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Effect of the molecular weight of chitosan on its antifungal activity against *Candida* spp. in planktonic cells and biofilm

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ABSTRACT

Difficulties in the treatment of *Candida* spp. invasive infections are usually related to the formation of biofilms. The aim of this study was to determine the effects of molecular weight (MW) of chitosan (using high (HMW), medium (MMW) and low (LMW) molecular weight chitosan) on *Candida albicans*, *Candida tropicalis* and *Candida parapsilosis sensu stricto*. The deacetylation degree (DD) and molecular weight M were measured by potentiometric titration and viscosimetry, respectively. The planktonic shape activity was quantified by broth microdilution, and the activity against biofilm was quantified by metabolic activity through XTT 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]- 2H-tetrazolium hydroxide and biomass formation (crystal violet). The influence of chitosan MW on the planktonic form of *Candida spp*. was strain dependent. Fungal growth decreased with increasing chitosan MW for *C. tropicalis* and *C. parapsilosis,* while chitosan MW did not modulate the effect for *C. albicans*. With regard to the formation of biofilms, in both the adhesion and mature phases, the biomass and metabolic activities of *Candida* spp. were reduced by about 70% and 80%, respectively for each phase.

1. Introduction

Candida spp. are opportunistic pathogens, commonly isolated in a hospital environment that are responsible for causing systemic infections, mainly in immune-compromised patients. These microorganisms can colonize the surface of implant devices, producing a cellular aggregate embedded within a self-produced matrix of extracellular polymeric substances (EPSs), also known as biofilms. These biofilms are largely associated with infections, limiting the lifetime of the device and increasing the risk of infection (Leonhard & Schneider-Stickler, 2015).

The major *Candida* species associated with candidiasis infections is *C. albicans*, a normal constituent of the human intestinal, oral cavity, and vaginal microflora. *C. albicans* is one of the most important causes of nosocomial fungemia (Lahkar et al., 2017). On the other hand, the number of infections caused by non-*C.albicans* species, such as *C. tropicalis* and *C. parapsilosis sensu stricto*, has also increased, mainly due to their high resistance to azole antifungal agents following biofilm production (Deorukhkar, Saini, & Mathew, 2014). As such, the development of new antifungal agents against *Candida* spp. biofilms has been sought, such examples include essential oils (Souza et al., 2016), flavonoids (Seleem, Pardi, & Murata, 2017), and polysaccharides (Silva-Dias et al., 2014).

Chitosan, a linear polysaccharide, obtained from the partial deacetylation of chitin, has been used against planktonic and biofilm cells for different microorganisms (Costa et al., 2017; Sun, Shi, Wang, Fang, & Huang, 2017). This biopolymer has been largely used as an antimicrobial agent, due to its chemical properties, biocompatibility, biodegradability and low toxicity (Muzzarelli et al., 2012). The antimicrobial activity of chitosan is influenced by a number of factors, one

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of the most important being the molecular weight. Differences in molecular weight can alter the properties of chitosan in two manners: firstly, high molecular weight (HMW) chitosan presents increased adsorption on cell walls, leading to the coverage of cell walls, membrane weakening, disruption and cell leakage; secondly low molecular weight (LMW) chitosan can penetrate living cells, leading to the inhibition of various enzymes and the disruption of protein synthesis, interfacing with the synthesis of mRNA. Chitosan also inhibits microbial growth by the chelation of nutrients and essential metals (Yuan, Lv, Tang, Zhang, & Sun, 2016). Metal ions that combine with the cell wall molecules of the microorganism are fundamental for cell wall stability. Therefore, the chelation of these ions by chitosan has been proposed to represent a possible mechanism of its action.

Kulikov et al. (2014) carried out a study to correlate the molecular weight and antifungal activity of eight oligochitosan samples, with molecular weights in the range 0.73–19.99 kDa, against planktonic cells of *Candida* spp. Authors found that oligochitosans displayed activity against yeast cell multiplication and caused severe cell wall modifications. The anti-biofilm activity of carboxymethyl chitosan was recently demonstrated against non-*C. albicans* species by Tan, Leonhard, Ma, Moser, and Schneider-Stickler (2018), who showed that 2.5 mgL^{-1} of carboxymethyl chitosan inhibited 73.4% of multi-species biofilm formation.

While some studies have correlated the molecular weight of chitosan with its antifungal activity against planktonic cells or biofilm formation, the effects of the molecular weight of chitosan, on antifungal activity against the adhesion and development of *Candida* spp. biofilms are still not well established. Due to the exopolymeric matrix produced by biofilms and the defense mechanisms attributed to these communities, the behavior of chitosan against biofilms is different from that against planktonic cells. The aim of this study was initially to chemically characterize chitosans with regard to their molecular weight and deacetylation degree, and subsequently to investigate their antifungal activity against planktonic cells and biofilms of *C.* albicans, *C. tropicalis* and *C. parapsilosis* sensu stricto. The effect of chitosan on the morphology and structure of mature *C. tropicalis* biofilms was also observed using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Chitosan characterization

2.1.1. Molecular weight (MW) determination

This study used three kinds of chitosan obtained from Sigma-Aldrich (Sigma Chemical Corporation, USA): high molecular weight (HMW – 419419), medium molecular weight (MMW – 448877) and low molecular weight (LMW – 448869) chitosan. The molecular weights of the chitosans were determined by viscosimetry, as previously reported in the literature (Huei & Hwa, 1996). Chitosan samples were prepared in buffer solution (0.2 mol L^{-1} of sodium acetate and 0.3 mol L^{-1} of acetic acid – pH ~4.5). The relative viscosity, η, of chitosan samples was measured using a Canon Fensk capillary viscometer at 30 ± 0.5 °C. Specific viscosity was determined using Eq. (1)

$$\eta_{sp} = (\eta_{solution} - \eta_{solvent})/\eta_{solvent}) \quad (1)$$

Intrinsic viscosity, $[\eta]$, is defined as reduced viscosity, η_{red}, extrapolated to a chitosan concentration, C, of zero by Eq. (2):

$$[\eta] = (\eta_{sp}/C)_{c\rightarrow 0} = (\eta_{red})_{c\rightarrow 0} \quad (2)$$

Viscosity average molecular weight was calculated based on the Mark–Houwink equation Eq. (3):

$$[\eta] = KM_V{}^a \quad (3)$$

with K = 0.074 and a = 0.76 (Rinaudo, Milas, & Le dung, 1993).

2.1.2. Deacetylation degree (DD) determination

Deacetylation degree was determined by potentiometric titration. A known amount (25 mL–0.15 molL^{-1}) of hydrochloric acid solution was mixed with chitosan mass (~ 0.20 g), soaked for 24 h for amino group protonation, and then titrated with 0.1 molL^{-1} sodium hydroxide solution. At each known increase in NaOH volume, the potential in millivolts was measured, to produce a typical potentiometric titration curve. Based on the first derivative of the titration curve, it was possible to observe two inflexion points, which correspond to the volumes required to neutralize the HCl excess and the amino groups protonated in chitosan samples. Using these two inflexion point values from the derivate curve, it was possible to determine the percentage of amino groups on the chitosan chain by Eq. (4) (Vieira & Beppu, 2006).

$$\%NH_2 = \left[\frac{M_{NaOH} \times (V_2 - V_1) \times 161}{W_1}\right] \quad (4)$$

in which M_{NaOH} is the molarity of the NaOH solution (mol L^{-1})), V_1 and V_2 are, respectively, the volume (L) of NaOH used to neutralize the excess of HCl and the volume (L) of the protonated chitosan sample, 161 is the molecular weight of the monomeric unit of chitosan and W_1 is the mass (g) of the sample in a dry state before titration.

2.2. Microorganisms

This study included 6 strains of *C. albicans* and 12 non-C. *albicans* (6 strains of *C. tropicalis* and 6 strains of *C. parapsilosis sensu stricto* from the Fungal Culture Collection of the Specialized Medical Mycology Center (CEMM, Federal University of Ceara). The procedures were performed in a class II biological safety cabinet.

2.3. Preparation of chitosan and drugs

Chitosan solutions (10 mg/ml) were prepared in 1% (w/v) glacial acetic acid 99% (Panreac, Barcelona, Spain) and stored under refrigeration. Control drugs, amphotericin B (AMB) and itraconazole (ITC) (Sigma Chemical Corporation, USA), were prepared with DMSO (Sigma-Aldrich) as solvent, according to the Clinical and Laboratory Standards Institute (CLSI, 2008). Subsequently, AMB and ITC were prepared in RPMI 1640 medium (Sigma, St. Louis) buffered at pH 7.0 with 0.165 M MOPS.

2.4. Susceptibility testing

2.4.1. Planktonic form

The minimum inhibitory concentration (MIC) of HMW, MMW, LMW, AMB and ITC against the *Candida* spp. planktonic cells was determined by a broth microdilution method (de Aguiar Cordeiro et al., 2012; de Medeiros et al., 2017) as described in M27-A3 (CLSI, 2008; CLSI, 2012). *C. parapsilosis* ATCC 22019 was included as a control for each test (CLSI, 2008; CLSI, 2012). All strains were tested in duplicate. Chitosan was used in concentrations of 2–512 μg/ml (Kulikov et al., 2014). The antifungal concentrations, AMB and ITC, ranged from 0.03125–16 μg/ml. For chitosan samples, the MICs were defined as the lowest concentrations able to inhibit 50% ($MIC_{50\%}$), 80% ($MIC_{80\%}$) and 100% ($MIC_{100\%}$) (Brilhante et al., 2014; Gadelha Rocha et al., 2011) of fungal growth, compared to the drug-free control well. For AMB and ITC, the MIC was defined as the lowest drug concentration that inhibited 100% and 50% of fungal growth, respectively (CLSI, 2008).

2.4.2. Biofilm

2.4.2.1. Evaluation of the effect of chitosan on the initial adhesion of Candida spp. biofilms. For biofilm experiment assays (biomass and metabolic activity), the inoculum was prepared as described by

Brilhante et al. (2016). Briefly, *Candida* spp. were cultivated on Sabouraud dextrose agar for 48 h at 30 °C. Subsequently, a loop full of cells was transferred to Sabouraud dextrose broth and incubated for 24 h at 30 °C in a rotary shaker at 150 rpm. The cells were collected by centrifugation (3000 rpm, 10 min) and the pellet was washed twice with PBS. Suspensions were adjusted to 1×10^6 cells/ml in RPMI medium. One-hundred µL of the inoculum was then transferred to flat bottomed 96-well polystyrene plates with 100 µL of chitosan solution. To determine the activity of chitosan against biofilms, the microorganisms were exposed to four different growth conditions; RPMI culture medium with the inoculum, without added chitosan (considered as the positive growth control; Brilhante et al., 2015) and in the presence of three different chitosan concentrations, which were determined based on planktonic cell experiments, the MIC that was able to inhibit 100% of growth ($MIC_{100\%}$), $4xMIC_{100\%}$ and $8xMIC_{100\%}$. The plates were incubated at 37° C for 90 min. The supernatants were then removed and the wells were washed with PBS-Tween 20 (Brilhante et al., 2016). Afterwards, the chitosan effect on biomass and metabolic activity was determined as described previously (Brilhante et al., 2016). All experiments were conducted in triplicate.

For biomass evaluation, the wells were washed three times with PBS (pH = 7.4) and 0.05% (v v^{-1}) Tween 20 to remove non-adhered cells. Subsequently, wells were washed with 100 µL of 100% methanol and the supernatant was aspirated. An aliquot of 100 µL of 0.3% crystal violet (w v^{-1}) was added to each well. After 20 min at 25 °C, the dye solution was aspirated and the wells were washed twice with 200 µL of sterile distilled water. Finally, 150 µL of 33% acetic acid (v v^{-1}) were added to stained wells and left for 30 s. After this time, the volume was transferred to another plate and the optical density (OD) of the acetic acid was immediately measured using a spectrophotometer at 590 nm (Brilhante et al., 2016).

The metabolic activity of the biofilm was evaluated by metabolic assay using (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma). The wells were washed twice with PBS with 0.05% (v v^{-1}) Tween 20 to remove non-adhered cells. One-hundred-and-thirty-one µL of XTT solution (50 µL sterile PBS, 75 µL XTT and 6 µL menadione) (Sigma) were added to each well with the aid of a multichannel pipette and the plates were incubated at 35 °C, for 24 h in the dark, with stirring at 80 rpm. Afterwards, the contents of each well were transferred to another 96-well plate, which was immediately submitted to spectrophotometric reading at 492 nm. RPMI medium was included a negative control in all experiments (Brilhante et al., 2016).

2.4.2.2. Evaluation of the effect of chitosan effect on mature biofilms of Candida spp.. Inocula were prepared as described in Section 2.4.2.1. To allow biofilm formation, aliquots of 200 µL of the fungal suspension were added to flat bottom 96-well plates and incubated for 48 h at 37 °C. The supernatants were then removed and the wells were washed with PBS-Tween 20 (Brilhante et al., 2016). To determine the activity of chitosan against mature biofilms, the biofilms were exposed to four different growth conditions; RPMI culture medium with the inoculum, without added chitosan (considered as the positive growth control; Brilhante et al., 2015) and in the presence of three different chitosan concentrations, which were determined based on planktonic cell experiments, the MIC that was able to inhibit 100% of growth ($MIC_{100\%}$), $4xMIC_{100\%}$ and $8xMIC_{100\%}$. Plates were re-incubated at 37 °C and, after 48 h, the plates were washed with PBS-Tween 20 for the evaluation of biomass and biofilm metabolic activity, as previously described. All experiments were conducted in triplicate.

2.5. Evaluation of the morphology and structure of Candida spp. biofilms

The effect of chitosan of low molecular weight (LMW) on the morphology and structure of *C. tropicalis* mature biofilms was investigated using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Mature biofilms were formed as previously described in the previous section on Thermanox™

2.5.1. Confocal laser scanning microscopy (CLSM)

The *C. tropicalis* biofilms were analyzed by confocal microscopy to correlate the reduction in XTT with visually evaluated effects on biofilm metabolism and structure. The CLSM was analyzed according to Brilhante et al. (2016). Mature biofilms were formed on Thermanox™, as previously described. After incubation, biofilms were washed with PBS and stained using LIVE/DEAD™ fluorescent dye (Invitrogen, USA). The biofilms were analyzed using a Nikon C2 microscope at 488 nm for the detection of SYTO 9 fluorescent dye, which identifies live cells, and at 561 nm for the detection of propidium iodide, which identifies dead or damaged cells. Several sections were obtained in the XY plane at 1 µm intervals along the Z axis. Three-dimensional reconstructions of biofilms were obtained using the resident software and images were processed with Photoshop software (Adobe Systems, San Jose, USA).

2.5.2. Scanning electron microscopy (SEM)

To visualize architectural differences between untreated and chitosan-treated *C. tropicalis* mature biofilms, the *C. tropicalis* biofilms were also evaluated by scanning electron microscopy (SEM), according to the methodology described by Brilhante et al. (2016), with minor modifications. Biofilms were formed directly on Thermanox™ coverslips using 12-well tissue culture plates. After 24 h of growth, the coverslips were washed with PBS and different LMW concentrations ($MIC_{100\%}$, $4xMIC_{100\%}$ and $8xMIC_{100\%}$) were added to the samples.

2.5.3. Statistical analyses

Experimental results were expressed as means ± standard deviations (SD). Student's *t*-Test and one-way analysis of variance (ANOVA) were applied. Differences were considered to be statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Characterization of chitosan

The mean values of chitosan deacetylation degree (Table 1) were 81.8%, 84.2% and 79.0%, for HMW, MMW and LMW, respectively. These are within the range reported in the literature (Kumar, 2000), and within the range informed by Sigma-Aldrich (75–85%). This parameter is important for correlation with antimicrobial activity, since one of the mechanisms of action of chitosan is the interaction of negative cell walls with protonated amino groups from the chitosan chain (Hosseinnejad & Jafari, 2016).

Another important property measured was molecular weight. The values of MW found (Table 1) for HMW, MMW and LMW were 247,795.2 (g mol^{-1}), 140,469.4 (g mol^{-1}) and 75,774.77 (g mol^{-1}), respectively. These values were within the molecular weight range accepted for chitosan, which is 10^4–10^6 g mol^{-1} (Canella & Garcia, 2001). We defined low, medium and high molecular weight chitosan as relating to one type of chitosan or another. This same classification has been used in other studies described in the literature for different

Table 1
Characterization of high, medium and low molecular weight chitosan (degree of deacetylation and molecular weight).

Sample	Degree of deacetylation (% mol)	Molecular weight	
		Intrinsic viscosity (h)	Molecular weight
LMW	79.0 ± 1.0	13.94	75,554.8 ± 0.01
MMW	84.2 ± 0.3	7.90	140,469.4 ± 0.01
HMW	81.8 ± 0.1	4.25	247,795.2 ± 0.03

Table 2
Minimum Inhibitory Concentration (MIC) of high, medium and low molecular weight chitosan against *Candida* spp. in the planktonic form.

Chitosan									
	HMW (μg/ml)			MMW (μg/ml)			LMW (μg/ml)		
Species	50% Range	80% Range	100% Range	50% Range	80% Range	100% Range	50% Range	80% Range	100% Range
C. albicans CEMM-01-05-004	128	256	512	128	256	512	128	256	512
C. albicans CEMM 01-05-005	64	256	512	32	128	512	128	256	512
C. albicans CEMM 01-05-006	128	256	512	128	256	512	128	256	512
C. albicans CEMM 02-01-070	128	256	512	128	256	512	128	256	512
C. albicans CEMM 01-03-037	64	256	512	64	256	512	64	256	512
C. albicans ATCC 10231	64	256	512	64	256	512	64	256	512
C. parapsilosis CEMM-01-01-165	1	2	4	1	2	4	1	2	4
C. parapsilosis CEMM-01-01-193	4	8	16	8	16	32	16	32	128
C. parapsilosis CEMM-05-01- 054	64	128	256	64	128	256	128	256	512
C. parapsilosis CEMM-01-01-168	64	128	256	64	128	256	128	256	512
C. parapsilosis CEMM-01-01-166	8	16	64	8	16	64	16	64	128
C. parapsilosis ATCC 22019	2	4	8	4	8	16	4	8	16
C. tropicalis CEMM-03-06-076	8	16	32	8	16	64	16	128	256
C. tropicalis CEMM-03-06-068	4	8	16	8	32	64	16	128	256
C. tropicalis CEMM-03-06-072	128	256	512	128	256	512	128	256	512
C. tropicalis CEMM-03-06-069	64	128	256	64	128	256	128	256	512
C. tropicalis CEMM-03-06-077	64	128	256	64	128	256	128	256	512
C. tropicalis CEMM-03-06-079	64	128	512	64	128	512	64	128	512

Antifungals						
Species	AMB (μg/ml)			ITC (μg/ml)		
	50%	80%	100%	50%	80%	100%
C. parapsilosis ATCC 22019	–	–	0.03	0.5	–	–

applications. Li et al. (2015) worked with chitosan of 2, 5 and 50 kDa, and considered chitosan to be of low, medium and high molecular weight, respectively. Additionally, Alakayleh et al. (2016) used chitosan of 8 and 88 kDa, and described these as of low and high molecular weight, respectively.

3.2. Planktonic form

The influence of molecular weight on antifungal activity was evaluated and results are presented in Table 2. Data demonstrate that the efficacy of chitosan depends not only on its molecular weight but also on the microorganism studied. Within the same species, significant differences were found between the MIC values for the different chitosans. LMW chitosan presented a MIC range of 4–512 μg/ml for *C.parapsilosis* sensu strictu and HMW chitosan demonstrated a range of 16–512 μg/ml for *C. tropicalis*. Therefore, our study shows that the activity of chitosan is strain-dependent.

The differences found in the MIC values for the different *Candida* species may be based on the composition and negative charge density present in the cell wall. The major antimicrobial mechanism action of chitosan is the electrostatic interaction between the positive charges of the protonated amino groups of chitosan and the negative charges of the cell wall, causing its disruption and the release of intracellular components (Li, Yang, & Yang, 2015; Severino et al., 2015). Palmeira-de-Oliveira et al. (2011) evaluated the surface charge density of *Candida* species and subsequently related a chitosan sensitivity profile. They showed that *C. albicans* has lower a negative charge density at the cell surface, followed by *C. tropicalis* and *C. parapsilosis*, presenting inversely proportional MIC values.

Another study carried out by Palma-Guerrero et al. (2010) demonstrated that chitosan-resistant fungi had lower amounts of unsaturated fatty acids present in the cell membrane. This was demonstrated by testing the antimicrobial activity of chitosan against a mutant of *Neurospora crassa* with a reduced amount of unsaturated fatty acids, leading to a decrease in the antimicrobial activity of chitosan, compared to the wild type of *Neurospora crassa*. These findings demonstrated association with the amount of charges present in the membrane, since larger amounts of fatty acids conferred greater negative charge on the membrane, facilitating the action of chitosan.

Based on our results and in accordance with the results of Palmeira-de-Oliveira et al. (2011) and Palma-Guerrero et al. (2010), we suggest that the strains that presented higher MIC values are chitosan-resistant strains, due to a lower fatty acid content or fewer negative charges in the cell membrane. A large variation in MIC values for the same species were demonstrated by Alburquenque et al. (2010). They found MIC values of low molecular weight chitosan ranging from 4.8–2500 mg/l against strains of *C. glabrata*. This result agrees with the idea that chitosan activity is strain-dependent. The influence of molecular weight on the antifungal activity of chitosan was observed for *C. tropicalis* and *C. parapsilosis sensu stricto,* with the highest activity for the HMW sample. Numerous investigations have suggested that a variation in the molecular weight of chitosan leads to two different mechanisms of action. The mechanism of action high molecular weight chitosan occurs via the deposition of chitosan on the cell wall; since the molecule cannot pass through the membrane, the membrane becomes fragilized and ruptures, resulting in cell leakage. On the other hand, low molecular weight chitosan penetrates the microbial cell and causes the inhibition of some enzymes and the disruption of protein synthesis (Kong, Chen, Xing, & Park, 2010).

The relationship between molecular weight and the antifungal activity of chitosan is not well understood; thus, we aimed to determine the association between the size of the polymer chain of the chitosan and the type of microorganism. With regard to the activity of chitosan against planktonic cells, an increase in molecular weight led to an increase in antifungal activity, although no correlation was observed between molecular weight and the antifungal activity of chitosan against *Candida* spp. Biofilms. Chien and Chou (2006) also demonstrated a relationship between molecular weight and antifungal activity. The authors demonstrated that chitosan of a high molecular weight (MW = 357.3 kDa) and of low molecular weight

(MW = 92.1 kDa), at the concentration of 20,000 µg/ml, was able to inhibit the growth of *Penicillium italicum* by 90.5% and 78.6%, respectively. However, Qiu et al. (2014) observed the converse for the growth of *Fusarium concentricum,* which was inhibited by 89% and 74% for low molecular weight (viscosity of 20 mPa s^{-1}) and high molecular weight (viscosity of 92.5 mPa s^{-1}), respectively. The antifungal activity of chitosan against *B. cinerea* also decreased with increasing molecular weight (Badawy & Rabea, 2009). As such, data show that numerous factors such as fungal species, degree of deacetylation and the molecular weight of chitosan, as well as differences in the methods used to obtain chitosan, can significantly influence the effects of chitosan on antifungal activity.

3.3. Biofilm

One of the major virulence factors of *Candida* spp. is the ability to form biofilms, which are the microbial communities linked to a matrix of extracellular polymer substances that can form on biotic or abiotic surfaces. The biofilm acts as a physical barrier and prevents the entry and expression of the activity of drugs or toxic substances (Araújo, Henriques, & Silva, 2017). Biofilms contribute to therapeutic failure due to their resistance to antimicrobial agents and cause an increase in mortality rates (De Vita et al., 2016). The biofilms of *Candida* spp. present resistance to a broad spectrum of available antifungal drugs. Azoles, including voriconazole, have no activity against pre-formed *Candida* spp. biofilms (Uppuluri et al., 2011). For this reason, studies of the agents that have activity against biofilm are necessary, as are investigations to determine which factors can influence their activity, in order to improve the mode of application of these molecules. Although the activity of chitosan against *Candida* spp. is known, it is important to evaluate the parameters that can influence such activity. We worked with different types of chitosan (high, medium and low molecular weight), different microorganisms *C. albicans* and *C.* non-*albicans*, different stages of biofilm formation (adhesion and mature biofilm) and used different activity parameters of evaluation (biomass and metabolic activity).

3.3.1. Adhesion bioforms

Candida spp. biofilm formation begins with the adherence of round yeast cells to a solid surface, which is crucial for all later stages of biofilm development (Gulati & Nobile, 2016). Fig. 1 represents the effects of HMW, MMW and LMW on biomass (Fig. 1A) and metabolic activity (Fig. 1B) during the adhesion phase of *Candida* spp. biofilm formation (based on the percentage of reduction). Reductions in both biomass and metabolic activity were observed, as compared to the growth of the positive control, for all the concentrations used ($MIC_{100\%}$, $4xMIC_{100\%}$ and $8xMIC_{100\%}$). By increasing the chitosan concentration, progressive reductions in biomass and metabolic activity were observed. At 4 x $MIC_{100\%}$, the reductions were statistically significant (* $p < 0.05$ in comparing the control group) for both biomass and metabolic activity. For HMW, MMW and LMW chitosan, when the concentration was increased to a maximum of $8xMIC_{100\%}$, the biomass and metabolic activities of the *Candida* spp. were inhibited by approximately 70%.

Several factors influence the adhesive capacity of yeasts, including cellular hydrophobicity and electrostatic interactions (zeta potential) between microbial cells and substrate surfaces. The phenomenon of adhesion on inert surfaces (polystyrene) is commanded by the physicochemical properties of yeast cell surfaces (Rotrosen, Calderone, & Edwards, 1986). It is probable that HMW, MMW and LMW chitosan reduce the relative hydrophobicity of the cell surface. Panagoda, Ellepola, and Samaranayake (2001) demonstrated that there is a relationship between the adhesion of microorganisms to buccal epithelial cells and acrylic surfaces and the relative hydrophobicity of the microorganism cell surface. The material used in this study to evaluate biofilm adhesion was polystyrene (PS), while Poly(methyl methacrylate) (PMMA) was used in the study conducted by Panagoda et al. (2001) and different materials may affect the interaction of the yeast cell with the surface. The interactions that occur between the PMMA surface and yeast cells may be of the dipole-dipole type or via hydrogen bonds. Since polystyrene is more hydrophobic than PMMA or cell membranes, it is possible that no charge or dipole interactions occur between the surface and the yeast surface or chitosan. A study carried out with different types of polymers (polytetrafluorethylene, polyethyleneterephthalate and polystyrene) demonstrated that *C. albicans* cells present a greater adhesion on polystyrene surfaces due to its higher hydrophobicity (Klotz, Drutz, & Zajic, 1985). Given that polystyrene favors the adhesion process, the efficacy of chitosan for preventing biofilm adhesion is further supported in this study.

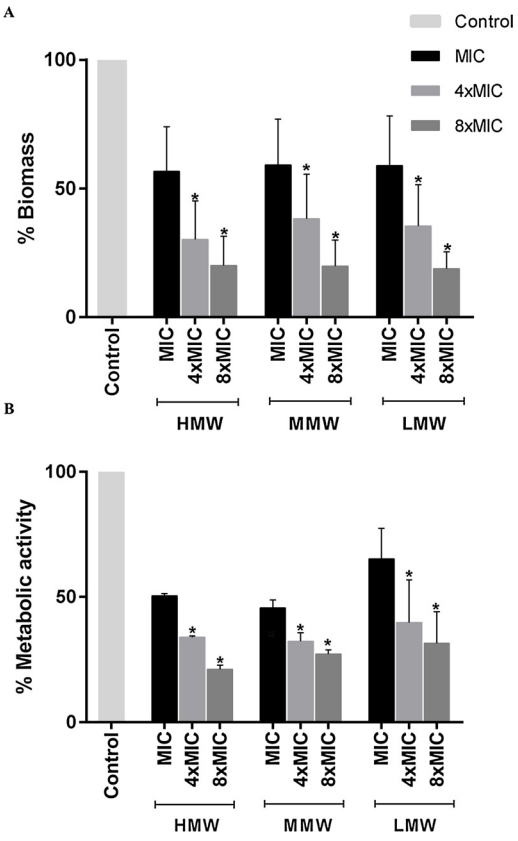

Fig. 1. Inhibitory effect of HMW, MMW and LMW chitosan on the biomass (A) and the metabolic activity (B) of the biofilm adhesion phase of *Candida* spp. Cells were co-incubated in 96-well plates with various concentrations ($MIC_{100\%}$, $4xMIC_{100\%}$, $8xMIC_{100\%}$) of HMW, MMW and LMW chitosan for 90 min and biofilm production was compared to that of fungal cells incubated without chitosan. *, $P < 0.05$, compared to the control groups. Values obtained are given as the percentage of biofilm formation. Results are expressed as mean ± SD.

Another important factor affecting the adhesion of *Candida* is its expression of peripheral proteins called adhesins. Several *Candida* adhesins have been identified and play an important role in adhesion, both on mammalian cells (HeLa cells) and on polystyrene surfaces (Li & Palecek, 2003). Some of these adhesins are present on the surface of the cell wall (Chaffin et al., 1998). Therefore, based on the mechanism of action of chitosan, which consists of inducing cell wall damage, we suggest that chitosan is capable of compromising the cell adhesion process.

The present study revealed the ability of HMW, MMW and LMW chitosan to affect an important virulence factor of *Candida* species, i.e. surface colonization.

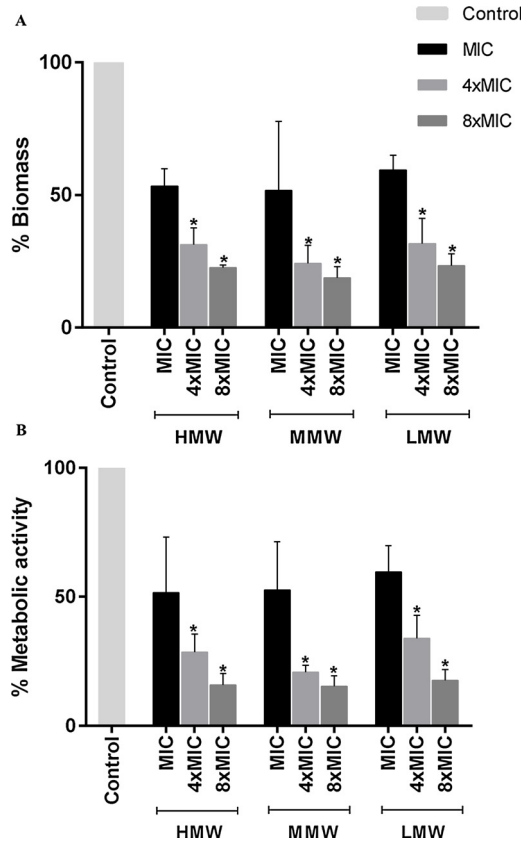

Fig. 2. Inhibitory effects of HMW, MMW and LMW chitosan on the biomass (A) and the metabolic activity (B) of the mature biofilm phase of *Candida* spp. Cells were co-incubated in 96-well plates with various concentrations ($MIC_{100\%}$, $4xMIC_{100\%}$, $8xMIC_{100\%}$) of HMW, MMW and LMW chitosan for 48 h and their biofilm production was compared to that of fungal cells incubated without chitosan. *, $P < 0.05$, compared to the control groups Values obtained are given as the percentage of biofilm formation. Results are expressed as mean ± SD.

3.3.2. Mature biofilms

As shown in Fig. 2, the activities of HMW, MMW and LMW chitosan against mature *Candida* spp. biofilms were measured based on the percentage reduction of biomass (Fig. 2A) and metabolic activity (Fig. 2B). Significant reductions in growth control (compared to biofilms that were not exposed to chitosan) were observed when biofilms were exposed to the concentration of $4xMIC_{100\%}$. The percentage of biomass and metabolic activity decreased in association with the increase in HMW, MMW and LMW chitosan concentrations. At the highest concentration used ($8xMIC_{100\%}$), about 18.7% of the biomass and 15.31% of the metabolic activity were observed, indicating a percentage reduction in these parameters of more than 80% for the three kinds of chitosan studied. The results obtained in this study demonstrated no correlation between molecular weight and the antifungal activity of chitosan against biofilms of *Candida* spp. The three different chitosans showed statistically similar activities against mature biofilms that were independent of molecular weight. These results contrast with those obtained for planktonic cells, since the mature biofilms of *Candida* spp. produce an exopolymeric matrix that hinders the penetration of antimicrobial agents.

The mechanisms of action reported for chitosan activity against the biofilms of Candida spp. are not as well described as they are for planktonic cells. The action of chitosan on the extracellular biofilm matrix can be attributed to the attraction of chitosan, due to its cationic charges, to the exopolymeric components of the biofilm matrix that consists of glycoproteins (55%), carbohydrates (25%), lipids) and nucleic acids (5%) (Nobile & Johnson, 2015; Zarnowski et al., 2014). These components of the matrix have a predominantly anionic character, facilitating the action of chitosan in the biofilm matrix (Donlan & Costerton, 2002). The use of substances capable of destroying the physical integrity of the biofilm matrix is an attractive approach as the consequent loss of the highly protective barrier, represented by the exopolysaccharide matrix, exposes the sessile microbial cells to the antifungal agents. It is believed that this mechanism of action occurs for all the chitosan used in this study, due to the relatively high molecular weight of the chitosans used. Chitosan penetration of the biofilm matrix probably did not occur in this study and the activities of the types of chitosan used were probably mediated by charge effects. Some studies have reported that chitosan has an optimal antimicrobial activity in a range of MW from 10k to 50k. Chitosans in this molecular weight range have a better absorption profile and are able to penetrate the biofilm matrix and reach the cell more efficiently. It is assumed that statistical differences would be found if we worked with chitosans that presented a broader range of MW, which would probably have different mechanisms of action.

With regards to DD, previous studies have shown that the antimicrobial activity of chitosan against planktonic cells increases in association with the increase in DD (Chien, Yen, & Mau, 2016; Chung & Chen, 2008; Tsai, Su, Chen, & Pan, 2002). Increasing the deacetylation degree leads to more available amino groups, increasing the electrostatic interaction with the fungal cell wall. Knowing that the exopolysaccharide matrix of biofilms contains components that give it a negative charge, it is believed that chitosan with higher DDs will also be more effective against biofilms. The DDs of chitosan used in this study were close and it was not possible to evaluate the influence of DD on chitosan activity against biofilms. The close values of DD may have contributed to the similar activities of the chitosans studied herein.

The efficacy of chitosan against biofilms of *C. albicans, C. glabrata, C. parapsilosis, and C. tropicalis* was previously reported by Silva-Dias et al. (2014). The authors showed that chitosan with a low molecular weight (50 kDa), at a concentration of 1×10^4 mg/l, was able to reduce biofilm biomass and metabolic activity for all *Candida* species investigated up to 90%. However, in their study, Silva-Dias et al. (2014) demonstrated only the activity of low molecular weight chitosan. In this study, the activities of medium and high molecular weight chitosan against biofilms of *Candida* spp. were also demonstrated.

3.4. Morphology and structure of biofilms

3.4.1. Confocal laser scanning microscopy (CLSM)

Confocal microscopy was used to correlate XTT reduction assays with the visual effects on biofilm metabolism and structure (Fig. 3A–D). The regions of green fluorescence correspond to metabolically-active cells, while red fluorescence represents metabolically-inactive or non-viable cells. The biofilms of *C. tropicalis* cultivated in the absence of chitosan showed regions of high metabolic activity (Fig. 3A), while biofilms treated with LMW chitosan at concentrations of $MIC_{100\%}$, $4xMIC_{100\%}$ and $8xMIC_{100\%}$ showed a decrease in metabolic activity (Fig. 3(B–D)). The decrease in metabolic activity reflects the stress caused by LMW chitosan in the biofilm

3.4.2. Scanning electron microscopy (SEM)

SEM images were performed to show structural differences between the biofilms of *C.tropicalis* treated with chitosan and those which were untreated (Fig. 3E–H). In the absence of LMW, biofilms of *C. tropicalis* showed blastoconidia, long and short hyphae that were organized in dense structures and composed of multilayers of associated cells, as observed in Fig. 3E. In the presence of chitosan, at the $MIC_{100\%}$ obtained for planktonic cells, a reduced number of cells were observed associated with the biofilms of *C. tropicalis* (Fig. 3F). Biofilms treated with chitosan at concentrations of $4xMIC_{100\%}$ and $8xMIC_{100\%}$ presented wrinkled and collapsed yeast cells (Fig. 3G and H), distinguishing them

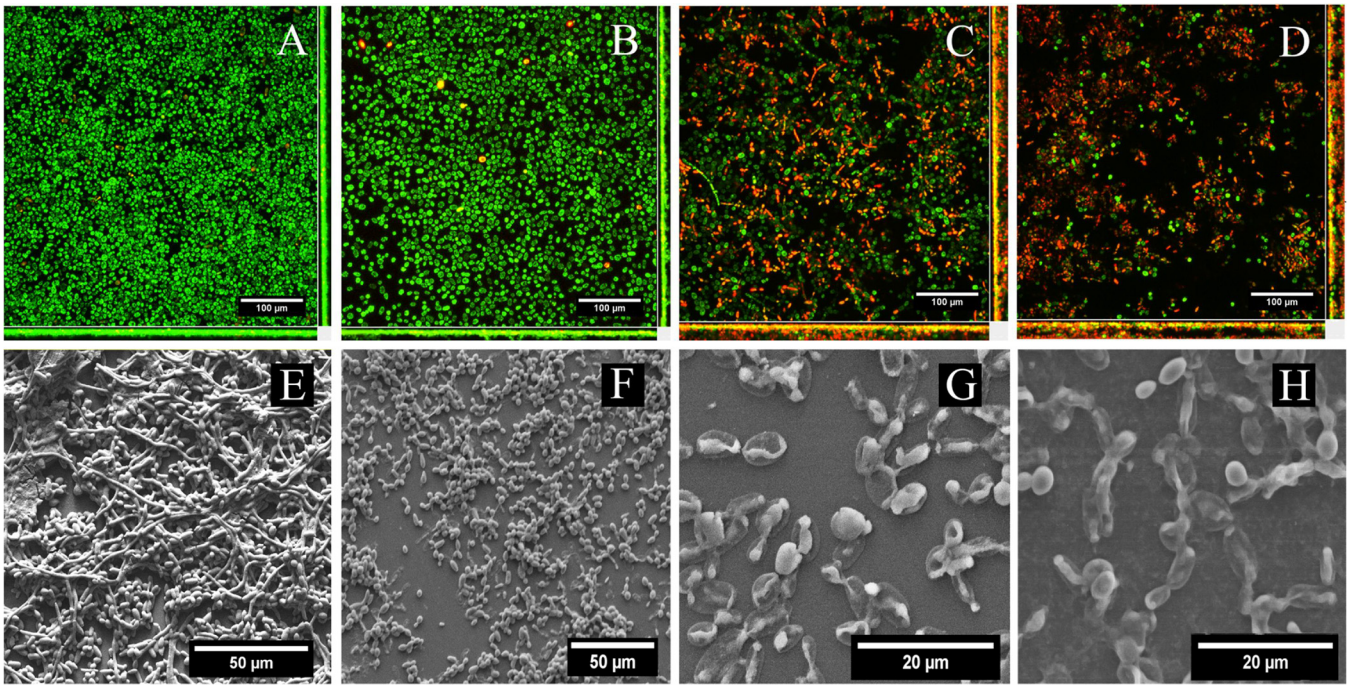

Fig. 3. Confocal microscopy (A–D) images and Scanning electron microscopy (E − H) of *C. tropicalis* biofilm exposed to different concentrations of LMW. Biofilms developed without chitosan (A and E), and biofilms exposed to $MIC_{100\%}$ (B and F), 4x $MIC_{100\%}$ (C and G) and 8x $MIC_{100\%}$ (D and H). The green color corresponds to metabolically-active cells while the red areas represent metabolically-inactive or nonviable cells. Scalebars: 100 µm (Figure A–D), 50 µm (Figure E and F), 20 µm (Fgures G e H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from the regular and smooth surface observed in the yeasts of the control biofilm.

4. Conclusion

With regard to planktonic cells, the effect of MW was observed for the strains of *C. parapsilosis sensu stricto* and *C. tropicalis*, with HMW displaying the highest antimicrobial activity. With regard to biofilms, HMW, MMW and LMW chitosan reduced biomass and metabolic activity both in the adhesion phase and in the mature biofilms. The MW had no influence on the activity of chitosan against biofilms and the three MWs displayed statistically similar effects. Therefore, it can be concluded that chitosan showed promising results in the search for new agents with antifungal activity against *Candida* spp. However, the function of chitosan in biofilm control *in vivo* deserves further study.

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