

Laís Salomão Arias

**Síntese e avaliação dos efeitos de um
nanocarreador de miconazol sobre
microrganismos orais**

ARAÇATUBA - SP

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nanocarreador de miconazol sobre
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Tese apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, como parte dos requisitos para a obtenção do título de Doutora em Ciência Odontológica – Área Saúde Bucal da Criança.

Orientador: Prof. Dr. Douglas Roberto Monteiro

Coorientadores: Prof. Tit. Alberto Carlos Botazzo Delbem

Prof. Assoc. Juliano Pelim Pessan

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Dedicatória

Dedico este trabalho

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*“A educação é para a alma humana, o que a escultura é para um
bloco de mármore”*

Joseph Addison

Resumo Geral

ARIAS, L.S. **Síntese e avaliação dos efeitos de um nanocarreador de miconazol sobre microrganismos orais**. 2020 149f. Tese (Doutorado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

RESUMO GERAL

A presente tese teve como objetivo geral preparar, caracterizar e avaliar os efeitos antimicrobianos de um nanocarreador de miconazol (MCZ) à base de nanopartículas magnéticas de óxido de ferro (NPsMOF) funcionalizadas com quitosana (QS). Assim, dois subprojetos (SP1 e SP2) foram desenvolvidos e apresentaram os seguintes objetivos específicos: SP1) Preparar, caracterizar e avaliar os efeitos do nanocarreador NPsMOF-QS-MCZ sobre células planctônicas e biofilmes simples e misto de *Candida albicans* e *Candida glabrata*; SP2) Avaliar o efeito do nanocarreador na composição de três diferentes modelos de biofilmes polimicrobianos patogênicos orais (gingivite, prótese total e cárie dentária). A primeira etapa do SP1 consistiu em revestir as NPsMOF com QS e carregar este *core-shell* com MCZ, a fim de caracterizar este nanocarreador por métodos físico-químicos. As concentrações inibitórias mínimas (CIMs) do nanocarreador foram determinadas pelo método da microdiluição em caldo, usando o índice da concentração inibitória fracionária a fim de avaliar se houve interação sinérgica entre os compostos. Ainda, biofilmes simples e mistos de *Candida* foram formados no fundo de placas de 24 ou 96 poços por 48 h e, em seguida, tratados por 24 h com NPsMOF-QS-MCZ carreando MCZ a 31,2 e 78 µg/ml, na presença e ausência de um campo magnético externo. Os biofilmes foram avaliados quantitativamente por biomassa total, atividade metabólica, contagem de unidades formadoras de colônias (UFCs) e composição da matriz extracelular. Os dados foram analisados por ANOVA a dois fatores, seguida pelo teste de Holm-Sidak ($p < 0,05$). Ainda, a estrutura dos biofilmes foi avaliada qualitativamente por microscopia eletrônica de varredura e microscopia confocal. Os resultados do SP1 mostraram que o nanocarreador apresentou diâmetro menor que 50 nm e valores de CIM menores do que aqueles encontrados para MCZ sozinho, com efeito sinérgico sobre *C. albicans*. NPsMOF-QS-MCZ a 78 µg/ml foi mais eficaz que MCZ sozinho na redução de UFCs e atividade metabólica de biofilmes misto e simples de *C. albicans*. A biomassa total dos biofilmes e a matriz extracelular não foram afetadas pelo nanocarreador, e a aplicação de um campo magnético externo não melhorou seu efeito antibiofilme. As imagens de

microscopia confirmam que tratamentos com o nanocarreador, principalmente na maior concentração, apresentaram maior atividade antibiofilme. Com relação ao SP2, as CIMs de NPsMOF-QS-MCZ foram determinadas para diferentes espécies microbianas, e todos os biofilmes polimicrobianos foram desenvolvidos por 5 dias e tratados por 24 h com NPsMOF-QS-MCZ a 64 µg/ml. Após o tratamento, os biofilmes foram avaliados quanto à biomassa total, atividade metabólica, contagem de UFCs e análise composicional por PCR quantitativo. Microscopia eletrônica de varredura foi usada para analisar a estrutura do biofilme. As diferenças entre os grupos foram determinadas por teste t não pareado ($p < 0,05$). Os resultados do SP2 mostraram que NPsMOF-QS-MCZ foi mais eficaz que MCZ sozinho contra a maioria das células fúngicas e bacterianas testadas. Ainda, este nanocarreador foi capaz de reduzir a atividade metabólica, biomassa total e UFCs ($p < 0,05$) dos biofilmes, além de alterar a sua ultraestrutura. Por fim, NPsMOF-QS-MCZ afetou a composição dos três biofilmes polimicrobianos avaliados, reduzindo principalmente os números de *Streptococcus* spp. e alterando a prevalência das espécies nos biofilmes. Em suma, os resultados dos SP1 e SP2 permitiram concluir que o nanocarreador melhorou o efeito antimicrobiano do MCZ, dependendo da espécie e parâmetro de biofilme avaliados. O nanocarreador também mostrou potencial para interferir nas interações sinérgicas entre células fúngicas e bacterianas dentro de biofilmes polimicrobianos.

Palavras-chaves: Biofilmes; *Candida*; Miconazol; Nanopartículas; Óxido ferroso-férrico; Quitosana.

General Abstract

ARIAS, L.S. **Synthesis and evaluation of the effects of a miconazole nanocarrier on oral microorganisms.** 2020 149f. Tese (Doutorado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

GENERAL ABSTRACT

The present thesis aimed to prepare, characterize and evaluate the antimicrobial effects of a miconazole (MCZ) nanocarrier based on iron oxide magnetic nanoparticles (IONPs) functionalized with chitosan (CS). Thus, two subprojects (SP1 and SP2) were developed and had the following specific objectives: SP1) To prepare, characterize and evaluate the effects of the IONPs-CS-MCZ nanocarrier on planktonic cells and single- and dual-species biofilms of *Candida albicans* and *Candida glabrata*; SP2) To evaluate the effect of IONPs-CS-MCZ on the composition of three different models of oral pathogenic biofilms (gingivitis, denture and dental caries). The first step of SP1 was to coat IONPs with CS and to load this *core-shell* association with MCZ, in order to characterize this nanocarrier by physicochemical methods. The minimum inhibitory concentrations (MICs) of the nanocarrier were determined by the microdilution method, using the fractional inhibitory concentration index in order to assess whether there was synergistic interaction between the compounds.. In addition, single- and dual-species biofilms of *Candida* species were formed at the bottom of 24- or 96-well plates for 48 h and, in sequence, treated for 24 h with IONPs-CS-MCZ carrying MCZ at 31.2 and 78 µg/ml, in both the presence and absence of an external magnetic field. Biofilms were quantitatively evaluated by total biomass, metabolic activity, counting of colony forming units (CFUs) and extracellular matrix components. Data were analyzed by two-way ANOVA, followed by Holm-Sidak test ($p < 0.05$). In addition, the structure of biofilms was qualitatively evaluated by scanning electron microscopy and confocal microscopy. The results from SP1 showed that IONPs-CS-MCZ presented diameter smaller than 50 nm, and MIC values lower than those found for MCZ alone, with synergistic effect on *C. albicans*. Moreover, 78 µg/ml IONPs-CS-MCZ was more effective than MCZ alone in reducing CFUs and metabolic activity of single biofilms of *C. albicans* and dual-species biofilms. Total biofilm biomass and extracellular matrix were not affected by the nanocarrier, and the application of an external magnetic field did not improve the nanocarrier effects. Microscopy images confirm that treatments with the nanocarrier, mainly in the highest concentration, exhibited greater antibiofilm activity. Regarding SP2, the MICs of IONPs-CS-MCZ were determined for different

microbial species, and all polymicrobial biofilms were developed for 5 days and treated for 24 h with IONPs-CS-MCZ at 64 µg/ml. After treatment, the biofilms were evaluated for total biomass, metabolic activity, counting of CFUs and quantitative PCR analysis. Scanning electron microscopy was used to analyze the biofilm ultrastructure. Differences between groups were determined by unpaired t-test ($p < 0.05$). Results from SP2 showed that IONPs-CS-MCZ was more effective than MCZ alone against most fungal and bacterial cells tested. Moreover, this nanocarrier was able to reduce the metabolic activity, total biomass and CFUs ($P < 0.05$) of the biofilms, besides altering their ultrastructure. Finally, IONPs-CS-MCZ affected the composition of the three evaluated biofilms, mainly reducing the numbers of *Streptococcus* spp. and changing the prevalence of species in the biofilms. From the results obtained by SP1 and SP2, it was possible to conclude that the nanocarrier improved the antimicrobial effect of MCZ, depending on the species and biofilm parameter evaluated. Nanocarrier also showed potential to interfere in the synergistic interactions among fungal and bacterial cells within polymicrobial biofilms.

Keywords: Biofilms, *Candida*; Miconazole; Nanoparticles; Ferrosulfate Oxide; Chitosan.

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Introdução geral

Introdução geral

O ecossistema oral é um ambiente único que proporciona nichos favoráveis à colonização e proliferação de diferentes microrganismos, os quais se encontram, na maioria das vezes, em associações comunitárias complexas formando biofilmes¹. Os desequilíbrios causados pela falta de higiene oral, uso de certos medicamentos, próteses orais, alterações imunológicas e doenças sistêmicas como diabetes e síndrome da imunodeficiência adquirida geram alterações na composição do microbioma oral, levando ao desenvolvimento de doenças orais como gengivite, cárie dentária e candidíases²⁻⁵.

As leveduras do gênero *Candida* colonizam a cavidade oral de 25 a 75% dos indivíduos saudáveis⁶⁻⁸. No entanto, em estado de desequilíbrio, fungos oportunistas contribuem significativamente para o desenvolvimento de patologias orais⁷. Como um exemplo, *Candida albicans* seguido por *Candida glabrata* são os microrganismos mais frequentemente detectados em candidíases orais, como a estomatite protética relacionada à *Candida*^{9,10}. Ainda, *C. albicans* promove associações sinérgicas com outros microrganismos patogênicos, como *Streptococcus mutans*¹¹. Tal associação é mediada principalmente pela secreção de glicosiltransferases por *S. mutans*¹², já que estas exoenzimas produzem α -glucanos que aumentam a adesão de *S. mutans* à parede celular de *C. albicans*^{12,13}. Além disso, a associação destas espécies resulta na secreção de baixas concentrações (25-50 μ M) da molécula de *quorum sensing* farnesol por *C. albicans*, o que estimula a formação de biofilmes de *S. mutans*¹⁴.

Dentre os agentes antifúngicos empregados no tratamento das candidíases destaca-se o miconazol (MCZ), um fármaco de amplo espectro do grupo imidazol, capaz de combater fungos e algumas bactérias¹⁵. MCZ tem atividade superior à nistatina

no combate à candidíase oral¹⁶ e é superior em termos de eficácia em relação a outros azóis, uma vez que apresenta atividade fungicida e fungistática¹⁷. Entretanto, esta droga pode interagir com outros fármacos, reduzindo sua eficácia, bem como apresentar efeitos colaterais como irritação da mucosa, vômito, diarreia e dores de cabeça^{16,18}.

O conhecimento do microbioma oral como um conjunto heterogêneo de espécies que modulam umas às outras, o seu ambiente e também o hospedeiro, auxilia a compreender não apenas como ocorre a progressão das doenças, mas também como os microrganismos se adaptam às intempéries físicas e químicas¹⁹. A literatura tem relatado com maior frequência o desenvolvimento de resistência microbiana aos fármacos convencionais, como o MCZ, e os principais fatores envolvidos são a super-expressão de bombas de efluxo, alteração do alvo celular da droga e a formação de biofilmes²⁰⁻²³.

A fim de resolver esta problemática, os pesquisadores vêm estudando novas estratégias que possam contornar os mecanismos de resistência sem estimular o desenvolvimento de microrganismos ainda mais resistentes e de difícil erradicação. O uso de nanopartículas como sistemas controlados de entrega de drogas tem sido bastante investigado, já que os nanocarreadores têm potencial para contornar barreiras físicas e químicas, bem como entregar a droga na célula alvo, reduzindo o tempo de exposição e a quantidade de fármaco empregada. Como resultado, o uso de nanocarreadores pode diminuir os efeitos colaterais dos fármacos, bem como as chances de desenvolvimento de mecanismos de resistência por parte dos microrganismos²⁴⁻²⁶.

Nessa perspectiva, as nanopartículas magnéticas de óxido de ferro (NPsMOF) permitem fácil manipulação com ajuda de um campo magnético externo e a entrega de drogas de modo ativo e passivo, além de apresentar alta receptividade pelo tecido ou

célula alvo²⁷. Ainda, a capacidade de funcionalização do núcleo ou '*core*' de óxido de ferro com compósitos ou '*shell*' de diversas naturezas (ex.: polímeros, surfactantes, ouro, silanos, entre outros)²⁸ permite reduzir a oxidação das nanopartículas e torná-las mais biocompatíveis, favorecendo sua aplicabilidade clínica²⁸. A quitosana (QS) é um polímero derivado da quitina (obtida da carapaça de crustáceos) que tem se mostrado como revestimento apropriado às NPsMOF, já que apresenta propriedades antimicrobianas e hemostáticas, além de ser biocompatível e biodegradável^{29,30}. Ainda, a QS permite a ligação química e o carreamento de diferentes drogas, diversificando o campo de atuação dos nanocarreadores³⁰⁻³².

Embora os nanocarreadores à base de NPsMOF tenham sido testados em diversos estudos *in vitro* e *in vivo*, revestidos ou não por QS, sua aplicabilidade tem sido quase inteiramente voltada para terapias de tratamento do câncer^{28,33,34}. Entretanto, estudos recentes têm investigado a ação antibiofilme de nanocarreadores de diferentes drogas convencionais baseados em NPsMOF, mostrando resultados favoráveis em comparação às drogas aplicadas sozinhas^{30,32,35}. Por fim, uma outra aplicação é a utilização de um campo magnético externo guiado a fim de mover as NPsMOF pelo biofilme e criar microcanais que facilitam a penetração de drogas nas camadas mais internas do biofilme, promovendo ação antimicrobiana sobre um número maior de células^{36,37}.

Diante de todo o contexto supracitado e tendo em vista que a eficácia antimicrobiana de um nanocarreador de MCZ com base em NPsMOF revestidas com QS permanece desconhecida, os objetivos do presente estudo foram: 1) preparar e caracterizar um novo nanocarreador de MCZ com base em NPsMOF revestidas com QS (NPsMOF-QS-MCZ), bem como avaliar seu efeito antimicrobiano sobre biofilmes

simples e mistos de *C. albicans* e *C. glabrata*, na presença e ausência de um campo magnético externo; 2) avaliar o efeito *in vitro* do nanocarreador NPsMOF-QS-MCZ na composição de modelos polimicrobianos de biofilmes orais patogênicos de gengivite, prótese total e cárie dentária.

Para alcançar os objetivos propostos, o presente estudo foi dividido em dois capítulos, como detalhado abaixo:

- Capítulo 1: “Novel miconazole nanocarrier based on chitosan-coated iron oxide nanoparticles as a nanotherapy to fight *Candida* biofilms” (artigo nas normas do periódico Colloids and Surfaces B: Biointerfaces).

- Capítulo 2: “Effect of a miconazole nanocarrier on the composition of three *in vitro* models of pathogenic oral biofilms” (artigo nas normas do periódico Journal of Oral Microbiology).

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Capítulo 1

Novel nanocarrier of miconazole based on chitosan-coated iron oxide nanoparticles as a nanotherapy to fight *Candida* biofilms

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Abstract

Overexposure of microorganisms to conventional drugs has led to resistant species that require new treatment strategies. This study aimed to prepare and characterize a nanocarrier of miconazole (MCZ) based on iron oxide nanoparticles (IONPs) functionalized with chitosan (CS), as well as to test its antifungal activity against biofilms of *Candida albicans* and *Candida glabrata*. IONPs-CS-MCZ was prepared by loading MCZ on CS-covered IONPs and characterized by physicochemical methods. Minimum inhibitory concentration (MIC) of the nanocarrier was determined by the microdilution method. Biofilms were developed (48 h) in microtiter plates and treated with MCZ-carrying nanocarrier at 31.2 and 78 $\mu\text{g/mL}$, in both the presence and absence of an external magnetic field (EMF). Biofilms were evaluated by total biomass, metabolic activity, cultivable cells (CFU), extracellular matrix components, scanning electron microscopy and confocal microscopy. Data were analyzed by two-way ANOVA and Holm-Sidak test ($p < 0.05$). A nanocarrier with diameter lower than 50 nm was obtained. Nanocarrier's MIC values were lower than those found for MCZ, and showed synergism for *C. albicans* and indifference for *C. glabrata*. IONPs-CS-MCZ did not affect total biomass and extracellular matrix. IONPs-CS-MCZ containing 78 $\mu\text{g/mL}$ MCZ showed superior antibiofilm effect to MCZ in reducing CFU and metabolism for single biofilms of *C. albicans* and dual-species biofilms. The EMF did not improve the nanocarrier effects. Microscopy images confirmed the antibiofilm effect of the nanocarrier. IONPs-CS-MCZ was more effective than MCZ mainly against *C. albicans* planktonic cells and number of CFU and metabolism of the biofilms.

Keywords: Antifungal agents; Biofilms; *Candida albicans*; *Candida glabrata*; Chitosan; Ferric oxide; Miconazole; Nanoparticles.

1. Introduction

Candida species are opportunistic yeasts that usually colonize oral cavity, vagina, respiratory and intestinal tracts of humans [1, 2]. In fact, around 23 to 49% of women over 50 years-old are likely to present vulvovaginal candidiasis [3], while the mortality rate of hospitalized patients with invasive candidiasis ranges from 14 to 80% [4-6]. Moreover, the *Candida* genus is present in the oral microbiome of 25 to 75% of healthy people [7-9]. However, clinical imbalances caused by immunologic shifts, use of dentures, antibiotics, corticosteroids, diabetes, HIV syndrome and other factors can lead to oral candidiasis [7, 10-12]. This clinical condition is characterized by erythematous, pseudomembranous and hyperplastic lesions, and *Candida*-related lesions may also be present, such as angular cheilitis, rhomboid glossitis and denture stomatitis (DS) [7]. *Candida albicans* followed by *Candida glabrata* are frequently detected in cases of oral candidiasis, besides being the most prevalent species in DS, an oral disease that affects from 15 to 70% of complete denture wearers [13-15].

Miconazole (MCZ) is an imidazole with topical indication for candidiasis, acting against fungi and a large set of bacteria [16, 17]. Noteworthy, MCZ has shown antimicrobial effect on fluconazole-resistant *Candida* species [18, 19]. On the other hand, MCZ can interact with other drugs, reducing its antimicrobial efficacy, apart from presenting local irritation for some patients [5]. *C. albicans* sessile cells (1 to 10%) may also survive even when exposed to high concentrations of MCZ [20], and azole cross resistance of *C. albicans* and *C. glabrata* to this antifungal has been reported [21, 22].

In order to find a solution for the above-mentioned limitations, drug delivery systems have been developed to increase drug effectiveness and reduce therapeutic

concentrations compared to conventional treatments [23]. At this point, iron oxide magnetic nanoparticles (IONPs) were approved by the World Health Organization and by the Food and Drug administration for clinical use in different biomedical applications, including drug hyperthermia, magnetic resonance and design of drug delivery systems [24]. Although there are few studies on the antibiofilm activity of IONPs, a recent study showed that the magnetic properties of these nanoparticles may be exploited by using an external magnetic field to conduct IONPs within the biofilm, creating artificial channels that enhance drug penetration, thereby increasing the cell death [25].

IONPs surface may be coated with different organic and inorganic compounds (*i.e.*: surfactants, polymers, gold, silica, peptides and others), which makes these nanoparticles more biocompatible and prevents their aggregation and oxidation [26]. In this sense, natural polymers such as chitosan (CS) have been used as a nanoparticle coating since this polymer has biocompatibility, mucoadhesive, hemostatic and antimicrobial properties [26-29]. IONPs-based nanocarriers functionalized with CS proved to be effective against *C. albicans* and different bacteria [30], as well as successfully favored the efficacy of broad spectrum drugs such as chlorhexidine against oral pathogenic microorganisms *in vitro* [27]. However, no other work has investigated the use of CS-coated IONPs as a therapeutic tool to improve the antifungal effect of MCZ. Therefore, the aim of this study was to prepare and characterize a MCZ nanocarrier based on CS-coated IONPs, as well as to test its *in vitro* antifungal activity against single- and dual-species biofilms of *C. albicans* and *C. glabrata*, in both the presence and absence of an external magnetic field. The null hypothesis of the study was that the antifungal effect of the IONP-CS-MCZ nanocarrier would not differ from

that observed for MCZ alone, regardless of the presence of an external magnetic field during the treatment period.

2. Materials and methods

2.1. Preparation and characterization of the IONPs-CS-MCZ nanocarrier

Colloidal IONPs (Fe_3O_4) were supplied by nChemi Engenharia de Materiais (São Carlos, São Paulo, Brazil), while CS and MCZ were purchased from Sigma-Aldrich (St. Louis, MO, USA). The preparation and characterization of the IONPs-CS-MCZ nanocarrier were conducted as described elsewhere [27]. In brief, CS was solubilized in 2% acetic acid under constant stirring during 24 h at room temperature. CS-coated IONPs were obtained by mixing equal volumes of IONPs and CS, both at 1400 $\mu\text{g}/\text{mL}$ of these components. In order to obtain the IONP-CS-MCZ nanocarrier, 500 μg MCZ were added to the IONP-CS compound (700 $\mu\text{g}/\text{mL}$), followed by a 1-h solubilization process under constant magnetic stirring at room temperature. Next, the nanocarrier was characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), X-ray powder diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA)

2.2. Strains and growth conditions

The present study used two reference strains from American Type Culture Collection (ATCC): *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030. Stock cultures (-80°) of both strains were aerobically cultivated on Sabouraud Dextrose Agar (SDA; Difco, Le Pont de Claix, France) plates at 37°C . After 24 h, colonies of both

stains from SDA plates were separately inoculated overnight in Sabouraud Dextrose Broth (SDB; Difco) at 37°C. To adjust the inoculum concentration, the fungal cells were harvested by centrifugation (8000 rpm, 5 min) and washed twice in phosphate buffered saline (PBS; pH 7, 0.1 mol/l). Artificial saliva (AS; pH 6.8)[31] was the medium used to resuspend the cells at 1×10^7 cells/mL or 2×10^7 cells/mL, respectively, for single- and dual-species biofilms.

2.3. Determination of the minimum inhibitory concentration (MIC) on planktonic cells

The broth microdilution method was employed to determine the MIC of the IONPs-CS-MCZ nanocarrier against the studied strains, as detailed elsewhere [32]. Briefly, cell suspensions of *C. albicans* and *C. glabrata* were adjusted in saline solution to a concentration correspondent to the standard 0.5 of McFarland scale (0.5 to 2.5×10^3 cells/mL) and then diluted in saline solution (1:5), and subsequently in Roswell Park Memorial Institute (RPMI 1640; Sigma-Aldrich) medium (1:20). Then, 100 μ L of each yeast suspension were added to the wells of a 96-well flat bottom plate (Costar, Tewksbury, USA) containing 100 μ L of each specific concentration of the nanocarrier (0.09-50 μ g/mL) previously diluted in RPMI 1640. IONPs (0.13-70 μ g/mL), CS (0.13-70 μ g/mL) and MCZ (0.09-50 μ g/mL) alone were tested as controls. After incubation for 48 h at 37 °C, the MIC values were visually established as the lowest concentrations capable of completely (100%) inhibiting the yeasts growth. In order to evaluate the type of interaction among the nanocarrier's compounds, the fractional inhibitory concentration (FIC) indexes were calculated, based on the MIC results, as detailed elsewhere [33]. MIC assays were repeated in triplicate on three independent occasions.

2.4. Biofilm formation and treatment

For single-species biofilm formation, 200 μL of each yeast suspension (1×10^7 cells/mL in AS) were added into wells of 96-well flat bottom plates, whereas for dual-species biofilms 100 μL of each microbial suspension (2×10^7 cells/mL for *C. albicans* + 2×10^7 cells/mL for *C. glabrata*) were incorporated into each well. The microtiter plates were then aerobically incubated at 37°C. After 48-h biofilm formation (with refreshment of the AS medium after the first 24 h), single- and dual-species biofilms were treated during 24 h with the MCZ-containing nanocarrier at two different concentrations: 31.2 (IONPs-CS-MCZ31.2) and 78 $\mu\text{g/mL}$ (IONPs-CS-MCZ78). These concentrations correspond to 20- and 50-fold the nanocarrier MIC for *C. glabrata* (1.56 $\mu\text{g/mL}$). All biofilm assays had appropriate controls, including 110 $\mu\text{g/mL}$ IONPs, 110 $\mu\text{g/mL}$ CS, 78 $\mu\text{g/mL}$ MCZ and untreated biofilms as negative control (NC). Biofilm treatment was performed in both the presence and absence of an external magnetic field. For the set of experiments involving magnetic field, magnetic plates (Supergauss Prod. Magnéticos Ltda., São Paulo, Brazil) of 10 x 100 x 150 mm with magnetic flux density between 3900~4000 G were positioned under the 96 well-plates during the 24-h treatment period.

2.5. Biofilm quantification

Subsequently to treatment period, all biofilms were rinsed once with PBS to remove non-adhered cells. Following, crystal violet (CV) staining and XTT reduction assay were employed, respectively, to quantify the total biofilm biomass and metabolic activity of biofilm cells, as previously described [34]. For quantification of colony-forming units (CFUs), the treated biofilms were scraped from the bottom of the plates,

suspended in PBS (1 ml) and homogenized in vortex (90 s). Then, serial dilutions in PBS were plated on SDA for single biofilms, and on CHROMagar *Candida* (Difco) for dual-species biofilms. The agar plates were incubated at 37°C and the CFUs, counted after 24 to 48h [33]. The results of total biomass, metabolic activity and number of CFUs were expressed as a function of the well area (Abs/cm² and log₁₀ CFU/cm²).

The compositional analysis of the biofilms' extracellular matrix was also performed. Briefly, single- and dual-species biofilms were developed in 24-well plates containing 1 mL of cell suspension, and treated with the nanocarrier and controls, as detailed above. For matrix extraction, the resulting biofilms were scraped from the wells, resuspended in PBS, sonicated on ice (30 s; 30 w) and vortexed for 2 min. Afterwards, biofilm suspensions were centrifuged at 3000 *g* for 10 min and the supernatant, filtered through a nitrocellulose filter (0.22 μm) [35, 36]. The protein content of the extracellular matrix was determined by the bicinchoninic acid method (BCA kit; Sigma-Aldrich), using bovine serum albumin as standard [37], while the total carbohydrate content was estimated using glucose as standard [35, 36, 38]. To assess the content of DNA from matrix biofilm, 1.5 μL of the supernatant was pipetted into a nanodrop device (Eon Microplate Spectrophotometer; Bio Tek, Winooski, USA) and spectrophotometrically analysed at 260 nm and 280 nm [39]. Total contents of protein, carbohydrate and DNA were expressed as a function of the liquid phase of the extracellular matrix (mg/mL).

2.6. Structural analysis of biofilms

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were employed to visualize the ultrastructure of dual-species *Candida* biofilms

treated with IONPs-CS-MCZ nanocarrier and controls. For this, biofilms were formed at the bottom of 24-well plates for SEM, and on sterile coverslips into 24-well plates for CLSM. Biofilm treatment was performed in the absence of an external magnetic field, as described above. For SEM preparation, the samples were serially washed in ethanol for dehydration (70% for 10 min, 95% for 10 min and 100% for 20 min), air-dried in a desiccator, and cut from the bottom of the plates. Samples were then positioned onto aluminium stubs before being coated with gold, and qualitatively analyzed by SEM (FEG-VP Supra 35; Carl Zeiss, Jena, Thüringen, Germany)[32]. As for CLSM analysis, the resulting biofilms were stained with 200 μ L of a solution containing 3 μ g/mL SYTO9 green fluorescent dye and 3 μ g/mL propidium iodide for 20 to 30 min at room temperature, protected from light [35]. After, biofilm samples were gently rinsed with sterile water and analyzed under a confocal microscope (Nikon C2/C2si, Tokyo, Japan) at 488/500-570 nm for SYTO9 dye and 561/570-1000 nm for propidium iodide. Fluorescent green and red colors represent living and dead cells, respectively.

2.7. Statistical analysis

All biofilm assays were conducted in triplicate on three separate occasions. Data presented normal (Shapiro-Wilk test) and homogeneous (Cochrane test) distribution, except for DNA (*C. glabrata* and dual-species biofilms) and protein (*C. albicans* and dual-species biofilms). Results were submitted to 2-way ANOVA, considering the different compounds and the presence of an external magnetic field as variation factors. Holm-Sidak test was applied for multiple-comparisons when applicable. SigmaPlot software (version 12.0; Systat Software Inc., San Jose, USA) was used, adopting $p < 0.05$ as statistically significant.

3. Results

3.1. Characterization of the IONPs-CS-MCZ nanocarrier

As the characterization results of IONPs alone and CS-coated IONPs have been described in a previous study[27], this section presents only the characterization of the IONPs-CS-MCZ nanocarrier. TEM and DLS analyzes were used to estimate the morphology and average size of the nanocarrier. By TEM, it was possible to observe a predominantly spherical shape for IONPs and MCZ particles, with a diameter lower than 50 nm for the IONPs-CS-MCZ nanocarrier (Fig. 1a). It was also possible to note the MCZ particles adhered to the CS-coated IONPs, thus forming the nanocarrier (Fig. 1a). In turn, DLS analysis showed that the average hydrodynamic size of the nanocarrier was close to 180 nm (Fig. 1b). Regarding the nanocarrier's crystalline structure, the XRD pattern obtained was similar to that seen for IONPs alone, and revealed a spinel-type structure [27].

FTIR analysis revealed the chemical constitution of the IONPs-CS-MCZ nanocarrier. Characteristic bands of IONPs[27] and CS[27] were seen in the nanocarrier's FTIR spectrum (Fig. 1c). Absorption peaks around 1585 cm^{-1} , 1473 cm^{-1} (C-C of two dichlorobenzene), 1385 cm^{-1} (C-C and C-H of imidazole) and 1327 cm^{-1} (C-H of two dichlorobenzene), which are indicative of MCZ [40], were also detected (Fig. 1c). For TGA, a marked mass loss was seen from 200 degrees Celsius, suggesting CS degradation and MCZ melting (Fig. 1d). Analyzing the temperature range from 400 to 800°C , the thermogravimetric curve for IONPs-CS-MCZ (Fig. 1d) showed a similar mass loss pattern to that previously found for IONPs e CS-coated IONPs[27], thus

videncing that all MCZ incorporated was successfully conjugated to the IONPs-CS compound.

3.2. Determination of the MIC

Planktonic cell susceptibility results are shown in Table 1. For *C. albicans*, the nanocarrier promoted an 8-fold reduction in the MCZ MIC value compared to the antifungal applied alone, while for *C. glabrata* this reduction was 2- to 4-fold. Furthermore, *C. albicans* was slightly more susceptible to MCZ and nanocarrier than *C. glabrata*, whereas IONPs and CS alone did not inhibit the growth of both strains at 140 µg/mL. The association of compounds forming the nanocarrier was then classified as synergistic and indifferent, respectively for *C. albicans* and *C. glabrata*.

3.3. Biofilm quantification

For single- and dual-species biofilms treated in the presence or absence of an external magnetic field, all assessed compounds were not able to promote significant decreases in total biomass compared to NC groups (Fig. 2).

Regarding the metabolic activity of biofilms of *C. albicans* and mixed, MCZ and IONPs-CS-MCZ78 were the only compounds that led to significant reductions compared to NCs, both in the presence and in absence of an external magnetic field (Fig. 3a and c). IONPs-CS-MCZ78 significantly differed from all other groups and produced the highest reductions in biofilm metabolism compared to NCs (93.04-94.40%; Fig. 3a and c), regardless of the use of a magnetic field. For single biofilm of *C. glabrata*, MCZ, IONPs-CS-MCZ31.2 and IONPs-CS-MCZ78 did not differ from each other and promoted significant reductions in the metabolic activity (ranging from

64.15 to 98.27%) compared to the NC (Fig. 3b). The use of an external magnetic field did not influence the metabolism results for all biofilms (Fig. 3).

CFU enumeration results for single-species biofilms of *C. albicans* and *C. glabrata* showed that CS, MCZ and IONPs-CS-MCZ31.2 were able to significantly reduce the number of cultivable cells compared to NCs (Fig. 4a and b). However, IONPs-CS-MCZ78 was the most effective treatment, differing significantly from all groups and promoting cell number decreases in comparison to the NCs ranging from 1.21- to 1.42- \log_{10} , in both the presence and absence of an external magnetic field (Fig. 4a and b). For *C. albicans* in dual-species biofilms, IONPS-CS-MCZ78 also exhibited the highest reductions compared to the NCs (1.85-2.05- \log_{10} ; Fig. 4c). As for *C. glabrata* in dual-species biofilms, MCZ and IONPs-CS-MCZ78 were the most effective compounds in reducing the number of CFUs, without significant differences between them (Fig. 4d). The presence of an external magnetic field influenced only the CFU quantification of *C. albicans* in dual-species biofilms treated with IONPs (Fig. 4c).

For all evaluated biofilms, treatments with IONPs-CS-MCZ and controls did not affect the protein (Table 2), carbohydrate (Table 3) and DNA (Table 4) contents of the extracellular matrix. In addition, the use of an external magnetic field during biofilm treatment did not interfere with the results of matrix composition.

3.4. Structural analysis of biofilms

SEM images revealed that the untreated dual-species biofilm consisted of a dense and robust network of interconnected yeasts and hyphae, forming a multilayer structure (Fig. 5a). Biofilms treated with IONPs, CS and IONPs-CS-MCZ31.2 exhibited the same structural pattern observed for the NC group (Fig. 5b, c and e). On the other

hand, MCZ and IONPs-CS-MCZ78 produced ruptures in the biofilms, generating less dense structures, with more visualization of polystyrene surface areas (Fig. 5d and f). Some particle agglomerates (CS and IONPs) were also seen in biofilms exposed to CS (Fig. 5c), IONPs-CS-MCZ31.2 (Fig. 5e) and IONPs-CS-MCZ78 (Fig. 5f). According to CLSM images, biofilms treated with CS, MCZ, IONPs-CS-MCZ31.2 and IONPs-CS-MCZ78 showed higher number of non-viable cells compared to the NC group (Fig. 6).

4. Discussion

The constant exposure of pathogenic microorganisms to conventional drugs has stimulated the development of improved mechanisms of resistance, making it difficult to treat diseases while creating a generation of over-resistant microorganisms, known as superbugs[41]. Within this context, the present study investigated the potential of a nanocarrier based on CS-coated IONPs to improve the antifungal efficacy of MCZ against pathogenic fungal biofilms. The study's null hypothesis was partially rejected, since the nanocarrier effects on planktonic cells and on some biofilm parameters (CFUs for all biofilms, and metabolic activity for single biofilms of *C. albicans* and dual-species biofilms) were superior to those found for MCZ alone.

The results of characterization shown in Fig. 1 evidenced the successful assembly of IONPs-CS-MCZ as a functional nanocarrier. TEM results confirmed a diameter lower than 50 nm for the nanocarrier, which ensures that this conjugate can be explored as an alternative nanotherapy. In contrast, DLS results indicated a large hydrodynamic diameter (around 180 nm; Fig. 1b). DLS is an indirect method that calculates sample size by the frequency of movement of particles in aqueous medium [42]. Thus, this technique is very sensitive to aggregation and may generate different

results from those obtained for imaging techniques of dried samples, as TEM [42]. Due to its biocompatibility, biodegradability and stability in acidic pH, CS is frequently used as a coating for IONPs as this polymer usually adsorbs to these nanoparticles through glycosidic bonds[43, 44]. In turn, MCZ probably bound to CS via electrostatic attraction between the amine groups of CS and the negative charge of MCZ [45]. Taken together, these physicochemical phenomena explain how the nanocarrier was formed.

As for MIC determination, IONPs-CS-MCZ was more effective than MCZ alone on *C. albicans* and *C. glabrata* planktonic cells (Table 1). For planktonic *C. albicans*, the nanocarrier effect was the result of a synergistic interaction among the compounds present in the nanocarrier, as shown by the FIC index. MCZ causes accumulation of reactive oxygen species (ROS) in the fungal cytoplasm, which can lead to a fungicidal effect. Moreover, a previous study showed that even before ROS can cause cell death, the majority of *C. albicans* cells exposed to MCZ were already necrotic [20]. It was suggested that prior to ROS production, MCZ affects the fungal actin cytoskeleton and creates channels in the mitochondrial membrane [20]. Regarding the mechanisms of antimicrobial action of CS, it was hypothesized that its positive charge interacts with the negatively charged cell membrane phospholipids, leading to increase in the membrane permeability, leakage of cell contents and, consequently, cell death [46]. CS also operates against *C. albicans* antagonizing the SAGA complex, which coordinates Ada2 and ABC transporter-encoding genes such as *CDR1* and *MDR1*, and altering the integrity of cell surface [47]. In addition, IONPs-CS may increase ROS production [48]. All the above-mentioned context could explain the synergistic effect found for IONPs-CS-MCZ on *C. albicans* in planktonic culture, since the nanocarrier combines drugs with different mechanisms of action.

However, *C. glabrata* showed to be less susceptible to MCZ and nanocarrier, with MIC values 2- to 8-fold higher than those found for *C. albicans* (Table 1). Exposure to MCZ may lead to mitochondrial injuries in *C. glabrata* that cause upregulation of efflux pumps genes (*Cdr1*, *Cdr2*, *Snq2* and *Qndr2*) associated with azole resistance, even before ROS accumulation causes its effect, thus increasing resistance to MCZ for this fungal pathogen [49, 50]. Furthermore, although *Ada2* controls antifungal drug tolerance and cell wall integrity in *C. glabrata*, it has a different role from that observed in *C. albicans* and does not regulate ABC transporter-encoding genes such as *CDR1*, *CDR2 (PDH1)* or *SNQ2* [51]. Thus, even if CS may attack *Ada2* in *C. glabrata*, a different mechanism is involved, which could justify the indifference found for IONPs-CS-MCZ on *C. glabrata* planktonic cells.

Regarding biofilm assays, promising results were found for metabolic activity and CFU counting, considering that the IONPs-CS-MCZ78 nanocarrier was more effective than 78 $\mu\text{g/mL}$ MCZ in reducing these parameters (Figs 3 and 4). These results are in agreement with SEM observations, which demonstrated greater ruptures and ultrastructure alterations in dual-species biofilms promoted by IONPs-CS-MCZ78, compared to other groups (Fig. 5). All treatments also generated biofilms with visually higher presence of dead cells in comparison to NC and IONPs alone, as displayed by CLSM (Fig. 6). As CS and MCZ alone were able to promote significant reductions in CFU and metabolism for some biofilms, the results found for IONPs-CS-MCZ78 may reflect a combined action of the antimicrobial effects of CS and MCZ, as justified for MIC results. The antibiofilm activity of IONPs-CS-MCZ78 was also dependent on the presence of MCZ at 78 $\mu\text{g/mL}$, since a dose-dependent effect was noted in comparison to its counterpart containing 31.2 $\mu\text{g/mL}$ MCZ (IONPs-CS-MCZ31.2). On the other

hand, nanocarrier and controls did not affect the total biofilm biomass (Fig. 2) and extracellular matrix components (Tables 2, 3 and 4). Therefore, all biofilm results analyzed together indicate that the IONPs-CS-MCZ78 nanocarrier was able to cross the extracellular matrix without changing it, exclusively acting at cellular level (affecting cell viability and metabolism). Indeed, nanocarriers are told built for the purpose of circumventing the physical barriers and penetrating at the most profound layers of the biofilm, due to its size advantages [26].

Magnetic nanoparticles may be additionally guided by external magnetic forces to the cell target [52]. Inside biofilms, magnetic fields create artificial channels and increase drug penetration, improving the antimicrobial effect [25, 53]. In this sense, the current study tested whether the presence of a static one-side magnetic field (positioned at the bottom of 96-well plates) would add any benefit to the nanocarrier's antibiofilm effect. However, in general, the quantitative results showed no differences between biofilms treated in both the presence and absence of an external magnetic field. Probably, the prolonged time of treatment used (24 h) was sufficient for nanocarrier penetration into the deeper layers of biofilms treated in the absence of magnetic field. Furthermore, this study did not use a switched magnetic field, which could create additional channels and improve drug efficacy [54].

Another interesting result was the higher effectiveness of the IONPs-CS-MCZ78 nanocarrier compared to MCZ alone, mainly for *C. albicans* in single- and dual-species biofilms (Figs 3 and 4). From a clinical perspective, these findings highlight the potential of the MCZ nanocarrier as a topical treatment to fight recurrent oral candidiasis in which *C. albicans* play a major role, such as DS. However, to broaden the knowledge about this nanocarrier, future studies assessing the MCZ release profiles,

different treatment periods, as well as toxicity to human cells caused by IONPs-CS-MCZ are needed.

5. Conclusion

The IONPs-CS-MCZ nanocarrier showed superior antifungal effect to MCZ alone on planktonic cells of *C. albicans* and *C. glabrata*, as well as on some parameters of single- and dual-species biofilms (cultivable cells and metabolism). Additionally, the nanocarrier advantage was more evident for *C. albicans*, and the presence of an external magnetic field during biofilm treatment did not potentiate the nanocarrier's antibiofilm effect.

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Conflict of Interest

The authors declare no conflict of interest.

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Table and figure captions

Table 1. Minimum inhibitory concentration (MIC) values of iron oxide magnetic nanoparticles (IONPs), chitosan (CS) and miconazole (MCZ), alone or in association, for the tested *Candida* strains

Table 2. Mean values (standard deviation) of the protein content obtained from the extracellular matrix of single- and dual-species *Candida* biofilms after treatment with different compounds

Table 3. Mean values (standard deviation) of the carbohydrate content obtained from the extracellular matrix of single- and dual-species *Candida* biofilms after treatment with different compounds

Table 4. Mean values (standard deviation) of the DNA content obtained from the extracellular matrix of single- and dual-species *Candida* biofilms after treatment with different compounds

Figure 1. Transmission electron microscopy image (a), size distribution histogram (b), Fourier-transform infrared spectrum (c) and thermogravimetric analysis (d) for the iron oxide magnetic nanoparticles-chitosan-miconazole nanocarrier. Enlarged image in (a) represents the core of a chitosan-coated nanoparticle, and miconazole particles bound to chitosan. The red circle in (c) shows the region of characteristic bands of the presence of miconazole.

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Figure 3. Quantification of metabolic activity (mean absorbance values per cm^2) for single- (a and b) and dual-species (c) biofilms of *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030 treated with 110 $\mu\text{g}/\text{ml}$ iron oxide magnetic nanoparticles (IONPs), 110 $\mu\text{g}/\text{ml}$ chitosan (CS), 78 $\mu\text{g}/\text{ml}$ miconazole (MCZ) and MCZ-containing nanocarrier at 31.2 (IONPs-CS-MCZ31.2) and 78 $\mu\text{g}/\text{ml}$ (IONPs-CS-MCZ78), in the absence or presence of an external magnetic field. Negative control denotes non-treated biofilms (NC). Different uppercase and lowercase letters show significant differences among the treatments for biofilms treated in the absence and presence of an external magnetic field, respectively (2-way ANOVA and Holm-Sidak test, $p < 0.05$).

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Figure 5. Scanning electron microscopy images of dual-species biofilms of *Candida albicans* ATCC 10231 and *C. glabrata* ATCC 90030 treated with 110 $\mu\text{g}/\text{ml}$ iron oxide magnetic nanoparticles (b), 110 $\mu\text{g}/\text{ml}$ chitosan (c), 78 $\mu\text{g}/\text{ml}$ miconazole (d) and miconazole-containing nanocarrier at 31.2 (e) and 78 $\mu\text{g}/\text{ml}$ (f). Negative control denotes non-treated biofilm (a). Magnification: 2500x. Bars: 10 μm . The red arrows in images (c), (e) and (f) indicate particle clusters.

Figure 6. Confocal laser scanning microscopy images of dual-species biofilms of *Candida albicans* ATCC 10231 and *C. glabrata* ATCC 90030 treated with 110 $\mu\text{g}/\text{ml}$ iron oxide magnetic nanoparticles (b), 110 $\mu\text{g}/\text{ml}$ chitosan (c), 78 $\mu\text{g}/\text{ml}$ miconazole (d) and miconazole-containing nanocarrier at 31.2 (e) and 78 $\mu\text{g}/\text{ml}$ (f). Negative control denotes non-treated biofilm (a). Magnification: 20x. Bars: 100 μm .

Table 1. Minimum inhibitory concentration (MIC) values of iron oxide magnetic nanoparticles (IONPs), chitosan (CS) and miconazole (MCZ), alone or in association, for the tested *Candida* strains

Species	MIC ($\mu\text{g/ml}$)						FICI	Classification
	Alone			In association (Nanocarrier)				
	IONPs	CS	MCZ	IONPs	CS	MCZ		
<i>C. albicans</i> ATCC 10231	> 140	> 140	1.56	0.27	0.27	0.19	< 0.12	Synergism
<i>C. glabrata</i> ATCC 90030	> 140	> 140	3.12– 6.25	2.18	2.18	1.56	< 0.53	Indifference

Note: FICI, fractional inhibitory concentration indices for the nanocarrier

Table 2. Mean values (standard deviation) of the protein content obtained from the extracellular matrix of single- and dual-species *Candida* biofilms after treatment with different compounds

Biofilms	Proteins (mg/ml)					
	NC	IONPs	CS	MCZ	IONPs-CS-MCZ31.2	IONPs-CS-MCZ78
Presence of an external magnetic field						
<i>C. albicans</i> ATCC 10231	0.10 (0.05)	0.09 (0.00)	0.08 (0.01)	0.09 (0.02)	0.11 (0.03)	0.07 (0.02)
<i>C. glabrata</i> ATCC 90030	0.04 (0.01)	0.07 (0.01)	0.08 (0.02)	0.04 (0.00)	0.06 (0.01)	0.06 (0.03)
<i>Dual-species biofilm</i>	0.12 (0.09)	0.09 (0.04)	0.11 (0.04)	0.09 (0.05)	0.11 (0.02)	0.08 (0.00)
Absence of an external magnetic field						
<i>C. albicans</i> ATCC 10231	0.09 (0.02)	0.12 (0.08)	0.12 (0.06)	0.11 (0.05)	0.13 (0.05)	0.08 (0.01)
<i>C. glabrata</i> ATCC 90030	0.06 (0.03)	0.10 (0.04)	0.09 (0.04)	0.05 (0.01)	0.06 (0.03)	0.05 (0.00)
<i>Dual-species biofilm</i>	0.12 (0.08)	0.06 (0.03)	0.07 (0.02)	0.05 (0.01)	0.08 (0.04)	0.09 (0.06)

Note: there were no statistically significant differences among the compounds, regardless of the presence of a magnetic field (2-way ANOVA; $p < 0.05$). Negative control (NC); 110 $\mu\text{g/ml}$ iron oxide magnetic nanoparticles (IONPs); 110 $\mu\text{g/ml}$ chitosan (CS); 78 $\mu\text{g/ml}$ miconazole (MCZ); MCZ-containing nanocarrier at 31.2 (IONPs-CS-MCZ31.2) and 78 $\mu\text{g/ml}$ (IONPs-CS-MCZ78).

Table 3. Mean values (standard deviation) of the carbohydrate content obtained from the extracellular matrix of single- and dual-species *Candida* biofilms after treatment with different compounds

Biofilms	Carbohydrates (mg/ml)					
	NC	IONPs	CS	MCZ	IONPs-CS-MCZ31.2	IONPs-CS-MCZ78
Presence of an external magnetic field						
<i>C. albicans</i> ATCC 10231	0.74 (0.24)	0.57 (0.13)	0.56 (0.07)	0.75 (0.46)	0.74 (0.09)	0.63 (0.14)
<i>C. glabrata</i> ATCC 90030	0.47 (0.40)	1.09 (0.66)	1.16 (0.78)	0.42 (0.35)	0.82 (0.24)	0.59 (0.59)
<i>Dual-species biofilm</i>	0.77 (0.26)	0.65 (0.11)	0.62 (0.19)	0.74 (0.27)	0.74 (0.04)	0.65 (0.21)
Absence of an external magnetic field						
<i>C. albicans</i> ATCC 10231	0.68 (0.26)	0.48 (0.17)	0.62 (0.42)	0.33 (0.09)	0.53 (0.15)	0.38 (0.31)
<i>C. glabrata</i> ATCC 90030	0.81 (0.76)	1.15 (0.97)	1.50 (0.72)	0.80 (0.48)	0.80 (0.20)	0.85 (0.40)
<i>Dual-species biofilm</i>	0.55 (0.37)	0.83 (0.15)	0.52 (0.21)	0.67 (0.03)	0.84 (0.55)	0.48 (0.25)

Note: there were no statistically significant differences among the compounds, regardless of the presence of a magnetic field (2-way ANOVA; $p < 0.05$). Negative control (NC); 110 $\mu\text{g/ml}$ iron oxide magnetic nanoparticles (IONPs); 110 $\mu\text{g/ml}$ chitosan (CS); 78 $\mu\text{g/ml}$ miconazole (MCZ); MCZ-containing nanocarrier at 31.2 (IONPs-CS-MCZ31.2) and 78 $\mu\text{g/ml}$ (IONPs-CS-MCZ78).

Table 4. Mean values (standard deviation) of the DNA content obtained from the extracellular matrix of single- and dual-species *Candida* biofilms after treatment with different compounds

Biofilms	DNA (mg/ml)					
	NC	IONPs	CS	MCZ	IONPs-CS-MCZ31.2	IONPs-CS-MCZ78
Presence of an external magnetic field						
<i>C. albicans</i> ATCC 10231	0.03 (0.02)	0.03 (0.00)	0.02 (0.00)	0.04 (0.02)	0.04 (0.01)	0.04 (0.01)
<i>C. glabrata</i> ATCC 90030	0.01 (0.01)	0.03 (0.01)	0.03 (0.00)	0.02 (0.00)	0.02 (0.00)	0.04 (0.02)
<i>Dual-species biofilm</i>	0.04 (0.04)	0.03 (0.01)	0.03 (0.00)	0.04 (0.01)	0.04 (0.01)	0.04 (0.01)
Absence of an external magnetic field						
<i>C. albicans</i> ATCC 10231	0.03 (0.01)	0.03 (0.01)	0.02 (0.01)	0.03 (0.01)	0.04 (0.00)	0.03 (0.01)
<i>C. glabrata</i> ATCC 90030	0.02 (0.01)	0.04 (0.03)	0.04 (0.02)	0.02 (0.00)	0.03 (0.02)	0.02 (0.00)
<i>Dual-species biofilm</i>	0.03 (0.01)	0.03 (0.01)	0.03 (0.00)	0.03 (0.00)	0.04 (0.01)	0.03 (0.01)

Note: there were no statistically significant differences among the compounds, regardless of the presence of a magnetic field (2-way ANOVA; $p < 0.05$). Negative control (NC); 110 $\mu\text{g/ml}$ iron oxide magnetic nanoparticles (IONPs); 110 $\mu\text{g/ml}$ chitosan (CS); 78 $\mu\text{g/ml}$ miconazole (MCZ); MCZ-containing nanocarrier at 31.2 (IONPs-CS-MCZ31.2) and 78 $\mu\text{g/ml}$ (IONPs-CS-MCZ78).

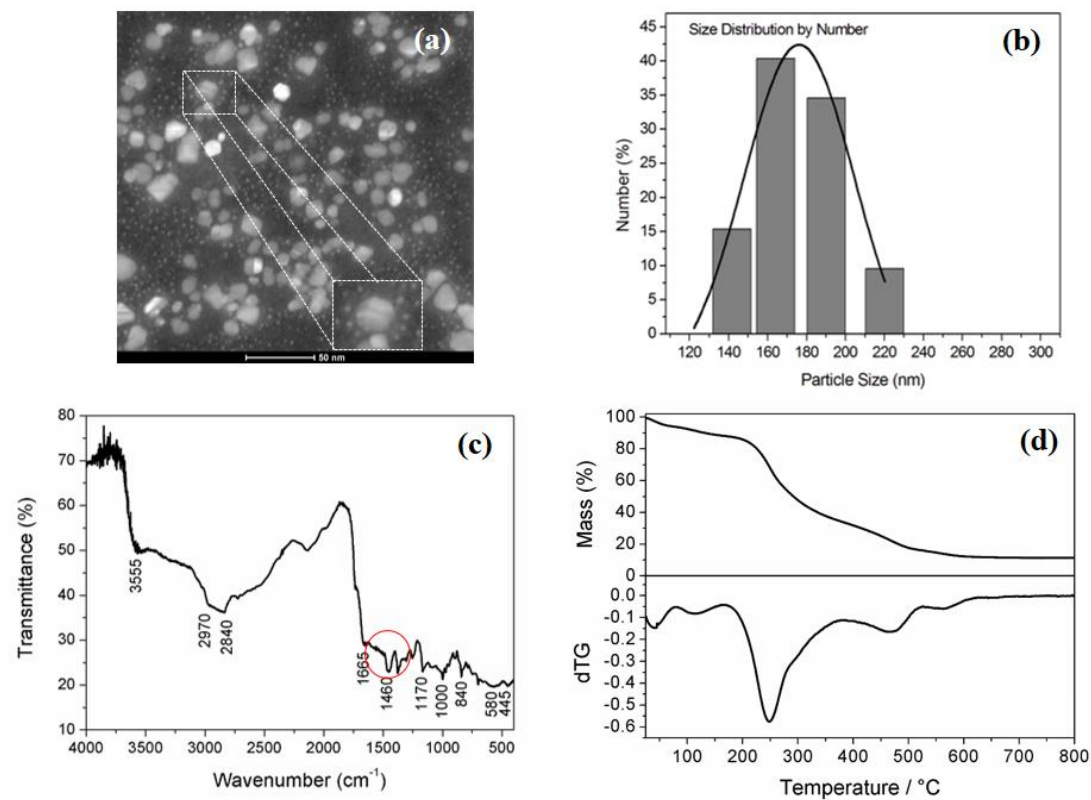


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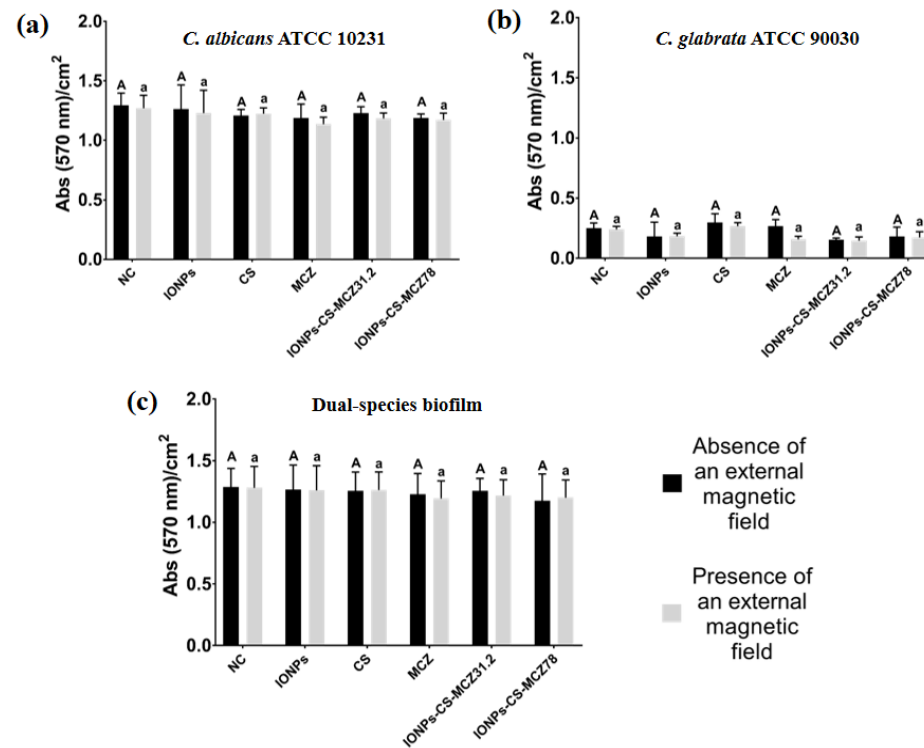


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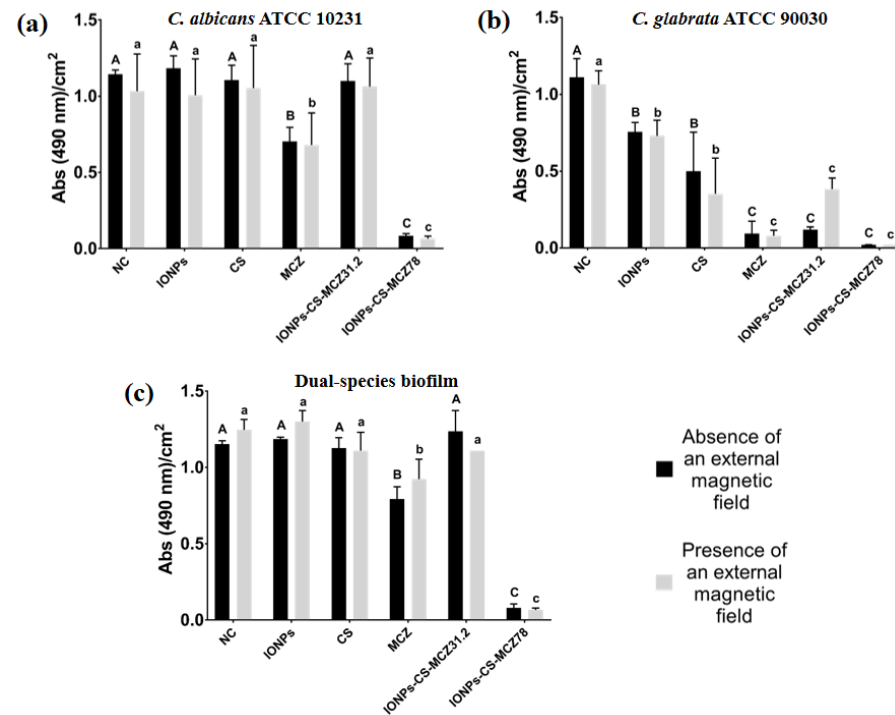


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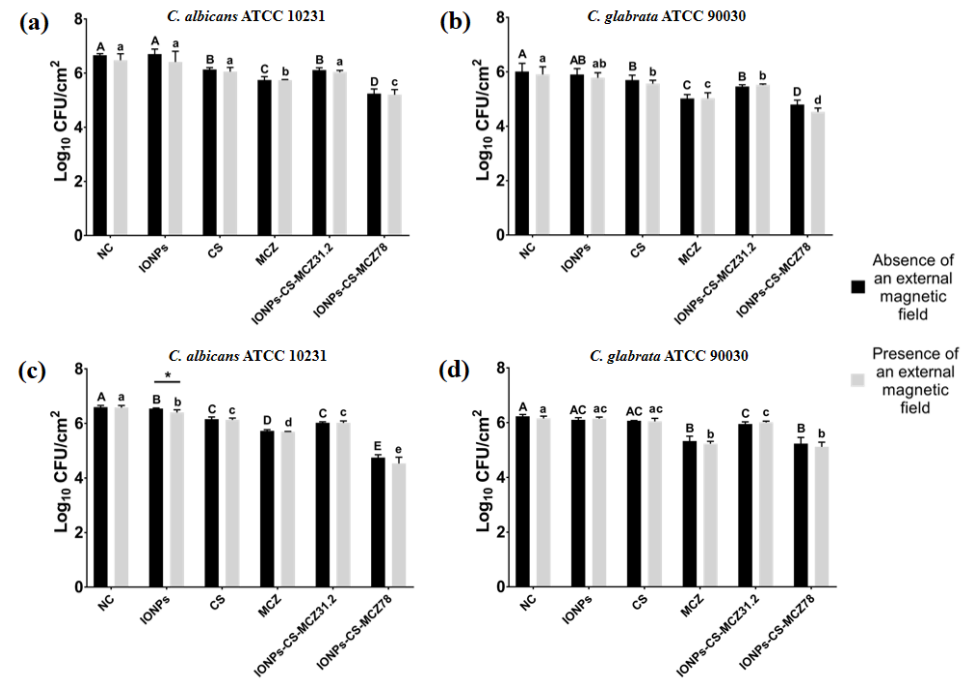


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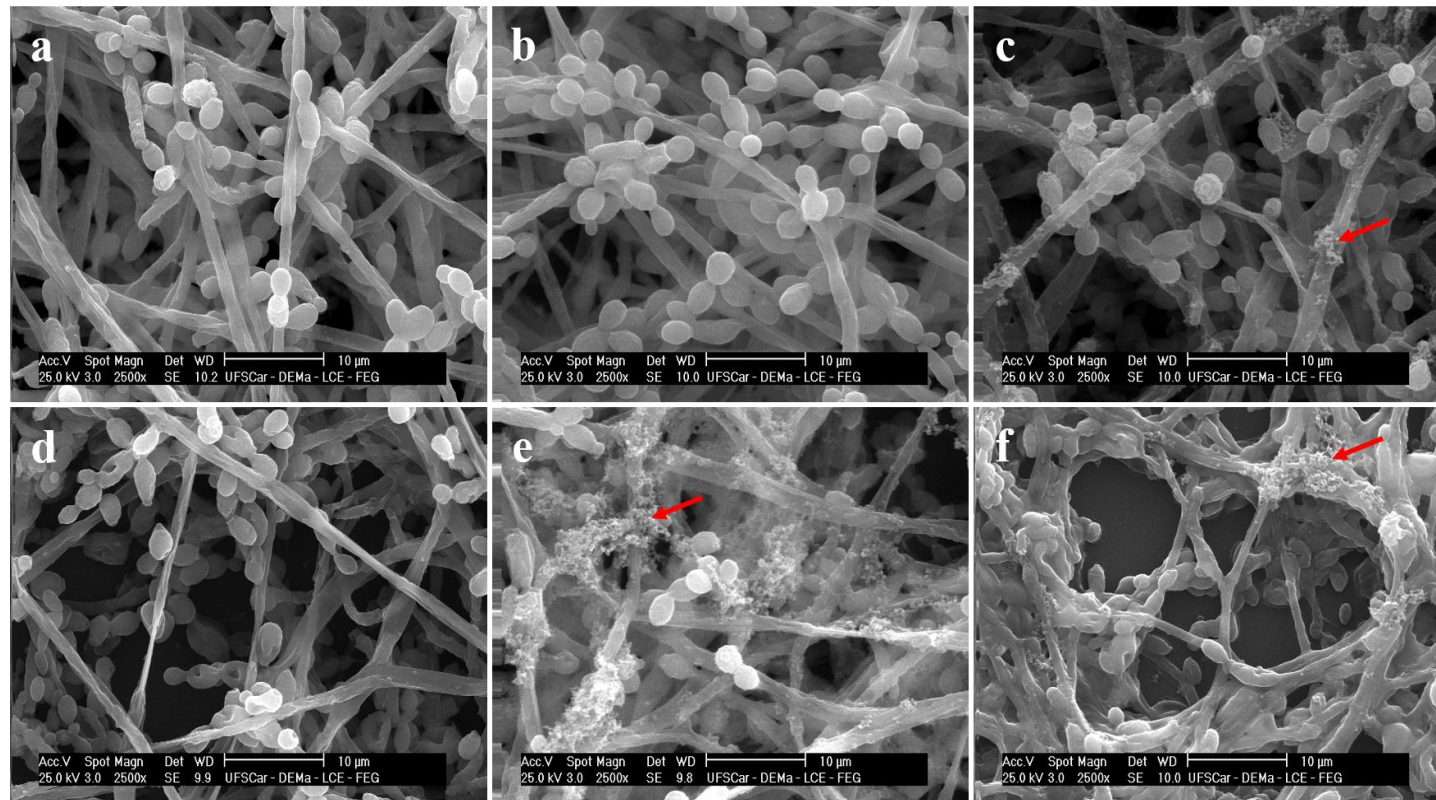


Figure 5. Scanning electron microscopy images of dual-species biofilms of *Candida albicans* ATCC 10231 and *C. glabrata* ATCC 90030 treated with 110 µg/ml iron oxide magnetic nanoparticles (b), 110 µg/ml chitosan (c), 78 µg/ml miconazole (d) and miconazole-containing nanocarrier at 31.2 (e) and 78 µg/ml (f). Negative control denotes non-treated biofilm (a). Magnification: 2500x. Bars: 10 µm. The red arrows in images (c), (e) and (f) indicate particle clusters.

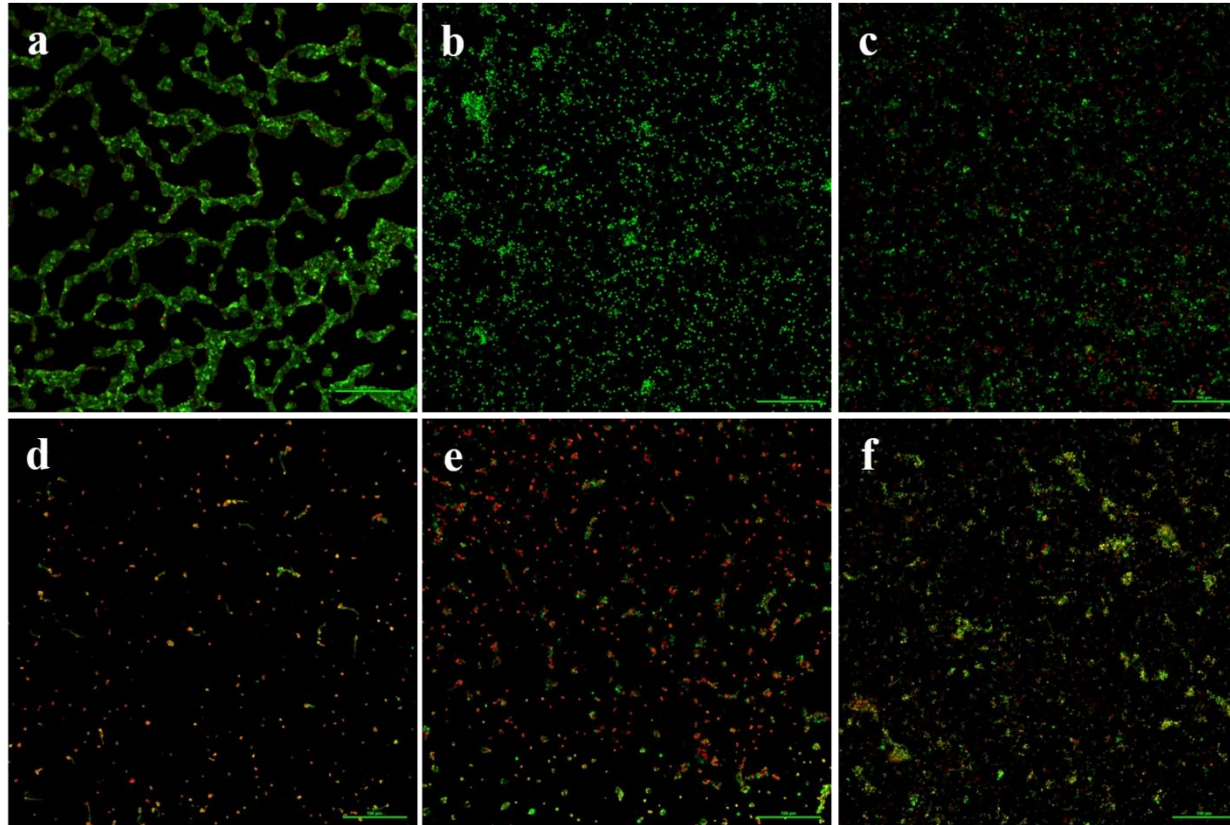


Figure 6. Confocal laser scanning microscopy images of dual-species biofilms of *Candida albicans* ATCC 10231 and *C. glabrata* ATCC 90030 treated with 110 µg/ml iron oxide magnetic nanoparticles (b), 110 µg/ml chitosan (c), 78 µg/ml miconazole (d) and miconazole-containing nanocarrier at 31.2 (e) and 78 µg/ml (f). Negative control denotes non-treated biofilm (a). Magnification: 20x. Bars: 100 µm.

Capítulo 2

Effect of a miconazole nanocarrier on the composition of three *in vitro* models of pathogenic oral biofilms

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Abstract

Objective: This study aimed to evaluate the effect of a miconazole (MCZ) nanocarrier, based on iron oxide nanoparticles (IONPs) and chitosan (CS) (IONPs-CS-MCZ), on the composition of three *in vitro* models of pathogenic oral biofilms (*i.e.*, dental caries, denture and gingivitis) formed by *Candida albicans* and different bacterial species.

Materials and methods: The planktonic and sessile minimum inhibitory concentrations (MICs) of MCZ alone and IONPs-CS-MCZ against different *C. albicans* strains were determined, as well as against all species that formed the three biofilm models. All biofilms were developed for 5 days and subsequently treated for 24 hours with IONPs-CS-MCZ containing MCZ at 64 mg/L. Following treatment, biofilms were assessed for total biomass, metabolic activity (XTT reduction assay), colony forming units (CFUs) and compositional analysis using quantitative PCR. Scanning electron microscopy (SEM) was used to analyze biofilm structure. Differences between groups were determined by unpaired t-test ($p < 0.05$). **Results:** In general, MIC results showed that IONPs-CS-MCZ was more effective than MCZ alone against *C. albicans* and most bacterial species investigated. IONPs-CS-MCZ also promoted significant reductions in the number of CFUs, total biomass and metabolic activity of the biofilms, as well as altering biofilm ultrastructure. Furthermore, IONPs-CS-MCZ affected the composition of the three evaluated biofilms, given that the death of *Candida* cells was followed by a higher number of dead bacterial cells, specially *Streptococcus spp.* After exposure to the nanocarrier, *Veillonella dispar*, *Actinomyces naeslundii* and *C. albicans* were the most prevalent species respectively for gingivitis, denture and dental caries models. **Conclusion:** In conclusion, the IONPs-CS-MCZ nanocarrier was efficient against three

in vitro models of pathogenic oral biofilms, showing potential to interfere in the synergistic interactions among fungal and bacterial cells within polymicrobial consortia by changing biofilm composition.

Keywords: biofilms; polymicrobial; antimicrobials; miconazole; nanocarriers.

Introduction

Oral diseases are extremely frequent around the globe, and the misdiagnosis or non treatment can incur in severe systemic consequences. As an example, gingivitis is considered by many a common and easy to treat alteration, but when left untreated, can lead to periodontitis, a disease that affects 10% of global population, being a major cause for tooth loss next to dental caries (1). According to World Health Organization (WHO) surveys, dental caries is the most common disease in humans, affecting 49 to 83.4% of children at scholar age (1). Similarly, the prevalence of denture stomatitis has also been increasing along with the numbers of elderly people and denture wearers, as well as immunocompromised patients (2-4).

Treatment or control of different oral diseases require the understanding of their pathogenesis, including identification of causative agents and the implicated environmental factors that culminate in development of polymicrobial biofilm formation; the key driving factor in pathogenesis of gingivitis, caries and denture stomatitis (5). Frequently, *Candida albicans* participates in this cross-kingdom correlation as an opportunistic fungus, whose association with different oral bacteria favors the virulence and resistance of these species to conventional treatment (6-8). The production of adhesins and carbon sources by *Candida* allows a synergistic relationship, supporting the growth of early and later colonizers of the oral biofilms through well-characterized interactions between bacterial and fungal surface receptors/proteins (6-9). Moreover, *C. albicans* alone is considered by the WHO as one of the greatest pathogenic microorganisms, whose antimicrobial resistance causes major concerns to public health (10).

Once the polymicrobial interactions phenomena are understood, circumventing the problem of antimicrobial resistance and innovating treatments through the discovery and administration of new drugs is becoming increasingly risky, given that constant exposure of microorganisms to drugs, even at low levels, generates mutations and new resistance mechanisms (11). Azoles are considered a major problem in this sense. Oral candidiasis is frequently treated by routine antifungals such as azoles of topical use (12). Among those, miconazole (MCZ) has proven to be a good antimicrobial choice since its broad-spectrum activity affects both fungi and different bacteria species (13). Such activity is clinically desirable for a polymicrobial clinical challenge but reports of microbial resistance and azole cross resistance alert us to the risk of over-reliance on definitive treatments (14, 15). Thus, alternative strategies such as the use of drug nanocarriers enhances the action of conventional drugs by facilitating their delivery to target cells and overcoming the biofilm physical barriers, which aims to reduce the exposure time and response of microorganisms to drugs (16, 17).

To this end, iron oxide magnetic nanoparticles (IONPs) have recently been studied for different clinical applications, including hyperthermia, magnetic resonance imaging, tissue repair and drug delivery (17). More recently, *in vitro* studies have presented IONPs-based nanosystems with antibiofilm purposes, whose nano and magnetic properties promise to meet the optimization needs of antimicrobial treatments, interfering with the composition of pathogenic biofilms while bringing balance to patient's health (18, 19).

Therefore, the aim of the present study was to investigate the effect of a MCZ nanocarrier based on IONPs and chitosan (CS), on the composition of three *in vitro* models representative of pathogenic oral biofilms (*i.e.*, dental caries, denture and

gingivitis) composed by *C. albicans* and different bacterial species. Furthermore, the minimum inhibitory concentrations (MICs) of MCZ and MCZ nanocarrier system for planktonic and sessile cells of different *C. albicans* and bacterial strains were determined. The effect of IONPs-CS-MCZ was assessed by XTT reduction assay, quantification of total biomass (crystal violet assay), counting of colony forming units (CFUs), compositional analysis of the biofilms (qPCR) and scanning electron microscopy (SEM) analysis.

Materials and methods

Assembly and characterization of the IONPs-CS-MCZ nanocarrier

A stock solution of the nanocarrier was prepared by the mixture of equal volumes of a IONPs colloidal suspension (700 mg/L) and CS (700 mg/L) followed by the MCZ solubilization (500 mg/L) into the *core-shell* compound IONPS-CS, as previously described for a chlorhexidine-carrier nanosystem (18). Transmission electron microscopy, X-ray powder diffraction, fourier-transform infrared spectroscopy and thermogravimetric analysis to confirm the effective incorporation of MCZ into the IONPs-CS compound, forming a nanocarrier with a diameter smaller than 50 nm while maintaining the nanoparticles' crystalline structure.

Strains and growth conditions

Twelve strains of *Candida albicans* were utilised in the current study, including 11 clinical oral isolates (BC020, BC023, BC037, BC038, BC039, BC117, BC136, BC145, BC044, BC146, 3153A) from the Oral Sciences Research Group, University of

Glasgow, Glasgow, United Kingdom, and 1 reference strain from American Type Culture Collection (ATCC 10231). Stock cultures of all *Candida* strains kept at -80°C were reactivated on Sabouraud dextrose agar (SAB agar) previously to the microbiological assays. For the MIC and biofilm assays, a loopful of *Candida* colonies was suspended in 10 mL of Yeast Extract Peptone Dextrose (YPD) medium and grew aerobically overnight at 37°C, with broths constantly stirring at 120 rpm. Following centrifugation, the fungal cells were adjusted with the aid of a haemocytometer to 2×10^4 , 1×10^6 cells/mL and 1×10^7 cells/mL for planktonic, sessile and mature biofilm evaluations respectively (15).

For the multi-species biofilm models, the following bacterial strains were employed: *Streptococcus mitis* NCTC 12261, *Streptococcus oralis* NCTC 11427, *Streptococcus intermedius* DSM 20573, *Streptococcus mutans* NCTC 10449, *Veillonella dispar* NCTC 11831, *Actinomyces naeslundii* DSM 17233, *Lactobacillus casei* DSMZ 20011, *Lactobacillus zae* DSM 20178, *Rothia dentocariosa* DSM 43762, *Fusobacterium nucleatum* ATCC 10953 and *Fusobacterium nucleatum vicentii* DSM 19507. From those, *Streptococcus* and *Rothia* species were seeded on Columbia Blood Agar base containing 10% defibrinated horse blood (CBA; Sigma–Aldrich, Dorset, UK) in 5% CO₂ and in aerobiosis, respectively. Strict and facultative anaerobic strains (*V. dispar*, *A. naeslundii*, *F. nucleatum*, *F. nucleatum vicentii*) were cultivated on Fastidious Anaerobe Agar base containing 10% defibrinated horse blood (FAA; Sigma–Aldrich, Dorset, UK) in anaerobic incubator, while *Lactobacillus* strains were propagated on De Man Rogosa Sharpe Agar (MRS agar; Sigma–Aldrich, Dorset, UK) in 5% CO₂. The temperature of incubation for all aforementioned bacterial strains was 37°C. Following growth on solid agar, a loopful from each bacterial strain was suspended in 10 mL of

specific media (Tryptic Soy Broth (TSB; Sigma–Aldrich, Dorset, UK) for *Streptococcus* species, Brain Heart Infusion broth (BHI; Difco) for *A. naeslundii*, *V. dispar* and *R. dentocariosa*, Schaedler’s anaerobic broth (SCH; Oxoid) for *Fusobacterium* and MRS broth for *Lactobacillus* species) and incubated overnight at 37°C as previously described (20). After the incubation period, the bacterial cells concentration was adjusted to 1×10^8 cells/mL.

Determination of the MICs

The broth microdilution method was employed to determine the MCZ and IONPs-CS-MCZ MICs for planktonic (pMIC) and sessile (sMIC) cells of the studied fungal and bacterial strains, as detailed elsewhere (15). For planktonic cells, 100 µL of standardized cell suspensions (2×10^4 cells/mL for *Candida* and 2×10^5 cells/mL for bacterial cells) were pipetted in round-bottom wells of 96-well plates containing 100 µL of a 2-fold serial dilution of MCZ and IONPs-CS-MCZ with concentration ranging from 0.5-256 mg/L, and the pMIC values were visually determined after 24-48 h. To determine the sMIC, 200 µL of the microbial inoculum (1×10^6 cells/mL for fungal cells and 1×10^7 for bacterial cells) were pipetted in flat-bottom wells of 96-well plates to allow the biofilm formation during 24 h. The biofilms were treated with 2-fold serial dilutions of MCZ and IONPs-CS-MCZ ranging from 0.5-256 mg/L and incubated at 37°C under specific aerobic or anaerobic conditions dependent on microbial species. After 24 h, the resulting biofilms were gently washed with phosphate buffered saline (PBS) and the wells received 100 µL of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt] (Sigma Aldrich). After 2h-incubation at 37°C, the absorbance values were read at 490 nm and the sMIC₅₀ and sMIC₈₀ were

considered the concentrations that lead respectively to 50% and 80% reduction of XTT readings, when compared to the non-treated positive control.

Multi-species biofilm models and treatment

To assess the effect of IONPs-CS-MCZ on pathogenic biofilms, three different *in vitro* multi-species biofilm models were tested, representative of caries, denture and gingivitis developed as previously described (4, 21, 22). The microbial composition of each biofilm model is represented in Table 2. For all biofilm models, the bacterial and fungal cells were adjusted to 1×10^7 cells/mL in Todd Hewitt Broth (THB) medium supplemented with 0.01 mg/mL hemin and 2 μ g/mL menadione in a similar manner to as previously described (20, 23). Biofilms were formed at the bottom of wells of 24-well plates for XTT reduction and biomass quantification assays, and on coverslips for CFU counting, qPCR assays and SEM analysis. Biofilm formation consisted of inoculating *Streptococcus* species and *Candida* strains (500 μ L) on the first day to promote primary colonization, followed by the addition of the remaining species on the second day. All biofilms developed anaerobically during additional 4 days, and the culture media was replenished every 24 h. Afterwards, the stock solution of the IONPs-CS-MCZ nanocarrier was diluted in THB supplemented media to the concentration of 64 mg/mL of MCZ which was used to treat the biofilms for 24 h. Biofilms minus treatment were considered as controls.

XTT reduction, total biomass and SEM analysis

After the treatment period, the biofilms were gently washed thrice with PBS then incubated with 250 μ L of XTT (Sigma Aldrich). The plates were then protected from

light and incubated for 2 h at 37°C as previously described (15). Next, 100 µL of each well's content was transferred to 96-well plates and the absorbance read at 490 nm to determine the biofilms' metabolic activity. To quantify biofilm biomass, the crystal violet method was applied as discussed elsewhere (15). In short, biofilms received 250 µL of 0.05% crystal violet for 20 minutes. Then, the remaining dye was removed and the biofilms were washed with water to remove any excess dye. The microtiter plates were dried at room temperature and biofilms destained with 99% ethanol. Finally, 100 µL of each well's content were transferred to 96-well microtiter plates and the results were read at 570 nm.

To analyse the biofilms ultrastructure after exposure to the IONPs-CS-MCZ nanocarrier, the coverslips were prepared as reported previously (24). Briefly, the resulting biofilms were washed with PBS and fixed in a solution containing 2% glutaraldehyde, 2% paraformaldehyde, 0.15% alcian blue and 0.15 M sodium cacodylate (pH 7.4), and further processed for SEM analysis. The samples were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope at a magnification of 1,000 and 3,500 x (JEOL Ltd, Hertfordshire, UK).

CFU counting and live/dead compositional analysis of biofilms

Following treatment, the coverslips containing biofilms were gently washed three times with PBS, removed from the wells and transferred into bijoux tubes containing 1mL of PBS for sonication at 35 kHz for 10 min (20). For CFU counting, 20 µL of each sample went through serial dilution in PBS for plating on specific agar media (SAB for *Candida*, and CBA and FAA supplemented with 0.025 mg/mL of Amphotericin B for aerobic and anaerobic growth respectively). The CFUs were counted after 48 h (20).

A qPCR viability method was used to count the microbial cells and determine the composition of the biofilm models, as previously described (4, 20). In short, the sonicated samples were split into 2 Eppendorf tubes containing each one 450 μL . For each group, one of the Eppendorfs received 50 μM of propidium monoazide (PMA; SigmaAldrich, Dorset, United Kingdom) to quantify the viable cells, prior to incubation of all samples in the dark for 10 min. Next, the samples were exposed to 650 W halogen light for 5 min. Samples without PMA served as controls to quantify biofilm total cells. The DNA was then extracted from the samples by using the kit QIAmp mini DNA Extraction Kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's recommendations, and real-time quantitative PCR (qPCR) was employed to quantify the total and viable cells of the biofilms. For qPCR 1 μL of sample DNA was added to a mastermix solution containing 10 μL of SYBR GreenERTM, 7 μL of UV-treated RNase-free water and 1 μL of 10 μM forward/reverse primers for each microbial species. Table 3 displays the primers used in the study (4, 20, 22). A total volume of 20 μL was added to MicroAmp fast-optical 96-well 0.1 ml reaction plates (Applied Biosystems, California, USA) and loaded into the StepOnePlusTM real time system (Applied Biosystems, California, USA). The following thermal cycle was used: 95°C for 2 minutes, 40 amplification cycles of 95°C for 3 seconds followed by 55°C for 30 seconds. All samples were run in duplicate. Colony forming equivalents (CFEs) were quantified using a standard curve of bacterial and fungal CFUs ranging from 1×10^3 to 10^8 CFU/mL.

Statistical analysis

All microbiological assays were performed in triplicate on two independent occasions. Data underwent unpaired t-test with significance level of 5%, using the statistical program GraphPad Prism (version 7; La Jolla, CA, United States).

Results

Determination of the MICs

The results of pMICs for all *C. albicans* strains revealed the same susceptibility pattern to MCZ and IONPs-CS-MCZ, with values ranging from 1.9 to 3.9 mg/L (Table 1). For sMIC results, it was possible to observe lower MIC values for the biofilms in response to the nanocarrier compared to the MCZ, for both sMIC₅₀ and sMIC₈₀, respectively. The analysis of susceptibility of oral strains to MCZ showed that concentrations of 15.6 to 125 mg/L were needed to inhibit the biofilms, which corresponds to 4- to 64-fold change in comparison to planktonic cells (Table 1). As for nanocarrier, lower concentrations were required under the same conditions, where fold change ranged from 0.125 to 8, compared to planktonic condition. A similar pattern was seen for the reference strain of *C. albicans* (ATCC 10231), in which sMIC₈₀ showed fold change of 16 and 8 compared to planktonic cells, respectively for MCZ and IONPs-CS-MCZ (Table 1).

Table 2 shows the MIC values of the strains included in the composition of the three studied biofilm models (Gingivitis, Denture and Dental caries). For all bacterial species, MCZ and IONPs-CS-MCZ displayed the same pMIC values, except for *R. dentocariosa*, for which the nanocarrier showed lower values (>32-fold reduction) compared to MCZ. Regarding sMIC₅₀, IONPs-CS-MCZ showed similar or slightly

lower values compared to MCZ, except for *A. naeslundii*, whose sMIC₅₀ value for the nanocarrier was 4 times higher than that found for MCZ. In addition, the nanocarrier showed sMIC₈₀ values lower than MCZ (2- to 8-fold change), except for *L. casei*, *L. zae* and *R. dentocariosa*. Comparing pMIC with sMIC₈₀, 4- to 512- and 1- to 128-fold changes were found for MCZ and IONPs-CS-MCZ, respectively. Taken together, the results from the preceding section indicate that IONPs-CS-MCZ nanocarrier was more effective than MCZ alone against *C. albicans* and the majority of bacteria species investigated in this study.

XTT reduction, total biomass and SEM analysis

For all evaluated models, the resulting biofilms after exposure to IONPs-CS-MCZ resulted in significant reductions in the metabolic activity and total biomass compared to the control group, with values ranging from 28 to 86.6%, except for gingivitis model biomass, which did not differ from the control group. (Figures 1, 2 and 3). Regarding the SEM observation, the control group showed a robust scaffold with dense network of interconnected cells. Bacteria were visibly adhered to the yeast and hyphae of *C. albicans*. In turn, exposure to the nanocarrier resulted in a reduction in biofilm thickness with certain areas of the coverslips now visible specifically in the gingivitis and caries model, as well as reduced bacterial adhesion to yeast. In addition, cells with altered morphology were visualized (collapsed and damaged cell wall structures), suggesting cell death (Figures 1C, 2C and 3C).

CFU counting and compositional analysis of the biofilm models

The Figure 4 shows the effect of the nanocarrier IONPs-CS-MCZ on the cells that form the gingivitis biofilm model. Overall, CFU and CFE quantifications revealed that the number of all microorganisms treated with IONPs-CS-MCZ were significantly reduced ($p < 0.05$) in comparison to the control group (Figures 4A and B). The compositional analysis of the gingivitis biofilm model showed that treatment with the nanocarrier promoted reductions in the total number of cells, as well as in the proportion of the microorganisms within the biofilm (Figure 4C and Table 4). The greatest change occurred for the *Streptococcus* spp., which was the most predominant consortia in the biofilm from the control group (representing 46% of biofilm total cells), yet only the third most prevalent in the treated group (15% of the total).

In the denture model, the number of CFUs and CFEs of all species after treatment with the nanocarrier decreased ($p < 0.05$) compared to the control group, except for the CFE numbers of *R. dentocariosa* (Figures 5A and B). The percentage of microorganisms within the denture biofilm was modified after treatment with IONPs-CS-MCZ (Table 4). Interestingly, the numbers of *Streptococcus* spp. and *V. dispar* (first and second most prevalent genus/species in the non-treated biofilms) were drastically reduced and replaced by *A. naeslundii* and *L. zae* as the most prevalent cells in the final composition of the treated biofilms (Figure 5C).

Analyzing the caries model biofilm, results of CFU showed that *Candida* cells significantly reduced when exposed to the nanocarrier in comparison to the control group ($p = 0.0234$). However, the same pattern was not shown for the CFE counting as *C. albicans* cells reductions were not significant. For all the other microorganisms included in this model, there were reductions ($p < 0.05$) in the CFU and CFE counts, except for the CFE of *L. casei* (Figures 6A and 6B). These results are reflected in the

percentages of the biofilm composition (Table 4), where *C. albicans* and *L. casei* were the only species that increased their prevalence in the total number of cells for treated biofilms (Figure 6C).

Discussion

In recent decades, our understanding that microorganisms form complex communities and polymicrobial interactions has helped to explain pathogenesis of diverse chronic diseases, as well as the biofilms resistance to antimicrobials (25, 26). Furthermore, our understanding of polymicrobial biofilms allows us to perceive how an appropriate therapeutic intervention can re-establish health while balancing the mechanisms of synergism and antagonism among the microorganisms (27). Here, we report for the first time the effect of a MCZ nanocarrier against three different pathogenic biofilm models, first evaluating the MICs of MCZ alone and IONPs-CS-MCZ for all studied microorganisms, before assessing the changes in number of cells, biofilm ultrastructure and species composition for the biofilm models.

The results of planktonic and sessile MICs of MCZ and IONPs-CS-MCZ against several *C. albicans* strains showed that the planktonic cells were equally affected by the tested drugs (Table 1). However, according to the sMIC₈₀ results, it was possible to obtain higher antimicrobial effect at lower concentrations of MCZ against a more resistant sessile biofilm, by using a nanocarrier (Table 1). Miconazole has been used for over 30 years and is prescribed specially against oral fungal infections for topical use (28, 29). Different from other azoles, MCZ has a dual mechanism of action. Besides interfering with ergosterol synthesis by inhibition of lanosterol demethylase, it also inhibits fungal catalase and peroxidase, therefore increasing intracellular reactive

oxygen species and leading to cell death (30). As for CS, literature suggests that the interaction between the positively charged drug and the negatively charged bacterial cell membranes results in the leakage of intracellular constituents (31, 32). Therefore, the lower MIC values found for the IONPs-CS-MCZ could be explained due to the synergistic action between CS and MCZ or to the very recurrent hypothesis that drug-delivery nanosystems are designed to break through physical barriers and meet the cell of interest (17, 33)

Previous work from our group has led to the development of three different biofilm models used in this study (gingivitis, denture and dental caries), taking into consideration the main early and later colonizers involved in the development of the aforementioned oral diseases (4, 20, 22). Since *C. albicans* is the flagship of many biofilm-related diseases frequently aggravating their progression and virulence, it was specially incorporated in all three biofilm models.

Data presented in Table 2 showed that IONPs-CS-MCZ was able to act effectively against planktonic and sessile cells of most microbial species that make up the three studied biofilm models, confirming that it has both antifungal and antibacterial activity. Indeed, literature data reports the antibacterial potential of topical use concentrations of MCZ, specially against gram-positive bacteria (13).

Significant reductions in the number of CFUs and metabolic activity for all biofilms, as well as in the total biomass for denture and dental caries biofilm models were corroborated by SEM observations, whereby the groups treated with IONPs-CS-MCZ nanocarrier, less dense biofilms were observed. These findings also indicate that the equilibrium of a complex system such as biofilm can be broken by attacking its main support system, in this case, the *Candida* cells (Figures 1, 2 and 3). Tentatively, it

could be proposed that bacterial cells were more exposed to nanocarrier action as reductions in the number of hyphae occurred. The compositional analysis confirms this hypothesis. For all biofilm models, a drastic change in the predominance of the different species after treatment with the MCZ nanocarrier was observed. In general, the most prevalent species in all biofilm models were aerobic and anaerobic-facultative bacteria, such as *Streptococcus* spp., *Veillonella* spp. and *Actinomyces* spp. (Figures 4, 5 and 6). After exposure to IONPs-CS-MCZ, it becomes noticeable that, proportionally, the reduction in the number of most bacterial cells was much higher than that noted for *C. albicans*, which increased its prevalence within the total number of the biofilm cells (Figures 4C, 5C, 6C and Table 4). This trend was expected, especially considering the role of *Candida* in the dimensional correlation within the bacterial community. The *Candida* yeast cell form has around 6.6 μm diameter, but its hyphae are much larger in size (34). Thus, they serve as a scaffold for the smaller bacterial cells to attach, which is directly correlated to the results observed in the current study.

For gingivitis and denture models (Figures 4 and 5), low or moderate reductions in the number of *Candida* cells were accompanied by significantly higher reductions in the number of bacteria. This occurrence may not be directly associated with the MCZ antibacterial effect alone, since one less hyphal cell within the biofilm represents lack of support and protection for several small bacterial cells, leading to a cascading reducing effect. Interestingly, for the dental caries biofilm model (Figure 6), *S. mutans* was the most affected species, suffering the highest reduction after treatment with the MCZ nanocarrier (Figure 6B). This result suggests that MCZ-based formulations may be an interesting method of treatment for polymicrobial biofilms that contain recognized microbial synergistic associations. In this model, a low reduction of *Candida* cells was

observed, which can be explained by the beneficial *Candida-Streptococcus* interaction. Possibly, *S. mutans*-derived α -glucans surrounding the fungal cells formed an additional "drug capture matrix" that prevented uptake of antifungals, therefore reducing *Candida* cell death (35).

These interactions between *Candida* and bacteria are largely reported, especially *Candida-Streptococcus* interactions. Briefly, *C. albicans* can promote streptococcal proliferation by providing growth stimulating factors and reducing oxygen tension (7). On the other hand, some *Streptococcus spp.* such as *S. mutans* can produce glucosyltransferases that bind to the fungal surface. In addition, this Gram-positive bacteria generates extracellular polysaccharides (EPS) in the presence of sucrose, which provide binding site for *S. mutans* while allowing *C. albicans* to adhere and colonize abiotic surfaces (6, 8).

Apart from the discussed correlations, other synergistic interactions that occur between bacteria are fundamental for the establishment and maintenance of the biofilms. Namely, in dental caries biofilm, the production of glucans by *S. mutans* favors the adhesion of *L. casei* (36). Moreover, the presence of *Fusobacterium spp.* in the dental caries and gingivitis model is only possible due to the early presence of *A. naeslundii*, at the same time that the inclusion of *S. oralis* possibly stimulates the growth of *Fusobacterium spp.* (37). This may explain how the reductions in the numbers of *A. naeslundii* were followed by even higher reductions in the numbers of *Fusobacterium spp.* cells after treatment with the MCZ nanocarrier (Figures 4B and 6B).

In general, IONPs-CS-MCZ nanocarrier showed a great versatility since it was able to significantly reduce the number of cells, biomass and metabolism of the three

pathogenic biofilm models tested, as well as to promote changes in their composition. This suggests that the nanocarrier has potential to fight important oral diseases (gingivitis, caries and denture stomatitis) associated with biofilms formed by microbial species that establish beneficial interactions with each other. Nevertheless, future studies should be conducted to evaluate the effect of the nanocarrier on microcosm biofilms, as well as tests that beneficially explore the magnetic properties of the nanoparticles to improve drug delivery accuracy. These analyzes will bring new knowledge about the antibiofilm effect of the IONPs-CS-MCZ nanocarrier and will favor the delivery of MCZ to the target cells, reducing the drug concentration used. In addition, cytotoxicity tests on human cells should also be performed.

In summary, IONPs-CS-MCZ nanocarrier affected the composition of the three evaluated biofilms, causing high diminution of *Streptococcus* cells proportion for all biofilms and major prevalence of *V. dispar*, *A. naeslundii* and *C. albicans* respectively for gingivitis, denture and dental caries models. This alteration was directly related to the antibiofilm effect of this compound. Moreover, lower reductions of *C. albicans* resulted in accentuated reductions of bacterial cells for all biofilms.

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Figure captions

Figure 1. Results of XTT reduction assay (A), quantification of total biomass (B) and scanning electron microscopy (SEM) observation (C) for the gingivitis biofilm model non-treated (control) and treated with the nanocarrier containing miconazole at 64 mg/L (IONPs-CS-MCZ). Magnification of the SEM images: 1,000x and 3,500x; Bars: 10 and 5 μ m. Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). White arrows represent adhesion of bacteria to yeasts/hyphae. Yellow arrow represents loss of cellular membrane integrity.

Figure 2. Results of XTT reduction assay (A), quantification of total biomass (B) and scanning electron microscopy (SEM) observation (C) for the denture biofilm model non-treated (control) and treated with the nanocarrier containing miconazole at 64 mg/L (IONPs-CS-MCZ). Magnification of the SEM images: 1,000x and 3,500x; Bars: 10 and 5 μ m. Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). White arrows represent adhesion of bacteria to yeasts/hyphae. Yellow arrow represents loss of cellular membrane integrity.

Figure 3. Results of XTT reduction assay (A), quantification of total biomass (B) and scanning electron microscopy (SEM) observation (C) for the caries biofilm model non-treated (control) and treated with the nanocarrier containing miconazole at 64 mg/L (IONPs-CS-MCZ). Magnification of the SEM images: 1,000x and 3,500x; Bars: 10 and 5 μ m. Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p <$

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Figure 4. Results of counting of colony forming units (A), colony forming equivalents of viable cells (B) and biofilm percentage composition (C) for gingivitis biofilm model non-treated (control group) and treated with nanocarrier containing 64 mg/L miconazole (IONPs-CS-MCZ). Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 5. Results of counting of colony forming units (A), colony forming equivalents of viable cells (B) and biofilm percentage composition (C) for denture biofilm model non-treated (control group) and treated with nanocarrier containing 64 mg/L miconazole (IONPs-CS-MCZ). Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 6. Results of counting of colony forming units (A), colony forming equivalents of viable cells (B) and biofilm percentage composition (C) for caries biofilm model non-treated (control group) and treated with nanocarrier containing 64 mg/L miconazole (IONPs-CS-MCZ). Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Table 1. Values of minimum inhibitory concentration (MIC) of miconazole (MCZ) and MCZ nanocarrier (IONPs-CS-MCZ) for planktonic (pMIC) and sessile (sMIC₅₀ e sMIC₈₀) cells of different *Candida albicans* strains

	MCZ (mg/L)			IONPs-CS-MCZ (mg/L)		
	pMIC	sMIC ₅₀ (fold change)	sMIC ₈₀ (fold change)	pMIC	sMIC ₅₀ (fold change)	sMIC ₈₀ (fold change)
Clinical oral strains (n=10)	1.9-3.9	<0.24-31.25 (≤0.125-16)	15.6-125 (4-64)	1.9-3.9	<0.24-3.9 (≤0.125-2)	<0.24-15.6 (0.125-8)
ATCC (n=1)	3.9	15.6(4)	62.5 (16)	3.9	1.9 (0.5)	31.25 (8)

Table 2. Values of minimum inhibitory concentration (MIC) of miconazole (MCZ) and MCZ nanocarrier (IONPs-CS-MCZ) for planktonic (pMIC) and sessile (sMIC₅₀ e sMIC₈₀) cells of the species included in the different studied biofilm models

	Biofilm model	pMIC [mg/l]		sMIC ₅₀ [mg/l] (fold change)		sMIC ₈₀ [mg/l] (fold change)	
		MCZ	IONPs-CS-MCZ	MCZ	IONPs-CS-MCZ	MCZ	IONPs-CS-MCZ
<i>C. albicans</i> 3153A	C/G/D	4	4	8 (2)	<0.5 (0.125)	16 (4)	4 (1)
<i>S. mitis</i> 12261	G/D	4	4	16 (4)	16 (4)	>256 (>64)	64 (16)
<i>S. oralis</i> 11427	G/D	4	4	4 (1)	4 (1)	>256 (>64)	64 (16)
<i>S. intermedius</i> 20573	G/D	<0.5 - 1	<0.5 - 1	64 (64-128)	64 (64-128)	256 (256-512)	64 (64-128)
<i>S. mutans</i> 10449	C	4	4	128 (32)	64 (16)	256 (64)	64 (16)
<i>V. dispar</i> 11831	C/G/D	8-16	8-16	32 (2-4)	16 (1-2)	>256 (>32)	32 (2-4)
<i>A. naeslundii</i> 17233	C/G/D	8	8	16 (2)	64 (8)	256 (32)	128 (16)
<i>F. nucleatum</i> 10953	C/G	4	4	32 (8)	16 (4)	32 (8)	16 (4)
<i>F. nucleatum vincentii</i> 10507	G	2	2	16 (8)	4 (2)	32 (16)	8 (4)
<i>L. casei</i> 20011	C/D	16	16	>256 (>16)	256 (16)	>256 (>16)	>256 (>16)
<i>L. zaeae</i> 20178	D	16	16	>256 (>16)	64 (4)	>256 (>16)	>256 (>16)
<i>R. dentocariosa</i> 43762	D	>128	4-8	32 (<0.125)	32 (4-8)	64 (<0.25)	64 (8-16)

Note: Capital letters C, G and D represent, respectively, the Dental caries, Gingivitis and Denture biofilm models.

Table 3. Bacterial and fungal primer sequences for real time PCR

Target	Primer sequence (5' - 3')	Reference
<i>C. albicans</i>	F - GGGTTTGCTTGAAAGACGGTA	Sherry et al., 2018
	R - TTGAAGATATACGTGGTGGACGTTA	
<i>Streptococcus spp.</i>	F - GATACATAGCCGACCTGAG	Zhou et al., 2018
	R - CCATTGCCGAAGATTCC	
<i>A. naeslundii</i>	F - GGCTGCGATACCGTGAGG	Zhou et al., 2018
	R - TCTGCGATTACTAGCGACTCC	
<i>V. dispar</i>	F - CCGTGATGGGATGGAAACTGC	Zhou et al., 2018
	R - CCTTCGCCACTGGTGTTCCTTC	
<i>Fusobacterium</i>	F - GGATTTATTGGGCGTAAAGC	Sherry et al., 2018
	R - GGCATTCCTACAAATATCTACGAA	
<i>L. casei</i>	F - TGCACTGAGATTCGACTTAA	Zhou et al., 2018
	R - CCCACTGCTGCCTCCCGTAGGAGT	
<i>L. zeae</i>	F - TGCATCGTGATTCAACTTAA	Ramage et al., 2019
	R - CCCACTGCTGCCTCCCGTAGGAGT	
<i>R. dentocariosa</i>	F - GGGTTGTAAACCTCTGTTAGCATC	Ramage et al., 2019
	R - CGTACCCACTGCAAAAACCAG	

Table 4. Mean % composition values for each microorganism from the three different biofilm models (Gingivitis, Denture and Dental caries).

	Gingivitis model		Denture model		Dental caries model	
	Control	IONPs-CS-MCZ	Control	IONPs-CS-MCZ	Control	IONPs-CS-MCZ
CFE/mL	2.1 x 10 ⁹	3.1 x 10 ⁸	6.8 x 10 ⁸	6.4 x 10 ⁶	5.9 x 10 ⁸	6.2 x 10 ⁷
<i>Candida</i>	0.1%	0.3%	2.3%	6.5%	4.6%	35.4%
<i>Streptococci</i>	46.1%	15.4%	49.4%	9%	17.3%	0.8%
<i>V. dispar</i>	27.3%	43.6%	25.9%	4.9%	44.8%	33.4%
<i>A. naeslundii</i>	24.4%	40.5%	21.0%	58.0%	31.6%	13.2%
<i>Fusobacterium</i>	2.1%	0.2%	—	—	0.6%	0.04%
<i>L. casei</i>	—	—	0.4%	3.8%	1.1%	17.1%
<i>L. zeae</i>	—	—	0.9%	17.6%	—	—
<i>R. dentocariosa</i>	—	—	0.1%	0.2%	—	—

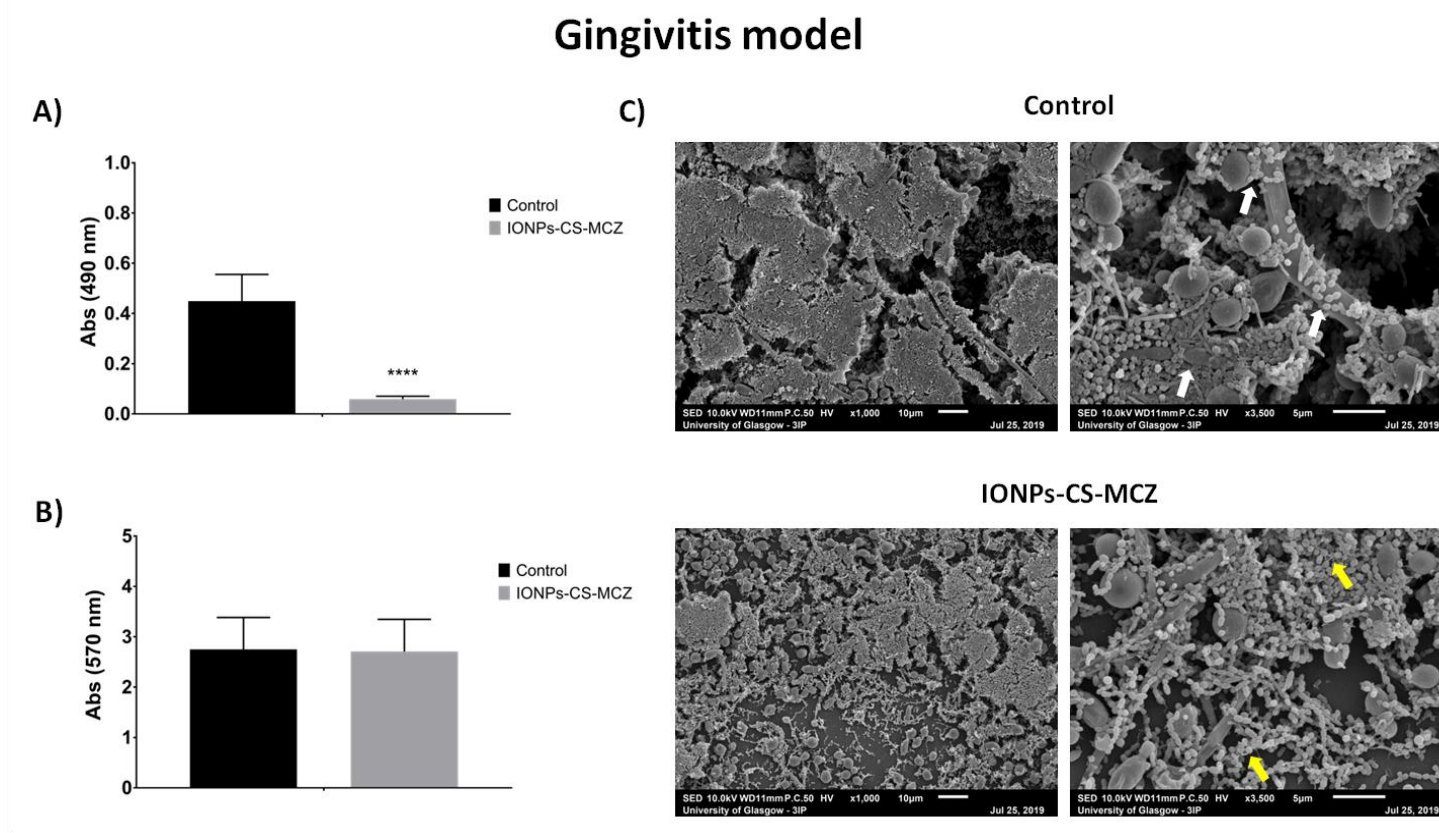


Figure 1. Results of XTT reduction assay (A), quantification of total biomass (B) and scanning electron microscopy (SEM) observation (C) for the gingivitis biofilm model non-treated (control) and treated with the nanocarrier containing miconazole at 64 mg/L (IONPs-CS-MCZ). Magnification of the SEM images: 1,000x and 3,500x; Bars: 10 and 5 μ m. Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). White arrows represent adhesion of bacteria to yeasts/hyphae. Yellow arrow represents loss of cellular membrane integrity.

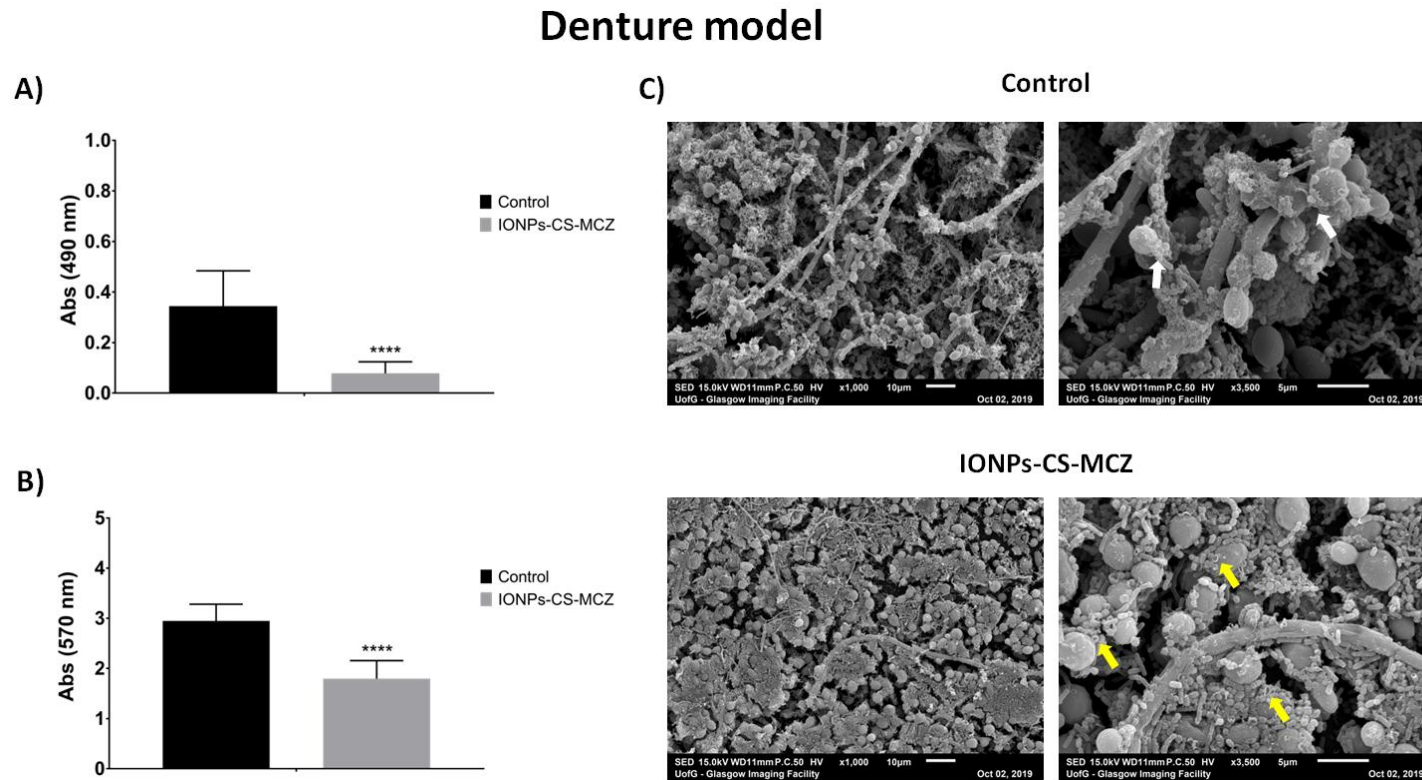


Figure 2. Results of XTT reduction assay (A), quantification of total biomass (B) and scanning electron microscopy (SEM) observation (C) for the denture biofilm model non-treated (control) and treated with the nanocarrier containing miconazole at 64 mg/L (IONPs-CS-MCZ). Magnification of the SEM images: 1,000x and 3,500x; Bars: 10 and 5 μ m. Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). White arrows represent adhesion of bacteria to yeasts/hyphae. Yellow arrow represents loss of cellular membrane integrity.

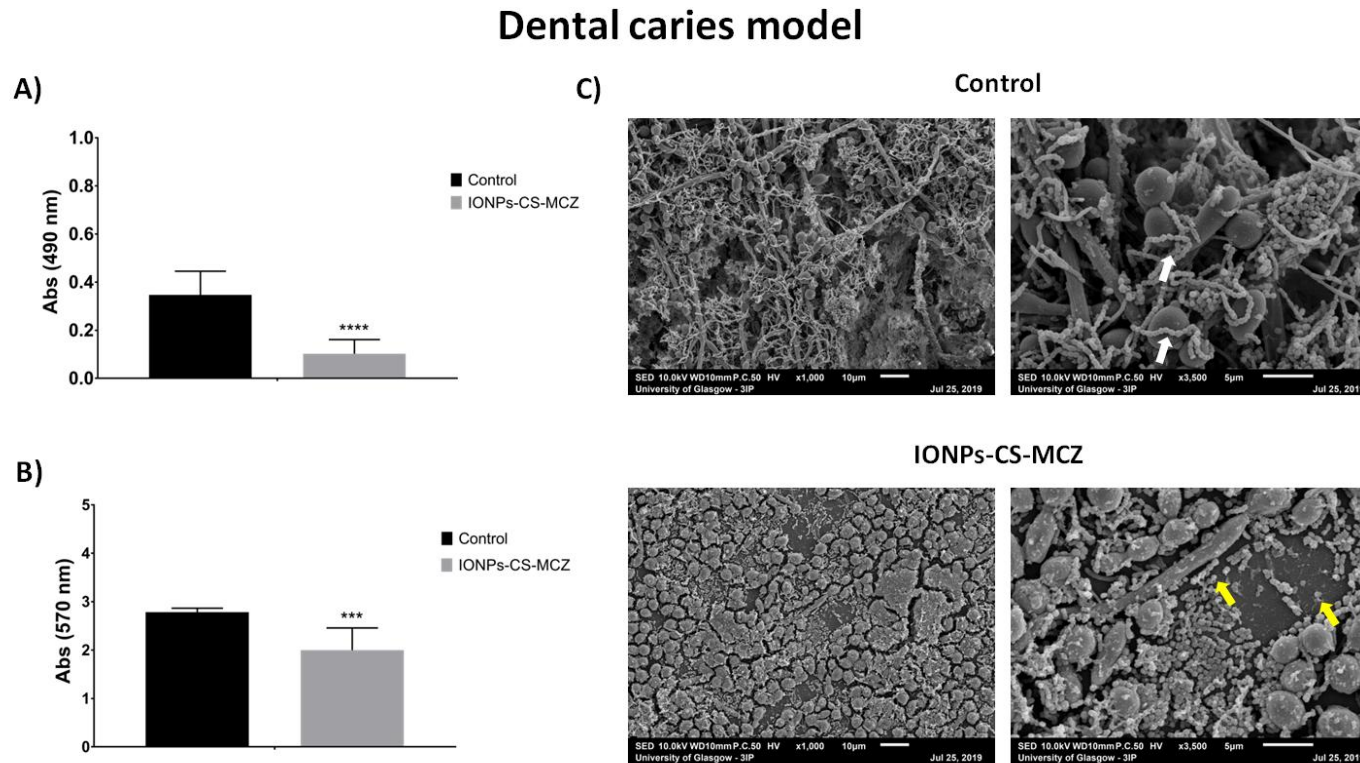


Figure 3. Results of XTT reduction assay (A), quantification of total biomass (B) and scanning electron microscopy (SEM) observation (C) for the caries biofilm model non-treated (control) and treated with the nanocarrier containing miconazole at 64 mg/L (IONPs-CS-MCZ). Magnification of the SEM images: 1,000x and 3,500x; Bars: 10 and 5 μ m. Significant differences between the groups were calculated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). White arrows represent adhesion of bacteria to yeasts/hyphae. Yellow arrow represents loss of cellular membrane integrity.

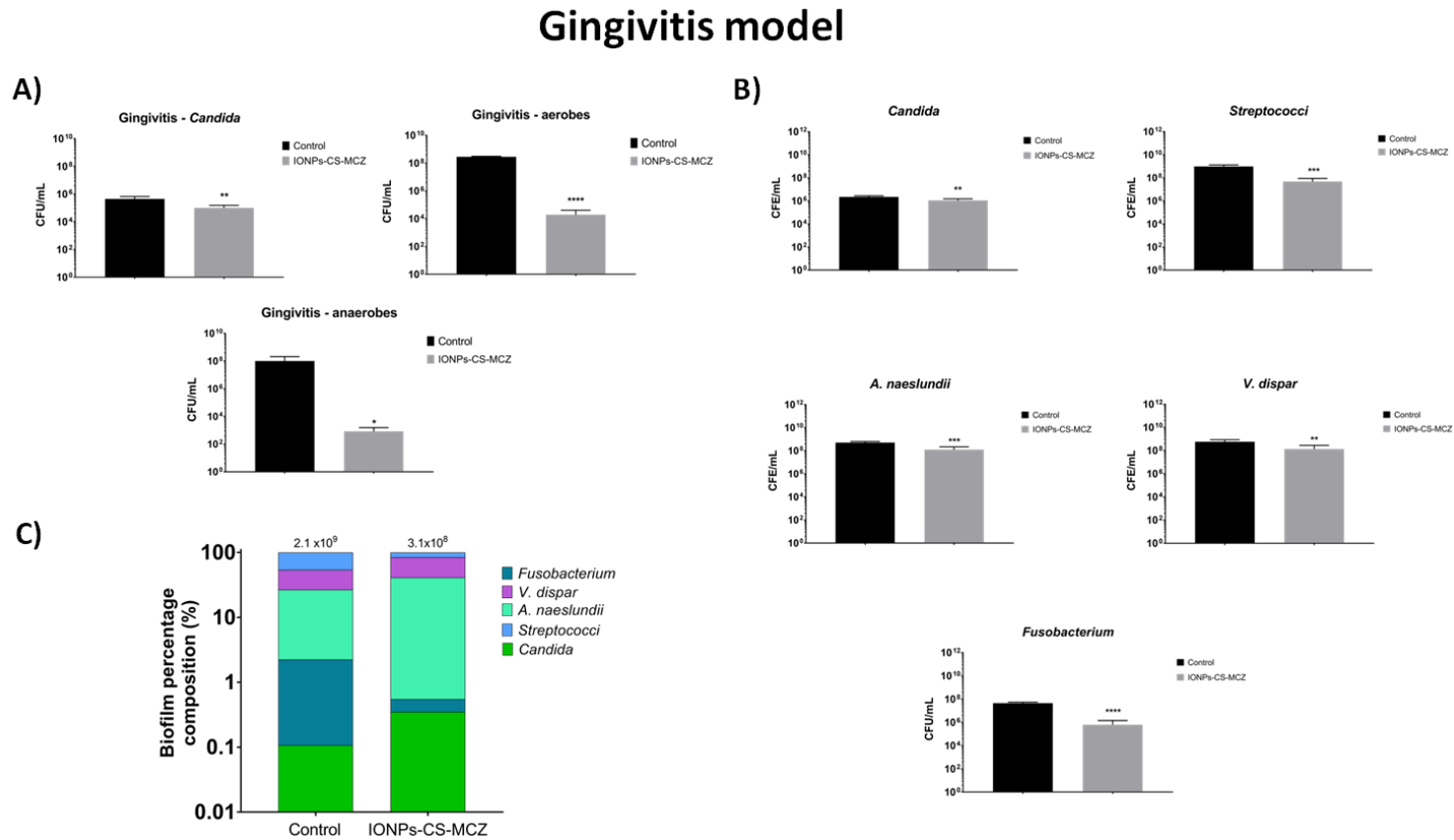


Figure 4. Results of counting of colony forming units (A), colony forming equivalents of viable cells (B) and biofilm percentage composition (C) for gingivitis biofilm model non-treated (control group) and treated with nanocarrier containing 64 mg/L miconazole (IONPs-CS-MCZ). Significant differences between the groups were calculated by unpaired t-test (* p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001).

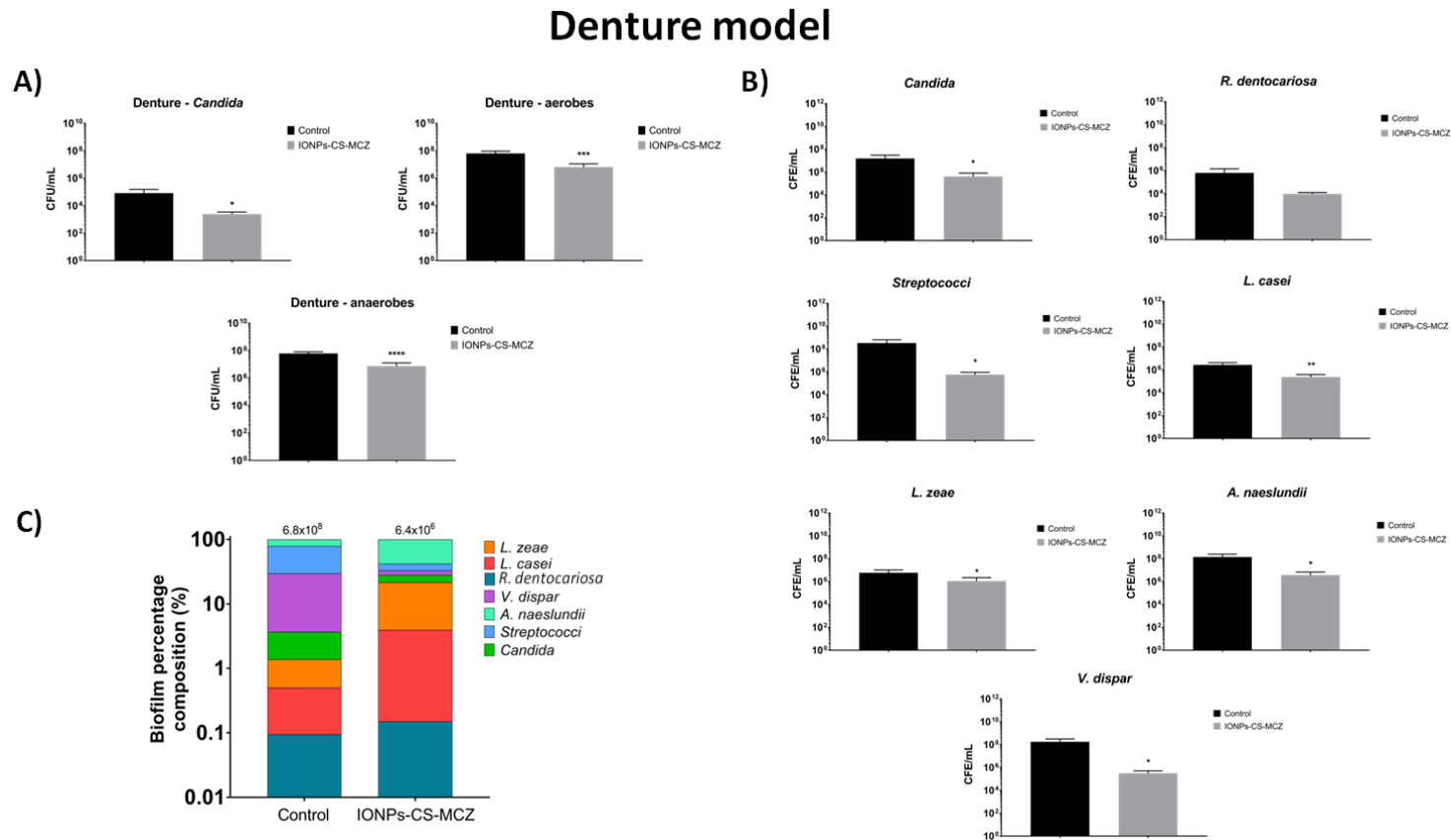


Figure 5. Results of counting of colony forming units (A), colony forming equivalents of viable cells (B) and biofilm percentage composition (C) for denture biofilm model non-treated (control group) and treated with nanocarrier containing 64 mg/L miconazole (IONPs-CS-MCZ). Significant differences between the groups were calculated by unpaired t-test (* p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001).

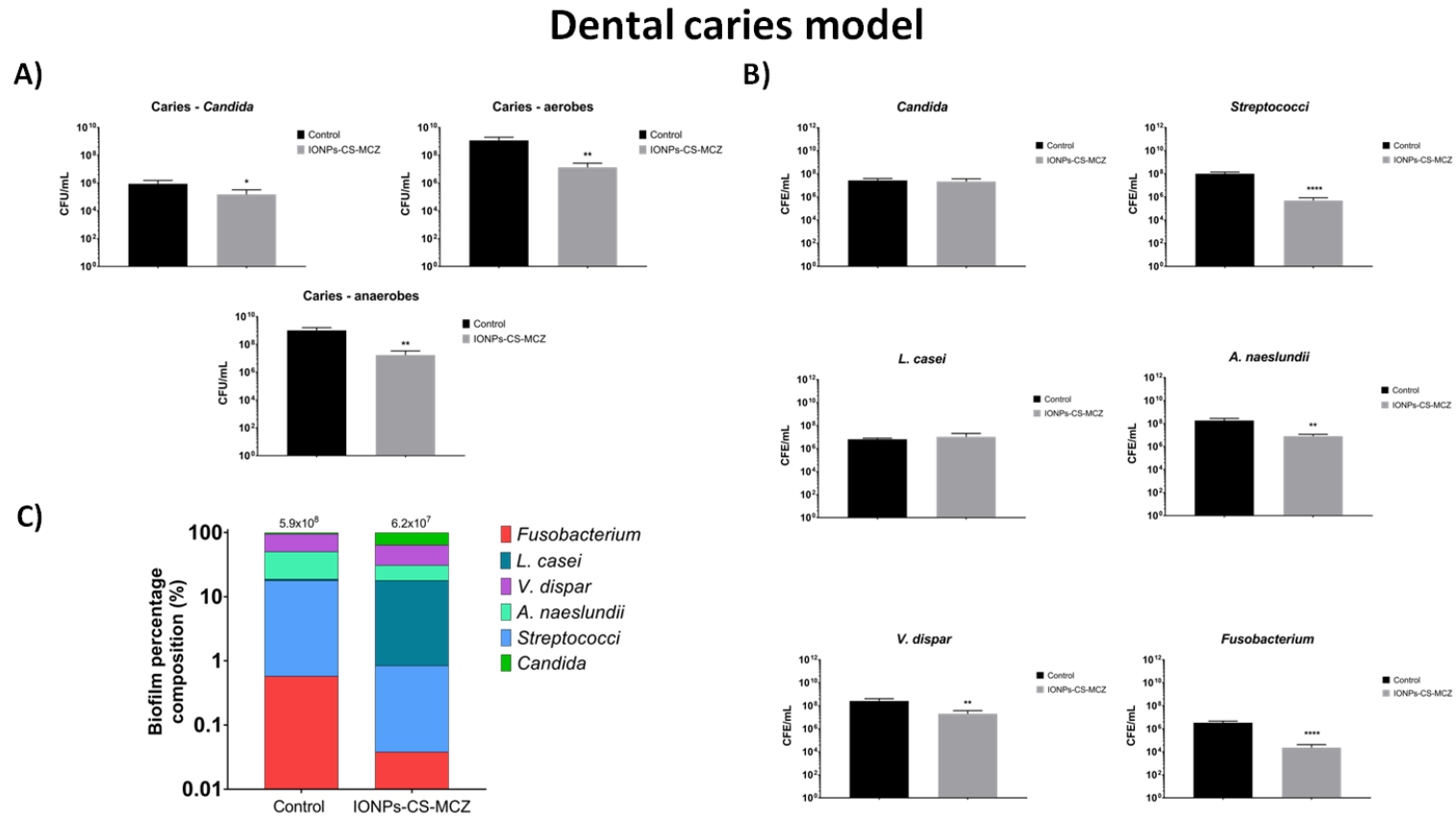


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Anexos

ANEXO A

*Normas da revista na qual será submetido o artigo referente ao **Capítulo 1** desta tese (Colloids and Surfaces B: Biointerfaces).

Author Guidelines (Colloids and Surfaces B: Biointerfaces)

Colloids and Surfaces B: Biointerfaces

An International Journal Devoted to Fundamental and Applied Research on Colloid and Interfacial Phenomena in Relation to Systems of Biological Origin

Editors: Henk Busscher, Hong Chen, Dganit Danino, Deborah Leckband, Alyssa Panitch, Yongfeng Zhou

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