

WILMER RAMÍREZ CARMONA

**Nanocarreador dual de clorexidina e miconazol baseado em
nanopartículas de óxido de ferro revestidas com quitosana:
caracterização físico-química e efeito antibiofilme**

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Tese apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista (Unesp), como parte dos requisitos para a obtenção do título de Doutor em Ciências, área de concentração Saúde Bucal da Criança.

Orientador: Prof. Assist. Dr. Douglas Roberto Monteiro

Coorientador: Prof. Dr. Caio Sampaio

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“Educar é dar ao homem as chaves do mundo, que são a independência e o amor, e prepará-lo com forças para percorrê-lo sozinho, com o passo alegre do homem natural e livre”

José Martí

RESUMO

CARMONA, W. R. **Nanocarreador dual de clorexidina e miconazol baseado em nanopartículas de óxido de ferro revestidas com quitosana: caracterização físico-química e efeito antibiofilme.** 2024. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2024.

Este estudo preparou e caracterizou um novo nanocarreador dual de clorexidina (CLX) e miconazol (MCZ), usando nanopartículas de óxido de ferro (NPsFeO) revestidas com quitosana (QTS), bem como avaliou seu efeito sobre biofilme microcosmo de candidíase bucal. CLX e MCZ foram imobilizados sobre NPsFeO previamente funcionalizadas com QTS. Microscopia eletrônica de transmissão, análise termogravimétrica e espectroscopia de infravermelho com transformada de Fourier foram usadas para caracterizar o novo nanocarreador dual. A concentração inibitória mínima (CIM) do nanocarreador dual sobre cinco isolados clínicos de *Candida albicans* foi avaliada pelo método da microdiluição em caldo, seguida pela determinação do índice de concentração inibitória fracionada. A mistura das salivas de cinco usuários de próteses removíveis com estomatite protética foi o inóculo usado na formação de biofilmes microcosmos. Os biofilmes foram formados por 72 horas sobre espécimes de resina acrílica posicionados no modelo de adesão ativa de Amsterdam e tratados por 24 horas com NPsFeO-QTS carreando, isoladamente, 4 diferentes concentrações de CLX-MCZ (7,8, 19,5, 39 ou 78 µg/mL). NPsFeO, QTS, CLX e MCZ sozinhos foram testados como controles, enquanto o biofilme não tratado foi o controle negativo (CN). Os ensaios quantitativos de biomassa total, atividade metabólica, contagem de unidades formadoras de colônias (UFCs) e produção de ácido láctico foram conduzidos para se avaliar o efeito antibiofilme. A viabilidade celular e a espessura do biofilme também foram analisadas por microscopia confocal de varredura a laser. Todos os resultados foram analisados por ANOVA ou teste de Kruskal-Wallis, seguidos pelo teste *post hoc* de Student-Newman-Keuls ($\alpha=0,05$). O nanocarreador mostrou forma prevalentemente esférica, com diâmetro médio de 6,6 nm. Para todos os isolados clínicos de *C. albicans*, os valores de CIM do nanocarreador dual foram menores do que aqueles encontrados para CLX e MCZ na forma isolada, e o efeito sobre células planctônicas foi classificado como sinérgico. O nanocarreador dual na maior concentração testada (78 µg/mL) aumentou significativamente a biomassa total do biofilme em comparação ao CN. Por outro lado, esse composto reduziu significativamente a atividade metabólica e a produção de ácido láctico, superando o efeito promovido por CLX-MCZ. Para as cargas microbiana total e de *Streptococcus mutans*, o nanocarreador contendo a maior concentração dos antimicrobianos

comportou-se semelhantemente ao composto CLX-MCZ, e ambos levaram a reduções significativas em relação ao CN. Por microscopia confocal, o nanocarreador dual e CLX-MCZ reduziram e aumentaram significativamente a viabilidade celular e a espessura do biofilme polimicrobiano, respectivamente, comparados ao CN. Conclui-se que o nanocarreador dual foi efetivo na inibição de células planctônicas de *C. albicans*. Esse composto também mostrou efeito antimicrobiano sobre biofilme microcosmo de candidíase bucal igual ou superior à combinação de CLX com MCZ sem nanocarreador, dependendo do método analisado. A nanoterapia testada neste estudo tem potencial para ser usada em diversas aplicações clínicas em Odontologia.

Palavras-chave: biofilmes; candidíase bucal; clorexidina; miconazol; nanopartículas magnéticas de óxido de ferro; quitosana.

ABSTRACT

CARMONA, W. R. **Dual nanocarrier of chlorhexidine and miconazole based on chitosan-coated iron oxide nanoparticles: physicochemical characterization and antibiofilm effect.**

2024. Thesis (PhD) - Faculty of Dentistry, São Paulo State University, Araçatuba, 2024.

This study prepared and characterized a new dual nanocarrier of chlorhexidine (CHX) and miconazole (MCZ) using chitosan (CS)-coated iron oxide nanoparticles (IONPs), as well as evaluated its effect on an oral candidiasis microcosm biofilm. CHX and MCZ were immobilized on IONPs previously functionalized with CS. Transmission electron microscopy, thermogravimetric analysis, and Fourier transform infrared spectroscopy were used to characterize the novel dual nanocarrier. The minimum inhibitory concentration (MIC) of the dual nanocarrier on five clinical isolates of *Candida albicans* was evaluated by the broth microdilution method, followed by the determination of the fractional inhibitory concentration index. The mixture of saliva from five removable denture wearers with denture stomatitis was the inoculum used in the formation of microcosm biofilms. The biofilms were formed for 72 hours on acrylic resin specimens positioned in the Amsterdam active attachment model and treated for 24 hours with IONPs-CS carrying, alone, 4 different concentrations of CHX-MCZ (7.8, 19.5, 39, or 78 µg/mL). IONPs, CS, CHX, and MCZ alone were tested as controls, while untreated biofilm was the negative control (NC). Quantitative assays of total biomass, metabolic activity, colony-forming unit (CFU) count, and lactic acid production were conducted to evaluate the antibiofilm effect. Cell viability and biofilm thickness were also analyzed by confocal laser scanning microscopy. All results were analyzed by ANOVA or Kruskal-Wallis test, followed by the Student-Newman-Keuls *post hoc* test ($\alpha=0.05$). The nanocarrier showed a predominantly spherical shape, with an average diameter of 6.6 nm. For all clinical isolates of *C. albicans*, the MIC values of the dual nanocarrier were lower than those found for CHX and MCZ in isolated form, and the effect on planktonic cells was classified as synergistic. The dual nanocarrier at the highest concentration tested (78 µg/mL) significantly increased the total biofilm biomass compared to NC. On the other hand, this compound significantly reduced metabolic activity and lactic acid production, surpassing the effect promoted by CHX-MCZ. For the total microorganism and *Streptococcus mutans* loads, the nanocarrier containing the highest concentration of antimicrobials behaved similarly to the CHX-MCZ compound, and both led to significant reductions in CFUs compared to NC. By confocal microscopy, the dual nanocarrier and CHX-MCZ significantly reduced and increased cell viability and polymicrobial biofilm thickness, respectively, compared to NC. It was possible to conclude that the dual

nanocarrier was effective in inhibiting *C. albicans* planktonic cells. This compound also showed an antimicrobial effect on oral candidiasis microcosm biofilm equal to or greater than the combination CHX-MCZ without nanocarrier, depending on the method analyzed. The nanotherapy tested in this study has the potential to be used in several clinical applications in Dentistry.

Keywords: biofilms; chitosan; chlorhexidine; magnetic iron oxide nanoparticles; miconazole; oral candidiasis.

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J and K, respectively. Different lowercase letters in images J and K show significant differences among groups. Data were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* test (for biofilm thickness ($n = 6$; $\alpha = 0.05$); K) or Kruskal-Wallis and Student-Newman-Keuls *post hoc* test (for percentage of dead cells ($n = 6$; $\alpha = 0.05$); J)_____44

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LISTA DE SIGLAS

CFU	colony-forming units
CHX	chlorhexidine
CIM	concentração inibitória mínima
CLSM	confocal laser scanning microscopy
CLX	clorexidina
CN	controle negativo
CS	chitosan
CV	crystal violet
FICI	fractional inhibitory concentration index
FTIR	fourier transform infrared spectroscopy
IONPs	iron oxide nanoparticles
LAc	lactic acid
MCZ	Miconazol/miconazole
MIC	minimum inhibitory concentration
NC	negative control
NPsFeO	nanopartículas de óxido de ferro
QTS	quitosana
TEM	transmission electron microscopy
TG	termogravimetric analysis
UFCs	unidades formadoras de colônias
XTT	sodium 3-[1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate

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Dual nanocarrier of chlorhexidine and miconazole based on chitosan-coated iron oxide nanoparticles: physicochemical characterization and antibiofilm effect*

Abstract

Objectives: This study used chitosan (CS)-coated iron oxide nanoparticles (IONPs) to create a dual nanocarrier of chlorhexidine (CHX) and miconazole (MCZ), and assessed its impact on a denture stomatitis microcosm biofilm.

Methods: CHX and MCZ were immobilized on IONPs functionalized with CS, and the nanocarrier was characterized by physicochemical tests. Biofilms originating from the saliva of denture stomatitis patients were formed on acrylic surfaces and treated with IONPs-CS carrying different concentrations of CHX-MCZ (7.8, 19.5, 39, or 78 µg/mL). Untreated biofilm served as negative control (NC), whereas IONPs, CS, and CHX-MCZ were used as controls. Biomass, metabolism, lactic acid generation, number of cultivable cells, cell viability, and biofilm thickness assays were conducted. ANOVA or Kruskal-Wallis tests were used to examine all results, and then the Student-Newman-Keuls *post hoc* test was applied ($\alpha=0.05$).

Results: The nanocarrier showed spherical shape and diameter of 6.6 nm. The nanocarrier at 78 µg/mL increased the biomass compared to NC. This compound also reduced metabolism and lactic acid generation, surpassing the effect promoted by CHX-MCZ. For the total microorganism and *Streptococcus mutans* loads, IONPs-CS-CHX-MCZ behaved similarly to the CHX-MCZ compound, and both led to significant reductions relative to NC. The nanocarrier reduced and increased cell viability and biofilm thickness, respectively, compared to NC.

Conclusion: The dual nanocarrier showed antibiofilm effect equal to or greater than the combination CHX-MCZ, depending on the variable analyzed.

Clinical significance: This nanotherapy has the potential to be used as a mouthwash, denture cleaning solution, or antibiofilm mucoadhesive coating.

Keywords: Biofilms; Chitosan; Chlorhexidine; Magnetic Iron Oxide Nanoparticles; Miconazole; Oral Candidiasis.

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1. INTRODUCTION

Fungal infections in immunocompromised patients frequently affect the oral cavity, and *Candida albicans* stands out as the main pathogen [1]. Reduced immunity, imbalance of the oral microenvironment, and resistance characteristics are factors that lead to the *Candida* spp. proliferation and the appearance of oral candidiasis [2]. Denture stomatitis is a type of oral candidiasis that affects up to 80% of immunocompromised patients and elderly denture wearers, mainly after denture colonization by *Candida* species and subsequent biofilm formation [3,4]. *C. albicans* forms multilayered polymicrobial biofilms favored by its structure called hyphae. This infectious inflammatory pathology is characterized by persistent redness in the support area of a removable prosthesis, preferably the palatal area [3,4].

The treatment of denture stomatitis consists of relining the denture base with a resilient material, instructions for using and cleaning the dentures, and prescribing antimicrobials. Regarding antimicrobials, chlorhexidine (CHX) at 0.12% and miconazole (MCZ) at 2% are widely used to treat denture stomatitis due to their easy handling and antimicrobial effects [5,6]. However, CHX may cause tooth staining and oral dysbiosis when used daily [5]. Likewise, MCZ may induce adverse effects such as burning, itching, nausea, and vomiting [6]. Furthermore, a growing microbial resistance to traditional antifungal therapies has been observed in recent decades [7]. Resistance to azoles from *C. albicans*, *Candida glabrata*, and *Candida auris*, for example, is due to a lower affinity to the Erg11 protein and the positive regulation of transporter genes in the ergosterol pathway [1]. Specifically, overexpression of Cdr1 and Cdr2 that act on the ABC transporter pump and changes in the enzyme encoded by the *erg11* gene are described as mechanisms of resistance to azoles [1,8]. CHX has also a limited effect on *C. albicans* biofilm, achieving a 50% reduction in metabolic activity at a concentration of 128 µg/mL [9]. In addition, the expression of Als1, Als3 and Hwp1 on the cell surface, secretion of hydrolytic enzymes (aspartyl proteinases, phospholipases, and lipases), and degradation of E-cadherins increase *Candida* antimicrobial resistance [10], making the management of oral candidiasis difficult.

In this scenario of drug-resistant pathogens, new ideas emerge and are being developed seeking to reverse this challenge. Nanotechnology constitutes a real alternative in several fields of science [11,12], including the development of nanopharmaceuticals. In particular, metal nanoparticles have electrical, magnetic, and optical properties, making them capable of carrying drugs more easily into tissues or biofilms [11,13-15]. Iron oxide nanoparticles (IONPs) based

on maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and/or magnetite (Fe_3O_4) particles are used in biomedical applications such as contrast agents for magnetic resonance imaging, drug and gene carriers, therapeutic agents for cancer treatments, magnetic sensing probes for diagnosis, and neoadjuvant therapy for the production of vaccines and antibodies [16,17]. IONPs have a diameter ranging from 1 to 100 nanometers and may be synthesized by aqueous/hydrolytic or non-aqueous/non-hydrolytic routes. The coprecipitation method is a hydrolytic route that has advantages over other methods, especially for its simplicity and easy reproducibility [18]. However, the poor stability of these nanoparticles is an eminent concern and has been controlled with the help of polymers like chitosan (CS), a compound that reduces clumping and maintains nanoparticle properties [19]. CS is biodegradable, biocompatible, non-toxic, and binds extraordinarily well to IONPs, also presenting an antibiofilm effect [19-21].

CS-coated IONPs acting as carriers of CHX or MCZ have been tested on oral pathogens in different biofilm models. For mono- or dual-species biofilms of *C. albicans* and *Streptococcus mutans*, the IONPs-CS-CHX nanocarrier exhibited comparable or higher antimicrobial effects to those found for free (non-carried) CHX [22]. When IONPs-CS carried 156 $\mu\text{g/mL}$ CHX, greater reductions in total biomass and metabolism of mono-species biofilms of *C. glabrata* and *Enterococcus faecalis* were found, compared to free CHX at the same concentration [23]. On the other hand, a nanocarrier of MCZ could surpass the antifungal effects of free MCZ on mono- or dual-species biofilms of *C. albicans* and *C. glabrata*, mainly for the quantification of cultivable cells and metabolism [24]. For a polymicrobial biofilm model that mimicked an oral candidiasis microcosm, the IONPs-CS-MCZ nanocarrier surpassed the antibiofilm effect of free MCZ, generating the greatest reductions in *Candida*, *Streptococcus*, and *Lactobacillus* loads [20].

Based on the results obtained for the isolated nanocarriers of CHX and MCZ, the combination of these two antimicrobials in a single nanocarrier could improve their antibiofilm effect. Furthermore, despite the undeniable advantages of the biofilm models used in the aforementioned studies, microcosm biofilm models originating from the saliva of denture stomatitis patients would potentially be more representative of clinical conditions of dysbiosis found in these patients. Consequently, this study aimed to prepare and characterize a new dual nanocarrier of CHX and MCZ, using CS-coated IONPS, as well as to evaluate its effect on a denture stomatitis microcosm biofilm.

2. MATERIALS AND METHODS

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

IONPs at 5000 $\mu\text{g/mL}$ as colloidal suspension were generously donated by the nChemi company (Engenharia de Materiais, São Carlos, São Paulo, Brazil), while CS ($\text{C}_{12}\text{H}_{24}\text{N}_2\text{O}_9$), CHX ($\text{C}_{22}\text{H}_{30}\text{Cl}_2\text{N}_{10}$), and MCZ ($\text{C}_{18}\text{H}_{14}\text{Cl}_4\text{N}_2\text{O}$) were acquired from Sigma-Aldrich (Saint Louis, MO, USA). To assemble the dual nanocarrier, the protocols already published by Vieira *et al.* [22] and Arias *et al.* [24] were adopted, with slight modifications. Succinctly, the IONPs colloid was diluted in sterile deionized water to obtain a suspension at 1400 $\mu\text{g/mL}$. In turn, a CS solution at 1400 $\mu\text{g/mL}$ was prepared in deionized water containing 2% acetic acid. The CS solution remained under magnetic stirring at room temperature until the polymer was completely solubilized. Next, 5 mL of the CS solution (1400 $\mu\text{g/mL}$) were pipetted into a falcon-type tube containing the same volume of IONP suspension (1400 $\mu\text{g/mL}$), enabling the nanoparticles to be coated with the polymer. The dual nanocarrier was then obtained by incorporating 500 μg of each antimicrobial (CHX and MCZ) per mL of the IONPs-CS system (700 $\mu\text{g/mL}$ IONPs + 700 $\mu\text{g/mL}$ CS), followed by vortexing until complete solubilization of the antimicrobials. Three dual nanocarrier characterization assays were employed, including transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), and thermogravimetric analysis (TG). Sample preparation and methodological details of the characterization assays were previously reported [22,24,25]. The characterization results of IONPs alone and CS-coated IONPs were previously published [22].

2.2. Saliva collection and identification of *Candida* species

This research was previously approved by the Human Research Ethics Committee of the São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil (CAAE: 71285123.5.0000.5420)[†]. Human saliva samples were the same as those used in a previous study [21], in which five volunteers (average age 65.8 years) using removable dentures and suffering from denture stomatitis signed the Informed Consent Form to participate in the study. Additionally, the patient selection criteria for inclusion and exclusion were previously described [21]. After masticatory stimulation with a plastic paraffin film (Sigma-Aldrich) [26],

[†]ANEXO A: PARECER CONSUBSTANCIADO DO CEP

saliva was collected in polypropylene tubes and kept on ice. Volunteers were required not to clean their dentures or remaining teeth the night before and on the day of collection, and were required to wait two hours after their last meal and drink to donate saliva. Likewise, the consumption of alcoholic beverages was prohibited the day before collection [27]. When each volunteer's saliva was obtained, a volume of 100 μ L was deposited onto CHROMagar *Candida* plates (Difco, Difco Laboratories, Becton, Dickinson and Company, France) and incubated at 37 °C for 48 h. This procedure was performed in triplicate. Among *Candida* species, only *C. albicans* was detected in all samples [21]. Saliva samples were mixed with glycerol, pipetted (1 mL) into polypropylene microtubes, and stored at -80 °C [28].

2.3. Effect of the dual nanocarrier on planktonic cells of *C. albicans* clinical isolates

To verify the effect of IONPs-CS-CHX-MCZ on the five clinical isolates of *C. albicans* in the planktonic state, the broth microdilution assay was employed [29]. In summary, two dilutions of the dual nanocarrier suspension were prepared, the first in deionized water (in geometric order) and the second (1:5) in Roswell Park Memorial Institute 1640 culture medium (Sigma-Aldrich). Regarding the fungal inoculum, a suspension of each *C. albicans* strain was prepared in 0.85% saline solution and standardized to turbidity 0.5 on the McFarland scale. Subsequently, two dilutions of the previously obtained fungal suspensions were performed, the first (1:5) in 0.85% saline solution and the second (1:20) in RPMI 1640. In a 96-well U-bottom plate, equal volumes (100 μ L) of each nanocarrier suspension and fungal inoculum were inserted into the wells, totaling a final volume of 200 μ L per well. The *Candida* concentration within each well was $0.5\text{--}2.5 \times 10^3$ colony-forming units (CFUs)/mL. On the other hand, CHX and MCZ conjugated to the nanocarrier presented concentrations ranging from 25 to 0.048 μ g/mL, after the last dilution. The effects of IONPs, CS, CHX, and MCZ in their free forms were also determined as controls. The tested concentrations ranged from 500 to 0.97, 1400 to 2.73, 25 to 0.048, and 64 to 0.12 μ g/mL, respectively for IONPs, CS, CHX, and MCZ. Visual analysis of the plates after incubation for 48 h at 37 °C was used to detect wells with a complete absence of fungal growth, making it possible to determine the minimum inhibitory concentration (MIC). Additionally, the fractional inhibitory concentration index (FICI) was calculated using the MIC values as detailed previously [30]. MIC assays were conducted in triplicate on three different days (n = 9).

2.4. Effect of the dual nanocarrier on denture stomatitis microcosm biofilm

2.4.1. Obtainment, characterization, and sterilization of acrylic resin specimens

Acrylic resin specimens measuring $10 \times 10 \times 3$ mm were obtained by pressing the thermopolymerizable acrylic resin QC-20 (Dentsply Ind. e Com. Ltda, Rio de Janeiro, Brazil) onto stainless steel molds previously included in a metal muffle with type III stone, as described elsewhere [21]. After finishing with sequential grit sandpaper, the resin specimens were immersed in deionized water for two days (37 °C) to release the residual monomer. Then, surface roughness was determined, following previously published parameters [21]. This step was crucial to uniform the acrylic surfaces for subsequent microbiological tests. The specimens exhibited an average roughness of 0.21 ± 0.02 μm . For sterilization, the specimens were first arranged in an upright position on the lid of the Amsterdam Active Attachment (AAA) model. Subsequently, the lid + specimen assembly was subjected to ultraviolet radiation inside a laminar flow for 60 min (30 min/specimen side) [31]. Sterility was verified after 72 h of incubation of the specimens in RPMI 1640 and plating of the resulting medium on Trypticase Soy Agar (TSA; Difco) enriched with 5% fresh sheep blood. The lack of microbial growth on TSA confirmed the effectiveness of this sterilization method.

2.4.2. Microcosm biofilm formation and treatment protocol

The saliva pool from the five patients was diluted (1:15) in McBain culture medium [32] containing 0.2% sucrose to constitute the final microbial inoculum. A volume of 1.5 mL of this inoculum was deposited inside the wells of a 24-well plate, and the lid of the AAA model with the vertically positioned resin specimens was used to cap this plate. The plate + lid set was placed in a bacteriological incubator for 8 h at 37 °C, in microaerophilia (5% CO₂). Afterwards, the AAA model lid was used to close a new 24-well plate containing fresh McBain medium supplemented with 0.2% sucrose. This new set was incubated at 37 °C under microaerophilic conditions (5% CO₂) for 16 h. Subsequently, two new renewals of the culture medium were performed every 24 h, generating a 72-h-old mature microcosm biofilm.

To prepare the treatment suspensions, the dual nanocarrier was diluted in McBain medium supplemented with 0.2% sucrose to reach concentrations of 7.8 (IONPs-CS-CHX7.8-MCZ7.8), 19.5 (IONPs-CS-CHX19.5-MCZ19.5), 39 (IONPs-CS-CHX39-MCZ39) and 78

$\mu\text{g/mL}$ of CHX and MCZ (IONPs-CS-CHX78-MCZ78), giving rise to 4 dual nanocarriers. These experimental groups represent 20-, 50-, 100-, and 200-fold the highest MIC value found ($0.39 \mu\text{g/mL}$) for CHX and MCZ conjugated to the core-shell structure (IONPs-CS). The following compounds were tested as controls: $110 \mu\text{g/mL}$ IONPs, $110 \mu\text{g/mL}$ CS, and CHX + MCZ both at $78 \mu\text{g/mL}$ (CHX-MCZ). The mature biofilm exposed to pure McBain medium constituted the negative control (NC) group. Regarding the treatment, a volume of 1.5 mL of each treatment suspension was pipetted into the wells of a 24-well plate, which was then closed with the AAA model lid containing the resin specimens. The treatment was performed in a microaerophilic environment (5% CO_2) for 24 h at 37°C .

After exposure to the different compounds, the resulting microcosm biofilms were analyzed by quantitative assays of biomass production, metabolic activity, lactic acid production, CFU enumeration, and viability and thickness by confocal microscopy.

2.4.3. Total biomass production

To evaluate the total biomass production, the crystal violet (CV; Sigma-Aldrich) staining assay was applied, as detailed in a previous study [21]. Briefly, biofilms were rinsed once with phosphate-buffered saline (PBS; 0.1 M, pH 7.0) and fixed with 99% methanol (Sigma-Aldrich) for 15 min. After the drying step, the biofilms were stained with 1% CV solution for 5 min. The stained biofilms were then washed with sterile deionized water and immersed in a 33% acetic acid solution (Sigma-Aldrich), and the absorbance was read at 570 nm (absorbance/mL). The final absorbance values were those obtained for the acrylic specimens containing biofilms subtracted from the blanks. The blank values represented the acrylic specimens exposed to McBain medium without human saliva and treatment suspensions.

2.4.4. Assessment of metabolic activity

To assess the metabolism of biofilm cells, the XTT reagent (2,3-(2-methoxy-4-nitro-5-sulfophenyl) 5-[(phenylamino)carbonyl] 2H-tetrazolium hydroxide; Sigma-Aldrich) in combination with phenazine metasulfate (Sigma-Aldrich) was employed, as specified elsewhere [21]. When the treatment period was completed, the biofilms were washed with PBS and placed in contact with a solution of XTT at 150 mg/L + phenazine metasulfate at 10 mg/L . The 24-well plate with biofilms was then wrapped with aluminum foil and placed on an orbital

shaker at 120 rpm for 3 h, at a controlled temperature (37 °C). Finally, the absorbances of the resulting solutions were read at 490 nm (absorbance/mL) and subtracted from the blank values.

2.4.5. Analysis of lactic acid production

This assay was performed using a 24-well plate filled with 1.5 ml of buffered peptone water (BPW) supplemented with 0.2% glucose, which was capped with the lid of the AAA model containing the resin specimens with the resulting biofilms after treatment. The plate + lid assembly was placed in a bacteriological incubator for 3 h at 37 °C in microaerophilic conditions. Subsequently, the optical density at 340 nm of the resulting BPW solution mixed with lactate dehydrogenase (Sigma-Aldrich) was read, as specified by van Loveren *et al.* [33]. Sodium L-lactate (Sigma-Aldrich) solutions at concentrations ranging from 0 to 20 mM were used to construct a standard curve, which allowed for determining the lactate concentrations in the BPW solutions.

2.4.6. Determination of microbial load

The protocols for enumeration of CFUs and supplementation of agar media were based on a previous study [20]. In essence, biofilms were detached from acrylic surfaces by vortexing (1 min) with PBS and the suspensions generated were serially diluted and plated in 4 culture media. To determine total microorganisms, *C. albicans*, *S. mutans*, and *Lactobacillus* spp., biofilm suspensions were plated on TSA with 5% fresh sheep blood, CHROMagar *Candida*, Mitis Salivarius Agar (Difco), and Rogosa Agar (Difco), respectively. CHROMagar *Candida* plates were aerobically incubated, while plates of the other media were incubated in a microaerophilic atmosphere (5% CO₂). After a period of 48-72 h at 37 °C, CFUs were counted and represented as Log₁₀ CFU/mL.

2.4.7. Biofilm viability and thickness analysis by confocal microscopy

After exposure to the treatment compounds, the microcosm biofilms were rinsed once with PBS and immersed in wells of a 24-well plate containing 500 µL of the mixture of the fluorescent dyes SYTO9 and propidium iodide from the FilmTracer™ Live/Dead™ kit (Invitrogen, Life Technologies Corporation, Eugene, OR, USA). After 20 min, the biofilms

were rinsed again with PBS and examined under a confocal microscope (STELLARIS 5, Leica, Germany) at 10× objective. The emission and excitation wavelengths for each dye were detailed previously [24]. This analysis was conducted in duplicate for the NC, CHX-MCZ, and IONPs-CS-CHX78-MCZ78 groups, and three areas of each specimen were analyzed (at center and at two ends; n = 6). The values of green (live cells) and red (dead cells) fluorescence intensity were used to calculate the percentage of dead cells for each experimental group. This was performed according to the following formula: $(\text{red fluorescence intensity} / (\text{green} + \text{red fluorescence intensity})) \times 100$ [20,34]. In addition, 3D images were also obtained, allowing the average thickness of each biofilm to be calculated.

2.5. Statistical Analysis

The quantitative assays of biomass production, metabolic activity, lactic acid production, and CFU enumeration were conducted in triplicate on three different occasions (n = 9). Total biomass, metabolic activity, and lactic acid production data were cubic root transformed. All results passed the normality test (Shapiro-Wilk) and were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* test, except for the data on percentage of dead cells, which did not pass normality and were analyzed by Kruskal-Wallis and Student-Newman-Keuls *post hoc* test. A p-value < 0.05 was assumed to be statistically significant. Version 12.0 of the SigmaPlot program (Systat Software Inc., San José, USA) was used in all analyses.

3. RESULTS

3.1. Characterization of the IONPs-CS-CHX-MCZ dual nanocarrier

According to the TEM image, the dual nanocarrier presented a mostly spherical shape and some particle clusters were also observed (Fig. 1A). The size distribution histogram revealed that the dual nanocarrier had minimum and maximum sizes of 2.8 and 15.3 nm, respectively, with an average of 6.6 nm (Fig. 1B).

The thermal analysis of the nanocarrier was performed in a temperature range from 0 to 800 °C. At 100 °C, the nanocarrier lost 6.4% of its mass, and when the temperature reached 200 °C the loss was 17.8% (Fig. 1C). The mass losses were 40.7 and 57.8%, respectively at 300

and 400 °C. At 500 °C, there was a mass loss of 91.2%, followed by a less pronounced decline in loss, which stabilized around 600 °C. The total mass loss in the temperature range tested was 96.1% (Fig. 1C). Mass losses occurred mainly due to evaporation of residual water and decomposition of organic compounds (polyethylene glycol) on the surface of IONPs (around 100 °C), followed by pyrolytic decomposition of CS (around 300 °C) (Fig. 1C) [35-37]. In turn, the mass loss associated with CHX decomposition occurred in a temperature range from 100 to 400 °C [38]. As for MCZ, mass loss showed two stages of degradation. The first occurred around 200 °C and was associated with $C_3H_3N_2$ and HCl release in gaseous form. At temperatures above 300 °C, the $C_{15}H_{10}Cl_3O$ molecule from MCZ was degraded (Fig. 1C) [39]. For temperatures above 700 °C, mass loss related to the change from maghemite ($\gamma\text{-Fe}_2\text{O}_3$) to hematite ($\alpha\text{-Fe}_2\text{O}_3$) occurred in the IONPs. Since the mass loss of IONPs-CS was 73% [25], the dual nanocarrier presented about 23.1% of CHX and MCZ effectively bound to IONPs-CS. Finally, the IONPs-CS-CHX-MCZ dual nanocarrier remained stable up to 50 °C.

FTIR was used to characterize the dual nanocarrier by identifying its infrared absorption spectrum. Characteristic vibration peaks of IONPs were detected in the range of 540-585 cm^{-1} and at 1337 cm^{-1} , corresponding to the Fe-O bond (Fig. 1E). An additional Fe-O vibrational peak was detected at 530 cm^{-1} [40-42]. Typical CS peaks were also evident, including a peak at 1646 cm^{-1} , representing the NH group, and two peaks at 1419 and 1374 cm^{-1} , corresponding to the primary alcohol groups C-N and C-O, respectively [41] (Fig. 1D). Other CS-related peaks were noted at 3461, 1653, 1599 and 1377 cm^{-1} , indicating the axial elongation of the O-H and N-H bonds, axial deformation of the C=O carbonyl of the acetamide group, angular deformation of the -N-H bond of the amide, and stretching of the -C-N group, respectively [43].

For CHX, characteristic peaks were identified at 3415 cm^{-1} , attributed to O-H stretching, a band at 1612 cm^{-1} corresponding to the stretching vibration of the C=N imine group, and a band at 1638 cm^{-1} assigned to the -C=N bond (Fig. 1D) [43-45]. Regarding MCZ, the characteristic bands observed at 1311, 1510, and 1330 cm^{-1} correspond to the -C-N stretching and C-C stretching vibrations of the imidazole group, CH bending of the imidazole group (aliphatic moiety), and C=N stretching vibration, respectively [39,46,47]. These characterization data proved the successful incorporation of CHX and MCZ and the formation of the dual nanocarrier.

3.2. MIC of the dual nanocarrier on clinical isolates of *C. albicans*

The MIC values for IONPs, CS, CHX, and MCZ in free form were >500, 600-800, 3.12-12.50, and 4-8 µg/mL, respectively (Table 1). When these compounds formed the dual nanocarrier, the MIC values were reduced to 0.13-0.54 µg/mL for IONPs and CS and 0.09-0.39 µg/mL for CHX and MCZ (Table 1). The FICIs ranged from 0.04 to 0.16, showing synergistic effects of the dual nanocarrier on planktonic *C. albicans* isolates (Table 1).

3.3. Quantitative biofilm results

The CV assay outcomes showed that none of the tested compounds reduced the total biofilm biomass. In turn, exposure of the microcosm biofilm to the IONPs-CS-CHX78-MCZ78 nanocarrier led to a significant 41.5% increase in biomass compared to NC ($p = 0.040$; Fig. 2A). In contrast, CHX-MCZ and IONPs-CS-CHX78-MCZ78 promoted reductions of 58.06 ($p = 0.002$) and 82.25% ($p < 0.001$) in biofilm metabolism relative to NC, respectively, and the IONPs-CS-CHX78-MCZ78 nanocarrier surpassed the effect promoted by CHX-MCZ ($p = 0.004$; Fig. 2B).

Regarding lactic acid production, microcosm biofilm treatments with CS, CHX-MCZ, and all dual nanocarriers led to significant reductions ranging from 66.41 to 97.27% relative to NC ($p < 0.001$; Fig. 2C). IONPs-CS-CHX19.5-MCZ19.5, IONPs-CS-CHX39-MCZ39, and IONPs-CS-CHX78-MCZ78 overcame the reducing effect generated by CHX-MCZ ($p \leq 0.019$; Fig. 2C).

For total microbial load, 2.72-log_{10} ($p < 0.001$) and 2.09-log_{10} ($p < 0.001$) CFU reductions compared to NC were seen after biofilm treatment with CHX-MCZ and IONPs-CS-CHX78-MCZ78, respectively, and these two compounds did not differ from each other (Fig. 3A). A similar behavior was found for the *S. mutans* load (Fig. 3C). For *C. albicans* load, however, CHX-MCZ was the only compound capable of generating significant reduction compared to NC (1.9-log_{10} reduction; $p = 0.003$; Fig 3B). No tested compound affected the number of *Lactobacillus* CFU (Fig. 3D).

3.4. Biofilm viability and thickness by confocal microscopy

Two- and three-dimensional images of biofilms from the CHX-MCZ (Fig. 4D and E) and IONPs-CS-CHX78-MCZ78 (Fig. 4G and H) groups revealed denser and more compact

structures, with the presence of larger aggregates of microbial cells, compared to NC (Fig. 4A and B). The percentages of dead cells after exposure to CHX-MCZ and IONPs-CS-CHX78-MCZ78 were 53.53 and 56.86%, respectively, and these values were significantly higher than that found for NC ($p < 0.05$; Fig. 4J). By three-dimensional analysis of the biofilm thickness, it was possible to notice a greater intensity of red fluorescence in the different layers of the biofilm treated with the IONPs-CS-CHX78-MCZ78 nanocarrier than for the NC and CHX-MCZ groups (Fig. 4C, F and I). Compared to NC, increases in biofilm thickness of 37.44 ($p = 0.001$) and 38.71% ($p = 0.002$) were found after exposure to CHX-MCZ and IONPs-CS-CHX78-MCZ78, respectively (Fig. 4K). There were no differences in biofilm viability and thickness between CHX-MCZ and IONPs-CS-CHX78-MCZ78.

4. DISCUSSION

Candida-associated denture stomatitis frequently affects most denture wearers due to imbalances in oral microbial ecology and compromised immunity. In these patients, polymicrobial biofilm formation on denture surfaces presents a major clinical challenge, mainly due to tolerance to traditional antifungals, making it essential to find alternatives for managing oral candidiasis [2-4]. Given this scenario, the nanotherapy consisting of CS-coated IONPs simultaneously carrying CHX and MCZ was tested on *C. albicans* in the planktonic state or forming microcosm biofilms derived from the saliva of denture stomatitis patients.

Physicochemical analysis information confirmed the creation of a stable dual carrier nanosystem (Fig. 1), which was generated through a network of chemical bonds or electrostatic interactions. Likely, the hydrogen bonds between the primary amino group ($-NH_2$) of CS and the oxygen of IONPs, as well as the electrostatic interactions between positively charged CS and negative hydroxyl groups present on the surface of IONPs were responsible for maintaining the CS coating on IONPs [48-50]. When CHX and MCZ were added to IONPs-CS, the antimicrobials bound to the outer layer of the core-shell system, which was formed by CS. CHX established stable chemical bonds with CS from covalent bonds [51,52], primarily between CS amino radicals and CHX imine nitrogen groups [23]. Conversely, MCZ negative charges were electrostatically attracted by CS amino radicals [53,54].

Regarding the impact of the dual nanocarrier on clinical isolates of *C. albicans* in a planktonic state, the MIC outcomes showed synergistic effects (Table 1), indicating that the nanocarrier surpassed the antifungal effects of each compound analyzed in its free form. IONPs

possess molecular mechanisms that deregulate the hypha-specific genes *als1* and *hwp1* in *C. albicans*, preventing their development as planktonic cells [55]. Furthermore, IONPs reduce *erg11* gene expression in fluconazole-resistant *C. albicans* isolates, affecting the ergosterol pathway [55]. On the other hand, a previous study showed that a CS oligosaccharide was successfully incorporated into the cell wall of *Candida* cells and exhibited antifungal activity, harming the integrity of the fungal wall [56]. In this particular case, the mechanism of action of CS was related to its interference with β -(1,3)-glucanase transferases, affecting the assembly of cell wall polysaccharides [56].

CHX, however, exhibits an antimicrobial effect on planktonic *C. albicans* [57]. When this antimicrobial binds to negatively charged groups on the fungal cell wall, inhibition of its synthesis occurs, followed by leakage of intracellular components and microbial death [58]. The mechanism of action of MCZ is associated with 14- α -sterol demethylase inhibition, compromising ergosterol biosynthesis in the cytoplasmic membrane and generating 14- α -methyl sterols accumulation. These methyl sterols disrupt the compact arrangement of the acyl chains of phospholipids, compromising the functions of membrane-bound enzyme systems and consequently inhibiting fungal growth [59,60]. Interestingly, when all these different targets of antimicrobial action discussed above were present in a single compound (IONPs-CS-CHX-MCZ), there was an amplification of antifungal effects, leading to synergistic effects on *C. albicans* planktonic cells.

When the dual nanocarrier was tested on oral candidiasis microcosm biofilms, the evaluated biofilm parameters showed different outcomes (Figs. 2 and 3). The IONPs-CS-CHX78-MCZ78 nanocarrier was found to significantly enhance overall biomass (Fig. 2A) and decrease total microorganism and *S. mutans* loads (Fig. 3) in comparison to NC. Because the CV cannot distinguish between living and dead cells, it readily integrates into the cells and extracellular matrix components of the polymicrobial biofilm, resulting in the greatest overall biomass levels [61,62]. Furthermore, the *C. albicans* cells, which are larger in size and retain more CV than bacterial cells, may have generated more extracellular matrix after exposure to IONPs-CS-CHX78-MCZ78 as a defense mechanism, which raised the total biomass value. This fact is a consequence of the absence of a reducing effect of the dual nanocarrier on the *C. albicans* count in the microcosm biofilm (Fig. 3B). These results coincide with those from confocal microscopy analysis, which showed significant increases in biofilm thickness after exposure to CHX-MCZ and IONPs-CS-CHX78-MCZ78 (Fig. 4K), presumably due to extracellular matrix production as a biofilm defense mechanism. Some defensive mechanisms

include the production of glucan polymers from carbohydrates mediated by glycosyltransferases from *S. mutans*, the fermentative metabolic cooperation between *Lactobacillus* and *S. mutans*, and the production of hyphae and pseudohyphae by *C. albicans*, generating a more robust and complex architecture for the biofilm [63,64].

In contrast, the dual nanocarrier resulted in a significant decrease in biofilm metabolism compared to NC (Fig. 2B), surpassing the effect caused by CHX-MCZ, and these findings are in agreement with those observed for lactic acid (Fig. 2C). Therefore, biofilm cells exposed to the dual nanocarrier generated reduced amounts of lactic acid due to the decrease in their metabolism. The observed reductions in metabolism and acid production may also be attributed to the significant reduction in the *S. mutans* load promoted by the nanocarrier (Fig. 3C), as this bacterium is a large producer of lactic acid. This finding has great clinical relevance since acid production is a crucial virulence factor linked to oral candidiasis microcosm biofilms. The most logical explanation for the best effects of IONPs-CS-CHX78-MCZ78 in reducing metabolism and lactic acid production is due to the synergistic action of the different compounds that make up the dual nanocarrier, resulting from the combination of different mechanisms of action, as mentioned previously for MIC results. Furthermore, the dual nanocarrier may have penetrated more effectively into the deeper layers of the microcosm biofilm [65], altering the pharmacokinetics of the carried antimicrobials and leading to better reducing effects compared to CHX-MCZ. The confocal microscopy findings corroborate this assumption, as in the three-dimensional image of the biofilm treated with the dual nanocarrier (Fig. 4I), a greater intensity of red fluorescence (dead cells) is observed in depth compared to the biofilm treated with CHX-MCZ (Fig. 4F).

As for CFU enumeration, CHX-MCZ and IONPs-CS-CHX78-MCZ78 behaved similarly and led to significant decreases in total microorganism and *S. mutans* loads (Fig. 3A and 3C), and these findings are consistent with those of cell viability by confocal microscopy (Fig. 4J). For this biofilm parameter tested (CFU), these results show that the antibiofilm effect of the dual nanocarrier is dependent on the presence of CHX and MCZ, without the participation of IONPs and CS in the antimicrobial action. CHX-MCZ may have increased the number of metal ions and reactive oxygen species (ROS) within microbial cells. The transcription factors Mac1 (copper), Sfu1, and Sef2 (iron), and the transporter Ctr1 (copper) regulate the intracellular homeostasis of these ions and ROS [66]. Thus, the removal of *mac1*, *sfu1*, or *sef2* genes increases ROS production and susceptibility of *C. albicans* to CHX [66]. In turn, MCZ inhibits

the enzyme lanosterol 14- α -demethylase, which is involved in ergosterol synthesis, compromising the integrity of the fungal cell wall [59].

Specifically for *C. albicans* load, only CHX-MCZ generated a significant reduction (Fig. 3B). This outcome contrasts with a prior investigation into the antibiofilm activity of a dual nanocarrier of CHX and fluconazole (FLZ), which found that IONPs-CS-CHX-FLZ was significantly superior to CHX-FLZ in lowering *C. albicans* and *C. glabrata* loads of the polymicrobial biofilm [25]. In this previous study, saliva from healthy volunteers was supplemented with reference strains of *Candida* [25]. In contrast, the saliva samples used in the present investigation came from denture stomatitis patients, which is why the *C. albicans* isolates in microcosm biofilms were resistant to IONPs-CS-CHX-MCZ. A similar trend was noted for *Lactobacillus* spp., as no treatment was able to affect its load compared to NC (Fig. 3D), which suggests specific resistance characteristics of the clinical isolates present in the saliva of the donors concerning the compounds evaluated.

In the present study, a discrepancy between the results of CFU quantification and metabolic activity was identified. This could be linked to the fact that only three microbial species were quantified in the CFU assay, which could be underestimating the real impact of the dual nanocarrier in lowering cell viability. Therefore, the dual nanocarrier may have overcome the effect of CHX-MCZ on species that were not selected for quantification. *C. albicans*, *S. mutans*, and *Lactobacillus* spp. are known to be closely associated with cariogenic diets frequently found in patients with caries and denture stomatitis [67-69], which is the rationale behind their selection for CFU measurement. *C. albicans* has the ability to interact with other pathogenic microorganisms within biofilms. The synergistic or non-competitive interrelations between these pathogens can be chemical, metabolic, or by direct contact through fungal hyphae [70]. Glycosyltransferases B (GtfB) produced by *Streptococcus* spp. bind to mannans in the cell wall and hyphae of *C. albicans*, allowing the production of glucan and increasing the ability of *S. mutans* to adhere to these fungi. Furthermore, *S. mutans* mutants lacking GtfB and GtfC show a reduced ability to form polymicrobial biofilms with *C. albicans* [70]. Additionally, the contact between *C. albicans* and *Streptococcus* spp. is mediated via the hypha-specific adhesin Als3 and cell wall-associated secreted aspartyl proteinase Sap9 [70]. On the other hand, *Lactobacillus* spp. isolates significantly inhibit *C. albicans* when co-incubated, downregulating the expression of *als3*, *hwp1*, *efg1*, and *cph1* [71]. The downregulation of GtfB of *S. mutans* by *Lactobacillus casei* is also known to be another possible mechanism of interaction between these species [72].

The results described in this work have important clinical implications in the prevention and control of oral fungal diseases in patients wearing dentures. IONPs-CS-CHX78-MCZ78 could be used as an oral mouthwash, as a denture cleaning solution, or as an antibiofilm mucoadhesive coating. However, before translating the research into clinical practice, several additional studies are needed, including the assessment of how the dual nanocarrier impacts other microbial species in the microcosm biofilm and their intra- and interkingdom interactions. Understanding the possible toxic effects on models of reconstituted oral epithelium, in addition to analyzing the antimicrobial efficacy in experimental candidiasis models in rats, is also necessary to open avenues of clinical applications of nanocarriers in Dentistry.

5. CONCLUSION

A dual nanocarrier of CHX and MCZ formed by CS-functionalized IONPs was successfully engineered. This nanotherapy showed a synergistic antimicrobial effect on *C. albicans* strains isolated from denture stomatitis patients, being more effective than CHX and MCZ applied alone. Furthermore, IONPs-CS-CHX78-MCZ78 showed an antimicrobial effect on oral candidiasis microcosm biofilm equal to or greater than the combination CHX-MCZ without nanocarrier, depending on the variable analyzed.

Declaration of Competing Interest

The authors declare that they have no financial interests or personal relationships that could have influenced the results presented in this paper.

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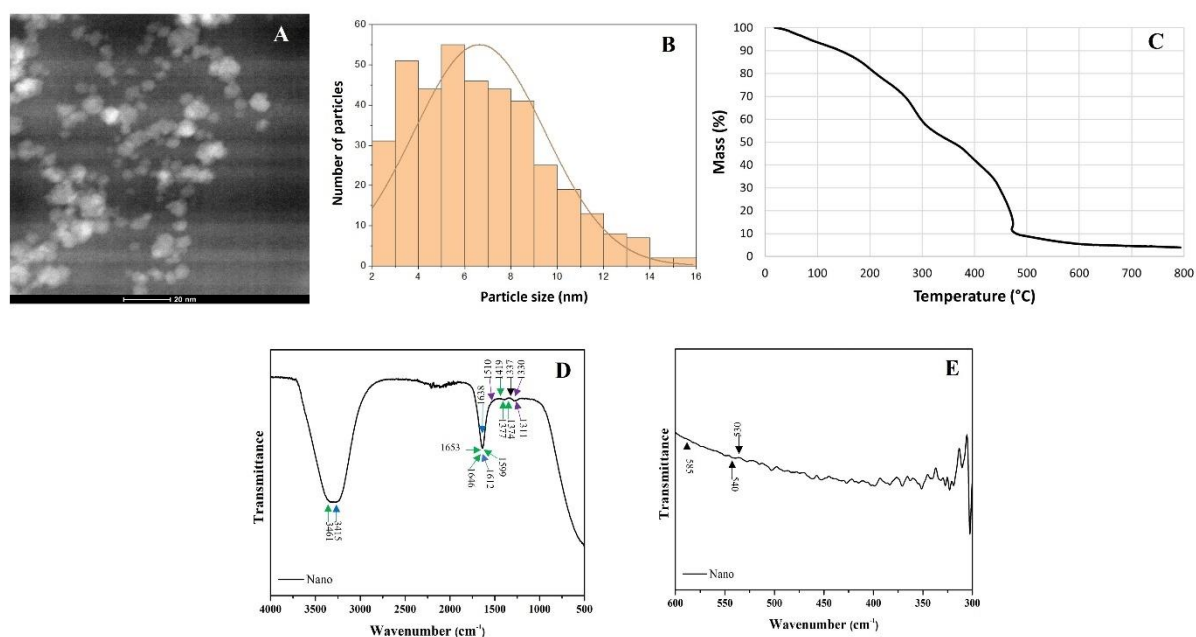


Fig. 1. Physicochemical assays of the dual nanocarrier. Characterization assay of the dual nanocarrier IONPs-CS-CHX-MCZ obtained by transmission electron microscopy (A) and average size of the nanocarrier (B), thermogravimetric analysis (C), and Fourier transform infrared spectroscopy (D and E). The black, green, blue, and purple arrows identify the peaks of IONPs, CS, CHX, and MCZ, respectively.

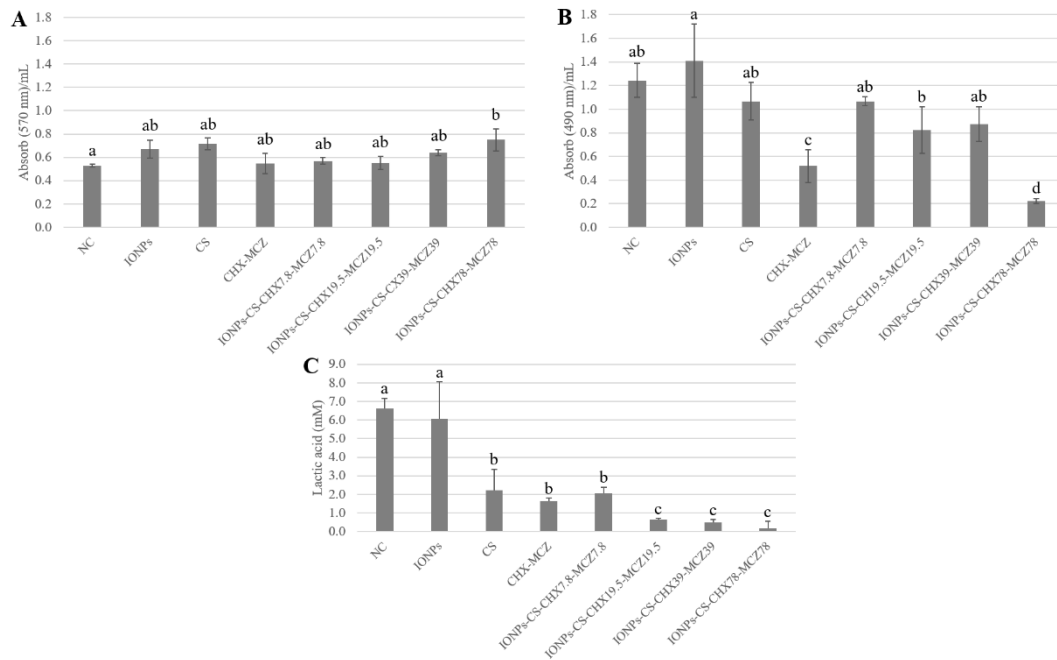


Fig. 2. Effects of the compounds on total biomass (A), metabolic activity (B), and lactic acid production (C) by microcosm biofilms. NC = negative control; IONPs = iron oxide nanoparticles; CS = chitosan; CHX = chlorhexidine; MCZ = miconazole; CHX-MCZ = chlorhexidine-miconazole; IONPs-CS-CHX7.8-MCZ7.8 = dual nanocarrier containing 7.8 $\mu\text{g/mL}$ CHX and 7.8 $\mu\text{g/mL}$ MCZ; IONPs-CS-CHX19.5-MCZ19.5 = dual nanocarrier containing 19.5 $\mu\text{g/mL}$ CHX and 19.5 $\mu\text{g/mL}$ MCZ; IONPs-CS-CHX39-MCZ39 = dual nanocarrier containing 39 $\mu\text{g/mL}$ CHX and 39 $\mu\text{g/mL}$ MCZ; IONPs-CS-CHX78-MCZ78 = dual nanocarrier containing 78 $\mu\text{g/mL}$ CHX and 78 $\mu\text{g/mL}$ MCZ. Different lowercase letters show significant differences among groups (One-way analysis of variance and Student-Newman-Keuls *post hoc* test ($n = 9$; $\alpha = 0.05$)).

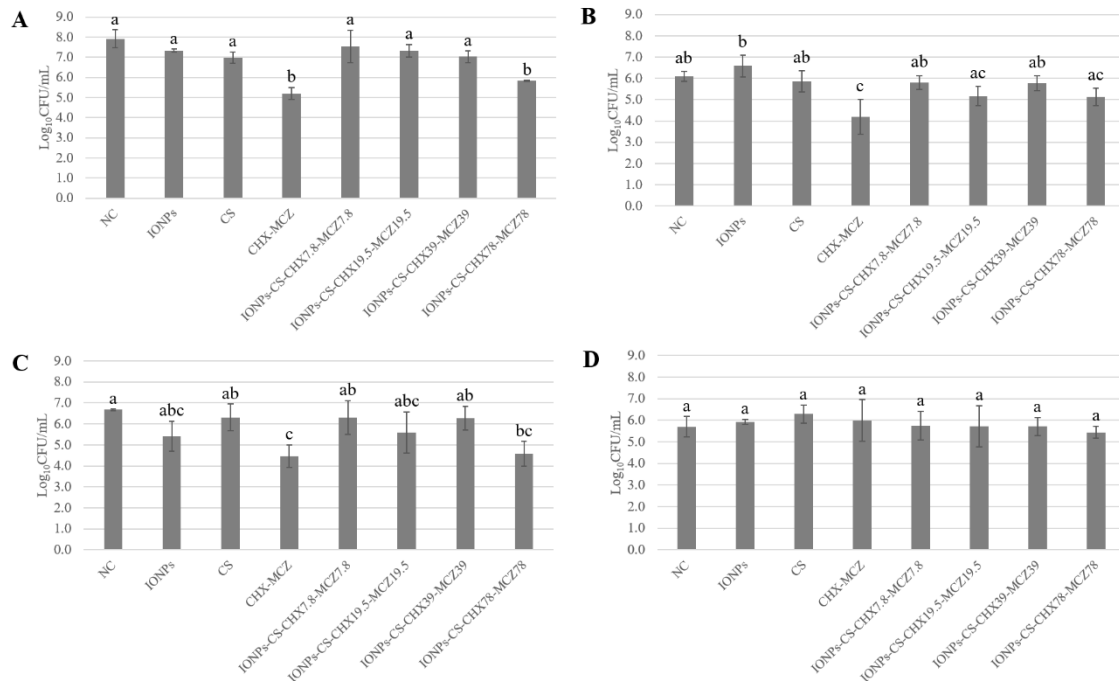


Fig. 3. Effects of the compounds on the number of colony-forming units of total microorganisms (A), *Candida albicans* (B), *Streptococcus mutans* (C), and *Lactobacillus* spp. (D) of microcosm biofilms. NC = negative control; IONPs = iron oxide nanoparticles; CS = chitosan; CHX = chlorhexidine; MCZ = miconazole; CHX-MCZ = chlorhexidine-miconazole; IONPs-CS-CHX7.8-MCZ7.8 = dual nanocarrier containing 7.8 $\mu\text{g/mL}$ CHX and 7.8 $\mu\text{g/mL}$ MCZ; IONPs-CS-CHX19.5-MCZ19.5 = dual nanocarrier containing 19.5 $\mu\text{g/mL}$ CHX and 19.5 $\mu\text{g/mL}$ MCZ; IONPs-CS-CHX39-MCZ39 = dual nanocarrier containing 39 $\mu\text{g/mL}$ CHX and 39 $\mu\text{g/mL}$ MCZ; IONPs-CS-CHX78-MCZ78 = dual nanocarrier containing 78 $\mu\text{g/mL}$ CHX and 78 $\mu\text{g/mL}$ MCZ. Different lowercase letters show significant differences among groups (One-way analysis of variance and Student-Newman-Keuls *post hoc* test ($n = 9$; $\alpha = 0.05$)).

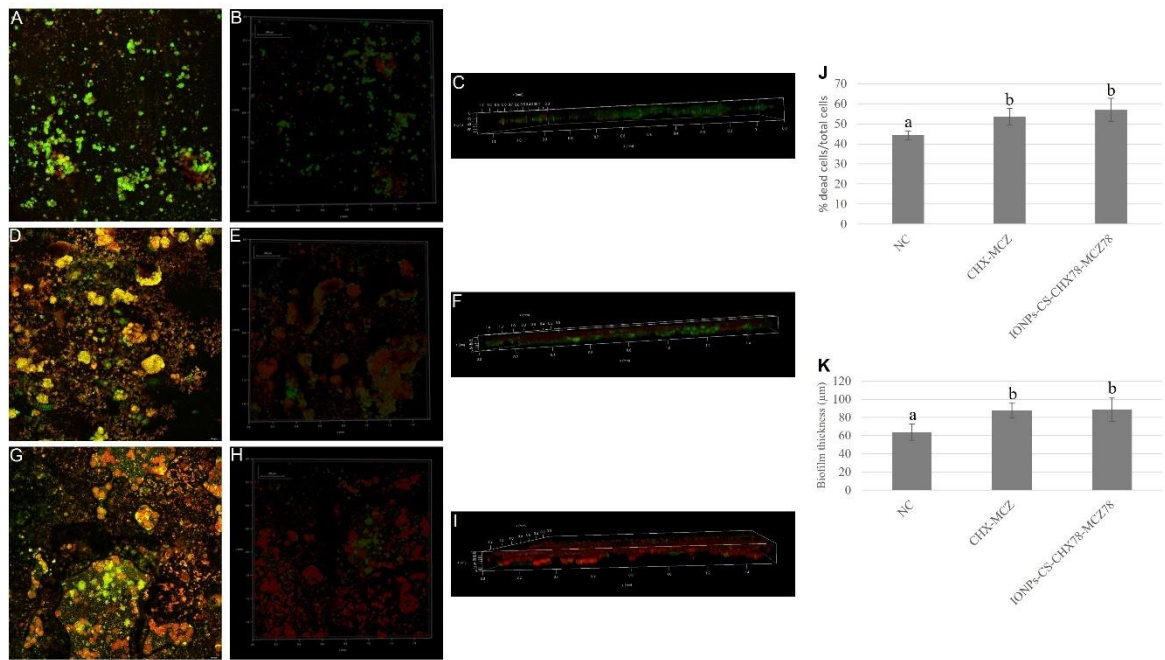


Fig. 4. Confocal microscopy analysis of the microcosm biofilm. Structure and viability of microcosm biofilms from the negative control (NC) group (A, B, and C), biofilm treated with chlorhexidine-miconazole (CHX-MCZ) (D, E, and F), and biofilm treated with the dual nanocarrier containing chlorhexidine and miconazole at 78 $\mu\text{g/mL}$ (IONPs-CS-CHX78-MCZ78) (G, H, and I). Live and dead cells are represented by green and red fluorescence, respectively. Quantitative analyses of cell viability and biofilm thickness are shown in images J and K, respectively. Different lowercase letters in images J and K show significant differences among groups. Data were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* test (for biofilm thickness ($n = 6$; $\alpha = 0.05$); K) or Kruskal-Wallis and Student-Newman-Keuls *post hoc* test (for percentage of dead cells ($n = 6$; $\alpha = 0.05$); J).

Table 1. Minimum inhibitory concentration (MIC) values obtained in the analysis of the susceptibility of *Candida albicans* planktonic cells exposed to iron oxide nanoparticles (IONPs), chitosan (CS), chlorhexidine (CHX), and miconazole (MCZ), alone or as part of the dual nanocarrier.

<i>Candida</i> species/Patient (P)	MIC (µg/mL)									
	Alone				In combination (dual nanocarrier)					
	IONPs	CS	CHX	MCZ	IONPs	CS	CHX	MCZ	FICI	Classification
<i>C. albicans</i> /P ₁	>500	600	6.25	4	0.27	0.27	0.19	0.19	0.07	Synergism
<i>C. albicans</i> /P ₂	>500	600	3.12	4	0.27	0.27	0.19	0.19	0.10	Synergism
<i>C. albicans</i> /P ₃	>500	600	3.12	4	0.13- 0.27	0.13- 0.27	0.09- 0.19	0.09- 0.19	0.10	Synergism
<i>C. albicans</i> /P ₄	>500	800	6.25	4	0.27- 0.54	0.27- 0.54	0.19- 0.39	0.19- 0.39	0.16	Synergism
<i>C. albicans</i> /P ₅	>500	600	12.50	8	0.13- 0.27	0.13- 0.27	0.09- 0.19	0.09- 0.19	0.04	Synergism

FICI: Fractional inhibitory concentration indices for the dual nanocarrier

ANEXO

ANEXO A - PARECER CONSUBSTANCIADO DO CEP

<p>UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"</p>	
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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Nanocarreador dual de clorexidina e miconazol baseado em nanopartículas de óxido de ferro revestidas com quitosana: caracterização físico-química e efeitos antibiofilme.

Pesquisador: Juliano Pelim Pessan

Área Temática:

Versão: 2

CAAE: 71285123.5.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 6.666.487

Apresentação do Projeto:

O nanocarreador IONPs-QTS-CLX-MCZ será obtido por meio da mistura de uma solução de CLX e MCZ com um peso conhecido do sistema IONPs - QTS (40 mg/mL). A caracterização do nanocarreador será realizada pelos métodos físico-químicos de microscopia eletrônica de transmissão, análise termogravimétrica, difração de raios-X e espectroscopia de infravermelho com transformada de Fourier. O efeito antibiofilme do nanocarreador será avaliado sobre biofilmes microcosmos de candidíase oral, os quais serão formados sobre espécimes de resina acrílica, usando o Amsterdam Active Attachment Model. Após 72 horas de formação, os biofilmes serão tratados durante 24 horas com o nanocarreador IONPs-QTS-CLX-MCZ. A combinação de CLX com MCZ (sem IONPs e QTS), IONPs e QTS sozinhas serão usadas como controles. Biofilme não tratado será o controle negativo. O efeito antibiofilme será analisado através da quantificação das células cultiváveis e da biomassa total, produção de ácido láctico e análise da estrutura dos biofilmes. Os dados serão submetidos ao teste de normalidade e, a seguir, será aplicada análise estatística adequada para cada resultado, com nível de significância de 5%. O objetivo do presente estudo é preparar um novo nanocarreador dual de clorexidina (CLX) e miconazol (MCZ) baseado em nanopartículas magnéticas de óxido de ferro (IONPs) revestidas por quitosana (QTS) e avaliar seu

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Continuação do Parecer: 6.666.487

efeito sobre biofilmes microcosmos de candidíase oral. O nanocarreador IONPs-QTS-CLX-MCZ será obtido por meio da mistura de uma solução de CLX e MCZ com um peso conhecido do sistema IONPs- QTS (40 mg/mL). A caracterização do nanocarreador será realizada pelos métodos físico-químicos de microscopia eletrônica de transmissão, análise termogravimétrica, difração de raios-X e espectroscopia de infravermelho com transformada de Fourier. O efeito antibiofilme do nanocarreador será avaliado sobre biofilmes microcosmos de candidíase oral, os quais serão formados sobre espécimes de resina acrílica, usando o Amsterdam Active Attachment Model. Após 72 horas de formação, os biofilmes serão tratados durante 24 horas com o nanocarreador IONPs-QTS-CLX-MCZ. A combinação de CLX com MCZ (sem IONPs e QTS), IONPs e QTS sozinhas serão usadas como controles. Biofilme não tratado será o controle negativo. O efeito antibiofilme será analisado através da quantificação das células cultiváveis e da biomassa total, produção de ácido lático e análise da estrutura dos biofilmes. Os dados serão submetidos ao teste de normalidade e, a seguir, será aplicada análise estatística adequada para cada resultado, com nível de significância de 5%.

Objetivo da Pesquisa:

Objetivo Primário:

O presente trabalho tem os objetivos de preparar um novo nanocarreador dual de CLX e MCZ baseado em IONPs revestidas por QTS e avaliar seu efeito sobre biofilmes microcosmos de candidíase oral.

Avaliação dos Riscos e Benefícios:

Riscos:

Ao participar da pesquisa o voluntário terá o desconforto de mastigar um pedaço de filme plástico flexível e cuspir a saliva em um tubo estéril tipo falcon. Assim, o risco será mínimo.

Benefícios:

Ao participar desta pesquisa os voluntários não terão nenhum benefício direto resultante da mesma. Esperamos que este estudo resulte em informações importantes sobre os efeitos de um nanocarreador dual de clorexidina e miconazol sobre biofilmes microcosmos de candidíase oral. Os pesquisadores se comprometem a divulgar os resultados obtidos na pesquisa.

Comentários e Considerações sobre a Pesquisa:

PESQUISA APRESENTA-SE APTA PARA A SUA REALIZAÇÃO.

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Continuação do Parecer: 5.565.487

Considerações sobre os Termos de apresentação obrigatória:

TODOS OS TERMOS FORAM ADICIONADOS DE ACORDO COM A RESOLUÇÃO 466/12 DO CONSELHO NACIONAL DE SAÚDE.

Recomendações:

NÃO HÁ.

Conclusões ou Pendências e Lista de Inadequações:

PESQUISA APRESENTA-SE APTA PARA A SUA REALIZAÇÃO.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, de-vendo o primeiro relatório ser enviado até 01/08/2024.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_P ROJETO_2178479.pdf	21/02/2024 23:28:52		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	solicitacaodedispensadotcle.pdf	13/07/2023 10:37:30	Juliano Pelim Pessan	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_CEP.pdf	12/07/2023 18:35:58	Juliano Pelim Pessan	Aceito
Declaração de Pesquisadores	Declaracaodepesquisadores.pdf	12/07/2023 16:54:33	Juliano Pelim Pessan	Aceito
Folha de Rosto	Folhaderostro.pdf	12/07/2023 16:47:23	Juliano Pelim Pessan	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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Continuação do Parecer: 6.666.487

ARACATUBA, 23 de Fevereiro de 2024

Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador(a))

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