

Endereço: Rodovia Raposo Tavares, Km 572
Bairro: Bairro Limoeiro **CEP:** 19.067-175
UF: SP **Município:** PRESIDENTE PRUDENTE
Telefone: (18)3229-2079 **Fax:** (18)3229-2080 **E-mail:** cep@unoeste.br

ANEXO C

Solicitação de Dispensa do Termo de Consentimento Livre e Esclarecido

Solicito a dispensa da aplicação do Termo de consentimento livre e esclarecido do projeto de pesquisa intitulado “Efeito de nanocarreadores de drogas antifúngicas sobre espécies de Candida em biofilmes microcosmos”, com a seguinte justificativa:

1. As amostras de saliva serão coletadas do pesquisador responsável pelo estudo

Atenciosamente,

Presidente Prudente, 05 de Julho de 2019.

Anne C. M. Caldeirão

Anne Caroline Morais Caldeirão

Pesquisador responsável