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Fallon dos Santos Siqueira

ESTUDOS QUÍMICOS, MOLECULARES, MICROBIOLÓGICOS E TOXICOLÓGICOS DE NOVAS MOLÉCULAS EFICAZES CONTRA BIOFILMES DE *Pseudomonas aeruginosa* E MICOBACTÉRIAS DE CRESCIMENTO RÁPIDO

Santa Maria, RS
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Orientadora: Profª Drª. Marli Matiko Anraku de Campos
Coorientador: Prof. Dr. Alencar Kolinski Machado

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Fallon dos Santos Siqueira

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Aprovado em 12 de Julho de 2021:

Marli Matiko Anraku de Campos, Dra. (UFSM-RS)
(Presidente/Orientador)

Alencar Kolinski Machado, Dr. (UFN-RS)
(Coorientador)

Danielle Ferreira Dias, Dra. (UNIFAL - MG) - Parecer

Thiago Belarmino De Souza, Dr. (UFOP- MG) - Parecer

Mirian Salvador, Dra. (UCS- RS) - Parecer

Sydney Hartz Alves, Dr. (UFSM- RS) - Parecer

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2021

DEDICATÓRIA

Dedico este trabalho a minha família:
meu marido, futuros filhos, mãe, pai e irmã.

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“O conhecimento destrói mitos”.

Autor desconhecido

RESUMO

ESTUDOS QUÍMICOS, MOLECULARES, MICROBIOLÓGICOS E TOXICOLÓGICOS DE NOVAS MOLÉCULAS EFICAZES CONTRA BIOFILMES DE *Pseudomonas aeruginosa* E MICOBACTÉRIAS DE CRESCIMENTO RÁPIDO

AUTOR: Fallon dos Santos Siqueira
ORIENTADORA: Marli Matiko Anraku de Campos
COORIENTADOR: Alencar Kolinski Machado

Sabe-se que as infecções microbianas causadas por microrganismos na forma séssil são uma questão mais desafiadora, no que diz respeito à patogênese e tratamento, do que as infecções causadas por microrganismos planctônicos. A maturação e dispersão do biofilme envolve mecanismos complexos, influenciados por estímulos genéticos e ambientais. Biofilmes de micobactérias contêm uma matriz extracelular rica em ácidos micólicos livres, que abriga as populações bacterianas, conferindo uma maior resistência dessa estrutura a fármacos anti-micobacterianos, mesmo quando expostos a altas concentrações. Além disso, espécies patogênicas como *Pseudomonas aeruginosa* e espécies do grupo das Micobactérias de Crescimento Rápido (MCR) passam a apresentar intensa comunicação celular mediada por moléculas sinalizadoras, acoplando a transcrição de genes específicos com a densidade celular bacteriana. Esse processo é conhecido como *quorum sensing (QS)* e induz ao crescimento e agrupamento tridimensional das bactérias, proporciona um aumento da aderência da estrutura à superfície e a formação de canais aquosos para a troca de água e nutrientes com o meio externo. O presente estudo visa contribuir para a elucidação e melhor compreensão dos mecanismos envolvidos na virulência e resistência associada à formação dos biofilmes de *P. aeruginosa* e MCR, bem como auxiliar na busca de novas opções terapêuticas para prevenção e erradicação de infecções associadas à formação dessas películas microbianas. Nos últimos anos a derivação de antimicrobianos clássicos vem demonstrando excelente atividade contra uma variedade de microrganismos, estimulando assim o desenvolvimento de compostos orgânicos e inorgânicos através de diferentes metódos de síntese química. Para avaliar as atividades biológicas dos derivados do sulfametoxazol, foram utilizados ensaios *in vitro* de inibição do crescimento bacteriano, tanto na forma planctônica quanto na forma de biofilme. Além disso, ensaios *in silico* de docagem molecular e *in vitro* de inibição do *QS* foram realizados para sugerir o mecanismo de ação dessas moléculas. A ação dos compostos foi observada através da Microscopia de Força Atômica e o perfil de segurança determinado em modelo de células mononucleadas de sangue periférico (PBMC), através de ensaios colorimétricos e fluorimétricos. Os resultados obtidos nesse trabalho indicaram potencial atividade antimicrobiana de complexos de sulfametoxazol-prata, bem como promissora atividade antibiofilme através da inibição do *QS*. O composto demonstrou atuar sobre os sistemas *las* e *Pqs*, principais reguladores da formação de biofilmes em *P. aeruginosa*. Além disso, uma Base de Schiff inédita derivada do sulfametoxazol foi sintetizada e caracterizada, demonstrando potencial atividade antimicobacteriana e antibiofilme frente a MCR. As duas moléculas apresentaram segurança em modelo de PBMC. Os dados dos perfis de segurança foram obtidos através de ensaios citotóxicos, genotóxicos e de modulação nitrosativa e oxidativa.

Palavras-Chave: Biofilme; Resistência; Base de Schiff; Complexos metálicos; Sulfametoxazol;

ABSTRACT

CHEMICAL, MOLECULAR, MICROBIOLOGICAL AND TOXICOLOGICAL STUDIES OF NEW EFFECTIVE MOLECULES AGAINST BIOFILMS OF *Pseudomonas aeruginosa* AND RAPIDLY GROWING MYCOBACTERIA

AUTHOR: Fallon dos Santos Siqueira
ADVISOR: Marli Matiko Anraku de Campos
CO-ADVISOR: Alencar Kolinski Machado

It is known that microbial infections caused by microorganisms in sessile form are a more challenging issue, with regard to pathogenesis and treatment, than infections caused by planktonic microorganisms. Biofilm maturation and dispersion involves complex mechanisms, influenced by genetic and environmental stimuli. Mycobacterial biofilms contain a cell wall rich in free mycolic acids, which houses bacterial populations, providing greater resistance of this structure to anti-mycobacterial drugs, even when exposed to high concentrations. In addition, pathogenic species such as *Pseudomonas aeruginosa* and species in the Rapidly Growing Mycobacteria (RGM) group are now showing intense cellular communication mediated by signaling molecules, coupling the transcription of specific genes with bacterial cell density. This process is known as *quorum sensing* (QS) and induces the growth and three-dimensional grouping of bacteria, provides an increase in the adhesion of the structure to the surface and the formation of aqueous channels for the exchange of water and nutrients with the external environment. he present study aims to contribute to the elucidation and better understanding of the mechanisms involved in virulence and resistance associated with the formation of *P. aeruginosa* and RGM biofilms, as well as assisting in the search for new therapeutic options for the prevention and eradication of infections associated with the formation of biofilms microbial. In recent years, the derivation of classic antimicrobials has demonstrated excellent activity against a variety of microorganisms, thus stimulating the development of organic and inorganic compounds through different methods of chemical synthesis. To evaluate the biological activities of sulfamethoxazole derivatives, *in vitro* bacterial growth inhibition assays were used, both in planktonic and biofilm form. Furthermore, *in silico* molecular docking assays and *in vitro* QS inhibition assays were performed to suggest the mechanism of action of these molecules. The action of the compounds was observed through Atomic Force Microscopy and the safety profile determined in a model of peripheral blood mononuclear cells (PBMC), through in vitro colorimetric and fluorimetric assays. The results obtained in this work indicated potential antimicrobial activity of sulfamethoxazole silver complexes, as well as promising antibiofilm activity through the inhibition of *QS*. The compound has been shown to act on the *las* and *Pqs* systems, the main regulators of biofilm formation in *P. aeruginosa*. In addition, an unprecedented Schiff's Base derived from sulfamethoxazole was synthesized and characterized, demonstrating potential antimicrobial and antibiofilm activity against RGM. The two molecules were safe in peripheral blood monocucleated cells. The safety profiles data were obtained through cytotoxic, genotoxic and nitrosative and oxidative modulation tests.

Key words: Biofilm; Resistance; Schiff's base; Metal complexes; Sulfamethoxazole;

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

ARTIGO CIENTÍFICO 1

MANUSCRITO I

MANUSCRITO II

LISTA DE TABELAS

REVISÃO BIBLIOGRÁFICA

ARTIGO CIENTÍFICO I

MANUSCRITO I

MANUSCRITO II

SUMÁRIO

APRESENTAÇÃO

A presente **Tese de Doutorado** foi redigida de acordo com as normas do Manual de Dissertações e Teses (MDT) da Universidade Federal de Santa Maria (UFSM). O trabalho conta com **3 produções científicas,** que apresentam e discutem os resultados obtidos. O estudo está redigido contendo as seções **INTRODUÇÃO**, **OBJETIVOS, REVISÃO DE LITERATURA, PRODUÇÕES CIENTÍFICAS**, **DISCUSSÃO** e **CONCLUSÃO**. A seção **INTRODUÇÃO** conta com uma breve revisão e apresentação do tema. A secão **REVISÃO DE LITERATURA** fornece um compilado da literatura científica, a fim de contextualizar o tema e fornecer conteúdo necessário para a compreensão da relevância do tema e dos resultados obtidos. As metodologias utilizadas para a realização dos estudos, os resultados e as discussões pertinentes dos mesmos, bem como as referências utilizadas encontram-se na seção **PRODUÇÕES CIENTÍFICAS,** que é composta pelo **Artigo Científico I, Manuscrito I** e **Manuscrito II**. Todos os trabalhos estão formatados de acordo com o periódico aos quais foram publicados e/ou submetidos. Os tópicos **DISCUSSÃO** e **CONCLUSÕES** apresentam interpretações e comentários gerais acerca do conteúdo abordado nesta tese. As **REFERÊNCIAS** referem-se somente às utilizadas no item **INTRODUÇÃO e REVISÃO DE LITERATURA**. Em anexo ao conteúdo desse trabalho é possível encontrar os pareceres de aprovação do Comitê de Ética e Pesquisa da Universidade Federal de Santa Maria (UFSM), a carta de aceite para publicação do **Artigo científico e** os comprovantes de submissão do **manuscrito 1** e **manuscrito 2**.

1. INTRODUÇÃO

De alto impacto para a saúde pública, a formação de biofilmes causa grande preocupação devido à baixa suscetibilidade aos antimicrobianos, sua variabilidade genética e a capacidade de colonizar diferentes superfícies (FLEMMING et al., 2016). Microrganismos patogênicos são capazes de formar biofilmes em dispositivos médico-hospitalares e tecidos vivos, podendo causar infecções crônicas graves, atuando como potenciais fontes de infecção (TRENTIN et al., 2013). Os biofilmes são estruturas que apresentam uma variável distribuição de células e agregados, aos quais constituem um modo protegido de crescimento em ambientes severos e de estresse para o microrganismo (LEWIS, 2001).

Diferentes fatores podem ser considerados no estudo da resistência microbiana associada aos biofilmes, como a penetração restrita de antimicrobianos, a diminuição da taxa de crescimento dos microrganismos e a expressão de genes de resistência (LEWIS, 2012). A formação e a dispersão do biofilme são processos controlados a nível genético e por sinais do ambiente. O "*quorum sensing*" aparece como o principal regulador nos biofilmes, principalmente em gram-negativos (FAZLI et al., 2014).

Já em microrganismos do gênero *Mycobacterium*, a formação de biofilme é uma estratégia bem-sucedida de sobrevivência para organismos muito hidrofóbicos e um nível elevado de ácidos micólicos livres está intimamente associado a maturação de biofilmes de micobactérias (OJHA et al., 2008). Embora a capacidade de desenvolvimento de biofilme não seja uma característica geral presente em todos os isolados clínicos de MCR, inúmeras espécies de MCR já foram associadas a biofilmes em dispositivos biomédicos, sistemas de abastecimento de água e ambientes hospitalares, apresentando grande impacto na saúde humana (MARTÍN-DE-HIJAS et al., 2009; PITOMBO et al., 2009).

Desta forma, é de extrema relevância a compreensão dos fatores metabólicos e genéticos envolvidos na formação do biofilme das diferentes espécies, bem como a relação desses processos no estudo de novas opções terapêuticas para a prevenção e tratamento desse tipo de infecção. Nesse contexto, a derivação de antimicrobianos clássicos e a coordenação de antimicrobianos a metais representa uma alternativa promissora na tentativa de encontrar compostos efetivos contra biofilmes (LEMIRE et al., 2013).

Recentemente, compostos de coordenação contendo sulfonamidas como ligantes vem destacando-se e demonstrando um amplo espectro de atividade antibacteriana, inclusive contra microrganismos gram-negativos, gram-positivos e do *gênero Mycobacterium* (SIQUEIRA et al. 2018; AGERTT et al., 2016; MIZDAL, 2017).

Além disso, Bases de Schiff derivadas de aminas e aldeídos aromáticos têm uma ampla variedade de aplicações (SILVA et al, 2011). Muitas bases de Schiff biologicamente importantes foram relatadas na literatura, possuindo atividades antibacteriana, antifúngica, anti-inflamatória, anticonvulsivante e antitumoral (ZOUBI, 2013). Esses achados aumentam o interesse no estudo de novas moléculas através da derivação química de antimicrobianos clássicos, principalmente no que diz respeito a moléculas que sirvam de abordagem terapêutica contra biofilmes microbianos, visto que as infecções microbianas causadas por microrganismos na forma séssil são uma questão mais desafiadora, no que diz respeito a patogênese e tratamento, do que as infecções causadas por microrganismos planctônicos (FLEMMING, 2016; ZOUBI, 2013; SILVA et al, 2011).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a atividade antimicrobiana e anti-biofilme de moléculas derivadas do sulfametoxazol.

2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Sintetizar e realizar a caracterização química de uma nova base de Schiff derivada do sulfametoxazol.

2.2.2 Avaliar a atividade inibitória de uma nova base de Schiff derivada do sulfametoxazol frente a cepas padrões e isolados clínicos de micobactérias na forma planctônica.

2.2.3 Avaliar a atividade anti-biofilme de uma nova base de Schiff derivada do sulfametoxazol frente a biofilmes formados por micobactérias.

2.2.4 Avaliar o perfil de segurança de uma nova base de Schiff derivada do sulfametoxazol através de ensaios citotóxicos e genotóxivos em modelo de cultura celular.

2.2.5 Reproduzir a síntese de complexos metálicos derivados do sulfametoxazol.

2.2.6 Determinar a atividade de complexos de Sulfametoxazol com Au, Cd, Ni, Hg, Ag, e Cu frente a cepas padrões e isolados clínicos de *Psudomonas aeruginosa*.

2.2.7 Avaliar a atividade antibiofilme de complexos metálicos do sulfametoxazol frente a biofilmes formados por cepas de *Pseudomonas aeruginosa*.

2.2.8 Avaliar o perfil de segurança do complexo sulfametoxazol-Ag através de ensaios citotóxicos e genotóxivos em modelo de cultura celular.

2.2.9 Avaliar a atividade antifúngica dos complexos metálicos do sulfametoxazol.

3 REVISÃO DE LITERATURA

3.1 RESISTÊNCIA MICROBIANA

Embora todos os microrganismos possam adquirir resistência, as bactérias resistentes são, atualmente, segundo a Organização Mundial da Saúde (OMS), uma das três ameaças mais importantes à saúde pública do século XXI (WHO, 2018). As bactérias, como grupo ou espécie, não são necessariamente suscetíveis ou resistentes a qualquer agente antimicrobiano em particular. Os níveis de resistência podem variar muito dentro dos grupos bacterianos relacionados. A suscetibilidade e a resistência são geralmente medidas em função da concentração inibitória mínima (CIM), que se apresenta como a concentração mínima de uma droga que inibirá o crescimento da bactéria (MARTINEZ, 2014).

A resistência microbiana é resultado de uma imensa plasticidade genética apresentada por diferentes patógenos bacterianos (CHUNG; KHANUM, 2017). Ao desencadear respostas específicas, os microrganismos tornam-se resistentes por meio de um ou mais mecanismos, isolados ou em conjunto, que podem ter origem natural ou adquirida (ALI; RAFIQ; RATCLIFFE, 2018).

A resistência natural pode ser intrínseca, quando está sempre expressa na espécie, ou induzida, quando os genes ocorrem naturalmente na bactéria, mas só são expressos em níveis de resistência após exposição a um antibiótico. Redução da permeabilidade da membrana externa, atividade natural de bombas de efluxo e atividade de múltiplas bombas induzidas são mecanismos bacterianos comuns de resistência intrínseca, que não estando associadas a transferência horizontal de genes (REYGAERT, 2018). Na tabela 1 podemos observar exemplos de bactérias com resistência intrínseca a diferentes antimicrobianos.

A resistência adquirida pode ser observada em situação em que a bactéria sofre mutações temporárias ou permanentes em seu prório DNA cromossômico, geralmente durante a etapa de sua reprodução, ou em condições em que bactérias adquirem material genético através da transferência horizontal de gênes (transformação, transposição e conjugação). A conjugação é um processo que requer o contato célula a célula via pili ou adesinas da superfície celular, através do qual o DNA é transferido da célula doadora para a célula receptora. A transformação caracteriza a transmissão de genes de resistência mediada por plasmídeo e é a rota mais comum para aquisição de material genético externo. A transdução ocorre através de bacteriófados que podem transferir DNA de uma célula doadora a uma

célula receptora, contudo a transmissão por bacteriófago é bastante rara (ARZANLOU; CHAI; VENTER, 2017; VON WINTERSDORFF et al., 2016).

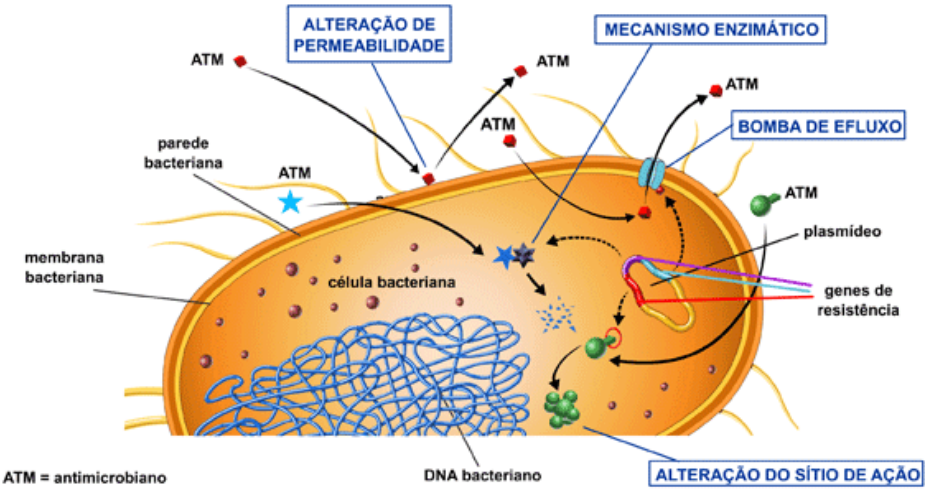
Tabela 1 – Exemplos de resistência intrínseca apresentada por diferentes microrganismos.

Microrganismo	**Resistência Intrínseca**
Bacteroides (anaeróbicos)	Aminoglicosideos, β-lactamicos e quinolonas
Gram-positvos	Aztreonam
Enterococci	Aminoglicosideos, cefalosporinas e lincosamidas
Gram-negativos	Glicopeptideos e lipopeptideos
Escherichia coli	Macrolídeos
Klebsiella spp.	ampicilina
Serratia marcescens	macrolideos
Pseudomonas aeruginosa	Sulfonamidas, ampicilina, cefalosporinas de primeira e segunda geração, cloranfenicol e tetraciclinas
Stenotrophonomas maltophilia	Aminoglicosidios, β- lactaminos, carbapenemicos e quinolonas
Acinetobacter spp.	Ampicilina e glicopeptideos

Fonte: Adaptado de REYGAERT (2018).

Os mecanismos principais de resistência dos microrganismos dividem-se em quatro principais categorias: (1) alteração da permeabilidade ou absorção do medicamento; (2) expressão de bombas de efluxo, levando ao efluxo de medicamentos ativos; (3) inativação do fármaco; e (4) modificação do alvo do medicamento, através da redução do número ou da afinidade aos locais de ligação. É interessante observar que a resistência intrínseca pode limitar a absorção da droga, inativar moléculas e levar ao efluxo de substâncias antibacterianas, enquanto que os mecanismos de resistência adquiridos utilizados por diferentes microrganismos podem ser modificação do alvo da droga, inativação da molécula e efluxo através de múltiplas bombas (ANVISA, 2007). A Figura 1 mostra a representação dos principais mecanismos de resistência aos antimicrobianos.

Figura 1 – Mecanismos de resistência antimicrobiana.



Fonte: ANVISA (2007).

3.2 BIOFILMES BACTERIANOS

Os microrganismos podem apresentar-se na forma planctônica, dispersos no meio, ou na forma séssil, formando comunidades com diferentes graus de complexidade, denominadas biofilmes. Os biofilmes são grupos de microrganismos cercados por um polímero autoproduzido, aderidos a uma superfície, que conferem alta resistência dos microrganismos a antimicrobianos (WOOLHOUSE; FARRAR, 2014; SINGH et al., 2017). Estima-se que, mesmo uma estrutura séssil altamente sensível, sem qualquer composição genética inerente à resistência a antibióticos, desenvolve uma resistência 1000 vezes superior quando comparado ao microrganismo disperso na forma livre (DAVENPORT; CALL; BEYENAL, 2014; VENKATESAN; PERUMAL; DOBLE, 2015).

Na última década, há uma crescente apreciação de que o modo de crescimento do biofilme é o estilo de vida mais comum adotado pelas bactérias (HOTTERBEEKX et al., 2017) e estima-se que não exista superfície, sintética ou natural, que não sirva de base para a formação dessas comunidades (MORENO, 2010). A colonização de superfícies para a formação de biofilmes é uma estratégia universal das bactérias para a sobrevivência e pode ocorrer naturalmente (SANTOS et al., 2018).

Microrganismos patogênicos são capazes de formar biofilmes em dispositivos médico-hospitalares e tecidos vivos, podendo causar infecções crônicas graves, atuando como potenciais fontes de infecção (TRENTIN et al., 2013). De acordo com o “National Institutes

of Health", dos Estados Unidos da América, biofilmes estão associados, em algum grau, a 80% de todas as infecções no mundo, incluindo endocardites, otites, prostatites, periodontites, conjuntivites, vaginites, infecções relacionadas à fibrose cística, sendo colonizadores de implantes biomédicos, tais como cateteres venosos, arteriais e urinários, dispositivos intrauterinos, lentes de contato e próteses (NIH, 2018; HOIBY et al., 2011). Na tabela 2 observamos as doenças associadas a formação de biofilmes, nos diferentes sistemas do corpo humano.

Tabela 2 - Doenças associadas ao biofilme de diferentes sistemas corporais e seus órgãos afetados.

Sistema do corpo	Órgão afetado	Doença
Auditivo	Ouvido médio	Otite média
Cardiovascular	Valvas cardiacas	Endocardite infecciosa
	arterias	arterioesclerose
Digestivo	Glandulas salivares	Sialolitíase (cálculos do ducto salivar)
	Vesícula biliar	Febre tifóide recalcitrante e predisposição para cânceres hepatobiliares
	Intestino delgado e grosso	Doença inflamatória intestinal e câncer colorretal
Tegumentar	Pele e tecido subjacente	Infecções de feridas
Reprodutivo	Vagina	Vaginose bacteriana
	Útero	Endometrite crônica
	Glândulas mamárias	Mastite
Respiratório	Cavidade nasal e seios paranasais	Rinossinusite crônica
	Garganta, ou seja, faringe com amígdalas e adenóides e laringe com cordas vocais	Faringite e laringite
	Vias aéreas superiores e inferiores	Coqueluche (tosse convulsa) e outras infecções por *Bordertella* sp. / Fibrose Cística
Urinário	Próstata	Prostatite bacteriana crônica
	Uretra, bexiga, uretra, rins,	infecções do trato urinário

Fonte: Adaptado de VESTBY et al. (2020).

Além disso, estima-se que não haja superfície sintética que não seja viável a adesão de microrganismos para o desenvolvimento de biofilme (SANTOS et al., 2018. Na tabela 3, apresentamos as diferentes superfícies médico-hospitalares onde biofilmes de diferentes microrganismos são capazes de aderir.

Tabela 3 – Formação de biofilmes em diferentes dispositivos médicos.

Dispositivos médicos	Bactérias formadoras de biofilme	Referências
Lentes de contato	*P. aeruginosa, S. aureus, S. epidermidis, S. saprophyticus, Klebsiella spp.*	El-Ganiny et al., 2017
Cateter venoso central	*Staphylococcus* CN, *S. aureus*, Enterobactérias	Gominet et al., 2017
Cateter urinário	*S. aureus, Enterococcus faecalis, P. aeruginosa*	Murugan et al., 2016
Cateteres de diálise peritoneal	*S. epidermidis, P. acnes, S. warneri, S. lugdunensis, R. mucilaginosa*	Pihl et al., 2013
Válvulas cardíacas mecânicas	*Streptococcus spp., S. aureus, S. epidermidis*, Bacilos Gram-negativos, *Enterococcus*	Jamal et al., 2018
Shunts do líquido cefalorraquidiano	*S. aureus, S. epidermidis, Enterococcus faecalis, Enterococcus faecium*	Bayston et al., 2012
Implantes mamários	*S. epidermidis, Coagulase-negative Staphylococci, Propionibacterium acnes*	Pajkos et al., 2003; Rieger et al., 2013
Implantes ortopédicos	*S. aureus, S. epidermidis, P. aeruginosa, E. coli, S. haemolyticus*	Arciola et al., 2015
Implantes dentários	Cocos Gram positivos, *Actinomyces spp.*, Bactérias orais Gram-negativas anaerobicas	Dhir, 2013; Veerachamy et al., 2014
Próteses vocais	*S. aureus, P. aeruginosa, Klebsiella spp., Enterobacterspp., R. dentocariosa e Proteus spp.*	Somogyi-Ganss et al., 2017
Marcapassos cardíacos	*S. aureus, S. epidermidis*	Santos et al., 2011
Dispositivos intrauterinos	*E. coli, Streptococcus agalactie, S. aureus, Enterococcus faecalis, Lactobacillus spp., Prevotella spp., Porphyromonas spp., Bacteroides, Fusobacterium spp.*	Pal et al., 2005
Stents biliares	*Pseudomonas, Citrobacter, Klebsiella, Staphylococcus, Enterococcus, Aeromonas, Proteus, Enterobacter*	Vaishnavi et al., 2018

Fonte: Adaptado de VESTBY et al. (2020).

3.2.1 Composição e Ciclo Biológico dos biofilmes bacterianos

Em relação a sua composição, os biofilmes podem ser definidos como grupos de microrganismos, sejam ou não da mesma espécie, cercados por um polímero autoproduzido de substâncias poliméricas extracelulares (SPE), aderidos a uma superfície (VERT et al., 2012; FLEMMING; WINGENDER, 2010). São constituídos, por água, microrganismos, SPE (hidrogel constituído de polissacarídeos, proteínas, lipídeos, DNA, RNA e íons) – que são responsáveis por características morfológicas, biológicas e físico-químicas dos biofilmes- e resíduos do ambiente colonizado, aderidos a uma superfície sólida (GUPTA et al, 2016).

A matriz polimérica é produzida pelos próprios microrganismos ao qual o biofilme está incorporado e é responsável pela morfologia, estrutura, coesão, integridade funcional dos biofilmes e a sua composição determina a maioria das propriedades físico-químicas e biológicas dos biofilmes (MACHADO, 2005). Na tabela 4, destacamos algumas das funções desempenhadas pelas EPS no biofilme bacteriano.

Um modelo de processo de cinco estágios foi sugerido para o desenvolvimento de biofilmes. As etapas envolvidas foram (i) a fixação inicial das células à superfície, (ii) a fixação irreversível das células, (iii) o desenvolvimento inicial da arquitetura do biofilme, (iv) a maturação do biofilme e (v) a dispersão de células individuais. O ciclo biológico para a formação de um biofilme passa por diferentes etapas: contato, adesão microbiana a superfície (reversível e irreversível), formação de microcolônias, maturação e desprendimento/dispersão (MA et al., 2017). Após o contato e a adesão dos microrganismos a superfície, à medida que a população microbiana em sua proximidade aumenta, as bactérias se organizam em microcolônias dispostas em monocamadas, ocorrendo um aumento na produção, na liberação e na detectação de moléculas sinalizadoras que ativam e transcrevem genes específicos que alteram o fenótipo de bactérias planctônicas para o fenótipo de biofilme (TRENTIN et al., 2013). As etapas da formação do biofilme podem ser observadas na figura 2.

Tabela 4 - Funções de substâncias poliméricas extracelulares (EPS) no biofilme bacteriano.

Função	Relevância para biofilmes
Adesão	Permite os passos iniciais da colonização de superfícies pelas células planctônicas e a fixação dos biofilmes as superfícies.
Agregação	Permite comunicação entre as células, a imobilização temporária de populações bacterianas, o desenvolvimento de densidades celulares elevadas e reconhecimento de célula a célula.
Coesão dos biofilmes	Forma uma rede de polímero, mediando a estabilidade mecânica do biofilme e, através da estrutura das EPS, determina a arquitetura do biofilme e permite a comunicação célula a célula.
Retenção de água	Mantém um microambiente altamente hidratado em torno dos microrganismos do biofilme, levando à tolerância a dessecação em ambientes deficientes em água.
Barreira protetora	Confere resistência às defesas do hospedeiro durante infecções e confere tolerância a vários agentes antimicrobianos, como desinfetantes e antimicrobianos.
Atividade enzimática	Permite a digestão de macromoléculas exógenas para aquisição de nutriente e a degradação de EPS estruturais, permitindo a liberação de células dos biofilmes.
Fonte de nutrientes	Fornece suprimento de carbono, nitrogênio e fósforo para utilização pelo biofilme.
Exportação de componentes celulares	Libera material celular como resultado da atividade metabólica.
Troca de informação genética	Facilita a transferência horizontal de genes entre as células do biofilme.

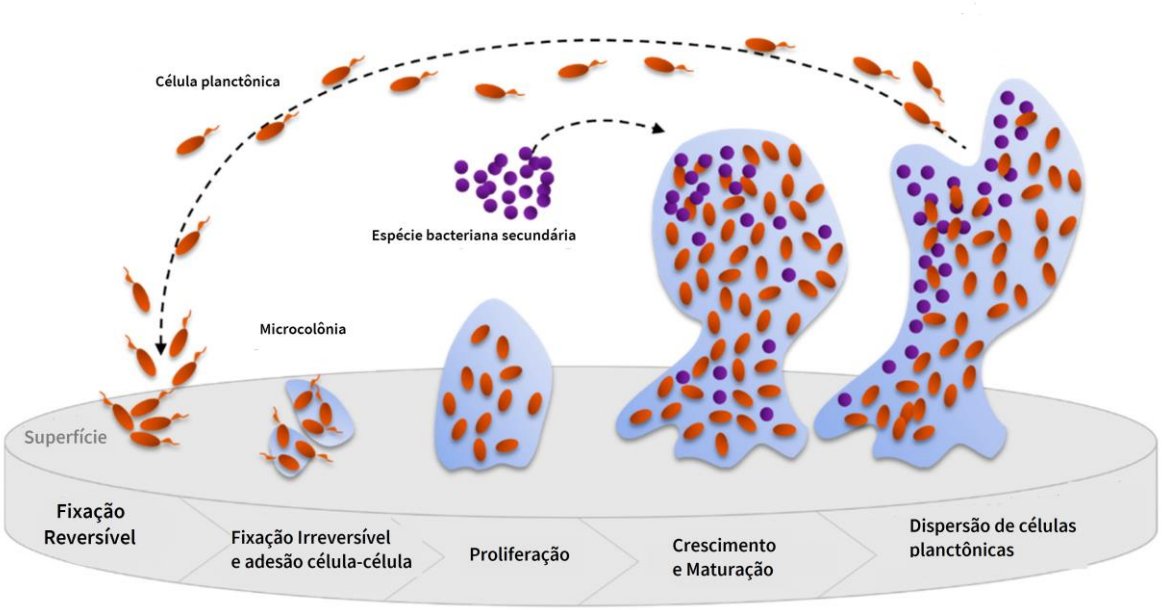
Fonte: Adaptado de FLEMMING; WINGENDER (2010).

Essas moléculas são autoindutoras e responsáveis pela regulação da motilidade, virulência, produção de SPE e pela formação e maturação do biofilme. Esse processo de intensa comunicação celular mediado por moléculas sinalizadoras, que acopla a transcrição de genes específicos com a densidade celular bacteriana, é conhecido como *quorum sensing* (QS) e é encontrado em muitas espécies de bactérias patogênicas (BHARATI; CHATTERJI, 2013).

A ativação desses genes induz ao crescimento e agrupamento tridimensional das bactérias, proporciona um aumento da aderência da estrutura à superfície e a formação de canais aquosos para a troca de água e nutrientes com o meio externo. Sendo assim, o biofilme pode permanecer aderido à superfície por longo período ou então se dispersar, liberando microrganismos na forma planctônica para que iniciem um novo ciclo de formação de biofilme (TAMASHIRO et al., 2013).

Acredita-se que a fixação das células à superfície durante os primeiros estágios conduza a alterações fisiológicas das células e seja acompanhada pela produção de EPS, fixando as células à superfície. Após o processo de fixação inicial, as células se dividem e formam microcolônias. Durante o processo de maturação, as células continuam se dividindo e produzem mais EPS. Nesse estágio, o biofilme pode se estender da superfície, construindo uma estrutura tridimensional. Após a maturação, as células podem deixar o biofilme para colonizar novas regiões (MAUNDERS; WELCH, 2017).

Figura 2 – Formação do biofilme bacteriano.



Fonte: adaptado de MAUNDERS; WELCH (2017).

3.2.2 Resistência microbiana associada a formação dos biofilmes bacterianos

Quando o biofilme está aderido e maduro, do ponto de vista clínico, tornam-se ineficientes as terapias desenvolvidas para erradicar microrganismos planctônicos (SCHMIDT et al., 2015). Além disso, sabe-se que infecções associadas a biofilmes tornam-se um desafio diagnóstico e terapêutico, quando comparadas a infecções causadas por microrganismos planctônicos (ISLAM et al., 2012). Isso ocorre porque, microrganismos inclusos em biofilmes, apresentam resistência aumentada a antimicrobianos, o que dificulta a erradicação dessa estrutura e desencadeia em doenças persistentes (SINGH et al., 2017).

Independentemente de se o biofilme é formado por uma espécie específica de patógeno ou por diversas espécies, os microrganismos ficam protegidas do ambiente externo e comunicam-se através de caminhos de transdução de sinal (*quorum sensing*), levando a mudanças na expressão de genes, aumentando a virulência e acelerando a aquisição de resistência a diferentes antimicrobianos (SCHROEDER et al., 2017).

Isolados ou em combinação, diferentes fatores são responsáveis pela resistência elevada a antimicrobianos. Os mecanismos propostos para o aumento da resistência podem ser atribuídos a alguns parâmetros que incluem: (I) A produção de SPE; (II) A população heterogênea com variada taxa de crescimento; (III) A alta frequência de mutação devido a transmissão horizontal de genes e a (IV) a falha no reconhecimento dos biofilmes pela defesa imunológica humana (LEWIS, 2012; TRENTIM et al., 2013).

3.2.2.1 Produção de SPE

A produção de SPE reduz expressivamente a capacidade de penetração de antimicrobianos e biocidas na estrutura do biofilme. Essa matriz polimérica retém a maior quantidade dos agentes químicos agressores, formando uma barreira física que impede a atuação dessas substâncias sobre as células. Além disso a matriz exopolimérica pode ligar-se a antimicrobianos, modificando suas características e inativando-os. Isso proporcionará resistência efetiva para células microbianas do biofilme contra grandes moléculas, como os antimicrobianos, lisozimas e sistema complemento (TRENTIN et al., 2013; LEWIS, 2012; LEWIS 2001).

3.2.2.2 *População heterogênea*

A população heterogênea com variada taxa de crescimento e suscetibilidade que constitui o biofilme é, sem dúvida, outro fator a ser considerado quando se estuda a resistência de biofilmes bacterianos (STEWART; FRANKLIN, 2008). Na base estrutural do biofilme as células estão em estado de latência metabólica, diferentemente das células da camada mais externa, que se apresentam em alta atividade. Tendo em vista que a maioria dos antimicrobianos são mais eficazes contra microrganismos em alta atividade metabólica, o baixo metabolismo dessas células persistentes garante a sua resistência ao tratamento. Sendo assim, é possível erradicar uma grande parte da população do biofilme após a terapêutica, porém a fração de células dormentes não atingidas seguirão atuando como uma fonte de reinfecção (LEWIS, 2012; LEWIS, 2001).

A organização dos biofilmes bacterianos permite que uma diversidade de microrganismos interaja, em estreita proximidade. Isso permite a troca de metabólitos, moléculas de sinalização, material genético e compostos defensivos. Além disso, a heterogeneidade, tal como na forma de células com diferentes capacidades metabólicas ou gradientes fisiológicos, oferece oportunidades de cooperação entre os microrganismos (FLEMMING et al., 2016).

3.2.2.3 Transmissão horizontal de genes

Decorrente da proximidade dos microrganismos em biofilmes, as bactérias podem apresentar alta frequência de mutação devido a transmissão horizontal de genes, adquirindo através de plasmídeos os genes responsáveis pela codificação de inúmeros mecanismos de resistências a variados antimicrobianos. Como exemplo, pode-se enumerar a produção enzimática, a modificação do alvo primário de atividade antimicrobiana e a produção de bombas de efluxo (MADSEN et al., 2012; SOUSA et al., 2015).

Além de ser capaz de adquirir rapidamente um fenótipo resistente a múltiplos fármacos, a combinação de mecanismos de resistência levou os cientistas a descrever os biofilmes como tendo resistência para uma vasta gama de classes de antimicrobianos (CAMPOCCIA et al., 2010).

3.1.2.4 Falha no reconhecimento dos biofilmes

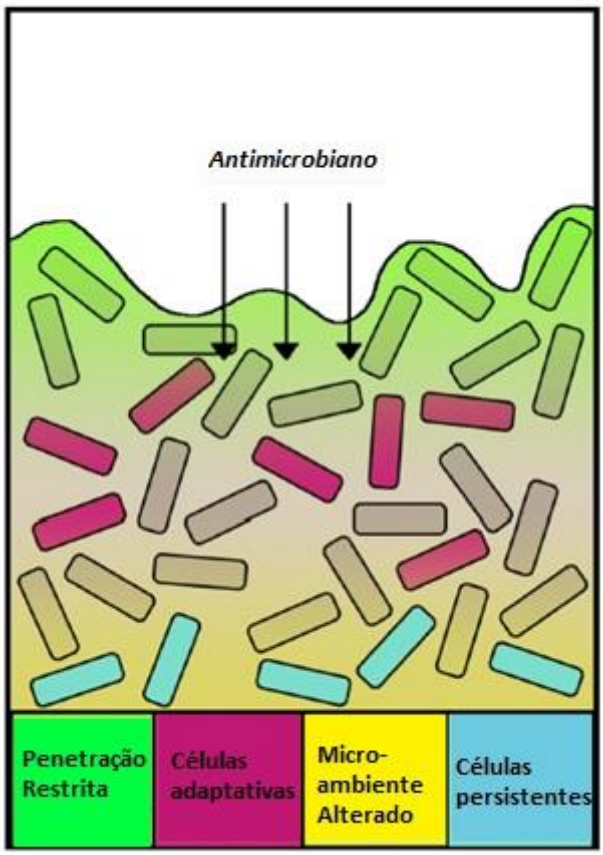
Isso ocorre devido à presença de grande quantidade de SPE que mascara os microrganismos, impedindo assim a ação de anticorpos, radicais livres e fagócitos, dificultando expressivamente o combate à infecção pelo sistema imunitário do hospedeiro (BRYERS, 2008).

As principais características de biofilmes *in vivo* são bactérias agregadas, que toleram a defesa do hospedeiro e concentrações elevadas de agentes antimicrobianos (BJARNSHOLT, et al., 2018). Mesmo em indivíduos que possuem o sistema imune competente, infecções que apresentam biofilmes como a base são raramente resolvidas apenas com intervenção medicamentosa. Os tecidos adjacentes ao biofilme podem sofrer danos colaterais por complexos imunes e pela atividade de neutrófilos. Testes de suscetibilidade em modelos de biofilme *in vitro* demonstraram a sobrevivência das bactérias de biofilmes após o tratamento com antimicrobianos em concentrações centenas ou mesmo milhares de vezes maiores que a CIM em populações planctônicas (STEWART; FRANKLIN, 2008).

In vivo, os antimicrobianos podem suprimir sintomas de infecção por erradicar as bactérias de livre flutuação, que são liberadas pela população séssil, mas não conseguem erradicar as células bacterianas ainda incorporadas aos biofilmes. No momento em que se suspende a quimioterapia antimicrobiana, o biofilme pode agir como um nicho para a recorrência da infecção (STEWART; COSTERTON, 2001). Por esta razão, as infecções causadas por biofilmes tipicamente mostram sintomas recorrentes, após ciclos repetidos de tratamento com antimicrobianos, até que a população séssil seja removida cirurgicamente do corpo (GHOSH, et al., 2017).

O mecanismos de resistência a antibióticos mediada pela formação do biofilmes (Figura 3) faz com que os antimicrobianos penetrem lentamente no biofilme (verde) e algumas células do biofilme expressem uma resposta e se adaptem ao estresse, permitindo a sobrevivência em condições adversas (rosa). Além disso, o microambiente alterado (amarelo) dentro do biofilme induz ao crescimento lento de bactérias, o que reduz a absorção de antibióticos. Já as células persistentes (azul), além de tornarem-se tolerantes a múltiplas drogas, funcionam como uma potencial fonte de re-infecção, uma vez que permanecem inclusas nas partes mais profundas do biofilme, onde quase que a totalidade dos antimicrobianos tem baixa ou nenhuma capacidade de alcançar (PANGA et al., 2020).

Figura 3 - Mecanismos de resistência a antibióticos mediada pela formação de biofilmes.



Fonte: Adaptado de PANGA et al. (2020).

3.3 ***Pseudomonas aeruginosa***

As espécies de *Pseudomonas* são ubíquuas no meio ambiente e podem causar doenças em animais e plantas. Das diferentes espécies de *Pseudomonas*, *P. aeruginosa* destaca-se por ser agente etiológico de diversas infecções humanas (CDC, 2016).

Embora cause apenas infecções localizadas leves em pacientes saudáveis, como otite média ou otite externa, é um dos principais responsáveis por diversas infecções em pacientes imunocomprometidos. Além de ser a principal causa de mortalidade em pacientes com fibrose cística, causa pneumonia bacteriana, endocardite, meningite, infecções de feridas de queimaduras e sépse. Todas essas infecções estão associadas a alta mortalidade (LEE; YOON, 2017).

Nas UTIs brasileiras é o terceiro patógeno mais frequente, estando envolvido em 30% das infecções de corrente sanguínea relacionadas ao uso de cateteres (NEVES et al., 2011), enquanto que nos Estados Unidos da América (EUA) é o agente causador mais comum de

infecções hospitalares e a segunda mais causa comum de pneumonia associada ao ventilador mecânico (GELLATLY et al., 2013). Em hospitais do Brasil, a produção de metalobetalactamases do tipo SPM-1, a superexpressão de bombas de efluxo e a perda de porina do tipo PprD podem justificar o grande número de isolados clínicos com fenótipos multiresistêntes, bem como explicar os altos índices de resistência aos aminoglicosídeios e carbapenêmicos. (NEVES et al., 2011).

Esse bacilo gram negativo é conhecido por produzir numerosos e variados fatores de virulência. Os fatores de virulência que *P. aeruginosa* produz, contribuindo para sua patogenicidade, incluem flagelos, pili tipo IV, alcalino protease, elastase , lipopolissacarídeo, fosfolipase, exotoxina, pioverdina, piochelina, piocianina, Pseudomonas quinolona sinal (PQS) e muito mais (LEE; YOON, 2017) .

Quando comparamos o genoma de *P. aeruginosa* com o genoma de microrganismos como *Mycobacterium tuberculosis*, *Bacillus subtilis* e *Escherichia coli*, observamos que esse bacilo gram negativo apresenta o maior gene em relação a outros procariotos já sequenciados. Esse grande número de genes reguladores permite grande versatilidade e uma adaptação contínua a diferentes condições ambientais, conferindo a esse microrganismo uma grande facilidade no desenvolvimento de resistência aos antimicrobianos (KLOCKGETHER et al., 2011).

A resistência intrínseca de *P. aeruginosa* a torna tolerante a maioria de antibióticos, devido a permeabilidade restrita da sua membrana externa, a produção de enzimas como β-lactamases e a expressão de bombas de efluxo (BREIDENSTEIN et al., 2011). Além disso, através da resistência adquirida, *P. aeruginosa* desenvolve-se facilmente em cepas multiresistentes, dificultando a erradicação desse patógeno oportunista e levando ao desenvolvimento de casos de infecções persistentes (HENRICHFREISE et al., 2007).

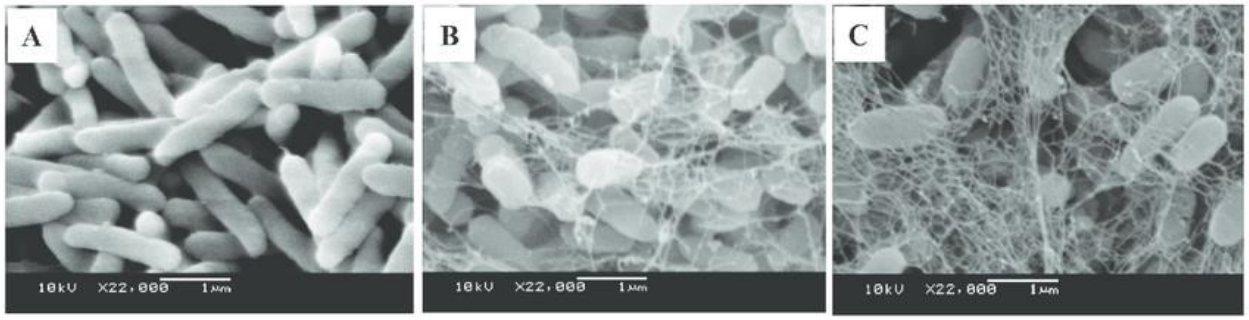
3.3.1 Biofilmes formados por *P. Aeruginosa*

Devido sua capacidade de em desenvolver-se na forma séssil, *P. aeruginosa* tem se tornado a principal causa de infecções relacionadas a assistência a saúde em hospitais. O biofilme de *P. aeruginosa* altera a expressão gênica padrão, levando a um aumento na taxa de transferência horizontal de genes, desencadeando em uma resistência elevada aos agentes químicos externos (MULCAHY et al., 2010; HENGZHUANG et al., 2012).

Infecções agudas provocadas por *P. aeruginosa* estão associadas a células planctônicas enquanto que as infecções crônicas estão associadas ao modo séssil de crescimento

(MAUNDERS; WELCH, 2017). Esse microrganismo tem a capacidade de formar biofilme em uma série de superfícies biomédicas sintéticas e em tecidos vivos, como cateteres, tubos, sondas, pulmões, feridas e queimaduras (WALKER; MOORE, 2014). Além disso, isolados clínicos multirresistentes apresentam alta capacidade de formar biofilmes (SHARMA et al., 2014). O biofilme de *P. aeruginosa* é rico em uma EPS, composta de polissacarídeos, proteínas e DNA extracelular. Essa substância mantém a célula unida, o que é necessário para a comunicação celular. Também permite a formação de estruturas tridimensionais que dão às bactérias maior acesso aos nutrientes e às vantagens da vida multicelular (PANGA et al., 2020).

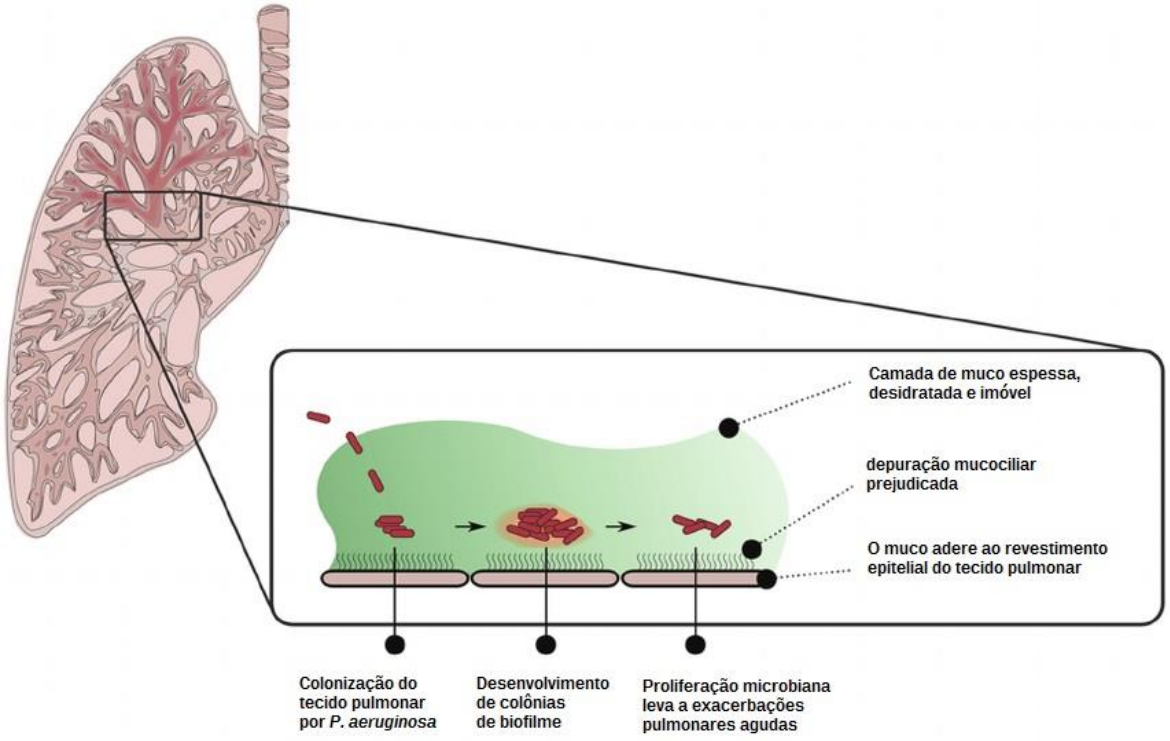
Figura 4 – Processo de formação de biofilme, observado por microscopia eletrônica de varredura, de *P. aeruginosa* em 6 (A), 24 (B) e 48 (C) horas, com progressiva produção de EPS, a 37ºC.



FONTE: adaptado de TRENTIN et al. (2013).

Em pacientes com FC, *Pseudomonas aeruginosa* causa infecções crônica, principalmente devido a formação de biofilme e forma biofilme na superfícies das células epiteliais (TAYLOR et al., 2014). A Figura 5, apresenta as etapas de formação do biofilme *in vivo*, em pulmão de pacientes com fibrose cística.

Figura 5 – Fomação de biofilme de *P. Aeruginosa* em tecido pulmonar de pacientes com Fibrose Cística.



Fonte: adaptado de LUND-PALAU et al. (2016).

Pseudomonas aeruginosa utiliza o sistema quórum sensing para regular a virulência e a formação de biofilmes (CHRISTIAEN et al., 2014). Esse sistema caracteriza um processo de intensa comunicação celular, mediada por moléculas sinalizadoras, que acopla a transcrição de genes específicos com a densidade celular bacteriana. (BHARATI; CHATTERJI, 2013).

Considerando que a secreção de moléculas sinalizadoras aumenta à medida que a densidade de bactérias aumenta, quando uma molécula sinalizadora atinge um certo limiar, a molécula liga-se ao receptor correspondente da molécula e ativa o receptor. O receptor ativado então ativa os reguladores transcricionais relevantes para sintetizar polissacarídeos extracelulares, fatores tóxicos, alginatos, entre outros, fazendo com que as bactérias formem biofilmes (ZHAO et al., 2020).

Las e Rhl são dois grandes sistemas do QS de *P. aeruginosa*, que são responsáveis pela síntese da n-acyl homoserina lactona (AHL), N-(3-oxododecanoyl)-L-homoserina lactona ($_3$O-C$_{12}$-HSL) e N-butanoyl-L-homoserina lactona (C$_4$-HSL) (GLESSNER et al., 1999; RUTHERFORD; BASSLER, 2012; ZHAO et al., 2020). $_3$O-C$_{12}$-HSL e C$_4$-HSL se ligam e ativam seus fatores de transcrição cognato *LasR* e *RhlR*, respectivamente, induzindo a

formação de biofilme e expressão de vários fatores de virulência incluindo elastase, proteases, piocianina, lectinas, raminolipídeos e toxinas (RUTHERFORD; BASSLER, 2012).

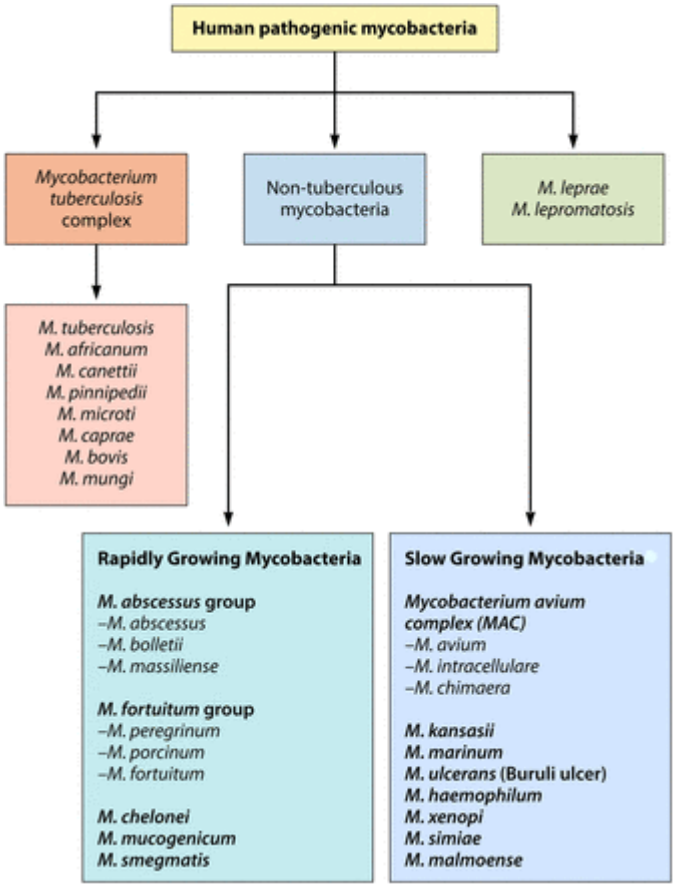
3.4 MICOBACTÉRIAS DE CRESCIMENTO RÁPIDO

Micobactérias são bacilos aeróbicos, álcool ácido resistentes, não formadores de esporos, que apresentam uma morfologia reta ou ligeiramente curva e suportam variações de 30°C a 45°C na temperatura e de 6 a 8 no pH (SILVA, 2013).

As Micobactérias Não Tuberculosas (MNT) apresentam uma patogenicidade variável e existem mais de 140 espécies de MNT conhecidas, das quais 50 espécies foram identificadas como patógenos oportunistas em humanos (KIM et al, 2014). Sendo oriundas do ambiente, as infecções causadas pelas MNT não são contagiosas e ocorrem de forma oportunista (MARINHO et al., 2008). Suas fontes incluem desde reservatórios como água, solos, animais e produtos lácteos até equipamentos médicos como endoscópio e soluções cirúrgicas. São responsáveis por infecções de pele e tecidos moles, infecções pulmonares, crônicas e progressivas, linfadenite cervical pediátrica e infecções disseminadas, especialmente em casos avançados de SIDA (WILDNER et al., 2011).

As MNT foram classificadas em 1959 por Runyon de acordo com o tempo do seu crescimento, a semelhança de suas características morfológicas, e a capacidade de produzir pigmentos após a exposição à luz e/ou obscuridade. A partir desse estudo, os grupos foram denominados I, II, III e IV. Os grupos I, II e III são as espécies de crescimento lento, que levam mais de uma semana para crescer, e o grupo IV constitui as espécies de crescimento rápido, que se desenvolvem em tempo inferior a uma semana (MOTA, 2011). A classificação, em grupos, dos microrganismos do gênero *Mycobacterium*, pode ser observada na figura 6.

Figura 6 - Classificação das principais micobactérias patogênicas.



Fonte: Adaptado de FRANCO-PAREDES et al. (2019).

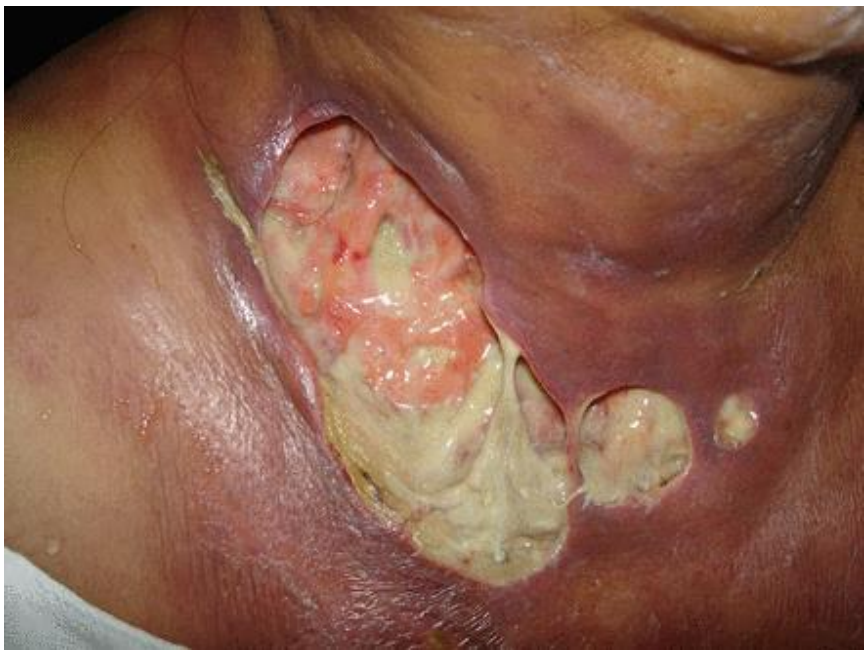
As doenças que as MNT ocasionam são denominadas de micobacterioses, independentemente de qual espécie é o agente causador da patologia (BRASIL, 2008). O tratamento das micobacterioses é complexo, uma vez que elas são naturalmente resistentes ou têm pouca sensibilidade aos fármacos tuberculostáticos, sendo que a sensibilidade difere entre as espécies. Muitos são os esquemas utilizados para o tratamento. Em um tratamento eficiente, é necessária inicialmente a identificação do agente causal, para que, de acordo com a sua sensibilidade, sejam escolhidos os fármacos a serem utilizados (WILDNER et al., 2011). Para alguns fármacos e espécies, não há correlação da suscetibilidade in vitro com a resposta efetiva aos antimicrobianos in vivo (GRIFFITH, 2010).

Os antimicrobianos indicados para o tratamento de infecções causadas por MCR são amicacina, azitromicina, cefoxitina, ciprofloxacino, claritromicina, clofazimina, doxiciclina, imipenem, linezolida, minociclina, sulfametoxazol, tigeciclina e trobamicina (GRIFFITH et

al., 2007). Para o acompanhamento e terapêutica de pacientes com micobacterioses é importante o estudo do perfil de suscetibilidade a antimicrobianos. O Clinical and Laboratory Standards Institute (CLSI) recomenda como padrão-ouro a microdiluição em caldo para a realização do teste de suscetibilidade (TS). O teste pode ser aplicado em qualquer micobactéria com significado clínico: isolados de sangue, líquidos corporais estéreis, tecidos, e amostras oriundas de lesões de pele e tecidos moles (CLSI, 2011).

Isolada pela primeira vez em 1950 em paciente com infecção e abscessos subcutâneos no joelho, *M. abcessus* é a espécie de MCR mais comum em doenças pulmonares (FRANCO-PAREDES et al.,2019). Já *M. fortuitum*, *M. chelonae*, *M. massiliense* e *M. boletti* são as principais micobactérias associadas a infecções cutâneas. A FIGURA 7 mostra um adulto com infecção por *Mycobacterium abscessus*, apresentando escrofuloderma com extensa destruição de tecido nas áreas cervical e supraclavicular direita.

Figura 7 – Infecção cutânea causada por *M. abcessus*.



Fonte: Adaptado de FRANCO-PAREDES et al. (2019).

3.4.1 Biofilmes formados por Micobactérias de Crescimento Rápido

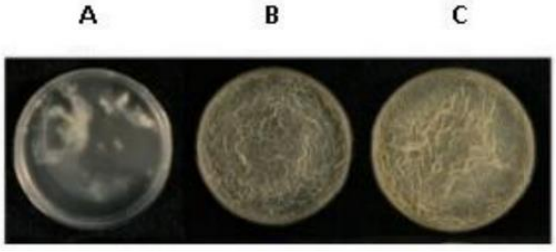
Os microrganismos desse gênero apresentam uma parede celular composta de quatro camadas bem delimitadas de uma superfície cérea, sendo a mais interna de peptideoglicano, que confere rigidez a parede micobacteriana. A segunda camada composta de arabinoglicano

e a camada seguinte por grande quantidade de um componente presente apenas em bactérias do gênero *Mycobacterium*, os ácidos micólicos. A camada mais externa contém uma grande variedade de lipídeos, incluíndo glicolipídeos, sulfolipídeos, fenolglicolipídeos, peptideoglicolipídeos e lipoarabinomanano (DULBERGE et al., 2020). Essa complexidade de lipídeos constituintes confere a esses microrganismos uma grande resistência à água, dessecação, a antimicrobianos e a desinfetantes (JOHNSON; ODELL, 2014).

A resistência em micobactérias é conferida por sua parede celular altamente lipofílica e existem vários mecanismos que controlam o conteúdo da parede celular, o que inclui um baixo número de porinas, ampla gama de bombas de efluxo, biotransformação ativa por enzimas citossólicas e induzíveis mecanismos de resistência (VAN et al., 2012). A presença de uma parede celular impermeável, constituída por uma espessa camada cérea, atua como barreira física e química, devido a sua hidrofobicidade. O envelope celular e o seu alto teor lipídico, até 60% do peso seco da bactéria, é considerado o principal fator que contribui à sua baixa permeabilidade (NESSAR et al. 2012).

Os biofilmes de micobactérias contêm uma matriz extracelular rica em ácidos micólicos livres, que abriga as populações de bactérias, conferindo uma maior resistência do biofilme aos fármacos nocivos às micobactérias, apesar da exposição a altos níveis de antimicrobianos (OJHA et al., 2008). A figura 8 mostra o desenvolvimento *in vitro* de biofilme micobacteriano.

Figura 8 – Biofilme formado *in vitro*, por *M. smegmatis* em meio de cultura M63. Biofilme em superfície ar-líquido com 3 dias (a), 4 dias (b) e 5 dias (c) de incubação.



Fonte: Adaptadp de OJHA; HATFULL (2007).

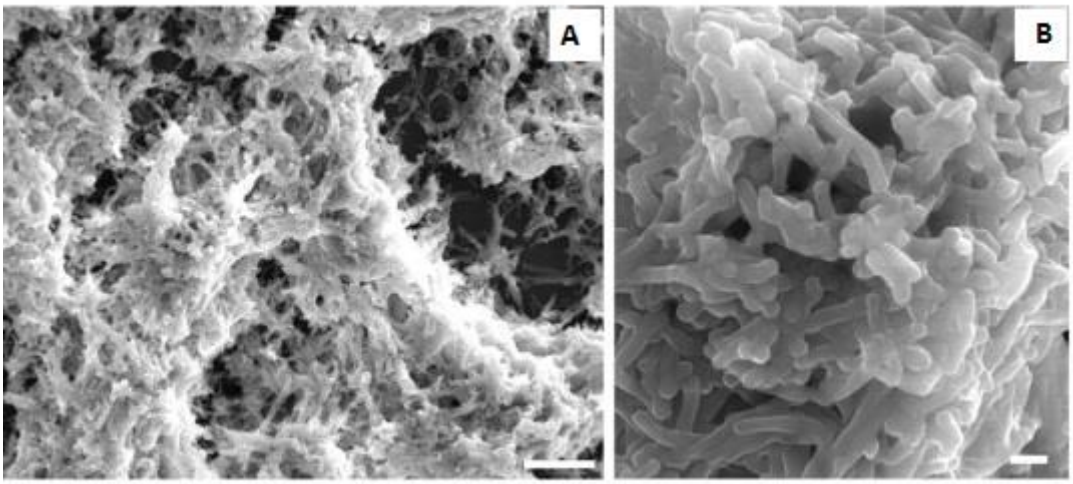
Embora a capacidade de desenvolvimento de biofilme não seja uma característica geral presente em todos os isolados clínicos de MCR, inúmeras espécies de MCR já foram associadas a biofilmes em dispositivos biomédicos e sistemas de água em ambientes hospitalares, apresentando grande impacto na saúde humana (MARTÍN-DE-HIJAS et al., 2009; PITOMBO et al., 2009). Além disso, estudos já relataram que biofilmes de

micobactérias contribuem para a patogênese das infecções sanguíneas relacionadas ao uso de cateteres (EL HELOU et al., 2013).

Pacientes com micobacterioses decorrente de implantes de próteses articulares contaminadas com MCR apresentaram falha no tratamento medicamentoso, obtendo cura total somente após remoção das próteses associadas a biofilmes maduros (EID et al., 2007). Em outro caso, o exame microscópico de uma válvula aórtica protética retirada de um paciente permitiu a identificação de uma estrutura composta por MCR, juntamente a uma fina camada de matriz de fibrina associada a macrófagos CD38 e um baixo número de plaquetas, consistentes em um biofilme. Neste caso, a endocardite da válvula protética teve como agente etiológico *M. fortuitum* (BOSIO et al., 2012).

Sousa e colaboradores (2018), mostraram o desenvolvimento cinético da biomassa de *M. smegmatis, M. fortuitum* e *M. chelonae* durante 5 dias. Todas as cepas de MNT foram capazes de montar biofilmes em placas de cultura de células. *M. fortuitum* e *M. smegmatis* demonstratam aumentos semelhantes na biomassa ao longo do tempo. Já *M. chelonae* apresentou um crescimento mais lento, por apresentar uma fase de adaptação mais longa. Uma visão panorâmica de biofilme de *M. smegmatis* (A) e um detalhe de uma estrutura tipo torre (B), característico de biofilmes desse gênero, foram obtidos por microscopia eletrônica de varredura e podem ser observados na figura 9.

Figura 9 – Microscopia eletrônica de varredura de biofilme formado por *M. smegmatis*.

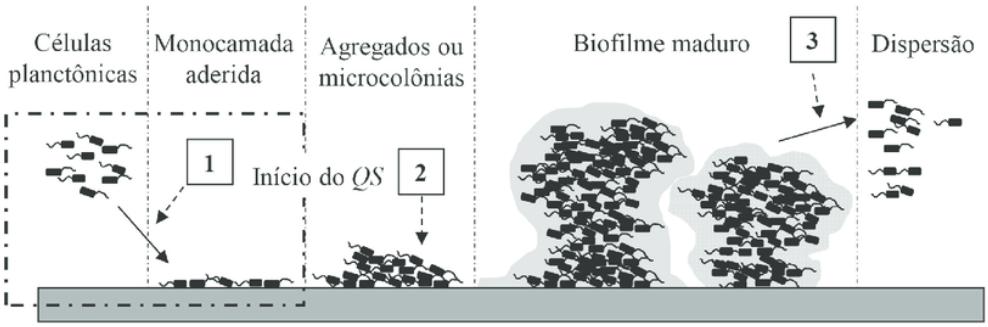


Fonte: adaptado de SOUSA et al. (2018).

3.5 NOVAS ESTRATÉGIAS PARA O TRATAMENTO DE INFECÇÕES POR BIOFILME

A alta resistência dos biofilmes ao tratamento antimicrobiano requer o desenvolvimento de terapêuticas específicas para prevenção e tratamento de infecções relacionadas a esse ecossistema (FUENTE-NÚNEZ et al., 2013). Atualmente, a inibição da formação de biofilmes via bloqueio da adesão bacteriana à superfície (etapa 1) ou o rompimento da comunicação celular bacteriana - *Quorum sensing* (etapa 2) e a erradicação ou tratamento de biofilmes já formados (etapa 3), surgem como principais alvos no desenvolvimento de novas moléculas (Figura 10).

Figura 10 – Alvos de combate a biofilmes bacterianos.



Fonte: Adaptado de MACEDO; ABRAHAM (2009).

Na tabela 5, podemos conhecer algumas das principais estratégias, que vem ganhando interesse na comunidade clínica e científica, para tratamento e prevenção de infecções associadas por biofilmes microbianos.

Tabela 5 – Estratégias de prevenção e erradicação de biofilmes bacterianos.

ESTRATÉGIA	OBJETIVO	EXEMPLO
Perturbação da aderência celular às superfícies	Modificar a superfície externa desses materiais para dificultar a adesão e colonização Microbiana.	Revestindo de superfícies médicas com partículas de prata, que demonstraram algum sucesso em limitar a formação de biofilmes de superfície (Mijnendonckx et al., 2013).
Aumento dispersão de células em biofilmes	Aumentar a dispersão de células planctônicas do biofilme, através da utilização de agentes de dispersão que serão usados em combinação com antibióticos convencionais para erradicar as células livres.	Óxido Nítrico, que atua como um sinal para desencadear a dispersão do biofilme. Mecanicamente, foi sugerido que a sinalização através de NO dispara a sinalização de detecção de quorum e reduz a síntese de c-di GMP promovendo a dispersão de células do corpo maduro biofilme (Barraud et al., 2009).
Digestão direta da matriz extracelular de biofilmes.	tratamento direto de biofilmes com moléculas capazes de degradas a EPS e deixar os microrganismos inclusos no biofilme mais suscetivies	Tratamento direto de biofilmes com desoxirribonúcleo ase (DNase) leva à digestão enzimática do eD NA que é um importante componente estrutural da matriz extracelular de biofilmes (Shakir et al., 2012).
Inibidores de quorum sensing	Interferência na comunicação bacteriana, reduzindo assim a mudança do fenótipo planctônico para o fenótipo de biofilme ao bloquear moléculas sinalizadoras que ativam e transcrevem genes específicos envolvidos na maturação e manutenção do biofilme.~	Inibidores naturais ou sintéticos para sistemas Las e Rhl, que são capazes de reduzir a atividade da acil-homoserina lactona (AHL) sintase, inibir a produção de AHL, degradar AHLs ou competir pela ligação de Receptores de AHL (Kalia, 2013).
Terapia de bacteriófago	Uso de fagos para matar bactérias em infecções de biofilme com base na proximidade de bactérias células dentro de um biofilme que permite altos títulos virais locais e rápido disseminação de infecções fágicas em uma colônia de biofilme (Ryan et al., 2011)	-

Fonte: elaborado pelo autor.

3.6 ANTIMICROBIANOS CLÁSSICOS E DERIVAÇÃO QUÍMICA

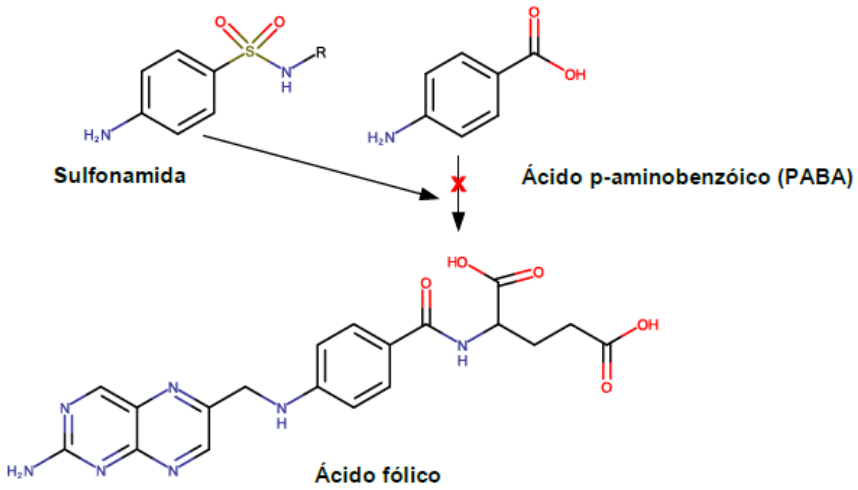
3.6.1 Sulfonamidas

As sulfonamidas são antibacterianos, bacteriostáticos, que atuam pela inibição competitiva da enzima dihidropteroato sintetase, devido sua semelhança estrutural com o Ácido-p-aminobenzóico (PABA). Dessda maneira, não ocorre a síntese de hidrofolato, um intermediário da rota de produção do ácido fólico, componente essencial para a síntese de purinas de DNA bacteriano. A similaridade estrutural entre as sulfonamidas, o PABA e o ácido fólico está representada na Figura 11 (NUNES, 2015).

Além disso, foram os primeiros fármacos sistêmicos eficazes utilizados para o tratamento de infecções humanas (GARCÍA-GALÁN et al., 2008) O grupo das sulfonamidas compreende seis drogas principais: sulfanilamida, sulfisoxazol, sulfacetamida, ácido para-aminobenzóico, sulfadiazina e sulfametoxazol, sendo as duas últimas de maior importância clínica (ANVISA, 2013).

O sulfametoxazol é uma sulfonamida de amplo espectro, considerada de grande importancia no controle de infecções causadas por diferentes patógenos, incluindo MCR, como *M. abscessus* e *M. fortuitum* (BRASIL, 2008) e a combinação do sulfametoxazol ao trimetropim já foi uma alternativa muito sugerida no tratamento de infecções causadas por microrganismos do gênero Mycobacterium. A indicação clínica para o sulfametoxazol isolado também persiste, porém é constatado que sua atividade diminui expressivamente quando o agente infeccioso se apresenta na forma de biofilmes ou no interior de macrófagos do hospedeiro (FLORES et al., 2016; DAVIES et al., 2014).

Figura 11 – Semelhanças estruturais entre sulfonamidas, PABA e ácido fólico.



Fonte: Adaptado de NUNES (2015).

No estudo de Flores e colaboradores (2016) foi demonstrado que, após tratamento com sulfametoxazol, biofilmes de MCR apresentaram quase nenhuma modificação em sua película microbiana já formada, apresentando alto índice de resistência ao fármaco. Quando tratando de sua capacidade de inibir a formação de biofilmes de MCR, o sulfametoxazol apresentou leve atividade apenas contra *M. fortuitum*. Além disso, a utilização clínica do sulfametoxazol, isolado ou associado ao Trimetropim, vem sendo diminuída e esse fármaco tem sido utilizado na prática clínica com grande restrição. Isso é decorrente do desenvolvimento de resistência a esse agente e a sua rápida disseminação, fazendo com que novos antimicrobianos substituam essa sulfonamida na maioria de suas indicações clínicas (HOUVINEN, 2001).

3.6.2 Compostos de coordenação

Já é constatado que metais são capazes de interromper a formação de biofilmes, exercem atividade sinérgica com antimicrobianos e biocidas, inibem diferentes vias metabólicas e, dessa forma, são capazes de matar bactérias resistentes a diversos antimicrobianos através do estresse oxidativo, disfunção de proteínas ou danos a membrana celular (LEMIRE et al., 2013; MIZDAL, 2017). Compostos de coordenação utilizando antimicrobianos como ligantes e explorando as características intrínsecas dos íons metálicos vem permitindo a obtenção de uma ampla variedade de complexos a serem explorados como potenciais agentes antimicrobianos. Recentemente, compostos de coordenação contendo

sulfonamidas como ligantes vem destacando-se e demonstrando um amplo espectro de atividade antibacteriana frente a gram-negativos e frente a microrganismos do gênero *Mycobacterium* (ROCHA et al., 2011; AGERTT, 2016).

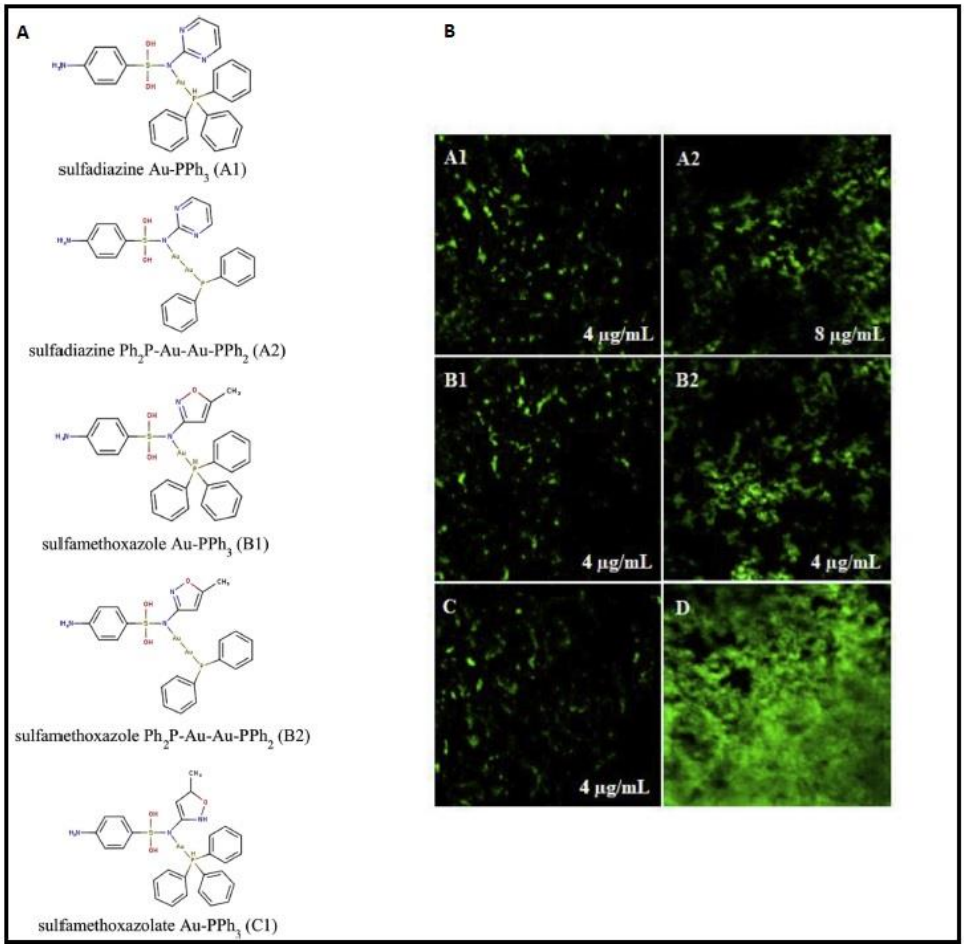
No estudo de Marques (2007) foram sintetizados compostos a partir da interação do sulfametoxazol com diferentes metais, demonstrando seus comportamentos coordenativos e sua atividade antimicrobiana. Os resultados demonstraram que os complexos com Au e Ag foram expressivamente mais ativos frente a *E. coli* e *P. aeruginosa*. Neste caso, os compostos foram 32 vezes mais ativos que o fármaco não coordenado.

Agertt e colaboradores (2013) avaliaram a atividade dos mesmos compostos e o sinergismo com o Trimetropim frente a *M. smegmatis*, demostrando que as Concentrações Inibitórias Mínimas (CIM) obtidas foram expressivamente baixas, ressaltando a potencialização dos compostos inorgânicos como uma alternativa promissora no combate à resistência ao sulfametoxazol. Além disso, o composto coordenado ao Au apresentou alto sinergismo ao trimetropim e potencial atividade antimicobacteriana frente a 9 isolados clínicos de *M. tuberculosis* na forma planctônica e frente a Micobacterias de Crescimento Rápido (MCR) (AGERTT et al, 2016).

Complexos metálicos de prata, cobre, mercúrio e cádmio contendo sulfametoxazol sintetizados pelo mesmo grupo de pesquisa tiveram atividade inibitória em biofilmes de *E. coli* (MIZDAL et al, 2017). Além disso, Siqueira e colaboradores (2018) destacou em seu estudo a atividade antibiofilme do sulfametoxazol complexado com ouro frente a MCR. O composto foi capaz de inibir completamente a formação do biofilme micobacteriano.

No estudo de Mizdal e colaboradores (2018), foi avaliada a atividade antibacteriana de sulfadiazina Au-PPh_3, sulfadiazina Ph_2P-Au-Au-PPh_2, sulfametoxazol Au-PPh_3, sulfametoxazol Ph_2P-Au-Au-PPh_2 e sulfametoxazol Au-PPh_3 contra *Pseudomonas aeruginosa.* A atividade antibacteriana da sulfonamida foi testada a através do ensaio MIC, e análise quantitativada inibição do biofilme. A observação da formação do biofilme foi acompanhada com microscopia de fluorescência. Os compostos apresentaram notável inibição da formação de biofilme de *P. aeruginosa.* Na figura 12 estão representadas as estruturas químicas e nomenclatura das sulfonanamidas complexadas com ouro (A), bem como a inibição de biofilme de *Pseudomonas aeruginosa* PA01 detectado por microscopia de fluorescência (B), após 24 h, tratados com sulfadiazina Au-PPh_3 (A1), sulfadiazina Ph_2P-Au-Au- PPh_2 (A2), sulfametoxazol Au-PPh_3 (B1), sulfametoxazol Ph_2P-Au-Au-PPh_2 (B2), sulfametoxazolato Au-PPh_3 (C1), e sem tratamento (D) também estão representados na figura adaptada do autor.

Figura 12 – Estrutura química e formação do biofilme de *P. aeruginosa* observada por microscopia de fluorescência



Fonte: MIZDAL et al. (2018).

3.6.3 Bases de Schiff

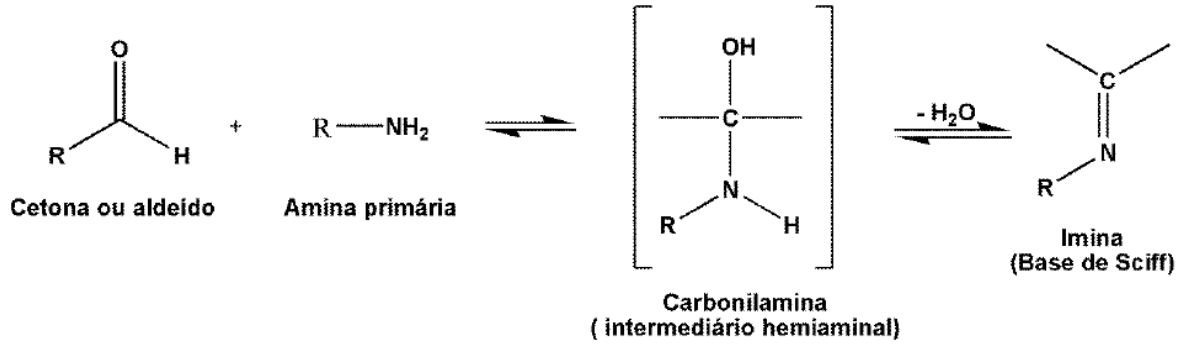
Bases de Schiff derivadas de aminas e aldeídos tem se apresentado como alternativa promissora no campo da química de coordenação e da bio-inorgânica (HUSSAIN et al., 2016). Bases de Schiff com atividades antibacterianas e antifúngicas tem desencadeado interesse nos pesquisadores como estruturas biologicamente ativas (HUSSAIN et al., 2016; HICKEY, J. et al., 2011). Ligantes contendo Bases de Schiff (imina) apresentam um grupo funcional formado por uma ligação dupla carbono-nitrogênio (C=N) e a sua obtenção ocorre através da reação de condensação entre uma amina primária (R-N_H2) e uma cetona ou aldeído. As Bases de Schiff são utilizadas na química de coordenação, pois apresentam afinidade por íons metálicos levando na maioria dos casos, a formação de complexos polidentados com variado número de coordenação e geometria (SILVA et al, 2011).

Esstas iminas apresentam características estruturais importantes aos quais estacam-se a possibilidade de atuação como grupo aceptor de hidrogênio, doador de densidade eletrônica para anéis aromáticos, e ainda o aumento da permeabilidade celular, devido a sua maior lipofilicidade quando comparado ao grupo amino correspondente (BREAK et al., 2013).

A primeira rota sintética de obtenção de grupos imínicos foi realizada por Hugo Schiff em 1864, e por essa razão estes compostos, também são chamados de bases de Schiff. Esta síntese ocorre através da condensação entre compostos carbonílicos (aldeídos e cetonas) e aminas primárias (SCHIFF, 1864).

A obtenção da base de Schiff parte da adição nucleofílica da amina sobre o carbono eletrofílico do aldeído, assim obtendo um intermediário hemiaminal, além de água como subproduto. O esquema de síntese pode ser observado na figura 13.

Figura 13 - Mecanismo de formação da Base de Schiff.



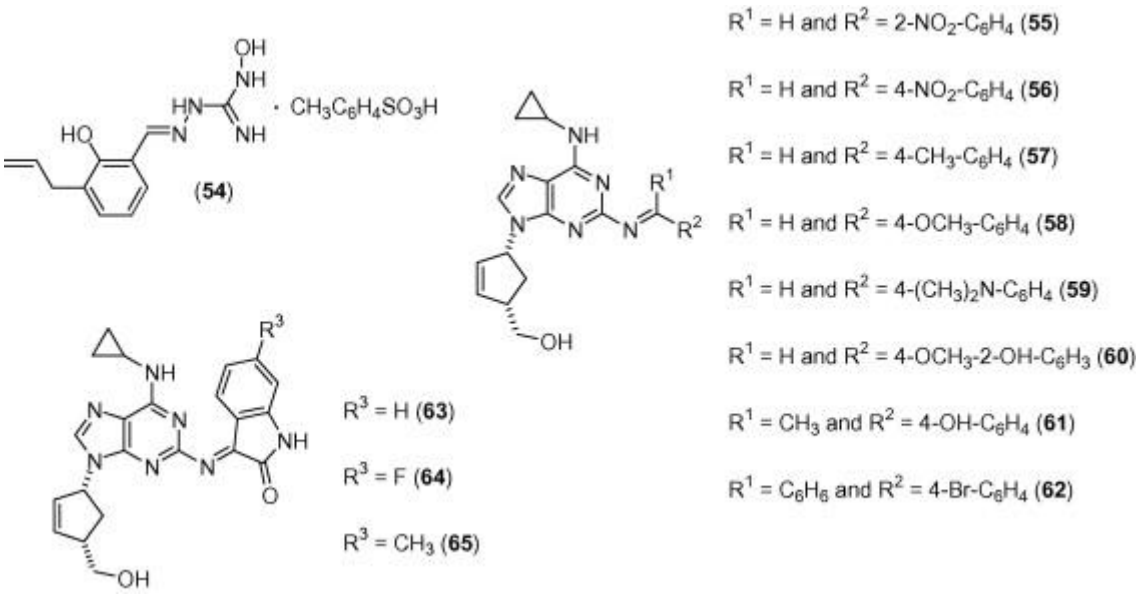
R = H ou CH_3

Fonte: Adaptado de PELLEGRIN (2019).

Bases de schiff derivadas de diferentes produtos naturais e sintéticos apresentam atividade antimalárica, sendo capaz de agir com potencial promissor frente a 4 espécies de *Plasmodium* (*P. falciparum, P. vivax, P. ovale e* P. *malariae*), atividade antibacteriana frente a *Mycobacterium tuberculosi Pseudomonas fluorescence, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Vibrio choleraenon, Enterococcus faecalis, Proteus shigelloides, Micrococcus luteus, Streptococcus epidermidis, Bacillus cereus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Micrococcus flavus* e *Sarcina lutea*, atividade antifúngica frente a *Aspergillus fumigatus, Aspergillus flavus, Trichophyton mentagrophytes, Penicillium marneffei, Trichophyton rubrum, Epidermophyton floccosum Microsporum audouinii, Microsporum gypseum, Candida albicans, Aspergillus niger, Cryptococcus neoformans, T.mentagrophytes, E. floccosum* e *Histoplasma capsulatuma* (SILVA, et a., 2011).

Sriram e colaboradores (2006) relataram a síntese e a atividade antiviral das bases de Schiff derivadas do abacavir (Figura 14). Esses compostos são uma nova série de pró-drogas abacavir. O abacavir é um análogo de nucleosídeo capaz de inibir a atividade da transcriptase reversa. É usado para tratar o vírus da imunodeficiência humana (HIV) e AIDS. Os compostos foram significativamente eficazes contra o vírus da imunodeficiência humana tipo 1 (HIV-1).

Figura 14 – Bases de Schiff sintéticas derivadas do abacavir.



Fonte: adaptado de SRIRAM et al. (2006).

Embora as pesquisas sobre o assunto sejam incipientes, recentemente vem crescendo uma série de relatórios divulgando os efeitos das bases de Schiff nos patógenos de interesse clínico. Os compostos de base de Schiff têm se mostrado promissores para o projeto de agentes antimicrobianos mais eficientes avanços neste campo exigirão análises das relações estrutura-atividade das bases de Schiff, bem como a elucidação do mecanismo de ação desses compostos.

4 PRODUÇÕES CIENTÍFICAS

4.1 Artigo Científico 1

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Molecular docking, quorum quenching effect, anti-biofilm activity and safety profile of silver-complexed sulfonamide on *Pseudomonas aeruginosa*

Fallon dos Santos Siqueira[a], Camilla Filippi dos Santos Alves[a], Alencar Kolinski Machado[b], Josiéli Demétrio Siqueira[c], Thiago dos Santos[d], Caren Rigon Mizdal[a], Kelly Schneider Moreira[c], Diogo Teixeira Carvalho[e], Pauline Codernonsi Bonez[a], Carolina Gonzalez Urquhart[a], Roberto Christ Vianna Santos[a], Michele Rorato Sagrillo[b], Davi Fernando Back[c*], Lenice de Lourenço Marques[f], Marli Matiko Anraku de Campos[a*]

[a]*Graduate Program in Pharmaceutical Sciences, Federal University of Santa Maria, Avenue Roraima 1000, zip code: 97105-900, Santa Maria, Brazil.*
[b]*Graduate Program in Nanoscience, Franciscan University, Santa Maria, Brazil, Andradas Street, 1614, zip code: 97010-032, Santa Maria, Brazil*
[c]*Graduate Program in Chemistry, Federal University of Santa Maria, Avenue Roraima 1000, zip code: 97105-900, Santa Maria, Brazil.*
[d] *Research Center on Natural and Synthetic Products, University of Sao Paulo, Rectory Street, zip code:05508-220, Ribeirão Preto, Brazil*
[e] *Pharmaceutical Chemistry Research Laboratory, Federal University of Alfenas, Gabriel Monteiro da Silva Street 700, zip code: 37130-001, Alfenas, Brazil.*
[f]*Farroupilha Federal Institute, São Vicente do Sul Campus, Zip code: 97420-000, São Vicente do Sul, Brazil*

**Corresponding Authors*: Marli Matiko Anraku de Campos and Davi Fernando Back
E-mail: laboratoriomycobacteriologia@gmail.com
Federal University of Santa Maria
Avenue Roraima 1000 - Zip code: 97105-900
Santa Maria - RS –Brazil
*Corresponding Author Email: laboratoriomycobacteriologia@gmail.com

ABSTRACT

Microbial infections caused by microorganisms in sessile form are known to be a more challenging issue than infections caused by planktonic microorganisms. *Pseudomonas aeruginosa* is an opportunistic pathogen and biofilm-forming agent. This species presents intense cellular communication mediated by signaling molecules. This process is known as quorum sensing (QS) and induces the transcription of specific genes that favors cell density growth and three-dimensional bacteria grouping. In this context, the discovery of compounds capable of inhibiting the action of the QS signaling molecules seems to be a promising strategy against biofilms. This work aimed to evaluate the antibiofilm action and the *in vitro* safety profile of a sulfamethoxazole-Ag complex. The obtained results indicated potential antibiofilm activity through QS inhibition. *In silico* tests have shown that the compound acts on the las and pqs systems, which are the main regulators of biofilm formation in *P. aeruginosa*. Additionally, the molecule proved to be safe for human peripheral blood mononuclear cells.

Key words: quorum-sensing; resistance; inorganic compounds; sulfamethoxazole.

Introduction

Biofilms play a very important role in the virulence of the microorganism, delaying the penetration of drugs and increasing resistance to antimicrobial drugs (Bouyahya et al. 2017; Sharma et al. 2013). This has become a public health problem, resulting in several deaths from persistent infections and a high financial demand for health services. In the process of biofilm formation, an intracellular signaling occurs. This mechanism is called quorum sensing (QS) and has a regulatory role on the bacterial population density. These interactions activate or repress different bacteria genes, inducing biofilm production from the synthesis of extracellular polysaccharides, alginates, toxic factors, and other essential elements (Zhao et al. 2020).

In theory, 80% of the microorganism species currently known are capable of forming biofilms. Therefore, in this study, *Pseudomonas aeruginosa*, an opportunistic Gram-negative bacterium often associated with nosocomial infections related to biofilm, was highlighted. The QS in this bacterium modulates the expression of various genes related to motility, biofilm formation, immune evasion, iron scavenging, and antibiotic resistance (Jakobsen et al. 2013). It includes, for example, the PqsABCDE/PqsR system, which influences the production of extracellular DNA (eDNA), an important polymeric matrix component that covers the biofilm structure, and also the RhlI/RhlR signalizing that enhances colonization and microbial resistance (Pérez-Pérez et al. 2017; Allesen- Holm et al. 2006). In addition, the QS of this microorganism presents the las system, which contains the transcriptional regulator LasR, an active transcription factor in the presence of 3O-C12-HSL, which is able to bind the DNA and regulate the transcription of multiple genes inside the biofilm (Lee et al. 2015).

Microbial resistance has also turned into a subject of interest to the clinical and scientific community, leading them to develop strategies to combat the progressive increase of this phenomenon (Hwo, 2012). Since the QS system plays an important role in mechanisms

relating to bacterial drug resistance, which regulate the formation of biofilms, the discovery of compounds capable of inhibiting the action of QS mechanism signaling appears to be a promising strategy for the preventive treatment of microbial resistance associated with the formation and virulence of biofilms (Zhao et al. 2020; An et al. 2019; Lee et al. 2015).

Thus, this research investigated the effectiveness of a sulfamethoxazole-silver complex (SMTZAg) in inhibiting planktonic and sessile growth of *P. aeruginosa* (PAO1) and multidrug-resistant (MDR) clinical isolates obtained from the patients of an intensive care unit, determining its anti-bacterial and anti-biofilm activities. In addition, the potential of this compound as a promising QS inhibitory molecule was evaluated using *in vitro* and molecular docking analyzes, with the main QS regulator in *P. aeruginosa*, the LasR and PqsR. Finally, the *in vitro* safety profile of this molecule was evaluated using cytotoxicity and genotoxicity assays in human peripheral blood mononuclear cells (PBMC).

Material and methods

Pseudomonas aeruginosa strains and multidrug-resistant clinical isolates

In this study, the standard strain *P. aeruginosa* (ATCC 27853) and the standard biofilm-forming strain *P. aeruginosa* (PAO1) were used. In addition, twenty-six MDR clinical isolates of *P. aeruginosa* were obtained from the University Hospital of Santa Maria (UHSM), Santa Maria, Rio Grande do Sul state, Brazil. The isolates were classified as MDR according to the results of susceptibility tests conducted in the Clinical Analysis Laboratory of the UHSM and through the definitions established by Magiorakos et al. (2012). The microorganisms were kept on a BHI broth supplemented with glycerol (HiMedia Laboratories Pvt. Ltd, India) at -20ºC until required. This work was conducted with authorization from the Research Ethics Committee of the Federal University of Santa Maria (FUSM) and approved by the Presentation for Ethics Assessment Certificate number 12114713.1.0000.5346.

Synthesis of sulfonamide complexes

Synthesis of the sulfonamide complexes, sulfamethoxazole-mercury complex (SMTZHg), sulfamethoxazole-copper complex (SMTZCu), sulfamethoxazole-nickel complex (SMTZNi), sulfamethoxazole-gold complex (SMTZAu), sulfamethoxazole-cadmium complex (SMTZCd), and SMTZAg complex, was performed in the bio-inorganic laboratory of the chemistry department of the FUSM, according to routes previously described by Marques (2007).

Sulfamethoxazole (SMTZ) (Sigma-Aldrich, Saint Louis, MO, USA) was used as a ligand in the synthesis of the metal complexes and was also used as a standard in the microbiological assays, in order to compare the activity of the classic antimicrobial with the compound coordinated to silver. SMTZAg, the focus of this study, was synthesized as shown in Figure 1.

Susceptibility tests

The susceptibility tests were conducted using the broth microdilution method, according to the standard protocol CLSI M100eS23 (CLSI 2013), while the inoculum density standardization was performed according to the McFarland 0.5 scale. Initial stock solutions were made of each compound in dimethyl sulfoxide (DMSO) at 50 $mg.mL^{-1}$. From the stock solution, concentrations ranging from 8.0 – 4102.0 $\mu g.mL^{-1}$ were prepared. To perform this evaluation, 96-well plates were used with 100 µL of Mueller–Hinton broth (Hi Media Laboratories Pvt. Ltd, India), 100 µL of each specific concentration from each compound, and 10 µL of the inoculum per well. Plates were then incubated for 24 hours at 37°C. The minimal inhibitory concentration (MIC) was considered as the lowest concentration capable of inhibiting visible bacterial growth. 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec®, Rio de Janeiro, RJ, Brazil) was used as an indicator of microbial growth.

Time-kill assay

The death curve assay was performed using a concentration of 10^5 colony forming units (CFU).mL^{-1} of *P. aeruginosa* (PAO1). Concentrations of MIC and 1/2 MIC were used for the evaluated compounds. The plates were incubated at 37°C for 24 h. During this period, samples were removed from colonies and counted at different time intervals (0, 6, 12, 18, and 24 h). The inoculated medium without drug was plated as the growth control. Based on the CFU, the inhibitory effect of the compounds was calculated. Bactericidal activity was defined as the count of the number of viable colonies treated with the compounds compared to the untreated control at a specific time point. The procedure was repeated three times (Bonez et al. 2016).

Antibiofilm activity assays

Biofilm formation inhibition

This assay used conventional 96-well polystyrene plates as an adhesion material. Culture broth was used as the negative control. *P. aeruginosa* (PAO1) inoculum was used as the positive control. Bacterial suspensions were prepared in Mueller Hinton broth and visually adjusted to the 0.5 McFarland scale. Serial dilutions (ultrapure water and 1% DMSO as diluents) of the silver-complexed sulfonamide were prepared in MIC and sub-inhibitory concentrations (32 µg.mL^{-1} and 8 µg.mL^{-1}). Each dilution was pipetted to four wells in a sterile flat-bottomed microtiter plate, along with 100 µL of the inoculum. The plates were incubated for 24 h at 37°C to promote adhesion. The formed biofilm was stained using the crystal violet technique. After incubation, the contents of the plate were removed, and successive washes were performed with physiological solution to remove the weakly adhered cells. Then, 200 µL of 0.1% crystal violet suspension were added to the wells and allowed to stand for 10 min. The plates were subsequently washed again, 200 µL of 95% ethanol added,

and the plates allowed to stand for 15 min. Finally, this ethanolic solution was transferred to another plate to read the optical density (OD) at 570 nm using a TP-Reader® (Thermo Plate, Inc, Ohio, USA) (Bonez et al. 2017). Biofilm formation of the testing groups was determined in comparison to the mean OD obtained in the positive control (culture medium and bacteria) and the negative control (culture media only).

Biofilm removal test

Bacterial suspensions of *P. aeruginosa* (PAO1), adjusted to the 0.5 McFarland scale, were added to 96-well polystyrene plates and incubated at 37°C for 24 h for biofilm formation. After the adhesion and biofilm maturation, 100 µL of SMTZ or the silver-complexed sulfonamide was added to each well, at a concentration equal to or higher than the MIC. The plate was incubated at 37°C for an additional 24 h (Bonez et al. 2017). The biofilm was stained and quantified as previously described in the biofilm formation inhibition assay.

Quorum sensing inhibition assay

Inhibition of QS was evaluated according to Burt et al. (2014), with some modifications. The production of violacein, the violet pigment produced by *Chromobacterium violaceum* (ATCC 12472), was quantitatively determined as a result of QS activity. Initially, the MIC for the microorganism was determined. Then, 1 mL of the subinhibitory concentrations of the silver-complexed sulfonamide was added to portions of 2 mL *C. violaceum* brain heart infusion (BHI) inoculated (adjusted to D.O 600 nm) and incubated for 24 h. After 24 h, a 2 mL sample was centrifuged for 5 min at 3000 rpm, and the supernatant was discarded. After discarding the violacein, the pellet was resuspended in 150 µL of BHI, to which 150 µL of 10% sodium dodecyl sulfate (SDS) was added to lyse the bacteria. After 5 min at room temperature, 675 µL of water-saturated *n*-butanol was added, and the mixture

vortexed to dissolve the violacein and centrifuged for 5 min at 13000 g. The supernatant was transferred to a 96-well plate, and the OD was measured at 540 nm on a plate reader. A blank reading (sterile BHI) was subtracted from the measurements. The assay was performed in triplicate for each concentration of the compound evaluated. The absorbances obtained in the wells treated with the compound were compared with the absorbance of the positive control.

Biofilm atomic force microscopy (AFM)

Samples for the biofilm atomic force microscopy (AFM) analysis were prepared according to the method described by Bonez et al. (2017). In this assay, high-density polyethylene (HDPE) surfaces (Braskem, Sao Paulo, Brazil) of ~ 1 cm^2 were inoculated into 12-well plates containing 1 mL of BHI broth and 100 mL of a suspension of *P. aeruginosa* PAO1 on the 0.5 McFarland scale per well. The substrates (HDPE surfaces) were inoculated in the absence or presence of SMTZAg at 1/2 MIC (16 $\mu g.mL^{-1}$) and the highest concentration tested in the semi-quantitative assay (128 $\mu g.mL^{-1}$). After 24 h of incubation at 37°C, the surfaces were fixed in absolute methanol for 1 min and then analyzed via AFM to visualize the three-dimensional structure of the biofilm. Images of the formed biofilms on the HDPE substrates, with and without SMTZAg, were captured. Topography maps were recorded on a Park NX10 microscope (Park Systems, Suwon – Korea) equipped with SmartScan software version 1.0.RTM11a. The measurements were conducted using a TAP300G probe with a nominal resonance frequency of 300 kHz and 40 N/m force constant. All measurements were made at room temperature (about 21±5°C) and relative humidity (55±10%), with a scanning rate of 0.7 Hz. Images were offline treated using XEI software version 4.3.4Build22.RTM1.

Molecular docking

The crystallographic structures of *P. aeruginosa* LasR and PqsR were obtained from the Protein Data Bank (IDs = 3IX3, 1.40 Å, and 4JVI, 2.90 Å). Water molecules and the co-

crystallized ligand (N-3-oxo-dodecanoyl-L-homoserine lactone) were removed via UCSF Chimera 1.14 (Yang et al. 2012). The tridimensional structures of the ligands (SMTZ and SMTZAg) were generated via MarvinSketch 19.24.0 (Marvin). AutoDockTools 1.5.6 (Morris et al. 2009) was employed for the preparation of the .pdbqt files of the receptor by the addition of polar hydrogens and computation of Kollman chargers and ligands with the definition of the number of torsions and the addition of Gasteiger charges. The metal charge was manually assigned by editing the related pdbqt file to SMTZAg. The grid box was set to 60x60x60 points with a grid spacing of 0.375 Å and centered on the ligand from the crystal structure complex. The parameters regarding Ag were set in the AD4 parameters.dat to be considered by Autodock4 and AutoGrid4 as follows: Van der Waals radius of the atom/ion (Rii = 3.15 Å), epsilon or energy well depth (epsii = 0.036 kcal/mol), atomic solvation volume (vol = 12.000 Å^3), atomic solvation parameter (solpar = -0.00110), hydrogen bonding radius (0.0 Å), hydrogen bonding energy well depth (0.0 kcal/mol), hydrogen bonding type (0), receptor type index (-1), grid map index (-1), and bond type index (4). By selecting the Lamarckian genetic algorithm, the studies comprised 250 GA runs with a population size of 300 per ligand using AutoDock 4.2. In order to validate the docking protocol, a re-docking of the co-crystalized ligand was performed prior to all other docking analyses (Figure 2), with a RMSD (Root Mean Square Deviation) of 0.703 Å (PDBid = 3IX3) and 1.480 Å (PDBid = 4JVI). Additionally, the same docking studies were performed via Autodock Vina 1.1.2 (Trott et al. 2010), keeping the grid box and the other parameters set to default, apart the exhaustiveness, which was set to 14, and the number of modes, which was set to 100. For the re-docking processes, RMSDs of 1.110 Å (PDBid = 3IX3) and 1.590 Å (PDBid = 4JVI) were obtained. Since the silver atom is not recognized by Autodock Vina 1.1.2, the copper atom was considered as a replacement. All the images were generated via UCSF Chimera or BIOVIA Discovery Studio Visualizer v19.1.0.18287 (Biovia, 2019).

Silver-complexed sulfonamide in vitro safety profile evaluation

Cell culture and treatments

PBMCs derived from discarded total blood samples of healthy adults were obtained from the Clinical Analysis Laboratory of the Franciscan University (UFN) (experimental protocol approved by UFN ethics committee for research with human beings, CAAE number: 31211214.4.0000.5306). Blood samples were processed for the separation of PBMCs through the procedure based on density gradient difference using Ficoll Histopaque-1077® reagent (Sigma-Aldrich, St. Louis, MO, USA). After blood disposition into the reagent (1:1), samples were centrifuged for 30 min at room temperature. After centrifugation, PBMCs were isolated and plated in 96-well plates at a final cellular density of $2x10^5$ cells.mL^{-1} (Botton et al. 2015). RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum and supplemented with 1% of antibiotics and antimycotic was used as the cellular medium. Cells were then exposed to each compound tested for the efficacy protocol described in this research, at the same concentrations over 24 h, to evaluate their effect on cellular modulation through different colorimetric and fluorimetric assays. All treatments and assays were performed at least in triplicate. Hydrogen peroxide (H_2O_2) at 200 µM was used as a positive control for all assays.

Cellular viability assay

After the treatment period, cellular viability was evaluated. The first assay performed was the MTT assay using MTT bromide salt {[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]} reagent (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg.mL^{-1} diluted in phosphate buffer (PBS, 1X, pH 7.4), following the instructions of Mosmann et al. (1983). After 4 h of incubation at 37^{o}C, the intracellular formazan crystals were solubilized

with DMSO. Absorbance was determined at 570 nm using Anthos 2010 microplate reader equipment (Anthos, London, UK).

Genotoxicity assay

Evaluation of the genotoxicity of the SMTZAg complex was performed by the cometa assay. The cells were treated with concentrations ranging from 2 $\mu g.mL^{-1}$ to 128 $\mu g.mL^{-1}$ over 24 h. After incubation, treated cells were mixed with low melting point agarose and deposited on a glass slide precoated with a layer of 1.5% agarose. The material was immersed in a lysis solution (lysis solution:10 mL of DMSO, and 1 mL of Triton X-100). Subsequently, electrophoresis was performed for 30 min under 25 V and 300 mA in an alkaline (pH 13) buffer (300 $mmol.L^{-1}$ NaOH and 1 $mmol.L^{-1}$ EDTA in distilled water). Then, the processes of neutralization (neutralizing buffer pH 7.5), fixation (15% trichloroacetic acid), and staining (acridine orange) were conducted before microscopic analysis.

One hundred cells were analyzed under an optical microscope (10x and 40x magnification) and classified according to the length of the comet tail (formed by DNA fragments). The cells received scores from 0 (no damage) to 4 (maximum damage). The test was performed in triplicate, and the data were transformed into a damage index, which was analyzed statistically according to Filho et al. (2013).

Determination of total levels of reactive oxygen species

Quantification of total levels of reactive oxygen species (ROS) was performed using the 2',7'-dichlorofluorescein diacetate assay (DCFH-DA) (Sigma-Aldrich, Saint Louis, MO, USA), as described by Ahn et al. (1996). DCFH-DA reagent has the ability to cross the cellular membrane. Inside the cell, it is deacetylated by mitochondrial enzymes, giving rise to 2',7'-dichlorodihydrofluorescein, which reacts with ROS, mainly H_2O_2, and produces DCFH,

which emits fluorescence. Treated and untreated PBMCs were mixed with DCFH-DA reagent for 1 h at room temperature. Fluorescence was then determined in a spectrofluorimeter (SpectraMax Solutions Ltd, London, UK) based on the wavelengths of 488 nm for excitation and 525 nm for emission (Esposti 2002).

Indirect nitric oxide level quantification

Nitric oxide level evaluation was performed through an indirect assay used to detect the presence of organic nitrites in the sample, following the instructions published by Choi et al. (2012). Nitrite is detected and analyzed by the formation of a pinkish color when Griess reagent is added to a sample containing NO_2^-. The sulfanilamide present in Griess reagent is responsible for the formation of diazonium in the sample. When the compound *N*-1-naphthylenediamino-bichlorohydrate interacts with this diazonium salt, an azo compound with pink color appears in the sample. Supernatants of PBMCs, both exposed and unexposed to the compounds in question, were transferred to another clear 96-well plate containing 100 µL of Griess reagent. After an incubation of 15 min at room temperature, absorbance was determined at 540 nm using an Anthos 2010 microplate reader (Anthos, London, UK).

Determination of hemolytic activity

The hemolytic activity of the compound was determined according to the protocol described by Dobrovolskaia et al. (2009). Whole blood sample was washed three times using PBS (1x, pH 7.4) (1:1, v:v). Then, 400 µL of red blood cells were added to 1 mL of PBS (1x, pH 7.4) and 80 µL of the SMTZAg complex treatment. Red blood cells (400 µL) and PBS (1.080 mL) were used as the negative control. Red blood cells (400 µL), PBS (1 mL), and 200 µM H_2O_2 (80 µL) were used as the positive control. Treatments were incubated at 37°C for 1

h. Samples were then centrifuged for 5 min at 1000 rpm, and the supernatants were read at 405 nm using an Anthos 2010 microplate reader (Anthos, London, UK).

Statistical analysis

Biofilm formation and QS inhibition were determined by a significant difference between the averages of absorbance obtained in the positive control and the average obtained in the negative control. The experiment was performed in triplicate. The OD readings obtained in the biofilm formation assay were recorded as mean ± standard deviation (SD) and were submitted to a *t*-test (compared with the positive control). Values with $p < 0.05$ were considered to indicate statistical significance.

The *in vitro* safety profile results obtained are presented as a percentage of the untreated control group (NC). The results of the hemolysis assay are expressed as a percentage of the positive control (100%). Data were expressed as mean ± SD. Analyses were performed by 1-Way ANOVA followed by Dunnett's post hoc. Values with $p < 0.05$ were considered statistically significant, being * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Graphs were prepared using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA).

Results

Susceptibility tests

The susceptibility profile of *P. aeruginosa* (PAO1) against SMTZ, metal derivatives of SMTZ, and the salts used for their synthesis can be seen in table 1.

From the results, it is possible to affirm that a high concentration of free SMTZ was necessary to completely inhibit the planktonic growth of *P. aeruginosa*. When compared to SMTZ alone, the complexes with different metals presented a greater inhibitory activity with

smaller MICs, especially for the SMTZAg. This silver complex showed an activity that was 64 times bigger than the free drug, with the lowest MIC found.

From the results mentioned above and considering the promising activity presented by the SMTZAg complex, the MIC of this compound was determined against 26 MDR clinical isolates and against *P. aeruginosa* (ATCC 27853). The values obtained can be seen in table 2.

Time kill assay

The kinetic profile of the *P. aeruginosa* time of death after exposure to SMTZAg showed a reduction in the number of viable cells in a few hours. The compound inhibited the bacterial growth within the first 12 h of treatment with MIC. When the bacteria were exposed to sulfonamide in 1/2 MIC concentration, the compound was not able to kill the bacteria during 24 h of treatment (Figure 3).

Antibiofilm activity assays

Biofilm formation inhibition test and biofilm removal test

The results of the efficacy of SMTZ and SMTZAg in inhibiting the formation of *P. aeruginosa* biofilm (PAO1), as well as in eradicating the biofilm previously formed by the microorganism, are shown in Figure 4.

SMTZ showed a slight ability to inhibit bacterial biofilm in the range of 512.75- 2051 $\mu g.mL^{-1}$ and was not able to completely eradicate the previously formed biofilm at the higher concentrations used (2051 $\mu g.mL^{-1}$, 4102 $\mu g.mL^{-1}$, and 8204 $\mu g.mL^{-1}$). On the other hand, the SMTZAg complex was able to inhibit biofilm formation with subinhibitory concentrations, much lower than the concentrations evaluated for SMTZ (8 $\mu g.mL^{-1}$, 16 $\mu g.mL^{-1}$, and 32 $\mu g.mL^{-1}$), leading to results of biofilm formation similar to the values obtained in the negative

control of the assay. In addition, this metal complex eradicated the *P. aeruginosa* biofilm in all tested concentrations (32 μg.mL^{-1}, 64 μg.mL^{-1}, and 128 μg.mL^{-1}), showing a significant difference in biofilm formation when compared to the positive control, destroying the sessile structure through much lower concentrations than those of the non-complexed drug.

Quorum sensing inhibition

Initially, the MIC of the SMTZAg complex was determined against *C. violaceum* (ATCC 12472), in order to determine the concentrations to be used in the QS inhibition assay. The concentration capable of completely inhibiting the microorganism growth was 32 μg.mL^{-1}. Therefore, the ability of the compound to inhibit QS in subinhibitory concentrations was evaluated, and the obtained results are shown in Figure 5, which represents significant QS inhibition in concentrations of 32 μg.mL^{-1}, 16 μg.mL^{-1}, and 8 μg.mL^{-1}.

Atomic force microscopy (AFM) of biofilm

Figure 6 shows the AFM results of the HDPE substrates that were not treated with the SMTZAg compound and other substrates treated with SMTZAg at the MIC (16 μg.mL^{-1}) and a concentration of 128 μg.mL^{-1} for *P. aeruginosa* PAO1 strain (highest concentration tested here). The image also shows the negative control (A) and the positive control (B) for biofilm formation; there is considerable variation in topography. This difference is displayed by comparing the two and three-dimensional images, in which the mass and density of the biofilm formed can be visualized, appearing smaller in the negative control (A). Images in panels C and D demonstrate the action of SMTZAg on biofilm formation, although the three-dimensional structures in images C and D show some dense and higher peaks. The activity of SMTZAg at 32 μg.mL^{-1} (MIC) was effective, presenting a roughness (*Ra*) value of 13.635 nm, which was substantially less than the highest peak found in the positive control (*Ra* =

30.355 nm). The same is observed in the panel D, which represents the SMTZAg activity at 128 μg.mL^{-1}, where the determined *Ra* value was only 8.461 nm. The three-dimensional image analysis shows how the SMTZAg compound is effective against the formation of the biofilm at both tested concentrations. Topographies of SMTZAg treatment groups, as well as the negative control, show the formation of a small biofilm.

Molecular docking

Results of the molecular docking predictions can be found in table 3. Considering a lower binding energy for a better predicted affinity profile, SMTZ and its silver (I) complex were evaluated in comparison with the binding mode of N-3-oxo-dodecanoyl-L-homoserine lactone (OdDHSL) in LasR and a known PqsR inhibitor (3NH2-7Cl-C9QZN). Regarding the LasR receptor from *P. aeruginosa* and the binding poses of SMTZ and SMTZAg (Figure 7 A and B, respectively), the establishment of an important hydrogen bonding is noted, comprising Tyr56, Thr75, Tyr93, and Ser129 with the aniline and sulfonamide moieties plus the oxygen from the oxazole ring for both ligands. Considering the predicted affinity of SMTZ and SMTZAg towards the binding pocket in PqsR (PDBid = 4JVI) (Figure 7 C and D), the nitrogen heteroatom from the oxazole ring also participates in hydrogen bonding besides the sulfonamide group, including residues such as Leu197, Ser196, Gln194, and Ile236. Even with the additional volume from Ag in the metallic complex, the best docked structures of SMTZAg and SMTZ basically occupy the region of the co-crystalized ligands, O-DDHSL and 3NH2-7Cl-C9QZN in LasR and PqsR from *P. aeruginosa*, respectively (Figure 8 A-D).

Safety profile of the sulfamethoxazole-silver complex

Regarding the *in vitro* safety profile analyses, it was possible to observe by the MTT technique (Figure 9A) that none of the silver complex concentrations tested caused any

modification relating to cellular viability since PBMCs retained viability levels similar to the negative control after 24 h of incubation. As expected, H_2O_2 significantly decreased cellular viability, to about 45%. No DNA damage was not observed through the comet assay (Figure 9B) when cells were treated with 2-16 $\mu g.mL^{-1}$ of SMTZAg. However, the highest concentrations (32-128 $\mu g.mL^{-1}$) were capable of inducing some DNA damage when compared to untreated cells.

Results showed that 200 µM H_2O_2 expressively increased levels of ROS (Figure 9C) in PBMCs after 24 h of exposure. On the other hand, none of the SMTZAg concentrations used were able to modify ROS production in comparison to the negative control. Additionally, most of the SMTZAg concentrations tested also kept NO levels (Figure 9D) similar to those found for untreated PBMCs, except for concentrations under 128 $\mu g.mL^{-1}$.

Finally, none of the SMTZAg concentrations caused any change in terms of erythrocyte hemolysis, since all treatments presented significantly smaller levels of hemoglobin release when compared to the positive control (Figure 9E).

Discussion

The World Health Organization has named antimicrobial resistance as one of the three most important threats to public health in the 21st century (WHO, 2018). In addition, strains of *P. aeruginosa* are considered to be one of the most common causes of infections in hospitalized patients, being one of the main microorganisms isolated in intensive care units (ICUs) (Bălăşoiu et al. 2014).

Because of the rapid spread of bacterial resistance and the reduced number of antimicrobials approved by the Food and Drug Administration (FDA), in recent years, it has become extremely necessary to search for new substances with antibacterial potential (Burnside et al. 2012). In this context, coordination compounds using antimicrobial drugs as

ligands and investigation of the intrinsic characteristics of metal ions have allowed a large variety of complexes to be explored as potential antimicrobial agents (Rocha et al. 2011; Agertt et al. 2016).

SMTZ complexes coordinated with Hg, Cu, Ni, Au, Cd, and Ag were investigated in this study for their ability to inhibit the planktonic growth of *P. aeruginosa* (PAO1). The MIC obtained in the evaluations demonstrated that sulfonamides complexed with metals showed greater antibacterial activity than free SMTZ, with the emphasis on the SMTZAg complex, which presented the lowest MIC against *P. aeruginosa*. Previous research has shown that sulfonamides complexed with metals are a promising alternative in the combat of planktonic mycobacterium microorganisms, both against the group of mycobacteria belonging to the *Mycobacterium tuberculosis* complex (Agertt et al. 2013) and against pathogens belonging to the group of non-tuberculous mycobacteria (NTM) (Siqueira et al. 2018; Agertt et al. 2016). In addition, studies have reported the antimicrobial effect of these complexes against gram-negative microorganisms, such as *Escherichia coli* (Mizdal et al. 2017) and *P. aeruginosa* (Mizdal et al. 2019), and gram-positive microorganisms, such as MRSA (methicillin-resistant *Staphylococcus aureus*) (Mizdal et al. 2018; Marques et al. 2007). Chohan et al. (2010) also studied the complexes of sulfonamide with Co, Cu, Ni, and Zn and described the antifungal activity of these substances against *Candida albicans*, *Aspergillus niger*, *Microsporum canis*, and *Trichophyton mentagrophytes*, demonstrating the importance of exploring sulfonamides coordinated to metals as possible anti-infective agents. In addition, the MIC values determined for the salts used in the synthesis of the compounds used in this work confirm that the increase in biological activity is due to the complexation with the metal and not to the salt itself.

Looking forward, to explore the antibacterial activity of the silver complex, MIC values were determined against 26 MDR clinical isolates. By the results presented in table 2,

it was possible to reinforce the promising activity presented by this substance against *P. aeruginosa*. The results suggest that the lower MIC of the silver complex is due to the broad biological activity of the silver ion, acting on different bacterial targets in order to inhibit the growth of the microorganism (Barras et al. 2018). Before the inclusion of modern antibacterial drugs in the clinic field, silver was possibly the most important known antimicrobial compound (Mijnendonckx et al. 2013). However, the mechanism underlying the silver ions' antimicrobial activity is not yet fully understood. It is known that silver ions can act as adjuvants to increase the antibiotic activity of antibacterials, targeting prokaryotic DNA, macromolecules as proteins, free amino acids as cysteine, and small molecules as glutathione. Also, silver ions could mediate changes in the cellular membrane, modifying the permeability of bacteria (Barras et al. 2018).

The time-kill assay provides a dynamic picture of the action of antibiotics over time (Lee et al. 2013). The broth microdilution method was confirmed by the time-kill assay. In the presence of the evaluated compound, the death curve showed a decrease in the survival of *P. aeruginosa* in the first 12 h of treatment with under 32 $\mu g.mL^{-1}$ of SMTZAg. It is known that some antibiotics, such as rifampicin for gram-positive bacteria and fluoroquinolones for gram-negative bacteria, act as an alternative in the treatment of chronic infections associated with biofilms; however, a total eradication of these sessile structures is still a challenge in the treatment, for a huge variety of cases (Bjarnshol et al. 2018; Kinnaritj et al. 2015). The ability of *P. aeruginosa* to form biofilms in medical devices and living tissues after a few hours of insertion makes this virulence factor a highly effective survival and resistance mechanism for this pathogen (Xu et al. 2015; Fazli et al. 2014). Also, the formation of *P. aeruginosa* biofilm can cause serious complications in patients with cystic fibrosis, once antibiotic therapy becomes highly ineffective when fixing these films in the lung tissue (Davies, 2002; Høiby et al. 2010).

In this study, the SMTZAg complex was shown to be highly effective in inhibiting the formation of *P. aeruginosa* biofilm in concentrations equal to and less than the MIC initially determined. The bacterial viability of PAO1 treated with 1/2 MIC was confirmed through the death curve. While planktonic cells are viable from the beginning of the biological cycle of biofilm formation, by using sub-inhibitory concentrations, the silver complex was shown to play a role in inhibiting the sessile structure formation process. Additionally, this substance was able to destroy the previously formed biofilm in concentrations equal to and greater than the MIC. On the other hand, free SMTZ was not able to inhibit or destroy biofilms of *P. aeruginosa* as effectively as the complex, presenting a discreet inhibition and destruction at higher concentrations than the effective concentrations of the metal complex. The results of SMTZAg antibiofilm activity were confirmed by AFM, proving the low absorbance obtained in the semi-quantitative assay for the *P. aeruginosa* PAO1 standard strain. Moreover, a significant difference was observed in the topography of the treated biofilms, with reduced values of roughness for both and reduced biomass production, compared to the positive control.

The formation of the exopolysaccharide matrix, an extracellular material made up of different biopolymers, occurs during the stages of biofilm formation (Garnett and Matthews, 2013). This material causes an increase in impermeability characteristics, making it difficult for antimicrobial agents to enter the deeper biofilm cells (Koo et al. 2017). It is believed that the greater capacity for removal of the *P. aeruginosa* biofilm by the silver complex can be explained by the activity of the Ag^{+} ion in damaging this matrix, destroying it more easily in the low concentrations used, allowing the dispersion of the biofilm and facilitating the access of the SMTZAg complex within the sessile structure (Roizman et al. 2017). Thus, SMTZ could easily penetrate the microbial structure, acting even against the microorganisms in the innermost part of the biofilm.

Due to the involvement of QS in the biofilm formation, maintenance and dispersion, QS inhibitors have been proposed as promising antibiofilm agents. The phenomenon of QS inhibition or "quorum quenching" can be achieved through enzymatic degradation of the autoinducer compound in two ways: 1) blockage of autoinducer production; or 2) the blockage of their reception through the addition of some compound "inhibitors" that can mimic them (Turan and Engin, 2018; Smith and Iglewski, 2013).

Once a high and efficient *P. aeruginosa* biofilm formation inhibition was induced by the complex, inhibition of the QS could be suggested as the potential mechanism by which this process occurs. QS performs a very important role in the late development of the biofilm and in its dispersion, controlling the synthesis of ramnolipids that are essential in the development of the biofilm of this pathogen (Fazli et al. 2014; Wolska et al. 2015).

To evaluate this hypothesis, an *in vitro* quantification test was performed for the violacein produced by *C. violaceum*. This substance is involved in the QS through the self-induction of CviL synthase. This self-inducer binds to the transcriptional regulator CviR and participates in the expression of specific genes, one of which is responsible for violacein production. This is released into the environment and diffuses when the QS is reached (Burt et al. 2019; Vasavi et al. 2017). Once the SMTZAg complex was able to inhibit the production of violacein in subinhibitory concentration, it was possible to consider that the compound acts on the QS and may be a possible inhibitor of this cellular communication process.

P. aeruginosa uses three known QS systems, named as las, rhl, and pqs, and they serve to regulate many genes, including virulence factors, antibiotic resistance, biofilm formation, and modulation of host immune response (Mizdal et al. 2018). QS stimulus is directly related to bacterial pathogenicity, LasR being the protein that plays this role in *P. aeruginosa*. LasR is activated by lactone *N*-3-(oxo)-dodecanoyl-L-homoserine (OdDHL), a native ligand. Under an infection by *P. aeruginosa*, a secretion of OdDHL occurs and while bound with the LasR

protein, it promotes the expression of bacterial virulence factors (Mohanvel et al. 2019; Shah et al. 2019).

In addition, *P. aeruginosa* las regulatory circuitry is linked to a second QS signaling system, which employs 2-heptyl-3-hydroxy-4(1H)-quinolone, the pseudomonas quinolone signal (PQS). PQS also regulates the production of virulence determinants including elastase, rhamnolipids, the galactophilic lectin, LecA, and pyocyanin (a blue-green phenazine pigment), influencing biofilm development. Expression of the *pqsABCDE* operon, and hence 2-alkyl-4-quinolones (AHQ) production, is controlled by the LysR-type regulator PqsR(MvfR), which binds directly to the pqsA promoter (Diggle et al. 2015).

Negative binding affinity values ($kcal.mol^{-1}$), obtained from the molecular docking analysis, show that the binding of SMTZ and SMTZAg complex to LasR and PqsR is favorable. Analyzing the interaction energies of SMTZ compounds and OdDHL it is possible to suggest a competitive action between these ligands. SMTZAg was found to have a comparable or better stable bond with LasR compared to SMTZ. It is worth mentioning that none of the ligands have lower energy than the native ligand.

The study results show that SMTZ and SMTZAg can be accommodated in an active site where OdDHSL binds, suggesting a possible competition of both molecules for binding to LasR. The binding of SMTZ and SMTZAg with the two essential residues Tyr56 and Ser129 promotes an interruption of binding activity by the native ligand (OdDHSL). The SMTZ compounds revealed positive patterns of interaction with the LasR quorum receptor, as demonstrated by the biofilm inhibition assays. The results suggest that the compounds showed LasR antagonistic activities in *P. aeruginosa* due to the blocking of the protein binding site, resulting in a decreased pathogenicity.

Additionally, the capability of the studied SMTZ compounds to interact with the binding pocket in PqsR was evaluated. The most stable conformations of both ligands were

seen to be able to occupy the region explored by PqsR inhibitors, such as 3NH2-7Cl-C9QZN, displaying an important hydrogen bond via Gln194 (Ilangovan et al. 2013). Therefore, it is possible that the SMTZ and SMTZAg could also contribute to the QS inhibition of *P. aeruginosa* by affecting the transcriptional regulatory activity of the PqsR protein, which can be related to the observed antibiofilm formation proprieties via assay.

From the point of view of technological design, the synthesis and development of metallodrugs (metal-based drugs) is a great challenge, especially when it comes to inorganic compounds. The bioaccumulation of metal ions can cause serious adverse side effects, which require accurate pharmacological investigation, also considering physiological and toxicological aspects (Rocha et al. 2011; Sagrillo et al. 2015). Therefore, the SMTZAg *in vitro* safety profile was investigated, in order to prove the safety of this compound before a possible *in vivo* evaluation. PBMCs were used for this, since these cells have been an excellent experimental model in the field of cyto-genotoxic understanding (Boligon et al. 2009; Boligon et al. 2012; Sagrillo et al. 2015). After 24 h of SMTZAg exposure, cells were tested to assess the cellular viability index, genotoxic effects, and possible hemolysis induction, as well as some oxidative parameters, including the determination of NO and ROS production. The concentrations of the compound chosen to evaluate the cellular effects were selected based on the MICs previously obtained.

Through the MTT assay, the cellular viability of the PBMCs was evaluated. The colorimetric test showed that all the compound concentrations maintained cellular viability in comparison to untreated cells. In a study with nanostructures, Oliveira (2009) established a classification to standardize cellular viability indexes, considering the percentage of modification as: non-cytotoxic (viability >90%), slightly cytotoxic (viability ~80–89%), moderately cytotoxic (viability ~50–79%), or highly cytotoxic (viability <50%). Considering this classification, the obtained results show that the majority of the tested concentrations of

the complex were considered non-cytotoxic, with the exception of concentrations of 32 $\mu g.mL^{-1}$, 64 $\mu g.mL^{-1}$, and 128 $\mu g.mL^{-1}$, which were considered as slightly cytotoxic (Filho et al. 2015; Oliveira, 2009). In addition, the cell viability pattern obtained in the tests can be compared to that of drugs with a proven safety profile, such as meropenem, a carbapenem used in infections associated with *P. aeruginosa* (Sueke et al. 2015).

Furthermore, following 24 h exposure to the silver complex, no DNA damage was detected in concentrations from 2 $\mu g.mL^{-1}$ to 16 $\mu g.mL^{-1}$, through the comet assay. However, the concentration of 32 $\mu g.mL^{-1}$ showed slight damage to DNA, being classified as damage index 1. The highest concentrations evaluated (64 $\mu g.mL^{-1}$ and 128 $\mu g.mL^{-1}$) showed the highest levels of DNA damage in PMBCs, indicating a potential genotoxicity effect of this compound in high concentrations (Filho et al. 2015).

It should be highlighted that the comet assay works as a gold standard method for assessing DNA damage of different cells, given the ultra-sensitivity of this assay and its precision (Lu et al. 2017). Furthermore, Itoh et al. (2006) demonstrated that antimicrobials such as ciprofloxacin showed greater genotoxicity when evaluating the genotoxic potential of quinolone antimicrobials through the comet assay.

Another important point for the safe administration of a new antimicrobial drug is its influence on the oxidative and nitrosative modulation of an organism. Although oxidative stress can be an adjunct to antimicrobial activity, and NO production is related to the activation of inflammatory responses associated with fighting bacterial infections, both processes must be controlled (Wang et al. 2017; Dhand et al. 2015).

All concentrations of SMTZAg used in the treatment of PBMCs led the cells to show non-significant levels of ROS production. These levels of oxidative molecules were similar to the negative control and are an important safety result of this new molecule, since eukaryotic oxidative stress can damage cells and decrease cell viability (Wang et al. 2017). Furthermore,

considering that NO is a reactive nitrogen species (RNS) related to the synthesis of superoxide anion and peroxynitrite, it is important to highlight that there was no cellular modulation in the production of NO that reinforces SMTZAg safety. Additionally, low or null toxicity for erythrocytes is also important when considering a new antimicrobial application (Oddo et al. 2016). In this sense, healthy erythrocytes were exposed to the different concentrations of SMTZAg to measure possible hemolysis. Fortunately, the complex has low hemolytic capacity in the tested concentrations.

All the obtained results suggest that SMTZAg is an effective molecule against planktonic growth and sessile cells of *P. aeruginosa* at the same concentrations in which it maintains the cellular viability of PBMCs, presenting a great *in vitro* safety profile.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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FIGURES

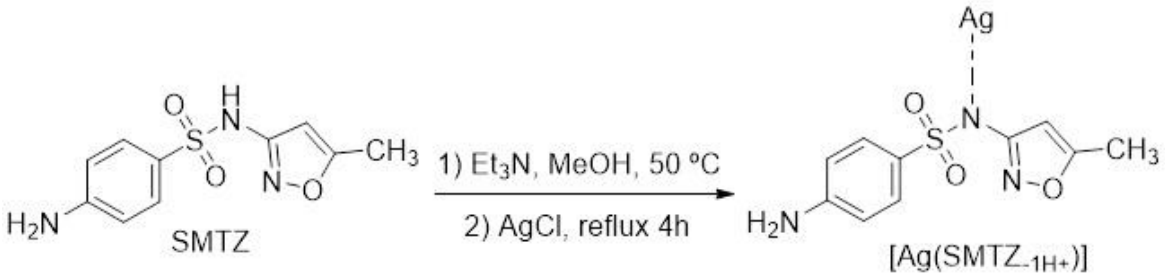

Figure 1. Synthesis, chemical structure and nomenclature of sulfamethoxazole-silver complex.

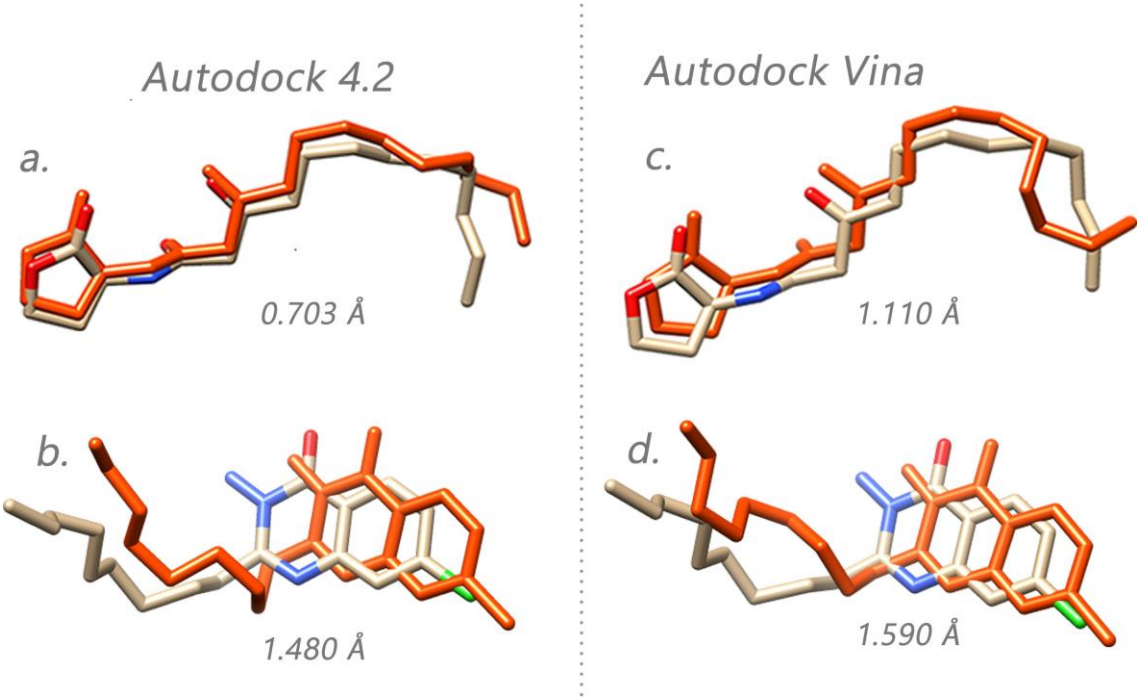

Figure 2. Docking validation via redocking of the co-crystalized ligands (a. and c. for 3IX3, b. and d. for 4JVI). The original conformations of the co-crystalized ligands are highlighted in gray, while docked poses are in orange. UCSF Chimera 1.14 was applied to create these images.

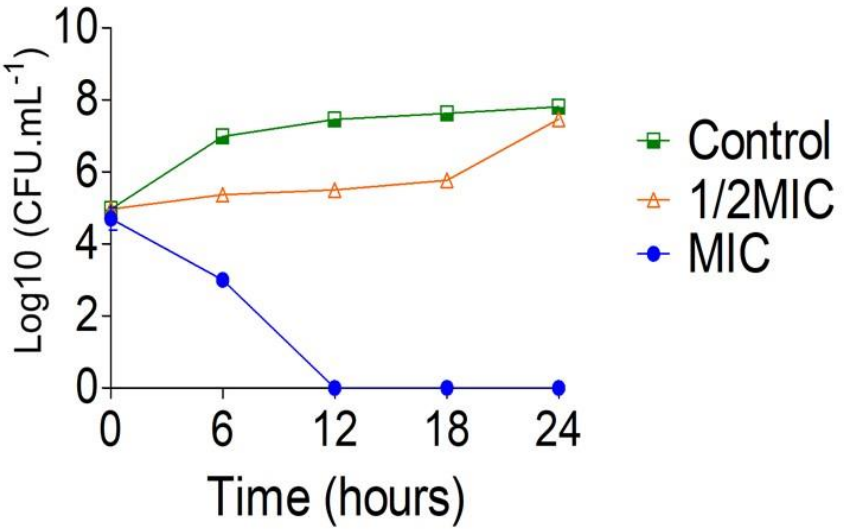

Figure 3. Time-kill curves of STMZAg against *Pseudomonas aeruginosa* PA01. The results are expressed using the means ± S.E.M., n = 4.

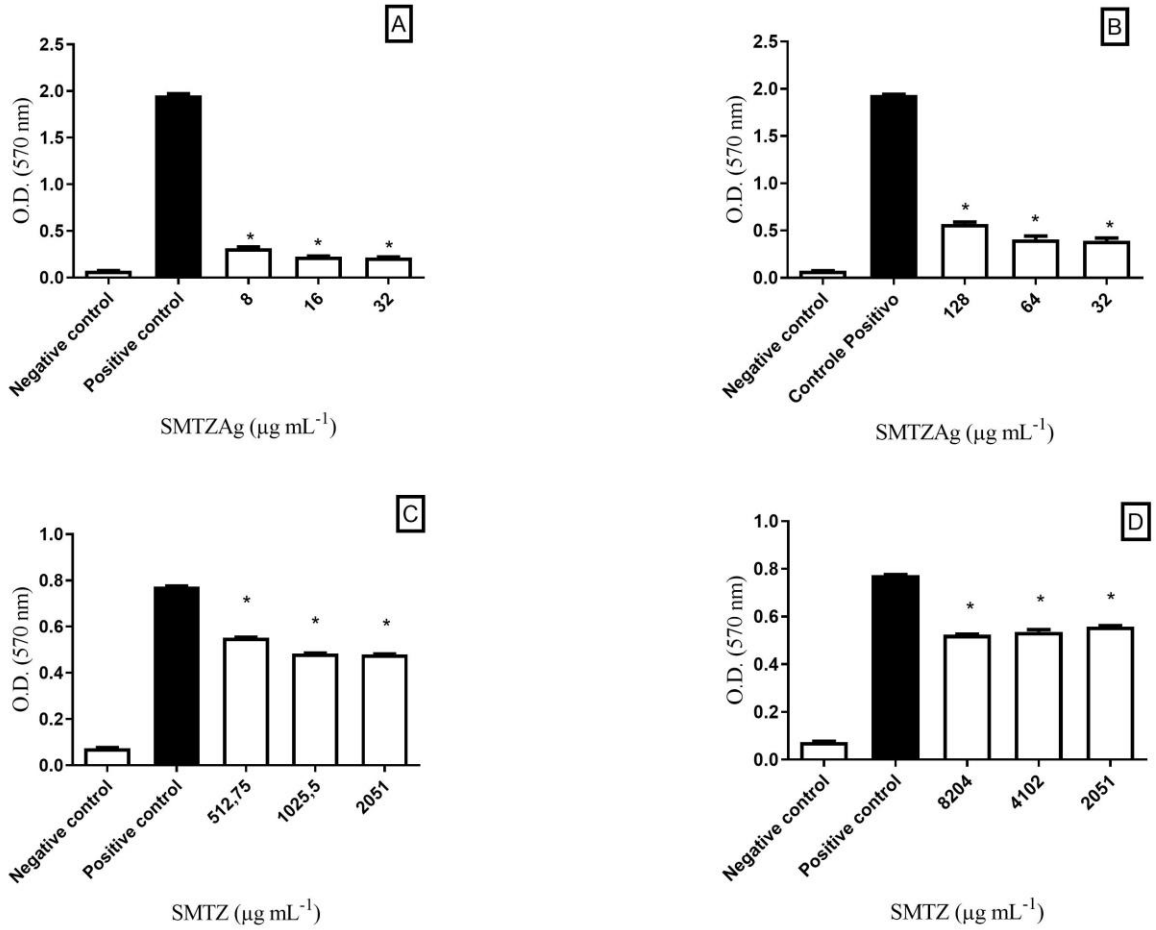

Figure 4. Effect of different concentrations of sulfamethoxazole-silver complex on the inhibition (A) and removal (B) and different concentrations of Sulfamethoxazole on inhibition (C) and removal (D) of the biofilm from *Pseudomonas aeruginosa* (PA01). * Represents significant difference between the concentration tested and the positive control (PC). The tests were subjected to the Student t-test (n = 3), considering statistical differences when $p < 0.05$.

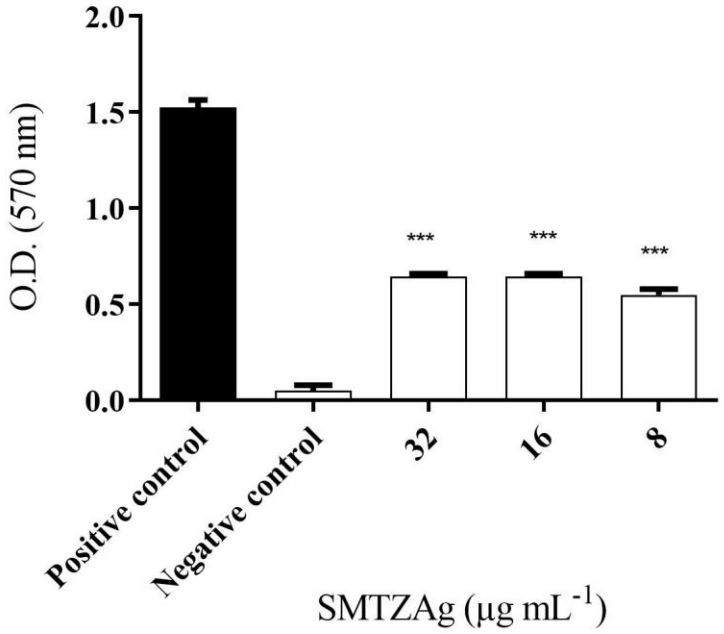

Figure 5. Effect of different concentrations of sulfamethoxazole-silver complex on the inhibition of *quorum sensing* by inhibiting the violacein produced by *C. violacium.* *** Represents significant difference between the concentration tested and the positive control (PC). The tests were subjected to the Student t-test (n = 3), considering statistical differences when $p < 0.01$.

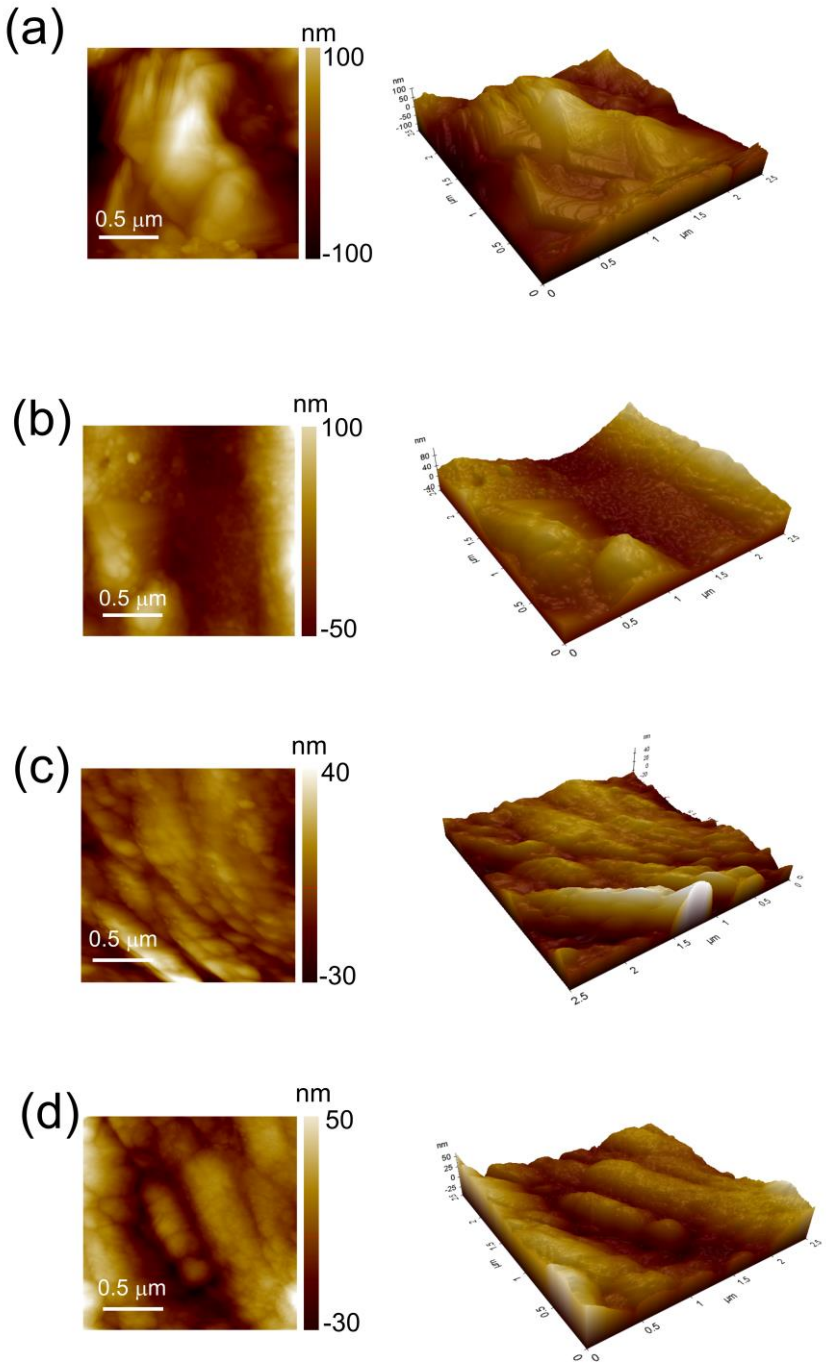

Figure 6. Images of *P. aeruginosa* biofilms adhered to an HDPE surface, obtained through AFM, after 24 h of incubation at 37ºC. (A) Negative biofilm control image (Mueller Hinton broth culture medium only); the image demonstrates the HDPE surface without any sessile structure attached. (B) Image of the positive control of the formation of the biofilm of *P. aeruginosa* (bacterial inoculum and Mueller Hinton broth culture medium); the image shows a dense and rough structure adhered to the HDPE surface. (C-D) Significant reduction in the biomass formed from the biofilm, through the inhibitory action of SMTZAg in the concentrations of (C) 16 µg.mL^{-1} and (d) 128 µg.mL^{-1}.

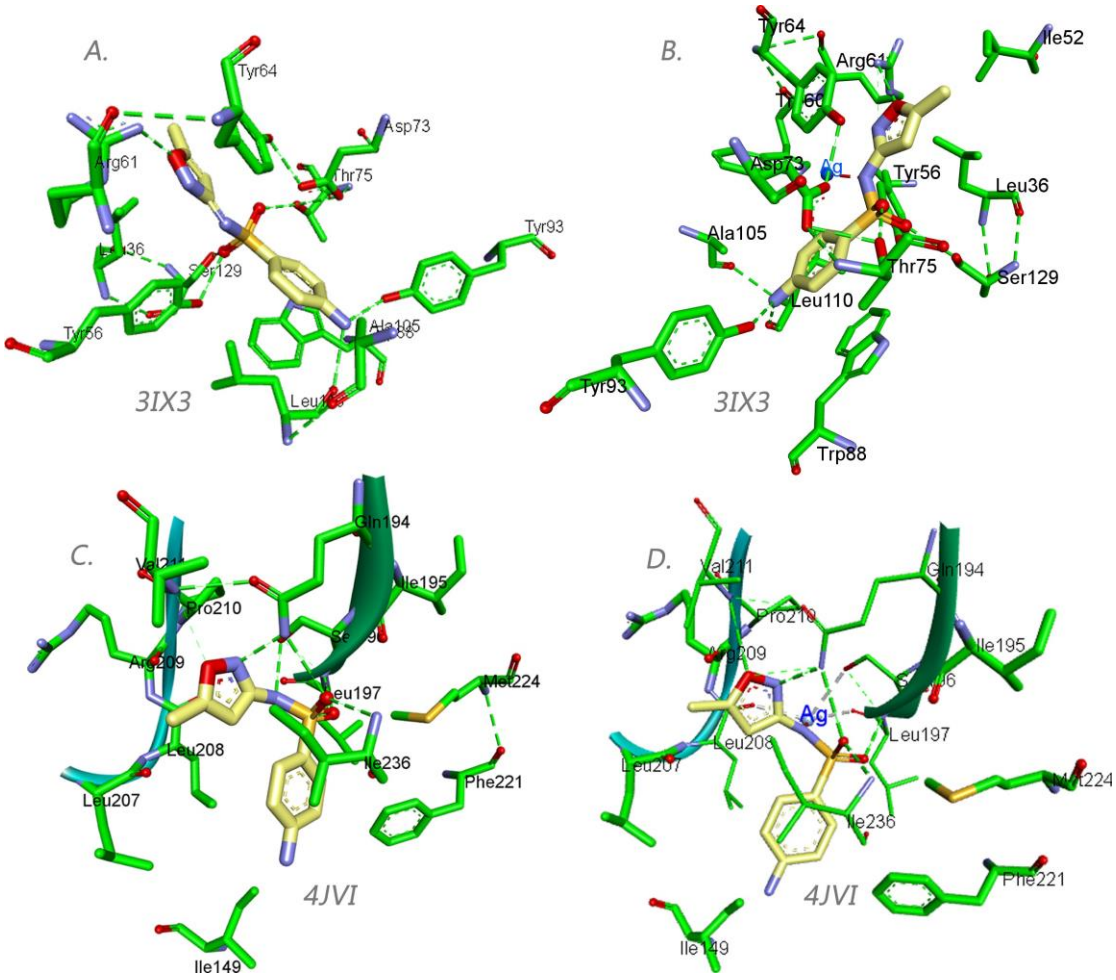

Figure 7. Binding modes of SMTZ (A and C) and SMTZAg (B and D). The aminoacid residues are highlighted in green and the docked poses via AutoDock 4.2 in yellow. BIOVIA Discovery Studio Visualizer was employed to generate these images.

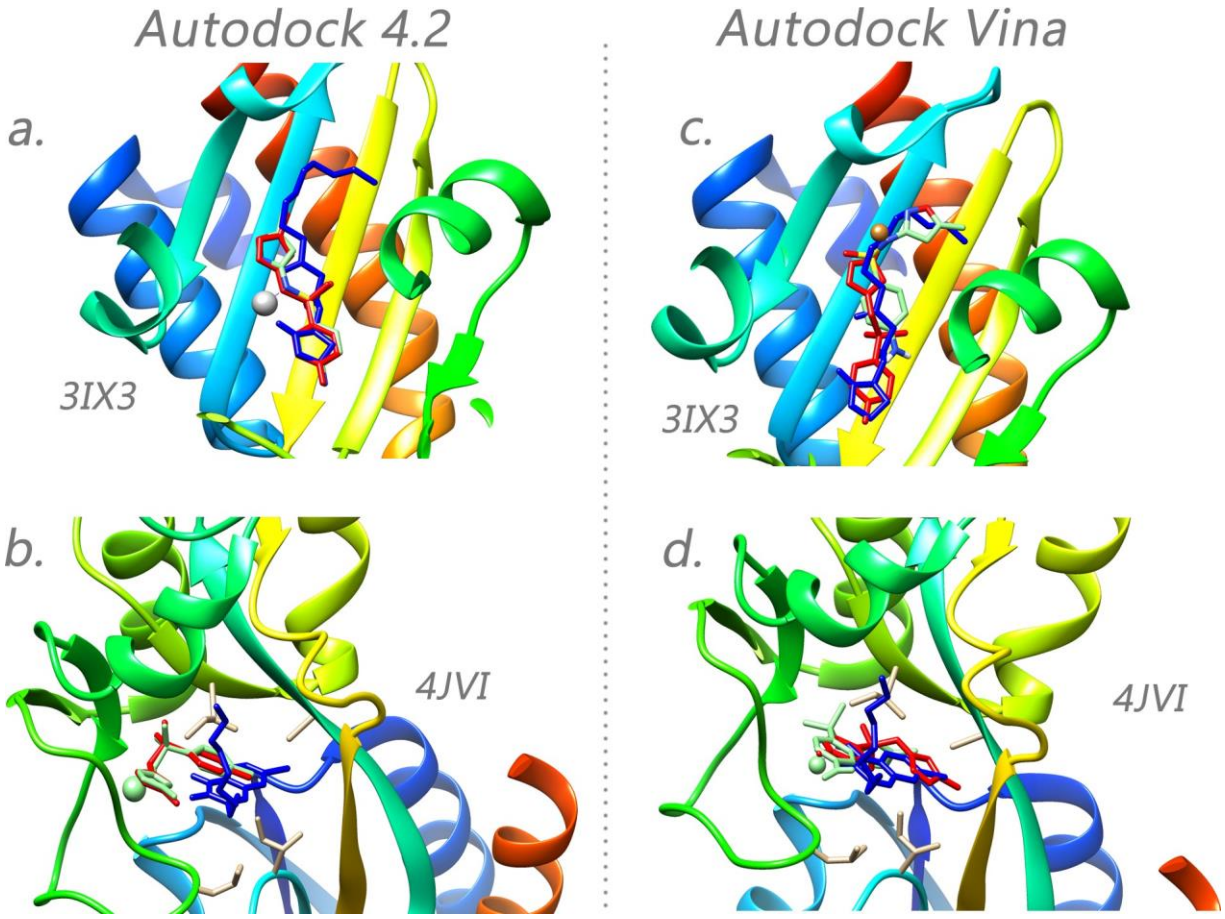

Figure 8. Superposition of the best docked structures for SMTZ (red) and SMTZAg (light green) that were obtained via AutoDock 4.2 and AutoDock Vina. O-DDHSL in LasR and PqsR inhibitor 3NH2-7Cl-C9QZN are in blue. UCSF Chimera 1.14 was applied to create these images.

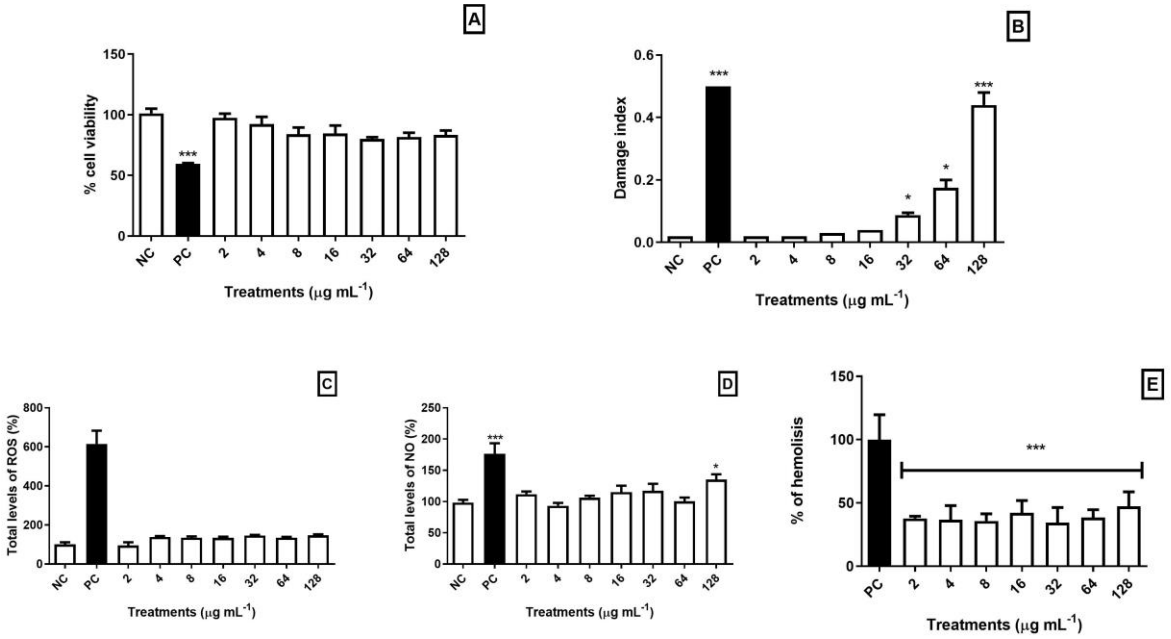

Figure 9. Cyto and Genotoxic analysis in PBMCs exposed to sulfamethoxazole-silver complex during 24h. Cellular viability analysis (MTT assay) (A), DNA damage evaluation (Comet assay) (B), total levels of ROS (dcfh-da assay) (C), and indirect quantification of NO (D). Results are expressed as percentage of the negative control (100%). Hemolysis assay (E). Results are expressed as percentage of the positive control (100%) (200 μM H_2O_2). Data were expressed as mean ± standard deviation (SD). Analyzes were performed by 1-Way ANOVA followed by Dunnett's post hoc. Values with $p < 0.05$ were considered statistically significant. Being * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

TABLES

Table 1. Values of the minimum inhibitory concentration for the standard strain of *Pseudomonas aeruginosa* (PA01) exposed to different sulfamethoxazole derivatives, different metal salts and sulfamethoxazole.

Compound	Minimal Inhibitory Concentrations (μg mL^{-1})
SMTZHg	64
Mercury acetate (II)	512,8
SMTZCu	128
Copper acetate (II).H_2O	512,8
SMTZNi	512,8
Nickel acetate II	1025,6
SMTZAu	64
Gold chloride. PPh_3	256
SMTZCd	64
Cadmium Acetate (II)	512,8
SMTZAg	32
Silver chloride	256
SMTZ	2051

Table 2. Values of the minimum inhibitory concentration for the MDR clinical isolates of *Pseudomonas aeruginosa* and *P. aeruginosa* (ATCC 27853) exposed to sulfamethoxazole-silver complex.

Microrganism	Minimal Inhibitory Concentrations (μg mL^{-1})
ATCC 27853	16
I	32
II	64
III	32
IV	32
V	32
VI	32
VII	32
VIII	32
IX	32
X	32
XI	32
XII	64
XIII	32
XIV	32
XV	32
XVI	64
XVII	64
XVIII	32
XIX	64
XX	64
XXI	32
XXII	32
XXIII	64
XXIV	32
XXV	32
XXVI	32

Table 3. Results of the docking predictions.

Receptor PDBId= 3IX3	Ligand	Scoring values kcal/mol	Important residues
AutoDock 4.2	SMTZ	-7.90	Tyr56, Arg61, Thr75, Tyr93, Ser129
	SMTZAg	-7.96	Tyr56, Thr75, Tyr93, Ser129
	O-DDHSL	-8.23	Tyr56, Trp60, Asp73, Ser129
AutoDock Vina	SMTZ	-9.0	Tyr56, Tyr93, Thr75, Ser129
	SMTZAg*	-8.9	Thr75, Ser129
	O-DDHSL	-9.8	Tyr56, Trp60, Asp73, Ser129
Receptor PDBId= 4JVI			
Autodock 4.2	SMTZ	-8.70	Leu197, Ser196, Gln194, Ile236
	SMTZAg	-8.18	Val211, Gln194, Ile236, Leu197, Ser196
	3NH2-7Cl-C9QZN	-9.02	Gln194, Ser196
AutoDock Vina	SMTZ	-6.8	Ile236, Arg209
	SMTZAg*	-6.9	Leu197, Ser196
	3NH2-7Cl-C9QZN	-7.7	Ile236, Ala168, Leu208

4.2 Manuscrito 1

O manuscrito 1 encontra-se submetido no periódico **Chemistry and biodiversity** e está formatado de acordo com as normas exigidas para publicação no período.

Synthesis and Biological Evaluation of New Sulfamethoxazole Derivative Schiff Base as antimicrobial and antibiofilm Agent on Rapidly Growing Mycobacteria.

Fallon dos Santos Siqueira[1], Josiéli Demétrio Siqueira[2], Alencar Kolinski Machado[3], Kelly Schneider Moreira[5], Michele Rorato Sagrillo[3], Thiago Augusto Lima Burgo[5], Bernardo Almeida Iglesias[4], Davi Fernando Back[2*], Marli Matiko Anraku de Campos[1]

[1] Mycobacteriology Laboratory, Graduate Program in Pharmaceutical Sciences, Department of Clinical and Toxicological Analysis, Federal University of Santa Maria, Roraima Avenue 1000, zip code: 97105-900, Santa Maria, Brazil.

[2] Inorganic Materials Laboratory, Department of Chemistry, Federal University of Santa Maria, Roraima Avenue 1000, zip code: 97105-900, Santa Maria, Brazil.

[3]Graduate Program in Nanosciences, Franciscan University, Andradas' Street, 1614, zip code: 97010-032, Santa Maria, Brazil

[4] Bioinorganic and Porphyrinoid Materials Laboratory, Department of Chemistry, Federal University of Santa Maria, Roraima Avenue 1000, zip code: 97105-900, Santa Maria, Brazil.

[5] Coulomb Electrostatic and Mechanochemical Laboratory, Graduate Program in Chemistry, Department of Chemistry, Federal University of Santa Maria, Roraima Avenue 1000, zip code: 97105-900, Santa Maria, Brazil.

[*]*Corresponding Author*: Davi Fernando Back
E-mail: daviback@gmail.com
Universidade Federal de Santa Maria
Roraima Avenue 1000 - Zip code: 97105-900
Santa Maria - RS –Brazil

ABSTRACT

Rapidly Growing Mycobacteria (RGM) are microorganisms dispersed in nature, with variable pathogenicity, that cause different clinical forms of mycobacteriosis, especially in immunocompromised patients. They can form structured communities in liquid-air interface and adhere to animate and inanimate solid surfaces, characterizing one of its most powerful mechanisms of resistance and survival, named biofilms. Considering the difficulty in implementing an adequate treatment in cases of RGM infections, we synthesized and characterized a new Sulfamethoxazole Derivative Schiff Base (SMTZ-SB), obtained by the condensation of sulfamethoxazole primary amine, with pyridoxal hydrochloride (Vitamin B6). Antimicrobial activity assays were performed using the broth microdilution technique and the antibiofilm activity of this compound was evaluated using a semiquantitative macro-technique. The synthetized Schiff base showed significant ability to inhibit the growth of RGM, with Minimum inhibitory concentrations (MIC) of 1.22 μg mL^{-1} and 0.61 μg mL^{-1}, in both plankton and biofilm forms. In addition, the molecule was much more effective than sulfamethoxazole. The effectiveness of the compound was further observed using atomic force microscopy, which showed great changes on topography, electrical force and nanomechanical properties of the microorganism. The cytotoxic assays with peripheral blood mononuclear cell model (PBMC) suggest that the new compound may be considered as an alternative antimicrobial, as well as a safe substance, since cytotoxic concentrations were higher than determined MICs, showing selectivity indexes in the range of efficacy. Thus, this new derivative could be considered a good candidate for further studies that would lead it to be used as a potential therapeutic agent for bacterial and biofilm elimination.

Keywords: sulfonamides, antimicrobial, mycobacteria, biofilm, atomic force microscopy.

INTRODUCTION

Rapidly Growing Mycobacteria (RGM) compose a subgroup of Nontuberculous mycobacteria (NTM) that is responsible for a large number of infections reported worldwide.[1] These microorganisms are dispersed in nature, have variable pathogenicity and causes different clinical forms of mycobacteriosis, especially in immunocompromised patients. [2] The high rates of morbidity and mortality associated with infections caused by RGM species are aggravated by imprecise identification of the etiologic agent of the mycobacteriosis, leading to difficulties in the implementation of adequate drug therapy.[3]

Furthermore, the RGM species, such as *M. fortuitum*, *M. massiliense* and *M. abscessus*, are able to form structured communities in liquid-air interface and adhere to solid surfaces, characterizing one of its most powerful mechanisms of resistance called biofilms.[4, 5] Biofilms are considered important sources of infection due to their increased antimicrobial resistance and their ability to adhere on different natural and synthetic surfaces.[5] Some studies suggest that biofilm development capacity is related to virulence profile, pathogenicity, resistance to antimicrobial agents and environmental survival.[4]

Despite the increase in the number of cases of microbial resistance, few antimicrobial drugs are approved. According to data from the Food and Drug Administration (FDA), between 2003 and 2012, there were only seven new antimicrobials made available.[6] This scarcity, threat the treatment of common infectious diseases, such as pneumonia and tuberculosis, making the post-antibiotic era, where banal infections can cause death, even closer.[7] In this regard, it has been observed an increasing emphasis in terms of the screening of new and more effective antimicrobial drugs.

In this field of investigation, an important research target is the organic compounds. In this present study we highlight Schiff bases. Schiff bases are widely used organic compounds, due to its structural chemical characteristics, that allow high interest of use in coordination chemistry, and for their biological activities.[8, 9] Structurally, Schiff bases are condensation products of primary amines with an active carbonyl, forming a carbon-nitrogen double bond (C=N).[10] These compounds are derived from different sulfonamide and have shown potential antimicrobial activity against Mycobacterium tuberculosis complex microorganisms and non-tuberculous mycobacteria (*Mycobacterium avium* e *Mycobacterium kansasii*), as well as against gram-negative microorganisms.

Regarding to that difficulty in terms of treatment of these RGM infections, in this study we synthesize and characterized a Schiff bases derived from sulfamethoxazole (SMTZ),

condensed with pyridoxal (analogous to vitamin B6), and evaluated its antimycobacterial and antibiofilm activities against different standard strains of mycobacteria and seven clinical isolates from patients diagnosed with mycobacteriosis admitted to a hospital in southern Brazil. In addition, we have evaluated the safety *in vitro* profile of this compound using a peripheral blood mononuclear cell model (PBMC).

RESULTS AND DISCUSSION

Chemistry

The term vitamin B6 corresponds to a set of 6 molecules found in nature, which can be interconverted in metabolic processes and are important in the metabolism of amino acids (Figure 1).[11-15] These molecules participate in reactions of dehydration, decarboxylation and racemization of amino acids, among others.

Schiff bases have the ability to form stable complexes with a large number of transition metals[16, 17], in addition, they can be synthesized with relative ease, and modified in order to adjust steric, electronic factors and induce chirality. [18, 19] The most widespread synthesis for obtaining imines is the condensation of a carbonyl compound (aldehyde or ketone) with a primary amine. [20] The reaction basically consists of the attack of the amine to carbonyl carbon, leading to the tetrahedral intermediate, and the subsequent elimination of water and formation of the double bond $R_1R_2C{=}NR_3$. Azometinic nitrogen presents sp^2 hybridization, being typically recognized as a Lewis base of an intermediate nature. Its association with other molecules is of great interest for several areas related to coordination chemistry, such as bioinorganic chemistry [21, 22] and catalysis. [23, 24]

Our work resulted in obtaining a Schiff base through the condensation of the primary amine of the sulfonamide group present in sulfamethoxazole with the aldehyde present in pyridoxal hydrochloride (Vitamin B6), as we can see in Figure 2.

After all the synthesis processes here performed, we have developed different experimental analysis looking forward to investigate the compound characterization, antibiofilm activity, microbial inhibitory potential as well as the *in vitro* safety profile. The proposed mechanism for the Sulfamethoxazole Derivative Schiff Base (SMTZ-SB) is illustrated in Scheme 1. The first step involves the nucleophilic attack of the amine to the carbonyl carbon of the aldehyde forming the intermediate zwiterionic specie **I**. In the second step the alkoxide acts as a base capturing a proton of the protonated amine forming the amino-alcohol intermediate **II**. In the third stage, prototropism occurs (transfer of a hydrogen atom

bound to nitrogen to the oxygen atom), forming another zwitterionic intermediate **III** with a good leaving group. In the fourth step it occurs the migration of an electron pair located in nitrogen to form the π bond between carbon and nitrogen and the elimination of a water molecule leading to the formation of the imine ligand **IV.**

Susceptibility tests

The susceptibility profile of the clinical isolates against the indicated drugs in the treatment of mycobacteriosis was evaluated using the gold standard technique indicated by the CLSI (2015)[25]. The broth microdilution, and the profiles results of each clinical isolate can be observed in Table 1. The results show that clinical isolates presented a higher resistance index to sulfamethoxazole (four resistant isolates), followed by clarithromycin (two resistant isolates and three isolates with intermediate resistance), ciprofloxacin (two isolates with resistance), doxycycline (one isolate with resistance and one isolate with intermediate resistance), amikacin and imipenem (with one clinical isolate with intermediate resistance each).

The different results obtained for the susceptibility tests clearly demonstrate that RGM require targeted treatment based on *in vitro* susceptibility determination. Thus, for the implementation of an efficient treatment it is necessary initially to identify the etiological agent. After identification it is necessary to determine resistance profile and according to it the ideal drug treatment can be selected. [3]

Another strongly important aspect regarding to bacterial infection therapy is antimicrobial drugs resistance. [26] In this context, our results showed that no one of the tested clinical isolate samples was sensitive to all the antimicrobial here tested, presenting at least an intermediate response for one of them. Macrolides, such as clarithromycin, for example, appear as first-line drugs for the treatment of mycobacterioses in which the etiologic agent is a RGM.[3,26] The clarithromycin susceptibility evaluation showed that from all studied samples, only 2 isolates could be classified as drug-sensitive, with a MIC of 2.0 μg mL^{-1} or less. In addition, it can be observed that isolate number VII was classified as multidrug resistant, showing resistance to clarithromycin, doxycycline and sulfamethoxazole. [27] Overall, among RGM, studies have been demonstrating and describing resistance to a broad spectrum of antimicrobials that are often used to treat infections caused by these microorganisms.[26] It should be noted that the great variability of RGM susceptibility profile, combined with high

resistance rates, is in turn a result of the limited effectiveness of current treatment regimens for diseases caused by these microorganisms.[28]

Thus, considering the high resistance profile of microorganisms to sulfamethoxazole, standard strains were added to the study in order to determine the inhibitory activity of the new compound. The results of the MICs of sulfamethoxazole and its derivative against the standard strains of RGM, as well as the new compound against clinical isolates demonstrate that the new molecule has significantly greater efficacy in planktonic inhibition of all microorganisms compared to sulfamethoxazole itself, with MICs ranging from 1.22 μg mL^{-1} and 0.61 μg mL^{-1}. These results can be seen in Table 2.

The clinical use of sulfamethoxazole, isolated or associated with Trimetropim, has been decreasing and this drug has been used in clinical practice with great restrictions. This is due to the development of resistance against this agent and its rapid spread, causing new antimicrobials to replace this sulfonamide in most of its clinical indications. [29] On the other hand, numerous biological activities, including antibacterial activity, have been described in the literature for several Schiff bases and several research groups have been involved in the synthesis and biological screenings of these organic compounds.[9]

Anacona *et al.* [30] synthesized, characterized and determined the antibacterial activity of a sulfathiazole-derived Schiff base, proving that the formed Schiff base had antimicrobial activity potential against *S. aureus* e *E. coli*. Sulfamerazine and salicylaldehyde were used to synthesize a Schiff base in the study of Barnabas *et al.* [31] The prepared hybrid materials showed good antimicrobial activity against both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria.

In the study of Krátký *et al.* [32], a series of Schiff bases derived from sulfadiazine were synthesized. All compounds evaluated were able to inhibit more effectively *M. Tuberculosis*, *M. avium* and *M. Kansasii*, with MIC values in the range of 8.0 to 1000 μM. In another study containing sulfonamide-derived Schiff bases, Schiff base has been synthesized by equimolar reaction (condensation) of sulfonamide by Kanh *et al.* [33] The *S. aureus* and *E. coli* bacterial strains were used in this study for antibacterial activity, showing varied levels of activity.

Atomic force microscopy (AFM)

The effect of the treatment with sulfamethoxazole derivative Schiff base on the topography, electrostatic and adhesion force properties of a *M. smegmatis*. As seen in Figure

3A and 3B, topography images display the expected feature for untreated mycobacteria, a rod-shaped morphology structure. On the other hand, as shown in Figure 3C and 3D the bacilli are significantly modified after treatment, now exhibiting a non-specific morphology, but can be viewed as ellipsoids of revolution with withered regions. In fact, the membranes of mycobacteria very susceptible to the treatment undergoing to large deformations and displaying many holes all over their surface.

In addition to morphological changes, the treatment with the compound also affects the adhesion force on mycobacterial walls. As shown in Figure 3B and 3D, adhesive forces on untreated bacteria membrane is roughly 0.140 μN, but increasing up to 0.29 μN. At this time, we speculate that the treatment with the derivative damage the mycobacteria by changing the organic functions on the membrane structure. This facilitates the AFM tip nanoindentation on the bacteria wall, inducing a higher contact area and following a subsequent increase in the adhesive forces (pull-off force), where a similar effect was also observed by Laskowski *et al.*[34] and Rahnamaeian *et al.*[35]. Moreover, the adhesion force map shown in Figure 3B shows a higher adhesive force at the border between the bacteria surface and substrate, but after treatment the adhesive force at the mycobacteria perimeter is decreased (Figure 3D). These evidences suggest that the compound also reduces the bacteria interfacial adhesion, what it must contribute to decreasing bacterial growth rates on the substrates.

Figure 3A and 3C shows the topography and electric force maps for the mycobacteria untreated and treated with SMTZ-SB, respectively. In EFM maps, dark-colored regions are relatively more negative, while brighter ones indicate positively charged regions. Thus, the electric force maps in Figure 3A indicate that the mycobacterial membrane is much more negative than the substrate, indicating that electrostatic forces must also mediate the adhesion of the bacteria to the substrate.[56, 57] The electrostatic component between mycobacteria membrane and substrate does not show significant changes after the treatment with SMTZ-SB, but many negative regions were found on the bacterial walls. In fact, most of the higher topography dots correspond to negative regions, while on withered spots the bacterial membrane is positively charged, highlighting its destabilization due to the SMTZ-SB treatment.[56,57]

The AFM measurements on mycobacteria are timely: the effect of the treatment with SMTZ-SB is easily observed on topography, adhesion and electric force images. The AFM 3D image reconstruction feature is even more suitable to evidence the deformation of the bacterial wall after the treatment. In fact, Figure 4 shows 3D topographic images of

Mycobacterium smegmatis, where a withered region is clearly observed after SMTZ-SB treatment (B) (0.61 µg mL-1).

Biofilm formation inhibition test

A large and growing body of evidence offers convincing arguments that the persistence of most microbial species is achieved through biofilm formation. In very hydrophobic microorganisms, such as those of the genus *Mycobacterium*, biofilm formation is a successful survival strategy. [1, 36] Biofilms formed by mycobacteria have been reported in environmental studies, especially in water systems. Another important biofilm-related group of infections are those associated with biomaterial. Biofilms are important sources of infections on biomedical surfaces, and most infections involving biofilm formation are associated with medical devices implants such as catheters and prostheses. [1, 36, 37] In addition, acute infections caused by microorganisms of the genus *Mycobacterium* are normally associated with contamination by planktonic organisms, whereas chronic infections seem to be strongly associated with biofilm formation.[38]

In our study, from the seven clinical isolates, three formed highly dense and compact biofilms. It is known that RGM are able to form biofilms *in vitro*, being this structure of great importance in the pathogenesis of their infections.[39] Also, biofilm development capacity is a property that is not uniformly present among clinical isolates of RGM, which corroborates to the results here described, since four clinical isolates did not exhibit this ability when exposed to the same experimental conditions as clinical isolates that were able to form biofilm.[40] Besides to representing a prominent factor in the pathogenesis of mycobacteriosis, the presence of mycobacteria in biofilm form is also a public health problem because it is present in water pipes, including public supplies, making the biofilms of these microorganism potential sources of infection. [1, 5]

M. abcessus, M. massiliense and *M. fortuitum* and clinical isolate samples number IV, V and VI were able to form biofilms on the air-liquid surface and on all surface of polystyrene tubes. The results of inhibition of the mycobacterial biofilms formation by sulfamethoxazole and its new derivative can be seen in Figures 5 and 6, respectively.

Subinhibitory concentrations of sulfamethoxazole and the Schiff base derived from sulfamethoxazole were used to perform the tests in order to maintain bacterial cell viability. Thus, the microorganisms present in inoculum remained, after treatment with the drug and the derivative, available to adhere to the tube surface and form the sessile structure.

For all microorganisms evaluated, sulfamethoxazole showed low efficacy in inhibiting the biofilm formation. The drug inhibited the formation of the biofilm of *M. abcessus* only at the highest concentration. In addition, it showed mild inhibition in the biofilm formation by *M. fortuitum* and *M. massiliense*. These results corroborate with the studies performed by Flores *et al.* [26] and Siqueira *et al.* [41]. Both authors report low efficacy of sulfamethoxazole in inhibiting the sesile structure formed by *M. abcessus*, *M. fortuitum* and *M. massiliense*. Thus, this demonstrates that the drug has low ability to eradicate mycobacterial biofilm.

Sulfamethoxazole is a broad-spectrum sulfonamide, considered of great importance in the control of infections caused by different pathogens, including RGM, as *M. abscessus* e *M. fortuitum* and the combination of sulfamethoxazole to trimetropim has already been a much-suggested alternative in the treatment of infections caused by microorganisms of the genus *Mycobacterium*.[42] The clinical indication for sulfamethoxazole persists, but it is found that activity decreases significantly when the infectious agent presents itself in the form of biofilms or inside host macrophages.[26, 29]

On the other hand, synthetized Schiff base inhibited significantly the formation of biofilms by the three strains used in the study, inhibiting, to some degree, the formation of the biofilm in all concentrations tested. Additionally, the concentrations used for the new compound were lower than the concentrations used for sulfamethoxazole for all the performed assays, demonstrating the potential activity of the new molecule.

Several studies have reported the antibiofilm activity from different Schiff bases, but the present work reports for the first time the antibiofilm activity of a Schiff base derived from sulfamethoxazole against RGM. More *et al.* [20] showed the antibiofilm activity of thiazole Schiff bases against gram-positive microorganisms. Arshia *et al.* [43] also evaluated the antibiofilm potential of synthetic 2-amino-5-chlorobenzophenone Schiff against biofilms formed by gram-negative microorganisms. When evaluating antibiofilm activity of Novel Chitosan Derivative Schiff Base, synthesized by Ali *et al.* [8], it was found that this organic compound completely instilled the formation of biofilms of *P. aeruginosa.*

The inhibition of biofilm formation of *M. abcessus*, *M. fortuitum* e *M. massiliense* suggest that the Schiff base synthesized in our work can act mainly in two different ways: I) avoiding the microorganism's surface, by inhibiting physical-chemical interactions that mediate this process; and/or II) as an inhibitor of cellular communication of the sessile community, known as *Quorum sensing*.[44] Thus, the molecule would act by inhibiting the signaling of stages of the biological cycle of the formation of mycobacterial biofilm.

However, new *in vitro* and *in vivo* experimental trials should be performed to elucidate the exactly mechanism by which this Schiff base inhibits the biofilms formation.

Moreover, since the Schiff base formed is structurally presented as an imine functional group, we highlight the greater lipophilicity of the organic compound when compared to the corresponding amino group.[10] This is a limiting factor in the antibacterial activity of new molecules, since microorganisms of the *Mycobacterium* genus have a thick, highly hydrophobic and lipid cell wall rich in mycolic acids, making them waterproof to disinfectants and antimicrobials, thus hindering its inhibition, both in the plankton form and in the sessile form.[45]

The promising activity of our molecule as an inhibitor of the formation of mycobacterial biofilms is extremely relevant, since biologically active substances against this sessile structure are increasingly scarce. Flores *et al.* [26] evaluated the Antibiofilm effect of antimicrobials used in the therapy of mycobacteriosis and concluded that Biofilms formed by rapidly growing mycobacteria (RGM) are resistant to antimicrobials commonly used in mycobacteriosis therapy. The antibiotics tested included amikacin, ciprofloxacin, Clarithromycin, doxycycline, imipenem and sulfamethoxazole.

Cytotoxicity assessment of SMTZ-SB in PBMC cell culture

The concentrations chosen for compound to evaluate cellular effects were selected based on previously obtained MICs results. Cell viability after treatment with the Schiff base was evaluated using the MTT technique and the free dsDNA quantification using the Picogreen® reagent. Both results demonstrate a safe and similar profile of cellular viability. When comparing the results with the positive control, the treatments with the new molecule tested presented a satisfactory safety profile in the cell viability of the model used. While the positive control (H_2O_2 group) presented a significant decrease of cellular viability, all SMTZ-SB tested concentrations kept cellular viability index in a similar way than negative control. Additionally, the treatments did not significantly change the dsDNA release compared to untreated cells. Cells treated with H_2O_2 showed an increased amount of extracellular dsDNA, as expected, possibly due to membrane damages (Figures 7A and B). These results are in concordance with the pharmacological principle that a formulation which has effective biological properties and safety profile at small concentrations is preferable.[46]

Also, it is important to highlight that some microorganisms, such as *M. tuberculosis*, can induce cellular death through mitochondrial modulation, directly modulating cellular oxidative metabolism.[47] However, although some antimicrobial molecules act by inducing

oxidative stress. In this regard, we must take into account that high production of ROS can be highly disastrous to eukaryote cells, thus limiting their use as antimicrobial agent.[48] In view of this, after the incubation period, the PBMCs were also evaluated in terms of ROS levels and NO production. No one of the concentrations here analyzed was able to modify ROS levels compared to the negative control. Moreover, most of the concentrations also kept NO production to similar amount found for untreated cells, except the except the highest concentrations tested, which caused a slight increase in NO levels. As expected, H_2O_2 exposure cause an increased production of ROS and NO compared to negative control (Figure 7C and D).

CONCLUSION

Our work confirms that the modification of sulfonamide structure has proven highly effective. The synthetized Schiff base showed significant ability to inhibit the growth of RGM, in both plankton and biofilm forms. In addition, the molecule was much more effective than sulfamethoxazole and showed safety *in vitro* profile. We emphasize that the modifications that were made do not exhaust other possibilities for changes that can be made to improve potency and efficacy of these sulfonamides.

EXPERIMENTAL SECTION

Synthesis reaction of the New Sulfamethoxazole Derivative Schiff Base

In a round bottom flask, 0.100 g of sulfamethoxazole (3.94×10^{-4} mol) was diluted in 20 mL of methanol. Then 0.100 g (3.94×10^{-4} mol) of pyridoxal hydrochloride was added. The orange solution remained under stirring and heating at 50°C for 3 hours. After evaporation of the solvent, an orange solid material was obtained. For purification, a 1:1 column (hexane: acetate) was made. The solvents methanol, ethanol, dimethylformamide (DMF) used in the synthesis of the organic compound, as well as sulfamethoxazole (Sigma) were used without previous any treatment.

New sulfamethoxazole derivative Schiff Base: Yield: 92.8%. M.W. = 402 g mol^{-1}. FTIR (KBr pellets, cm^{-1}): 3452.76 [m, υ $(O–H)_{alcohol}$]; 1619.51 [s, δ$(C=N)_{imine}$]; 2947.34 [m, υ $(C–H)_{aromatic}$]. ^{1}H-RMN (DMSO-d_6) 400 MHz (δ em ppm): 4.52 s {3H} CH_3; 5.58-6.61 m {8H} } CH $_{(aromático)}$; 6.96 {1H} CH $_{(imina)}$; 10.10 s {1H} $NH_{(hidrazida)}$.

General Instrumentation

CHN% elemental analyses were performed at a Shimadzu EA112 microanalysis instrument. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DPX-400 spectrometer. DMSO-d_6 and $CDCl_3$ were used as the solvent and TMS as the internal reference. Chemical shifts are reported in parts per million (δ, ppm) and were referenced to residual solvent peak. With the multiplicities expressed as: s, singlet; t, triplet; q, quartet; quintet; sextet, m, multiplet and br, broad). FTIR spectra were recorded on a Bruker Vertex 70 spectrometer equipped with Platinum ATR accessory in the 4000-30 cm^{-1} region and on a Tensor 27 Bruker spectrometer with KBr pellets in the 4000-400 cm^{-1} region. UV-vis absorption spectra were recorded on a UV-2600 Shimadzu spectrometer and U-1800, Hitachi. Cyclic voltammograms were recorded with a potenciostat/galvanostat AutoLab Eco Chemie PGSTAT 128N system at room temperature and under argon atmosphere, in dry DMF solution. Electrochemical grade tetrabutylammonium hexafluorophosphate (0.1 M $TBAPF_6$) was used as supporting electrolyte. Employing a standard three-component system carried out these CV experiments: a glassy carbon working electrode; a platinum wire auxiliary electrode and a platinum wire *pseudo*-reference electrode. To monitor the reference electrode, the ferrocenium/ferrocene redox couple was used as an internal reference [49].

Microorganisms strains and clinical isolates

The standard strains *M. abscessus* (ATCC 19977), *M. fortuitum* (ATCC 6841), *M. smegmatis* (ATCC 700084) and *M. massiliense* (ATCC48898) were used in this work. In addition, for the accomplishment of this study we used 7 clinical isolates of patients hospitalized at the University Hospital of Santa Maria (HUSM) obtained from the Clinical Analysis Laboratory in 2020, characterized as acid-alcohol resistant bacillus (AARB positive) and that have grown in Lowenstein-Jensen (LJ) medium (HiMedia Laboratories Pvt Ltd, Mumbai, India). The microorganisms were kept at −80 °C and grown on Löwenstein-Jensen agar (HiMedia Laboratories Pvt. Ltd, India). This work was conducted with experimental protocol approved by UFSM ethics committee for research with clinical isolates, CAAE number: 71795417.6.0000.5346.

Susceptibility tests

The susceptibility tests were performed by broth microdilution method according to the standard protocol established by Clinical & Laboratory Standards Institute (CLSI), document M24-A2.[25] The inoculum density for this susceptibility test was 5.0 x 10^5 CFU

mL^{-1}. The antimicrobial agents were evaluated at the following concentrations: amikacin (Fluka) 1.0–128 μg mL^{-1}, ciprofloxacin (Fluka) 0.125–16 μg mL^{-1}, clarithromycin (Sigma) 0.06–64 μg mL-1, doxycycline (Sigma) 0.25–32 μg mL^{-1}, imipenem (Fluka) 1–64 μg mL^{-1} and sulfamethoxazole (Sigma) 1.0–128 μg mL^{-1}. The New Sulfamethoxazole Derivatives Schiff Base were evaluated at concentrations of 0.150 to 1250 μg mL^{-1}.

After 72 hours of incubation at 30°C, treatment plates were read and the minimum inhibitory concentrations (MIC) were determined using 2,3,5-triphenyltetrazolium chloride (Vetec VR, Rio de Janeiro, Brazil) as microbial growth indicator. [41]

Atomic force microscopy (AFM) analysis

The effect and interaction of New Sulfamethoxazole Derivative Schiff Base on the mycobacteria was investigated by AFM. *M. smegmatis* (ATCC 700084) was used as a non-pathogenic agent from the RGM group, although it presented a similar response to the strains/Sulfamethoxazole Derivative Schiff Base in preliminary studies.[50] The inoculum preparation for microscopy was performed in 96-well microplates and its density was 5.0 x 10^5 CFU mL^{-1}. After 24 hours of incubation at 30°C in the presence of the compound at the concentration previously determined as MIC (0.61 μg mL^{-1}), ATCC 700084 samples were diluted in water and deposited on small square pieces of freshly cleaved mica. AFM characterization was done using a Park NX10 microscope (Park 200 Systems, Suwon – Korea) equipped with a SmartScan software version 1.0.RTM11a. Topography and adhesion maps were acquired simultaneously using the PinPoint Nanomechanical mode and a high frequency rotated monolithic silicon probe (TAP300-G Budget Sensors, Sofia – Bulgaria) with a nominal resonance frequency of 300 kHz and 40 Nm−1 force constant. Electric force mode was performed using the standard non-contact AFM setup with a gold-coated n-type silicon probe (NSC14 Cr/Au Mikromasch, Sofia – Bulgaria), nominal resonance frequency of 160 kHz, and 5.0 N m^{-1} force constant. All measurements werecarried out under ambient conditions at room temperature (21 ± 5°C) and relative humidity (55 ± 10%). Images were treated offline using XEI software version 4.3.4 Build22.RTM1.

Biofilm formation inhibition test

The standard strains *M. abcessus*, *M. fortuitum* and *M. massiliense* and biofilm-forming clinical isolates were used to evaluate the ability of the compound to inhibit biofilm formation. The biofilms formation and quantification were determined as described by Flores *et al*. [26] In polystyrene test tubes it was added 1.0 mL of Middlebrook 7H9 medium (BD®, Le

Pont de Claix, France) containing 1.0 x 10^7 CFU mL^{-1} of clinical isolates culture. So, 1.0 mL of treatment per tube was added for each compound concentration. The concentrations used were equal and lower than the MIC according to the methodology proposed by Flores *et al*. [26] The tubes were topped with parafilm® and incubated at 30 °C for 7 days. After this period, the biofilm quantification was performed using 0.1% crystal violet. The absorbance was measured in spectrophotometer in optical density (OD) of 570 nm (Spectrophotometer U-1800, Hitachi, Berkshire, United Kingdom). The sulfamethoxazole mycobacterial biofilm inhibitory activity was also determined for comparison and the tests were performed in triplicate. The microorganism biofilms formation inhibition caused by the compound was determined by the significant difference between the means of the obtained absorbance for the positive control (culture medium and bacterium) and the mean obtained for the negative control (culture medium).

In vitro cytotoxicity evaluations

Cell culture and treatments

Peripheral blood mononuclear cells (PBMCs) derived from discarded total blood samples of healthy adults were obtained from the Clinical Analysis Laboratory (LEAC) of the Franciscan University (UFN) (experimental protocol approved by UFN ethics committee for research with human beings, CAAE number: 31211214.4.0000.5306). Blood samples were processed for PBMC separation through the procedure based on density gradient difference using Ficoll Histopaque-1077VR reagent (Sigma-Aldrich). After blood disposition into the reagent (1:1, v:v), samples were centrifuged for 30 min at room temperature. PBMCs were plated in 96-well plates containing RPMI 1640 cellular medium (Sigma-Aldrich) containing 10% fetal bovine serum, and supplemented with 1.0% of antibiotics (penicillin/streptomycin) and antimycotic (amphotericin B). Cells were cultured at an initial density of 2.0 x 10^5 cells/mL per well.[51] Then, cells were exposed to the compound here tested for the efficacy protocol described in this research at the same concentrations for 24h, looking forward to evaluate their effect on cellular viability and oxidative homeostasis through different colorimetric and fluorimetry assays. All treatments and assays were performed in at least triplicate to ensure statistical coherence; hydrogen peroxide (H_2O_2) at 200 μM was used as a positive control for all *in vitro* cellular assays.

Cell viability measurements

After the period of treatment, cellular viability was evaluated. The first assay performed was the MTT assay using MTT bromide salt {[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]} reagent (Sigma-Aldrich) at 5.0 mg mL^{-1} diluted in phosphate buffer solution at pH 7.4.[52] The plates were incubated for 4h at 37ºC and the intracellular formazan crystals formed were solubilized with dimethyl sulfoxide (DMSO). Absorbance was determined at 570 nm using Anthos 2010 microplate reader equipment (Anthos, London, UK). To complement the results obtained from the MTT assay, extracellular dsDNA measurement was performed, as a parameter of cellular mortality through lysis and dsDNA release. This evaluation was accomplished using PicoGreen® reagent.[53] This reagent has high dsDNA affinity even when it is present at peak concentrations. Treatment's supernatants were plated on dark 96-well microplates, and mixed with PicoGreen® reagent diluted with T.E 1X buffer. After incubation for 5 min at room temperature, fluorescence was determined at 480 nm excitation and 520 nm emission using a SpectraMax microplate reader (SpectraMax Solutions Ltd, London, UK).

Quantification of total levels of reactive oxygen species

Quantification of total levels of reactive oxygen species (ROS) was determined using the 2',7'-dichlorofluorescein diacetate assay (DCFH-DA) (SigmaAldrich), as described by Ahn *et al*. [54] DCFH-DA reagent has the ability to cross the cellular membrane. Inside the cell, it is deacetylated by mitochondrial enzymes, giving rise to 2' ,7'-dichlorodihydrofluorescein which reacts with ROS, mainly hydrogen peroxide (H_2O_2), and produces DCF which emits fluorescence. Treated and untreated PBMCs were mixed with DCFH-DA reagent for 1h at room temperature. Thus, fluorescence was determined in a spectrofluorometer (SpectraMax Solutions Ltd) based on the wavelengths of 488 nm excitation and 525 nm emission.

Indirect determination of nitric oxide production

The determination of nitric oxide (NO) production was determined by using an indirect method described by Choi *et al*. [55] Through this colorimetric assay it is possible to quantify NO metabolites nitrate and nitrite. After incubating supernatant samples with Greiss reagent for 15 minutes, absorbance was measured at 540 nm in a plate-reader (SpectraMax Solutions Ltd).

Statistical Analysis

Biofilm formation inhibition were determined by a significant difference between the averages of absorbance obtained in the positive control and the average obtained in the negative control. The experiment was performed in triplicate. The OD readings obtained in the biofilm formation assay were recorded as means ± standard deviation (SD) and were submitted to a t-test (compared with the positive control). Values with $p < 0.05$ were considered to indicate statistical significance.

The *in vitro* safety profile results obtained are presented as a percentage of the untreated control group (NC). Data were expressed as means ± SD. Analyses were performed by 1-way ANOVA followed by Dunnett's post hoc. Values with $p < 0.05$ were considered statistically significant, being * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Graphs were prepared using GraphPad Prism version 9.01 (GraphPad Software, La Jolla, CA, USA).

AUTHOR CONTRIBUTION STATEMENT

The conception and design of the work was carried out by F. S. Siqueira, A. K. Machado, D. F. Back and M. M. A. Campos. The synthetic experimental work and structural characterization was performed by F. S. Siqueira, D. F. Back, J. D. Siqueira and B. B. Iglesias. Data collection was carried out by F. S. Siqueira. Analysis and interpretation of the data was undertaken by F. S. Siqueira, J. D. Siqueira, D. F. Back and A. K. Machado. Citotoxicity studies were conducted by F. S. Siqueira, A. K. Machado and M. R. Sagrillo. The antimicrobial/antibiofilm evaluation and subsequent data analysis was done by F. S. Siqueira and A. K. Machado. Atomic force microscopy images were performed by F. S. Siqueira, T. A. L. Burgo and K. S. Moreira. Drafting of the manuscript was conducted by F. S. Siqueira, A. K. Machado and Davi F. Back. The critical revisions of the manuscript were conducted by F. S. Siqueira, A. K. Machado, D. F. Back. All authors approved the submitted manuscript.

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FIGURES

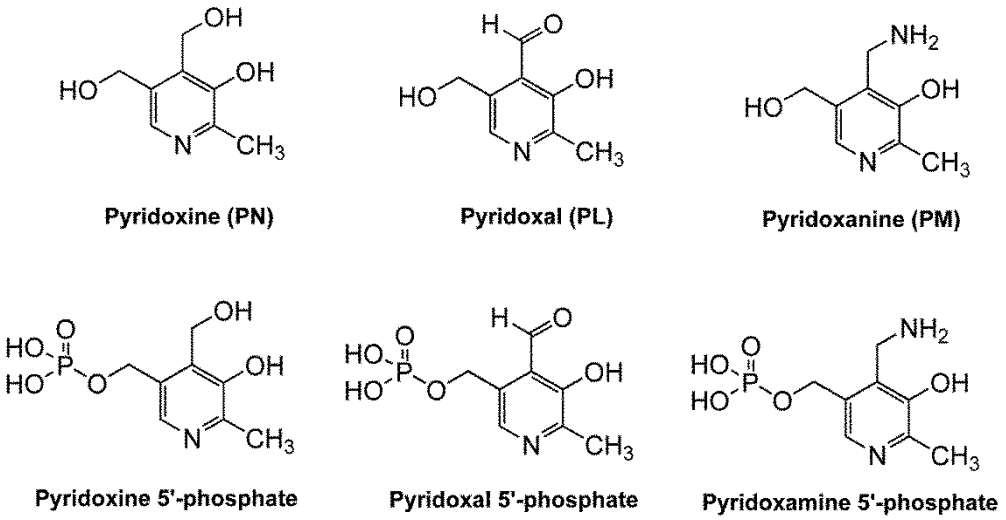


Figure 1. Variations of vitamin B6 found in nature.

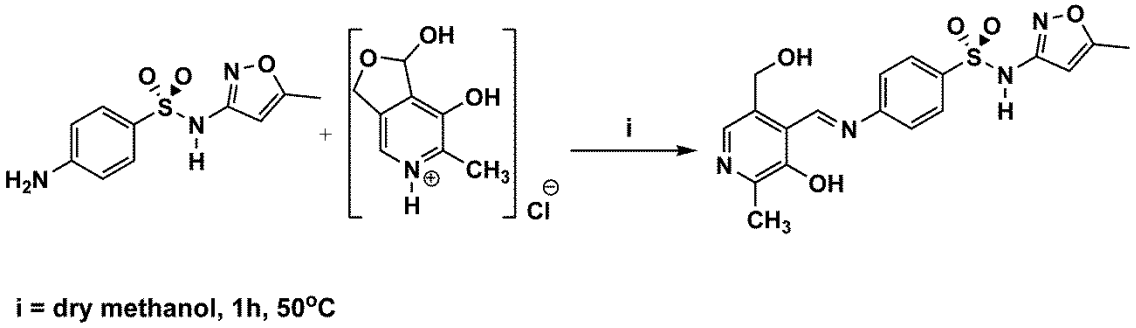


Figure 2. Scheme of new sulfamethoxazole derivative Schiff base (SMTZ-SB), obtained through condensation of the primary amine of the sulfonamide group present in sulfamethoxazole with the aldehyde present in pyridoxal hydrochloride (Vitamin B6).

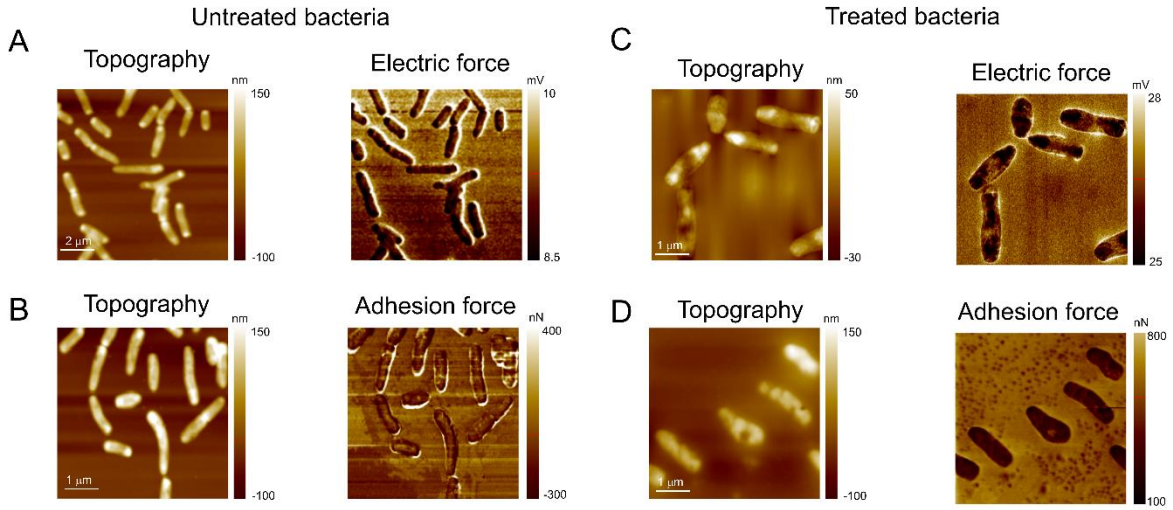


Figure 3. AFM images of *Mycobacterium smegmatis* after incubation for 24 h at 30ºC. **(A-B)** Negative control image (bacterial inoculum and Mueller Hinton broth culture medium); the images demonstrate the microorganism's electrical force **(A)** and adhesion force **(B)** parameters, as well as their topographies, without treatment with SMTZ-SB. **(C-D)** Images of *M. smegmatis* after treatment with minimal inhibitory concentration provided for the compound SMTZ-SB (0.61 µg mL^{-1}); the images demonstrate the altered parameters of electrical force **(C)** and adhesion force **(D)** of the microorganism, as well as their topographies damaged by the action of the compound.

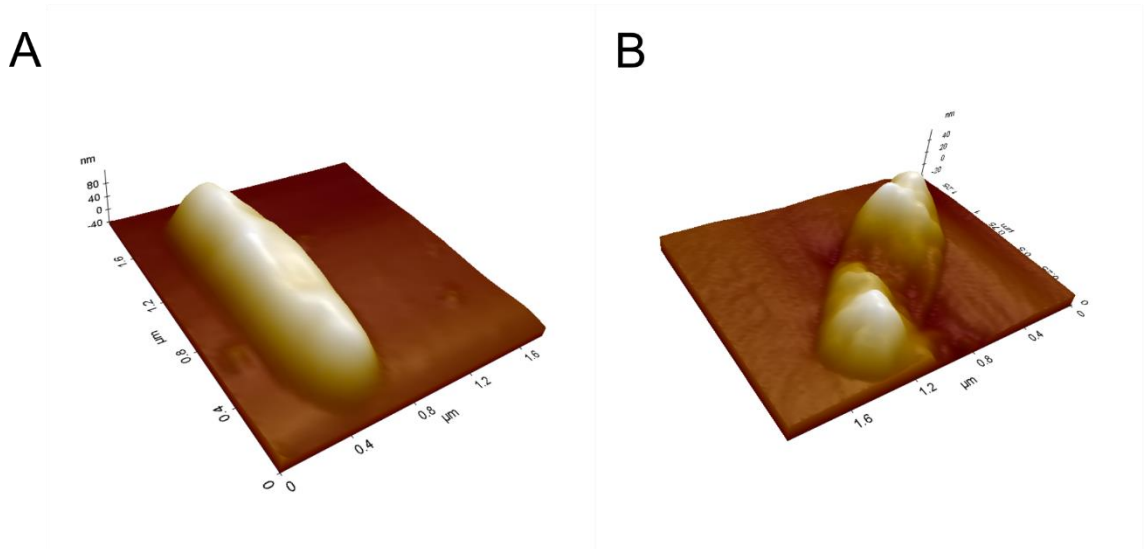


Figure 4. 3D topographic images of *Mycobacterium smegmatis* before (A) and after (B) SMTZ-SB treatment (0.61 µg mL^{-1}).

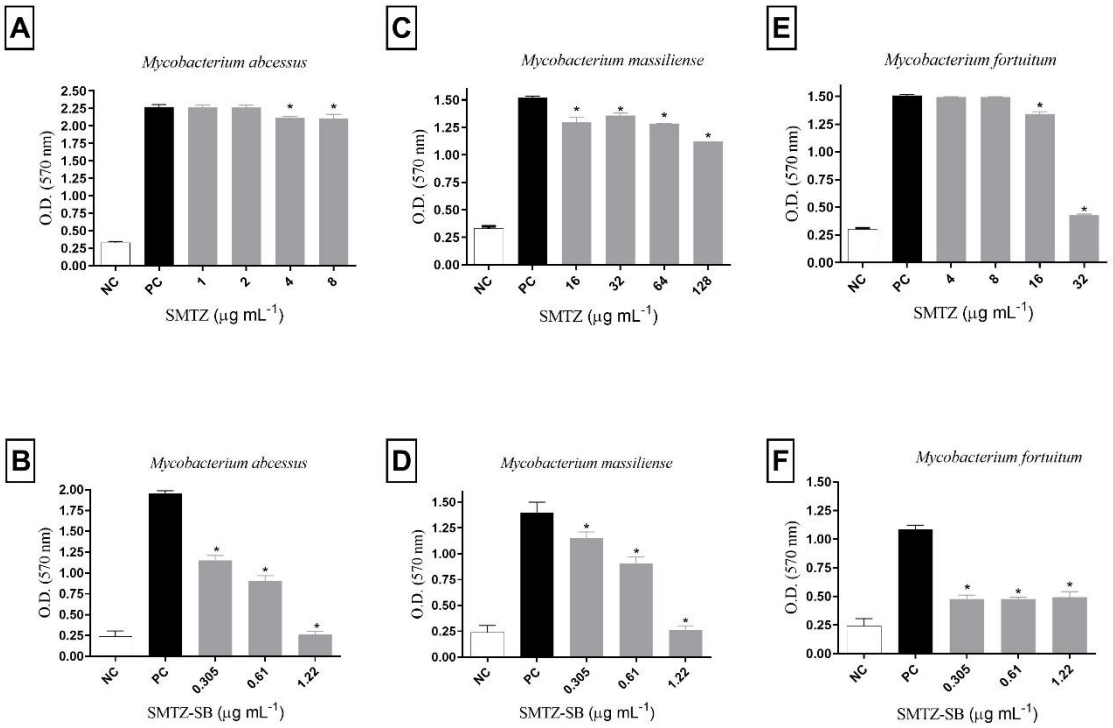


Figure 5. Effect of subinhibitory concentrations of SMTZ (A, C and E) and SMTZ-SB (B, D and F) on the biofilm inhibition from *Mycobacterium abscessus* (A and B), *Mycobacterium massiliense* (C and D*) and Mycobacterium fortuitum* (E and F).* Represents significant difference between the concentration tested and the positive control (PC). The tests were subjected to the Student t-test (n = 3), considering statistical differences when $p < 0.05$.

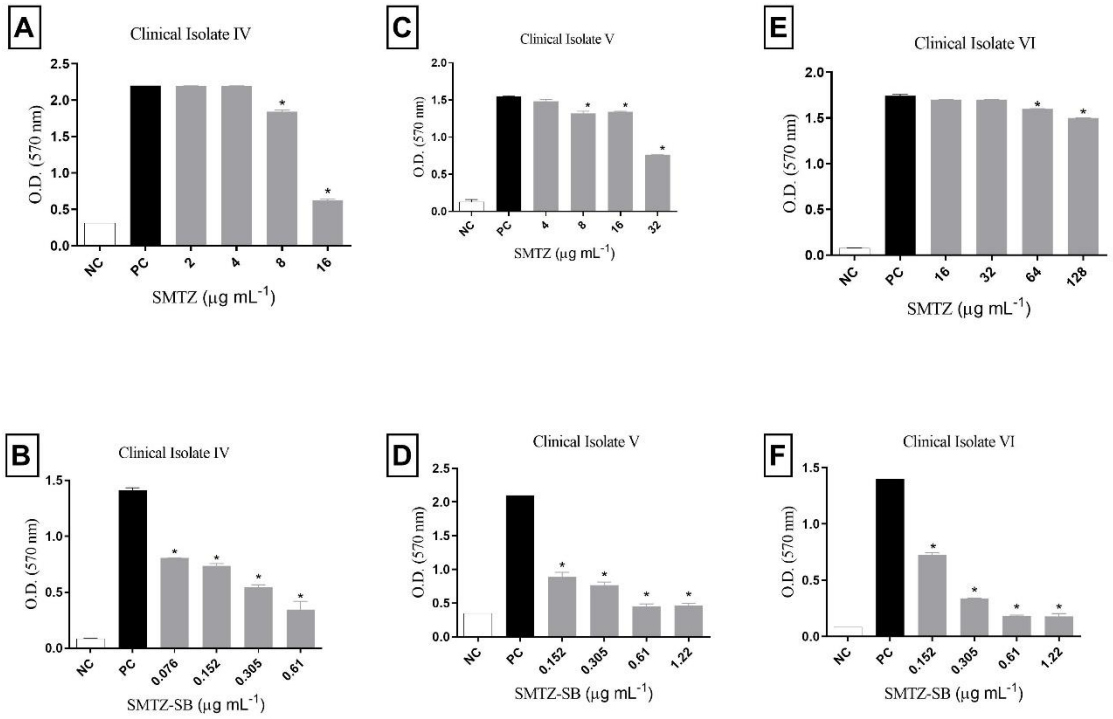


Figure 6. Effect of subinhibitory concentrations of SMTZ (A, C and E) and SMTZ-SB (B, D and F) on the biofilm inhibition from Clinical Isolate IV (A and B), Clinical Isolate V (C and D) and Clinical Isolate VI (E and F).* Represents significant difference between the concentration tested and the positive control (PC). The tests were subjected to the Student t-test (n = 3), considering statistical differences when $p < 0.05$.

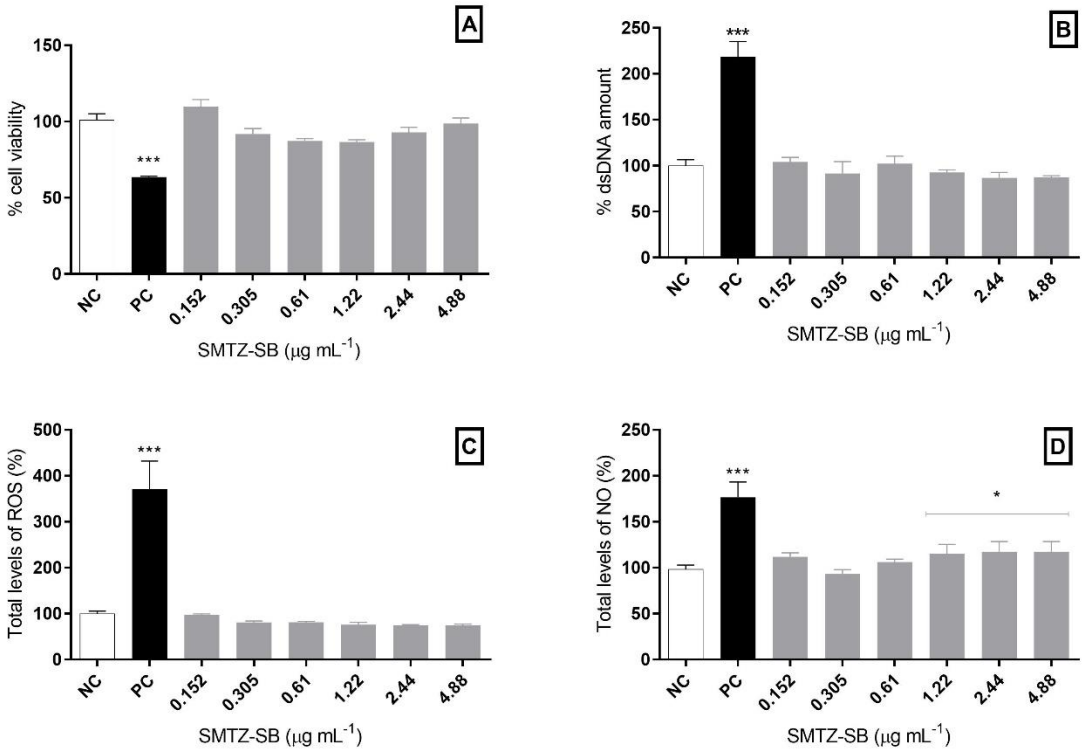
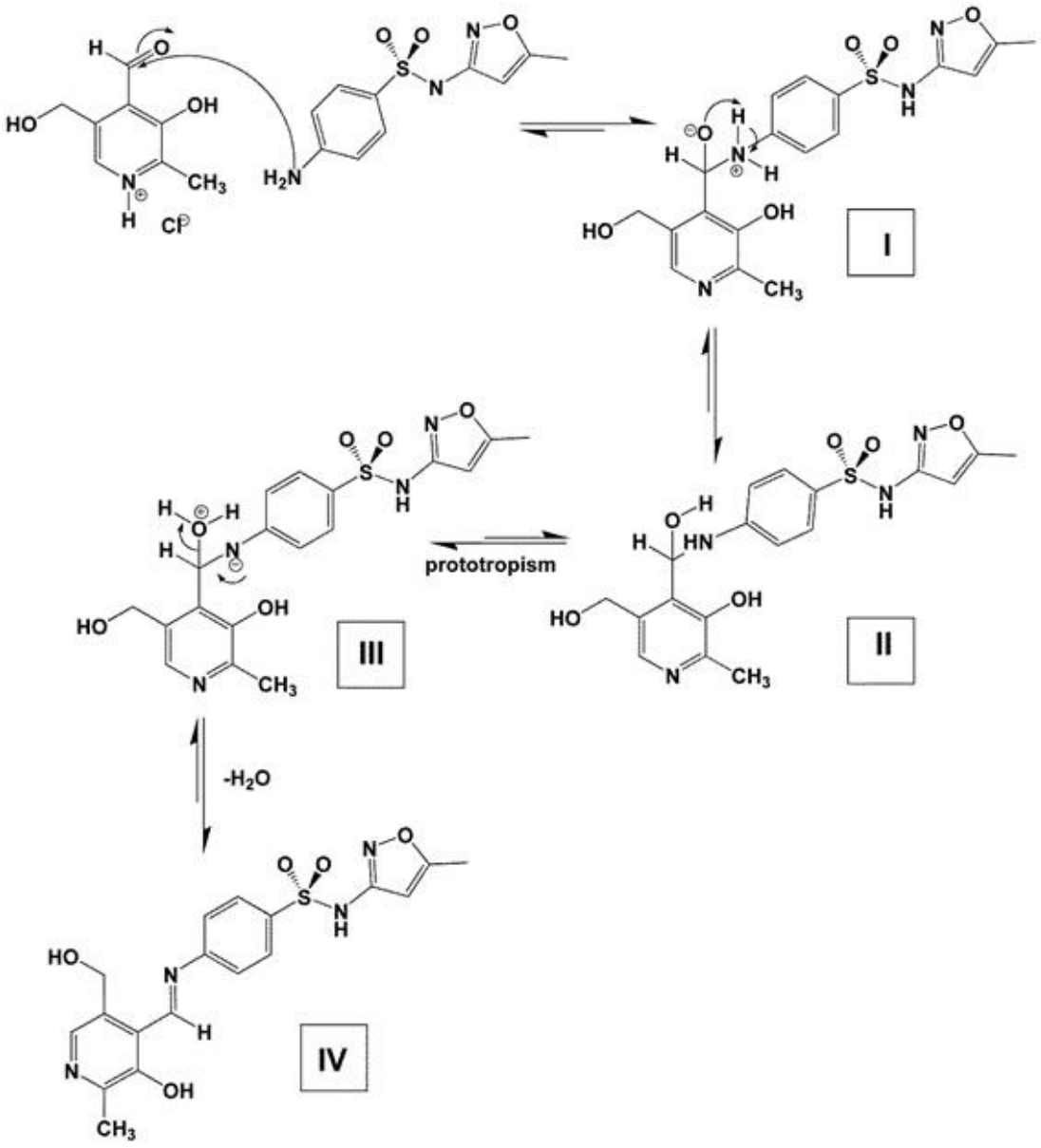


Figure 7. Cytotoxic analysis in PBMCs exposed to SMTZ-SB for 24 h. Cellular viability analysis (MTT assay) (A), Free dsDNA quantification amount (B), total levels of ROS (dcfh-da assay) (C), and indirect quantification of NO (D). The results are expressed as percentages of the negative controls (100%). Data are expressed as means ± SD. Analyses were performed by 1-way ANOVA followed by Dunnett's post hoc. Values with $p < 0.05$ were considered statistically significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

SCHEMES



Scheme 1. Schematic image of Sulfamethoxazole Derivative Schiff Base obtaining proposal.

TABLES

Table 1. *In vitro* susceptibility of clinical isolates of rapidly growing mycobacteria to different antimicrobial agents.

	Minimum inhibitory concentrations (μg mL^{-1})					
Clinical Isolate	Sulfamethoxazole	Clarithromycin	Doxycycline	Amikacin	Imipenem	Ciprofloxacin
I	128^{c}	4.0^{b}	2.0^{a}	1.0^{a}	0,5^{a}	1.0^{a}
II	32^{a}	4.0^{b}	0.250^{a}	0.5^{a}	1.0^{a}	0.125^{a}
III	128^{c}	4.0^{b}	4.0^{b}	1.0^{a}	2.0^{a}	0.250^{a}
IV	16^{a}	16^{c}	0.250^{a}	32 ^{b}	1.0^{a}	0.125^{a}
V	32^{a}	2.0^{a}	0.250^{a}	2.0^{a}	8.0^{b}	4.0^{c}
VI	128^{c}	0.5^{a}	0.5^{a}	2.0^{a}	4.0^{a}	4.0^{c}
VII	128^{c}	64^{c}	32^{c}	8.0^{a}	0.25^{a}	0.03125^{a}

^{a}Susceptible; ^{b} Intermediate; ^{c} Resistant

Table 2. MIC values for the standard strains and clinical isolates of rapidly growing mycobacteria exposed to sulfamethoxazole (SMTZ) and sulfamethoxazole derivative Schiff base (SMTZ-SB).

	Minimal Inhibitory Concentrations (μg mL^{-1})	
Microrganism	**SMTZ**	**SMTZ-SB**
M. abscessus	8.0	1.22
M. fortuitum	32	1.22
M. massiliense	128^{c}	0.61
M. smegmatis	64	0.61
Clinical Isolate I	128^{c}	1.22
Clinical Isolate II	32^{a}	1.22
Clinical Isolate III	128^{c}	1.22
Clinical Isolate IV	16^{a}	0.61
Clinical Isolate V	32^{a}	1.22
Clinical Isolate VI	128^{c}	1.22
Clinical Isolate VII	128^{c}	1.22

^{a}Susceptible; ^{b} Intermediate; ^{c} Resistant

Supporting Information

Synthesis and Biological Evaluation of New Sulfamethoxazole Derivative Schiff Base as antimicrobial and antibiofilm Agent on Rapidly Growing Mycobacteria.

Fallon dos Santos Siqueira[1], Josiéli Demétrio Siqueira[2], Alencar Kolinski Machado[3], Kelly Schneider Moreira[5], Michele Rorato Sagrillo[3], Thiago Augusto Lima Burgo[5], Bernardo Almeida Iglesias[4], Davi Fernando Back[2*], Marli Matiko Anraku de Campos[1]

CONTENTS

FIGURES AND TABLES

1. Chemistry

1.1 ^{1}H- and ^{13}C-NMR spectra.

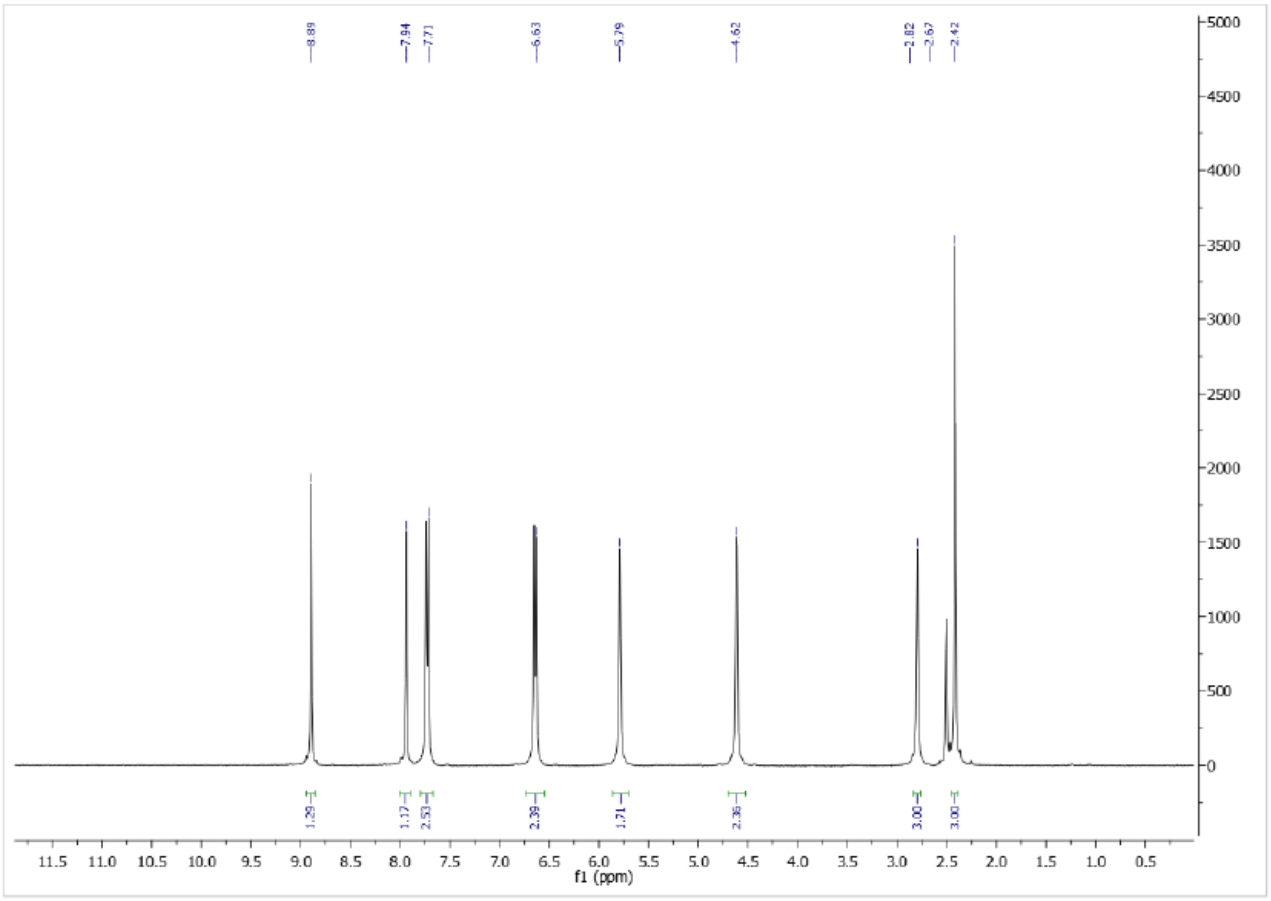


FIGURE S1. ^{1}H-NMR spectrum of new sulfamethoxazole derivative Schiff base.

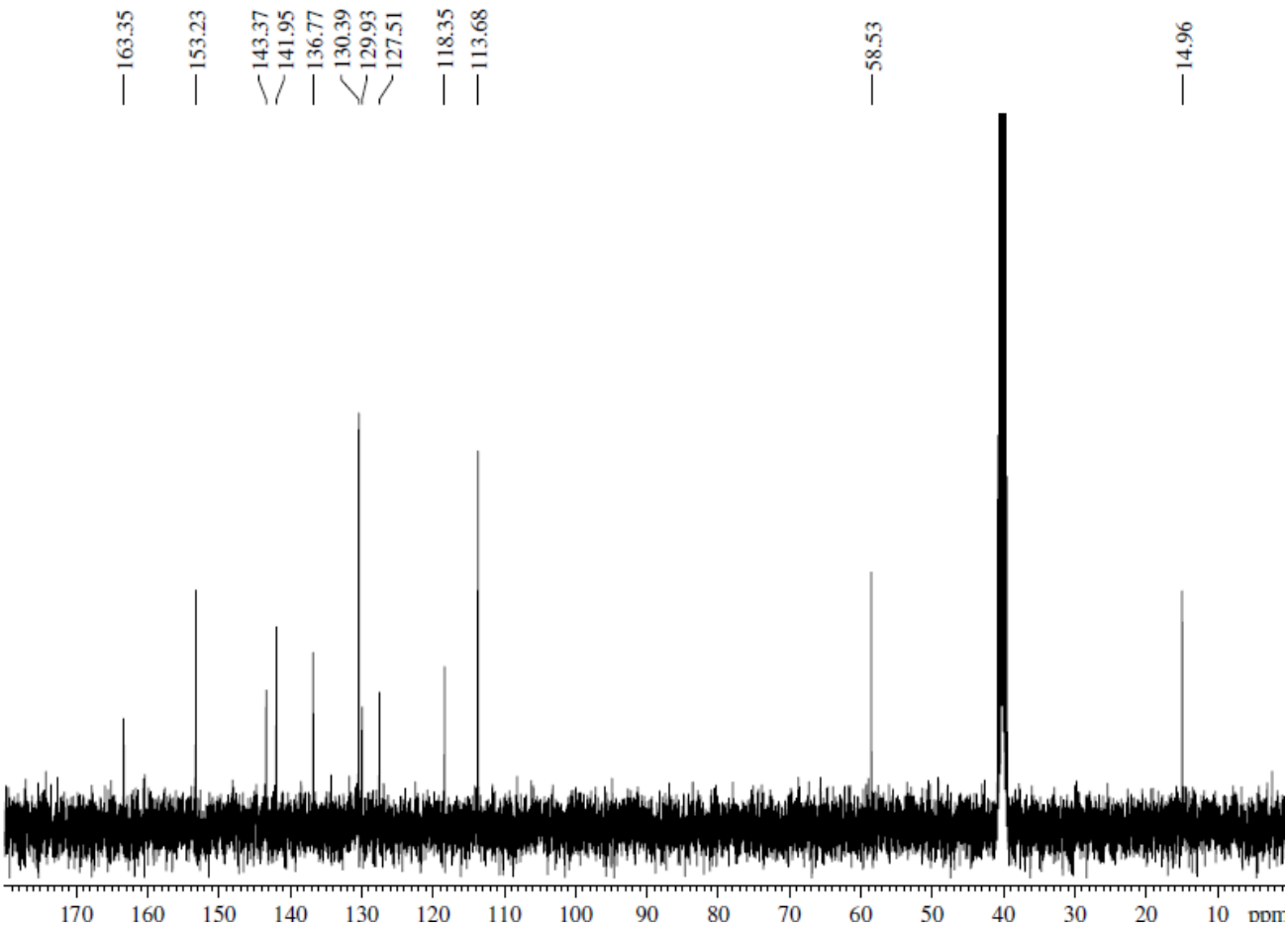


FIGURE S2. ^{13}C-NMR spectrum of new sulfamethoxazole derivative Schiff base.

1.2 UV-Vis analysis

UV-Vis absorption spectra of compounds are conducted in DMF solution on a UV-2600 Shimadzu spectrometer, using stock solutions at [ ] = 10^{-5} M range. The UV-Vis absorption spectra for compounds were carried out both in the DMF solution, revealing absorption bands in the 250–600 nm range (Table X and Figure X). In a DMF solution, both derivatives have transition bands in the ultraviolet region, where the starting material (black solid line – Figure X) presents an electronic transition of the type π→π* and for the ligand containing the pyridoxal moiety, a transition of lower energy of the n→π* type is observed, referring to the presence of the imine portion in the molecule (red solid line – Figure X).

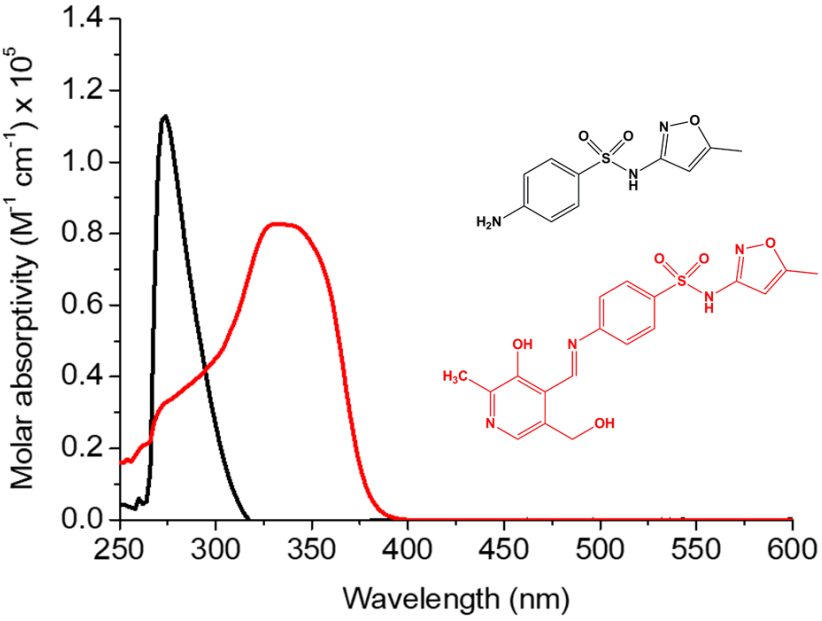


Figure S3. UV–Vis absorption spectra for compounds in DMF solution ([ ] = 10^{-5} M range).

Table S1. UV-Vis absorption data of compounds in DMF solution.

Compound	**λ, nm (ε; M^{-1} cm^{-1})**
sulfamethoxazole	273 (112,951)
Schiff base	334 (82,796)

1.3 FTIR - Infrared analysis

The sulfamethoxazole starting material, primary amine bands (-NH2) located at 3341 cm^{-1} and 3200 cm^{-1}, aromatic C-H around 3000 cm^{-1} and 2900 cm^{-1}, showed a connection C=N (oxazolic ring) are observed in the region of 1643cm^{-1}, C=C aromatic in 1600 cm^{-1}, N-H split in the region close to 1501 cm^{-1} and the band referring to the C-N connection of the ring (oxazolic ring) located in the band of 1227 cm^{-1}.

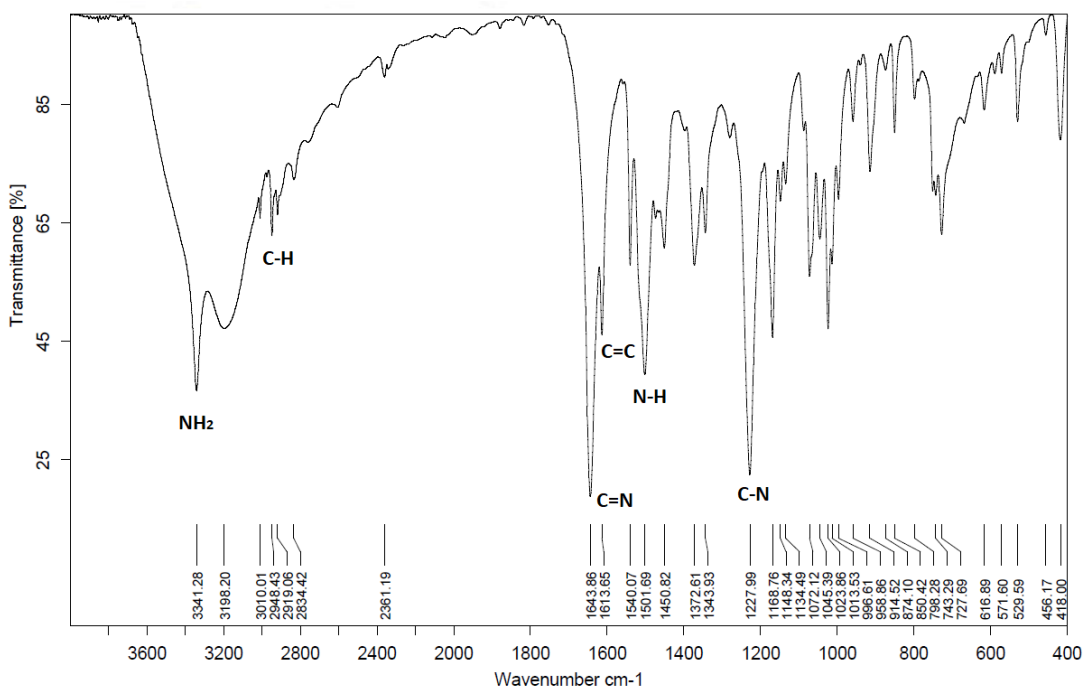


Figure S4. FTIR spectrum of sulfamethoxazole.

Analyzing the infrared spectrum of pyridoxal hydrochloride, the band referring to the carbonyl (C=O) of the aldehyde between 1643 cm^{-1} is identified. Another information is the stretches related to OH bonds, observed in the region close to 3600-3400 cm^{-1} and the stretches of C=N (pyridine), C-N (pyridine) bonds are observed in the regions of 1480 cm^{-1} and 1227 cm^{-1} respectively, [1, 2].

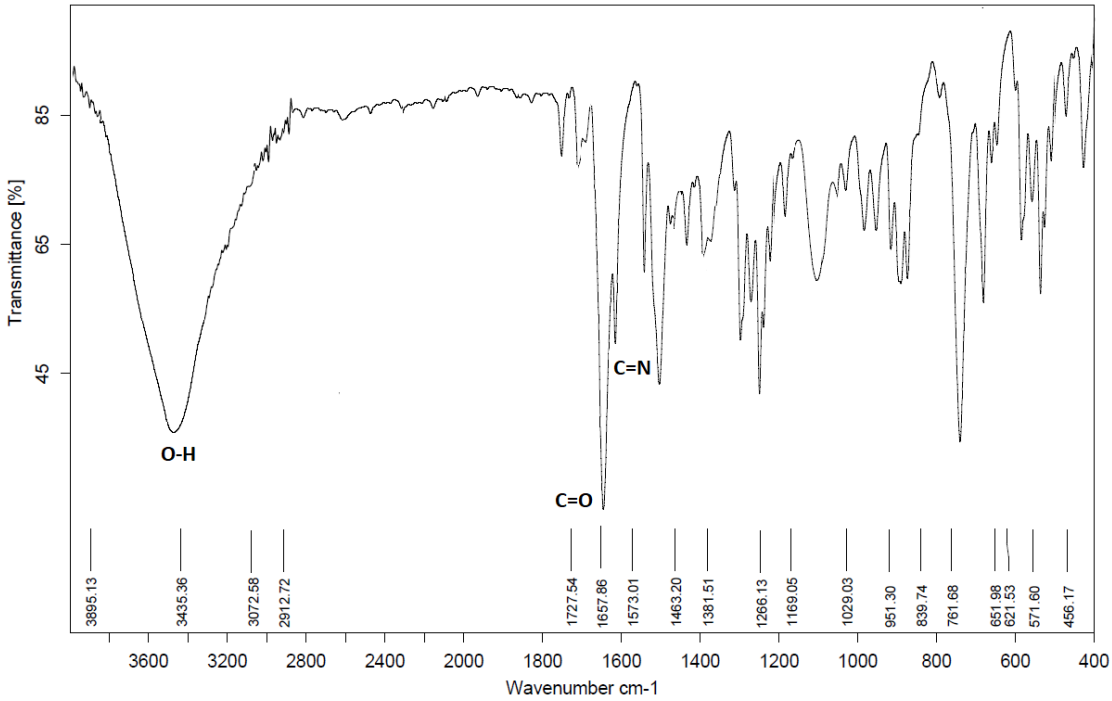


Figure S5. FTIR spectrum of pyridoxal hydrochloride.

The characterization of the Schiff base ligand showed the absence of stretch bands characteristic of primary amines and the aldehyde function. The presence of bands related to O-H bonds was observed, observed in the region close to 3600-3400 cm^{-1}, C-H of aromatic around 3000 cm^{-1} and 2900 cm^{-1}, band referring to imine function (C=N) , observed in the region 1600 cm^{-1}, C-N from pyridine in the region 1451 cm^{-1} and the C-O band of alcohol 1260 cm^{-1}.

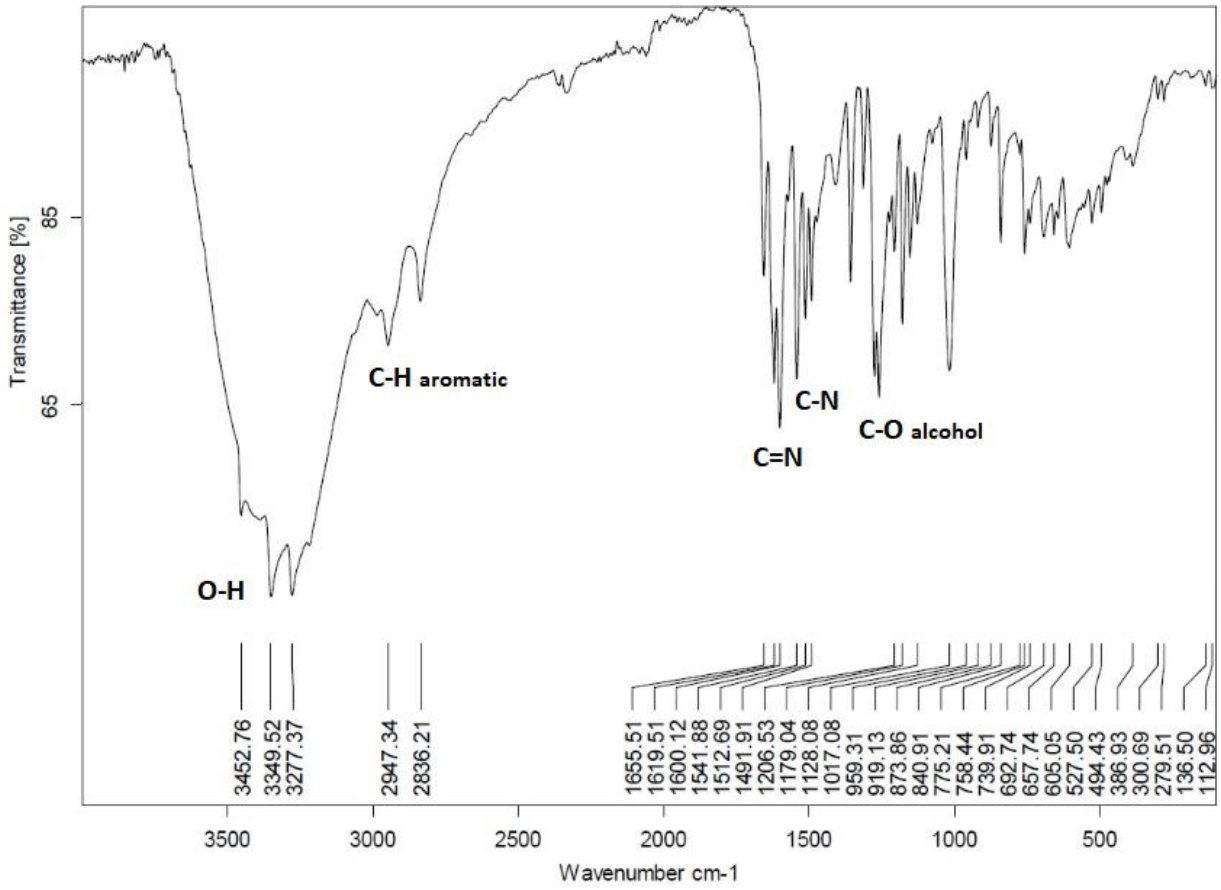


Figure S6. FTIR spectrum of new sulfamethoxazole derivative Schiff base.

1.4 Cyclic voltammetry analysis

Cyclic voltammograms were recorded with a potenciostat/galvanostat AutoLab Eco Chemie PGSTAT 128N system at room temperature and under argon atmosphere, in dry DMF solution. Electrochemical grade tetrabutylammonium hexafluorophosphate ($TBAPF_6$, 0.1 M) was used as supporting electrolyte. Employing a standard three-component system carried out these CV experiments: a glassy carbon working electrode; a platinum wire auxiliary electrode and a platinum wire *pseudo*-reference electrode. To monitor the reference electrode, the ferrocenium/ferrocene redox couple was used as an internal reference [4].

The redox profiles of compounds in DMF solution at the −1.8 to +0.90 V range (*versus* Fc/Fc^+) are shown in Figure S7. In general, both cyclic voltammogram of the derivatives showed one reduction process during cathodic scans at the −0.3 to −1.80 V window, which may be probably attributed to the formation of π-anion radical species in solution (Table S2). Moving to the positive side, all compounds presents one oxidative process at the 0.0 to +0.90 V range, which can be assigned to the ligand oxidation processes (Table S2) [5].

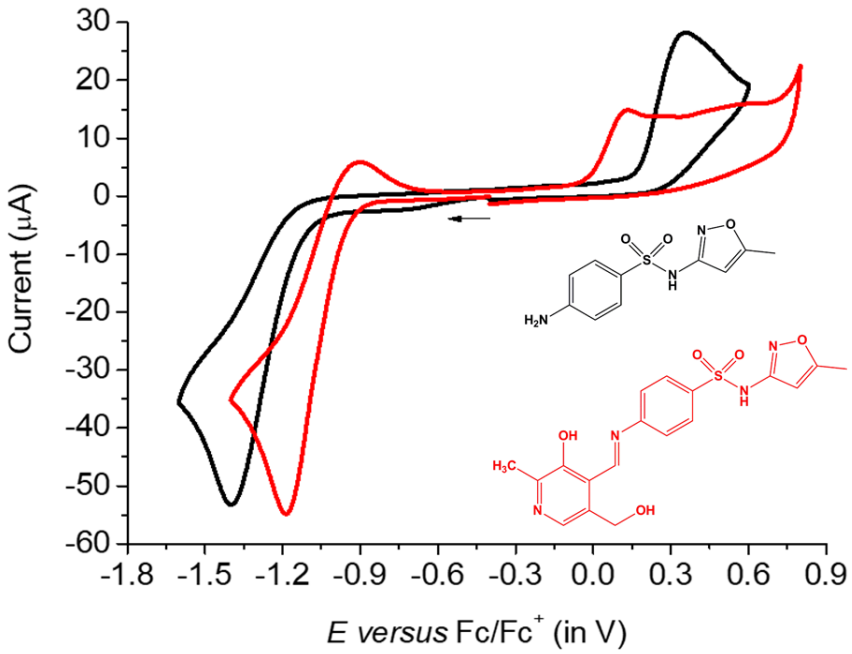


Figure S7. Cyclic voltammograms of compounds in DMF solution using 0.1 M $TBAPF_6$ as support electrolyte and scan rate of 100 mV/s.

Table S2. Redox potentials of compounds in dry DMF solution (*E* vs Fc/Fc^+).

Compound	E_{red1}	E_{oxid1}	HOMO (eV)[d]	LUMO (eV)[e]	ΔE (eV)[f]
mp	−1.40 V[a]	+0.35 V[b]	−5.80	−4.75	1.05
Shiff base	−1.04 V[c]	+0.13 V[b]	−5.44	−4.53	0.91

[a]E_{pc} = cathodic peak; [b]E_{pa} = anodic peak; [c]$E_{1/2} = E_{pc} + E_{pa}/2$; [d]$E_{HOMO} = -[4.4 + E_{oxid}\,(versus\ Fc/Fc^+)]$; [e]$E_{LUMO} = -[4.4 + E_{red}\,(versus\ Fc/Fc^+)]$; [f]$\Delta E = E_{LUMO} - E_{HOMO}$.

2. Clinical isolates Phenotypical identification

The Phenotypical identification were performed as recommended in the Manual of Laboratory Surveillance of Tuberculosis and other Mycobacteria [3]. Clinical isolates (CI) were differentiated between NTB and *M. tuberculosis* through the culture growth method in LJ plus 500 µg/ml p-nitrobenzoic acid (PNB) (HiMedia Laboratories). By determining the colonies growth time, the mycobacteria that grow in the LJ medium in up to 7 days were classified RGM and those that took more than 7 days to grow colonies in LJ were classified as slow-growing mycobacteria and excluded from the study. Besides, the ability of pigment

production by clinical isolates was evaluated in the absence and presence of incident light within 7 days.

Through the phenotypic identification tests it was observed that all the seven clinical isolates used in the study were classified as RGM, considering growth in PNB, growth time and pigment production. Clinical isolates presented positive growth in the presence of PNB, growing in less than seven days, without pigment production in their colonies.

Table S3. Phenotypic Identification of Clinical Isolates.

Clinical Isolate	Growth in PNB	Growth Time	Pigment Production
I	Positive	< 7 Days	Negative
II	Positive	< 7 Days	Negative
III	Positive	< 7 Days	Negative
IV	Positive	< 7 Days	Negative
V	Positive	< 7 Days	Negative
VI	Positive	< 7 Days	Negative

3. Breakpoints for interpreting the susceptibility tests

Breakpoints for interpreting the susceptibility of microorganisms to antimicrobial agents can be seen in Table 3.

Table 4. Breakpoints for determining the susceptibility of RGM according to *CLSI* (2015) [6].

	Minimum inhibitory concentrations (µg/mL)		
Antimicrobial agent	Susceptible	Intermediate	Resistant
Amikacin	≤ 16	32	≥ 64
Ciprofloxacin	≤ 1	2	≥ 4
Clarithromycin	≤ 2	4	≥ 8
Doxycycline	≤ 1	2-4	≥ 8
Imipenem	≤ 4	8-16	≥ 32
Sulfamethoxazole	≤ 38	-	≥ 76

4. Formation of Clinical isolate biofilms

The formation and quantification of the clinical isolates biofilm were determined as described by Flores *et al.* (2016) [7]. In polystyrene test tubes it was added 1 mL of Middlebrook 7H9 medium (BD®, Le Pont de Claix, France) containing $1x10^7$ CFU/mL of clinical isolates culture. The tubes were topped with parafilm® and incubated at 30°C for 7 days. After this period, the biofilm quantification was performed using 0.1% crystal violet. The absorbance was measured in spectrophotometer in optical density (OD) of 570 nm (Spectrophotometer U-1800, Hitachi, Berkshire, United Kingdom).

The clinical isolates were submitted to the same experimental conditions to evaluate the capacity of biofilm formation. It was used *M. massiliense* standard strain (ATCC 48898) as the positive control of biofilm formation, while culture medium was used as the negative control. From seven clinical isolates, three were classified as biofilm formers. The biofilm formation quantification of the clinical isolate samples number IV, V and VI are shown in figure S8.

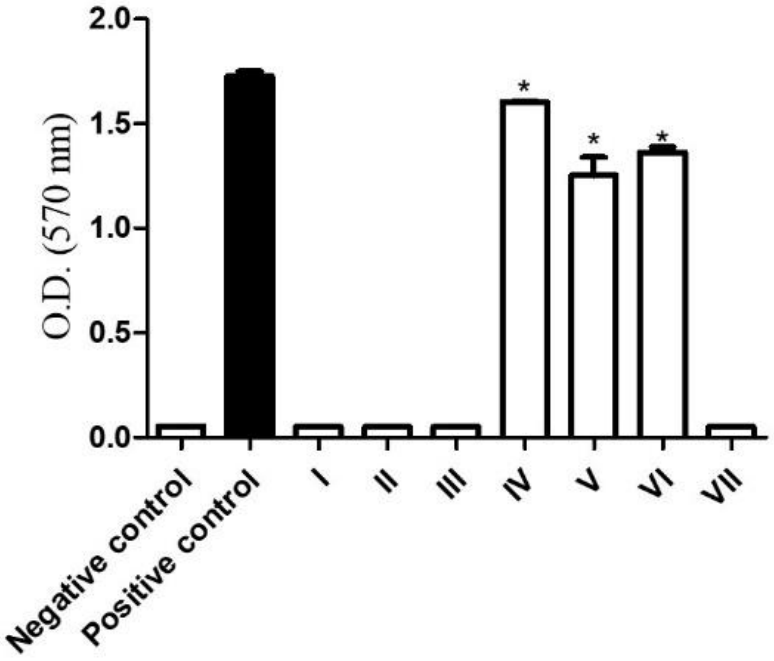


Figure S8. Quantification of biofilm formation of clinical isolates. Positive control: *M. Massiliense* (ATCC 48898); Negative Control: sterile culture medium.

4. References

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4.3 Manuscrito 2

O manuscrito 2 encontra-se formatado de acordo com as normas exigidas para publicação no período **Letters in Drug Design & Discovery**.

Antibacterial, antifungal and anti-biofilm effects of sulfamethoxazole-derived compounds against pulmonary infection agents

Fallon dos Santos Siqueira[1], Camilla Filippi dos Santos Alves[1], Josiéli Demétrio Siqueira[2], Laura Bedin Denardi[1], Alencar Kolinski Machado[3], Sydney Hartz Alves[1], Roberto Christ Vianna Santos[1], Davi Fernando Back[2*], Lenice de Lourenço Marques[4], Marli Matiko Anraku de Campos[1*]

[1]*Graduate Program in Pharmaceutical Sciences, Federal University of Santa Maria, Avenue Roraima 1000, zip code: 97105-900, Santa Maria, Brazil.*
[2]*Graduate Program in Chemistry, Federal University of Santa Maria, Avenue Roraima 1000, zip code: 97105-900, Santa Maria, Brazil.*
[3]*Graduate Program in Nanoscience, Franciscan University, Santa Maria, Brazil, Andradas Street, 1614, zip code: 97010-032, Santa Maria, Brazil*
[4]*Farroupilha Federal Institute, São Vicente do Sul Campus, Zip code: 97420-000, São Vicente do Sul, Brazil*

*Corresponding Author: Marli Matiko Anraku de Campos and Davi Fernando Back
E-mail: laboratoriomycobacteriologia@gmail.com
Universidade Federal de Santa Maria
Roraima Avenue 1000 - Zip code: 97105-900
Santa Maria - RS –Brazil

ABSTRACT

Antibiotic resistance associated with agents of pulmonary infection has become a public health problem, being one of the priorities for immediate resolution, with the objective of reducing this phenomenon. Aiming to increase therapeutic options in combating resistant microorganisms, the synthesis of molecules from pre-existing drugs has been shown to be a promising alternative. To determine the biological activity of sulfamethoxazole-metal complexes (gold, silver, nickel, cadmium, mercury and copper) against fungal and bacterial agents that cause lung infections. We used the minimum inhibitory concentration (MIC) technique to evaluate the compound antifungal and antibacterial properties. In addition, we verified the antibiofilm capacity of the new molecules derived from sulfamethoxazole. Biofilm was stained using crystal violet and quantified using a spectrophotometer. The results demonstrate the potentiation of the new compounds in the antibacterial and antifungal activity, mainly in the silver-complex, which was able to inhibit the growth of microorganisms that cause lung diseases with concentrations in the range of 2 μg mL^{-1} and 8 μg mL^{-1}. Furthermore, metallic derivatives of sulfamethoxazole are capable of inhibiting the formation and destroying bacterial biofilms, at significantly lower concentrations than free sulfamethoxazole. The present study presents promising results that demonstrate a possibility of derivatization of sulfamethoxazole and its use as a possible antimicrobial and antifungal agent against causative agents of lung infections. Our results suggest the complexation of sulfamethoxazole with metals as an alternative to drug-resistant strains.

1. INTRODUCTION

Since 1980, a steady increase in acute lower respiratory tract diseases has been observed globally [1]. Pneumonia is an infection that settles in the lungs, caused by the penetration of an infectious or irritating agent (bacteria, viruses, fungi and allergic reactions) into the alveolar space, where gas exchange occurs. It can affect the region of the pulmonary alveoli where the terminal ramifications of the bronchi and, sometimes, the interstitium flow out [2].

Together, influenza and pneumonia, caused by different etiologic agents, represent the sixth leading cause of death in the United States [1, 3]. In 2012, the World Health Organization (WHO) announced that the disease is the one that most kills children under the age of five, with an estimated 1.2 million worldwide, more than the deaths caused by AIDS, malaria and tuberculosis gathered [4]. Of these deaths from pneumonia, more than 99% would be registered in developing countries such as Brazil, which makes the WHO has reinforced the request to these governments to prioritize prevention and combat the disease. According to the organization, pneumonia is one of the problems with the greatest possible solution in the global health scenario [5].

Antibiotics and the availability of mechanical ventilation significantly changed the natural history of pneumonia and promoted a decrease in its lethality [6]. At the same time, the widespread use of antimicrobial and antifungal agents has led to the emergence of drug-resistant strains and thus has altered and expanded the range of pathogens responsible for pneumonia, especially among hospitalized patients. Thus, antibiotic resistance associated with the etiological agents of pneumonia has become a public health problem that needs great attention from the clinical and scientific community, being one of the priorities for immediate resolutions, with the objective of reducing this phenomenon [7].

In order to increase the therapeutic options in combating resistant microorganisms, the synthesis of molecules from pre-existing drugs has been shown to be a promising alternative [8, 9]. In this context, coordination compounds using antimicrobials as ligands and exploring the intrinsic characteristics of metallic ions have allowed the obtainment of a wide variety of complexes to be explored as potential antimicrobial agents. Recently, coordination compounds containing sulfonamides as ligands have been standing out and demonstrating a broad spectrum of biological activity against different microorganisms [10, 11, 12, 13]. The use of low molecular weight molecules, such as sulfamethoxazole, allows the complexation of this sulfonamide with hard metal centers, due to the presence of important coordination sites (S, N and O), which makes this drug, according to Pearson's Theory , a good candidate for the synthesis of biologically active compounds compatible with the body's metabolic activities [14].

Considering the need to develop new molecules effective against pulmonary infectious agents, this work aims to investigate the biological activity of sulfamethoxazole derivatives complexed with Au, Ag, Hg, Cd, Ni and Cu against fungal pulmonary infectious agents such as *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*, as well as against bacterial pulmonary infectious agents such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*.

2. MATERIALS AND METHODS

2.1 Microorganisms

To carry out the study, the following microorganisms were used: one clinical strains of *A. fumigatus*, *A. fumigatus* CBS 13361 strain, two clinical strains of *H. capsulatum*, two clinical strains of *P. brasiliensis*, one clinical strain *of P. aeruginosa* multidrug resistance (MDR) and *M. tuberculosis* H37RV strain. Fungal agents come from the Laboratory of Mycological Research of the Federal University of Santa Maria and the bacterial agents are from the mycobacteriology laboratory of the Federal University of Santa Maria. Fungal agents were kept in yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, 2 dextrose and 1.5% agar) agar in filamentous form (M) at room temperature (25 to 28 °C) with monthly subculture, *P. aeruginosa* was kept on a BHI broth supplemented with glycerol (HiMedia Laboratories Pvt. Ltd, India) at -20ºC and *M. tuberculosis* Standard strain was maintained on Lowenstein-Jensen (HiMedia Laboratories Pvt. Ltd, India) agar until needed.

2.2 Sulfamethoxazole-derived compounds

The synthesis of compounds derived from sulfamethoxazole was reproduced according to routes previously described by Marques (2007) [15, 16]. Derivatives complexed with Ag (SMTZAg), Au (SMTZAu), Cu (SMTZCu), Ni (SMTZAg), Cd (SMTZCd) and Hg (SMTZHg) were synthesized in the Bio-Inorganic Laboratory of the Federal University of Santa Maria.

Sulfamethoxazole (SMTZ) (Sigma-Aldrich, St Louis, MO, USA) was used as a ligand in the synthesis of the derivatives and was also used as a standard in the microbiological and antifungal assays. Amphotericin (AMB) (Sigma-Aldrich, São Paulo, Brazil), itraconazole (ITZ) (Leadiant Biosciences, Maryland, USA) and fluconazole (FLU) (Leadiant Biosciences, Maryland, USA) were used following the recommendations of the CLSI M38-A2 protocol for filamentous fungi [17].

Initial stock solutions of these drugs and Sulfamethoxazole-derived compounds were made in dimethyl sulphoxide (DMSO) at 50 mg ml^{-1} from which further dilutions were made in Mueller–Hinton (Merck) broth for M. *tuberculosis* and *P. aeruginosa* and RPMI medium for antifungal agents. The chemical structure of the sulfamethoxazole derivatives used in this study can be seen in Figure 1.

2.3 Determination of Minimum Inhibitory Concentration (MIC)

2.3.1. In vitro activity of antifungal drugs, sulfamethoxazole, and its derived compounds (SMTZ-Ag, SMTZ-Hg, SMTZ-Au, SMTZ-Cu, SMTZ-Cd, SMTZ-Ni) against fungi pulmonary infection agents.

The susceptibility tests were conducted using the broth microdilution method with RPMI 1640 medium (standard medium), according to the standard protocol CLSI M38-A2 [17] for *Aspergillus* spp., *H. capsulatum* strains and *P. brasiliensis.* The drugs and the compounds were dissolved in 100% dimethyl sulfoxide (DMSO) (Vetec) and serial 2-fold dilutions were prepared according to CLSI M38-A2 protocol. Antifungal drugs and the compounds derived

from sulfamethoxazole were tested at the concentrations of 0.015 μg mL^{-1} to 128 μg mL^{-1}. The standardized fungal inoculum suspensions were diluted 1:50 in RPMI in order to obtain a cell number ranging from $0.5x10^5$ to 2.5 $x10^5$ cel/mL. To perform this evaluation, 96-well plates were used with 100 μL of each specific compound concentration and 100 μL of the inoculum per well. The microdilution plates were incubated at 37°C and read after 48 h (*A. fumigatus*), 120 h (*H. capsulatum*) or 15 days (*P. brasiliensis*) of incubation. Readings were performed visually based on the comparison of the growth in the wells containing the drug with that of the growth control (100 μL of the medium plus 100 μL of inoculum suspension.

2.3.2. *In vitro* activity of sulfamethoxazole and its derived compounds against *Mycobacterium tuberculosis*.

The MIC of sulfamethoxazole and its derivatives was performed by the broth microdilution method [18]. Stock solutions of each compound were made in DMSO and then diluted in MD7H9 to the desired concentrations for evaluation. Sulfamethoxazole, as well as its inorganic derivatives, were tested at concentrations of 9 μg mL^{-1} to 2500 μg mL^{-1}. For bacterial suspension, MD7H9 (Middlebrook 7H9) base medium supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) and 0.2% glycerol, which were incubated at 35ºC for 7 days, was used. The bacterial suspension was adjusted to MacFarland scale 1 and then diluted 1/25 in MD7H9 at the time of use. To perform this evaluation, 96-well plates were used with 100 μL of each specific compound concentration and 100 μl of the inoculum per well. After 72 hours of incubation at 30°C, treatment plates were read and the minimum inhibitory concentrations were determined using 2,3,5-triphenyltetrazolium chloride (Vetec VR, Rio de Janeiro, Brazil) as microbial growth indicator [12].

2.3.3 *In vitro* activity of sulfamethoxazole and its derived compounds against *Pseudomonas aeruginosa*.

The susceptibility tests were conducted using the broth microdilution method, according to the standard protocol CLSI M100eS23 [19], while the inoculum density standardization was performed according to the McFarland 0.5 scale. Initial stock solutions were made for each compound in dimethyl sulfoxide (DMSO). From the stock solution, concentrations ranging from 9 μg ml^{-1} and 2.500 μg ml^{-1} were prepared. To perform this evaluation, 96-well plates were used with 100 μl of Mueller–Hinton broth (Hi Media Laboratories Pvt. Ltd, India), 100 μl of each specific concentration from each compound, and 10 μl of the inoculum per well. Plates were then incubated for 24 h at 37°C. The minimal inhibitory concentration (MIC) was considered as the lowest concentration capable of inhibiting visible bacterial growth. The tetrazolium salt, 2,3,5- triphenyltetrazolium chloride (TTC) (Vetec ®, Rio de Janeiro, RJ, Brazil) was used as an indicator of microbial growth indicator [12].

2.4 Antibiofilm activity assays on *Pseudomonas aeruginosa*

The metal complexes were evaluated for their activity to inhibit the formation of biofilms and remove biofilms already formed from clinical strain MDR of *P. aeruginosa*. For the following assays, *P. aeruginosa* (PAO1) inoculum was used as the positive control and culture broth was used as the negative control. Biofilm biomass by the tested groups was determined in comparison with the mean OD obtained in the positive control (culture medium and bacteria) and the negative control (culture medium only).

2.4.1 Inoculum

Bacterial suspensions were prepared in Mueller Hinton broth and visually adjusted to the 0.5 McFarland scale [20].

2.4.2 Biofilm formation inhibition

This assay used conventional 96-well polystyrene plates as an adhesion material. Serial dilutions (ultrapure water and 1% DMSO as solvents) of the metals-complexed sulfonamide and SMTZ were prepared at the MIC and sub-inhibitory concentrations. Each dilution were pipetted into four wells in a sterile flat-bottomed microtiter plate, along with 100 µl of the inoculum. The plates were incubated for 24 h at 37°C to promote adhesion. The biofilm was stained and quantified [20].

2.4.3 Biofilm removal test

100 µl of the inoculum was added to 96-well polystyrene plates and incubated at 37°C for 24 h for biofilm formation. After adhesion and biofilm maturation, 100 µl of SMTZ and the metals-complexed sulfonamide were added to each well, at a concentration equal to or higher than the MIC. The plate was incubated at 37°C for an additional 24 h (Bonez et al. 2017). The biofilm was stained and quantified [20].

2.4.4 Biofilm quantification

The biomass of biofilm was stained using crystal violet. After incubation, the contents of the plate were removed, and successive washes were performed with physiological solution to remove weakly adhered cells. Then, 200 µl of 0.1% crystal violet were added to the wells and allowed to stand for 10 min. The plates were subsequently washed again, then 200 µl of 95% ethanol were added, and the plates allowed to stand for 15 min. Finally, this ethanolic solution was transferred to another plate to read the optical density (OD) at 570 nm using a TP-Reader® (Thermo Plate, Inc, Ohio, USA) [20].

2.4.3 Statistical Analysis

Biofilm formation was determined by a significant difference between the averages of absorbance obtained in the positive control and the average obtained in the negative control. The experiment was performed in triplicate. The OD readings obtained in the biofilm formation assay were recorded as means ± standard deviation (SD) and were submitted to a t-test (compared with the positive control). Values with $p<0.005$ were considered to indicate

statistical significance. Graphs were prepared using GraphPad Prism version 9.01 (GraphPad Software, La Jolla, CA, USA).

3 RESULTS

3.1 Determination of Minimum Inhibitory Concentration (MIC)

The MIC values determined for the metal complexes derived from SMTZ can be seen in table 1. For comparison purposes, we used uncomplexed sulfamethoxazole in the broth microdilution assays. The results obtained allow us to highlight that, in order to inhibit the planktonic growth of all fungal and bacterial species, high concentrations of sulfamethoxazole were necessary in comparison with the lowest necessary concentrations of the metal complexes.

Among the metal complexes, SMTZAg was able to inhibit pulmonary fungi agents at low concentrations, with MICs between 8 μg mL^{-1} and 2 μg mL^{-1}. Against the three fungal species, SMTZAg has an inhibitory activity equal to or better than the inhibitory activity shown by fluconazole, a drug used as an antimycotic, belonging to the class of triazole antifungals. Against bacterial agents, SMTZAg inhibited *M. tuberculosis* at concentrations 8 times lower than the required concentration of SMTZ and inhibited *P. aeruginosa* at concentrations 64 times lower than the required concentration of sulfonamide.

3.2 Antibiofilm activity assays on *Pseudomonas aeruginosa*

Biofilms formed by MDR clinical isolate of P. aeruginosa were slightly destroyed/dispersed after the action of 2500 μg mL^{-1}, 5000 μg mL^{-1} and 10000 μg mL^{-1} of SMTZ. In addition, sub-inhibitory concentrations of this sulfonamide had a weak ability to inhibit biofilm formation, resulting in slight inhibition in the concentrations evaluated.

Through the quantification of biofilm biomass, it was observed that all SMTZ derivatives complexed with the different metals were able to inhibit and destroy the bacterial biofilm to some degree. Among the metal complexes, SMTZAg destroyed a large part of the sessile structure formed by the clinical isolate, with the lowest concentrations among those tested (39 μg mL^{-1}, 78 μg mL^{-1} and 156 μg mL^{-1}). In addition, SMTZNi, SMTZAu, SMTZCu and SMTZCd showed higher activity than SMTZ. The destruction of the biofilm by SMTZ and its derivatives can be seen in Figure 2.

A similar profile of action can be observed for the inhibition of biofilm formation, highlighting SMTZAg, which almost completely inhibited *P. aeruginosa* biofilm formation, showing the best activity among the metal complexes. All metal complexes were able to inhibit bacterial biofilm formation at concentrations lower than the concentration of SMTZ, which discretely inhibited the biofilm at concentrations of 2500 μg mL^{-1}, 1250 μg mL^{-1} and 625 μg mL^{-1}. The inhibition of the biofilm by SMTZ and its derivatives can be seen in Figure 3.

4 DISCUSSION

Developing potential new treatments for acute and chronic respiratory infections is one of the main issues for reducing morbidity and mortality rates related to these conditions.

Currently, respiratory tract infections represent the greatest morbi-mortality around the world [21]. Contributing to this phenomenon, it is possible to highlight one of the most alarming public health problems in the clinical and scientific community: the evolution of resistance of different microorganisms to drugs used in the treatment of infections [7]. This is because resistance to anti-infective drugs limits the treatment of invasive infections, making the arsenal of effective drugs to fight different etiological agents limited [22].

In this work, SMTZ complexed with different metals were evaluated against different microorganisms causing lung infections, in order to contribute to the development of new molecules with antimicrobial and antifungal activity. SMTZ presented MIC > 32 μg mL^{-1} against clinical isolates of *A. fumigatus*, *P. brasiliensis* and *H. capsulatum*. The same susceptibility profile was observed for the metallic complexes, with the exception of SMTZAg, which presented MICs < 8 μg mL^{-1}. Different compounds with Ag have shown promising antifungal activities. N Khatoon *et al.* 2018 highlighted the action of Ag nanoparticles in inhibiting the growth of microorganisms of the candida genus, from the decrease of ergosterol synthesis [23]. Longhi *et al.* 2016, proved that the combination of fluconazole with Ag improves the antifungal effect against drug-resistant *Candida albicans* planktonic cells [24]. Furthermore, D'Adamio *et al*. 2019 suggested in their study that silver coating on tumor endoprostheses is a reliable and effective implementation for an antifungal purpose, in addition to its widely known and demonstrated antibacterial potential [25].

The MIC values obtained for SMTZAg against *H. capsulatum*, *P. brasiliensis* and *A. fumigatus*, fungi that cause different clinical forms of infections, including pulmonary and systemic infections, suggest that the complexation with Ag potentiated the effect of sulfamethoxazole, which, added to the intrinsic biological characteristics of the silver ion, it inhibited fungal growth with very low concentrations [26]. The development of new promising and effective molecules against *H. capsulatum* is of great importance, since this fungus is the most common endemic pulmonary mycosis in the USA [27]. In acute or epidemic histoplasmosis, symptoms are high fever, cough, asthenia, retrosternal pain, accompanied by enlarged cervical lymph nodes, liver, and spleen [28]. Just as important, new eradication strategies for *P. brasiliensis* and *A. fumigatus* must be considered. Chronic paracoccidioidomycosis can diffusely affect the lungs. Even after antifungal therapy, patients may present with residual respiratory abnormalities due to fungus-induced lung fibrosis [29]. Furthermore, the importance of invasive pulmonary aspergillosis has increased with the increasing number of patients with impaired immune status associated with the treatment of malignancy, organ transplantation, autoimmune and inflammatory diseases; critically ill patients and those with chronic obstructive pulmonary disease [30].

When it comes to pulmonary infection, co-infection with different infectious agents becomes common, especially in hospitalized patients [31]. Co-infection with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* in cystic fibrosis and fungal infection in pulmonary tuberculosis are some examples of recurrent co-infections [31-33]. Considering the recurrence of pulmonary infections of this type, we evaluated the activity of SMTZ derivatives against the MDR clinical isolate of *P. aeruginosa* and against the standard strain of *M. tuberculosis*.

The results obtained show that SMTZAg was the most efficient complex against the two bacterial species, presenting MICs lower than free sulfamethoxazole and lower than

SMTZ complexed with the other metals. Studies have demonstrated the activity of inorganic derivatives of SMTZ against different bacterial agents.

Siqueira *et al* 2018 demonstrated that metal-complexed Sulfonamides are a promising alternative in combating fast-growing mycobacteria [12]. In addition, Agertt *et al* 2013 proved that Au-complexed sulfamethoxazole is effective against clinical isolates of *M. tuberculosis* [18]. Against mycobacterial biofilms, Bonez *et al* 2021 proved that sulfonamides complexed with metals are efficient in inhibiting the formation of this structure [33]. In the case of microorganisms of the *Mycobacterium* genus, the high hydrophobicity of the cell wall becomes a limiting factor in the activity of drugs such as sulfamethoxazole [12, 18, 31].

It is suggested that the potentiated activity, observed in our results, of SMTZAg in inhibiting the growth of *M. tuberculosis* can be better understood considering Overtone's concept and Tweedy's chelation theory [35]. According to Overtone's concept of cellular permeability, the lipid membrane surrounding the mycobacterial cell favors the passage only of liposoluble materials. In Tweedy's chelation theory, the polarity of the metal ions is reduced to a greater degree due to overlapping of the ligand orbitals, and the partial sharing of the positive charge of the metal ion with the donor groups. In addition, delocalization of p-electrons on the chelate ring is increased, which consequently increases the liposolubility of the complexes [36-38]. Therefore, sulfamethoxazole derivatives could easily penetrate the microbial structure, to perform its activity together with the silver ions [39,40].

The susceptibility profile obtained for the clinical isolate MDR of *P. aeruginosa* confirms that SMTZAg was the complex with the most promising activity. The MIC obtained was the lowest when compared to the MIC obtained for the other complexes and was extremely lower than the MIC obtained for the SMTZ. Several studies demonstrate the activity of SMTZ metal complexes against gram negative and positive microorganisms. Mizdal *et al.* 2017 demonstrated that SMTZAg is potentially effective against *Escherichia Coli* [41]. Furthermore, Mizdal *et al* 2018 concluded that gold-complexed sulfonamides are potential antibacterial agents against methicillin-resistant *Staphylococcus aureus* [42]. Furthermore, Siqueira *et al.* 2021 demonstrated that SMTZAg inhibits the planktonic and sessile growth of a standard strain of *P. aeruginosa* (PAO1) [13]. Whereas *P. aeruginosa* can cause Chronic lung infection by *Pseudomonas aeruginosa* biofilm e to investigate the anti-biofilm activity of metal complexes, we evaluated the effectiveness of SMTZ and derivatives in inhibiting and destroying the biofilm formed by *P. aeruginosa*.

Biofilms endow the bacteria a unique resistance against antibiotics and other anti-microbial agents, making treatments for infections related to this structure more difficult from a treatment perspective [43]. Currently, biofilms are defined as the result of a microbiological ecosystem attached to a surface, formed by populations developed from a single or multiple species. Therefore, these structures can be constituted by bacteria, fungi and/or protozoa. As a result of the association between microorganisms, a matrix of organic polymers is formed [44]. Also part of this structure are residues from the colonized environment, genetic material, proteins, lipids, enzymes, ions and water. All this covered by layers of Extracellular Polymeric Substances (SPE) forming a porous and hydrated structure [45].

All metallic compounds inhibited the biofilm formation of MDR clinical isolate of *P. aeruginosa.* In addition, all compounds destroyed the previously formed biofilm, with the exception of SMTZHg. Studies suggest that the release of metal ions in the medium interferes

with bacterial adhesion on the surface, which prevents the microorganism from starting the biological cycle of biofilm formation [13, 37, 42]. Furthermore, it suggests that SMTZ-metal complexes interfere in cell communication, preventing the microorganisms that make up the biofilm from maturing and adequately completing the formation of the sessile community. Ultimately, the sub-inhibitory concentrations used in the biofilm formation inhibition assays prove that the plactonic cells were viable for adhesion and subsequent maturation of the sessile structure.

It has already been shown that metals exert synergistic biological activities with antimicrobials and biocides, inhibiting different pathways microorganisms and thus are able to kill bacteria resistant to various antimicrobials through oxidative stress, protein dysfunction or cell membrane damage [37]. Therefore, it is suggested that the metallic ions present in the structure damage the extracellular matrix formed by the biofilm, allowing the dispersion/destruction of bacterial cells of this sessile structure to occur [46].

Finally, all sulfamethoxazole derivatives used in this study were analyzed in previous research, regarding their cytotoxicity, using the peripheral blood mononuclear cell (PBMC) model [12, 13]. The results revealed in the literature by our research group ensure that SMTZ derivatives are safe molecules that do not decrease cell viability, do not trigger the production of reactive oxygen and nitrogen species, and do not damage the DNA of cells. Thus, sulfamethoxazole metal complexes are promising molecules which could be used as potential therapeutic agents for bacterial, fungal and biofilm elimination.

5 **CONCLUSION**

The present study provides promising results that demonstrate the possibility of derivatizing sulfamethoxazole and its use as a possible antimicrobial and antifungal agent against causative agents of lung infections. All metallic derivatives of sulfamethoxazole proved to be effective alternatives in inhibiting *M. tuberculosis* and *P. aeruginosa*, with emphasis on SMTZAg, which in addition to presenting antibacterial activity, showed the best results in inhibiting the growth of *H. capsulatum*, *P. brasiliensis* and A. *fumigatus*. Furthermore, all metal complexes proved to be potent antibiofilm agents, becoming an alternative to sulfamethoxazole against drug-resistant strains.

CONSENT FOR PUBLICATION

The authors confirm that this article content has no conflict of interest.

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FIGURES

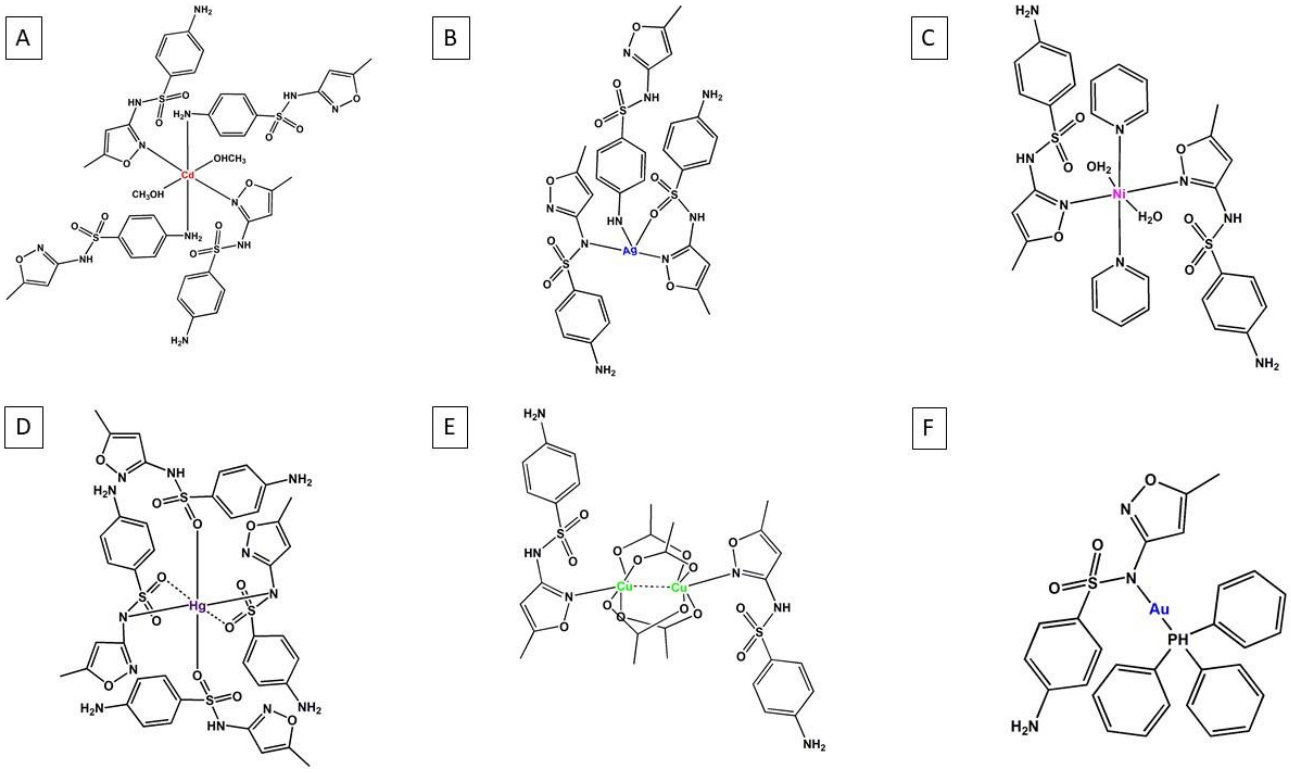

Figure 1. Structural representation of sulfamethoxazole derivatives. Chemical structure of SMTZCd (A), SMTZAg (B), SMTZNi (C) SMTZHg (D), SMTZCu (E) and SMTZAu (F). Adapted from Marques et. al (2007).

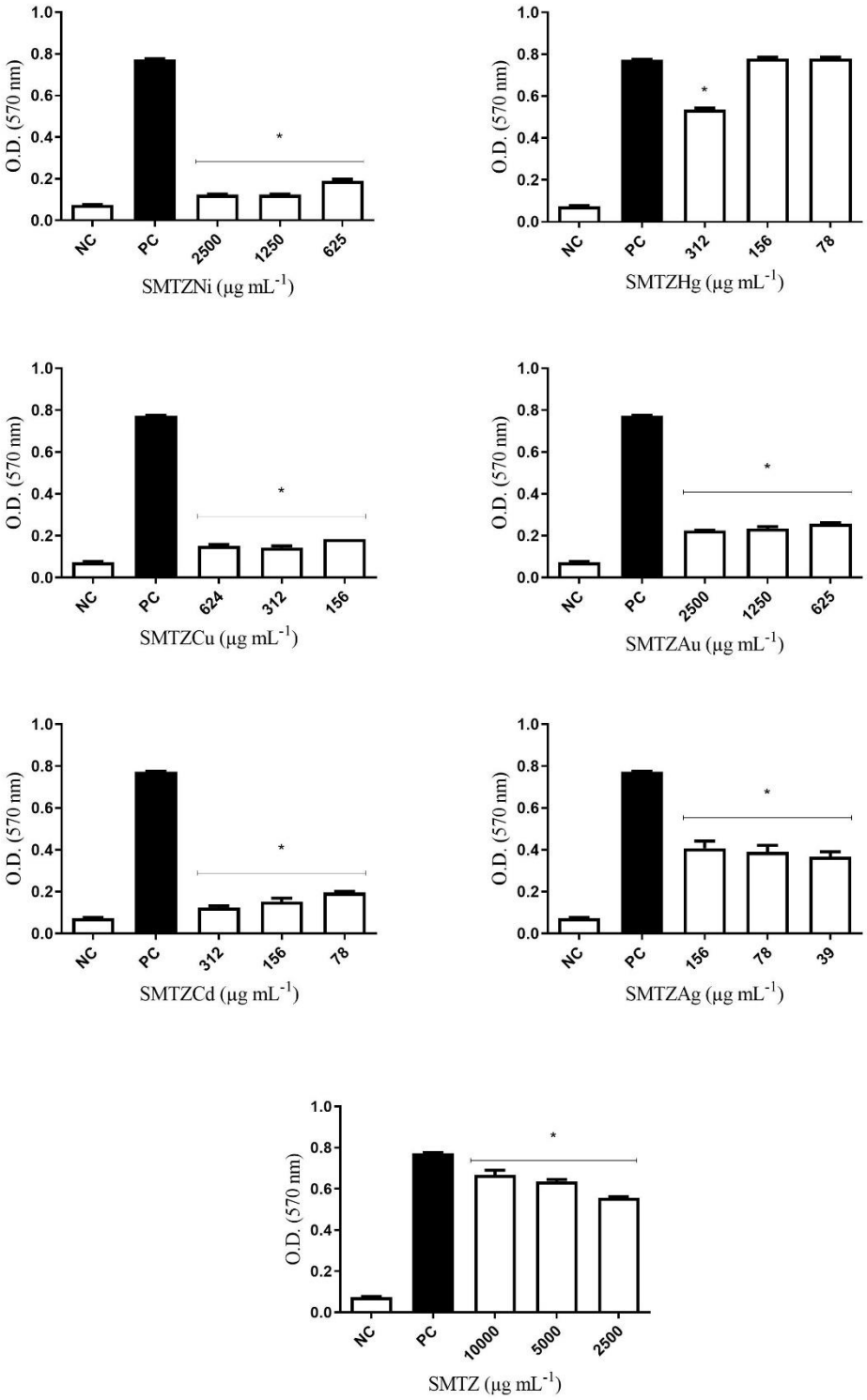

Figure 2. Effect of SMTZNi, SMTZHg, SMTZCu, SMTZAu, SMTZCd, SMTZAg and SMTZ on destruction/removal of *P. aeruginosa* biofilms. * Represents a significant difference between the concentration tested and the positive control (PC). The tests were subjected to the Student t-test (n = 3), considering statistical differences when $p < 0.05$.

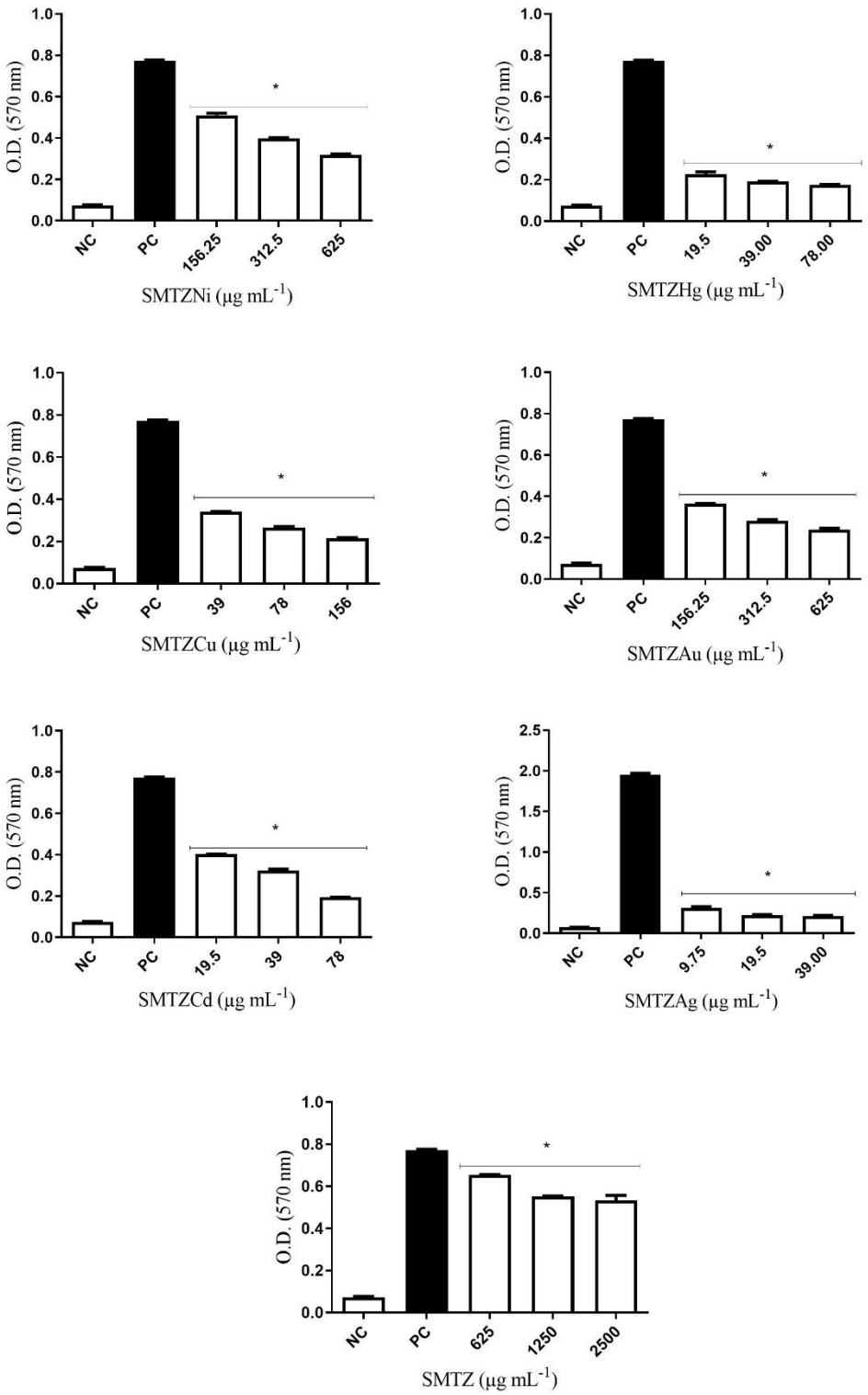

Figure 3. Effect of SMTZNi, SMTZHg, SMTZCu, SMTZAu, SMTZCd, SMTZAg and SMTZ on inhibition of *P. aeruginosa* biofilms. * Represents a significant difference between the concentration tested and the positive control (PC). The tests were subjected to the Student t-test (n = 3), considering statistical differences when $p < 0.05$.

TABLES

Table 1. *In vitro* activity of sulfamethoxazole and its derived compounds (SMTZ-Ag, SMTZ-Hg, SMTZ-Au, SMTZ-Cu, SMTZ-Cd, SMTZ-Ni) against pulmonary infection agents.

Compounds	*Minimal Inhibitory Concentration (μg mL^{-1})*							
	H. capsulatum 01	*H.capsulatum 02*	*P. brasiliensis 01*	*P. brasiliensis 02*	*A. fumigatus 01*	*A. fumigatus CBS 13361*	*M. tuberculosis* H37RV	*P. aeruginosa 01*
SMTZ-Ag	8	8	4	2	2	2	78	39
SMTZ-Hg	16	16	8	8	>32	>32	312.5	78
SMTZ-Au	>32	>32	>32	>32	>32	>32	156.25	64
SMTZ-Cu	>32	>32	>32	>32	>32	>32	312.5	156.25
SMTZ-Cd	>32	>32	>32	>32	>32	>32	156.25	78
SMTZ-Ni	>32	>32	>32	>32	>32	>32	312.5	625
SMTZ	>32	>32	32	32	>32	>32	625	2500
AMB	0.25	0.125	1	1	0.25	0.125	-	-
ITZ	0.031	0.015	0.031	0.031	0.125	0.25	-	-
FLU	64	64	4	2	>64	>64	-	-

SMTZ, sulfamethoxazole; AMB, amphotericin B; ITZ, itraconazole; FLU, fluconazole.

5 DISCUSSÃO

Os recentes avanços nas estratégias de modificação estrutural de fármacos conhecidos têm ganhado grande destaque na área de desenvolvimento de novas moléculas bioativas. Conhecidos como fármacos "*me-too*", essas moléculas são baseadas em modificações estruturais de fármacos pré-existentes que, através de diferentes processos de derivação química, resultam em novos compostos com estrutura química muito similar e pequenas diferenças físico-químicas em comparação ao fármaco original (BARREIRO; FRAGA, 2005).

No contexto de resistência antimicrobiana, o desenvolvimento de novas moléculas eficazes no combate a cepas fármaco-resistêntes, causadoras de diferentes processos infecciosos, tornou-se prioridade, uma vez que a resistência aos antimicrobianos é, atualmente, uma das ameaças mais significativas à saúde pública do século XXI (WHO, 2018).

Nesse contexto, o uso de sulfonamidas como ligantes tem cada vez mais atraído atenção na química supramolecular. Isso ocorre porque essas moléculas são capazes de combinar o potencial biológico para diferentes atividades com a capacidade de coordenar metais a partir de seus grupos fenilamino e sulfonil-ácido (SHAH et al., 2013). A utilização de moléculas de baixo peso molecular, como o sulfametoxazol, permite a complexação desta sulfonamida com centros metálicos duros, devido à presença de importantes sítios de coordenação (S, N e O), o que torna este fármaco, segundo a Teoria de Pearson, um bom candidato para a síntese de compostos biologicamente ativos compatíveis com as atividades metabólicas do organismo (DIPANKAR et al., 2014).

Nesta tese, foram relatadas importantes atividades biológicas de compostos derivados do sulfametoxazol. Os resultados forneceram evidências concretas de que os derivados estudados apresentam atividade antibacteriana e antibiofilme, sendo eficazes contra microrganismos do gênero *mycobacterium*, bactérias gram-negativas e gram-positivas, bem como frente a diferentes agentes fúngicos causadores de infecções pulmonares.

Os resultados descritos nesse trabalho vêm ao encontro de diversos estudos já realizados, que descrevem as atividades antimicrobianas e antibiofilme dos mesmos complexos do sulfametoxazol com Au, Ag, Cu, Hg, Cd e Ni, frente a diferentes espécies bacterianas patogênicas (AGERTT et al., 2013; AGERTT et al., 2016; MIZDAL et al., 2017; MIZDAL et al., 2018; SIQUEIRA et al., 2018; BONEZ et al., 2021). As produções científicas deste trabalho apresentam que essas moléculas, sintetizadas e caracterizadas inicialmente por Marques e colaboradores (2007), possuem capacidades inibitórias frente a *P. aeruginosa*

muito superiores quando comparadas as atividades do sulfametoxazol livre. Esse fenômeno de potencialização da atividade dos complexos pode ser observado tanto frente ao microrganismo na forma planctônica (livre), quanto frente ao microrganismo na forma séssil (em biofilmes).

Os resultados apresentados no **Artigo científico I** demonstram que, dentre os complexos metálicos, o complexo sulfametoxazol-Ag apresenta destaque quanto a atividade inibitória da cepa padrão de *P. aeriuginosa* (PAO1). O complexo com Ag apresentou atividade anti-biofilme *in vitro*, através da inibição do QS e, além disso, estudos *in silico* de docagem molecular sugeriram que essa molécula atua na inibição dos dois principais reguladores da comunicação celular, envolvidos na formação do biofilme de *P. aeruginosa*, o sistema *las* e o sistema *pqs*.

O QS em *P. aeruginosa* é formado por três sistemas de comunicação: *las*, *rhl* e *pqs*, e esses sistemas são controlados por moléculas autoindutoras como: N-3-oxo-dodecanoil homoserina lactona, N-butanoil-homoserina lactona e 2-heptil-3-hidroxi-4-quinolona (KARIMINIK et al., 2017). O QS é o exemplo mais bem caracterizado de comunicação química entre os microrganismos, sendo responsável por modular uma variedade de funções celulares, incluindo a formação do biofilme, a patogênese, a aquisição de nutrientes, a motilidade e a produção de metabólitos secundários (RENNER; WEIBEL, 2011). Ao utilizar abordagens que inibam a comunicação celular bacteriana mediada por esses sistemas, ocorre uma diminuição na expressão de diversos fatores de virulência envolvidos na formação e maturação do biofilme bacteriano. Sendo assim, sugere-se que o sulfametoxazol com Ag atua como uma promissora molécula inibidora do QS. A descoberta de compostos capazes de inibir a ação de moléculas sinalizadoras de QS parece ser uma estratégia promissora para controlar a formação e virulência dos biofilmes.

Avaliar a ação antibiofilme do sulfametoxazol complexado com os diferentes metais configurou uma importante estratégia, uma vez que a produção de biofilme é um mecanismo importante para a sobrevivência de *Pseudomonas aeruginosa* e sua relação com a resistência antimicrobiana representa um desafio para a terapêutica dos pacientes (LIMA et al., 2018). Corroborando com os resultados apresentados no **Artigo Cientfíco I**, o **Manuscrito II** apresentou resultados promissores de atividade anti-biofilme dos complexos frente a isolado clínico de *P. aeruginosa* resistente a múltiplas drogas, sugerindo que essas moléculas possam ser consideradas boas candidatas para estudos futuros que levem esses compostos a serem utilizados como agentes terapêuticos para eliminação de bactérias e biofilmes.

As atividades antimicrobianas, antifungicas e antibiofilmes apresentadas pelo complexo de prata, superiores aos demais complexos e ao sulfametoxazol livre, já eram esperadas, uma vez que o íon prata surge na atualidade como uma substância reconhecida a respeito da sua atividade biológica frente a diferentes microrganismos. Embora o mecanismo pelo qual a prata atua como um potente antimicrobiano não seja totalmente esclarecido, sabe-se que os íons prata podem atuar como adjuvantes para aumentar a atividade antibiótica dos fármacos, tendo como alvo o DNA procariótico, proteínas, aminoácidos livres e moléculas menores. Além disso, os íons prata podem mediar mudanças na membrana celular, modificando a sua permeabilidade (BARRAS et al., 2018).

O sulfametoxazol é uma sulfonamida de amplo espectro, considerada de grande importância no controle de infecções causadas por microrganismos do gênero *Mycobacterium*, porém a sua atividade diminui significativamente quando o agente infeccioso se apresenta na forma de biofilmes (BRASIL, 2008). Sendo assim, o **Manuscrito I** apresenta a síntese e a caracterização das propriedades químicas e estruturais de uma base de Schiff derivada do sulfametoxazol. O híbrido sulfametoxazol-piridoxal foi avaliado quanto a sua atividade antimicobacteriana e antibiofilme frente a Micobactérias de Crescimento Rápido (MCR) e a relevância dos resultados obtidos pode ser destacada, uma vez que a resistência antimicrobiana é um tópico importante no estudo das micobactérias, principalmente quando associada a formação de biofilmes (MACEDO et al., 2009).

Estudos sugerem que a capacidade de desenvolvimento de biofilmes por microrganismos do gênero *Mycobacterium* está relacionada à sobrevivência ambiental do microrganismo, patogenicidade em humanos, virulência e resistência a agentes antimicrobianos (LEWIS, 2012; ARAI ET AL., 2013). Além disso, inúmeras espécies de MCR já foram associadas a biofilmes em dispositivos biomédicos, próteses estéticas, próteses articulares e sistemas de água em ambientes hospitalares, apresentando grande impacto na saúde humana (MARTÍN-DE-HIJAS et al., 2009; PITOMBO et al., 2009). Mais recentemente, relatou-se que biofilmes de micobactérias contribuem para a patogênese das infecções sanguíneas relacionadas ao uso de cateteres (EL HELOU et al., 2013).

Os resultados obtidos neste trabalho e apresentados no **Manuscrito I** mostram que a base de Schiff derivada do sulfametoxazol foi capaz de inibir o crescimento das MCR na forma livre, em concentrações baixíssimas, signinificativamente inferiores que as concentrações necessárias de sulfametoxazol. Além disso, a atividade antibiofilme dessa molécula superou, expressivamente, a atividade antibiofilme do sulfametoxazol livre, sendo a

molécula capaz de inibir a formação dos biofilmes micobacterianos em todas as concentrações testadas.

As características estruturais das micobactérias permitem que essa atividade potencializada da base de Schiff seja melhor entendida. Considerando que a parede celular micobacteriana é altamente lipofílica devido a grande quantidade de ácidos micólicos, a hidrofobicidade dessa estrutura torna-se um fator limitante na permeabilidade celular dos fármacos (JOHNSON; ODELL, 2014).

Visto isso e considerando que a base de Schiff formada apresenta-se como um grupo funcional imina, é possível destacar a maior lipofilicidade do composto orgânico quando comparado ao grupo amino correspondente (BREAK et al., 2013). Esse aumento da lipossolubilidade do derivado do sulfametoxazol nos permite sugerir, com base no conceito de overtone, que essa molécula apresenta maior facilidade em atuar sobre as células lipofílicas micobacterianas. De acordo com o conceito de permeabilidade celular de Overtone, a membrana lipídica que envolve a célula bacteriana favorece a passagem apenas de materiais lipossolúveis, o que torna a lipossolubilidade um fator importante no controle da atividade antimicobacteriana e antibiofilme (RAMAN et al., 2004).

Ambas as produções científicas apresentam imagens de microscopia de força atômica, que comprovam os efeitos biológicos das moléculas na topografia dos microrganismos. O **Artigo Científico I** apresenta a diminuição da biomassa dos biofilmes de *P. aeruginosa* na presença do complexo sulfametoxazol-Ag, enquanto o **Manuscrito I** demonstra os efeitos danosos que a nova base de schiff derivada do sulfametoxazol causa na topografia de *M. smegmatis*. Ademais, o **Artigo Científico I** e o **Manuscrito I** provaram que, além de eficazes, as moléculas avaliadas nos respectivos trabalhos podem ser consideradas moléculas seguras.

O perfil de segurança das substâncias foi avaliado em modelo de células mononucleadas de sangue periférico. Os resultados obtidos nos ensaios de cito e genotoxicidade *in vitro* sugeren que tanto o complexo sulfametoxazol-Ag, quanto a nova base de Schiff derivada do sulfametoxazol manteram a viabilidade celular, não apresentaram potencial genotóxico e não desencadearam a produção de espécies reativas de nitrogênio e oxigênio. Esses resultados estão em concordância com o princípio farmacológico que atualmente é defendido, onde uma formulação que tem propriedades biológicas eficazes e perfil de segurança em pequenas concentrações é preferível (RANG; DALE, 2016; MENDES et al., 2015).

6 CONCLUSÕES

Em função dos objetivos propostos neste trabalho e considerando os resultados experimentais obtidos, pode-se concluir que:

6.1 Uma Base de Schiff derivada do sulfametoxazol foi sintetizada e caracterizada em relação a suas propriedades químicas e estruturais, demonstrando o sucesso da síntese orgânica.

6.2 A base de Schiff sintetizada inibiu o crescimento planctônico de espécies de MCR, com valores de MIC entre 1,22 µg/mL e 0,61µg/mL, valores expressivamente menores do que os valores determinados para o sulfametoxazol livre.

6.3 A base de Schiff derivada do sulfametoxazol inibiu a formação de biofilmes de MCR em todas as concentrações testadas e em concentrações significativamente menores do que as concentrações avaliadas de sulfametoxazol.

6.4 Complexos de sulfametoxazol com Ag, Cu, Hg, Cd, Ni e Au apresentaram atividade antibacteriana e antibiofilme superior a do sulfametoxazol frente a cepas padrões e isolados clínicos resistentes a múltiplas drogas de *P. aeruginosa*, bem como atividade antifúngica frente a agentes fúngicos de infecções pulmonares, com destaque para as atividades apresentadas do complexo com Ag.

6.5 O complexo de sulfametoxazol com Ag apresentou atividade anti-biofilme *in vitro* frente a *P. aeruginosa*, através da inibição do QS.

6.6 Testes *in silico* sugerem que o complexo sulfametoxazol-Ag atua nos sistemas *las* e *pqs* do QS bacteriano, que são os principais reguladores da formação de biofilme em *P. aeruginosa*.

6.7 A avaliação toxicológica *in vitro*, realizada em PBMCs, demonstrou que a base de Schiff derivada do sulfametoxazol e o complexo sulfametoxazol-Ag são alternativas seguras, apresentando resultados semelhantes aos encontrados nos controles negativos dos ensaios de cito e genotoxicidade.

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ANEXOS

ANEXO A – DADOS DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA (UFSM)

UNIVERSIDADE FEDERAL DE SANTA MARIA/ PRÓ-REITORIA DE PÓS-GRADUAÇÃO E

COMPROVANTE DE ENVIO DO PROJETO

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: IDENTIFICAÇÃO MOLECULAR E ESTUDO DO PERFIL DE SUSCETIBILIDADE DE ISOLADOS CLÍNICOS DE MICOBACTÉRIAS DE CRESCIMENTO RÁPIDO

Pesquisador: MARLI MATIKO ANRAKU DE CAMPOS

Versão: 2

CAAE: 71795417.6.0000.5346

Instituição Proponente: Universidade Federal de Santa Maria/ Pró-Reitoria de Pós-Graduação e Pesquisa

DADOS DO COMPROVANTE

Número do Comprovante: 082876/2017

Patrocinador Principal: Financiamento Próprio

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Pesquisa de compostos que atuam sobre biofilmes microbianos

Pesquisador: MARLI MATIKO ANRAKU DE CAMPOS

Área Temática:

Versão: 1

CAAE: 12114713.1.0000.5346

Instituição Proponente: Universidade Federal de Santa Maria/ Pró-Reitoria de Pós-Graduação e

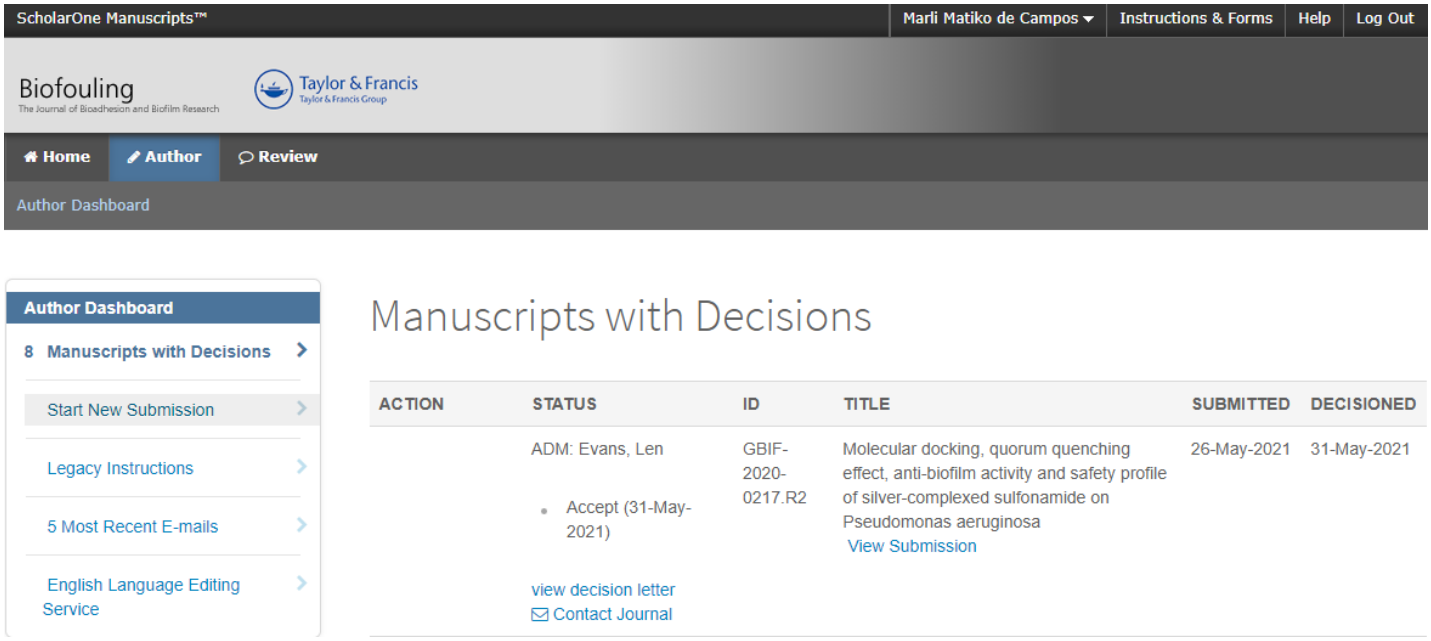
Patrocinador Principal: Financiamento Próprio

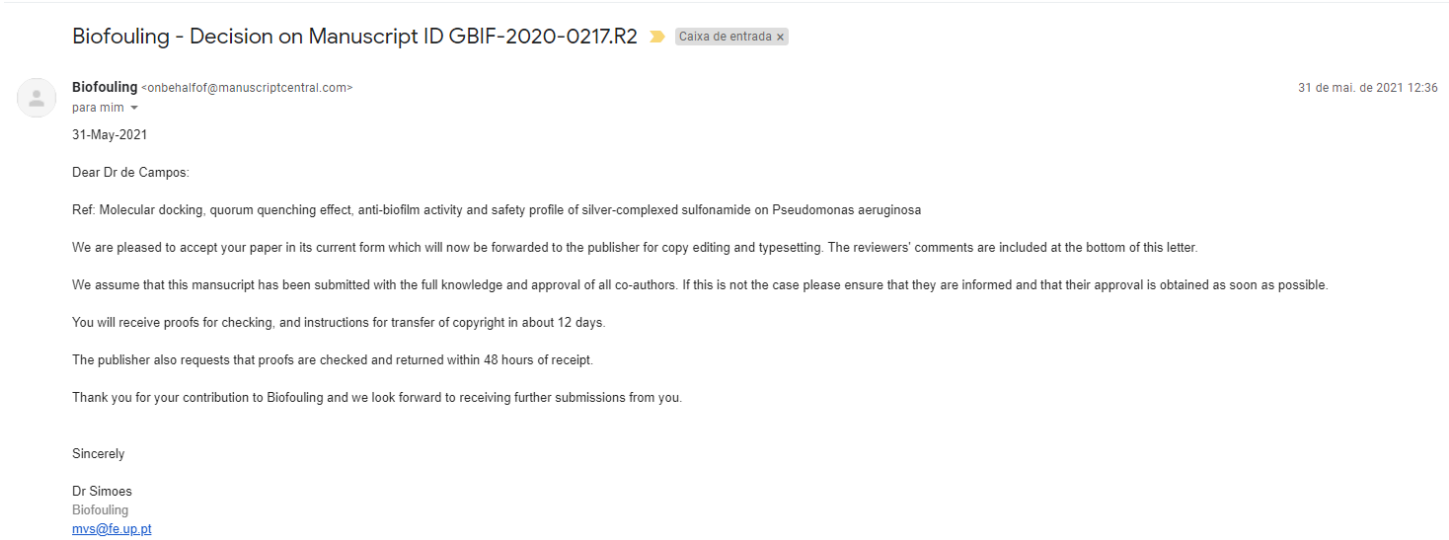
DADOS DO PARECER

Número do Parecer: 203.802

Data da Relatoria: 19/02/2013

ANEXO B – COMPROVANTE DE PUBLICAÇÃO





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31-May-2021

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Sincerely

Dr Simoes
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ANEXO C – COMPROVANTE DE SUBMISSÃO DO MANUSCRITO I

Submission Confirmation for Synthesis and Biological Evaluation of New Sulfamethoxazole Derivative Schiff Base as antimicrobial and antibiofilm Agent on Rapidly Growing Mycobacteria.

Caixa de entrada ×

Chemistry and Biodiversity 14:18 (há 8 horas)

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Dear Dr Back,

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