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MAYARA ITALA GERONIMO DE AZEVEDO

ESTUDO DO MECANISMO DE AÇÃO ANTIFÚNGICA DE QUITOOLIGOSSACARÍDEOS DE QUITOSANA E PROSPECÇÃO DE OUTRAS APLICAÇÕES BIOTECNOLÓGICAS

FORTALEZA
2024

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutora em Bioquímica. Área de concentração: Bioquímica.

Orientador: Prof. Dr. Thalles Barbosa Grangeiro.

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BANCA EXAMINADORA

_____
Prof. Dr. Thalles Barbosa Grangeiro (Orientador)
Universidade Federal do Ceará (UFC)

_____
Prof. Dr. Daniele de Oliveira Bezerra de Sousa
Universidade Federal do Ceará (UFC)

_____
Prof. Dr. Luciana de Siqueira Oliveira
Universidade Federal do Ceará (UFC)

_____
Prof. Dr. Valdirene Moreira Gomes
Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF)

_____
Prof. Dr. Bruno Lopes de Sousa
Universidade Estadual do Ceará (UECE)

À minha família.

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“A ciência e a vida cotidiana não podem e não devem ser separadas” (Rosalind Franklin)

RESUMO

A quitosana é um polissacarídeo natural derivado da quitina que possui diversas aplicações biotecnológicas. Entretanto, a insolubilidade em valores neutros de pH e alta viscosidade são fatores que limitam a aplicabilidade desse polímero. Em contrapartida, os oligossacarídeos obtidos da hidrólise de quitosana, denominados de quitooligossacarídeos (QOS), não possuem as limitações físico-químicas da mesma e apresentam atividades biológicas que podem ter aplicações práticas, como ação antimicrobiana, inseticida, antitumoral, e anti-HIV, bem como elicitação de defesas de plantas. QOS podem ser produzidos por métodos físicos, químicos e enzimáticos, porém o método enzimático é mais eficiente na produção de moléculas com grau de polimerização bem definidos e não traz malefícios ao meio ambiente. O presente trabalho teve como objetivo explorar o potencial de QOS produzidos por via enzimática pela quitosanase *Cv*Csn46 de *Chromobacterium violaceum* frente as seguintes atividades biológicas: ação antifúngica, proteção de frutos e ação inseticida. A investigação sobre a atividade antifúngica contra diferentes espécies de *Candida* (*C. albicans*, *C. krusei*, *C. parapsilosis* e *C. tropicalis*) mostrou que os QOS produzidos apresentaram atividade inibitória contra 8 das 10 estirpes utilizadas, com a MIC variando de 78 a 1.250 µg/mL e atividade letal contra 6 as 10 estirpes, com CLM variando de 156 a 625 µg/mL. Aprofundando as análises com duas espécies não-albicans (*C. krusei* ATCC 6258 e *C. parapsilosis* ATCC 22019), foi observado que o tempo de morte de *C. krusei* quando em contato com QOS na concentração letal (312 µg/mL) é de 4 horas e de *C. parapsilosis* (CLM = 312 µg/mL) é de apenas 2 horas. Resultados dos testes relacionados a formação e degradação de biofilme apontaram que apesar de QOS não apresentar efeito sobre a formação do biofilme das duas espécies testadas, essas moléculas foram capazes de degradar 41.5% do biofilme de *C. krusei* e 22.5% do biofilme de *C. parapsilosis* quando o dobro da MIC foi utilizado. Análises sobre o modo de ação dos QOS mostraram que essas moléculas afetam severamente a membrana celular dessas leveduras, além de estimular a produção de espécies reativas de oxigênio e provocar danos morfológicos, evidenciados por análises de citometria de fluxo, microscopia de fluorescência e microscopia eletrônica de varredura. Nas análises sobre a capacidade de QOS na proteção de frutos na fase de pós-colheita, foi utilizado o melão Cantaloupe minimamente processado. As frutas tratadas com QOS (1 g/L) foram capazes de manter a sua firmeza, pH e conteúdo de vitamina C quando comparadas com o controle, além de demonstrar melhor capacidade antioxidante total e enzimática antioxidante. No que concerne ao efeito inseticida, apesar de QOS não demonstrar atividade larvicida contra larvas de *Aedes aegypti* quando essas moléculas foram utilizadas

sozinhas, QOS foi capaz de potencializar a atividade larvicida das toxinas Bt. A ação conjunta da CL_{20} (11.01 ng/mL) das toxinas Bt com QOS em diferentes concentrações (de 500 a 32 µg/mL) causaram severos danos ao intestino médio das larvas tratadas. O presente trabalho demonstrou a atividade de quitooligossacarídeos produzidos pela quitosanase de *C. violaceum* contra fungos, na proteção de frutos pós-colheita e contra insetos, evidenciando o potencial de QOS em diversas áreas.

Palavras-chave: espécies de *Candida*; larvicida; defesa de plantas.

ABSTRACT

Chitosan is a natural polysaccharide derived from chitin that has several biotechnological applications. However, insolubility at neutral pH values and high viscosity are factors that limit the applicability of this polymer. On the other hand, the oligosaccharides obtained from chitosan hydrolysis, called chitooligosaccharides (QOS), do not have the same physicochemical limitations and present biological activities that can have practical applications, such as antimicrobial, insecticidal, antitumor, and anti-HIV action. as well as elicitation of plant defenses. QOS can be produced by physical, chemical and enzymatic methods, however the enzymatic method is more efficient in producing molecules with a well-defined degree of polymerization and does not cause harm to the environment. The present work aimed to explore the potential of QOS produced enzymatically by chitosanase *Cv*Csn46 from *Chromobacterium violaceum* against the following biological activities: antifungal action, fruit protection and insecticidal action. The investigation into the antifungal activity against different *Candida* species (*C. albicans, C. krusei, C. parapsilosis* and *C. tropicalis*) showed that the QOS produced presented inhibitory activity against 8 of the 10 strains used, with the MIC varying from 78 to 1,250 µg/mL and lethal activity against 6 to 10 strains, with CLM ranging from 156 to 625 µg/mL. Deepening the analyzes with two non-albicans species (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019), it was observed that the death time of *C. krusei* when in contact with QOS at the lethal concentration (312 µg/mL) is 4 hours and for *C. parapsilosis* (CLM = 312 µg/mL) it is only 2 hours. Results of tests related to biofilm formation and degradation showed that although QOS had no effect on biofilm formation in the two species tested, these molecules were able to degrade 41.5% of the *C. krusei* biofilm and 22.5% of the *C. parapsilosis* biofilm when twice the MIC was used. Analysis of the mode of action of QOS showed that these molecules severely affect the cell membrane of these yeasts, in addition to stimulating the production of reactive oxygen species and causing morphological damage, evidenced by flow cytometry, fluorescence microscopy and electron microscopy analyses. scan. In the analyzes on the ability of QOS to protect fruits in the post-harvest phase, minimally processed Cantaloupe melon was used. Fruits treated with QOS (1 g/L) were able to maintain their firmness, pH and vitamin C content when compared to the control, in addition to demonstrating better total antioxidant and enzymatic antioxidant capacity. Regarding the insecticidal effect, although QOS did not demonstrate larvicidal activity against *Aedes aegypti* larvae when these molecules were used alone, QOS was able to enhance the larvicidal activity of Bt toxins. The joint action of LC_{20} (11.01 ng/mL) of Bt toxins with QOS in different

concentrations (from 500 to 32 µg/mL) caused severe damage to the midgut of treated larvae. The present work demonstrated the activity of chitooligosaccharides produced by C. violaceum chitosanase against fungi, in the protection of post-harvest fruits and against insects, highlighting the potential of QOS in several areas.

Keywords: *Candida* species; plant defense; insecticidal.

SUMÁRIO

1 INTRODUÇÃO

A quitosana é um polissacarídeo natural, desacetilado e catiônico derivado da quitina. A biodegradabilidade, não-toxicidade e não-antigenicidade são características da quitosana que fazem com que esse polissacarídeo seja alvo de diversas aplicações (MUXIKA et al., 2017). Além disso, há vários estudos sobre as atividades biológicas da quitosana, principalmente antibacteriana e antifúngica (KE et al., 2021). Porém, as propriedades físico-químicas da quitosana, como insolubilidade em valores de pH neutros e alta viscosidade, dificultam a sua utilização (TABASSUM; AHMED; ALI, 2021).

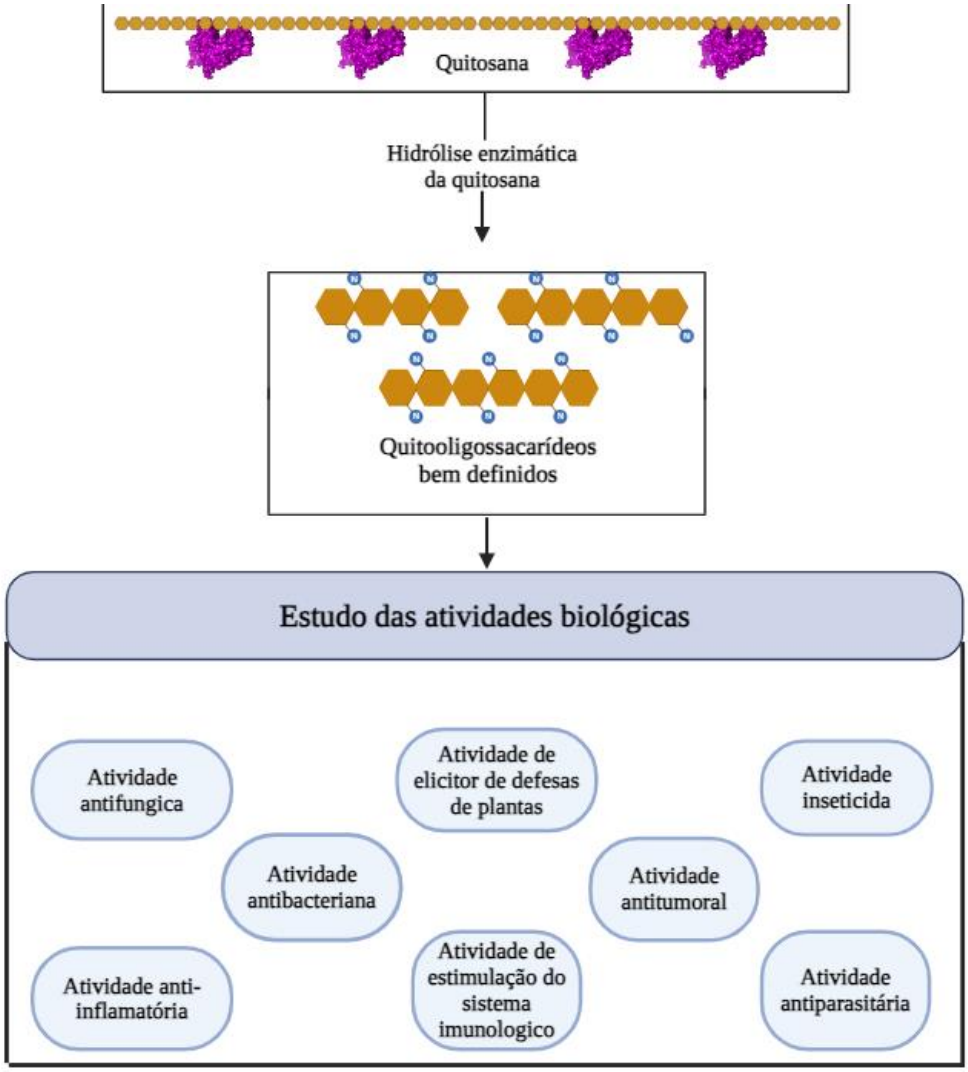
Uma possibilidade para o aproveitamento do potencial biotecnológico da quitosana é a sua hidrólise, transformando-a em oligossacarídeos que, diferente da quitosana, são solúveis em valores de pH neutros e apresentam baixa viscosidade, uma vez que têm baixa massa molecular e apresentam grupamentos NH_2 livres (NAVEED et al., 2019). A produção de quitooligossacarídeos (QOS) em larga escala geralmente é feita por meio de hidrólise química, mas esse tipo de tratamento gera subprodutos tóxicos e produtos não uniformes, fazendo com que a hidrólise enzimática seja uma alternativa (YUAN et al., 2019). QOS também são biodegradáveis, não tóxicos e não alergênicos, além de apresentar atividade antibacteriana, antifúngica, antitumoral, antioxidante, anti-HIV, entre outras. Muitas das atividades biológicas são relacionadas a carga positiva dos QOS, proveniente dos grupamentos NH_2, que são protonados (NH_3^+) em pH < 6,0 (LIANG; SUN; DAI, 2018).

A atividade antifúngica dos QOS é uma das atividades biológicas mais promissoras dessas moléculas, não apenas pela importância das infecções fúngicas, mas também porque o último grupo antifúngico aprovado pela FDA foi caracterizado há 20 anos. O mercado carece de moléculas que possam conter a infecções com sucesso (FAUSTO; RODRIGUES; COELHO, 2019). A ação de QOS contra fungos já foi reportada em vários trabalhos, tanto contra fungos fitopatogênicos como patogênicos a humanos. Entretanto, ainda há divergências

em relação ao potencial de QOS em ser um agente antifúngico devido à falta de caracterização das moléculas utilizadas e das condições em que os ensaios foram realizados. Por isso, é necessário que tanto a caracterização de QOS quanto as condições de atividade serão bem descritas e estudadas (LIAQAT; ELTEM, 2018).

Em 2020, uma quitosanase de *Chromobacterium violaceum* se mostrou capaz de produzir QOS bem definidos e que demonstraram atividade contra o fungo fitopatogênico *Lasiodiplodia theobromae*, provando o potencial dessa enzima em produzir QOS bioativos (AZEVEDO *et al.*, 2020). Sabendo disso e potencial de QOS contra fungos, além das suas outras potenciais aplicações biotecnológicas (FIGURA 1), o presente trabalho teve como objetivo avaliar a ação de QOS produzidos por via enzimática com a quitosanase de *C. violaceum* contra diferentes espécies de *Candida*, assim como explorar outras aplicações dessas moléculas, como proteção de frutos na fase pós-colheita e ação inseticida.

Figura 1. Estudo das potenciais atividade biológicas de quitoologissacarídeos produzidos por via enzimática.



Fonte: Própria autora.

2 REVISÃO DE LITERATURA

A revisão de literatura do presente trabalho está dividida em duas partes:

- A primeira parte (Tópicos 2.1, 2.2 e 2.3) está relacionada a todos os capítulos da tese, oferecendo informações necessárias para o entendimento da razão dos quitooligossacarídeos produzidos por via enzimática terem sido escolhidos como as moléculas de estudo do trabalho.
- A segunda parte (Tópicos 2.4, 2.5 e 2.6) o oferece a base para cada um dos três capítulos da tese, onde o tópico 2.4 está relacionado ao capítulo I, o tópico 2.5 está relacionado ao capítulo II e o tópico 2.6 está relacionado a capítulo III.

2.1 Quitosana

O prefixo “bio” no termo “biopolímeros” significa que esse polímero é produzido por seres vivos. O desenvolvimento e uso de biopolímeros gerou uma competição em relação aos polímeros baseados em combustíveis fósseis em termos de funcionalidades e custos e, nos últimos tempos, utilizações variáveis de biopolímeros foram realizadas, variando de aditivos e misturas em bioplásticos a higiene pessoal, bens comestíveis e produtos médicos, oferecendo o benefício da degradação ecofriendly (GEORGE *et al.*, 2020). Biopolímeros degradáveis naturais incluem polissacarídeos (tais como celulose, quitina/quitosana, amido) e polímeros à base de proteínas (por exemplo, colágeno, gelatina e albumina) (RANGANATHAN *et al.*, 2020).

A quitosana é um polissacarídeo biodegradável, não antigênico, não tóxico e biocompatível derivado da quitina, constituído por unidades de β-*D*-glicosamina e N-acetil-β-*D*-glicosamina conectadas por ligações β-1,4. Mesmo tendo essa definição, a maioria das unidades que constituem esse polímero são de β-*D*-glicosamina, sendo a fração molar desse monossacarídeo definida como grau de desacetilação. O grau de desacetilação (GD) da quitosana é o que a difere da quitina, polissacarídeo formado por unidades de N-acetil-β-*D*-

glicosamina, sendo um parâmetro crítico na avaliação das características da quitosana (KOU; PETERS; MUCALO, 2021). Pode ser encontrada na parede celular de fungos, principalmente do filo Zygomycota, mas é geralmente obtida em grande quantidade pela desacetilação química da quitina (DE LIMA BATISTA; DE SOUZA NETO; DE SOUZA PAIVA, 2018).

A quitosana é um polímero catiônico insolúvel em água e em solventes orgânicos, sendo solúvel apenas em soluções ácidas. A solubilidade e viscosidade desse polissacarídeo é diretamente afetada pelo grau de desacetilação e peso molecular: quanto maior o GD e menor o peso molecular, mais fácil se torna sua solubilização. Um alto peso molecular faz com que o número de ligações de hidrogênio intra e intermolecular seja maior, tornando as cadeias poliméricas emaranhadas entre si. Além disso, muitos grupos acetil também favorecem interações moleculares que formam agregados mais difíceis de dissolver (ROY *et al.*, 2017; WANG, Wenjie; XUE; MAO, 2020).

Muitos estudos têm mostrado que a quitosana possui uma potente atividade antioxidante e antimicrobiana, além de já ser usada em várias aplicações biomédicas e biológicas, incluindo transporte de drogas, tratamento de água e na engenharia de tecidos (ABD EL-HACK *et al.*, 2020). Também foi relatado que a quitosana tem propriedades de cicatrização de feridas e pode ser utilizada no tratamento do solo para biossorção de metais pesados, na imobilização enzimática e ajudar na redução da severidade de doenças fúngicas de plantas quando aplicada no solo (PAL *et al.*, 2021).

Apesar da diversidade de aplicações da quitosana, suas características físico-químicas, como baixa solubilidade em água e alta viscosidade, limitam o uso desse polissacarídeo em vários campos, tornando a hidrólise da quitosana para transformá-la em oligossacarídeos uma alternativa para a exploração do seu potencial biotecnológico (TABASSUM; AHMED; ALI, 2021).

Os principais métodos de hidrólise da quitosana são os químicos e enzimáticos. Os métodos químicos, principalmente a hidrólise ácida, têm sido amplamente utilizados para a transformação em larga escala de quitosana em oligossacarídeos, mas esse tipo de reação traz diversos problemas como a alta utilização de reagentes químicos, geração de produtos não definidos e subprodutos tóxicos. A hidrólise enzimática tem se revelado atrativa pelas suas características positivas: geração de produtos definidos, o processo não envolve o uso de reagentes tóxicos e é facilmente controlável (YUAN *et al.*, 2019).

2.2 Quitosanases

As quitosanases (EC 3.2.1.132) são caracterizadas como enzimas que catalisam a hidrólise de ligações glicosídicas da quitosana para formar quitooligossacarídeos e podem ser encontradas em uma variedade de organismos como bactérias, fungos e plantas. De acordo com informações de suas sequências de aminoácidos e estruturas tridimensionais, essas enzimas estão incluídas em sete famílias de hidrolases de glicosídeos (GH3, GH5, GH7, GH8, GH46, GH75 e GH80), de acordo com a classificação do banco de dados CAZy (Carbohydrate-Active Enzyme database). As famílias que contêm apenas quitosanases são GH46, GH75 e GH80, sendo a família GH46 a mais estudada (CHENG *et al.*, 2021).

A família GH46 foi construída com base na estrutura primária das duas primeiras quitosanases descritas na literatura: as quitosanases de *Bacillus circulans* MHK1 e de *Streptomyces* sp. N174. Essas enzimas apresentam uma catálise ácido-base com um mecanismo de inversão, tendo como aminoácidos catalíticos um resíduo de ácido glutâmico e um outro de ácido aspártico (VIENS; LACOMBE-HARVEY; BRZEZINSKI, 2015). Cada quitosanase tem um produz oligossacarídes com um perfil de polimerização definido dependendo das características da sua ação enzimática, tamanho do seu sítio catalítico, preferências de perfil de desacetilação, ação endo ou exoenzimática e presença de domínio de ligação a carboidratos (SHINYA, Shoko; FUKAMIZO, 2017). Apesar de alguns membros dessa família terem

apresentado atividade antifúngica (PANG et al., 2021), os estudos envolvendo essas enzimas geralmente têm o objetivo de produzir oligossacarídeos de quitosana (DING *et al.*, 2019; LUO *et al.*, 2020; YANG, Guosong *et al.*, 2020).

2.3 Quitooligossacarídeos

Quitosanas com grau de polimerização entre 2 e 20 são consideradas quitooligossacarídeos ou oligossacarídeos de quitosana, e sua composição pode variar em grau de polimerização, grau de acetilação e padrão de acetilação (LIAQAT; ELTEM, 2018). Os quitooligossacarídeos (QOS) têm como principais características solubilidade em meio aquoso a pH neutro, baixa viscosidade, biodegradabilidade, biocompatibilidade e baixa toxicidade, tornando-os mais adequados que a quitosana para aplicações em diversos campos (HAO; LI; LI, 2021; MARMOUZI *et al.*, 2019). Por causa das suas propriedades físico-químicas favoráveis, os QOS e seus derivados já foram testados para aplicações farmacêuticas, produtos nutracêuticos e cosméticos. Esses oligossacarídeos são extensivamente estudados e já apresentaram atividade antimicrobiana, anti-inflamatória, antioxidante, antitumoral, antidiabética, antiobesidade, anti-hipertensiva, anti-HIV, além de estimular o aumento da ação do sistema imunológico (NAVEED *et al.*, 2019).

2.4 Aplicações biotecnológicas dos quitooligossacarideos

2.4.1 Quitooligossacarídeos como agentes antimicrobianos

Os quitooligossacarídeos já são conhecidos por sua atividade antimicrobiana, tanto contra bactérias Gram-positivas como Gram-negativas, bem como contra fungos. Essa atividade está relacionada à massa molecular, grau de desacetilação e pH do meio e natureza catiônica, podendo ser alterada de acordo com essas características (LIANG; SUN; DAI, 2018). O modo de ação dos QOS ainda não é totalmente conhecido, mas há indícios de que as cargas positivas dos grupos NH_2 protonados são responsáveis pela atividade antimicrobiana, podendo agir de duas maneiras: 1) interagindo com os componentes de carga negativa da membrana

plasmática microbiana, alterando sua permeabilidade e impedindo a entrada de solutos essenciais; 2) adentrando a célula, interagindo com o DNA e bloqueando a transcrição (PHIL *et al.*, 2018).

Há vários estudos reportando a atividade antifúngica dos quitooligossacarídeos, tanto contra fungos patogênicos a humanos como contra fitopatógenos (JAFARI *et al.*, 2020). Essa atividade também parece estar ligada a carga positiva dos QOS. Azevedo e colaboradores observaram alterações morfológicas nas hifas de *Lasiodiplodia theobromae* quando esse fungo foi tratado com uma solução de quitooligossacarídeos, o que fez os autores teorizassem que os QOS teriam interagido com os fosfolipídeos da membrana celular do fitopatógeno e impedido o alongamento das células (AZEVEDO *et al.*, 2020).

Ainda há contradições em relação ao potencial antimicrobiano dos QOS devido aos diferentes métodos e microrganismos usados, além da caracterização pobre dos quiooligossacarídeos utilizados nesses estudos. Por essa razão, é necessário que haja uma maior exploração de novas técnicas de produção, purificação e caracterização dessas moléculas (LIAQAT; ELTEM, 2018).

2.4.1.1 Doenças causadas por fungos

As infecções causadas por fungos têm aumentado nos últimos anos, junto com o número de fungos multirresistentes, causando mortalidade significativa principalmente em pacientes imunossuprimidos. Dentre as espécies que causam infecções, as pertencentes aos gêneros *Candida*, *Aspergillus*, *Cryptococcus* e *Pneumocystis* mostram grandes taxas de resistência aos medicamentos utilizados (ARASTEHFAR *et al.*, 2020; CHANG, Zanetta *et al.*, 2019; LOCKHART; GUARNER, 2019). Ademais, as infecções fúngicas também oferecem risco a pacientes com COVID-19, acarretando piora principalmente em casos críticos da doença e tornando o uso de novos antifúngicos imprescindível para o tratamento (LAI; YU, 2021; MARR *et al.*, 2021). Na Índia, o número de mortes por mucormicose ou “fungo preto” entre pacientes recém tratados para COVID-19 está crescendo, levantando um alerta nas autoridades

responsáveis, uma vez que essa doença também pode causar a perda dos olhos dos enfermos (DYER, 2021).

A caracterização do último grupo de antifúngicos aprovado para o uso em humanos foi feita há quase vinte anos, o que faz com que aja poucos antifúngicos no mercado. Isso provavelmente está ligado a falta de investimento em relação as doenças causadas por fungos quando comparado a doenças causadas por bactérias e protozoários, como tuberculose e malária (FAUSTO; RODRIGUES; COELHO, 2019). Isso faz com que haja a necessidade da prospecção e aprovação de novas moléculas com atividade antifúngica (VAN DAELE *et al.*, 2019)

Apesar da necessidade de novos agentes antifúngicos, a utilização de ferramentas químicas e sintéticas para combater esses microrganismos pode acarretar efeitos tóxicos para a planta, levando à deterioração da qualidade do fruto e poluição ambiental, acompanhada de efeitos colaterais na saúde humana, o que faz com que os produtos naturais sejam uma boa alternativa para superar doenças causadas por esses fitopatógenos (SANTRA; BANERJEE, 2020).

2.4.1.2 Leveduras patogênicas

Leveduras são consideradas organismo unicelulares, porém são capazes de ativar diversos processes de diferenciação, mudando o seu fenótipo celular de levedura para pseudo-hifas em resposta a estímulos no ambiente. Esses organismos também são capazes de criar estruturas multicelulares, como colônias e biofilmes (PALKOVÁ; VÁCHOVÁ, 2016). As quatro espécies de leveduras patogênicas que apresentam maior risco para a saúde humana são *Candida*, *Cryptococcus, Malassezia* e *Trichosporon* (TAKASHIMA; SUGITA, 2022). Entre essas quatro espécies citadas, *Candida* e *Cryptococcus* spp são importantes patógenos oportunistas: enquanto *Cryptococcus* spp se aproveitam da baixa imunidade de células de pacientes imunossuprimidos, *Candida* spp dependem do acesso a mucosa e ruptura da pele

(WHITNEY; BICANIC, 2015).

Cryptococcus é um gênero muito difundido na natureza e afeta severamente indivíduos imunossuprimidos, geralmente causando infecções no sistema nervoso. É estimado a taxa de mortalidade dos pacientes com HIV que desenvolvem meningite causada por *Cryptococcus* é de mais de 81% todo ano (YANG, Chen *et al.*, 2022). Já o gênero *Candida* é o mais comum patógeno fúngico de humanos, sendo o responsável por mais de 80% de todas as infecções hospitalares causadas por fungos e tendo a espécie *Candida albicans* como o seu principal representante (PENDLETON; HUFFNAGLE; DICKSON, 2017).

2.4.1.3 Espécies de Candida não-albicans

Apesar da maioria dos casos de infecções fúngicas causadas pelo gênero *Candida* ainda serem relacionadas a *Candida albicans*, o número de infecções envolvendo outras espécies de *Candida* vem aumentando a cada ano. Esse número crescente é uma consequência da seleção de espécies em virtude do uso de antifúngicos, visto que muitas espécies não-albicans apresentam resistência (SILVA, Sónia *et al.*, 2011).

Todas as espécies de *Candida* causam infecções semelhantes, porém existem diversas variações fenotípicas que podem alterar a interação patógeno-hospedeiro, como tamanho celular, morfologia, composição da parede celular e fatores de virulência. Essas variações causam respostas imunes distintas das células que compõem o nosso sistema imunológico, principalmente dos neutrófilos, que são cruciais na resposta imune a infecções sistêmicas causadas por *Candida* spp (WHIBLEY; GAFFEN, 2015). Por essa razão, e da resistência a drogas azólicas geralmente associada a espécies não-albicans, testes de suscetibilidade são frequentemente necessários para o tratamento de infecções causadas por essas espécies, porém, em várias partes do mundo, esses tipos de testes são escassos, o que leva a variação do espectro dessas leveduras assim como os seus perfis de suscetibilidade (SEYOUM; BITEW; MIHRET, 2020).

As espécies não-albicans mais recorrentes são *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, e *C. krusei*. Com *C. glabrata* sendo o causador mais comum de candidíase vulvovaginal e *C. parapsilosis* o maior causador de fungemia em crianças e recém-nascidos. Já *C. tropicalis* e *C. krusei* estão associadas a infecções em pacientes com doenças hematológicas (WHALEY *et al.*, 2017), além disso, *C. krusei* demonstra grande potencial para adquirir resistência a múltiplos antifúngicos em função da sua resistência intrínseca a fluconazol, com mais de 97% dos seus isolados apresentando algum tipo de resistência, e da sua rápida evolução para adquirir resistência a outros antifúngicos (JAMIU *et al.*, 2021). Além disso, *C. auris*, uma nova espécie de *Candida* descoberta em 2009, se tornou uma séria ameaça a saúde humana devido a sua alta taxa de mortalidade (entre 30 e 60%) e multirresistência a antifúngicos (CHOWDHARY; JAIN; CHAUHAN, 2023).

Por essas razões, há uma grande pressão para o estudo de compostos com potencial antifúngico que possam servir como alternativas para o tratamento de infecções causadas por espécies não-albicans (GÓMEZ-GAVIRIA; RAMÍREZ-SOTELO; MORA-MONTES, 2022).

2.4.2 Quitooligossacarídeos como tratamento pós-colheita de frutos

As plantas usam uma resposta inata de defesa para lidar contra patógenos como fungos, insetos, identificando padrões moleculares relacionados ao ataque de patógenos como componentes da parede celular. Assim que esses padrões são reconhecidos as plantas ativam respostas de defesa chamadas de imunidade desencadeada por padrão (*Pattern triggered immunity* ou PTI em inglês) e estimulam diversos mecanismos para que a planta se defenda contra estresses bióticos e abióticos. Os QOS são capazes de ativar o PTI e agem como elicitores de defesa de plantas (SHINYA, Tomonori *et al.*, 2022).

Vários trabalhos já mostraram que a capacidade dos QOS de ativar defesas de plantas se revelou efetiva em suprimir diversas doenças no pós-colheita de frutas cítricas, tomate, pera e maçã, aumentando a qualidade e o tempo de prateleira dessas frutas por meio da indução de

respostas de defesa (CHEN et al., 2018). Entre as respostas que QOS pode estimular em plantas estão a regulação positiva de hidrolases e enzimas antioxidantes (LAN *et al.*, 2016), lignificação da parede celular (SIRIWONG *et al.*, 2021), ativação do metabolismo dos fenilpropanóides (YU *et al.*, 2022), aumento do conteúdo de vitamina C, entre outros (SALACHNA; GRZESZCZUK; SOBÓL, 2017).

O aumento de hidrolases e da lignificação da parede celular está diretamente ligado a adaptação e fortalecimento da parede celular para conferir resistência a estresses (LE GALL *et al.*, 2015), já as enzimas oxidantes, a vitamina C e outras moléculas antioxidantes são fatores importantes na minimização de espécies reativas de oxigênio que são produzidas em alta quantidade em condições de estresse e podem danificar o metabolismo da planta quando não controladas com sucesso (RAJPUT *et al.*, 2021; VENKATESH; PARK, 2014). O metabolismo dos fenilpropanóides está ligado a síntese de diversas moléculas que participam da defesa das plantas contra estresses bióticos e abióticos, como flavonoides e antocianinas, além de também participar do processo de lignificação da parede celular (DONG; LIN, 2021).

Todas as respostas de defesa induzidas pelos QOS no pré e no pós-colheita de plantas estão relacionadas a capacidade de QOS de se ligar a receptores de membrana e então estimular a produção de espécies reativas de oxigênio que servirão como moléculas sinalizadoras para a ativação de uma via chamada de MAP quinase (*Mitogen Activated Protein Kinases* - Proteínas-quinases ativadas por mitógenos)(BRULÉ *et al.*, 2019), que é capaz de regular o metabolismo celular por meio de alteração na expressão gênica em resposta a estímulos externos (PATIL; NANDI, 2020).

2.4.2.1 Pós-colheita de frutos

Há estimativas que, até 2050, a produção de frutas e vegetais teria que dobrar para que a população de aproximadamente 10 bilhões de pessoas possa ter uma vida saudável (BRUMMELL; BOWEN; GAPPER, 2022). Depois da colheita, as frutas continuam

biologicamente ativas e sofrem processos metabólicos, como amadurecimento e senescência, que levam a perda da qualidade e as tornam altamente perecíveis, o que faz com que a qualidade e segurança alimentar sejam os principais parâmetros que determinam a competitividade do mercado no período do pós-colheita de frutos (BELAY; JAMES CALEB, 2022).

A Organização das Nações Unidas para Alimentação e Agricultura (*Food and Agriculture Organization* - FAO) estima que 33% de toda produção alimentícia para consumo humano é perdida devido a deterioração no pós-colheita. (PALUMBO *et al.*, 2022). Além disso, doenças fúngicas no período pós-colheita são um problema para a saúde humana e animal, tendo em vista que fungos que comumente infectam alimentos como *Aspergillus*, *Penicillium*, *Alternaria* e *Fusarium* produzem micotoxinas que são nefrotóxicas, genotóxicas, teratogênicas, cancerígenas e citotóxicas (HUANG *et al.*, 2021).

A aplicação de fungicidas e outros aditivos sintéticos pode causar danos à saúde humana, além de ir de encontro com a tendencia dos consumidores de preferir produtos sem a adição de produtos sintéticos. Consequentemente, o desenvolvimento de medidas alternativas que visão a manutenção da qualidade das frutas bem como as protejam de doenças fúngicas é imprescindível (ROMANAZZI *et al.*, 2017).

2.4.2.2 Frutos minimamente processados

Frutas e vegetais são uma fonte essencial de micronutrientes e minerais e a procura por esses produtos vem aumentando, tendo em vista que há várias evidências de que o seu consumo promove a prevenção de muitas doenças degenerativas, doenças coronárias, cancro e, em geral, do envelhecimento (DE CORATO, 2020). Além disso, a comodidade e facilidade de consumo trazida pelos frutos minimamente processados faz com que o mercado desses produtos esteja em ascensão (CHANG, Shuaidan *et al.*, 2023).

De acordo com a Associação Internacional de Produtos Minimamente Processados (*International Fresh-Cut Produce Association* - IFPA), produtos minimamente processados

(MP) são qualquer fruto ou vegetal que tenha sido submetido a alteração física da sua forma original, mas continua em um estado fresco e pronto para consumo. Frutas MP podem ser lavadas, descascadas e picadas, criando um produto de fácil consumo que mantem todas as características da fruta que não passou por processamento (AGRIOPOULOU *et al.*, 2020).

Em consequência do processamento, as frutas MP se tornam extremamente perecíveis e com um menor tempo de prateleira devido a rápida queda de qualidade. Características indesejadas como alterações de cor, escurecimento e amolecimento podem causar rejeição e devem ser evitadas, pois resultam em perdas econômicas e problemas éticos devido à produção e acúmulo de alta concentração de resíduos agroindustriais (RAMOS *et al.*, 2021).

Novas estratégias e tecnologias para aumentar o tempo de prateleira e manter a qualidade de frutos MP sem alterar suas características precisam ser desenvolvidas (WILSON *et al.*, 2019). Por essa razão, estudos baseados na produção de revestimentos naturais e seguros para a saúde humana estão sendo realizados, dispondo de diversos tipos de materiais baseados em biopolímeros, compostos fenólicos, óleos essenciais, entre outros (IÑIGUEZ-MORENO; RAGAZZO-SÁNCHEZ; CALDERÓN-SANTOYO, 2021; SINGH; KIM; LEE, 2022; WICOCHEA-RODRÍGUEZ *et al.*, 2019).

2.4.3 Quitooligossacarídeos contra insetos

Insetos são uma das formas mais bem-sucedidas de vida do planeta. Na agricultura, algumas espécies de inseto podem trazer diversos benefícios por meio da polinização e dispersão de sementes, porém, outras espécies que se alimentam de importantes culturas agrícolas causando perdas de até 15.1% de toda produção anual e, portanto, são chamadas de pestes (VAN DEN BOSCH; WELTE, 2017). Além disso, alguns insetos também podem ser vetores de várias doenças humanas, como malária, dengue, Zika e Chikungunya. Essas doenças podem ser controladas usando abordagens baseadas em inseticidas, porém esse controle está ameaçado pela resistência aos inseticidas já utilizados. Por isso, o desenvolvimento de novos

inseticidas é necessário (SHAW; CATTERUCCIA, 2018).

A quitina está presente na estrutura do exoesqueleto e na cutícula de insetos, portanto quitinases são necessárias para esses organismos para degradação e remodelação de tecidos, tornando-as possíveis alvos na busca de novos inseticidas, visto que já foi provado que a regulação negativa dessas enzimas traz severos problemas aos insetos, como distúrbio de ecdise, inibição do crescimento, falha na pupação e morte (CHEN; YANG, 2020).

Os QOS podem ser utilizados como inibidores da atividade de enzimas quitinolíticas, sendo capazes de se ligarem ao sítio catalítico dessas hidrolases por terem estrutura similar aos oligosaccharídeos de quitina, porém, agindo como inibidores, uma vez que os resíduos catalíticos precisam interagir com os grupos acetil da N-acetil-glicosamina da quitina para realizar sua função catalítica e eles estão total ou parcialmente ausentes nos QOS. Apesar de haver poucas informações da atividade de QOS contra insetos, um trabalho que usou quitinases da família GH18, a mesma das quitinases de insetos, provou que QOS totalmente desacetilados e de baixo peso molecular são capazes de inibir essas enzimas por meio de inibição competitiva (CHEN, Lei *et al.*, 2014).

2.4.3.1 Doenças causadas por arbovírus

Arbovírus são um grupo altamente diversificado de vírus que são transmitidos de hospedeiros infectados para hospedeiros susceptíveis por meio de grande variedade artrópodes (mosquitos, carrapatos, flebotomíneos e culioides) que funcionam como vetores. Após a ingestão do sangue de um hospedeiro infectado, os vírus se multiplicam no intestino médio do inseto, resultando em uma carga viral elevada particularmente nas glândulas salivares, o que leva a transmissão do vírus quando o vetor morder um hospedeiro susceptível (YOUNG, 2018).

Dependendo do vírus, as infecções causadas por arbovírus podem causar febre alta, dor nas articulações, encefalite, febre hemorrágica, coma, convulsões e, às vezes, morte (PRINCE *et al.*, 2023). As doenças causadas por arbovírus são responsáveis por 17% das doenças

infecciosas, além de provocarem 700 mil mortes anualmente. As principais doenças causadas por arbovírus são dengue, Zika, Chikungunya e febre amarela (TAJUDEEN *et al.*, 2021).

A dengue é a doença viral transmitida por mosquito mais relevante no contexto da saúde humana, causando infecção viral com quatro diferentes sorotipos (DENV1 a DENV4). Essa doença é geralmente se manifesta em sintomas leves, semelhantes a gripe, porém, também pode progredir até a síndrome do choque da dengue, também conhecida como dengue hemorrágica, uma síndrome potencialmente fatal (HARAPAN *et al.*, 2020). É estimado que a dengue causa 100 milhões de infecções anualmente, sendo considerada uma grande preocupação pública apesar de ser historicamente uma doença negligenciada pela indústria farmacêutica (ISLAM; MUBARAK, 2020).

Chikungunya é uma doença causada pelo arbovírus CHIKV e pode ser transmitida pelo mosquito *Aedes aegypti*, caracterizada por promover uma febre aguda e dores nas articulações. A infecção desse vírus geralmente dura poucos dias, mas as dores nas articulações podem persistir por semana ou meses, e, em alguns casos, até anos (RABELO; PAIXÃO; ABREU, 2020). Além disso, complicações severas como miocardite, meningite, encefalite e paralisia flácida são associadas a Chikungunya (APPASSAKIJ *et al.*, 2020). Apesar da mortalidade dessa doença ser baixa (1%), as sequelas deixadas por ela incluem astenia e mudanças de humor, gerando um grande impacto na qualidade de vida das pessoas que são infectadas (WEAVER; CHEN; DIALLO, 2020).

Assim como a Chikungunya e a Dengue, Zika é outra doença causada por arbovírus (ZIKV) e que pode ser transmitida pelo mosquito *Aedes aegypti* (MARTINS; MEDRONHO; CUNHA, 2021). Essa doença era associada a sintomas brandos, como febre baixa, manchas vermelhas na pele e dor de cabeça, mas durante um surto de Zika no período de 2015/2016, foi descoberto que a infecção pelo ZIKV durante a gravidez pode resultar em defeitos congênitos, como microcefalia, levando à declaração de uma emergência de saúde global (RAPER;

CHAHROUDI, 2021).

O vetor das principais doenças causadas por arbovírus geralmente é o mosquito *Aedes aegypti* e já foi constatado que a melhor forma de conter a transmissão e surtos dessas doenças é o controle desses agentes transmissores (OWINO, 2021).

2.4.3.2 Aedes aegypti

O mosquito *Aedes aegypti* (Diptera: Culicidae) é o agente responsável pela transmissão de várias doenças causadas por arbovírus, como febre amarela, dengue, Chikungunya e Zika e, por isso, é uma das espécies de vetores mais estudadas em todo o mundo. O controle desse mosquito e, consequentemente, das doenças transmitidas por ele é do interesse de vários países, principalmente os de clima tropical e subtropical, que oferecem o clima propício para a sua reprodução (MCGREGOR; CONNELLY, 2021). *Ae. aegypti* possui características que o fazem ser um vetor extremamente eficiente: é altamente antropofílico, vive principalmente em ambientes urbanos e é ativo durante o dia todo, sendo capaz de picar humanos a qualquer hora (LUPICA; PALUMBO, 2020).

O aumento de surtos sazonais das doenças que têm *Ae. aegypti* como vetor é relacionado ao aumento do número de fêmeas quando as condições de temperatura e de chuvas estão favoráveis para a reprodução (PLIEGO PLIEGO; VELÁZQUEZ-CASTRO; FRAGUELA COLLAR, 2017). De acordo com o Ministério da Saúde do Brasil, o ciclo de vida desse mosquito é composto por quatro estágios: ovo, larva, pupa e adulto. Após a fêmea se alimentar de sangue, ela deposita ovos em locais propícios, como recipientes com água parada. Os ovos eclodem em larvas em 2 a 3 dias. Durante o estágio larval, que pode variar de 4 a 14 dias, as larvas passam por quatro instares, alimentando-se de micro-organismos presentes na água. Depois, inicia-se o estágio de pupa, que dura de 1 a 4 dias, no qual ocorre a metamorfose da larva para o mosquito adulto (AEDES AEGYPTI — MINISTÉRIO DA SAÚDE, 2020).

A Organização Mundial da Saúde (OMS) recomenda que o controle do *Ae. aegypti* seja

feito tanto na sua fase imatura (Ovo, larva e pulpa), quanto na sua forma adulta. Para esse controle, são utilizados três métodos: biológico, mecânico ou ambiental e químico. Para o controle biológico, têm sido empregados predadores naturais de larvas de mosquitos. O controle ambiental ou mecânico consiste na eliminação ou redução ativa dos criadouros do mosquito por meio da promoção da conscientização. O controle químico envolve o uso de inseticidas contra larvas e/ou mosquitos adultos (SILVA et al., 2020).

2.4.3.3 Toxina Bt no combate a insetos

Bacillus thuringiensis (Bt) é uma bactéria Gram positiva formadora de esporos que sintetiza corpos de inclusão cristalinos contendo proteínas Cry e Cyt, algumas das quais são tóxicas contra uma ampla variedade de insetos, nematoides e células cancerígenas humanas, essas proteínas são conhecidas como toxinas Bt (PALMA *et al.*, 2014). As toxinas Bt são um dos biopesticidas mais conhecidos do mercado. As toxinas Cry são geralmente o grupo de toxinas Bt mais exploradas como biopesticida, devido a características como: extremamente específicas em relação ao grupo-alvo e à segurança, biodegradáveis e não prejudiciais ao meio ambiente (SANTOS, Edclécia N. *et al.*, 2022). As toxinas Bt são seletivas para insetos, tendo ação em nas ordem Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Orthoptera e Mallophaga, mas não apresentam perigo para humanos, vertebrados e plantas. Por isso, Bt é responsável por cerca de 90% da produção e uso de biopesticidas e tornou-se o biopesticida mais amplamente utilizado em todo o mundo (LI, Yujie *et al.*, 2022).

Estudos revelaram que as toxinas Bt são ativas contra larvas de mosquitos, sendo altamente tóxicas para *Ae. aegypti*, interagindo com receptores das células epiteliais do intestino médio, o que resulta na oligomerização das toxinas e na formação poros nas membranas do intestino das larvas (ALAM *et al.*, 2022). Ademais, é recomendado que misturas de inseticidas sejam usadas no lugar de um único produto ativo. Essa abordagem é feita para evitar que os insetos desenvolvam resistência contra os inseticidas, principalmente quando os componentes

individuais da mistura têm mecanismos de ação diferente. A ação sinergética de dois inseticidas, quando bem aplicada, pode reduzir custos, tendo em vista que as doses aplicadas serão menores e a atividade de ambos será potencializada (KONECKA *et al.*, 2020).

3 HIPÓTESE

Os quitoolitossacarídeos produzidos pela quitosanase de *Chromobacterium violaceum* *Cv*Csn46 possuem atividade antifúngica contra diferentes espécies de *Candida*, sendo capazes de causar dano a membrana celular dessas leveduras, além de apresentar potencial para serem utilizados em outras aplicações biotecnológicas, como proteção de frutos na fase pós-colheita e ação inseticida.

4 OBJETIVOS

4.1 Objetivo geral

Produzir oligossacarídeos de quitosana por via enzimática com atividade antifúngica contra diferentes espécies de *Candida* e estudar o mecanismo de ação dessas moléculas contra espécies não-albicans, além de avaliar outras aplicações biotecnológicas dos quitooligossacarídeos.

4.2 Objetivos específicos

- Definir a concentração inibitória mínima e letal mínima dos quitooligossacarídeos contra diferentes espécies de *Candida*.
- Estimar o tempo de morte de espécies de *Candida* não-albicans quando em contato com os quitooligossacarídeos
- Analisar a ação dos quitooligossacarídeos sobre o biofilme de *Candida* não-albicans
- Investigar o mecanismo de ação dos quiooligossacarídeos quanto a ação sobre a membrana celular, produção de espécies reativas de oxigênio e alterações morfológicas em espécies de *Candida* não-albicans
- Avaliar a ação de quitooligossacarídeos sobre melão minimamente processado quanto a sua atividade protetiva e aumento do tempo de prateleira
- Avaliar a ação de quitooligossacarídeos sobre larvas de *Aedes aegypti* quanto a sua atividade inseticida e capacidade de potencializar a atividade das toxinas Bt
- Avaliar a toxicidade de quitooligossacarídeos utilizando o organismo modelo *Danio rerio* (Zebra fish)

5 ARTIGO I: Targeting non-albicans *Candida* with enzymatically produced chitooligosaccharides: Insights into antifungal mechanisms

Mayara I. G. Azevedo [a], Bruno R. S. Queiroz [b], Nadine M. S. Araujo [a], Daniele O. B. Sousa [a], Celso S. Nagano [c], Rafael G. G. Silva [d], José E. Monteiro-Júnior [e], Thalles B. Grangeiro [e,*]

[a] Departamento de Bioquímica e Biologia Molecular, Centro de Ciências, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

[b] Departamento de Bioquímica, Universidade de São Paulo, São Paulo, Brazil.

[c] Departamento de Engenharia de Pesca, Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

[d] Laboratórios Integrados de Pesquisa em Botânica – LIB, Departamento de Biologia, Centro de Ciências, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

[e] Laboratório de Genética Molecular, Departamento de Biologia, Centro de Ciências, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

*Corresponding author

Prof. Dr. Thalles B. Grangeiro

Laboratório de Genética Molecular, Departamento de Biologia, Centro de Ciências, Universidade Federal do Ceará, Campus do Pici, Fortaleza-CE, 60.440-900, Brazil

E-mail: thalles@ufc.br

Abstract

Among the fungal species that cause human diseases, the *Candida* genera is one of the most relevant. The number of infections and deaths caused by non-albicans species (NAC) is rapidly increasing because of their growing resistance to antifungals. Chitooligosaccharides (COS) are chitosan derivatives that present several biological activities, including antifungal activity. In this work, the activity of enzymatically produced and 100% water soluble COS (degree of polymerization of 1 to 9) against *Candida* species was evaluated. Minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) varied among yeast species and two NAC (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) were chosen to further analysis. COS was able to kill the strains tested in a maximum of four hours and degrade the biofilms formed by them. Mode of action analysis showed that the cell membrane is probably COS main target of activity, disturbing its integrity and favoring the ROS production and morphology alterations. Thus, the COS produced in this work have the potential to become an alternative therapy for non-albicans species.

Keywords: Antifungal, infection, yeast

5.1 Introduction

Fungal infections affect more than 1.5 billion people and kill more than one and a half million every year, representing a worldwide health care problem and an economic burden (STRICKLAND; SHI, 2021). Among the fungal species that cause invasive infections, the ones belonging to the *Candida*, *Aspergillus*, *Cryptococcus* and *Pneumocystis* genera are the most clinically relevant (ARASTEHFAR *et al.*, 2020). The Candida genera has at least 15 species that cause human diseases, but more of 95% of the invasive infections are caused by only 6 species: *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei* and, more recently, *Candida auris* (MCCARTY; WHITE; PAPPAS, 2021). *Candida albicans* is the main cause of the diseases caused by the genera, but this has been changing in the recent years, with a growing number of infections caused by non-albicans species (NAC) due their increasing resistance to antifungals. *C. krusei* infections results in the lowest 90-day survival rate when compared to other *Candida* species and *C. parapsilosis* is the second to third more isolated *Candida* specie in the ICU, accounting for approximately 80% of newborns infections caused by NAC (JAMIU *et al.*, 2021; TÓTH *et al.*, 2019).

The characterization of the last group of antifungals approved for use in humans was carried out almost twenty years ago, which means that there are few antifungals on the market. This is probably related to the lack of investment in diseases caused by fungi when compared to diseases caused by bacteria and protozoa, such as tuberculosis and malaria (FAUSTO; RODRIGUES; COELHO, 2019). Because of that, it is necessary to prospect and approve new molecules with antifungal activity (VAN DAELE *et al.*, 2019).

Chitosan is a natural, deacetylated, cationic polysaccharide derived from chitin. Biodegradability, non-toxicity and non-antigenicity are characteristics of chitosan that make this polysaccharide the target of several applications (MUXIKA *et al.*, 2017). Furthermore, there are several studies on the biological activities of chitosan, mainly antibacterial and

antifungal (KE *et al.*, 2021). However, the physicochemical properties of chitosan, such as insolubility at neutral pH values and high viscosity, make its use difficult (TABASSUM; AHMED; ALI, 2021).

A possibility to exploit the biotechnological potential of chitosan is its hydrolysis, transforming it into oligosaccharides which, unlike chitosan, are soluble at neutral pH values and have low viscosity, given that they have low molecular mass and have free NH_2 groups (NAVEED *et al.*, 2019). Large-scale production of chitooligosaccharides (COS) is usually done through chemical hydrolysis, but this type of treatment generates toxic by-products and non-uniform products, making the enzymatic hydrolysis a more viable alternative (YUAN *et al.*, 2019). COS are also biodegradable, non-toxic, and non-allergenic, in addition to having antibacterial, antifungal, antitumor, antioxidant, anti-HIV activities, among others. Many of their biological activities are related to the positive charge of QOS, originating from NH_2 groups, which are protonated (NH_{3+}) at pH < 6.0 (LIANG; SUN; DAI, 2018). In this work, enzymatically produced COS were evaluated on their antifungal activity against *Candida* species, especially NAC. Their capability of contain and degrade biofilm, the time they take to kill the yeast cells and the mode of action of their antifungal activity was assessed.

Hypotheses:

The enzymatically produced COS have the ability to inhibit the growth and kill Non-albicans *Candida* species, interacting with the cell membrane of the yeasts, thus disturbing their metabolism.

5.2 Material and methods

5.2.1 Materials

Chitosan powder (degree of deacetylation, DDA = 93%) was purchased from Êxodo Científica (Sumaré, SP, Brazil). All other reagents were of high purity, analytical grade.

5.2.2 Enzymatic COS production

The enzymatic production of chitosan oligosaccharides was performed using a recombinant chitosanase from *Chromobacterium violaceum* (AZEVEDO *et al.*, 2020). The reaction was carried out by dissolving 1% (w/v) of chitosan powder in 50 mM of acetic acid and mixing it with the chitosanase (5 μg of protein for every milliliter of the solution). The mixture was incubated for 24 h in 50 °C under constant agitation and the reaction was stopped by immersing the tubes in boiling water for 10 min. After cooling in room temperature, the mixture was centrifugated (10000 g for 20 min at 10 °C) and the pellet was discarded. The supernatant was freeze dried and kept at 4 °C until used.

5.2.3 Average molecular mass of COS

The average molecular mass and the degree of polymerization were determined by mass spectrometry using ESI-MS as described by Azevedo et al. (2020). Data collection and processing were controlled by Mass Lynx 4.1 software (Waters).

5.2.4 Water solubility

The water solubility of COS compared to chitosan was determined according to Cano-Chauca et al. (2005) with modifications. Briefly, 0.5 g of the dry samples (COS and chitosan) were added 50 mL of water and shaken for 30 min (180 rpm). The mixtures were submitted to centrifugation (8.000 g for 20 min at 10 °C) and 25 mL of the supernatant was transferred to pre-weighted petri dishes and immediately dried at 105 °C for 3 h. The water solubility was calculated by the weight difference.

5.2.5 Yeast strains and growth conditions

The yeast strains used in this work (*C. albicans* ATCC 10231, *C. albicans* ATCC 44858, *C. albicans* ATCC 64129, *C. albicans* ATCC 90028, *C. albicans* ATCC 90029, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. parapsilosis* ATCC 90018, *C. tropicalis* ATCC 750 and *C. tropicalis* ATCC 13803) were provided by Departamento de Análises Clínicas e Toxicológicas (UFC). The strains were kept frozen in Sabouraud dextrose broth and glycerol at -80 ˚C until testing. For each experiment, yeasts were subcultured on Sabouraud agar and incubated for 24 h at 35 ˚C.

5.2.6 Minimal inhibitory concentration and minimal lethal concentration

To determinate the Minimal inhibitory concentration (MIC) of COS against the *Candida* species the microdilution method was applied using 96 well plates according to the norm M27-A2, vol. 23, nº 2 (CSLI, 2008), with modifications. The yeasts were cultivated in Sabouraud dextrose broth at 35 °C for 24 h and, after this period, the cell suspensions were diluted to prepare the inoculums (0.5×103 to 2.5×103 CFU/mL). Aliquots of the cell suspensions (0.1 mL) were incubated with the same volume of COS diluted in sterile water (2500 to 19 µg/mL) for 24 h at 35 °C. Fluconazole (64 µg/mL) and distilled water were used was positive and negative controls, respectively. The minimal inhibitory concentration was defined as the lowest COS concentration at which there was no visible growth after 24 h at 35 °C.

The minimal lethal concentration (MLC) was determined by incubating 5 µL aliquots from wells where there was no growth observed in the MIC determination assay onto Sabouraud agar. After 24 h of incubation at 35 °C, the colony forming units (CFU) were counted and the MLC was considered as the lowest concentration of COS capable of killing 99.9% of the microbial strains tested (TILLE, 2022).

The following methods were performed with 2 out of the 4 *Candida* spp. used in this work. The strains were chosen by taking into consideration the MIC and MLC values as well

as the relevancy of the species for health care system.

5.2.7 Evaluation of antifungal activity using an agar plate

Agar diffusion testing was performed as described in CLSI document M44-A2 (CLSI, 2009), with modifications. In brief, cell density of *Candida* cultures was adjusted to match the turbidity of a 0.5 McFarland standard and inoculated onto Sabouraud agar plates using a sterile cotton swab that was saturated with the inoculum suspension. COS (0.5%) was incorporated in an agarose gel (0.2%) and applied on the medium surface. The plates were incubated at 35 ◦C for 24 h and the diameters of growth inhibition zones were measured using a pachymeter and rounded off to the closest millimeter. The antifungal activity of COS-gel was compared with ketoconazole-based antifungal cream.

5.2.8 Time-kill essay

The inoculums for the time-kill essay were prepared as previously described and incubated with different concentrations of COS (1/2 MIC, MIC and 2xMIC) for 24 h at 35 °C. Samples were of each treatment were regularly incubated onto Sabouraud dextrose agar in defined intervals (0, 2, 4, 6, 8, 10, 12 and 24 h) and after 24 h of each incubation the colony forming units (CFU) were counted.

5.2.9 Assessment of biofilm formation and degradation

The essays to evaluate the effect of COS on biofilm formation and degradation were performed by measuring the biomass produced by the *Candida* species as described by (ARAÚJO *et al.*, 2019). The essays were carried out with different concentrations of COS (1/2 MIC, MIC and 2xMIC), fluconazole (64 μg/mL) and distilled water (negative control).

5.2.10 Fluorescence microscopy

5.2.10.1 Evaluation of membrane integrity

Aliquots of 1 mL of yeast cell suspensions (10^6 CFU/mL) were incubated with COS (MIC), water (negative control) and ethanol 50% (positive control) for 1h. After this period, the

cells were centrifugated (5000 g for 10 min at 4 °C), resuspended in 1 mL of 1.5×10^5 μM NaCl and incubated with 1 μM propidium iodide for 10 min. The evaluation of the integrity of the cell membranes was visualized by the fluorescence microscope Olympus System BX 60 with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

5.2.10.2 Evaluation of the reactive oxygen species (ROS) generation

This essay was carried out by incubating yeast cell suspensions (10^6 CFU/mL) were incubated with COS (MIC), water (negative control) for 1h. The cell suspensions were also incubated with H_2O_2 10% (positive control) for 15 min. After each period, the cells were centrifugated (5000 g for 10 min at 4 °C) and incubated with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 30 min. The cells were washed twice with 1.5×10^5 μM NaCl to reduce any background fluorescence and the ROS generation was visualized in fluorescence microscope (Olympus System BX 60 at excitation wavelength of 490 nm and emission wavelength of 520 nm.

5.2.11 Flow cytometry

The flow cytometry experiments were carried by using aliquots of 3 mL of yeast cell suspensions (10^6 CFU/mL) treated with COS (MIC), water (negative control) and ethanol 50% (positive control) for 1h. After this period, the cells were centrifugated (5000 g for 10 min at 4 °C), resuspended in 2 mL of 1.5×10^5 μM NaCl and incubated with 1 μM propidium iodide for 10 min. The samples were analyzed by PARTEC CyFlow cytometer and approximately 150,000 cells were analyzed from each sample. The graphs were obtained by the server Floreada.io.

5.2.12 Effect of NaCl on the anticandidal activity of COS

The effect of COS on the growth of *Candida* spp. in the presence of increasing ionic strength (0, 150, 300, 450 and 600 mM of NaCl) was performed using the microdilution method as previously described on item 2.5. The yeast growth was monitored at 600 nm using an

automated microplate reader (Epoch, BioTek, USA).

5.2.13 Effect of pH on the anticandidal activity of COS

The effect of different pH values (4.5, 5.5 and 7.5) on the antifungal activity of COS on *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 was investigated by using the microdilution method as previously described on item 2.5. The yeast growth was monitored at 600 nm using an automated microplate reader (Epoch, BioTek, USA).

5.2.14 Evaluation of cell morphological alterations

The assay to verify possible morphological changes in the yeast cells cells treated with COS was conducted by incubating COS (MIC) with the yeast cells for the period determined in the time-kill essay described on 2.5 item. After the incubation period, the cells were centrifugated, washed three times with NaCl 150 mM and fixated for 16 h with glutaraldehyde 2,5% in sodium phosphate buffer 150 mM pH 7,2. When the fixation period was finished, the cells were washed with the mentioned buffer, fixated again with osmium tetroxide 10%, treated with crescent concentrations of ethanol (0, 30, 50, 70 and 100%) to perform the dehydration of the cells. The yeasts were then dried with hexamethyldisilazane (HMDS) and metalized with gold. The COS-treated and control cells (Fluconazole and water) were observed under a Quanta FEG-450 Scanning Electron Microscope instrument equipped with a low energy detector (Everhart-Thornley detector).

5.3 Results

5.3.1 Molecular weight profile and solubility of the enzymatically produced COS

When the low molecular mass products released from colloidal chitosan incubated with the recombinant chitosanase were analyzed by ESI-MS, an array of positive ion clusters with a degree of polymerization (DP) ranging from 1 to 9 was obtained (Figure 1). The enzymatic hydrolysis of chitosan produced COS with 100% of solubility in water while chitosan, as expected, presented 0% of solubility.

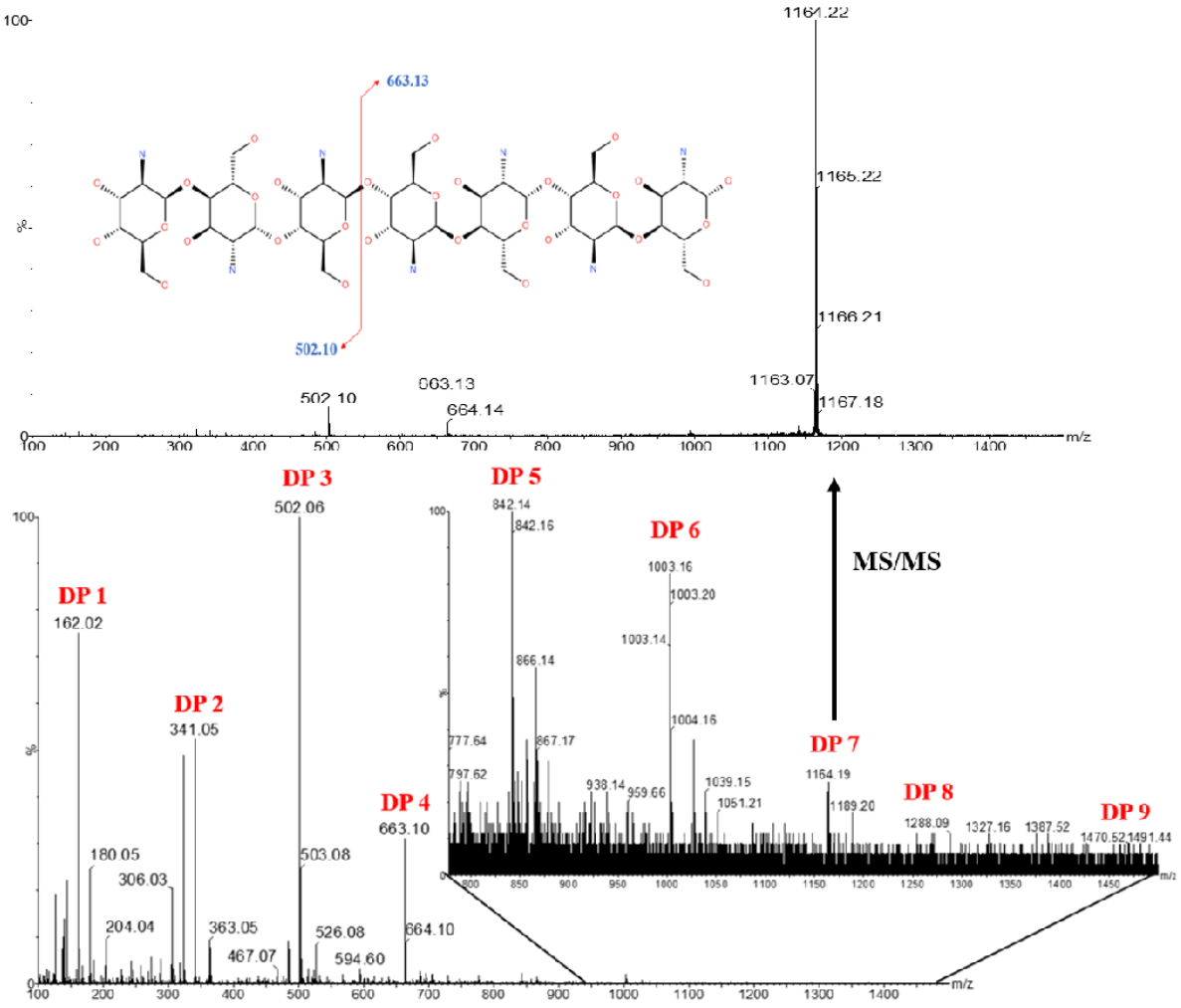


Fig. 1. ESI-MS analysis (positive ion mode) of enzymatic products released from colloidal chitosan incubated with CvCsn46. Bottom image: mass spectrum of products released from 1% colloidal chitosan incubated with 5 µg/mL CvCsn46 for 24 h at pH 6.0 and 50 °C. Top image:MS/MS spectrum of product atm/z 1164.2. The schematic fragmentation of $(GlcN)_7$ (calculated mass = 1146 Da), and resulting product ions, are shown next to m/z 1164.2.

5.3.2 Minimal inhibitory and lethal concentrations of COS against Candida species

The chitooligosaccharides exhibited activity against eight out of ten *Candida* strains used in this work. The MIC and MLC are shown in Table 1. COS were active against three out of the five *C. albicans* strains tested, with MIC values ranging from 1250 to 156 µg/mL and with only one of the strains (ATCC 90028) suffering a lethal effect on the concentration of 312 µg/mL. Both C. *parapsilosis* and *C. tropicalis* strains as well as the *C. krusei* strain used in this

work were affected by the COS treatment, with MIC values ranging from 78 to 312 μg/mL and MLC ranging from 156 to 312 μg/mL**.**

Table 1.

Minimal inhibitory concentration and minimal lethal concentration values towards *Candida* species.

***Candida* species**	**MIC**	**MLC**
C. albicans ATCC 10231	Not identified	-
C. albicans ATCC 44858	Not identified	-
C. albicans ATCC 64129	1250 μg/mL	-
C. albicans ATCC 90028	1250 μg/mL	-
C. albicans ATCC 90029	156 μg/mL	312 μg/mL
C. krusei ATCC 6258	312 μg/mL	312 μg/mL
C. parapsilosis ATCC 22019	312 μg/mL	312 μg/mL
C. parapsilosis ATCC 90018	312 μg/mL	625 μg/mL
C. tropicalis ATCC 750	78 μg/mL	156 μg/mL
C. tropicalis ATCC 13803	156 μg/mL	156 μg/mL

5.3.3 Antifungal activity against non-albicans strains on a surface

The antifungal activity of the COS gel containing was compared to a commercial ketoconazole-based antifungal cream (Fig. 2). Ketocanozole, like fluconazole, is an azole antifungal mainly used in personal products, like in anti-dandruff shampoos, gels, creams, and tablets, for the treatment of fungal infections (BHAGAT *et al.*, 2021). The mean sizes of the

inhibition zones surrounding the gel samples containing COS (dose = 15 mg) were comparable to those of the commercial antifungal cream (dose = 12 mg) containing 2% (m/m) ketoconazole (Table 2). These results demonstrate that COS are a promising antifungal agent that could be further investigated as a potential candidate for the treatment of infections caused by *Candida* species.

Since most of the research related with COS activity against *Candida* species focus on *C. albicans* strains, from now on our work will deepen the studies on activity of COS on other two clinically important species of this yeast: *Candida krusei* and *Candida parapsilosis*.

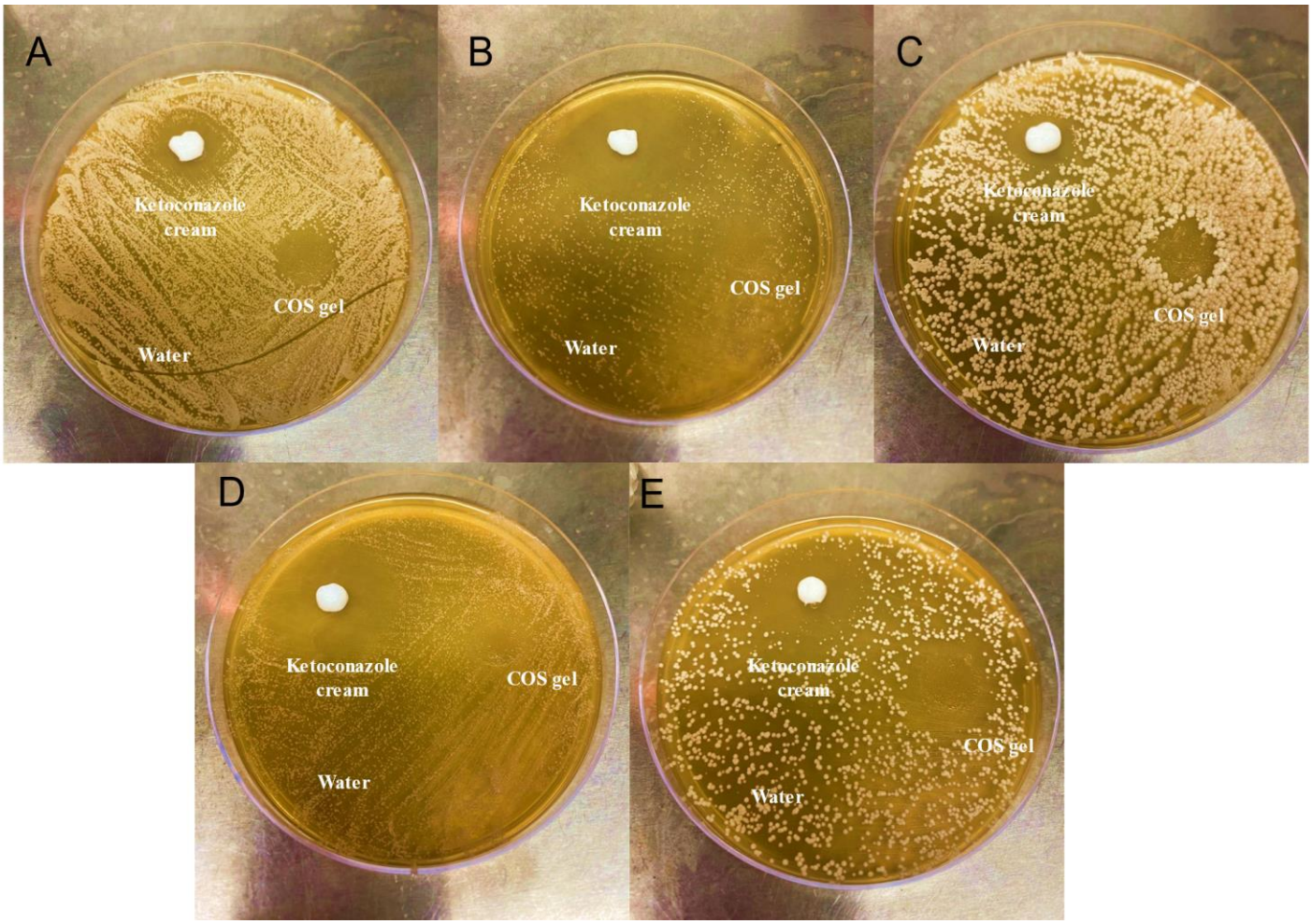


Fig. 2. Diffusion test on Sabouraud agar medium using COS-gel containing against *Candida* strains. The following strains were used: *C. tropicalis* ATCC 13803 (A), *C. parapsilosis* ATCC 90018 (B), *C. krusei* ATCC 6258 (C), *C. parapsilosis* ATCC 22019 (D), and *C. tropicalis*

ATCC 750 (E). The samples loaded onto the plates were: sterile ultrapure water, COS-gel, and commercial ketoconazole-based antifungal cream (dose = 12 mg; CIMED Pharmaceutical Industry, Pouso Alegre, MG, Brazil).

Table 2.

Evaluation of antifungal activity on a surface using a COS gel.

Strains	COS-gel	Commercial antifungal	Water
Candida krusei ATCC 6258	10±1	10±1	0
Candida parapsilosis ATCC 22019	13±2	25±1	0
Candida parapsilosis ATCC 90018	11±1	28±1	0
Candida tropicalis ATCC 750	12±1	14±2	0
Candida tropicalis ATCC 13803	24±1	20±1	0

All the values mentioned the table are expressed in millimeters.

5.3.4 Time-kill

The time-kill essay was performed to determine how long it takes for COS to kill the yeast samples at the concentration used in the MIC essays. On Figure 3A. it is possible to observe that COS (MIC/MLC) took four hours to kill *C. krusei* ATCC 6258 and could affect the growth of this yeast even with a lower concentration (MIC/2) when compared to the control. For *C. parapsilosis* ATCC 22019 (Figure 3B), COS was able to kill the yeast cells even faster, taking only two hours to completely exterminate the cell growth using MIC/MLC and, like it was observed for *C. krusei*, a lower concentration could also affect the cell growth when compared to the control.

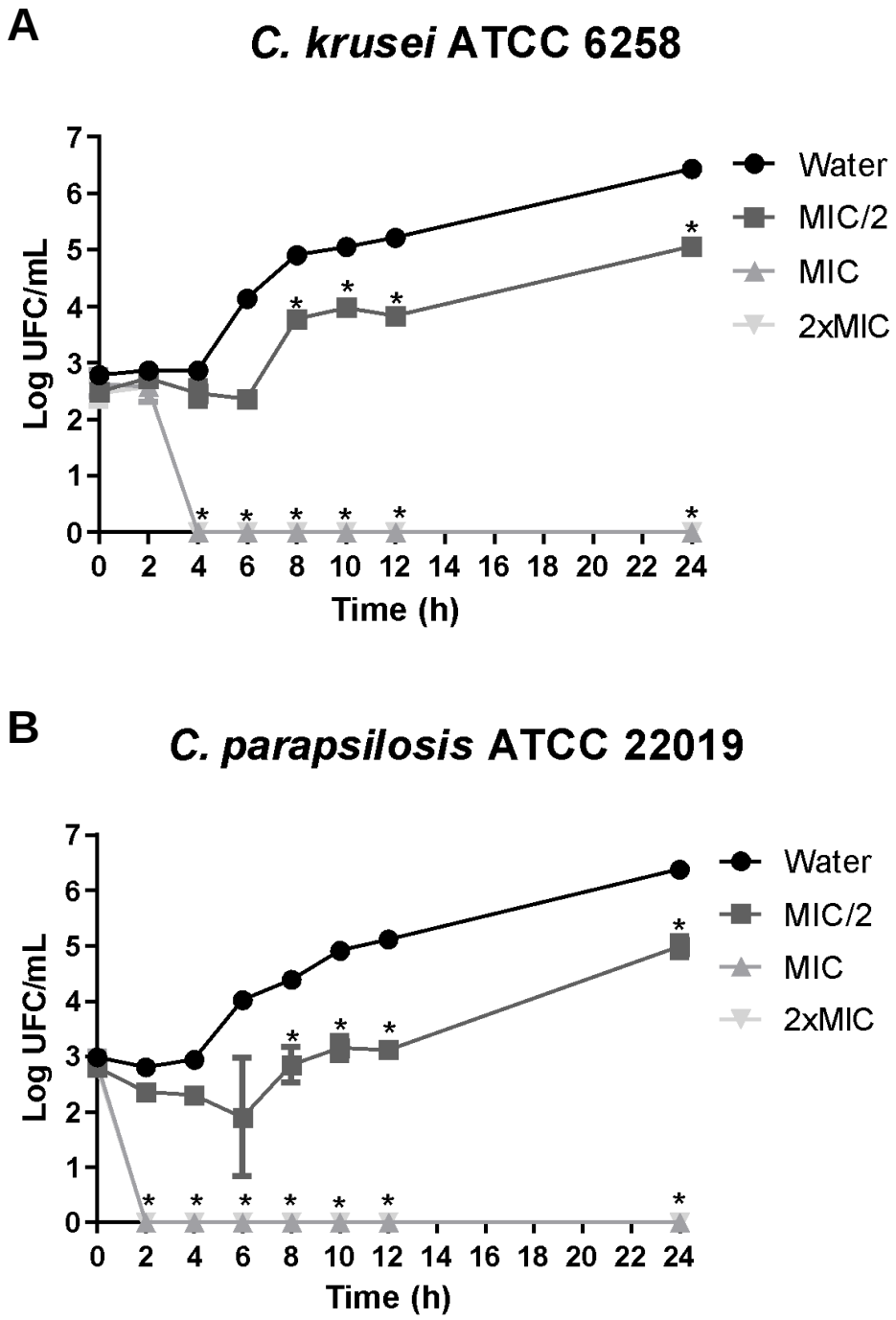
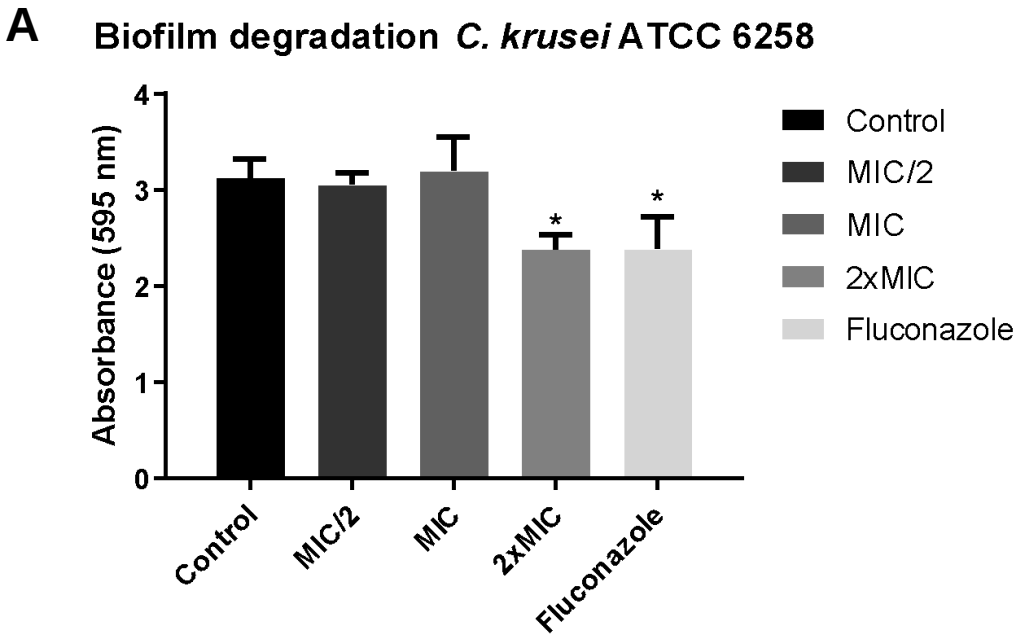


Fig. 3. Effect of the time of exposure of different COS concentrations on the viability of *Candida* strains, determined by the microdilution technique in culture broth and viable cell count. (A) Time-kill curve of COS effect on *C. krusei* ATCC 6258. (B) Time-kill curve of COS effect on *C. parapsilosis* ATCC 22019. The cells were exposed to ultrapure water, 156 µg/ml of COS (MIC/2) 312.5 µg/ml (MIC) of COS and 625 µg/ml (2xMIC) of COS. The values are expressed as mean ± SEM of three experiments, with three replicates. Data analysis was

performed by ANOVA, with Dunnet post-test and $p < 0.05$ (*).

5.3.5 Formation and degradation of biofilm

The inhibition of the formation of biofilm of both *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 wasn't observed with the concentrations used in this essay. However, the degradation of the biofilm of both yeast species (Figure 4), for *C. krusei* ATCC 6258, the degradation was only observed using 2xMIC (41.66%) and for *C. parapsilosis* ATCC 22019 22.5% of the biofilm was degraded using the MIC and 40.41% was degraded with 2xMIC.



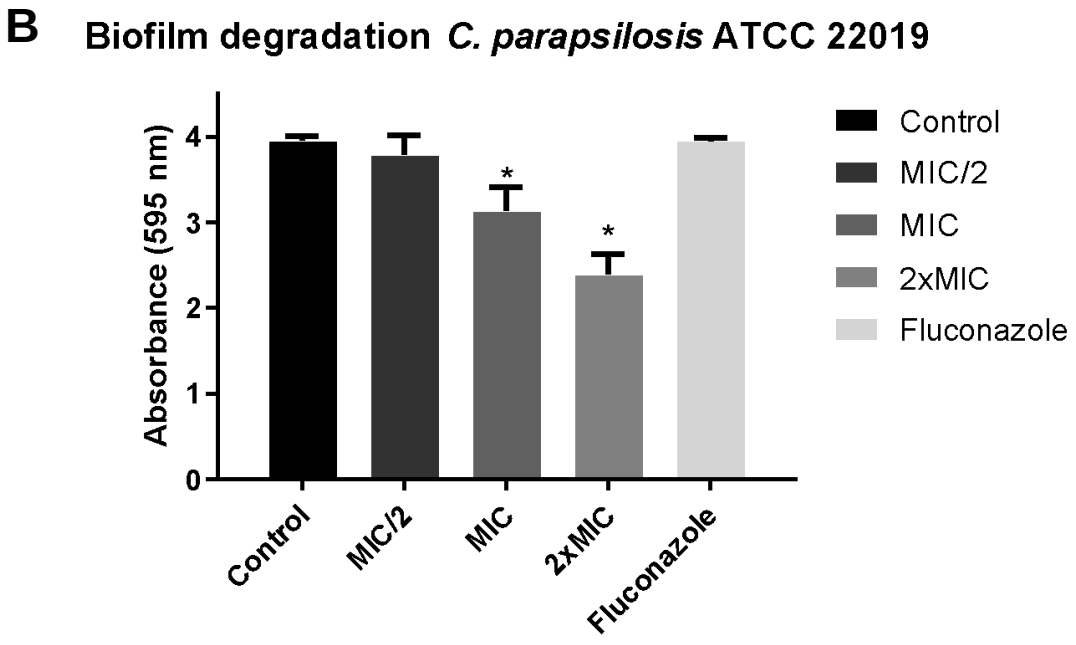


Fig. 4. Effect of COS on the biomass degradation of the *C. krusei* ATCC 6258 (A) and *C. parapsilosis* ATCC 22019 (B) biofilm. The cells were exposed to 1.5×10^5 µM NaCl (negative control), 156 µg/ml of COS (MIC/2) 312.5 µg/ml (MIC) of COS, 625 µg/ml (2xMIC) of COS, and 128 µg/ml of fluconazole. Data are expressed as the mean ± standard deviation of three independent experiments. Different letters indicate significant differences between treatments ($p < 0.05$).

5.3.6 Mode of action of COS on Candida spp.

5.3.6.1 Cell membrane integrity

The cell membrane integrity of the *Candida* strains treated with COS is shown on Figures 5 and 6. It is possible to observe that COS directly affected the membrane permeability of both *C. krusei* and *C. parapsilosis* strains with results compared to the positive control (Ethanol 50%).

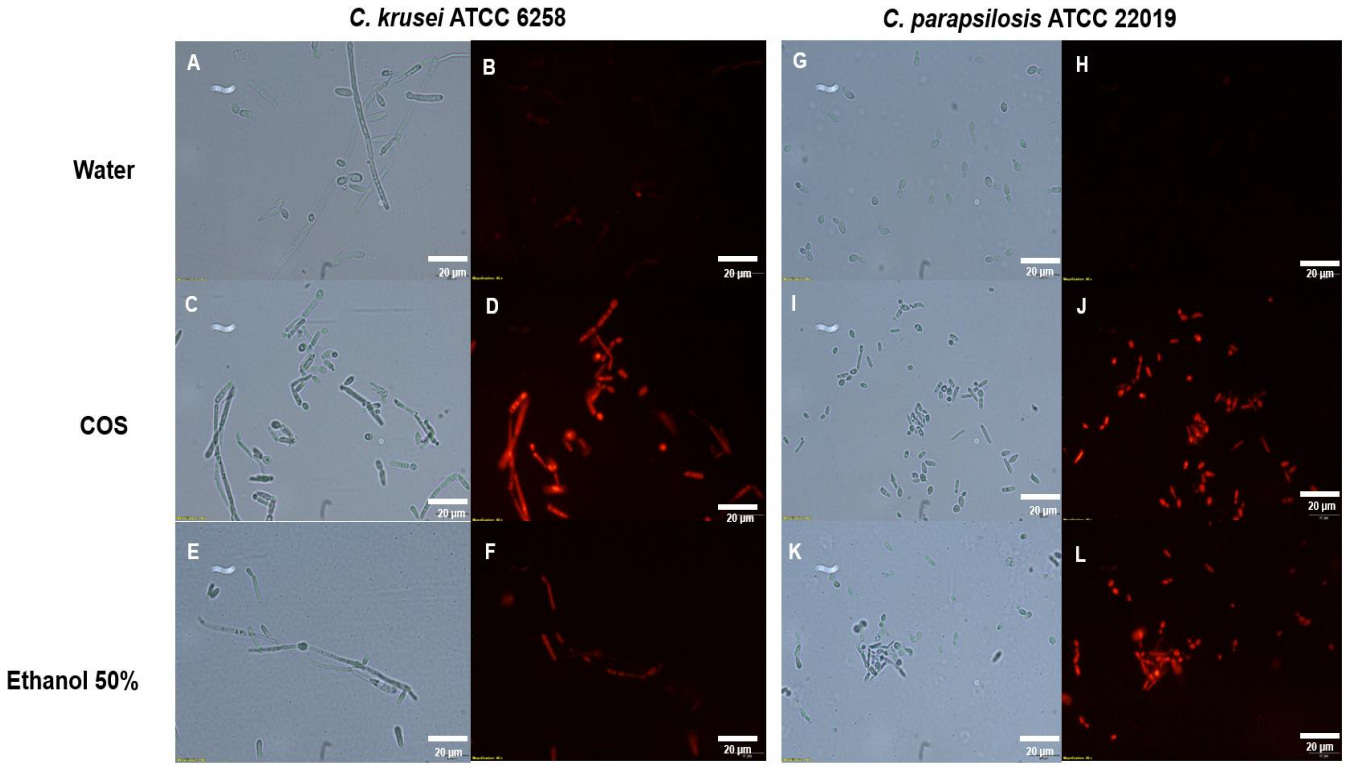


Fig. 5. Micrographs of *C. krusei* and *C. parapsilosis* cells to assess membrane permeabilization. In (A) and (B), *C. krusei* cells were treated with water; in (C) and (D), cells were treated with 312.5 µg/ml; in (E) and (F), cells were treated with Ethanol 50%. In (G) and (H), *C. parapsilosis* cells were treated with water; in (I) and (J), cells were treated with 312.5 µg/ml of COS (MIC); in (K) and (L), cells were treated with ethanol 50%. Fluorescence microscope micrographs: (B); (D); (F); (H); (J); and (L).

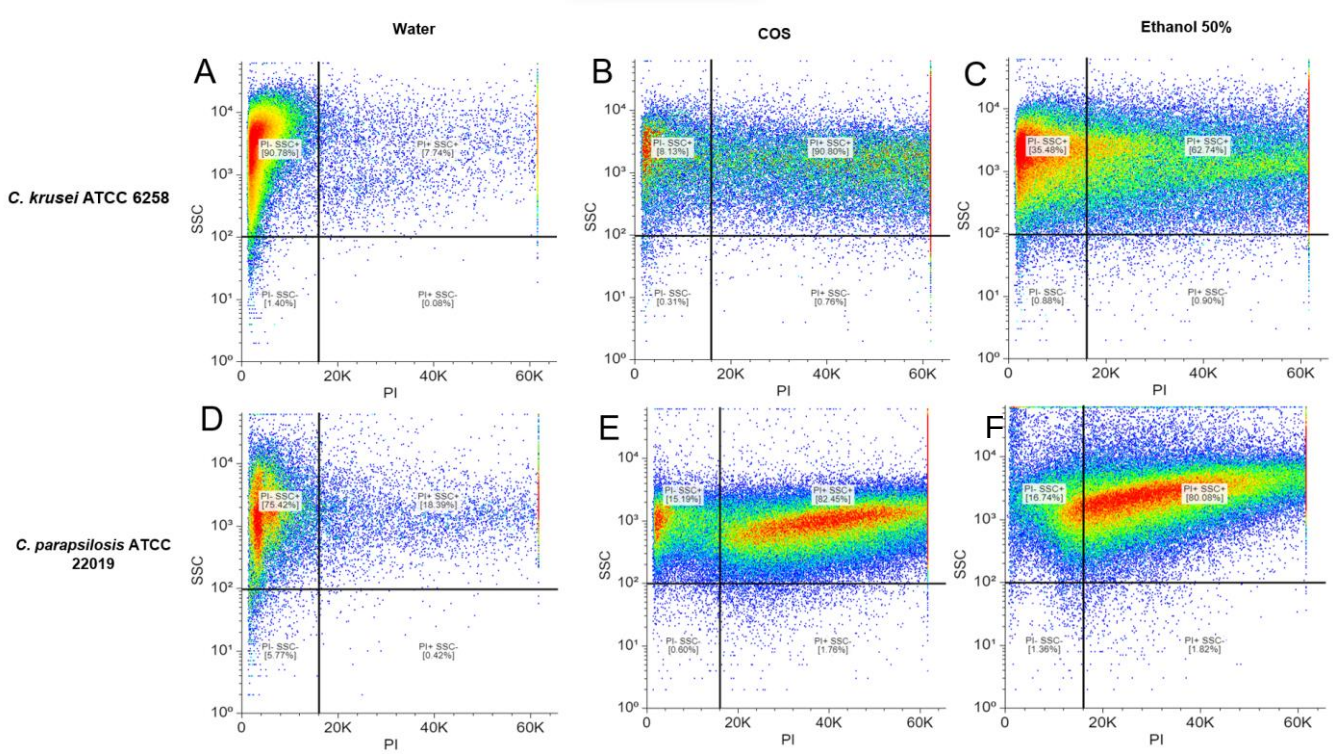


Fig. 6. Flux cytometry analysis of C. krusei and C. parapsilosis cells to assess membrane permeabilization. In (A), *C. krusei* cells were treated with water; in (B), cells were treated with 312.5 µg/ml of COS (MIC); in (C), cells were treated with Ethanol 50%. In (D), *C. parapsilosis* cells were treated with water; in (E), cells were treated with 312.5 µg/ml of COS (MIC); in (F), cells were treated with ethanol 50%. Images were obtained on Floreada.io server.

5.3.6.2 Formation of reactive oxygen species

COS are able to increase the levels of reactive oxygen species on the *Candida* cells when compared to the negative control cells (Figure 7). Figures 6 C, D, I and J shows that the treatment with COS using the MIC produces a stronger fluorescence than the peroxide hydrogen used as positive control (Figures 7 E, F, K and L) for both *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019.

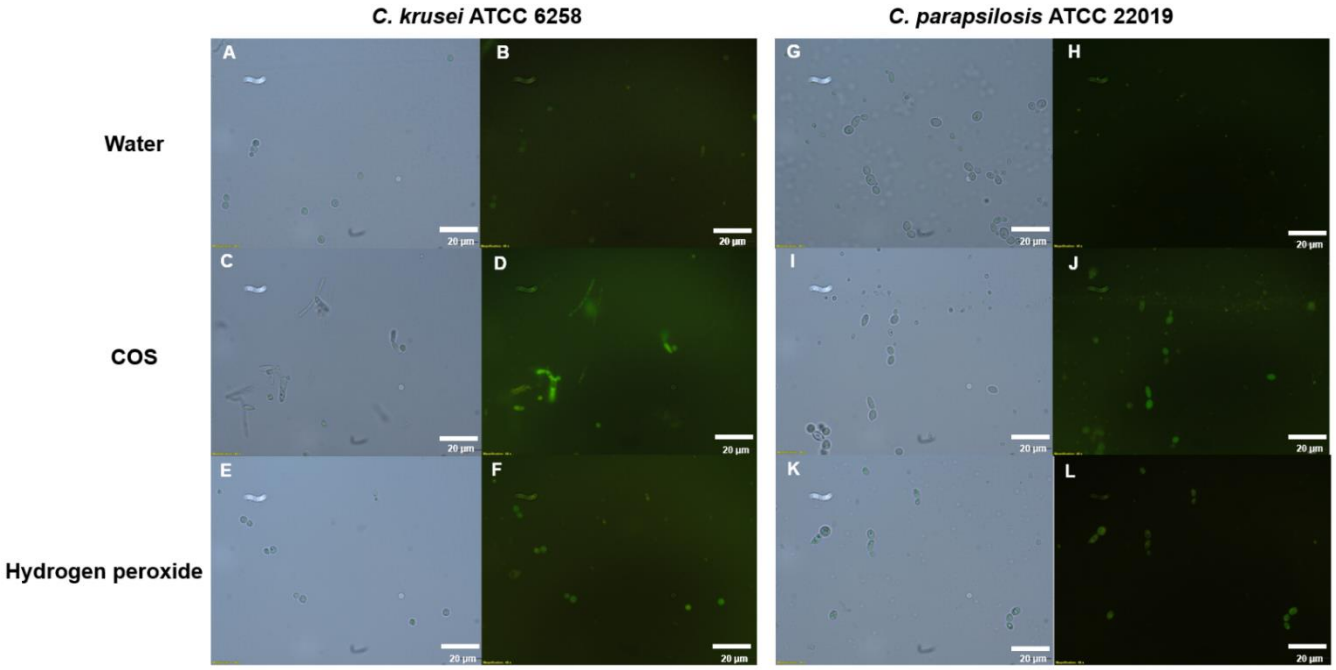


Fig. 7. Effect of COS in ROS generation in *C. krusei* and *C. parapsilosis* cells. In (A) and (B), *C. krusei* cells were treated with water; in (C) and (D), cells were treated with 312.5 µg/ml of COS (MIC); in (E) and (F), cells were treated with hydrogen peroxide 10%. In (G) and (H), *C. parapsilosis* cells were treated with water; in (I) and (J), cells were treated with 312.5 µg/ml of COS (MIC); in (K) and (L), cells were treated with hydrogen peroxide 10%. Fluorescence microscope micrographs: (B); (D); (F); (H); (J); and (L).

5.3.6.3 Ionic strength and pH influence on the antifungal activity

The influence of the ionic force on the antifungal activity of COS was tested by using increasing concentrations of NaCl. The activity against *C. krusei* ATCC 6258 was affected with 0.3 M of NaCl (Figure 8A) while the activity against *C. parapsilosis* ATCC 22019 was only affected with 0.45 M of NaCl (Figure 8B). Different pH values didn't affect the MIC for *C. krusei*, but it was proven to be of great importance for *C. parapsilosis*, showing that low pH values improve COS antifungal activity (Figure 8C).

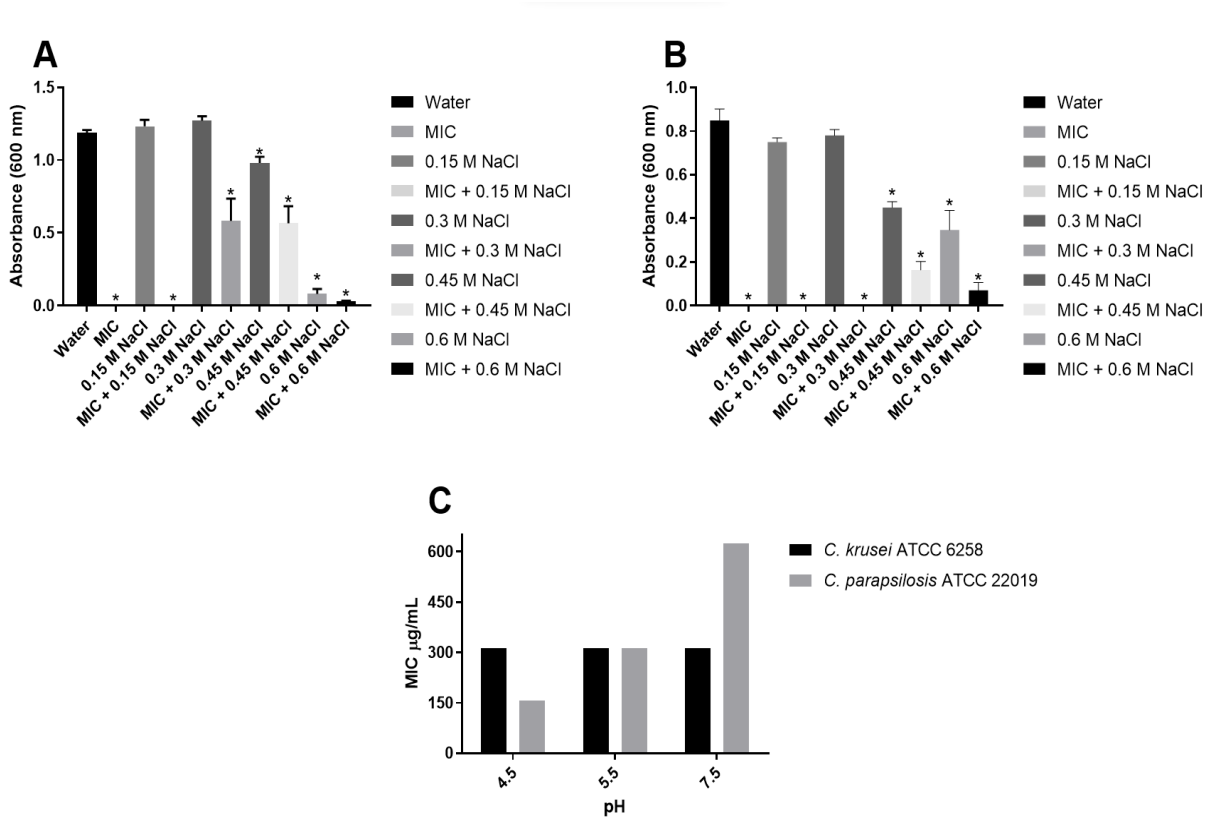


Fig. 8. Effect of ionic strength and pH on COS antifungal activity against *C. krusei* and C. *parapsilosis*. (A) Effect of different concentrations of NaCl on the MIC of COS against *C. krusei* ATCC 6258. (B) Effect of different concentrations of NaCl on the MIC of COS against *C. parapsilosis* ATCC 22019. (C) Effect of different pH values on the MIC of COS against *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

5.3.6.4 Morphological alterations on the yeast cells

To understand the mechanism of action of COS on the cell wall of non-albicans *Candida* species, SEM studies were performed with cells of *C. krusei* and *C. parapsilosis* and the changes in the morphology of cell wall of these two strains were examined. Figures 9 A, B, G and H showed the morphology of the untreated cells (control). The C. krusei cells treated with COS (Figure 9 C and D) presented clear wrinkling and shrinking, losing their usual morphology, while the *C. parapsilosis* cells (Figure 8 I and J) not only lost their usual shape, but also presented pore formation, with pores ranging from 0.8 to 1.1 nm.

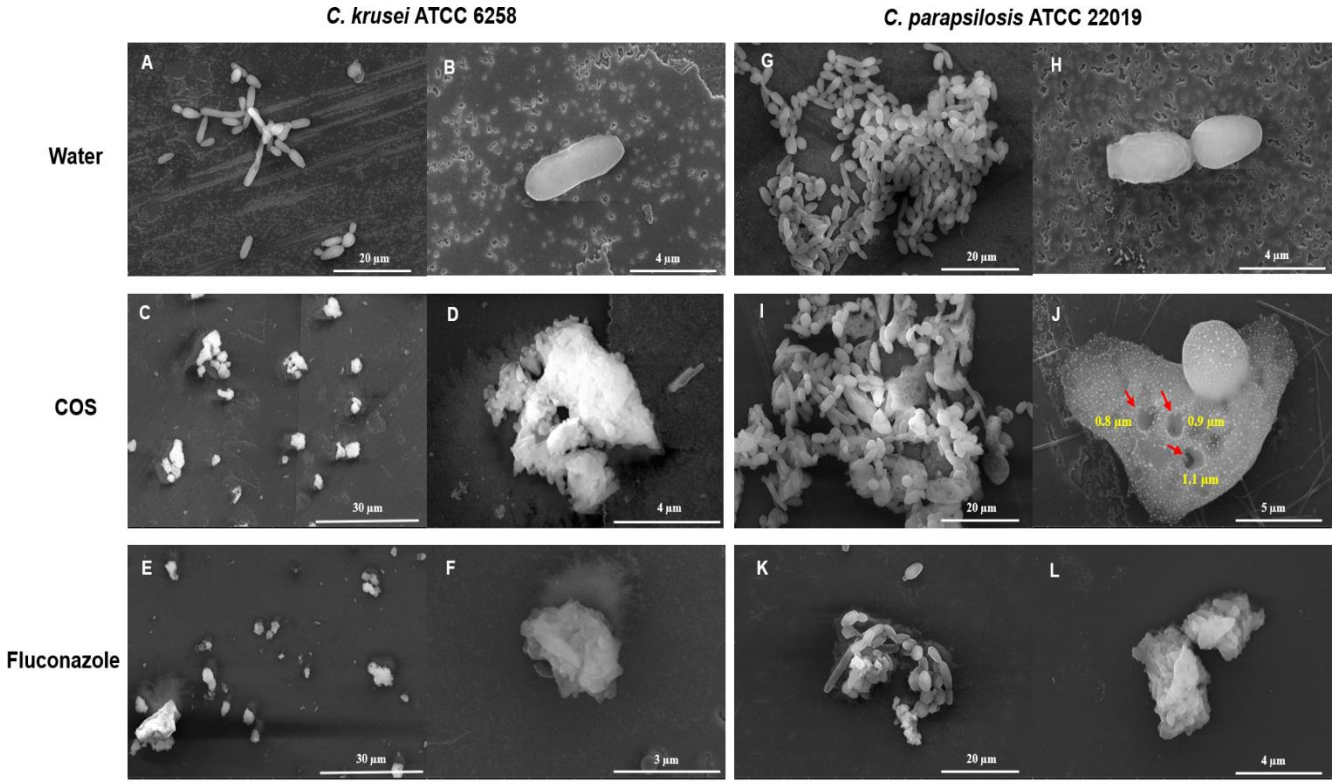


Fig. 9. Morphological alterations of C. krusei and C. parapsilosis after COS treatment. In (A) and (B), *C. krusei* cells were treated with water; in (C) and (D), cells were treated with 312.5 µg/ml of COS (MIC); in (E) and (F), cells were treated with 128 µg/ml of fluconazole. In (G) and (H), *C. parapsilosis* cells were treated with water; in (I) and (J), cells were treated with 312.5 µg/ml of COS (MIC); in (K) and (L), cells were treated with 128 µg/ml of fluconazole. Red arrows point to pore formation.

5.4. Discussion

The profile of the COS obtained differs from what was observed on another work using the same enzyme, where the DP ranged from 1 to 6 (AZEVEDO *et al.*, 2020). The difference between the DP was probably caused by the distinct preparation method, in which the present work's method diminishes the loss of larger molecules by abolishing the ultrafiltration step and substituting it for centrifugation, presenting a more teeming sample of COS.

The of MIC and MLC of *Candida* strains results go against what was observed by (KULIKOV *et al.*, 2014) where the *Candida* species, specially the ATCC 90028, wasn't affected by low molecular COS, only showing activity with MW higher than 9 kDa. In another work by Silva et al. (2021), the COS profile is similar to the present work with DP ranging from 1 to 6 and when the same strain of *C. albicans* (ATCC 90028) was treated with COS the MIC observed was 500 μg/mL. These differences might be caused either by the production process of COS or by the chitosan used since both factors are extremely important to determinate the acetylation degree and pattern of COS which directly affects its biological activities (LIAQAT; ELTEM, 2018). Another factor that might have contributed in the different MIC values are: solubility, since the COS that presented activity in Kulikov et al. (2014) have higher molecular weight, they might not be as soluble as the COS used in the present work, and pH, since the medium used in (SILVA, Nayara Sousa da *et al.*, 2021) was Muller Hinton broth, which presents a pH value around 7.2, higher than the pI of COS amino groups.

When Ganan et al. (2019) derivatized COS with 2-aminoacridone, the time-kill curves showed a very different result for a strain of *C. guilliermondii*: the number of the surviving cells was only reduced after 8 h and after this period the yeast cells presented a dose-dependent recovery, only keeping its reduced surviving cell rate when concentrations five or ten times higher than the MIC were used. The authors attribute the dose-dependent recovery to the metabolization of COS, which clearly doesn't happen in the present work, where the

concentration of the yeast cells of both *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 doesn't increase once it's severely reduced. It is interesting to notice that the COS used in Ganan et al. (2019) are higher than 3.5 KDa, with a DP around 54 and this might interfere in the cell incorporation of COS. Sun et al. (2021) showed that the cell uptake of chitosan is an important process for its antifungal activity against *Penicillium expansum*, which is probably also true for COS.

A study using *C. albicans* biofilms on PMMA resin immersed the biofilm samples in COS and chitosan solutions to test the remaining viability of the yeast cells and it showed positive results for both in 1 and 12 h (SRIMANEEPONG *et al.*, 2021). Another study using low molecular mass chitosan against the biofilm formation and degradation of several species of *Candida*, including *C. krusei* and *C. parapsilosis*, presented satisfactory results for both tests (SILVA-DIAS *et al.*, 2014). The higher concentration that was used in the present work was 732 μg/mL while the concentrations used by Srimaneepong et al. (2021) was 3000 and 6000 μg/mL and the one used in Silva-Dias et al. (2014) ranged from 10 to 10000 μg/mL, showing a percentage of degradation similar to the ones found in this work (~40%) with concentrations around 1250 μg/mL for *C. krusei* ATCC 6258 and 310 μg/mL for *C. parapsilosis* ATCC 22019.

To assess the mode of action of COS activity against *Candida* species, the cell membrane integrity was analyzed using the membrane-impermeable DNA intercalating agent propidium iodide (PI). PI is an indicator of the cell membrane integrity because specifically penetrates and stains DNA in dead cells or those with compromised cell membranes (ROSENBERG; AZEVEDO; IVASK, 2019) As it's shown on figures 4 and 5, the fluorescence levels of both *Candida* species treated with COS were higher than the control treatment, which suggests that COS significantly affects the *Candida* cells integrity. Kaloriti et al. (2014) observed that cationic fluxes can interfere with the activation of the ROS detoxification system in *Candid*a cells, which means that the fact COS have cationic properties along with the fact

the lack membrane integrity of the cells probably triggered ROS formation, might explain the high levels of ROS shown in figure 7. It is widely believed that the antimicrobial action of cationic molecules is due the interaction and permeabilization of the membrane of microorganisms (HELANDER; LATVA-KALA; LOUNATMAA, 1998) and the pore formation observed on the SEM analysis of the *Candida parapsilosis* cells treated with COS (Figure 9 J and I) is another clear indication of the effect of these molecules on the cell membrane of *Candida* species. A similar effect was seen by Vishu Kumar et al. (2005) when *B. cereus* cells were treated with COS, the authors observed pore formation and alteration on the cell morphology and suggested this antimicrobial activity could be due the cationic nature of COS and its interaction with the cell membrane.

Although the MIC and MLC were the same for both *C. krusei* ATCC 6258 and C. *parapsilosis* ATCC 22019, the results of the ionic strength and pH influence on the antifungal activity of COS were quite opposite (Figure 8). This result demonstrates that, despite of the expectation of the ionic force and pH of the medium to interfere with COS antifungal potential, since its activity is mostly related to its positive charge, the influence of this parameters depends on the species. *Candida parapsilosis* has been classified as salt tolerant (KRAUKE; SYCHROVA, 2010), while *Candida krusei* has shown morphology (JAMIU *et al.*, 2021) and cell wall components profile (ALVARADO *et al.*, 2023) different from other *Candida* species, these factors can influence on how medium characteristics such as ionic strength and pH will affect the COS and other molecules ability to inhibit the growth of these yeasts.

5.5 Conclusion

In the present work, chitooligosaccharides with a polymerization degree of 1 to 9 and 100% of solubility in water were successfully produced by an enzymatic method. COS has showed antifungal potential against several *Candida* species, presenting the capacity of degrading biofilms and rapidly kill the non-albicans *Candida* species tested in this work. The

analysis related to the mechanism of action of COS revelated that their antifungal activity is probably related to their ability to interact with negative components of the cell membrane, disturbing its integrity, inducing ROS production and causing morphological alterations in the cells. Further investigations about COS feasibility as a new therapy for the treatment of *Candida* infections are needed.

Autor statement

The authors declare that the manuscript entitled “Targeting non-albicans Candida with enzymatically produced chitooligosaccharides: Insights into antifungal mechanisms”, has not been published previously and that it is not under consideration for publication elsewhere. Authors also declare that they do not have any potential conflicts of interest.

Acknowledgments

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6 ARTIGO II: Optimizing quality and shelf life of minimally processed melons through chitooligosaccharides treatment

Mayara I. G. Azevedo [a], Luciana de Siqueira Oliveira [b], Thalles B. Grangeiro [c,*]

[a] Departamento de Bioquímica e Biologia Molecular, Centro de Ciências, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

[b] Departamento de Tecnologia de Alimentos, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil.

[c] Laboratório de Genética Molecular, Departamento de Biologia, Centro de Ciências, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil

*Corresponding author

Prof. Dr. Thalles B. Grangeiro

Laboratório de Genética Molecular, Departamento de Biologia, Centro de Ciências, Universidade Federal do Ceará, Campus do Pici, Fortaleza-CE, 60.440-900, Brazil

E-mail: thalles@ufc.br

Highlights

- COS treatment improves quality attributes of minimally processed melons.
- At day 4 of storage, the vitamin C content drastically drops in the control fruits and is maintained stable with the COS treatment.
- COS was able to activate an early stress response in MP melons, triggering their antioxidant system.

Abstract

The consumption of minimally processed fruits (MPF) has been increasing. Because of that, new strategies to improve the quality and shelf life of MPF are needed. Natural products with biological activities that cause little to no alterations on the fruits usual characteristics are a good alternative for this task. Thus, the aim for this study is to evaluate the effectiveness of a chitooligosaccharide (COS) treatment on the quality of minimally processed melons (*Cucumis melo* var. Cantalupensis Naud.) stored for 6 days at 10 °C. The COS treatment was able to improve quality attributes such as firmness, titratable acidity, and pH, therefore showing no softening and pH decrease during storage time. The treatment was also able to maintain vitamin C content of the melon, while the control showed a 41.3% decrease from day 4 of storage, and was also able to activate an early response from the antioxidant system, improving total antioxidant activity from day 2 and showing a higher enzymatic activity of catalase and ascorbar-peroxidase from day 4 of storage. These results obtained indicate that COS treatment can trigger a early stress response on minimally processed melons and consequently improve their quality and shelf-life.

Keywords: fresh-cut, shelf-life, fruit quality

6.1 Introduction

A diet enriched with fruit and vegetables has constantly been associated with the reduced risks of several diseases (WOODSIDE *et al.*, 2023), making the World Health Organization (WHO) recommend the consumption of 400 g of fruits per capita (LAFARGA *et al.*, 2019). Lately, the search for convenience has made the demand for minimally processed fruits (MPF) grow. MPF also known as "ready-to-eat" and "fresh cut" are fruits submitted to physical changes but remain fresh and keep the natural properties of the original fruit. The MPF undergo to several operations such as selection, cleaning, washing, trimming, peeling, cutting/mashing, sanitizing, and packing, but usually don't last as much as a regular fruit, due the exposure to microorganisms and loss of quality (MELO; QUINTAS, 2023; SAEBI; MORADINEZHAD; ANSARIFAR, 2023). Despite of this, the MPF market has been expanding, reaching an income of 320 billion USD in 2022 and bringing to light the need to find ways to maintain MPF quality and increase their shelf-life ("Processed Fruits & Vegetables Market Share Report 2023-2032")

Melon (*Curcumis melo* L.) is one of the most popular tropical fruits available as a minimally processed product and its consumption has been related to healthy habits due its bioactive compounds, including vitamin c and flavonoids (LUCIANO *et al.*, 2022). The processed melon portions have a shelf-life of 10 to 15 days at 2 °C, but this is rarely the temperature encountered at markets and at higher temperatures its quality and nutritional parameters decline much faster (SPADAFORA *et al.*, 2019). Natural bioactive products are an alternative for this task, since many of them are classified as generally recognized as safe (GRAS), causing minimal to no alterations on the product usual characteristics (SANTOS, Maria Isabel *et al.*, 2023).

Chitooligosaccharides (COS) are low molecular oligosaccharides with a polymerization degree ranging from 2 to 20 derived from chitosan's hydrolysis. COS has shown several

biological activities such as antimicrobial, antitumor, plant defense elicitor, and many others (LIAQAT; ELTEM, 2018). These molecules present high solubility in neutral pH, low viscosity, biodegradability, biocompatibility, and low toxicity, which makes them good candidates for several applications that involve their biological activities, presenting little to no setbacks when it comes to physicochemical attributes (HAO; LI; LI, 2021). Therefore, the aim of this work was to evaluate the effects of a COS treatment on minimally processed melon during storage, analyzing quality parameters and the antioxidant potential of the melons at different time periods.

6.2 Materials and Methods

6.2.1 Chitooligosaccharides production

The enzymatic production of chitosan oligosaccharides was performed using a recombinant chitosanase from *Chromobacterium violaceum* (AZEVEDO *et al.*, 2020). The reaction was carried out by dissolving 1% (w/v) of chitosan powder in 50 mM of acetic acid and mixing it with the chitosanase (5 μg of protein for every milliliter of the solution). The mixture was incubated for 24 h in 50 °C under constant agitation and the reaction was stopped by immersing the tubes in boiling water for 10 min. After cooling in room temperature, the mixture was centrifugated (10000 g for 20 min at 10 °C) and the pellet was discarded. The supernatant was freeze dried and kept at 4 °C until used.

6.2.2 Plant material

Ripe cantaloupe melons (*Cucumis melo* var. *Cantalupensis*) were purchased from a local market (Fortaleza – CE) and selected for the uniformity of the maturity stage, size, and absence of warning signs. After selection and washing with chlorinated water (150 μL/L, pH 7.0) for disinfection, the fruits were subjected to minimal processing.

6.2.3 Processing and COS application

Processing was carried out under refrigeration (20 °C). Before processing, all utensils

and the environment were previously sanitized with chlorinated water (150 µL/L, pH 7.0). Operators were protected with latex gloves, aprons, caps, and masks, to avoid possible contamination during processing. After peeling the fruits, proceed with processing according to the following steps: cutting the ends, cross-section dividing the fruit into equivalent halves, removing the seeds, and cutting into cubes (50 g).

After minimal processing, the minimally processed fruits in cubes were immersed for 1 minute in the following treatments: COS at 1 g/L (Dissolved in distilled water) and distilled water (Control treatment) and then left to drain the excess for 3 minutes. The coated cubes were packed in PET trays wrapped in PVC film. The trays with coated or uncoated processed fruits (control) were stored at 10 °C for 6 days and were analyzed for quality and antioxidant potential on days 0, 2, 4 and 6.

6.2.4 Quality attributes

6.2.4.1 Color and soluble solids

Fruit color was determined using the Minolta CR-300 colorimeter and two measurements were made per cube to obtain average luminosity values (L). The soluble solids (SS) content was determined with a digital refractometer (Model PR-100 Pallete) according to Instituto Adolfo Lutz (IAL, 2008) and the results were expressed in ºBrix.

6.2.4.2 Firmness

The firmness of the processed and treated melon cubes was measured using a digital bench penetrometer (Solilcontrol, model DD – 200). The measurement was carried out on opposite sides of each cube and the results were expressed in Newtons (N).

6.2.4.3 Titratable acidity and pH

For the following analysis, the melon cubes for each treatment were macerated into pulp. The pH was determined with a pHmeter and the titratable acidity was determined by titration with a 0.1 M NaOH solution with the results expressed in g of acidic content/100 g of

fruit.

6.2.4.4 Determination of soluble protein content

Concentration of soluble proteins was determined using the Bradford method (BRADFORD, 1976). The results were expressed in mg of protein per g of pulp and were used to calculate the enzymatic activities mentioned in the following sessions.

6.2.5 Antioxidant potential

6.2.5.1 Vitamin C

Total vitamin C content was determined by titration with 0.02% 2,6-dichloro-indophenol (DFI) (Strohecker and Henning, 1967), with one gram of pulp homogenized in 100 mL of 0.5 % oxalic acid. Then, 5 mL of this solution was diluted to 50 mL with distilled water, titrated, and results were expressed as mg kg 1on a fresh weight basis.

6.2.5.2 Yellow flavonoids

The yellow flavonoids content was determined as described by Francis (1982). Briefly, 1 g of the fruit pulp of each treatment was homogenized in 50 mL of an extracting solution (1.8% HCl in ethanol 95%) and left to rest for 16 h in 4 °C. After this period, the material was filtered through filter paper and immediately read in a spectrometer at an absorbance of 374 nm.

6.2.5.3 Carotenoids

The total carotenoids content was measured as described by Lichtenthaler and Wellburn (1983), based on the chlorophyll a and b concentrations and using the following formulas: chlorophyll a (g/L) = 12.25x(A663) - 2.81(A646); chlorophyll b (g/L) = 20.31x(A646) - 2.81x(A663) and carotenoids (g/L) = (1000x(A470) - 3.27x[chlorophyll a] - (104x[chlorophyll b])/227. The results were expressed in mg of carotenoids per kg of fresh fruit.

6.2.5.4 Polyphenols

Total polyphenols content was determined according to the Folin-Ciocalteu method.

Total phenolic amount was calculated by a five-point calibration curve obtained with different quantities of gallic acid standard solution ranging from 0 to 50 µg. The results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

6.2.5.5 Total antioxidant activity

Total antioxidant activity using the FRAP method was determined according to (PULIDO; BRAVO; SAURA-CALIXTO, 2000). Three dilutions were made from the extract, and an aliquot of 90 µL of each dilution was transferred to test tubes and 270 µL of distilled water and 2700 µL of FRAP reagent were added (25 mL of 0.3 M acetate buffer, 2.5 mL of 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) 10 mM, 2.5 mL of 20 mM ferric chloride. The suspensions were homogenized and kept in a water bath at 37° C for 30 minutes, when the absorbance was measured at 595 nm. A standard curve used was obtained from ferrous sulfate with concentrations ranging from 500 to 1500 µM and the results were expressed as µM ferrous sulfate 100 g^{-1} MF.

6.2.5.6 Antioxidant enzymes

The catalase activity (CAT, EC 1.11.1.16) was determined according to Beers and Sizer (1952). The enzymatic activity was measured by monitoring the decrease of hydrogen peroxide concentration through absorbance at 240 nm and quantified by its molar extinction coefficient (36 mol L^{-1} cm^{-1}). The results were expressed in 1 µmol H_2O_2 kg^{-1} min^{-1}. The ascorbate peroxidase (APX, EC 1.11.1.1) activity was determined according to Nakano and Asada (1981), using 96-well plates. The enzymatic activity was measured using the molar extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}), considering that 1 µmol of ascorbate is required for a reduction of 1 µmol H_2O_2. The results were expressed in 1 µmol H_2O_2 kg^{-1} min^{-1}.

6.2.6 Statistical analysis

The experimental design was completely randomized in a 2x6 factorial (treatment x storage time), with three replications consisting of three trays with eight melon cubes each. The data

were subjected to analysis of variance (ANOVA) using a statistical program GraphPad Prism 8.4.3, and the means were compared using the Tukey test at 5% probability.

6.3 Results and discussion

6.3.1 Effects of COS on quality and physicochemical characteristics

6.3.1.1 Fruit color and firmness

Fresh cut melons treated with enzymatically produced COS, as well as untreated control fruit showed no signs of decay, and therefore were acceptable for consumption for up to six days, during which they were evaluated for quality attributes. The COS treatment did not affect the fruit color when compared to the control (Fig. 1A), as both treatments had the lightness (L*) decreasing around 16% at the end of storage time. For melon fruits, this parameter might only be affected by a longer storage period. A work used a chitosan coating with trans-cinnamaldehyde to treat minimally processed melon and observed significant difference between coated and uncoated fruits only after 10 days of treatment (CARVALHO, Roberta Lopes *et al.*, 2016).

The firmness was positively affected by the COS treatment (Fig. 1B). While the control fruits firmness decreased 47% from day 2, the treated fruits were able to maintain their firmness through the whole period of storage with no statistical difference between the treated fruit from the beginning (day 0) to the end of storage time (day 6). Firmness is an indicator of fruit quality and is directly related to the mechanical and physical characteristics of the cell wall (SUN, Qinming *et al.*, 2023). Maintaining a desirable firmness is extremely important, since melon consumers consider texture one of the most important quality attributes for acceptability of this fruit (LESTER, 2006). Fruit softening occurs due the disassociation of the cell wall and solubilization of the pectin in the middle lamella, which drastically reduces cell adhesion (MOLINA-HIDALGO *et al.*, 2013). COS is a known plant elicitor and can trigger defense mechanisms like the thickening of the plant cell wall with the accumulation of pectin and lignin

(SIRIWONG *et al.*, 2021), which probably increased the firmness of the treated fresh melon.

6.3.1.2 Soluble solids, pH, and titratable acidity

The soluble solids content showed no significant difference between treated and not treated melons (Fig. 1C). As it was suggested for color, this parameter might only be influenced by treatments when it's observed during a longer storage period when it comes to melon fruits. Treatments with chitosan (ZHANG, Qin *et al.*, 2022) and curcumin-based photodynamic sterilization (LIN, Yilin *et al.*, 2019) only showed statistically significant differences in fresh cut melon's soluble solids after 12 and 9 days, respectively.

The pH of the untreated fruits decreased through the storage period from 7.2 (Day 0) to 6.5 (day 6), however the COS treatment was able to control this phenomenon and kept the pH stable around 7.2 during storage (Fig. 1D). The titratable acidity is directly related to the pH and showed a similar pattern (Fig. 1E), increasing through the storage period for the untreated fruits, while the showing stability for the COS treated ones. Acidity, measured by titratable acidity and pH, is considered an important factor in fruit organoleptic quality, and is greatly influenced by organic acids in ripe fruits (ETIENNE *et al.*, 2013). Melon has low levels of acidity, with pH around neutral values, and citrate is its main organic acid (COHEN *et al.*, 2014). ATP-citrate lyase (ACL) is a key regulator of acetyl-CoA pathway and plays a critical role in citric acid accumulation in melon (REN *et al.*, 2023). ACL can be upregulated when plants are under stress (HU *et al.*, 2015). COS can trigger the MAPK (mitogen-activated protein kinases) pathway by interacting with membrane receptors and stimulating a ROS burst in several plants activating early stress responses (BRULÉ *et al.*, 2019; KOZYULINA *et al.*, 2023). The MAPK pathway is capable to regulate immunity responses in plants and can elicit several mechanisms to handle stresses and upregulate enzymes to deal with high acid levels and malate/citrate metabolism (LIN, Li *et al.*, 2021; PATIL; NANDI, 2020). Therefore, we hypothesize that the COS treatment may induce the MAPK pathway, activating immunity

responses on fresh cut melons, thus regulating enzymes like ACL to maintain low acidity levels.

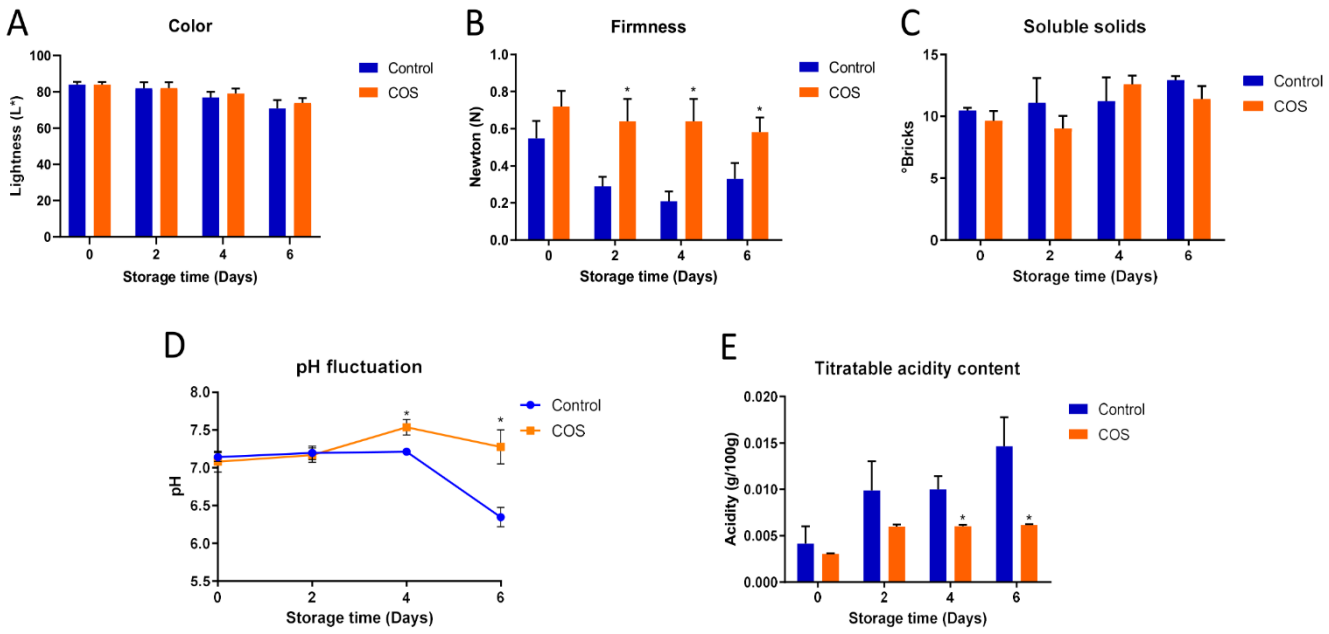

Fig. 1. Color (A), firmness (B), soluble solids (C) pH (D), and titratable acidity (E) contents of fresh-cut melon treated with COS and stored at 10 °C for 6 days. Asterisks (*) indicate significant differences at $P < 0.05$ between treatment and control.

6.3.2 Antioxidant system

6.3.2.1 Antioxidant compounds

Bioactive antioxidant compounds were evaluated. The results showed that while the polyphenols (Fig. 2A) and yellow flavonoids (Fig. 2B) content remained unchanged for treated and untreated fruits, with no statistic difference between the first and last day of storage, the carotenoids content (Fig. 2C) displayed decay for both treatments. However, the vitamin C content had a significant drop of 41.3% from the day 4 of storage time in the untreated melon while it was kept stable in the ones treated with COS (Fig 2. D). COS treatment was also able to increase vitamin C levels in passion fruit (ZHANG, Liqun *et al.*, 2023), potato (XIE, Pengdong *et al.*, 2022) and strawberry (HE *et al.*, 2018). High concentrations of reactive oxygen species (ROS) can damage the cells and reduce the quality of fruits. Antioxidant compounds such as polyphenols, flavonoids, carotenoids, and vitamin C are used by plants to manage ROS

concentration, but during postharvest storage the content of compounds decreases, presenting the demand for a suitable treatment or method that can maintain a high antioxidant content (MEITHA; PRAMESTI; SUHANDONO, 2020).

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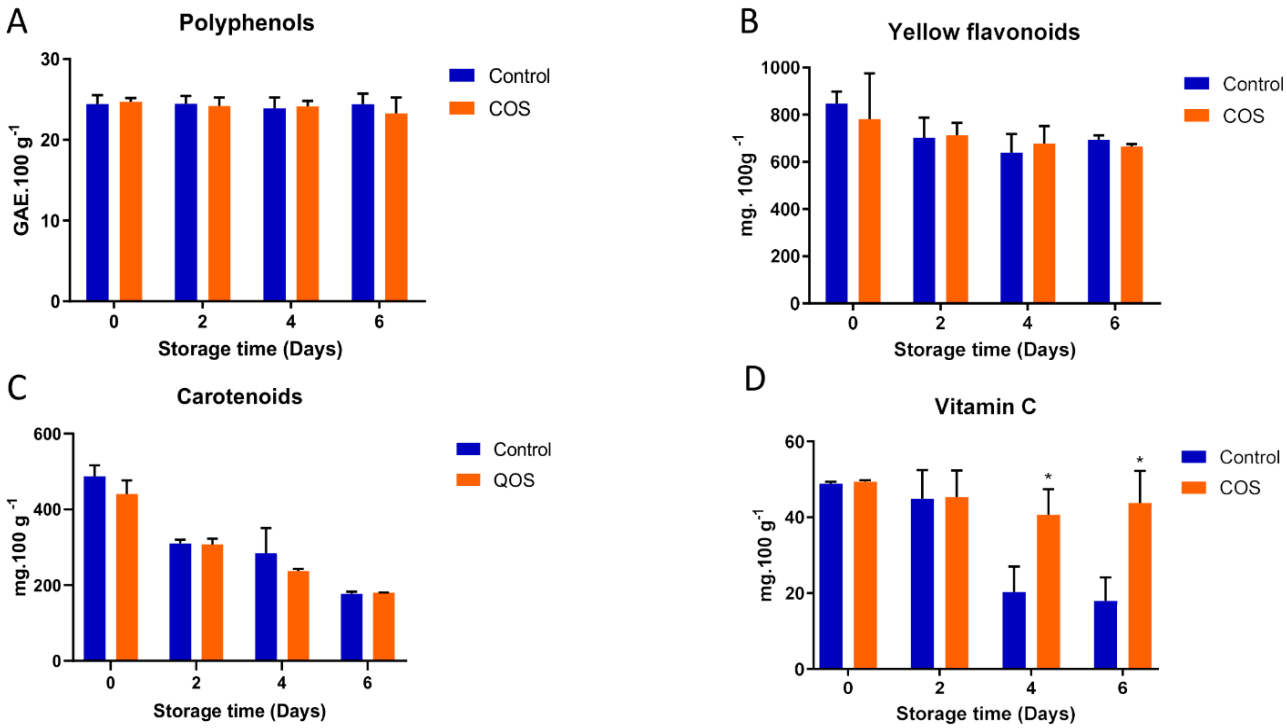

Fig. 2. Polyphenols (A), yellow flavonoids (B), total carotenoid (C) and total vitamin C (D) contents of fresh-cut melon treated with COS and stored at 10 °C for 6 days. Asterisks (*) indicate significant differences at $P < 0.05$ between treatment and control.

6.3.2.2 Antioxidant enzymes and total antioxidant activity

COS treatment showed positive impact on both catalase (CAT) and ascorbate peroxidase (APX). As it was shown on Fig. 3A, while CAT enzymatic activity of the control fruits had a 58.3% decay from day 0 to day 6, the samples treated with COS were able to maintain most of their enzymatic activity during storage time. The APX activity (Fig. 3B) of the control fruits showed similar pattern of what it was observed for CAT, losing 25% of its activity in the last day of storage. The COS treatment not only kept APX activity stable, but it

was able to enhance it by 10% from the day 4 of storage, keeping this increase at day 6. The abiotic stress related to the wounding caused by minimally processing of fruits and vegetables has a significant impact on quality of the shelf-life of the products (HODGES; TOIVONEN, 2008). Under biotic and abiotic stress, the redox balance of the plants is disturbed, leading to an exacerbated accumulation of ROS and causing oxidative stress. The antioxidant system through its enzymes acts to mitigate the ROS levels and maintain the plant homeostasis, which makes the antioxidant enzymes an essential tool to improve the response against stresses (LIU, Runqiang *et al.*, 2021). COS can effectively ameliorate the activity of antioxidant enzymes such CAT and APX in plants, improving the resistance against several biotic and abiotic stresses (LI, Jingchong *et al.*, 2022). In this work, the COS treatment was clearly able to stimulate the antioxidant system to manage the stress caused by minimal processing of melon.

The total antioxidant activity (TAA) of the fresh melons (Fig. 3C) had also showed an improvement caused by the COS treatment. COS provoked a higher antioxidant activity from day 2 of storage time, keeping it at day 4 and only returning to values similar to the control treatment on day 6. This result indicates that the COS treatment induces faster antioxidant response when compared to the control.

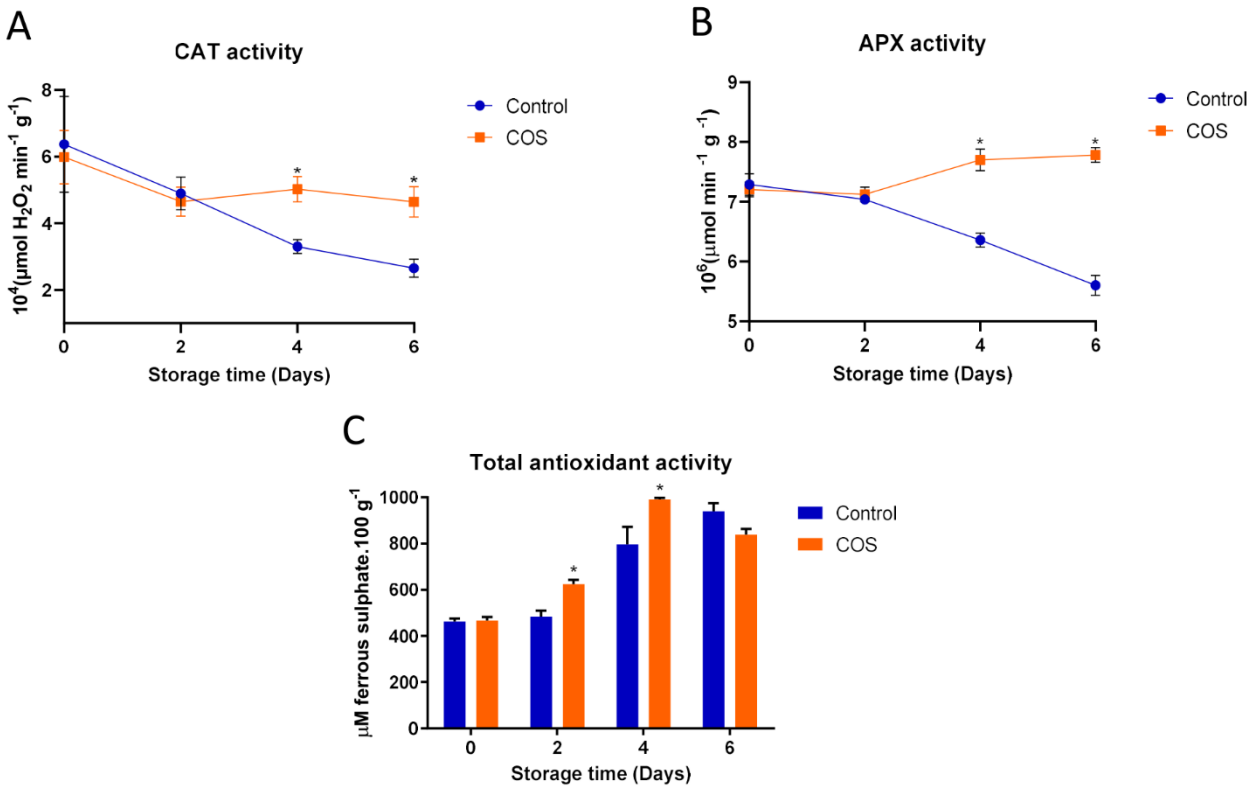

Fig. 3. Activity of antioxidant enzymes catalase-CAT (A) and ascorbate peroxidase-APX (B) and total antioxidant activity of fresh cut melon treated with COS and stored at 10 °C for 6 days. Asterisks (*) indicate significant differences at $P < 0.05$ between treatment and control.

COS has the ability of stimulating the antioxidant system in plants, increasing the activity and content of ROS scavenging enzymes to protect the plants against several stresses (LIU, Yao *et al.*, 2023). Like it has been mentioned in the previous section, COS can trigger the MAPK pathway by using ROS as signaling molecules, activating early stress responses. The results obtained in this work are consistent with this, given that the TAA was enhanced by day 2 of storage time, while the control showed a late response and both CAT and APX activities were improved by day 4, while the control treatment only showed decay.

6.4 Conclusion

The 1g/L COS treatment was able to maintain the quality of minimally processed Cantaloupe melons during storage at 10 °C for 6 days by preserving firmness, vitamin C and

their neutral pH and activating their antioxidant system. It is proposed that COS, due its ability to interact with membrane receptors on the cellular membrane of plants and trigger the MAPK pathway by using ROS as signaling molecules, stimulate an early response to the stress caused by the minimal processing (Fig. 4), resulting in fruits with better quality and shelf-life than the ones that didn't go through COS treatment.

These results provide evidence that COS can be used as a protective treatment for Cantaloupe melons to maintain the quality of the minimally processed fruits during storage.

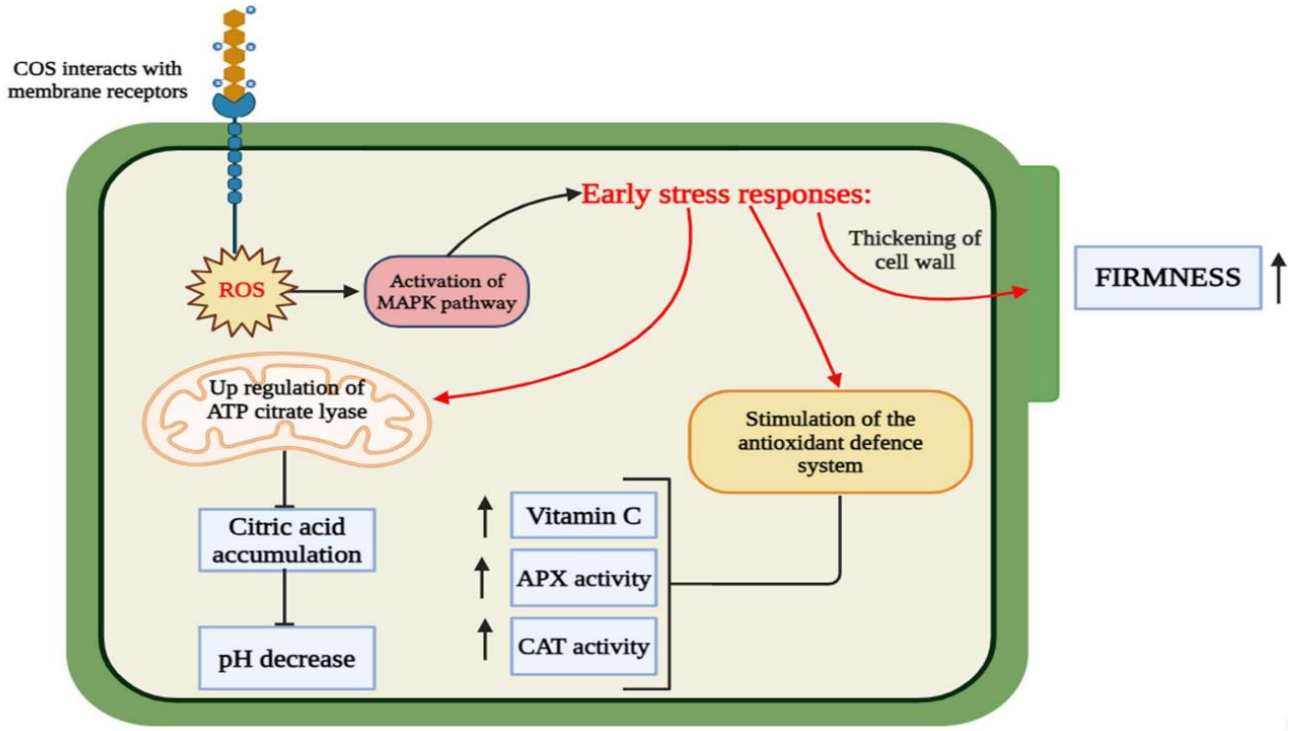

Fig. 4. Proposed mode of action of the COS treatment on fresh cut melon physiology and cell metabolism.

Autor statement

The authors declare that the manuscript entitled "Optimizing quality and shelf life of minimally processed melons through chitooligosaccharides treatment, has not been published previously and that it is not under consideration for publication elsewhere. Authors also declare that they do not have any potential conflicts of interest.

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7 ARTIGO III: Assessment of the potential of chitooligosaccharides to enhance the activity of *Bacillus thuringiensis* against *Aedes aegypti*

Mayara I. G. Azevedo [a], Rafael X. Martins [b], Maria E. S. Maia [b], Davi Farias [b], Luiz C. P. Almeida Filho [c], Pedro M. S. Tabosa [c], Ana F. F. U. Carvalho, Thalles B. Grangeiro [c,*]

[a] Department of Biochemistry and Molecular Biology, Federal University of Ceará, Fortaleza, CE, Brazil

[b] Laboratory for Risk Assessment of Novel Technologies (LabRisk), Department of Molecular Biology, Federal University of Paraiba, Campus I, 58051-900, João Pessoa, PB, Brazil

[c] Department of Biology, Science Center, Science Center, Federal University of Ceará, Fortaleza, CE, Brazil

*Corresponding author

Prof. Dr. Thalles B. Grangeiro

Laboratório de Genética Molecular, Departamento de Biologia, Centro de Ciências, Universidade Federal do Ceará, Campus do Pici, Fortaleza-CE, 60.440-900, Brazil

E-mail: thalles@ufc.br

Abstract

Aedes aegypti is a vector for several important diseases, such as dengue and yellow fever. Many insecticides have been used over the years to control this vector. However, *Ae. aegypti* has acquired resistance, revealing the need for new strategies to contain this mosquito. The use of combined molecules could be an interesting alternative for this problem. COSs are chitosan derivatives that present many biological activities, such as antimicrobial, antitumour and plant defense elicitation, as well as solubility at neutral pH and low viscosity. COS could be a good candidate for combination with a well-known insecticide, such as Bt toxins. Thus, the aim of this work was to study COS activity against *Ae. aegypti* larvae, both alone and in combination with Bt toxins, and to evaluate the potential of COS for toxicity to Zebra fish. The results showed that COS was not active against *Ae. aegypti* larvae but was able to enhance the activity of the Bt toxin LC_{20} at different concentrations (500 to 16 µg/mL), and their combined action caused severe damage to the larval midgut, probably impairing food absorption. COS did not present any toxicity to Zebra fish embryos. Therefore, the combination of COS and Bt toxins could be an interesting and safe alternative for controlling *A. aegypti* larvae.

Keywords: Insecticide, dengue fever, Bt toxins

7.1 Introduction

Mosquitos are globally distributed and are only sensitive to extreme temperatures. Mosquito-borne diseases are responsible for more than 700 million infections every year, most of which are caused by arboviruses (COTTIS *et al.*, 2023). Arboviruses are viruses that replicate in the cells of hematophagous arthropod vectors and infect the human host through the arthropod vector bite. The most important arboviruses are those that cause dengue fever, yellow fever, Zika and chikungunya, and they are all transmitted by the mosquito *Aedes aegypti* (ALCORN; KLIMSTRA, 2022). *Aedes aegypti* (Diptera: Culicidae) is a highly efficient vector because it can be commonly found in urbanized areas of tropical and subtropical countries due to its strong ability to live in close association with humans (LEANDRO *et al.*, 2022). *Aedes* is an animal taxon that causes more economic consequences, costing 150 billion USD every year because of damage and management, demonstrating the need for investments in vector control strategies (BONDS; COLLINS; GOUAGNA, 2022).

The usual control strategies for *Ae. aegypti* focus on reducing larval habitats by increasing awareness of the population and reducing adult populations using insecticides. Nevertheless, there are several reports of insecticide resistance in *Ae. aegypti*, urging the use of management approaches, as well as new potential insecticides (DUSFOUR *et al.*, 2019). The Bt toxins, a group of toxins produced by the gram-positive bacteria *Bacillus thuringiensis*, are among the most commonly used insecticides in the world. Bt toxins are extremely selective for insects, acting against the Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Orthoptera and Mallophaga orders without harming humans, animals, or plants (LI, Yujie *et al.*, 2022). Bt toxins act against *Ae. aegypti* larvae by interacting with midgut receptors and forming pores, which makes these toxins the most effective and recommended larvicides against Aedes spp. (CARVALHO, Karine da Silva *et al.*, 2021). However, constant exposure to Bt toxins can cause larvae to develop moderate resistance after 30 generations (TETREAU

et al., 2012). Therefore, the combination of Bt toxins with other molecules that could work synergistically or could potentiate their activity might be an alternative to prevent resistance and augment insect control.

Among the molecules that could be used to potentiate the activity of Bt toxins against *Ae. aegypti*, chitooligosaccharides (COSs) are a good alternative. COSs are oligosaccharides of chitosan and, like chitosan, have several biological activities, such as antimicrobial, antitumor, and antiparasitic activities, among others (KOWALCZUK; ANIL, 2022). These oligosaccharides could have potential insecticidal activity because they can act as inhibitors of the chitinases present in the insect midgut during their development stages, such as in larvae, by working as competitive inhibitors and disturbing larval growth and nutrient absorption (CHEN, Lei *et al.*, 2014). In this context, the present study aimed to evaluate the larvicidal activity of COS and its ability to potentiate the larvicidal activity of Bt toxins against *Ae. aegypti* larvae.

7.2 Materials and Methods

7.2.1 Reagents

Chitosan powder with a deacetylation degree of 85% was purchased from Êxodo Científica (Sumaré, SP, Brazil). All other chemical reagents were of analytical grade.

7.2.2 Recombinant protein

The chitosanase *Cv*Csn46 from *C. violaceum* ATCC 12472 was produced in *Escherichia coli* BL21(DE3) and purified as described by Azevedo et al. (2020). The purity of the samples was verified by polyacrylamide gel electrophoresis under denaturing conditions (SDS–PAGE) according to the methods of Laemmli (LAEMMLI, 1970).

7.2.3 Chitosan oligosaccharides

Chitooligosaccharides were generated from polymeric chitosan by enzymatic digestion with the recombinant chitosanase *Cv*Csn46 following a previously described protocol. The

molecular masses of the oligosaccharides present in the COS preparations were determined by electrospray ionization mass spectrometry (ESI-MS) according to previously described protocols (AZEVEDO *et al.*, 2020).

7.2.4 Toxins from B. thuringiensis

Total Cry toxins from *B. thuringiensis* subsp. *israelensis* were prepared from sporulating cultures as described by (TABOSA *et al.*, 2020a).

7.2.5 Toxicity testing on larvae of Aedes aegypti

Eggs of *Ae. aegypti* (Rockefeller strain) were maintained at 27 °C ± 2 °C under a photoperiod of 12 h light/12 h dark. To evaluate the effects of proteins and chitooligosaccharides on egg hatching, 5 mL of distilled water containing the compounds (10 eggs/replicate and 3 replicates/treatment) was added to the eggs, which were incubated under standard conditions, and the number of larvae that hatched until 48 h was recorded. Toxicity tests to evaluate larvicidal activity were performed using 4th instar larvae, which were transferred to 5 mL of distilled water (pH = 6.5) containing the compound to be evaluated. Six replicates were used per treatment, with 10 larvae per replicate. Larvae transferred to distilled water alone or to distilled water containing *Bt* toxins were used as controls. Larval lethality data were subjected to one-way analysis of variance (ANOVA), and the means were compared by Tukey's test (significance level threshold = 0.01).

7.2.5.1 Mosquitoes

The eggs of *Aedes aegypti* (Rockefeller strain) were provided by Núcleo de Controle de Vetores (Nuvet) and maintained at 27 ± 2 °C under a photoperiod of 12 h of light. The turtle food Alcon Club Reptolife (Camboriú, SC, Brazil) was used to feed the larvae. The same conditions were used in all assays with the larvae.

7.2.5.2 Larvicidal activity of Bt toxins

The larvicidal assay against *Ae. aegypti* was performed with ten initial 4th instar larvae

as previously described (FARIAS *et al.*, 2009). Ten larvae were collected and exposed to toxin solutions (5 mL) at concentrations ranging from 278.7 × 10-3 to 0.544 × 10-3 μg.mL^{-1} for 24 h. Distilled water was used as a negative control. The tests were performed in sextuplicate, and the lethal concentrations of toxins that cause 20% mortality (LC20) were determined from the observed data via logistic survival analysis.

7.2.5.3 Potentiating action of chitooligosaccharides on Bt toxins and histological analysis

The effect of COS on the larvicidal activity of toxins produced by Bt was determined according to Tabosa et al. (2020). Ten larvae were incubated in the previously determined Bt LC20 combined with COS (125; 62.5; 31.125; 15.56 μg.mL^{-1}) for 24 h. The tests were performed in sextuplicate. Distillated water, Bt CL20 and COS (1000 μg.mL^{-1}) were used as controls. The data obtained were used to determine the cooperative effect of COS and the activity of Bt toxins by comparison to those of the controls.

Morphological changes in the gut epithelial cells of insect larvae were observed according to Viana et al. (2020). After 24 h of exposure to the highest concentrations of COS, protoxin solutions and their combinations, live larvae were individually collected, placed into sterile tubes containing paraformaldehyde (4%) and kept at room temperature for 48 h. Afterwards, the biological material was incubated in alcohol (70%) for 24 h, after which the larvae were subjected to serial dehydration with ethanol (80 to 95%). The dehydrated samples were subjected to preinfiltration with a Leica Historesin Embedding Kit® (50, 75 and 100%) and finally blocked. Histological sections of 5 μm were made using a microtome, stained with toluidine blue and analyzed under an optical microscope at final magnifications of 40 and 100 times.

7.2.6 Statistical analysis

To determine significant differences among treatments, analyses of variance (ANOVAs) were used, followed by Tukey's test ($p < 0.01$). The results are expressed as the

mean ± standard deviation (SD) of each result.

7.2.7 Zebrafish embryos

Zebrafish (*Danio rerio*) embryos (AB wild-type strain) at approximately 1 hpf (hour postfertilization) were obtained from the Production Unit for Alternative Model Organisms (UniPOM), Federal University of Paraiba, João Pessoa, Brazil. The parents were maintained in a recirculation system with regular monitoring of water quality parameters (pH, ammonia and nitrite levels) in a room with controlled temperature (26 ± 1 °C) and photoperiod (14:10 light/dark cycle). Fishes were fed daily with commercial food (Color Bits Tetra, Melle, Germany) and freeze-dried spirulina (Fazenda Tamanduá, Patos, Brazil) and were also monitored for abnormal behavior or disease development.

The day before the experiment, adult zebrafish (male to female ratio of 2:1) were transferred to a 7-L spawning tank with a bottom mesh and a quick-opening valve for embryo collection. Embryos were collected on the day of the experiment and cultured in adapted E3 embryonic media (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, and 0.33 mM MgSO4) supplemented with 0.005% methylene blue. Only spawning plants with a fertilization rate ≥ 90% were used. Viable embryos (with a normal cleavage pattern and without morphological changes) were selected under an inverted light microscope (Televal 31, Zeiss, Germany) at 50× magnification.

The experiments conducted with zebrafish in this study were approved by the Ethics Committee on the Use of Animals at UFPB, with authorization documented by protocol no. 4460140920.

7.2.8 Acute toxicity test

The fish embryo acute toxicity (FET) test was conducted with COS according to OECD's guideline number 236 (OECD, 2013), which was adapted for 96-well plates by Muniz et al. (2021). Zebrafish embryos at up to 3 hpf were exposed to 100 mg/L COS, and this

concentration was used as a limit test. Twenty wells were filled individually with 0.3 mL of solution and 1 embryo. Under the same experimental conditions, controls were also prepared with only E3 medium or 4 mg/L 3,4-dichloroaniline (DCA) (positive control).

Lethal and nonlethal effects were observed daily for 96 h. Embryos showing lethality endpoints (embryo coagulation, lack of somite formation, nondetachment of the tail bud and lack of heartbeat) were considered dead. This number was used to determine the survival rate % (number of living organisms/total number of organisms x 100) per tested sample. The exposures were under static conditions (without renovation of the exposure solution). Observations were performed under a stereomicroscope (Olympus SZX7, Japan) at 56× magnification, and the samples were photographed (Moticam 5+, China). After 96 h, the surviving larvae were euthanized with eugenol and properly discarded.

7.3 Results and Discussion

7.3.1 Activity against eggs and larvae of Ae. aegypti

In this work, we assessed the toxicity of a GH46 chitosanase from *C. violaceum* ATCC 12472 (*Cv*Csn46) and a mixture of chitooligosaccharides obtained from the digestion of polymeric chitosan by this enzyme to *Ae. aegypti* eggs and larvae. When chitooligosaccharides (concentrations ranging from 0.03125 to 1 mg/mL) or *Cv*Csn46 (concentrations varying from 0.03125 to 0.5 mg/mL) were evaluated alone against third instar larvae, no toxicity was observed, as evidenced by a 100% larval survival rate after 24 h of exposure. Under the same experimental conditions, *Bt* toxins, at a concentration of 13.09 μg/mL (LC_{50}), caused a mean lethality of 60% ± 10%. Furthermore, neither chitosanase nor COS alone, at concentrations ranging from 0.0625 to 0.5 mg/mL or from 0.03125 to 1 mg/mL, respectively, affected egg hatching, even after 48 h of exposure to the recombinant enzyme.

COS could have potential activity against *Ae. aegypti* larvae since it can function as an inhibitor of insect chitinases. Chitin is present in the exoskeleton of insects and in their gut

lining; for this reason, chitinases are key enzymes for insects since they are secreted during development stages (CHEN, Lei *et al.*, 2014). Considering that the COS used in this work did not affect *Ae. aegypti* larvae on their own, in the next section, we tested their ability to enhance the well-known larvicidal activity of Bt toxins.

7.3.2 Increased Bt toxin activity in Ae. aegypti larvae

To verify whether these compounds potentiate the larvicidal activity of *Bt* toxins, they were tested against third instar larvae of *Ae. aegypti* in the presence of *Cry* toxins at 0.01309 µg/mL (LC_{50}). The mean mortality of larvae exposed to *Bt* toxins alone (LC_{50}) was 40%, whereas the average mortality of larvae exposed to *Bt* toxins (LC_{50}) in the presence of different concentrations of *Cv*Csn46 was significantly greater ($P < 0.01$), ranging from 53.3% to 83.3% (Fig. 1). When third-instar larvae were exposed to *Bt* toxins in the presence of COS, the mean lethality rates were significantly greater ($P < 0.01$) (93.3% and 96.6%; Fig. 2) than the mean lethality percentage of larvae treated with *Bt* toxins alone. These data showed that *Cv*Csn46 and COS potentiate the larvicidal action of Cry toxins.

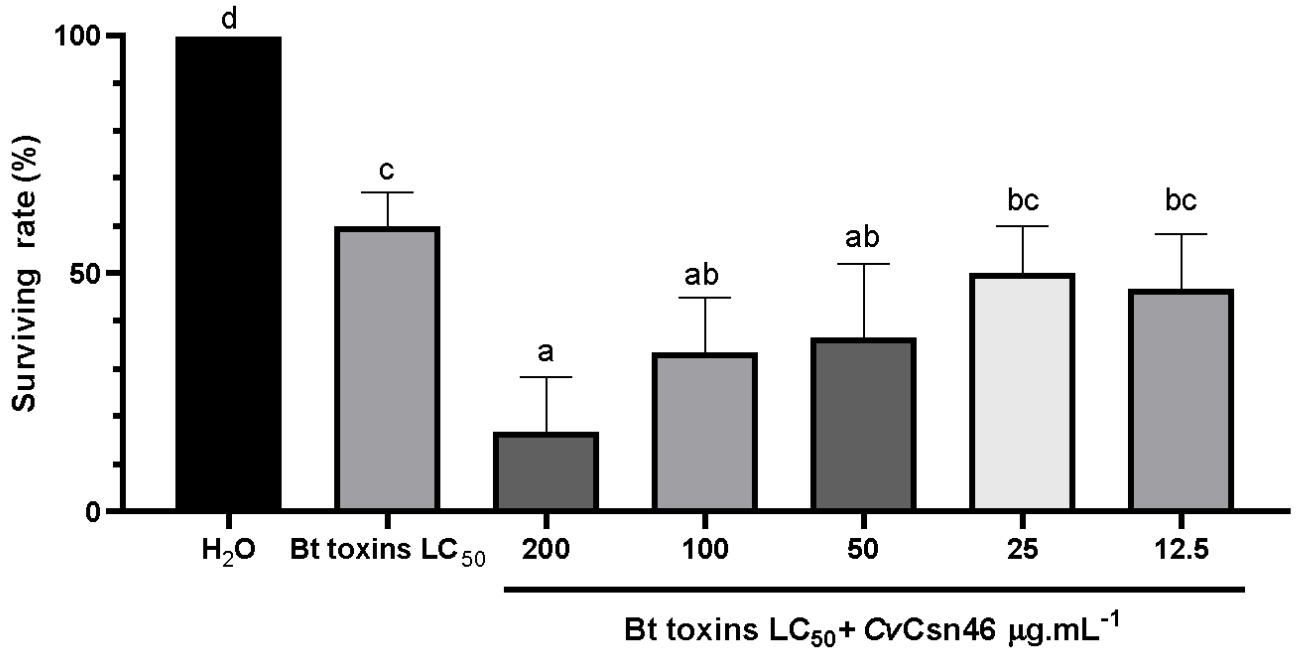


Fig. 1. *Cv*Csn46 enhanced the activity of Bt toxins against *Ae. aegypti* larvae.

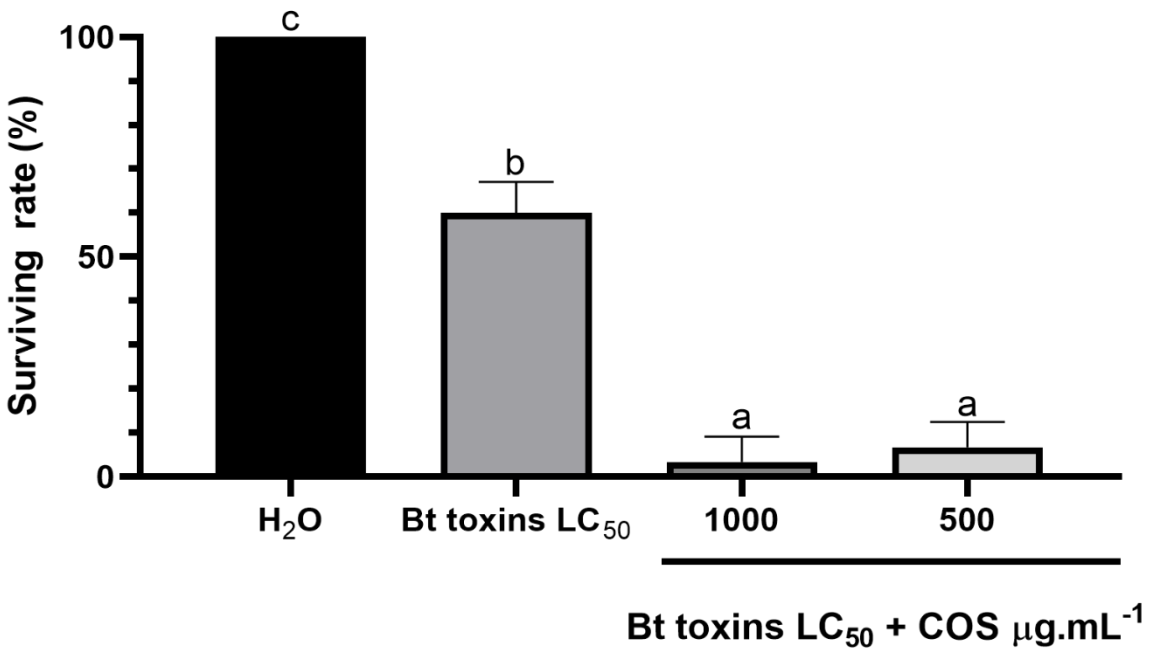


Fig. 2. COS-mediated increase in the activity of Bt toxins at the LC50 against *Ae. aegypti* larvae.

To further support the evidence that COS can significantly potentiate the larvicidal activity of *Bt* toxins, third instar larvae of *Ae. aegypti* were treated with *Bt* toxins at 11.01 ng/mL (LC_{20}) in the absence or presence of COS at concentrations ranging from 15.56 to 125.0 μg/mL (Fig. 3). In this assay, the mean mortality of third instar larvae exposed to *Bt* toxins alone was 32%, whereas the average mortality of larvae treated with a mixture of *Bt* toxins (LC_{20}) and COS was significantly greater ($P < 0.01$), ranging from 62.5% (11.01 ng/mL *Bt* toxins + 15.56 μg/mL COS) to 95% (11.01 ng/mL *Bt* toxins + 125.0 μg/mL COS).

Both COS and chitosan (3 mg/ml) were used against the insect *Helicoverpa armigera* (Lepidoptera: Noctuidae), and both showed insecticidal activity when sprayed on this leaf worm. However, COS presented a very low activity (only 3.3% after 48%), while chitosan presented a mortality rate of 40%. The authors suggested that chitosan might stimulate the production of chitosanases in the insect body and cause its insecticidal activity, while COS might not have this function (ZHANG, M I *et al.*, 2003). In this work, neither COS nor the chitosanase *Cv*Csn46 had activity against *Ae. aegypti* larvae. However, both have shown the

capacity to potentiate the larvicidal activity of Bt toxins, with COS displaying great results at low concentrations with the LC_{20} of Bt toxins. Several studies have used other molecules to enhance Bt toxin activity against insects, such as proteinase inhibitors, enzymes, and peptides (REGEV *et al.*, 1996; TABOSA *et al.*, 2020b; XIE, Ruiyu *et al.*, 2005), but COS has never been used for this purpose, highlighting the novelty of this work.

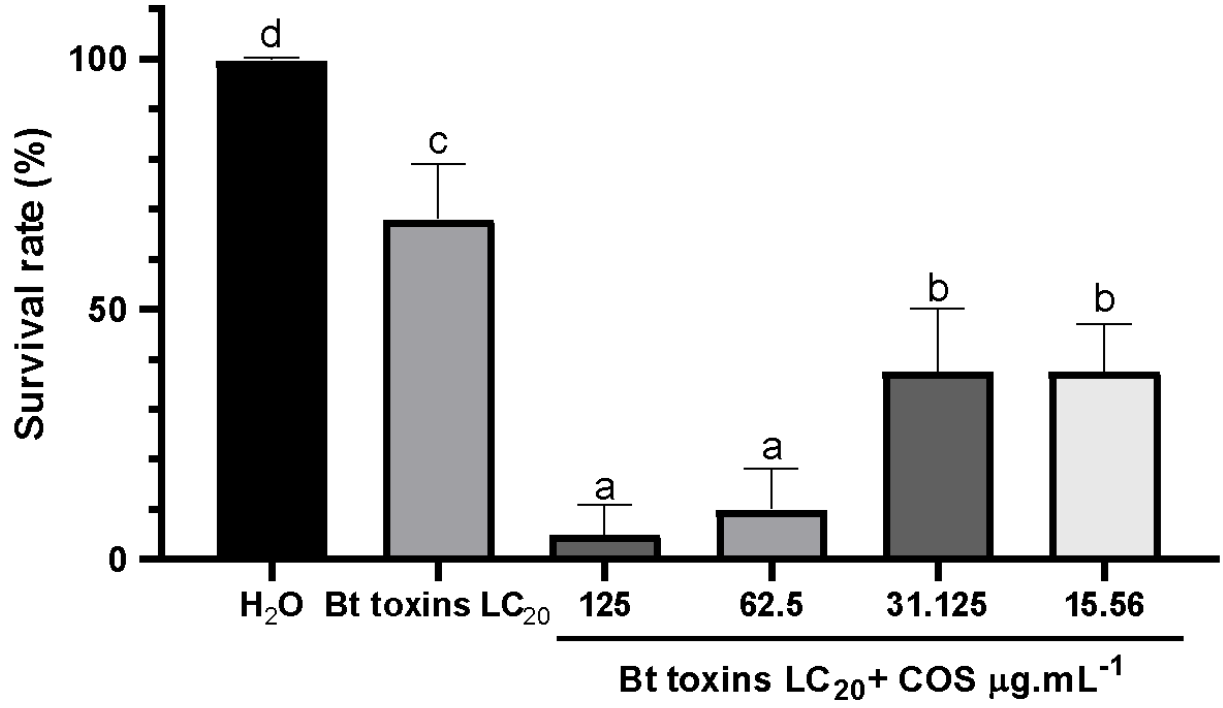


Fig. 3. COS-enhanced activity of the LC_{20} Bt toxin against *Ae. aegypti* larvae

7.3.3 Morphological alterations

To assess the morphological alterations caused by treatment with the Bt toxin LC_{20} and different concentrations of COS to *Ae. aegypti* larvae, seven treatments were analyzed. In the negative control (water) (Fig. 4A) as well as in the treatments with the Bt toxin LC_{20} (Fig. 4B) and 500 µg/ml COS (Fig. 4C), the larval gut remained intact, showing no signs of damage. However, severe increases in midgut and epithelial cell swelling were observed in the larvae treated with the Bt toxin LC_{20} together with COS at 500 (Fig. 4D), 125 (Fig. 4E) and 31 µg/ml (Fig. 4F). Treatment with the Bt toxin LC_{20} + COS 16 µg/ml did not affect the midgut of the

larvae.

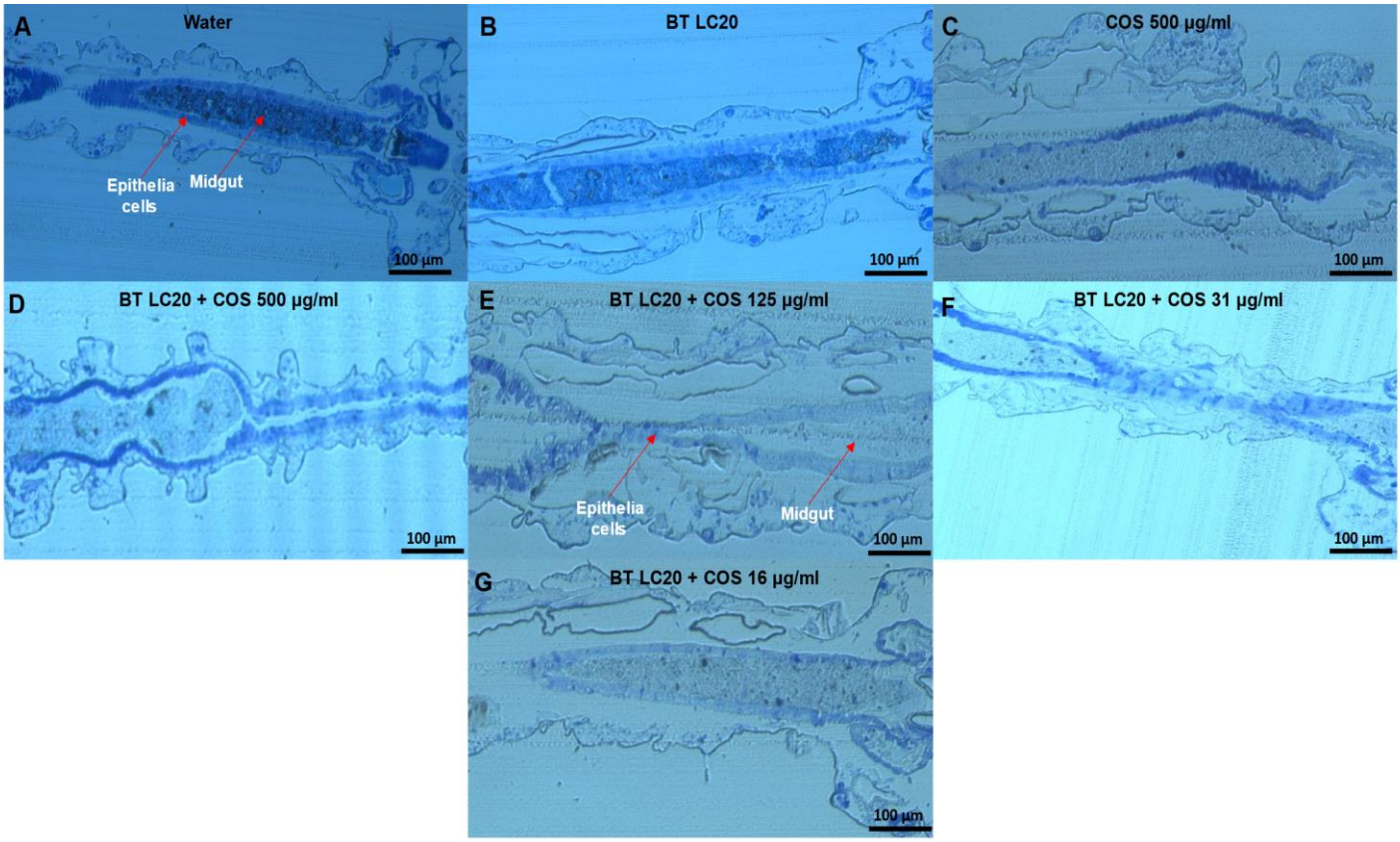


Fig 4. Effect of the Bt toxin LC_{20} with different concentrations of COS on the morphology of *Ae. aegypti* third instar larvae. (A) Control; (B) Bt toxin LC_{20}; (C) COS 500 µg/ml; (D) Bt toxin LC_{20} + COS 500 µg/ml; (E) Bt toxin LC_{20} + COS 125 µg/ml; (F) Bt toxin LC_{20} + COS 31 µg/ml; (G) Bt toxin LC_{20} + COS 16 µg/ml (magnification 20x). The red arrows indicate the midgut and epithelial cells of the larvae.

The digestive system of *Ae. aegypti* larvae has three segments: the stomodeum or foregut, mesenteron or midgut, and proctodeum or hindgut. The midgut is also fractionated into three segments: anterior, middle, and posterior regions. In the midgut of healthy larvae, these regions are well divided and easily distinguishable (LEMOS *et al.*, 2018), as shown in Fig. 4A, B and C. Aswin Jeno & Nakkeeran (2022) treated *Ae. aegypti* larvae with neem oil-loaded noisomes and observed damage similar to that observed in this work. Since the midgut is

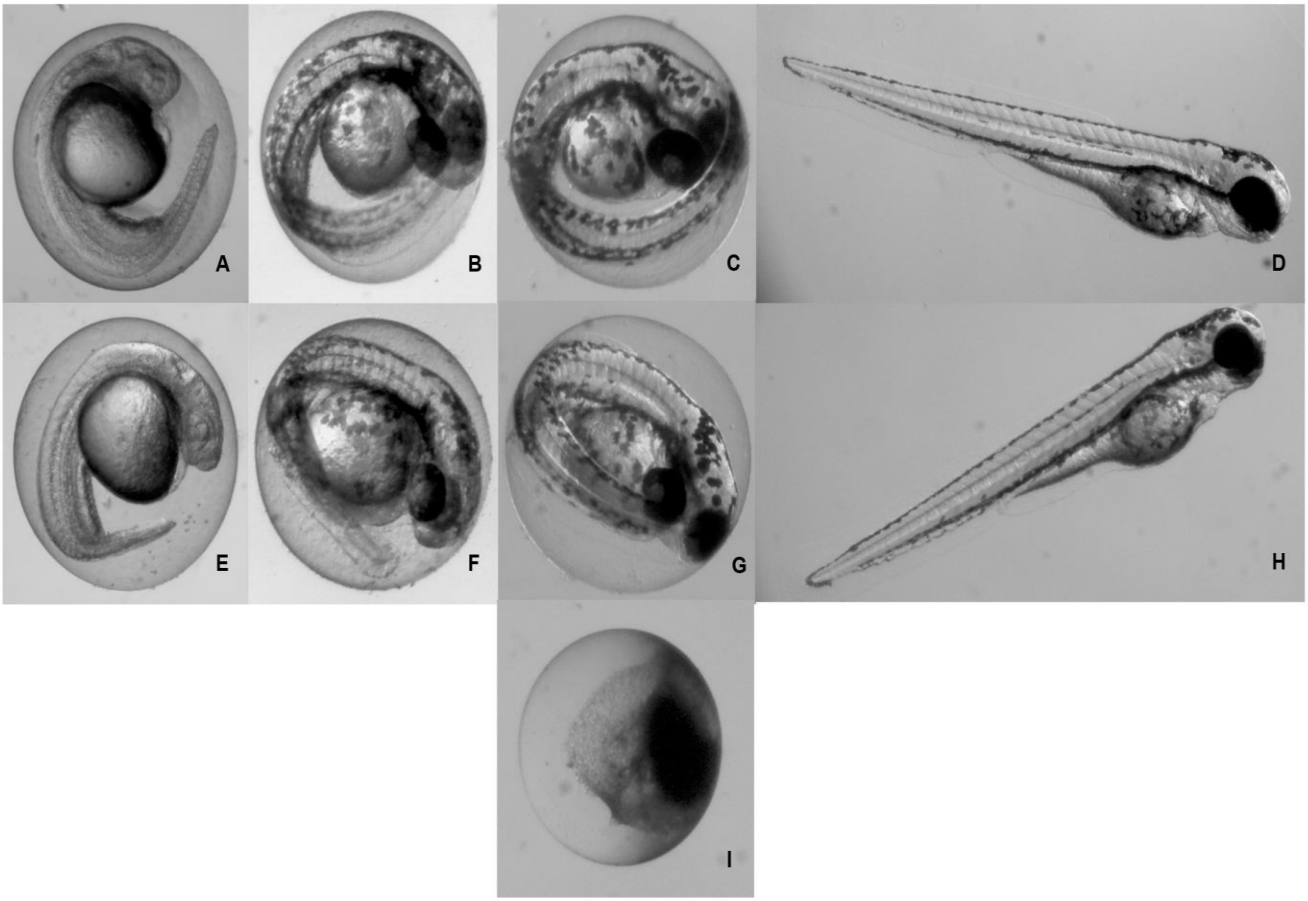
essential for the absorption of food, these authors considered damage to the midgut region to be the major cause of death (ASWIN JENO; NAKKEERAN, 2022). The epithelial cells in the midgut are revested in chitin, serving as a permeability barrier between the food bolus and the midgut and enhancing digestive processes (PARTHIBAN *et al.*, 2021). COS, facilitated by Bt toxins, could interact with the larval chitinases responsible for chitin remodeling and function as inhibitors; hence, damage and tightening, as well as epithelial cell swelling, were observed in the midgut of the treated larvae.

7.3.4 Safety assessment

The survival rate of zebrafish embryos after exposure to 100 mg/L COS and E3 media for 96 h was 100%, while the group treated with 4 mg/L DCA died all treated embryos, as expected. In addition to not showing lethality endpoints, COS did not induce any morphological changes (nonlethal effects) in the embryos or larvae. The only lethal effect observed in the DCA treatment group was egg clotting (Fig. 5).

COSs are usually considered generally recognized as safe (GRASs) and show no mutagenic potential, cytotoxicity or acute toxicity (WANG, Yuanyuan *et al.*, 2023). However, since different methods of COS production can result in toxic byproducts, it is necessary to evaluate the toxicity potential of each preparation (MITTAL *et al.*, 2023). The use of Zebra fish for toxicity tests has been increasing widely because it is a fast, efficient, reproducible, and cost-effective method that is similar to that used for mammals and has the advantage of being a whole-organism model (NIKAM *et al.*, 2020).

Fig. 5. Survival rates of zebrafish embryos and larvae treated with 100 mg/L COS and control E3 medium or 4 mg/L 3,4-dichloraniline-DCA after 96 h of exposure. (A - D) Zebrafish embryos and larvae at 24, 48, 72 and 96 hpf, respectively, exposed to E3 medium showing normal development; (E – H) zebrafish embryos and larvae at 24, 48, 72 and 96 hpf, respectively, exposed to 100 mg/L COS also showing normal development; and (I) coagulated



zebrafish embryos after 24 h of exposure to the positive control DCA (4 mg/L).

Given the abovementioned findings, it is possible to affirm that COS is not toxic to the early life stages of zebrafish development under the conditions tested, with an LC50 >100 mg/L (96 h of exposure). This result provides important information about the safety of this substance since the zebrafish embryotoxicity test is a good predictor of chemical toxicity to vertebrate animals.

7.4 Conclusions

The present work aimed to evaluate the larvicidal activity of COS against *Aedes aegypti* larvae, the ability of COS to enhance the larvicidal activity of Bt toxins, and the safety of COS by assessing its toxicity in Zebra fish. Despite the lack of larvicidal activity of COS on its own, it greatly enhanced Bt toxin activity against *Ae. aegypti* larvae. The combined action of the Bt

toxin LC_{20} and different concentrations of COS caused damage to the larval midgut, while COS did not cause any toxicity to the embryos of Zebra fish. Thus, the results presented in this work suggest the potential use of COS combined with Bt toxins for the control of *Ae. aegypti* without affecting nontarget organisms.

Conflict of interest

The authors declare that they have no conflicts of interest.

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