UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

WILLIAM GUSTAVO DE LIMA

PEPTÍDEOS ANTIMICROBIANOS DERIVADOS DA TOXINA DE ARTRÓPODES COMO POTENCIAIS FÁRMACOS CONTRA BACTÉRIAS MULTIRRESISTENTES: Eficácia *in vitro* e *in vivo*, toxicidade e formulação farmacêutica

Belo Horizonte

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Orientadora: Profa. Simone Odília Antunes Fernandes

Coorientador: Prof. Valbert Nascimento Cardoso

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FOLHA DE APROVAÇÃO

"Peptídeos antimicrobianos derivados da toxina de artrópodes como potenciais fármacos contra bactérias multirresistentes: eficácia in vitro e in vivo, toxicidade e formulação farmacêutica"

WILLIAM GUSTAVO DE LIMA

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“A resistência antimicrobiana é uma das maiores ameaças que enfrentamos como comunidade global. Uma resposta rápida a essa emergência em saúde pública é essencial para conter sua ascensão e proteger um século de progresso em saúde. Isso enfatiza, com razão, que não há tempo para esperar e eu peço a todas as partes interessadas que ajam de acordo com suas recomendações e trabalhem urgentemente para proteger nossa população e planeta, assegurando um futuro sustentável para todos”

Amina Mohammed, secretária-geral adjunta da ONU

“Parece que eu era apenas um garoto brincando à beira-mar e me desviando de vez em quando para encontrar uma pedra mais lisa ou uma concha mais bonita do que o normal, enquanto o grande oceano da verdade estava por descobrir antes de mim. Este mundo é grandioso e existe um oceano de descobertas desconhecidas que aguardam mentes ansiosas e curiosas”

Isaac Newton

RESUMO

O surgimento de bactérias resistentes a antibióticos juntamente com a relativa estagnação no desenvolvimento de antibióticos eficientes, levou a enormes problemas econômicos e de saúde pública, exigindo a necessidade de desenvolver novos agentes antimicrobianos. Nessa direção, o veneno de artrópodes representa um rico repertório de componentes farmacologicamente ativos que podem ser explorados no desenvolvimento de novos antibióticos. Como um componente majoritário no veneno da abelha melífera (*Apis mellifera*), a melitina tem um grande potencial em aplicações médicas, sobretudo como agente antibacteriano. Além disso, derivados da toxina da aranha-de-jardim (*Lycosa erythrognatha*), como o peptídeo LyeTx I mnΔK, tem se revelado altamente ativo contra diversas bactérias. Nesse estudo, nós objetivamos realizar uma caracterização profunda do efeito anti-estafilocóccico e anti-*Escherichia coli* da melitina usando isolados clínicos multirresistentes desses patógenos. Em adição, nós objetivamos também descrever o perfil antibacteriano do peptídeo LyeTx I mnΔK contra *Acinetobacter baumannii* resistente aos carbapenêmicos (CRAB). A melitina revelou um potente efeito antibacteriano contra *Staphylococcus* spp. (CIM 0.12-4 μM) e *Escherichia coli* uropatogênica (UPEC) (CIM 0,5-8 μM). O efeito contra *S. aureus* foi bactericida (CBM 0,12-16 μM) e rápido (0,5 h), sendo que a melitina foi hábil em atuar contra células planctônicas, estacionárias e persistentes desse patógeno. Similarmente, a melitina exibiu a capacidade de eliminar células de UPEC resistentes as quinolonas e produtoras de beta-lactamase de espectro ampliado (ESBL) *in vitro* (CBM 1-32 μM). Na faixa de concentração testada (8-32 μM) a melitina reduz significativamente o biofilme maduro de *S. aureus* resistente a meticilina (MRSA), e ela foi hábil em lisar as células desse patógeno. A combinação da melitina com antibióticos convencionais resultou em efeito sinérgico, e esse peptídeo re-sensibilizou o MRSA para ação da amoxicilina, ceftzoxima e vancomicina. Para UPECs resistentes as quinolonas e produtoras de ESBL os resultados foram similares, com a melitina inibindo a formação de biofilmes em sondas uretrais e re-sensibilizando a ação de beta-lactâmicos contra essa bactéria. O tratamento de feridas induzidas por MRSA com uma pomada contendo melitina resultou em uma expressiva redução da carga bacteriana na ferida e redução significativa de citocinas pró-inflamatórias (TNF-α, IL-6, IL-1β). Por sua vez, o peptídeo LyeTx I mnΔK apresentou uma considerável atividade antibacteriana contra isolados clínicos extensivamente resistentes de *A. baumannii,* apresentando CIM e CBM nas faixas de 1-16 μM e 2-32 μM, respectivamente. De maneira similar a melitina, o LyeTx I mnΔK interage sinergicamente e re-sensibiliza células de CRAB aos efeitos de antibióticos comerciais, além de romper biofilmes maduros formados por essa bactéria gram-negativa. A concentração citotóxica do LyeTx I mnΔK (CC_{50} = 9.40±2.84 μM) contra células Vero foi similar ao da colistina (CC_{50}=15.42±2.73 μM), e sua atividade hemolítica foi consideravelmente baixa (HC_{50}=77.07±4.00 μM). Estudos *in vitro* deram conta que sua estabilidade plasmática e oral é muito baixa. Entretanto, o uso desse peptídeo por via inalatória mostrou-se efetivo para reduzir significativamente a carga bacteriana pulmonar em um modelo de infecção por CRAB. Feito juntos, os resultados desse estudo destacam para a melitina e o peptídeo LyeTx I mnΔK como potenciais protótipos no desenvolvimento de novos agentes antibacterianos contra os patógenos multirresistentes de relevância clínica.

Palavras-chave: Peptídeos antimicrobianos; *Staphylococcus aureus* resistente a meticilina (MRSA); *Acinetobacter baumannnii* resistente aos carbapenêmicos (CRAB); *Escherichia coli* resistente as quinolonas; Infecções urinárias; Feridas; Pneumonia

ABSTRACT

The emergence of antibiotic-resistant bacteria, dubbed superbugs, together with relative stagnation in developing efficient antibiotics has led to enormous health and economic problems, necessitating the need for discovering and developing novel antimicrobial agents. In this respect, animal venoms represent a rich repertoire of pharmacologically active components that can be exploited in the development of new antibiotics. Melittin is the major component in bee venom (*Apis mellifera*) and has great potential in medical applications, especially as an antibacterial agent. In addition, derivatives of the toxin of the *Aranha-de-Jardim* (Lycosa erythrognatha), such as the peptide LyeTx mnΔK, are highly active against multidrug-resistant Gram-negative bacteria. In this study, we aimed to carry out an in-depth characterization of the anti-staphylococcal and anti-*Escherichia coli* effect of melittin using multidrug-resistant clinical isolates of these pathogens. We also aim to describe the antibacterial spectrum of LyeTx I mnΔK against carbapenem-resistant *Acinetobacter baumannii* (CRAB) and to prospect the use of this compound in pneumonia caused by this pathogen. Melittin revealed a potent antibacterial effect against *Staphylococcus* spp. (MIC 0.12-4 μM) and uropathogenic *Escherichia coli* (UPEC) (MIC 0.5-8 μM). Furthemore, it showed a fast (0.5h) bactericidal activity, being active against planktonic, stationary, and persistent cells. Similarly, melittin exhibited the ability to kill quinolone-resistant and extended-spectrum beta-lactamase (ESBL) producing UPEC cells *in vitro* (CBM 1-32 μM). In the tested concentration range (8-32 μM), melittin significantly reduces the mature MRSA biofilm, and it was able to lyse the cells of this pathogen. The combination of melittin with conventional antibiotics produced a synergistic effect, and this peptide re-sensitize MRSA to the action of amoxicillin, ceftizoxime, and vancomycin. For quinolone-resistant and ESBL-producing UPECs, the results were similar, with melittin inhibiting the formation of biofilms in urethral catheter and re-sensitizing the action of beta-lactams against this bacterium. The treatment of MRSA-induced wounds with an ointment containing melittin resulted in a significant reduction in the bacterial load and a considerable reduction of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β). LyeTx I mnΔK also showed considerable antibacterial activity against extensively resistant *A. baumannii*, with MIC and MBC ranging from 1 to 16 μM and 2 to 32 μM, respectively. Similar to melittin, LyeTx I mnΔK interacts synergistically and re-sensitize CRAB cells to the effects of commercial antibiotics, in addition to disrupting mature biofilms formed by this Gram-negative pathogen. The cytotoxic concentration of LyeTx I mnΔK (CC_{50} = 9.40±2.84 μM) against Vero cells was similar to that of colistin (CC_{50} = 15.42±2.73 μM), and its hemolytic activity was considerably low (HC_{50} = 77.07±4.00 μM). The oral and plasmatic stability of LyeTx I mnΔK is very low. Nevertheless, the inhalation of this peptide proved to be effective in the reduction of pulmonary bacterial load in a mouse model of CRAB infection. Together, the results of this study highlight that the melittin and the peptide LyeTx I mnΔK are potential prototypes in the development of new antibacterial agents against multidrug-resistant pathogens of clinical relevance.

Keywords: Antimicrobial peptides; Methicillin-resistant *Staphylococcus aureus* (MRSA); Carbapenem-resistant *Acinetobacter baumannnii* (CRAB); Quinolone-resistant *Escherichia coli*; Urinary infections; Wounds; Pneumonia

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LISTA DE ABREVIATURAS, SIMBOLOS E UNIDADES

%	Porcentagem
% (v/v)	*Concentration (volume per volume)*
% (w/v)	*Concentration (weight per volume)*
µCi	*Microcurie*
µm	Micrômetro
^{99m}Tc	Tecnécio-99 metaestável
AATF	*Antimicrobial Availability Task Force*
ABSSSIs	*Acute bacterial skin and skin structure infections*
AMP	*Antimicrobial peptides*
APD	*Antimicrobial Peptides Database*
ATCC	*American Type Culture Collection*
BFS	*Bovine fetal serum*
BSA	*Bovine serum albumine*
$CaCl_2$	Cloreto de cálcio
CBM	Concentração Bactericida Mínima
CC_{50}	*Cytotoxic concentrations for 50% of the cells*
CDC	Centro de Controle e Prevenção de Doenças
CEUA	Comitê de ética em uso de animais
CFT	*Ceftizoxime*
CFU	*Colony forming unit*
CFU/g	*Colony forming unit per gram*
CFU/mL	*Colony forming unit per milliliter*
CIM	Concentração Inibitória Mínima
CLSI	*Clinical and Laboratory Standards Institute*
CO_2	Dióxido de carbono
COVID-19	Doença do coronavírus de 2019
CRAB	*Acinetobacter baumannii* resistente aos carbapenêmicos
Da	*Daltons*
DMEM	*Dulbecco's Modified Eagle Medium*
ECDC	Centro Europeu de Controle de Doenças
ELISA	*Enzyme-Linked Immunosorbent Assay*
ESBL	Beta-lactamase de espectro ampliado
ESKAPE	***E**nterococcus faecium*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, **P***seudomonas aeruginosa*, **E**nterobacteriaceae
EUA	Estados Unidos da América
FIC	*Fractional inhibitory concentration*
FICI	*Fractional inhibitory concentration index*
FIOCRUZ	Fundação Oswaldo Cruz
FDA	*Food and Drug Administration*
h	Hora
HIV/AIDS	Vírus da imunodeficiência humana/Síndrome da imunodeficiência adquirida
HPLC	*High performance liquid chromatography*
i.p.	Intraperitoneal

IASS	Infecções associadas ao sistema de saúde
IDSA	*Infection Diaseas Socity of American*
IL-1β	*Interleukin-1 beta*
IL-6	*Interleukin-6*
KCl	Cloreto de potássio
LAMIA	Laboratório de microbiologia clínica
MALDI-TOF-MS	*Matrix-assisted laser desorption ionization time of flight mass spectrometer*
MBC	*Minimal bactericidal concentration*
MBq	*Megabequerel*
mCi	*Milicurie*
MDR	Multirresistente
mg	Miligrama
MHA	*Mueller-Hinton agar*
MHB	*Mueller-Hinton broth*
MIC	*Minimal inhibitory concentration*
mL	Mililitro
mL/min	Mililítro por minuto
mm	Milímetro
MRSA	*Staphylococcus aureus* resistente a meticilina
MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium*
NaCl	Cloreto de sódio
nm	Nanômetro
°C	Graus Celsius
OD	Optical density
OMS	Organização Mundial da Saúde
OXA	Oxacilinases
P&D	Pesquisa e desenvolvimento
PAMs	Peptídeos antimicrobianos
PBP2a	*Penicillin-binding protein 2a*
PDR	Pan-resistente
pH	Potencial hidroxigeniônico
PLP	Proteínas ligadoras de penicilina
r.p.m.	Rotação por minuto
SEM	*Scanning electron microscopy*
TNF-α	*Tumor necrosis factor-*α
UFMG	Universidade Federal de Minas Gerais
UFSJ	Universidade Federal de São João del-Rei
UPEC	Escherichia coli uropatogênica
Vero	*Epithelial cells from kidney of Cercopithecus aethiops*
VISA	*Vancomycin- intermediate resistant S. aureus*
XDR	Extensivamente resistente
µg	Micrograma
Mg/mL	*Micrograma por mililitro*
µM	Micromol por litro

SUMÁRIO

Introdução e Justificativa

Introdução e Justificativa

O sucesso dos antimicrobianos em meados do século XX gerou o falso presságio de que as doenças infecciosas tinham sido eficientemente controladas (ANTIMICROBIAL RESISTANCE COLLABORATORS, 2022; RODRÍGUEZ-VERDUGO et al., 2020). Entretanto, após quase 50 anos, vemos um aumento alarmante na incidência de doenças infecto-contagiosas, tornando-as a segunda causa de mortalidade em países subdesenvolvidos e a quarta mundialmente (WORLD HEALTH ORGANIZATION, 2022). A resistência dos microrganismos às terapias convencionais contribui para a intensificação e posterior cronificação deste cenário. Anualmente, morrem 700.000 pessoas por infecções causadas por microrganismos resistentes no mundo (RODRÍGUEZ-VERDUGO et al., 2020). Um estudo encomendado pelo governo britânico ao economista Jim O'Neill revelou que, caso nenhuma medida efetiva seja tomada pelos órgãos de vigilância sanitária dos diferentes países, em 2050 bactérias multirresistentes matarão 10 milhões de pessoas anualmente no mundo, superando doenças crônicas como o câncer e o diabetes (O´NEILL, 2016). No Brasil, o panorama é também preocupante. O Ministério da Saúde estima que das 720.000 infecções relacionadas à assistência à saúde que ocorrem anualmente no país mais de 70% envolvem bactérias que são resistentes a pelo menos um dos antimicrobianos comumente utilizados para o tratamento desses pacientes (BARROS et al., 2012).

Entretanto, devido a pandemia da doença do coronavírus de 2019 (COVID-19), iniciada em março de 2020, as previsões de Jim O'Neill fazem-se atualmente desatualizadas. Uma nova estimativa aponta que o perfil previsto para 2050 tenha sido acelerado em pelo menos 10 anos (LIMA et al., 2020a). Esse fato se justifica pela utilização exagerada, e muitas vezes irracional, de grandes quantidades de antibióticos nos pacientes internados com COVID-19. Vale destacar o uso profilático, nesses pacientes, de agentes terapêuticos de “último recurso” que são quase sempre

reservados para infecções ocasionadas por patógenos pan-resistentes (LIMA et al., 2020b; BRITO et al., 2020). Esse cenário assustador tem levado muitos autores a apontarem para uma próxima pandemia com bactérias multirresistentes. A “era das trevas” da antibioticoterapia já se põe como um desafio a humanidade hoje e em sua expressão mais tenebrosa vemos a morte de pacientes por infecções a pouco tempo consideradas triviais como feridas e infecções do trato urinário (CRUZ et al., 2022).

Na contra mão da re-emergência de doenças infecciosas de etiologia bacteriana em função do rápido aumento e expansão de linhagens resistentes, observa-se uma negligência das grandes indústrias farmacêuticas nos investimentos voltados a pesquisa e desenvolvimento (P&D) de novos agentes antimicrobianos (THEURETZBACHER et al., 2009; SPELLBERG et al., 2004; BOUCHER et al., 2009; TALBOT et al., 2006; ANTIMICROBIAL RESISTANCE COLLABORATORS, 2022; THE LANCET, 2020). De 1998 a 2002 a agência americana de controle sanitário *Food and Drug Administration* aprovou apenas 7 fármacos voltados ao tratamento de doenças infecciosas, o que equivale a apenas 3% de todos os 225 novos fármacos licenciados neste período. Se comparado ao período de 1983 a 1987, por exemplo, observa-se uma redução de 56% no número de antimicrobianos aprovados (SPELLBERG et al., 2004). O cenário futuro também é pouco promissor. Dos 315 novos fármacos em fase de pesquisa pelas 15 maiores indústrias farmacêuticas do mundo apenas 31 (cerca de 10%) são anti-infectivos, dos quais 5 (menos de 2%) são novos antibacterianos pertencentes a classes já disponíveis no mercado e somente 3 estão em fase clínica avançada (fase III) (THEURETZBACHER et al., 2009; TALBOT et al., 2006; ANTIMICROBIAL RESISTANCE COLLABORATORS, 2022).

A indústria, no seu modelo de mercado, espera um rápido retorno do capital investido para colocar um novo fármaco na clínica o qual gira em torno de 400 a 800 milhões de dólares por agente licenciado (DIMASSA et al., 2003; ANTIMICROBIAL RESISTANCE COLLABORATORS, 2022; THE LANCET, 2020). Essa logística não é compatível com os esquemas terapêuticos curtos normalmente associados às doenças infecto-contagiosas de curso agudo (THEURETZBACHER et al., 2009). O quadro que se apresenta, associado ao aumento da incidência e prevalência das doenças crônicas não transmissíveis mais rentáveis e da maior pressão por parte dos prescritores para escolha das primeiras linhas de terapia corroboram para o desinteresse das grandes farmacêuticas em investir seus recursos orçamentários e

humanos em P&D voltados a novos antimicrobianos (SPELLBERG et al., 2004). Desta forma o conhecimento acumulado nesse campo acaba se dispersando e sendo transferidos para indústrias de menor porte, as quais muitas vezes contam com infraestrutura física e recursos financeiros incompatíveis com a conclusão de todas as etapas voltadas a P&D e comercialização de novos antimicrobianos (BOUCHER et al., 2009; THEURETZBACHER et al., 2009).

Com o objetivo de estimular os investimentos e esforços das indústrias farmacêuticas para o desenvolvimento de novos antibióticos, a Organização Mundial da Saúde (OMS) publicou em fevereiro de 2017 uma lista das principais espécies de bactérias multirresistentes que ameaçam a saúde global e necessitam urgentemente de novos agentes terapêuticos (WORLD HEALTH ORGANIZATION, 2017). Dentre os microrganismos abordados nessa listagem o *Acinetobacter baumannii* resistente aos carbapenêmicos (CRAB) e *Enterobacteriales* (*e.g.*, *Escherichia coli*) produtoras de beta-lactamase de espectro ampliado (ESBL) são apontadas como os microrganismos de mais alta prioridade para o desenvolvimento de novos antibióticos. Em adição, a lista aponta também a importância em se desenvolver novos agentes terapêuticos contra o *Staphylococcus aureus* resistente a meticilina (MRSA), o qual é definido como uma espécie de alta prioridade para novos fármacos (WORLD HEALTH ORGANIZATION, 2017). Assim torna-se urgente e extremamente importante à busca de novos antimicrobianos para o combate desses microrganismos. Entretanto, devido aos reduzidos recursos investidos pelas indústrias nesse ramo farmacêutico, as universidades passam a ter papel central no aprimoramento e execução da P&D de novos agentes antimicrobianos (THEURETZBACHER et al., 2009; SPELLBERG et al., 2004; BOUCHER et al., 2009; TALBOT et al., 2006).

Nessa direção, os peptídeos antimicrobianos (PAMs) se destacam como uma abordagem promissora contra esses e outros patógenos multirresistentes (FOSGERAU & HOFFMANN, 2015; KANG et al., 2012). Os PAMs são as moléculas responsáveis pela imunidade inata de organismos multicelulares, sendo expressos de forma constitutiva ou induzida, apresentam um grande número de estruturas e conformações, possuindo com frequência entre 12 à 50 aminoácidos (SCHMIDTCHEN et al., 2013; ZHANG & GALLO, 2016). Atualmente existem oito PAMs aprovados para uso clínico pela *Food and Drug Administration* (FDA) (*i.e.*,

gramicidina, colistina, polimixina B, daptomicina, vancomicina, oritavancina, dalbavancina e telavancina) todos eles considerados antibióticos de “ultima linha” e ativo mesmo contra exemplares multirresistentes (CHEN & LU, 2020). Esses agentes são conhecidos por agirem sobre a membrana celular de patógenos bacterianos estimulando a lise e liberação do conteúdo citoplasmático. Além disso, eles podem atravessar a membrana inibindo a síntese de diferentes produtos do metabolismo bacteriano e são capazes de modular a resposta imune antibacteriana (EPAND & EPAND, 2011; SCHMIDTCHEN et al., 2013; ZHANG & GALLO, 2016).

Os PAMs apresentam uma série de vantagens em relação aos antibióticos convencionais como rápido efeito bactericida, baixa capacidade de selecionar resistência, ausência de formação de resíduos após sua excreção e espectro de atividade ampliado (CHEN & LU, 2020). Essas características tornam os PAMs uma estratégia promissora no desenvolvimento de novos antibióticos, e o aumento vertiginoso no número de patentes depositadas com essa estratégia terapêutica comprova o interesse crescente dos PAMs como fármacos antibacterianos.

Uma das principais fontes de PAMs com potencial antibiótico trata-se da peçonha de animais, com destaque ao veneno de artrópodes (HAEBERLI et al., 2000; VASSILEVSKI et al., 2009; SANTOS et al., 2010; KUHN-NENTWIG et al., 2013; ZHANG, 2015; BORGES et al., 2016; GOPALAKRISHNAKONE et al., 2016; REIS et al., 2018). Como exemplo, a melitina, um peptídeo de 26 resíduos de aminoácidos presente no veneno da Abelha-Europeia (*Apis mellifera*), tem se mostrado como um potente agente antibacteriano contra *S. aureus* e *E. coli* multirresistente, inclusive em modelos *in vivo* (MEMARIANI et al., 2019; CHOI et al., 2015). Em adição, o LyeTx mnΔK, um peptídeo sintético derivado da toxina da aranha-de-jardim (*Lycosa erythrognatha*), têm revelado promissora atividade contra inúmeras bactérias de interesse médico, incluindo o *Acinetobacter baumannii* (JÚNIOR, 2015; FUSCALDI et al., 2021).

Nessa direção, esses peptídeos se destacam como promissores protótipos de agentes farmacológicos antibacterianos, tendo potencial como princípio ativo em formulações direcionadas a terapia de infecções por patógenos multirresistentes. Além disso, eles podem atuar como agentes ativos contra importantes fatores de virulência microbiana, como os biofilmes. Assim, o objetivo do presente trabalho é

avaliar a viabilidade do uso desses dois peptídeos na remissão clínica de infecções superficiais e invasivas ocasionadas por bactérias insensíveis às terapias convencionais e verificar o efeito anti-biofilme dessas moléculas.

Revisão da literatura

Revisão da literatura

Infecções bacterianas e resistência aos antibióticos

Em meados do século XX, graças aos trabalhos pioneiros de Paul Ehrlich e Alexandre Fleming, vivemos a “era de ouro” dos antibióticos. Nesse período foi lançada a maior parte das classes terapêuticas de antibacterianos que temos atualmente disponíveis na clínica, e desde então poucas novas classes têm sido disponibilizadas (ANTIMICROBIAL RESISTANCE COLLABORATORS, 2022; THE LANCET, 2020). Assim, o sucesso da antibioticoterapia durante esse período gerou o falso presságio de que as doenças infecciosas de etiologia bacteriana tinham sido eficientemente controladas. No entanto, a resistência aos antibióticos atualmente disseminada de maneira irreversível têm pressionado os órgãos de controle sanitário no mundo inteiro, tornando as doenças infecciosas como graves problemas de saúde pública re-emergentes (THE LANCET, 2020; RODRÍGUEZ-VERDUGO et al., 2020).

Segundo a Organização Mundial de Saúde (OMS), as doenças infecciosas são a quarta principal causa de mortes no mundo e a segunda entre países de baixa renda (**Figura 1**) (WORLD HEALTH ORGANIZATION, 2022). As infecções do trato respiratório inferior (especialmente as pneumonias bacterianas) são atualmente a doença infecciosa mais letal, causando 2,6 milhões de mortes em todo o mundo em 2019. A taxa de mortalidade por doenças diarréicas infecciosas diminuiu quase 1,1 milhão entre 2000 e 2019, mas ainda foi responsável por 1,5 milhões de mortes em 2019. Nessa direção, as doenças infecciosas bacterianas têm atualmente uma mortalidade superior à de doenças crônicas não transmissíveis, sendo que somente a pneumonia bacteriana, por exemplo, supera as mortes causadas por diabetes e doenças renais combinadas. Outras doenças infecciosas que estavam entre as 10 principais causas de morte em 2000 não estão mais na lista. O HIV/AIDS é uma delas. As mortes por HIV/AIDS caíram 51% nos últimos 20 anos, passando da 8ª principal

causa de morte no mundo em 2000 para a 19ª em 2019 (WORLD HEALTH ORGANIZATION, 2022).

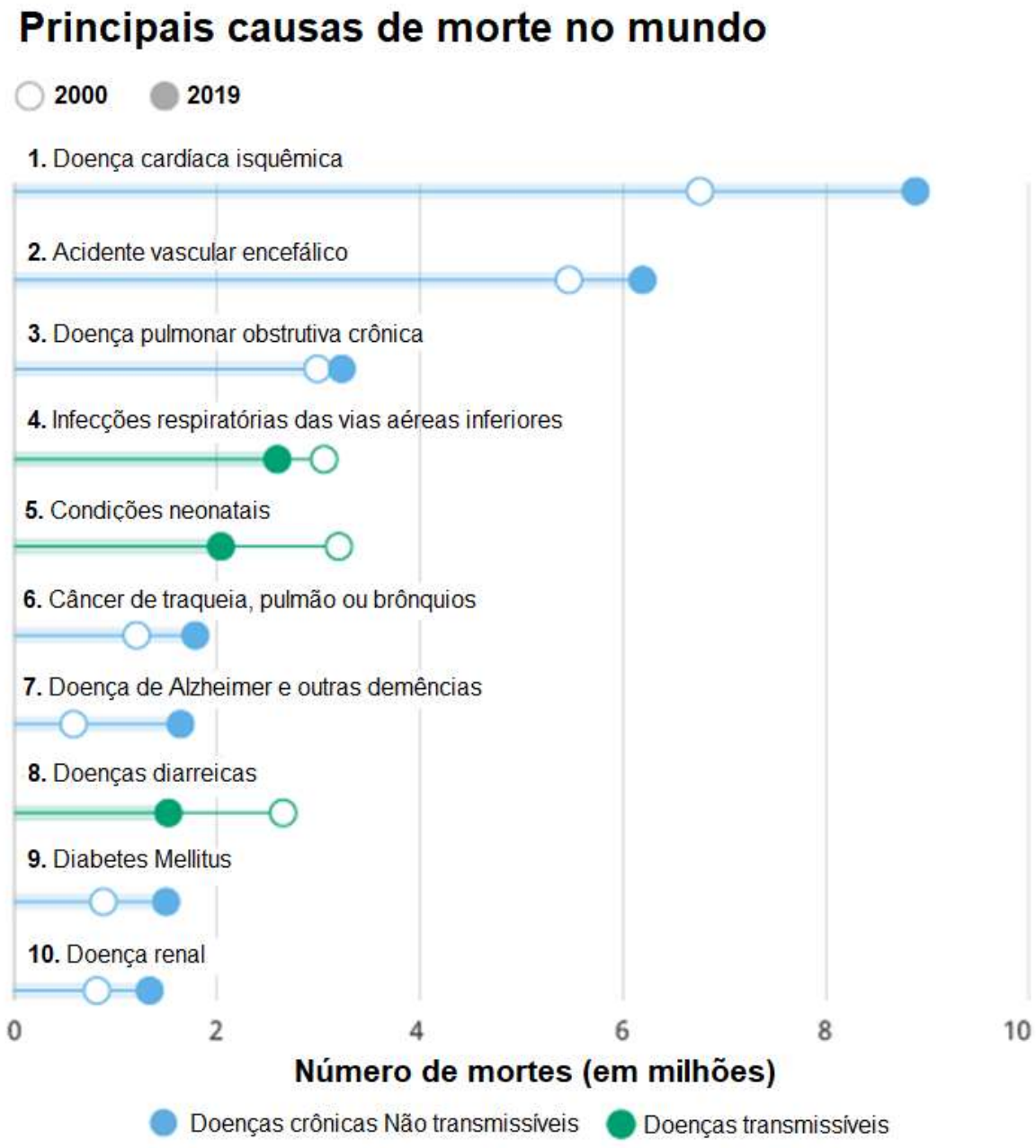

Figura 1: Principais causas de morte no mundo em 2019 segundo estimativas da Organização Mundial da Saúde. FONTE: WORLD HEALTH ORGANIZATION, 2022.

Nas últimas duas décadas, têm sido observado um aumento alarmante da resistência aos antibióticos por bactérias patogênicas, fato que pode ajudar a explicar o porquê às doenças infecciosas de etiologia bacteriana não reduziram em incidência global ao nível do HIV/AIDS (RODRÍGUEZ-VERDUGO et al., 2020). De fato, enquanto inúmeros antirretrovirais vêm sendo lançado na clínica a cada ano, o número de novos antibióticos é significativamente menor do que a capacidade das bactérias se tornarem resistentes a eles (HAMERS et al., 2018; THE LANCET, 2020).

Para padronizar a nomenclatura referente à resistência aos antibióticos de maneira a garantir uma terminologia internacional, o Centro Europeu de Controle de Doenças (ECDC) e o Centro de Controle e Prevenção de Doenças (CDC) dos Estados Unidos da América tem adotado a classificação de MAGIORAKOS e colaboradores (2012). De acordo com esse sistema, os patógenos resistentes podem ser categorizados em três classes: multirresistente (MDR), extensivamente resistente (XDR) e pan-resistente (PDR) (MAGIORAKOS et al., 2012). Os patógenos MDR foram definidos como aqueles resistentes á ao menos um antibiótico de três diferentes classes terapêuticas. Já os patógenos XDR foram definidos como aqueles sensíveis a apenas um ou dois antibióticos, sendo resistentes a todas as outras classes. Por sua vez, os patógenos PDR são aqueles resistentes a todas as classes terapêuticas disponíveis (MAGIORAKOS et al., 2012).

Em se tratando somente das mortes associadas por infecções por patógenos MDR, anualmente, são reportados 700.000 óbitos em todo o mundo (WORLD HEALTH ORGANIZATION, 2018). Um estudo encomendado pelo governo britânico ao economista Jim O'Neill revelou que, caso nenhuma medida efetiva seja tomada pelos órgãos de vigilância sanitária dos diferentes países, em 2050 bactérias multirresistentes matarão 1 pessoa a cada 3 segundos no mundo. Isso totalizará 10 milhões de mortes anuais, número que irá superar doenças crônicas como o câncer (8,2 milhões de óbitos) e o diabetes (1,5 milhões de óbitos), e causas externas como os acidentes de trânsito (1,2 milhões de óbitos) (**Figura 2**) (O´NEILL, 2016). Em adição, o estudo tem apontado também que em 2050 os gastos associados somente às internações por bactérias resistentes atingirá o patamar de 100 bilhões de dólares anualmente no mundo (O´NEILL, 2016). O aumento nas taxas de resistência observado atualmente tem se refletido também no consumo global de antibióticos.

Nesse sentido, um estudo projeta que em 2030, presumindo nenhuma mudança na política de comercialização global desses fármacos, o consumo de antibióticos ficará em torno de 82 bilhões de doses diárias o qual é o dobro do que foi utilizado no ano de 2015 (KLEIN et al., 2018).

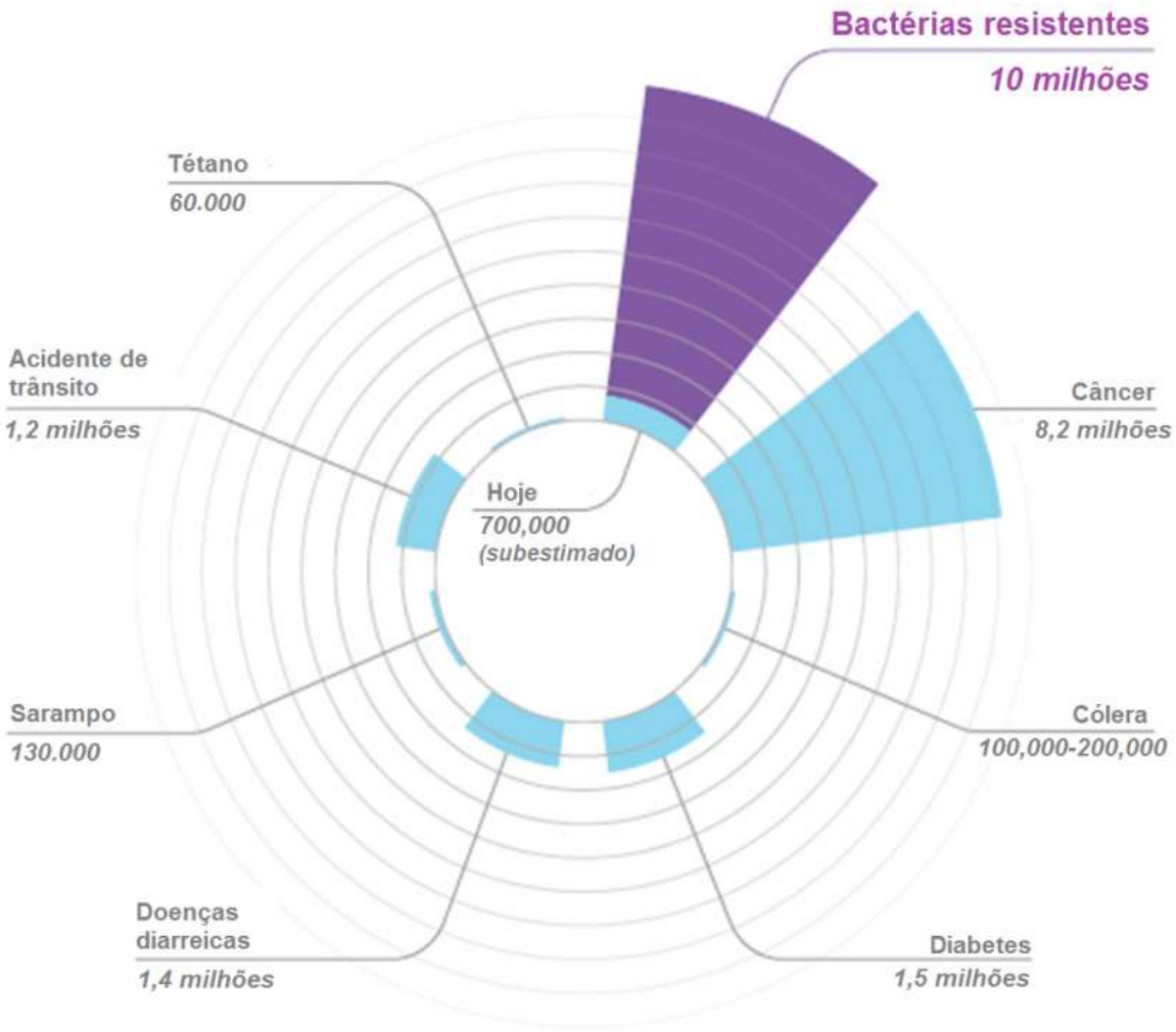

Figura 2: Representação gráfica das previsões referente ao número de óbitos por diferentes causas para 2050 conduzido pelo economista britânico Jim O´Neill como parte do estudo *Review on Antimicrobial Resistance* encomendado pelo governo britânico. FONTE: Adaptado de O´NEILL, 2016

No Brasil, o panorama também é preocupante. O Ministério da Saúde estima que das 720.000 infecções relacionadas à assistência à saúde que ocorrem anualmente no país (BARROS et al., 2012) mais de 70% envolvem bactérias que são resistentes a pelo menos um dos antimicrobianos comumente utilizados para o tratamento desses pacientes. O custo hospitalar atribuível à ocorrência dessas infecções é de 651 dólares por dia, elevando-se para 1.780 dólares na UTI, o que contribui para a elevação expressiva dos custos em saúde pública no país (SANCHEZ-VELAZQUEZ et al., 2006).

Patógenos multirresistentes de relevância clínica

Durante o ano de 2003, a sociedade americana de doenças infecciosas (IDSA, do inglês *Infection Diaseas Socity of American*) criou a força tarefa para disponibilidade de antimicrobianos (AATF, do inglês *Antimicrobial Availability Task Force*). Esse grupo se encarregou de avaliar as tendências relacionadas à P&D de novos antibióticos bem como fiscalizar o valor destes medicamentos assegurando sua ampla disponibilização (TALBOT *et al*., 2006). Deste trabalho resultou o relatório intitulado "*Bad Bugs, No Drugs: As antibiotic R&D stagnates, a public health crisis brews"* que além de mostrar os problemas elencados propôs uma série de potenciais soluções (INFECTIOUS DISEASES SOCIETY OF AMERICA, 2004). Dentre os assuntos levantados no presente documento, a AATF listou as espécies de bactérias MDR mais relevantes na clínica médica. A listagem elencada pela AATF compreendia bastonetes Gram-negativos frequentemente relacionados a infecções relacionadas à assistência à saúde como o *Acinetobacter baumanni*, *Pseudomonas aeruginosa* e *Enterobacteriales* produtoras de beta-lactamases de espectro ampliado (ESBL); cocos Gram-positivos resistentes à vancomicina como o *Staphylococcus aureus*, *Enterococcus faecium* e *Enterococcus faecalis;* e fungos pertencentes ao gênero *Penicillium* que causam infecções oportunistas graves (cujas taxas de mortalidade podem alcançar 90% dos infectados) (TALBOT *et al*., 2006; INFECTIOUS DISEASES SOCIETY OF AMERICA, 2004).

Com base neste relatório e em uma revisão de outros trabalhos sobre o perfil de resistência de diferentes espécies de microrganismos, RICE (2008) empregou pela

primeira vez a sigla ESKAPE para designar as iniciais dos principais patógenos que "escapam" das terapias convencionais (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, **P***seudomonas aeruginosa*, **E***nterobacter*). Essas bactérias podem expressar resistência intrínseca (inerente), ou seja, mecanismos de resistência naturais de um gênero ou espécie bacteriana, ou podem expressar resistência adquirida, ou seja, aquela originada a partir de mutações nos próprios genes ou pela aquisição dos genes de resistência de outras bactérias (conjugação com troca de plasmídeo e transposon), via bacteriófago (transdução) ou via ambiente (transformação) (LIMA et al., 2019).

De fato, os microrganismos do grupo ESKAPE se relacionam com infecções oportunistas que em quase a totalidade dos casos não respondem as primeiras linhas de tratamento, tornando necessário o uso de fármacos das últimas linhas (RICE, 2008; PENDLETON et al., 2013). Estes fármacos, no entanto, são muito mais caros e tóxicos que os antibióticos de primeira escolha, o que contribui para a elevação dos gastos públicos em saúde (KARAISKOS et al., 2019). Desde então, os esforços para P&D de novos antibióticos vêm focando nessas espécies de microrganismos, nos quais a necessidade de novos agentes farmacológicos é crítica (LUEPKE & MOHR, 2017).

Staphylococcus aureus resistente a meticilina

O *S. aureus* era extremamente sensível à ação da penicilina na década de 1940 (10 anos após sua descoberta por Alexandre Flaming). Entretanto, na década de 1950, muitas infecções já não respondiam adequadamente ao uso deste fármaco (JOSEPH, 2020; THE LANCET et al., 2020). Foi constatado que algumas linhagens começaram a produzir penicilinases, uma enzima que clivava o anel beta-lactâmico da penicilina, o que acarreta a sua inativação (BITRUS et al., 2018; JOSEPH, 2020). Essas alterações a nível gênico reduziram expressivamente a susceptibilidade aos beta-lactâmicos, em especial a penicilina, cujas taxas de resistência podem chegar a até 90% dos isolados de *S. aureus* hoje em dia (HASAN et al., 2016).

Com o avanço da resistência as penicilinas a indústria farmacêutica foi pressionada para desenvolver congêneres resistentes as penicilinases, culminando no lançamento do protótipo meticilina, que acabou sendo substituído pela oxacilina e

cloxacilina em muitos países (inclusive no Brasil) (JOSEPH, 2020). O uso indiscriminado destes agentes no tratamento de infecções dérmicas não bacterianas, no entanto, estimulou a seleção de linhagens genericamente chamadas de *S. aureus* resistente a meticilina (MRSA), que apesar do nome, são resistentes a todos os representantes da classe dos beta-lactâmicos (ANSARI et al., 2019; CHIPOLOMBWE et al., 2016).

A resistência a meticilina em *S. aureus* deve-se principalmente a alteração de alvo (BITRUS et al., 2018; PEACOCK & PATERSON, 2015). Os antibióticos beta-lactâmicos de uma maneira geral atuam inibindo a síntese de parede. Isso ocorre quando esses antibióticos se ligam a proteínas responsáveis pela formação das ligações cruzadas que mantém a integridade e resistência dessa estrutura celular. As proteínas recebem o nome genérico de proteínas ligadoras de penicilina (PLP), e quando inibidas pelos beta-lactâmicos perdem sua capacidade funcional, reduzindo assim a síntese da parede celular bacteriana (BUSH & BRADFORD, 2016). No entanto, algumas cepas de *S. aureus* podem codificar um PLP alterada (chamada de PLP2a) a qual apesar de manter sua capacidade funcional, torna-se insensível a ligação dos beta-lactâmicos. Essa PLP é normalmente codificada pelo gene *mecA* em *S. aureus*, que quando expresso, produz o fenótipo conhecido como MRSA (PEACOCK & PATERSON, 2015).

Nos Estados Unidos, estima-se que ocorram anualmente mais de 94.000 casos de infecções invasivas por MRSA dos quais 18.000 evoluem para óbito, superando assim o número de mortes causadas por HIV/AIDS, tuberculose e homicídeos combinados nesse país (KLEVENS et al., 2006; BOUCHER & COREY, 2008; GAROY et al., 2019). Na união europeia, por sua vez, os países contabilizam mais de 150.000 infecções anuais por MRSA, as quais geram 380 milhões de euros em custos hospitalares extras para os sistemas de saúde dos países europeus (KÖCK et al., 2010; GAROY et al., 2019). No Brasil, o estudo multicêntrico SENTRY, realizado em hospitais do país durante os anos de 2005 a 2008, coloca o *S. aureus* como principal agente de infecção da corrente circulatória (20,2%), principal agente de infecções de pele e tecidos moles (28,1%) e segundo agente mais comum de pneumonia em pacientes hospitalizados (24,9%) (GALES et al., 2009). Além disso, o estudo aponta

para o preocupante aumento dos achados de MRSA, o qual representou 31% dos isolados identificados como *S. aureus* nesse período (GALES et al., 2009).

A resistência à vancomicina entre *S. aureus*, a qual é considerada o último recurso terapêutico em infecções por MRSA, vem avançando de maneira expressiva em todo mundo e contribuindo para tornar o cenário atual ainda mais crítico (CONG et al., 2019; TENOVER & MOELLERING-JUNIOR, 2007; GARDETE & TOMASZ, 2014). Em 2002, a primeira cepa de *S. aureus* resistente à vancomicina (VRSA) foi recuperada em Michigan (EUA). No mesmo ano, a segunda cepa de VRSA foi isolada na Pensilvânia (EUA). Desde então, um total de 52 cepas de VRSA foram relatadas nos EUA (14 isolados), Índia (16 isolados), Irã (11 isolados), Paquistão (9 isolados), Brasil (1 isolado) e Portugal (1 isolado) (CONG et al., 2019). Além disso, linhagens com resistência intermediária (VISA) aos glicopeptídeos (classe da vancomicina) são ainda mais frequentes, principalmente após a redução do ponto de corte pelo *Clinical and Laboratory Standards Institute* (CLSI) de 4 para 2 µg/mL em 2007 (TENOVER & MOELLERING-JUNIOR, 2007; GARDETE & TOMASZ, 2014).

Escherichia coli resistente as quinolonas e produtora de beta-lactamase de espectro ampliado (ESBL)

Escherichia coli, um membro da família *Enterobacteriales*, é um bastonete gram-negativo fermentador de glicose que normalmente habita o trato gastrointestinal de humanos e animais (VILA et al., 2016). Como comensal, a *E. coli* vive em uma associação mutuamente benéfica com os hospedeiros. No entanto, algumas cepas dessa bactéria são comumente implicadas em infecções clínicas relevantes, como infecções do trato urinário (ITU) (KAPER et al., 2004). De fato, as ITUs adquiridas na comunidade (CA-ITU) causadas por *E. coli* uropatogênica (UPEC) são as infecções bacterianas mais comuns, afetando aproximadamente 150 milhões de pessoas anualmente em todo o mundo (principalmente mulheres) (KUCHERIA et al., 2005). Os antibióticos fosfomicina e sulfas são as primeiras escolhas para tratar CA-ITU; entretanto, as quinolonas têm sido o antibiótico mais prescrito nesses casos. Esse uso indiscriminado de quinolonas aumentou a pressão seletiva sobre os uropatógenos, elevando significativamente a incidência de UPECs resistentes a essa classe (LEE et

al., 2016; MOREIRA DA SILVA et al., 2017; VIEIRA et al., 2020). As quinolonas atuam inibindo a ação das subunidades A da topoisomerase II (também chamada de DNA girase) nas bactérias gram-negativas, a qual participa da replicação gênica. Já nos estafilococos e pneumococos, o local primário de ação é a topoisomerase IV, e, a DNA-girase se torna um local secundário (PAIVA et al., 2019; VIEIRA et al., 2020).

Portanto, o principal mecanismo que confere alto nível de resistência às quinolonas em UPECs são as mutações cromossômicas envolvendo o gene da subunidade A da DNA-girase (*gyrA*), especificamente na região que codifica os aminoácidos 67-106, conhecida como região determinante da resistência às quinolonas (QRDR) (VIEIRA et al., 2020; LEE et al., 2016). No entanto, desde 1998, quando o primeiro gene de resistência às quinolonas mediado por plasmídeo (PMQR) foi identificado em *Klebsiella pneumoniae* (MARTÍNEZ-MARTÍNEZ et al., 1998), a transferência horizontal tem sido vista como a principal via na disseminação da resistência às quinolonas em UPECs (VIEIRA et al., 2020). Esses determinantes de resistência conferem um aumento sutil nas concentrações inibitórias mínimas (CIM) das fluoroquinolonas, o que normalmente mascara sua detecção *in vitro*. Além disso, PMQRs podem contribuir para um aumento na ocorrência de mutações espontâneas em QRDRs, permitindo o desenvolvimento de resistência de alto nível (MOREIRA DA SILVA et al., 2017). Atualmente, são conhecidos três principais mecanismos de resistência às quinolonas associados aos PMQRs os quais induzem resistência por bloquear o sítio de ligação do fármaco na DNA-girase (gene *qnr*), modificar enzimaticamente o antibiótico (gene *aac(6`)-Ib-cr*), ou expelir o composto de seu local de ação pela codificação de bombas de efluxo (genes *oqxAB* e *qepA*) (VIEIRA et al., 2020).

Infecções causadas por UPECs resistentes as quinolonas geralmente evoluem para complicações graves, como pielonefrite, bacteremia e urossepse. Essas complicações são tratadas com antibioticoterapia intravenosa, na qual apenas algumas opções estão disponíveis (e.g., polimixinas, aminoglicosídeos, cefalosporinas) (NICOLLE et al., 2005; YAMAMOTO, 2016). As cepas de UPECs resistentes a quinolonas normalmente carregam enzimas que codificam resistência a outros antibióticos com destaque a beta-lactamases de espectro ambliado (ESBL) (HALAJI et al., 2020).

ESBLs são um grupo de β-lactamases que compartilham a capacidade de hidrolisar penicilinas, cefalosporinas de primeira, segunda e terceira geração, e aztreonam (mas não as cefamicinas, como cefoxitina ou carbapenêmicos), e são inibidos por inibidores de β-lactamase, como ácido clavulânico (HALAJI et al., 2020). A maioria das ESBLs são derivadas das β-lactamases de amplo espectro TEM e SHV. Existem também outras famílias de ESBLs, incluindo as enzimas do tipo CTX-M e OXA, bem como outras β-lactamases não relacionadas (por exemplo, PER-1, PER-2, VEB-1, CME-1, TLA-1, GES- 1) (CASTANHEIRA et al., 2021). As ESBLs são amplamente distribuídas e podem se espalhar facilmente, pois os genes que as codificam geralmente são transferidos por meio de elementos genéticos móveis, como plasmídeos, transposons e integrons. No cenário clínico, as UPECs produtoras de ESBL estão associadas a maiores taxas de mortalidade e altos custos de saúde (YAMAMOTO, 2016; VIEIRA et al., 2020). Portanto, o desenvolvimento de novos agentes farmacológicos contra UPECs resistentes a quinolonas e produtoras de ESBL é urgentemente necessário.

Acinetobacter baumannii **resistente aos carbapenêmicos**

O gênero *Acinetobacter* compreende muitas espécies intimamente relacionadas, incluindo aquelas que formam o complexo *Acinetobacter baumannii-Acinetobacter calcoaceticus* (por exemplo, *A. baumannii*, *A. nosocomialis*, *A. pittii*, e *A. calcoaceticus*) (ALMASAUDI, 2018). O *A. baumannii*, no entanto, é reconhecido como a espécie mais relevante clinicamente deste complexo microbiano, sendo ela frequentemente identificada em infecções relacionadas à assistência à saúde (JUNG & PARK, 2015; LEE et al., 2017). Essa bactéria é um coco-bacilo não móvel, gram-negativo, aeróbico, não fermentador de glicose, oxidase negativa, e pleomórfica que possui tipicamente um tamanho de 1,5-2,5 mm (ALMASAUDI et al., 2018; JUNG & PARK, 2015; LEE et al., 2017). Inicialmente descrita como uma bactéria oportunista de baixa patogenicidade, o *A. baumannii* emergiu como um importante patógeno em infecções nas unidades de terapia intensivas (UTIs) durante a última década, estando envolvido em doenças como pneumonia (especialmente na pneumonia associada a ventilação mecânica), osteomielite, peritonite, endocardite, septicemia, meningite e

infecções de ferida, pele, tecidos moles, trato urinário, ouvido e olhos (ALMASAUDI et al., 2018).

Estudos têm revelado que o *A. baumannii* pode aderir a dispositivos médicos (inclusive no sistema de ventilação mecânica) e sobreviver até 35 dias em superfícies secas (HOWARD et al., 2012; JUNG & PARK, 2015). Em adição para sua notável resistência na ausência de nutrientes, o *A. baumannii* comumente não responde a desinfecção com o uso de agentes químicos convencionais, sendo esse efeito muitas vezes associado a produção de uma cápsula de polissacarídeo e a formação de biofilmes por essa bactéria (HOWARD et al., 2012). Além disso, a aquisição de resistência a múltiplos antibióticos, especialmente aos carbapenêmicos e polimixinas, tornou esse patógeno um grande problema de saúde pública na atualidade (LIMA et al., 2018; LIMA et al., 2019; LIMA et al., 2020c).

Os antibióticos carbapenêmicos (*e.g.*, imipenem, doripenem, meropenem e ertapenem) são considerados a última opção terapêutica para o tratamento de infecções causado por *A. baumannii* com fenótipo MDR (LIMA et al., 2019). No entanto, desde 1991, quando foi relatado o primeiro caso de *A. baumannii* resistente aos carbapenêmicos (CRAB) (URBAN et al., 1993), um aumento considerável no número dessas cepas XDR foi documentado em todo o mundo (KIM et al., 2014). Na Grécia, por exemplo, as taxas de resistência ao imipeném aumentaram de 90,3% em 2010 para 94,5% em 2015, enquanto as taxas de resistência ao meropeném aumentaram de 82,6% em 2010 para 94,8% em 2015 (DAFOPOULOU et al., 2018). REDDY et al. (2010), por sua vez, mostraram que em hospitais norte-americanos as taxas de resistência aos carbapenêmicos em *A. baumannii* também aumentaram, passando de 1,0% em 2003 para 58,0% em 2008. No Brasil, um estudo multicêntrico envolvendo quatro estados (Rio Grande do Sul, Rio de Janeiro, Paraná e São Paulo) mostrou uma taxa de resistência de 61% entre os isolados de *A. baumannii* avaliados (ROCHA et al., 2017). Em conjunto, esses dados revelam que as taxas de resistência aos carbapenêmicos são expressivamente altas, o que pressiona os clínicos para a prescrição de antibióticos de último recurso como as polimixinas (LIMA et al., 2020).

A resistência aos carbapenêmicos entre *A. baumannii* pode ser associada com a presença de ao menos um dos quatro mecanismos a seguir: (i) bombas de efluxo (*e.g.*, AdeABC) (MARCHAND et al., 2004); (ii) alterações nas proteínas de ligação à

penicilina (GEHRLEIN et al., 1991); (iii) perda de proteínas da membrana externa (*e.g.*, CarO) (LIMANSKY et al., 2002); e principalmente (iv) expressão de β-lactamases (carbapenemases) que hidrolisam os carbapenêmicos (BUSH, 2013).

Até o momento, três tipos de carbapenemases foram relatados em *A. baumannii*, sendo elas as β-lactamases da classe A de Ambler (*e.g.*, GES-14 e KPC-1), metalo-β-lactamases da classe B de Ambler (*e.g.*, IMP, VIM, SIM-1, e NDM) e oxacilinases da classe D de Ambler (OXAs) (BUSH, 2013; LIMA et al., 2019; MOUBARECK & HALAT, 2020). Segundo relatos de diferentes países, a expressão de carbapenemases do tipo OXA é o principal determinante de resistência aos carbapenêmicos em *A. baumannii*. Nessa direção, têm sido descritos sete diferentes tipos de oxacilinases nesta espécie que têm sido divididas em dois grandes grupos, os quais (MOUBARECK & HALAT, 2020):

► Oxacilinases intrínsecas: São encontradas em virtualmente todos os exemplares da espécie *A. baumannii* (sendo frequentemente empregado para identificar essa espécie) e podem ser encontradas no cromossomo ou em plasmídeos dentro da bactéria. É representada por um único tipo chamado de OXA-51-like.

► Oxacilinases adquiridas: São carbapenemases que a bactéria pode adquirir por conjugação, transformação ou transdução. Essas carbapenemases são encontradas em plasmídeos e atualmente têm sido reportadas seis tipos em *A. baumannii*: OXA-23-like, OXA-24/40-like, OXA-48-like, OXA-58-like, OXA-143-like e OXA-235-like.

Além resistência aos carbapenêmicos associados a expressão das oxacilinases (OXAs), sabe-se que a presença de sequências de inserção (IS) imediatamente a montante dos gene bla_{OXA} contribui para induzir a superexpressão de algumas oxacilinases como a OXA-51, OXA-23 ou OXA-58, gerando assim resistência a alto nível contra os carbapenêmicos em *A. baumannii* (LIMA et al., 2019; MOUBARECK & HALAT, 2020).

Peptídeos antimicrobianos como fonte de novos antibióticos

Uma das alternativas como opção terapêutica contra os patógenos multirresistentes como o MRSA, CRAB e UPEC resistente a quinolona e produtora de ESBL trata-se dos peptídeos antimicrobianos (PAM) (SEO et al., 2012). Os PAMs ocorrem naturalmente como um componente da resposta imune inata de praticamente todas as espécies, desde vertebrados á bactérias e plantas (FOSGERAU & HOFFMANN, 2015; KANG et al., 2012). Seu proeminente efeito antimicrobiano vem sendo explorado pela indústria farmacêutica no desenvolvimento de novos fármacos, em especial aqueles desenvolvidos contra patógenos resistentes as terapias convencionais (SEO et al., 2012; FOSGERAU & HOFFMANN, 2015). Nessa direção, atualmente oito PAMs têm sido aprovados como antibióticos pela *Food and Drug Administration* (FDA), *i.e.*, gramicidina, colistina, polimixina B, daptomicina, vancomicina, oritavancina, dalbavancina e telavancina, e vêm sendo empregados na clínica médica. As estruturas desses fármacos são mostradas na **Figura 3** (CHEN & LU, 2020).

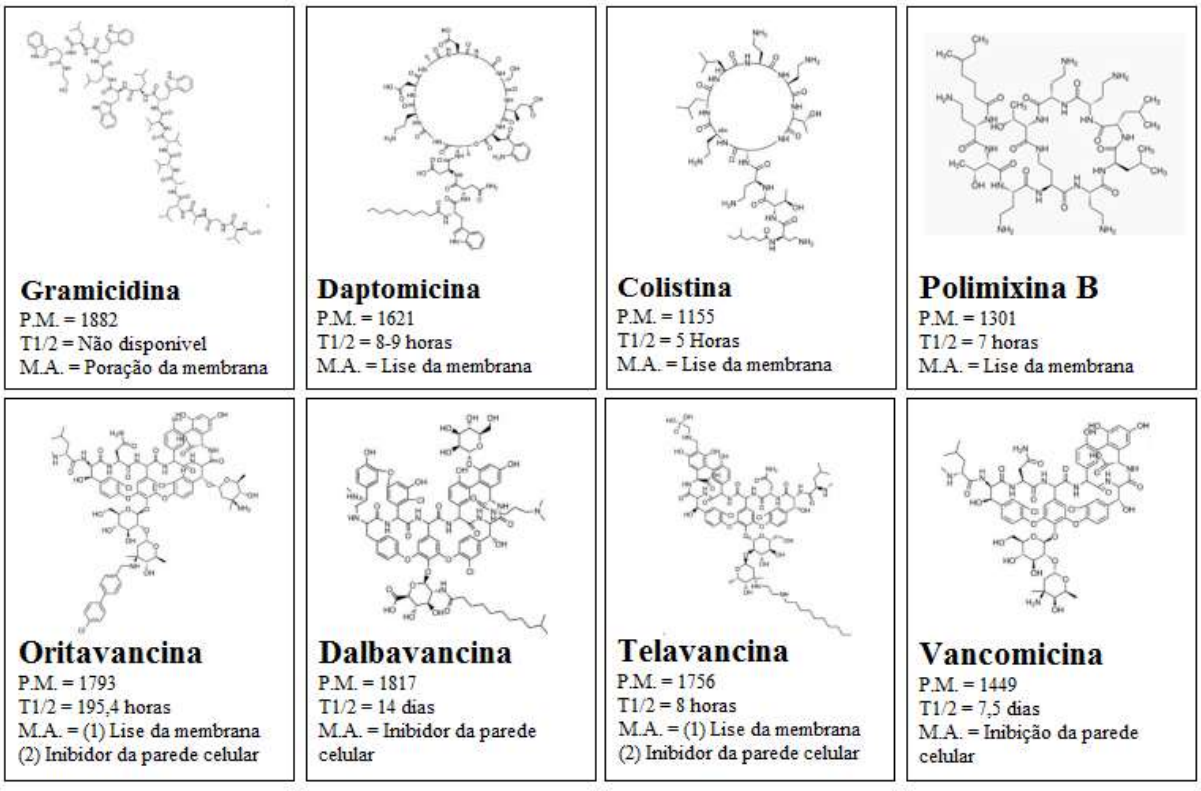

Figura 3: Estrutura química dos oito peptídeos antimicrobianos (PAMs) atualmente aprovados para o uso clínico. FONTE: Adaptado de LIMA et al*.*, 2018.

Os PAM são compostos cujos seus monômeros, resíduos de aminoácidos, são conjugados por ligações peptídicas e os pontos fundamentais que os diferenciam são o número de resíduos de aminoácidos, a composição e a ordem dos mesmos na sequência peptídica (WIELAND & BODANSZKY, 2012). Todos os PAMs que estão atualmente sendo desenvolvidos como potenciais antibióticos podem ser acessados utilizando-se o *Antimicrobial Peptides Database* (APD), uma iniciativa internacional que busca registrar todas as atividades de pesquisa centradas nesses biopolímeros. Atualmente, um total de 3.156 PAMs está listado no APD, a maioria dos quais foi descoberta na natureza (WANG et al., 2016). Uma análise recente com 2.700 PAMs presentes na APD mostrou que um terço dos peptídeos são derivados do veneno de sapos, o comprimento médio é de 33 aminoácidos (sendo que 90% dos peptídeos não têm mais que 50 resíduos e são chamados peptídeos pequenos), o conteúdo hidrofóbico médio é de 54% e a carga líquida média é +3. Em adição, cerca de 45% dos peptídeos não contêm cisteína e entre os que apresentam esse resíduo 21% e 17% deles têm duas cisteínas e seis cisteínas, respectivamente; o que revela o potencial para a formação de ligações dissulfeto (CHEN & LU, 2020).

Os PAMs possuem um espectro de vantagens em relação aos antimicrobianos tradicionais. Essas vantagens podem ser definidas pelo seu efeito majoritariamente bactericida, seu potencial anti-inflamatório intrínseco (que pode beneficiar os pacientes com inflamação associada ao processo infeccioso), baixo potencial de elicitar o desenvolvimento de resistência, atividade antibacteriana potente (normalmente em baixas concentrações), rápido efeito bactericida (o que restringe a probabilidade da infecção evoluir para quadros mais graves), ausência de formação de resíduos (devido sua rápida hidrólise no ambiente), e atividade em patógenos multirresistentes (FOSGERAU & HOFFMANN, 2015; KANG et al., 2012; CHEN & LU, 2020). No entanto, algumas desvantagens também podem ser destacadas como o alto custo de produção, a alta instabilidade plasmática (são sensíveis à ação de protease plasmáticas), a alta toxicidade, baixa estabilidade na presença de sal, eliminação renal rápida e graves problemas farmacocinéticos (principalmente envolvido com a baixa penetração nos tecidos) (FOSGERAU & HOFFMANN, 2015; CHEN & LU, 2020).

A atividade antibacteriana associada aos PAMs esta relacionada a diferentes mecanismos de ação (ZHANG & GALLO, 2016). No entanto, a principal atividade dos PAMs se refere à habilidade que esses compostos têm de interagir seletivamente com a membrana ou parede celular bacteriana. Geralmente, os PAMs têm carga positiva e grandes quantidades de resíduos hidrofóbicos, o que permite sua interação com a membrana bacteriana carregada negativamente. Essa interação permite a formação de poros na membrana com consequente lise celular, produzindo um efeito bactericida (LAZAREV & GOVORUN, 2010; BAHAR & REN, 2013; MALMSTEN, 2014). Além disso, alguns PAMs conseguem atravessar a bicamada lipídica e induzir seu efeito antibacteriano ao inibir enzimas essenciais ou reduzir a síntese de ácidos nucleicos e proteínas (BROGDEN, 2005; ZHANG & GALLO, 2016). Os efeitos diretos exercidos pelos PAMs podem também ser complementados pela sua atividade indireta. É conhecido que eles envolvem para um importante efeito imunomodulador que auxilia na resposta imune antibacteriana, como a indução do recrutamento/ativação de imunócitos e o auxílio ao reconhecimento de produtos bacterianos (LPS e ácido nucleico) pelos receptores do tipo *Toll* (EPAND & EPAND, 2011; SCHMIDTCHEN et al., 2013; ZHANG & GALLO, 2016). A **Figura 4** resume os principais efeitos biológicos dos PAMs que justifica sua potente atividade antibacteriana.

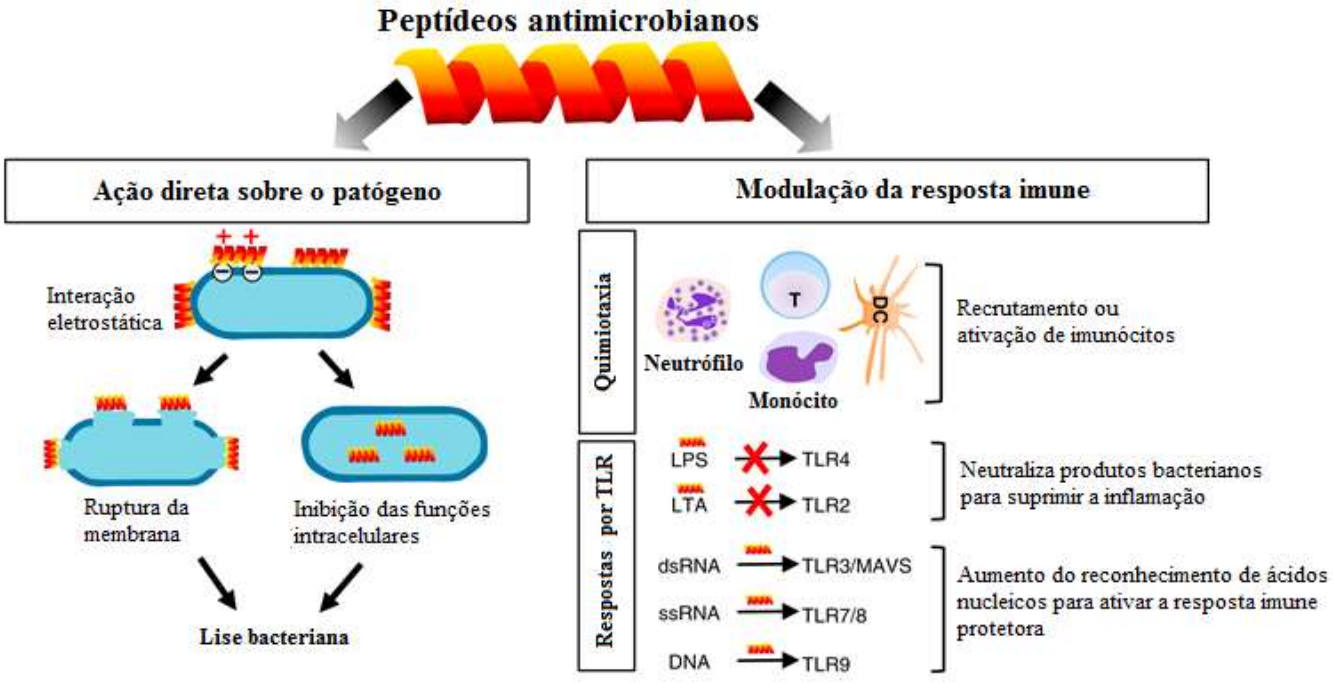

Figura 4: Representação esquemática dos principais mecanismos de ação antibacteriana dos PAMs. T: Linfócito T; DC: Células dendríticas; LPS: Lipopolissacarídeo; LTA: Ácido lipoteicoico; TLR: Receptores do tipo Toll; dsRNA: Ácido ribonucleico de fita dupla; ssRNA: Ácido ribonucleico de fita simples; DNA: Ácido desoxirribonucleico. FONTE: Adaptado de ZHANG & GALLO, 2016.

Uma das principais fontes de PAMs na natureza trata-se da peçonha de animais (LEI et al., 2019). Os venenos são matrizes complexas repleto de moléculas com uma ampla faixa de atividades biológicas, muitas das quais podem ser exploradas como potenciais medicamentos. Entre esses compostos se destacam os PAMs, os quais geralmente apresentam uma atividade de espectro entendido com ação contra bactérias gram-positivas e gram-negativas MDR (ZHANG, 2015). Nesse contexto, uma das fontes mais ricas em PAMs trata-se das toxinas de artrópodes como aranhas, vespas, formigas, abelhas, centopeias e escorpiões (HAEBERLI et al., 2000; VASSILEVSKI et al., 2009; SANTOS et al., 2010; KUHN-NENTWIG et al., 2013; ZHANG, 2015; BORGES et al., 2016; GOPALAKRISHNAKONE et al., 2016; REIS et al., 2018). Assim, o estudo direcionado a caracterização do potencial antimicrobiano de PAMs derivados da toxina de artrópodes pode fornecer subsídios importantes para o desenvolvimento de novos antibióticos ou compostos protótipos contra patógenos resistentes que ameaçam a saúde pública global.

Melitina

A melitina é um dos principais componentes presentes no veneno da Abelha melífera (*Apis mellifera*) (**Figura 5**), representando 40 a 60% do peso seco do veneno (RADY et al., 2017; HOSSEN et al., 2017). Quimicamente, ela é caracterizada como um peptídeo composto de 26 resíduos de aminoácido sem nenhuma ponte dissulfeto no qual a porção *N*-terminal é predominantemente hidrofóbica enquanto a *C*-terminal é hidrofílica e fortemente básica. A massa molar da melitina é de 2846.46266 g/mol e a sua análise sequencial revela a seguinte configuração: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-GlnNH_2 (HOSSEN et al., 2017). Sua estrutura secundária revela uma predominância de α-hélice na porção *N*-terminal, o que está de acordo com as características hidrofóbicas dessa região (**Figura 6**).

Figura 5: Abelha melífera (*Apis mellifera*). FONTE: http://canalpontoazul2.blogspot.com/2013/01/abelha-europeia-apis-mellifera.html

Em um contexto biológico, a melitina encontra-se armazenada nas vesículas de veneno da *Apis mellifera* em uma conformação tetramérica de baixa toxicidade, a qual protege as células do inseto (**Figura 6**) (CARDOSO et al., 2003). Entretanto, quando o veneno é liberado após a picada, a melitina se dissocia em sua forma monomérica e promove um aumento na atividade catalítica das fosfolipases também presentes no veneno da abelha, contribuindo assim para seu potente efeito tóxico (CARDOSO et al., 2003; KOUMANOV et al., 2003). Além disso, a melitina possui efeito tóxico direto agindo ao desestabilizar membranas celulares e ao induzir a liberação do conteúdo citoplasmático de diversos tipos celulares (PICOLI et al., 2018).

No entanto, devido ao fato de a carga global da melitina ser de +6 em pHs neutros, a afinidade desse peptídeo em membrana com excesso de carga negativa, como as das células microbianas, é cerca de 100-vezes maior que para membrana com características zwitteriônicas, como aquelas encontradas em células de mamíferos (HOSSEN et al., 2017; PICOLI et al., 2018). Essa característica faz com que a melitina seja amplamente estudada devido suas propriedades antibacterianas e antifúngicas (PICOLI et al., 2018).

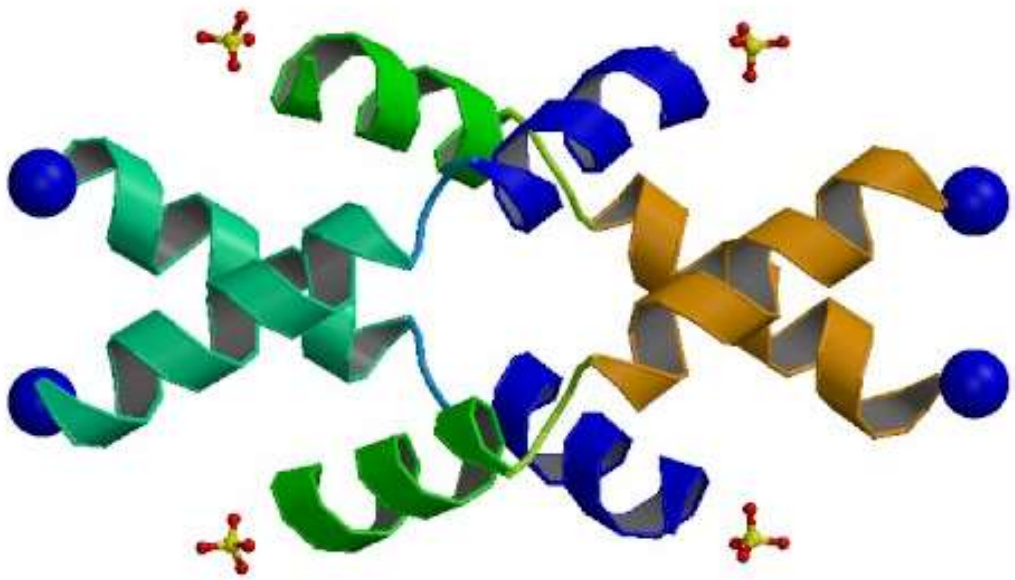

Figura 6: Estrutura tetramérica da melitina encontrada naturalmente nas vesículas da Abelha (*Apis mellifera*). FONTE: Protein Data Bank (PDB) 2MLT.

De fato, inúmeros trabalhos têm reportado os efeitos antimicrobianos desse peptídeo natural. Historicamente, a atividade antibacteriana do veneno da abelha foi primeiro reportado por Schmidt-Lange em 1941 (SCHMIDT-LANGE, 1941). A seguir, no início dos anos 1950 a melitina foi descoberta e caracterizada a partir da separação do veneno da abelha por eletroforese, e alguns anos mais tarde FENNELL et al. (1967) identificou esse peptídeo como o constituinte antibacteriano ativo dessa matriz (FENNELL et al., 1967). Nesse estudo pioneiro, os autores identificaram que a melitina exerce efeito inclusive em linhagens de *S. aureus* resistente às penicilinas, e foi reportado que sua ação antibacteriana é preferencialmente relacionada com bactérias

Gram-positivas (FENNELL et al., 1967). A partir de então inúmeros outros trabalhos *in vitro* destacou a atividade desse peptídeo contra várias espécies de bactérias e fungos de importância médica, inclusive contra linhagens multirresistentes (MEMARIANI et al., 2019).

Dentre as espécies mais susceptíveis a ação antibacteriana da melitina, se destaca o *Staphylococcus aureus*. Estudos *in vitro* prévios que avaliaram a atividade antibacteriana da melitina contra essa espécie revelou que a concentração inibitória mínima (CIM) varia de 0.5 á 8 μM contras cepas referência e isolados clínicos de *S. aureus* (MEMARIANI et al., 2019). Interessantemente, esses estudos apontam que os efeitos antibacterianos da melitina são ainda mais evidentes em cepas de MRSA (CHOI et al., 2015). A atividade antibacteriana pode ser também reportada em isolados de *S. aureus* com resistência intermediária e completa a vancomicina, destacando a atividade contra patógenos PDR que causam infecções consideradas de difícil tratamento (DESLOUCHES et al., 2015). Em adição as evidências *in vitro*, um estudo empregando um modelo de infecção dérmica com MRSA em camundongos mostrou que a melitina é também ativa em infecções *in vivo* (CHOI et al., 2015). Nesse caso, o uso tópico da melitina (10 μg) reduziu a área das feridas causadas pela inoculação dérmica dessa bactéria (CHOI et al., 2015).

Outro patógeno particularmente sensível aos efeitos antibacterianos da melitina é a *Escherichia coli*. PICOLI et al. (2017) mostraram que a melitina tem um valor de CIM de 40-42,5 μg/mL (~13 μM) e de concentração bactericida mínima (CBM) na faixa de 64-128 μg/mL (~20-40 μM) contra *E. coli* ATCC 8739. HAN et al. (2009), por sua vez, destacaram que a melitina purificada de veneno de abelha apresentou CIM de 0,125 μg/mL (~0,04 μM) contra *E. coli* ATCC 25922. No entanto, para o melhor do nosso conhecimento, nenhum estudo tem se dedicado a verificar o efeito antibacteriano da melitina contra exemplares clínicos de *E. coli* multirresistente.

Para a translação à testes clínicos, algumas informações da eficácia e segurança da melitina ainda necessitam ser elucidados. Nessa direção a estabilidade do peptídeo em condições frequentemente encontradas em regiões de infecção ativa (*e.g.*, pHs reduzidos, alta concentração salina, grande quantidade de proteínas), seu efeito anti-inflamatório em modelos de infecção, o potencial desse peptídeo em induzir resistência e sua atividade após a incorporação em formulações farmacêuticas

disponíveis comercialmente permanecem ainda para ser determinado em estudos futuros (MEMARIANI et al., 2019).

LyeTx I mnΔK

Outro peptídeo antimicrobiano promissor derivado da toxina de artrópodes trata-se do LyeTx I. Em 2010, SANTOS e colaboradores isolaram um peptídeo contendo 25 resíduos de aminoácidos, com uma modificação amida natural na extremidade *C*-terminal e sem modificação na porção *N*-terminal cuja sequência foi definida como H-IWLTALKFLGKNLGKHLAKQQLAKL-NH_2 (M.M. = 2831,1 Da). Esse peptídeo foi isolado por técnicas cromatográficas do veneno da aranha *Lycosa erythrognatha*, popularmente conhecida como tarântula, aranha-de-jardim ou aranha-de-grama (**Figura 7**) (SANTOS et al., 2010).

Figura 7: Aranha *Lycosa erythrognatha*, conhecida por tarântula. Fonte do autor

Estudos de caracterização da estrutura terciária do peptídeo LyeTx I revelou que ele possui uma orientação preferencial em α-hélice, devido a composição majoritariamente hidrofóbica da porção *C*-terminal (SANTOS et al., 2010). Em virtude do fato de que a maior parte dos peptídeos antimicrobianos apresenta essa conformação (ZHANG & GALLO, 2016; CHEN & LU, 2020), estudos *in vitro* foram conduzidos e revelou uma importante atividade desse peptídeo contra bactérias e fungos de interesse médico. Nessa direção, o LyeTx I revelou-se ativo contra *Escherichia coli* ATCC 25922 (CIM: 7,81 μM), *Staphylococcus aureus* ATCC 33591 (CIM: 3,79 μM), *Candida krusei* (CIM: 26,30 μM) e *Cryptococcus neoformans* (CIM: 13,20 μM) (SANTOS et al., 2010).

Posteriormente, a segurança do peptídeo LyeTx I foi estudada *in vitro* pelos ensaios de atividade hemolítica e capacidade de lise de membranas. Os resultados revelaram que o peptídeo foi capaz de provocar hemólise apenas quando utilizado em concentrações bastante elevadas (DE_{50} = 130 μM). Ainda, o peptídeo é capaz de alterar, de maneira dose-dependente, a permeabilidade de lipossomas constituídos por *L*-α-fosfatidilcolina, mimetizando sua ação na membrana celular. É importante ressaltar que a adição de colesterol na constituição dos lipossomas desfavoreceu a ação de LyeTx I, reduzindo em cinco vezes a permeabilidade das vesículas. Por outro

lado, a adição de ergosterol na formulação lipossomal não prejudicou a ação do peptídeo (SANTOS et al., 2010). Sabe-se que o colesterol está presente na constituição das membranas celulares de mamíferos, enquanto que o ergosterol está presente nas membranas das células fúngicas. Portanto, o perfil de alteração da permeabilidade de vesículas lipossomais e a reduzida atividade hemolítica de LyeTx I sugerem que este peptídeo apresenta baixa afinidade por células zwitteriônicas. Assim, os resultados relevantes encontrados para esse peptídeo renderam um depósito de patente no ano de 2012: BR n. PI2348A – “Composições farmacêuticas compreendendo peptídeos catiônicos incluídos e/ou associados à ciclodextrinas e usos” (DOS SANTOS et al., 2012).

Entretanto, a viabilidade farmacológica do LyeTx I esbarra no alto custo de produção, baixa estabilidade em contextos biológicos devido a alta susceptibilidade para a hidrólise por proteases e/ou peptidases plasmáticas e na toxicidade pronunciada muitas vezes associada a peptídeos com mais de 20 resíduos (FOSGERAU & HOFFMANN, 2015; CHEN & LU, 2020). Assim, no ano de 2015, com o objetivo de aperfeiçoar o seu emprego como um agente terapêutico, um derivado truncado e modificado foi proposto e denominado LyeTx I mnΔK (JÚNIOR, 2015). O peptídeo proposto foi sintetizado pelo emprego da técnica de síntese em fase sólida e a resolução da sua estrutura secundária por ressonância magnética nuclear revelou uma orientação em α-hélice (**Figura 8**) (BARBOSA, 2016). A sequência do LyeTx I mnΔK apresenta 16 resíduos de aminoácidos dispostos na seguinte conformação: H-IWLTKALKFLGKNLGK-NH_2 (JÚNIOR, 2015).

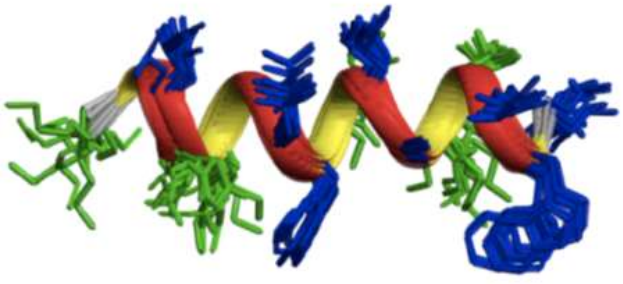

Figura 8: Estrutura secundária do peptídeo LyeTx I mnΔK resolvida por ressonância magnética nuclear. FONTE: Adaptado de BARBOSA, 2016.

Posteriormente, o peptídeo LyeTx I mnΔK foi testado contra diferentes espécies de microrganismos de importância médica e apresentou uma significativa atividade antibacteriana e antifúngica, especialmente contra *S. aureus* (JÚNIOR, 2015). Em adição, o uso intra-articular desse peptídeo mostrou-se promissor para o tratamento de artrite séptica por *S. aureus* á concentração de 0.8 μM, sendo ele hábil em reduzir a carga bacteriana significativamente em relação ao controle não tratado. Além disso, o LyeTx I mnΔK reduziu o infiltrado inflamatório na cápsula articular significativamente, mostrando um potente efeito anti-inflamatório nesse modelo (FUSCALDI, 2019).

Assim, os resultados prévios demostram a promissora atividade antibacteriana do peptídeo LyeTx I mnΔK, revelando-o como um potencial antibiótico. Entretanto falta ainda ser determinada para esse peptídeo sua atividade contra cepas multirresistentes e isolados clínicos de bactérias patogênicas, sua toxicidade *in vitro* e *in vivo*, sua atividade hemolítica, a estabilidade plasmática, sua habilidade de interagir sinergicamente com antibióticos comerciais e o seu potencial em selecionar cepas resistentes. Nessa direção, estudos futuros devem ser conduzidos para elucidar esses parâmetros.

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Objetivos

Objetivo geral

Avaliar a atividade antibacteriana e a toxicidade *in vitro* e *in vivo* de dois peptídeos derivados da toxina de artrópodes contra *Staphylococcus aureus* resistente a meticilina, *Escherichia coli* resistente as quinolonas e produtoras de ESBL, e *Acinetobacter baumannii* resistente aos carbapenêmicos, além de desenvolver formulações contendo esses peptídeos para o tratamento de infecções superficiais ou invasivas.

Objetivos específicos

Capítulo I

● Avaliar a atividade antibacteriana *in vitro* da melitina contra isolados clínicos de *Escherichia coli* uropatogênica (UPEC) resistente a quinolona e produtora de ESBL.

● Avaliar o efeito da melitina sobre a cinética de morte de células logarítmicas de um exemplar de UPEC resistente a quinolona e produtora de ESBL.

● Avaliar o efeito re-sensibilizante da combinação da melitina com antibióticos convencionais contra UPEC resistente a quinolona e produtora de ESBL.

● Avaliar o efeito bioativo de uma sonda vesical sensibilizada com melitina contra biofilmes formados por um isolado de UPEC resistente a quinolona e produtora de ESBL.

Capítulo II

● Avaliar a atividade antibacteriana *in vitro* da melitina contra isolados clínicos de *Staphylococcus* spp.

● Avaliar o efeito da melitina sobre a cinética de morte de células logarítmicas, estacionárias e persistentes de MRSA e a habilidade desse peptídeo em romper biofilme maduros de *S. aureus* e *S. epidermidis.*

● Investigar o mecanismo de ação da melitina frente à MRSA.

● Avaliar o efeito sinérgico e re-sensibilizante da combinação da melitina com antibióticos convencionais contra o MRSA.

● Avaliar a estabilidade da atividade antibacteriana da melitina em diferentes condições.

● Avaliar o potencial de indução de resistência pela melitina frente ao MRSA.

● Formular a melitina em uma pomada para uso externo.

● Avaliar o potencial terapêutico da pomada contendo melitina contra feridas induzidas por MRSA através da quantificação da carga bacteriana, do processo inflamatório (dosagem de citocinas) e da varredura da ferida com antibiótico radiomarcado.

Capítulo III

● Avaliar a atividade antibacteriana *in vitro* do LyeTx I mnΔK contra isolados clínicos de *Acinetobacter baumannii* resistente aos carbapenêmicos (CRAB).

● Investigar o mecanismo de ação do LyeTx I mnΔK frente à CRAB.

● Avaliar o efeito sinérgico e re-sensibilizante da combinação do LyeTx I mnΔK com antibióticos convencionais contra CRAB.

● Avaliar a estabilidade da atividade antibacteriana do LyeTx I mnΔK em diferentes condições.

● Avaliar o potencial de indução de resistência pelo LyeTx I mnΔK frente ao CRAB.

● Investigar a habilidade do LyeTx I mnΔK de romper biofilme bem estabelecidos de CRAB.

● Avaliar a toxicidade do LyeTx I mnΔK *in vitro* sobre células epiteliais de mamíferos e hemácias humanas.

● Formular o CRAB em uma suspensão para inalação.

● Avaliar o potencial terapêutico da suspensão contendo LyeTx I mnΔK contra pneumonia induzidas por CRAB através da quantificação da carga bacteriana pulmonar.

Capítulo I

Purificação da melitina e avaliação de sua atividade contra isolados clínicos de *Escherichia coli* uropatogênica resistentes as quinolonas e produtoras de beta lactamase de espectro ampliado

Antibacterial, anti-biofilm, and anti-adhesive activities of melittin, a honeybee venom-derived peptide, against Quinolone-resistant Uropathogenic *Escherichia coli* (UPEC)

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Abstract: Here, we demonstrated the *in vitro* and *in vivo* antibacterial and anti-biofilm activities of melittin, a peptide derived from honeybee venom, against uropathogenic *E. coli* (UPEC) resistant to quinolones. The minimum inhibitory concentration (MIC) of melittin varied from 0.5 to 8 µM. The bactericidal effect was considered rapid and potent (ranging from 3.0 to 6.0 h after incubation) against a quinolone-resistant and Extended Spectrum Beta-lactamase (ESBL)-producing UPEC strain. Prior exposure to melittin did not reduce the MIC of the quinolones tested, but it decreased the MIC of ceftizoxime by 8-fold due to its ability to form pores in the membrane. Furthermore, melittin disrupted mature biofilms (39.58% at 32 µM) and inhibited the adhesion of this uropathogen to the surfaces of urethral catheter. These results show that melittin is a promising molecule that can be incorporated into invasive urethral medical devices to prevent urinary infections caused by multidrug-resistant UPECs.

Keywords: *Escherichia coli*; Urinary Tract Infections; Urethral catheter; Antimicrobial peptides; Biofilm

1. Introduction

Escherichia coli, a member of the *Enterobacteriales* family, is a Gram-negative glucose-fermenting rood that typically inhabits the gastrointestinal tract of humans and animals (Vila et al. 2016). As a commensal, it lives in a mutually beneficial association with hosts. However, some *Escherichia coli* strains are commonly implicated in relevant clinical infections, such as urinary tract infections (UTI) (Kaper et al. 2004). Indeed, community-acquired UTIs (CA-UTI) caused by uropathogenic *E. coli* (UPEC) are the most common bacterial infection, affecting approximately 150 million people annually worldwide (mainly women) (Kucheria et al. 2005). The antibiotics fosfomycin and sulfas are the first choices to treat CA-UTI; however, quinolones have been the most frequently prescribed antibiotic in these cases. This indiscriminate use of quinolones has increased the selective pressure on uropathogens, significantly increasing the incidence of quinolone-resistant UPECs (Lee et al. 2016; da Silva et al. 2017; Vieira et al. 2020).

Infections caused by quinolone-resistant UPECs usually evolve to life-challenge complications, such as pyelonephritis, bacteremia, and florid urosepsis. These complications are treated with intravenous antibiotic therapy, in which only a few options are available (*e.g.*, polymyxins, aminoglycosides, cephalosporins) (Nicolle et al. 2005; Yamamoto 2016). To make this scenario even more critical, quinolone-resistant UPECs strains typically carry enzymes that encode resistance to other antibiotics, mainly broad-spectrum beta-lactamases (ESBL) associated with resistance against cephalosporins, and the aminoglycosides acetyltransferase that modifies gentamicin, amikacin, and tobramycin (Ali et al. 2016; Halaji et al. 2020). Therefore, the development of new pharmacological agents against quinolone-resistant UPECs is urgently needed. In this context, antimicrobial peptides stand out as a promising source of new anti-UPECs antibiotics. These agents are known to possess a potent bactericidal effect, low capacity to induce resistance, good efficacy in low concentrations, absence of waste generation after the use of conventional doses, and potent immunomodulatory effect (Bechinger & Gorr 2017; Chen & Lu 2020; Lima et al. 2021). Moreover, antimicrobial peptides are usually highly hydrophilic and thereby captured by the urinary tract after an intravenous administration (Kang et al. 2014;

Chen & Lu 2020). The latter is a significant pharmacokinetic advantage in the case of UTIs.

One of the best consolidated sources of antimicrobial peptides is the toxin of venomous animals (Wu et al. 2018), especially arthropods (Samy et al. 2017). Honeybee (*Apis mellifera* L.) venom contains a complex mixture of therapeutic compounds, including antimicrobial peptides, allowing bees to defend their hives against predators and external threats (Leandro et al. 2015; El-Seedi et al. 2020). Several biological and pharmacological studies have examined the peptide melittin, which is the major component of bee venom (40–48%, w/w) (Memariani et al. 2019). This bee venom-derived peptide has been extensively investigated and exhibits potent cytolytic and antimicrobial activities (Choi et al. 2015; Lima et al. 2021). However, little is known about its antibacterial potential against clinically relevant multi-resistant species, such as UPECs (Lima et al. 2021). Therefore, this study aims to evaluate the antibacterial effect of melittin against several planktonic quinolone-resistant UPECs, as well as to investigate its effect on the biofilms formed by these pathogens.

2. Material and Methods

2.1. Reagents

Colistin, Gentamicin (Inlab, São Paulo, SP, Brazil), ciprofloxacin, levofloxacin, α-cyano-4-hydroxycinnamic acid, trifluoroacetic acid (TFA), acetonitrile (Sigma-Aldrich, Frankfurt, Germany), crystal violet, 95% ethanol, glucose, and sodium chloride (Synth, São Paulo, SP, Brazil) were purchased from commercial suppliers and used without further purification. Mueller–Hinton broth (MHB) and agar (MHA), nutrient agar, MacConkey agar, and trypticase soy broth (TSB) were purchased from Kasvi (São José do Pinhais, PR, Brazil). Apitoxin was provided by Ezequiel Dias Foundation (FUNED) and was collected following Benton's procedure (Benton et al. 1963). Meal-worm beetle (*Tenebrio molitor*; Coleoptera) larvae were purchased from a company that is specialized in agricultural products (Mercado Central de Belo Horizonte, Minas Gerais, Brazil). Polyvinyl chloride (PVC) urethral catheters (Embramed®; São Paulo, SP, Brazil) were obtained from stores specialized in hospital-medical supplies.

2.2. Microorganisms

The antibacterial activity was determined against forty-one clinical isolates of quinolone-resistant UPEC kindly provided by Profª. Drª. Magna Cristina Paiva (Laboratory of Clinical Microbiology, Universidade Federal de São João Del Rei, Divinpolis, MG, Brazil). All isolates were recovered from patients with community-acquired or hospital-acquired UTI. The bacterial identification was performed by the automated system Vitek®2 (bioMerieux, Hazelwood, MO). The susceptibility profile to several antimicrobials was determined using Kirby-Bauer assay, as showed in a previous study. (Paiva et al. 2012) In addition, one reference strain from the American Type Culture Collection (ATCC) (*E. coli* ATCC 8739) was kindly provided by the Microbiology Reference Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ-RJ, Brazil) and included in this study.

2.3. Melittin purification and characterization

Honey bee venom (100 mg) was solubilized in 5 mL of purified water and then filtered through a 0.22 µm syringe filter. The filtered product was purified by high-performance liquid chromatography (HPLC; Shimadzu® LC20AD HPLC with SPDM20A and RID 20A detector) on a C18 column (Shimadzu® C18 column, 10 µm, 250 mm, 20 mm), previously balanced with 0.1% v/v trifluoroacetic acid (TFA) in water - phase A - and eluted in a flow of 3 mL/min by the following linear gradient of 0.08% v/v TFA in acetonitrile - phase B: 00 to 08 minutes - 10% phase B, 08 to 48 minutes - 100% phase B, 48 to 50 minutes - 10% phase B, 48 to 55 minutes - 10% phase B and 55 to 65 minutes - 10% phase B.

The fractions of the bands with higher absorptions were collected and evaluated by ionization-time analysis by laser desorption, assisted by matrix, in a flight time mass spectrometer (MALDI-TOF-MS) in AutoFlex III (Bruker Daltonics®, Germany). Briefly, the samples were placed on a plate (MTP 384 Anchorchip Bruker Daltonics®, Germany) mixed with a saturated solution of cyano-4-hydroxycinic acid (alpha-cyano) and left to dry at room temperature. These and the standards were read in MALDI-

TOF-MS with the pepmix method (up to 4 kDa). Mass spectra (MS) were acquired in positive reflector mode with an external calibrator (Peptide Calibration Standard II, Bruker Daltonics®, Germany) (Strohalm et al. 2008).

2.4. Antibacterial activity

Inoculum preparation: The bacterial inoculum employed in the susceptibility tests was standardized according to the Clinical Laboratory and Standard Institute (CLSI) document M07 (Clinical and Laboratory Standards Institute 2018). Approximately three to five isolated colonies collected from a 24 h UPEC culture in nutrient agar were suspended in 10 mL sterile saline (0.9% NaCl). The resulting suspension was then adjusted to the McFarland 0.5 scale (*i.e.*, 10^8 colony forming units (CFU)/mL) using a spectrophotometer (Nova Instruments, Sao Paulo, SP, Brazil), which correspond to an optical density (OD) of ~ 0.2 at 625 nm. Then, 50 µL of the bacterial suspension was transferred to 10 mL of MHB, resulting in a final inoculum of 10^6 CFU/mL.

Minimum inhibitory concentration (MIC): The bacteriostatic activity was evaluated by the determination of the MIC using the broth microdilution method according to M07 document of CLSI for bacteria that grow aerobically (Clinical and Laboratory Standards Institute 2018), with minor modifications (Lima, Alves-Nascimento, et al. 2019). 100 µL from a UPEC inoculum (10^6 CFU/mL) was added to sterile microplates previously filled with 100 µL of a two-fold serial dilution (0.5–32 µg/mL) of melittin in MHB. The plates were subsequently incubated at 35±2°C for 18 h. The MIC was then defined as the lowest concentration of the peptide that inhibited the visible growth of bacteria. Colistin and Gentamicin were used as positive controls (0.5 to 32 µg/mL), and the media was supplemented with 0.002% polysorbate 80 to prevent drug binding to plastic surfaces of microplates.

Minimum bactericidal concentration (MBC): The bactericidal activity was determined by pipetting 10 µL from the wells where no growth was detected in the MIC plates and incubating these samples in MHA at 35±2°C for 24 h, as previously described (Lima, dos Santos, et al. 2019). The MBC was considered the lowest concentration of melittin/controls that completely inhibits bacterial growth.

Time-kill curve: Initially, a pre-inoculum of a quinolone-resistant and ESBL-producing UPEC strain (EC49) at 10^8 CFU/mL was prepared as previously described. Then, 50 µL of the pre-inoculum was added to test tubes containing 10 mL of MHB (*i.e.*, resulting in a bacterial suspension of 10^6 CFU/mL). Bacteria were then challenged with melittin at the 2x, 5x, and 10x MIC. Untreated cells and colistin at 10x MIC were used as the negative and positive controls, respectively. The tubes were incubated at 35±2°C with aeration at 225 rpm. At intervals of 0, 0.5, 1, 3, 6, 12, and 24 h, 100 µL of the samples were serially diluted (10^{-1}-10^{-6}) in sterile saline (0.9% NaCl) and plated onto MHA. The plates were incubated for 24 h, and the CFU/mL was determined (Andrade et al. 2018).

2.5. Re-sensitization assay

Re-sensitization of a quinolone-resistant and ESBL-producing UPEC strain (EC49) to quinolones (ciprofloxacin and levofloxacin) and cephalosporin (ceftizoxime) was performed as previously described (Lima et al. 2021). Colistin was used as the positive control. The results were expressed as the fold-change of re-sensitization, calculated by the ratio of the MIC of the antibiotic alone and the MIC of the antibiotic after re-sensitization with ½ × MIC of melittin or colistin.

2.6. Anti-biofilm assay

Mature biofilm: The effects of melittin on the disruption of the established biofilm of a quinolone-resistant and ESBL-producing UPEC strain (EC49) was evaluated using the microtiter plate biofilm formation assay as previously described (Herrera et al. 2020). The UPEC inoculum at 1x10^6 CFU/mL was incubated at 35±2°C for 24 h in tryptic Soy Broth (TSB) supplemented with 1 mM glucose to allow biofilm formation and adhesion. Then, the medium was removed, and microtiter plates were washed three times with sterile saline (0.9% NaCl). Biofilms were treated with melittin diluted in TSB with 1 mM glucose at 32, 16, or 8 µM, and the microtiter plates were incubated for 24 h at 35±2°C. After incubation, the medium was removed, all wells were washed three times with sterile saline (0.9% NaCl), and the biofilms were stained with 0.1% w/v crystal violet for 30 minutes at room temperature. The wells were then washed three times, air dried,

and biofilm masses were solubilized in 95% ethanol. The OD at 595 nm was measured using a spectrophotometer (Bio-Tek Instruments, Winooski, VT), and the results were expressed graphically as a function of the percentage of reduction of mature biofilm *vs.* the concentration of extract/controls.

Catheter sensitization: One centimeter (1 cm) sterile PVC urethral catheter segments were directly immersed in a stirred solution containing melittin at 1 mg/mL or 10 mg/mL for 90 min. The segments were dried overnight and rinsed with 1 mL sterile distilled water to remove any unbound compound.

Biofilm colonization of catheters: Biofilm formation on sterile catheter segments was carried out according to Sousa et al. (de Sousa et al. 2019), with modifications. Briefly, the segments were immersed in tubes containing a bacterial suspension of a quinolone-resistant and ESBL-producing UPEC strain (EC49) at 10^6 CFU/mL in TBS supplemented with 1 mM glucose. All tubes were incubated at 35±2^{o}C for 24 h to allow biofilm formation and adhesion on the catheter surface. Then, the bacterial cultures were discarded, and all catheter segments were added in tubes with 5 mL of sterile saline (0.9% NaCl) and remained under shaking for 30 min. The segments were removed with sterilized dissection forceps, transferred to another tube containing 5 mL of sterile saline (0.9% NaCl), and sonicated for 5 min at 40 KHz (Soniclean, New York, NY, USA). The samples were then homogenized, and 100 µL was aliquoted, serially diluted (10^{-1} - 10^{-6}) in sterile saline (0.9% NaCl), and plated onto MacConkey agar. The plates were incubated at 35±2^{o}C for 24 h, and the CFU/cm of the catheter was determined by colony counting.

2.7. Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation of the replicates. One-way analysis of variance (ANOVA) followed by Tukey's multiple analysis were used to compare differences between treated biofilms and controls. Furthermore, the results of the *in vivo* experiment were arranged in a survival curve using the Kaplan–Meier method, and statistical analysis

was performed using the log-rank test. All statistical analyses were assessed using GraphPad Prism 5.03 (GraphPad Software Inc., LaJolla, CA), and p values <0.05 were considered statistically significant.

3. Results and Discussion

Community-acquired urinary tract infections (CA-UTIs) are caused mainly by UPECs (70-90% of all cases) and exhibit a high recurrence rate, which increases morbidity rates worldwide, especially in women (Kucheria et al. 2005). The treatment of CA-ITUs is based on the use of various classes of antimicrobials, of which quinolones are considered the second-line treatment against UPECs. However, due to the indiscriminate use of this class of antibiotics, strains of quinolone-resistant UPEC have been extensively reported in the last decades (Lee et al. 2016; da Silva et al. 2017; Vieira et al. 2020). In this context, the development of new antibacterial agents against quinolone-resistant UPECs is crucial. Antimicrobial peptides derived from animal toxins are a rich source of new biologically active compounds, and the potential of honeybee venom needs to be evaluated (Primon-Barros & Macedo 2017; El-Seedi et al. 2020). Thus, the antibacterial activity of melittin, a peptide derived from honeybee venom, was investigated in this study against 41 clinical isolates of quinolone-resistant UPECs.

Initially, melittin was purified and characterized from honeybee venom. The chromatogram of the apitoxin presented several peaks, in which the retention time of melittin was 42.735 minutes (**Figure 1**). In addition, this peak was confirmed by MALDI-TOF mass spectrometry and showed a peak of 2,845.88Da, a value equivalent to the molar mass of melittin (Strohalm et al. 2008). Since more than 90% of the chemical composition of the dry mass of the bee venom is peptides, enzymes, or proteins, the concentration of melittin was estimated in apitoxin as 45%, which is within the expected

range and varies between 40% and 60% of the dry weight of apitoxin (Son et al. 2007). The final purification yield was 90%, and its purity was 93%.

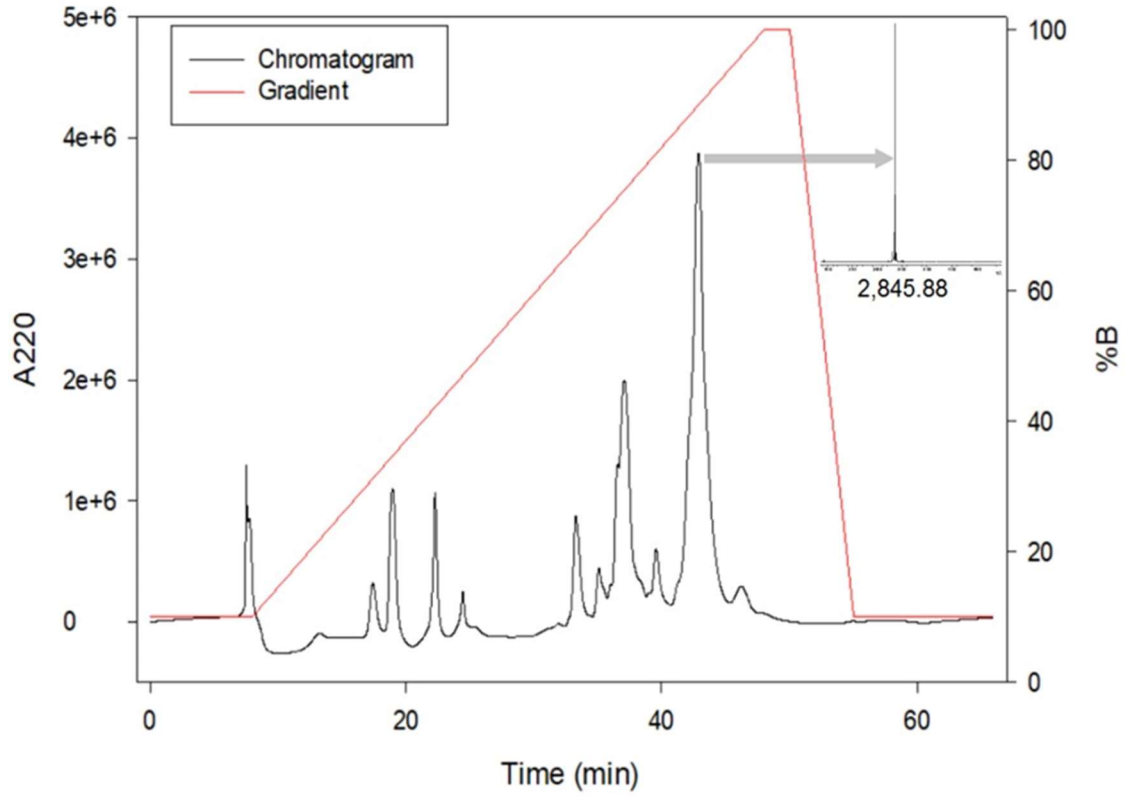

Figure 1: Chromatogram of apitoxin showing the peak of the collected melittin and the result of MALDI-TOF/MS

Melittin was highly active against clinically relevant quinolone-resistant UPECs, inhibiting the growth of all isolates tested at concentrations ranging from 0.5 to 8 μM (**Table 1**). The concentrations of this peptide required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were 4 μM and 8 μM, respectively, while colistin presented an MIC_{50} and MIC_{90} of 1 μM and 1.9 μM, respectively. Importantly, melittin kept its antibacterial activity against UPECs strains resistant to several classes of antibiotics, including cephalosporin, amino-penicillin, amino-penicillin+β-lactamase inhibitors, and aminoglycosides, suggesting that cross-resistance between these particular antibiotics and melittin is unlikely to occur (Mohamed et al. 2016). Furthermore, the antibacterial concentration of melittin against UPECs was lower than the cytotoxic concentration against mammalian kidney cells (MDCK cells: CC_{50} 15,8 μg/mL), suggesting a good selectivity for prokaryotic cells in relation to zwitterionic eukaryotic cells (Alsafar et al. 2020).

The MICs reported in this study were similar to those found in previous studies. Stocker and Trayno, in a pioneer study conducted in 1986, showed that melittin was active against *Escherichia coli* NCIR 9552 at 7 µg/mL (~2 µM) (Stocker & Trayn or 1986). Picoli et al. (2017) found that melittin has MIC of 40–42.5 µg/mL (~13 µM) and MBC of 64-128 µg/mL (~20-40 µM) against *E. coli* ATCC 8739 (Picoli et al. 2017). In another study, Han et al. (2009) demonstrated that the MIC of melittin purified from honeybee venom against *E. coli* ATCC 25922 was 0.125 µg/mL (~0.04 µM) (Han et al. 2009). However, to the best of our knowledge, this is the first study to point out the effect of melittin against a group of 41 isolates of clinically relevant multidrug-resistant (MDR) *Escherichia coli*. Melittin has been well explored as an antibacterial agent; however, a gap still exists regarding its spectrum of antimicrobial activity. One of the most relevant questions is: Does melittin maintain its potent antibacterial effect against MDR strains? Although a considerable effort has been made to answer this question, most of the studies have focused on MDR Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), and vancomycin-resistant *Enterococcus* (Choi et al. 2015; Memariani et al. 2019). However, little is known about the antibacterial effect of melittin against Gram-negative multi-resistant pathogens.

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of melittin and controls (colistin and Gentamicin) against ciprofloxacin-resistant uropathogenic *Escherichia coli* (UPEC) strains

Strains	Resistance profile	Phenotype/ Genotype	Melittin (μM)		Gentamicin (μg/mL)		Colistin (μg/mL)	
			MIC	MBC	MIC	MBC	MIC	MBC
ATCC 8739	-	-	2	4	≤ 0.5 (S)	1	1 (S)	1
SM17	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	1	1	≤ 0.5 (S)	≤ 0.5	0.5 (S)	0.5
SJ19	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	1	2	≤ 0.5 (S)	≤ 0.5	0.25 (S)	0.25
SJ16	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	1	2	1 (S)	1	1 (S)	8
SJ4	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	2	4	≤ 0.5 (S)	≤ 0.5	2 (S)	2
SM16	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	8	32	1 (S)	2	2 (S)	2
SM26	AMC, ATM, CRO, CAZ, CTX, CFO, CIP, GEN	ESBL	8	8	> 32 (R)	-	1 (S)	2
SM4	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	4	4	≤ 0.5 (S)	2	1 (S)	8
SJT1	AMC, ATM, CRO, CAZ, CTX, CFO, CIP, GEN	ESBL	4	8	16 (R)	32	1 (S)	1
SM2	AMC, ATM, CRO, CAZ, CTX, CFO, CIP, GEN	ESBL	4	8	32 (R)	> 32	1 (S)	2

Strains	Resistance profile	Phenotype/ Genotype	Melittin (μM)		Gentamicin (μg/mL)		Colistin (μg/mL)	
			MIC	MBC	MIC	MBC	MIC	MBC
EC50	CIP, AMC, ATM, CRO, CAZ, CTX, CFO	ESBL	4	16	≤ 0.5 (S)	0.5	1 (S)	1
SM23	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	2	2	≤ 0.5 (S)	1	1 (S)	2
SM10	AMC, ATM, CRO, CAZ, CTX, CFO, CIP, GEN	ESBL	4	4	> 32 (R)	-	1 (S)	1
SL5	AMC, ATM, CRO, CAZ, CTX, CFO, CIP, GEN	ESBL	2	2	8 (R)	8	1 (S)	1
SM1	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	4	4	≤ 0.5 (S)	2	1 (S)	1
SL2	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	8	8	0,5 (S)	0.5	1 (S)	8
SJ6	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	4	2	≤ 0.5 (S)	≤ 0.5	0.5 (S)	0.5
SJ15	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	8	16	≤ 0.5 (S)	≤ 0.5	1 (S)	1
SJ17	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	4	4	≤ 0.5 (S)	≤ 0.5	2 (S)	2
SM25	AMC, ATM, CRO, CAZ, CTX, CFO, CIP	ESBL	8	16	≤ 0.5 (S)	1	2 (S)	2
EC81	NAL, OFX, NOR, CIP, LVX, GEN	GyrA (S83L/D87N)	4	4	> 32 (R)	-	0.5 (S)	2
EC20	MER, GEN, SUT, NAL, OFX, NOR, CIP, LVX	GyrA (S83L/D87N)	4	8	≤ 0.5 (S)	0.5	1 (S)	1

Strains	Resistance profile	Phenotype/ Genotype	Melittin (μM)		Gentamicin (μg/mL)		Colistin (μg/mL)	
			MIC	MBC	MIC	MBC	MIC	MBC
EC100	CIP, GEN	Undetermined	4	16	> 32 (R)	-	1 (S)	2
EC68	CIP	Undetermined	2	16	≤ 0.5 (S)	0.5	1 (S)	2
EC93	CIP	Undetermined	4	16	1 (S)	1	1 (S)	1
EC59	CIP	Undetermined	8	16	1 (S)	2	0.1 (S)	0.5
EC90	NAL, OFX, NOR, CIP, LVX, AMP	GyrA (S83L/D87N)/ParC (S80L) and QnrS1	8	16	1 (S)	2	1 (S)	1
EC18	CRO, CTX, CIP	Undetermined	4	8	0.5 (S)	1	1 (S)	1
EC83	CIP	Undetermined	2	2	0.5 (S)	1	0.1 (S)	1
EC92	CIP	Undetermined	8	8	≤ 0.5 (S)	8	1 (S)	4
EC5	CFO, CIP	Undetermined	4	16	0.5 (S)	1	0.5 (S)	0.5
EC55	CIP	Undetermined	0.5	8	≤ 0.5 (S)	1	1 (S)	2
EC66	CIP	Undetermined	2	8	≤ 0.5 (S)	1	1 (S)	4
EC30	CIP, AMC, ATM, CRO, CAZ, CTX, CFO	ESBL	4	4	≤ 0.5 (S)	1	1 (S)	2

Strains	Resistance profile	Phenotype/ Genotype	Melittin (µM)		Gentamicin (µg/mL)		Colistin (µg/mL)	
			MIC	MBC	MIC	MBC	MIC	MBC
EC31	NAL, OFX, NOR, CIP, LVX,GEN	GyrA (S83L/D87N) mutations	2	2	16 (R)	32	2 (S)	2
EC62	CIP	Undetermined	4	8	≤ 0.5 (S)	1	0.5 (S)	1
EC65	CIP	Undetermined	4	4	≤ 0.5 (S)	1	1 (S)	1
EC13	CIP	Undetermined	2	16	0.5 (S)	1	2 (S)	4
EC11	NAL, OFX, NOR, CIP, LVX	GyrA (S83L/D87N) mutations	8	8	0.5 (S)	1	0.1 (S)	0.1
EC97	NAL, OFX, NOR, CIP, LVX	GyrA (S83L/D87N/E153A/K154T/P156L)	2	16	≤ 0.5 (S)	1	1 (S)	1
EC99	CIP, GEN	Undetermined	2	16	8 (R)	16	2 (S)	8
EC58	NAL, OFX, NOR, CIP, LVX	GyrA (S83L/D87N) mutations	8	8	≤ 0.5 (S)	1	2 (S)	2
EC49	CAZ, CTX, CFO, NAL, OFX, NOR, CIP, LVX	ESBL and GyrA (S83L/D87N) mutation	8	8	0.5 (S)	1	2 (S)	2

MIC_{50}: Concentration required to inhibit 50% of isolates; MIC_{90}: Concentration required to inhibit 90% of isolates; MBC_{50}: Concentration required to kill 50% of isolates; MBC_{90}: Concentration required to kill 90% of isolates. MEM: Meropenem ; AMC: Amoxicillin / clavulanic acid ; ATM: Aztreonam; CRO: Ceftriaxone; CAZ: Ceftazidime; CTX: Cefotaxime; CFO: Cefoxitin; NAL: Nalidixic acid; OFX: Ofloxacin; NOR: Norfloxacin; CIP: Ciprofloxacin; LVX : Levofloxacin; GEN: Gentamicin. S: Sensitive; I: Intermediate ; R: Resistant according to CLSI (2018)

Melittin presented a predominantly bactericidal effect (**Table 1**). This peptide was able to kill quinolone-resistant UPECs isolates at concentrations ranging from 2 to 16 µM, showing MBC_{50} and MBC_{90} of 8 µM and 16 µM, respectively. Cationic AMPs, such as melittin, are known to possess mainly bactericidal action since they can interact electrostatically with the bacterial anionic membrane, forming pores and leading to lysis and microbial death (Pandey et al. 2010; Memariani et al. 2019; Lima et al. 2021).

Moreover, the time-kill curve study confirmed the potent bactericidal effect of melittin (**Figure 2**). In this assay, for a substance to be considered bactericide a reduction in the number of CFU of 3 log_{10} should be obtained (Alder & Eisenstein 2004). Melittin presented a rapid bactericidal effect, eliminating a high microbial load (1 x 10^6 CFU/mL) of quinolone-resistance and ESBL-producing UPEC strain within 3 h at 5x and 10x MIC. At 2x the MIC, melittin took 6 h to sterilize the culture medium, while colistin at 10x MIC has maximum effect after 30 min of incubation (**Figure 2**). The bactericidal effect of melittin on *E. coli* was slower and less potent than that presented against methicillin-resistant *Staphylococcus aureus* in a previous study conducted by our group (Lima et al. 2021), indicating that the microbicidal action of this peptide is more powerful in Gram-positive bacteria compared to Gram-negative species. However, the fast and efficient elimination of UPEC showed in this work minimizes the risk of complications of urinary infection, reduces the antimicrobial concentration required to produce the desired effect, decreases the likelihood of resistance induction during clinical use, and restricts treatment time (Alder & Eisenstein 2004).

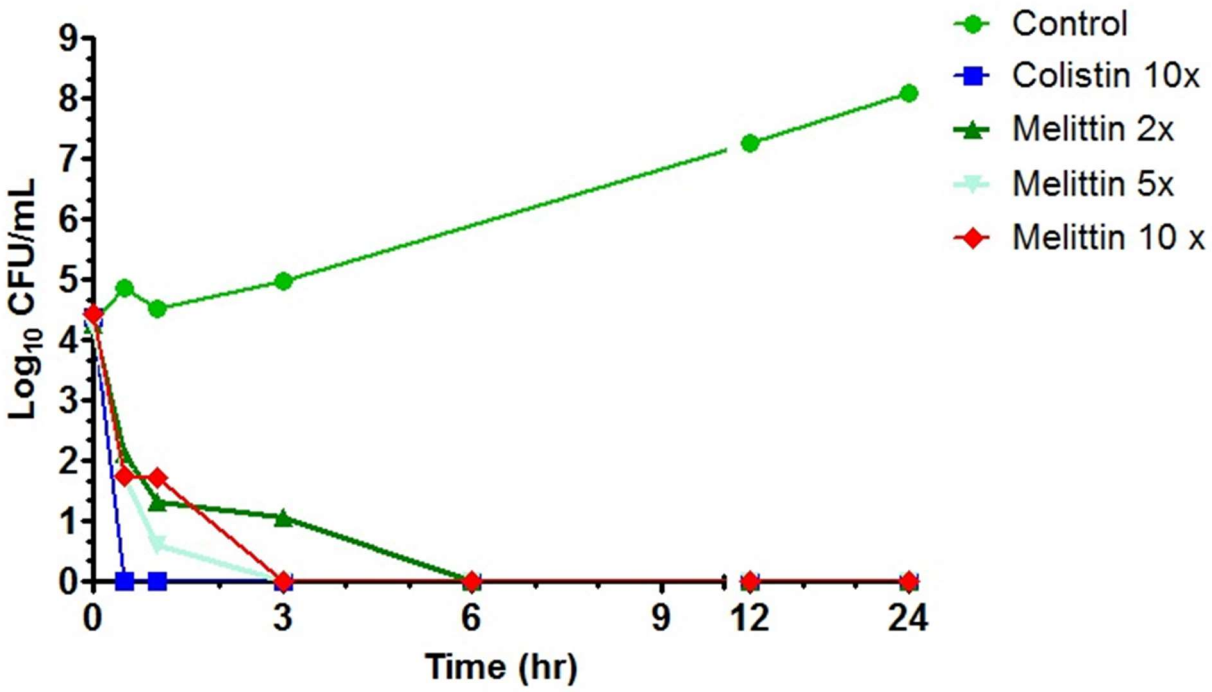

Figure 2: Time-Kill curve of melittin against a quinolone-resistant and ESBL-producing UPEC. The plot shows the number of logarithmic colony forming units per milliliter (Log_{10}CFU/mL). Bacterial cells untreated were used as negative control (Green circles). Colistin was employed as the positive control at 10x MIC (20 µg/mL) (Blue square). Melittin was tested at concentrations of 2x (16 µg/mL) (green triangle), 5x (40 µg/mL) (clear green invertible triangle), or 10x MIC (80 µg/mL) (red diamond).

The combination of melittin with clinically available drugs is an effective way to reduce its toxic effect since it reduces the concentration of each of the agents during therapy (Tängdén 2014). In this study, we evaluated whether prior exposure to sub-inhibitory concentrations of melittin reduces the MIC of quinolones and beta-lactams of a quinolone-resistant and an ESBL-producing UPEC. As shown in **Table 2**, while previous exposure to melittin reduces the MIC of ceftizoxime by 8-folds, this effect was not detected for the quinolones tested. The mechanism of resistance to cephalosporin in this isolate is through ESBL production, a saturable system (Rawat & Nair 2010). As melittin forms pores in the bacterial cell membrane (Pandey et al. 2010), it allows the entry of large amounts of the drug, which saturates the enzyme, and then the compound can perform its antibacterial action. The mechanism of resistance to

quinolone, on the other hand, is by target modification. Indeed, quinolone resistance in *Enterobacteriales* was initially attributed to chromosomal mutations in the quinolone-resistance-determining regions (QRDRs) harboring the genes encoding gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), which codes for antibiotic-insensitive molecular targets (Vieira et al. 2020). Thus, even though the intracellular concentration of quinolones increases, they will not perform their antimicrobial action since the molecular target is not affected by the compound.

Table 2: Re-sensitization of a quinolone-resistant and ESBL-producing Uropathogenic *Escherichia coli* (UPEC) to ceftizoxime, levofloxacin, and ciprofloxacin using a sub-inhibitory concentration (½ × MIC) of melittin.

Strain ID	**Antibiotics**	**Fold of re-sensitization**	
		Melittin (MIC After → Before exposition)	**Colistin** (MIC After → Before exposition)
E. coli EC49	Ceftizoxime	**8** (MIC 4 µg/mL → 0.5 µg/mL)	**2** (MIC 4 µg/mL → 2 µg/mL)
	Ciprofloxacin	**No sensitization effect** (MIC 128 µg/mL → 128 µg/mL)	**No sensitization effect** (MIC 128 µg/mL → 128 µg/mL)
	Levofloxacin	**No sensitization effect** (MIC 64 µg/mL → 64 µg/mL)	**No sensitization effect** (MIC 64 µg/mL → 64 µg/mL)

Fold of re-sensitization: It is the ratio of the MIC of antibiotic alone divided by the MIC of antibiotic after re-sensitization with (½ × MIC) of melittin.

In addition to being the microorganism most frequently involved in CA-UTIs, *E. coli* is also the most common bacteria in catheter-associated UTIs in hospital settings (Jacobsen et al. 2008). Catheter-associated UTIs are currently recognized as the most common type of nosocomial infection and account for over 1 million cases annually (Jacobsen et al. 2008) and over 40% of all nosocomial infections in hospitals worldwide (Warren 1997). In these cases, the infection is directly associated with the formation of bacterial biofilm on the inert surface of catheters. This bacterial community produces a mucopolysaccharide coating that prevents the penetration of antibiotics and keeps the microorganisms inside it in a stationary phase of growth (*i.e.*, a phase insensitive to the action of most antibiotics, since they are often designed to act on logarithmic growing bacteria) (Mittal et al. 2015). Thus, to assess the potential application of melittin against biofilms, we assessed the ability of this antibacterial peptide to disrupt

mature biofilms and inhibit colonization/biofilm formation on the surface of the urethral catheter. According to **Figure 3A**, the bacterial load of urethral catheters treated with melittin at 1 mg/mL (2.02 ± 0.41 Log_{10}CFU/cm) and 10 mg/mL (0.76 ± 0.53 Log_{10}CFU/cm) was significantly lower than that observed in untreated catheter (3.76 ± 0.48 Log_{10}CFU/cm). These results show for the first time that melittin inhibits the adhesion and colonization of bacteria to the surfaces of invasive medical equipment. As shown in our study, urethral catheter functionalized with melittin has increased resistance to bacterial colonization, which can be associated with a reduced frequency of latent infections. These infections are often difficult to treat and can be potentially fatal, especially among critically ill patients.

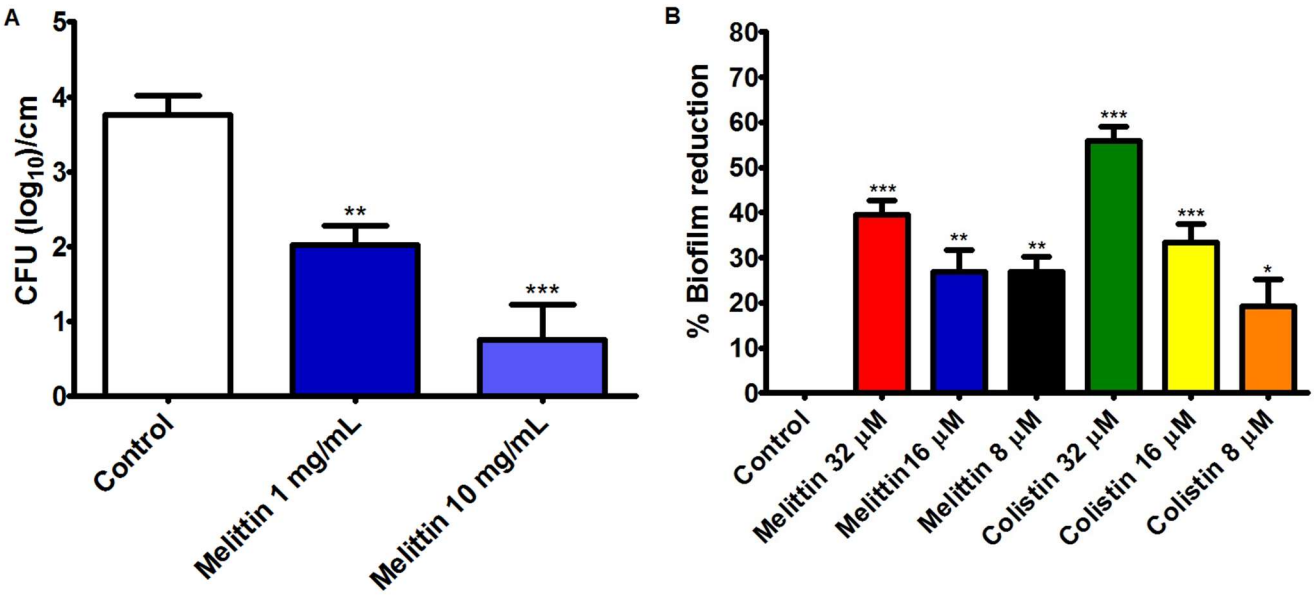

Figure 3: Activity of melittin or colistin against biofilms formed by a quinolone-resistant and ESBL-producing UPEC strain on PVC urethral catheter surface (A) and against preformed structures (B). All experiments were done in triplicate for statistical significance. Two asterisks (**) indicate statistically different compared to the control with $p < 0.01$. Three asterisks (***) indicate statistically different compared to the control with $p < 0.0001$. The results were analyzed by One-way ANOVA with Dunnett post-hoc.

The effect of melittin was also investigated against pre-established biofilms. This peptide significantly reduced the UPEC biofilm biomass at 32 µM and 8 µM by 39.58% and 26.78%, respectively. The anti-biofilm effect of melittin was similar to the effect observed for colistin, which reduced the preformed UPEC biofilm by 55.91% 19.21% at 32 µM and 8 µM, respectively (**Figure 3B**). Similarly, other studies have confirmed

the activity of melittin against biofilms formed by reference strains of *E. coli* (Han et al. 2009; Picoli et al. 2017). Indeed, antimicrobial peptides are known for their high activity against mature bacterial biofilms because they induce the disruption or degradation of the membrane potential of biofilm embedded cells, inhibit the signaling systems of bacteria by downregulation the genes responsible for biofilm formation and transportation of binding proteins, and degrade the polysaccharide and biofilm matrix (Yasir et al. 2018).

4. Conclusion

The results show that melittin has a potent antibacterial effect against UPECs, regardless of their resistance to clinically available antibiotics. Melittin also showed an anti-biofilm effect, breaking mature biofilm and inhibiting the adhesion of bacteria to sensitized urethral catheter. Taken together, these data indicate that melittin is a promising prototype for the development of more effective therapies against urinary tract infections by quinolone-resistant UPECs.

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6. Compliance with Ethical Standards

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Capítulo II

Atividade terapêutica da melitina contra feridas induzidas por *Staphylococcus aureus* resistente a meticilina

In-depth characterization of antibacterial activity of melittin against *Staphylococcus aureus* and use in a model of non-surgical MRSA-infected skin wounds

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Abstract: Skin infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) require the development of new and effective topical antibiotics. In this context, melittin, the main component of apitoxin, has a potent antibacterial effect. However, little is known regarding the anti-inflammatory potential this peptide in infection models, or its ability to induce clinically important resistance. Here, we aimed to conduct an in-depth characterization of the antibacterial potential of melittin in vitro and evaluate the pharmaceutical potential of an ointment containing melittin for the treatment of non-surgical infections induced by MRSA. The minimum inhibitory concentration of melittin varied from 0.12 to 4 μM. The antibacterial effect was mainly bactericidal and fast (approximately 0.5 h after incubation) and was maintained even in stationary cells and mature MRSA biofilms. Melittin interacts synergistically with beta-lactams and aminoglycosides, and its ability to form pores in the membrane reverses the resistance of vancomycin-intermediate *Staphylococcus aureus* (VISA) to amoxicillin, and vancomycin. Its ability to induce resistance in vitro was absent, and melittin was stable in several conditions often associated with infected wounds. In vivo, a ointment containing melittin reduced bacterial load and the content of pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6 (IL-6), and IL-1 beta. Collectively, these data point to melittin as a potential candidate for topical formulations aimed at the treatment of non-surgical infections caused by MRSA.

Keywords: Methicillin-resistant *Staphylococcus aureus*; Melittin; Wound; Antimicrobial peptides

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most successful modern pathogens and is currently responsible for 10-fold more infections than all multi-drug resistant (MDR) Gram-negative bacteria combined (Bernier-Lachance et al., 2020; Correia et al., 2019; Craft et al., 2019). It is estimated that 11,000 people die each year from an MRSA-related infection in the USA, representing nearly half of all fatalities caused by MDR pathogens in the country (Graber, 2007; Mohamed et al., 2016; Turner et al., 2019). Methicillin resistance is mediated mainly by the *mecA* gene in *S. aureus* (Turner et al., 2019). The *mecA* gene encodes the penicillin-binding protein 2a, an enzyme responsible for the synthesis of bacterial cell walls that have low affinity for β-lactams, resulting in resistance to virtually all drugs of this class of antibiotics (Aguayo-Reyes et al., 2018; Turner et al., 2019). Because MRSA can infect almost any body site, it can cause several clinical conditions, such as bacteremia, endocarditis, skin and soft tissue infections, bone and joint infections, and hospital-acquired infections (Gajdács, 2019; Turner et al., 2019). Additionally, *S. aureus* is the pathogen most frequently isolated from human skin and non-surgical wound infections, leading to recurring/chronic infections, prolonging inflammation, and hindering wound healing (Graber, 2007; Mohamed F Mohamed et al., 2016).

Currently, the definition of non-surgical wounds includes chronic skin ulcers (such as pressure sores or diabetic ulcers), burns, and traumatic wounds (Gurusamy et al., 2013). MRSA can be present in 7% to 30% of acute bacterial skin and skin structure infections (ABSSSIs) associated with non-surgical causes (Álvarez et al., 2019). Without the implementation of correct therapeutic management in these cases, MRSA from ABSSSIs may spread to the bloodstream, causing a life-threatening illness (Pulido-Cejudo et al., 2017). Worryingly, resistance to other antibiotics (mupirocin and fusidic acid) and complete resistance to vancomycin is currently emerging in MRSA worldwide (Watkins et al., 2019). Therefore, the failure of antibiotics to kill MRSA due to bacterial resistance highlights the urgent need to develop novel, potent, and less toxic topical agents (Pulido-Cejudo et al., 2017; Turner et al., 2019). In this context, the antimicrobial peptides (AMPs) represent potential alternatives in the treatment of ABSSSIs caused by MRSA (Rončević et al., 2019). Interestingly, several studies have revealed that a decrease in the production of AMPs in the derma (especially of β-

defensins produced by keratinocytes) is associated with increased susceptibility to skin infection with *S. aureus* in humans (Afshar and Gallo, 2013). Furthermore, AMPs are a potential alternative to conventional antibiotics due to their relatively low potential to elicit resistance, a broad spectrum of activity, ability to neutralize virulence, and ability to modulate the host immune response (Brady et al., 2019; Mwangi et al., 2019). Based on these characteristics, AMPs provide an important strategy for combating ABSSSIs caused by MRSA or other Gram-positive and Gram-negative MDR pathogens.

Among the remarkable sources of AMPs with known anti-MRSA activities are venoms from insects, such as ants, bees, centipedes, scorpions, spiders, and wasps (Primon-Barros and Macedo, 2017; Wu et al., 2018). Such molecules are promising candidates for novel venom-based drugs against MDR pathogens (Perumal Samy et al., 2017). Bee venom holds promise as a rich source of AMPs effective against *S. aureus*, as is the case with melittin (Memariani et al., 2019). Melittin is the major component in the venom of the European honeybee (*Apis mellifera*) and is characterized by a small cationic linear peptide composed of 26 amino acid residues (GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$) (Rady et al., 2017). At high concentrations, melittin can induce pain and inflammation in humans; however, concentrations of up to ~35 mM have been shown to have several pharmacological properties in animal models, such anti-inflammatory, nociceptive, and antitumor effects (Maiden et al., 2019). Moreover, several studies have shown that melittin exhibits a potent antibacterial effect against *S. aureus* at concentrations significantly below toxic concentrations and is effective against both planktonic and biofilm-embedded MRSA strains at concentrations ranging from 0.5 to 8 μM (Choi et al., 2015; Dosler and Alev Gerceker, 2012; Picoli et al., 2017). Therefore, melittin stands out as a potential pharmacological agent for the development of new topical formulations directed toward the treatment of MRSA-infected wounds.

In fact, Choi et al. (2015) showed that the diameters of the abscesses formed after intradermal administration of MRSA in mice (1×10^7 CFU/mouse) were significantly reduced with the use of melittin (10 μg) (Choi et al., 2015). However, the authors did not investigate the effects of this AMP on the bacterial load of the lesion or its anti-inflammatory potential in this model. Furthermore, with regards to the effect of melittin against *S. aureus*, the activity of this peptide on persister and stationary cells,

the potential for resistance induction, the lytic effect in bacterial cells, and their stability in several conditions frequently found in infected wounds (e.g., lower pH, high protein concentration, and presence of organic salts) remains to be understood. In addition, the viability of the potent antibacterial action of melittin after incorporation into commercially available formulation remains to be clarified (Memariani et al., 2019). In this regard, the paraffin-based ointments are good options as a delivery vehicle to melittin because its form an occlusive barrier over the wound, which can increase both skin hydration and percutaneous absorption of this AMP. Their occlusive, water-free nature protects the skin from aqueous irritants, reduces the risk of sensitization through the lack of preservatives, and provides a longer contact time than creams or lotions (Harrison and Spada, 2018).

Therefore, in this study, we performed an in-depth characterization of the antibacterial effects of melittin against *S. aureus*. Moreover, we developed a melittin-containing ointment and evaluated its effectiveness on microbiological and inflammatory parameters associated with non-surgical MRSA-infected wounds in mice.

Material and Methods

Bacterial strains and reagents

The bacterial strains examined in the present study were obtained from the American Type Culture Collection (ATCC) and kindly provided by the Microbiology Reference Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ-RJ, Brazil). Antibacterial activity assays were performed with the following microorganisms: *S. saprophyticus* ATCC 15305, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 16538, *S. aureus* ATCC 29213, and *S. aureus* MRSA USA300. In addition, 11 clinical isolates of *S. aureus* were used, which were provided by the Laboratory of Clinical Microbiology of the Federal University of São João Del-rei (UFSJ). Identification of these isolates was initially performed with traditional biochemicals (i.e., mannitol salt agar growth and fermentation, catalase, coagulase, and DNase) and morphological (Gram staining) assays, followed by mass spectrometry-based techniques using matrix-assisted laser

desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Biotype Bruker®, Berlin, BA, Germany). All characteristics of clinical isolates employed are shown in Table S1 (supplementary file).

The melittin was obtained by purification from apitoxin and provided by the *Serviço de Opoterápicos*, *Fundação Ezequiel Dias* (FUNED, Belo Horizonte, Brazil) with a purity of 90%, which was confirmed by spectrometric and chromatographic analysis. Purified ceftizoxime, ciprofloxacin, levofloxacin, nitrofurantoin, gentamicin, and vancomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amoxicillin+clavulanate was obtained from Prati-Donaduzi (Maringá, PR, Brazil). Lanolin and solid Vaseline were both obtained from Pharma Nostra® Co., Inc. (Rio de Janeiro, RJ, Brazil).

Antibacterial activity

Inoculum preparation: The bacterial inoculum employed in susceptibility testing was standardized according to the Clinical Laboratory and Standard Institute (CLSI) document M07 (CLSI, 2018). Approximately three to five colonies isolated from a 24 h *Staphylococcus* culture were suspended in 10 mL saline (0.9% NaCl; Synth, São Paulo, SP, Brazil). The resulting suspension was then adjusted to the McFarland 0.5 scale, which corresponds to 10^8 colony forming units (CFU)/mL, using a spectrophotometer (OD_{625nm} of ~0.2; Nova Instruments, Sao Paulo, SP, Brazil). Next, 50 µL of resultant suspension was transferred to 10 mL of Mueller-Hinton broth (MHB; Himedia, Mumbai, MH, India) resulting in an inoculum of 10^6 CFU/mL, which was employed in the posterior assays.

Bacteriostatic activity: MHB (Himedia, Mumbai, MH, India) (Lima et al., 2019) was used to determine the minimal inhibitory concentration (MIC) by broth microdilution according to the CLSI (CLSI, 2018). Melittin and vancomycin (positive control) were solubilized in sterile water and serially diluted two-fold ranging from 0.25 to 32 µM. The results were visualized after 24 h of incubation at 37°C and the MIC value was considered the lowest concentration of tested compound able to prevent visible bacterial growth. All test media were supplemented with 0.002% polysorbate 80 to prevent drug binding to plastic surfaces.

Bactericidal activity: Minimum bactericidal concentration (MBC) was determined by pipetting 10 µL from the wells that showed no growth in the MIC assay and incubating these samples in Mueller-Hinton agar (MHA; Himedia, Mumbai, MH, India) for 24 h, as described previously (Lima et al., 2019). All data were recorded following at least three independent experiments.

Time-kill curve

Initially, a pre-inoculum of MRSA USA300 at 10^8 CFU/mL was prepared as described. Next, 50 µL of the pre-inoculum was added to test tubes containing 10 mL of MHB (Himedia, Mumbai, MH, India). Bacteria were then challenged with melittin at the 2x, 5x, and 10x MIC. Untreated cells and vancomycin at 10x MIC were used as controls. The tubes were incubated at 37°C with aeration at 225 rpm. At intervals of 0, 0.5, 1, 3, 6, 12, and 24 h, 100 µL was serially diluted (10^{-1} to 10^{-5}) in sterile saline (0.9% NaCl; Synth, São Paulo, SP, Brazil) and plated onto MHA (Himedia, Mumbai, MH, India). The plates were finally incubated for 24 h and the CFU/mL was calculated (Andrade et al., 2018).

Activity against stationary cells

The effect of melittin and vancomycin on *S. aureus* stationary cells was investigated as described previously (Mohamed et al., 2016). Briefly, an overnight culture of MRSA USA300 (1 x 10^8 CFU/mL in MHB) was treated with the test agents (melittin and vancomycin) at a concentration of 2x, 5x, and 10x MIC. The tubes were then incubated at 37°C with aeration at 225 rpm. To determine the bacterial burden after treatment, samples were collected after 0, 1, 3, 6, 24, and 48 h, diluted in sterile saline, and transferred to MHA plates.

Activity against persister cells that demonstrated tolerance to ciprofloxacin

The effect of melittin and conventional antibiotic (vancomycin) on *S. aureus* MRSA USA300 persister cells was investigated as described previously (Mohamed et al., 2016), with modifications. Briefly, an overnight culture of MRSA USA300 (1 x 1010 CFU/mL) was incubated with ciprofloxacin at 80x MIC (10 µg/mL) for 6 h at 37°C. Next,

the test agents (melittin, vancomycin, and ciprofloxacin) were added at concentrations of 5x, 10x, and 20x MIC. Bacteria were incubated with test agents at 37°C for 48 h. Finally, samples were collected after 7, 10, 20, and 40 h, diluted in sterile saline, and transferred to MHA plates in order to determine the bacterial border (Log_{10} UFC/mL).

Biofilm eradication assessment

Melittin and vancomycin were examined for their ability to disrupt well-established mature MRSA USA300 and *S. epidermidis* ATCC 12228 biofilm using the microtiter disk biofilm formation assay (O'Toole, 2010), with minor modifications. Bacteria (1 x 10^6 CFU/mL) in tryptic soy broth (Himedia, Mumbai, MH, India) supplemented with 1% glucose (Isofar, Rio de Janeiro, RJ, Brazil) were incubated at 37°C for 24 h. After, the mature biofilm attached to the wells was treated with the compounds (melittin or vancomycin at 8, 16, or 32 µg/mL) and the microtiter plates were again incubated at 37°C for 24 h. Finally, the biofilm was revealed using 0.1% crystal violet (Isofar, Rio de Janeiro, RJ, Brazil) and labeling intensity was measured using a microplate reader (Bio-Tek Instruments, New York, NY, USA) at 595 nm. Untreated biofilm was considered a control. The result was then graphically expressed as a function of % inhibition of biofilm *vs.* concentration of compound.

Combination therapy analysis

The synergistic effects associated with the combination of melittin and commercial antibiotics (amoxicillin+clavulanate, ceftizoxime, levofloxacin, nitrofurantoin, gentamicin, and vancomycin) were assessed using the checkerboard test (Orhan et al., 2005). For the assay, MRSA USA300 was incubated with melittin, control antibiotics, or a combination of melittin plus a control antibiotic at concentrations ranging from 0.25 to 8 µg/mL (or µM to melittin) for 24 h. Next, the absence of bacterial growth was evaluated visually. The fractional inhibitory concentration index (FIC index) was calculated as the interpretation criterion of results by the sum of the FICs of each drug only, which is defined by the following equation:

$$FIC\ melittin = \frac{MIC\ mellitin_{\ Combined}}{MIC\ mellitin_{\ Only}} \qquad FIC\ antibiotic = \frac{MIC\ antibiotic_{\ Combined}}{MIC\ antibiotic_{\ Only}}$$

In accordance with Orhan et al. (2005), an FIC index of 0.5 or less was defined as synergism. A FIC index between 0.5 and 2.0 was defined as additive and between 2.0 and 4.0 as indifferent. A FIC index of more than 4.0 was defined as antagonism. In addition, the results were interpreted by plotting the FIC values of each compound involved in the combinations. Therefore, as isobologram graph was generated, whereby agents were considered synergistic when orthogonal projection was concave; additive or indifferent, if the projection behaved like a straight line, and antagonistic in cases where the projection took a convex form.

Resensitization of vancomycin-intermediate *Staphylococcus aureus* (VISA) and MRSA to antibiotics in the presence of sub-inhibitory concentrations of melittin

Resensitization of VISA (*S. aureus* H4 strains) and MRSA USA300 to antibiotics (amoxicillin, Gentamicin, levofloxacin, ceftizoxime, and vancomycin) was performed as described previously (Mohamed et al., 2016), with modifications. Briefly, ½ × MIC of melittin was incubated with the bacterial strain (1 × 10^6 CFU/mL) in MHB at 37°C for 60 min. After incubation, peptide-treated bacteria were added to 96-well plates and the MIC of each antibiotic was determined. Bacteria peptide-untreated and vancomycin-treated bacteria served as negative and positive controls, respectively. The result was expressed as the fold-change of resensitization, calculated by the ratio of the MIC of antibiotic alone and the MIC of the antibiotic after resensitization with ½ × MIC of melittin or vancomycin.

Evaluation of bacterial lysis

The potential of melittin and vancomycin to lyse MRSA USA300 cells was studied by visible and ultraviolet spectrophotometric methods.

Lysis using visible spectrum: For lysis analysis via the visible spectrum, 10 mL of culture at OD_{600} of 1.0 was treated with 10 ×MIC of compounds (melittin and

vancomycin) for 24 h, after which each culture was added to glass test tubes and photographed (Ling et al., 2015). Furthermore, the OD_{600} of bacterial suspensions after treatment was determined.

Lysis using ultraviolet spectrum: The release of intracellular material with absorbance at 260 nm was quantified according to the modified method of Bruin and Birnboim (de Bruin and Birnboim, 2016). Aliquots of 5 mL obtained from bacterial suspensions (10^8 CFU/mL) prepared in sterile saline were treated with the compounds (melittin and vancomycin) at 10x MIC for 24 h. After treatment, cells were centrifuged at 2,500 ×*g* for 5 min, and the absorbance of the supernatant at 260 nm was determined using an ultraviolet spectrophotometer (Hitachi U-1100, Lancashire, UK).

Scanning electron microscopy (SEM)

To evaluate the morphological changes induced by melittin and vancomycin on MRSA USA300 cells, SEM analysis was performed according to Ravensdale et al. (Ravensdale et al., 2016). The stubs containing the samples to be analyzed were coated by sputtering with a 10 nm layer of gold and viewed using a scanning electron microscope (Jeol JSM-6010Plus/LA, Germany).

Multi-step resistance study

The ability of MRSA USA300 to develop resistance to melittin and vancomycin was investigated via a multi-step resistance study, as described previously (Bogdanovich et al., 2005). Bacteria were exposed to two dilutions below the MIC of each compound for 21 days. The MIC of melittin and vancomycin was set daily during the experimental period, and the results were expressed graphically as a function of the time of MIC increase vs. exposure time (in days).

Stability of melittin

The activity of melittin in different environmental conditions, including salts (NaCl, $CaCl_2$, and KCl), acid and basic pH, serum, plasma, and plasmatic proteins,

was evaluated using the broth microdilution assay described above. *S. aureus* MRSA USA300 was treated with compounds (melittin and vancomycin) under specific conditions frequently found in infected wounds and healthy skin. In addition, to examine the ability of compounds to resist proteolytic digestion by mammalian proteases, melittin and vancomycin were incubated with trypsin at a molar ratio of 500:1 (peptide:enzyme).

Ointment

For the delivery of melittin and positive control (vancomycin), the drugs were formulated in a simple ointment according to the Brazilian Pharmacopoeia Sixth Edition (Table S2, Supplementary file). After formulation of the base, melittin was diluted in 100 µL of dimethyl sulfoxide (DMSO) and incorporated at concentrations of 0.25%, 0.5%, and 1.0%. Vancomycin, in turn, was incorporated at a concentration of 1.0%. A base ointment containing 100 µL DMSO was used as a negative control.

In vivo assay

Six-week-old male BALB/c mice (Biotério Central da UFMG, Belo Horizonte, MG, Brazil) were used in this study. The animals were kept in polypropylene boxes measuring 30 x 19 x 13 cm in an environment with a controlled temperature of 25 ± 2°C, 40% humidity, and a 12/12 h light/dark cycle. All experimental procedures strictly followed the international protocols for laboratory animal management, and the methods were approved by the Laboratory Animal Research Ethics Committee of the Federal University of Minas Gerais (CEUA-UFMG: 299/2019).

Murine model of non-surgical MRSA-infected wounds: An in vivo model of MRSA USA300 skin infection was conducted according to the methods of Thangamani et al. (2016). Following the induction of general anesthesia (60 mg/kg ketamine + 8 mg/kg xylazine, i.p.), the dorsal hair was removed with a trichotomizer (REF 9681 Cirúrgicos 3M™, São Paulo, SP, Brazil), and the skin was cleaned with 70% (v/v) ethanol. Inside the biological security cabin (Veco® Bioseg 18, Campinas, SP, Brazil), a bacterial suspension at 10^8 CFU/mL was prepared in sterile saline from an overnight culture of

S. aureus MRSA USA300. Next, 50 µL of this suspension was aspirated and injected subcutaneously into the dorsal area of the animals. After infection, the animals were placed in previously sterilized polypropylene boxes and kept in this environment with access to autoclaved water and feed *ad libitum*. After 48 h, an open wound/abscess was observed at the injection site and treatment with the different ointments was started.

Treatment of animals: After induction of MRSA skin infection, the animals were divided into seven experimental groups (n = 5). These groups were treated topically with the previously formulated ointment containing melittin at 0.25%, 0.5%, or 1.0%. The positive control received 1.0% vancomycin-containing ointment. Groups of animals treated topically with saline (untreated control) and base ointment (base control) were also included. The animals were treated for three consecutive days with two daily administrations.

Bacterial burden: First, 24 h after the last topical treatment, the mice were humanely euthanized via cervical dislocation under anesthesia. The region around the skin wound was slightly swabbed with 70% ethanol and the wound/abscess was precisely excised, homogenized, serially diluted in saline (0.9% NaCl; 10^{-1}-10^{-5}), and transferred to mannitol salt agar plates (Kasvi, São José dos Pinhais, PR, Brazil). Plates were then incubated at 37°C for 24 h, and the CFU in each sample were determined.

Cytokine determination: Skin homogenates obtained from the murine non-surgical MRSA-infected wound were centrifuged. The supernatant was collected and used to quantify the levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 by ELISA Kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

Technetium-99m labeled ceftizoxime (^{99m}Tc-CFT) wound imaging: To follow the regression of the infectious process after treatment with the ointments, the mice wounds were analyzed using ^{99m}Tc-CFT. Radiolabeling of the CFT using ^{99m}Tc was conducted in accordance with the procedure described by Diniz et al. (2005). Briefly, a kit containing 2.5 mg of CFT and 6.0 mg of sodium dithionite was reconstituted with 1.0 mL of sodium ^{99m}Tc-pertechnetate solution ($Na^{99m}TcO_4$) containing 74 MBq (2 mCi) of activity, which was obtained from a ^{99}Mo/^{99m}Tc generator (Ipen, São Paulo, SP,

Brazil). The solution was then boiled for 10 min and cooled in running water for 5 min. Finally, the solution was filtered through a cellulose ester filter (0.22 mm) (Millipore, Burlington, MA, USA) and used for quality control, as previously described (Diniz et al., 2005).

After the ^{99m}Tc-CFT preparation, 3.7 MBq (100 μCi) was administered to the tail vein of previously anesthetized mice, and scintigraphic images of each animal were obtained after 1 and 6 h (n = 3). During scintigraphic imaging, the animals were anesthetized and placed in the prone position on a gamma-camara for small animals equipped with a low-energy collimator (Nuclide TH22, Mediso, Hungary). Images were acquired using 256 × 256 × 16 pixels matrix size with a ± 10% energy window set at 140 keV for 10 min. For quantitative analysis of ^{99m}Tc-CFT uptake by infected wounds, scintigraphic images were assessed by target to non-target ratio determination. The infected (right) and contralateral (left) wounds were delimited, followed by radioactivity determination in each area. Finally, the target to non-target ratio was calculated as follows:

$$\text{Target to non - target ratio} = \frac{\text{counts}_{\text{(infected flank / right flank)}}}{\text{counts}_{\text{(contralateral flank / left flank)}}}$$

Results and Discussion

In vitro antibacterial activity of melittin: As shown in **Table 1**, melittin is highly active against several clinically relevant *S. aureus* strains. Melittin inhibited the growth of all isolates tested, with MIC ranging from 0.12 to 4 μM. The antimicrobial effect observed was mostly bactericidal, and it was shown that melittin could kill *S. aureus* cells at concentrations ranging from 0.12 to 16 μM. The activity profile of melittin was very similar to that of vancomycin, which showed MIC in the range of 0.25-8 μM and CBM between 0.25 and 16 μM. The CBM required to inhibit 50% of *Staphylococcus* isolates (CBM_{50}) was 1.5 μM for both compounds. The MICs reported in this study were similar to those found in previous studies, which also determined the effect of melittin via broth

microdilution assay (Blondelle and Houghten, 1991; Dosler and Alev Gerceker, 2012; Ebbensgaard et al., 2015; Han et al., 2009; Leandro et al., 2015; Moerman et al., 2002; Pandey et al., 2010). Interestingly, MRSA strains were more sensitive to the bacteriostatic (MIC range: 0.12-4 μM) and bactericidal (CBM: 0.5-4 μM) effects of melittin than MSSA isolates (MIC range: 0.12-4 μM; CBM range: 0.5-16 μM). For example, the *S. aureus* MRSA2 and *S. aureus* H3 strain melittin was twice as potent as vancomycin. Therefore, the results suggest that isolates of *S. aureus* with the MDR phenotype are more sensitive to the antibacterial effects of melittin, which is consistent with the results of a previous study (Choi et al., 2015). In addition, melittin was active against the VISA isolate tested (MIC 2 μM). This result is of particular importance because vancomycin is considered one of the "last-line" classes of antibiotics for the treatment of infections caused by MDR Gram-positive microorganisms (Wijesekara et al., 2017).

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of melittin and vancomycin against clinical and drug-resistant staphylococci isolates.

	Strain ID	Melittin (μM)		Vancomycin (μM)	
		MIC	MBC	MIC	MBC
	S. saprophyticus ATCC 15305	1	2	1	1
	S. epidermidis ATCC 12228	2	2	0.5	1
MSSA	*S. aureus* ATCC 16538	0.5	1	0.5	16
	S. aureus ATCC 29213	1	1	0.5	2
	S. aureus 8	4	16	0.5	1
	S. aureus IC1	0.5	0.5	0.5	1
	S. aureus IC2	4	4	0.5	0.5
	S. aureus H1	1	16	4	4
	S. aureus ST1	1	1	4	4
	S. aureus ST3	1	1	2	2
MRSA	*S. aureus* MRSA USA 300	1	1	0.5	0.5
	S. aureus MRSA2	0.12	0.12	0.25	0.25
	S. aureus H2	4	4	1	4
	S. aureus H3	0.5	4	1	8
	S. aureus ST2	0.5	0.5	1	1
VISA	*S. aureus* H4	2	4	8	8
	MIC_{50}	1		0.75	
	MBC_{50}	1.5		1.5	

MSSA: Methicillin-sensitive *Staphylococcus aureus;* MRSA: Methicillin-resistant *Staphylococcus aureus*; VISA: Vancomycin-intermediate *Staphylococcus aureus.*

Melittin exhibits rapid bactericidal effects on stationary, persister, and logarithmic cells of MRSA: Because of the potent antibacterial effect of melittin in vitro, we aimed to evaluate the bacterial killing kinetics of this AMP in cells in the logarithmic and stationary phases as well as in persistent cells of MRSA. Melittin had a rapid bactericidal effect against cells under logarithmic growth and completely eliminated a high microbial load (1 x 10^6 CFU/mL) of MRSA USA300 within 30 min at all concentrations tested (2x, 5x, and 10x MIC) (**Fig. 1**). Fast and efficient pathogen elimination is required in compounds with antibacterial activity. This is because the rapid bactericidal effect minimizes the risk of spreading and the progression of infection, reduces the antimicrobial concentration required to produce the desired effect, decreases the likelihood of resistance induction during clinical use, and restricts treatment time (Alder and Eisenstein, 2004). Despite the melittin, the bactericidal effect of vancomycin was less potent. After 6 h of incubation, vancomycin produced a 4.02-log reduction and required 24 h to completely eliminate MRSA USA300 (**Fig. 1A**), which has also been found in previous studies (Mohamed et al., 2016). Clinical use of vancomycin often leads to therapeutic failure due to its limited bactericidal capacity (Wijesekara et al., 2017), which makes melittin advantageous because of its efficient microbicidal property.

Persister cells are phenotypic variants that are highly resistant to conventional antibiotics and contribute to chronic and latent infections. They are especially significant when the pathogen is shielded from the immune system by biofilms, or in sites where the immune components are limited in the nervous system, stomach, or inside macrophages (Keren et al., 2004; Wood et al., 2013). To evaluate the effect of melittin on persister cells, an inoculum of MRSA USA300 that demonstrates ciprofloxacin tolerance was treated with this AMP or a conventional antibiotic (vancomycin). The subsequent addition of vancomycin had no considerable effect on the persister cell number, as has been shown previously (Mohamed et al., 2016; Thangamani et al., 2016). In contrast, melittin completely eradicated persistent cells after 7 and 20 h at concentrations of 10x, 20x, and 5x at MIC, respectively (**Fig. 1C**). Next, the antibacterial effect of melittin was evaluated on stationary cells, which characterize bacterial populations present inside biofilms and are normally insensitive to the action of conventional antibiotics (Keren et al., 2004). In fact, as shown in Fig. 1B, vancomycin exhibits a discrete effect on the stationary cells of MRSA USA300, in

accordance with a previous study (Mohamed et al., 2016). However, melittin resulted in complete eradication of stationary cells after 30 min and 48 h at concentrations of 10x and 5x MIC, respectively (**Fig. 1**). The most effective antibacterial effect of melittin, when compared to vancomycin, can be explained by the mechanism of action of these AMPs. Conventional antibiotics, such as vancomycin, act primarily on metabolically active cells, such as those recovered from patients with active infections (Kapoor et al., 2017). However, their action on cells in dormant or quiescent states (such as stationary and persistent cells) is severely compromised. In contrast, AMPs act by stimulating cell lysis by interacting with the cell membrane (Memariani et al., 2019; Picoli et al., 2017; Wu et al., 2018). This mechanism does not require the cells to be metabolically active, causing the action of AMPs to be conserved even in stationary and persister cells, as shown in this study.

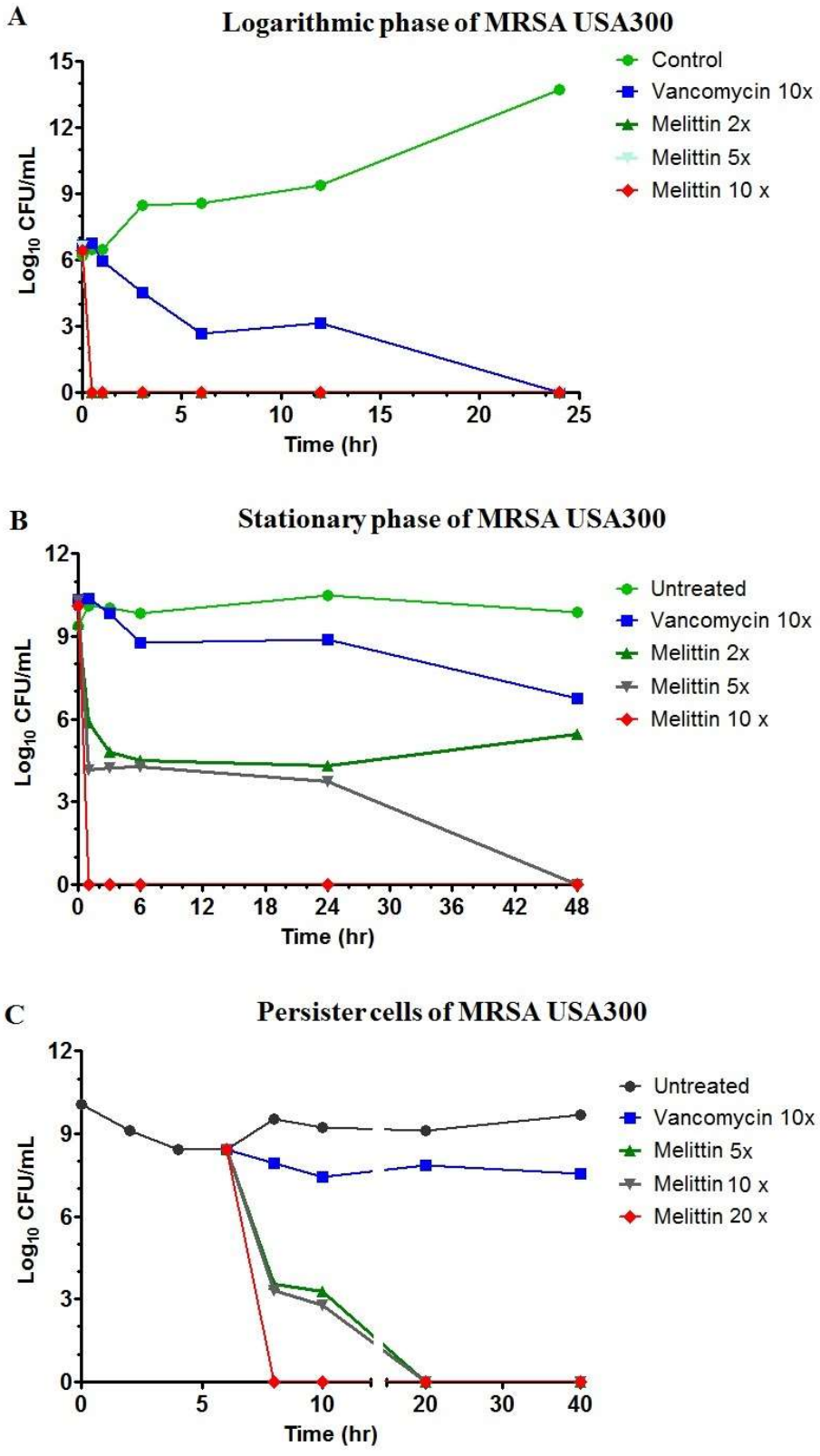

Fig. 1: The kinetics of killing of melittin and vancomycin against logarithmic, stationary and persister cells of MRSA USA300.

Melittin reduces preformed biofilms: In addition to its potential to acquire antibiotic resistance, S. aureus exhibits the ability to form biofilms on organic and inanimate surfaces (Craft et al., 2019). When biofilms are established in wounds, they exhibit resistance to destruction by conventional antimicrobials, which involves a high risk of inflammation and/or purulence (Percival et al., 2015). Therefore, to assess the potential of melittin against wounds complicated by the onset of mature biofilms, we studied the capacity of this AMP to disrupt preformed staphylococcal biofilms. As shown in **Fig. 2**, melittin reduced the MRSA USA300 biofilm by more than 50% at all concentrations tested (8, 16, and 32 μM) after 24 h of incubation. This effect was similar to that observed with vancomycin, which significantly disrupted the preformed *S. aureus* biofilm (**Fig. 2**). However, it has been shown that many conventional antibiotics do not maintain this effect after 24 h. This is because the cells remaining in the biofilm after treatment proliferate again and establish a new exopolymer matrix (Wolcott et al., 2010). Therefore, we also studied the effects of vancomycin and melittin on mature biofilms after 48 h of incubation. As expected, the anti-biofilm effect was reduced; however, the decrease was more prominent in the cells receiving vancomycin. In addition, 16 μM of melittin reduced the preformed MRSA biofilm by 55%, while vancomycin decreased biomass by 38% compared to untreated cells at the same concentration. These results indicate that the anti-biofilm effects of melittin are more stable and last longer than those of conventional antibiotics.

In accordance with this study, a recent review highlighted the potent effects of melittin on well-established biofilms formed by Gram-positive (*S. aureus, Streptococcus agalactiae, Listeria monocytogenes*) and Gram-negative (*Pseudomonas aeruginosa*, *Acinetobacter baumannii, Borrelia burgdorferi*) pathogens, including MRSA (Memariani et al., 2019). Therefore, in order to evaluate whether melittin is also active for other *Staphylococcus* species frequently recovered in non-surgical wounds, we determined the potential of this AMP against biofilm-embedded *S. epidermidis* cells. Similar to that observed with MRSA, melittin had a potent effect on *S. epidermidis* biofilms after 24 h of incubation. However, while 32 μM of vancomycin had its activity against *S. epidermidis* biofilm reduced by 28% after 48 h of incubation, the effect of melittin remained constant (**Fig. 2**). This reinforces the good stability of melittin against biofilms compared to conventional antibiotics. The activity profile shown in this study is relevant since *S. aureus* and *S. epidermidis* are the two

species most frequently recovered from contaminated wounds (Gurusamy et al., 2013; Percival et al., 2015; Pulido-Cejudo et al., 2017), and the biofilm formed by these species results in severe clinical complications.

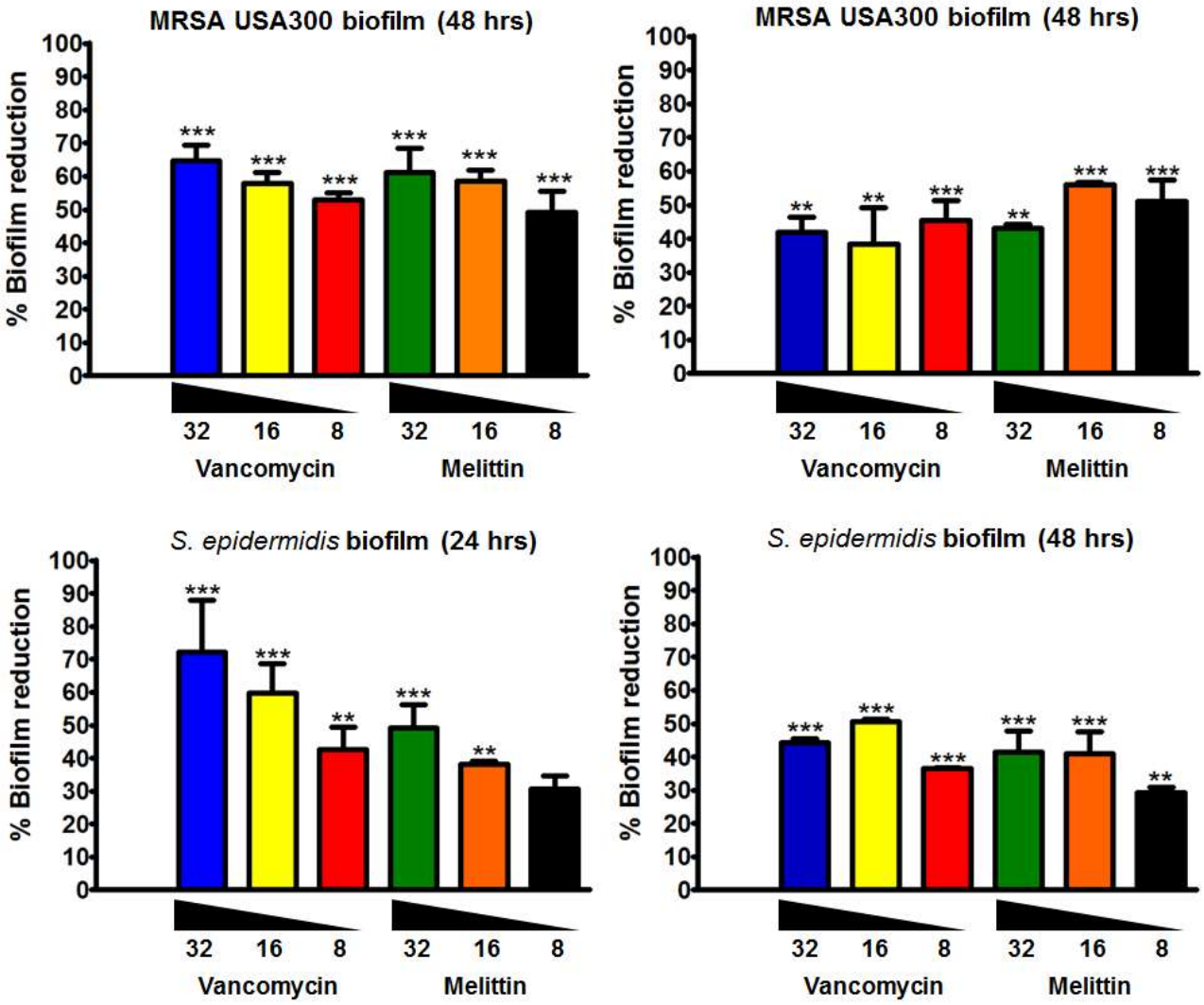

Fig. 2: Anti-biofilm activity of melittin and vancomycin against 24 and 48 hrs old biofilms of *S. aureus* MRSA USA300 and *S. epidermidis* ATCC 12228. The adherent biofilm stained with crystal violet, then the dye was extracted with ethanol, measured at 595nm absorbance and presented as percentage of biofilm reduction compared to untreated cells. All experiments were done in quadruplicate for statistical significance. Two asterisks (**) indicate statistically different than control with $p < 0.01$. Three asterisks (***) indicate statistically different than control with $p < 0.0001$. The results were analyzed by One-way ANOVA with Dunnett post-hoc.

Melittin exhibits synergistic effects with beta-lactams and aminoglycosides against MRSA: A combination of different therapeutic agents is common for the treatment of infected wounds. Many of the topical options currently available in the pharmaceutical market relate to the combination of two or more antibacterial agents, such as Nebacetin® (bacitracin and neomycin), Neosporin® (bacitracin, neomycin, and polymyxin B sulfate), and Polysporin® (bacitracin, polymyxin B sulfate, and gramicidin) (Pulido-Cejudo et al., 2017). Combination therapies are often stimulated in the therapeutic management of clinically relevant infections as they have several advantages over monotherapy. These advantages include increasing the spectrum of drug-affected microorganisms, reducing the risk of resistance selection, reducing treatment time, and reducing the concentration of each drug employed in order to minimize its adverse effects (Rybak and McGrath, 1996). Furthermore, in some cases, the combinations act synergistically and might, thus, accelerate pathogen clearance in wounds with high bacterial loads (Pletz et al., 2017). Therefore, we studied the synergistic potential of melittin in combination with antibiotics currently employed in clinical practice.

Table 2 shows that melittin interacts synergistically with amoxicillin+clavulanate (FICI 0.50), ceftizoxime (FICI 0.16), and gentamicin (FICI 0.14), and exerts an additive effect when combined with levofloxacin (FICI 0.75), nitrofurantoin (FICI 1.00), or vancomycin (FICI 0.75) against MRSA USA300. Additionally, no antagonistic effects were observed. These results indicate that melittin has a strong synergistic effect when combined with beta-lactams and aminoglycosides, as confirmed by the concave projections of gentamicin, amoxicillin+clavulanate, and ceftizoxime on isobolograms (Supplementary file, Fig. S1). Since aminoglycosides have considerable nephrotoxic and ototoxic effects (including those associated with irreversible cochlear lesions) at high concentrations, decreasing their therapeutic dose by combination with melittin may promote greater clinical safety (Krause et al., 2016). According to Akbari et al. (Akbari et al., 2019), melittin exhibits a highly synergistic effect with beta-lactams (doripenem and ceftazidime) against *P. aeruginosa* and *A. baumannii*, despite being indifferent when combined with other antibiotic classes, such as tetracyclines (doxycycline) and polymyxins (colistin). This result corroborates our findings and indicates that the synergistic effects of melittin are produced as a result of the defects induced by this AMP in the microbial cell, consequently increasing the

membrane permeability and concentration of antibiotics in the pathogen (Akbari et al., 2019; Memariani et al., 2019).

Table 2: The fractional inhibitory concentration (FIC) and FIC index (FICI) range of melittin in combination with antibiotics (beta-lactams, aminoglycoside, fluorquinolone, nitrofuran and glycopeptide) against methicillin-resistant *Staphylococcus aureus* (MRSA USA 300).

Class	Antibiotics	FIC		FICI (ΣFIC)	Effect
		Melittin	Antibiotic		
Beta-lactam+Beta-lactamase inhibitor	Amoxicillin+Clavulanate	0.25	0.25	0.50	Sinergy
Beta-lactam	Ceftizoxime	0.13	0.03	0.16	Sinergy
Aminoglycoside	Gentamicin	0.01	0.13	0.14	Sinergy
Fluorquinolone	Levofloxacin	0.25	0.50	0.75	Additive
Nitrofuran	Nitrofurantoin	0.50	0.50	1.00	Additive
Glycopeptide	Vancomycin	0.25	0.50	0.75	Additive

ΣFICI: Fractional inhibitory concentration index. FIC index was interpreted as follows: An FIC index of ≤ 0.5 is considered to demonstrate synergy. Additive effect was defined as an FIC index between 0.6 and 1. An FIC index between 1.1 and 4 was considered such as indifferent effect. Antagonism was defined as an FIC index of > 4.

Prior exposure to sub-inhibitory concentrations of melittin reduces the MIC of conventional antibiotics against MRSA and VISA: As a result of the potent synergistic effects observed after the combination of melittin with beta-lactams and aminoglycosides, we aimed to assess whether prior exposure to this AMP reduces the resistance of MRSA USA300 to conventional antibiotics. Treatment of MRSA cells for 1 h with melittin at ½ × MIC reduced the MIC of amoxicillin, ceftizoxime, and vancomycin by 16-fold compared to unexposed cells (Table 3). Vancomycin, in turn, reduced the MIC of amoxicillin by only four-fold after exposure under the same conditions, and it had no sensitization effect on ceftizoxime. Melittin also resensitized vancomycin-intermediate *S. aureus* to vancomycin and beta-lactams (**Table 3**). Therefore, this study showed, for the first time, that melittin can be used as an antimicrobial agent alone or in combination with antibiotics in the treatment of staphylococcal infections, and that it has the potential to suppress the resistance of MRSA and VISA to conventional antibiotics. Similar to synergism, the effect of melittin

on the bacterial membrane justifies its resensitizing potential. Consistent with this hypothesis, Mohamed et al. (Mohamed et al., 2016) reported that two short synthetic AMPs (WR12 and D-IK8) demonstrated the ability to resensitize vancomycin-resistant *S. aureus* (VRSA) strains to the effects of vancomycin, teicoplanin, and oxacillin because they permeabilize the membrane, leading to increased antibiotic access to their targets.

Table 3: Re-sensitization of methicillin-resistant *S. aureus* (MRSA USA 300) and vancomycin-intermediate *Staphylococcus aureus* (VISA) to amoxicillin, Gentamicin, levofloxacin, Ceftizoxime, and vancomycin using a sub-inhibitory concentration (½ × MIC) of melittin.

Strain ID	Antibiotics	Fold of re-sensitization	
		Melittin	Vancomycin
S. aureus MRSA USA 300	Amoxicillin	16	4
	Gentamicin	4	2
	Levofloxacin	4	2
	Ceftizoxime	16	No sensitization effect
	Vancomycin	16	-
S. aureus VISA	Amoxicillin	8	-
	Gentamicin	2	-
	Levofloxacin	2	-
	Ceftizoxime	8	-
	Vancomycin	4	-

Fold of re-sensitization: It is the ratio of the MIC of antibiotic alone divided by the MIC of antibiotic after re-sensitization with (½ × MIC) of melittin.

Melittin does not induce resistance in MRSA: According to the World Health Organization, bacterial resistance to conventional antibiotics is the second most serious global public health problem (WHO, 2019). Estimates indicate that if no action is taken today, in 2050, approximately 10 million people will die annually from infections caused by MDR microorganisms, overtaking chronic diseases, such as cancer and diabetes (O' Neil, 2014). To assess whether melittin induces resistance in MRSA, a multi-step resistance selection study was conducted. The MIC values of melittin and vancomycin remained consistent and did not change throughout the 21 passages (**Fig. 3**). Therefore, the results from the multi-step resistance study indicated that MRSA was unable to develop rapid resistance against melittin. In fact, AMPs are known for their low propensity for resistance development, which has been associated with the ability

of peptides to induce rapid cell death as a result of membrane damage (Wang et al., 2016). This propriety of AMPs lessens development of bacterial resistance because membrane redesign by bacteria would be a “costly” solution for most microbial species (Lima et al., 2018; Wang et al., 2016).

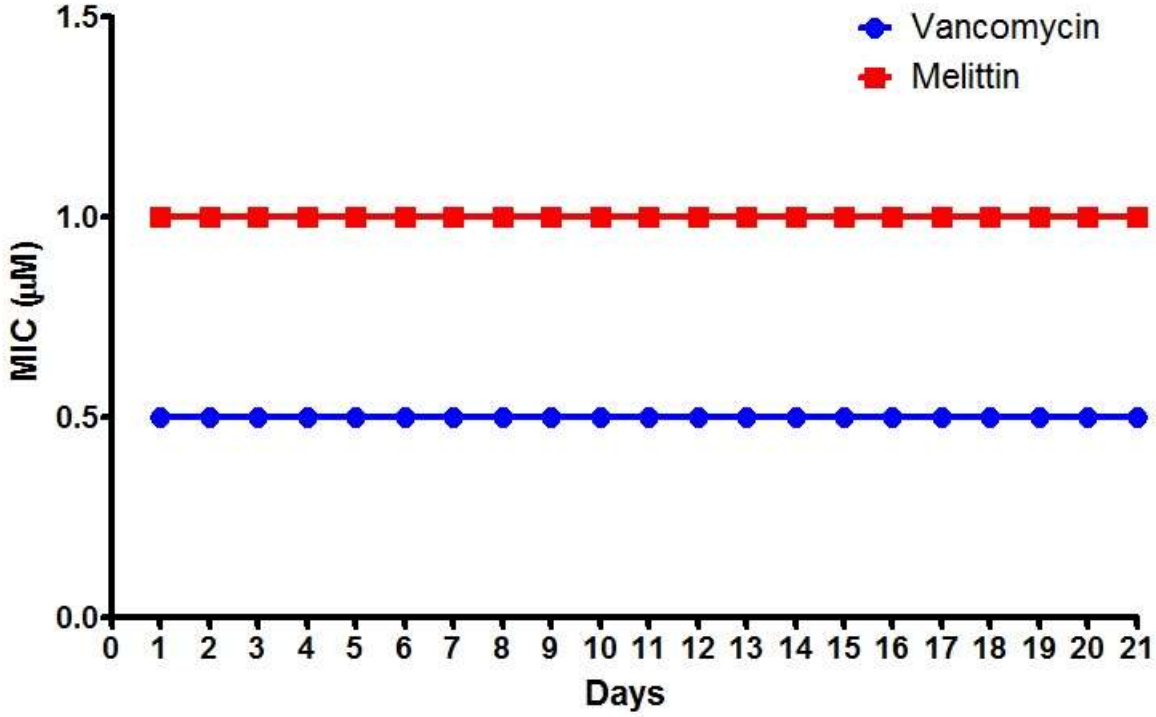

Fig. 3: Multi-step resistance study of melittin and vancomycin against methicillin-resistant *S. aureus* (MRSA USA300). Bacteria were serially passaged over a 21-day period, and the broth microdilution assay was used to determine the minimum inhibitory concentration (MIC) of each compound after each successive passage.

Stability of the antibacterial activity of melittin under several conditions: Despite the potent antimicrobial effect generally associated with AMPs, these compounds generally have low stability. AMPs have high susceptibility to degradation by proteases, extensive binding to biological proteins, loss of activity in the presence of different organic salts (even at physiological concentrations), and low stability at pH variations (Mwangi et al., 2019). This is of particular concern when targeting the compound to treat infected wounds, which are usually accompanied by changes in pH, protein concentration, availability of salt, and the presence of some serum components

(especially in burn wounds) (Tiwari, 2012). Therefore, we aimed to assess the stability of melittin and vancomycin under different biological and physical chemistry conditions. As shown in **Table 4**, melittin was stable in the presence of all tested salts (KCl, $CaCl_2$, and NaCl), while vancomycin activity was reduced in the presence of NaCl. This can be explained by the presence of tryptophan (one residue; Trp19) and arginine (two residues; Arg22 and Arg24) in the chemical structure of melittin, which are amino acids that increase the stability of peptides at high salt concentrations (Chan et al., 2006). Similarly to vancomycin, the MIC of melittin against MRSA increases in the presence of bovine albumin in a dose-dependent manner, which suggests that a fraction of these compounds can interact and bind to biological proteins (**Table 4**). Furthermore, melittin, but not vancomycin, was stable in fetal bovine serum, human serum, and human plasma, highlighting the stability of this AMP in the presence of serum components (**Table 4**). However, although vancomycin remains stable in the presence of mammalian proteases, such as trypsin, the biological activity of melittin is lost (**Table 4**). This is due to the peptide nature of melittin, which makes it a potential substrate for this protease. In intact skin, α1-anti-trypsin is produced, which inhibits the proteolytic action of trypsin. However, in lesions and wounds, the production of this natural inhibitor is reduced and the activity of trypsin is increased, which can compromise the therapeutic effect of melittin (Afshar and Gallo, 2013). To overcome this challenge, topical ointments containing melittin can be supplemented with trypsin inhibitors; however, the viability of this approach must be clarified in future studies.

Table 4: Minimal inhibitory concentration (MIC) of melittin and vancomycin in the presence of salts (NaCl, $CaCl_2$, and KCl), different pH ranges (4, 5, and 8), trypsin (1:500), fetal bovine serum (FBS, 2%, 5% or 10%), bovine serum albumin (BSA, 2%, 5% or 10%), human serum (HS; 2% or 5%) and plasma from human donors (HP; 2% or 5%) against methicillin-resistant *Staphylococcus aureus* (MRSA USA 300).

	Control	**NaCl 100mM**	**$CaCl_2$ 8µM**	**KCl 1mM**	**pH 4**	**pH 5**	**pH 8**	**FBS 2%**	**FBS 5%**	**FBS 10%**	**BSA 50 mg/mL**	**BSA 30 mg/mL**	**BSA 10 mg/mL**	**HS 2%**	**HS 5%**	**HP 2%**	**HP 5%**	**Trypsin 1:500**
Melittin	1	1	1	1	2	2	1	1	1	1	8	4	2	1	1	1	1	>32
Vancomycin	0.5	2	0.5	0.5	0.5	0.5	0.5	0.5	1	2	1	2	2	1	1	1	1	0.5

NaCl: Sodium chloride; $CaCl_2$: Calcium chloride; KCl: Potassium chloride; pH: Hydrogen potential; FBS: Fetal bovine serum; BSA: Bovine serum albumin; HS: Human serum; HP: Human plasma

Melittin results in the lysis of MRSA: The mechanism of action of melittin on MRSA USA300 was assessed by a lysis assay. After incubation with melittin at 10x MIC (10 μM), a significant reduction in the optical density of the treated bacterial suspension was observed in relation to the untreated control (**Fig. 4A and 4B**). The lytic effect of melittin was significantly greater than that of vancomycin (p-value < 0.05). The lysis of MRSA after melittin exposure was also confirmed by the release of 260 nm-absorbing intracellular material. As shown in **Fig. 4C**, treatment of bacterial suspensions with melittin at 10x MIC for 24 h induced a marked increase in the release of cellular material, suggesting that this peptide can disrupt the integrity of the bacterial membrane. Corroborating with this hypothesis, SEM analysis showed that melittin induced alterations in the membrane of MRSA USA300. Herein, the microorganisms treated with this AMP showed multiple cellular anomalies, such as asymmetric division, inclusions and projections on the cellular surface, and large amounts of cellular debris in the field (indicating bacterial lysis) (**Fig. 5C**). In contrast, a few changes were observed in cells treated with the positive control (vancomycin) (**Fig. 5B**), and no alterations were founded in untreated bacteria (**Fig. 5A**). Similar to this study, Pandey et al. (Pandey et al., 2010) showed that melittin can lyse bacterial cells, which is associated with the ability of this peptide to induce the formation of pores of variable sizes (~23 nm) onto bacterial membranes.

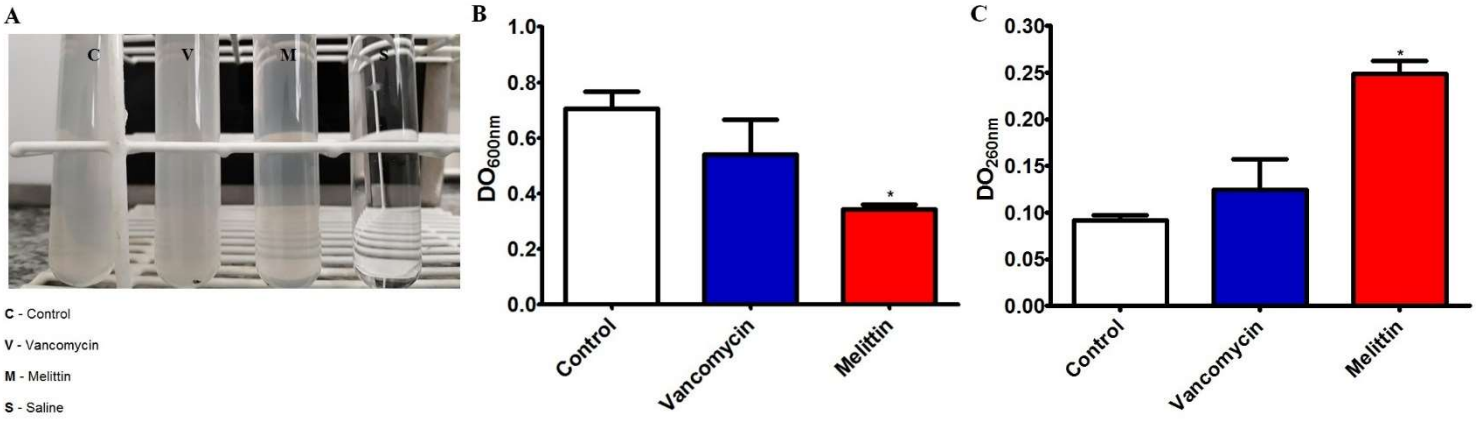

Fig. 4: Melittin treatment resulted in lysis of MRSA USA300. Analysis of lysis by visible spectrum (OD_{600nm}) (A and B). The figure is representative of 3 independent experiments (A). Release of 260-nm-absorbing intracellular material assay (C).

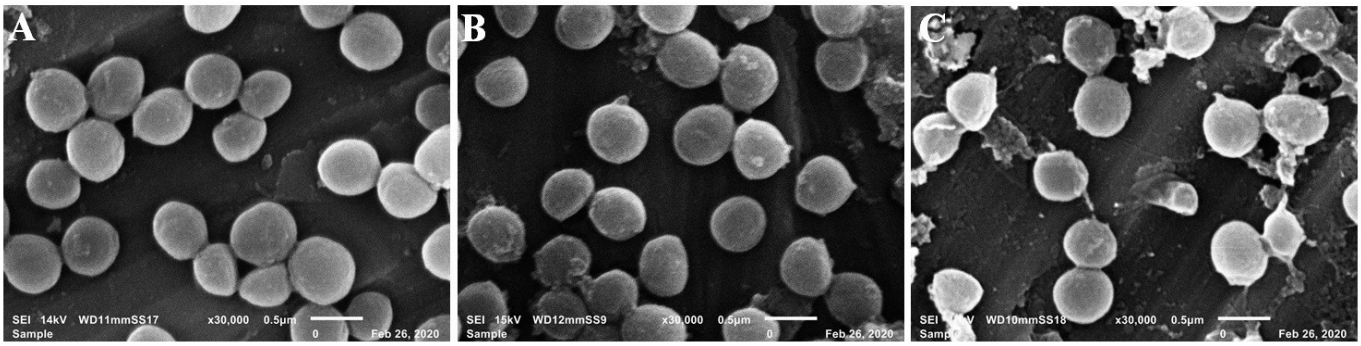

Fig. 5: Scanning electron microscopy (SEM) images of untreated and treated (melittin or vancomycin) methicillin-resistant *S. aureus* (MRSA USA300) cells. Control of untreated cells (A). Cells treated with the positive control (vancomycin) (B). Cells treated with the melittin (C).

Melittin is superior to conventional antibiotics in reducing bacterial load in a murine model of MRSA-infected wound: Despite several in vitro studies describing the biological activities of melittin (Blondelle and Houghten, 1991; Dosler and Alev Gerceker, 2012; Ebbensgaard et al., 2015; Han et al., 2009; Leandro et al., 2015; Moerman et al., 2002; Pandey et al., 2010), few studies have shown the antibacterial effectiveness of this AMP in animal models (Choi et al., 2015; Maiden et al., 2019). To assess the potential of melittin as a topical pharmacological agent, mice were infected with *S. aureus* MRSA USA 300 by intradermal injection allowing the formation of an open wound/abscess (Supplementary file, Fig. S2). After treatment, a significant reduction in the bacterial load from lesions was observed when compared to the untreated groups (6.28 ± 0.77 Log10 CFU/wound) or those treated with base ointment (7.25 ± 0.79 Log10 CFU/wound) ($p < 0.05$; **Fig. 6**). Mice treated with ointment containing 1% melittin showed the greatest reduction in bacterial load (3.79 ± 0.15 Log10 CFU/wound), followed by those that received 0.5% melittin (5.05 ± 0.78 Log10 CFU/wound), melittin 0.25% (5.57 ± 0.20 Log10 CFU/wound), and 1% vancomycin (6.09 ± 0.05 Log10 CFU/wound) (**Fig. 6**). Therefore, treatment with melittin, even at the lowest concentration tested (0.25%), was more effective than the antibiotic vancomycin. These results corroborate the finding that melittin is a promising molecule for topical administration and is, thus, superior to reference drugs.

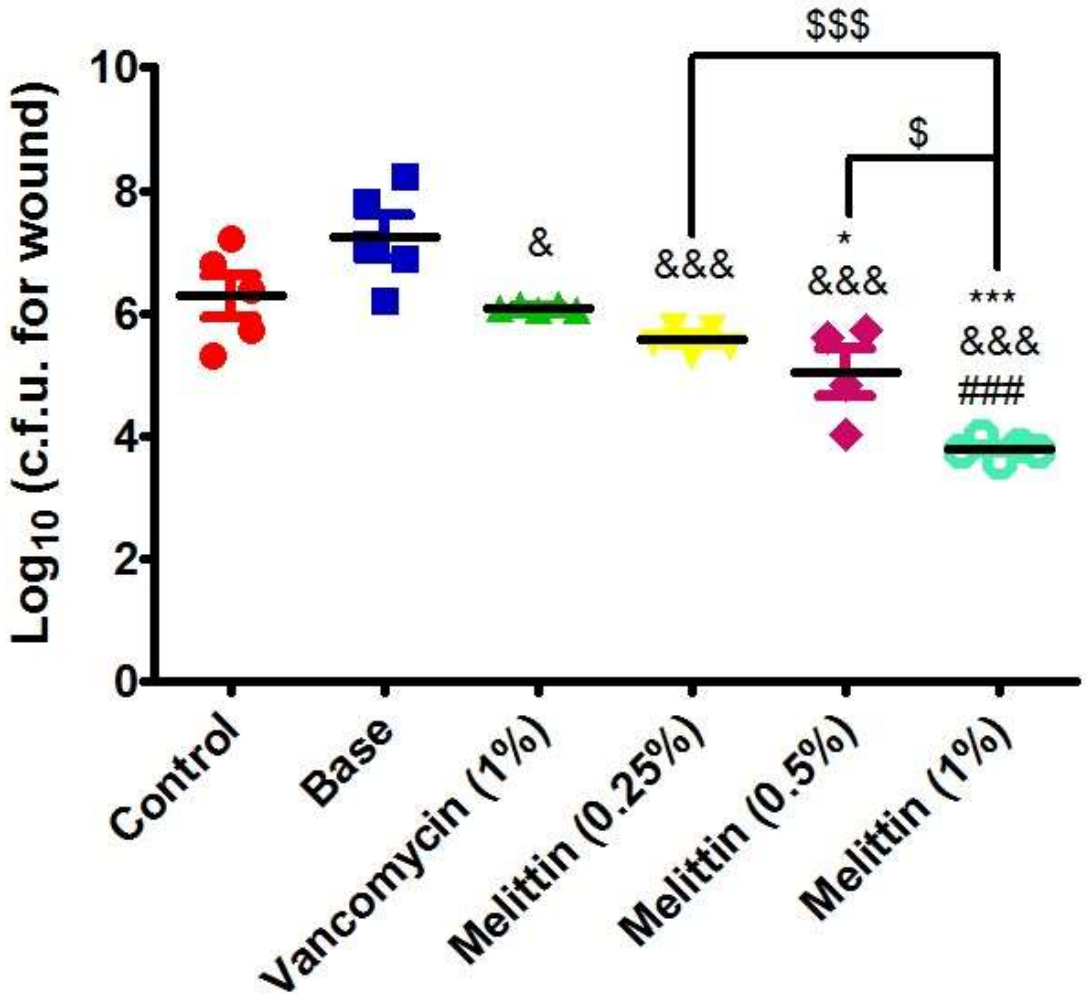

Fig. 6: Bacterial load (Log10 CFU/wound) after the topical treatment of non-surgical meticillin-resistant *Staphylococcus aureus* (MRSA USA300)-infected wounds with ointment contained melittin 0.25%, 0.5%, or 1%, vancomycin 1%, base ointment or saline (controls) (n = 5). Mice were intradermally injected with 10^7 CFU of highly virulent MRSA USA300. Followed 48 h after injection, the mice developed an open wound/abscess at the local site of injection and were treated twice daily for 3 days. One asterisk (*) indicates statistically different than control with $p < 0.05$. Three asterisks (***) indicates statistically different than control with $p < 0.0001$. One ampersand (&) indicates statistically different than base with $p < 0.05$. Three ampersands (&&&) indicates statistically different than base with $p < 0.0001$. Three hashtags (###) indicates statistically different than vancomycin (1%) with $p < 0.0001$. One dollar sign ($) indicates statistically different than melittin (0.5%) with $p < 0.05$. Three dollar signs ($$$) indicates statistically different than melittin (0.25%) with $p < 0.0001$. All results were analyzed by One-way ANOVA with Tunkey post-hoc.

In addition to the plating of the wound, the bacterial load was also evaluated by nuclear techniques employing ^{99m}Tc-CFT. As shown in **Fig. 7A**, uptake of ^{99m}Tc-CFT in the wound region was considerably lower among animals that received melittin compared to untreated controls. Vancomycin, in turn, reduced ^{99m}Tc-CFT uptake in the affected region only slightly. The target-non-target ratio was significantly lower in animals treated with melittin at 0.5% (1.03 ± 0.09) and 1% (0.98 ± 0.24) compared to those treated with base-ointment (1.36 ± 0.13). This shows that the bacterial load in the infected region was similar to that found in the healthy contralateral region (Fig. 7C). As the skin of animals has an indigenous microbiota, the uptake is expected even in regions not infected with MRSA USA300. Scintigraphic imaging of wounds revealed the potent dose-dependent effect of melittin. **Fig. 7B** shows that uptake of ^{99m}Tc-CFT in wounds that received saline, base, or vancomycin was considerably greater than in wounds treated with melittin-containing ointment. Taken together, these results indicate that conventional melittin-based ointments result in high efficacy against skin infections by MRSA USA300 and can be explored as topical bacterial agents.

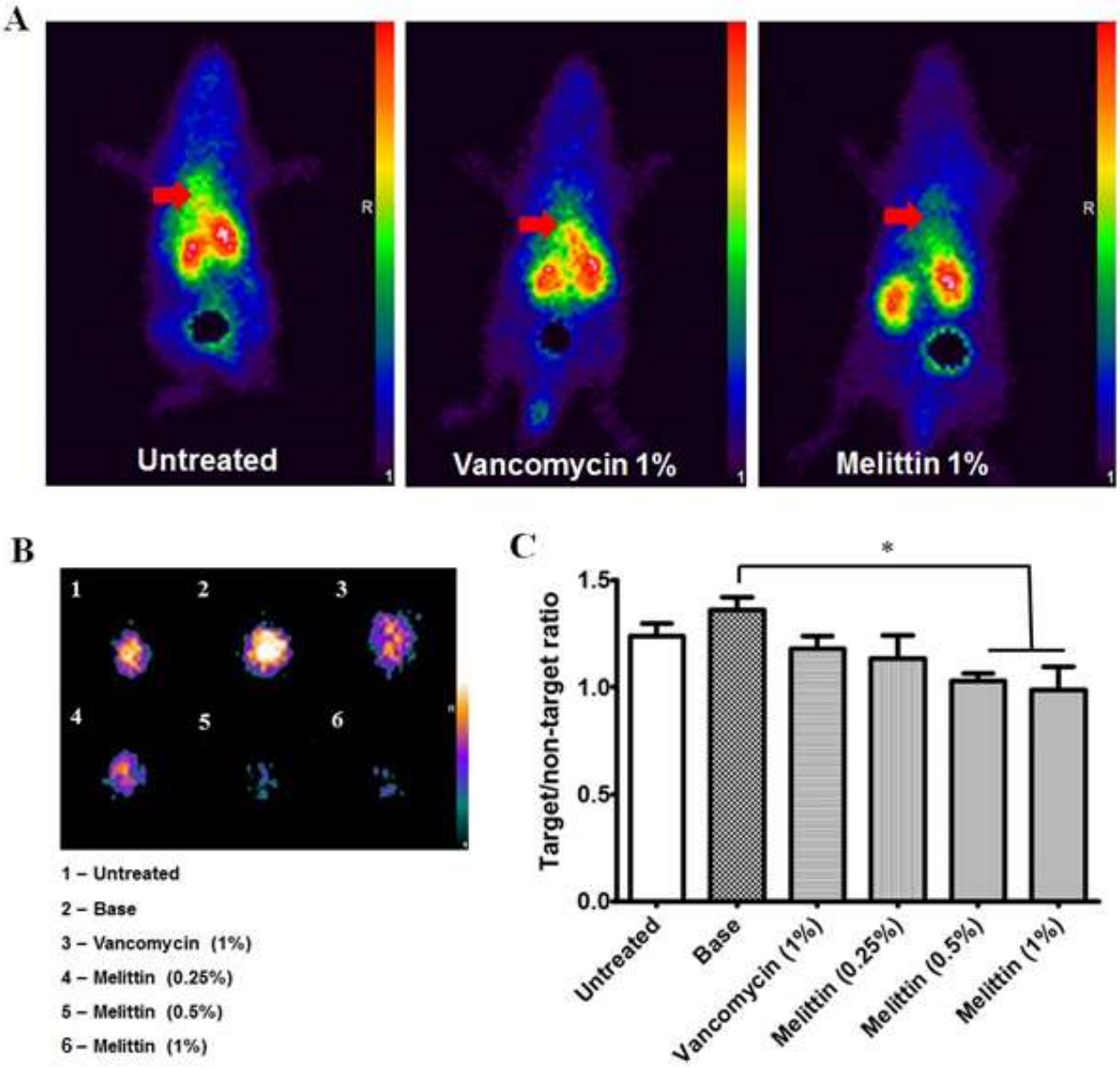

Fig. 7: Scan of non-surgical meticillin-resistant *Staphylococcus aureus* (MRSA USA300)-infected wounds after the topical treatment with melittin (0.25%, 0.5%, or 1%), vancomycin (1%), base ointment or saline (control) using technetium-99m labeled ceftizoxime (^{99m}Tc-CFT) (n = 3). Scintigraphic images from biodistribuition of ^{99m}Tc-CFT in MRSA wound-bearing mice at 6 hours post-intravenous injection of 3.7 MBq of radiolabeled antibiotic. The red arrow points to the wound site (A). Representative planar gamma images of wound recovered from of infected mice 6 hour after i.v. injection of ^{99m}Tc-CFT (B). Target-to-non-target ratios determined by ROI for the ^{99m}Tc-CFT in MRSA wound-bearing mice at 1 hours post-intravenous injection of 3.7 MBq of radiolabeled antibiotic. Statistical analysis was performed by the One-way ANOVA with Dunnett´s post-hoc. * P-values of ≤ 0.05 were considered significant (C).

Melittin reduces skin inflammation associated with MRSA infection: Dermal inflammation associated with MRSA-induced infections is more harmful than the bacterial load itself. This is because inflammation has been associated with delayed healing processes, scarring with aesthetic damage to the skin, and clinical complications (Percival et al., 2015; Tiwari, 2012). Conventional topical treatments, mupirocin and linezolid, used against wounds caused by MRSA work effectively against ongoing infections but have little or no anti-inflammatory action in this context (Gurusamy et al., 2013). Therefore, we aimed to evaluate the potential of melittin in the control of the inflammatory process associated with dermal MRSA-infection by measuring pro-inflammatory cytokines using ELISA. As shown in **Fig. 8**, the 0.25% and 1% melittin ointments significantly reduced the levels of cytokine TNF-α, IL-1β, and IL-6 in the wounds of infected animals. In addition, at a dose of 0.5%, melittin significantly reduced the levels of cytokines IL-6 and TNF-α in the analyzed samples. Similar to what has been observed with mupirocin and linezolid in previous studies (Mohamed et al., 2016; Thangamani et al., 2016, 2015), vancomycin did not reduce the levels of any pro-inflammatory cytokine studied.

This dual antibacterial and anti-inflammatory activity of melittin indicates its potential as a new topical therapeutic option for the treatment of wounds infected with MRSA. The anti-inflammatory mechanism of melittin has been shown to evolve transcription factors that regulate the expression of genes associated with pro-inflammatory cytokines. Therefore, melittin inhibits the release of iκB through the inhibition of IKKs, resulting in the inactivation of NF-κB and inhibition of the generation of inflammatory mediators (Son et al., 2007).

This study has some limitations. First, despite the advantages of using ointments as a delivery vehicle for melitin in the treatment of infected wounds, this formulation has some drawbacks that can compromise treatment adherence and effectiveness. Ointments tend to be greasy and difficult to remove however, which may impact patient compliance, and they lack the ability to provide a cooling effect through surface evaporation, potentially exacerbating discomfort. They also prevent excessive exudate from escaping from a wound, which may cause maceration of healthy skin (Harrison and Spada, 2018). Second, even though it remains active after incorporation in the ointment, the stability of melittin

in this formulation needs to be elucidated in future studies. Third, data on the release/penetration of melittin when included in ointment are absents and should be the subject of further studies. These results can help in discussing antimicrobial activity of formulation proposed in this work.

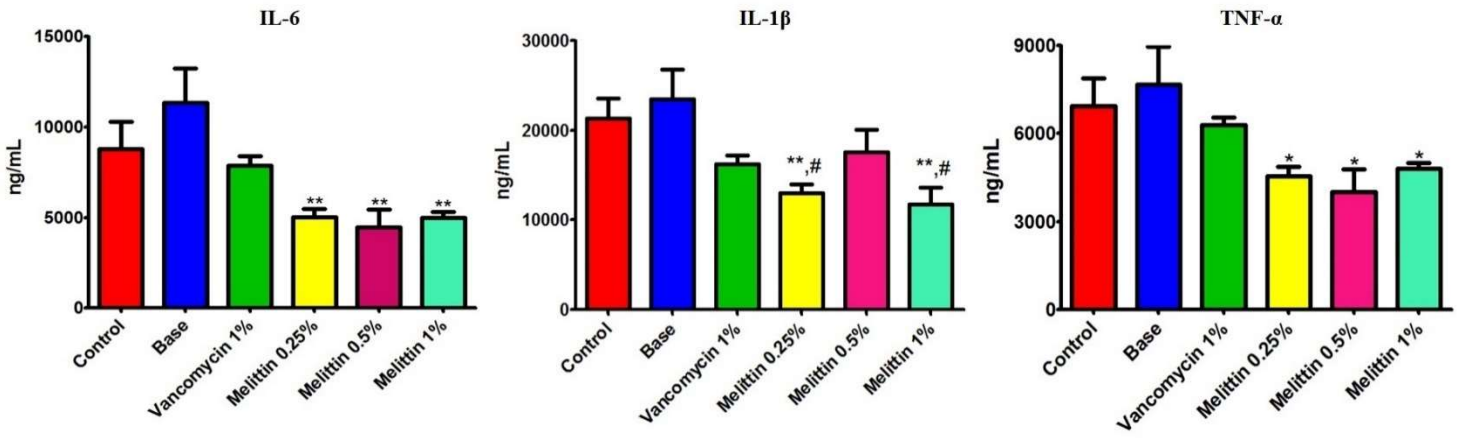

Fig. 8: Inflammatory cytokine levels in non-surgical meticillin-resistant *Staphylococcus aureus* (MRSA USA300)-infected wounds treated with melittin 0.25%, 0.5% and 1%, vancomycin (1%), base ointment or saline (controls) (n = 5). Statistical analysis was performed by the One-way ANOVA with Dunnett´s post-hoc. * P-values of ≤ 0.05 were considered significant. IL-6: Interleukin-6; IL-1β: Interleukin-1 beta; TNF-α, Tumor necrosis factor alpha.

Conclusion

In summary, melittin, the main component of apitoxin, has good antibacterial and anti-inflammatory activities against non-surgical MRSA-induced wound models. In vitro tests showed that melittin has a potent antibacterial effect even in strains highly resistant to antibiotics. Due to its ability to lyse *S. aureus* cells, melittin has a rapid bactericidal activity (within 0.5 h of incubation). This effect is maintained even in stationary and persister cells, which are generally insensitive to currently available antibiotics. The ability to interact synergistically with conventional antibiotics as well as the possibility of reverting the drug-resistant phenotype in bacteria highlights the potential of combining melittin with other pharmacological agents against MRSA dermal infections. Over 21 days, no isolate with acquired resistance after exposure to sub-inhibitory concentrations of melittin was recovered, indicating its low resistance-inducing capacity. Its stability

in the face of different conditions frequently found in infected wounds as well as its capacity to effectively reduce bacterial load and inflammation in this context highlights the pharmacological potential of melittin. Collectively, these data indicate that melittin is a good potential candidate for topical formulations aimed at the treatment of wounds caused by MRSA.

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Compliance with Ethical Standards

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Conflict of Interest: All authors report that they do not have any conflicts of interest.

Ethical approval: The study was approved by the Laboratory Animal Research Ethics Committee of the Federal University of Minas Gerais (CEUA-UFMG: 299/2019).

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SUPPLEMENTARY FILE

In-depth characterization of antibacterial activity of melittin against *Staphylococcus aureus* and use in a model of non-surgical MRSA-infected skin wounds

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Table S1: Main phenotypic characteristics of the employed microorganisms

Microorganisms	Reference	Clinical origin	Phenotypic characteristic
S. saprophyticus ATCC 15305	ATCC	Urine	-
S. epidermidis ATCC 12228	ATCC	-	-
S. aureus ATCC 6538	ATCC	Wound	MSSA
S. aureus ATCC 29213	ATCC	Wound	MSSA
S. aureus MRSA USA 300	ATCC	Wound	MRSA
S. aureus 8	LAMIA collection	-	MSSA
S. aureus IC1	LAMIA collection	Wound	MSSA
S. aureus IC2	LAMIA collection	Wound	MSSA
S. aureus MRSA2	LAMIA collection	Blood (Hemoculture)	MRSA
S. aureus H1	LAMIA collection	Blood (Hemoculture)	MSSA
S. aureus H2	LAMIA collection	Blood (Hemoculture)	MRSA
S. aureus H3	LAMIA collection	Blood (Hemoculture)	MRSA
S. aureus H4	LAMIA collection	Blood (Hemoculture)	VISA
S. aureus ST1	LAMIA collection	Abscesses	MSSA
S. aureus ST2	LAMIA collection	Abscesses	MSSA
S. aureus ST3	LAMIA collection	Abscesses	MRSA

MRSA: Methicillin-resistant *Staphylococcus aureus*; MSSA: Methicillin-sensible *Staphylococcus aures*; VRSA: Vancomycin-intermediate *Staphylococcus aureus*

Table S2: Composition of the ointment employed for melittin and positive control (Vancomycin) delivery.

Components	Quantity	Function
Lanoline	30%	Base
Vaseline	70%	Base
Butyl hydroxy toluene (BHT)	0.02%	Antioxidant
Dimethyl sulfoxide	q.s.*	Dispersant

*The maximum quantity employed was 100 µL.

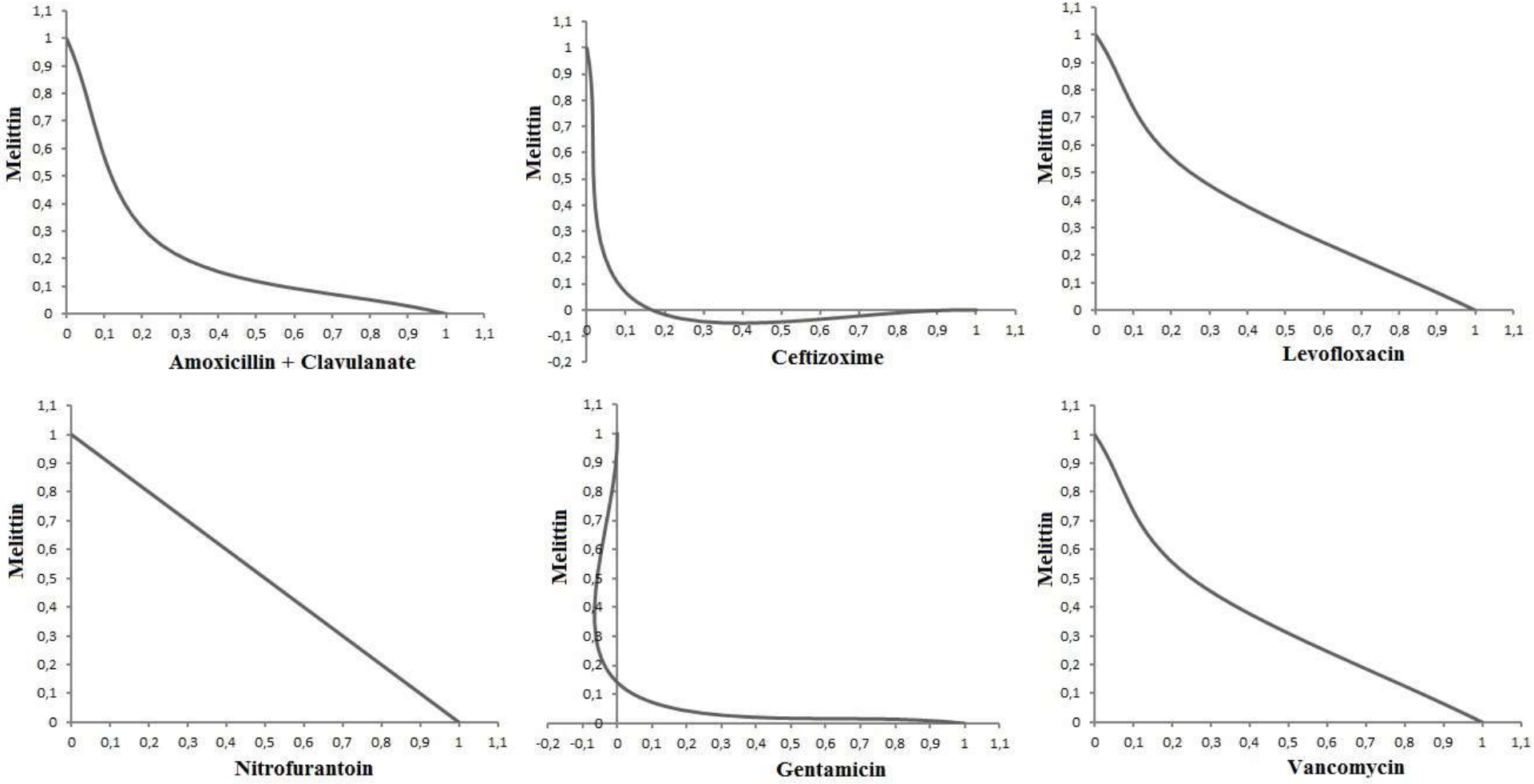

Figure S1: Isobolograms of melittin in combination with antibiotics (beta-lactams, aminoglycosides, quinolones and glycopeptides) against methicillin-resistant *Staphylococcus aureus* (MRSA USA 300).

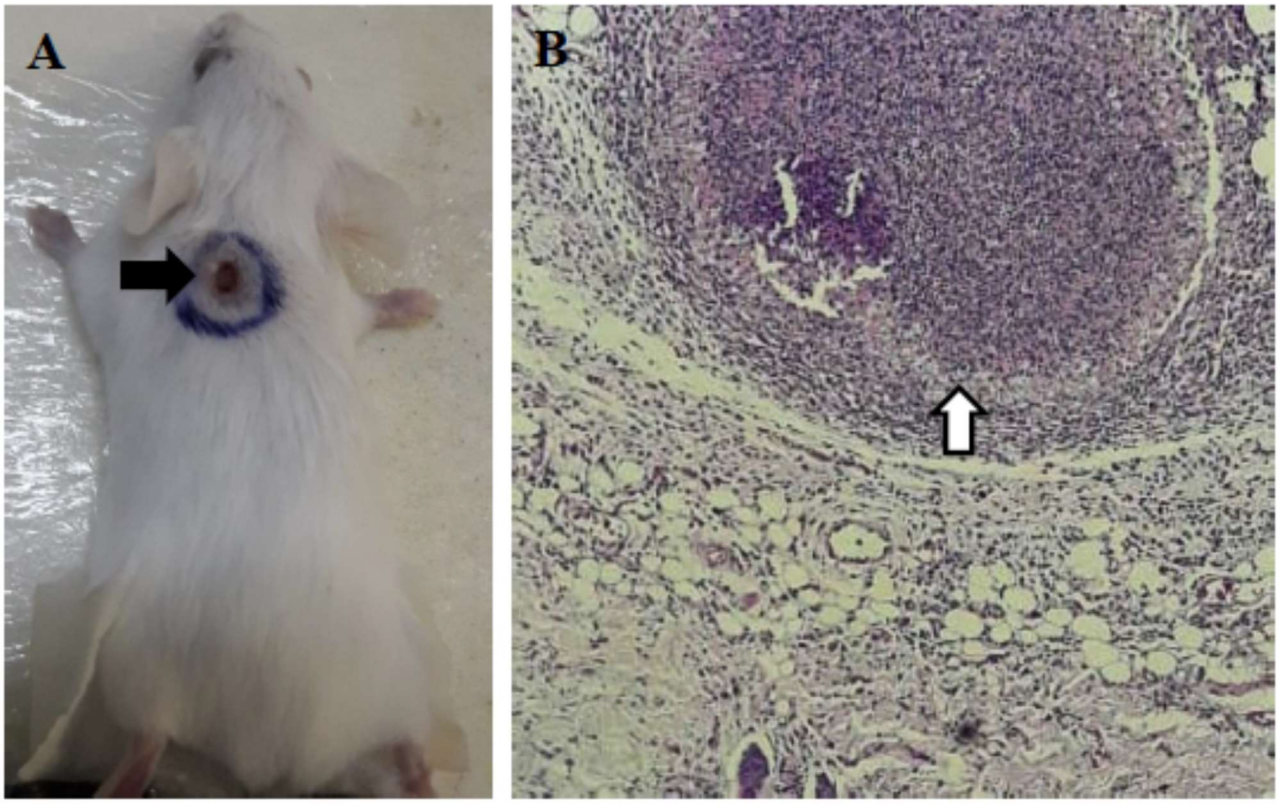

Figure S2: Macroscopic and histopathological aspects of the open wound induced by subcutaneous injection of methicillin-resistant *Staphylococcus aureus* (MRSA USA300) in male BALB/c mice. (A) Open wound originating from the intense inflammatory process associated with dermal infection of MRSA USA300. (B) Micrograph of histological sections of the skin area infected by MRSA USA300. It is possible to verify a large abscess in the sub-epidermal region filled with polymorphonuclear cells. For the histological analysis, the samples of wounds were fixed in 10% buffered formalin and then dehydrated and processed, followed by paraffin embedding. Next, were obtained sections of 5 µm thickness for hematoxylin-eosin staining. The sections were analyzed using a light microscope (Carl Zeiss AG, Oberkochen, B-W, Germany).

Capítulo III

Atividade terapêutica do peptídeo LyeTx I mnΔK contra pneumonia induzida por *Acinetobacter baumannii* resistente aos carbapenêmicos

A short synthetic peptide, based on LyeTx I from *Lycosa erythrognatha* **venom, shows potential to treat pneumonia caused by carbapenem-resistant** *Acinetobacter baumannii* **without detectable resistance**

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Abstract: The emergence of antibiotic-resistant bacteria, especially carbapenem-resistant *Acinetobacter baumannii* (CRAB), together with relative stagnation in the development of effective antibiotics, has led to enormous health and economic problems. In this study, we aimed to describe the antibacterial spectrum of LyeTx I mnΔK, a short synthetic peptide based on LyeTx I from *Lycosa erythrognatha* venom, against CRAB. LyeTx I mnΔK showed considerable antibacterial activity against extensively resistant *A. baumannii*, with minimum inhibitory and bactericidal concentrations ranging from 1 to 16 μM and 2 to 32 μM, respectively. This peptide significantly increased the release of 260 nm-absorbing intracellular material from CRAB, suggesting bacteriolysis. LyeTx I mnΔK was shown to act synergistically with meropenem and colistin against CRAB. The cytotoxic concentration of LyeTx I mnΔK against Vero cells (CC_{50} = 9.40±2.84 μM) and its hemolytic activity (HC_{50} = 77.07±4.00 μM) were considerably low; however, its antibacterial activity was significantly reduced in the presence of human and animal serum and trypsin. Nevertheless, the inhalation of this peptide was effective in reducing pulmonary bacterial load in a mouse model of CRAB infection. Altogether, these results demonstrate that the peptide LyeTx I mnΔK is a potential prototype for the development of new effective and safe antibacterial agents against CRAB.

Keywords: Antimicrobial peptides; Carbapenem-resistant *Acinetobacter baumannii* (CRAB); Pneumonia; Venoms; *Lycosa erythrognatha*; Biopharmaceutical innovation.

1. Introduction

Acinetobacter baumannii is an aerobic, gram-negative, opportunistic, non-fermentative, and non-motile coccobacillus commonly found in hospital environments [1]. This bacterium can adhere to medical devices (including mechanical ventilation systems) and survive for up to 33 days on dry surfaces [2,3]. Furthermore, the acquisition of multiple-drug resistance, especially to carbapenem, has made this pathogen a major public health concern [1]. Since the first carbapenem-resistant *A. baumannii* (CRAB) was reported in 1991, a considerable increase in the number of these resistant strains has been documented worldwide [4,5]. Thus, the World Health Organization (WHO) has classified CRAB as a top-priority organism for research and development of new antibiotics [6].

Antimicrobial peptides (AMPs) have shown significant promise in recent years as novel therapeutic agents for the treatment of infections caused by extensively drug-resistant (XDR) bacteria, such as CRAB [7]. AMPs occur naturally as a component of the innate immune response of virtually all species, from vertebrates to bacteria and plants. Their prominent antimicrobial effects have been explored by the pharmaceutical industry for the development of new biopharmaceuticals, especially those developed against pathogens resistant to conventional therapies [7,8]. In addition to possessing potent antibacterial activity, AMPs have several unique advantages over traditional antibiotics, including a primarily bactericidal effect, low potential to elicit the development of resistance, potent antibacterial activity, and absence of training waste (because of its rapid hydrolysis in the environment) [7,8]. However, there are several limitations in utilizing naturally derived AMPs, particularly for the treatment of invasive infections. These limitations include high production costs, high toxicity, low stability in the presence of salt or serum components, and rapid renal elimination [9]. Thus, these limitations need to be addressed, and short AMPs may hold potential for utilization as antibacterial agents. In fact, the simplified sequences should facilitate the rapid production of AMPs, decrease synthesis costs, increase stability, and accelerate their translational clinical applications [10].

In the present study, the in vitro and in vivo antibacterial activity of a unique AMP, LyeTx I mnΔK, was investigated against CRAB. LyeTx I mnΔK (H-IWLTKALKFLGKNLGK-NH_2; Molecular weight: 1828.13 Da) is a *de novo*-designed

short peptide comprising 16 residues modified from the *C*-terminal portion of LyeTx I (H-IWLTALKFLGKNLGKHLAKQQLAKL-NH_2; Molecular weight: 2830.73 Da), a natural peptide derived from *Lycosa erythrognatha* spider venom that has been shown to be active against pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*) and fungi (*Candida krusei* and *Cryptococcus neoformans*)[11–13]. Thus, this study aimed to investigate the antibacterial spectrum of LyeTx I mnΔK against a collection of important CRAB strains isolated from clinical settings and to explore their antibiofilm activity and ability to induce the emergence of bacterial resistance after serial passage with the peptide. In addition, we investigated whether LyeTx I mnΔK could be used in combination with conventional antibiotics and assessed its efficacy in a CRAB-induced pneumonia model using immunocompetent mice. Furthermore, we aimed to investigate toxicity *in vitro* by using cytotoxicity assays in mammalian cells and assessing hemolytic activity.

2. Material and Methods

2.1. Microorganisms and reagents

The chemicals and bacterial strains used in this study are presented in the supplementary file.

2.2. Antibacterial assay

Mueller Hinton broth[14] supplemented with Tween-80 (0.002%) was used to determine the minimal inhibitory concentration (MIC) using the broth microdilution technique according to the Clinical and Laboratory Standards Institute [15]. The peptide (LyeTx I mnΔK) and controls were dissolved in autoclaved distilled water and serially diluted two-fold to concentrations of 0.25–32 µM. The results were visualized after 24 h of incubation at 35±2°C, and the MIC value was considered as the lowest concentration of the tested compound able to prevent visible growth. Furthermore, the minimal bactericidal concentration (MBC) was determined by plating 10 µL of the optically free growth wells in the MIC assay on Mueller-Hinton agar (MHA). After incubation for 24 h at 35±2 °C, MBC was defined as the lowest concentration of

compounds that killed at least 99.9% of the initial inoculum in relation to the untreated control.

2.3. Release of cellular material

The release of intracellular material with absorbance at 260 nm was quantified according to the modified methods of Bennis et al. [16]. Aliquots of 5 mL obtained from CRAB (AC37 strain) suspensions (10^8 cells/mL) prepared in 0.9% saline were treated with LyeTx I mnΔK or colistin at 10× their respective MICs for 24 h. After treatment, cells were centrifuged at 2,500 × g for 5 min, and the absorbance of the supernatant at 260 nm was determined using an ultraviolet spectrophotometer (Hitachi U-1100, Lancashire, UK). A solution containing only the peptide was considered as blank for treated cells. Saline and colistin solutions were used as a blank for the negative and positive controls, respectively.

2.4. Anti-biofilm assay

The effect of LyeTx I mnΔK on the preformed biofilm of CRAB (AC37 strain) was evaluated according to the methodology described by Herrera et al. [17]. Initially, CRAB suspensions (10^6 CFU/mL) were cultivated in 96-well microplates at 35±2 °C for 24 h in Tris-buffered saline supplemented with 100 mM glucose to allow biofilm adhesion. Then, the cells were washed and treated with the compounds (8–32 μM), and the microplates were incubated for 24 h at 35±2 °C. After incubation, the microplates were washed, treated with 125 μL of 0.1% crystal violet, and incubated for 30 min at room temperature. Next, the microplates were washed to remove the excess crystal violet and left to dry inverted for 1 h. Finally, the crystal violet was diluted with 125 μL of 95% ethanol, and the biofilms were quantified by measuring the optical density at 570 nm in a microplate reader (Bio-Tek Instruments, New York, NY, USA). Colistin was used as a positive control. The results were expressed graphically as a function of the percentage of reduction of mature biofilm *vs.* the concentration of compounds.

2.5. Synergism assay

The interactions of LyeTx I mnΔK with the antibiotics meropenem, levofloxacin, colistin, and gentamicin were evaluated by the checkerboard assay as described by Orhan et al. [18]. The fractional inhibitory concentration (FIC) index (FICI) was determined as the sum of the FIC of the compound and the FIC of the antibiotic (*i.e.*, $FICI = FIC_{LyeTx\ mn\Delta K} + FIC_{antibiotic}$, where $FIC = MIC_{compound\ in\ combination}/MIC_{compound\ alone}$). According to Oroojalian et al. (2010), the effect of the combination is considered as synergic if FICI ≤ 0.5, additive if 0.5 < FICI ≤ 1.0, indifferent if 1.0 < FICI ≤ 4.0, and antagonistic if FICI > 4.0. In addition, the results were interpreted by plotting the FIC values of each compound involved in the combinations. Thus, a graph called an isobologram was generated, where agents are considered synergistic when the orthogonal projection is concave, additive or indifferent if the projection behaves like a straight line, and antagonistic in cases where the projection takes a convex form.

2.6. *Stability of the antibacterial action of LyeTx I mnΔK*

The activity of LyeTx I mnΔK against CRAB (AC37 strain) in the presence of different conditions, such as high saline concentrations (NaCl, $CaCl_2$, and KCl), acidic and basic pH, presence of human serum and plasma, and physiologic concentrations of plasmatic proteins, were evaluated using the broth microdilution assay described previously. In addition, to examine the ability of compounds to resist proteolytic digestion by mammalian proteases, LyeTx I mnΔK was incubated with trypsin at a molar ratio of 500:1 (peptide:enzyme).

2.7. Multi-step resistance study

The ability of CRAB (AC37 strain) to develop resistance to LyeTx I mnΔK and colistin was investigated via a multi-step resistance study, as described previously [19]. Bacteria were exposed to two dilutions below the MIC of each compound for 14 days. The MIC of LyeTx I mnΔK and colistin was set daily during the experimental period, and the results were expressed graphically as a function of the time of MIC increase *vs.* exposure time (in days).

2.8. *Toxicity*

Toxicity was determined by assays for cytotoxicity in mammalian cells,[20] hemolytic activity,[21] and antibacterial activity against a probiotic species (*Lactobacillus fermentum* ATCC14932).[14] All methodological details of toxicity assay are described in the supplementary file.

2.9. In vivo assay

Forty-six-week-old female BALB/c mice (Central vivarium of the UFMG, Belo Horizonte, MG, Brazil) were used in this study. All experimental procedures strictly followed the international protocols for laboratory animal management, and the methods were approved by the Laboratory Animal Research Ethics Committee of the Federal University of Minas Gerais (CEUA-UFMG: Protocol number = 367/2019). A murine pneumonia model induced by CRAB (AC37 strain) was prepared as described elsewhere[22] with minor modifications. Briefly, mice (five mice per group) after anesthetization (60 mg/kg ketamine + 8 mg/kg xylazine, intraperitoneally) were infected by intranasal instillation of a suspension containing ~10^8 CFU of CRAB (40 µL). Two hours after animals were infected, treatment was initiated under new anesthesia. Five groups of mice were treated intranasally with LyeTx mnΔK (1, 5, and 10 mg/kg) or colistimethate sodium (10 mg/kg). The control group received 0.9% saline solution intranasally. Treatments were administered once. Then, 22 h after the treatment, the mice were humanely euthanized *via* cervical dislocation under anesthesia. The lungs were precisely excised, weighed, homogenized, serially diluted in 0.9% saline (10^{-1}-10^{-6}), and then transferred to MHA plates. Plates were incubated at 35±2 °C for 24 h for posterior colony counting and determination of bacterial load (*i.e.*, Log_{10} CFU/g of lung).

3. Results and Discussion

3.1. Antibacterial activity of LyeTx I mnΔK against CRAB

The antibacterial activity of LyeTx I mnΔK against a panel of clinically relevant strains of extensively drug-resistant *A. baumannii* was evaluated by determining MICs and MBCs. As shown in **Table 1**, LyeTx I mnΔK was active against all tested *A. baumannii* isolates, with MICs in the range of 1–16 μM. The concentrations of this peptide required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates tested were 4 μM and 8 μM, respectively. With regard to colistin, the MIC_{50} and MIC_{90} values were 0.8 μM and 1.6 μM, respectively. Interestingly, LyeTx I mnΔK retained its antibacterial activity against CRAB strains that were resistant to several antibiotic classes, including tetracycline, cephalosporin, penicillin, macrolides, quinolones, and aminoglycosides, suggesting that cross-resistance between these particular antibiotics and LyeTx I mnΔK is unlikely to occur [10].

The antibacterial effect of LyeTx I mnΔK was mainly bactericidal. This short peptide was able to kill *A. baumannii* isolates at concentrations within the range of 2–32 μM, showing MBC_{50} and MBC_{90} values of 8 μM and 32 μM, respectively. In fact, cationic AMPs such as LyeTx I mnΔK are known to be mainly bactericidal, since they have the ability to interact electrostatically with the bacterial anionic membrane, thus causing lysis and consequent microbial death [9,23,24]. When compared to the original natural peptide (LyeTx I), the truncated derivative (LyeTx I mnΔK) is less active against *A. baumannii* ATCC 19606 (MIC: LyeTx I mnΔK 8 μM *vs.* LyeTx I 0.70 μM; and MBC: LyeTx I mnΔK 8 μM *vs.* LyeTx I 2 μM)[13]. It may be related to reduced hydrophobicity and positive charge after the restriction in the peptide structure. Indeed, highly hydrophobic and positively charged peptides interact better with the bacterial membrane and are therefore more microbicidal[10].

	Microorganisms	Origen	LyeTx I mnΔK (μM)		Colistin (μM)	
			MIC	MBC	MIC	MBC
CSAB	*A. baumannii* ATCC19606	-	8	8	0.4	3.2
	A. baumannii AC09	Wound	2	2	0.4	0.4
	A. baumannii AC36	Tracheal aspirate	8	8	0.2	0.4
CRAB	*A. baumannii* AC10	Wound	2	2	0.8	1.6
	A. baumannii AC37	Central venous catheters	4	4	0.4	0.4
	A. baumannii AC30	Bloodstream infections	8	8	0.4	1.6
	A. baumannii AC31	Bloodstream infections	16	16	0.8	1.6
	A. baumannii AC35	Tracheal aspirate	4	4	0.02	0.02
	A. baumannii AC03	Liquor	4	4	0.4	0.4
	A. baumannii AC04	Tracheal aspirate	4	16	0.05	0.4
	A. baumannii AC25	Wound	4	8	1.6	12.8
	A. baumannii AC26	Tracheal aspirate	2	16	0.4	1.6
	A. baumannii AC52	Tracheal aspirate	2	32	0.8	0.8
	A. baumannii AC40	Tracheal aspirate	16	32	0.8	0.8
	A. baumannii AC43	Sputum	2	2	0.8	0.8
	A. baumannii AC47	Urine	1	8	1.6	1.6
	A. baumannii AC45	Tracheal aspirate	4	32	1.6	6.4
	A. baumannii AC39	Urine	4	8	0.8	3.2
	A. baumannii AC55	Wound	4	4	1.6	12.8
	A. baumannii AC23	Bloodstream infections	8	8	0.2	0.8
	A. baumannii AC24	Bloodstream infections	8	8	1.6	1.6
		MIC_{50}	4		0.8	
		MIC_{90}	8		1.6	
		MBC_{50}	8		1.6	
		MBC_{90}	32		6.4	

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of LyeTx I mnΔK and colistin against clinical and drug-resistant *Acinetobacter baumannii*

CSAB: Carbapenem-susceptible *Acinetobacter baumannii*; CRAB: Carbapenem-resistant *Acinetobacter baumannii*; MIC_{50}: Concentration required to inhibit 50% of isolates; MIC_{90}: Concentration required to inhibit 90% of isolates; MBC_{50}: Concentration required to kill 50% of isolates; MBC_{90}: Concentration required to kill 90% of isolates.

3.2. Bacteriolysis induced by LyeTx I mnΔK

Overall, AMPs are known membrane-active agents. In fact, although intracellular targets such as DNA, RNA, proteins, enzymes, and/or lipids have been described as AMPs, the primary target of these molecules appears to be the cell membrane [25]. Several membrane experiments using in vitro models clearly showed the interaction of AMPs with the lipid components of this cellular structure as well as the disruption of lipid bilayers, which correlates well with the lysis of bacterial cells exposed to these agents [9,23–25]. To determine whether disruption of the cell membrane was induced by LyeTx I mnΔK, the loss of intracellular material with absorption at 260 nm was evaluated. As shown in **Figure 1**, the exposure of CRAB to LyeTx I mnΔK at 10× MIC for 24 h induced a marked increase in the release of cellular material compared to untreated cells (0.09±0.02 vs. 0.25±0.07; $p<0.05$), suggesting that this peptide is able to disrupt the integrity of the bacterial membrane. Colistin, a polymyxin that competitively displaces divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids, which leads to the disruption of the outer cell membrane and leakage of intracellular contents [26], also increased the loss of 260 nm-absorbing material (0.25±0.03), thereby validating the experimental conditions employed in this study.

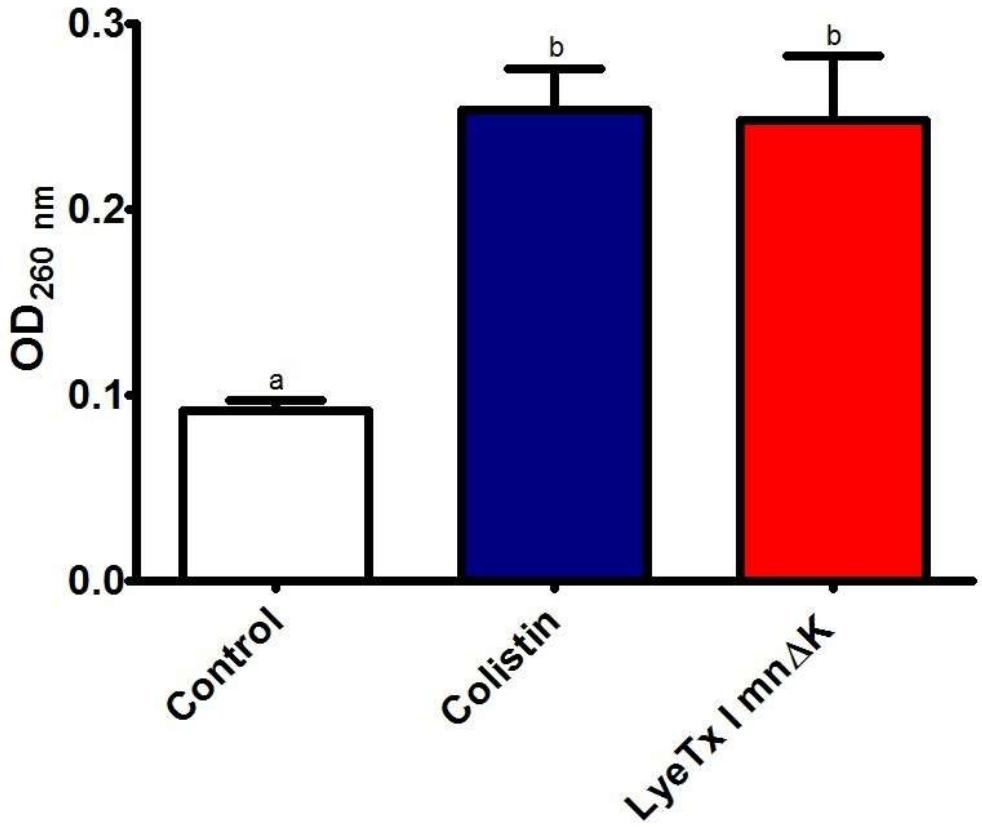

Figure 1: Release of 260 nm-absorbing intracellular materials in carbapenem-resistant *Acinetobacter baumannii* induced by exposure to LyeTx I mnΔK or colistin during 24 h. Different letters represent a statistically significant difference ($p < 0.05$) determined by one-way analysis of variance with Dunnett's post-hoc test (all groups were compared with untreated cells).

3.3. LyeTx I mnΔK reduces preformed biofilms of CRAB

A. baumannii is capable of forming biofilms, which are aggregates of microbial cells that are encompassed by self-produced exopolysaccharide matrices [27]. Biofilms demonstrate greater protection against antibiotics, host immune defense, and adverse environmental conditions than free-living cells [28], and the inability of conventional antimicrobials to target and disrupt adherent bacterial biofilms is a great challenge for current antibiotic therapies [29,30]. Thus, the potential of LyeTx I mnΔK to disrupt the preformed CRAB biofilm was evaluated using the crystal violet method. The peptide LyeTx I mnΔK showed a potent anti-biofilm effect in a dose- and time-dependent manner. As shown in **Figure 2**, LyeTx I mnΔK at 32 μM significantly reduced the CRAB biofilm mass by 46.68% and 63.22% after 24 h and 48 h of incubation, respectively.

The anti-biofilm effect of LyeTx I mnΔK was similar to the effect observed with colistin at 32 μM, which was able to reduce the preformed CRAB biofilm by 50.87% and 69.48% after 24 h and 48 h of incubation, respectively (**Figure 2**). These results demonstrate that LyeTx I mnΔK is capable of reducing adherent CRAB biofilms, thus enabling future studies to evaluate this peptide as a novel treatment option for biofilm-related *Acinetobacter* infections.

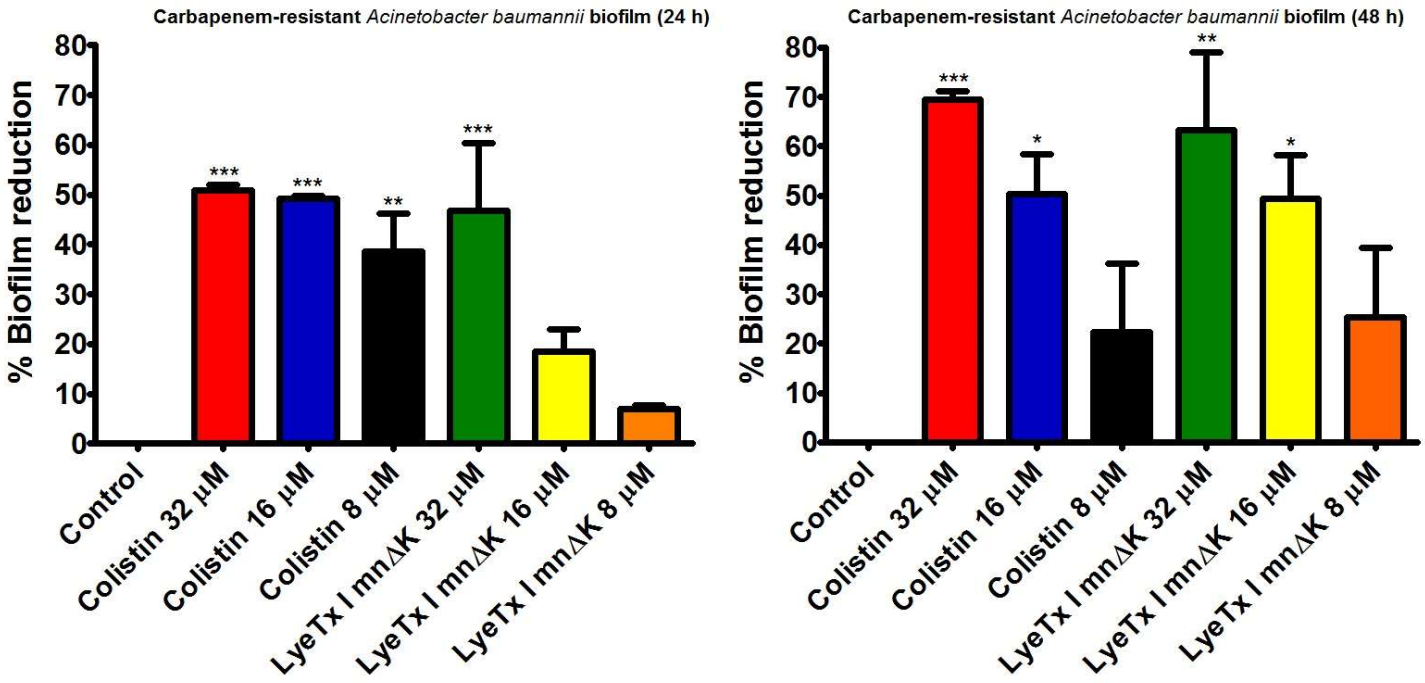

Figure 2: Anti-biofilm activity of LyeTx I nmΔK or colistin against 24- and 48-hour-old biofilms of carbapenem-resistant *Acinetobacter baumannii*. The adherent biofilm was stained by crystal violet; then, the dye was extracted with ethanol, and absorbance was measured at 595 nm. The results were presented as the percentage of biofilm reduction compared to that of untreated cells. All experiments were conducted in quadruplicate for statistical significance. One asterisk (*) indicate a statistically significant difference compared to the control with $p < 0.05$. Two asterisks (**) indicate a statistically significant difference compared to the control with $p < 0.01$. Three asterisks (***) indicate a statistically significant difference compared to the control with $p < 0.0001$. The results were analyzed by one-way analysis of variance with Dunnett's post-hoc test.

3.4. Combination therapy of LyeTx I mnΔK with conventional antimicrobials

Combinations of antibiotics offer a productive strategy to address the widespread emergence of antibiotic-resistant strains, especially XDR bacteria such as CRAB. Therapies based on combinations of different antimicrobial agents are mostly utilized because this strategy broadens the antibacterial spectrum, reduces the concentration necessary for each drug to generate its therapeutic effect, decreases the side effects associated with high doses of antibiotics in monotherapy, and increases the synergism between different treatments [31,32]. Furthermore, the chance of resistance emerging against two or more drugs is considerably lower than that of a single drug.[30] To study the effect of combinations of LyeTx I mnΔK with conventional antibiotics (meropenem, Gentamicin, levofloxacin, and colistin), we employed checkerboard assay. In combination with LyeTx I mnΔK, meropenem (FICI 0.19) and colistin (FICI 0.37) showed synergic effects, and gentamicin revealed an additive effect (FICI 0.75). Meanwhile, an indifferent effect was observed when combined with levofloxacin (FICI 3.0) (**Table 2**). These results indicate that the peptide has a strong synergistic effect when combined with carbapenem and polymyxins, as confirmed by the concave projections of meropenem and colistin in the isobolographic analysis (Figure S1, Supplementary file). The synergistic effects of LyeTx I mnΔK with these antibiotics are probably due to damage induced by this peptide on the bacterial membrane, consequently increasing the permeability to the antibiotic and its access to bacterial cells [10,23].

Table 2: The fractional inhibitory concentration (FIC) and FIC index (FICI) range of LyeTx I mnΔK in combination with different antibiotics against carbapenem-resistant *Acinetobacter baumannii*

Class	Antibiotics	FIC		FICI (ΣFIC)	Effect
		LyeTx I mnΔK	Antibiotics		
Carbapenem	Meropenem	0.13	0.06	0.19	Sinergy
Aminoglycosides	Gentamicin	0.50	0.25	0.75	Additive
Quinolones	Levofloxacin	1.0	0.24	3.0	Indifferent
Polymyxin	Colistin	0.13	0.24	0.37	Sinergy

ΣFICI: Fractional inhibitory concentration index. FIC index was interpreted as follows: An FIC index of ≤ 0.5 is considered to demonstrate synergy. Additive was defined as an FIC index between 0.6 and 1. An FIC index between 1.1 and 4 was considered such as indifferent effect. Antagonism was defined as an FIC index of > 4.

3.5. LyeTx I mnΔK is unable to generate resistance in vitro

According to the WHO, antibiotic resistance is an increasingly serious threat to global public health that requires action across all government sectors and society [33]. This phenomenon is particularly common among clinical isolates of *A. baumannii*, which are generally likely to carry intrinsic and acquired genes that encode resistance to various conventional antibiotics [34,35]. Therefore, to assess whether LyeTx I mnΔK induces resistance in CRAB, a multi-step resistance selection study was conducted. As shown in **Figure 3**, during the 14 days of exposure to a sub-inhibitory concentration (two-fold below the MIC) of LyeTx I mnΔK, no resistant strains were identified, and the MICs remained constant throughout the experiment. In contrast, the MIC of colistin increased two-fold after the eighth day of passage and four-fold on the fourteenth day, indicating that the antibiotic induced resistance in vitro. These results highlight that LyeTx I mnΔK has a low potential to elicit resistance. In fact, peptides are known for their stability in relation to microbiological resistance due to their mechanism of action on the bacterial membrane. This is because resistance mechanisms that involve the cellular membrane are usually accompanied by a considerable reduction in the virulence or even viability of pathogens, which is considered a “costly” solution for most microbial species [34,36].

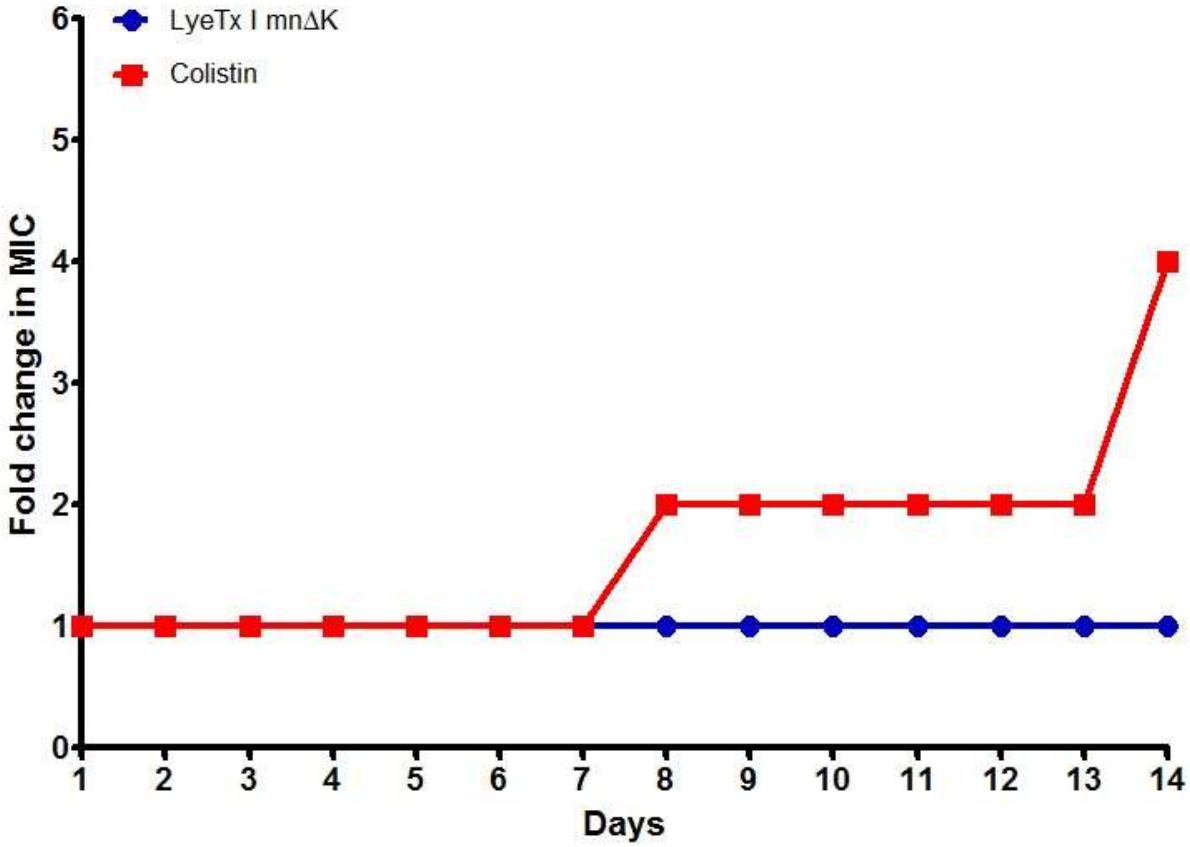

Figure 3: Multi-step resistance study of LyeTx I nmΔK or colistin against carbapenem-resistant *Acinetobacter baumannii*. Bacteria were serially passaged over a 14-day period, and the broth microdilution assay was used to determine the minimum inhibitory concentration (MIC) of each compound after each successive passage.

3.6. In vitro toxicity study

Several early AMP candidates underwent clinical development for more than a decade without reaching regulatory approval, mainly due to nonspecific cytotoxicity [37]. Regarding the toxicity of AMPs, numerous studies have shown that mammalian cells could also be the targets of these molecules because some peptides are able to bind to various host components, such as extracellular surfaces, the extracellular matrix, and the host cellular membrane [24]. Here, we investigated the toxic potential of the peptide LyeTx I mnΔK using a cytotoxicity assay with Vero cells and assessed hemolytic activity in human blood cells. The concentration of LyeTx I mnΔK required to kill 50% of mammalian cells (CC_{50}) and to induce lysis in 50% of human blood cells (HC_{50}) were 55.31±5.0 μM and 77.07±4.1 μM, respectively. The original natural

peptide (LyeTx I) was significantly more hemolytic (HC_{50} 32.35 μM) and cytotoxic (CC_{50} of 1.06 μM to Lund Human Mesencephalic (LUHMES) cells in development and CC_{50} of 0.31 μM against mature LUHMES cells) than its derivative (LyeTx I mnΔK)[13], suggesting that the truncation reduced toxicity. It can also be justified by the reduction in the hydrophobicity of LyeTx I mnΔK, since very lipophilic peptides interact indiscriminately with the lipid membranes of prokaryotic and eukaryotic cells.

Colistin, in turn, presented a CC_{50} of 105.46±4.5 μM against Vero cells. However, this polymyxin revealed no hemolytic activity even at a higher concentration (512 μM). The selectivity index was then calculated. As shown in Table S1 (Supplementary File), the peptide LyeTx I mnΔK is considerably more toxic against bacterial cells than against mammalian cells, showing a mean selectivity index of 16.46 and 22.94 in relation to Vero and human blood cells, respectively. This higher selectivity in relation to pathogens can be justified because bacterial cell membranes contain negatively charged lipids, while the human cell membrane possesses zwitterionic lipids [9,23,24]. Thus, cationic AMPs such as LyeTx I mnΔK use this difference to exert selective antimicrobial activity by interacting preferentially with the negatively charged bacterial membrane.

Administration of broad-spectrum AMPs can eliminate the entire indigenous microflora found in humans, which can cause other severe adverse side effects, such as superinfections, intense diarrhea, and predisposition to intestinal disturbances [24]. To study the potential effect of the peptide LyeTx I mnΔK on indigenous microflora, we determined the MIC against *Lactobacillus fermentum*, an important gram-positive species with probiotic potential found in normal microbiota. The MIC against *L. fermentum* was 32 μM, and the mean selectivity index was calculated to be 9.52, suggesting that the peptide LyeTx I mnΔK mainly targets pathogenic bacteria (Table S1; Supplementary file).

3.7. The plasmatic and oral stability of LyeTx I mnΔK is predicted to be low

Although AMPs show high antimicrobial activities, some of them are also highly unstable in the presence of inorganic salts, lose activity at different pH levels, and are subject to proteolytic degradation by human and bacterial proteases, which limit their

pharmaceutical uses [9,23,24]. Therefore, we aimed to assess the stability of the anti-CRAB activity of LyeTx I mnΔK under different biological and physicochemical conditions. The synthetic peptide showed an important reduction in its antibacterial activity when incubated in the presence of fetal bovine serum and human plasma; however, it was relatively stable in the presence of albumin, different pH values, and high concentrations of salts (**Table 3**). The significant reduction in activity in the presence of plasma indicates that this peptide is strongly susceptible to degradation by plasma peptidases because the activity was maintained in the presence of albumin, indicating that the reduction was not associated with binding to plasma proteins. Corroborating this presupposition, the activity of the LyeTx I mnΔK peptide was completely lost after incubation with trypsin, a protease produced in large quantities in the digestive tract [38]. In contrast, colistin showed good stability under all conditions studied. Taken together, these results suggest that LyeTx I mnΔK has low stability against plasma and gastro-enteric proteases, which limits its use as an intravenous or oral drug.

Table 3: Minimal inhibitory concentration (MIC) of LyeTx I mnΔK or colisin in the presence of salts (NaCl, $CaCl_2$, and KCl), different pH ranges (4, 5, and 8), trypsin (1:500), fetal bovine serum (FBS, 2%, 5% or 10%), bovine serum albumin (BSA, 10, 30, and 50 mg/mL), and human plasma (HP; 2% or 5%) against carbapenem-resistant *Acinetobacter baumannii*

	Control	**NaCl 100mM**	**$CaCl_2$ 8µM**	**KCl 1mM**	**pH 4**	**pH 5**	**pH 8**	**FBS 2%**	**FBS 5%**	**FBS 10%**	**BSA 10 mg/mL**	**BSA 30 mg/mL**	**BSA 50 mg/mL**	**HP 2%**	**HP 5%**	**Trypsin 1:500**
LyeTx I mnΔK	4	8	4	8	8	4	8	16	32	32	4	4	4	8	16	>32
Colistin	0.4	0.4	0.4	0.4	>13.3	0.4	0.4	0.8	0.8	1.6	1.6	0.8	0.8	0.4	0.8	0.8

All results are express as µM. NaCl: Sodium chloride; $CaCl_2$: Calcium chloride; KCl: Potassium chloride.

3.8. Intranasal use of LyeTx I mnΔK reduces the bacterial load in a mouse model of CRAB lung infection at a similar level as colistin

Confirmation of the potent in vitro anti-CRAB activity of LyeTx I mnΔK led us to investigate the efficacy of this drug in treating pneumonia induced by CRAB. *Acinetobacter baumannii*, especially CRAB, is one of the most prevalent healthcare-associated pneumonia-causing pathogens, and it is involved in up to 47% of cases in some geographic regions, especially in humid climates [39]. However, the limitations associated with high intravenous and oral instability need to be addressed, and new avenues should be pursued in order to transform LyeTx I mnΔK into potential therapeutic agents capable of clinical use. One avenue that is gaining momentum is the utilization of this peptide as an inhaled antibacterial agent against CRAB-induced pneumonia, in which case systemic instability is not a problem. Interestingly, a recent meta-analysis showed that nebulized antibiotics seem to be associated with higher clinical cure rates in the treatment of bacterial pneumonia, suggesting that this route is more effective and safer than the intravenous route [40]. Thus, we evaluated the intranasal administration of LyeTx I mnΔK for the treatment of mice with CRAB-induced lung infection. Mice that received the peptide at doses of 1 mg/kg (6.81±0.72 CFU log10/g tissue), 5 mg/kg (6.96±0.58 CFU log10/g tissue), and 10 mg/kg (6.50±0.94 CFU log10/g tissue) showed a significant reduction in bacterial load in the lungs compared to the untreated controls (7.79±0.34 CFU log10/g tissue) ($p<0.05$) (**Figure 4**). The in vivo antibacterial effect found with the use of this peptide was similar to that observed with the inhaled colistin (6.55±0.39 CFU log10/g tissue) (Figure 4), which is the antibiotic considered as a last resort in the treatment of lower airway infections by carbapenem-resistant gram-negative bacteria [26]. Thus, LyeTx I mnΔK shows promise for use as an inhaled antimicrobial and, in this study, was similar to conventional antimicrobials against pneumonia caused by CRAB.

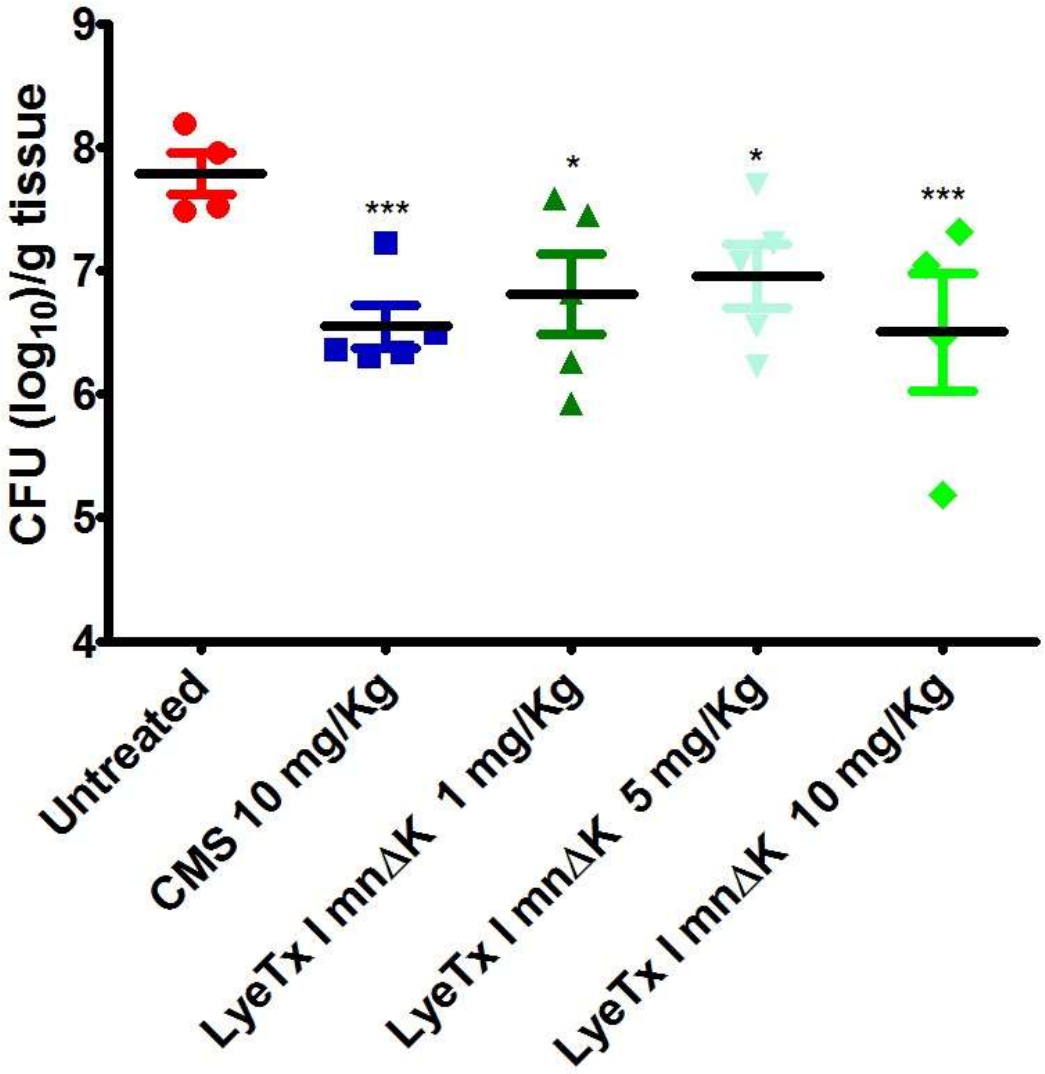

Figure 4: Bacterial load (Log_{10}CFU/g of lung) after intranasal administration of carbapenem-resistant *Acinetobacter baumannii*-induced pneumonia with LyeTx nmΔK (1, 5, and 10 mg/kg), colistimethate sodium (10 mg/kg), or saline (control). One asterisk (*) indicates a statistically significant difference compared to the control with $p < 0.05$. Three asterisks (***) indicate a statistically significant difference compared to the control with $p < 0.0001$. All results were analyzed by one-way analysis of variance with Tukey's post-hoc test.

4. Conclusion

The LyeTx I mnΔK peptide showed promising antibacterial activity against clinical isolates of CRAB from different origins. The results of this study reveal that the antimicrobial effect is likely associated with the ability of LyeTx I mnΔK to lyse CRAB cells, although we cannot discard the possibility that other mechanisms may also be involved. In addition, the peptide had a potent anti-biofilm effect and did not elicit resistance in vitro after 14 days of

exposure. We suggest that the activity of LyeTx I mnΔK on the membrane ensured an increase in the uptake of conventional antibiotics, thereby generating a synergistic effect with meropenem and colistin. Although LyeTx I mnΔK presented lower activity than the natural peptide (LyeTx I) against *A. baumannii*, it was significantly less toxic. In fact, the toxicity to mammalian cells was reduced, and LyeTx I mnΔK was demonstrated to have limited impact on physiological microbial flora. Due to its high instability when administered intravenously and orally, the use of the LyeTx I mnΔK peptide by the intranasal route was determined to be effective against CRAB-induced pneumonia in mice. Taken together, the results of this study highlight that the peptide LyeTx mnΔK is a potential prototype for the development of new effective and safe antibacterial agents against extensively resistant pathogens of clinical relevance.

5. Acknowledgments

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6. Compliance with Ethical Standards

Funding: CNPq, CAPES and FAPEMIG.

Conflict of Interest: All authors report that they do not have any conflicts of interest.

Ethical approval: The study was approved by the Laboratory Animal Research Ethics Committee of the Federal University of Minas Gerais (CEUA-UFMG: 367/2019).

Author contributions: All authors contributed to the development, analysis, and drafting of this article.

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SUPPLEMENTARY FILE

A short synthetic peptide, based on LyeTx I from *Lycosa erythrognatha* venom, shows potential to treat pneumonia caused by carbapenem-resistant *Acinetobacter baumannii* without detectable resistance

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Microorganisms

Antibacterial activity was determined against twenty clinical isolates of *A. baumannii*, which were kindly provided by Magna Cristina Paiva (Laboratory of Clinical Microbiology, Universidade Federal de São João Del Rei, Divinopolis, MG, Brazil). All isolates were identified by the automated system Vitek®2 (bioMerieux, Hazelwood, MO) and confirmed with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), using a Microflex LT spectrometer (BrukerDaltonics, MA, USA). The minimal inhibitory concentrations (MICs) of several antimicrobials were determined using Vitek®2 (Table S1).

In addition, two reference bacterial obtained from the American Type Culture Collection (ATCC) (*A. baumannii* ATCC 19606 and *Lactobacillus fermentum* ATCC 14932) was kindly provided by the Microbiology Reference Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ-RJ, Brazil) and included in study.

Reagents

Colistin, meropenem, levofloxacin, Gentamicin, serum bovine albumin (Inlab, São Paulo, SP, Brazil), colistimethate sodium (ABL, São Paulo, Brazil), crystal violet, 95% ethanol, dimethyl sulphoxide (DMSO) (Synth, São Paulo, SP, Brazil), trypsin, fetal bovine serum (Gibco, ThermoFisher Scintific, Frankfurt, HE, Germany) and MTT assay reagent (Sigma-Aldrich, San Louis, USA) were all purchased from commercial suppliers. The peptide LyeTx mnΔK was obtained by solid phases synthesis and acquired from GL Biochem (Shangai, China) with a purity of 99%, which was confirmed by spectrometric analysis [1]. Mueller–Hinton broth and agar and trypticase soy broth (TSB) were purchased from Kasvi (São José do Pinhais, PR, Brazil) and Dulbecco's Modified Eagle Medium (DMEM) was purchased from CultLab (São Paulo, SP, Brazil).

Table S1: Identification, clinical origin and resistance profile of the isolates of *Acinetobacter baumannii* used in the study.

Isolate	Sample type	Identification		MIC (µg/mL)/Susceptibility profile														
		VITEK2®	MALDI-TOF	APS	PPT	CAZ	CPM	IMP	MER	AMI	GEN	CIP	AMP	CFX	CEFT	TIG	CFR	CFR/AX
ATCC	-	ACB complex	AB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AC09	Wound	ACB complex	AB	≤2/S	≤4/S	4/S	2/S	≤0,25/S	≤0,25/S	≤2/S	≤1/S	≤0,25/S	8/R	≥64/R	16/I	≤0,5/S	32/R	32/R
AC36	Tracheal aspirate	ACB complex	AB	≤2/S	NT	16/I	2/S	≤0,25/S	0,5/S	NT	≤1/S	≤0,25/S	≥32/R	≥64/R	16/I	≤0,5/S	32/R	32/R
AC10	Wound	ACB complex	AB	16/I	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	32/R	≥16/R	≥4/R	≥32/R	≤4/S	≥64/R	2/I	≥64/R	≥64/R
AC37	Venous catheters	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	32/R	8/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC30	Bloodstream infections	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	16/I	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC31	Bloodstream infections	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	NT	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC35	Tracheal aspirate	ACB complex	AB	16/I	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	NT	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	2/S	≥64/R	≥64/R
AC03	Liquor	ACB complex	AB	16/ I	≥ 128/ R	≥64/ R	≥ 64/ R	≥ 16/ R	≥ 16/ R	8 / S	≥ 16/ R	≥ 4/ R	≥32/ R	≥64/R	≥64/R	1/S	≥64/R	≥64/R
AC04	Tracheal aspirate	ACB complex	AB	≤2/ S	32/ I	16/ I	8/ S	≤ 0,25/ S	0,5/ S	≤2/ S	≤1/ S	0,5/ S	NT	NT	NT	NT	NT	NT
AC25	Wound	ACB complex	AB	16/I	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	4/S	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	1/S	≥64/R	≥64/R
AC26	Tracheal aspirate	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	16/I	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC52	Tracheal aspirate	ACB complex	AB	≤2/S	NT	16/I	4/S	≤0,25/S	1/S	≤2/S	2/S	≤0,25/S	≥32/R	≥64/R	32/I	≤0,5/S	32/R	32/R
AC40	Tracheal aspirate	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	16/I	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC43	Sputum	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	16/I	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	≤0,5/S	≥64/R	≥64/R
AC47	Urine	ACB complex	AB	NT	≥128/R	NT	≥64/R	NT	≥16/R	16/I	≥16/R	≥4/R	≥32/R	NT	≥64/R	NT	≥64/R	≥64/R

AC45	Tracheal aspirate	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	16/I	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	2/S	≥64/R	≥64/R
AC39	Urine	ACB complex	AB	NT	≥128/R	≥64/R	≥64/R	NT	≥16/R	≥64/R	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	NT	≥64/R	≥64/R
AC55	Wound	ACB complex	AB	≥32/R	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	16/I	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC23	Bloodstream infections	ACB complex	AB	16/I	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	NT	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R
AC24	Bloodstream infections	ACB complex	AB	16/I	≥128/R	≥64/R	≥64/R	≥16/R	≥16/R	NT	≥16/R	≥4/R	≥32/R	≥64/R	≥64/R	4/I	≥64/R	≥64/R

ACB complex: *Acinetobacter calcoaceticus-baumannii* complex; AB: *Acinetobacter baumannii*; APS: Ampicillin/Sulbactam; PPT: Piperacillin/Tazobactam; CAZ: Ceftazidime; IMP: Imipenem; MER: Meropenem; AMI: Amikacin; GEN: Gentamicin; CIP: Ciprofloxacin; AMP: Ampicillin; CFX: Cefoxitin; CEFT: Ceftriaxone; TIG: Tigecycline; CFR: Cefuroxime; CFR/AX: Cefuroxime/Axetil

NT: Not tested; A: Resistant; I: Intermediate; S: Sensitive. All results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) 2018, except for tigecycline wich we used the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Cytotoxicity

The cytotoxicity of compounds (LyeTx mnΔK and colistin) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [2]. African green monkey kidney epithelium (Vero, ATCC CCL-81) cells were grown in DMEM supplemented with 5% fetal bovine serum and 0.3% penicillin-streptomycin-amphotericin solution (10,000 U/mL + 10 mg/mL + 2 mg/mL). The culture was incubated at 37°C in a humidified atmosphere containing 5% CO_2. In total, 5×10^5 cells were added to each well of a 96-well plate and incubated for 24 h at 37°C. Following this, cells were exposed to different dilutions of compounds (0.25–32 μM) and the microplates were incubated for 48 h at 37°C. Finally, the cell viability was determined using MTT (5 mg/mL) and the cytotoxic concentration for 50% of the cells in culture (CC_{50}) was calculated by linear regression analysis [3].

Hemolytic activity

Hemolytic activity was determined by incubating suspensions of human red blood cells with serial dilutions of LyeTx mnΔK [4]. Red blood cells were rinsed several times in 0.9% saline by centrifugation for 3 min at 3,000 *g* until the OD of the supernatant reached the OD of the control (saline only). Red blood cells were counted by a hemocytometer and adjusted to 10^6 cells/mL. Red blood cells were then incubated at room temperature for 1 h with saline (blank) or LyeTx mnΔK at concentrations in range of 0.5-512 μM. Next, the samples were centrifuged at 10,000 *g* for 5 min, the supernatant was separated from the pellet, and its absorbance measured at 570 nm in a microplate reader (Bio-Tek Instruments, New York, NY, USA). The hemolytic concentration for 50% of the blood cells (HC_{50}) was calculated by linear regression analysis.

Selectivity index

The selectivity index (SI) value was determined by the ratio of the CC_{50}/HC_{50} to the MIC (antibacterial assay) [3], indicating the specificity of compounds toward pathogens and Zwitterionic cells. Furthermore the MIC of LyeTx I nmΔK was

determined against *Lactobacillus fermentum* ATCC 14932 in order to determine the selectivity against probiotic bacteria from physiologic microbiote.

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Conclusões gerais

Conclusões gerais

Os resultados reveleram que a melitina possui um potente efeito antibacteriano contra *S. aureus* e *E. coli* uropatogênica resistentes às terapias convencionais. Além disso, esse peptídeo interagiu de maneira sinérgica com antibióticos convencionais, re-sensibilizou exemplares de MRSA e UPECs resistentes as quinolonas e produtoras de ESBL para a atividade de classes terapêuticas as quais esses patógenos eram inicialmente resistentes, e revelou um efeito microbicida potente e rápido para ambos. A melitina possui a habilidade de lisar células de MRSA, além de interferir com biofilmes maturos. Sua estabilidade em diferentes concentrações salinas, pHs e na presença de proteínas plasmáticas foi substancial, mas ela é sensível a hidrólise por proteases como a tripsina. A incorporação da melitina em uma pomada e o uso dessa formulação revelou-se uma estratégia terapêutica promissora contra feridas induzidas por MRSA *in vivo*. A pomada formulada foi capaz de reduzir expressivamente a carga bacteriana na ferida além de produzir um potente efeito anti-inflamatório. Além disso, uma sonda vesical sensibilizada com melitina previniu a formação de biofilmes por UPECs multirresistentes, destacando-se como um promissor biomaterial contra infecções urinárias associadas a cateteres.

A avaliação do efeito antibacteriano do peptídeo LyeTx I mnΔK, por sua vez, revelou que esse agente é um promissor biofármaco contra *A. baumannii* resistente aos carbapenêmicos. Esse peptídeo interagiu sinergicamente com antibióticos convencionais e possui a habilidade de romper biofilme maturo de CRAB *in vitro*. Seu efeito bactericida foi rápido e potente, e sua capacidade de induzir a lise de CRAB foi reportada. Sua toxicidade *in vitro* se revelou baixa, entretanto o peptídeo foi altamente instável na presença de sais e de proteínas plasmáticas, o que motivou seu uso inalatório. A exposição prévia a esse peptídeo não foi capaz de elicitar resistência *in vitro* entre CRAB. Ademais o uso inalatório do LyeTx I mnΔK foi capaz de reduzir a carga bacteriana pulmonar em um modelo de pneumonia induzida por CRAB.

Esses resultados confirmam que PAMs isolados (*e.g.*, melitina) ou inspirados (*e.g.*, LyeTx I mnΔK) em componentes do veneno de artrópodes apresentam potente atividade antibacteriana. Concluímos, portanto, que o veneno desses animais se destaca como uma promissora fonte de agentes bioativos contra bactérias multirresistentes. Talvez essa matriz complexa e rica em compostos bioativos, que são as peçonhas de insetos, seja de fato a luz que buscamos para iluminar a “era das trevas” da antibioticoterapia que estamos vivendo hoje!

Apêndices

Apêndice

Lista de **ARTIGOS CIENTÍFICOS**, ligados a matéria da tese, publicados durante o doutorado

Título	**Ano da Publicação**	**Periódico**	**Fator de impacto**	**Link de acesso**
Plasmid-mediated quinolone resistance (PMQR) among Enterobacteriales in Latin America: a systematic review	2019	Molecular Biology Reports	2,316	https://doi.org/10.1007/s11033-019-05220-9
Pharmacologic potential of new nitro-compounds as antimicrobial agents against nosocomial pathogens: design, synthesis, and in vitro effectiveness	2019	Folia Microbiologica	2,099	https://doi.org/10.1007/s12223-019-00747-7
Carbapenem-resistant *Acinetobacter baumannii* in patients with burn injury: A systematic review and meta-analysis	2019	Burns	2,744	https://doi.org/10.1016/j.burns.2019.07.006
Ventilator-associated pneumonia (VAP) caused by carbapenem-resistant *Acinetobacter baumannii* in patients with COVID-19: Two problems, one solution?	2020	Medical Hypothesis	1,538	https://doi.org/10.1016/j.mehy.2020.110139
Rate of polymyxin-resistance among *Acinetobacter baumannii* recovered from hospitalized patients: A systematic review and meta-analysis	2020	European Journal of Clinical Microbiology & Infectious Diseases	3,267	https://doi.org/10.1007/s10096-020-03876-x
Antibacterial and antibiofilm activities of synthetic analogs of 3-alkylpyridine marine alkaloids	2020	Medicinal Chemistry Research	1,965	https://doi.org/10.1007/s00044-020-02549-w
Synthesis, characterization and antimicrobial activity of new Cu (ii), Co (ii) and Sn (ii) complexes with the sodium hydrotris(2-mercaptothiazolyl)borate ligand	2020	Química Nova	0,961	http://dx.doi.org/10.21577/0100-4042.20170526
PEGylated LyeTx I-b peptide is effective against carbapenem-resistant *Acinetobacter baumannii* in an in vivo model of pneumonia and shows reduced toxicity	2021	International Journal of Pharmaceutics	5,875	https://doi.org/10.1016/j.ijpharm.2021.121156
A short synthetic peptide, based on LyeTx I from Lycosa erythrognatha venom, shows potential to treat pneumonia caused by carbapenem-resistant *Acinetobacter baumannii* without detectable resistance	2021	Journal of Antibiotics	2,649	https://doi.org/10.1038/s41429-021-00421-6

Título	**Ano da Publicação**	**Periódico**	**Fator de impacto**	**Link de acesso**
In-depth characterization of antibacterial activity of melittin against *Staphylococcus aureus* and use in a model of non-surgical MRSA-infected skin wounds	2021	European Journal of Pharmaceutical Sciences	4,384	https://doi.org/10.1016/j.ejps.2020.105592
Pristimerin isolated from Salacia crassifolia (Mart. Ex. Schult.) G. Don. (Celastraceae) roots as a potential antibacterial agent against Staphylococcus aureus	2021	Journal of Ethnopharmacology	4,360	https://doi.org/10.1016/j.jep.2020.113423
Netzahualcoyonol from Salacia multiflora (Lam.) DC. (Celastraceae) roots as a bioactive compound against gram-positive pathogens	2022	Natural Product Research	2,861	https://doi.org/10.1080/14786419.2021.2023865
Anti-biofilm, and anti-adhesive activities of melittin, a honeybee venom-derived peptide, against quinolone-resistant uropathogenic *Escherichia coli* (UPEC)	2022	Natural Product Research	2,861	https://doi.org/10.1080/14786419.2022.2032047

Lista de **PATENTES** depositadas durante o doutorado

Título	Número do registro	Tipo de patente	Local do depósito
Composição farmacêutica com atividade contra pneumonia bacteriana e uso	BR1020200168487	Privilégio de Inovação	Instituto Nacional da Propriedade Industrial
Processo de revestimento de cateter urinário	BR10202101949	Privilégio de Inovação	Instituto Nacional da Propriedade Industrial

Lista de **TRABALHOS APRESENTADOS EM EVENTOS CIENTÍFICOS** durante o doutorado

Título	Nome do evento	Classificação	Ano
A peptide derived from Lycosa erythrognatha spider venom shows high activity against pneumonia induced by carbapenem-resistant *Acinetobacter baumannii*	31º Congresso Brasileiro Microbiologia	Nacional	2021
Atividade antiviral do mel de *Astronium urundeuva* (M. Allemão) ENGL. (Anacardiaceae) (aroeira) in natura frente ao Zika virus	31º Congresso Brasileiro Microbiologia	Nacional	2021
Therapeutic effect of hydroethanolic extract of *Fridericia chica* (Bonpl.) L. G. Lohmann leaves against vulvovaginal candidiasis	31º Congresso Brasileiro Microbiologia	Nacional	2021
A melitina mostra potente efeito antifúngico contra *Candida* spp. com ação em fatores de virulência	31º Congresso Brasileiro Microbiologia	Nacional	2021
mportante ação antifúngica de peptídeo derivado do veneno da aranha *Lycosa erythrognatha*	31º Congresso Brasileiro Microbiologia	Nacional	2021
Potential activity of phenolic-enriched ethanolic extract from Campomanesia lineatifolia leaves against Mayaro virus	4th Brazilian Conference on Natural Products (BCNP)	Internacional	2021
Antiviral activity of extracts from *Fridericia* sp. against Mayaro virus	XXXII Congresso Brasileiro de Virologia	Nacional	2021

PRÊMIOS E TÍTULOS recebidos durante o doutorado

Highlighted article in the 2021 July issue of The Journal of Antibiotics (Vol. 74, Issue 7), *The Journal of Antibiotics*

From Editorial Offie: Journal of Antibiotics - Decision on JA-24153R

Você respondeu em Seg, 24/05/2021 10:31

HI Hisayo Ito <ito@antibiotics.or.jp>
Para: williamgustavofarmacia@hotmail.com
Seg, 24/05/2021 08:25

Dear Dr. Lima,

This is Hisayo Ito from Editorial Office of the Journal of Antibiotics.

Thank you very much for contributing your manuscript entitled A short synthetic peptide, based on LyeTx I from *Lycosa erythrognatha* venom, shows potential to treat pneumonia caused by carbapenem-resistant *Acinetobacter baumannii* without detectable resistance.

I am writing to let you know that your article has been booked in the 2021 July issue of JA. Also your article was selected as the highlighted article.

In each issue, three highlighted articles are selected by the Managing Editors that want to feature the most from the issue. These articles will be featured by making short copies for each title for the cover and making these articles free for one month usually. Please see the attached cover image for an example.

Therefore, we would appreciate it if you could provide the short copies (about 50-60 characters) the most relevant findings of their study by 25th May, 2021. We are so sorry to rush you.

We look forward to receiving it.
Thank you for your cooperation in this matter.

Sincerely yours,
Hisayo

Anexos

Anexo

CEUA Capítulo II

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o projeto intitulado "Avaliação de peptídeos derivados de toxinas de artrópodes como potenciais agentes terapêuticos tópicos contra infecções cutâneas por Staphylococcus aureus resistentes a meticilina (MRSA)", protocolo do CEUA: 299/2019 sob a responsabilidade de Simone Odilia Antunes Fernandes que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE MINAS GERAIS, em reunião de 02/12/2019.

Vigência da Autorização	02/12/2019 a 01/12/2024
Finalidade	Pesquisa
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério Central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério Central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério central da UFMG
***Espécie/linhagem**	Camundongo isogênico / Camundongos BALB/c
Nº de animais	15
Peso/Idade	18g / 4(semanas)
Sexo	masculino
Origem	Biotério Central da UFMG

Considerações posteriores:

02/12/2019	Aprovado na reunião do dia 02/12/2019. Validade: 02/12/2019 à 01/12/2024

Belo Horizonte, 04/05/2020.

Atenciosamente,

CEUA capítulo III

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto intitulado "Biodistribuição e avaliação da atividade de peptídeos contra pneumonia induzida por Acinetobacter baumannii", protocolo do CEUA: 367/2019 sob a responsabilidade de Simone Odilia Antunes Fernandes que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE MINAS GERAIS, em reunião de 16/03/2020.

Vigência da Autorização	16/03/2020 a 15/03/2025
Finalidade	Pesquisa
*Espécie/linhagem	Camundongo isogênico / BALBL/c
Nº de animais	25
Peso/Idade	20g / 6(semanas)
Sexo	feminino
Origem	Biotério central da UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	110
Peso/Idade	20g / 6(semanas)
Sexo	feminino
Origem	Biotério central da UFMG
*Espécie/linhagem	Camundongo isogênico / BALB/c
Nº de animais	60
Peso/Idade	20g / 6(semanas)
Sexo	feminino
Origem	Biotério central da UFMG

Considerações posteriores:

16/03/2020	Aprovado na reunião do dia 16/03/2020. Validade: 16/03/2020 à 15/03/2025.

Belo Horizonte, 04/05/2020.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

Universidade Federal de Minas Gerais

SISGEN

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso
Cadastro nº AC2C2F8

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	AC2C2F8
Usuário:	FUNED
CPF/CNPJ:	17.503.475/0001-01
Objeto do Acesso:	Patrimônio Genético
Finalidade do Acesso:	Pesquisa e Desenvolvimento Tecnológico

Espécie

Impossibilidade de identificação

Título da Atividade:	Melitina como antimicrobiano

Equipe

Júlio César Moreira de Brito	FUNED
Willian Gustavo de Lima	UFMG
Bárbara Gatti Cardoso	UFMG
SIMONE ODÍLIA ANTUNES FERNANDES	UFMG

Parceiras Nacionais

17.217.985/0001-04 / Universidade Federal de Minas Gerais

Data do Cadastro:	16/09/2019 09:35:06
Situação do Cadastro:	Concluído

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em 9:35 de 16/09/2019.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN