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**ATIVIDADE ANTIMICROBIANA E MECANISMO DE AÇÃO DE PEPTÍDEOS
SINTÉTICOS BIOINSPIRADOS EM *JcTI-I*, UM INIBIDOR DE TRIPSINA DE
*Jatropha curcas***

FORTALEZA

2023

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curcas

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 Vegetal.

Orientador: Profa. Dra. Daniele de Oliveira Bezerra de Sousa.

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A Deus.

À minha família e aos meus amigos.

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“Existem muitas hipóteses em ciência que estão erradas. Isso é perfeitamente aceitável, elas são a abertura para achar as que estão certas” (CARL SAGAN).

RESUMO

Nos últimos anos, o aumento da resistência microbiana tem causado preocupação e representa um grave problema de saúde pública, causando milhares de mortes mundialmente. Assim, é emergencial a descoberta de novos agentes antimicrobianos que possam ser utilizados no controle desses microrganismos resistentes. Dentro desse contexto, os peptídeos antimicrobianos, sobretudo os sintéticos, têm se destacado no controle dessas infecções, utilizando diversos mecanismos de ação. Dessa forma, o objetivo do trabalho foi desenhar e sintetizar peptídeos inspirados na sequência de um inibidor de tripsina purificado da torta de *Jatropha curcas* (*JcTI-I*) o qual já se mostrou antimicrobiano, além de investigar seus mecanismos de ação frente a diferentes espécies de leveduras e bactérias. A estratégia experimental compreendeu três blocos: Bloco 1) desenho e análise *in silico* dos peptídeos, a fim de avaliar suas características físico-químicas bem como prever a sua atividade antimicrobiana, Bloco 2) investigação, *in vitro*, dos mecanismos de ação antimicrobiana dos peptídeos e Bloco 3) análises, *in vitro* e *in silico*, para avaliar a segurança de uso dos peptídeos. Os resultados obtidos revelaram que um dos peptídeos desenhados, denominado *JcTI-PepI*, se mostrou altamente eficaz contra diferentes espécies de *Candida*. *JcTI-PepI* foi mais eficaz contra *C. krusei*, causando a permeabilização da membrana das células e uma superprodução de Espécies Reativas de Oxigênio. Além disso, a capacidade de acidificação do meio foi severamente comprometida nas células tratadas. *JcTI-PepI* também foi eficaz contra o biofilme pré-formado de *C. krusei* e não foi tóxico contra eritrócitos humanos e células Vero. Sendo assim, essas características indicam que *JcTI-PepI* é seguro e eficaz contra *C. krusei*, uma cepa intrinsecamente resistente que causa sérios problemas de saúde e é frequentemente negligenciada, apresentando um alto potencial para uso como um novo agente antimicrobiano no futuro.

Palavras-chave: peptídeos antimicrobianos; *C. krusei*; modo de ação; resistência microbiana.

ABSTRACT

In recent years, the increase in microbial resistance has caused concern and represents a serious public health problem, causing thousands of deaths worldwide. Thus, the discovery of new antimicrobial agents to control these resistant microorganisms is urgent. Within this context, antimicrobial peptides, especially synthetic ones, have been highlighted in the control of these infections, using different mechanisms of action. Therefore, the objective of this work was to design and synthesize peptides inspired in the sequence of a purified trypsin inhibitor from *Jatropha curcas* cake (*JcTI-I*), which has already shown to be antimicrobial, in addition to investigating its mechanisms of action against different species of yeasts and bacteria. The experimental strategy comprised three sections: Section 1) *in silico* design and analysis of the peptides, in order to evaluate their physicochemical characteristics as well as predict their antimicrobial activity, Section 2) investigation, *in vitro*, of the peptides' antimicrobial action mechanisms and Section 3) analyses, *in vitro* and *in silico*, to assess the safety of using the peptides. The results revealed that one of the designed peptides, called *JcTI-PepI*, proved to be highly effective against different species of *Candida*. *JcTI-PepI* was especially active against *C. krusei*, causing cell membrane permeability and an overproduction of Reactive Oxygen Species. Furthermore, the ability to acidify the medium was severely compromised in the treated cells. *JcTI-PepI* was also effective against preformed *C. krusei* biofilm and was non-toxic against human erythrocytes and Vero cells. Therefore, these characteristics indicate that *JcTI-PepI* is safe and effective against *C. krusei*, an intrinsically resistant strain that causes serious health problems and is often neglected, presenting a high potential for use as a new antimicrobial agent in the future.

Palavras-chave: antimicrobial peptides; *C. krusei*; mode of action, microbial resistance.

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LISTA DE ABREVIATURAS E SIGLAS

ABNT	Associação Brasileira de Normas Técnicas
PAMs	Peptídeos Antimicrobianos
ANVISA	Agência Nacional de Vigilância Sanitária
FDA	Federal Drug Administration
<i>JcTI-I</i>	<i>Trypsin Inhibitor from Jatropha curcas</i>
ATCC	<i>American Type Culture Collection</i>
DMSO	Dimetilsulfóxido

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1 INTRODUÇÃO

Os agentes antimicrobianos têm desempenhado um papel de alta relevância no combate às doenças infecciosas. No entanto, ao longo de várias décadas tem sido observado um aumento dos casos de infecções causadas por microrganismos resistentes (ROOPE *et al.*, 2019; SEKYERE; ASANTE, 2018; ZHU, Yiwen *et al.*, 2022). A resistência microbiana é atualmente uma das questões mais alarmantes no contexto de saúde pública mundialmente, especialmente devido ao aparecimento rápido de mecanismos de resistência ainda pouco elucidados e a demora na liberação de novas drogas antimicrobianas (BLASKOVICH; BUTLER; COOPER, 2017; BROWN; WRIGHT, 2016; KOK *et al.*, 2022; VARELA *et al.*, 2021).

Baseando-se nas informações relatadas na literatura já se sabe que a resistência aos tratamentos disponíveis atualmente pode ser alcançada através de diferentes mecanismos. As bactérias, por exemplo, podem sofrer mutações em genes intrínsecos ou adquirí-las através de DNA exógeno via plasmídeos (DUJIKEREN *et al.*, 2018). Essas mutações podem levar então a ativação de diversas respostas de defesa, como alterações na membrana, modificações no sítio de reconhecimento da droga, inibição da captação da droga e promoção do efluxo do medicamento para o meio extracelular (DUJIKEREN *et al.*, 2018; MUTUKU *et al.*, 2022).

Os fungos, por sua vez, também têm se destacado no contexto de resistência e o problema é agravado visto que as opções de tratamento são limitadas, existindo poucas classes de antifúngicos disponíveis para o tratamento das infecções, sendo as mais comuns: os azóis, as equinocandinas e os polienos (GUPTA *et al.*, 2021; PERFECT, 2017). Além dessa baixa disponibilidade de drogas eficientes, os fungos apresentam diversos mecanismos de resistência aos agentes antimicrobianos, incluindo alteração do alvo da droga, alterações na atividade de transportadores e em vias celulares (MARTINEZ-ROSSI *et al.*, 2021).

A resistência de microrganismos aos tratamentos disponíveis é responsável por causar aproximadamente 700.000 mortes/ano no mundo. Além disso, estima-se ainda que cerca de 10 milhões de mortes devido a essa resistência irão ocorrer todos os anos após 2050, causando mais óbitos que o câncer (ALVES *et al.*, 2021; PULINGAM *et al.*, 2022).

Com o intuito de minimizar os impactos causados pela resistência antimicrobiana, é urgente e imperativa a busca por novas moléculas que apresentem uma ação potente contra esses agentes infecciosos. Nesse contexto, nos últimos anos os peptídeos antimicrobianos (PAMs) têm sido cada vez mais estudados e têm se mostrado eficientes sobretudo no combate a infecções causadas por microrganismos resistentes (GAN *et al.*, 2021; TAN *et al.*, 2021). Esses peptídeos, além de apresentarem uma potente ação antimicrobiana, apresentam também vantagens em comparação com agentes antimicrobianos tradicionais. Por exemplo, esses peptídeos possuem um amplo espectro de ação, habilidade de neutralizar processos de virulência e de modular respostas imunes no hospedeiro (WANG *et al.*, 2018; YANG *et al.*, 2021). No entanto, os PAMs de origem natural apresentam algumas limitações de uso, especialmente no tratamento de infecções invasivas. Essas limitações incluem elevada toxicidade, alta susceptibilidade a degradação por proteases, instabilidade em diferentes condições de pH e um alto custo de obtenção através dos processos de purificação clássicos (GIULIANI; RINALDI, 2011; SARKAR *et al.*, 2021; VANZOLINI *et al.*, 2022). Para contornar essas limitações de uso dos peptídeos naturais, peptídeos sintéticos têm sido desenvolvidos com base na sequência de PAMs naturais ou proteínas antimicrobianas isoladas de plantas, animais e microrganismos. Esses peptídeos sintéticos bioinspirados normalmente apresentam toxicidade baixa ou ausente frente às células de mamíferos, baixos índices de imunogenicidade e maior estabilidade tanto em condições fisiológicas quanto em condições de armazenamento (OMARDIEN *et al.*, 2018; SARKAR, T.; CHETIA; CHATTERJEE, 2021). Diante dessas vantagens, diversos estudos têm avaliado a ação desses PAMs sintéticos

no controle de microrganismos não-resistentes e/ou resistentes aos tratamentos convencionais (DE CESARE *et al.*, 2020; DIAS *et al.*, 2020; OLIVEIRA, J. T. A. *et al.*, 2019; PEREZ-RODRIGUEZ *et al.*, 2022). Além disso, muitos desses peptídeos já estão em estágio de testes clínicos e têm se mostrado eficientes no tratamento e prevenção de infecções (KOO; SEO, 2019; DIJKSTEEL *et al.*, 2021).

Nesse contexto de atividade antimicrobiana, há alguns anos nosso grupo de pesquisa purificou, a partir da torta do pinhão-manso, uma proteína que apresentou atividade contra fungos e bactérias e que foi denominada *JcTI-I* (COSTA, *et al.*, 2014). *JcTI-I* é uma proteína de 10,252 kDa e dados de espectrometria de massas e de sequenciamento por degradação de Edman permitiram sua classificação como sendo uma albumina 2S. Essa proteína apresenta uma elevada especificidade pela tripsina ($K_i = 2 \times 10^{-11}M$), inibindo sua atividade através de um mecanismo do tipo não competitivo. No entanto, tendo em vista a dificuldade de obtenção do material bem como o baixo rendimento da purificação, foi proposto o desenho de peptídeos inspirados na sequência primária de *JcTI-I*. Sendo assim, esse trabalho foi elaborado com base nas seguintes perguntas de partida:

- Os peptídeos sintéticos inspirados na sequência de *JcTI-I* apresentarão atividade antimicrobiana frente às diferentes espécies de leveduras e bactérias?
- Quais características físico-químicas e bioquímicas desses peptídeos podem favorecer sua atividade antimicrobiana?
- Quais mecanismos de ação justificam a sua ação?
- Vislumbrando um futuro uso *in vivo*, esses peptídeos sintéticos seriam tóxicos contra células animais?

2 REVISÃO DE LITERATURA

2.1 Resistência Microbiana

Na era pré-antibióticos, a alta mortalidade por doenças causadas por microrganismos, como catapora, cólera e pneumonia era comum no mundo todo (ADEDEJI, 2016). A descoberta da penicilina por Alexander Fleming em 1928, transformou a medicina e continua sendo uma das mais importantes causas do aumento da expectativa de vida (MAUGERI *et al.*, 2019). Por exemplo, a descoberta dos antibióticos reduziu os índices de mortalidade para pneumonia, endocardite e meningite em 30%, 75% e 60%, respectivamente (DHINGRA *et al.*, 2020).

No entanto, nas últimas décadas tem sido observado um retrocesso no combate a infecções causadas por microrganismos (DHINGRA *et al.*, 2020). Isso acontece, pois apesar da disponibilidade de agentes antimicrobianos que vêm sendo utilizados há décadas, a resistência microbiana emergiu como um dos principais problemas de saúde pública, sobretudo no século XXI (JAMPILEK, 2022). Essa resistência pode ter sido desencadeada por múltiplos fatores como o uso incorreto e indiscriminado desses medicamentos, sobretudo decorrente da automedicação (JANI *et al.*, 2021; ZEB *et al.*, 2022). É importante destacar que estudos já têm relatado os impactos decorrentes do aumento no uso indiscriminado de antibióticos durante a pandemia de COVID-19 (DARIA; ISLAM, 2022; KNIGHT *et al.*, 2021). Os resultados desse mau uso já estão sendo observados em todo o mundo. Artigos recentes têm mostrado um aumento nos casos de infecções causadas por microrganismos resistentes após o início da pandemia causada pelo SARS-CoV-2 (do inglês, “*severe acute respiratory syndrome coronavirus 2*”) (CHUNG; KHANUM, 2017; NORI *et al.*, 2020; TIRI *et al.*, 2020). Um dos estudos relatou que a incidência de infecções por enterobactérias

resistentes aos carbapenêmicos (considerados medicamentos de última escolha no tratamento de infecções causadas por bactérias multirresistentes) aumentou de 6,7% em 2019 para 50% em 2020 na Itália, um dos países mais fortemente atingidos pelos efeitos da pandemia (TIRI *et al.*, 2020)

Além disso, o uso de agentes antimicrobianos no setor agropecuário para promover o crescimento e prevenir doenças nos animais usados na alimentação pode ser apontado como outro grande indutor de resistência (OLIVEIRA, N. A. *et al.*, 2020). O uso de antibióticos como promotores de crescimento já foi completamente banido na Europa desde 2006, no entanto, em outros países esse uso ainda é permitido e continua contribuindo para o desencadeamento desse problema (DUTTA; YADAV; CHATTERJEE, 2019).

Esse problema é extremamente grave quando se considera, por exemplo, a resistência de muitas bactérias aos antibióticos. Nos últimos anos tem sido registrado um aumento no número de mortes decorrentes de infecções ocasionadas por bactérias multirresistentes (MDR, do inglês “*multidrug resistant bacteria*”). De acordo com a Organização Mundial da Saúde, estima-se que em 2018 cerca de 408.000 pessoas tiveram tuberculose causada por bactérias resistentes à rifampicina, um medicamento de primeira linha considerado altamente eficaz (OMS, 2020). Considerando as mortes causadas por microrganismos resistentes, de modo geral, os dados são ainda mais preocupantes. Estima-se que aproximadamente 4,95 milhões de pessoas morreram em 2019, por causas associadas à resistência microbiana (MURRAY *et al.*, 2022). As infecções do trato respiratório inferior causadas por bactérias foram responsáveis por mais de 1,5 milhão de mortes em 2019, tornando-se a mais grave síndrome infecciosa.

Considerando o cenário brasileiro os números também preocupam. De 2010 a 2019, foram registrados mais de 1 milhão de casos de sepse no Brasil. Essas infecções resultaram em mais de 463 mil óbitos, com uma média 23 óbitos a cada 100 mil habitantes (ALMEIDA *et al.*, 2022). Entre os anos de 2014 e 2017 foram registrados 10.210 casos de infecções

ocasionadas por bactérias MDR em hospitais pediátricos no estado do Rio de Janeiro, sendo *Klebsiella* sp. e *Escherichia coli* as duas mais prevalentes (DE OLIVEIRA, P. M. N. *et al.*, 2019). Além disso, um estudo recente avaliou a susceptibilidade de cepas de *Staphylococcus aureus* isoladas de pacientes em tratamento em hospitais públicos do Maranhão e os resultados são alarmantes. Dos isolados avaliados, 78% mostraram resistência a oxacilina e a clindamicina. Além disso, também foi verificada uma elevada incidência de isolados resistentes a outras drogas muito utilizadas, como a eritromicina (70%), a ciprofloxacina (61%) e a gentamicina (52%) (MONTEIRO *et al.*, 2019).

O impacto da resistência bacteriana também é econômico. Nos Estados Unidos os custos para o tratamento de infecções causadas por apenas 6 espécies bacterianas chegam a quase \$ 5 bilhões de dólares (NELSON *et al.*, 2021).

Assim com as bactérias, os fungos também têm apresentado resistência aos agentes antimicrobianos. As infecções fúngicas têm sido um problema de saúde pública mundialmente, gerando preocupação, sobretudo, com os pacientes imunocomprometidos os quais são altamente susceptíveis a essas infecções (HOENIGL *et al.*, 2022; MEI-SHENG RILEY, 2021; OLIVIER-GOUGENHEIM *et al.*, 2021). As infecções fúngicas sistêmicas estão comumente associadas à elevadas taxas de mortalidade e têm sido associadas ao aumento da incidência de fungos patogênicos como os do gênero *Aspergillus* e as leveduras do gênero *Candida* (BORJIAN BOROUJENI *et al.*, 2021). As infecções causadas por espécies desse gênero representam um problema de saúde pública. No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) inclui as leveduras do gênero *Candida* sp na lista dos principais microrganismos notificados como agentes etiológicos de Infecção Primária de Corrente Sanguínea Laboratorial (IPCSL) em pacientes adultos hospitalizados em UTIs no Brasil (ANVISA, 2013).

A candidemia, uma forma mais invasiva de candidíase, causa preocupação também

nos Estados Unidos (MAGILL *et al.*, 2018). Entre os anos de 2013 e 2017 estima-se que a incidência da doença foi de 9 infectados para cada 100.000 pessoas. O Centro de Controle e Prevenção de Doenças estima que aproximadamente 25.000 casos de candidemia acontecem anualmente nos EUA (TSAY *et al.*, 2020).

Dessas infecções invasivas causadas por *Candida*, cerca de 95% são desencadeadas por apenas cinco espécies: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. Tropicalis* e *C. krusei*. A proporção de infecções causadas por cada espécie varia com a localização geográfica, porém de modo geral, *C. albicans* é a espécie mais comumente isolada de pacientes com infecções invasivas (MCCARTY; WHITE; PAPPAS, 2021). No entanto, o número de infecções causadas por essa espécie tem diminuído e as espécies não-albicans já são responsáveis por cerca de 50% das infecções por *Candida* (PFALLER *et al.*, 2019).

Essas infecções causam muitas mortes em todo o mundo. No caso das infecções invasivas, a taxa de mortalidade é de cerca de 20% e geram também um impacto econômico relevante visto que cada caso de candidemia pode gerar um custo total de até \$ 150,000, por paciente (WAN ISMAIL *et al.*, 2020).

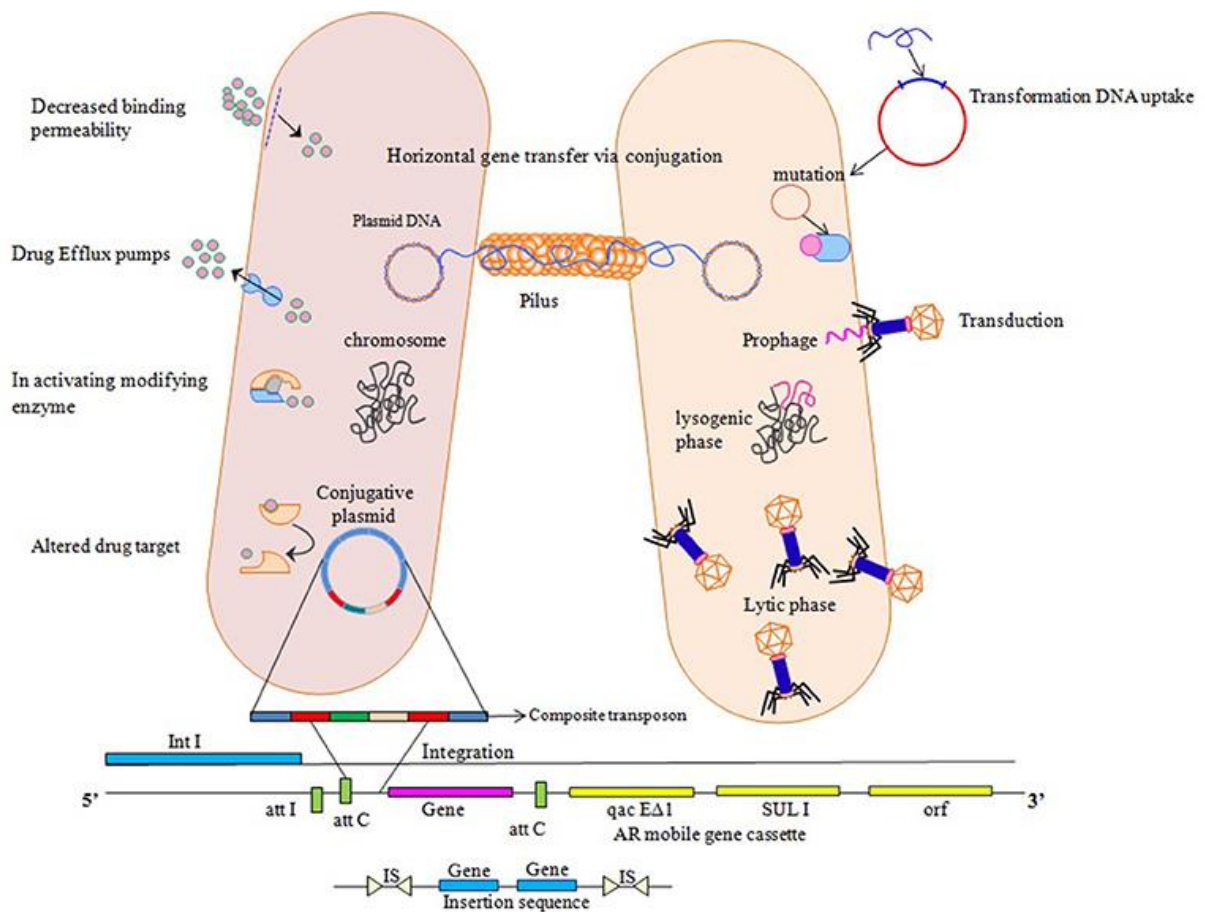
Essa resistência reportada para diferentes espécies de fungos e bactérias pode ser desencadeada por diferentes mecanismos, os quais serão abordados a seguir.

2.1.1 Resistência microbiana em bactérias

Antibióticos são comumente utilizados no tratamento de infecções causadas por bactérias e têm sua ação baseada, por exemplo, na inibição da síntese da parede celular, de proteínas ou de ácidos nucleicos (ALI; RAFIQ; RATCLIFFE, 2018). Os principais mecanismos de resistência que culminam na baixa eficácia dos antibióticos incluem os seguintes: a) inativação da droga via ação de enzimas; b) modificação do sítio de ação da

droga; e c) redução da permeabilidade dos medicamentos (SULTAN *et al.*, 2018). Uma visão geral desses mecanismos de resistência em bactérias pode ser observada na Figura 1.

Figura 1 – Diferentes mecanismos de resistência aos antibióticos em bactérias. A resistência pode ser intrínseca ou adquirida, como na imagem abaixo, através da transferência de elementos gênicos.



Fonte: Sultan et al., 2018.

Mudanças no ambiente, como radiação, alterações na iluminação ou no pH podem contribuir para o surgimento da resistência (WELLINGTON *et al.*, 2013). Essa resistência pode ainda ser adquirida através da transmissão de genes de resistência contidos em elementos genéticos móveis, como plasmídeos, *transposons* e *integrons* (SULTAN *et al.*, 2018). Esses mecanismos de resistência, muitas vezes em conjunto, permitem que as bactérias

sobrevivam e se multipliquem mesmo durante o tratamento com antibióticos (VARELA *et al.*, 2021).

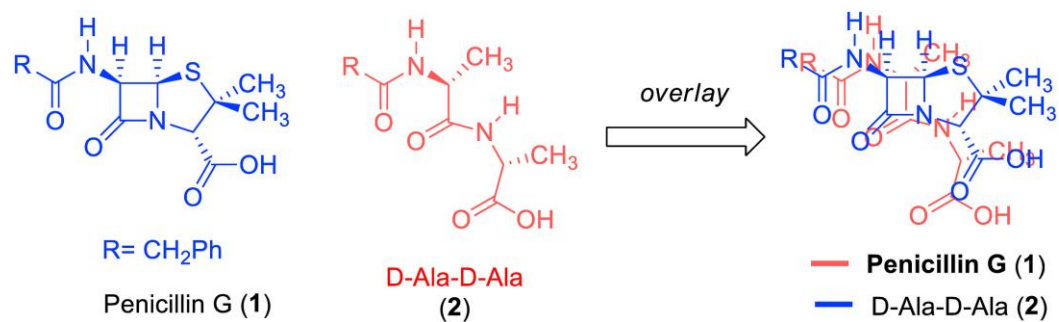
Para entender como as bactérias resistentes agem, é importante conhecer também os principais mecanismos de ação das classes de antibióticos mais importantes. Sendo assim, serão abordadas a seguir as características das principais classes de antibióticos disponíveis e as informações já disponíveis sobre resistência bacteriana a esses medicamentos.

2.1.1.1 Interferência na síntese da parede celular

A parede celular é uma estrutura essencial para manter a forma e a integridade da célula bacteriana, além de proteger contra a lise por pressão osmótica (ABUSHAHEEN *et al.*, 2020). Sua estrutura é formada longas cadeias de N-acetilglucosamina (GlcNAc) e de ácido N-acetilmurâmico (MurNAc) que são interligados em ligações cruzadas pela ação de transpeptidases e carboxipeptidases, enzimas conhecidas como proteínas de ligação à penicilina (PBPs, do inglês “Penicillin Binding Proteins). A parede celular das bactérias é um dos principais alvos da ação de diversos antibióticos, como os β -lactâmicos (penicilinas, carbapenêmicos e cefalosporinas) e também dos glicopeptídeos (vancomicina e teicoplanina) (NIKOLAIDIS; FAVINI-STABILE; DESSEN, 2014).

Os antibióticos β -lactâmicos têm como alvos de ação as PBPs, enzimas envolvidas na síntese do peptidoglicano, um elemento chave da parede celular. A inativação dessas proteínas impede a formação do arcabouço estrutural da parede celular, o que resulta na morte da célula (MLYNARCZYK-BONIKOWSKA *et al.*, 2022). A atividade de inibição enzimática se baseia em similaridades estruturais e geométricas entre os antibióticos, como a penicilina, e o substrato das PBPs, um dipeptídeo D-Ala-D-Ala (Figura 2) (LIMA *et al.*, 2020).

Figura 2 – Similaridade estrutural entre o antibiótico penicilina e o substrato das enzimas transpeptidases, o dipeptídeo D-Ala-D-Ala. Essa similaridade leva ao reconhecimento da penicilina pelas PBPs, resultando na inibição da sua atividade.



Fonte: Lima et al., 2020.

O reconhecimento do antibiótico pela enzima, gera um impedimento estérico que torna impossível a catálise das ligações cruzadas que formam a parede celular. Consequentemente, as ligações enfraquecidas no arcabouço da parede celular a tornam mais suscetível a danos (LIMA *et al.*, 2020).

A resistência aos antibióticos β -lactâmicos é adquirida sobretudo pela produção de β -lactamases nas bactérias. Essas enzimas hidrolisam o anel β -lactâmico dos antibióticos, impedindo assim o seu reconhecimento pelas PBPs (PAPP-WALLACE *et al.*, 2020). Além disso, já foram reportados outros mecanismos de resistência a esses antibióticos. A resistência aos carbapenêmicos, por exemplo, pode acontecer por mutações nas PBPs ou através da exclusão do medicamento para o espaço periplasmático pela ação de bombas de efluxo (SCHWEIZER, 2003; SULTAN *et al.*, 2018).

Outra classe de antibióticos que também tem a parede celular como alvo é a dos glicopeptídeos. A vancomicina, o primeiro glicopeptídeo descoberto, surgiu em uma época na qual a resistência aos β -lactâmicos já tinha começado a ser reportada. Desde então, essa tem

sido a última opção de tratamento para infecções causadas por bactérias gram-positivas multirresistentes (SARKAR *et al.*, 2020). Outros glicopeptídeos, naturais e semissintéticos, foram aprovados para uso nos últimos anos.

A atividade antibacteriana dos glicopeptídeos se baseia, principalmente, na sua alta capacidade de ligação a um precursor na síntese da parede celular, o lipídeo II (VAN GROESEN; INNOCENTI; MARTIN, 2022). Esses antibióticos interagem com a região terminal D-Ala-D-Ala do precursor através de ligações de hidrogênio. Essa interação impede a formação do arcabouço da parede celular, comprometendo a integridade da célula (BLASKOVICH *et al.*, 2018). Uma mutação comum que confere resistência aos glicopeptídeos envolve a alteração do resíduo D-Ala-D-Ala para um resíduo D-Ala-D-Lac ou D-Ala-D-Ser. Com isso, a interação entre o antibiótico e o precursor lipídeo II não acontece e a biossíntese da parede celular não é comprometida (JAMES *et al.*, 2012).

2.1.1.2 Inibição da síntese de proteínas

Após as etapas de replicação do DNA e da sua transcrição em uma molécula de RNAm ocorre a síntese de proteínas, conhecida como tradução. Os ribossomos são os agentes responsáveis pelo processo de síntese proteica tanto em procariontes quanto em eucariontes (BHATTACHARJEE, 2016). Em bactérias, o ribossomo 70S é formado pelas subunidades 30S e 50S e os antibióticos que atuam sobre a síntese proteica se ligam a uma dessas subunidades, bloqueando o processo e interrompendo o crescimento das células (BECKERT *et al.*, 2021).

Os macrolídeos, aminoglicosídeos e a tetraciclina são exemplos de antibióticos que bloqueiam a síntese proteica pela ligação à subunidade 30S. Esses medicamentos possuem carboidratos carregados positivamente em sua superfície os quais interagem com a membrana

plasmática negativa, permitindo a entrada na célula. Dentro da célula eles se ligam à subunidade menor do ribossomo e geram um pareamento incorreto entre o RNAm e o RNAr, resultando no bloqueio da síntese (VLADAR *et al.*, 2015).

Outros medicamentos, como o cloranfenicol, se ligam à subunidade 50S e impedem a adição de novos aminoácidos na cadeia proteica em formação. Dessa forma, o processo de alongação é interrompido e a síntese proteica é comprometida (ABUSHAHEEN *et al.*, 2020).

Diferentes mecanismos de resistência aos antibióticos citados acima têm sido reportados na literatura. Um dos mecanismos de resistência aos aminoglicosídeos, por exemplo, envolve a modificação de enzimas, como acetiltransferases, fosfotransferases e nucleotidiltransferases (VLADAR *et al.*, 2015). Para o cloranfenicol e a tetraciclina já foi reportada a resistência por inativação do medicamento via enzimática e também pelo efluxo através de bombas (GASPARRINI *et al.*, 2020; SCHWARZ *et al.*, 2004).

2.1.1.3 Inibição da síntese de ácidos nucleicos

Duas classes principais de antibióticos têm sua ação antibacteriana baseada na inibição da síntese de ácidos nucleicos: as quinolonas e as fluoroquinolonas. As quinolonas são antibióticos sintéticos e a primeira quinolona descoberta, o ácido nalixídico, se trata de um subproduto da síntese da cloroquina (BISACCHI, 2015). As quinolonas são inibidores das topoisomerasas II ou IV, enzimas que catalisam a interconversão de diferentes formas topológicas do DNA (relaxo ou superenrolado) que possibilita a ocorrência de processos como a replicação e a transcrição. Após essa ligação, o relaxamento ou a torção do DNA são comprometidos e o processamento do DNA, processo vital para a célula, é interrompido e culmina na morte celular (BUSH *et al.*, 2020).

A otimização das quinolonas levou a uma nova classe de antibióticos, as fluoroquinolonas, obtidas pela adição de um átomo de flúor no C-6 (BALL, 2000). As fluoroquinolonas, por sua vez, atuam como inibidores da DNA girase em bactérias gram-negativas e da topoisomerase IV em gram-positivas, bloqueando processos como a replicação e afetando a segregação dos cromossomos homólogos (BUSH *et al.*, 2020). Esses medicamentos têm sido amplamente utilizados no combate a infecções como a candidíase esofágica, a peritonite e infecções do trato urinário. No entanto, a sua elevada toxicidade e o aumento nos casos de resistência têm levado à contraindicação do seu uso em infecções leves e moderadas (MROCZYŃSKA; BRILLOWSKA-DĄBROWSKA, 2020).

Os mecanismos de resistência reportados para esses antibióticos incluem a diminuição da sua entrada na célula, o seu efluxo através de bombas e também mutações em seus alvos moleculares, as DNA girases e topoisomerasas (SULTAN *et al.*, 2018).

2.1.2 Resistência microbiana em fungos

Assim como as bactérias, os fungos também têm mecanismos de resistência já bem definidos contra as principais classes de antifúngicos: os azóis, os polienos e as equinocandinas.

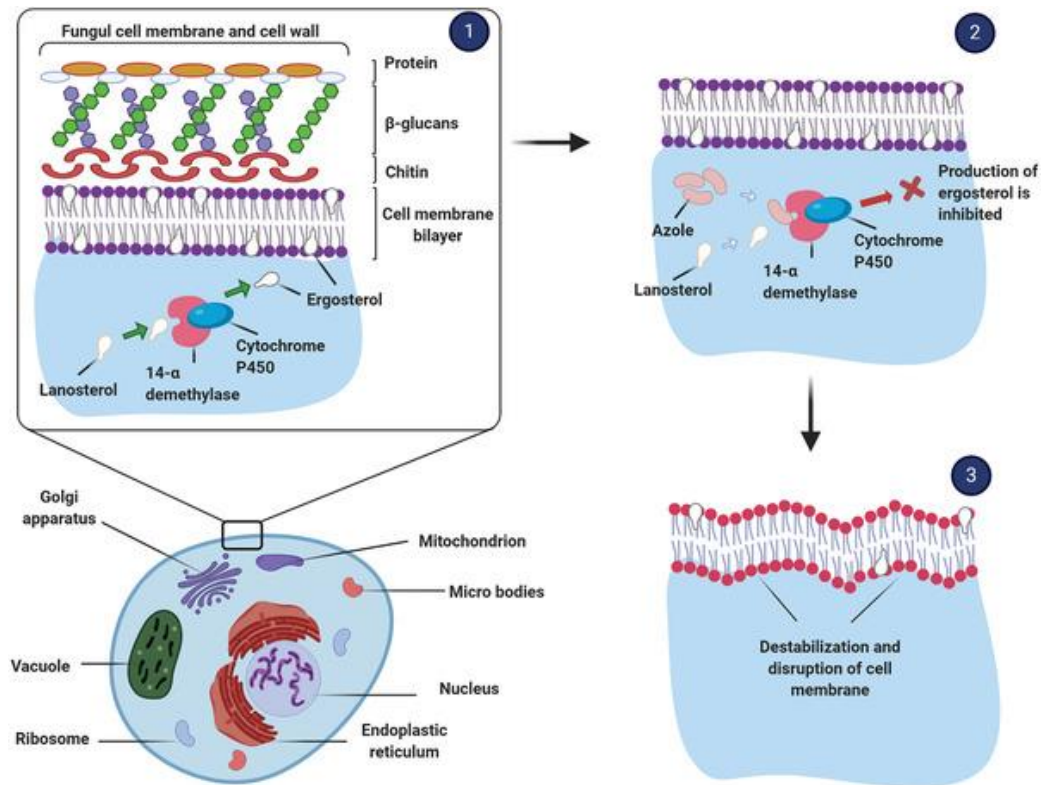
2.1.2.1 Resistência aos azóis

Os azóis são drogas que inibem a via de biossíntese do ergosterol, um componente estrutural importante da membrana plasmática das leveduras. De modo mais específico, essas drogas têm como alvo a enzima lanosterol 14- α -demetilase, codificada pelo gene *ERG11* em leveduras (Figura 3) (PRISTOV; GHANNOUM, 2019). A inibição dessa via resulta no

acúmulo de esteróis tóxicos que promovem instabilidade na membrana e impedem o crescimento do fungo (MAKVANDI *et al.*, 2021; NAMI *et al.*, 2019; ROBBINS; CAPLAN; COWEN, 2017). Os azóis não prejudicam as células do paciente durante o tratamento pois os seus principais alvos estão ausentes. Por exemplo, nas células do paciente o colesterol vai ser parte estrutural da membrana, e não o ergosterol (PRISTOV; GHANNOUM, 2019).

Os azóis são a classe de antifúngico com mais medicamentos disponíveis comercialmente e têm sido usados há décadas no combate a diversas infecções fúngicas superficiais e invasivas (SHAFIEI *et al.*, 2020). O primeiro azol, o clormidazol, foi liberado comercialmente na década de 50 e inúmeras pesquisas foram conduzidas ao longo dos anos a fim de otimizar a sua atividade antifúngica bem como melhorar outros parâmetros como a segurança de uso. Atualmente, existem aproximadamente 40 drogas e candidatos a drogas dessa classe, divididos em mais de três gerações (SHAFIEI *et al.*, 2020).

Figura 3 – Mecanismo de ação dos azóis. (1) Inibição da conversão do lanosterol em ergosterol; (2) inibição na síntese do ergosterol e (3) desestabilização e ruptura da membrana plasmática.



Fonte: Makvandi et al., 2021.

A primeira geração inclui medicamentos como o clotrimazol e o miconazol e tem sido usada sobretudo no tratamento de infecções superficiais como as dermatomicoses e a candidíase vaginal (FROMTLING, 1988). Os azóis de segunda geração foram melhorados em termos de segurança, espectro de ação e características farmacocinéticas. O cetoconazol, por exemplo, foi obtido pela substituição de uma cetona por um dioxolano heterocíclico. A sua atividade antifúngica foi melhorada em relação aos medicamentos de primeira geração, mas ele se mostrou extremamente tóxico e pouco seletivo, causando danos graves nas células dos pacientes. Por essa razão, o cetoconazol foi removido da lista de medicamentos aprovados pelo FDA (do inglês, *Federal Drug Administration*) para o tratamento de micose sistêmica em

2013 (SHAFIEI *et al.*, 2020). Outra droga de segunda geração é o fluconazol que também teve modificações estruturais que resultaram em uma maior estabilidade metabólica, maior solubilidade e menos efeitos colaterais (RICHARDSON *et al.*, 1990).

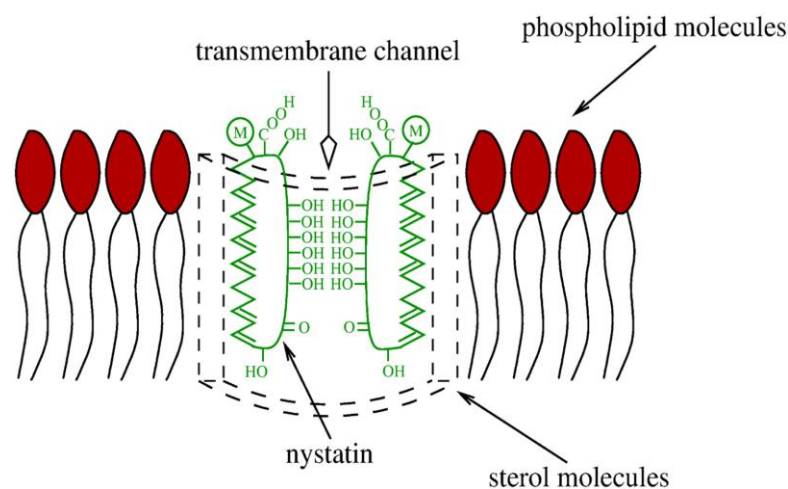
Com o aumento nos casos de resistência, novos medicamentos foram sintetizados com base na estrutura dos azóis de segunda geração. Esses medicamentos, de terceira geração, tiveram alterações estruturais que resultaram em características farmacocinéticas e de segurança melhores, além de um espectro de ação ampliado e um maior potencial para controlar infecções mais graves (SHAFIEI *et al.*, 2020). Sendo assim, em 2002 o FDA aprovou o voriconazol, um azol com um anel fluorpirimídico, que se mostrou 30 vezes mais potente contra *C. albicans*, 20 vezes mais potente contra *C. glabrata* e completamente ativo contra *Aspergillus* sp. em comparação com o fluconazol (SARAVOLATZ; JOHNSON; KAUFFMAN, 2003; SABATELLI *et al.*, 2006). Continuando as melhorias, drogas de quarta geração já estão em fase clínica de testes, como o quilseconazol e o VT-1598 (WIEDERHOLD *et al.*, 2018).

Apesar dos esforços em desenvolver novas drogas, os fungos continuam burlando a ação desses medicamentos. Um dos mecanismos de resistência aos azóis envolve alterações na expressão do gene *ERG11* e em espécies de *Candida* e *Aspergillus* resistentes já foi reportada a substituição de aminoácidos em regiões próximas ao sítio de ligação da enzima lanosterol 14- α -demetilase, causando alterações que impedem a ação da droga (ROBBINS; CAPLAN; COWEN, 2017). Além disso, em *C. albicans*, *C. neoformans* e *A. fumigatus* já foi relatada a superexpressão de bombas de efluxo que retiram os azóis da célula e mutações em canais que impedem a entrada do medicamento para o espaço intracelular (SHAPIRO; ROBBINS; COWEN, 2011).

2.1.2.2 Resistência aos polienos

O principal alvo de ação dos polienos, os quais possuem uma estrutura anfipática, é justamente a membrana plasmática, onde são formados poros que resultam no colapso da célula (Figura 4) (KRISTANC *et al.*, 2019). Esses poros variam bastante em tamanho e podem permitir a passagem de diferentes solutos pela membrana.

Figura 4 – Representação do mecanismo de ação dos polienos. As drogas dessa classe formam poros na membrana plasmática dos fungos, resultando na morte das células.



Fonte: Kristanc *et al.*, 2019.

A formação desses poros gera uma perturbação nos gradientes eletroquímicos da célula, culminando em sua morte (SERHAN *et al.*, 2014). Além disso, os polienos podem causar uma superprodução de espécies reativas de oxigênio (EROs), peroxidação lipídica e podem impedir o transporte de certas moléculas importantes como aminoácidos e carboidratos (BOLARD, 1986; SANGALLI-LEITE *et al.*, 2011; SERHAN *et al.*, 2014).

Além disso, essas drogas têm uma alta afinidade por esteróis presentes na membrana (CAROLUS *et al.*, 2020). Sendo assim, diversos estudos já mostraram que os polienos adsorvem o ergosterol presente nas membranas dos fungos, o que gera uma perda de estabilidade estrutural que culmina na morte celular (FALCÓN-GONZÁLEZ *et al.*, 2017;

GRAY *et al.*, 2012). Alguns pesquisadores, inclusive têm como hipótese que o efeito potente dos polienos contra diferentes espécies de fungos se dá mais pela sua alta interação com o ergosterol do que pela formação de poros na membrana (GRAY *et al.*, 2012; TE WELSCHER *et al.*, 2008).

Um dado que corrobora com essa hipótese é o de que um dos mecanismos de resistência já reportados aos polienos é justamente o aumento no conteúdo de ergosterol na membrana, burlando assim a ação do medicamento (KRISTANC *et al.*, 2019). Além disso, uma maior proporção de colesterol, um análogo ao ergosterol, na membrana de mamíferos poderia reduzir os efeitos colaterais dos polienos (KAMIŃSKI, 2014).

Alterações de componentes da via de biossíntese do ergosterol também já foram relacionadas à resistência aos polienos. Por exemplo, mutações no gene *ERG3* em fungos provocam a perda de função da enzima D-5,6-desaturase, culminando na diminuição do teor de ergosterol e no acúmulo de esteróis alternativos que não são reconhecidos (COWEN *et al.*, 2015). Ainda assim, os polienos são amplamente utilizados devido ao seu amplo espectro de ação e a resistência a eles é menos comum em comparação com os azóis e as equinocandinas (WIEDERHOLD, 2017).

2.1.2.3 Resistência às equinocandinas

Até o final do século 20, os azóis e os polienos eram os agentes antifúngicos mais utilizados. Entretanto, o uso dessas drogas pode causar uma série de efeitos colaterais que afetam principalmente o fígado e os rins. Além disso, como relatado acima, diversos casos de resistência já tinham sido reportados para essas duas classes de antifúngicos (WALL; LOPEZ-RIBOT, 2020). Além disso, por terem um alvo em comum, o ergosterol da membrana,

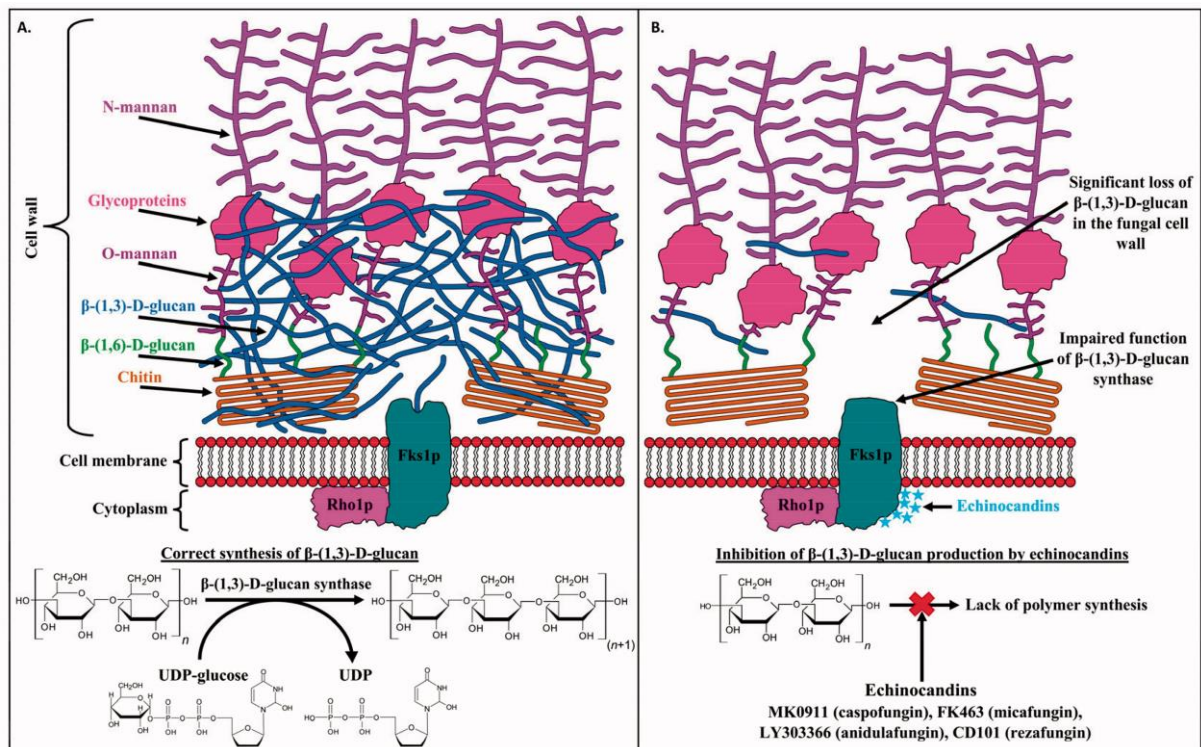
diversos casos de resistência cruzada aos azóis e aos polienos já foram reportados (HULL *et al.*, 2012).

Sendo assim, a busca por novos agentes antifúngicos que contornassem os problemas citados acima resultou na descoberta das equinocandinas, uma classe de antifúngico com efeito fungicida contra *Candida* sp. e fungistático contra *Aspergillus* sp. Além disso, as equinocandinas causam efeitos colaterais mais brandos em comparação com outras classes de antifúngicos (MROCZYŃSKA; BRILLOWSKA-DĄBROWSKA, 2020; SZYMAŃSKI *et al.*, 2022).

As equinocandinas têm como alvo a parede celular dos fungos, inibindo a (1,3)- β -D glicano sintase (EC 2.4.1.34), codificada pelos genes *FKS1* e *FKS2* em *Candida*, induzindo um estresse na parede celular, com posterior perda da integridade (Figura 5) (SUCHER; CHAHINE; BALCER, 2009).

A recomendação de utilização se dá no tratamento de infecções invasivas causadas principalmente por fungos dos gêneros *Candida* e *Aspergillus*. Essas drogas são também eficientes na prevenção da formação de biofilmes e promovem um efeito fungicida que causa um aumento e uma distorção das células, o que contribui para a inibição da proliferação celular (SHERRY *et al.*, 2017).

Figura 5 – Mecanismo de ação das equinocandinas. (A) produção normal do β -(1,3)-D-glicano e (B) ação da droga na subunidade FKS1p com a inibição da glicano sintase, o que resulta na morte do fungo causada pela instabilidade na parede celular.



Fonte: Szymański et al., 2022.

Diversos casos de resistência às equinocandinas têm sido reportados nos últimos anos. Diversas espécies de *Candida*, como *C. albicans*, *C. tropicalis*, *C. glabrata* e *C. krusei* têm se tornado cada vez mais resistentes a ação dessas drogas (SZYMAŃSKI *et al.*, 2022). Em resposta a essas drogas, os fungos desenvolvem continuamente mecanismos de reparo da parede celular que permitem o seu crescimento mesmo na presença desses agentes antimicrobianos. A maioria dos estudos relaciona a resistência a mutações na subunidade catalítica da glicano-sintase, a Fks. Essa subunidade é codificada por três genes: *FKS1*, *FKS2* e *FKS3*, os quais são alvos de mutações nas cepas resistentes (SUWUNNAKORN *et al.*, 2018). Essas mutações levam a alterações na subunidade catalítica, como substituição ou deleção de

aminoácidos, o que dificulta o reconhecimento dessa proteína pelo medicamento (ARENDRUP; PERLIN, 2014).

Todos esses diversos mecanismos moleculares de resistência aos agentes antimicrobianos relatados acima, ressaltam a importância da descoberta de novos agentes que atuem no controle dessas doenças infecciosas.

2.2 Peptídeos antimicrobianos

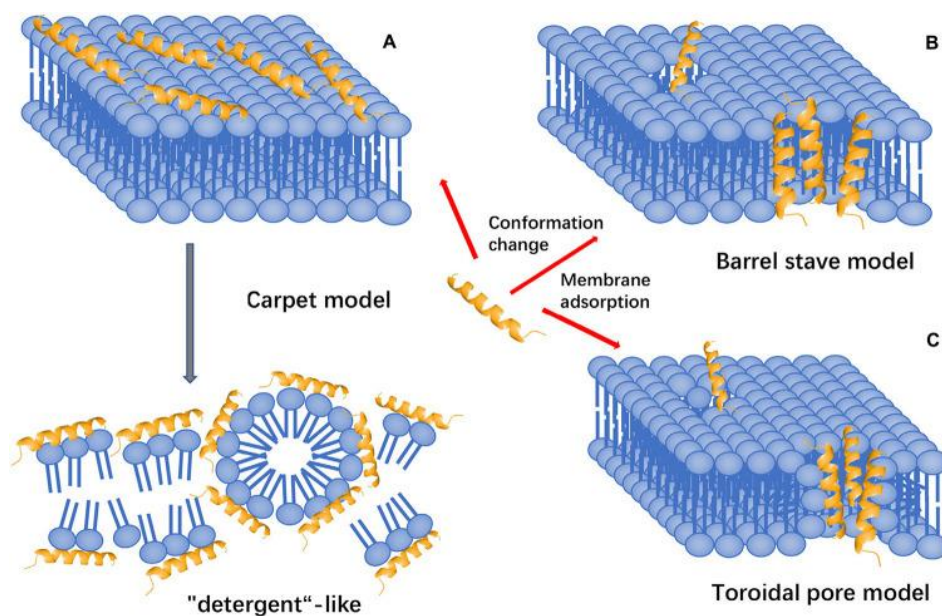
Uma das estratégias mais antigas no combate a infecções bacterianas e fúngicas em diversos organismos é a produção de peptídeos antimicrobianos (PAMs) (MAGANA *et al.*, 2020). Essas moléculas são produzidas, de forma constitutiva ou induzida, por bactérias (bacteriocinas), plantas (defensinas), protozoários e por praticamente todos os animais (LEI *et al.*, 2019). Esses PAMs são normalmente catiônicos e por isso, possuem uma elevada afinidade com a membrana plasmática (KUMAR; KIZHAKKEDATHU; STRAUS, 2018).

Em plantas, os peptídeos podem ser encontrados em flores, folhas, frutos, raízes, tubérculos e sementes. Eles normalmente apresentam baixa massa molecular, carga positiva em pH fisiológico, são anfipáticos e estáveis (MAGANA *et al.*, 2020). Esses PAMs apresentam um amplo espectro de ação e uma baixa taxa de indução de resistência quando comparados aos antimicrobianos convencionais (MAHLAPUU *et al.*, 2016).

Entretanto, o uso dos PAMs de origem natural enfrenta algumas limitações como o alto custo, baixa estabilidade e a elevada citotoxicidade (LEI *et al.*, 2019). Sendo assim, os PAMs sintéticos têm sido cada vez mais estudados por apresentarem vantagens como atividade melhorada, menos susceptibilidade à proteólise e baixa toxicidade (BROWNE *et al.*, 2020). Nos últimos anos, diversos trabalhos têm mostrado o elevado potencial antimicrobiano de peptídeos sintéticos, os quais atuam através de diversos mecanismos de ação.

Apesar da existência de muitos estudos que abordam a forma como esses peptídeos antimicrobianos agem, ainda não existe nenhuma teoria ou modo de ação que possa ser utilizado universalmente por todos os PAMs. Os estudos têm focado mais na interação desses peptídeos com a membrana dos microrganismos e no que ocorre após essa interação. Esse processo envolve, normalmente, duas etapas. Primeiramente, os PAMs catiônicos se ligam de forma ativa à superfície negativa das membranas, destruindo-as pela formação de poros (LEI *et al.*, 2019). Existem 3 modelos principais de mecanismos de ação de peptídeos por ruptura de membrana: formação de carpete (carpet-like), modelo de barril (barrel-stave) ou poro toroidal (toroidal pore) (Figura 6) (BROGDEN, 2005; HUAN *et al.*, 2020).

Figura 6 – Modelos de interação de peptídeos antimicrobianos (PAMs) com a membrana plasmática.



Fonte: Huan et al., 2020.

No modelo *carpet-like*, o peptídeo interage com fosfolipídeos da membrana, recobrando toda a sua superfície. Quando é atingida uma concentração crítica, a membrana sofre alterações morfológicas e se rompe formando agregados de peptídeos e micelas (HE;

LAZARIDIS, 2013). Não há necessariamente a formação de poros por esse mecanismo, mas pode haver a formação de poros associada à cobertura da membrana plasmática.

Pelo modelo de barril (barrel-stave), a interação do peptídeo com a membrana depende de sua concentração e se inicia com um acúmulo de peptídeo na superfície da membrana, o que reduz a sua espessura. Quando a concentração crítica é alcançada, os peptídeos formam poros transmembrana do tipo barril, no qual a porção hidrofóbica fica voltada para os fosfolipídeos e a porção hidrofílica fica protegida na parte interna do poro (SHAI, 2002).

O modelo toroidal também envolve a formação de poros e tem sido mais aceito que o modelo em barril. Nesse caso, os peptídeos interagem com as cabeças polares dos fosfolipídeos de membrana, induzindo uma curvatura em sua estrutura que permite que o peptídeos se insiram perpendicularmente, formando o poro ao mesmo tempo que permanecem ligados aos fosfolipídeos (PRIYADARSHINI *et al.*, 2022).

Além de exercer atividade antimicrobiana através da formação de poros na membrana, os peptídeos também podem causar outras alterações nas células que resultam na morte do microrganismo. Por exemplo, a ação pode se dar via inibição da síntese de ácidos nucleicos ou de proteínas e também pela ativação de bombas de efluxo na membrana plasmática (ARMENGOL; DOMENECH; VINAS, 2019; SIERRA *et al.*, 2017).

Outros estudos, como o de Dias *et al.* (2020), também reportam os mecanismos por trás da atividade antimicrobiana de peptídeos sintéticos. No referido trabalho, dois peptídeos sintéticos foram desenhados a partir da sequência de uma albumina 2S purificada das sementes de *Ricinus communis* (Rc-2S-Alb) e denominados RcAlb-PepI e RcAlb-PepII. Esses peptídeos promoveram uma alta inibição no crescimento de *Klebsiella pneumoniae* e de *Candida parapsilosis*, através de mecanismos como alterações na morfologia, indução de poros na membrana e geração de espécies reativas de oxigênio. Além disso, os peptídeos apresentaram baixa atividade citotóxica e atividade hemolítica ausente

(DIAS *et al.*, 2020). Outros peptídeos, como *Mo*-CBP₃-PepIII, bioinspirado na sequência de uma proteína de *Moringa oleifera*, também apresentaram atividade antimicrobiana e induziram uma permeabilização na membrana plasmática (OLIVEIRA, *et al.*, 2019).

Esses peptídeos, sobretudo os sintéticos, têm elevado potencial para serem utilizados como uma alternativa ao uso de antimicrobianos convencionais a fim de diminuir os impactos gerados pela resistência microbiana (KUMAR; KIZHAKKEDATHU; STRAUS, 2018). Muitos dos peptídeos estudados já estão em fase de testes clínicos e podem ser utilizados na formulação de medicamentos de uso tópico ou de uso oral e intravenoso o que reforça o seu potencial como agente terapêutico (ZHU, YIYUN, 2022).

Nesse contexto, estudos preliminares conduzidos por nosso grupo de pesquisa reportaram a atividade antimicrobiana de uma albumina 2S com atividade inibitória de tripsina purificada da torta de *Jatropha curcas* (denominada *JcTI-I*) (COSTA, *et al.*, 2014). No entanto, devido a algumas limitações na purificação, como baixo rendimento de purificação e dificuldade na obtenção do material de partida, foi então proposto o objetivo desse trabalho de prospectar peptídeos sintéticos baseados na sequência dessa proteína de modo a contribuir para o entendimento acerca do potencial terapêutico dos PAMs.

3 HIPÓTESE

Os peptídeos sintéticos bioinspirados em *JcTI-I* apresentam uma potente atividade antimicrobiana frente à diferentes espécies de leveduras e bactérias de interesse clínico, causando efeitos deletérios nesses microrganismos que resultam na inibição do seu crescimento.

4 OBJETIVOS

4.1 Objetivo Geral

Desenhar peptídeos sintéticos inspirados na sequência de um inibidor de tripsina de *Jatropha curcas* (JcTI-I) e avaliar sua ação contra bactérias e leveduras de interesse clínico, determinar suas propriedades estruturais, seu modo de ação e sua toxicidade *in vitro* e *in silico*.

4.2 Objetivos Específicos

- Desenhar e analisar as características físico-químicas e estruturais dos peptídeos sintéticos bioinspirados *in silico*;
- Analisar a citotoxicidade e a alergenicidade dos peptídeos sintéticos mais promissores *in vitro* e *in silico*.
- Investigar a atividade antimicrobiana dos peptídeos sintéticos contra diferentes espécies de bactérias e leveduras;
- Investigar a atividade antibiofilme dos peptídeos sintéticos;
- Caracterizar os mecanismos que justificam a ação antimicrobiana desses peptídeos;

5 ENSAIO COM BACTÉRIAS DE INTERESSE CLÍNICO

A partir da sequência primária de *JcTI-I*, um inibidor de tripsina de *Jatropha curcas*, foram selecionados três peptídeos com características mais promissoras, os quais foram sintetizados e denominados *JcTI-PepI*, *JcTI-PepII* e *JcTI-PepIII*. Os três peptídeos sintéticos foram testados contra diferentes espécies de bactérias de interesse clínico.

As bactérias patogênicas Gram-positivas (*Bacillus subtilis* (ATCC 6633) e *Staphylococcus aureus* (ATCC 25923)) e Gram-negativas (*E. Coli* (ATCC 8739), *K. pneumoniae* (ATCC 10031) e *Salmonella choleraesuis* (ATCC 14028)) foram fornecidas pelo Laboratório de Toxinas Vegetais.

Para o ensaio de inibição de crescimento das bactérias foi utilizada a técnica de microdiluição usando caldo Mueller Hinton para bactérias como descrito anteriormente (OMARDIEN *et al.*, 2018). Os peptídeos foram testados contra as bactérias citadas anteriormente em placas de microtitulação de 96 poços de poliestireno de fundo chato. Uma alíquota de 100 µL do meio de cultura caldo Mueller Hinton contendo células bacterianas (5×10^5 UFC/mL) foi incubada (8 h, 37 °C, no escuro) com os peptídeos sintéticos solubilizados em DMSO 5% em NaCl 0,15 M (concentrações finais que variaram de 500 µM a 0,25 µM). Após 24 h de incubação, foram realizadas leituras de absorvância a 600 nm usando um leitor de microplacas (Epoch, BioTek Instruments, Inc., EUA). Os controles negativo e positivo foram DMSO 5% em NaCl 0,15 M e Ciprofloxacino (780 µM), respectivamente. O meio de cultura contendo apenas as células bacterianas foi usado como referência (100% de crescimento). A CIM (Concentração Inibitória Mínima) foi determinada por comparação das absorvâncias das células tratadas com os peptídeos e das células tratadas com o controle negativo.

Como resultado do ensaio de inibição do crescimento bacterianos, foi observado que nenhum dos três peptídeos desenhados a partir de *JcTI-I* se mostrou eficaz contra as bactérias testadas. Devido a ausência de atividade antibacteriana, os dados referentes a esses experimentos não foram inseridos no artigo publicado no periódico internacional *Biochimie* no ano de 2022, o qual apresentou apenas os resultados da atividade antifúngica dos peptídeos sintéticos bioinspirados no inibidor de tripsina de *Jatropha curcas*.

6 CAPÍTULO 1

Revista científica

O seguinte artigo referente à tese foi publicado no Periódico Internacional *Biochimie* (ISSN: 0300-9084), volume 200, páginas 107-118, em 2022.

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Título

JcTI-PepI, a synthetic peptide bioinspired in the trypsin inhibitor from *Jatropha curcas*, presents potent inhibitory activity against *C. krusei*, a neglected pathogen

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***JcTI-PepI*, a synthetic peptide bioinspired in the trypsin inhibitor from *Jatropha curcas*, presents potent inhibitory activity against *C. krusei*, a neglected pathogen**

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Abstract

Antimicrobial resistance has been increasing globally, posing a global public health risk. It has prompted the scientific community to look for alternatives to traditional drugs. Antimicrobial Peptides (AMPs) have stood out in this context because they have the potential to control infectious diseases while causing no or little harm to mammalian cells. In the present study, three peptides, *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII*, were designed and tested for antimicrobial activity based on the primary sequence of *JcTI-I*, a 2S albumin with trypsin inhibitory activity from *Jatropha curcas*. *JcTI-PepI* strongly inhibited *C. krusei* growth, and it caused severe disruptions in cellular processes and cell morphology. *C. krusei* cells treated with *JcTI-PepI* showed indicative of membrane permeabilization and overproduction of Reactive Oxygen Species. Moreover, the yeast's ability to acidify the medium was severely compromised. *JcTI-PepI* was also effective against pre-formed biofilm and did not harm human erythrocytes and Vero cells. Overall, these characteristics indicate that *JcTI-PepI* is both safe and effective against *C. krusei*, an intrinsically resistant strain that causes serious health problems and is frequently overlooked. It implies that this peptide has a high potential for use as a new antimicrobial agent in the future.

Keywords: AMPs, *C. krusei*, antimicrobial resistance.

6.1 Introduction

In the fight against infectious diseases, antimicrobial agents have played an important role. However, there has been an increase in infections caused by resistant microorganisms over the last several decades [1]. In this context of resistance, fungi have stood out, and the problem is concerning because there are only a few classes of antifungals available to treat infections, with the most common being: azoles, echinocandins, and polyenes [2].

In humans, fungal infections are classified as superficial or deep, systemic or disseminated infections such as invasive aspergillosis and candidemia [3]. Infections caused by *Candida* species are a public health concern. Candidemia, a more invasive form of candidiasis, is a source of concern in a number of countries [4]. It is estimated that nine people were infected with the disease for every 100,000 people between 2013 and 2017. In addition, the Center for Disease Control and Prevention estimates that more than 25,000 cases of candidemia occur in the United States each year [5]. Systemic fungal infections are considered medical emergencies and present high mortality rates. In this scenario, azole resistance among *Candida* species, along with echinocandin and multidrug resistance, is one of the main challenges in medical care [6].

C. albicans, *C. parapsilosis*, *C. Tropicalis*, *C. glabrata*, and *C. krusei* cause more than 95% of invasive infections. The proportion of infections caused by each species varies with geographic location. In general, *C. albicans* is the most commonly isolated from patients with invasive infections [7]. However, the number of infections caused by *C. albicans* has been decreasing, and non-*albicans* species are now responsible for about 75% of *Candida* infections in the USA [7].

Among these species, *C. krusei* has emerged as a serious pathogen, accounting for 1.5–8% of candidemia cases. This is especially concerning because this species is intrinsically resistant to fluconazole (FLC) and has decreased susceptibility to other azoles and polyenes [[7], [8], [9]]. Echinocandins are still an important treatment option for invasive *C. krusei* infection. Nonetheless, several studies have shown that resistance develops quickly during caspofungin and micafungin treatment [10,11]. In addition, infections caused by *C. krusei* have a higher mortality rate (20%–67%) when compared to several non-*albicans* species, which is likely related to their poor response to standard antifungal therapies [12]. To minimize the impacts caused by antimicrobial resistance, the search for new molecules with potent antimicrobial activity against these infectious agents is critical.

In this context, antimicrobial peptides (AMPs) have emerged as particularly effective in combating infections caused by resistant microorganisms. AMPs are low-molecular-mass defense peptides with the ability to interact with cell membranes. They have a wide range of action, including the ability to neutralize virulence processes and modulate immune responses in the host [13]. However, natural AMPs have some limitations in their use, such as high toxicity, high susceptibility to degradation by proteases, and loss of microbial activity under physiological salts conditions [14]. Synthetic peptides based on the sequence of naturally occurring peptides and proteins obtained from animals, plants, and microorganisms have been developed to overcome these limitations. As a result, several studies have been conducted to investigate the role of synthetic AMPs in the control of non-resistant and resistant microorganisms [15].

Recently, our research group purified a protein from *Jatropha curcas* seed cake, which was named *JcTI-I* [16]. *JcTI-I* is a 2S albumin with trypsin inhibitory activity that showed high antimicrobial potential against several fungi and bacteria species. However, the low purification yield (1.2%) impeded the use of this protein as an antimicrobial molecule. Thus, the design of peptides inspired by the primary sequence of *JcTI-I* was proposed. The designed peptides were tested against several species of *Candida*, and the most promising had its mode of action investigated against *C. krusei*.

6.2 Materials and Methods

6.2.1 Materials

C. parapsilosis (ATCC 22019, ATCC 7330, and ATCC 90018), *C. krusei* (ATCC 6258 and ATCC 14243), *C. albicans* (ATCC 10231, ATCC 14053, and ATCC 18804), and *C. tropicalis* (clinical isolate, ATCC 750, and ATCC 13803) were acquired from the Laboratory of Plant Toxins at the Department of Biochemistry and Molecular Biology of the Federal University of Ceara (UFC), Brazil. Human blood samples (ABO system) were obtained from healthy donors in the blood bank HEMOCE (Hemotherapy Center of Ceara, Brazil). All other chemicals and culture mediums were purchased from Sigma-Aldrich Co. (St. Louis, USA) and Himedia® Laboratories Pvt. Ltd. (Mumbai, India).

6.2.2 Prediction, *in silico* analysis, and peptide synthesis

To design the peptides, the amino acid sequence of *JcTI-I* was loaded into the freely accessible tool CAMP_{R3} (<http://www.camp3.bicnirrh.res.in/index.php>) [17]. Among the suggested peptides, those with a low molecular weight (600 at 1200 Da), positive net charge ($\geq +1$), total hydrophobic ratio $\geq 30\%$, and Boman index ≤ 4.5 were selected [18]. These properties were assessed using the Antimicrobial Peptide Database tool (<https://aps.unmc.edu/prediction>) [19]. The iAMPpred (<http://cabgrid.res.in:8080/amppred/>) was used to predict the antimicrobial potential of the designed peptides [20], with results displayed as a score ranging from 0 to 1, with values close to 1 indicating peptides with a high probability of exhibiting antimicrobial activity.

The peptide's ability to penetrate the cell membranes (http://crdd.osdd.net/raghava/cellppd/multi_pep.php) [21], its resistance to proteolysis (http://web.expasy.org/peptide_cutter/), and half-life in an intestine-like environment (<http://crdd.osdd.net/raghava/hlp/help.html>) [22] were also evaluated. Furthermore, some biological activities were predicted using the following *in silico* tools: the allergenic (<http://imed.med.ucm.es/Tools/antigenic.pl>), hemolytic (<https://webs.iiitd.edu.in/raghava/hemopi/>) [23], and toxic (<https://webs.iiitd.edu.in/raghava/toxinpred/design.php>) [24] potentials. Also, the anticancer activity was evaluated *in silico* (<https://webs.iiitd.edu.in/raghava/anticp/>) [25].

Following all the above analyses, three peptides, *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII*, showed the most promising antimicrobial properties *in silico*. They were chosen and chemically synthesized by GenOne (Rio de Janeiro, Brazil). The purity of the peptides ($\geq 95\%$) was assessed by high-pressure liquid chromatography (RP-HPLC) and mass spectrometry analysis.

6.2.3 Structural characterization

The PEP-FOLD online server (<http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/>) was used to predict the 3D structure of *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII* [26]. PEP-FOLD was also used to predict T_m and sOPEP scores, which were then used to determine the best structure model. The 3D structural analysis was executed using the *PyMol* Molecular Graphics System, version 1.7.4, Schrodinger, LLC. The electrostatic

maps were obtained using APBS [27] and PDB 2PRQ [28]. Helical wheel plots were created using the software Kamal v 2.1 (<http://www.cenargen.embrapa.br/kamal/>) [29].

6.2.4 Anticandidal activity of *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII*

The effect of the peptides on yeast cells growth was evaluated in polystyrene flat-bottom 96-well microtiter plates, as reported previously (DA SILVA NETO *et al.*, 2020). Aliquots (100 μL) of *C. parapsilosis*, *C. krusei*, *C. albicans*, and *C. tropicalis* cells suspensions (5×10^5 CFU/mL), prepared in Sabouraud broth, were incubated with 100 μL of *JcTI-PepI*, *JcTI-PepII* or *JcTI-PepIII* at different concentrations (500 μM to 0.25 μM final concentrations, prepared in 0.15 M NaCl containing 5% DMSO). After incubation (37 $^{\circ}\text{C}$, 24 h), yeast growth was evaluated by absorbance at 600 nm in an automated microplate reader (Epoch, BioTek Instruments Inc., USA). The commercial antifungal Nystatin (122.7 μM) was used as the positive control (100% inhibition), and the 0.15 M NaCl solution containing 5% DMSO was used as the negative (100% growth). Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the peptide that inhibited the visible *in vitro* growth. Furthermore, to determine the Minimum Fungicidal Concentration (MFC), aliquots (10 μL) from the wells that showed a high inhibition were incubated in Petri dishes containing agar-Sabouraud medium, for 24 h at 37 $^{\circ}\text{C}$. MFC was defined as the lowest concentration of peptide that reduces the viability of the initial microorganism inoculation by $\geq 99.9\%$, causing its death. Three independent experiments were performed (with three biological replicates).

6.2.5 Assessment of antibiofilm effect of *JcTI-PepI*

The effect of *JcTI-PepI* on *C. krusei* biofilms was evaluated in polystyrene flat-bottom 96-well microtiter plates, according to the method described by Dias *et al.* [31]. Aliquots (100 μL) of *JcTI-PepI* (at concentrations ranging from 250 μM to 15.6 μM) were incubated (37 $^{\circ}\text{C}$, 48 h) with 100 μL of *C. krusei* cell suspensions (10^6 cells mL^{-1} , prepared in Sabouraud broth). After incubation, the supernatant was removed, and the biofilm formed was stained with 0.1% (m/v) crystal violet for 15 min. After removing the excess stain with 0.15 M NaCl, 250 μL of 95% (v/v) ethanol was added to solubilize the crystal violet bound to

the cells. Then, the absorbances were measured at 570 nm. Inhibition of biofilm formation was calculated using the following formula: %Inhibition = $100 - [\text{Abs (cells treated with } JcTI\text{-PepI)} \times 100 / \text{Abs (cells treated with 5\% DMSO in 0.15 M NaCl)}]$.

To assess the effect of *JcTI-PepI* on the degradation of preformed yeast biofilm, two hundred microliters of *C. krusei* cell suspension were incubated at 37 °C for 24 h. Next, the supernatant was removed, and 100 µL of Sabouraud broth and 100 µL of *JcTI-PepI* (at the same concentrations described above) were added. The supernatant was removed after 24 h of incubation at 37 °C, and the remaining biofilm was stained with 0.1% (m/v) crystal violet. The above-mentioned procedure was followed by quantification. Nystatin (122.7 µM) and 5% DMSO in 0.15 M NaCl solution were used as positive and negative control, respectively.

6.2.6 Mechanisms of the anticandidal action of *JcTI-PepI* on *C. krusei* cells

6.2.6.1 Assessment of cell membrane integrity

The *C. krusei* cells (5×10^5 CFU/mL) were incubated with 100 µL of 15.62 µM *JcTI-PepI* (prepared with 5% DMSO in 0.15 M NaCl), 8.6 µM amphotericin B (positive control), or 5% DMSO in 0.15 M NaCl (negative control), for 24 h at 37 °C. After incubation, aliquots (100 µL) of the treated cells were incubated with 1 mM propidium iodide at 37 °C for 30 min, under moderate agitation (75 rpm). The visualization of the cells was done using a fluorescence microscope (Olympus BX 60 System, excitation wavelength, 490 nm, emission wavelength, 520 nm) [32]. To determine the dimensions of the pores formed, the treated cells were incubated with calcein (0.994 kDa) and fluorescein isothiocyanate-conjugated dextran (FD10 and FD20, with molecular masses of 10 kDa and 20 kDa, respectively). After incubation (30 min, 37 °C), the visualization was performed using a fluorescence microscope (Olympus System BX 60; excitation wavelength, 490 nm; emission wavelength, 520 nm).

*6.2.6.2 Effect of *JcTI-PepI* on Reactive Oxygen Species (ROS) production*

After the treatment (24 h at 37 °C) with 15.62 µM *JcTI-PepI* (prepared in 5% DMSO in 0.15 M NaCl), 8.6 µM amphotericin B (positive control), or 5% DMSO in 0.15 M NaCl (negative control), the *C. krusei* cells (5×10^5 CFU/mL) were incubated with 10 µM 2',7'-

Dichlorofluorescein diacetate (DCFH-DA), at 37 °C for 30 min. Next, these cells were washed with 100 µL 0.15 M NaCl and visualized under a fluorescence microscope (Olympus System BX 60; excitation wavelength, 488 nm; emission wavelength, 525 nm) [33].

6.2.6.3 Scanning electron microscopy analyses

C. krusei cells (5×10^5 CFU/mL) were incubated with 15.62 µM *JcTI-PepI* for 24 h, under the conditions described in the 2.4 section. After incubation, the cells were fixed and prepared for scanning electron microscopy (SEM) analysis, as previously described by Dias et al. [31]. Briefly, after incubation, the cells were centrifuged (Mikro 200 R centrifuge, Hettich, Germany) at 3000g, for 5 min at 20 °C, and the supernatant was discarded. Cell pellets were fixed at room temperature (20-24 °C) for 5 h with 100 µL of the fixation solution [1% (v/v) glutaraldehyde – 4% (v/v) formaldehyde prepared in 0.15 M Na-phosphate buffer, pH 7.2]. The material was centrifuged, and the cells were washed (3 ×) with 0.15 M Na-phosphate buffer, pH 7.2. Then, the cells were treated with 0.2% (m/v) osmium tetroxide, followed by another centrifugation at the conditions described above. Then, the cells were dehydrated by serial incubation in a range of ethanol concentrations (30-10%) for 10 min each. Next, the samples were immersed in 50/50 ethanol/hexamethyldisilazane (HMDS) for 10 min, followed by centrifugation. The pellet was treated with 100% (v/v) HMDS and placed on a coverslip which was mounted on stubs and coated with a gold layer (20 nm).

SEM images were obtained in the Center of Advanced Microscopy and Microanalysis (Central Analytical facilities) at Federal University of Ceará, Brazil, using an Inspect™ 50 FEI Scanning Electron Microscope (Oregon USA) equipped with a low energy detector (Everhart-Thornley detector).

6.2.6.4 Effect of *JcTI-PepI* on glucose-stimulated acidification of the extracellular medium

This assay was carried out in accordance with the methodology previously described by Gomes et al. [34]. 100 mL of a *C. krusei* cells suspension (with absorbance at 600 nm = 0.200) was centrifuged (2000 g, 5 min, 4 °C), and the pellets were resuspended with 5 mL of 5 mM Tris-HCl buffer, pH 7.1. Next, these pellets were incubated (for 30 min) with 15.62 µM, 7.81 µM or 3.9 µM *JcTI-PepI* (final concentrations), 122.7 µM nystatin (positive control), or 5% DMSO in 0.15 M NaCl (negative control). After incubation, 1 mL of 0.5 M

glucose was added, and the acidification of the medium was monitored every 10 min, for 1 h using a pH meter (Marconi, MA PA 200, São Paulo, Brazil). The experiments were conducted in triplicate.

The results were expressed in terms of pH variation over time, and the inhibition after 1 h was calculated using the following formula:

$$\% \text{ inhibition} = 100 - [(\text{pH variation}_{\text{treatment}} / \text{pH variation}_{\text{negative control}}) \times 100]$$

6.2.7 Haemolytic activity of *JcTI-PepI*

To evaluate the haemolytic potential, 100 μL of *JcTI-PepI* (prepared in 5% DMSO in 0.15 M NaCl, with concentrations ranging from 250 μM to 7.81 μM) were incubated with 100 μL of 2.5% suspension of the red blood cells (type A, B, and O), as described by Dias et al., with modifications [31]. In addition, the blood cells were also incubated with 5% DMSO in 0.15 M NaCl (negative control, 0% haemolysis) or 0.1% (v/v) Triton X-100 (positive control, 100% haemolysis), without *JcTI-PepI*. After incubation (1 h at 37 °C), the mixtures were centrifuged (10 min at 300 g at 10 °C, Mikro 200R centrifuge, Hettich, Germany), and the supernatants were transferred to a polystyrene flat-bottom 96-well microtiter plate. Then, the supernatant absorbance was measured at 414 nm in an automated microplate reader (Epoch, BioTek Instruments Inc., USA). The percentage of haemolysis was determined as follows: $[(\text{Abs}_{414\text{nm}}$ of the red blood cells treated with *JcTI-PepI*) - ($\text{Abs}_{414\text{nm}}$ of the red blood cells treated with the negative control)] / ($\text{Abs}_{414\text{nm}}$ of the red blood cells treated with the positive control) - ($\text{Abs}_{414\text{nm}}$ of the red blood cells treated with the negative control)] $\times 100$ [35].

6.2.8 Cytotoxicity of *JcTI-PepI*

The cytotoxic activity of *JcTI-PepI* was assessed against a Vero cell suspension prepared in Leibovitz L-15 medium, supplemented with 10% fetal bovine serum (Cultilab, Brazil) at 37 °C, in humidified air containing 5% CO_2 [36]. Cells were seeded at 1×10^4 per well in 96-well plates and were cultured at 37 °C in the presence of 5% CO_2 . 24 h later, samples in triplicate were treated with different concentrations of the *JcTI-PepI* (final concentrations ranging from 1 mM to 7.81 μM). The plates were incubated for further 72 h under the above incubation conditions. A solution of 5% DMSO in 0.15 M NaCl solution was

used as negative control (100% cell viability). Then, each well received 10 μ L of MTT dye solution (1 mg/mL in 0.15 M NaCl). The plates were incubated for additional 4 h at 37 °C. After removing the medium, the formed formazan crystals were solubilized in 100 μ L DMSO. The absorbance was measured at 495 nm in an automated microplate reader (Epoch, BioTek Instruments Inc., USA). The cytotoxicity potential was calculated compared to the values obtained from the control wells (only treated with 5% DMSO in 0.15 M NaCl). Cell viability percentages were calculated in comparison to control using the formula: $(\text{Absorbance}_{\text{treated cells}}/\text{Absorbance}_{\text{negative control}}) \times 100$. The CC50, defined as the peptide concentration required for reducing the cell viability by 50%, was calculated by linear regression analysis of concentration–response curve generated by the data.

6.2.9 *In vitro* digestibility of JcTI-PepI

JcTI-PepI was incubated (at 37 °C for 2 s, 5 min, 30 min, and 1 h) with a simulated gastric fluid (SGF: 0.034 M NaCl, 0.07% HCl and 3.2 mg/mL pepsin) and a simulated intestinal fluid (SIF: 0.05 M potassium phosphate and 10 mg/mL pancreatin) to assess the *in vitro* digestibility. After incubation, the mixtures and the intact peptide were loaded in Liquid Chromatography coupled to an Electrospray Ionization – Mass Spectrometry (LC-ESI-MS). Five microliters of the samples were applied in a reverse phase C-18 column (0.075 \times 100 mm) coupled to a nanoAcquity system. Next, the eluted material was applied in a mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp, MA, USA). The analysis conditions were as follows: positive mode, temperature of 363 K, and capillary voltage of 3.5 kV [31].

6.2.10 Statistics

All the experiments were carried out in triplicate, and the experimental results were represented as error bars in the figures. Statistical significance analyzes were performed using one-way ANOVA followed by Tukey's test of multiple comparisons ($p < 0.05$), using GraphPad Prism 5.0 software.

6.3 Results

6.3.1 Peptide design, in silico analysis, and structural characterization

Several putative antimicrobial peptides were identified using the CAMP_{R3} tool. The most promising, analyzed *in silico*, were the *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII*. They were further characterized, and their molecular mass, Boman index, charge, hydrophobicity, and pI were determined. *In silico*, neither of the chosen peptides had a penetrating potential. Furthermore, none of these peptides were predicted to have an allergic or toxin potential (Table 1).

Table 1. Physicochemical characterization and prediction of biological properties of the synthetic peptides *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII* by bioinformatics tools.

Analyses	Peptide		
	<i>JcTI-PepI</i>	<i>JcTI-PepII</i>	<i>JcTI-PepIII</i>
Physicochemical Properties			
Amino acid sequence	ⁿ KRRKLISC ^c	ⁿ LKKGIVGS ^c	ⁿ LLGKVGSKD ^c
¹ Calculated molecular mass (Da)	1003.274	800.99	916.079
² Experimental molecular mass (Da)	1003.280	800.98	916.078
³ Isoelectric point (pI)	11.01	10.00	8.59
¹ Boman Index	4.15	-0.15	0.82
¹ Total hydrophobicity (%)	37	37	33
¹ Net charge	+4	+2	+1
⁴ T _m	0.48	0.51	0.47
⁴ sOPEP	-7.21	-14.13	-8.62
Biological Properties			
⁵ Penetrating peptide potential	No	No	No
⁶ Allergic potential	No	No	No
⁷ Antimicrobial potential score			
Antibacterial	0.94	1.00	0.99
Antifungal	0.94	0.98	0.83
⁸ Cleavage sites			
Trypsin (high pH)	4	2	2
Pepsin (pH 1.3)	0	1	2
Pepsin (pH >2)	0	1	2
⁹ Half-life time	2.299	1.172	1.544
⁹ Stability	High	High	High
¹⁰ Hemolytic potential	0.48	0.51	0.50
¹¹ Toxin potential	No	No	No
¹² Anticancer activity prediction	AntiCP	AntiCP	AntiCP

¹Calculated using the Antimicrobial Peptide Database.

²Data obtained by mass spectrometry conducted by GenOne (Sao Paulo, Brazil).

³Calculated using the ExPASy ProtParam toll.

⁴T_m and sOPEP scores were calculated using the PEP-FOLD server.

⁵Penetrating Peptide (CPP) capacity was predicted using the CellPPD tool.

⁶Allergic potential was predicted using the Antigenic prediction tool.

⁷Antimicrobial potential was calculated using the iAMPpred tool.

⁸Cleavage sites were determined using the Peptide Cutter tool.

⁹Half-life time in the intestinal environment and stability were determined using the HLP tool.

¹⁰Hemolytic potential was predicted by HemoPI tool.

¹¹Toxin potential was calculated using the ToxinPred tool.

¹²Anticancer activity was predicted using the AntiCP tool.

ⁿN-terminal end of the peptide.

^cC-terminal end of the peptide.

JcTI-PepI, *JcTI-PepII*, and *JcTI-PepIII* presented high stability, *in silico*, to proteolysis in the intestinal environment. Despite the structure's cleavage sites, their half-life times were greater than 1.5 s. The antimicrobial potential of the three synthetic peptides was also investigated using the iAMPpred tool, and the results showed that the three synthetic peptides had a high potential to be antibacterial or antifungal, with scores greater than 0.8. In addition, it was observed that the three-dimensional models obtained from *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII* presented high-quality structures, being composed of secondary α -helix type structures (Fig. 1A–C). All the peptides' models showed N-terminal regions with predominantly positive charges (blue) and C-terminal regions with predominantly negative charges (red) (Fig. 1D–F). However, the *JcTI-PepI*, model had the largest negative electrostatic potential at pH 7.0 (Fig. 1D). The Schiffer-Edmundson helical wheel projection of the three peptides showed that the molecular arrangement of the peptides did not generate amphipathic structures (Fig. 1G–I).

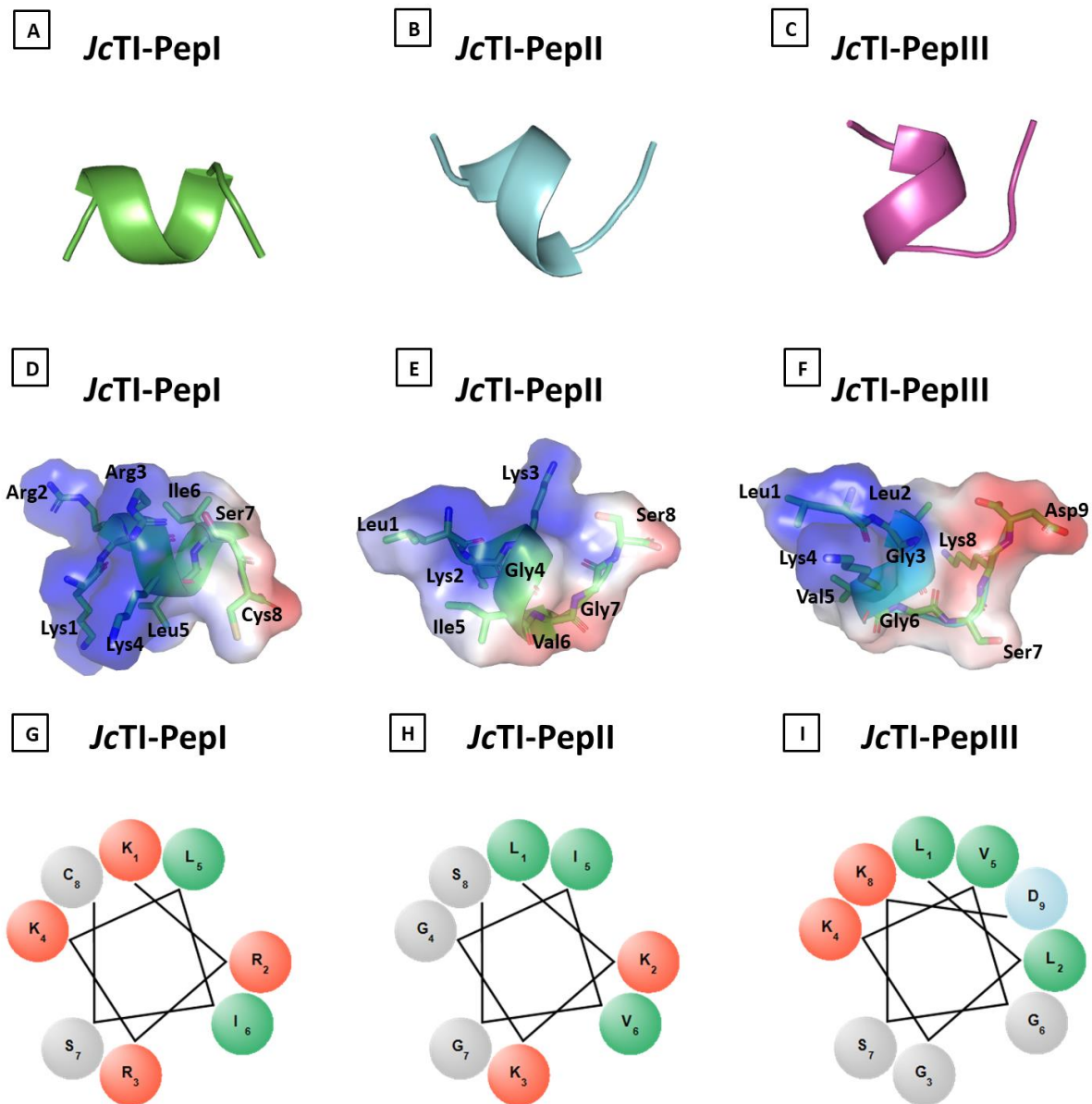


Fig. 7. Three dimensional structures models of *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII*. Cartoon representations (A, B, and C). Electrostatic potential representations (D, E, and F). Positively and negatively charged residues are represented in blue and red, respectively. Schiffer–Edmundson helical wheel representation (G, H, and I). Hydrophobic residues are represented in green, positively charged residues are represented in red, negatively charged residues are represented in light blue and polar uncharged residues in gray.

6.3.2 Anticandidal activity

JcTI-I was the only peptide that inhibited *Candida* species growth. When compared to *C. tropicalis*, the strongest activity was obtained against *C. krusei*, with a lower MIC value

(48.87 μM) (Table 2). In addition, the MFC value (93.75 μM) was also lower for this species. *C. krusei* ATCC 6258 was more susceptible to the treatment with *JcTI-PepI* (MIC: 31.25 μM /MFC: 62.5 μM). Therefore, *JcTI-PepI* was chosen for further characterization and research into its antimicrobial activity and mode of action against *C. krusei* ATCC 6258. On the other hand, *JcTI-PepII* and *JcTI-PepIII* had MICs and MFCs greater than 500 μM to all the tested strains, so they were not further studied.

Table 2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII* against different *Candida* species.

Microorganism	Peptide					
	<i>JcTI-PepI</i> (μM)		<i>JcTI-PepII</i> (μM)		<i>JcTI-PepI</i> (μM)	
	MIC ^a	MFC ^b	MIC ^a	MFC ^b	MIC ^a	MFC ^b
Yeast						
<i>C. albicans</i>						
ATCC 10231	ND	ND	ND	ND	ND	ND
ATCC 14053	250	ND	ND	ND	ND	ND
ATCC 18804	ND	ND	ND	ND	ND	ND
<i>C. tropicalis</i>						
Clinical isolate	62.5	125	ND	ND	ND	ND
ATCC 750	62.5	62.5	ND	ND	ND	ND
ATCC 13803	125	250	ND	ND	ND	ND
<i>C. krusei</i>						
ATCC 6258	31.25	62.5	ND	ND	ND	ND
ATCC 142243	62.5	125	ND	ND	ND	ND
<i>C. parapsilosis</i>						
ATCC 22019	ND	ND	ND	ND	ND	ND
ATCC 7330	ND	ND	ND	ND	ND	ND

ATCC 90018	ND	ND	ND	ND	ND	ND
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^a Values were defined as the lowest concentration that totally inhibits the growth of the tested organism.

^b The MFC is the peptide lowest concentration required to kill a particular fungal.

ND means that the MIC or MFC value was greater than the maximum concentration tested (500 μ M). So, these values could not be determined in this study.

6.3.3 Antibiofilm activity of *JcTI-PepI*

JcTI-PepI was active against planktonic yeast cells, but it did not prevent the formation of *C. krusei* biofilm (data not shown). On the other hand, *JcTI-PepI*, at 62.5 μM , degraded the preformed biofilm of *C. krusei* in about 63%, similar to the result obtained with 122.7 μM nystatin (68%) (Fig. 2). The difference was not statistically significant. Higher concentrations of *JcTI-PepI* (250-125 μM) did not result in an enhanced antibiofilm activity, as the inhibition was similar to what was obtained using 62.5 μM *JcTI-PepI*.

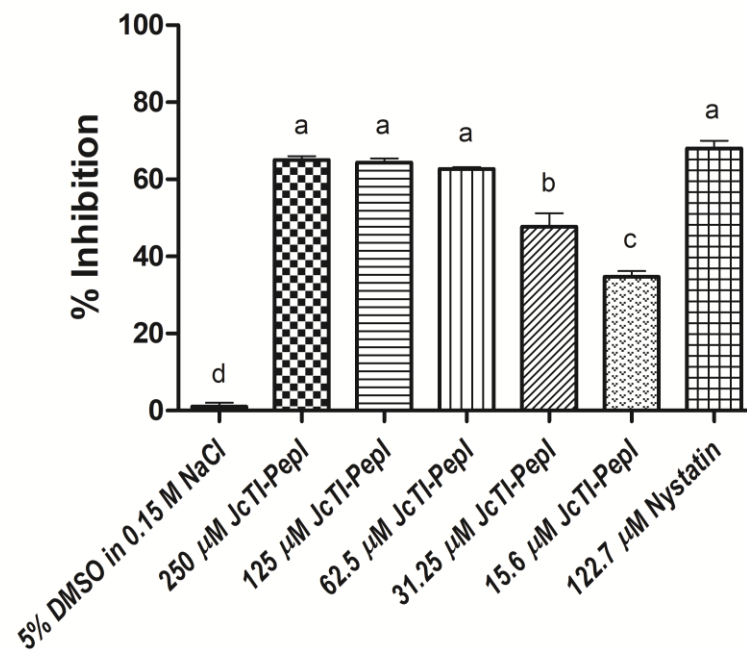


Fig. 8. Antibiofilm activity of *JcTI-PepI*. *JcTI-PepI*, at doses higher than 62.5 μM , degraded more than 60% of the preformed *C. krusei* biofilm, similar to what was found using Nystatin (68%). The biofilm was stained with 0.1% crystal violet followed by washing with 0.15 M NaCl and 95% (v/v) ethanol. The absorbances were measured at 570 nm using an automated microplate reader (Epoch, BioTek Instruments Inc., USA). The experiment was performed in triplicate, and error bars represent standard deviation. Different letters indicate significant differences between treatments. The results were analyzed using one-way ANOVA followed by Tukey's test of multiple comparisons ($p < 0.05$).

6.3.4 Mode of action of *JcTI-PepI* on *C. krusei*

6.3.4.1 Effect of *JcTI-PepI* on ROS production, membrane integrity, and cell morphology

DCFH-DA is a cell-permeant dye, which can be oxidized by ROS and turned into a fluorescent molecule 2,7-dichlorofluorescein. Our results demonstrate that *JcTI-PepI* induced overproduction of ROS in *C. krusei* cells as indicated by the observation of green fluorescence (Fig. 3C and D). Furthermore, *C. krusei* cells treated with 15.62 μM *JcTI-PepI* internalized propidium iodide, which is indicative of membrane disruption (Fig. 3I and J). Such effects were not observed in control cells treated with 5% DMSO in 0.15 M NaCl. In addition, the treated cells incorporated calcein after the incubation with *JcTI-PepI* (Fig. 4C and D), but they did not internalize FITC-Dextran of 10 kDa (Fig. 4E and F) and 20 kDa (Fig. 4G and H). These results indicated that *JcTI-PepI* formed pores of less than 10 kDa in *C. krusei* membrane.

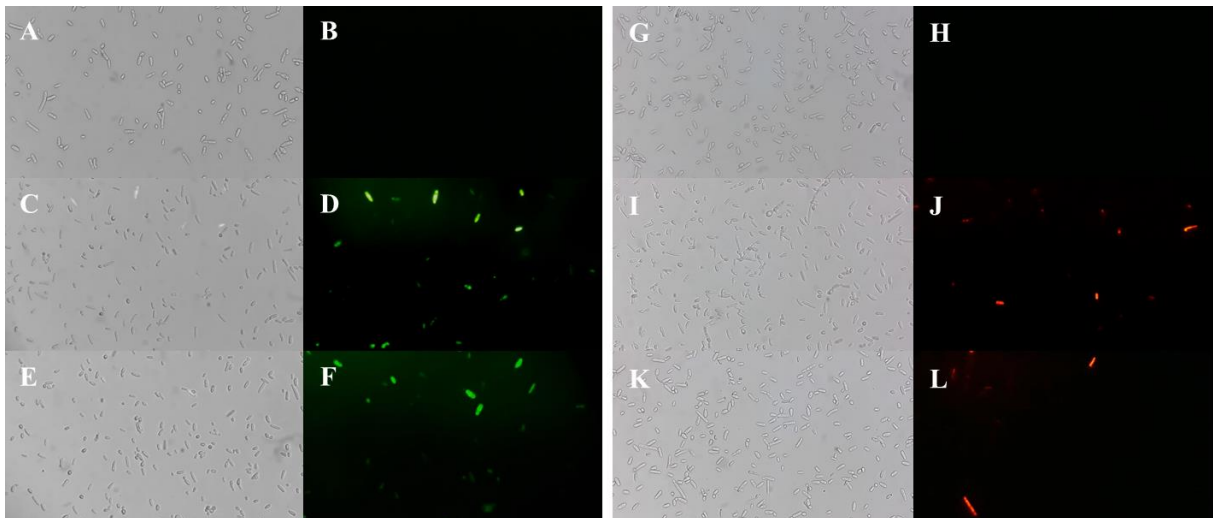


Fig. 9. Effects of *JcTI-PepI* treatment on ROS production (A–F) and membrane integrity (G–L) of *C. krusei* cells. Cells were treated with 5% DMSO in 0.15 M NaCl (A and B), 15.62 μM *JcTI-PepI* (C–D), or 8.6 μM amphotericin B (E–F), followed by incubation with DCFH-DA for 30 min at 37 °C. Next, these cells were visualized under a fluorescence microscope (Olympus System BX 60; excitation wavelength, 488 nm; emission wavelength, 525 nm). In (G) and (H), cells were treated with 5% DMSO in 0.15 M NaCl; in (I) and (J) with 15.62 μM *JcTI-PepI*; and in (K) and (L) with 8.6 μM amphotericin B, followed by incubation with propidium iodide (PI) for 30 min at 37 °C. Next, these cells were washed with 0.15 M NaCl and visualized under a fluorescence microscope (Olympus System BX 60; excitation wavelength, 488 nm; emission wavelength, 525 nm). The scale bars represent 10 μm . ROS overproduction can be seen as a green fluorescence, and membrane permeabilization can be observed as a red fluorescence.

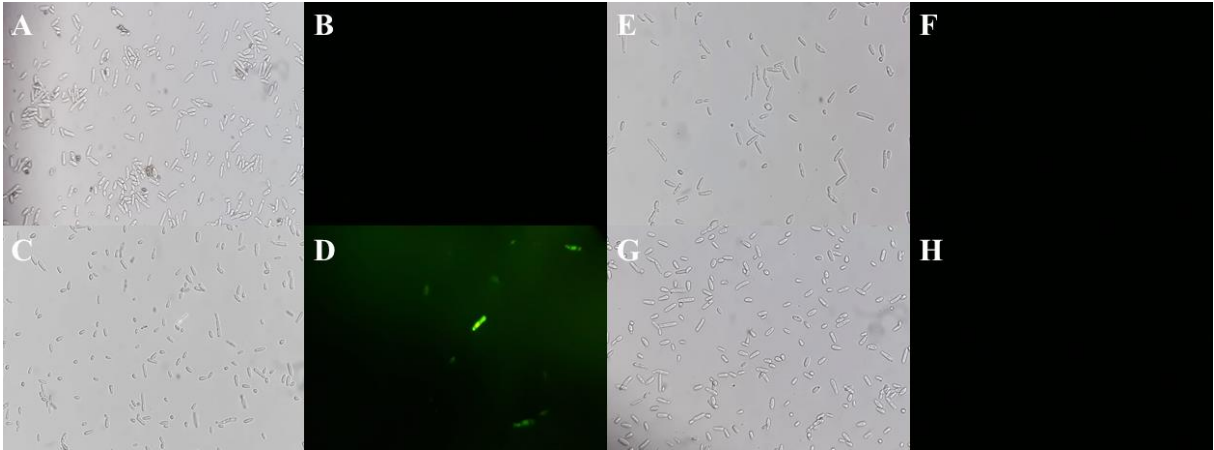


Fig. 10. Micrographs of *C. krusei* cells showing the dimensions of the pores formed. In (A) and (B) cells were treated with 5% DMSO in 0.15 M NaCl, followed by incubation with calcein (0.994 kDa) and fluorescein isothiocyanate-conjugated dextrans (FD10 and FD20, molecular masses of 10 kDa and 20 kDa respectively). In addition, cells were treated with 15.62 μ M *JcTI-PepI* followed by incubation with calcein (C and D), FD10 (E and F), and FD20 (G and H). After incubation (30 min at 37 °C), the cells were visualized under a fluorescence microscope (Olympus System BX 60; excitation wavelength, 490 nm; emission wavelength, 520 nm). The scale bars represent 10 μ m.

A scanning electron microscope was used to examine and compare the morphology of treated and untreated *C. krusei* cells. The *C. krusei* cells treated with 5% DMSO in 0.15 M NaCl presented a smooth surface and no changes in cell size or format (Fig. 5A and D). Besides, there were no pores or ruptures in the plasma membrane. On the other hand, treatment with 15.62 μ M *JcTI-PepI* caused several damages. The treated cells had uneven surfaces, loss of internal content, and large pores in the plasma membrane (Fig. 5B, C, E, and F). Also, the cells were more elongated (pseudo hypha growth) than those of the control.

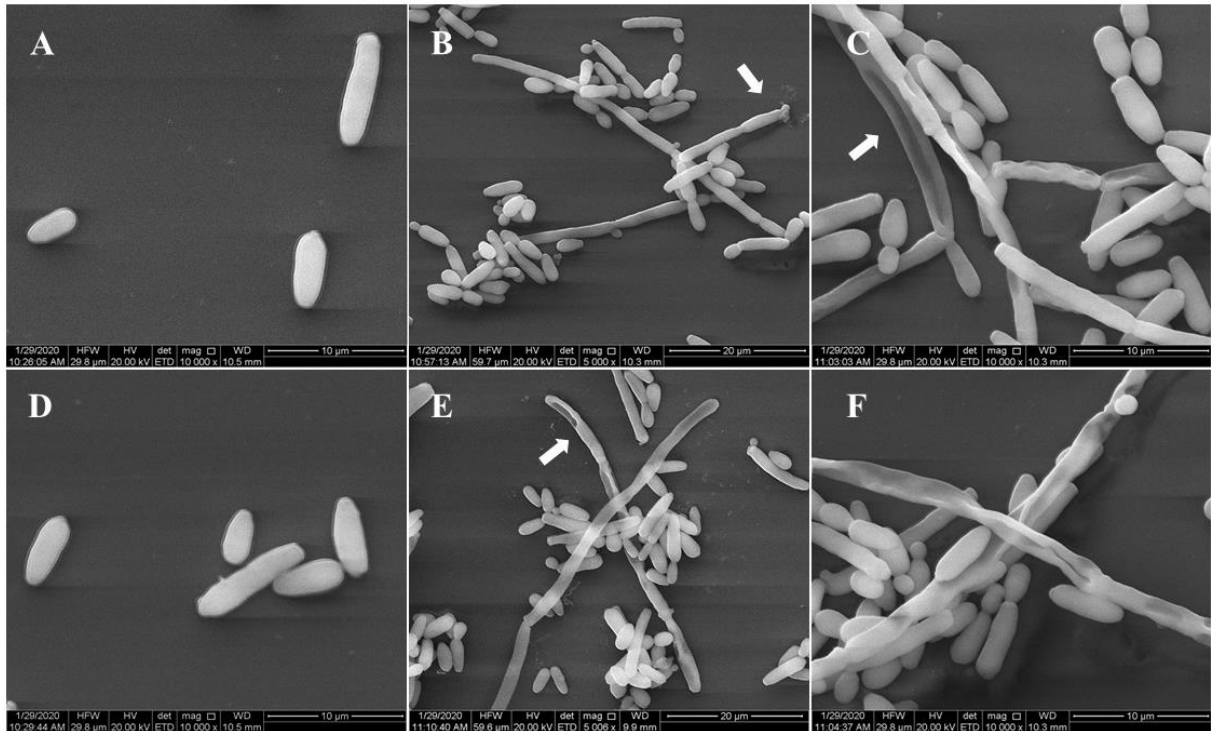


Fig. 11. Scanning Electron Microscopy (SEM) images of *C. krusei* cells. In (A) and (D) cells were treated with 5% DMSO in 0.15 M NaCl. In (B), (C), (E), and (F) cells were treated with 15.62 μM *JcTI-PepI*. The cells treated with the peptide had uneven surfaces, loss of internal content, and large pores in the plasma membrane, as indicated by the arrows.

6.3.4.2 Assessment of glucose-stimulated acidification of the extracellular medium

JcTI-PepI and Nystatin (positive control) at concentrations of 15.62 μM and 122.7 μM inhibited the acidification by 73% and 98%, respectively, when compared to the results for 5% DMSO in 0.15 M NaCl (negative control) (Fig. 6). Even at lower doses (7.81 and 3.9 μM), the treatment with *JcTI-PepI* reduced almost 60% of medium acidification compared to the negative control.

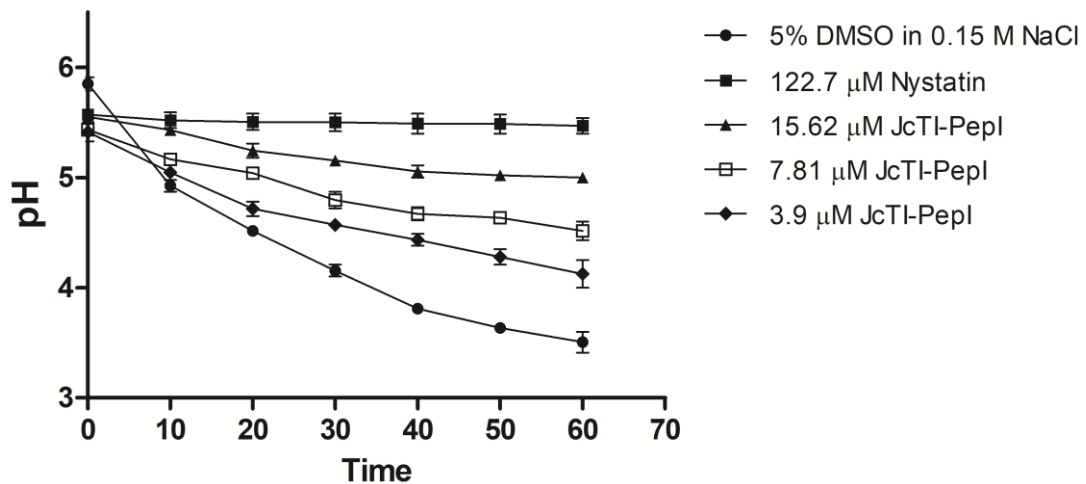


Fig. 12. Inhibition of glucose-stimulated acidification of the extracellular medium by *JcTI-PepI*. *C. krusei* cells were exposed to 5% DMSO in 0.15 M NaCl (negative control), 3.9–15.62 μM *JcTI-PepI*, or 122.7 μM Nystatin (positive control). After incubation, the medium acidification was monitored every 10 min, for 1 h, using a pHmeter. All the experiments were performed in triplicate, and error bars represent standard deviation. The %inhibition was calculated comparing the pH variation of the treatments with the pH variation of the negative control.

6.3.4.3 *In vitro* haemolysis, cytotoxic activity, and digestibility of *JcTI-PepI*

Even at concentrations four times higher than the MFC for *C. krusei*, *JcTI-PepI* had no adverse effect on human erythrocytes. Similarly, 0.15 NaCl (as a negative control) had no effect on human erythrocytes. On the other hand, 0.1% Triton X-100 caused 100% haemolysis (data not shown). Regarding the cytotoxic activity, the CC50 for *JcTI-PepI* was 704.28 μM , which is more than 20 \times the MIC value against *C. krusei* (Fig. 7). There was no significant reduction in Vero cell viability at 31.25 μM and 62.5 μM (MIC and MFC). The ion $[\text{M}+\text{H}]^+$ at 1003.55 was not detected in the MS spectrum after the treatment with either pepsin or pancreatin, indicating that *JcTI-PepI* was completely digested in both SGF (Fig. 8A–F) and SIF (Fig. 8G–L) at all the evaluated times (2 s, 5 min, 30 min, and 1 h).

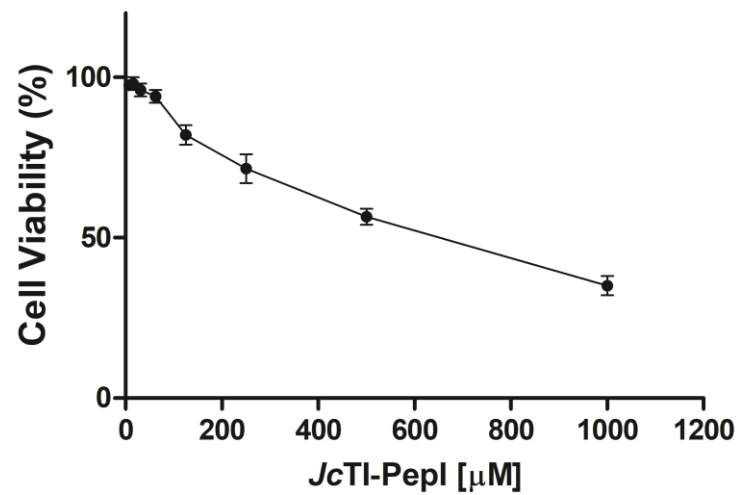


Fig. 13. The cytotoxic activity of *JcTI-PepI* at different concentrations [7.81–1000 μM] was assessed against a Vero cell suspension. All the experiments were performed in triplicate, and error bars represent standard deviation. The CC50, defined as the peptide concentration required for reducing the cell viability by 50%, was calculated by linear regression analysis of concentration–response curve generated by the data.

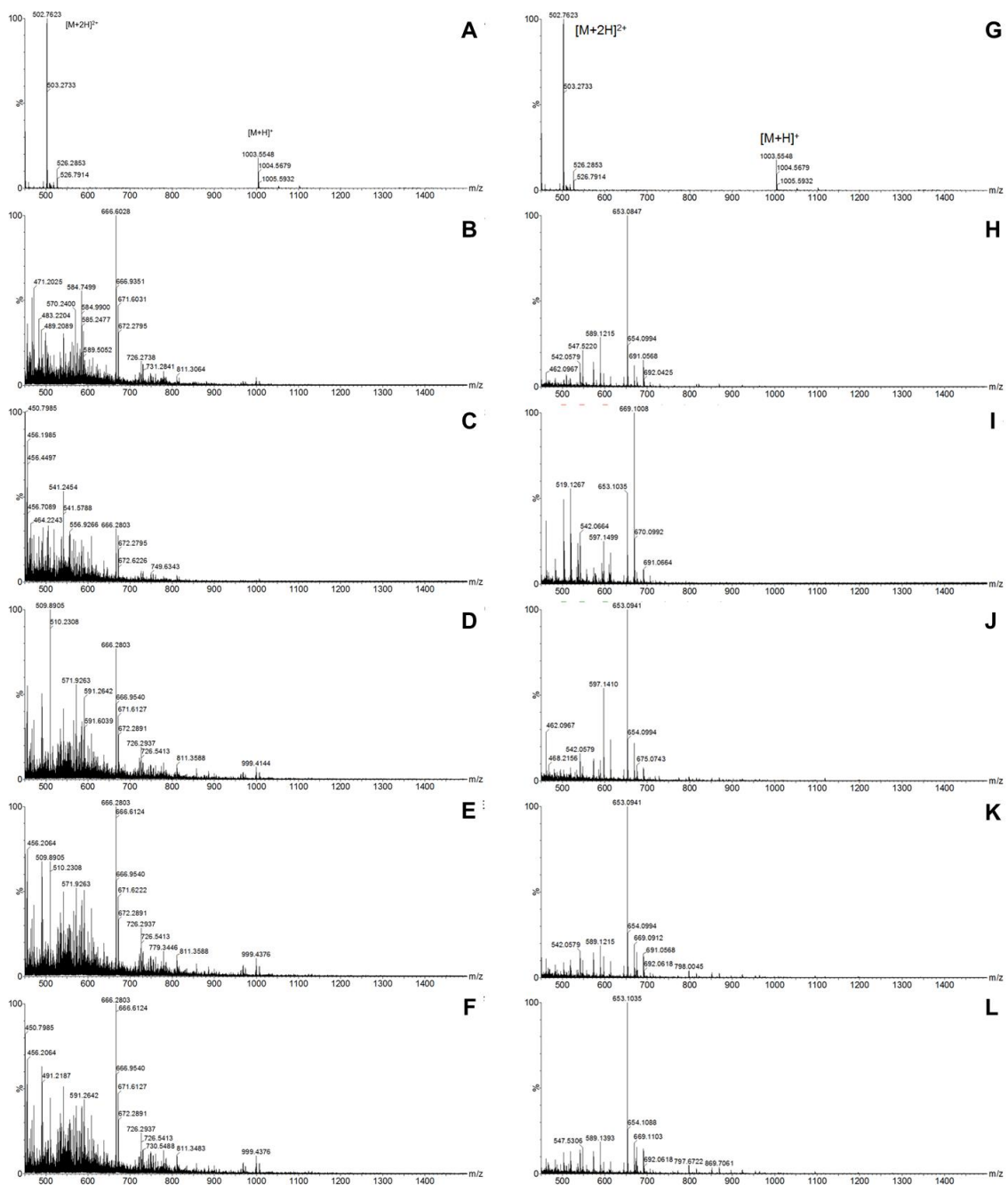


Fig. 8. Mass spectra of *Jc*TI-PepI digested with SIF and SGF. (A) Intact *Jc*TI-PepI, (B) SGF, (C), (D), (E), and (F) correspond to *Jc*TI-PepI incubated with SGF for 2 s, 5 min, 30 min, and 1 h, respectively. (G) Intact *Jc*TI-PepI, (H) SIF, (I), (J), (K), and (L) correspond to *Jc*TI-PepI incubated with SIF for 2 s, 5 min, 30 min, and 1 h, respectively.

6.4 Discussion

The list of the etiological agents of candidiasis has increased in recent years as new species have been added. However, most cases are caused by only five species: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* [37]. Although *C. albicans* is the most common cause of candidemia, infections caused by *C. krusei* are on the rise and pose a growing public health risk worldwide. *C. krusei* produces cylindrical cells, and the infections caused by these species have a high mortality rate (40–58%) and poor response to standard antifungal therapies [38].

The Infectious Disease Society of America published updated guidelines for the treatment of candidiasis in 2016. The choice of the antifungal agent is based on the area affected by the infection, the immune status of the patients, the species of *Candida*, and the susceptibility of the *Candida* species [39]. Currently, the five major classes of antifungal agents used to treat infections caused by *Candida* sp. are azoles, polyenes, echinocandins, allylamines, and nucleoside analogs [40]. Fluconazole is one of the most commonly used drugs for the treatment of candidiasis. However, more than 95% of clinical and veterinary *C. krusei* isolates are fluconazole-resistant, indicating that this species is resistant by nature [41,42]. Moreover, some strains are also resistant to itraconazole and voriconazole, representing a severe public health threat [41,43]. Therefore, finding new molecules with inhibitory activity towards *C. krusei* is imperative.

Antimicrobial peptides (AMPs) stood out as potential candidates for use in the age of resistance in this context. AMPs are a class of bioactive small proteins that play a role in the immune system's defense against a variety of pathogens. They can disrupt cell membranes, modulate the immune response, and regulate inflammation [44]. Several AMPs have shown broad-spectrum activity against Gram-positive and Gram-negative bacterial strains, and they have also been used in the treatment of Candidiasis [45,46]. Most natural antimicrobial peptides have long sequences, which can make commercialization difficult due to the high cost of purification [47]. In addition, these natural AMPs are generally sensitive to protease digestion and might present cytotoxicity towards mammalian cells [15,48,49]. As a result, research has focused on the use of synthetic AMPs, a chemical class of promising new drugs with biomimetic mechanisms of action that are easily obtained and inexpensive [47]. These benefits explain why synthetic antimicrobial peptides are gaining interest as potential therapeutic agents [[50], [51], [52]].

In the present study, several peptides were designed based on the sequence of *JcTI-I*, a trypsin inhibitor purified from *Jatropha curcas*. Among the designed peptides, three (*JcTI-PepI*, *JcTI-PepII*, and *JcTI-PepIII*) were chemically synthesized as they presented the best *in silico* features, such as great stability, resistance to proteolysis, and high antimicrobial potential (Table 1). These peptides were tested against *Candida* sp., but only *JcTI-PepI* presented activity against all the tested strains. However, the inhibitory action was higher towards *C. krusei* (Table 2). Therefore, *JcTI-PepI* was chosen for further characterization and investigation of its antimicrobial activity and mode of action against *C. krusei*. The three-dimensional model of *JcTI-PepI* (Fig. 1A and D) revealed that this peptide is formed by an α -helix type structure, and it has a positive charge. Positively charged antifungal peptides may show strong interaction with negatively charged fungal membranes due to the higher phosphatidylinositol and phosphatidic acid content [53]. However, positive charges are not the only determining factor for the fungicidal activity of peptides. Furthermore, the amino acid composition of the peptide and the effects of its side chain in the interaction with the fungal membrane must be also considered [54]. These characteristics could explain the non-fungicidal effect for the *JcTI-PepII* and *JcTI-PepIII* peptides even though they present a positive charge at pH 7.0.

Regarding the antifungal activity, *JcTI-PepI* showed both fungistatic (31.25 μ M) and fungicide (62.5 μ M) effects against *C. krusei* (Table 2). A similar result was found for protonectin, a synthetic AMP, with a MIC value of 32 μ M against *C. krusei* and an MFC 2 \times or 4 \times the MIC [55]. The antimicrobial polybia-CP, isolated from the venom of the social wasp *Polybia paulista*, also presented a MIC value of 32 μ M against *C. krusei*. The MFC value was also 32 μ M, which was lower than what was observed using *JcTI-PepI* [56].

JcTI-PepI was active against planktonic yeast cells, but it did not prevent the formation of *C. krusei* biofilm. On the other hand, the preformed biofilm of *C. krusei* was degraded in 62% by 62.5 μ M *JcTI-PepI* (Fig. 2). The concentration of *JcTI-PepI* was even lower than the concentration of nystatin, which caused a comparable reduction (122.7 μ M). In a study with the antimicrobial peptide VLL-28, isolated from an archaeal transcription factor, Roscetto et al. obtained similar results [57]. VLL-28, as *JcTI-PepI*, did not prevent the formation of *C. krusei* biofilm. The preformed biofilm, however, was degraded in 62.5% at a concentration of 50 μ M, which was similar to our results with *JcTI-PepI*. The synthetic AMP protonectin also degraded the pre-formed biofilm of *C. krusei*, but this inhibition was only observed at higher doses of the peptide (128 μ M) [55]. Microbial biofilms can be defined as

complex structured communities immersed in an extracellular matrix (ECM) that is attached to a surface [58]. *Candida* is known to form biofilms, and these communities of packed fungi can complicate treatment and lead to persistent fungemia [59,60]. According to the National Institutes of Health (NIH), more than 80% of all human infections caused by microorganisms are associated with biofilms [61]. Infections caused by *Candida* biofilm have mortality rates higher than 40% [62]. Biofilms protect fungal cells while frequently increasing drug resistance [63]. As a result, the importance of discovering new compounds that disrupt biofilms, such as *JcTI-I*, is clear, making this peptide a promising candidate to treat biofilm-associated infections or invasive infections caused by intrinsically resistant *C. krusei*.

As the antimicrobial results were promising, *JcTI-PepI* was characterized for its mode of action against *C. krusei*. *JcTI-PepI* induced ROS overproduction in *C. krusei* cells (Fig. 3C and D). ROS play a relevant role in the cellular environment and, under stress, the cellular redox balance is generally disturbed. Therefore, ROS production increases intensely and causes damages to cellular proteins, lipids, and DNA [56]. Our results indicate that the treatment with *JcTI-PepI* increased ROS production, and the excessive amounts of ROS might have interfered with cellular events, which led to yeast death. Other studies have also demonstrated that endogenous ROS is a critical mediator in antifungal effects [31,55,64].

The treatment with *JcTI-PepI* also disrupted *C. krusei* membrane as observed by the uptake of propidium iodide (PI) (Fig. 3I and J). Indeed, the pores formed by *JcTI-PepI* allowed the passage of calcein, a 0.994 kDa compound (Fig. 4C and D). PI is a fluorescent molecule that binds to DNA with little or no sequence preference [56]. Once the dye binds to nucleic acid, its fluorescence is enhanced and can be observed under a fluorescent microscope. PI is membrane impermeable and generally excluded from viable cells, so it is commonly used as a marker for dead cells. Other studies reported that the treatment with antimicrobial peptides also caused membrane permeabilization [55,[64], [65], [66]]. Besides inducing ROS overproduction and membrane permeabilization, *JcTI-PepI* also caused several morphological alterations in *C. krusei* cells. SEM images revealed that cells treated only with 5% DMSO in 0.15 M NaCl presented a regular shape, with no deformities and a smooth surface (Fig. 5A and D). On the other hand, cells treated with *JcTI-PepI* had uneven surfaces, loss of internal content, and large pores in the plasma membrane (Fig. 5B, C, E, and F), which corroborates with the uptake of PI. Also, there were observed elongated cells at the early stage of pseudohyphal formation (Fig. 5B, C, E, and F). Morphological changes in *Candida* cells after treatment with AMPs have also been previously reported, for example, when studying

the effects of ToAP2 and NDBP-5.7 against *C. albicans* [67], cationic peptides against *C. albicans* [68], and RcAlb-PepII against *C. parapsilosis* [31].

The medium acidification mediated by glucose was also strongly inhibited (73%) by JcTI-PepI (Fig. 6). This peptide possibly inhibited the plasma membrane (PM) H⁺-ATPase activity. Fungal PM-H⁺-ATPase is a transmembrane enzyme that plays an important role in cell metabolism, including the maintenance of a transmembrane electrochemical proton gradient, which is very important for the uptake of nutrients [30]. This ATPase has become a target for the action of antifungal agents [30,[69], [70], [71]].

It is clear that this peptide causes numerous alterations in the treated cells, which results in their death. A potential antimicrobial drug must have potent inhibitory activity, but that is not the only requirement. In order to foresee a future application for JcTI-PepI, its effects on mammalian cells were also investigated. *In silico* analyses predicted that JcTI-PepI did not have a toxin or allergic potential (Table 1). Our *in vitro* results showed that JcTI-PepI did not cause haemolysis of human blood cells and was not cytotoxic to Vero cell lines at MIC and MFC values (Fig. 7). *In silico* predictions were also used to evaluate the resistance to proteolysis, and the results indicated high stability (Table 1). According to *in silico* analyses, JcTI-PepI has four trypsin cleavage sites, which is confirmed by the complete digestion in SIF (Fig. 8A–F), which contains a mixture of several proteases, including trypsin. On the other hand, no pepsin cleavage site was predicted, but the peptide was completely digested in SGF (Fig. 8G–L). Similarly, RcAlb-PepII, a synthetic peptide designed based on a protein from *Ricinus communis*, was also completely digested in SIF after 2 s [31].

Altogether, these results indicate that JcTI-PepI presents a potent activity against *C. krusei*, a generally neglected pathogen.

6.5 Conclusions

JcTI-PepI had a strong inhibitory effect on *C. krusei* cells, disrupting structures and cell processes. Furthermore, the synthetic peptide was non-toxic to mammalian cells. These characteristics suggest that JcTI-PepI has the potential to be used as an antimicrobial agent in the future, assisting in the treatment of infections caused by *C. krusei*, a global public health threat.

Author's contributions

LAL, LPD, JTAO, and DOBS participated in the conception and design of the study. LAL, LPS, NMSA executed most of the experimental procedures and acquisition of data. RGGS obtained the fluorescence images. CST, RFC, and CSN obtained and analyzed the structural data and the *in vitro* digestibility. LAL, LPD, and DOBS interpreted the data. LAL, LPD, and DOBS contributed with the manuscript preparation. LPD and DOBS revised the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare no competing financial interest.

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7 CONSIDERAÇÕES FINAIS

Os resultados apresentados neste trabalho confirmaram o potencial antimicrobiano de peptídeos sintéticos. Assim como em outros estudos já reportados na literatura, peptídeos bioinspirados na sequência de uma proteína antimicrobiana, *JcTI-PepI*, se mostraram ativos contra diferentes espécies de microrganismos de interesse clínico.

Os três peptídeos sintetizados com base no inibidor de tripsina de *Jatropha curcas* foram ativos contra quatro espécies de *Candida*. Porém, a melhor atividade foi observada para *JcTI-PepI*, com os menores valores de MIC observados. O potencial desse peptídeo foi ainda melhor quando utilizado contra *C. krusei*, uma espécie que gera preocupação na comunidade médica e científica por sua elevada resistência aos medicamentos disponíveis atualmente. Além disso, é importante ressaltar que, em comparação a outras espécies de *Candida*, ainda existem poucos estudos utilizando *C. krusei* como modelo.

JcTI-PepI foi capaz de inibir tanto as células planctônicas como também foi ativo contra o biofilme de *C. krusei*, exibindo um efeito fungicida sobre essa levedura. Os mecanismos por trás dessa atividade envolvem a permeabilização da membrana plasmática, a superprodução de EROs, o comprometimento da acidificação do meio e alterações morfológicas extremamente deletérias. Esses achados revelam que esse peptídeo apresenta um potencial a ser considerado e explorado em aplicações futuras como molécula antifúngica.

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APÊNDICE A – ARTIGO EM COLABORAÇÃO

Revista científica

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Role of membrane sterol and redox system in the anti-candida activity reported for *Mo*-CBP₂, a protein from *Moringa oleifera* seeds

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Role of membrane sterol and redox system in the anti-candida activity reported for Mo-CBP₂, a protein from *Moringa oleifera* seeds



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ABSTRACT

Plant proteins are emerging as an alternative to conventional treatments against candidiasis. The aim of this study was to better understand the mechanism of action of Mo-CBP₂ against *Candida* spp, evaluating redox system activity, lipid peroxidation, DNA degradation, cytochrome c release, medium acidification, and membrane interaction. Anti-candida activity of Mo-CBP₂ decreased in the presence of ergosterol, which was not observed with antioxidant agents. *C. albicans* treated with Mo-CBP₂ also had catalase and peroxidase activities inhibited, while superoxide dismutase was increased. Mo-CBP₂ increased the lipid peroxidation, but it did not alter the ergosterol profile in live cells. Estemal medium acidification was strongly inhibited, and cytochrome c release and DNA degradation were detected. Mo-CBP₂ interacts with cell membrane constituents, changes redox system enzymes in *C. albicans* and causes lipid peroxidation by ROS overproduction. DNA degradation and cytochrome c release suggest apoptotic or DNase activity. Lipid peroxidation and H⁺-ATPases inhibition may induce the process of apoptosis. Finally, Mo-CBP₂ did not have a cytotoxic effect in mammalian Vero cells. This study highlights the biotechnological potential of Mo-CBP₂ as a promising molecule with low toxicity and potent activity. Further studies should be performed to better understand its mode of action and toxicity.

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1. Introduction

Candida genus yeasts are members of a fungal group commonly found in normal human microbiota, colonizing the skin, oral cavity, esophagus, gastrointestinal tract and vagina. Nevertheless, *Candida* spp. such as *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* can cause serious infections, mainly in hospitalized patients [1–3]. Invasive *Candida* infections cause a significant impact on morbidity and overall mortality, especially among immunocompromised patients [4,5], with *C. albicans* being the most common pathogen in clinical cases. The indiscriminate use of antibiotics, diabetes mellitus type 1 and 2, chemotherapy, probe implantation in surgery, organ transplant, and hemodialysis are risks factors for the development of these infections [6–9].

The impact of fungi on human health is associated with the limited availability of antifungal drugs. Basically, only three classes of antifungals

(azoles, polyenes, and echinocandins) are used in the treatment of *Candida* infection. In addition, antifungal drugs commonly used can lead to the development of resistant yeast strains [10–12]. The main drug resistance mechanisms are: (1) reduction of intracellular drug content; (2) drug target alteration and overexpression; and (3) metabolic bypasses [10,13,14]. In addition, it is reported that many of these compounds exhibit various toxic effects, such as nephrotoxicity [15], teratogenicity, and cardiotoxicity [16,17]. Based on the reduced number and toxicity of current drugs, it is important to find new antifungal agents as alternatives to current treatments [18,19]. In the search for new alternatives, plants are a large reservoir of biological compounds against *Candida* spp.

Various protein molecules of plant origin with inhibitory effect against *Candida* spp. have been isolated, such as lectin [20], lipid transfer protein [21], trypsin inhibitor [22], and peptides [23,24]. Another protein class isolated from plants with anti-candida activity is the chitin-binding proteins (CBPs). These molecules reversibly bind with chitin, an important structural polysaccharide present in many organisms, including the fungal cell wall. However, this polysaccharide does not

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APÊNDICE B – ARTIGO EM COLABORAÇÃO

Revista científica

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Insights into the structure and mechanism of action of the anti-candidal lectin *Mo*-CBP₂ and evaluation of its synergistic effect and antibiofilm activity

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Insights into the structure and mechanism of action of the anti-candidal lectin Mo-CBP₂ and evaluation of its synergistic effect and antibiofilm activity

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ABSTRACT

Plant bioactive compounds are extensively used in traditional medicine to treat diabetes, inflammation, hypertension, liver disease, and microbial infections. In this context, plant proteins are promising molecules in the treatment of candidiasis, being an alternative to the classical antifungals currently in use. This study aimed to better understand the structural characteristics of Mo-CBP₂, a chitin-binding protein isolated from *Moringa oleifera* seeds. Its mode of action against *Candida* spp. was investigated, evaluating protein oligomerization, pore formation, secondary structure, cell wall interaction, and biofilm inhibition. Mo-CBP₂ has a trimeric and hexameric structure, which may explain its aggregation effect against *Candida albicans* cells. Both yeast aggregation and anti-candida activity were depleted in the presence of N,N,N'-acrylychitosane and laminarin, indicating that Mo-CBP₂ interacts with the fungal cell wall. Mo-CBP₂ showed high stability at extreme temperatures (100 °C) and pH (2, 4 and 10). Furthermore, treatment with Mo-CBP₂ caused pores, severe morphological damage, and the release of cytoplasmic material in *Candida*. Mo-CBP₂ also showed a synergistic effect with azole, polyene, and echinocandin antifungals. Finally, Mo-CBP₂ strongly inhibited biomass production in both mature and non-mature biofilms of *C. albicans*. This study highlights the biotechnological potential of Mo-CBP₂ as a promising anti-candida molecule against planktonic and sessile *C. albicans* cells.

1. Introduction

The *Candida* genus includes members such as *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. guilliermondii*, and *C. lusitanae*. These species are part of the natural human microbiota that colonizes the gastrointestinal tract, oral cavity, esophagus, skin, and vagina. This

yeast genus, however, can cause serious infections, particularly in hospitalized patients [1,2]. Invasive fungal infections caused by *Candida* spp. have a significant impact on morbidity and overall mortality rates, with *C. albicans* being identified as the major cause of infections [3,4]. Several factors, including indiscriminate use of antibiotics, mechanical ventilation, use of catheters, diabetes, and chemotherapy, increase the

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