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NILTON ARARIPE DOS SANTOS NETO

UNITED WE STAND, DIVIDED WE FALL: ANTIBIOFILM ACTIVITY AND MECHANISMS OF ACTION OF SYNTHETIC PEPTIDES IN COMBINATION WITH CIPROFLOXACIN AGAINST *Staphylococcus aureus* BIOFILM

FORTALEZA

2023

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Coorientador: Prof. Dr. Pedro Filho Noronha de Souza.

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We all make choices, but in the end, our choices make us. (Andrew Ryan, Bioshock, 2007).

RESUMO

A bactéria patogênica *Staphylococcus aureus* é conhecida desde meados do século XX por ser uma bactéria resistente aos antibióticos. Com o passar dos anos, essa resistência expandiu para antibióticos além daqueles encontrados na classe das penicilinas. Por conta disso, *S. aureus* é uma bactéria de grande relevância clínica, sendo um dos principais organismos causadores de infecções relacionadas à assistência à saúde. Ademais, *S. aureus* também é capaz de formar biofilme, uma matriz extracelular polimérica capaz de conferir uma maior proteção às células bacterianas. Para contornar tal problemática, a prospecção por tratamentos alternativos eficazes contra biofilmes de bactérias resistentes se faz necessária. Os peptídeos antimicrobianos (PAMs) são uma alternativa promissora, por serem moléculas capazes de contornar os mecanismos de resistência bacteriano. Porém, peptídeos naturais são economicamente inviáveis, pois são encontrados em quantidades diminutas, além de sua instabilidade e toxicidade aos hospedeiros. Como alternativa, o uso de peptídeos antimicrobianos sintéticos, tem se mostrado vantajoso, pois através de alterações pontuais em sua sequência, é possível excluir desvantagens como a toxicidade e até aumentar a atividade antimicrobiana dos mesmos. O presente trabalho teve como objetivo analisar a atividade antibiofilme de oito peptídeos sintéticos, em combinação com o antibiótico ciprofloxacina, além de elucidar seus mecanismos de ação por microscopia de fluorescência e análises proteômicas. Os resultados mostraram que quatro dos oito peptídeos, combinados com ciprofloxacina, inibiram a formação do biofilme em até 76%, que foi obtido da combinação do peptídeo *Mo*-CBP$_3$-PepIII + ciprofloxacina, e reduziram a biomassa de biofilme pré-formado em até 65%, que foi obtido da combinação *Rc*Alb-PepII + ciprofloxacina. As análises de microscopia de fluorescência mostraram que os peptídeos agem formando poros na membrana da célula, além de estimularem a superprodução de espécies reativas de oxigênio (EROs). As análises proteômicas com *Mo*-CBP$_3$-PepI + ciprofloxacina, cuja combinação obteve 50% de redução de biomassa do biofilme, revelaram alteração no perfil de proteínas relacionadas ao metabolismo de DNA, metabolismo de proteínas, síntese de parede celular, metabolismo redox, *quorum sensing* e também relacionadas à formação de biofilme. Além disso, foi possível observar um acúmulo diferenciado em células tratadas com a combinação das moléculas e tratadas apenas com as moléculas isoladas, revelando um possível efeito sinérgico.

Palavras-chave: EROs; proteômica; ciprofloxacina; biofilme.

ABSTRACT

Staphylococcus aureus is a pathogenic bacterium known as resistant to antibiotics since the mid-20th century and, since then; your resistance did not stop developing resistance, even towards new pharmaceuticals. *S. aureus* has a huge medical importance, since it is a major organism related to hospital-acquired infections (HAI). Furthermore, *S. aureus* is capable of forming biofilm, a polymeric extracellular strutcture that confers even more resistance to antibiotics. To overcome this problem, the search for alternative and effective treatments against biofilms of resistant bacteria is necessary. Antimicrobial peptides (AMPs) gained attention as promising alternative molecules, since their mechanisms of action can trespass bacterial resistance mechanisms. Even so, natural AMPs are economically unviable, given their low yield, low stability and toxicity to hosts. As an alternative, synthetic antimicrobial peptides (SAMPs), bioinspired in plant proteins, have been proving advantageous. These synthetic peptides, through punctual modifications into their sequence, can lost their disadvantages like toxicity to host, and can have their antimicriobial activity enhanced. The present work aimed at the analysis of the antibiofilm activity of eight SAMPs, in combination with the antibiotic ciprofloxacin, and elucidate its mechanisms of action through fluorescence microscopy and proteomic analysis. Out of eight, four peptides, in combination with ciprofloxacin, were capable of inhibiting *S. aureus* biofilm formation up to 76%. The best combination was made by *Mo*-CBP$_3$-PepIII and ciprofloxacin, and reduced the biomass of preformed biofilm up to 65%, with *Rc*Alb-PepII + ciprofloxacin combination. Fluorescence microscopy analysis revealed that the peptides act by forming pores onto the cell membrane and stimulating reactive oxygen species (ROS) overproduction. In addition, proteomic analysis with the combination of *Mo*-CBP$_3$-PepI + ciprofloxacin, which achieved 50% of biomass reduction of biofilm, showed an alteration at the proteins profile, especially with proteins related with DNA and protein metabolism, cell wall biosynthesis, redox metabolism, *quorum* sensing and biofilm formation. Moreover, it was possible to observe a differential accumulation in cells treated with both molecules and cells treated with only one of them, showing a possible synergistic effect.

Keywords: ROS; proteomics; ciprofloxacin; biofilm.

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1 INTRODUÇÃO

De acordo com a Organização Mundial da Saúde – OMS (2020), a resistência bacteriana a antibióticos é uma das principais ameaças à humanidade do século XXI. De acordo com os estudos de Pulingam *et al.* (2022), é estimado que a resistência bacteriana cause 10 milhões de mortes anuais no ano de 2050. Além dos danos à vida da população, a resistência bacteriana também pode ser responsável por danos econômicos (NAYLOR *et al.*, 2018).

Atualmente, os patógenos ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* e *Enterobacter species*) são descritos como os patógenos bacterianos que mais apresentam resistência a antibióticos. Dentre eles, *S. aureus* é um dos principais causadores de infecções adquiridas em hospitais. Além de possuir diversos mecanismos de patogenicidade, e ser capaz de formar biofilme, o que dificulta seu tratamento, *S. aureus* possui o potencial de desenvolver resistência a diversos antimicrobianos, como penicilinas, cefalosporinas, carbapenêmicos, quinolonas, macrolídeos, tetraciclinas, dentre outros. O potencial de aquisição de resistência antimicrobiana, associado aos mecanismos de patogenicidade, contribuem para que esse micro-organismo se torne um importante patógeno humano (GUO *et al.*, 2020; VESTERGAARD; FREES; INGMER, 2019).

Diante desse cenário preocupante, o desenvolvimento de terapias alternativas faz-se necessário, com destaque ao uso de peptídeos antimicrobianos (PAMs). Os PAMs são pequenas moléculas de natureza protéica com atividade antimicrobiana. A principal vantagem dos PAMs é principalmente o seu mecanismo de ação mais comum, que é a interação com a bicamada lipídica da membrana plasmática do patógeno, e alterar sua permeabilidade. Porém, os PAMs naturais possuem alguns empecilhos em sua utilização, como toxicidade ao hospedeiro e baixa seletividade ao patógeno. Para contornar essas desvantagens, o desenho racional de peptídeos sintéticos se dá como uma saída interessante. Com o desenho racional, através de ferramentas *in silico*, é possível obter sequências peptídicas com elevada atividade antimicrobiana e com baixa toxicidade (LEI *et al.*, 2019; LIMA *et al.*, 2021b).

Este estudo visa avaliar a atividade antibiofilme de peptídeos sintéticos, em combinação com o antibiótico ciprofloxacina, analisar a toxicidade dos peptídeos na combinação com o antibiótico, além de elucidar os mecanismos de ação envolvidos na atividade antibiofilme através de microscopia de fluorescência e análises proteômicas.

2 CAPÍTULO I – REFERENCIAL TEÓRICO

2.1 Antibióticos e a resistência bacteriana

Desde antes do início da era dos antibióticos, há registros de que a humanidade já se utilizava de sabedoria popular para o tratamento de infecções associadas a ferimentos, milhares de anos atrás. Mais recentemente, no início do século XX, foi desenvolvido o Salvarsan, mais especificamente para o tratamento de sífilis, seguido do Prontosil, a primeira sulfonamida. Já em 1928, por um acaso, Alexander Fleming descobriu a penicilina e assim deu-se início a era da pesquisa e desenvolvimento de medicamentos para o controle e tratamento de doenças infecciosas (HUTCHINGS; TRUMAN; WILKINSON, 2019; MA *et al.*, 2021).

Nos anos de 1940-1960, a chamada "era de ouro" da descoberta de antibióticos naturais, houve uma explosão de prospecção e descoberta desses novos agentes antimicrobianos, a maioria deles contra bactérias Gram positivas, como a própria *S. aureus*, entre eles estão os β-lactâmicos, tetraciclinas, macrolídeos, aminoglicosídeos e até mesmo peptídeos. (GUIMARÃES; MOMESSO; PUPO, 2010; HUTCHINGS; TRUMAN; WILKINSON, 2019; NICOLAOU; RIGOL, 2018).

Já nos anos de 1960-1980, os antibióticos semi-sintéticos ganharam mais evidência, e o diferencial deles para os já existentes era o de amplo espectro, ou seja, possuem atividade contra bactérias Gram-positivas e Gram-negativas. Grande parte desses novos antibióticos foram derivados a partir de antibióticos já existentes, como os derivados β-lactâmicos e análogos de tetraciclinas. Além disso, foi durante esse período em que foram identificadas as primeiras cepas de *S. aureus* resistente à meticilina (MRSA) (GUIMARÃES; MOMESSO; PUPO, 2010; NICOLAOU; RIGOL, 2018).

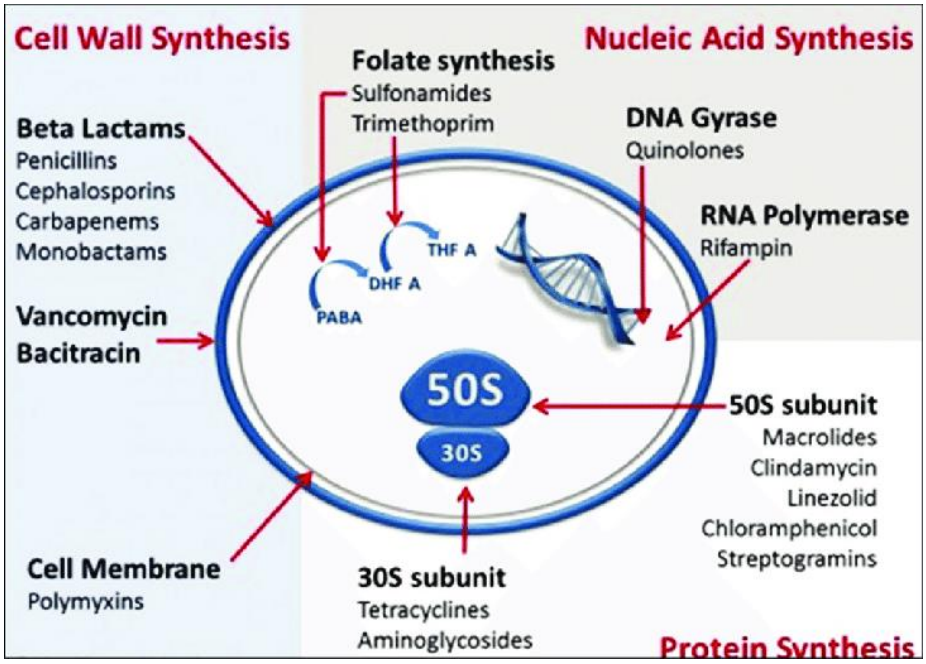
Entre 1980-2000, houve uma redução drástica da descoberta e desenvolvimento de novos antibióticos, mesmo com ferramentas mais avançadas e recentes da época, como a genômica. Atrelado à essa redução no desenvolvimento de novos antibióticos, o crescimento da resistência microbiana acabou por piorar a situação. Porém, a introdução de antibióticos sintéticos, como as Fluoroquinolonas, foi um marco que modificou o mercado de antibióticos da época, amenizando a problemática da resistência (GUIMARÃES; MOMESSO; PUPO, 2010; NICOLAOU; RIGOL, 2018).

A partir dos anos 2000, a introdução de novos antibióticos no mercado continuou a

diminuir, mesmo com a crescente resistência bacteriana aos fármacos já existentes. (GUIMARÃES; MOMESSO; PUPO, 2010). Neste período, houve a documentação de isolados de *Streptococcus pneumoniae* não sensível à penicilina (PNSSP), *Enterococcus* resistente à vancomicina (VRE) e *Enterobacteriaceae* produtoras de β-lactamases de amplo espectro (ESBL) (SANTOS, 2004).

Os antibióticos podem ser divididos em 4 grupos principais (Figura 1), relacionados ao seu alvo no patógeno, podem (1) atuar na inibição da síntese, ou *turnover*, da parede celular; (2) em enzimas relacionadas ao metabolismo ácidos nucléicos; (3) inibindo a síntese protéica; e (4) podem também atuar em vias de síntese de alguns metabólitos essenciais, como o ácido fólico (KAPOOR; SAIGAL; ELONGAVAN, 2017).

Figura 1 – Mecanismos de ação de antibióticos



Fonte: Kapoor, Saigal e Elongavan, 2017

A crescente identificação de microrganismos cada vez mais resistentes têm se mostrado como um dos maiores problemas de saúde mundial dos últimos anos (NAINU *et al.*, 2021). Esse crescimento acelerado da resistência bacteriana se dá, principalmente, por dois fatores principais: uso indiscriminado e indevido de antibióticos pela sociedade, seja pela indústria agropecuária ou pela sociedade em geral, o que gera uma pressão seletiva nesses micro-organismos, selecionando os organismos resistentes aos antibióticos, os quais acabam se perpetuando com mais facilidade. Além disso, a baixa disponibilidade de novas terapias, sendo necessário o uso de moléculas com os mesmos mecanismos de ação por vários anos, acaba em condicionar os patógenos a adquirirem resistências a esses tratamentos (NAINU *et al.*, 2021).

2.2 Fluoroquinolonas e a Ciprofloxacina

As fluoroquinolonas,também conhecidas como quinolonas, são uma classe de antibióticos sintéticos que foi introduzida para uso clínico em 1965 com a introdução do ácido nalidíxico. Porém, o ácido nalidíxico apresentou algumas limitaçõs quanto ao seu uso clínico, como baixa absorção via administração oral, e baixa tolerância por parte dos pacientes(GAUBA; SAXENA, 2023; GIGUÈRE; DOWLING, 2013).

Nesse período entre 1960-1980, houve a introdução de diversas quinolonas para uso clínico, como a flumaquina e o ácido oxolínico. Já em meados dos anos 1980, algumas modificações foram feitas nesses antibióticos, como a adição de átomos de flúor, de modo a melhorar algumas características desses fármacos. Essa modificação veio a aumentar a atividade antibacteriana, melhorar a abosrção oral, e a distribuição pelos tecidos. A partir disso, esses antibióticos começaram a ser chamados de “fluoroquinolonas” que, entre eles, está a ciprofloxacina(GIGUÈRE; DOWLING, 2013; SANDERS, 1988; WILES *et al.*, 2006).

A ciprofloxacina é uma fluoroquilonona de segunda geração que foi introduzida para uso clínico em 19XX. O alvo intracelular desse antibiótico pode variar dependendo do organismo contra qual é usado. Em *S. aureus*, a ciprofloxacina tem como alvo a subunidade A da enzima DNA topoisomerase IV, uma enzima que catalisa o relaxamento da torção do DNA cromossômico, para posterior segregação ou replicação do mesmo(SANDERS, 1988; WATKINS; HOLUBAR; DAVID, 2019).

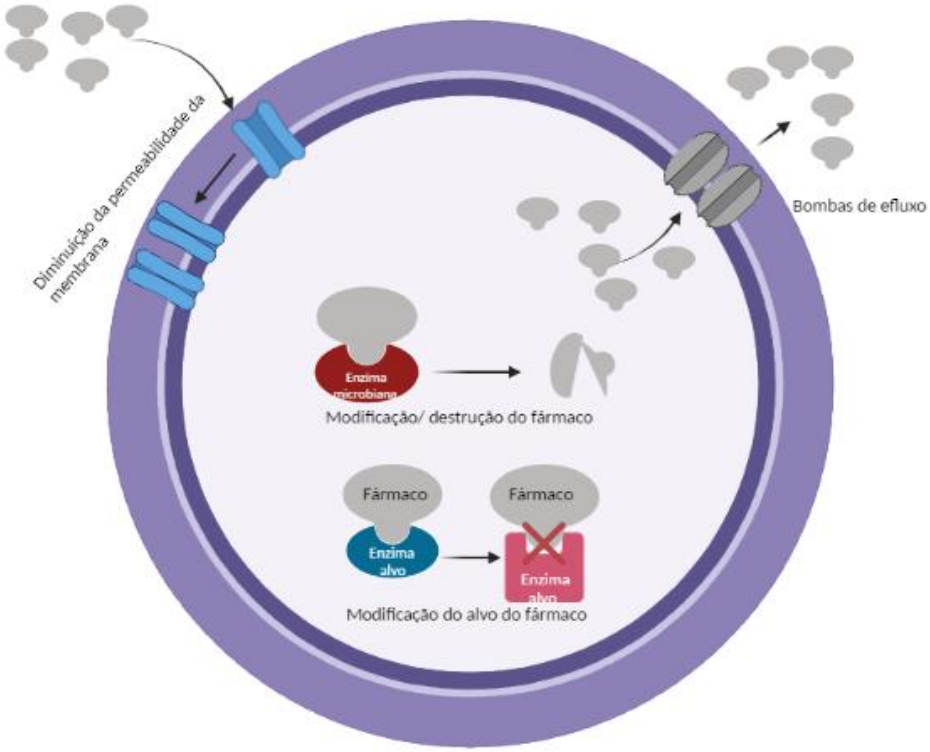
Apesar de ser um antibiótico com atividade antibacteriana satisfatória e seguro, sua utilização ainda pode acarretar em efeitos colaterais que podem variar desde tendinopatia, até quadros mais graves como anemia autoimune. Além disso, a ciprofloxacina tamém apresenta algumas implicações ecológicas quanto a sua utilização, pois esa droga não facilmente degradada no ambiente, pode afetar o sistema reprodutivo de peixe e anfíbios, além de afetar a fisiologia de plantas(GAUBA; SAXENA, 2023; PARASTAN *et al.*, 2020; WILES *et al.*, 2006).

2.3 Mecanismos de resistência em *Staphylococcus aureus*

A resistência a antibióticos em *Staphylococcus aureus* ocorre, principalmente, por ação humana. Essa resistência, como mostrado na figura 2, pode ser adquirida através de novas mutações randômicas e seleção natural, quando em contato com o agente antimicrobiano; adquirindo, de outros microrganismos, a informação genética que confere tal resistência (transferência horizontal de genes) e também através de assimilação de material genético

disponível no ambiente (TENOVER, 2006).

Figura 2 - Mecanismos de resistência a antibióticos



Fonte: elaborado pelo autor.

Em se tratando dos mecanismos de resistência, as bactérias podem: (1) alterar o alvo proteico do antibiótico em questão, podendo ocorrer a modificação ou deleção do sítio de ligação, (2) aumentar o acúmulo de enzimas que alteram e inativam o agente antimicrobiano, (3) diminuir a permeabilidade da membrana a tais agentes, ou (4) aumentar o acúmulo de bombas de efluxo responsáveis por lançar para o meio extracelular o agente antibacteriano (TENOVER, 2006).

As β-lactamases são um exemplo bem conhecido de hidrolases relacionadas à resistência. Essa classe de enzimas atua hidrolisando a ligação amida do anel β-lactâmico e, desse modo, fazendo com que o antibiótico perca a capacidade de interagir com proteínas importantes no *turnover* da parede celular bacteriana (EGOROV; ULYASHOVA; RUBTSOVA, 2018). Algumas enzimas N-acetiltransferases são responsáveis por conferir resistência a patógenos contra antibióticos da classe dos aminoglicosídeos, adicionando grupos acetil ou metil na molécula antimicrobiano, impedindo-o de se ligar ao ribossomo e inibir a síntese proteica (EGOROV; ULYASHOVA; RUBTSOVA, 2018; SEFTON, 2002).

Em *S.* aureus, a resistência a antibióticos da classe dos beta-lactâmicos possui maior

prevalência. *S. aureus* possui dois mecanismos que podem conferir resistência: (1) a produção de enzimas beta-lactamases e (2) mudanças estruturais na proteínas de ligação a penicilina (PBPs), reduzindo a afinidade com os fármacos dessa classe. As β-lactamases são um exemplo bem conhecido de hidrolases relacionadas à resistência. Essa classe de enzimas atua hidrolisando a ligação amida do anel β-lactâmico e, desse modo, fazendo com que o antibiótico perca a capacidade de interagir com proteínas importantes no *turnover* da parede celular bacteriana (EGOROV; ULYASHOVA; RUBTSOVA, 2018).

Para a remoção dessas moléculas antimicrobianas, os microrganismos também vieram a se utilizar de bombas de efluxo, que possui como função a regulação do ambiente intracelular removendo metabólitos, moléculas relacionadas com *quorum sensing* e também de susbstâncias tóxicas para a célula, como os antibióticos. Em bactérias, existem cinco classes de bombas de efluxo que têm relação com a resistência a antibióticos, com dois principais mecanismos de efluxo: protomotriz (H^+, Na^+) e por hidrólise de ATP. Essas bombas de efluxo estão relacionadas com a resistência A diversas classes de antibióticos, como as fluoroquinolona, tetraciclinas e aminoglicosídeos. Alguns exemplos de organismos com esse mecanismo de resistência são: *Pseudomonas aeruginosa*, *S. typhimurium* e *S. aureus* (ARCHER *et al.*, 2011; EGOROV; ULYASHOVA; RUBTSOVA, 2018; WEBBER, 2003).

A resistência descrita em *S. aureus* contra antibióticos da classe das fluoroquinolonas, como a ciprofloxacina, pode ocorrer através de dois mecanismos principais: (1) o alto acúmulo de bombas de efluxo para diminuir a concentração intracelular do fármaco e (2) a mudança no sítio alvo do antibiótico nas enzimas DNA girase e DNA topoisomerase IV, que são importantes enzimas no processo de replicação do DNA cromossômico. Além disso, bombas de efluxo são capazes de reduzir a concentração intracelular desses fármacos, reduzindo sua atividade.

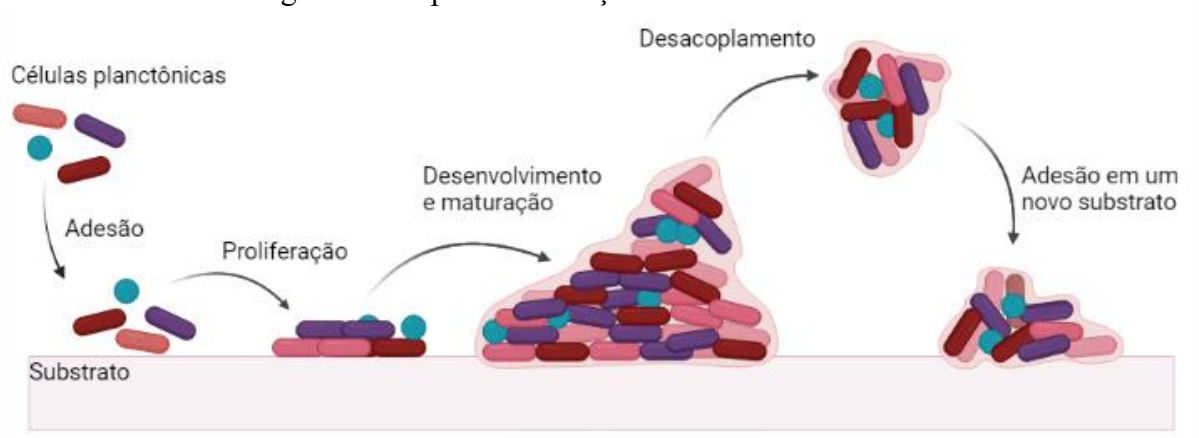
Outro mecanismo de resistência *que S.* aureus pode apresentar, é a redução da permeabilidade da membrana à certos tipos de moléculas. *S.* aureus pode reduzir a abundância de proteínas membranares por onde os agentes são transportados. Um exemplo dessas proteínas são as aquaporinas, as quais transportam antibióticos hidrofílicos. Além disso, o próprio biofilme, mesmo não sendo um mecanismo de resistência, é capaz de reduzir a permeabilidade da membrana, podendo atrasar, ou até impedir, a penetração dos antibióticos na célula. Além disso, células mais internas no biofilme, apresentam metabolismo reduzido, o que vem a dificultar a ação de alguns fármacos. Esse biofilme é conhecido por ser composto de diversas células amontoadas umas nas outras que, por sua vez, formam uma matriz extracelular polimérica. No caso das bactérias, essa matriz é composta por uma variedade de secreções

celulares, como proteínas, carboidratos e DNA. *S. aureus* é um exemplo de micro-organismo capaz de formar biofilme (NAINU *et al.*, 2021; PAGÈS; JAMES; WINTERHALTER, 2008).

2.4 Biofilme bacteriano

Um biofilme é composto, principalmente, por um consórcio estruturado de células bacterianas envolvidas em uma matriz extracelular de polissacarídeos, proteínas e DNA extracelular. Além dos mecanismos de resistência já apresentados, a maioria das bactérias, se não todas, são capazes de formar biofilme. O biofilme bacteriano é responsável por promover a sobrevivência das bactérias na natureza. Atrelada à questão da sobrevivência em ambientes, o biofilme também confere resistência a antibióticos, desinfetantes químicos e à fagocitose e outros componentes do sistema imunológico. Para que se chegue ao estágio de biofilme maduro, sua produção pelas bactérias passa por algumas etapas, como mostrado na figura 3: (1) o acoplamento reversível das células bacterianas à superfície; (2) ligação irreversível à superfície, multiplicação das células, formação das microcolônias, produção da matriz extracelular que envolve a microcolônia; (3) e dispersão de células bacterianas para colonização de novos locais onde seja possível a formação de biofilme (HØIBY *et al.*, 2011; MOORMEIER; BAYLES, 2017).

Figura 3 - Etapas de formação de biofilme microbiano



Fonte: elaborado pelo autor.

O biofilme bacteriano é causador de diversas doenças, e o biofilme pode ser formado em diversas superfícies diferentes, como dentes, válvulas do coração, ouvido médio e em cateteres intravenosos. As infecções causadas por biofilme podem ser divididas em duas categorias, (1) infecções associadas a dispositivos e (2) infecções não associadas a dispositivos.

A primeira categoria envolve a colonização e formação de biofilme em objetos como cateteres, próteses dentárias, dispositivos intrauterinos, entre outros, onde as células bacterianas podem se desacoplar dessas superfícies e se disseminarem aos tecidos próximos e até mesmo para a corrente sanguínea, podendo causar infecções severas. Já a segunda se trata da infecção diretamente nos tecidos do hospedeiro, onde pode levar a diferentes doenças dependendo de onde está instalado, como otite crônica, infecção recorrente do trato urinário e endocardite (SEGEV-ZARKO *et al.*, 2015; YASIR; WILLCOX; DUTTA, 2018). Como o biofilme se apresenta como uma barreira física, dificultando a ação de antibióticos, é de extrema importância a descoberta de novas moléculas e tratamentos alternativos que possibilitem eliminar esses biofilmes bacterianos.

2.5 *Staphylococcus aureus*

Em se tratando especificamente das bactérias do gênero *Staphylococcus*, elas são bactérias gram positivas, esféricas, com diâmetro entre 0,5 e 1,5 μm, não esporulam, e são imóveis. Os estafilococos podem se arranjar no ambiente de diversas formas, sozinhas, em pares, cadeias curtas e agrupamentos irregulares. A primeira descrição de *S. aureus* é datada de 1883, por Alexander Ogston, quando foi encontrada em pus de alguns abscessos cirúrgicos. Desde sua descoberta, e até antes disso, *S. aureus* vem sendo um grande problema de saúde pública sendo causadora de diversas doenças, como espinhas, furúnculos, e até pneumonias e meningites, e desde a descoberta dos primeiros antibióticos, nas décadas de 1920 e 1930, *S. aureus* vem evoluindo concomitantemente à disponibilização de novos antibióticos desde o uso da penicilina durante a Segunda Guerra Mundial, e com o tempo vem adquirindo resistência aos mesmos e, por conta disso, tornou-se uma bactéria de alta importância hospitalar (FAREED KAHN, 2017; LAKHUNDI; ZHANG, 2018).

A patogenicidade de *S. aureus* depende do sucesso das quatro etapas principais: (1) O início da infecção; (2) o estabelecimento, (3) a manutenção e (4) persistência da mesma. Durante essas etapas, é necessário que o patógeno seja capaz de se evadir dos diversos mecanismo que o hospedeiro possui para se livrar da infecção (CHEUNG; BAE; OTTO, 2021; KWIECINSKI; HORSWILL, 2020).

Durante todas essas etapas, *S. aureus* se dispõe de diversos mecanismos para escapar dos mecanismos de defesa do hospedeiro. Mecanismos para atravessar a barreira epitelial no início da infecção, pelo uso de toxinas; inibir a ativação dos neutrófilos, impedindo sua adesão; degradando receptores de quimiocinas responsáveis pela quimiotaxia dos neutrófilos; ativação de mecanismos que induzem a morte celular dos neutrófilos; e também

pela formação de macroestruturas como biofilme e abcessos (CHEUNG; BAE; OTTO, 2021; KWIECINSKI; HORSWILL, 2020; LAARMAN *et al.*, 2012).

Além de todos esses mecanismos que auxiliam a combater as defesas do hospedeiro, diversas cepas de *S. aureus* possuem resistência a vários tipos de antibióticos, o que dificulta ainda mais o seu tratamento. O histórico de resistência a antibióticos de *S. aureus* data de 1948, quando alguns isolados produtores de beta-lactamases foram identificados. Em apenas duas décadas, os números de isolados resistentes à penicilina chegaram a 80%. Atualmente, 99% dos isolados clínicos de *S. aureus* são resistentes à penicilina. Já no início dos anos 1960, a meticilina foi introduzida nos tratamentos para conter a onda de bactérias resistentes à penicilina. Porém, com menos de um ano, isolados de *S. aureus* resistentes à meticilina (MRSA) foram identificados em hospitais europeus. A resistência à meticilina se dá pela presença do gene *mecA*, que codifica uma transpeptidase (PBP) com menor afinidade para a maioria dos antibióticos beta-lactâmicos (SANTAJIT; INDRAWATTANA, 2016; VESTERGAARD; FREES; INGMER, 2019).

Mais tarde, nos anos 1990, isolados de MRSA iniciaram a apresentar uma resistência intermediária à vancomicina (VISA), um antibiótico da classe dos glicopeptídeos que, na época, era amplamente utilizado para o tratamento de infecções por MRSA e, posteriormente, em 2002, isolados que apresentavam resistência à vancomicina (VRSA). A resistência à vancomicina se dá pelo operon *vanA*, operon responsável pela expressão de um maquinário enzimático responsável pela hidrólise de precursores D-ala-D-ala, alvo da vancomicina e a síntese do precursor D-ala-D-lac, ao qual a vancomicina não é capaz de se ligar (CONG; YANG; RAO, 2020; VESTERGAARD; FREES; INGMER, 2019).

A resistência de *S. aureus* a antibióticos inibidores da replicação do DNA, como as fluoroquinolonas, é considerada comum, dada a alta prevalência de até 60%. A resistência a essa classe de antibiótico se dá por modificações ao sítio alvo das enzimas-alvo, que podem ser causadas pelos genes *gyrA* e *glrA*, que promovem modificações na enzima topoisomerase IV e DNA girase, respectivamente. Genes da família *nor* codificam bombas de efluxo responsáveis pela expulsão do fármaco dentro da célula (JENSEN; LYON, 2009; VESTERGAARD; FREES; INGMER, 2019).

Há registros na literatura de que alguns isolados de *S. aureus* também possuem resistência associada a outras classes de antibióticos, como resistência a lipoglicopeptídeos, macrolídeos, tetraciclinas e aminoglicosídeos por diversos genes, mas são menos comuns. O gene *fosB* é capaz de conferir resistência a lipoglicopeptídeos; o gene *crf* codifica codifica uma transferase que confere resistência contra linezolida; genes da família *erm*, codificam rRNA

metilases, conferindo resistência a macrolídeos; e o gene *tetA*(K), que codifica uma bomba de efluxo responsável por expulsar antibióticos da classe das tetraciclinas de dentro da célula (JENSEN; LYON, 2009; VESTERGAARD; FREES; INGMER, 2019).

Dispondo de todo esse arsenal, cepas resistentes de *S. aureus* acabam por serem um problema muito sério, dado que estamos vivendo no período chamado de *Discovery Void*, um período que se dá desde 1987 (introdução das Oxazolidinonas) até os dias de hoje, onde nenhuma nova classe de antibióticos foi descoberta, enquanto cepas multirresistentes continuam acumulando resistências através dos mecanismos já supracitados (BROUGHTON *et al.*, 2016; MATSINGOS *et al.*, 2021).

2.6 Peptídeos antimicrobianos (PAMs)

Na tentativa de conter o crescimento de resistência de patógenos bacterianos a antibióticos, é necessário buscar e prospectar novas moléculas capazes de tratar infecções, ou até trazer de volta a efetividade de moléculas já ineficazes. Esse é o caso dos peptídeos antimicrobianos (PAMs) (ADEGOKE *et al.*, 2016; ZHANG; GALLO, 2016).

A escolha dos PAMs é muito interessante, e viável, por conta das diversas vantagens que eles trazem, como o fato do alvo dos PAMs serem as membranas celulares, parte da célula onde é muito difícil os microrganismos desenvolverem resistência, além do fato de que são produzidos por todos os seres vivos já estudados para esse propósito, tornando-os uma fonte para prospecção quase que infinita (GHOSH; HALDAR, 2015; ZHANG; GALLO, 2016).

2.6.1 Características dos PAMs

Mesmo possuindo uma ampla diversidade de PAMs, todos apresentam algumas características intrínsecas comuns entre eles, como serem oligopeptídeos, com a quantidade de aminoácidos variando entre 5 e 50. Além disso, os PAMs possuem características que conferem a eles essa capacidade de interagir com as membranas de microrganismos e, consequentemente, conferir essa atividade antimicrobiana. Entre essas características estão a presença de resíduos de aminoácidos com carga positiva (lisinas e argininas) e a presença de aminoácidos hidrofóbicos. A presença dessas duas classes de aminoácidos confere aos PAMs uma propriedade de muita importância para seu papel antimicrobiano: a anfipaticidade. Por serem moléculas anfipáticas, os PAMs são capazes de interagir com membranas celulares. Entretanto,

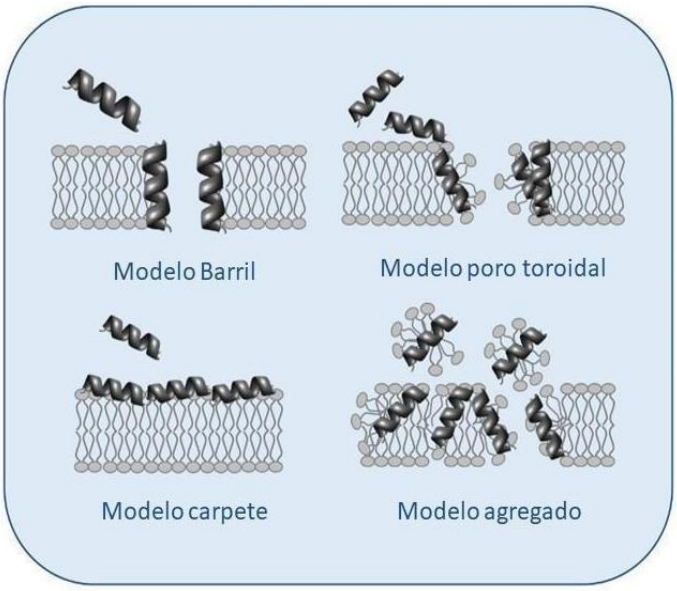
uma grande quantidade de aminoácidos com carga positiva pode causar um efeito indesejado, que é a baixa seletividade dos peptídeos e uma maior toxicidade às células do hospedeiro (BAHAR; REN, 2013; KANG *et al.*, 2017; LIMA *et al.*, 2021b; ZHANG; GALLO, 2016).

Os PAMs podem assumir diversas estruturas secundárias: folhas β, hélices α, e uma estrutura estendida. Essas estruturas estão diretamente ligadas à atividade biológica dos PAMs. A estrutura de folha β é caracterizada por pontes dissulfeto estabilizadas por resíduos Cys, como é o caso das defensinas e bacteriocinas. As hélices α são caracterizadas pela sua anfipaticidade, com a presença de aminoácidos catiônicos e hidrofóbicos para interação completa com a membrana, como é o caso do peptídeo humano LL37 e a Magainina de sapos. Além disso, esses peptídeos possuem uma peculiaridade, eles exibem pouca estrutura secundária em soluções aquosas, mas quando em um ambiente apolar (membrana bacteriana), eles assumem a estrutura de hélice α anfipática (ZHANG; GALLO, 2016).

2.6.2 Mecanismos de ação dos PAMs

Grande parte dos PAMs possuem um mecanismo de ação característico, que é a interação com a membrana bacteriana, formação de poros e morte celular subsequente. Já foram propostos quatro modelos de como os PAMs formam poros nas membranas, como pode ser visto na figura 3. São eles: poro toroidal, barril, carpete e modelo agregado (RAHEEM; STRAUS, 2019; YAN *et al.*, 2021).

Figura 4 - Mecanismos de ação dos PAMs



Fonte: (RAHEEM; STRAUS, 2019) (com modificações).

No modelo de poro toroidal, a parte hidrofílica dos PAMs interage com a cabeça polar da membrana lipídica, enquanto que a parte hidrofóbica interage com a cauda apolar. Então, quando a proporção entre moléculas de peptídeo e fosfolipídios chega a um certo valor, os PAMs são verticalmente embutidos na bicamada lipídica. Esse deslocamento faz com que a membrana lipídica dobre para dentro e que os PAMs interajam com o grupo polar dos fosfolipídios para formar poros na membrana, seguidos de extravasamento do conteúdo celular e da incapacidade de manter a pressão osmótica em níveis regulares. Um exemplo de PAM que segue esse modelo é o peptídeo LL-37 (SEYFI *et al.*, 2020; YAN *et al.*, 2021).

No modelo barril, quanto mais moléculas de PAMs interagem com a membrana, mais moléculas são atraídas a interagirem com a superfície da membrana celular. Desse modo, os PAMs são inseridos, verticalmente, na bicamada lipídica e se agregam (interação peptídeo-peptídeo) para formar um canal iônico que desestabiliza a membrana e provoca o extravasamento de conteúdo celular. O que difere o modelo barril do modelo de poro toroidal, é que no modelo de poro toroidal não há uma interação entre peptídeos, apenas uma interação polar entre peptídeo e fosfolipídio (SEYFI *et al.*, 2020; YAN *et al.*, 2021).

O modelo carpete difere um pouco dos outros supracitados, pois nele, os PAMs se dobram para formar uma estrutura anfifílica paralela à superfície da membrana e cobrindo-a, como um carpete. Com isso, haverá a formação de micelas e ruptura parcial da membrana celular, causando destruição da bicamada lipídica e extravasamento do conteúdo celular (SEYFI *et al.*, 2020; YAN *et al.*, 2021).

Também há o modelo de formação de agregados, onde PAMs se acumulam na superfície da membrana celular, sem nenhuma conformação específica. Após chegarem em uma determinada concentração, os PAMs embutidos na membrana, formam um complexo com as moléculas de fosfolipídios e cruzam a membrana na forma de agregados (SEYFI *et al.*, 2020; YAN *et al.*, 2021).

Apesar dos mecanismos de danos causados na membrana celular serem os mais comuns entre os PAMs, alguns deles possuem mecanismos que atuam no interior da célula, afetando a fisiologia basal para promover morte celular. A literatura mostra casos de PAMs com mecanismos de interferência nos processos de transcrição e replicação do DNA e RNA bacteriano, ocorrendo pela interação eletrostática do PAM com as moléculas de ácidos nucleicos. Os PAMs também podem inibir o processo de tradução e modificações pós-traducionais. Alguns PAMs também podem afetar a atividade de algumas enzimas, como enzimas essenciais no metabolismo de substâncias lipídicas, e assim afetando o metabolismo basal da bactéria. Por fim, os PAMs podem também afetar a divisão celular, bloqueando-a. Há

relatos na literatura de que, sob condições de extremo estresse, causada pelos PAMs, as bactérias assumem uma forma filamentosa, inibida de realizar divisão celular. Este último é o caso da diptericina, que faz com que células de *E. coli* se tornem mais alongadas, afetando o processo de divisão celular (YAN *et al.*, 2021).

2.7 PAMs sintéticos

Apesar dos PAMs serem ótimos candidatos para novas drogas alternativas no combate às bactérias resistentes, ainda existem alguns entraves em relação a sua utilização. PAMs naturais podem apresentar baixa seletividade, atividade hemolítica, toxicidade ao hospedeiro, baixa estabilidade relacionada à digestão por proteases *in vivo*, baixa solubilidade e problemas de biodistribuição. Além desses problemas, ainda há a questão do escalonamento, pois os PAMs naturais possuem um rendimento de obtenção baixíssimo, necessitando altas quantidades de material biológico para extração e purificação de alguns miligramas. Para contornar tais desvantagens, os peptídeos antimicrobianos sintéticos são ótimas alternativas (BOTO; PÉREZ DE LA LASTRA; GONZÁLEZ, 2018; SOUZA *et al.*, 2020a).

A produção dos peptídeos antimicrobianos sintéticos se dá pelo desenho racional dos mesmos, onde o tamanho do peptídeo, e quais aminoácidos serão utilizados, são minuciosamente selecionados, isso é chamado de desenho racional. O desenho racional é feito através de programas de bioinformática, onde é possível, com certo *softwares*, desenhar novos PAMs ou otimizar sequências já existentes, e obter diversos parâmetros do peptídeo desenhado, como carga líquida, hidrofobicidade, estrutura secundária, e índice de Boman, além de predizer uma possível atividade antimicrobiana, a qual pode ser melhorada com adições e remoções pontuais (KUNDU, 2020; SOUZA *et al.*, 2020a).

2.7.1 PAMs sintéticos desenhados a partir das proteínas Mo-CBP$_3$ e Rc-2S-Alb

Das sementes de *Moringa oleifera*, foi purificada, e caracterizada, a proteína *Mo*-CBP$_3$ (18 kDa), uma proteína de ligação à quitina da família das albuminas 2S, com alta atividade antifúngica. *In vitro*, *Mo*-CBP$_3$ apresentou atividade contra os fungos fitopatogênicos *Fusarium solani, Fusarium oxysporum, Colletotrichum musae* e *C. gloeosporioides*. Porém, ela não apresentou atividade antimicrobiana relevante contra patógenos humanos, como fungos do gênero *Candida*, mesmo em altas concentrações (BATISTA *et al.*, 2014; GIFONI *et al.*, 2012; NETO *et al.*, 2017).

Tabela 1 Propriedades físico-químicas dos peptídeos sintéticos *Mo*-CBP$_3$-PepI, *Mo*-CBP$_3$-PepII e *Mo*-CBP$_3$-PepIII

Propriedades físico-químicas	Peptídeos		
	Mo-CBP$_3$-PepI	*Mo*-CBP$_3$-PepII	*Mo*-CBP$_3$-PepIII
Sequência	CPAIQRCC	NIQPPCRCC	AIQRCC
[1]Massa molecular calculada (Da)	893.12	1033.26	692.86
[2]Massa molecular experimental (Da)	893.10	1033.23	692.85
[3]Ponto Isoelétrico (pI)	7.97	7.97	8.11
[1]Índice de Boman	1.23	2.03	1.86
[1]Razão Hidrofóbica Total (%)	62	44	66
[1]Carga líquida (+)	1	1	1
[4]Gráfico de Ramachandran (%)	100	67,7	83,3
[5]Tm	0,984	0,428	1,00
5sOPEP	-9.31	-8.16	-3.68

Fonte: OLIVEIRA *et al.* (2019)
[1]Calculado usando o banco de dados de peptídeos antimicrobianos.
[2]Dados obtidos por espectrometria de massa conduzida
[3]Calculado usando ExPASy ProtParam.
[4]Obtido usando Rampage.
[5]Obtido por Half Life Prediction.

A *Rc*-2S-*Alb* é uma proteína que foi purificada, e caracterizada, da torta das sementes de *Ricinus communis*, e também pertence à família das albuminas 2S, família cujas principais características são a baixa massa molecular e alto conteúdo de glutamina, arginina, asparagina e cisteína (SOUZA, 2020; SOUZA *et al.*, 2016). Ela mostrou possuir alta atividade antibacteriana contra *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* e *Bacillus subtilis*. Porém, ambas as proteínas apresentaram um problema em comum, um baixo rendimento de purificação, o mesmo problema recorrente de PAMs naturais.

Com o intuito de resolver o problema, foi feito o desenho racional de peptídeos sintéticos, baseados na sequência primária dessas proteínas, dando origem aos peptídeos *Mo*-CBP$_3$-PepI (CPAIQRCC), *Mo*-CBP$_3$-PepII (NIQPPCRCC) e *Mo*-CBP$_3$-PepIII (AIQRCC) (Tabela 1), a partir da proteína *Mo*-CBP$_3$; e *Rc*Alb-PepI (AKLIPTIAL), *Rc*Alb-PepII (SLRGCC) e *Rc*Alb-PepIII (AKLIPTIA) (Tabela 2), a partir da proteína *Rc*-2S-Alb (LIMA *et al.*, 2020; OLIVEIRA *et al.*, 2019; SOARES *et al.*, 2021). Esses peptídeos possuem como características principais a carga positiva (+1), razão hidrofóbica total entre 40-65% e índice de Boman ≤ 2,5. Diversos exemplos de peptídeos sintéticos antimicrobianos na literatura possuem

essas características, aumentam o potencial antimicrobiano *in silico* do peptídeo, o que aumenta a probabilidade de sucesso em ensaios *in vitro*. Os seis peptídeos já apresentaram atividade antimicrobiana *in vitro* contra patógenos bacterianos, fúngicos e também elmitos. (DIAS *et al.*, 2020; LIMA *et al.*, 2020; OLIVEIRA *et al.*, 2019; SOUZA *et al.*, 2020b).

Tabela 2 Propriedades físico-químicas dos peptídeos sintéticos *Rc*Alb-PepI, *Rc*Alb-PepII e *Rc*Alb-PepIII

Propriedades físico-químicas	Peptídeos		
	*Rc*Alb-PepI	*Rc*Alb-PepII	*Rc*Alb-PepIII
Sequência	AKLIPTIA	AKLIPTIAL	SLRGCC
[1]Massa molecular calculada (Da)	826.05	939.21	637.77
[2]Massa molecular experimental (Da)	826.03	939.17	637.75
[3]Ponto Isoelétrico (pI)	8.80	8.80	7.80
[1]Índice de Boman	-1.28	-1.68	1.65
[1]Razão Hidrofóbica Total (%)	62	66	50
[1]Carga líquida (+)	1	1	1
[4]Gráfico de Ramachandran (%)	98	100	66.7
[5]Tm	0.56	0.43	0.91
[5]sOPEP	-5.73	-7,95	-4,58

Fonte: Dias *et al.* (2020)

[1]Calculado usando o banco de dados de peptídeos antimicrobianos.

[2]Dados obtidos por espectrometria de massa conduzida

[3]Calculado usando ExPASy ProtParam.

[4]Obtido usando Rampage.

[5]Obtido por Half Life Prediction.

2.7.2 PAMs sintéticos desenhados a partir de quitinase de A. thaliana

Além dos seis peptídeos supracitados, foi feito o desenho racional de mais dois peptídeos. Porém, dessa vez, o desenho dos peptídeos foi feito a partir de uma sequência aleatória do banco de dados NCBI (https://www.ncbi.nlm.nih.gov/). No caso, a sequência escolhida foi de uma quitinase de *A. thaliana* (SOUZA *et al.*, 2020a).

O desenho foi feito seguindo um fluxograma descrito por Souza *et al.*, 2020, que consiste em quatro etapas: escolha da sequência protéica; uso de softwares para encontrar as melhores sequências de peptídeos aplicando alguns critérios, como a carga, índice de Boman e razão de hidrofobicidade; estabelecimento de outras propriedades fisico-químicas e biológicas, como potencial antimicrobiano e potencial antibiofilme; obtenção da estruturas 3D dos peptídeos. Com a avaliação desses critérios, foram obtidos dois peptídeos: PepGAT (GATIRAVNSR) e PepKAA (KAANRIKYFQ) (Tabela 3).

PepGAT e PepKAA já mostraram atividade antifúngica contra fungos como *C.*

neoformans, fungos do gênero *Tricophyton*, e atividade antibacteriana contra bactérias Gram-negativas como *Salmonella enterica*. Além disso, pep KAA aprensetou também atividade antimicrobiana contra Sars-Cov-2, o vírus causador da Covid-19 (LIMA *et al.*, 2021a; SOUZA *et al.*, 2020a).

Tabela 3 Propriedades físico-químicas dos peptídeos sintéticos PepGAT e PepKAA

Propriedades físico-químicas	Peptídeos	
	PepGAT	PepKAA
Sequência	GATIRAVNSR	KAANRIKYFQ
[1]Massa molecular calculada (Da)	1044.18	1238.44
[2]Massa molecular experimental (Da)	1044.90	1239.00
[3]Ponto Isoelétrico (pI)	12.00	10.29
[1]Índice de Boman	2.19	2.28
[1]Razão Hidrofóbica Total (%)	40	40
[1]Carga líquida (+)	2	2
[4]Gráfico de Ramachandran (%)	98	75
[5]Tm	0,360	0,414
[5]sOPEP	-7.39	-18.18

Fonte: Souza *et al.* (2020)

[1]Calculado usando o banco de dados de peptídeos antimicrobianos.

[2]Dados obtidos por espectrometria de massa conduzida

[3]Calculado usando ExPASy ProtParam.

[4]Obtido usando Rampage.

[5]Obtido por Half Life Prediction;

2.8 Sinergismo

O sinergismo entre agentes antimicrobianos acontece quando a combinação, de pelo menos dois deles, resulta em uma atividade antimicrobiana maior que atividade dos agentes somados. Os PAMs são ótimos candidatos a sinergismo com outros fármacos por conta do seu mecanismo de ação peculiar, de formar poros nas membranas celulares, esse tipo de mecanismo pode vir a facilitar a entrada e ação de fármacos aos quais os microrganismos possuam resistência relacionada à permeabilidade de membrana (HOLLMANN *et al.*, 2018; LI; FERNÁNDEZ-MILLÁN; BOIX, 2020). O sinergismo também pode levar a uma redução da dose efetiva do fármaco, reduzindo o custo de tratamento e podendo até reduzir a toxicidade. Com isso, o uso de PAMs em combinação com fármacos já presentes no mercado se torna bastante relevante (LI; FERNÁNDEZ-MILLÁN; BOIX, 2020).

3 HIPÓTESE

Os peptídeos sintéticos *Mo*-CBP$_3$-PepI, *Mo*-CBP$_3$-PepII, *Mo*-CBP$_3$-PepIII, *Rc*Alb-PepI, *Rc*Alb-PepII, *Rc*Alb-PepIII, pepGAT e pepKAA possuem atividade antibiofilme, em combinação com o antibiótico ciprofloxacina contra o biofilme da bactéria *Staphylococcus aureus*, por diversos mecanismos de ação.

4 OBJETIVOS

4.1 Objetivo geral

Avaliar o potencial antibiofilme e elucidar os mecanismos de ação dos peptídeos sintéticos *Mo*-CBP$_3$-PepI, *Mo*-CBP$_3$-PepII, *Mo*-CBP$_3$-PepIII, *Rc*Alb-PepI, *Rc*Alb-PepII, *Rc*Alb-PepIII, pepGAT e pepKAA contra o biofilme de *Staphylococcus aureus*, em combinação com o antibiótico ciprofloxacina.

4.2 Objetivos específicos

Avaliar a atividade antibioiflme *in vitro*, contra biofilme de *S. aureus*, de oito peptídeos sintéticos bioinspirados em proteínas vegetais, combinados com o antibiótico ciprofloxacina;

Avaliar a toxicidade das combinações peptídeo + ciprofloxacina a eritrócitos humanos;

Elucidar os mecanismos de ação dos peptídeos que obtiveram atividade antibiofilme satisfatória, através de microscopia de fluorescência;

Entender como os peptídeos, o antibiótico ciprofloxacina e ambos juntos alteram o perfil proteico das células do biofilme através de análises proteômicas.

5 CAPÍTULO II – ARTIGO CIENTÍFICO 1

Artigo científico publicado na revista *Antibiotics* (Fator de impacto: 4,5). Disponível em: (https://doi.org/10.3390/pathogens11090995)

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Article

Synergistic Antibiofilm Activity between Synthetic Peptides and Ciprofloxacin against *Staphylococcus aureus*

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Abstract: *Staphylococcus aureus* is a human pathogen known to be resistant to antibiotics since the mid-20th century and is constantly associated with hospital-acquired infections. *S. aureus* forms biofilms, which are complex surface-attached communities of bacteria held together by a self-produced polymer matrix consisting of proteins, extracellular DNA, and polysaccharides. Biofilms are resistance structures responsible for increasing bacterial resistance to drugs by 1000 times more than the planktonic lifestyle. Therefore, studies have been conducted to discover novel antibacterial molecules to prevent biofilm formation and/or degrade preformed biofilms. Synthetic antimicrobial peptides (SAMPs) have appeared as promising alternative agents to overcome increasing antibiotic resistance. Here, the antibiofilm activity of eight SAMPs, in combination with the antibiotic ciprofloxacin, was investigated in vitro. Biofilm formation by *S. aureus* was best inhibited (76%) by the combination of *Mo*-CBP$_3$-PepIII (6.2 μg mL^{-1}) and ciprofloxacin (0.39 μg mL^{-1}). In contrast, the highest reduction (60%) of the preformed biofilm mass was achieved with *Rc*Alb-PepII (1.56 μg mL^{-1}) and ciprofloxacin (0.78 μg mL^{-1}). Fluorescence microscopy analysis reinforced these results. These active peptides formed pores in the cellular membrane of *S. aureus*, which may be related to the enhanced ciprofloxacin's antibacterial activity. Our findings indicated that these peptides may act with ciprofloxacin and are powerful co-adjuvant agents for the treatment of *S. aureus* infections.

Keywords: synthetic peptides; biofilm; *Staphylococcus aureus*; synergism

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1. Introduction

Staphylococcus aureus resistance to antibiotics keeps evolving constantly. For example, penicillin was released for use in 1941. In 1943, the first *S. aureus* isolate resistant to penicillin was discovered. In 1944, 5% of *S. aureus* isolates were resistant to penicillin, and this number increased dramatically to 80% in 1959. In addition to resistance to penicillin, *S. aureus* acquired resistance genes such as *blaZ*, *blaI*, and *blaR1*, conferring resistance to other antibiotics [1–3].

In 1960, methicillin, a penicillin-class semi-synthetic antibiotic, was created to com-

Article

Synergistic antibiofilm activity between synthetic peptides and ciprofloxacin against *Staphylococcus aureus*

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Abstract: Staphylococcus aureus is a human pathogen known to be resistant to antibiotics since the mid-20th century and is constantly associated with hospital-acquired infections. S. aureus forms biofilms, complex surface-attached communities of bacteria held together by a self-produced polymer matrix consisting of proteins, extracellular DNA, and polysaccharides. Biofilms are resistance structures responsible for increasing bacterial resistance to drugs by 1000 times more than the planktonic lifestyle. Therefore, studies have been conducted to discover novel antibacterial molecules to prevent biofilm formation and/or degrade preformed biofilms. Synthetic Antimicrobial peptides (SAMPs) have appeared as promising alternative agents to overcome increasing antibiotic resistance. Here, the antibiofilm activity of eight SAMPs, in combination with the antibiotic ciprofloxacin, was investigated in vitro. Biofilm formation by S. aureus was best inhibited (76%) by the combination of Mo-CBP$_3$-PepIII (6.2 μg mL^{-1}) and ciprofloxacin (0.39 μg mL^{-1}). In contrast, the highest reduction (60%) of the preformed biofilm mass was achieved with RcAlb-PepII (1.56 μg mL^{-1}) and ciprofloxacin (0.78 μg mL^{-1}). Fluorescence microscopy analysis reinforced these results. These active peptides formed pores in the cellular membrane of S. aureus, which may be related to the enhanced ciprofloxacin's antibacterial activity. Our findings indicated that these peptides may act with ciprofloxacin and are powerful coadjuvant agents for the treatment of S. aureus infections.

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1. Introduction

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penicillin was released for use in 1941. In 1943, the first *S. aureus* isolate resistant to penicillin was discovered. In 1944, 5% of *S. aureus* isolates were resistant to penicillin, and this number increased dramatically to 80% in 1959. In addition to resistance to penicillin, *S. aureus* acquired resistance genes such as *blaZ, blaI,* and *blaR1* conferring resistance to other antibiotics (LOWY, 2003; PANTOSTI; SANCHINI; MONACO, 2007; SANTOS *et al.*, 2007).

In 1960, methicillin, a penicillin class semi-synthetic antibiotic, was created to combat resistant *S. aureus* by targeting beta-lactamase enzymes responsible for resistance to penicillin. Indeed, methicillin worked, but not for so long. In 1961, the first report of methicillin-resistant *S. aureus* (MRSA) was documented, and the genes *mecA* was reported as responsible for the resistance. Based on the resistance to methicillin, another antibiotic, vancomycin, was employed to treat *S. aureus* infections; however, later in the 60's emerged the Vancomycin-resistant *S. aureus* (VRSA). VRSA strains possess the operon vanA, which consists of a pool of genes involved in synthesizing a modified peptidoglycan precursor not affected by vancomycin treatment. Due to the falling use of vancomycin, fluoroquinolones were used against MRSA (KOURTIS *et al.*, 2019; LOWY, 2003; PANTOSTI; SANCHINI; MONACO, 2007; SANTOS *et al.*, 2007; SHARIATI *et al.*, 2020). After a few years, MRSA started to show resistance to these antibiotics, such as ciprofloxacin (LOWY, 2003; PANTOSTI; SANCHINI; MONACO, 2007).

Recently, the most common antibiotics used to treat MRSA infections are lipopeptides and oxazolidinones. Even though these antibiotics still being effective, recent studies already show MRSA strains resistant to these antibiotic classes (KOURTIS *et al.*, 2019; PANTOSTI; SANCHINI; MONACO, 2007; SHARIATI *et al.*, 2020). In 2017, an estimation of 119,247 *S. aureus* bloodstream infections occurred, with 19,832 associated deaths in the United States (SHARIATI *et al.*, 2020).

Besides the resistance to drugs, *S. aureus* can also form biofilms, which could increase resistance by 1,000 times (KOURTIS *et al.*, 2019; PANTOSTI; SANCHINI; MONACO, 2007; SHARIATI *et al.*, 2020). The biofilm matrix formed by *S. aureus* is composed of protein (either secreted and lysis-derived proteins), complex carbohydrates (e.g., N-acetyl-glucosamine), and extracellular DNA (eDNA) (LISTER; HORSWILL, 2014). These components vary according to the strain and environmental conditions (LISTER; HORSWILL, 2014). The biofilm matrix possesses a dispersal mechanism that reduces the effectiveness of drugs, increasing the resistance (IZANO *et al.*, 2008). All the ways by which *S. aureus* acquires resistance reinforce the need to seek alternative molecules related to treating infections caused by MRSA, especially hospital-acquired infections (KOURTIS *et al.*, 2019; PANTOSTI; SANCHINI; MONACO,

2007; SHARIATI *et al.*, 2020).

SAMPs are an alternative treatment to the infection caused by resistant *S. aureus* (BEZERRA *et al.*, 2022; KUNDU, 2020; OLIVEIRA *et al.*, 2019; SOUZA *et al.*, 2020a). SAMPs present many advantages since they are rationally designed based on antimicrobial proteins (SOUZA *et al.*, 2020a). Some of these advantages are their mechanism of action (interaction with the cell membrane and cell wall), low allergenic potential, and high yield since they can be obtained by chemical synthesis (KUNDU, 2020; SOUZA *et al.*, 2020a).

Recently, our research group has shown that SAMPs derived from plant antimicrobial proteins presented great activity against *S. aureus* planktonic cells (DIAS *et al.*, 2020; OLIVEIRA *et al.*, 2019; SOUZA *et al.*, 2020a). In this context, our research group evaluated the combined antibiofilm activity of those synthetic peptides with ciprofloxacin against *S. aureus* biofilm. In addition, the mechanism of action of peptides and ciprofloxacin alone or combined were evaluated by fluorescence microscopy. Altogether the results revealed synthetic peptides enhanced the potential of ciprofloxacin against *S. aureus* in both cases inhibiting biofilm formation and reduction the mass of preformed biofilm.

2. Materials and methods

2.1. Biologic Material

S. aureus (ATCC 25923) strain was obtained from the laboratory of toxic proteins (LabTox) from the department of biochemistry and molecular biology of the Federal University of Ceará (UFC).

2.2. Peptide Sequence

The synthetic peptides *Mo*-CBP$_3$-PepI, *Mo*-CBP$_3$-PepII, *Mo*-CBP$_3$-PepIII (Oliveira *et al.*, 2019), *Rc*Alb-PepI, *Rc*Alb-PepII, *Rc*Alb-PepIII (Dias *et al.*, 2020), PepGAT and PepKAA (Souza *et al.*, 2020) were chemically synthesized by ChemPeptide (Shanghai, China), where were analyzed purity and quality (≥ 95%) by reverse-phase high-performance liquid chromatography and mass spectrometry.

2.3. Antibiofilm Assay

The assay was made in flat-bottom 96-well polystyrene microplates as described by Bezerra *et al.* (BEZERRA *et al.*, 2022). The cell suspension was prepared using a single colony of *S. aureus* from a Petri dish with Mueller-Hinton broth (stock bacteria). The colony was transferred to Mueller-Hinton broth and incubated at 37 ºC for 24 h. After, the cell suspension was standardized to a concentration of 10^6 cells mL^{-1}. To the assay to observe the inhibition of

biofilm formation, 50 µL from the standardized cell suspension was incubated in contact with 25 µL of the peptide solution, diluted in 5% dimethyl sulfoxide (DMSO) prepared in NaCl 0.15 M (DMSO-NaCl), at different concentrations (1000 to 0.2 µg mL^{-1}); and 25 µL of ciprofloxacin solution, diluted in DMSO-NaCl, at different concentrations (1000 to 0.2 µg mL^{-1}) in the dark, at 37 ºC, for 48 h. The combination of peptide and antibiotic was made in a way the highest concentration of the peptide was combined with the lowest antibiotic concentration, and vice-versa.

After the incubation period, the supernatant was removed carefully from each well and washed one time with sterile NaCl 0.15 M solution. Then, biofilm was fixated with 100 µL of 99% methanol for 15 minutes, and after drying, biofilm was stained with 200 µL of 0.1% violet crystal solution. Then, the wells were washed three times with sterile 0.15 M NaCl solution, and the remaining crystal was solubilized with 200 µL of 33% acetic acid (v/v) solution. The absorbances were obtained via a microplate reader (BioTek™ Epoch, BioTek Instruments, Inc., USA) at 600 nm. The experiment was repeated three times.

The assay to observe the preformed biofilm reduction was conducted the same as described above, but with some adjustments. Initially, 50 µL of the standardized cell suspension were incubated in flat-bottom 96-well microplates in the dark, at 37 ºC, for 24 h, to biofilm formation. The preformed biofilm was incubated with 25 µL of the peptides or ciprofloxacin alone or in combination as described above. The microplates were incubated in the dark, at 37 ºC, for 24 h. After incubation, the biofilm was washed, dried, stained, and absorbance was taken as above.

2.4. Biofilm Integrity Determined by Fluorescence Microscopy

The assay was conducted as described by Bezerra *et al.* (BEZERRA *et al.*, 2022). Biofilm was produced, and the assay was conducted as described in the antibiofilm assay. Still, instead of using 96-well microplates, the assay was made in coverslips inside 6-well microplates. After incubation, the coverslips were washed three times with sterile 0.15 M NaCl. Afterward, the coverslips were incubated with an aqueous solution of propidium iodide (PI, 10 µM) in the dark, at 37 ºC, for 30 min. Then, they were washed with sterile 0.15 M NaCl three times to remove the excess PI. Then the coverslips were observed with a fluorescence microscope (Olympus System Bx 60) in a 535 nm excitation and 617 nm wavelength.

2.5. Overproduction of Reactive Oxygen Species (ROS)

The ROS overproduction was determined following the method described by Bezerra *et al.*

(ANDRADE *et al.*, 2022). The assays were conducted as the same for PI analysis. Then, 20 µL of 2′,7′ dichlorohfluorescein diacetate (DCFH-DA, Sigma, St. Louis, MI, USA) was added and incubated in the dark for 30 min at 24 °C. Finally, the biofilms were washed with 0.15 M NaCl and observed under a fluorescence microscope (Olympus System BX 41, Tokyo, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.6. Hemolytic assay

The hemolytic activities against A, B, and O types of human erythrocytes of peptides and ciprofloxacin alone or in combination were done following the methodology by Bezerra *et al.* [13]. The concentrations of all solutions were the same as used in the synergism assays. The blood types were provided by the Hematology and Hemotherapy Center of Ceará (Brazil). The blood was collected in a tube with heparin (5 IU mL^{-1}, Sigma Aldrich, São Paulo, Brazil), centrifuged at 300 x g for 5 min at 4 °C, washed with sterile 0.15 M NaCl, and diluted to a concentration of 2.5%. Each blood type was incubated (100 µL) with peptides and ciprofloxacin alone or in combination for 30 min at 37 °C. Then centrifuged 300 x g for 5 min at 4 °C (centrifuge Eppendorf 5810, Germany). Supernatants were collected and the absorbance read at 414 nm. DMSO-NaCl solution (0%) and 0.1% Triton X-100 (100%) as negative and positive controls for hemolysis, respectively. The equation calculated the hemolysis: [(Abs_{414nm} of the sample treated with peptides or drugs - Abs_{414nm} of samples treated with DMSO-NaCl) / [(Abs_{414nm} of samples treated with 0.1% TritonX-100-Abs_{414nm} of samples treated with DMSO-NaCl] x 100.

2.7. Statistical Analysis

All assays were performed in triplicate, and the values were expressed as the mean ± standard error. All quantitative data were submitted to one-way ANOVA followed by the Tukey test using GraphPad Prism 6.01 (GraphPad Software Company, Santa Clara, CA, USA), with a $p < 0.05$.

3. Results

3.1. Combined Antibiofilm Activity of Synthetic Peptides and Ciprofloxacin Against S. aureus

Synthetic peptides and ciprofloxacin presented different behaviors toward *S. aureus* biofilm. Ciprofloxacin alone could not successfully inhibit the formation or reduce the mass of preformed biofilms. In the case of synthetic peptides, the activity alone was very low but still present. However, combinations between some synthetic peptides and ciprofloxacin presented promising results (Fig. 1). Out of eight synthetic peptides, four presented great results in combination with ciprofloxacin Mo-CBP3-PepI, Mo-CBP3-PepIII, RcAlb-PepI, and RcAlb-PepII. All the effective combinations are presented in figure 1.

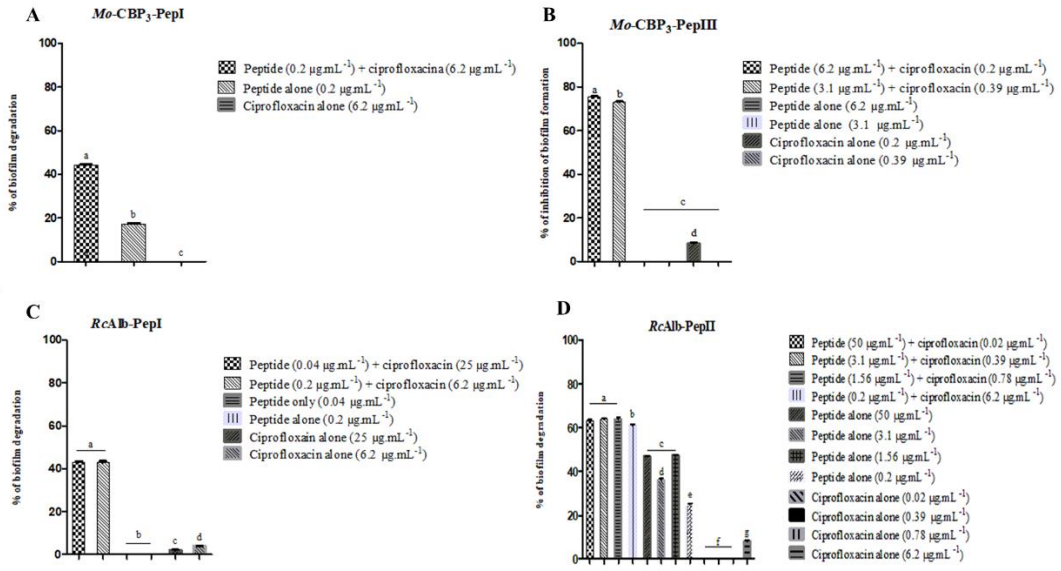

Figure 1. Combined antibiofilm activity of synthetic peptides and ciprofloxacin against *S. aureus* biofilm. (A) Mo-CBP3-PepI, (B) Mo-CBP3-PepIII, (C) RcAlb-PepI, and (D) RcAlb-PepII. *Different lowercase letters indicate statically significant difference compared to DMSO-NaCl by analysis of variance ($p < 0.05$).

Although twelve combinations of Mo-CBP3-PepI and ciprofloxacin have been tested, only one was effective in reducing the biomass of *S. aureus* preformed biofilm (Fig. 1A). In contrast, alone Mo-CBP3-PepI (0.2 µg mL-1) and ciprofloxacin (6.2 µg mL-1) reduced, respectively, 19 and 0% of preformed biofilm from *S. aureus*, combined the reduced of preformed biofilm increases to 50% (Fig. 1A). The combination between Mo-CBP3-PepIII and ciprofloxacin was best effective in the inhibition of biofilm formation (Fig. 1B). Alone, at concentrations of 6.2 µg mL^{-1} and 3.1 µg mL^{-1}, Mo-CBP3-PepIII did not inhibit the biofilm

formation of *S. aureus* at any extent. Ciprofloxacin alone, at concentrations of 0.2 μg mL^{-1} and 0.39 μg mL^{-1}, only inhibited the biofilm formation by 10% at 0.2 μg mL^{-1} (Fig. 1B). Two combinations of Mo-CBP3-PepIII and ciprofloxacin showed the best results. Mo-CBP3-PepIII (6.2 μg mL^{-1}) and Ciprofloxacin (0.39 μg mL^{-1}) and Mo-CBP3-PepIII (3.1 μg mL^{-1}) and Ciprofloxacin (0.2 μg mL^{-1}) inhibited the biofilm formation, respectively, in 73 and 76% (Fig. 1B).

RcAlb-PepI worked in two combinations with ciprofloxacin to reduce the mass o preformed biofilm (Fig. 1C). Two combinations made of RcAlb-PepI (0.04 μg mL^{-1}) and Ciprofloxacin (25 μg mL^{-1}) and RcAlb-PepI (0.2 μg mL^{-1}) and ciprofloxacin (6.2 μg mL^{-1}) reduced 40% the preformed biofilm of *S. aureus* (Fig. 1C). Interestingly, RcAlb-PepI and ciprofloxacin alone in both concentrations were not effective to reduce the biomass of biofilm from *S. aureus*. The combinations made by RcAlb-PepI (50 μg mL-1) and Ciprofloxacin (0.02 μg mL^{-1}), RcAlb-PepI (3.1 μg mL^{-1}) and Ciprofloxacin (0.39 μg mL^{-1}), RcAlb-PepI (1.56 μg mL^{-1}) and Ciprofloxacin (0.78 μg mL^{-1}), and RcAlb-PepI (0.2 μg mL^{-1}) and Ciprofloxacin (6.2 μg mL^{-1}) reduced around 65% of *S. aureus* biofilm biomass (Fig. 1D). At these concentrations alone, ciprofloxacin was not effective in reduced the biomass of *S. aureus* preformed biofilm.

3.2. Action Mechanisms of Synthetic Peptides

3.2.1. Membrane pore formation by propidium iodide uptake

The mechanisms of action behind the activity of synthetic peptides and ciprofloxacin, either alone or in combination, were evaluated by fluorescence microscopy. The best activities shown in Fig. 1 were analyzed by Fluorescence microscopy to observe the pore formation on the membrane of cells by the PI uptake. The combinations were, Mo-CBP3-PepI (0.2 μg mL^{-1}) and ciprofloxacin (6.2 μg mL^{-1}) against preformed biofilm (Fig. 1A), Mo-CBP3-PepIII (6.2 μg mL^{-1}) and ciprofloxacin (0.2 μg mL^{-1}) against the formation of biofilm (Fig. 1B), RcAlb-PepI (0.04 μg mL^{-1}) and ciprofloxacin (25 μg mL^{-1}) against preformed biofilm, and RcAlb-PepII (50 μg mL^{-1}) and ciprofloxacin (0.02 μg mL^{-1}) against preformed biofilm (Fig. 1D).

PI is a dye that interacts with nuclei acids releasing red fluorescence. However, PI can only through a damaged membrane; a healthy membrane did allow the movement of PI by it. The treatment presenting red fluorescence indicates damage to cell membranes. As expected, DMSO-NaCl and ciprofloxacin did not induce damage to the cell membrane, given the absence of red fluorescence (Figs. 2 and 3). The mechanism of action of ciprofloxacin does not involve damage to the membrane, which is confirmed by PI assay uptake. The Mo-CBP3-PepI (0.2 μg

mL^{-1}) alone was able to induce pore formation in S. aureus biofilm cells, and fluorescence was even higher in the combination of Mo-CBP3-PepI (0.2 µg mL^{-1}) and ciprofloxacin (6.2 µg mL^{-1}) (Fig. 2).

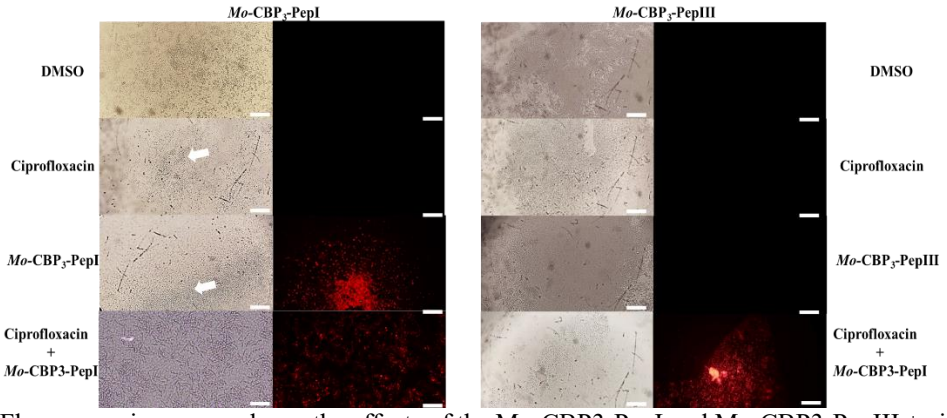

Figure 2. Fluoresce microscopy shows the effects of the Mo-CBP3-PepI and Mo-CBP3-PepIII + ciprofloxacin on the membrane of biofilm cells *S. aureus*. DMSO: Control treatment with DMSO-NaCl solution. Ciprofloxacin: at 6.2 µg mL^{-1} (left panel) and 0.2 µg mL-1 (right panel). Mo-CBP3-PepI alone at 0.2 µg mL^{-1}; Mo-CBP3-PepIII alone at 6.2 µg mL^{-1}; Mo-CBP3-PepI and ciprofloxacin (0.2 µg mL^{-1} and 6.2 µg mL^{-1}, respectively); Mo-CBP3-PepIII and ciprofloxacin (6.2 µg mL^{-1} and 0.2 µg mL^{-1}, respectively). In the left panel is the assay of reduction of preformed biofilm, and in the right panel is the inhibition of biofilm formation. Bars = 100 µm.

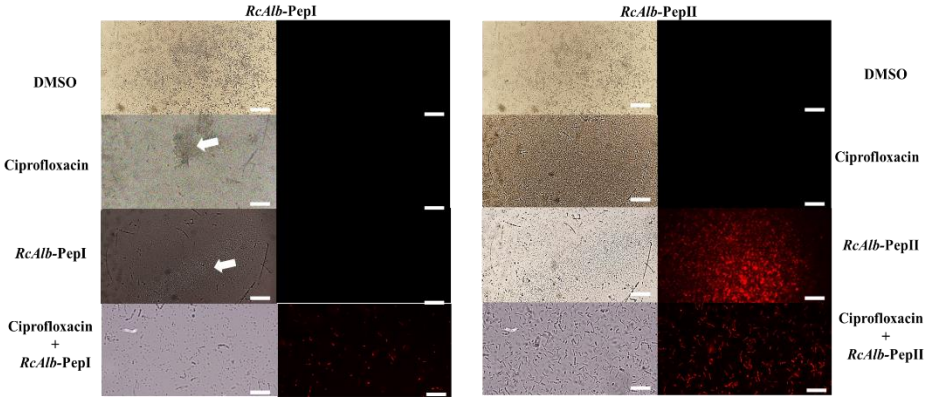

Figure 3. Fluoresce microscopy shows the effects of the RcAlb-PepI and RcAlb-PepIII and ciprofloxacin on the membrane of biofilm cells *S. aureus*. DMSO: Control treatment with DMSO-NaCl solution. Ciprofloxacin: at 25 µg mL^{-1} (left panel) and 0.02 µg mL^{-1} (right panel). RcAlbPepI alone at 0.04 µg mL^{-1}; RcAlb-PepIII alone at 50 µg mL^{-1}; RcAlb-PepI and ciprofloxacin (0.04 µg mL^{-1} and 25 µg mL^{-1}, respectively); RcAlbPepIII and ciprofloxacin (50 µg mL^{-1} and 0.02 µg mL^{-1}, respectively). Both panels are reduction of biofilm mass assay. Bars = 100 µm.

In contrast, Mo-CBP3-PepIII (6.2 µg mL^{-1}) alone was not able to induce pore formation of biofilm cells of *S. aureus* as no fluorescence was detected (Fig. 2). However, the combination made by Mo-CBP3-PepIII (6.2 µg mL^{-1}) and ciprofloxacin (0.2 µg mL^{-1}) and induced the releasing of red fluorescence, suggesting both Mo-CBP3-PepIII and ciprofloxacin work together to induce pore formation of biofilm cells of *S. aureus* (Fig. 2).

In the case of RcAlb-PepI, only in combination with RcAlb-PepI (0.04 µg mL^{-1}) and ciprofloxacin (25 µg mL^{-1}) was it possible to induce pore formation on the membrane of *S.*

aureus in biofilm (Fig. 3). Alone, DMSO, ciprofloxacin (alone) and RcAlb-PepI (at 6.2 µg mL^{-1} alone) did not induce pore formation on the membrane of *S. aureus* in biofilm (Fig. 3). In contrast, RcAlb-PepII alone (at 50 µg mL^{-1}) or in combination, RcAlb-PepII and ciprofloxacin (50 µg mL^{-1} and 0.02 µg mL^{-1}, respectively) was able to induced pore formation on the membrane as revealed by PI uptake. DMSO and ciprofloxacin could not induce pore formation (Fig. 3).

3.2.2. ROS overproduction

The evaluation of ROS overproduction revealed that DMSO-NaCl and ciprofloxacin did not induce ROS in any treatments (Figs. 4 and 5). Mo-CBP3-PepI slightly induced the ROS production in biofilm cells of *S. aureus* (Fig. 4). This ROS production was even higher in the combination of Mo-CBP3-PepI with ciprofloxacin (Fig. 4). In contrast, Mo-CBP3-PepIII only induced ROS overproduction in biofilm cells of *S. aureus* when combined with ciprofloxacin (Fig. 4). Alone, Mo-CBP3-PepIII, DMSO, and ciprofloxacin did not induce any ROS accumulation.

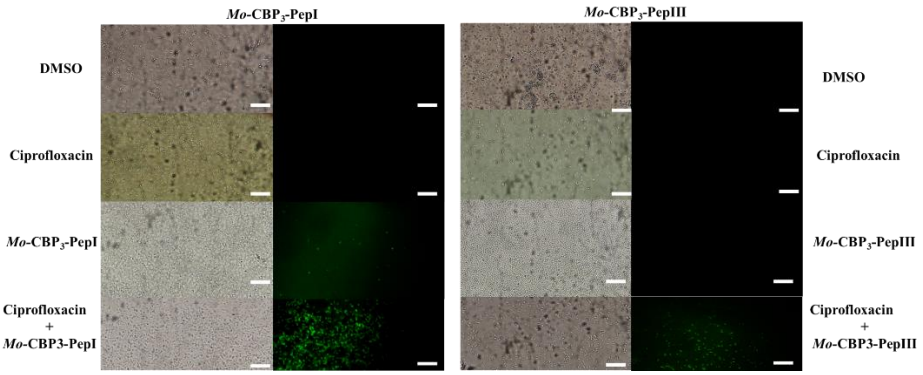

Figure 4. Fluoresce microscopy showed ROS overproduction induced by Mo-CBP3-PepI and Mo-CBP3-PepIII and ciprofloxacin ROS overproduction in *S. aureus* biofilm. DMSO: Control treatment with DMSO-NaCl solution. Ciprofloxacin: at 6.2 µg mL^{-1} (left panel) and 0.2 µg mL^{-1} (right panel). Mo-CBP3-PepI alone at 0.2 µg mL^{-1}; Mo-CBP3-PepIII alone at 6.2 µg mL^{-1}; Mo-CBP3-PepI and ciprofloxacin (0.2 µg mL^{-1} and 6.2 µg mL^{-1}, respectively); Mo-CBP3-PepIII + ciprofloxacin (6.2 µg mL^{-1} and 0.2 µg mL^{-1}, respectively). In the left panel is the assay of reduction of preformed biofilm and in the right panel is the inhibition of biofilm formation. Bars = 100 µm.

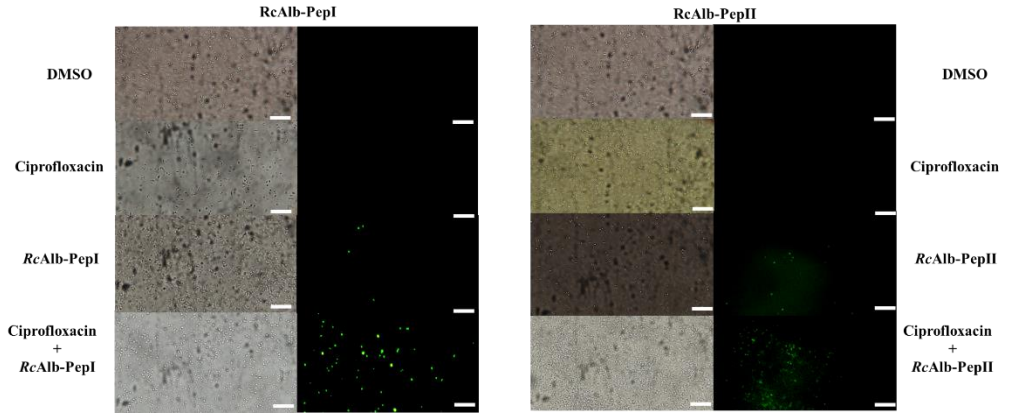

Figure 5. Fluoresce microscopy showed ROS overproduction induced by RcAlb-PepI, RcAlb-PepIII and ciprofloxacin *S. aureus* biofilm. DMSO: Control treatment with DMSO-NaCl solution. Ciprofloxacin: at 25 µg mL^{-1} (left panel) and 0.02 µg mL^{-1} (right panel). RcAlb-PepI alone at 0.04 µg mL^{-1}; RcAlb-PepIII alone at 50 µg mL^{-1}; RcAlb-PepI and ciprofloxacin (0.04 µg mL^{-1} and 25 µg mL^{-1}, respectively); RcAlb-PepIII and ciprofloxacin (50 µg mL^{-1} and 0.02 µg mL^{-1}, respectively). Both panels are reduction of biofilm mass assay. Bars = 100 µm.

Interestingly, both RcAlb-PepI and RcAlb-PepII induced ROS accumulation alone or in combination with ciprofloxacin. Both peptides presented similar behavior. Alone, they induced less ROS accumulation in biofilm cells of *S. aureus* than combined with ciprofloxacin (Fig. 5). In both cases, the combination with ciprofloxacin was effective in ROS accumulation.

3.3. Hemolytic Action

As reported in many other studies from our research group, synthetic peptides presented no toxicity to human red blood cells (HRBC) [10,12]. Additionally, it has been shown that during synergistic action, peptides, besides enhancing drugs effect, also reduce their toxicity to HRBC [11,13].

Here, the hemolytic potential of peptides and ciprofloxacin were assayed alone or in combination (Table 1). Positive control for hemolysis, 0.1% Triton X-100, induced 100% hemolysis in all three types of HRBC (Table 1). Ciprofloxacin in the highest concentration tested (1000 µg mL-1) hemolyzed 100% of all types of HRBC (Table 1). One of the concentrations of ciprofloxacin (25 µg mL^{-1}) used in combination with peptides, when alone, still induced 45, 51, and 34% of hemolysis, respectively, in type-A, -B, and -O of HRBC (Table 1). The other concentrations of ciprofloxacin were not toxic to HRBC. As expected, even at higher concentrations, peptides were not toxic to HRBC (Table 1).

Peptides/Combinations	% Hemolysis		
	Type-A Blood	Type-B Blood	Type-O Blood
0.1% Triton X-100	100 ± 0.001	100 ± 0.001	100 ± 0.005
DMSO-NaCl Solution	0	0	0
Ciprofloxacin (1000 μg mL^{-1})	100 ± 0.007	100 ± 0.003	100 ± 0.005
Ciprofloxacin (25 μg mL^{-1})	45 ± 0.004	51 ± 0.002	34 ± 0.006
Ciprofloxacin (0.2 μg mL^{-1})	0	0	0
Ciprofloxacin (0.02 μg mL^{-1})	0	0	0
Mo-CBP_3-PepI (1000 μg mL^{-1})	0	0	0
Mo-CBP_3-PepIII (1000 μg mL^{-1})	0	0	0
RcAlb-PepI (1000 μg mL^{-1})	0	0	0
RcAlb-PepII (1000 μg mL^{-1})	0	0	0
Mo-CBP_3-PepI (0.2 μg mL-1) and Ciprofloxacin (6.2 μg mL-1)	0	0	0
Mo-CBP_3-PepIII (6.2 μg mL^{-1}) and Ciprofloxacin (0.2 μg mL^{-1})	0	0	0
RcAlb-PepI (0.04 μg mL^{-1}) and Ciprofloxacin (25 μg mL^{-1})	0	0	0
RcAlb-PepII (50 μg mL^{-1}) and Ciprofloxacin (0.02 μg mL^{-1})	0	0	0

Table 1. Hemolytic activity of synthetic peptides, antifungal drugs, and their combination toward human red blood cells.

An interesting result came out during the hemolysis assay with combinations. All combinations of peptides and ciprofloxacin were not toxic to HRBC (Table 1). Even ciprofloxacin at 25 μg mL^{-1}, which was toxic alone, did not present to HRBC when combined with RcAlb-PepI (Table 1).

4. Discussion

There is no doubt that *S. aureus* is a major health issue due to the severity of hospital-acquired infections caused by *S. aureus*, which is worsened by its resistance to many antibiotics available nowadays. With the biofilm formation, the gravity of the situation is even more complicated because the biofilm is a well-evolved resistance structure formed to avoid potential threats. *S. aureus* biofilms cause concern due to their ability to ease form biofilms on many different surfaces [3–5,14–16].

As described above, *S. aureus* quickly accumulates mutations resulting in resistance to many drugs. Those are antibiotics from different groups with different modes of action, reinforcing the ability of *S. aureus* to acquire resistance [15,17]. This high acquisition of

mutation to many drugs results from a genetic variation associated with phenotypic plasticity presented by *S. aureus* in response to environmental insults, which allows *S. aureus* to adapt to environmental changes to keep growth, reproduction, and infection process [18]. For example, Gardete and Tomasz [19] analyzed that *S. aureus* cells cultivated in a media supplied with vancomycin presented a thickened cell wall compared to cells cultivated in a media vancomycin-free.

Based on that, developing new molecules or even the association of molecules could be a hopeful strategy to cope with *S. aureus* resistance. At this point, synthetic peptides could be a great alternative to be used alone to develop new drugs or even act as an adjuvant to improve drugs already used.

The synthetic peptides used in this work already had shown antimicrobial activity against many human pathogens, such as dermatophyte fungi, pathogenic yeasts, SARS-CoV-2, and bacteria such as *S. aureus* itself [10,12,20–22]. The hypothesis behind this work was whether these peptides could inhibit the formation or even reduce *S. aureus* biofilm biomass alone or in combination with ciprofloxacin.

Many studies have been carried out concerning the synergistic effect of antimicrobial peptides in combination with commercial drugs. Bessa *et al.* [23] and Martinez *et al.* [24] presented antimicrobial peptides with antibiofilm activity against *P. aeruginosa* resistant strains and synergistic effects in combination with antibiotics like meropenem and ciprofloxacin. However, the mechanisms wherewith this synergism happens are not well explained yet.

One of the proposed models most accepted by the scientific community is that the vast majority of antimicrobial peptides alter the membrane permeability, making it possible to enter such drugs inside cells and allow them to interact with their targets [25]. Our findings show an increase in inhibition and degradation of biofilms when ciprofloxacin is combined with peptides, even at very low concentrations. For example, ciprofloxacin at 25 μg mL^{-1} alone reduced only 8% of *S. aureus* preformed biofilm. However, the combination of ciprofloxacin at 25 μg mL^{-1} with RcAlb-PepI at 0.04 μg mL^{-1} increased this reduction up to 45% (Fig. 1). RcAlb-PepI at 0.04 μg mL^{-1} alone was able to induce pore formation in *S. aureus* biofilm cells, which could have facilitated the movement of ciprofloxacin to the cell cytoplasm, improving its action. It is essential to notice that our peptides increase the action of ciprofloxacin at very low concentrations (Table 1). While Bessa *et al.* [23] showed synergistic concentrations at 8, 16, 32, and 128 μg mL^{-1}, our peptides presented a synergistic effect at concentrations ten to hundreds of times lower than those.

Ciprofloxacin is antibiotic classified in the fluoroquinolone group. Its mechanism of

action relies on the inhibition of DNA replication by interacting and inhibiting the bacterial DNA topoisomerase IV enzyme [26]. Ciprofloxacin has been employed to treat abdominal infections, diarrhea, respiratory tract infections, and skin infections [26–31]. By attacking an intracellular protein, ciprofloxacin has to be transported to the cytoplasm passing by the membrane using porin transmembrane proteins as a channel [28]. The typical resistance mechanisms of *S. aureus* alter the cytoplasmic concentration of ciprofloxacin, such as increasing the concentration efflux pumps and developing mutations in the gene to produce a new or modified topoisomerase IV [32]. In this sense, combining ciprofloxacin with molecules that increase cytoplasmic concentration could be an excellent alternative to overcome the resistance of *S. aureus*.

Here, we showed that synthetic peptides Mo-CBP3-PepI, RcAlb-PepI, and RcAlb-PepI alone induced pore formation in biofilm cells of *S. aureus* (Figs. 2 and 3). PI in FM analysis helped us understand how mechanism peptides improve ciprofloxacin actions. Membrane pores induced by peptides allow the movement of PI, which has a molecular weight of 668.39 Da, by the membrane. So, the movement of PI by the membrane indicates, at least, the presence of a pore of that size (Figs. 2 and 3). Ciprofloxacin has a molecular weight of 331.34 Da. Based on that, it is feasible to suggest that the pores induced by peptides facilitate the movement of ciprofloxacin by the membrane, increasing its concentration in the cytoplasm and thus the activity. Recently, it has been shown that Mo-CBP3-PepI, RcAlb-PepI, and RcAlb-PepI can also induce the pore with a size of 6-kDa in the membrane of several pathogens [10,33]. This last data strengthens our hypothesis about how peptides enhance the action of ciprofloxacin against S. aureus.

Here, the second set of FM experiments revealed that all the combinations between peptides and ciprofloxacin lead to an overaccumulation of ROS in biofilm cells of *S. aureus* (Figs. 4 and 5). Only ciprofloxacin and Mo-CBP3-PepIII alone were not able to induce ROS accumulation. ROS, H2O2 essentially, is vital to the signaling process that leads to biofilm formation at earlier and later stages such as development and maturation [34]. However, cells need to strictly regulate the levels of ROS because from beneficial to lethal effects is a fine line easy to cross. Any imbalance in ROS levels leading to high accumulation is lethal because it destroys essential molecules to cell life, such as carbohydrates, nucleic acids, proteins, and lipids, triggering programmed cell death [35]. In addition, to facilitate the entry of ciprofloxacin into the cytoplasm, peptides induce additional stress via ROS accumulation on cells in biofilms of *S. aureus*, which makes it difficult for *S. aureus* to fight back against the combined action of peptides and ciprofloxacin.

Ciprofloxacin is known given the large number of collateral effects caused in patients during treatment [26–31,36]. Collateral effects caused by ciprofloxacin go from mild such as nausea and diarrhea, to severe such as seizures, neuropathy, photosensitivity, and hyper or hypoglycemia [26–31,36]. Additionally, it has been reported that treatment with ciprofloxacin induced interstitial nephritis and autoimmune hemolytic anemia [36].

Here, our data (Table 1) revealed that in addition to enhancing the action of ciprofloxacin, peptides reduced their hemolytic activity against HRBC. Alone, the ciprofloxacin at 25 μg mL^{-1} alone induced high levels of HRBC. However, the same concentration of ciprofloxacin in combination with peptide RcAlb-PepI at 0.04 μg mL^{-1} did not present any toxicity to HRBC. This is an exciting result because it reinforces the potential of peptides as adjuvants in drug formulations to treat infections caused by *S. aureus*. As revealed in other studies, the peptides used in this study were not toxic to human cells, and zebrafish embryos strengthen the clinical application of peptides [10,12,22].

5. Conclusion

Here, we presented four synthetic peptides that enhanced the activity of ciprofloxacin against biofilms of *S. aureus*. The mechanism of the combined effect is possible by increasing the cytoplasmatic concentration of ciprofloxacin supported by pores on the membrane of *S. aureus* cells. Additionally, peptides reduced the toxicity of ciprofloxacin to HRBC. Considering all these findings, it is possible to suggest that the peptides studied are a considerable option to surpass the resistance of S. aureus strains to antibiotics like ciprofloxacin.

Author Contributions: All authors made substantial contributions. The conception and design of the study and acquisition of data, analysis, and interpretation were performed by N.A.S.N., J.T.A.O., T.K.B.A, C.D.T.F., L.P.B., L.A.C.B., F.P.M., and P.F.N.S. Microscopic analyses were carried out by N.A.S.N., L.P.B., L.A.C.B., F.P.M., and T.K.B.A, Writing or revising the article was done by N.A.S.N., T.K.B.A., C.D.T.F., J.T.A.O., and P.F.N.S. Final approval and submission P.F.N.S. All authors have read and agreed to the published version of the manuscript.

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6 CAPÍTULO III – ARTIGO CIENTÍFICO 2

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United we stand, divided we fall: In-depth proteomic evaluation of the synergistic effect of *Mo*-CBP$_3$-PepI and ciprofloxacin against *Staphylococcus aureus* biofilm

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ABSTRACT

Staphylococcus aureus is a clinically important human pathogen, who is responsible for causing several kinds of infection. *S. aureus* forms biofilm, which is a structure capable of protecting bacterial cells, conferring antimicrobial resistance, making its treatment more difficult. Synthetic peptides appear as an alternative to overcome biofilm of bacterial pathogens. *Mo*-CBP$_3$-PepI, when combined with Ciprofloxacin, reduced preformed *S. aureus* biofilm by 50% at low concentrations (0.2 and 6.2 μg.mL^{-1}, respectively). Additionally, Fluorescence microscopy analysis revealed that the peptide induced pore formation and ROS overproduction, strengthened when combined with Ciprofloxacin. Here, proteomic analysis confirmed with more depth previously described mechanisms and revealed changes in accumulation of proteins related to DNA and protein metabolism, cell wall biosynthesis, redox metabolism, *quorum* sensing and biofilm formation. Some proteins related to DNA and protein metabolism were reduced, while other proteins, like redox system proteins, disappeared in Ciprofloxacin+*Mo*-CBP$_3$-PepI treatment. Our results indicated a synergistic effect of these two molecules, presenting several mechanisms against *S. aureus* biofilms, bringing perspectives for combined treatment with other drugs.

Keywords: Biofilm, Proteomics, ROS, Antibiotics

1. Introduction

The continuous spread of bacteria resistant to multiple drugs is becoming a major health issue worldwide. These superbugs and their associated resistance are responsible for millions of deaths in 2019 (CDC 2022), and this number will continue to grow if necessary measures are not taken. In 2050, antimicrobial resistance is estimated to extinguish approximately 300 million lives globally, and lead to trillions of economic losses (Xie *et al.* 2022). In addition, these superbugs can form biofilms, an extracellular structure that confers even more resistance to them. These microbial biofilms are responsible for about 4.1 million acquired healthcare-associated infections (HAIs) annually in European hospitals, leading to an associated death up to 37,000 (Rather *et al.* 2021).

Staphylococcus aureus is a Gram-positive bacterium, capable of forming biofilm that belongs to the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter speci*es) group of bacteria that frequently develop multidrug resistance, the so-called superbugs that are pathogenic to humans (De Oliveira *et al.* 2020). *S. aureus* is a commensal bacterium, but is also a human pathogen. More specifically, Methicillin-resistant *S. aureus* (MRSA) is resitant to nearly all ß-lactam antimicrobials and may be acquired in hospital (HA-MRSA) or in the community (CA-MRSA) and is among the most common causes of infective endocarditis that show a mortality rate up to 66% (Tong *et al.* 2015; De Oliveira *et al.* 2020).

In the face of this scenario, it is vital and indispensable the search for alternative molecules that could lead to a new treatment or even come to aid drugs that have lost their efficiency. Fulfilling these roles, antimicrobial peptides (AMPs) are great candidates to treat resistant bacteria. However, AMPs possess some crucial setbacks related to its clinical use, such as low selectivity, low stability, low solubility and hemolytic activity. To bypass these barriers, the use of synthetic antimicrobial peptides (SAMPs) showed up as a promising group of molecules that are conceived through rational design to own desirable properties such high antimicrobial activity, high stability and low toxicity to the host.(Boto *et al.* 2018; Souza *et al.* 2020).

Our research group has designed eight SAMPs, with one of them called *Mo*-CBP$_3$-PepI. *Mo*-CBP$_3$-PepI was designed based on a sequence of a chitin-binding protein from *Moringa oleifera* (Oliveira *et al.* 2019). *Mo*-CBP$_3$-PepI showed antibiofilm activity alone and in

combination with ciprofloxacin regarding the degradation of *S. aureus* biofilm. The antibiofilm assay revealed that *Mo*-CBP$_3$-PepI has a synergistic effect with ciprofloxacin and its mechanisms of action of *Mo*-CBP$_3$-PepI were revealed in *S. aureus* biofilms (Neto *et al.* 2022). Hence, this study provides an in-depth proteomic analysis to have a better comprehension of the antibiofilm synergistic effect of *Mo*-CBP$_3$-PepI combined with ciprofloxacin.

2. Material and methods

2.1 Biologic material

S. aureus (ATCC 25923) strain was obtained from the laboratory of toxic proteins (LabTox) at the department of biochemistry and molecular biology of the Federal University of Ceará (UFC)

2.2 Peptide sequence

The synthetic peptide *Mo*-CBP$_3$-PepI (Oliveira *et al.* 2019) were chemically synthesized by ChemPeptide (Shanghai, China), and submitted to analysis for purity and quality (95%) by reverse-phase high-performance liquid chromatography and mass spectrometry.

2.3 Antibiofilm assay

First, the antibiofilm assay was performed according to the method used by Neto *et al.* (Neto *et al.* 2022), using only the concentrations that showed combined antibiofilm activity. Briefly, a *S. aureus* cell suspension was prepared with Mueller-Hinton broth, incubated for 24 h in the dark, at 37º C and standardized to a concentration of 10^6 cells mL^{-1}. In sequence, the 50 µL of the standardized cells were incubated in a flat-bottom 96-well polystyrene microplate and incubated for 24 h in the dark at 37º C, for biofilm formation. After, the biofilm was incubated with 25 µL of the peptide solution (0.2 µg mL^{-1}) and 25 µL of the ciprofloxacin solution (6.2 µg mL^{-1}) combined, 50 µL of the peptide solution alone, 50 µL of the ciprofloxacin solution alone, and 50 µL of the control solution (DMSO 5% in NaCl 0.15 M) for more 24h.

2.4 Protein extraction and gel-free proteomic analysis

After the end of the assay, the protein extraction was performed according to Branco *et al.* (Branco *et al.* 2022), with some adjustments. The wells were washed twice with 50 mM sodium acetate pH 5.2, resuspended in 300 µL of the same buffer and frozen for 24 h. Then, the frozen samples were sonicated for 30 min to break the cell wall and membrane, and then the samples were centrifuged at 12,000x g for 15 min at 4º C, and the supernatant was collected.

Then, a 10 mM DTT solution was added to the samples and incubated for 1h at 37º C to reduce the proteins. After, iodoacetamide was added to a final concentration of 15 mM and incubated for 30 min in the dark for alkylation of the reduced proteins. The proteins were digested using trypsin gold (Promega, Madison, WI, USA) to a final concentration of 1:20 (*w/w)* as described by manufacturers. The trypsin digestion was performed for 16h at 37º C. Finally, the samples were dried in a speed vacuum (Eppendorf, Hamburg, Germany) for 3h and analyzed by ESI-QUAD-TOF mass spectrometer.

2.4 Protein identification

Tandem mass spectra were extracted into PKL files for all four samples. The proteins were searched using MASCOT MS/MS ions search from MATRIX SCIENCE (https://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS (accessed on 15 December 2022)) against UP625_E_coli_K12 (AA), UP808_M_Pneumoniae (AA) and SwissProt databases (the taxonomy was set in *B. subtilis* and "Other Firmicutes"). The terms for the search were: Carbamidomethyl (C) as fixed modification; Oxidation (M) as variable modification; the Peptide tolerance was set to 1.2 DA (with 1% FDR); the MS/MS tolerance was set to 0.6 DA; the peptide charge was set +2, +3 and +4; and the instrument was set to ESI-QUAD-TOF. The proteins identified in the samples were searched for in UNIPROT and separated into 6 sets (Unique from control, unique from cells treated with ciprofloxacin only, unique from cells treated with *Mo*-CBP$_3$-PepI only, unique from cells treated with ciprofloxacin + *Mo*-CBP$_3$-PepI, control x ciprofloxacin shared proteins, control x *Mo*-CBP$_3$-PepI shared proteins, control x ciprofloxacin + *Mo*-CBP$_3$-PepI shared proteins, ciprofloxacin x *Mo*-CBP$_3$-PepI shared proteins, ciprofloxacin only x ciprofloxacin + *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepI only x ciprofloxacin + *Mo*-CBP$_3$-PepI).

The proteins with a fold-change value ≥ 1.5 ($p < 0.05$, Tukey's test) were considered up-accumulated (increased abundance), and proteins with a fold-change value ≤ 0.5 ($p < 0.05$ Tukey's test) were considered down accumulated (decreased abundance), were taken into account for comparisons. For each protein, its corresponding FASTA file was downloaded. Then, the blast2go program (https://www.blast2go.com/) (accessed on 23 December 2022) was used to categorize the proteins detected by Gene Ontology (GO) annotation according to Molecular function, Biological process and subcellular location.

3. RESULTS AND DISCUSSION

3.1. Proteomic profile of S. aureus biofilms cells treated with combined drugs

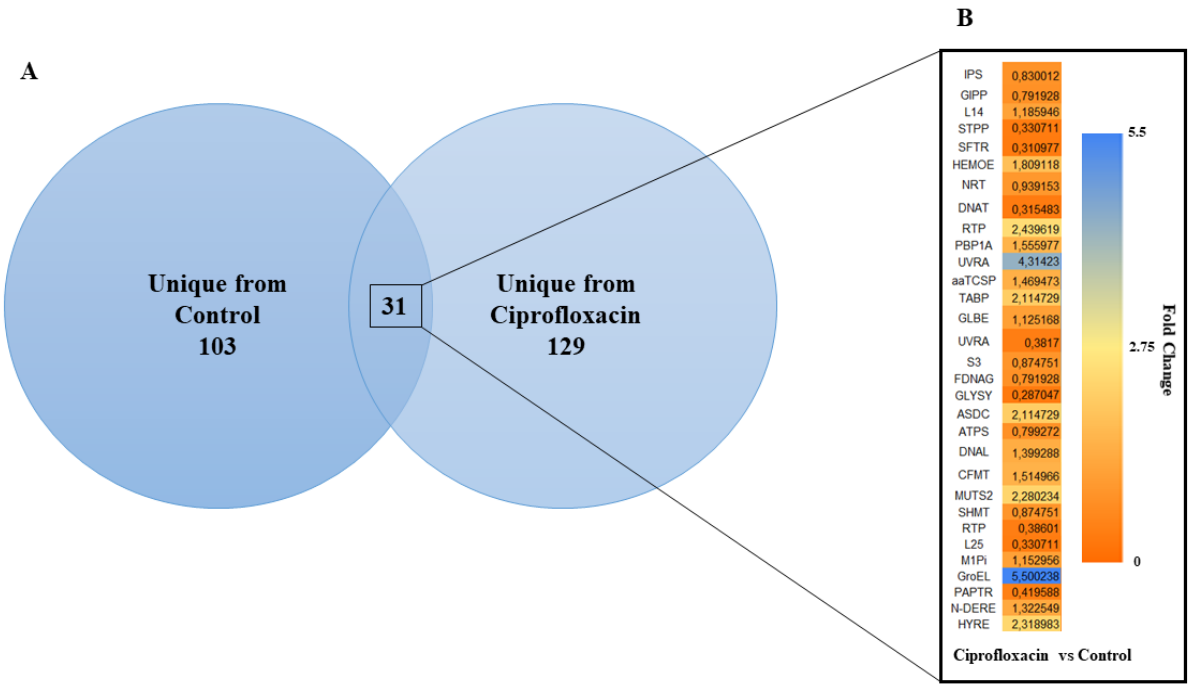
3.1.1 Overview

The proteomic analysis can be an interesting method to understand how the cells are responding to different treatments (Tsakou *et al.* 2020; Maaß *et al.* 2021), and is even employed to comprehend the effect of antimicrobials on biofilm cells (Sung *et al.* 2022). In this study, proteomic analysis was employed to overview protein changes in *S. aureus* biofilm cells after treatment with Mo-CBP_3-PepI, ciprofloxacin and both. 905 proteins were identified in total. 199 proteins were identified in the non-treated *S. aureus* biofilm cells, 237 proteins were identified in the *S. aureus* biofilm cells treated with ciprofloxacin, 211 proteins were identified in the *S. aureus* biofilm cells treated with *Mo*-CBP_3-PepI and 258 proteins were identified in the cells treated with both molecules. Regarding the exclusive proteins from each treatment, 103 proteins were exclusives to non-treated cells, 129 were exclusives to cells treated with ciprofloxacin, 117 proteins were exclusives to cells treated with *Mo*-CBP_3-PepI and 151 proteins were exclusive to cells treated with both (Figures 1-6, Supplementary tables 1-4).

Together with the exclusive proteins, the shared proteins between each treatment were also analyzed, since they are important proteins to understand how the antimicrobials modulate the gene expression. To understand these modulations, in each treatment, a fold-change rule was applied using the intensity of the following groups: Ciprofloxacin/Control (CiC); *Mo*-CBP_3-PepI/Control (MC); Ciprofloxacin+*Mo*-CBP_3-PepI / Control (CiMC); *Mo*-CBP_3-PepI / Ciprofloxacin (MCi); Ciprofloxacin/ Ciprofloxacin+*Mo*-CBP_3-PepI (CiMCi); and Ciprofloxacin+*Mo*-CBP_3-PepI / *Mo*-CBP_3-PepI (CiMM) (Tables 1-6).

Regarding the shared proteins, those proteins with a fold-change value ≥ 1.5 were considered as up-accumulated, and those with a fold-change value ≤ 0.5 were considered as down-accumulated. To illustrate the rule explained above, the 50S ribosomal protein L25 had a fold-change value of 0.33 between the cells treated with ciprofloxacin and the control cells. This indicates a down-accumulation of this protein considering these two treatments. In contrast, Endonuclease MutS2 had a fold-change value of 3.19 between cells treated with both molecules and control cells, showing an up-accumulation of this protein considering these two treatments.

For each comparison group, a different amount of shared proteins was found, as also the up and down accumulated proteins. The CiC group is composed of 31 shared proteins, with 10 up-accumulated proteins, 8 down-accumulated and 15 showing no alteration, comparing the Ciprofloxacin-treated cells with control cells (Fig 1-A). Through Gene Ontology, it was possible to find out that the shared proteins possess 11 groups of molecular function and 13 groups of biological process, (Fig 1-C). In molecular function, the most represented group were DNA-binding proteins, with a share of 26% from the total pool of proteins. Regarding the biological process, the most represented groups were the proteins involved in Energy and metabolism, representing 17%.



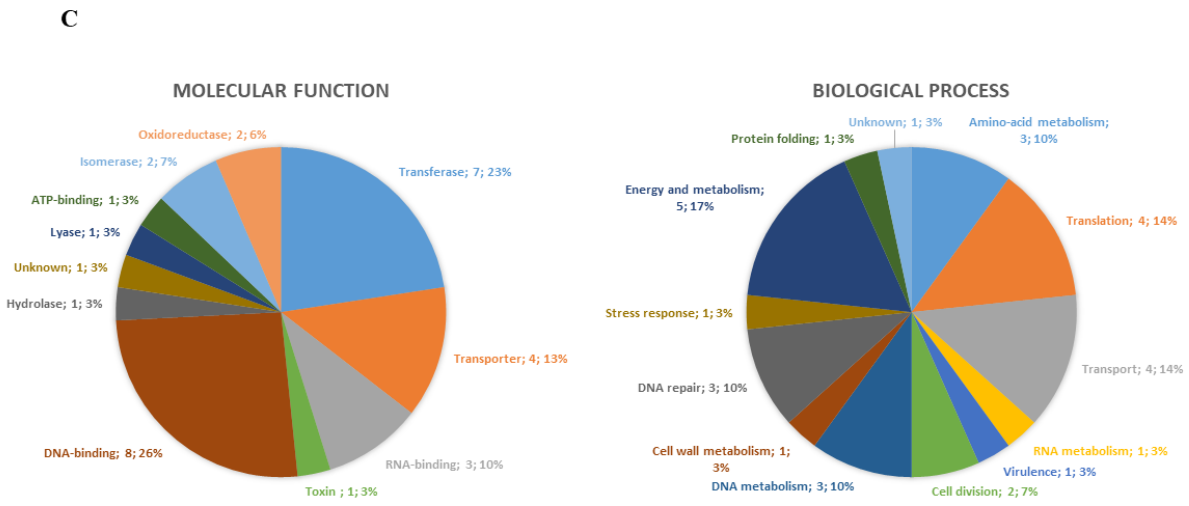
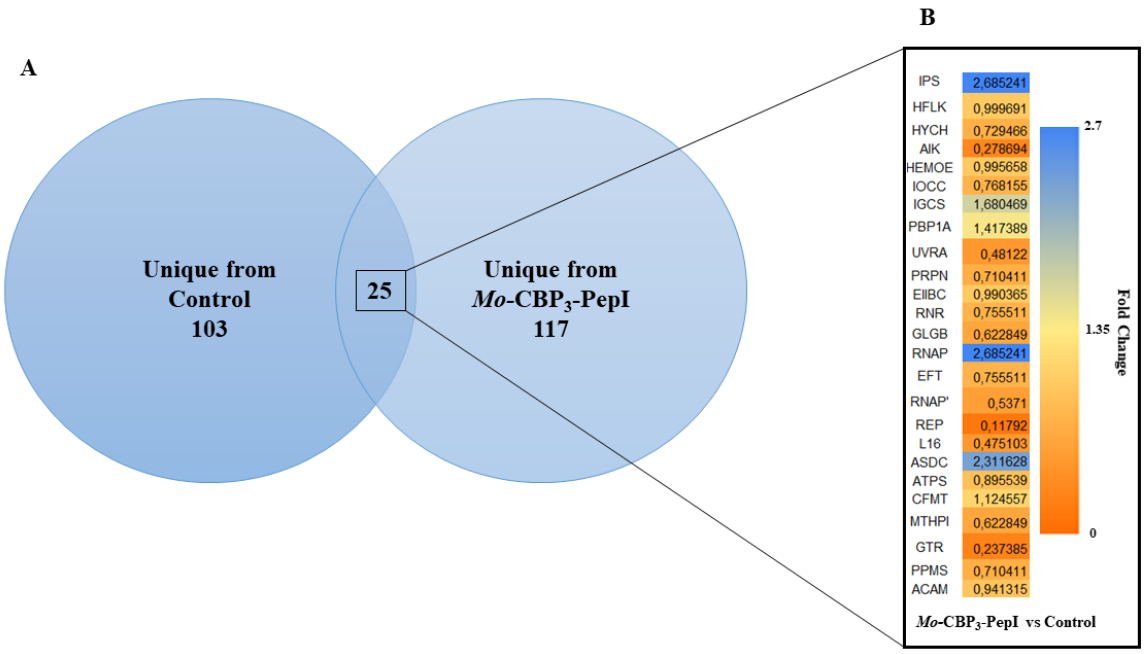


Figure 1. Distribution of S. aureus proteins obtained after treatment with Ciprofloxacin. (A) Venn diagram shows the distribution of proteins in control and Ciprofloxacin-treated cells. **(B)** Represents the fold-change value of shared proteins, which were found in both groups. The vertical bar indicates the color scale according to each protein's fold-change value. **(C)** Classification of proteins from *S.* aureus biofilm cells identified by LC-ESI-MS/MS analysis.

The MC group is composed of 25 shared proteins, with 4 up-accumulated proteins, 5 down-accumulated and 11 showing no alteration, comparing the *Mo*-CBP$_3$-PepI-treated cells with control cells (Fig 2-A). Through Gene Ontology, we observed that the shared proteins possess 13 groups for molecular function and 13 groups for biological process, (Fig 2-C). In molecular function, the most represented group were the Transferase proteins, with 32%. Regarding biological processes, the most represented groups were the proteins related to "energy and metabolism", representing 20%.



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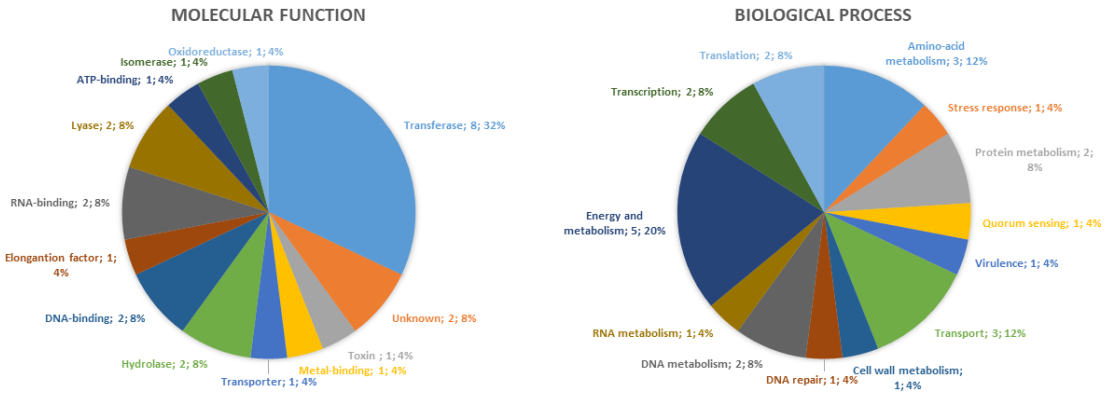
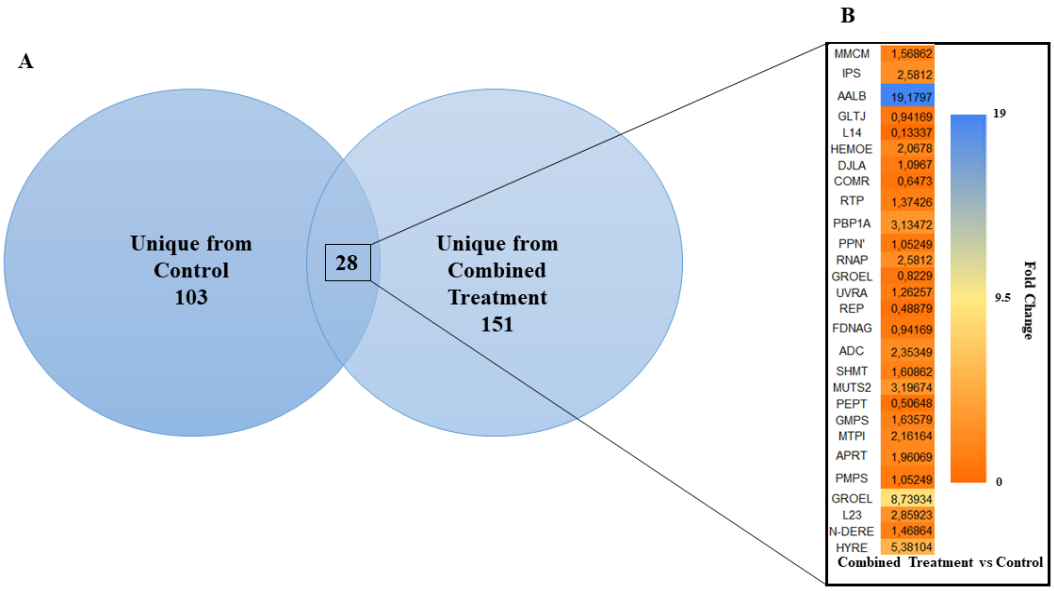


Figure 2. Distribution of S. aureus proteins obtained after treatment with Mo-CBP3-PepI. (A) Venn diagram shows the distribution of proteins in control and *Mo*-CBP$_3$-PepI-treated cells. **(B)** Represents the fold-change value of shared proteins, which were found in both groups. The vertical bar indicates the color scale according to each protein's fold-change value. **(C)** Classification of proteins from S. aureus biofilm cells identified by LC-ESI-MS/MS analysis.

The CiMC group is composed of 28 shared proteins, with 15 up-accumulated proteins, 3 down-accumulated and 10 which showing no alteration, comparing the Ciprofloxacin+*Mo*-CBP$_3$-PepI-treated cells with control cells (Fig 3-A). The analysis through Gene Ontology showed that the shared proteins possess 11 groups for molecular function and 12 groups for biological process, (Fig 3-C). In molecular function, the most represented group were the DNA-binding proteins, with 29%. Regarding the biological process, the most represented groups were the proteins related to Amino-acid and DNA metabolisms, representing 14% each.



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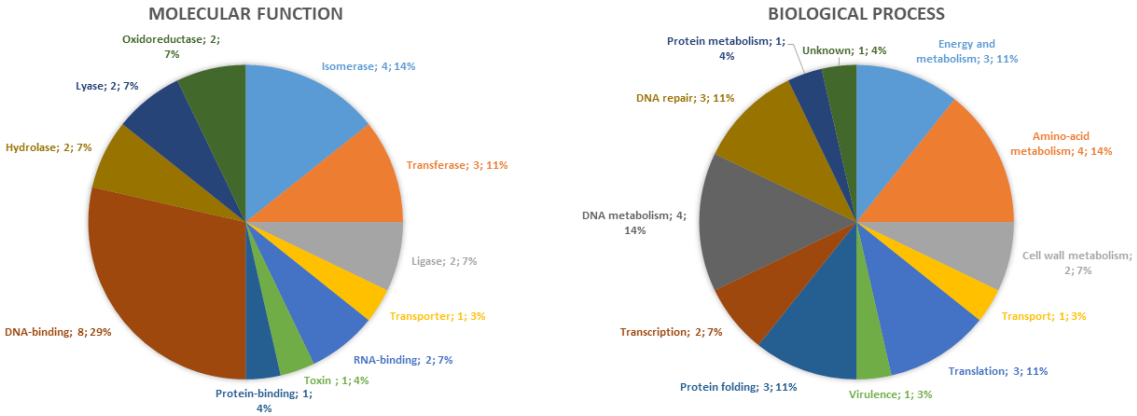
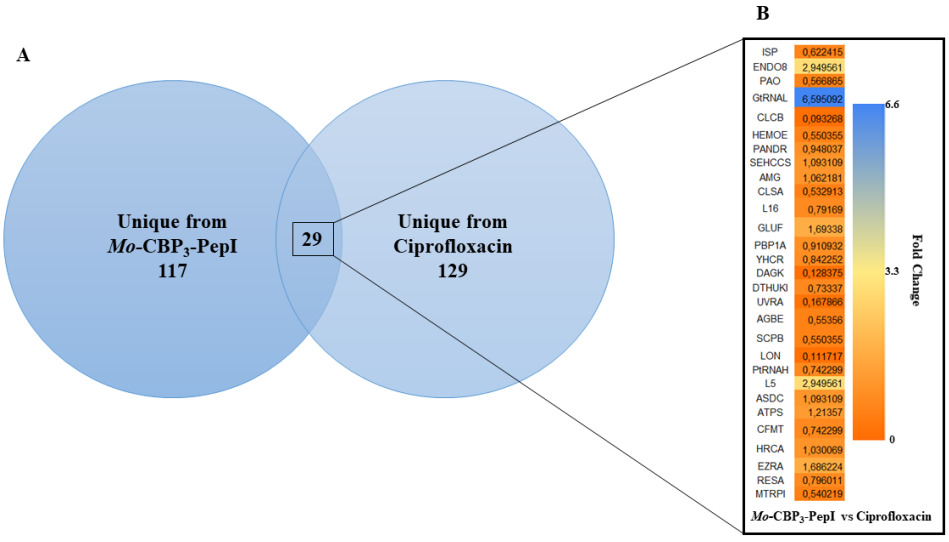


Figure 3. Distribution of S. aureus proteins obtained after Combined treatment. (A) Venn diagram shows the distribution of proteins in control and Ciprofloxacin+*Mo*-CBP$_3$-PepI-treated cells. **(B)** Represents the fold-change value of shared proteins, which were found in both groups. The vertical bar indicates the color scale according to each protein's fold-change value. **(C)** Classification of proteins from S. aureus biofilm cells identified by LC-ESI-MS/MS analysis.

The MCi group is composed of 29 shared proteins, with 5 up-accumulated proteins, 4 down-accumulated and 20 showing no alteration, comparing the *Mo*-CBP$_3$-PepI-treated cells with ciprofloxacin-treated cells (Fig 4-A). The analysis through Gene Ontology showed that the shared proteins possess 12 groups for molecular function and 12 groups for biological process, (Fig 4-C). In molecular function, the most represented group were the Transferase proteins, with 24%. Regarding the biological process, the most represented groups were the proteins related to energy and metabolism, representing 28%.



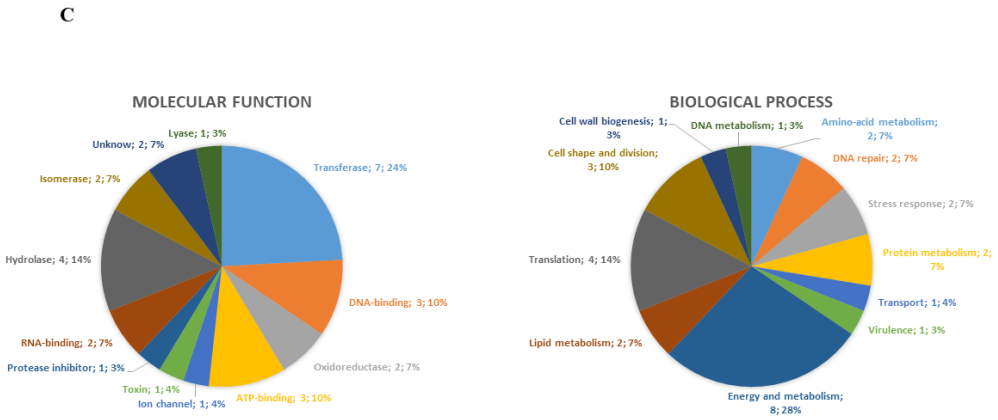
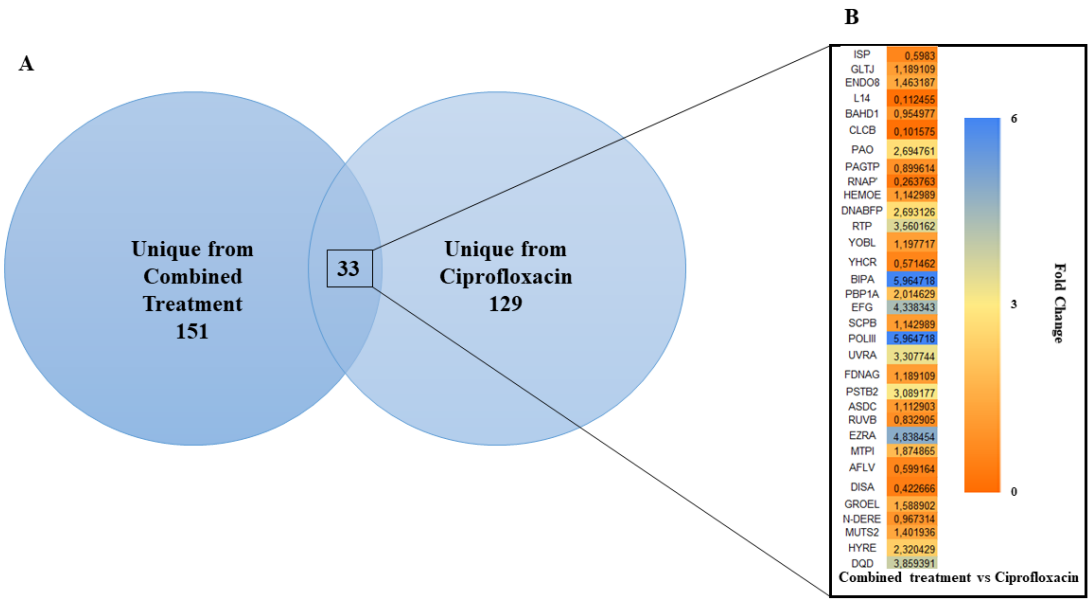


Figure 4. Distribution of S. aureus proteins obtained after Ciproflaxacin and *Mo*-CBP$_3$-PepI treatment. (A) Venn diagram shows the distribution of proteins in Ciprofloxacin and *Mo*-CBP$_3$-PepI-treated cells. **(B)** Represents the fold-change value of shared proteins, which were found in both groups. The vertical bar indicates the color scale according to each protein's fold-change value. **(C)** Classification of proteins from S. aureus biofilm cells identified by LC-ESI-MS/MS analysis.

The CiMCi group is composed of 33 shared proteins, with 13 up-accumulated proteins, 4 down-accumulated and 16 showing no alteration, comparing the ciprofloxacin-treated cells with *Mo*-CBP$_3$-PepI + ciprofloxacin-treated cells with control cells (Fig 5-A). The analysis through Gene Ontology showed that the shared proteins possess 12 groups for molecular function and 15 groups for biological process, (Fig 5-C). In molecular function, the most represented group were the DNA-binding proteins, with 22%. Regarding the biological process, the most represented groups were proteins involved in DNA repair, also representing 22%.



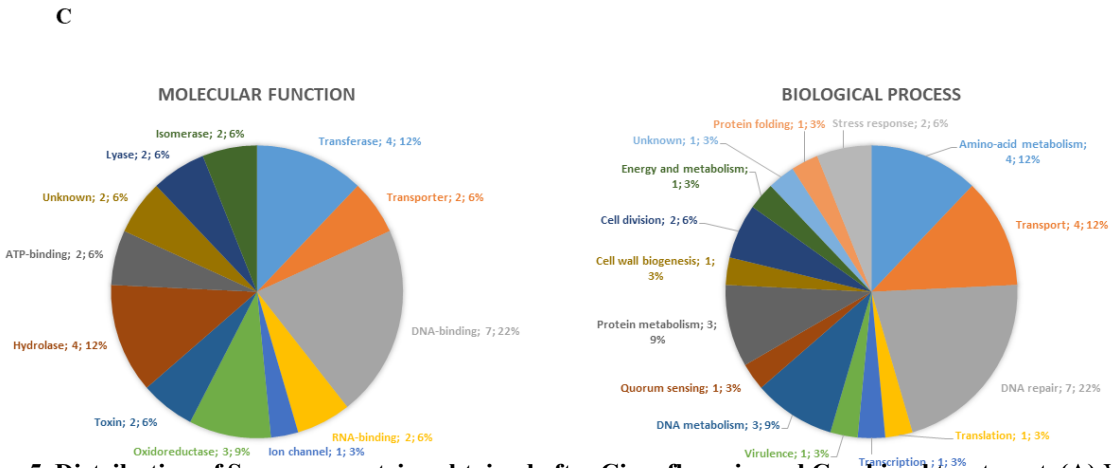
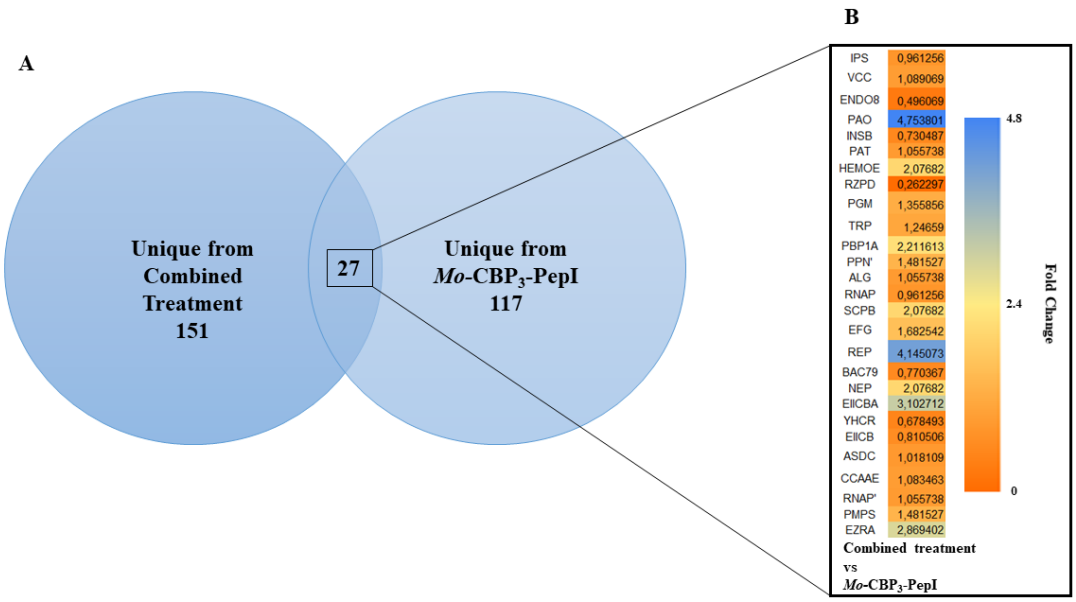


Figure 5. Distribution of S. aureus proteins obtained after Ciproflaxacin and Combined treatment. (A) Venn diagram shows the distribution of proteins in Ciprofloxacin and Ciprofloxacin+*Mo*-CBP$_3$-PepI-treated cells. **(B)** Represents the fold-change value of shared proteins, which were found in both groups. The vertical bar indicates the color scale according to each protein's fold-change value. **(C)** Classification of proteins from S. aureus biofilm cells identified by LC-ESI-MS/MS analysis.

The CiMM group is composed of 27 shared proteins, with 9 up-accumulated proteins, 2 down-accumulated and 16 showing no alteration, comparing the Combined treatment cells with *Mo*-CBP$_3$-PepI-treated cells (Fig 6-A). Gene Ontology classification showed that the shared proteins possess 10 groups for molecular function and 14 groups for biological process, (Fig 6-C). In molecular function, the most represented group were the Transferase proteins, with 26%. Regarding the biological process, the most represented groups were proteins related to Energy and metabolism, representing 18%.



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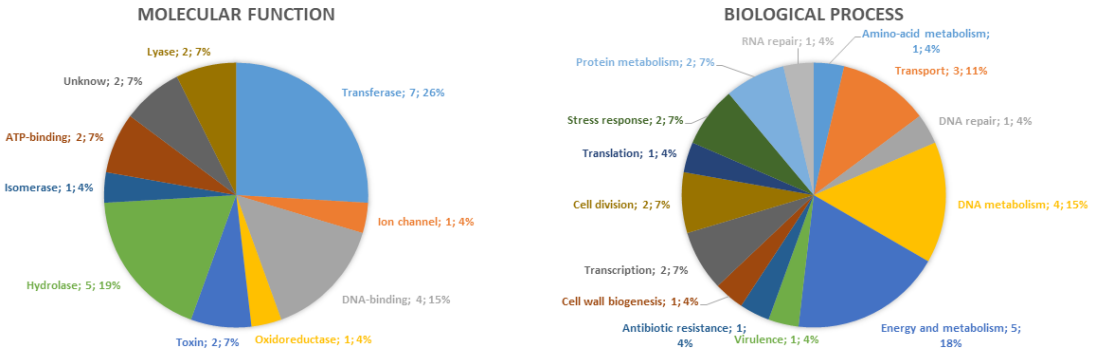

Figure 6. Distribution of S. aureus proteins obtained after *Mo*-CBP$_3$-PepI and Combined treatment. (A) Venn diagram shows the distribution of proteins in *Mo*-CBP$_3$-PepI and Ciprofloxacin+*Mo*-CBP$_3$-PepI-treated cells. **(B)** Represents the fold-change value of shared proteins, which were found in both groups. The vertical bar indicates the color scale according to each protein's fold-change value. **(C)** Classification of proteins from S. aureus biofilm cells identified by LC-ESI-MS/MS analysis.

3.2 DNA-related proteins

This group of proteins had different representatives in each comparison group. In the MC group (Table 1), three proteins were found: Replication protein, Primosomal protein N' and UvrABC system protein A. Primosomal protein N' is responsible for reactivating DNA replication (Merrikh *et al.* 2012). However, it showed no relevant fold-change. On the other hand, the Replication protein and the UvrABC system protein A showed a down-accumulation compared to control. This replication protein (repB) is involved in plasmid replication, and is well known that plasmids are responsible for antibiotic resistance and increased pathogenicity (DeNap and Hergenrother 2005; Novick *et al.* 2010; Haaber *et al.* 2017). The fold-change value of 0.11792 showed that *Mo*-CBP$_3$-PepI somehow affected the replication of plasmids in *S. aureus* cells and this could lead to a re-sensitization of these cells to antibiotic treatment. Alongside, the UvrABC system protein A also showed a down-accumulation. This protein is a component of the UvrABC nuclease system, which is responsible for the repair of UV-damaged DNA, but is also associated with DNA repair related to Reactive Oxygen Species (ROS) (Breimer 1991). Previous results revealed that *Mo*-CBP$_3$-PepI alone induced ROS overproduction (Neto *et al.* 2022). The down-accumulation of UvrABC could be associated with the ROS accumulation.

In the CiC group (Table 2), some DNA-related proteins were found: Replication Termination Protein, UvrABC system protein A, Formamidopyrimidine-DNA glycosylase,

DNA ligase, Endonuclease MutS2 and Cobalt-factor III methyltransferase. The DNA ligase and the Formamidopyrimidine-DNA glycosylase did not show significant fold-change value. However, the Replication Termination protein, UvrABC system protein A, Endonuclease MutS2 and Cobalt-factor III methyltransferase were up-accumulated. This up-accumulation in proteins related to DNA metabolism and repair could be related to the mechanism of action of Ciprofloxacin, who cause the inhibition of DNA-topoisomerase and DNA-gyrase (Thai *et al.* 2022). However, when we look into the exclusive Ciprofloxacin proteins (Supplementary table 3), we found that the Ciprofloxacin treatment stimulated the expression of multidrug efflux pumps, since Ciprofloxacin is a substrate to efflux pumps (Haaber *et al.* 2017).

Now, when we look to the CiMC group (Table 3), seven DNA-related proteins were observed: Replication Termination Protein, Primosomal Protein N', UvrABC system protein A, Replication protein, Formamidopyrimidine-DNA glycosylase, Endonuclease MutS2 and GMP synthase. Replication Termination Protein, Primosomal Protein N', UvrABC system protein A and Formamidopyrimidine-DNA glycosylase showed no significant fold-change value. In the meantime, Replication proteins repB was down-accumulated, while Endonuclease MutS2 and GMP synthase were up-accumulated. This scenario could imply that the ROS accumulation revealed in previous studies (Neto *et al.* 2022) could be related, but not only, to the reduced accumulation of DNA repair proteins such as the UvrABC nuclease system (Breimer 1991). Formamidopyrimidine-DNA glycosylase, a DNA mismatch repair protein, was down-accumulated as well, but Endonuclease MutS2 was up-accumulated. The non-accumulation of Formamidopyrimidine-DNA glycosylase could have been impactful on the ROS accumulation and subsequent DNA damage, since this protein is involved in the repair of Oxidative damage on DNA (Gilboa *et al.* 2002). The up-accumulation of Endonuclease MutS2 could also be related to the ROS accumulation. When we look at the Combined treatment exclusive proteins (Supplementary table 4), the proteins MutS and MutL were present, but the MutH was not. This lack of MutH could be related to the up-accumulation of MutS2, but without the MutH endonuclease this repair system is useless.

Now, when the Ciprofloxacin treatment and the *Mo*-CBP$_3$-PepI treatment were compared (Table 4), three shared DNA-related proteins: Endonuclease 8, UvrABC system protein A and Endonuclease YhcR. While Endonuclease YhcR did not present any significant fold-change value, Endonuclease showed an up-accumulation of 2.9495 (Table 4) and UvrABC system protein A showed a down-accumulation of 0.1678. Endonuclease 8 is a DNA repair protein involved in the cell response to oxidative DNA damage (Zharkov 2002). This up-accumulation is seen as a cell response to the ROS accumulation. However, UvrABC protein A

was down-accumulated somehow. Then, looking at the exclusive proteins, we found Superoxide dismutase (SOD) in Ciprofloxacin exclusive proteins (Supplementary table 3), but it was not found in *Mo*-CBP_3-PepI exclusive proteins. This lack of proteins responsible for the antioxidant defense could be leading to the ROS accumulation in *Mo*-CBP_3-PepI-treated cells, and subsequent DNA damage, even with accumulation of DNA repair proteins.

To finish, looking at the CiMM groups (Table 6), it was observed that the DNA-related proteins, at most part, did not reveal any up- or down-accumulation. Endonuclease 8 showed a down-accumulation and Replication protein showed an up-accumulation at the CiMM group. Interestingly, looking at the CiMCi group (Table 5), a differential accumulation of several DNA-related proteins was observed. Together with UvrABC protein A up-accumulation, the DNA base flipping protein YbaZ was also up-accumulated. YbaZ function is to bind with damaged DNA and flips the base out of the DNA duplex, which allows repair proteins, such as the UvrABC complex to act (Chen *et al.* 2008; Haaber *et al.* 2017). This coupled accumulation could have happened by the ROS overproduction inside the cell, which could lead to DNA damage.

Table 1 MC shared proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Reference Organism	Cellular Compartment	Fold Change *Mo*-CBP$_3$-PepI vs. Control
DNA metabolism				
Primosomal protein N'	P94461	*Bacillus subtilis* (strain 168)	Cytoplasm	0,710
Replication protein	P0A0C4	*Bacillus sp.*	Cytoplasm	0.117
DNA repair				
UvrABC system protein A	O34863	*Bacillus subtilis* (strain 168)	Cytoplasm	0.481
Transport				
Ion-translocating oxidoreductase complex subunit C	P77611	*Escherichia coli* (strain K12)	Cell membrane	0.768
Inner membrane metabolite transport protein YgcS	Q46909	*Escherichia coli* (strain K12)	Cell membrane	1.680
PTS system sucrose-specific EIIBC component	P05306	*Bacillus subtilis* (strain 168)	Cell membrane	0.990
Stress and Defense Response				
Modulator of FtsH protease HflK	P0ABC7	*Escherichia coli* (strain K12)	Periplasm space	0.999
Energy and Metabolism				

1,4-alpha-glucan branching enzyme GlgB	P39118	*Escherichia coli* (strain K12)	Cytoplasm	0.622
Aspartate 1-decarboxylase	B8I2Z2	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm	2.311
Cobalt-factor III methyltransferase	O87689	*Priestia megaterium (Bacillus megaterium)*	Cytoplasm	1.124
Phosphomethylpyrimidine synthase	A0Q1U9	*Clostridium novyi* (strain NT)	Cytoplasm	0.710
ATP synthase epsilon chain	A4W1V6	*Streptococcus suis* (strain 98HAH33)	Cell membrane	0.895
Translation and Protein metabolism				
Formate hydrogenlyase maturation protein HycH	P0AEV7	*Escherichia coli* (strain K12)	Cytoplasm	0.729
Elongation factor Ts	P78009	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	0.755
50S ribosomal protein L16	B1MW07	*Leuconostoc citreum* (strain KM20)	Cytoplasm	0.475
Glutamyl-tRNA reductase	Q3ACT4	*Carboxydothermus hydrogenoformans* (strain ATCC BAA-161 / DSM 6008 / Z-2901)	Cytoplasm	0.237
Transcription and RNA metabolism				
DNA-directed RNA polymerase subunit beta	P78013	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	2.685

DNA-directed RNA polymerase subunit beta'	P75271	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	0.537
Ribonuclease R	O32231	*Bacillus subtilis* (strain 168)	Cytoplasm	0.755
Amino-acid metabolism				
2-isopropylmalate synthase	P09151	*Escherichia coli* (strain K12)	Cytoplasm	2.685
Methylthioribose-1-phosphate isomerase	B1YIY4	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Cytoplasm	0.622
Acetylornithine aminotransferase	Q92BC0	*Listeria innocua serovar 6a* (strain ATCC BAA-680 / CLIP 11262)	Cytoplasm	0.941
Cell wall metabolism				
Penicillin-binding protein 1A/1B	P39793	*Bacillus subtilis* (strain 168)	Cell membrane	1.417
Quorum sensing				
Autoinducer-2 kinase	P77432	*Escherichia coli* (strain K12)	Cytoplasm	0.278
Virulence				
Hemolysin E, chromosomal	P77335	*Escherichia coli* (strain K12)	Secreted	0.995

Table 2 CiC shared proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment	Fold Change Ciprofloxacin vs. Control
DNA metabolism				
Replication termination protein	P0CI76	*Bacillus subtilis* (strain 168) *Bacillus spizizenii* (strain ATCC 23059 / NRRL B-14472 / W23)	Cytoplasm	2.439
DNA ligase	P0A0C4	*Staphylococcus aureus* (strain Mu3 / ATCC 700698)	Cytoplasm	1.399
DNA repair				
UvrABC system protein A	O34863	*Bacillus subtilis* (strain 168) *Mycoplasma pneumoniae* (strain ATCC 29342/ M129)	Cytoplasm	4.314
Endonuclease MutS2	Q3JZH6	*Streptococcus agalactiae* serotype Ia (strain 27591/A909/CDC SS700)	Cytoplasm	2.280
Transport				
Spermidine/putrescine transport system permease protein PotC	P0AFK6	*Escherichia coli* (strain K12)	Cell membrane	0.330

Nicotinamide riboside transporter PnuC	P0AFK2	*Escherichia coli* (strain K12)	Cell membrane	0.939
Glutamate/aspartate import permease protein GltJ	P0AER3	*Escherichia coli* (strain K12)	Cell membrane	0.791
Branched-chain amino acid transport system carrier protein BraB	O34545	*Bacillus subtilis* (strain 168)	Cell membrane	1.469
Stress and defense response				
Teichuronic acid biosynthesis protein TuaB	O32273	*Bacillus subtilis (strain 168)*	Cell membrane	2.114
Energy and Metabolism				
1,4-alpha-glucan branching enzyme GlgB	P39118	*Bacillus subtilis* (strain 168)	Cytoplasm	1.125
Aspartate 1-decarboxylase	B8I2Z2	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm	2.114
Glycogen synthase	O87689	*Desulfitobacterium hafniense*	Cytoplasm	0.287
Cobalt-factor III methyltransferase	A0Q1U9	*Priestia megaterium*	Cytoplasm	1.514
ATP synthase epsilon chain	A4W1V6	*Streptococcus suis* (strain 98HAH33)	Cell membrane	0.799
Translation and Protein metabolism				
50S ribosomal protein L14	P0ADY3	*Escherichia coli* (strain K12)	Cytoplasm	1.185

30S ribosomal protein S3	P78009	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	0.874
50S ribosomal protein L25	A8F919	*Bacillus pumilus* (strain SAFR-032)	Cytoplasm	0.330
NADPH-dependent 7-cyano-7-deazaguanine reductase	Q3ACT4	*Caldanaerobacter subterraneus subsp. tengcongensis* (strain DSM 15242 / JCM 11007 / NBRC 100824 / MB4)	Cytoplasm	1.322
Transcription and RNA metabolism				
Sulfurtransferase TusE	O32231	*Escherichia coli* (strain K12)	Cytoplasm	0.310
Amino-acid metabolism				
2-isopropylmalate synthase	P09151	*Escherichia coli* (strain K12)	Cytoplasm	0.830
Serine hydroxymethyltransferase	B1I6M4	*Desulforudis audaxviator* (strain MP104C)	Cytoplasm	0.874
Methylthioribose-1-phosphate isomerase	B1YIY4	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Cytoplasm	1.152
Cell wall metabolism				

Penicillin-binding protein 1A/1B	P39793	*Bacillus subtilis* (strain 168)	Cell membrane	1.555
Cell division				
DNA translocase FtsK	P46889	*Escherichia coli* (strain K12)	Cell membrane	0.315
Phospho-N-acetylmuramoyl-pentapeptide-transferase	A5VJ31	*Limosilactobacillus reuteri* (strain DSM 20016)	Cell membrane	0.419
Virulence				
Hemolysin E, chromosomal	P77335	*Escherichia coli* (strain K12)	Secreted	1.809
Protein folding				
Chaperonin GroEL	B8D0Z4	*Halothermothrix orenii* (strain H 168 / OCM 544 / DSM 9562)	Cytoplasm	5.500
Unknown				
Hydroxylamine reductase	C3L261	*Clostridium botulinum* (strain 657 / Type Ba4)	Cytoplasm	2.318

3.3 Cell wall metabolism proteins

Interestingly, in this group of proteins one stood out: Penicillin-binding protein (PBP). The PBP was found in every treatment and there was a considerable up-accumulation of this protein in most groups. At MC and CiC groups (Tables 1 and 2), the fold-change value was 1.4117 and 1.5559, respectively. The up-accumulation at the CiMC (Table 3) group was 3.1347, a significant up-accumulation. PBPs are transpeptidases or carboxypeptidases known for being involved at the peptidoglycan biosynthesis and being the target of penicillin-like antibiotics (Guinane *et al.* 2006; Zapun *et al.* 2008). The consortium between *Mo*-CBP_3-PepI and Ciprofloxacin, somehow, induced an up-accumulation in this protein, what could re-sensitize these cells for penicillin-like antibiotics that are resistant to β-lactamases and open up for a combined treatment attempt (Łęski and Tomasz 2005). Moreover, another interesting shared protein was found: the D-alanine--D-alanine ligase. This protein was found only at the CiMC (Table 3) group and presented a fold-change value of 19.1797, the highest of all fold-change values in any group. In the meantime, this protein was not found at the Ciprofloxacin-treated cells nor the *Mo*-CBP_3-PepI- treated cells (Supplementary tables 2 and 3). D-alanine–D-alanine ligase is an essential enzyme for cell wall biosynthesis; it is responsible for forming the D-alanine-D-alanine-dipeptide (Liu *et al.* 2006). The up-accumulation of these two proteins could mean that the consortium Ciprofloxacin+*Mo*-CBP_3-PepI is somehow affecting the cell wall biosynthesis or even the functioning of these two proteins. In addition, this up-accumulation could open new doors of combination with other antibiotics such as vancomycin ando D-cycloserine, whose targets are the D-alanine-D-alanine dipeptide and the enzyme D-alanine-D-alanine ligase, respectively (Bruning *et al.* 2011).

3.4 Quorum sensing and biofilm related proteins

In this group of specific biofilm-related proteins, only one shared protein was identified, an autoinducer-kinase. This protein was found in the MC group (Table 1) and had a fold-change value of 0.2786, revealing a down-accumulation. In addition, another autoinducer was found in Control cells exclusive proteins (Supplementary table 1), but it disappeared in the other treatments. The autoinducers are a group of proteins responsible for the communication between cells within the biofilm structure. Through the signaling of these proteins, the bacterial cells can experience coordinated behaviors (Mukherjee and Bassler 2019). Autoinducers in bacteria like S. aureus have the capacity of inhibiting biofilm formation in a bacterial

community (Mukherjee and Bassler 2019). Since the autoinducer suffered a down-accumulation in the MC group, and has not appeared at the Combined-treatment proteins, it could be an attempt of the cell community to increase biofilm's biomass, what was not enough to prevent cell death, due to our previous results (Neto *et al.* 2022).

In addition, an interesting family of proteins was found in all treatments, except at the Ciprofloxacin+Mo-CBP_3-PepI treated cells, the Clp ATPases family of proteins. The Clp ATPases are an important group of proteins for stress tolerance, virulence and biofilm formation (Frees *et al.* 2004). One protein from this family, ClpX, was found in both control cells and Ciprofloxacin-treated cells (Supplementary tables 1 and 3). This protein has chaperone-like functions, and is an important protein at the expression of virulence factors, biofilm formation, for growing under oxidative stress conditions and under high osmolarity (Frees *et al.* 2004). The absence of this protein in both Ciprofloxacin+Mo-CBP_3-PepI-treated and Mo-CBP_3-PepI-treated cells, could indicate a reduction in biofilm's biomass and ROS accumulation, what stay in consonance with our previous findings (Neto *et al.* 2022).

3.5 Cell Redox Homeostasis-Related Proteins

This set of proteins showed an interesting result in *S. aureus* biofilm cells. When looking at the unique proteins of each treatment (Supplementary tables 1-4), it was possible to detect the presence of proteins responsible for protecting the cell against ROS. In the control cells unique proteins (Supplementary table 1), the Flavodoxin/ferredoxin-NADP reductase was found, an enzyme that participates in the bacterial response to superoxide (Bianchi *et al.* 1995). In *Mo*-CBP_3-PepI-treated cells unique proteins (Supplementary table 2), the Catalase HPII was found. Catalases catalyzes the reaction that converts hydrogen peroxide to water and oxygen, being an important protein to handle oxidative stress (BarriÃ¨re *et al.* 2002). In ciprofloxacin-treated cells unique proteins (Supplementary table 3), the protein Superoxide dismutase was found. Superoxide dismutase is an important protein at the cell response to superoxide anion; it catalyzes the conversion of superoxide anion to oxygen and hydrogen peroxide, and can be related to virulence in some bacteria (Barnett *et al.* 2022). What is interesting with these results is that all these proteins disappeared in Ciprofloxacin+*Mo*-CBP_3-PepI- treated cells (Supplementary table 4); this phenomenon could have led to ROS accumulation and posterior damage in biomolecules due to oxidative stress.

Table 3 CiMC group shared proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment	Fold Change Combined treatment vs. Control
DNA metabolism				
Replication terminator protein	P0CI76	*Bacillus subtilis* (strain 168)	Cytoplasm	1.374
Replication protein	P0A0C4	*Bacillus sp.*	Cytoplasm	0.488
Primosomal protein N'	P94461	*Bacillus subtilis* (strain 168)	Cytoplasm	1.052
GMP synthase	Q92CU0	*Listeria innocua serovar 6a* (strain ATCC BAA-680 / CLIP 11262)	Cytoplasm	1.635
DNA repair				
UvrABC system protein A	P75176	*Bacillus subtilis* (strain 168)	Cytoplasm	1.262
Formamidopyrimidine-DNA glycosylase	P42371	*Lactococcus lactis subsp. cremoris*	Cytoplasm	0.941
Endonuclease MutS2	Q3JZH6	*Streptococcus agalactiae serotype Ia* (strain ATCC 27591 / A909 / CDC SS700)	Cytoplasm	3.196
Transport				
Glutamate/aspartate import permease protein GltJ	P0AER3	*Escherichia coli* (strain K12)	Cell membrane	0.941

Energy and Metabolism				
Methylmalonyl-CoA mutase	P27253	*Escherichia coli* (strain K12)	Cytoplasm	1.568
Aspartate 1-decarboxylase	B8I2Z2	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm	2.353
Phosphomethylpyrimidine synthase	A0Q1U9	*Clostridium novyi* (strain NT)	Cytoplasm	1.052
Translation and Protein metabolism				
50S ribosomal protein L14	P0ADY3	*Escherichia coli* (strain K12)	Cytoplasm	0.133
50S ribosomal protein L23	A6TWI0	*Alkaliphilus metalliredigens* (strain QYMF)	Cytoplasm	2.859
NADPH-dependent 7-cyano-7-deazaguanine reductase	Q8R9P3	*Caldanaerobacter subterraneus subsp. Tengcongensis* (strain DSM 15242/ JCM 11007/ NBRC 100824/ MB4)	Cytoplasm	1.468
Peptidase T	Q3ACT4	*Desulfitobacterium hafniense* (strain DSM 10664/ DCB-2)	Cytoplasm	0.506
Transcription and RNA metabolism				

DNA-directed RNA polymerase subunit beta	P78013	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	2.581
HTH-type transcriptional repressor ComR	P75952	*Escherichia coli* (strain K12)	Cytoplasm	0.647
Amino-acid metabolism				
2-isopropylmalate synthase	P09151	*Escherichia coli* (strain K12)	Cytoplasm	2.581
Methylthioribose-1-phosphate isomerase	B1YIY4	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Cytoplasm	2.161
Serine hydroxymethyltransferase	A0PZX4	*Clostridium novyi* (strain NT)	Cytoplasm	1.608
ATP phosphoribosyltransferase regulatory subunit	A7Z968	*Bacillus velezensis* (strain DSM 23117/ BGSC 10A6/ LMG 26770/ FZB42)	Cytoplasm	1.960
Cell wall metabolism				
Penicillin-binding protein 1A/1B	P39793	*Bacillus subtilis* (strain 168)	Cell membrane	3.134
D-alanine--D-alanine ligase B	P07862	*Escherichia coli* (strain K12)	Cytoplasm	19.179
Protein folding				
Chaperonin GroEL	B8D0Z4	Halothermothrix orenii (strain H 168 / OCM 544 / DSM 9562)	Cytosol	8.739

Co-chaperone protein DjlA	P31680	*Escherichia coli* (strain K12)	Cell membrane	1.096
Virulence				
Hemolysin E, chromosomal	P77335	*Escherichia coli* (strain K12)	Secreted	2.067
Unknown				
Hydroxulamine reductase	C3L261	*Clostridium botulinum* (strain 657 / Type Ba4)	Cytoplasm	5.381

Table 4 MCi group shared proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment	Fold Change (*Mo*-CBP$_3$-PepI vs. Ciprofloxacin)
DNA metabolism				
Endonuclease YhcR	P54602	*Bacillus subtilis* (strain 168)	Secreted	0.842
DNA repair				
Endonuclease 8	P50465	*Escherichia coli* (strain K12)	Cytoplasm	2.949
UvrABC system protein A	O34863	*Bacillus subtilis* (strain 168)	Cytoplasm	0.167
Transport				
Voltage-gated ClC-type chloride channel ClcB	P76175	*Escherichia coli* (strain K12)	Cell membrane	0.093
Stress and Defense Response				
Primary amine oxidase	P46883	*Escherichia coli* (strain K12)	Periplasm space	0.566
Heat-inducible transcription repressor HrcA	B1MZG8	*Leuconostoc citreum* (strain KM20)	Cytoplasm	1.030
Energy and Metabolism				
PanD regulatory factor	P37613	*Escherichia coli* (strain K12)	Cytoplasm	0.948

2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase	P17109	*Escherichia coli* (strain K12)	Cell membrane	1.093
Aspartate 1-decarboxylase	B8I2Z2	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm	1.093
Cobalt-factor III methyltransferase	O87689	*Priestia megaterium (Bacillus megaterium)*	Cytoplasm	0.742
Thiol-disulfide oxidoreductase ResA	A0RBT0	*Bacillus thurigiensis* (strain Al Hakam)	Cell membrane	0.796
ATP synthase epsilon chain	A4W1V6	*Streptococcus suis* (strain 98HAH33)	Cell membrane	1.213
4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase	P50843	*Bacillus subtilis* (strain 168)	Cytoplasm	0.733
1,4-alpha-glucan branching enzyme GlgB	P39118	*Bacillus subtilis* (strain 168)	Cytoplasm	0.553
Translation and Protein metabolism				
Peptidyl-tRNA hydrolase	P78034	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	0.742
50S ribosomal protein L5	Q5WLQ0	*Alkalihalobacillus clausii* (strain KSM-K16)	Cytoplasm	2.949
50S ribosomal protein L16	P0ADY7	*Escherichia coli* (strain K12)	Cytoplasm	0.791
Elongation factor G	P75544	*Mycoplasma pneumoniae* (strain ATCC 29342/ M129)	Cytoplasm	2.578

Glutamate--tRNA ligase	P04805	*Escherichia coli* (strain K12)	Cytoplasm	6.595
Alpha-2-macroglobulin	P76578	*Escherichia coli* (strain K12)	Secreted	1.062
Lipid metabolism				
Cardiolipin synthase A	P0A6H8	*Escherichia coli* (strain K12)	Cell membrane	0.532
Diacylglycerol kinase	P75271	*Bacillus subtilis* (strain 168)	Cytoplasm	0.128
Amino-acid metabolism				
2-isopropylmalate synthase	P09151	*Escherichia coli* (strain K12)	Cytoplasm	0.622
Methylthioribose-1-phosphate isomerase	B1YIY4	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Cytoplasm	0.540
Cell wall metabolism				
Penicillin-binding protein 1A/1B	P39793	*Bacillus subtilis* (strain 168)	Cell membrane	0.910
Cell shape and division				
Gluconeogenesis factor	O06974	*Bacillus subtilis* (strain 168)	Cytoplasm	1.693
Segregation and condensation protein B	P75477	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	0.550

Septation ring formation regulator EzrA	Q9CDR6	*Lactococcus lactis subsp. lactis* (strain IL1403)	Cell membrane	1.686
Virulence				
Hemolysin E, chromosomal	P77335	*Escherichia coli* (strain K12)	Secreted	0.550

1.6 Protein Biosynthesis and Metabolism Related Proteins

The analysis of the proteins involved in protein biosynthesis reveals a difficult situation for *S. aureus* cells. In every comparison group, translation-related proteins appeared. For example, in the MC group (Table 1) an abundance decrease of the 50S ribosomal protein L16 was detected. This protein is a key component of the 50S subunit and it plays a major role in peptidyl-transferase activity and association with the 30S subunit (Nishimura *et al.* 2004). The down-accumulation of this protein indicates a destabilization of bacterial ribosomes, leading to the inhibition of protein synthesis.

Now, looking at the CiC group (Table 2), it was possible to identify that translation-related proteins were not affected by the Ciprofloxacin treatment, like the 50S ribosomal protein L14 and the 30S ribosomal protein S3. Surprisingly, when we look at the CiMC group (Table 5) a large abundance decrease of L14 was shown, and S3 even disappeared, alongside L16. The L14 protein is one of the most conserved large subunit proteins in a large number of species, and the function of primarily bind to 23S rRNA and it is located in the subunit between the peptidyl transferase and GTPase centers (Davies *et al.* 1996). The S3 protein, together with S4 and S5 proteins forms the mRNA entrance, besides having helicase activity, necessary for melting the RNA entering region (Aseev and Boni 2011). The down-accumulation in these two proteins could be also leading to a destabilization of bacterial ribosomes.

Another interesting protein found at the comparison groups, more specifically at the MC group (Table 1), is the Glutamyl-tRNA reductase, which was down-accumulated at a fold-change value of 0.2373. Glutamyl-tRNA reductase is an important protein in heme biosynthesis, catalyzing the reduction of $tRNA^{Glu}$ to form glutamate-1-semialdehyde (Wang *et al.* 1999). Heme is an important enzymatic cofactor present in proteins of the respiratory chain, like cytochrome *c* (Wang *et al.* 1999; Anzaldi and Skaar 2010; Choby and Skaar 2016) and is necessary for redox system enzymes such as Catalase and peroxidases (Choby and Skaar 2016). In addition, Glutamyl-tRNA reductase is absent in Ciprofloxacin+*Mo*-CBP_3-PepI-treated cells (Supplementary table 4), which can be connected to ROS accumulation, since some redox system proteins need the heme cofactor to function properly.

Interestingly, looking at the exclusive proteins, it is possible to notice the emergence of tRNA ligase proteins in all treatments, but only a few of them appear in control cells. In control cells (Supplementary table 1), Threonine-tRNA ligase, Serine-tRNA ligase and glycine-tRNA ligase beta subunit were found. In the *Mo*-CBP_3-PepI-treated cells (Supplementary table 2),

Threonine-tRNA ligase, Serine-tRNA ligase, Valine-tRNA ligase and Phenylalanine-tRNA ligase beta subunit were found. In the Ciprofloxacin-treated cells (Supplementary table 3), Methionine-tRNA ligase, Glutamate-tRNA ligase, Threonine-tRNA ligase, Alanine-tRNA ligase, Proline-tRNA ligase, Glycine-tRNA ligase and Phenylalanine-tRNA ligase alpha subunit were found. In cells submitted to combined treatment (Supplementary table 4), Tryptophan-tRNA ligase, Histidine-tRNA ligase, Leucine-tRNA ligase, Glycine-tRNA ligase, Alanine-tRNA ligase, Aspartate-tRNA ligase, Phenylalanine-tRNA ligase beta subunit and Methionine-tRNA ligase were found. The combined treatment presented the wider variety of tRNA ligases, indicating the cell's attempt to increase the protein biosynthesis to fight back the stress imposed by the combination of *Mo*-CBP$_3$-PepI+Ciprofloxacin.

Protein synthesis is the major core of stress defense, since proteins are key for bacteria to understand the environment they are inserted in, and respond at an appropriate level (Starosta *et al.* 2014; Zhu and Dai 2020). Depending on which situation the cell is into, the protein synthesis must be adjusted; either decreasing for energy saving or increasing in harsh conditions, and this adjustment is made through ribosomes. Ribosomes are the “protein makers” in cells, and the translation capacity is measured through the number of active ribosomes and by the translational elongation rate (Zhu and Dai 2020). However, this adjustment made itself unviable, since treated cells had a reduction in abundance of ribosomal proteins and elongation factors. Then, the outcome is that the cell is incapable to respond accordingly to the stress imposed by Ciprofloxacin+*Mo*-CBP$_3$-PepI, leading to ROS accumulation and oxidative damage in biomolecules such as DNA and proteins, resulting in cell death as shown by Neto *et al.* (2022).

Table 5 CiMCi group shared proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment	Fold Change Combined treatment vs. Ciprofloxacin
DNA metabolism				
Replication terminator protein	P0CI76	*Bacillus subtilis* (strain 168)	Cytoplasm	3.560
Endonuclease YhcR	P54602	*Bacillus subtilis* (strain 168)	Secreted	0.571
DNA polymerase III subunit alpha	P75404	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129*)*	Cytoplasm	5.964
DNA repair				
UvrABC system protein A	P75176	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129*)*	Cytoplasm	3.307
Formamidopyrimidine-DNA glycosylase	P42371	*Lactococcus lactis subsp. cremoris*	Cytoplasm	1.189
Holliday junction ATP-dependent DNA helicase RuvB	B0TF70	*Heliobacterium modesticaldum* (strain ATCC 51547/ Ice1)	Cytoplasm	0.832
DNA integrity scanning protein DisA	Q18CB2	*Clostridioides difficile* (strain 630)	Cell membrane	0.422
Endonuclease MutS2	Q3JZH6	*Streptococcus agalactiae serotype Ia (strain ATCC 27591 / A909 / CDC SS700)*	Cytoplasm	1.401

DNA base-flipping protein	P0AFP2	*Escherichia coli* (strain K12)	Cytoplasm	2.693
Endonuclease 8	P50465	*Escherichia coli* (strain K12)	Cytoplasm	1.463
Transport				
Glutamate/aspartate import permease protein GltJ	P0AER3	*Escherichia coli* (strain K12)	Cell membrane	1.189
Voltage-gated ClC-type chloride channel ClcB	P76175	*Escherichia coli* (strain K12)	Cell membrane	0.101
p-aminobenzoyl-glutamate transport protein	P46133	*Escherichia coli* (strain K12)	Cell membrane	0.899
Phosphate import ATP-binding protein PstB 2	Q834B3	*Enterococcus faecalis* (strain ATCC 700802 / V583)	Cell membrane	3.089
Stress and Defense Response				
Hydroxylamine reductase	C3L261	*Clostridium botulinum* (strain 657 / Type Ba4)	Cytoplasm	2.320
Primary amine oxidase	P46883	*Escherichia coli* (strain K12)	Periplasmic space	2.694
Energy and Metabolism				
Aspartate 1-decarboxylase	B8I2Z2	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm	1.112

Translation and Protein metabolism				
50S ribosomal subunit assembly factor BipA	O07631	*Bacillus subtilis* (strain 168)	Cytoplasm	5.964
Elongation factor G	P75544	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	4.338
50S ribosomal protein L14	P0ADY3	*Escherichia coli* (strain K12)	Cytoplasm	0.112
NADPH-dependent 7-cyano-7-deazaguanine reductase	Q8R9P	*Caldanaerobacter subterraneus subsp. tengcongensis* (strain DSM 15242 / JCM 11007 / NBRC 100824 / MB4)	Cytoplasm	0.967
Transcription and RNA metabolism				
DNA-directed RNA polymerase subunit beta'	P0A8T7	*Escherichia coli (strain K12)*	Cytoplasm	0.263
Amino-acid metabolism				
2-isopropylmalate synthase	P09151	*Escherichia coli* (strain K12)	Cytoplasm	0.598
Bifunctional aspartokinase/homoserine dehydrogenase 1	P00561	*Escherichia coli* (strain K12)	Cytoplasm	0.954
Methylthioribose-1-phosphate isomerase	B1YIY4	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Cytoplasm	1.874

3-dehydroquinate dehydratase	Q92BC0	*Halalkalibacterium halodurans* (strain ATCC BAA-125 / DSM 18197 / FERM 7344 / JCM 9153 / C-125)	Cytoplasm	3.859
Cell wall metabolism				
Penicillin-binding protein 1A/1B	P39793	*Bacillus subtilis* (strain 168)	Cell membrane	1.417
Cell division				
Segregation and condensation protein B	P75477	*OS=Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	1.142
Septation ring formation regulator EzrA	Q9CDR6	*Lactococcus lactis subsp. lactis* (strain IL1403)	Cell membrane	4.838
Protein folding				
Chaperonin GroEL	B8D0Z4	*Halothermothrix orenii* (strain H 168 / OCM 544 / DSM 9562)	Cytoplasm	1.588
Virulence				
Hemolysin E, chromosomal	P77335	*Escherichia coli* (strain K12)	Secreted	1.142
Quorum sensing				
Toxin YobL	O34330	*Bacillus subtilis* (strain 168)	Secreted	1.197

Unknown

Nucleotide-binding protein Aflv_2526	B7GL38	*Anoxybacillus flavithermus* (strain DSM 21510 / WK1)	Cytoplasm	0.599

Table 6 CiMM group shared proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment	Fold Change Combined treatment vs.*Mo*-CBP$_3$-PepI
DNA metabolism				
Primosomal protein N'	P94461	*Bacillus subtilis* (strain 168)	Cytoplasm	1.481
Replication protein	P0A0C4	*Bacillus sp.*	Cytoplasm	4.145
Insertion element IS1 5 protein InsB	P0CF28	*Escherichia coli* (strain K12)	Cytoplasm	0.730
Endonuclease YhcR	P54602	*Bacillus subtilis* (strain 168)	Secreted	0.678
DNA repair				
Endonuclease 8	P50465	*Escherichia coli* (strain K12)	Cytoplasm	0.496
Transport				
Voltage-gated ClC-type chloride channel ClcB	P76175	*Escherichia coli* (strain K12)	Cell membrane	1.089
PTS system glucose-specific EIICBA component	P20166	*Bacillus subtilis* (strain 168)	Cell membrane	3.102
PTS system lactose-specific EIICB component	P23531	*Lactococcus lactis subsp. lactis*	Cell membrane	0.810
Stress and Defense Response				

Bacteriocin BAC79	C0HLU4	*Weissella confusa*	Secreted	0.770
Primary amine oxidase	P46883	*Escherichia coli* (strain K12)	Periplasmic space	4.753
Energy and Metabolism				
Alpha-galactosidase	O34645	*Bacillus subtilis* (strain 168)	Cytoplasm	1.055
Aspartate 1-decarboxylase	B8I2Z2	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm	1.018
Phosphoglucomutase	P18159	*Bacillus subtilis* (strain 168)	Cytoplasm	1.355
Phosphomethylpyrimidine synthase	A0Q1U9	*Clostridium novyi* (strain NT)	Cytoplasm	1.481
Phosphate acetyltransferase	P0A9M8	*Escherichia coli* (strain K12)	Cytoplasm	1.055
Translation and Protein metabolism				
Elongation factor G	P75544	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	1.682
Prophage Rz endopeptidase RzpD	P75719	*Escherichia coli* (strain K12)	Cytoplasm	0.262
Neutral endopeptidase	Q07744	*Lactococcus lactis subsp. lactis* (strain IL1403)	Cytoplasm	2.076

Transcription and RNA metabolism				
DNA-directed RNA polymerase subunit beta	P78013	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	0.961
DNA-directed RNA polymerase subunit beta'	Q5FM96	*Lactobacillus acidophilus* (strain ATCC 700396 / NCK56 / N2 / NCFM)	Cytoplasm	1.055
RNA repair				
CCA-adding enzyme	A0AK10	*Listeria welshimeri serovar 6b* (strain ATCC 35897 / DSM 20650 / CIP 8149 / NCTC 11857 / SLCC 5334 / V8)	Cytosol	1.083
Amino-acid metabolism				
2-isopropylmalate synthase	P09151	*Escherichia coli* (strain K12)	Cytoplasm	0.961
Cell wall metabolism				
Penicillin-binding protein 1A/1B	P39793	*Bacillus subtilis* (strain 168)	Cell membrane	2.211
Cell division				
Segregation and condensation protein B	P75477	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm	2.076
Septation ring formation regulator EzrA	Q9CDR6	*Lactococcus lactis subsp. lactis*	Cell membrane	2.869

Antibiotic resistance				
Tunicamycin resistance protein	P12921	*Bacillus subtilis* (strain 168)	Cell membrane	1.246
Virulence				
Hemolysin E, chromosomal	P77335	*Escherichia coli* (strain K12)	Secreted	2.076

3.7 Protein refolding- related proteins

The refolding of proteins is an important process that could mean the difference between life and death to a cell, since misfolded proteins cannot exercise their functions, leading to protein aggregation and cell death (Visick and Clarke 1995; Winter *et al.* 2005). Chaperones do the refold of proteins through the use of ATP and are known to be expressed under heat and other stress conditions, such as oxidative stress (Visick and Clarke 1995). The chaperonin GroEL was found in control cells, Ciprofloxacin-treated cells and Ciprofloxacin+*Mo*-CBP_3-PepI-treated cells. In the CiC comparison group (Table 2), GroEL showed a fold-change value of 5.5002, a considerable up-accumulation. Surprisingly, in the CiMC comparison group (Table 3), GroEL presented a fold value of 8.7393, an even higher up-accumulation. This increased abundance of GroEL indicates that the cells are under a considerable oxidative stress, which is causing both DNA damage and protein MISfolding.

3.8 Regulation Factor and RNA Processing Related Proteins

In this group of proteins, one protein in particular stood out, Elongation factor G, which increased in abundance with a fold-change value of 4.3383 in the CiMCi and 1.68 in the CiMM comparison group (Table 5-6). Elongation factor G is a key protein that uses GTP to interact with mRNA and tRNA during translocation in prokaryotic protein synthesis (Chen *et al.* 2016). This sudden increase in abundance is a desperate attempt to maintain protein synthesis even with the stress related to combined treatment, since protein synthesis is compromised as described above at the protein metabolism section.

On the overview, many mechanisms induced by the combination of Mo-CBP_3-PepI and Ciprofloxacin caused damage, cell death and reduction of biofilm biomass of S. aureus. As shown in previous studies, Mo-CBP_3-PepI alone was capable of inducing the pore formation in the membrane of S. aureus cells; the combination of Ciprofloxacin+Mo-CBP_3-PepI was capable of reducing the abundance of proteins related to the response to oxidative stress, leading to ROS accumulation inside the cell; ROS accumulation led to DNA and protein damage, leading to misfold and aggregation; the combined treatment led to up-accumulation of proteins related to cell wall biosynthesis that are targets to other antibiotics, opening a door for new combined treatments. Combined treatment also reduced the abundance of important ribosomal proteins, leading to reduction in protein biosynthesis, what can lead to a delayed response to stress. Also, the synthesis of proteins related to quorum sensing and biofilm formation were reduced, what

matches with previous results that the combination of Ciprofloxacin+Mo-CBP$_3$-PepI led to reduction of biomass in S. aureus biofilms.

4 Conclusions

The data presented in this study indicate a elaborated sequence of events induced by both *Mo*-CBP$_3$-PepI and Ciprofloxacin combined, that could have drove *S. aureus* biofilm to have its biomass reduced and cells to death. The number and complexity of mechanisms involved make us believe that it may be much more difficult for S. *aureus* cells to develop resistance against it. Therefore, it is possible to suggest that *Mo*-CBP$_3$-PepI could be helpful in combined treatment with antibiotics that have lower or no activity against *S. aureus* biofilm.

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Supplementary table 1 – DMSO exclusive - Biological Activity

Expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID uniprot	Organism Reference	Cellular Compartment
Carbohydrate metabolism			
Mannosylglycerate hydrolase	P54746	*Escherichia coli* (strain K12)	Cell membrane
Galactose-1-phosphate uridylyltransferase	Q1WUZ2	*Ligilactobacillus salivarius*	Cytoplasm
Phosphoglycerate kinase	A5CYN8	*Pelotomaculum thermopropionicum*	Cytoplasm
Hyaluronate lyase	Q59801	*Staphylococcus aureus*	Secreted
Cell division			
Trigger factor	Q9CI15	*Lactococcus lactis subsp. lactis*	Cytoplasm
UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	C5D4C2	*Geobacillus sp.* (strain WCH70)	Cell membrane
Cell division protein FtsZ	P75464	*Mycoplasma pneumoniae*	Cytoplasm
UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	Q81K13	*Bacillus anthracis*	Cytoplasm
Stress and defense response			
Acil-CoA desidrogenase putative AidB	P33224	*Escherichia coli* (strain K12)	Cytoplasm
Arsenate reductase	P0AB96	*Escherichia coli (strain K12)*	Unknown

Flavodoxin/ferredoxin--NADP reductase	P28861	*Escherichia coli (strain K12)*	Cytoplasm
CRISPR-associated protein Csn2	G3ECR4	*Streptococcus thermophilus*	Unknown

Energy and Metabolism

Aconitate hydratase A	P25516	*Escherichia coli* (strain K12)	Cytoplasm
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	P06959	*Escherichia coli* (strain K12)	Cytoplasm
Glycogen synthase	P39125	*Bacillus subtilis* (strain 168)	Cytoplasm
NADH-quinone oxidoreductase subunit I 1	Q67P14	*Symbiobacterium thermophilum*	Cell membrane
Enolase 2	Q88VW2	*Lactiplantibacillus plantarum*	Cytoplasm
Phosphoenolpyruvate carboxylase	Q1GBD4	*Lactobacillus delbrueckii subsp. bulgaricus*	Cytoplasm
NADH oxidase	P37061	*Enterococcus faecalis*	Cytoplasm
Glucose-6-phosphate isomerase	Q24VW9	*Desulfitobacterium hafniense*	Cytoplasm
ExodeoxyribonucleaseVII large subunit	P04994	*Escherichia coli (strain K12)*	Cytoplasm
ATP-dependent DNA helicase RecG	P24230	*Escherichia coli (strain K12)*	Cytoplasm
Nucleoid-associated protein YaaK	P24281	*Bacillus subtilis* (strain 168)	Cytoplasm

Holliday junction ATP-dependent DNA helicase RuvB	Q8RAN3	*Caldanaerobacter subterraneus subsp. tengcongensis* (strain DSM 15242 / JCM 11007 / NBRC 100824 / MB4)	Cytoplasm
Selenide, water dikinase	P16456	*Escherichia coli* (strain K12)	Cytoplasm
Amino-acid metabolism			
Glutamate 5-kinase	Q3K396	*Streptococcus agalactiae serotype Ia*	Cytoplasm
2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	Q5WF92	*Alkalihalobacillus clausii*	Cytoplasm
3-isopropylmalate dehydrogenase	Q02143	*Lactococcus lactis subsp. lactis*	Cytoplasm
Imidazole glycerol phosphate synthase subunit HisF	Q5KVD0	*Geobacillus kaustophilus*	Cytoplasm
ATP phosphoribosyltransferase	Q67KH5	*Symbiobacterium thermophilum*	Cytoplasm
Arginine repressor	A0Q0A7	*Clostridium novyi* (strain NT)	Cytoplasm
Aminodeoxychorismate synthase component 1	P28820	*Bacillus subtilis* (strain 168)	Cytoplasm
Anthranilate phosphoribosyltransferase	P03947	*Bacillus subtilis (strain 168)*	Cytoplasm
Acetolactate synthase small subunit	P37252	*Bacillus subtilis* (strain 168)	Cytoplasm
Ornithine carbamoyltransferase, catabolic	P75473	*Mycoplasma pneumoniae*	Cytoplasm
Regulation Factor			
Sensor histidina quinase YcbM	P42245	*Bacillus subtilis* (strain 168)	Cell membrane

Antibiotic biosynthesis			
Polyketide biosynthesis malonyl CoA-acyl carrier protein transacylase PksC	O34825	*Bacillus subtilis (strain 168)*	Cytoplasm
Transport			
Galactose/methyl galactoside import ATP-binding protein MglA	Q8XKQ2	*Clostridium perfringens*	Cell membrane
Sulfur carrier protein FdhD	A8FIA1	*Bacillus pumilus*	Cytoplasm
Nickel-binding periplasmic protein	P33590	*Escherichia coli* (strain K12)	Cell membrane
Xanthosine permease	P45562	*Escherichia coli* (strain K12)	Cell membrane
Autoinducer 2 ABC transporter ATP-binding protein LsrA	P77257	*Escherichia coli* (strain K12)	Cell membrane
D-galactonate MFS transporter	P0AA76	*Escherichia coli* (strain K12)	Cell membrane
Teichoic acids export ATP-binding protein TagH	P42954	*Bacillus subtilis* (strain 168)	Cell membrane
Low-affinity inorganic phosphate transporter PitA	P0AFJ7	*Escherichia coli* (strain K12)	Cell membrane
PTS system lactose-specific EIICB component	Q4L869	*Staphylococcus haemolyticus* (strain JCSC1435)	Cell membrane
Melibiose/raffinose/stachyose import permease protein MelC	O34518	*Bacillus subtilis* (strain 168)	Cell membrane
Ktr system potassium uptake protein C	P39760	*Bacillus subtilis* (strain 168)	Cell membrane
Na(+)/H(+) antiporter subunit D	O05229	*Bacillus subtilis* (strain 168)	Cell membrane

Iron(3+)-hydroxamate import ATP-binding protein FhuC	P49938	*Bacillus subtilis* (strain 168)	Cell membrane
DNA repair			
DNA mismatch repair protein MutS	P23909	*Escherichia coli* (strain K12)	Cytoplasm
Protein recombinase RecA	P0A7G6	*Escherichia coli* (strain K12)	Cytoplasm
UvrABC system protein C	Q8R8M9	*Caldanaerobacter subterraneus subsp. tengcongensis*	Cytoplasm
Transcription and RNA metabolism			
DNA-directed RNA polymerase subunit beta'	A5D5I3	*Pelotomaculum thermopropionicum*	Cytoplasm
Global transcriptional regulator CodY	Q49X40	*Staphylococcus saprophyticus subsp. saprophyticus*	Cytoplasm
Aspartyl-phosphate phosphatase Spo0E	P05043	*Bacillus subtilis* (strain 168)	Cytoplasm
Nus factor SuhB	P0ADG4	*Escherichia coli* (strain K12)	Cytoplasm
PtsGHI operon antiterminator	O31691	*Bacillus subtilis* (strain 168)	Cytoplasm
HTH-type transcriptional regulator GntR	P0ACP5	*Escherichia coli* (strain K12)	Cytoplasm
Transcriptional regulatory protein CusR	P0ACZ8	*Escherichia coli* (strain K12)	Cytoplasm

Nucleotide metabolism			
NAD kinase	A0A6M3ZF24	*Bacillus subtilis* (strain 168)	Cytoplasm
Polyribonucleotide nucleotidyltransferase	A0A6M4JGU2	*Bacillus subtilis* (strain 168)	Cytoplasm
Signal recognition particle receptor FtsY	P75362	*Mycoplasma pneumoniae*	Cell membrane
GTP 3',8-cyclase	Q9K9W9	*Halalkalibacterium halodurans*	Cytoplasm
GTPase Obg	A0A380F4Z7	*Staphylococcus carnosus*	Cytoplasm
Adenylate kinase	P99062	*Staphylococcus aureus*	Cytoplasm
tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	A9KLX8	*Lachnoclostridium phytofermentans*	Cytoplasm
S-adenosylmethionine synthase	Q8RCE4	*Caldanaerobacter subterraneus subsp. tengcongensis*	Cytoplasm
Virulance			
Sphingomyelinase C	Q9RLV9	*Listeria ivanovii*	Secreted
Perfringolysin O	P0C2E9	*Clostridium perfringens*	Cell membrane
Translation and protein metabolism			
30S ribosomal protein S7	P75545	*Mycoplasma pneumoniae*	Cytoplasm
Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	P75533	*Mycoplasma pneumoniae*	Cytoplasm

50S ribosomal protein L30	Q7ANU8	*Listeria innocua serovar 6a*	Cytoplasm
Ribosomal protein L11 methyltransferase	Q67S51	*Symbiobacterium thermophilum*	Cytoplasm
Ubiquinone U32 family peptidase	A0A1L5KM48	*Escherichia coli* (strain K12)	Cytoplasm
Protein FMN transferase	P0AB85	*Escherichia coli* (strain K12)	Cell membrane
UPF0758 protein YsxA	Q02170	*Bacillus subtilis* (strain 168)	Cytoplasm
Phosphoserine phosphatase RsbX	P17906	*Bacillus subtilis* (strain 168)	Cytoplasm
Elongation factor G 1	Q0AXN1	*Syntrophomonas wolfei subsp. wolfei*	Cytoplasm
Translation initiation factor IF-3	Q891T1	*Clostridium tetani*	Cytoplasm
Peptide chain release factor 2	A8AY61	*Streptococcus gordonii*	Cytoplasm
Threonine--tRNA ligase	Q38VT0	*Latilactobacillus sakei subsp. sakei*	Cytoplasm
Low molecular weight protein-tyrosine-phosphatase PtpA	Q4L7J1	*Staphylococcus haemolyticus*	Cytoplasm
Serine--tRNA ligase	B3W8V7	*Lacticaseibacillus casei*	Cytoplasm
Glycine--tRNA ligase beta subunit	Q1WTP1	*Ligilactobacillus salivarius*	Cytoplasm
Methionine aminopeptidase	P0AE18	*Escherichia coli* (strain K12)	Cytoplasm
Lipid metabolism			
Fatty acid oxidation complex subunit alpha	P21177	*Escherichia coli* (strain K12)	Cytoplasm

Isoprenyl transferase	Q92C39	*Listeria innocua serovar 6a*	Cytoplasm
Unknown			
4-aminobutirato aminotransferase GabT	P22256	*Escherichia coli* (strain K12)	Cytoplasm
UPF0713 protein YngL	O34506	*Bacillus subtilis* (strain 168)	Cell membrane
Bacilliredoxin BrxB	P54534	*Bacillus subtilis* (strain 168)	Unknown
3-phytase	O31097	*Bacillus subtilis*	Secreted
SPbeta prophage-derived uncharacterized protein YonE	O31953	*Bacillus subtilis* (strain 168)	Unknown
UPF0134 protein MPN_104	P75565	*Mycoplasma pneumoniae*	Unknown
Urease subunit gamma	Q733J4	*Bacillus cereus*	Cytoplasm
Protoheme IX farnesyltransferase	A4ILX0	*Geobacillus thermodenitrificans*	Cell membrane
2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	C5D3P3	*Geobacillus sp.* (strain WCH70)	Unknown
UPF0182 protein Csac_0864	A4XHU5	*Caldicellulosiruptor saccharolyticus*	Cell membrane

Supplementary table 2 *Mo*-CBP$_3$-PepI-treated exclusive proteins – Biological Activity
Expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment
DNA metabolism			
Arabinose operon regulatory protein	P0A9E0	*Escherichia coli* (strain K12)	Cytoplasm
Chromosomal replication initiator protein DnaA	P03004	*Escherichia coli* (strain K12)	Cytoplasm
Transcription-repair-coupling factor	P37474	*Bacillus subtilis* (strain 168)	Cytoplasm
Helicase/deoxyribonuclease subunit B	B2A611	*Natranaerobius thermophiles* (strain ATCC BAA-1301/DSM18059/JW/NM-WN-LF)	Cytoplasm
DNA topoisomerase 1	P39814	*Bacillus subtilis* (strain 168)	Cytoplasm
SPbeta prophage-derived endonuclease YokF	O32001	*Bacillus subtilis* (strain 168)	Secreted
DNA repair			
Transcription-repair-coupling factor	P37474	*Bacillus subtilis* (strain 168)	Cytoplasm
UvrABC system protein B	P0A8F8	*Escherichia coli* (strain K12)	Cytoplasm
DNA repair protein RecN	P05824	*Escherichia coli* (strain K12)	Cytoplasm

ABC transporter ATP-binding protein ModF	P31060	*Escherichia coli* (strain K12)	Cytoplasm
Transport			
Nickel transport system permease	P33591	*Escherichia coli* (strain K12)	Cell membrane
Arabinose-proton symporter	P0AE24	*Escherichia coli* (strain K12)	Cell membrane
Protein translocase SecA	A4IST9	*Eobacillus thermodenitrificans* (strain NG80-2)	Cell membrane
High-affinity branched-chain amino acid ABC transporter ATP-binding protein LivF	P22731	*Escherichia coli* (strain K12)	Cell membrane
nucleoside-specific channel-forming protein Tsx	P0A927	*Escherichia coli* (strain K12)	Cell membrane
Ferredoxin-type protein NapG	P0AAL3	*Escherichia coli* (strain K12)	Cell membrane
Cytochrome c biogenesis ATP-binding export protein CcmA	P33931	*Escherichia coli* (strain K12)	Cytoplasm
Spermidine/putrescine import ATP-binding protein PotA	P75059	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cell membrane
NADH-quinone oxidoreductase subunit NuoN	C5D978	*Geobacillus sp.* (strain WCH70)	Cell membrane
Electron transfer flavoprotein subunit beta	O85691	*Megasphaera elsdenii*	Cell membrane

Oligopeptide-binding protein OppA	Q9CEK0	*Lactococcus lactis subsp. lactis* (strain IL1403)	Cell membrane
Stress and Defense Response			
Catalase HPII	P21179	*Escherichia coli* (strain K12)	Cytoplasm
Stringent starvation protein A	P0ACA3	*Escherichia coli* (strain K12)	Cytoplasm
NADPH dehydrogenase	Q5KXG9	*Geobacillus kaustophilus* (strain HTA426)	Cytoplasm
Cell division inhibitor SulA	P0AFZ5	*Escherichia coli* (strain K12)	Cell membrane
L-cystine-binding protein TcyK	O34852	*Bacillus subtilis* (strain 168)	Cell membrane
Energy and Metabolism			
Phosphoenolpyruvate carboxylase	P00864	*Escherichia coli* (strain K12)	Cytoplasm
2-hydroxy-6-oxononatrienedioate hydrolase	P77044	*Escherichia coli* (strain K12)	Cytoplasm
Transketolase 1	P27302	*Escherichia coli* (strain K12)	Cytoplasm
Benzaldehyde dehydrogenase	O06478	*Bacillus subtilis* (strain 168)	Cytoplasm
Menaquinol-cytochrome c reductase iron-sulfur subunit	P46911	*Bacillus subtilis* (strain 168)	Cytoplasm
Scyllo-inositol 2-dehydrogenase	O05265	*Bacillus subtilis* (strain 168)	Cytoplasm
Uroporphyrinogen-III C-methyltransferase	P42437	*Bacillus subtilis* (strain 168)	Cytoplasm

Bacillopeptidase F	P16397	*Bacillus subtilis* (strain 168)	Cytoplasm
NAD kinase	P75508	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Thymidine phosphorylase	P75052	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Glycerol-3-phosphate oxidase	P75063	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
sulfate adenylyltransferase	A4J272	*Desulforamulus reducens* (strain ATCC BAA-1160 / DSM 100696 / MI-1)	Cytoplasm
glutamate-1-semialdehyde 2,1-aminomutase	A3DIF3	*Acetivibrio thermocellus* (strain ATCC 27405 / DSM 1237 / JCM 9322 / NBRC 103400 / NCIMB 10682 / NRRL B-4536 / VPI 7372)	Cytoplasm
Phosphoglycerate kinase	B0S1H0	*Finegoldia magna* (strain ATCC 29328 / DSM 20472 / WAL 2508)	Cytoplasm
GTP cyclohydrolase I	A4W1S9	*Streptococcus suis* (strain 98HAH33)	Cytoplasm
ATP synthase subunit gamma	A6LQH4	*Clostridium beijerinckii* (strain ATCC 51743 / NCIMB 8052)	Cell membrane
propionate--CoA ligase	P77495	*Escherichia coli* (strain K12)	Cytoplasm

Acryloyl-CoA reductase electron transfer subunit beta	G3KIM6	*Anaerotignum propionicum* (*Clostridium propionicum*)	Cytoplasm
2-succinylbenzoate--CoA ligase	P23971	*Bacillus subtilis* (strain 168)	Cytoplasm
6-phospho-5-dehydro-2-deoxy-D-gluconate aldolase	A7ZAH2	*Bacillus velezensis* (strain DSM 23117 / BGSC 10A6 / LMG 26770 / FZB42)	Cytoplasm
5-hydroxybenzimidazole synthase BzaB	A0A0K1TQ05	Eubacterium limosum	Cytoplasm
6-hydroxynicotinate reductase	Q0QLF7	*Eubacterium barkeri*	Cytoplasm
Translation and Protein metabolism			
Threonine--tRNA ligase	P0A8M3	*Escherichia coli* (strain K12)	Cytoplasm
Serine—tRNA ligase	P75107	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Phenylalanine--tRNA ligase beta subunit	Q92CI6	*Listeria innocua serovar 6a* (strain ATCC BAA-680 / CLIP 11262)	Cytoplasm
Thymidylate synthase	P0A884	*Escherichia coli* (strain K12)	Cytoplasm
50S ribosomal protein L15	C3P9S4	*Bacillus anthracis* (strain A0248)	Cytoplasm
Elongation factor G	B9DKV7	*Staphylococcus carnosus* (strain TM300)	Cytoplasm
Valine--tRNA ligase	Q05873	*Bacillus subtilis* (strain 168)	
30S ribosomal protein S7			Cytoplasm

	Q2RFP3	*Moorella thermoacetica* (strain ATCC 39073 / JCM 9320)	
GTPase Obg	B1YJR9	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Cytoplasm
Putative pyruvate, phosphate dikinase regulatory protein 2	Q4L6R3	*Staphylococcus haemolyticus* (strain JCSC1435)	Cytoplasm
Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	A5VLG2	*Limosilactobacillus reuteri* (strain DSM 20016)	Cytoplasm
Ribosome-recycling factor	Q8VS52	*Limosilactobacillus reuteri*	Cytoplasm
Transcription and RNA metabolism			
HTH-type transcriptional repressor AseR	P96677	*Bacillus subtilis* (strain 168)	Cytoplasm
2-iminobutanoate/2-iminopropanoate deaminase	P0AF93	*Escherichia coli* (strain K12)	Cytoplasm
Ribonuclease III	B1N016	*Leuconostoc citreum* (strain KM20)	Cytoplasm
23S rRNA (guanine(745)-N(1))-methyltransferase	P36999	*Escherichia coli* (strain K12)	Cytoplasm
UPF0714 protein YjqB	O34785	*Bacillus subtilis* (strain 168)	Cytoplasm
Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase TrmFO	C0M6C7	*Streptococcus equi subsp. equi* (strain 4047)	Cytoplasm

Carbohydrate metabolism			
Phosphoglucosamine mutase	A7FZ14	*Clostridium botulinum* (strain ATCC 19397 / Tipo A)	Cytoplasm
Galactarate dehydratase	P39829	*Escherichia coli* (strain K12)	Cytoplasm
Mannonate dehydratase	O34346	*Bacillus subtilis* (strain 168)	Cytoplasm
Cellobiose 2-epimerase	E6UB41	*Ruminococcus albus* (strain ATCC 27210 / DSM 20455 / JCM 14654 / NCDO 2250 / 7)	Cytoplasm
Thermostable beta-glucosidase B	P14002	*Acetivibrio thermocellus* (strain ATCC 27405 / DSM 1237 / JCM 9322 / NBRC 103400 / NCIMB 10682 / NRRL B-4536 / VPI 7372)	Unknown
Amino-acid metabolism			
D-serine ammonia-lyase	P00926	*Escherichia coli* (strain K12)	Cytoplasm
Cysteine synthase A	P63870	*Staphylococcus aureus* (strain Mu50 / ATCC 700699)	Cytoplasm
Methionine synthase	P13009	*Escherichia coli* (strain K12)	Cytoplasm
Aspartate carbamoyltransferase	P0A786	*Escherichia coli* (strain K12)	Cytoplasm
L-asparaginase 1	P26900	*Bacillus subtilis* (strain 168)	Cytoplasm
Pyrroline-5-carboxylate reductase	P0CI77	*Bacillus subtilis* (strain 168)	Cytoplasm

4-hydroxyphenylacetate decarboxylase activase	Q38HX2	*Clostridium escatologenes*	Cytoplasm
Shikimate kinase	Q49XY2	*Staphylococcus saprophyticus subsp. saprophyticus* (strain ATCC 15305 / DSM 20229 / NCIMB 8711 / NCTC 7292 / S-41)	Cytoplasm
Acetylglutamate kinase	B8D1G8	*Halothermothrix orenii* (strain H 168 / OCM 544 / DSM 9562)	Cytoplasm
4-hydroxy-tetrahydrodipicolinate synthase	Q97GI9	*Clostridium acetobutylicum* (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)	Cytoplasm
Phospho-2-dehydro-3-deoxyheptonate aldolase, Trp-sensitive	P00887	*Escherichia coli* (strain K12)	Cytoplasm
Methionine synthase	P13009	*Escherichia coli* (strain K12)	Cytoplasm
2-oxoisovalerate dehydrogenase subunit alpha	P37940	*Bacillus subtilis* (strain 168)	Cytoplasm
S-adenosylmethionine synthase	P78003	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Cell wall metabolism			
peptidoglycan DD-transpeptidase MrdA	P0AD65	*Escherichia coli* (strain K12)	Cell membrane
Amylopullulanase	P38939	*Thermoanaerobacter pseudethanolicus* (strain ATCC 33223 / 39E)	Cell wall

L-Ala--D-Glu endopeptidase	O32130	*Bacillus subtilis* (strain 168)	Cytoplasm
N-acetylmuramoyl-L-alanine amidase XylA	P39800	*Bacillus subtilis* (strain 168)	Secreted
Cell division			
Segregation/condensation protein A	P75478	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Nucleotide metabolism			
Nucleoside-diphosphate kinase	P0A763	*Escherichia coli* (strain K12)	Cell membrane
Bifunctional purine biosynthesis protein PurH	P12048	*Bacillus subtilis* (strain 168)	Cytoplasm
Lipid metabolism			
Acyl-CoA dehydrogenase	P45857	*Bacillus subtilis* (strain 168)	Cytoplasm
4'-phosphopantetheinyl transferase	P39144	*Bacillus subtilis* (strain 168)	Cytoplasm
Isopentenyl-diphosphate Delta-isomerase	Q65I10	*Bacillus licheniformis* (strain ATCC 14580 / DSM 13 / JCM 2505 / CCUG 7422 / NBRC 12200 / NCIMB 9375 / NCTC 10341 / NRRL NRS-1264 / Gibson 46)	Cytoplasm
Phosphate acyltransferase	Q8ER09	*Oceanobacillus iheyensis* (strain DSM 14371 / CIP 107618 / JCM 11309 / KCTC 3954 / HTE831)	Cytoplasm

Acetyl-CoA carboxylase carboxyltransferase	C4Z8P1	*Agathobacter rectalis* (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990)	Cytoplasm
Cardiolipin synthase C	P75919	*Escherichia coli* (strain K12)	Cytoplasm
DegV domain-containing protein MG326 homolog	P75312	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Protein folding			
Chaperone protein ClpB	P75247	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Trigger factor	P0A850	*Escherichia coli* (strain K12)	Cytoplasm
Unknown			
RNase adapter protein RapZ	P0A894	*Escherichia coli* (strain K12)	Cell membrane
Nucleoid-associated protein LBUL_1514	Q048W5	*Lactobacillus delbrueckii subsp. bulgaricus* (strain ATCC BAA-365 / Lb-18)	Cytoplasm
UPF0297 protein SGO_2042	A8AZT0	*Streptococcus gordonii (strain Challis / ATCC 35105 / BCRC 15272 / CH1 / DL1 / V288)*	Cytoplasm
N-carbamoyl-L-amino acid hydrolase	Q53389	*Geobacillus stearothermophilus*	Cytoplasm

Supplementary table 3 Ciprofloxacin treatment exclusive proteins – Biological Activity
Differentially expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment
DNA metabolism			
Purine nucleoside phosphorylase 2	P45563	*Escherichia coli* (strain K12)	Cytoplasm
Transposase InsF for insertion sequence IS3A	P0CF79	*Escherichia coli* (strain K12)	Unknown
Adenylate cyclase	P00936	*Escherichia coli* (strain K12)	Cytoplasm
Ribonucleoside-diphosphate reductase NrdEB subunit alpha	O31875	*Bacillus subtilis* (strain 168)	Cytoplasm
Type II restriction enzyme BsuBI	P33562	*Bacillus subtilis*	Cytoplasm
DNA-binding transcriptional activator AdeR	P71073	*Bacillus subtilis* (strain 168)	Cytoplasm
Sensor histidine kinase DesK	O34757	*Bacillus subtilis* (strain 168)	Cell membrane
DNA topoisomerase 4 subunit A	P75352	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129 / Subtype 1) (Mycoplasmoides pneumoniae)	Cell membrane
Exodeoxyribonuclease 7 large subunit	B9DUT9	*Streptococcus uberis* (strain ATCC BAA-854 / 0140J)	Cytoplasm

3'-5' exoribonuclease YhaM	Q65LT6	*Bacillus licheniformis* (strain ATCC 14580 / DSM 13 / JCM 2505 / CCUG 7422 / NBRC 12200 / NCIMB 9375 / NCTC 10341 / NRRL NRS-1264 / Gibson 46)	Unknown
DNA repair			
ATP-dependent DNA helicase RecG	Q5HPW4	*Staphylococcus epidermidis* (strain ATCC 35984 / RP62A)	Nucleus
Regulatory protein RecX	Q92AW7	*Listeria innocua serovar 6a* (strain ATCC BAA-680 / CLIP 11262)	Cytoplasm
Transport			
Multidrug efflux pump subunit AcrB	P31224	*Escherichia coli* (strain K12)	Cell membrane
PTS system mannitol-specific EIICBA component	P00550	*Escherichia coli* (strain K12)	Cell membrane
L-cystine-binding protein TcyJ	P0AEM9	*Escherichia coli* (strain K12)	Cell membrane
Cation/acetate symporter ActP	P32705	*Escherichia coli* (strain K12)	Cell membrane
SCP2 domain-containing protein YusD	O32170	*Bacillus subtilis* (strain 168)	Unknown
Sodium/proline symporter	P07117	*Escherichia coli* (strain K12)	Cell membrane
Protein translocase subunit SecA	P75559	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129 / Subtype 1)	Cell membrane
	P75957	*Escherichia coli* (strain K12)	Cell membrane

Lipoprotein-releasing system ATP-binding protein LolD			
Fe(3+) dicitrate transport ATP-binding protein FecE	P15031	*Escherichia coli* (strain K12)	Cell membrane
SCP2 domain-containing protein YusD	O32170	*Bacillus subtilis* (strain 168)	Unknown
Na(+)/H(+) antiporter subunit C	O05260	*Bacillus subtilis* (strain 168)	Cell membrane
L-cystine transport system permease protein TcyM	O34931	*Bacillus subtilis* (strain 168)	Cell membrane
Linearmycin resistance permease protein LnrM	P94441	*Bacillus subtilis* (strain 168)	Cell membrane
Ascorbate-specific PTS system EIIC component	P75291	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129 / Subtype 1)	Cell membrane
Potassium-transporting ATPase ATP-binding subunit	A0RA13	*Bacillus thuringiensis* (strain Al Hakam)	Cell membrane
Stress and defense response			
Superoxide dismutase [Fe]	P0AGD3	*Escherichia coli* (strain K12)	Cytoplasm
Universal stress protein E	P0AAC0	*Escherichia coli* (strain K12)	Cytoplasm
Chaperone protein DnaK	B0TAD7	*Heliobacterium modesticaldum* (strain ATCC 51547 / Ice1)	Cytoplasm
Subpeptin JM4-B	P83879	*Bacillus subtilis*	Secreted
Catalase	Q926X0	*Listeria innocua serovar 6a* (strain ATCC BAA-680 / CLIP 11262)	Cytoplasm
Energy and Metabolism			
			Cell membrane

Respiratory nitrate reductase 2 beta chain	P19318	*Escherichia coli* (strain K12)	
Hydrogenase-4 component B	P23482	*Escherichia coli* (strain K12)	Cell membrane
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	P21883	*Bacillus subtilis* (strain 168)	Cytoplasm
Glucose-6-phosphate isomerase	A9KHD9	*Lachnoclostridium phytofermentans* (strain ATCC 700394 / DSM 18823 / ISDg)	Cytoplasm
1-deoxy-D-xylulose-5-phosphate synthase	Q5WF63	*Alkalihalobacillus clausii* (strain KSM-K16) (Bacillus clausii)	Cytoplasm
Cobalt-precorrin-5B C(1)-methyltransferase	Q897K1	*Clostridium tetani* (strain Massachusetts / E88)	Cytoplasm
Glutamine—fructose-6-phosphate aminotransferase [isomerizing]	Q9CGT6	*Lactococcus lactis subsp. Lactis* (strain IL1403)	Cytoplasm
3-methyl-2-oxobutanoate hydroxymethyltransferase	A4XMZ4	*Caldicellulosiruptor saccharolyticus* (strain ATCC 43494 / DSM 8903 / Tp8T 6331)	Cytoplasm
ATP synthase subunit c	Q88UT8	*Lactiplantibacillus plantarum* (strain ATCC BAA-793 / NCIMB 8826 / WCFS1)	Cell membrane
Acetate kinase	Q5WED5	*Alkalihalobacillus clausii* (strain KSM-K16)	Cytoplasm

Homocitrate synthase, omega subunit	Q00852	*Clostridium pasteurianum*	Cytoplasm
GTP cyclohydrolase 1 type 2 homolog	Q8XIV9	*Clostridium perfringens* (strain 13 / Type A)	Cytoplasm
L-lactate dehydrogenase	Q5HL31	*Staphylococcus epidermidis* (strain ATCC 35984 / RP62A)	Cytoplasm
Hydroxyethylthiazole kinase	B2UY66	*Clostridium botulinum* (strain Alaska E43 / Type E3)	Cytoplasm
Transketolase	A0A0I9QGZ2	*Geobacillus stearothermophilus*	Cytoplasm
Galactokinase	Q5LYY7	*Streptococcus thermophilus* (strain CNRZ 1066)	Cytoplasm
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	B8FTK7	*Desulfitobacterium hafniense* (strain DSM 10664 / DCB-2)	Cytoplasm
Carbohydrate metabolism			
Dtdp-4-dehydrorhamnose reductase	P37760	*Escherichia coli* (strain K12)	Cytoplasm
Mannan endo-1,4-beta-mannosidase	O05512	*Bacillus subtilis* (strain 168)	Secreted
Alpha-ketoglutaric semialdehyde dehydrogenase	P42236	*Bacillus subtilis* (strain 168)	Cytoplasm
Methylglyoxal synthase	B1YKL9	*Exiguobacterium sibiricum* (strain DSM 17290 / CIP 109462 / JCM 13490 / 255-15)	Unknown

Xyloglucanase Xgh74A	A3DFA0	*Acetivibrio thermocellus* (strain ATCC 27405 / DSM 1237 / JCM 9322 / NBRC 103400 / NCIMB 10682 / NRRL B-4536 / VPI 7372)	Cytoplasm
Glycogen synthase	C4Z0G2	*Lachnospira eligens* (strain ATCC 27750 / DSM 3376 / VPI C15-48 / C15-B4)	Cytoplasm
Cobyric acid synthase	Q180T4	*Clostridioides difficile* (strain 630) (Peptoclostridium difficile)	Cytoplasm
Translation and Protein metabolism			
Elongation factor Ts	P0A6P1	*Escherichia coli* (strain K12)	Cytoplasm
Protein AsmA	P28249	*Escherichia coli* (strain K12)	Cell membrane
Chorismate pyruvate-lyase	P26602	*Escherichia coli* (strain K12)	Cytoplasm
Methionine—Trna ligase	P00959	*Escherichia coli* (strain K12)	Cytoplasm
Secretion monitor	P62395	*Escherichia coli* (strain K12)	Cytoplasm
Protease 2	P24555	*Escherichia coli* (strain K12)	Cytoplasm
Trna-2-methylthio-N(6)-dimethylallyladenosine synthase	O31778	*Bacillus subtilis* (strain 168)	Cytoplasm
Trna (guanine-N(1)-)-methyltransferase	Q5HPV1	*Staphylococcus epidermidis* (strain ATCC 35984 / RP62A)	Cytoplasm
Methionine aminopeptidase 2	O34484	*Bacillus subtilis* (strain 168)	Cytoplasm

50S ribosomal protein L32	Q97IA7	*Clostridium acetobutylicum* (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)	Cytoplasm
Methionine—Trna ligase	P00959	*Escherichia coli* (strain K12)	Cytoplasm
30S ribosomal protein S4	C4ZBU5	*Agathobacter rectalis* (strain ATCC 33656 / DSM 3377 / JCM 17463 / KCTC 5835 / VPI 0990) (Eubacterium rectale)	Cytoplasm
Pyrrolidone-carboxylate peptidase	A0A1Q9FFG5	*Bacillus licheniformis*	Cytoplasm
Threonine—Trna ligase	Q9K866	*Halalkalibacterium halodurans (strain ATCC BAA-125 / DSM 18197 / FERM 7344 / JCM 9153 / C-125)*	Cytoplasm
S-adenosylmethionine:Trna ribosyltransferase-isomerase	A9KK69	*Lachnoclostridium phytofermentans* (strain ATCC 700394 / DSM 18823 / ISDg)	Cytoplasm
Alanine—Trna ligase	Q88V10	*Lactiplantibacillus plantarum* (strain ATCC BAA-793 / NCIMB 8826 / WCFS1)	Cytoplasm
Protein-arginine kinase	B8D093	*Halothermothrix orenii* (strain H 168 / OCM 544 / DSM 9562)	Cytoplasm

Translation initiation factor IF-2	B1MZH4	*Leuconostoc citreum* (strain KM20)	Cytoplasm
Phenylalanine--tRNA ligase alpha subunit	Q9CEB4	*Lactococcus lactis subsp. lactis* (strain IL1403)	Cytoplasm
GTPase Obg	5D410	*Pelotomaculum thermopropionicum* (strain DSM 13744 / JCM 10971 / SI)	Cytoplasm
tRNA(Met) cytidine acetate ligase	B8CWV6	*Halothermothrix orenii* (strain H 168 / OCM 544 / DSM 9562)	Cytoplasm
Proline—Trna ligase	B0KCH5	*Thermoanaerobacter pseudethanolicus* (strain ATCC 33223 / 39E)	Cytoplasm
Glycine—Trna ligase beta subunit	A4J7C9	*Desulforamulus reducens* (strain ATCC BAA-1160 / DSM 100696 / MI-1) (Desulfotomaculum reducens)	Cytoplasm

Transcription and RNA metabolism

HTH-type transcriptional activator AaeR	P67662	*Escherichia coli* (strain K12)	Cytoplasm
Toxic protein SymE	P39394	*Escherichia coli* (strain K12)	Cytoplasm
Guanosine-inosine kinase	P0AEW6	*Escherichia coli* (strain K12)	Citoplasm
Anti-sigma-B factor antagonist	P17903	*Bacillus subtilis* (strain 168)	Cytoplasm
RNA polymerase sigma factor SigA	P06224	*Bacillus subtilis* (strain 168)	Cytoplasm

5'-nucleotidase SurE	A6TRH0	*Alkaliphilus metalliredigens* (strain QYMF)	Cytoplasm
Adenylosuccinate synthetase	A0PXA8	*Clostridium novyi* (strain NT)	Cytoplasm
Transcriptional regulator MraZ	A7Z4D6	*Bacillus velezensis* (strain DSM 23117 / BGSC 10A6 / LMG 26770 / FZB42)	Cytoplasm
Multifunctional CCA protein	P06961	*Escherichia coli* (strain K12)	Cytoplasm
Ribonuclease PH	Q5KWI5	Geobacillus kaustophilus (strain HTA426)	Cytoplasm
Amino-acid metabolism			
L-rhamnonate dehydratase	P77215	*Escherichia coli* (strain K12)	Unknown
5,10-methylenetetrahydrofolate reductase	P0AEZ1	*Escherichia coli* (strain K12)	Cytoplasm
Glutamate 5-kinase	P0A7B5	*Escherichia coli* (strain K12)	Cytoplasm
Pyrroline-5-carboxylate reductase 3	Q00777	*Bacillus subtilis* (strain 168)	Cytoplasm
N-acetyldiaminopimelate deacetylase	O34916	*Bacillus subtilis* (strain 168)	Cytoplasm
N-acetyl-gamma-glutamyl-phosphate reductase	C1KVN8	*Listeria monocytogenes serotype 4b (strain CLIP80459)*	Cytoplasm
ComE operon protein 4	P39696	*Bacillus subtilis* (strain 168)	Cytoplasm
Methylthioribose-1-phosphate isomerase	Q5L1E6	*Geobacillus kaustophilus* (strain HTA426)	Cytoplasm

4-hydroxy-3-methylbut-2-enyl diphosphate reductase	A7Z6T1	*Bacillus velezensis* (strain DSM 23117 / BGSC 10A6 / LMG 26770 / FZB42)	Cytoplasm
Tryptophanase 1	P31014	*Symbiobacterium thermophilum* (strain T / IAM 14863)	Cytoplasm
Oxygen-dependent choline dehydrogenase	Q5HL11	*Staphylococcus epidermidis* (strain ATCC 35984 / RP62A)	Cytoplasm
Histidinol dehydrogenase	Q8ESR8	*Oceanobacillus iheyensis* (strain DSM 14371 / CIP 107618 / JCM 11309 / KCTC 3954 / THE831)	Cytoplasm
3-isopropylmalate dehydratase small subunit	Q04DA2	*Oenococcus oeni* (strain ATCC BAA-331 / PSU-1)	Cytoplasm
Dihydroxy-acid dehydratase	A7GNQ7	*Bacillus cytotoxicus* (strain DSM 22905 / CIP 110041 / 391-98 / NVH 391-98)	Cytoplasm
Histidine decarboxylase proenzyme	P04193	*Lentilactobacillus buchneri (Lactobacillus buchneri)*	Cytoplasm
Imidazoleglycerol-phosphate dehydratase	Q88UE0	*Lactiplantibacillus plantarum (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) (Lactobacillus plantarum)*	Cytoplasm
Nucleic acid metabolism			
Phosphoribosylformylglycinamidine cyclo-ligase	B8FP03	*Desulfitobacterium hafniense* (strain DSM 10664 / DCB-2)	Cytoplasm

Orotate phosphoribosyltransferase	Q74J27	*Lactobacillus johnsonii* (strain CNCM I-12250 / La1 / NCC 533)	Cytoplasm
Cell wall metabolism			
Rhamnogalacturonan exolyase YesX	O31527	*Bacillus subtilis* (strain 168)	Secreted
D-alanyl-D-alanine carboxypeptidase DacF	P38422	*Bacillus subtilis* (strain 168)	Secreted
UDP-N-acetylmuramate--L-alanine ligase	C4L4P1	*Exiguobacterium sp.* (strain ATCC BAA-1283 / AT1b)	Cytoplasm
Cell division			
Chromosome partition protein MukB	P22523	*Escherichia coli* (strain K12)	Cytoplasm
UDP-N-acetylmuramate—L-alanine ligase	C4L4P1	*Exiguobacterium sp.* (strain ATCC BAA-1283 / AT1b)	Cytoplasm
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate—2,6-diaminopimelate ligase	Q9K9S4	*Halalkalibacterium halodurans (strain ATCC BAA-125 / DSM 18197 / FERM 7344 / JCM 9153 / C-125) (Bacillus halodurans)*	Cytoplasm
Cell division protein FtsA	O07672	*Enterococcus hirae*	Cell membrane
Virulence			
Trna(Glu)-specific nuclease WapA	D4G3R4	*Bacillus subtilis subsp. Natto* (strain BEST195)	Secreted
Collagenase ColH	Q46085	*Hathewaya histolytica (Clostridium histolyticum)*	Secreted
NAD(+) hydrolase TirS	P0DTS9	*Staphylococcus aureus* (strain MSSA476)	Secreted

Resistance			
Fosmidomycin resistance protein	P52067	*Escherichia coli* (strain K12)	Cell membrane
Protein folding			
Chaperonin GroEL 1	Q2RL13	*Moorella thermoacetica* (strain ATCC 39073 / JCM 9320)	Cytoplasm
Cell structure and fuction			
Glucans biosynthesis protein D	P40120	*Escherichia coli* (strain K12)	Periplasm
Phosphate acyltransferase	P27247	*Escherichia coli* (strain K12)	Cytoplasm
Lipid metabolism			
Bifunctional cytochrome P450/NADPH—P450 reductase 2	O08336	*Bacillus subtilis* (strain 168)	Cytoplasm
Carnitinyl-CoA dehydratase	P31551	*Escherichia coli* (strain K12)	Unknown
NTE family protein RssA	P0AFR0	*Escherichia coli* (strain K12)	Unknown
L-carnitine CoA-transferase	P31572	*Escherichia coli* (strain K12)	Cytoplasm
3-deoxy-D-manno-octulosonic acid transferase	P0AC75	*Escherichia coli* (strain K12)	Cell membrane
Glycerol-3-phosphate acyltransferase	P75428	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129 / Subtype 1) (Mycoplasmoides pneumoniae)	Cell membrane

Unknown			
PhoH-like protein	P46343	*Bacillus subtilis* (strain 168)	Cytoplasm
Nucleotide-binding protein SH2124	Q4L4J2	*Staphylococcus haemolyticus* (strain JCSC1435)	Cytoplasm

Supplementary table 4 Combined treatment exclusive proteins – Biological Activity
Expressed proteins identified by ESI-LC-MS/MS.

Protein Name	ID (Uniprot)	Organism Reference	Cellular Compartment
DNA metabolism			
DNA topoisomerase 1	P06612	*Escherichia coli* (strain K12)	Cytoplasm
Anaerobic ribonucleoside-triphosphate reductase	P28903	*Escherichia coli* (strain K12)	Cytoplasm
Replicative DNA helicase	P0ACB0	*Escherichia coli* (strain K12)	Cytoplasm
Chromosome partition protein Smc	P51834	*Bacillus subtilis* (strain 168)	Cytoplasm
Nuclease SbcCD subunit C	O06714	*Bacillus subtilis* (strain 168)	Cytoplasm
Beta sliding clamp	Q50313	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Exodeoxyribonuclease 7 large subunit	Q04KB0	*Streptococcus pneumoniae* serotype 2 (strain D39 / NCTC 7466)	Cytoplasm
DNA repair			
Phosphoglycolate phosphatase	P32662	*Escherichia coli* (strain K12)	Cytoplasm
Endonuclease MutS2	P94545	*Bacillus subtilis* (strain 168)	Cytoplasm
Holliday junction ATP-dependent DNA helicase RuvB	P75242	*Mycoplasma pneumonia* (strain ATCC 29342 / M129)	Cytoplasm

Formamidopyrimidine-DNA glycosylase	P75402	*Mycoplasma pneumonia* (strain ATCC 29342 / M129)	Cytoplasm
Transport			
Fe(3+) dicitrate transport protein FecA	P13036	*Escherichia coli* (strain K12)	Cell membrane
Formate channel FocA	P0AC25	*Escherichia coli* (strain K12)	Cell membrane
L-cystine transport system permease protein TcyL	P0AFT2	*Escherichia coli* (strain K12	Cell membrane
L-carnitine/gamma-butyrobetaine antiporter	P31553	*Escherichia coli* (strain K12)	Cell membrane
ABC transporter ATP-binding/permease protein YojI	P33941	*Escherichia coli* (strain K12)	Cell membrane
Multidrug export protein AcrF	P24181	*Escherichia coli* (strain K12)	Cell membrane
ESX secretion system protein YukB	C0SPA7	*Escherichia coli* (strain K12)	Cell membrane
Oligopeptide-binding protein OppA	P24141	*Bacillus subtilis* (strain K12)	Cell membrane
Magnesium transporter MgtE	O34442	*Bacillus subtilis* (strain 168)	Cell membrane
Phosphate transport system permease protein PstA homolog	P75185	*Mycoplasma pneumonia* (strain ATCC 29342 / M129)	Cell membrane
Na(+)/H(+) antiporter subunit A1	Q5HQL0	*Staphylococcus epidermidis* (strain ATCC 35984 / RP62A)	Cell membrane
NADH-quinone oxidoreductase subunit B	A4J659		Cell membrane

		Desulforamulus reducens (strain ATCC BAA-1160 / DSM 100696 / MI-1)	
Methionine import ATP-binding protein MetN 1	Q832Y6	*Enterococcus faecalis* (strain ATCC 700802 / V583)	Cell membrane
Manganese transport system membrane protein MntC	Q9KD29	*Halalkalibacterium halodurans* (strain ATCC BAA-125 / DSM 18197 / FERM 7344 / JCM 9153 / C-125)	Cell membrane
Multidrug efflux MFS transporter	A0A7L9L4W7	*Lactococcus lactis*	Cell membrane

Stress and Defense Response

RNA polymerase sigma factor RpoH	P0AGB3	*Escherichia coli* (strain K12)	Cytoplasm
Cold shock protein CspA	P0A9X9	*Escherichia coli* (strain K12)	Cytoplasm
Glycolate oxidase subunit GlcD	P0AEP9	*Escherichia coli* (strain K12)	Cytoplasm
Glutathione hydrolase proenzyme	P18956	*Escherichia coli* (strain K12)	Periplasm
Thiol management oxidoreductase	A0A6M4JHF0	*Bacillus subtilis* (strain 168)	Cell membrane
General TerD family protein	P80875	*Bacillus subtilis* (strain 168)	Cytoplasm

Energy and Metabolism

2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	P62707	*Escherichia coli* (strain K12)	Cytoplasm
Pyruvate kinase I	P0AD61	*Escherichia coli* (strain K12)	Cytoplasm
Fatty acid oxidation complex subunit alpha	P77399	*Escherichia coli* (strain K12)	Cytoplasm
NADP/NAD-dependent aldehyde dehydrogenase PuuC	P23883	*Escherichia coli* (strain K12)	Cytoplasm
Alpha-D-ribose 1-methylphosphonate 5-triphosphate synthase subunit PhnG	P16685	*Escherichia coli* (strain K12)	Unknown
ADP-ribose pyrophosphatase	Q93K97	*Escherichia coli* (strain K12)	Cytoplasm
Putative formate dehydrogenase YrhE	Q795Y4	*Bacillus subtilis* (strain 168)	Cytoplasm
6-phosphogluconate dehydrogenase, NADP(+)-dependent, decarboxylating	P80859	*Bacillus subtilis* (strain 168)	Cytoplasm
Glucokinase	P54495	*Bacillus subtilis* (strain 168)	Cytoplasm
Pantothenate synthetase	Q833S6	*Enterococcus faecalis* (strain ATCC 700802 / V583)	Cytoplasm
Phosphopantetheine adenylyltransferase	B1I2E4	*Desulforudis audaxviator* (strain MP104C)	Cytoplasm
Glucose-6-phosphate isomerase	Q83XM3	*Limosilactobacillus fermentum*	Cytoplasm
ATP-dependent dethiobiotin synthetase BioD	A7Z5B3	*Bacillus velezensis* (strain DSM 23117 / BGSC 10A6 / LMG 26770 / FZB42)	Cytoplasm
NAD-dependent malic enzyme	P16468	*Geobacillus stearothermophilus*	Cytoplasm

6,7-dimethyl-8-ribityllumazine synthase	Q897Q7	*Clostridium tetani* (strain Massachusetts / E88)	Cytoplasm
Heme-dependent peroxidase	P39645	*Bacillus subtilis* (strain 168)	Cytoplasm
Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase	B1I4H2	*Desulforudis audaxviator* (strain MP104C)	Cytoplasm

Translation and Protein metabolism

Tryptophan--tRNA ligase	P00954	*Escherichia coli* (strain K12)	Cytoplasm
Histidine--tRNA ligase	P60906	*Escherichia coli* (strain K12)	Cytoplasm
Ribosomal protein L11 methyltransferase	P0A8T1	*Escherichia coli* (strain K12)	Cytoplasm
Leucine--tRNA ligase	P07813	*Escherichia coli* (strain K12)	Cytoplasm
50S ribosomal protein L2	P60422	*Escherichia coli* (strain K12)	Cytoplasm
Elongation factor 4	P37949	*Bacillus subtilis* (strain 168)	Cytoplasm
Glycine--tRNA ligase alpha subunit	P54380	*Bacillus subtilis* (strain 168)	Cytoplasm
Alanine--tRNA ligase	Q24UT2	*Desulfitobacterium hafniense* (strain Y51)	Cytoplasm
Glutamyl aminopeptidase	Q48677	*Lactococcus lactis subsp. cremoris* (strain MG1363)	Cytoplasm

L-seryl-tRNA(Sec) selenium Transferase	O33277	*Moorella thermoacetica* (Clostridium thermoaceticum)	Cytoplasm
Aspartate--tRNA ligase	B8I3F1	*Ruminiclostridium cellulolyticum* (strain ATCC 35319 / DSM 5812 / JCM 6584 / H10)	Cytoplasm
Ribosome maturation factor RimM	B0TH66	*Heliobacterium modesticaldum* (strain ATCC 51547 / Ice1)	Cytoplasm
Extracellular elastase	P0C0Q3	*Staphylococcus epidermidis*	Secreted
Biotin/lipoate A/B protein ligase	D5WTA4	*Kyrpidia tusciae* (strain DSM 2912 / NBRC 15312 / T2)	Cytoplasm
Urease accessory protein UreG	Q4A0J8	*Staphylococcus saprophyticus subsp. saprophyticus* (strain ATCC 15305 / DSM 20229 / NCIMB 8711 / NCTC 7292 / S-41)	Cytoplasm
Phenylalanine--tRNA ligase beta subunit	Q38VV4	*Latilactobacillus sakei subsp. sakei* (strain 23K)	Cytoplasm
Glutamyl-tRNA(Gln) amidotransferase subunit A	B2GDS6	*Limosilactobacillus fermentum* (strain NBRC 3956 / LMG 18251)	Cytoplasm
Methionine--tRNA ligase	Q97EW5	*Clostridium acetobutylicum* (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)	Cytoplasm

Transcription and RNA metabolism

Dual-specificity RNA pseudouridine synthase RluF	P32684	*Escherichia coli* (strain K12)	Cytoplasm
Transcriptional regulatory protein PhoP	P23836	*Escherichia coli* (strain K12)	Cytoplasm
YebC/PmpR family DNA-binding transcriptional regulator	B1N016	Escherichia coli ISC7	Cytoplasm
LacI family transcriptional regulator	A0A6D1EPZ8	*Escherichia coli*	Cytoplasm
Acetoin dehydrogenase operon transcriptional activator AcoR	O31551	*Bacillus subtilis* (strain 168)	Cytoplasm
HTH-type transcriptional regulator DegA	P37947	*Bacillus subtilis* (strain 168)	Cytoplasm
DNA-directed RNA polymerase subunit beta'	P75271	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Polyribonucleotide nucleotidyltransferase	Q49X62	*Staphylococcus saprophyticus subsp. saprophyticus* (strain ATCC 15305 / DSM 20229 / NCIMB 8711 / NCTC 7292 / S-41)	Cytoplasm
Ribonuclease Z	Q1GAM2	*Lactobacillus delbrueckii subsp. bulgaricus* (strain ATCC 11842 / DSM 20081 / BCRC 10696 / JCM 1002 / NBRC 13953 / NCIMB 11778 / NCTC 12712 / WDCM 00102 / Lb 14)	Cytoplasm

Bifunctional protein PyrR	Q0AXG0	*Syntrophomonas wolfei subsp. wolfei* (strain DSM 2245B / Goettingen)	Cytoplasm
Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase	Q0AYP4	*Syntrophomonas wolfei subsp. wolfei* (strain DSM 2245B / Goettingen)	Cytoplasm
Pur operon repressor	P65832	*Streptococcus pneumoniae* serotype 4 (strain ATCC BAA-334 / TIGR4)	Cytoplasm
Endoribonuclease YbeY	A6TSK3	*Alkaliphilus metalliredigens* (strain QYMF)	Cytoplasm
tRNA(Met) cytidine acetate ligase	A9VU87	*Bacillus mycoides* (strain KBAB4)	Cytoplasm
Queuine tRNA-ribosyltransferase	B8FQV2	*Desulfitobacterium hafniense* (strain DSM 10664 / DCB-2)	Cytoplasm
Carbohydrate metabolism			
6-phospho-beta-glucosidase AscB	P24240	*Escherichia coli* (strain K12)	Cytoplasm
3-dehydro-D-guloside 4-epimerase	P76044	*Escherichia coli* (strain K12)	Cytoplasm
Aryl-phospho-beta-D-glucosidase BglA	P42973	*Bacillus subtilis* (strain 168)	Cytoplasm
Mannitol-1-phosphate 5-dehydrogenase	Q1WRQ9	*Ligilactobacillus salivarius* (strain UCC118)	Cytoplasm
L-arabinose isomerase	Q03XW2	*Leuconostoc mesenteroides subsp. mesenteroides* (strain ATCC 8293 / DSM 20343 / BCRC 11652 / CCM 1803 / JCM 6124 / NCDO 523 /	Cytoplasm

		NBRC 100496 / NCIMB 8023 / NCTC 12954 / NRRL B-1118 / 37Y)	
Inositol 2-dehydrogenase/D-chiro-inositol 3-dehydrogenase	Q5WKY6	*Alkalihalobacillus clausii* (strain KSM-K16)	Cytoplasm
Aerobic glycerol-3-phosphate dehydrogenase	P18158	*Bacillus subtilis* (strain 168)	Cytoplasm
Amino-acid metabolism			
N-succinylarginine dihydrolase	P76216	*Escherichia coli* (strain K12)	Cytoplasm
Alanine racemase, catabolic	P29012	*Escherichia coli* (strain K12)	Cytoplasm
Aspartate--ammonia ligase	P00963	*Escherichia coli* (strain K12)	Cytoplasm
Formate-dependent nitrite reductase complex subunit NrfG	P32712	*Escherichia coli* (strain K12)	Cell membrane
Constitutive lysine decarboxylase	P52095	*Escherichia coli* (strain K12)	Cytoplasm
Glutamine synthetase	P0A9C5	*Escherichia coli* (strain K12)	Cytoplasm
3-isopropylmalate dehydrogenase	Q38HX2	*Escherichia coli* (strain K12)	Cytoplasm
Pyrroline-5-carboxylate reductase	P54552	*Bacillus subtilis* (strain 168)	Cytoplasm
Indole-3-glycerol phosphate synthase	C4KZ66	*Exiguobacterium sp.* (strain ATCC BAA-1283 / AT1b)	Cytoplasm
Histidine decarboxylase proenzyme	P0C2E5	*Clostridium perfringens* (strain 13 / Type A)	Cytoplasm
Homoserine kinase	P04948	*Bacillus subtilis* (strain 168)	Cytoplasm

ATP phosphoribosyltransferase regulatory subunit	Q67KH4	*Symbiobacterium thermophilum* (strain T / IAM 14863)	Cytoplasm
Argininosuccinate lyase	Q2RG68	*Moorella thermoacetica* (strain ATCC 39073 / JCM 9320)	Cytoplasm
3-phosphoshikimate 1-carboxyvinyltransferase	B9MQK4	*Caldicellulosiruptor bescii* (strain ATCC BAA-1888 / DSM 6725 / Z-1320)	Cytoplasm
N-acetyl-gamma-glutamyl-phosphate reductase	C3P7R8	*Bacillus anthracis* (strain A0248)	Cytoplasm
Glutamate-1-semialdehyde 2,1-aminomutase	Q3ACS9	*Carboxydothermus hydrogenoformans* (strain ATCC BAA-161 / DSM 6008 / Z-2901)	Cytoplasm
Histidinol dehydrogenase	Q02136	*Lactococcus lactis subsp. lactis* (strain IL1403)	Cytoplasm
Asparagine synthetase [glutamine-hydrolyzing] 1	P54420	*Bacillus subtilis* (strain 168)	Cytoplasm
Cell wall metabolism			
Periplasmic beta-glucosidase	P33363	*Escherichia coli* (strain K12)	Periplasm
D-alanyl-D-alanine carboxypeptidase DacD	P33013	*Escherichia coli* (strain K12)	Cell membrane
D-alanine--D-alanine ligase	A4XJ89	*Caldicellulosiruptor saccharolyticus* (strain ATCC 43494 / DSM 8903 / Tp8T 6331)	Cytoplasm
Cell division			

Murein hydrolase activator EnvC	P37690	*Escherichia coli* (strain K12)	Cell membrane
Tyrosine recombinase XerC	P0A8P6	*Escherichia coli* (strain K12)	Cytoplasm
Cell division protein FtsZ	Q9K9T7	*Halalkalibacterium halodurans* (strain ATCC BAA-125 / DSM 18197 / FERM 7344 / JCM 9153 / C-125)	Cytoplasm

Nucleotide metabolism

Phosphoribosylformylglycinamidine cyclo-ligase	P08178	*Escherichia coli* (strain K12)	Cytoplasm
Aspartate carbamoyltransferase	P05654	*Bacillus subtilis* (strain 168)	Cytoplasm
Adenylate kinase	P16304	*Bacillus subtilis* (strain 168)	Cytoplasm
Ribose-phosphate pyrophosphokinase	P75044	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Purine nucleoside phosphorylase DeoD-type	P75053	*Mycoplasma pneumoniae* (strain ATCC 29342 / M129)	Cytoplasm
Inosine-5'-monophosphate dehydrogenase	P0C0H6	*Streptococcus pyrogenes*	Cytoplasm
Cytidylate kinase	Q97I08	*Clostridium acetobutylicum* (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)	Cytoplasm
Thymidylate synthase	P19368	*Lactococcus lactis subsp. lactis* (strain IL1403)	*Cytoplasm*

Adenine phosphoribosyltransferase	B9ML31	*Caldicellulosiruptor bescii* (strain ATCC BAA-1888 / DSM 6725 / Z-1320)	Cytoplasm
Lipid metabolism			
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	P0A9Q5	*Escherichia coli* (strain K12)	Cytoplasm
Long-chain-fatty-acid--CoA ligase	P69451	*Escherichia coli* (strain K12)	Cytoplasm
Octaprenyl diphosphate synthase	P0AD57	*Escherichia coli* (strain K12)	Cytoplasm
Acetyl-CoA acetyltransferase	P76461	*Escherichia coli* (strain K12)	Cytoplasm
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	P54482	*Bacillus subtilis* (strain 168)	Cytoplasm
1-deoxy-D-xylulose-5-phosphate synthase	B2TRM5	*Clostridium botulinum* (strain Eklund 17B / Type B)	Cytoplasm
Virulence			
Heme-degrading monooxygenase	Q8Y563	*Listeria monocytogenes serovar 1/2a* (strain ATCC BAA-679 / EGD-e)	Cytoplasm
Collagenase ColA	P43153	*Clostridium perfringens* (strain 13 / Type A)	Secreted
Streptopain	P0C0J1	*Streptococcus pyogenes serotype M1*	Secreted

Unknown			
UPF0401 protein YkfF	P75677	*Escherichia coli* (strain K12)	Unknown
TVP38/TMEM64 family protein	P76219	*Escherichia coli* (strain K12)	Cytoplasm
Protein RhsC	P16918	*Escherichia coli* (strain K12)	Unknown
Prophage bactoprenol glucosyl transferase homolog	P77293	*Escherichia coli* (strain K12)	Cell membrane
DUF421 domain-containing protein	A0A6M4JLS2	*Bacillus subtilis* (strain 168)	Cell membrane
UPF0750 membrane protein YitT	P39803	*Bacillus subtilis* (strain 168)	Cell membrane
Aminotransferase V	A0A0Q9Y4H1	*Staphylococcus sp.* NAM3COL9	Cytoplasm
Hemolysin family protein	A0A8F9SWT5	*Bacillus subtilis*	Cell membrane
UPF0145 protein Aflv_1588	B7GJU0	*Anoxybacillus flavithermus* (strain DSM 21510 / WK1)	Unknown
UPF0182 protein Moth_1139	Q2RJD6	*Moorella thermoacetica* (strain ATCC 39073 / JCM 9320)	Cell membrane
Flotillin-like protein FloA	A8FFC3	Bacillus pumilus (strain SAFR-032)	Cell membrane
Nucleoid-associated protein PTH_0052	A5D6B2	*Pelotomaculum thermopropionicum* (strain DSM 13744 / JCM 10971 / SI)	Cytoplasm

7. CONCLUSÃO

Os resultados encontrados ao longo deste estudo, mostraram o potencial antibiofilme de peptídeos sintéticos bioinspirados em proteínas vegetais de defesa. Além disso, foi visto que a combinação desses peptídeos com antibióticos, como a ciprofloxacina, podem reduzir a quantidade necessária dessas moléculas para apresentarem atividade antibiofilme, reduzindo possíveis efeitos colaterais e toxicidade.

As análises proteômicas vieram a adicionar mais uma camada de complexidade no mecanismo de ação da combinação peptídeo e fármaco, onde foi possível observar alterações no acúmulo de proteínas importantes, como proteínas relacionadas à tradução e ao metabolismo redox. Além disso, o alto acúmulo de algumas proteínas que são alvo de fármacos, podem dar novas perspectivas de combinações com outros fármacos além da ciprofloxacina,. Esses resultados mostram que os peptídeos utilizados nesse trabalho podem servir como adjuvantes ao tratamento de infecções por biofilme de *S. aureus*.

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ANEXO A – ARTIGO CIENTÍFICO 3

Artigo científico publicado na revista *Analytical Biochemistry* (Fator de impacto: 3,1).

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Luffa operculata seed proteins: Identification by LC-ESI-MS/MS and biotechnological potential against *Candida albicans* and *C. krusei*

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ARTICLE INFO

Keywords:
Candida
LC-ESI-MS/MS analysis
Seed proteins
Biotechnological potential

ABSTRACT

L: operculata is a plant commonly found in the North and Northeast of Brazil. Although the regional population knows its medicinal potential, there are few scientific studies about its antimicrobial potential. Thus, this study aimed to characterize the proteins from *L. operculata* seeds extracted using different solutions and evaluate their antimicrobial potentials. The protein extracts obtained with NaCl and sodium acetate buffer presented the best inhibitory activities against *Candida albicans* and *C. krusei*. The study of the mechanism of action revealed proteins from *L. operculata* seeds induced pore formation on the membrane and ROS overaccumulation. Scanning Electron Microscopy images also showed severe morphological changes in *Candida albicans* and *C. krusei*. Proteins from *L. operculata* seeds did not show antibacterial activity. The enzymatic assays revealed the presence of proteolytic enzymes, serine and cysteine protease inhibitors, and chitinases in both protein extracts. Proteomic analysis by LC-ESI-MS/MS identified 57 proteins related to many biological processes, such as defense to (a) biotic stress, energetic metabolism, protein folding, and nucleotide metabolism. In conclusion, the *L. operculata* seed proteins have biotechnological potential against the human pathogenic yeasts *Candida albicans* and *C. krusei*.

ANEXO B – ARTIGO CIENTÍFICO 4

Artigo científico publicado na revista *Journal of fungi* (Fator de impacto: 5,7).

Journal of *Fungi*

MDPI

Article

Essential Oil from *Croton blanchetianus* Leaves: Anticandidal Potential and Mechanisms of Action

Ellen A. Malveira [1], Pedro F. N. Souza [1,2,*], Nilton A. S. Neto [1], Tawanny K. B. Aguiar [1], Natanael S. Rodrigues [3], Carlos W. B. Henrique [3], Ayrles F. B. Silva [1], Leandro B. Lima [4], Cynthia C. Albuquerque [3] and Cleverson D. T. Freitas [1,*]

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Abstract: Antimicrobial drugs are becoming ineffective given the resistance acquired by microorganisms. As such, it is imperative to seek new antimicrobial molecules that could provide a basis for the development of new drugs. Therefore, this work aimed to evaluate the antimicrobial potential and the mechanisms of action of the essential oil extracted from leaves of *Croton blanchetianus* (named *Cb*EO) on different fungi and bacteria of clinical importance in both planktonic and biofilm lifestyles. GC-MS/MS analysis revealed the presence of twenty-two different compounds in the *Cb*EO, which were identified using the Kovats retention index. Among these, the most abundant were amorphene (20.03%), spathulenol (5%), bicyclogermacrene (1.49%), caryophyllene oxide (4.55%), and eucalyptol (5.62%). *Cb*OE (50 µg mL^{-1}) barely inhibited the growth of *Bacillus subtilis* (23%), *Pseudomonas aeruginosa* (27%), and *Salmonella enterica* (28%), and no inhibition was obtained against *Enterobacter aerogenes* and *Klebsiella pneumoniae*. Additionally, no activity against bacterial biofilm was detected. In contrast, *Cb*EO was active against *Candida* species. *C. albicans* and *C. parapsilosis* were inhibited by 78 and 75%, respectively. The antibiofilm potential also was favorable against *C. albicans* and *C. parapsilosis*, inhibiting 44 and 74% of biofilm formation and reducing around 41 and 27% of the preformed biofilm, respectively. *Cb*OE caused membrane damage and pore formation, overproduction of ROS, and apoptosis on *C. albicans* and *C. parapsilosis* cells, as well as not inducing hemolysis in human red cells. The results obtained in this work raise the possibility of using the essential oil of *C. blanchetianus* leaves as an alternative to fight infections caused by *C. albicans* and *C. parapsilosis*.

Keywords: essential oil; GC-MS/MS; biotechnological potential; *Candida* genus; antibiofilm activity

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ANEXO C – ARTIGO CIENTÍFICO 5

Artigo científico publicado na revista *Antibiotics* (Fator de impacto: 4,6).

antibiotics

MDPI

Article

New Insights into the Mechanism of Antibacterial Action of Synthetic Peptide *Mo*-CBP$_3$-PepI against *Klebsiella pneumoniae*

Levi A. C. Branco [1,†], Pedro F. N. Souza [1,2,*,†], Nilton A. S. Neto [1], Tawanny K. B. Aguiar [1], Ayrles F. B. Silva [1], Rômulo F. Carneiro [3], Celso S. Nagano [3], Felipe P. Mesquita [2], Luina B. Lima [2] and Cleverson D. T. Freitas [1]

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Abstract: *Klebsiella pneumoniae* is a multidrug-resistant opportunistic human pathogen related to various infections. As such, synthetic peptides have emerged as potential alternative molecules. *Mo*-CBP$_3$-PepI has presented great activity against *K. pneumoniae* by presenting an MIC$_{50}$ at a very low concentration (31.25 µg mL^{-1}). Here, fluorescence microscopy and proteomic analysis revealed the alteration in cell membrane permeability, ROS overproduction, and protein profile of *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI. *Mo*-CBP$_3$-PepI led to ROS overaccumulation and membrane pore formation in *K. pneumoniae* cells. Furthermore, the proteomic analysis highlighted changes in essential metabolic pathways. For example, after treatment of *K. pneumoniae* cells with *Mo*-CBP$_3$-PepI, a reduction in the abundance of protein related to DNA and protein metabolism, cytoskeleton and cell wall organization, redox metabolism, regulation factors, ribosomal proteins, and resistance to antibiotics was seen. The reduction in proteins involved in vital processes for cell life, such as DNA repair, cell wall turnover, and protein turnover, results in the accumulation of ROS, driving the cell to death. Our findings indicated that *Mo*-CBP$_3$-PepI might have mechanisms of action against *K. pneumoniae* cells, mitigating the development of resistance and thus being a potent molecule to be employed in producing new drugs against *K. pneumoniae* infections.

Keywords: multidrug-resistant bacteria; proteomic analysis; synthetic peptides; antibacterial peptides

Citation: Branco, L.A.C.; Souza, P.F.N.; Neto, N.A.S.; Aguiar, T.K.B.; Silva, A.F.B.; Carneiro, R.F.; Nagano, C.S.; Mesquita, F.P.; Lima, L.B.; Freitas, C.D.T. New Insights into the Mechanism of Antibacterial Action of Synthetic Peptide *Mo*-CBP$_3$-PepI against *Klebsiella pneumoniae*. *Antibiotics* **2022**, *11*, 1753. https://doi.org/10.3390/antibiotics11121753

Academic Editor: Maria Fernanda N. N. Carvalho

ANEXO D – ARTIGO CIENTÍFICO 6

Artigo científico publicado na revista *Antibiotics* (Fator de impacto: 4,6).

antibiotics

MDPI

Article

Synergistic Antifungal Activity of Synthetic Peptides and Antifungal Drugs against *Candida albicans* and *C. parapsilosis* Biofilms

Leandro P. Bezerra [1], Cleverson D. T. Freitas [1,*], Ayrles F. B. Silva [1], Jackson L. Amaral [1], Nilton A. S. Neto [1], Rafael G. G. Silva [2], Aura L. C. Parra [1], Gustavo H. Goldman [3], Jose T. A. Oliveira [1], Felipe P. Mesquita [4] and Pedro F. N. Souza [1,4,*]

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Citation: Bezerra, L.P.; Freitas, C.D.T.; Silva, A.F.B.; Amaral, J.L.; Neto, N.A.S.; Silva, R.G.G.; Parra, A.L.C.; Goldman, G.H.; Oliveira, J.T.A.; Mesquita, F.P.; et al. Synergistic Antifungal Activity of Synthetic Peptides and Antifungal Drugs against *Candida albicans* and *C. parapsilosis* Biofilms. *Antibiotics* **2022**, *11*, 553. https://doi.org/10.3390/antibiotics11050553

Academic Editor: Charlotte A. Huber

Abstract: *C. albicans* and *C. parapsilosis* are biofilm-forming yeasts responsible for bloodstream infections that can cause death. Synthetic antimicrobial peptides (SAMPs) are considered to be new weapons to combat these infections, alone or combined with drugs. Here, two SAMPs, called *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII, were tested alone or combined with nystatin (NYS) and itraconazole (ITR) against *C. albicans* and *C. parapsilosis* biofilms. Furthermore, the mechanism of antibiofilm activity was evaluated by fluorescence and scanning electron microscopies. When combined with SAMPs, the results revealed a 2- to 4-fold improvement of NYS and ITR antibiofilm activity. Microscopic analyses showed cell membrane and wall damage and ROS overproduction, which caused leakage of internal content and cell death. Taken together, these results suggest the potential of *Mo*-CBP_3-PepI and *Mo*-CBP_3-PepIII as new drugs and adjuvants to increase the activity of conventional drugs for the treatment of clinical infections caused by *C. albicans* and *C. parapsilosis*.

Keywords: antibiofilm activity; candidiasis; synergism; synthetic peptides; antifungal drugs

ANEXO E – ARTIGO CIENTÍFICO 7

Artigo científico publicado na revista *Antibiotics* (Fator de impacto: 4,6).

antibiotics

MDPI

Article

Behind the Curtain: In Silico and In Vitro Experiments Brought to Light New Insights into the Anticryptococcal Action of Synthetic Peptides

Tawanny K. B. Aguiar [1], Nilton A. S. Neto [1], Romério R. S. Silva [1], Cleverson D. T. Freitas [1], Felipe P. Mesquita [2], Luciana M. R. Alencar [3], Ralph Santos-Oliveira [4,5], Gustavo H. Goldman [6] and Pedro F. N. Souza [1,2,*]

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Citation: Aguiar, T.K.B.; Neto, N.A.S.; Silva, R.R.S.; Freitas, C.D.T.; Mesquita, F.P.; Alencar, L.M.R.; Santos-Oliveira, R.; Goldman, G.H.; Souza, P.F.N. Behind the Curtain: In Silico and In Vitro Experiments Brought to Light New Insights into the Anticryptococcal Action of Synthetic Peptides. *Antibiotics* **2023**, *12*, 153. https://doi.org/10.3390/antibiotics12010153

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Abstract: *Cryptococcus neoformans* is the pathogen responsible for cryptococcal pneumonia and meningitis, mainly affecting patients with suppressed immune systems. We have previously revealed the mechanism of anticryptococcal action of synthetic antimicrobial peptides (SAMPs). In this study, computational and experimental analyses provide new insights into the mechanisms of action of SAMPs. Computational analysis revealed that peptides interacted with the PHO36 membrane receptor of *C. neoformans*. Additionally, ROS (reactive oxygen species) overproduction, the enzymes of ROS metabolism, interference in the ergosterol biosynthesis pathway, and decoupling of cytochrome c mitochondrial membrane were evaluated. Three of four peptides were able to interact with the PHO36 receptor, altering its function and leading to ROS overproduction. SAMPs-treated *C. neoformans* cells showed a decrease in scavenger enzyme activity, supporting ROS accumulation. In the presence of ascorbic acid, an antioxidant agent, SAMPs did not induce ROS accumulation in *C. neoformans* cells. Interestingly, two SAMPs maintained inhibitory activity and membrane pore formation in *C. neoformans* cells by a ROS-independent mechanism. Yet, the ergosterol biosynthesis and lactate dehydrogenase activity were affected by SAMPs. In addition, we noticed decoupling of Cyt *c* from the mitochondria, which led to apoptosis events in the cryptococcal cells. The results presented herein suggest multiple mechanisms imposed by SAMPs against *C. neoformans* interfering in the development of resistance, thus revealing the potential of SAMPs in treating infections caused by *C. neoformans*.

Keywords: redox system; *Cryptococcus neoformans*; ROS metabolism; ergosterol; synthetic antimicrobial peptides

ANEXO F – ARTIGO CIENTÍFICO 8

Artigo científico publicado na revista *Pharmaceutics* (Fator de impacto: 6,0).

pharmaceutics

MDPI

Article

Antifungal Potential of Synthetic Peptides against *Cryptococcus neoformans*: Mechanism of Action Studies Reveal Synthetic Peptides Induce Membrane–Pore Formation, DNA Degradation, and Apoptosis

Tawanny K. B. Aguiar [1,†], Nilton A. S. Neto [1,†], Cleverson D. T. Freitas [1], Ayrles F. B. Silva [1], Leandro P. Bezerra [2], Ellen A. Malveira [1], Levi A. C. Branco [1], Felipe P. Mesquita [3], Gustavo H. Goldman [4], Luciana M. R. Alencar [5], Jose T. A. Oliveira [1], Ralph Santos-Oliveira [6,7] and Pedro F. N. Souza [1,3,*]

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Abstract: *Cryptococcus neoformans* is a human-pathogenic yeast responsible for pneumonia and meningitis, mainly in patients immunocompromised. Infections caused by *C. neoformans* are a global health concern. Synthetic antimicrobial peptides (SAMPs) have emerged as alternative molecules to cope with fungal infections, including *C. neoformans*. Here, eight SAMPs were tested regarding their antifungal potential against *C. neoformans* and had their mechanisms of action elucidated by fluorescence and scanning electron microscopies. Five SAMPs showed an inhibitory effect (MIC_{50}) on *C. neoformans* growth at low concentrations. Fluorescence microscope (FM) revealed that SAMPs induced 6-kDa pores in the *C. neoformans* membrane. Inhibitory assays in the presence of ergosterol revealed that some peptides lost their activity, suggesting interaction with it. Furthermore, FM analysis revealed that SAMPs induced caspase 3/7-mediated apoptosis and DNA degradation in *C. neoformans* cells. Scanning Electron Microscopy (SEM) analysis revealed that peptides induced many morphological alterations such as cell membrane, wall damage, and loss of internal content on *C. neoformans* cells. Our results strongly suggest synthetic peptides are potential alternative molecules to control *C. neoformans* growth and treat the cryptococcal infection.

Keywords: synthetic antifungal peptides; *Cryptococcus neoformans*; cryptococcal meningitis; inhibition; apoptosis induction

ANEXO G – ARTIGO CIENTÍFICO 9

Artigo científico publicado na revista *Future microbiology* (Fator de impacto: 3,1).

Future MICROBIOLOGY

Research Article

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Combined antibiofilm activity of synthetic peptides and antifungal drugs against *Candida* spp.

Leandro P Bezerra[‡,1], Ayrles FB Silva[‡,2], Ralph Santos-Oliveira[3], Luciana MR Alencar[4], Jackson L Amaral[1,2], Nilton AS Neto[1], Rafael GG Silva[5], Mônica O Belém[6], Claudia R de Andrade[6], Jose TA Oliveira[1], Cleverson DT Freitas[1] & Pedro FN Souza*[,1,7]

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Introduction: *Candida krusei* and *Candida albicans* are biofilm-forming drug-resistant yeasts that cause bloodstream infections that can lead to death. Materials & methods: nystatin and itraconazole were combined with two synthetic peptides, PepGAT and PepKAA, to evaluate the synergistic effect against *Candida* biofilms. Additionally, scanning electron and fluorescence microscopies were employed to understand the mechanism behind the synergistic activity. **Results:** Peptides enhanced the action of drugs to inhibit the biofilm formation of *C. krusei* and *C. albicans* and the degradation of mature biofilms of *C. krusei*. In combination with antifungal drugs, peptides' mechanism of action involved cell wall and membrane damage and overproduction of reactive oxygen species. Additionally, in combination, the peptides reduced the toxicity of drugs to red blood cells. **Conclusion:** These results reveal that the synthetic peptides enhanced the antibiofilm activity of drugs, in addition to reducing their toxicity. Thus, these peptides have strong potential as adjuvants and to decrease the toxicity of drugs.

Plain language summary: *Candida krusei* and *Candida albicans* are biofilm-forming, drug-resistant yeasts that cause bloodstream infections that can lead to death. In this study, biofilms of *C. krusei* and *C. albicans* were treated with a solution composed of synthetic peptides and antifungal drugs, none of which were effective alone. The synthetic peptides reduced the toxicity of drugs to red blood cells. These results may pave the way to the application of synthetic peptides as a beneficial additional to antifungal drugs to treat fungi that cannot be killed by drugs alone.

First draft submitted: 1 March 2022; Accepted for publication: 1 July 2022; Published online: 26 July 2022

Keywords: action mechanisms • antibiofilm activity • antifungal drugs • *Candida* • candidiasis • clinical application • nystatin and itraconazole • synergism • synthetic peptides

ANEXO H – ARTIGO CIENTÍFICO 10

Artigo científico publicado na revista *Future virology* (Fator de impacto: 1,8).

Future VIROLOGY

Review

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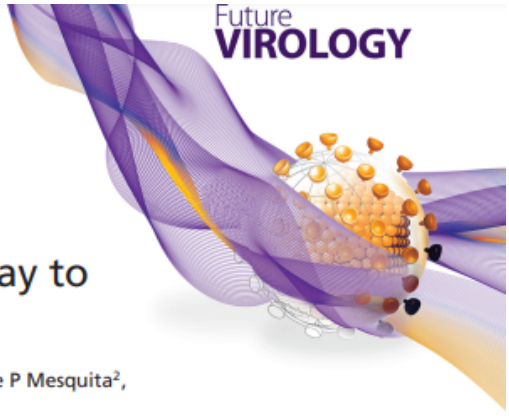

Synthetic antiviral peptides: a new way to develop targeted antiviral drugs

Aura LC Parra[1], Leandro P Bezerra[1], Dur E Shawar[1], Nilton AS Neto[1], Felipe P Mesquita[2], Gabrielly O da Silva[1] & Pedro FN Souza*[,1,2]

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The global concern over emerging and re-emerging viral infections has spurred the search for novel antiviral agents. Peptides with antiviral activity stand out, by overcoming limitations of the current drugs utilized, due to their biocompatibility, specificity and effectiveness. Synthetic peptides have been shown to be viable alternatives to natural peptides due to several difficulties of using of the latter in clinical trials. Various platforms have been utilized by researchers to predict the most effective peptide sequences against HIV, influenza, dengue, MERS and SARS. Synthetic peptides are already employed in the treatment of HIV infection. The novelty of this study is to discuss, for the first time, the potential of synthetic peptides as antiviral molecules. We conclude that synthetic peptides can act as new weapons against viral threats to humans.

First draft submitted: 21 October 2021; Accepted for publication: 6 May 2022; Published online: 26 May 2022

Keywords: antiviral activity • antiviral peptides • peptide-based drugs • synthetic peptides • viral infection

ANEXO I – ARTIGO CIENTÍFICO 11

Artigo científico publicado na revista *Antibiotics* (Fator de impacto: 4,6).

antibiotics

MDPI

Article

No Chance to Survive: *Mo*-CBP$_3$-PepII Synthetic Peptide Acts on *Cryptococcus neoformans* by Multiple Mechanisms of Action

Tawanny K. B. Aguiar [1], Felipe P. Mesquita [2], Nilton A. S. Neto [1], Francisco Í. R. Gomes [1], Cleverson D. T. Freitas [1], Rômulo F. Carneiro [3], Celso S. Nagano [3], Luciana M. R. Alencar [4], Ralph Santos-Oliveira [5,6], Jose T. A. Oliveira [1] and Pedro F. N. Souza [1,2,*]

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Abstract: Multidrug-resistant *Cryptococcus neoformans* is an encapsulated yeast causing a high mortality rate in immunocompromised patients. Recently, the synthetic peptide *Mo*-CBP$_3$-PepII emerged as a potent anticryptococcal molecule with an MIC$_{50}$ at low concentration. Here, the mechanisms of action of *Mo*-CBP$_3$-PepII were deeply analyzed to provide new information about how it led *C. neoformans* cells to death. Light and fluorescence microscopies, analysis of enzymatic activities, and proteomic analysis were employed to understand the effect of *Mo*-CBP$_3$-PepII on *C. neoformans* cells. Light and fluorescence microscopies revealed *Mo*-CBP$_3$-PepII induced the accumulation of anion superoxide and hydrogen peroxide in *C. neoformans* cells, in addition to a reduction in the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) in the cells treated with *Mo*-CBP$_3$-PepII. In the presence of ascorbic acid (AsA), no reactive oxygen species (ROS) were detected, and *Mo*-CBP$_3$-PepII lost the inhibitory activity against *C. neoformans*. However, *Mo*-CBP$_3$-PepII inhibited the activity of lactate dehydrogenase (LDH) ergosterol biosynthesis and induced the decoupling of cytochrome *c* (Cyt c) from the mitochondrial membrane. Proteomic analysis revealed a reduction in the abundance of proteins related to energetic metabolism, DNA and RNA metabolism, pathogenicity, protein metabolism, cytoskeleton, and cell wall organization and division. Our findings indicated that *Mo*-CBP$_3$-PepII might have multiple mechanisms of action against *C. neoformans* cells, mitigating the development of resistance and thus being a potent molecule to be employed in the production of new drugs against *C. neoformans* infections.

Keywords: alternative drugs; cryptococcosis; oxidative stress; synthetic peptides

Citation: Aguiar, T.K.B.; Mesquita, F.P.; Neto, N.A.S.; Gomes, F.Í.R.; Freitas, C.D.T.; Carneiro, R.F.; Nagano, C.S.; Alencar, L.M.R.; Santos-Oliveira, R.; Oliveira, J.T.A.; et al. No Chance to Survive: *Mo*-CBP$_3$-PepII Synthetic Peptide Acts on *Cryptococcus neoformans* by Multiple Mechanisms of Action. *Antibiotics* **2023**, *12*, 378. https://doi.org/10.3390/antibiotics12020378

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ANEXO J – ARTIGO CIENTÍFICO 12

Artigo científico publicado na revista *Food chemistry* (Fator de impacto: 9,2).

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journal homepage: www.elsevier.com/locate/foodchem

Use of *Calotropis procera* cysteine peptidases (CpCPs) immobilized on glyoxyl-agarose for cheesemaking

João P.B. Oliveira [a], Yandra A.P. Nascimento [a], Kímberle P.S. Amorim [b], Luciana R.B. Gonçalves [b], Larissa B.N. Freitas [a], Ayrles F.B. Silva [a], Odair P. Ferreira [c], Márcio V. Ramos [a], Pedro F.N. Souza [a], Jefferson S. Oliveira [d], Nilton A.S. Neto [a], Luciana G. Mendonça [e], Rafael A. Zambelli [e], Cleverson D.T. Freitas [a,*]

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ARTICLE INFO

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Dynamic light scattering (DLS)
Glyoxyl-agarose

ABSTRACT

Calotropis procera cysteine peptidases (CpCPs) have presented several potential biotechnological applications. Here, these enzymes were immobilized on glyoxyl-agarose (glyoxyl-CpCPs) with yields of 90–95 % and the recovered activities ranged from 10 % to 15 %, according to enzyme loadings (5, 10, 20, 40, and 50 mgBSAeq/g). Spectrophotometric assays and SDS-PAGE showed that the casein hydrolysis by glyoxyl-CpCPs was similar to soluble CpCPs. In addition, glyoxyl-CpCPs exhibited similar ratio of milk-clotting activity to proteolytic activity in comparison with soluble CpCPs and chymosin. Even after being stored for six months at 8 °C, the residual proteolytic activity of glyoxyl-CpCPs remained close to 100 %. Atomic force microscopy and dynamic light scattering techniques showed that the process of casein micelle aggregation after treatment with glyoxyl-CpCPs was very similar to its soluble form and chymosin. Glyoxyl-CpCPs performed well after five reaction cycles, producing cheeses with yield, moisture, protein, and fat similar to those produced with chymosin.

ANEXO K – ARTIGO CIENTÍFICO 13

Artigo científico publicado na revista *Antibiotics* (Fator de impacto: 4,6).

antibiotics

MDPI

Article

Giving a Hand: Synthetic Peptides Boost the Antifungal Activity of Itraconazole against *Cryptococcus neoformans*

Tawanny K. B. Aguiar [1], Ricardo M. Feitosa [1], Nilton A. S. Neto [1], Ellen A. Malveira [1], Francisco I. R. Gomes [1], Ana C. M. Costa [1], Cleverson D. T. Freitas [1], Felipe P. Mesquita [2] and Pedro F. N. Souza [1,2,*]

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Abstract: *Cryptococcus neoformans* is a multidrug-resistant pathogen responsible for infections in immunocompromised patients. Here, itraconazole (ITR), a commercial antifungal drug with low effectiveness against *C. neoformans*, was combined with different synthetic antimicrobial peptides (SAMPs), Mo-CBP3-PepII, RcAlb-PepII, RcAlb-PepIII, PepGAT, and PepKAA. The Mo-CBP3-PepII was designed based on the sequence of MoCBP3, purified from *Moringa oleifera* seeds. RcAlb-PepII and RcAlb-PepIII were designed using Rc-2S-Alb, purified from *Ricinus communis* seed cakes. The putative sequence of a chitinase from *Arabidopsis thaliana* was used to design PepGAT and PepKAA. All SAMPs have a positive liquid charge and a hydrophobic potential ranging from 41–65%. The mechanisms of action responsible for the combined effect were evaluated for the best combinations using fluorescence microscopy (FM). The synthetic peptides enhanced the activity of ITR by 10-fold against *C. neoformans*. Our results demonstrated that the combinations could induce pore formation in the membrane and the overaccumulation of ROS on *C. neoformans* cells. Our findings indicate that our peptides successfully potentialize the activity of ITR against *C. neoformans*. Therefore, synthetic peptides are potential molecules to assist antifungal agents in treating Cryptococcal infections.

Keywords: azole drugs; combined activity; membrane pore formation; synthetic antifungal peptides

Citation: Aguiar, T.K.B.; Feitosa, R.M.; Neto, N.A.S.; Malveira, E.A.; Gomes, F.I.R.; Costa, A.C.M.; Freitas, C.D.T.; Mesquita, F.P.; Souza, P.F.N. Giving a Hand: Synthetic Peptides