Veronica Rossetto

Understanding new microbial communication systems to combat antimicrobial resistance

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MSc of Molecular and Microbial Biology

Supervisors: Professor Dr Helena Galvão

Faculty of Science and Technology, Universidade do Algarve

Co-supervisor: Dr Jerry Reen

School of Microbiology, University College Cork

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluído.

Veronica Rossetto

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ABSTRACT

Bacterial biofilms provide an advantageous spatial structure for colonization and cell maintenance as a community. This multicellular behaviour is regulated by a bacterial quorum-dependent mechanism, called the quorum sensing (QS) system that regulates other diverse social behaviours such as toxin production and virulence factors. This mechanism is regulated by signal molecules that regulate intra-specific, inter-specific and inter-kingdom interactions. For these reasons, this mechanism is strongly studied, as well as signalling molecules and analogues, such as alkyl-quinolone based compounds, for the disarming of pathogenic bacteria resistant to multi-drugs that plague public health worldwide. The path to a complete understanding of how this occurs, what are the conditions for such biological responses and what machinery and mechanisms exist for the perception and modulation of these interactions is still far from reaching a conclusion. Therefore, the present work seeks to evaluate compounds against behaviours dependent on the quorum sensing mechanism, as well as the effect of these compounds on the growth of harmful pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Providing information to assist in understanding these microbial interactions, as well as the development of new anti-infectious strategies and the fight against antimicrobial resistance. The tested compounds confirmed activities such as anti-biofilm, anti-swarming and anti-pyocyanin production. Of the twenty-three analogous compounds to the alkyl-quinolones screened thirteen presented some type of interference between the three evaluated phenotypes, five with significant antagonistic activities against *P. aeruginosa* PA14 and three against staphylococcal strains, such as *S. aureus, Staphylococcus haemolyticus* and *Staphylococcus hominis*. Thus, it is concluded that small molecules based on alkyl-quinolones are effective bioactive against QS dependent behaviours and can assist in unravelling microbial communication and its impacts on human society.

Keywords: Interspecies Interactions; Small Molecule Mimics; Quorum Sensing Signalling Molecules; Bacterial Communication.

RESUMO

Microrganismos podem ser considerados organismos vastamente abundantes que colonizam nichos desde o fundo oceânico até ao interior do intestino humano. Esta alta diversidade pode ser explicada pela capacidade das bactérias se comportarem como uma estrutura espacial, chamada biofilme, auxiliando os processos de defesa, competição e cooperação bacteriana. Biofilmes bacterianos fornecem uma estrutura espacial vantajosa na colonização e manutenção celular como uma comunidade. Este comportamento multicelular é regulado por um mecanismo dependente de um quorum bacteriano, chamado sistema Quorum Sensing (QS), responsável por coordenar comportamentos de um organismo unicelular de tal forma que corresponda biologicamente a uma unidade multicelular ou comunidade.

O mecanismo deste sistema incorpora processos de produção, secreção e deteção de moléculas sinais ou autoindutores que, quando presentes em grande quantidade no meio exterior, penetram o interior celular e regulam a transcrição e expressão de múltiplos genes: formação de biofilme, motilidade, pigmentos, síntese de antibióticos, toxinas, fatores de virulência etc. Entretanto, autoindutores de bactérias gram-positivas são distintos de gram-negativas. O principal grupo autoindutor gram-negativo são os n-Acil-Homoserina Lactonas (AHLs) isto foi associado logo no começo das descobertas do QS em meados da década de 90, mas outras moléculas sinais são descritas, como Fator de Sinalização de Difusão (DSF) e Alquil-Quinolonas (AQ). *Pseudomonas aeruginosa* possui quatro sistemas distintos Las, Rhl, PQS e IQS. O sistema PQS possui como autoindutor a molécula nomeada Sinal de Quinolona de *Pseudomonas* (PQS), uma AQ que controla genes de virulência em conjunto com os sistemas Las e Rhl. Bactérias gram-positivas possuem oligo peptídeos ou também conhecidos como peptídeos autoindutores e consequentemente apresentam outros sistemas de QS. Um dos principais exemplos que podemos citar é o sistema Agr, em *Staphylococcus aureus*, que é responsável por fatores de virulência extracelulares, como hemolisinas, proteases e lipases.

Porém, ao contrário do que uma vez se imaginou as moléculas sinalizadoras AQ não possuem ação somente intraespecífica. Há relatos de comunicações por AQ em esferas intraespecíficas, interespecíficas e inter-reinos. Estas interações se mostraram relevantes principalmente em coinfecções de *P. aeruginosa* e *S. aureus*, muitas vezes dificultando o tratamento e a erradicação dos microrganismos patogênicos, principalmente quando há formações de biofilme bacteriano. Associadas a isto, temos uma problemática mundial, a resistência antimicrobiana. Bactérias conhecidas como *multidrug-resistant* encarecem os custos governamentais e aumentam os riscos de saúde pública numa escala mundial. As bactérias ESKAPEE que incluem *S. aureus* e *P. aeruginosa*, são exemplos de patógenos que apresentam grande ameaça, devido ao rápido desenvolvimento de mecanismos de resistência. Por sua relação com a formação de biofilme, virulência e mecanismos de resistência, o mecanismo de QS é fortemente estudado. Assim como as moléculas sinalizadoras e análogos destas moléculas, na busca pelo desarme do QS e consequentemente, da virulência e patogenicidade bacteriana. O caminho para total compreensão de como isso ocorre, quais as condições para tais respostas biológicas e quais maquinarias e mecanismos existem para a percepção e modulação destas interações ainda está longe de chegar em uma conclusão.

Por tanto, o presente trabalho teve como objetivo avaliar compostos contra comportamentos dependentes do mecanismo de QS, bem como o efeito destes compostos no crescimento de patógenos prejudiciais, como *P. aeruginosa* e *S. aureus*, de forma que forneçam

informações que auxiliem na compreensão destas interações microbianas, no desenvolvimento de novas estratégias anti-infecciosas e no combate da resistência antimicrobiana.

Triagens foram realizadas com a biblioteca de compostos disponibilizadas pelo departamento de microbiologia – University College Cork para a realização deste projeto. Na coleção bacteriana foram testadas *P. aeruginosa* PA14, uma gama de *Staphylococcus* spp. e *Bacillus atrophaeus*. Ensaios para verificação de inibição de biofilme foram avaliados seguindo o ensaio padrão de coloração com cristal violeta (0.1%), utilizando microplacas de 96 poços contendo 200 µL de cultura microbianas inoculadas com adição de compostos com concentração de 30 µM. Posteriormente, os compostos que demostraram atividade foram validados em novos testes padrão de coloração usando microplacas de 24 poços com volumes ajustados a 1 ml, com mesma concentração de composto adicionado. Análises de curvas de crescimento foram desenvolvidas em paralelo com os compostos (30 µM), utilizando o equipamento e sistema automatizado Bioscreen-C. Outros fenótipos biológicos dependentes de QS foram avaliados como motilidade bacteriana por enxameação, utilizando placas de Petri com baixas concentrações de ágar e adição de compostos a 10 µM. Assim como a quantificação da produção de piocianina com inserção dos compostos a 30 µM, através de ensaios de extração bifásica. Diferenças significativas foram determinadas por one-way ANOVA com o teste de comparação múltipla de Dunnett, bem como a geração de gráficos foram realizadas utilizando o software GraphPad Prism 8.

Perante os compostos testados foi confirmada atividades significativas como anti-biofilme, anti-enxameação e anti-piocianina. Dos vinte e três compostos triados treze apresentaram algum tipo de interferência entre os três fenótipos avaliados, cinco com atividades antagónicas significativas contra *P. aeruginosa* PA14 (dois compostos mostraram redução significativa na formação de biofilme e três na produção de piocianina) e três contra o biofilme de linhagens de estafilococos, como *S. aureus*, *Staphylococcus haemolyticus* e *Staphylococcus hominis*. Poucos compostos interferiram no crescimento bacteriano nos microrganismos testados. Somente os compostos 04 e 09 mostraram-se bactericidas em algumas linhagens de *Staphylococcus* spp. e *B atrophaeus*. Nenhum composto interferiu com *P. aeruginosa* PA14. O composto 09 foi o único a apresentar atividades antagónicas nos três gêneros testados e o composto 14 mostrou-se o mais promissor perante as três análises fenotípicas avaliadas sem apresentar efeitos bactericidas.

Devido ao papel fundamental nos sistemas de QS e na interação interespécie e interdomínio as AQ, bem como análogos destas moléculas, ganharam um espaço no *pipeline* de pesquisa e desenvolvimento de drogas antimicrobianas. Estudos anteriores demonstraram que a atividade anti-biofilme e anti-enxameação de análogos de AQ persiste após um amplo espectro de modificações das estruturas do anel de antranilato. Embora muitos trabalhos citem a resposta eficaz em moléculas com inserção ou substituição de grupos halogenados, os estudos deste trabalho revelam que a inserção nitro, corroborado por alguns trabalhos, e principalmente do grupo metil e metoxi apresentaram bons resultados invariavelmente da posição. Análises genómicas, bem como estudos polimicrobianos e avanços em estudos *in vivo* podem ser alvo de trabalhos futuros. Embora mais estudos necessitem ser realizados para compreensão profunda da comunicação microbiana, concluí-se que pequenas moléculas baseadas em autoindutores, tais como as AQ testadas no presente trabalho, são bioativos eficazes contra comportamentos QS dependentes e podem auxiliar no desvendamento da comunicação microbiana e seus impactos na sociedade humana.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AHL	n-Acyl-Homo-Serine Lactones
AI-1	Autoinducer-1
AI-2	Autoinducer-2
AQ	Alkyl-quinolones
CAI-1	(S)-3-hydroxytetradecanoe-4- ketone molecule
CF	Cystic Fibrosis
DMSO	Dimethyl Sulfoxide
DSF	Diffusion Signalling Factor
ESKAPEE	*Enterococcus faecium., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.* and *Escherichia coli*
HAQ	4-hydroxy-2-alkylquinoline molecule
HHQ	2-heptyl-4-quinolone molecule
HQNO	4-hydroxy-2-heptylquinoline-N-oxide molecule
IC_{50}	Half Maximum Inhibitory Concentration
MDR	Multidrug-resistant
MIC	Minimum Inhibitory Concentrations
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical Density
PQS	*Pseudomonas* Quinolone Signal (2-heptyl-3-hydroxy-4-quinolone) molecule
PSM	Phenol Soluble Modulin
QQ	Quorum Quenching
QS	Quorum Sensing
QSIs	Quorum Sensing Inhibitors
ROS	Reactive Oxygen Species
TCS	Two-component Systems
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

INTRODUCTION

The bacterial communication system, known as Quorum Sensing (QS), is an intercellular phenomenon based on the production, release and detection of one or several small signalling chemical molecules from cell to cell to coordinate group behaviour (WANG et al., 2020).

The description of this mechanism started in the mid-1960s and 1970s with studies on the genetic competence of *Streptococcus pneumoniae* (TOMASZ, 1965) and control of activity in luminescent bacteria, *Aliivibrio fischeri* (previously called *Vibrio fischeri*) and *Vibrio harveyi* (NEALSON; PLATT; HASTINGS, 1970) that observed the onset of a specific behaviour in cultures with a critical biomass. However, initially the scientific community did not readily accept this concept of microbial signalling and communication.

It was only in the end of 1970s that this subject began to be further studied and evidence such as the need for a minimum quorum of cell density for the behaviour of the community was described (NEALSON; HASTINGS, 1979). Subsequently, the structure of the self-inducing signal (N-3-oxohexanoil homoserine lactone) was described (EBERHARD et al., 1981) as well as the identification of which genes (*lux*) are involved in the luminescence process from the marine bacterium (ENGEBRECHT; NEALSON; SILVERMAN, 1983; ENGEBRECHT; SILVERMAN, 1984).

As a result of this discovery, the researchers added efforts to discover genes homologous to the *lux* genes in other bacteria. These included *Pseudomonas aeruginosa* (JONES et al., 1993; PASSADOR et al., 1993) and the description of a second circuit homologous to the LuxR-LuxI family. (BRINT; OHMAN, 1995). As well as in *Agrobacterium tumefaciens* whose *luxI/R* homologs (*traI/R*) located on a plasmid (FUQUA; WINANS, 1994). These data were compiled in a minireview addressing the metabolic pathways that portray the passive diffusion of the AHL (n-Acyl-Homo-Serine Lactones) autoindutor and its interaction with the *lux* genes in *A. fischeri*, as well as the homologous systems found in other bacterial species that led to the terminology "quorum sensing" be used for the first time by Fuqua and colleagues (FUQUA; WINANS; GREENBERG, 1994).

It was noted through the subsequent studies that LuxI homologs catalysed the synthesis of the autoinducer and LuxR homologs exhibited specificity for that molecule (WHITELEY; DIGGLE; GREENBERG, 2017) and this led to the concept of QS. This mechanism is based on the increase in the density of the bacterial population and the perception of a critical concentration of signalling molecules that provide a way by which bacterial species can

coordinate their behaviour as a community, sharing assets and protecting them from challenges. (WHITELEY; DIGGLE; GREENBERG, 2017). Specific genes are expressed and regulated, activating certain physiological functions and regulatory mechanisms (PIEWNGAM et al., 2020; WANG et al., 2020). Examples of these genes can be described as growth inhibition, antibiotic biosynthesis, motility and swarming, efflux pumps, biofilm development, and virulence factors such as pyocyanin etc. (AZIMI et al., 2020; GARCÍA-REYES, SOBERÓN-CHÁVEZ, COCOTL-YANEZ, 2020).

It is now known that QS in the microbial universe is widespread, occurring in gram-positive and gram-negative bacteria through distinct chemical signalling pathways. Importantly, it occurs in both pathogenic and non-pathogenic species (PIEWNGAM et al., 2020), making it a target for study in both clinical and environmental sectors.

1. Quorum Sensing in Gram-positive Bacteria

Gram-positive quorum-sensing systems are distributed across a wide variety of species, and a significant degree of species-specific has been observed. Gram-positive bacteria mainly use autoinducing peptides or modified oligopeptides as QS signalling molecules, that can be secreted and accumulated extracellularly like AHLs. However, these signals do not undergo passive diffusion. Rather, they need to be pumped in through the membrane via ABC transporters or other channel proteins (WANG et al., 2020). Usually, these transport systems are associated via two-component systems (TCS), which consist of a membrane-located signal receptor protein (histidine kinase). When the extracellular QS signal recognizes and bind in this receptor an activated process is started through a phosphorylation cascade that activates a response regulator inside the cell, which is a DNA-binding transcription factor in target genes (PIEWNGAM et al., 2020). Diverse functions of gram-positive bacterial QS systems have been reported, such as virulence factor production in the *Staphylococcus aureus* and *Bacillus cereus*, biofilm formation in *Bacillus subtilis*, and plasmid transfer in *Enterococcus faecalis* (WANG et al., 2020).

1.1. *Staphylococcus aureus*

This bacterium can reside as a normal commensal in up to two-thirds of healthy individuals (ARYEE; EDGEWORTH, 2017). However, if the skin's epithelial tissue is damaged, this bacterium can cause minor infections like atopic dermatitis (NAKAGAWA; HILLEBRAND; NUNEZ, 2020) or even can lead to pneumonia, bacteraemia, and sepsis (WANG et al., 2020). These diseases can be considered severe when infection is caused by the

"worst version" of *S. aureus*, the methicillin-resistant (MRSA), known as one of ESKAPEE opportunistic pathogens (ARYEE; EDGEWORTH, 2017). The differences in degrees of infection of this bacterium are dependent on the many combinations that can be generated from the expression of a range of adhesion molecules (which assist in the formation of biofilm, for example) toxins and compounds that affect the immune system (WANG et al., 2020). QS is a key element in controlling the expression of these molecules and compounds, thus facilitating the invasion and infection of the tissues of the human body.

The system responsible for the detection of QS in *S. aureus* is known as the Agr system, called the accessory gene regulator (PAULANDER et al., 2018). This functions as a TCS performs a cascade reaction responsible for the expression of extracellular virulence factors, e.g. hemolysins, proteases, lipases (QUAVE; HORSWILL, 2014). As well as the production of molecules affecting the immune system, such as δ toxin (a type of Modulin Soluble in Phenol - PSMs) and some cytotoxic peptides (NAKAGAWA; HILLEBRAND; NUNEZ, 2020).

Although the Agr system is involved in the production of biofilm in *Staphylococcus* spp., such as *S. aureus* and *S. edpidermidis in vitro, in vivo* it is closely linked to the surfactant function of PSM even in the low density activity of Agr. According to the authors, the influence of Agr on the infection associated with the biofilm is divergent: Agr is necessary for the structuring of the biofilm and the spread of the infection by the biofilm, but Agr's dysfunction leads to a more extensive formation of the biofilm and a decrease in the potential dissemination, which can be advantageous under certain conditions, such as greater success during persistent bacteraemia (LE; OTTO, 2015).

1.2. Quorum Sensing in *Bacillus* spp.

In the Firmicutes phylum, specifically in the Bacillales orders, a generalized QS system, in addition to TCS mentioned above, is a cytoplasmic receptor, called RRNPP (Rgg, Rap, NprR, PlcR and PrgX), a family of QS proteins (NEIDITCH et al., 2017). Like others, this mechanism has similarities in the process such as synthesis, secretion, processing and re-internalization of a signal peptide detectable by a receptor, the difference being that in the RRNPP family each receptor regulates different functions (VERDUGO-FUENTES et al., 2020 ; BABEL et al., 2020).

Some proteins in this system are interconnected to other QS-dependent systems, such as the NprR protein and the Spo0A system responsible for microbial sporulation. It is even believed that NprR probably plays a role in other processes of specialization and collective functions, for example, biofilm formation, virulence, propagation etc. in *B. cereus*

(VERDUGO-FUENTES et al., 2020). Like the NprR, a protein that also appears to have this ability to turn on / off from other systems and is involved in biofilm formation is the Rap protein. The peculiarity of this protein is the evidence that the same bacterial cell has more than one Rap protein. The authors mention that this overlap protein can provide advantages for *Bacillus* spp. in heterogeneous populations, activating genes in neighbouring cells and utilizing components produced by others without involving great energy costs (BABEL et al., 2020). Another detail is that Rap proteins seem to be related to the environment in which the species lives, providing advantages for different adaptations that the environment may require (VERDUGO-FUENTES et al., 2020). Although the *Bacillus* spp. has several systems, these are the most relevant for the present work. All mechanisms are not fully understood, but it is perceived that are connected to each other and strongly related to polymicrobial complexes and biological adaptations required in different environments.

2. Quorum Sensing in Gram-negative Bacteria

The main autoinducer in Gram-negative is the n-acyl homoserine lactones (AHL). This association was made around the 90s as previously described, through the species *P. aeruginosa* and *A. tumefaciens,* which had *A. fischeri* autoindutor analogues. This AHL signal, also known as AI-1, has a chemical composition of homoserine lactone rings with an additional fatty acid side chain - acyl (SAEKI; KOBAYASHI; NAKAZATO, 2020). This molecule mostly transits by simple diffusion in the cell membrane and remains free to accumulate in both the intracellular and extracellular portions (TAY; YEW, 2013). Traditionally considered as a signal molecule for intraspecific communication, studies suggest that AHLs can also be used to detect potential environmental competitors (SAEKI; KOBAYASHI; NAKAZATO, 2020). However, the AHL is not the only type of autoinducer utilized in gram-negative bacteria. Other molecules such as Quinolones, Diffusion Signalling Factor (DSF), (S)-3-hydroxytetradecanoe-4- ketone (CAI-1) have been identified and these tend to be species-specific (WANG et al., 2020).

In addition, there is another type of signalling that has been widely discussed as it presents itself as a non-specific signal molecule called Autoinducer-2 (AI-2). This molecule can be used by both gram-positive and gram-negative bacteria for both intra and interspecific communication through the LuxS system. Although the multifunctionality of AI-2 is still questioned, studies show that this molecule can regulate the luminescence of *V. harveyi*, the pathogenicity of *P. aeruginosa* and the biofilm formation of *E. coli* (PIEWNGAM et al., 2020; WANG et al., 2020; AZIMI et al., 2020).

2.1. *Pseudomonas aeruginosa*

An important example to elucidate the versatility of QS in gram-negative bacteria is *P. aeruginosa*. This bacterium has been studied for many years owing to its ability to infect the cystic fibrosis (CF) lung. In addition to its intrinsic resistance to antibiotics, *P. aeruginosa* is also a successful colonizer of the lung and of burn wounds, forming biofilms, a behaviour regulated by QS that favours persistence of the infection.

P. aeruginosa makes use of four interconnected QS systems, known as Las, Rhl, PQS and IQS. Autoinducers have three different chemotypes identified so far: AHLs used by the Las and Rhl systems, alkyl-quinolones (AQs) used by the PQS system and 2- (2-hydroxyphenyl) thiazol-4-carbaldehyde used by the IQS system (SCHÜTZ; EMPTING, 2018). There are other types of signalling molecules, but the most commonly studied are the AHL and AQ molecules (WANG et al., 2020). These systems, in conjunction with PQS and AHL, lead to the expression of numerous genes related to the production of proteases, exotoxins, biofilms, and virulence factors such as elastase, lecithin, rhamnolipids, and pyocyanin, one of the main virulence factors produced by this bacterium (GARCÍA-REYES, SOBERÓN-CHÁVEZ, COCOTL-YANEZ, 2020; SAEKI, KOBAYASHI, NAKAZATO, 2020).

Due to the ongoing search for new ways to mitigate the virulence of these bacteria, the PQS system has been gaining great attention from researchers. In this system, two molecules are fundamental to trigger virulence factors, the *Pseudomonas* Quinolone Signal - PQS (2-heptyl-3-hydroxy-4-quinolone) and its precursor 2-heptyl-4-quinolone (HHQ) (REEN et al., 2011). The genes encoding the biosynthesis of both molecules are involved in the synthesis of more than 50 different AQ molecules, which have been identified as being produced by *P. aeruginosa* (GARCÍA-REYES; SOBERÓN-CHÁVEZ; COCOTL-YANEZ, 2020). In addition to this context of signal quinolone autoinducer, data show that this molecule can also function to mediate iron acquisition, cytotoxicity, or to exercise host immunological modulatory activities, (LIN et al., 2018).

In this work, we will focus on discussions about the pathogenic bacterium *P. aeruginosa* because it is an antimicrobial resistant pathogen that requires further studies in an attempt to combat its emergence as a superbug of note.

3. Interspecies and Inter-kingdom Communication

Fossil evidence proves that 3500 million years ago bacteria already existed, with Negibacteria (now known as gram-negative bacteria) being the oldest species discovered to date (CAVALIER-SMITH, 2002). In addition to being older, the estimated magnitude of bacteria and archaea on Earth is 1.2×10^{30} cells, which live from ocean sediments to the interior of humans (FLEMMING; WUERTZ, 2019). If bacteria are such ancient organisms in the evolution line and if they are considered one of the most abundant organisms on the planet, how can we not think that exists an interaction between microbes - microbes and microbes with other organisms?

For several years researchers have been interested in the fact that microorganisms survive in adverse environmental conditions and now it is known that the majority of microorganisms live in a complex community called biofilm (microorganisms that live within a matrix of polymeric substances that adhere to several surfaces) through secretion of chemical signals to communicate with one another, without direct physical contact (SAEKI; KOBAYASHI; NAKAZATO, 2020). This interaction explains the spread of certain species in environments that are difficult to colonize, but it also strengthens the idea that different species cooperate for the survival of polymicrobial colonies.

According to Whiteley, Diggle, Greenberg (2017), bacteria can be distinguished as belonging to two types, "cooperative" bacteria, and "cheating" bacteria. Cooperative bacteria through the QS produce and release to the extracellular environment something that other microbial cells can take advantage of. Cheating bacteria, on the other hand, do not have the energy costs involved by the QS and take the same advantage of what was released by the cooperative bacteria. According to a study carried out with transcriptomes when some environmental variable is changed, such as the nutrient source, and quorum sensing needs to be activated for the structuring of differentiated enzymes, in response to this environmental change, only cooperatives will survive (WHITELEY; DIGGLE; GREENBERG, 2017). Thus, in this case, cheaters, who do not have the complete QS system, will suffer a penalty and, consequently, the population of these bacteria is controlled.

Initial hypotheses on this subject started with bacteria that perceive the signal molecules of others even if they don´t produce the same signals. A term utilized to explain this idea is "Orphan or solo LuxR homologs". Studies show that bacteria that contain only the receptor proteins (orphan LuxR) may have evolved in such a way as to perceive the host environment directly, rather than serving as native QS signal receptors (WHITELEY; DIGGLE; GREENBERG, 2017). As in the case of *E. coli* SdiA has been reported to respond to AHLs and

mammalian host-produced small molecules (HUGHES et al., 2010). Some plant-associated bacteria can activate transcription of specific genes in response to small molecule(s) produced by the plant or the plant microbiota (SUBRAMONI et al., 2011).

These two examples, both that of the cheating bacteria and of the interspecies perception through orphan LuxR, show us how the dynamics between microbial populations influence the ecological interactions of these microorganisms with each other and with the environment, as well as influencing the adaptation and evolution of signalling in polymicrobial communities.

And when it comes to controlling another population, a good example to cite is the bacterium *P. aeruginosa*, through AQ signalling molecules that control behaviour in other microbes. According to Reen, et al. (2018), AQ molecules, such as PQS and HHQ, can positively and negatively interfere with the behaviour of other microorganisms, such as growth, swarming motility, biofilm formation, interference with iron absorption (NGUYEN et al., 2016; NAZIK et al., 2020) and even in resistance to bacteriophages and antimicrobial resistance or modulate the virulence behaviour of a variety of bacterial pathogens (BISHT; BAISHYA; WAKEMAN, 2020).

3.1. *P. aeruginosa* and *S. aureus* interaction

The relationship between *P. aeruginosa* and *S. aureus* has existed for a long time, these bacteria are commonly found in mixed infections that are difficult to eradicate. According to Ibberson and Whiteley (2020), these bacteria often isolated in CF and chronic wound infections can exhibit both competitive and cooperative behaviours.

In vitro studies and a fly infection model carried out by Korgaonkar et al. (2013) showed that the perception of peptidoglycans released by gram-positives promotes the production of the pyocyanin virulence factor (by means of increased PQS) and several extracellular factors that show lytic activity against prokaryotic and eukaryotic cells. Protein A, produced by *S. aureus*, also appears to favor *P. aeruginosa* through the inhibition of neutrophil phagocytosis (ARMBRUSTER et al., 2016). Therefore, cooperative interactions through chronic polymicrobial infections accentuate virulence, can increase tolerance to antibiotics, which leads to an increase in the severity of the disease, making them difficult to treat and resolve (IBBERSON; WHITELEY, 2020).

These interactions could explain why wounds infected with *P. aeruginosa* are more severe and heal at a slower rate, as seen by Dalton et al. (2011) who presented in a model of chronic murine wound, more serious infections and recalcitrant to antimicrobial treatment in a coinfections by *P. aeruginosa* - *S. aureus* than monocultural infections by *P. aeruginosa*. Other

examples like this were observed in a model of rat lung infection and a model of *Drosophila* infection where *S. aureus* has been shown to increase the potential of *P. aeruginosa* virulence (DUAN et al., 2003; SIBLEY et al., 2008). According to the Nguyen et al. (2016) authors, the antimicrobial activity of *P. aeruginosa* against *S. aureus* is increased by iron depletion and is dependent on several AQ metabolites, such as PQS and HHQ. HHQ has an innate antimicrobial activity against *S. aureus* and depletion of iron potentiates this activity even from clinical CF isolates, showing that these antimicrobial effects are cumulative when there are multiple AQ metabolites.

However, studies such as the one by Michelsen et al. (2016) report differences in behaviour between these two bacteria when comparing a model laboratory and a clinical strains. According to the authors model organisms such as *P. aeruginosa* PA01 interacting with *S. aureus* effectively kills *S. aureus* in co-culture, through the production of a variety of molecules (e.g. pyocyanin, LasA, LasB, rhamnolipids and the 4-hydroxy-2-alkylquinoline (HAQ) molecule, 4-hydroxy-2-heptylquinoline-N-oxide (HQNO)). However, the interaction with a clinical strain provided a modulation of the profile of a molecule signal (HAQ), a morphological alteration of a thickened colony in *P. aeruginosa* and tolerance to antibiotics such as tobramycin in *S. aureus*, all of which was HAQ-mediated (MICHELSEN et al., 2016). It appears therefore that the interaction between these two bacteria can be antagonistic in a controlled coculture, but that in contrast these interactions can be considered protocooperative when it comes to mixed infections in the human host.

3.2. *P. aeruginosa* and *Bacillus atrophaeus* Interaction

Pseudomonas spp. and *Bacillus* spp. are commonly found in soil microbiomes (CAI et al., 2019). Both species have biofilm production and have the ability to swarming. Swarming as well as the production of biosurfactants that contribute to swarming (e.g. surfactins) are regulated by QS and are associated with behaviours of virulence and resistance to antibiotics (RÜTSCHLIN; BÖTTCHER, 2020). For this reason, swarming control is of great interest for the development of new anti-infectious strategies.

A good model for swarming studies is *B. atrophaeus* (also called *B. globigii*, formerly *B. subtilis*) (REEN et al., 2015). This bacteria presents some studies on biodefense as a substitute for investigations of *B. anthracis* (REEN et al., 2015), but mainly on its influence on agriculture and food security, as in the defence against disease-causing organisms and in the promotion of plant growth (RAJAOFERA et al., 2020).

Studies involving *B. atrophaeus* and AQ from *P. aeruginosa* are rare. But just as PQS at 50 μM inhibited the swarming of *Pseudomonas putida* and reduced the formation of biofilm, the same can occur in *B. atrophaeus* that presents the same behaviours (FERNÁNDEZ-PIÑAR et al., 2011). And in fact, it does. Although there are few studies, the research group of the laboratory where this work was carried out shows good results about this interaction. HHQ and PQS suppressed swarming in *B. subtilis* and HHQ interferes with pellicle and biofilm formation (REEN et al., 2011). PQS completely extinguished the swarming of *B. atrophaeus* by 10 μM, while HHQ at the same concentration only led to a minimal reduction in swarming (REEN et al., 2011; REEN et al., 2015). Still according to the authors the production of de synthetic derivatives of HHQ such as substitutions in anthranilate (the ring derived from the quinolone nucleus) and variations in the alkyl chain provide effective and potentiated anti-swarming compounds (REEN et al., 2015).

3.3. *P. aeruginosa* and Inter-kingdom Interactions

Contrary to what was previously thought, signal molecules like AQ have a signalling and interference capability across intraspecific and interspecific bacterial boundaries. Scientific evidence presented by Reen et al. (2011) demonstrated the ability of HHQ and PQS to influence social behaviours between gram-positive, gram-negative and inter-kingdoms, being the first to report such effects in different domains. These studies show that the biological response of these molecules has an extensive phenotypic variety and therefore high metabolic complexity. According to Nazik et al. (2020) in the realm of inter-microbial interactions, PQS can be used as signals to detect quorum by other bacteria, can interfere in biological processes of neighbouring cells, as well as the production of this molecule by other bacteria can be stimulated; In inter-kingdom interactions, fungi can inhibit the production of PQS, but in contrast PQS can modulate biofilm formation and present fungistatic properties in fungi and yeasts.

The description of interactions between microbes and other organisms is old. There, when the first indications of QS were described through *A. fischeri*, the inter-domain interactions in a certain way were already discussed, since these bacteria inhabited the interior of squids, but little was known about the interactions involving signal molecules. However, with the advancement of studies between molecular interaction, it was noticed that this microbial perception is beyond the inter-species barriers and that they encompass other spheres of communication reaching the degree of interdomain.

Organisms from this same environment have recently been described as being impacted due to the action of AQ. According to Whalen et al. (2019), the exposure of HHQ caused the abundances of the microbiota associated with particles and free living in these environments to be affected, such as nanoplankton and prokaryotic cells, gamma- and alpha-proteobacteria were favoured and Bacteroidetes decreased. In addition, it was found through genomic analysis that bacterial genera that had some genetic connection with HHQ showed an increase in relative abundance after exposure to HHQ, such as *Pseudoalteromonas* spp., known to produce HHQ (WHALEN et al., 2019). Prokaryotic and eukaryotic interactions better described involving AQ, in fact run away from the marine environment and occur between bacteria and fungi. However, the relationship between *P. aeruginosa* and fungi seems to be a two-way street, sometimes bacteria affect fungi, and other times fungi affect bacteria.

Initially, interactions between *P. aeruginosa* and single-celled eukaryotic cells, such as yeasts, have been reported. According to Cugini et al. (2007), a compound secreted by *Candida albicans* can suppress AQ signalling, but that in contrast this same compound can increase the production of PQS and restore pyocyanin (CUGINI; MORALES; HOGAN, 2010). Later, it was demonstrated that in this same yeast, HHQ antagonizes the formation of biofilms, while PQS induced a marginal increase (REEN et al. 2011). Lopez-Medina et al. (2015) described how *C. albicans* inhibits *P. aeruginosa* virulence through suppression of pyochelin and pyoverdine biosynthesis compounds, both of which involve the PQS system. Competition between these organisms has been shown to inhibit iron acquisition and bacterial virulence (LOPEZ-MEDINA et al., 2015).

Activity towards multicellular fungi has also been described. Reen et al. (2016) first reported the effect of the PQS and HHQ molecules on biofilm formation in the important fungal pathogen *Aspergillus fumigatus*. Through confocal microscopic analysis, it was confirmed that both molecules reduced the formation of biofilm mass and stopped the development of hyphae. The work also addressed the finding that structurally modified quinolone analogues maintain anti-biofilm activity and that one of these tested compounds even affected *A. fumigatus* preformed biofilms (REEN et al., 2016). A recent study showed that the same molecules give the ability to attenuate or accentuate the iron chelating response in the same species of fungus, depending on the concentration of iron present. In conditions of low iron content, PQS inhibits the chelation of *A. fumigatus* and in high concentrations stimulates the development of the fungus and the action of siderophores so that both cohabit in the same host, as in the case of patients with cystic fibrosis (NAZIK et al., 2020).

According to the authors, fungi can inhibit the production of PQS, but in contrast PQS can modulate the formation of biofilm and present fungistatic properties in fungi and yeasts. This demonstrates that the interaction between bacteria and fungi is not hierarchical and depends on other variables such as the influence by the host (NAZIK et al., 2020). A very clear example at this point is the compound farnesol produced by *C. albicans* which was first described as an antibacterial against *P. aeruginosa* and which when tested in mono-colonized mice with this bacteria had no effect, with no significant difference in survival time of infection, nor decreased virulence by *P. aeruginosa* (LOPEZ-MEDINA et al., 2015). This all shows that molecular interactions and physiological responses are more complex than we imagine, that controlled environments do not necessarily show behaviour in real life simply because there are many other variables that we disregard or ignore, mainly involving the host.

Finally, advances in the research of mammalian intestinal microbiomes are beginning to reveal these interactions better. According to Whiteley; Diggle; Greenberg (2017), the molecule AI-2, for example, can increase the abundance of Firmicutes on Bacteroidetes in intestinal colonization, as well as limit the infection caused by *Vibrio cholerae*.

In addition to changes in intestinal microbiomes, many signal molecules interfere with host cell homeostasis by providing a change in the response of the host's immune system. According to the review by Reen et al. (2018), PQS can be responsible for having direct effects on host cells, exerting immunomodulatory activities; interfering in the production of interleukins; regulating the expression of genes involved in the immune reaction and cytokines generation in different human cell types. As well as the collective effect of AQ, HHQ and PQS alter the responses of transcription factors used in cases of environmental stresses, such as the presence of viral and bacterial antigens (REEN et al., 2018). This shows that AQ molecules are fundamental to successful colonization by microbes, interfering in the organisms that cohabit the same host, be it prokaryotic or eukaryotic organisms, as well as in the defence responses of the host itself.

According to another review provided by Lin et al. (2018), PQS stimulates the generation of reactive oxygen species - ROS (compounds produced in response to environmental stress) *in vitro* in lung epithelial cells; can release factors of tumour necrosis; and stimulates neutrophil chemotaxis. PQS and HHQ negatively regulate the host's innate immune systems by preventing specific transcription factor signalling pathways; activate the bitter taste epithelial receptors in the airways; stimulate T2R-mediated immune responses (LIN et al., 2018).

Taken together, these findings corroborate the effect of deregulation of the host's immune reaction during infection by means of signalling molecules involved with the QS, especially AQ. All these adaptations of regulated intraspecies behaviours as well as intraspecies and interactions provide favourable conditions for colonization and propagation in environments that are constantly changing. It is important to distinguish that what we aim at here is the understanding of microbial language. We did not aim to study compounds that inhibit biofilm production, because a series of studies show that the link between QS and biofilm formation depends on environmental conditions, conditions that are not under evaluation in this work. We wanted to better understand how this communication works through the response effect caused by these compounds, so that in the future we can modulate "questions and answers" in this microbial language towards a potential therapeutic strategy to control virulence in a targeted way.

4. Antimicrobial Resistance

Antimicrobial resistance, also recognized as antibiotic resistance, is defined as the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals, and antimalarials) from working against it. As a result, standard treatments become ineffective, infections persist and may spread to others" (World Health Organization - WHO, 2019). The fact that these microorganisms, which can be harmful to human health, persist in the environment and disseminate among the population is one of the greatest challenges of the 21st century (ALANIS, 2005; TACCONELLI, PEZZANI, 2018; NEVILLE, JIA, 2019). In many cases, these infections are opportunistic, and their dissemination occurs predominantly within the hospital setting (hence the term nosocomial infections) where patients are immunocompromised (MARTÍNEZ, 2014; FARIA et al., 2015).

Estimates from a UK government review predicted that deaths in the world owing to antimicrobial resistance will be higher than cancer by the year 2050 (AHMED et al., 2019). According to the Organisation for Economic Co-operation and Development – OECD 2018 data 2.4 million people in Europe, North America, and Australia will die with resistant microorganisms infections in the next 30 years and could cost up to US$3.5 billion per year (HOFER, 2019). At this moment, the European Union has estimated >30,000 deaths due to antimicrobial resistance (BOOLCHANDANI; D'SOUZA; DANTAS, 2019). Furthermore, Southern European countries are predicted to attain the highest mortality rates, followed by developing countries, which already have high resistance rates (40-60% of infections) (HOFER,

2019). Countries such as Brazil, Indonesia, and Russia, will suffer a marked increase in cases, rising 4-7 times faster than in other OECD countries (HOFER, 2019). According to U.S. Centers for Disease Control and Prevention – CDC, in the U.S.A., ca. 3 million people are infected yearly with bacteria resistant to first-line antimicrobials and more than 35,000 people die as a result, costing US$20 billion in health-care costs (BOOLCHANDANI, D'SOUZA, DANTAS, 2019; CDC, 2019).

It is clear that antimicrobial resistance is not an isolated sporadic phenomenon but represents today one of the biggest threats to global health, food security, and development, leading to longer hospital stays, higher medical costs and increased mortality (WHO, 2018). In fact, the economic impact is considered severe because "Antibiotic resistance can affect anyone, of any age, in any country" (WHO, 2018). In the face of diminished interest from large pharmaceutical companies in the development of new antibiotics, many researchers propose we are entering a 'perfect storm' of global health significance (COOPER; SHLAES, 2011). Alternative approaches to antibiotic control are urgently needed. Molecules that can inhibit or kill bacteria are considered antimicrobials and when microorganisms develop the ability to defeat, grow and survive despite these therapeutic agents, antimicrobial resistance arises (BOOLCHANDANI, D'SOUZA, DANTAS, 2019; CDC, 2019).

Antimicrobial resistance is a globally widespread problem particularly when multi-resistance develops in bacterial strains. According to a recent report by the CDC (2019), 18 microbial pathogens require urgent attention and action, such as Carbapenem-resistant *Acinetobacter baumannii* and *Clostridioides difficile* for urgent threats, and multidrug-resistant *P. aeruginosa* and *S. aureus* - MRSA for serious threats. Due to the rapid acquisition and fast transmission in resistance, bacteria can defeat various classes of antibiotics simultaneously, creating what is called multidrug-resistant (MDR) strains or "superbugs" (ALANIS, 2005). Bacteria have shown the ability to develop resistance to all classes of antibiotics (VENTOLA, 2015; CDC, 2019; BOOLCHANDANI, D'SOUZA, DANTAS, 2019). According to Hofer (2019), 39% of all resistant infections were caused by bacteria that are resistant to last-resort antibiotics, demonstrating how threatening this situation has become.

Many researchers claim that overuse of antibiotics promotes selective pressure among microorganisms, which favours the emergence of resistance amongst them (CASSIR, ROLAIN, BROUQUI, 2014; CDC, 2019; NEVILLE, JIA, 2019). Past medical usage of antibiotics became widespread and routine, even if infections were non-bacterial (ALANIS, 2005). More recently, new guidelines were delineated in public health focusing on prevention, such as hygienic measures and basic sanitation for both people and domestic animals (WHO,

2018; CDC, 2019). In addition, recommendations were issued for cautious prescription of antibiotics and a shorter treatment duration by health professionals (VENTOLA, 2015; WHO, 2018; CDC, 2019; NIELSEN et al., 2019; HOFER, 2019).

However, these measures have affected an industry that thrives in the commercialization of these drugs; Newly launched antibiotics are narrow spectrum and are increasing market fragmentation without solving the overall problem (NIELSEN et al., 2019). Novel antibiotic development has not kept up with the increasing rates of antimicrobial resistance and its spread (HOFER, 2019). The development of new antibiotics has decreased steadily since the 1980s and the last two decades have been considered a discovery void with respect to antibiotic discovery (ALANIS, 2005; CASSIR, ROLAIN, BROUQUI, 2014; NIELSEN et al., 2019). According to CDC (2019), new health threats are emerging, such as common and deadly gram-negative infections, for which no new major antibiotics have been developed and/or approved between 1962 and 2000. In fact, due to lack of business incentives, 78% of major drug companies have scaled back or stopped antibiotic research since 1990; Past records of drug research and development, show that only 1 out of 5 experimental antibiotics that pass the initial phase of testing in humans will receive approval from the FDA to treat infectious disease (CDC, 2019).

In 1945, Sir Alexander Fleming while accepting the Nobel Prize for the discovery of penicillin, gave a warning on the use of this compound because it could lead to the selection of resistant "mutant forms" of *S. aureus* (ALANIS, 2005). One year later, a significant number of resistant strains had already emerged and a few years later, over 50% were no longer susceptible to this drug (ALANIS, 2005). Even though the overuse of antibiotics has been the main cause of the increased spread of antimicrobial resistance, these drugs have transformed modern medicine by helping people in need of urgent medical care, such as sepsis treatment, surgery, patients with chronic conditions (e.g., diabetes), organ transplants, dialysis, cancer care, and have saved millions of people (>33 million in the USA) as mentioned in the CDC (2019) report.

On the one hand, the use of antibiotics increases the selection of antimicrobial resistant populations. On the other hand, people are dying from infections for which effective antibiotics are no longer available. Therefore, it is necessary to find a balance between the use of existing antibiotic therapies and the development of new drugs. More effective stewardship has been highlighted as a key factor in addressing the global threat of antimicrobial resistance. In fact, some researchers believe that we are in a post-antibiotic era and maybe it is time to change the industry framework by establishing non-profit organizations for faster and cheaper research and development of these “life-saving drugs” (NIELSEN et al., 2019). This would offer the

possibility of improving our chances of surviving resistant infections, but of course there is no guarantee. In fact is important to note that, bacteria and fungi have natural resistance mechanisms through the development of new cell processes and/or cell walls, such as enzyme activity that destroys drugs, efflux pumping system to purge drugs or change antibiotic targets (CDC, 2019). Furthermore, these mechanisms are inherited from generation to generation via transduction, transformation or conjugation; The most frequent genetic recombination pathway is conjugation of a plasmid or lateral gene transfer that can easily and rapidly spread antibiotic resistance across the globe (ALANIS, 2005; MARTÍNEZ, 2014; BOOLCHANDANI, D'SOUZA, DANTAS, 2019; CDC, 2019). Therefore, resistance will likely exist even in the absence of antibiotic use. Overuse results in the emergence of these resistant clones through selection, at a scale that threatens human life.

In a recent study in the Norwegian Arctic region (MCCANN et al., 2019), 40 soil samples from 8 different stations were genetically analysed and showed that even in this inhospitable environment, 131 antibiotic resistance genes already existed. The disturbing surprise was that one of the genes (*blaNDM-1*) was first discovered a few years ago in New Delhi, India in 2008, geographically distant and ecologically distinct from the site of the Norwegian study; One possible explanation is that this gene was carried by human feces or guano from seabirds, then passed from feces to water (MCCANN et al., 2019). This study in the remote Arctic provides evidence that superbugs have become a truly global and pressing concern to human health. Moreover, this article raises the concern that the increased selection for antimicrobial resistant clones caused by antibiotic therapy will require a long time to be eliminated due to the fact that these compounds are immersed in our life cycle, including the disposal of such drugs in different environments such as aquatic/marine food webs, and even drinking water and marine sediments. (IAKOVIDES et al., 2019; XIA et al., 2019).

Although there is a significant market pull for new drugs, it is unlikely that "traditional" antibacterial chemicals are the key to resolving the current situation, other perspectives and truly innovative "platforms" need to be discussed and developed to address the clinical need (ALANIS, 2005). In this context, one new therapeutic approach would address the virulence itself, the features of the microbes that causes disease in the first place. It is important to note here that many of the infections that cause harm to humans, animals, and plants are opportunistic (MARTÍNEZ, 2014). Therefore, their existence is not a threat but rather when they enter a naïve host with a compromised natural defence system (VENTOLA, 2015). Therefore, anti-infection approaches offer the advantage of disarming the harmful effects of virulent (pathogenic) bacteria by disrupting the process of infection, but without directly

affecting bacterial growth or viability, thereby reducing selective pressure and injury on commensal species (AHMED et al., 2019; NEVILLE, JIA, 2019). This does not remove the fitness selection entirely but does significantly reduce the rate at which clones are selected.

4.1. Targeting the QS System: Anti-infective Strategies vs Signalling Molecules

QS interference occurs naturally with quorum sensing inhibitors (QSIs) that block the action of chemical signalling or with quorum quenching (QQ) with enzymes that degrade chemical signalling, known as autoinducers (RÉMY et al., 2018). According to recent studies, these strategies are promising routes to decrease bacterial pathogenicity and biofilm formation, as demonstrated in various animal models, by favouring bacterial susceptibility to antimicrobial agents (e.g. antibiotics) and constitutes a promising strategy to develop new medical devices against bacterial infections, such as antimicrobial dressings, and catheters (RÉMY et al., 2018; AHMED et al., 2019).

Quorum quenching research is focused nowadays on expanding the range of extracts and molecules with anti-virulence properties against MDR bacterial pathogens (GARCÍA-CONTRERAS; WOOD; TOMÁS, 2019). Common methods to verify this activity include inhibiting biofilm formation, swarming, and the production of specifics lipids, proteins, or pigments (GARCÍA-CONTRERAS; WOOD; TOMÁS, 2019). Biofilms are known to have antibiotic tolerance and are difficult to treat in clinical infections, and are frequently observed in *P. aeruginosa, Klebsiella pneumoniae* and *S. aureus* (RÉMY et al., 2018). Swarming behaviour is the ability of bacteria to migrate (motility) on semisolid surfaces and as with biofilm formation, it requires multi-cellular co-ordination of behaviour at the population level (RÜTSCHLIN; BÖTTCHER, 2020). This behaviour has been widely studied in *P. aeruginosa* and *Bacillus* spp. and is associated with enhanced virulence and antibiotic resistance of various human pathogens, such as *P. aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Salmonella typhimurium*, and *Clostridium septicum* (RÜTSCHLIN; BÖTTCHER, 2020).

Thus, the present research aimed to investigate small molecule compounds based on AQ signal molecules capable of acting specifically in inhibition of biofilm formation, swarming, and pyocyanin production, without affecting bacterial growth, as a contribution to the fight against microbial resistance.

OBJECTIVES

General:

To evaluate the antagonistic activity of twenty-three compounds analogous to alkyl-quinolones on QS-dependent bacterial social behaviours that assist in understanding and developing of new anti-infectious approaches.

Specifics:

- To screen compounds and validate for anti-biofilm activity in *P. aeruginosa* PA14, *B. atrophaeus* and a range of *Staphylococcus* spp.;
- To screen compounds and validate for anti-motility activity in *P. aeruginosa* PA14 and *B. atrophaeus*;
- To screen compounds and validate for anti-pyocyanin activity in *P. aeruginosa* PA14;
- To evaluate the effect of these compounds in the growth of tested microorganisms.

METHODOLOGY

Chemical Library and Bio-indicator Microorganisms:

The compounds that were tested and provided from the chemical library from the School of Microbiology - UCC with 23 synthetic compounds built (yet in sigil about its chemical structure) to improve biological activity. The microorganisms selected in this project are *Staphylococcus* spp. (clinical and model strains) and *B. atropheaus* as model for gram-positive bacteria, and *P. aeruginosa* PA14 as model for gram-negative bacteria.

Biofilm Assay:

Overnight cultures adjusted to an $OD_{600\ nm}$ of 0.05 in TSB (Tryptic Soy Broth) tested for presence and absence of each compound were used to evaluate inhibition of biofilm formation using a standard assay with crystal violet staining described by (FLYNN; REEN; O'GARA, 2019), with following modifications: The activity of 23 compounds (30 µM) was screened using 96-well plates containing 200 µL of inoculated medium, same volume (200 µL) was used to wash unattached biofilm, followed by staining with crystal violet (0.1% w/v) for 30 min, then solubilized with absolute ethanol (after removing excess stain by soaking the plate three times in a water gradient, tap and distilled water). Additionally, to check active compounds, different concentrations were used in 24-well plates applying the same method but with a final volume of 1 ml per well in each stage. Plates were incubated at 37°C and 30ºC for *Staphylococcus* spp. / *P. aeruginosa* PA14 and *B. atrophaeus,* respectively. The controls were TSB medium without inoculum and DMSO (dimethyl sulfoxide) as carrier control. Experiments were performed in triplicate and with at least three independent biological replicates. Biofilm formation was determined by measuring absorbance at 595 nm using the plate reader.

Analyses of Compounds Effects on Bacterial Growth Curves:

Isolates were cultured overnight in fresh TSB, $OD_{600\ nm}$ adjusted to 0.05, in the presence and absence of 30 µM of each testing compound, with DMSO as carrier control. Growth was measured spectrophotometrically at $OD_{600\ nm}$ in honeycomb plates (performed in triplicate and with at least three independent biological replicates for each strain) incubated at 37°C for *Staphylococcus* spp. and *P. aeruginosa* PA14, and 30ºC for *B. atrophaeus,* using a Bioscreen-C automated growth curve analyses system to capture the biomass data (Adapted from REEN et al., 2019).

Swarming Motility Assay:

Motility was measured on TSA (Tryptic Soy Agar) plates with 0.3% agar (w/v) for *B. atrophaeus* and on Eiken Agar (Eiken Chemical Tokyo) 0.8% (w/v) for *P. aeruginosa* PA14, in the presence and absence of compounds under study with 10 μM and 30 μM, respectively. Sterile tips were used to gently inoculate a single colony onto the surface of media plates with minimal pressure. DMSO and untreated plates were made as controls for the experiment. Plates were incubated overnight (37°C for *P. aeruginosa* PA14 / 30ºC for *B. atrophaeu*) and motility were observed and recorded the following day (Adapted from FLYNN; REEN; O'GARA, 2019).

Pyocyanin Assay:

Overnight cultures of *P. aeruginosa* PA14 were adjusted to an $OD_{600\ nm}$ of 0.05 in TSB, addition of compounds (30 μM) and incubated at 37°C with shaking at 180 rpm. After overnight (+/- 18h), cultures with 6 ml were centrifuged at 5000 rpm for 15 min to obtain a cell-free supernatant. In a new tube containing only the cell-free supernatant was added chloroform (3 ml), vortexed and centrifuged at 5000 rpm for 5 min. The bottom blue phase was transferred using a pipette to another tube avoiding the mixing of the phases and was added 2 ml of 0.2 M hydrochloric acid, vortexed, and centrifuged at 5000 rpm for 5 min. After the centrifugation, the absorbance of the top pink phase was read at $OD_{520\ nm}$ and corrected the concentration according to the formula: $(OD_{520\ nm} \times 17.072) \times 1.5$ (dilution factor of the acidic phase) (FLYNN; REEN; O'GARA, 2019).

Statistical Analyses:

Data presentation was performed using GraphPad Prism 8. The results were statistically analysed with one-way ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 8). Differences between tested compounds were considered significant at $P<0.05$ significance level.

RESULTS

Among the four types of known quorum sensing mechanisms in *P. aeruginosa*, the *Pseudomonas* Quinolone Signalling system (PQS) is regulated by the transcription factor 'multiple virulence factor regulator' (MvfR), also referred to as PqsR in non-PA14 strains (SCHÜTZ; EMPTING, 2018). The autoinducers of this mechanism are 4-Hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (PQS), being the two primary alkyl-quinolones (AQ) signals associated with QS regulation (RAMOS et al., 2020). A feature of this system that emerged in recent years has been its role as a modulator of interspecies and inter-kingdom behaviour in bacteria and fungi (REEN et al., 2011). It follows that mimics of these signals might be able to have a similar control effect, both in *P. aeruginosa* and in competing organisms.

Knowing the importance of these AQs, a compound structurally similar to HHQ, PQS, and HQNO, with a benzofuroquinoline framework containing a seven-carbon side chain (compound 13), was evaluated for the activities of anti-biofilm, growth and anti-swarming in *P. aeruginosa* PA14. The analyses revealed that this compound resulted in significantly reduced biofilm production but did not affect swarming activity. Both activities require multicellular cooperation but are governed by distinct regulatory systems. To further investigate the structural features responsible for the activity, two more molecules were tested, one with a side chain reduced to one carbon (compound 01) and another with an absent side chain (compound 23). The molecule with an absent chain behaved in the opposite way to the AQ analogue, causing a reduction in swarming motility. However, it did not affect biofilm production. The reduced-chain compound, on the other hand, had a satisfactory inhibition of biofilm and swarming activity (Figure 1).

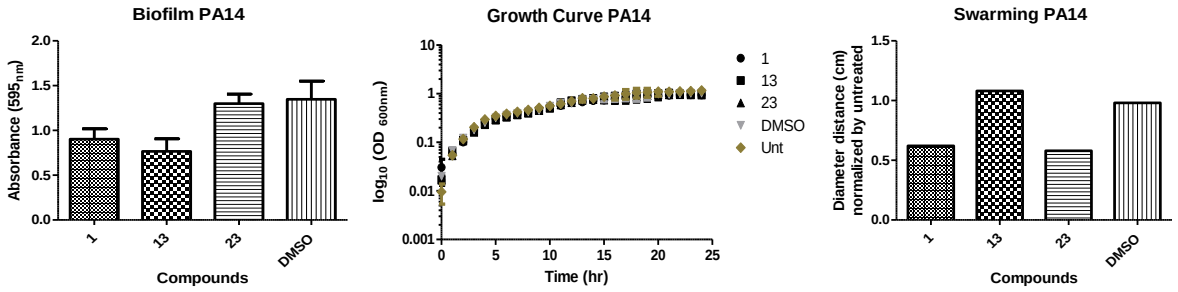

Figure 1. Activity analysis of three compounds against *P. aeruginosa* PA14 in three biological assays. In the first image, 96 well-plate biofilm assay with 03 biological replicates containing 03 sub replicates in each one (discounted the blank value and normalized relative to untreated), simultaneously with these assay was performed the growth curve of this bacteria represented in the second image. Third, swarming with just one biological replicate and normalized relative to untreated.

These positive results presented by the short side chain compound led us to question: what would happen with chemical modifications from this particular compound? Can the insertion of other substituents influence the response of *P. aeruginosa* to the benzofuroquinoline framework? For this purpose, 20 more benzofuroquinolines compounds with minor modifications were evaluated under the same activities mentioned before. Considering the 23 compounds, eight compounds showed a reduction in biofilm formation in preliminary tests using 96-well plates, compounds 01, 02, 03, 09, 13, 14, 15, and 21 (Figure 2A). Considering the mean values from the pilot tests the only compounds with values higher than 1.0 were the compounds 03 and 21 which were then excluded from further analysis.

Six compounds were selected from those that had been active against the *P. aeruginosa* PA14 biofilm, and these were validated using 24-well plates for anti-biofilm assays (Figure 2B). In parallel was tested the same compounds for their effects on pyocyanin production, a toxin linked to AQ signalling. In the validation test, five of the six compounds selected previously showed a reduction in biofilm, compounds 01, 02, 09, 14, and 15. In pyocyanin (Figure 2C), four compounds were identified with suppressive effects, compounds 01, 02, 13 and, 15. In summary, the compounds 09 and 14 presented the best reduction in the biofilm validation assay, with almost 50% of reduction, but they were also the least effective in the pyocyanin assay. Compound 13 presented lower absorbances in pyocyanin extraction, but unexpectedly, did not show reduction in biofilm as in the 96-well tests. This may reflect the different environments of the 96-well and 24-well plates from the perspective of microanaerobicity. Compounds 01, 02, and 15 although not the most potent inhibitors of the phenotypes tested, showed positive responses in both tests, biofilm and pyocyanin. It was noticeable that compound 01 shows good results for biofilm suppression, swarming motility and did not interfere with growth as shown previously and marks another point in the pyocyanin test with the biggest reduction, almost 50% compared with control (DMSO).

At this point, it is noted that some compounds had the ability to interfere with QS-related behaviours, and even more importantly without interfering with *P. aeruginosa* PA14 (data not shown), and it is this interference that we seek to understand.

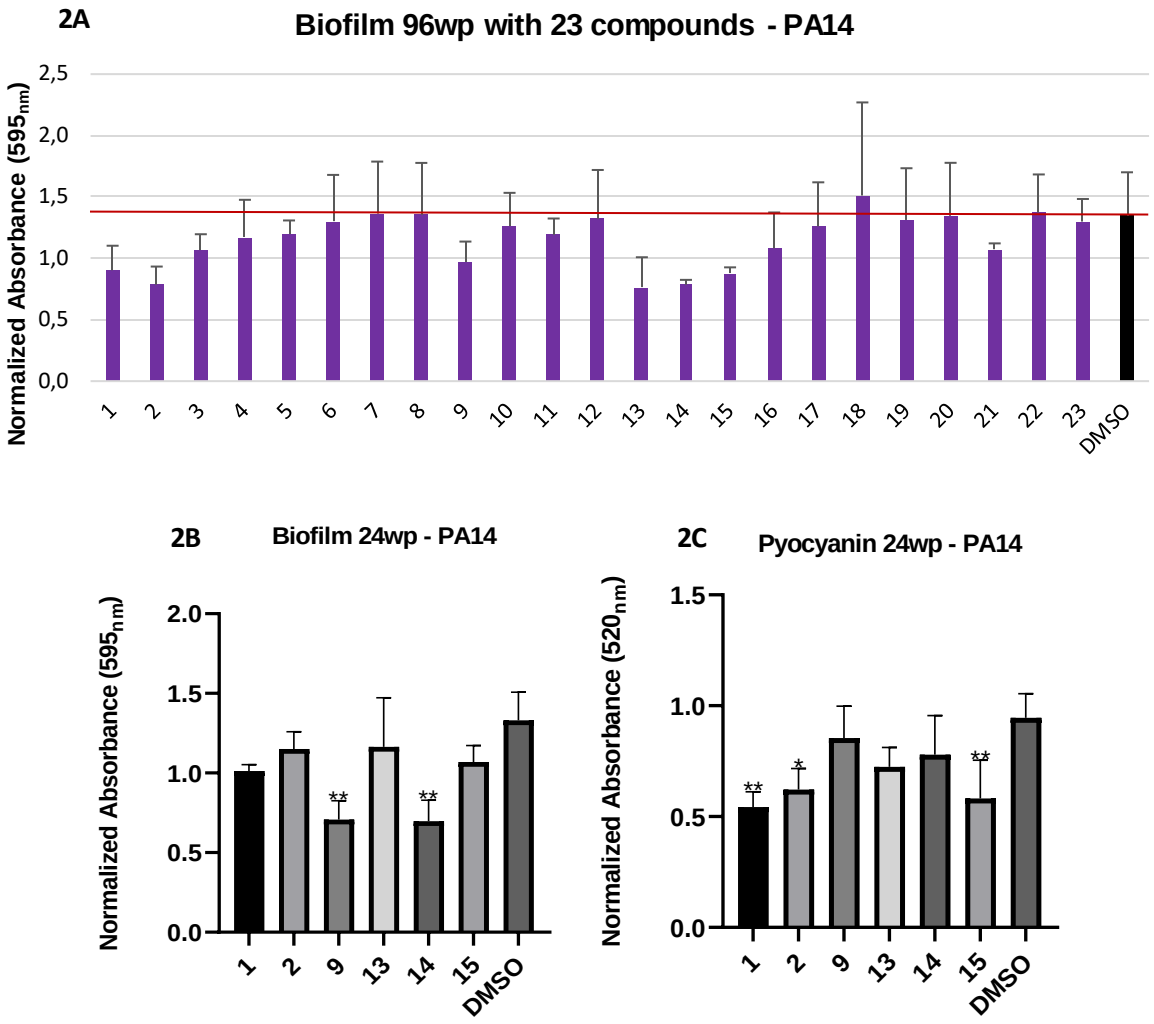

Figure 2. Biofilm screening against *P. aeruginosa* PA14, biofilm assay and pyocyanin extraction with lead compounds. (2A) 96-well plates biofilm assay with 23 compounds, considering 03 biological replicates with 03 sub replicates, discounted blank and normalized relative to untreated. The red line represents the limit of the mean effect of the control (DMSO). (2B) 24-well plates biofilm assay, considering 03 biological replicates (04 sub replicates), discounted blank and normalized relative to untreated. (2C) Measurement of absorbance of pyocyanin extraction with 03 biological replicates, corrected using the formula: *17.072*1.5, discounted blank and normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated bacteria to the control untreated ($*p \leq 0.05$, $**p \leq 0.005$).

Interspecies Interaction: Behavioural Modulation in Gram-positive Bacteria

Communication between bacteria has opened many doors to explain collective behaviours of the same species. However, it is known that this perception by signalling molecules occurs at interspecies as well. As previously mentioned, studies indicate that different species are not only recognized by other species, but their native signal molecules can also interfere with competing microbes by attenuating or accentuating collective behaviours, as is the case of the interaction between *P. aeruginosa* and *S. aureus* reported by co-existing in the lungs of patients with cystic fibrosis (IBBERSON; WHITELEY, 2020).

On the one hand, *P. aeruginosa* signal molecules such as PQS can attenuate swarming and biofilm formation in *S. aureus*, on the other hand, gram-positive communities like *S. aureus* accentuate virulence (FRYDENLUND MICHELSEN et al., 2016). That is, the existence of *S. aureus* in a co-infection is advantageous for *P. aeruginosa*. That is why investigating compounds that attenuate microorganisms such as *S. aureus* are also important for elucidating interspecies communication, as well as contributing to new treatment approaches against infections of this genus.

Therefore, the same compounds were tested with two strains of *S. aureus*. One of them, NCDO949 is a model strain routinely used in *S. aureus* assays, and the second, a clinical strain of *S. aureus* from a patient with cystic fibrosis to see if compounds similar to AQ would interfere in both or not, and whether there would be active compounds with specific action to strains of *S. aureus* or if the active compounds would be the same compounds presented in *P. aeruginosa* PA14.

The 23 compounds were tested for their effect on biofilm formation in 96-well plates. and the data revealed that none of the compounds affected biofilm formation in the model strain *S. aureus* NCDO949 (Figure 3A). However, in the case of the clinical *S. aureus* CUH strain, considering the standard deviations, there were two active compounds for the reduction of biofilm, compounds 04 and 14. Compound 04 showed approximately a 50% reduction and compound 14 showed a reduction greater than 80% (Figure 3B). In addition to compound 14 causing the largest reduction of biofilm with absorbencies close to 0.1, it is worth noting that this compound also showed anti-biofilm in *P. aeruginosa* PA14 (Figure 1).

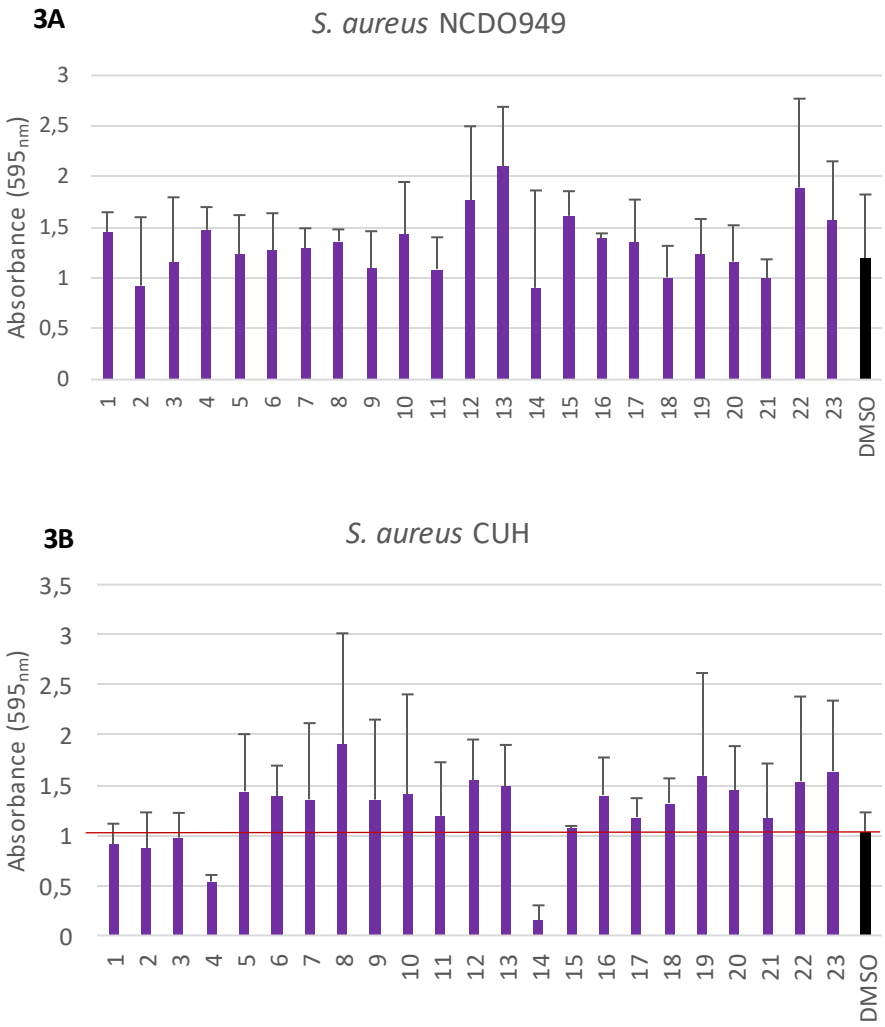

Figure 3. Biofilm screening against *S. aureus* strains. 96-well plates biofilm assay with strains a model, *S. aureus* NCDO949 (3A), and clinical, *S. aureus* CUH (3B), with 23 compounds, considering 03 biological replicates with 03 sub replicates, discounted blank and normalized relative to untreated. The red line represents the limit of the mean effect of the control (DMSO).

Parallel to the anti-biofilm activity tests, growth curve tests were performed with the addition of the 23 compounds to check if exists some interference in the growth curve. According to the averages for each strain, it is suggested that the compounds do not have major interferences in the growth curves in both strains, model and clinical, except compound 14 for the clinical strain that between 03 and 18 hours showed a lower growth rate when compared to untreated, but after this got nearer with the other rates.

Among the compounds tested, it is possible to visually identify groups among them, in such a way that compounds 01,13, 20, 23 are grouped by adding hydrocarbons; 02, 04, 15, 21 are grouped by adding methoxy group; 03, 05, 11, 12, 17, 18, 19, 22 by addition or replacement based on fluorine; 06, 07.16 by adding chlorine; 08, 10, 14, addition or replacement of nitrogen;

09 by adding naphthyl. The compounds that showed anti-biofilm results and were selected for further study were 04 and 14. Furthermore, the naphthyl compound is common in antimicrobial substances (VARGAS et al., 2008, 2009; SHAFIEE et al., 2009; LEITE et al., 2019). Therefore, due to its activity against biofilm formation in *P. aeruginosa* and due to the absence of other compounds with naphthyl groups among the compound library, it was decided to continue with compound 09 for the validation tests as well.

Due to the fact that there are effective compounds in the clinical lineage of *S. aureus*, it was decided to test the same compounds in other species of the same genus, mostly clinical strains, to see if lead compounds exhibited anti-biofilm activity in a broad sense within the genus.

Growth Curve and Anti-biofilm Validation Test against *Staphylococcus* spp.

After the screening phase, compounds 04, 09 and 14 were evaluated in 24-well plates against a panel of *Staphylococcus* spp. As in the previous phase, the biofilm tests were carried out in parallel to the growth curve tests for each strain.

In the case of the *S. aureus* NCDO949 strain (Figure 4), it is noteworthy that for the model strain, unexpectedly 09 has anti-biofilm activity as well as influencing the growth curve, while 04 on the other hand, promoted the formation of biofilm. For the clinical *S. aureus* CUH lineage, the three compounds presented a significant reduction of biofilm, and only 14, as seen in the screening phase, presented some interference in the growth curve. In the *S. aureus* Morph T strain, a significant reduction in biofilm formation can be seen only with 14 and for *S. aureus* LB Morph D strain, the enhancement of biofilm formation was noted for all three compounds tested. In fact, for this specimen, compound 09 presented a five-fold increase in biofilm compared with the control. No interference was observed in the growth curve of these latter two strains when compared to the carrier control.

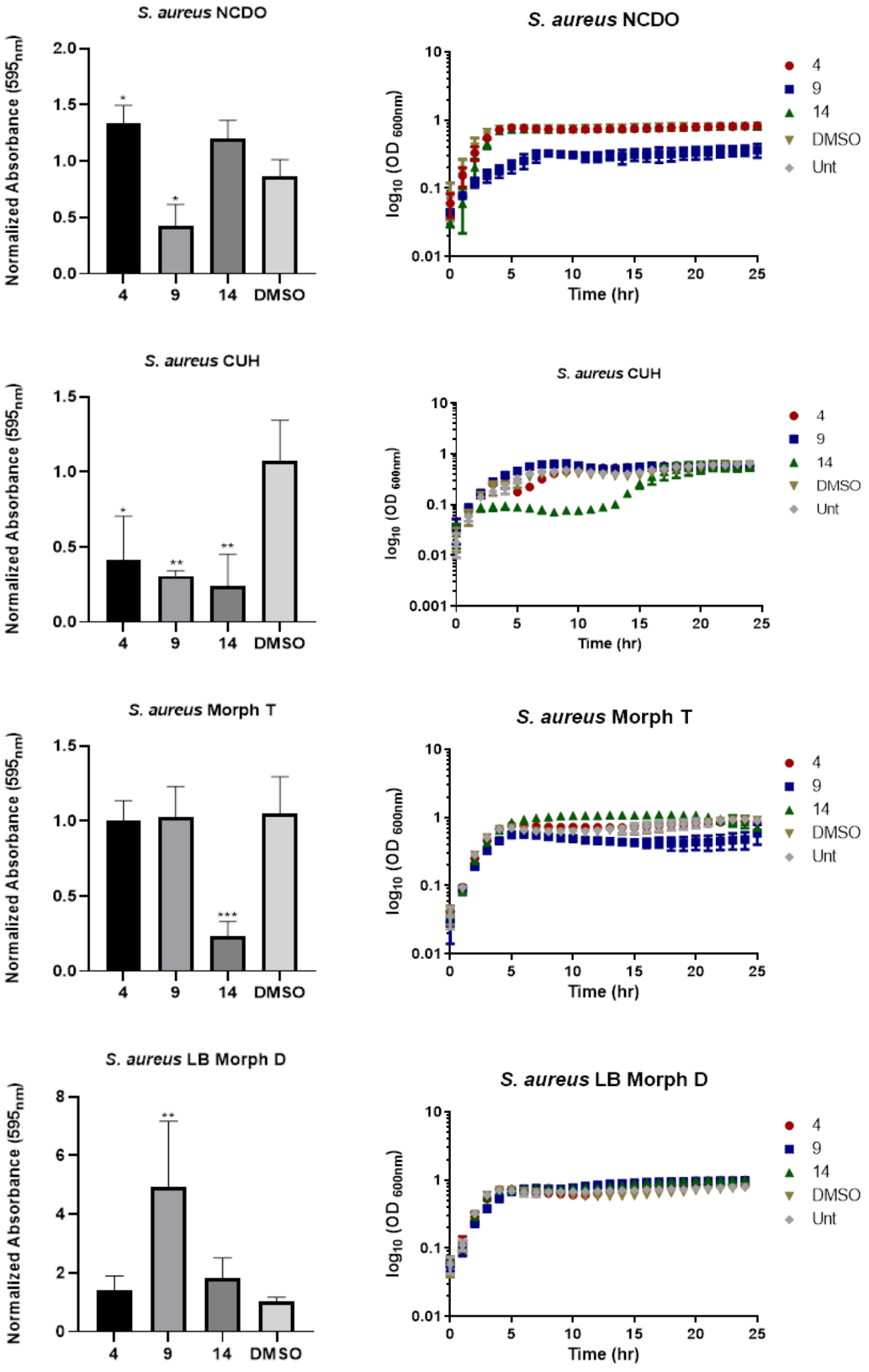

Figure 4. Biofilm assay with 24-well plates and respective growth curves with lead compounds against *S. aureus* strains. Considering at least 03 biological replicates (03 sub replicates) and discounted blank. Biofilm growth rates were normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated bacteria to the control untreated ($*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.001$).

Staphylococcus equorum strains (Figure 5), none of the three compounds tested proved to interfere with the formation of biofilm or on the respective growth curve.

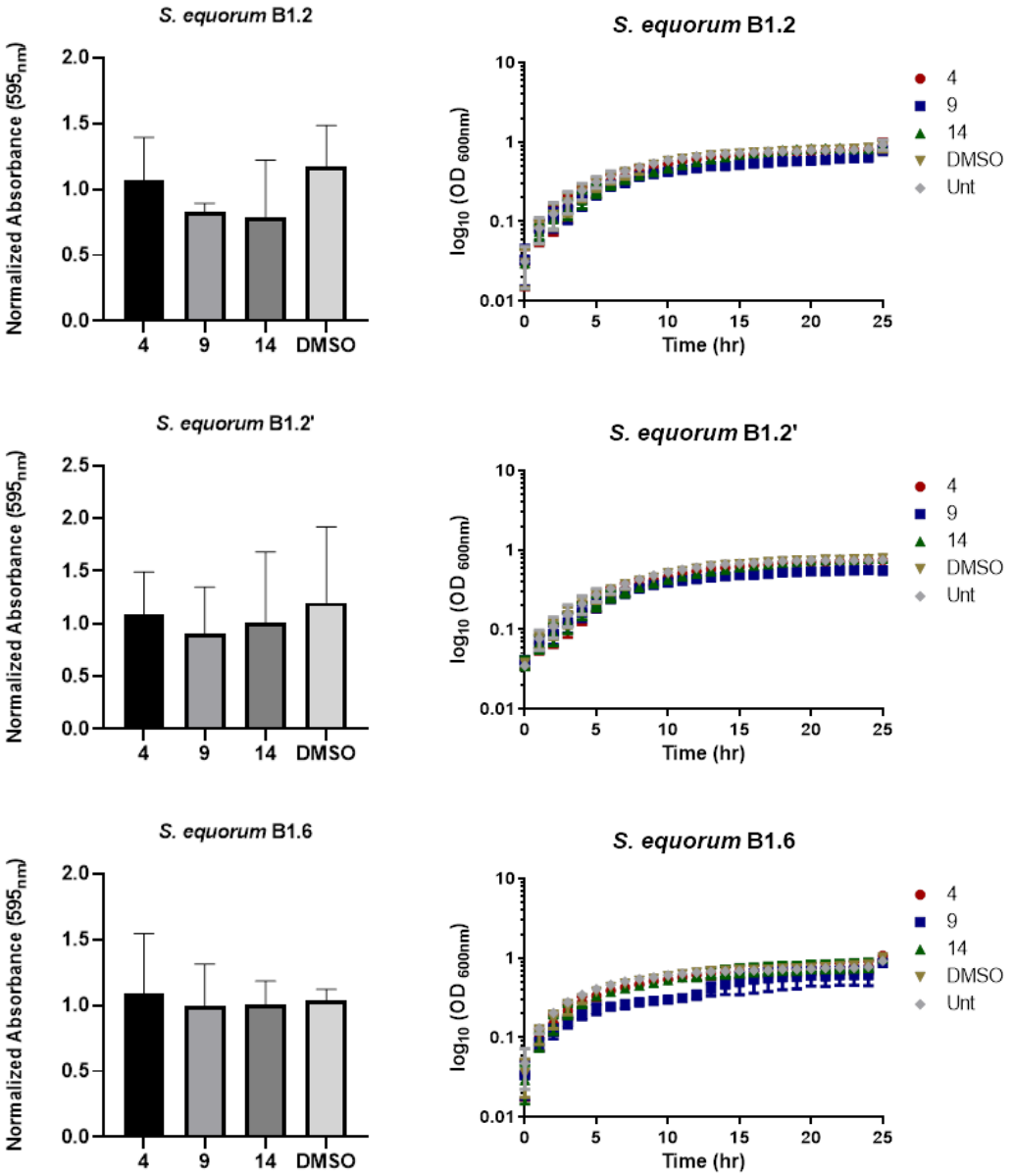

Figure 5. Biofilm assay with 24-well plates and respective growth curve with lead compounds against *S. equorum* strains. Considering at least 03 biological replicates (03 sub replicates), discounted blank. Biofilm rates were normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test.

For *Staphylococcus epidermidis* (Figure 6), three strains were tested and presented distinct data between themselves. Compound 09 showed to be the only active against the biofilm in the *S. epidermidis* B1.9, 14 increased the biofilm formation in *S. epidermidis* MS Morph D and 04 showed no activity. In the growth curve, none of the compounds showed significant interference.

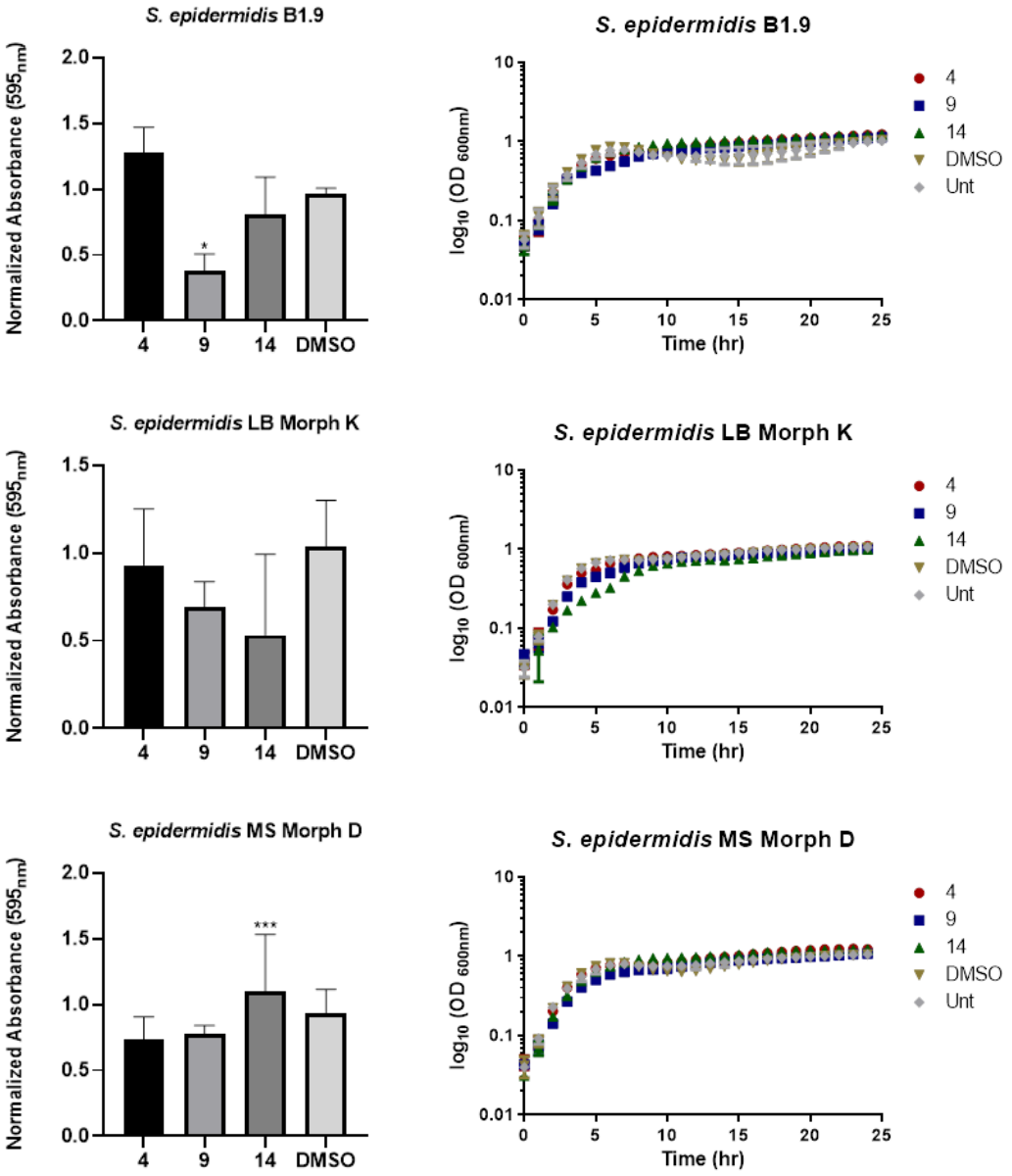

Figure 6. Biofilm assay with 24-well plates and respective growth curve with lead compounds against *S. epidermidis* strains. Considering at least 03 biological replicates (03 sub replicates), discounted blank. Biofilm rates were normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated bacteria to the control untreated ($*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.001$).

In *Staphylococcus haemolyticus* (Figure 7) the activity of compounds was more consistent. Compound 14 showed anti-biofilm activity significantly in both strains, being more evident in *S. haemolyticus* M-Staph, 04 presented a significant decrease only in the biofilm of the same strain and 09 showed no differences. In view of the growth curves, it was demonstrated that compound 14 affected both strains, with marked prolongation of lag phase.

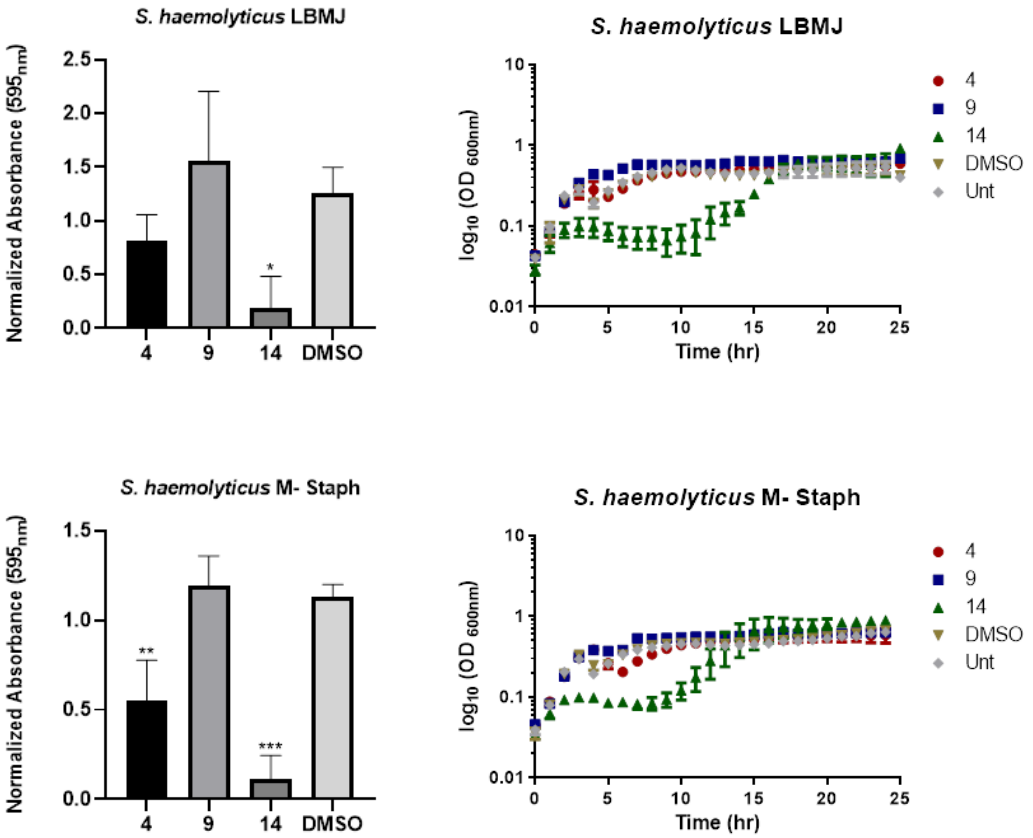

Figure 7. Biofilm assay with 24-well plates and respective growth curve with lead compounds against *S. haemolyticus* strains. Considering at least 03 biological replicates (03 sub replicates), discounted blank. Biofilm rates were normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated bacteria to the control untreated ($*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.001$).

Staphylococcus hominis was probably the specimen that showed the greatest reduction in face of the anti-biofilm activity with compound 14. Two compounds showed a marked reduction when compared to the control data (Figure 8). In microplates, the absence of biofilm formation and low turbidity in treatments with compound 14 were often perceived visually, suggesting a growth suppressive action and these indications can also be observed in the growth curve.

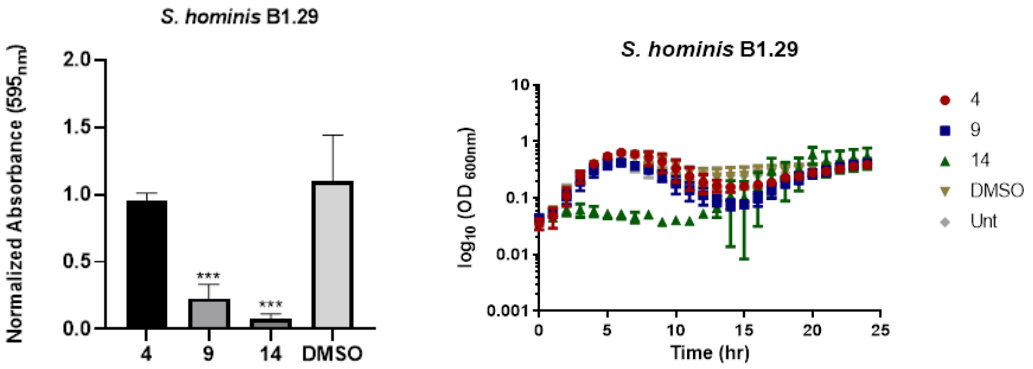

Figure 8. Biofilm assay with 24-well plates and respective growth curve with lead compounds against *S. hominis*. Considering at least 03 biological replicates (03 sub replicates), discounted blank. Biofilm rates were normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated bacteria to the control untreated ($*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.001$).

Summarizing the data presented in this phase of the study, the compound that most interfered in the formation of biofilm from thirteen strains tested was 14, anti-biofilm activity in five strains (*S. aureus* Morph T; both strains of *S. haemolyticus*; *S. hominis* and *S. aureus* CUH) and enhancers in a strain (*S. epidermidis* MSMD). However, compound 14 had the biggest impact on bacterial growth of tested strains, with lag phase prolongation for four strains (both strains of *S. haemolyticus*; *S. hominis* and *S. aureus* CUH). Compound 09 interfered with biofilm formation in five strains, four reductions (*S. aureus* NCDO949*; S. epidermidis* B1.9, *S. hominis* and *S. aureus* CUH) and an increase in biofilm formation (*S. aureus* Morph D). It showed a reduction in the growth curve of only *S. aureus* NCDO949. Compound 04 did not affect the growth of any of the strains tested and its activity was limited to increased biofilm in *S. aureus* NCDO949 and a reduction in *S. aureus* CUH.

From the perspective of species, it can be considered through these data that in *S. aureus* and *S. epidermidis* the compounds did not present same response in relation to the different strains. A good correlation between strains of the same species vs compounds was noted in *S. haemolyticus* in which compound 14 significantly reduced biofilm in both strains and 04,

although it only reached significance for one of the strains. Another species that proved to be consistent in the results was *S. equorum* with no one active compound for the three strains tested. The fact that phenotypic heterogeneity was observed was not surprising in light of the recent studies describing the extent to which genotypic heterogeneity exists in complex ecosystems such as the lungs of people with respiratory disease (LANGE et al., 2020).

Activity of Benzofuroquinoline Compounds against *B. atrophaeus*

The activity of lead compounds against *S. aureus* led us to investigate whether other species of organism found in common niches with *P. aeruginosa* and the AQ signalling framework were influenced for biofilm formation and motility. Screening of the 23 compounds against *B. atropheaus* biofilm formation, followed the same experimental pattern performed in 96-well microplates. Compounds 09, 20 and 23 exhibited anti-biofilm activity against *B. atropheaus*. It is worth mentioning that comparing the different gram-positive species 20 and 23 were active only for *B. atrophaeus* and compound 14 only for *S. aureus*.

Growth of *B. atropheaus* was attenuated by 04 and 09. Compound 04 showed a lower biomass than untreated cells over an extended period of time into stationary phase, but at the end of the 24 hours it shows levels close to the growth points of the other treatments in the curve. Compound 09 however had a clear impact on the growth of *B. atropheaus* and therefore its activity is growth limiting rather than anti-biofilm. This was also noticed in *S. aureus* NCDO949.

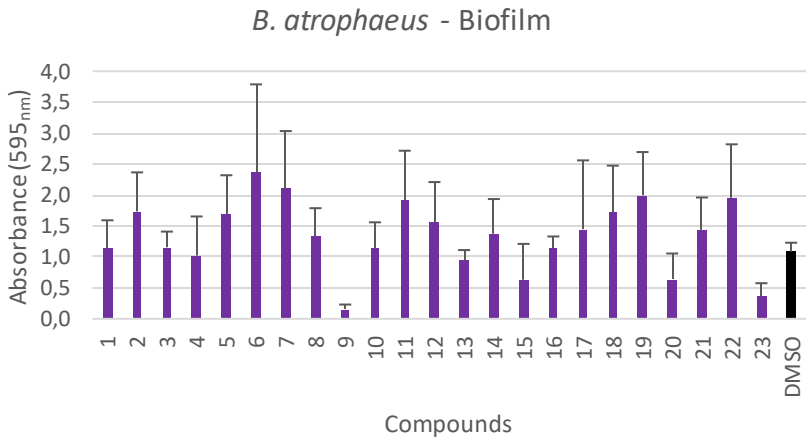

Figure 9. Biofilm screening against *B. atrophaeus*. 96-well plates biofilm assay with 23 compounds against *B. atrophaeus*, considering 03 biological replicates with 03 sub replicates, discounted blank and normalized relative to untreated.

Swarming Activity against *B. atrophaeus* and *P. aeruginosa* PA14

To check for swarming inhibition, the 23 compounds were screened only once in soft agar plates for *B. atrophaeus* (10 μM) and *P. aeruginosa* PA14 (30 μM). Additional replicates of the swarming motility assay were then performed to test compounds that demonstrated to be effective in the screening test (Figure 10). The data were obtained by measuring the diameter of the colony and recorded by photography to capture the tendril formations of the multicellular behaviour (Figure 11).

In the case of *B. atrophaeus* it was found that compounds 01, 04, 09, 15 and 20 had smaller diameters than the control (DMSO), with repression of swarming motility being most potent in plates treated with compounds 04 and 20. Compounds 04, 09, 15 and 20 reduced swarming significantly. However, it should be mentioned that compounds 04 and 09 had a negative influence on the growth curve of this bacteria, suggesting that the repression of swarming may simply be a result of antibacterial activity. *P. aeruginosa* PA14 screening revealed six possible interfering compounds in swarming, 01, 02, 14, 15, 18 and 20. However, according to statistical analysis, only 14 and 15 were effectively anti-swarming. In addition, a specific compound (18) showed unusual swarming behaviour, in this plate it was noticed that the swarming was completely irregular as if it were a blur of a drop instead of a growing tendril. This suggests that compound 18 disrupts the direction and control of microbial motility behaviour but does not repress it.

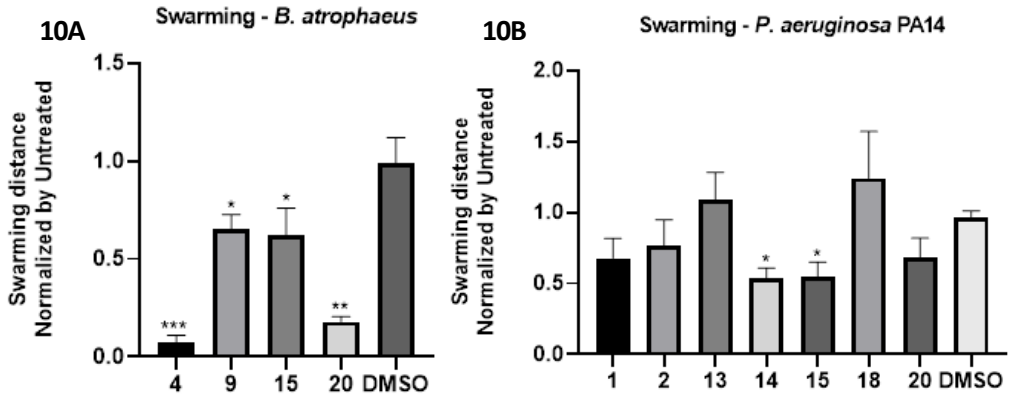

Figure 100. Swarming interference with lead compounds. **(10A)** *B. atrophaeus* results with 02 biological replicates. **(10B)** *P. aeruginosa* PA14 with 03 biological replicates. Data normalized relative to untreated. Significant differences were determined by one-way ANOVA with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated bacteria to the control untreated ($*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.001$).

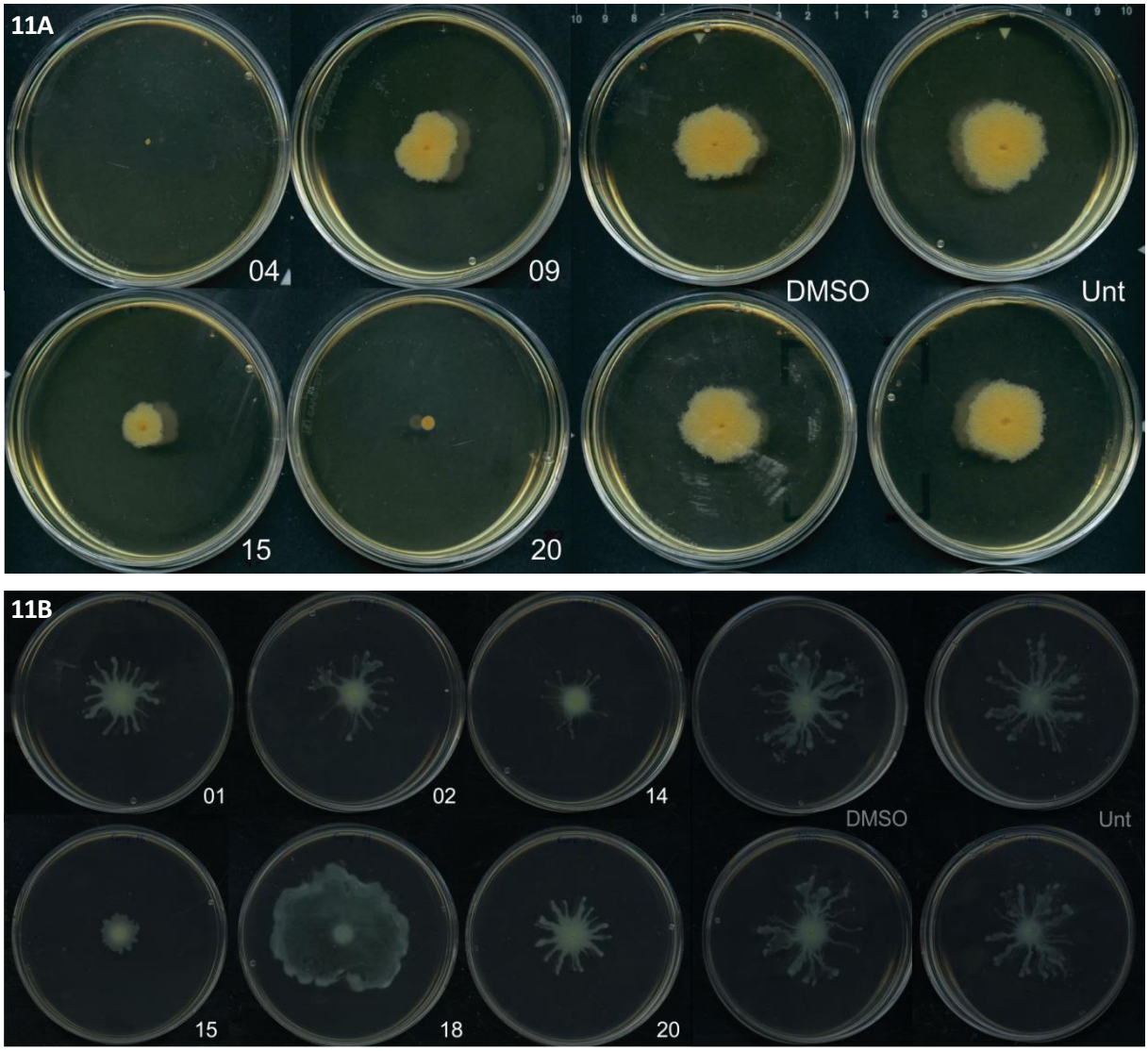

Figure 111. Photographic record of the swarming assay in soft agar plates. At the top, *B. atrophaeus* (11A) and at the bottom, *P. aeruginosa* PA14 (11B). The numbers on the figure represent the tested compounds with the most effect on swarming ability. The two columns on the right in both figures represent the controls (DMSO) and untreated (Unt) plates.

Most Effective Compounds

Biological tests were developed and funneled from screening to validation tests, to search correlations of compounds versus QS-dependent activities, and versus bacterial species (Table 1). From 23 compounds with structures based on AQ, it was noted that 09 and 14 interfered in the formation of biofilm independent of the bacterial genus or gram designation, being significant in some strains of *Staphylococcus* spp. and *P. aeruginosa* PA14. Compound 09 was the only one to cause reductions in biofilm among the three tested genera, besides presenting anti-swarming activity and interfering in the growth curve of *B. atrophaeus* and some *Staphylococcus* spp. strains. Compound 14, in addition to biofilm reductions, inhibited

swarming in *P. aeruginosa* PA14 and interfered with the growth curve in *Staphylococcus* spp. strains.

In the case of swarming activity, compound 15 inhibited both bacteria grams. Interestingly, this compound also significantly inhibited the production of pyocyanin. In addition to compound 09, 04 and 20, they inhibited the swarming specifically of *B. atrophaeus*. Preliminary studies also indicated that compound 20 inhibited the biofilm of this *B. atropheaus*. Due to the fact that compound 04 was able to interfere with biofilm formation in *Staphylococcus* spp. strains and also in the swarming of *B. atrophaeus*, a correlation of the response by gram-positives to this compound is suggested.

Table 1. Summary of biological assays versus 23 tested compounds. Biofilm and Growth Interference: Qualitative data shown in the screening phase and only significant quantitative data in validation. Pyocyanin interference and anti-swarming activity: only significant quantitative data. Symbols: (gray cell) Tested Compounds; (-) Reduction of biofilm; (+) Increase of biofilm; (- / -) Reduction of biofilm and growth curve.

Biofilm and Growth Interference																								
	Microbe / Compounds	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Screening	*P. aeruginosa* PA14	-	-	-		-				-		-		-	-	-						-		
	S. aureus NCDO949																							
	S. aureus CUH				-										-									
	B. atrophaeus									-											-			-
Validation	*S. aureus* NCDO949				+					-/-														
	S. aureus CUH				-					-					-/-									
	S. aureus LB Morph D									+														
	S. aureus Morph T														-									
	S. equorum B1.6																							
	S. equorum B1.2																							
	S. equorum B1.2'																							
	S. epidermidis B1.9									-														
	S. epidermidis LB Morph K																							
	S. epidermidis MS Morph D														+									
	S. haemolyticus LB Morph J														-/-									
	S. haemolyticus M- Staph				-										-/-									
	S. hominis B1.29									-					-/-									
	P. aeruginosa PA14									-					-									
Pyocyanin Interference																								
	Microbe / Compounds	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	P. aeruginosa PA14	-	-													-								
Anti-swarming Activity																								
	Microbe / Compounds	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
	P. aeruginosa PA14														-	-								
	B. atrophaeus				-					-						-					-			

DISCUSSION

Polymicrobial infections, especially chronic infections such as the co-infection of *S. aureus* and *P. aeruginosa* in cystic fibrosis, represent a significant burden on health systems and are often recalcitrant to antibiotic treatment (IBBERSON; WHITELEY, 2020). Alkyl-quinolones, such as HHQ and PQS are secondary metabolites that play a fundamental role in virulence and cell-communication through quorum sensing systems of *P. aeruginosa*. Therefore, it is believed that compounds similar to these metabolites can interrupt QS-dependent behaviours in bacteria, such as biofilm production, pyocyanin and swarming. Investigating ways of regulating these interactions and especially in combating biofilm is key to understanding the impacts of these interactions on human diseases and on the treatment of infected individuals.

A series of analogue compounds with the core framework of the quinolone ring structure were investigated for their potential as novel anti-infective pathoblockers (SCHÜTZ; EMPTING, 2018). For example, biofilm inhibitors in *P. aeruginosa* with phthalazine – quinolines (ZAHEER et al., 2016) and aminoquinolines (ALEKSIĆ et al., 2017) have exhibited good efficacy. Several other reports have described potent agents against staphylococcal nosocomial infectious bacteria, such as through small quinoline molecules against *S. aureus* dispersion and *S. epidermidis* biofilm formation (ABOUELHASSAN et al., 2014), and the action of halogenated quinolines against *S. epidermidis* (BASAK et al., 2018).

Due to the marked presence of a long alkyl chain in HHQ and PQS molecules that appear to be fundamental for this microbial modulation (REEN et al., 2011), AQs have gained an even greater focus in the research and development pipeline of antimicrobial drugs. The alkyl-quinolones quinolone class of signal molecule has shown itself to be an effective modulator of microbial behaviour, in both bacteria and fungi (REEN et al., 2011). It is known that these signalling molecules have already been confirmed to interfere with other bacteria such as *S. aureus* and *B. atrophaeus* (REEN et al., 2011; REEN et al., 2015). These relationships were most likely acquired through the coexistence of these microbes in clinical and environmental niches such as the plant ecosystem (REEN et al., 2016), exemplifying models of interspecies communication. In addition, it is known that these similar molecules can modulate behaviour in other spheres of kingdoms, being better known among fungi (REEN et al., 2011, 2016; ZAHEER et al., 2016; ZUO et al., 2016; KHAMKHENSHORNGPHANUCH et al., 2020).

More recently, studies such as Khamkhenshorngphanuch et al. (2020), verified 4-hydroxy-2-quinolinone analogues for minimum inhibitory concentrations (MICs) in *S. aureus*,

E. coli and half maximum inhibitory concentration (IC_{50}) for *Aspergillus flavus*. The structure-activity relationship revealed that the type of substituent has an impact on antimicrobial activities as well as the length of the alkyl chain, the nonyl side chain exhibited better results than tridecyl side chain, suggesting that there is an optimal action size for the alkyl chain (KHAMKHENSHORNGPHANUCH et al., 2020). As well as the work carried out by Khamkhenshorngphanuch et al. (2020), was demonstrated by Ritzmann et al. (2019) that brominated AQ analogues produce derivatives with modulated bioactivity. According to the authors AQs brominated in position C-3 have increased antibiotic activity against *S. aureus* and two strains of *Bacillus* sp. (RITZMANN et al., 2019).

Knowing the importance of the alkyl chain and the signalling potential of the HHQ and PQS molecules, it was decided to test a homologous analogue with heptyl side chain, compound 13, in the production of biofilm in *P. aeruginosa*. Good results have been achieved in the present work. Then, with the intuit of evaluating the action of this compound in another QS-dependent behaviour, that requires multicellular cooperation too even being from different regulatory systems, the potential for interference in swarming tests was evaluated aiming for a multi-active compound. Although, what would happen if we tested a compound with an inverse characteristic? That is, a short (compound 01) or absent (compound 23) alkyl chain. The two biological assays were repeated, and it was found that the short chain compound was the only one of the three to modulate both tested phenotypes of interest. As it has anti-biofilm and anti-swarming effects, in addition to not interfering with bacterial growth, it is suggested with these results that short-chain AQ may be promising for the antagonism of *P. aeruginosa*. Accordingly, studies such as by Espinosa-Valdés et al. (2019) show short alkyl chains also interfere in the reduction of *P. aeruginosa* biofilm.

However, considering that the insertion or substitution of different groups of radicals in AQ can alter the activity potential, 20 different compounds were evaluated using the same biological tests. Among these, six compounds stood out in the different evaluated phenotypes. However, among these compounds, 04, 09, and 14 showed variations with respect to interference in the growth curve. Compounds 04 and 09 showed bactericidal activity exclusively in *B. atrophaeus*, the latter compound also interfered negatively in *S. aureus* NCDO949. It must be noted that bactericidal effects cancel the perception of anti-biofilm and anti-swarming activity. Although, an extension of the lag phase was noted in compound 14 for four strains of *Staphylococcus* sp. this does not exclude it in relation to anti-infective analyses as they have no bactericidal effect.

Among the library compounds, six compounds were identified with anti-biofilm properties at 30 μM, five for *P. aeruginosa* and three for *S.aureus*, with compound 09 and 14 being active in both. Some of these compounds were also effective in decreasing the production of the pyocyanin virulence factor in *P. aeruginosa*, compounds 01, 02, and 15. Another social behaviour that was analysed and significantly interfered was swarming motility by compounds 14 in *P. aeruginosa*, 20 in *B. atrophaeus* and 15 in both.

Among the compounds that were selected by the preliminary tests (01, 02, 04, 09, 13, 14 and 15) three have radical groups insertion in other positions of the molecule outside the quinolone core rings, 02, 09 and 14. It is important to note that the last two showed activity anti-biofilm and anti-swarming in tested gram-positive and gram-negative bacteria. The remaining compounds showed changes in positions C-2 (01 and 13), C-6 (15) and C-7 (04). Several other studies show that a wide range of structural modifications in AQ analogues are capable of providing or maintaining anti-biofilm, anti-swarming and anti-pyocyanin production activities (REEN et al., 2011, 2015, 2016; SHANAHAN et al., 2017; ESPINOSA-VALDÉS et al., 2019; RAMOS et al., 2020). It is known that the C-3 position provides great stability and activity efficiency in PQS analogues (SHANAHAN et al., 2017) but in relation to this, no compound in the present work has substitution in this position, all of them has the same radical group, which is ideal according to Hartmann group to prevent antagonistic inactivation of the molecule by biological bacterial hydroxylation (LU et al., 2014).

Derivatization of the quinolone structure at positions C-6 and C-8 revealed that the anthranilate ring is particularly important in anti-biofilm activity (REEN et al., 2016). According to the work carried out by Ramos et al. (2020), the incorporation of decorations in the anthranilate ring at positions C-3, C-5, C-6, C-7 and C-8 can cause inactivity unless there is an extension of the alkyl chain from 7 to 9 carbons. According to the studies by Espinosa-Valdés et al. (2019), AQ analogues with an alkyl chain of more than 12 carbon atoms were the most active against biofilm formation in *P. aeruginosa* and *S. aureus*. However, with the exception of compound 13, which has an extensive side chain, we have seen in the present work that short alkyl chains with a carbon (methyl) or methoxy group, such as 01, 04 and 15, have activity against QS-dependent behaviours. Although it does not present the same chemical structures, it was analysed in simulations of molecular dynamics that a compound with a shorter alkyl chain has better protein binding to LasR protein than even the agonist signal molecule, which may be an explanation for the good results presented in this work (ESPINOSA-VALDÉS et al., 2019). However, the question still remains, how these molecules are operating in different

species as *Staphylococcus* and *Bacillus*. Since the gram-positive QS system is based on peptides and the perception of these molecules must be different due to the absence of LasR.

According to studies by Hodgkinson et al. (2010), who tested PQS analogues with an increase and decrease in the side chain length; insertion of fluorine and chlorine in different positions of the anthranilate ring, resulted in different biological responses, such as the production of pyoverdine, where both compounds had different activities. The structural analysis of the compounds versus the other tested phenotypes led the authors to indicate that almost all parts of the PQS molecule are required for full bioactive function in some regard (HODGKINSON et al., 2010). In view of the structural changes made to the compounds tested in the present work, it can be seen that chlorine and fluorine insertions were not very efficient in view of the tested antimicrobial activities. Exceptions to this were compound 03, which has fluorine substituent and interfered with biofilm formation of *P. aeruginosa*, and compound 18 which presented a destabilization in the formation and direction of the swarming movement, of the same bacteria, although this was not further explored. Compounds that contained the insertion of the methyl / methoxy group showed good results for the three activities tested, most notably in the reduction of pyocyanin, in which the three compounds that showed significant reduction contained these mentioned radicals. This suggests a correlation between the presence of these chemical groups and the suppression of pyocyanin production.

Studies such as that by Kamal et al. (2017) report that among a series of modified compounds based on HHQ with the introduction of nitro group or CF_3 at the C-6 position, providing antagonistic characteristics in the transcription regulatory protein (PqsR) of the operon responsible for the production of virulence factors (e.g. pyocyanin). Ramos et al. (2020) report that more than ten compounds with such modifications (NO_2 insertion) did not have the same activity characteristics in this position. Although in our compounds the position C-6 does not present any insertion of nitro group, it was noticed that of three compounds that had insertion of this group one presented significant results (14). According to Espinosa-Valdés et al. (2019), analogues of AQ with insertion of amino groups, such as compounds 14, present a molecular fit posture in specific regions of the active site of PqsD through the heterocyclic nucleus and the amine group in the C-2 position and the amide group of C-3 position. Therefore, is this nitro insertion capable of providing the same antagonistic characteristics through the removal of electrons as mentioned by Kamal et al. (2017) or did this insertion provide another advantageous feature? This question remains open for further studies to be done.

Few existing studies on AQ analogues and their microbial interferences showed that there is no concise recipe or idea about structure versus activity. According to studies by

Hodgkinson et al. (2010), different PQS analogues were tested for three phenotypes related to this QS system and it was not possible to achieve a single efficient structural profile, since the structure-activity profile was different for each of the three tested phenotypes. The analysis of the chemical structure of the analogues, led the authors to say that almost all parts of the PQS molecule have some function (HODGKINSON et al., 2010). Therefore, even small chemical changes in these molecules can lead to completely different biological responses and perhaps these could be the explanation for behavioural differences between targeted microbes. As described in the interactions of *P. aeruginosa* and *S. aureus* with some competitive relationships, such as cell lysis and other cooperative relationships, such as resistance to antibiotics (LIMOLI; HOFFMAN, 2019).

Recent studies (Ritzmann et al. 2019; Khamkhenshorngphanuch et al. 2020), investigating AQ analogues for bactericidal and fungicidal activities, showed promise but we need to emphasize that these compounds would interfere in the microbiome or influence dysbiosis and could have negative effects on human health or biological ecosystems. According to Reen et al. (2016) deciphering microbial chemical messages to control pathogens is an innovative approach in the age of molecular-based therapy if it does not interrupt the growth of adjacent microbiomes. In relation to the tested compounds, the majority did not interfere with bacterial growth, which fulfilled the objective of finding non-lethal compounds, that interfered in QS-dependent behaviours. The only compound that reduced growth rates was compound 09 in *B. atrophaeus* and a strain of *S. aureus* (NCDO949). Compounds 04 and 14 showed interference in growth with lag phase extension in *B. atrophaeus* and *Staphylococcus* spp. respectively. At the end of the cycle (approximately 24 hours), the bacterial biomass achieved rates comparable to the control. No compound interfered with the growth of *P. aeruginosa* in this study.

However, the present work demonstrated that AQ analogues, such as benzofuroquinolines, seem to be promising for such activities as anti-biofilm, anti-swarming and anti-pyocyanin. Among the compounds tested, 09 and 14 showed significant results. However, from the anti-infective point of view, the most valuable compound was 14, as it effected main pathogen groups causing co-infection of lung diseases (*P. aeruginosa* and *S. aureus*) and did not have bactericidal activity or effects on non-pathogenic bacteria (*S. aureus* NCDO949 and *B. atrophaeus).*

Among the limitations of this study, it should be mentioned the variation in the act of compounds on the screening phase (96-well plates) and on validated tests (24-well plates). A plausible explanation for the loss of detection of the activity of the compounds is that the gas

diffusion in microplates is directly related to the working volume and area of the well (ARAIN et al., 2005), may show significant variations in the results for the same assay of the same reagent.

The AQ signalling system is known to directly control the virulent potential of *P. aeruginosa*, but these molecules can also be perceived and trigger different behaviours in addition to intra-species interactions (BISHT; BAISHYA; WAKEMAN, 2020; IBBERSON; WHITELEY, 2020). Co-infection between *P. aeruginosa* and *S. aureus* promotes changes in genetic regulation resulting in a conversion of essential genes to expendable genes in co-infection, as observed in a murine wound model (IBBERSON et al., 2017). Therefore, an interesting area for the continuation of this study would be the genomic analysis of our tested bacteria to search for correlations between microbes and the specific action of these compounds. Namely, identifying binding site in proteins related to QS systems, such as protein signal molecule synthase PqsD and the PqsR receptor, would be of great value to explain how compounds act. Additionally, permanence of antagonistic activity in mixed cultures, as described in this work, should be further investigated.

Another interesting area for future studies would be to analyse polymicrobial cultures *in vitro* and *in vivo* in other niches of the host, such as the lung, since AQ signals and analogues may influence inter-species and inter-kingdom relationships, such as interactions between *P. aeruginosa* and *A. fumigatus* that are also frequently associated with co-infections in CF. Studies focusing on differences between mono-infections versus co-infections, as well as their regulatory mechanisms can be valuable for the development of new mechanisms of action to control and prevent infections. It has recently been shown that PQS interacts with non-PqsR proteins in the cell, suggesting that virulence modulation may extend to other indirectly related systems or even unknown systems (HODGKINSON et al., 2016; BAKER et al., 2017). This type of investigation would be useful, because in addition to AQ presenting interspecies and inter-kingdom activity, there may be other unknown mechanisms or metabolic that increase the range of action in controlling virulence of other microorganisms and other co-colonizing pathogens.

Although *in vitro* analyses are necessary initially, it is important to emphasize the need for *in vivo* studies as well. Mainly due to the fact that *in vitro* assays may not behave in the same way as *in vivo* assays (LOPEZ-MEDINA et al., 2015; IBBERSON; WHITELEY, 2020) and a molecule with therapeutic potential may have its activity lost in the *in vivo* environment. This can also occur with microorganisms with deficient QS systems. Some strains of *P. aeruginosa* isolated from CF lungs adapted to the host may present as mutants in *lasR* (ZHAO

et al., 2018) and this factor can present varied results from antagonistic compounds. Therefore, it is important that investigations with both *in vitro* and *in vivo* models are used to study bacterial interactions, with an awareness that *in vitro* studies using model isolates may be forcing interactions that are absent in human infections (IBBERSON; WHITELEY, 2020). Finally, host derived factors, such as nutrient availability, immune system, and microbial surface adhesion, can dramatically alter the effects of each molecule (REEN et al., 2016). Studies on interactions and models of infections provide information on the molecular basis of these interactions and on understanding of microbial interactions during chronic human infection (IBBERSON; WHITELEY, 2020), but remain challenging due to their complexity. In summary, unravelling bacterial communication and its regulatory mechanisms are the first steps towards the discovery of new methods for disrupting virulent agents.

CONCLUSIONS

Bacterial cell communication is mediated by signal molecules, such as HHQ and PQS in *P. aeruginosa*, alkyl-quinolones that enhance competitive behaviours for the producing community, and which can promote or suppress social behaviours towards other organisms in a species specific manner.

AQ analogues such as benzofuroquinolones have mimetic characteristics, these signalling molecules can also modulate interspecies behaviours in a similar way to HHQ and PQS. The activity of these compounds was shown to be sensitive to structural modification, considering different substitution positions and different radical groups. Considering the antagonistic activities towards the formation of biofilm, initiation of swarming motility and the production of pyocyanin pigment, these compounds seem promising in the development of new anti-effective strategies. However, the molecular mechanism through which these compounds are recognised by responsive species and strains is still poorly understood, requiring further studies. Specific conclusions of this work were:

- Of the twenty-three screened compounds thirteen showed some type of interference between the three phenotypes evaluated, five with significant antagonistic activities against *P. aeruginosa* PA14 and three against *Staphylococcus* spp.;
- Only compounds 04 and 09 showed interference with bacterial growth, and this was strain specific, being observed in only two of fifteen microorganisms tested;
- Compound 09, in which the lateral alkyl side chain is absent, was the only compound that modulated QS-dependent behaviours in the three tested genera, presenting antagonistic activity in gram-positive and gram-negative bacteria. It must be noted that QS signalling systems are distinct in gram-negative and gram-positive bacteria, thus highlighting the need to ascertain the molecular mechanisms trough which perception of the signal mimetic occurs;
- Compound 15 was shown to be effective as an anti-motility compound in both tested bacteria, in addition to presenting a significant reduction in pyocyanin production;
- Compound 14, with the presence of an amine group, showed anti-biofilm and anti-swarming activities in six out of fifteen strains tested, without showing bactericidal characteristics. Therefore, it can be considered the most promising anti-infective compound in view of the phenotypes studied in the present work.

REFERENCE & LITERATURE

ABOUELHASSAN, Y.; GARRISON, A. T.; BURCH, G. M.; WONG, W.; NORWOOD, V. M.; HUIGENS, R. W. Discovery of Quinoline Small Molecules with Potent Dispersal Activity against Methicillin-Resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms Using a Scaffold Hopping Strategy. **Bioorganic & Medicinal Chemistry Letters**, v. 24, n. 21, p. 5076–5080, Nov. 2014.

AHMED, S. A.; RUDDEN, M.; SMYTH, T. J.; DOOLEY, J. S. G.; MARCHANT, R.; BANAT, I. M. Natural quorum sensing inhibitors effectively downregulate gene expression of *Pseudomonas aeruginosa* virulence factors. **Applied microbiology and biotechnology**, v. 103, n. 8, p. 3521–3535, Mar. 2019.

ALANIS, A. J. Resistance to Antibiotics: Are We in the Post-Antibiotic Era? Archives of Medical Research, Infectious Diseases: Revisiting Past Problems and Addressing Future Challenges. v. 36, n. 6, p. 697–705, Nov. 2005.

ALEKSIĆ, I.; ŠEGAN, S.; ANDRIĆ, F.; ZLATOVIĆ, M.; MORIC, I.; OPSENICA, D. M.; SENEROVIC, L. Long-Chain 4-Aminoquinolines as Quorum Sensing Inhibitors in *Serratia marcescens* and *Pseudomonas aeruginosa*. **ACS Chemical Biology**, v. 12, n. 5, p. 1425–1434, May 2017.

ARAIN, S.; WEISS, S.; HEINZLE, E.; JOHN, G. T.; KRAUSE, C.; KLIMANT, I. Gas Sensing in Microplates with Optodes: Influence of Oxygen Exchange between Sample, Air, and Plate Material. **Biotechnology and Bioengineering**, v. 90, n. 3, p. 271–280, May 2005.

ARMBRUSTER, C. R.; WOLTER, D.J.; MISHRA, M.; HAYDEN, H. S.; RADEY, M. C.; MERRIHEW, G.; MACCOSS, M. J.; BURNS, J.; WOZNIAK, D. J.; PARSEK, M. R.; HOFFMAN, L. R. *Staphylococcus aureus* Protein A Mediates Interspecies Interactions at the Cell Surface of *Pseudomonas aeruginosa*. **mBio**, v. 7, n. 3, p. 1–9, May 2016.

ARYEE, A.; EDGEWORTH, J. D. Carriage, Clinical Microbiology and Transmission of *Staphylococcus aureus*. In: BAGNOLI, F.; RAPPUOLI, R.; GRANDI, G. (Ed.). ***Staphylococcus aureus*: Microbiology, Pathology, Immunology, Therapy and Prophylaxis**. Current Topics in Microbiology and Immunology. Springer International Publishing, p. 1–19, 2017.

AZIMI, S.; KLEMENTIEV, A. D.; WHITELEY, M.; DIGGLE, S. P. Bacterial Quorum Sensing During Infection. **Annual Review of Microbiology**, v. 74, n. 1, p. 201-219, Sep. 2020.

BABEL, H.; NARANJO-MENESES, P.; TRAUTH, S.; SCHULMEISTER, S.; MALENGO, G.; SOURJIK V.; BISCHOFS, I. B. Ratiometric Population Sensing by a Pump-Probe Signalling System in *Bacillus subtilis*. **Nature Communications**, v. 11, n. 1, p. 1176, Mar. 2020.

BAKER, Y. R.; HODGKINSON, J. T.; FLOREA, B. I.; ALZA, E.; GALLOWAY, W. R. J. D.; GRIMM, L.; GEDDIS, S. M.; OVERKLEEFT, H. S.; WELCH, M.; SPRING, D. R. Identification of New Quorum Sensing Autoinducer Binding Partners in *Pseudomonas aeruginosa* Using Photoaffinity Probes. **Chemical Science**, v. 8, n. 11, p. 7403–7411, Oct. 2017.

BASAK, A.; ABOUELHASSAN, Y.; KIM, Y. S.; NORWOOD, V. M.; JIN, S.; HUIGENS, R. W. Halogenated Quinolines Bearing Polar Functionality at the 2-Position: Identification of New Antibacterial Agents with Enhanced Activity against *Staphylococcus epidermidis*. **European Journal of Medicinal Chemistry**, v. 155, p. 705–713, Jul. 2018.

BISHT, K.; BAISHYA, J.; WAKEMAN, C. A. *Pseudomonas aeruginosa* Polymicrobial Interactions during Lung Infection. **Current Opinion in Microbiology**, v. 53, p. 1–8, Feb. 2020.

BOOLCHANDANI, M.; D'SOUZA, A. W.; DANTAS, G. Sequencing-based methods and resources to study antimicrobial resistance. **Nature Reviews Genetics**, v. 20, p. 356–370, 2019.

BRINT, J. M.; OHMAN, D. E. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. **Journal of Bacteriology**, v. 177, n. 24, p. 7155–7163, Dec. 1995.

CAI, P.; SUN, X.; WU, Y.; GAO, C.; MORTIMER, M. Soil Biofilms: Microbial Interactions, Challenges, and Advanced Techniques for Ex-Situ Characterization. **Soil Ecology Letters**, v. 1, n. 3–4, p. 85–93, Dec. 2019.

CASSIR, N.; ROLAIN, J.-M.; BROUQUI, P. A New Strategy to Fight Antimicrobial Resistance: The Revival of Old Antibiotics. **Frontiers in Microbiology**, v. 5, n. 551, p. 1–15, Oct. 2014.

CAVALIER-SMITH, T. The Neomuran Origin of Archaebacteria, the Negibacterial Root of the Universal Tree and Bacterial Megaclassification. **International Journal of Systematic and Evolutionary Microbiology**, v. 52, n. 1, p. 7–76, Jan. 2002.

CDC, U.S. CENTERS FOR DISEASE CONTROL AND PREVENTION. **Antibiotic Resistance Threats in the United States, 2019.** Atlanta, GA: U.S. Department of Health and Human Services, CDC, 2019.

COOPER, M. A.; SHLAES, D. Fix the Antibiotics Pipeline. **Nature**, v. 472, n. 7341, p. 32–32, Apr. 2011.

CUGINI, C.; CALFEE, M. W.; FARROW, J. M.; MORALES, D. K.; PESCI, E. C.; HOGAN, D. A. Farnesol, a Common Sesquiterpene, Inhibits PQS Production in *Pseudomonas aeruginosa*. **Molecular Microbiology**, v. 65, n. 4, p. 896–906, Aug. 2007.

CUGINI, C.; MORALES, D. K.; HOGAN, D. A. *Candida albicans*-Produced Farnesol Stimulates *Pseudomonas* Quinolone Signal Production in LasR-Defective *Pseudomonas aeruginosa* Strains. **Microbiology**, v. 156, n. 10, p. 3096–3107, Oct. 2010.

DALTON, T.; DOWD, S. E.; WOLCOTT, R. D.; SUN, Y.; WATTERS, C.; GRISWOLD, J. A.; RUMBAUGH, K. P. An i*n vivo* Polymicrobial Biofilm Wound Infection Model to Study Interspecies Interactions. **PLOS ONE**, v. 6, n. 11, p. 1-10, Nov. 2011.

DUAN, K.; DAMMEL, C.; STEIN, J.; RABIN, H.; SURETTE, M. G. Modulation of *Pseudomonas aeruginosa* Gene Expression by Host Microflora through Interspecies Communication. **Molecular Microbiology**, v. 50, n. 5, p. 1477–1491, Nov. 2003.

EBERHARD, A.; BURLINGAME, A. L.; EBERHARD. C.; KENYON, G. L.; NEALSON, K.H.; OPPENHEIMER J. Structural Identification of Autoinducer of *Photobacterium fischeri* Luciferase. **Biochemistry**, v. 20, n. 9, p. 2444–2449, Apr. 1981.

ENGEBRECHT, J.; NEALSON, K.; SILVERMAN, M. Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from *Vibrio fischeri*. **Cell**, v. 32, n. 3, p. 773–781, Mar. 1983.

ENGEBRECHT, J.; SILVERMAN, M. Identification of Genes and Gene Products Necessary for Bacterial Bioluminescence. **Proceedings of the National Academy of Sciences**, v. 81, n. 13, p. 4154–4158, Jul. 1984.

ESPINOSA-VALDÉS, M.; BORBOLLA-ALVAREZ, S.; DELGADO-ESPINOSA, A.; SÁNCHEZ-TEJEDA, J.; CERÓN-NAVA, A.; QUINTANA-ROMERO, O.; ARIZA-CASTOLO, A.; GARCÍA-DEL RÍO, D.; LOZA-MEJÍA, M. Synthesis, In Silico, and In Vitro Evaluation of Long Chain Alkyl Amides from 2-Amino-4-Quinolone Derivatives as Biofilm Inhibitors. **Molecules**, v. 24, n. 327, p. 1–18, Jan. 2019.

FARIA, R.; PEREIRA, C.; ALVES, R.; MENDONÇA, T.; FARINHA, F.; VASCONCELOS, C. Opportunistic Infections and Autoimmune Diseases. In: **Infection and Autoimmunity**. [s.l.] Elsevier, 2015. p. 251–277.

FERNÁNDEZ-PIÑAR, R.; CÁMARA, M.; DUBERN, J. F.; RAMOS, J. L.; ESPINOSA-URGEL, M. The *Pseudomonas aeruginosa* Quinolone Quorum Sensing Signal Alters the Multicellular Behaviour of *Pseudomonas putida* KT2440. **Research in Microbiology**, v. 162, n. 8, p. 773–781, Oct. 2011.

FLEMMING, H.-C.; WUERTZ, S. Bacteria and Archaea on Earth and their Abundance in Biofilms. **Nature Reviews Microbiology**, v. 17, n. 4, p. 247–260, Apr. 2019.

FLYNN, S.; REEN, F. J.; O'GARA, F. Exposure to Bile Leads to the Emergence of Adaptive Signalling Variants in the Opportunistic Pathogen *Pseudomonas aeruginosa*. Frontiers in Microbiology, v. 10, n. 2013, p. 1–13, Aug. 2019.

FRYDENLUND MICHELSEN, C.; KHADEMI, S. M. H.; JOHANSEN, H. K.; INGMER, H.; DORRESTEIN, P. C.; JELSBAK, L. Evolution of Metabolic Divergence in *Pseudomonas aeruginosa* during Long-Term Infection Facilitates a Proto-Cooperative Interspecies Interaction. **The ISME Journal**, v. 10, n. 6, p. 1323–1336, Jun. 2016.

FUQUA, W. C.; WINANS, S. C. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. **Journal of Bacteriology**, v. 176, n. 10, p. 2796–2806, May 1994.

FUQUA, W. C.; WINANS, S. C.; GREENBERG, E. P. Quorum Sensing in Bacteria: The LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulators. **Journal of Bacteriology**, v. 176, n. 2, p. 269–275, Jan. 1994.

GARCÍA-CONTRERAS, R.; WOOD, T. K.; TOMÁS, M. Editorial: Quorum Network (Sensing/Quenching) in Multidrug-Resistant Pathogens. **Frontiers in Cellular and Infection Microbiology**, v. 9, n. 80, p. 1–3, Apr. 2019.

GARCÍA-REYES, S.; SOBERÓN-CHÁVEZ, G.; COCOTL-YANEZ, M. The third quorum-sensing system of *Pseudomonas aeruginosa*: *Pseudomonas* quinolone signal and the enigmatic PqsE protein. **Journal of Medical Microbiology,** v. 69, n. 1, p. 25–34, Jan. 2020.

HODGKINSON, J. T.; GROSS, J.; BAKER, Y. R.; SPRING, D. R.; WELCH, M. A New *Pseudomonas* Quinolone Signal (PQS) Binding Partner: MexG. **Chemical Science**, v. 7, n. 4, p. 2553–2562, Mar. 2016.

HODGKINSON, J.; BOWDEN, S. D.; GALLOWAY, W. R. J. D.; SPRING, D. R.; WELCH, M. Structure-Activity Analysis of the *Pseudomonas* Quinolone Signal Molecule. **Journal of Bacteriology**, v. 192, n. 14, p. 3833–3837, Jul. 2010.

HOFER, U. The cost of antimicrobial resistance. **Nature Reviews Microbiology**, v. 17, n. 1, p. 3–3, Jan. 2019.

HUGHES, D. T.; TEREKHOVA, D. A.; LIOU, L.; HOVDE, C. J.; SAHL, J. W. Chemical Sensing in Mammalian Host-Bacterial Commensal Associations. **Proceedings of the National Academy of Sciences of the United States of America**, v. 107, n. 21, p. 9831–9836, May 2010.

IAKOVIDES, I. C.; MICHAEL-KORDATOU, I.; MOREIRA, N. F. F.; RIBEIRO, A. R.; FERNANDES, T.; PEREIRA, M. F. R.; NUNES, O. C.; MANAIA, C. M.; SILVA, A. M. T.; FATTA-KASSINOS, D. Continuous ozonation of urban wastewater: Removal of antibiotics, antibiotic-resistant *Escherichia coli* and antibiotic resistance genes and phytotoxicity. **Water Research**, v. 159, p. 333–347, Aug. 2019.

IBBERSON, C. B.; STACY, A.; FLEMING, D.; DEES, J. L.; RUMBAUGH, K.; GILMORE, M. S.; WHITELEY, M. Co-Infecting Microorganisms Dramatically Alter Pathogen Gene Essentiality during Polymicrobial Infection. **Nature Microbiology**, v. 2, n. 8, p. 1–6, May 2017.

IBBERSON, C. B.; WHITELEY, M. The Social Life of Microbes in Chronic Infection. **Current Opinion in Microbiology**, Host-Microbe Interactions: Bacteria. v. 53, p. 44–50, Feb. 2020.

JONES, S.; YU, B.; BAINTON, N. J.; BIRDSALL, M.; BYCROFT, B. W.; CHHABRA, S. R.; COX, A. J.; GOLBY, P.; REEVES, P. J.; STEPHENS, S. The Lux Autoinducer Regulates the Production of Exoenzyme Virulence Determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. **The EMBO journal**, v. 12, n. 6, p. 2477–2482, Jun. 1993.

KAMAL, A. A. M.; PETRERA, L.; EBERHARD, J.; HARTMANN, R. W. Structure–Functionality Relationship and Pharmacological Profiles of *Pseudomonas aeruginosa* Alkylquinolone Quorum Sensing Modulators. **Organic & Biomolecular Chemistry**, v. 15, n. 21, p. 4620–4630, May 2017.

KHAMKHENSHORNGPHANUCH, T.; KULKRAISRI, K.; JANJAMRATSAENG, A.; PLABUTONG, N.; THAMMAHONG, A.; MANADEE, K.; NA POMBEJRA, S.; KHOTAVIVATTANA, T. Synthesis and Antimicrobial Activity of Novel 4-Hydroxy-2-Quinolone Analogs. **Molecules**, v. 25, n. 13, p. 3059, Jan. 2020.

KORGAONKAR, A.; TRIVEDI, U.; RUMBAUGH, K. P.; WHITELEY, M. Community Surveillance Enhances *Pseudomonas aeruginosa* Virulence during Polymicrobial Infection. **Proceedings of the National Academy of Sciences**, v. 110, n. 3, p. 1059–1064, Jan. 2013.

LANGE, J.; HEIDENREICH, K.; HIGELIN, K.; DYCK, K.; MARX, V.; REICHEL, C. WAMEL, W. V.; REIJER, M. D.; GÖRLICH, D.; KAHL, B. C. *Staphylococcus aureus* Pathogenicity in Cystic Fibrosis Patients—Results from an Observational Prospective Multicenter Study Concerning Virulence Genes, Phylogeny, and Gene Plasticity. **Toxins**, v. 12, n. 5, p. 279, May 2020.

LE, K. Y.; OTTO, M. Quorum-Sensing Regulation in Staphylococci—an Overview. **Frontiers in Microbiology**, v. 6, n. 1174, p. 1–8, Oct. 2015.

LEITE, A.; BESSA, L. J.; SILVA, A. M. G.; GAMEIRO, P.; DE CASTRO, B.; RANGEL, M. Antibacterial Activity of Naphthyl Derived Bis-(3-Hydroxy-4-Pyridinonate) Copper(II) Complexes against Multidrug-Resistant Bacteria. **Journal of Inorganic Biochemistry**, v. 197, n. 110704, p. 1–8, Aug. 2019.

LIMOLI, D. H.; HOFFMAN, L. R. Help, Hinder, Hide and Harm: What Can We Learn from the Interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* during Respiratory Infections? **Thorax**, v. 74, n. 7, p. 684–692, Jul. 2019.

LIN, J.; CHENG, J.; WANG, Y.; SHEN, X. The *Pseudomonas* Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore. **Frontiers in Cellular and Infection Microbiology**, v. 8, n. 230, p. 1–9, Jul. 2018.

LOPEZ-MEDINA, E.; FAN, D.; COUGHLIN, L. A.; HO, E. X.; LAMONT, I. L.; REIMMANN, C.; HOOPER, L. V.; KOH, A. Y. *Candida albicans* Inhibits *Pseudomonas aeruginosa* Virulence through Suppression of Pyochelin and Pyoverdine Biosynthesis. **PLOS Pathogens**, v. 11, n. 8, p. 1-34, Aug. 2015.

LU, C.; KIRSCH , B.; MAURER, C. K.; DE JONG, J. C.; BRAUNSHAUSEN, A.; STEINBACH, A.; HARTMANN, R. W. Optimization of Anti-Virulence PqsR Antagonists Aqueous Solubility and Biological Properties Resulting in New Insights in Structure–Activity Relationships. **European Journal of Medicinal Chemistry**, v. 79, p. 173–183, May 2014.

MARTÍNEZ, J. L. Short-Sighted Evolution of Bacterial Opportunistic Pathogens with an Environmental Origin. **Frontiers in Microbiology**, v. 5, n. 239, p. 1–4, May 2014.

MCCANN, C. M.; CHRISTGEN, B.; ROBERTS, J. A.; SU, J.-Q.; ARNOLD, K. E.; GRAY, N. D.; ZHU, Y.-G.; GRAHAM, D. W. Understanding drivers of antibiotic resistance genes in High Arctic soil ecosystems. **Environment International**, v. 125, p. 497–504, Apr. 2019.

MICHELSEN, C.; KHADEMI, S. M. H.; JOHANSEN, H. K.; INGMER, H.; DORRESTEIN, P. C.; JELSBAK, L. Evolution of Metabolic Divergence in *Pseudomonas aeruginosa* during Long-Term Infection Facilitates a Proto-Cooperative Interspecies Interaction. **The ISME Journal**, v. 10, n. 6, p. 1323–1336, Jun. 2016.

NAKAGAWA, S.; HILLEBRAND, G. G.; NUNEZ, G. *Rosmarinus officinalis L.* (Rosemary) Extracts Containing Carnosic Acid and Carnosol are Potent Quorum Sensing Inhibitors of *Staphylococcus aureus* Virulence. **Antibiotics**, v. 9, n. 4, p. 149–160, Mar. 2020.

NAZIK, H.; SASS, G.; ANSARI, S. R.; ERTEKIN, R.; HAAS, H.; DÉZIEL, E.; STEVENS, D. A. Novel intermicrobial molecular interaction: *Pseudomonas aeruginosa* Quinolone Signal (PQS) modulates *Aspergillus fumigatus* response to iron. **Microbiology,** v. 166, n. 1, p. 44–55, 2020.

NEALSON, K. H.; HASTINGS, J. W. Bacterial Bioluminescence: Its Control and Ecological Significance. **Microbiological Reviews**, v. 43, n. 4, p. 496–518, Dec. 1979.

NEALSON, K. H.; PLATT, T.; HASTINGS, J. W. Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System1. **Journal of Bacteriology**, v. 104, n. 1, p. 313–322, 1970.

NEIDITCH, M. B.; CAPODAGLI, G. C.; PREHNA, G.; FEDERLE, M. J. Genetic and Structural Analyses of RRNPP Intercellular Peptide Signalling of Gram-Positive Bacteria. **Annual Review of Genetics**, v. 51, n. 1, p. 311–333, 27 Nov. 2017.

NEVILLE, N.; JIA, Z. Approaches to the Structure-Based Design of Antivirulence Drugs: Therapeutics for the Post-Antibiotic Era. **Molecules**, v. 24, n. 3, p. 378, Jan. 2019.

NGUYEN, A. T.; JONES, J. W.; CÁMARA, M.; WILLIAMS, P.; KANE, M. A.; OGLESBY-SHERROUSE, A. G. Cystic Fibrosis Isolates of *Pseudomonas aeruginosa* Retain Iron-Regulated Antimicrobial Activity against *Staphylococcus aureus* through the Action of Multiple Alkylquinolones. **Frontiers in Microbiology**, v. 7, n. 1171, p. 1–13, Jul. 2016.

NIELSEN, T. B.; BRASS, E. P.; GILBERT, D. N.; BARTLETT, J. G.; SPELLBERG, B. Sustainable Discovery and Development of Antibiotics — Is a Nonprofit Approach the Future? **The New England journal of medicine**, v. 381, n. 6, p. 503–505, Aug. 2019.

PASSADOR, L.; Cook, J. M.; Gambello, M. J.; Rust, L.; Iglewski, B. H. Expression of *Pseudomonas aeruginosa* Virulence Genes Requires Cell-to-Cell Communication. **Science (New York, N.Y.)**, v. 260, n. 5111, p. 1127–1130, May 1993.

PAULANDER, W.; VARMING, A. N.; BOJER, M. S.; FRIBERG, C.; BÆK, K.; INGMER, H. The Agr Quorum Sensing System in *Staphylococcus aureus* Cells Mediates Death of Sub-Population. **BMC Research Notes**, v. 11, n. 1, p. 503, Dec. 2018.

PIEWNGAM, P.; CHIOU, J.; CHATTERJEE, P.; OTTO, M. Alternative Approaches to Treat Bacterial Infections: Targeting Quorum-Sensing. **Expert Review of Anti-infective Therapy**, v. 18, n. 6, p. 499–510, Apr. 2020.

QUAVE, C. L.; HORSWILL, A. R. Flipping the switch: tools for detecting small molecule inhibitors of staphylococcal virulence. **Frontiers in Microbiology**, v. 5, 12 Dec. 2014.

RAJAOFERA, M. J. N.; WANG, Y.; JATOI, Z. A.; JIN, P.; CUI, H.; LIN, C.; MIAO, W. *Bacillus atrophaeus* HAB-5 Secretion Metabolites Preventing Occurrence of Systemic Diseases in Tobacco Plant. **European Journal of Plant Pathology**, v. 156, n. 1, p. 159–172, Jan. 2020.

RAMOS, A. F.; WOODS, D. F.; SHANAHAN, R.; CANO, R.; MCGLACKEN, G. P.; SERRA, C.; O'GARA, F.; REEN, F. J. A structure-function analysis of interspecies antagonism by the 2-heptyl-4-alkyl-quinolone signal molecule from *Pseudomonas aeruginosa*. **Microbiology,** v. 166, n. 2, p. 169–179, Feb. 2020.

REEN, F. J.; GUTIÉRREZ-BARRANQUERO, J. A.; MCCARTHY, R. R.; WOODS, D. F.; SCARCIGLIA, S.; ADAMS, C.; FOG NIELSEN, K.; GRAM, L.; O'GARA, F. Quorum Sensing Signalling Alters Virulence Potential and Population Dynamics in Complex Microbiome-Host Interactomes. **Frontiers in Microbiology**, v. 10, n. 2131, p. 1–13, Sep. 2019.

REEN, F. J.; MCGLACKEN, G. P.; O'GARA, F. The Expanding Horizon of Alkyl Quinolone Signalling and Communication in Polycellular Interactomes. **FEMS Microbiology Letters**, v. 365, n. 9, p. 1–10, May 2018.

REEN, F. J.; PHELAN, J. P.; WOODS, D. W.; SHANAHAN, R.; CANO, R.; CLARKE, S.; MCGLACKEN, G. P. Harnessing Bacterial Signals for Suppression of Biofilm Formation in the Nosocomial Fungal Pathogen *Aspergillus fumigatus*. **Frontiers in Microbiology**, v. 7, n. 2074, p. 1–12, 22 Dec. 2016.

REEN, F. J; MOOIJ, M. J.; HOLCOMBE, L. J.; MCSWEENEY, C. M.; MCGLACKEN, G. P.; MORRISSEY, J. P.; O'GARA, F. The *Pseudomonas* Quinolone Signal (PQS), and Its Precursor HHQ, Modulate Interspecies and Interkingdom Behaviour: Quinolone Signal Molecules Modulate Interkingdom Behaviour. **FEMS Microbiology Ecology**, v. 77, n. 2, p. 413–428, Aug. 2011.

REEN, J. F.; SHANAHAN, R.; CANO, R.; O'GARA, F.; MCGLACKEN, G. P. A Structure Activity-Relationship Study of the Bacterial Signal Molecule HHQ Reveals Swarming Motility Inhibition in *Bacillus atrophaeus*. **Organic & Biomolecular Chemistry**, v. 13, n. 19, p. 5537–5541, Apr. 2015.

RÉMY, B.; MION, S.; PLENER, L.; ELIAS, M.; CHABRIÈRE, E.; DAUDÉ, D. Interference in Bacterial Quorum Sensing: A Biopharmaceutical Perspective. **Frontiers in Pharmacology**, v. 9, n. 203, p. 1–17, Mar. 2018.

RITZMANN, N. H.; MÄHRLEIN, A.; ERNST, S.; HENNECKE, U.; DREES, S. L.; FETZNER, S. Bromination of Alkyl Quinolones by *Microbulbifer* sp. HZ11, a Marine Gammaproteobacterium, Modulates Their Antibacterial Activity. **Environmental Microbiology**, v. 21, n. 7, p. 2595–2609, May 2019.

RÜTSCHLIN, S.; BÖTTCHER, T. Inhibitors of Bacterial Swarming Behavior. **Chemistry – A European Journal**, v. 26, n. 5, p. 964–979, Jan. 2020.

SAEKI, E. K.; KOBAYASHI, R. K. T.; NAKAZATO, G. Quorum Sensing System: Target to Control the Spread of Bacterial Infections. **Microbial Pathogenesis**, v. 142, May 2020.

SCHÜTZ, C.; EMPTING, M. Targeting the *Pseudomonas* Quinolone Signal Quorum Sensing System for the Discovery of Novel Anti-Infective Pathoblockers. **Beilstein Journal of Organic Chemistry**, v. 14, n. 1, p. 2627–2645, Oct. 2018.

SHAFIEE, A.; EMAMI, S.; GHODSI, S.; NAJJARI, S.; SORKHI, M.; SAMADI, N.; FARAMARZI, M. A.; FOROUMADI, A. Synthesis and Antibacterial Activity of N-[2-(2-Naphthyl)Ethyl]Piperazinyl Quinolones. **Journal of the Iranian Chemical Society**, v. 6, n. 2, p. 325–333, Jun. 2009.

SHANAHAN, R.; REEN, F. J.; CANO, R.; O'GARA, F.; MCGLACKEN, G. P. The Requirements at the C-3 Position of Alkylquinolones for Signalling in *Pseudomonas aeruginosa*. **Organic & Biomolecular Chemistry**, v. 15, n. 2, p. 306–310, Jan. 2017.

SIBLEY, C. D.; DUAN, K.; FISCHER, C.; PARKINS, M. D.; STOREY, D. G.; RABIN, H. R.; SURETTE, M. G.; SCHNEIDER, D. S. Discerning the Complexity of Community Interactions Using a *Drosophila* Model of Polymicrobial Infections. **PLoS Pathogens**, v. 4, n. 10, p. 1-10, Oct. 2008.

SUBRAMONI, S.; GONZALEZ, J. F.; JOHNSON, A.; PÉCHY-TARR, M.; ROCHAT, L.; PAULSEN, I.; LOPER, J. E.; KEEL, C.; VENTURI, V. Bacterial Subfamily of LuxR Regulators That Respond to Plant Compounds. **Applied and Environmental Microbiology**, v. 77, n. 13, p. 4579–4588, Jul. 2011.

TACCONELLI, E.; PEZZANI, M. D. Public health burden of antimicrobial resistance in Europe. **The Lancet Infectious Diseases**, Issue 1, 4 - 6. v. 19, Nov. 2018.

TAY, S.; YEW, W. Development of Quorum-Based Anti-Virulence Therapeutics Targeting Gram-Negative Bacterial Pathogens. **International Journal of Molecular Sciences**, v. 14, n. 8, p. 16570–16599, Aug. 2013.

TOMASZ, A. Control of the Competent State in *Pneumococcus* by a Hormone-Like Cell Product: An Example for a New Type of Regulatory Mechanism in Bacteria. **Nature**, v. 208, n. 5006, p. 155–159, Oct. 1965.

VARGAS, F.; ZOLTAN, T.; RIVAS, C.; RAMIREZ, A.; CORDERO, T.; DÍAZ, Y.; IZZO, C.; CÁRDENAS, Y. M.; LÓPEZ, V.; GÓMEZ, L.; ORTEGA, J.; FUENTES, A. Synthesis, Primary Photophysical and Antibacterial Properties of Naphthyl Ester Cinoxacin and Nalidixic Acid Derivatives. **Journal of Photochemistry and Photobiology B: Biology**, v. 92, n. 2, p. 83–90, Aug. 2008.

VARGAS, F.; ZOLTAN, T; RAMIREZ, A.; CORDERO, T.; CHAVES, V.; IZZO, C.; LÓPEZ, V.; CÁRDENAS, Y. M.; Fernández, A.; Hincapie, L.; FUENTES, A. Studies of the photooxidant properties of antibacterial fluoroquinolones and their naphthalene derivatives. **Die Pharmazie - An International Journal of Pharmaceutical Sciences**, v. 64, n. 2, p. 116–122, Feb. 2009.

VENTOLA, C. L. The Antibiotic Resistance Crisis. **Pharmacy and Therapeutics**, v. 40, n. 4, p. 277–283, Apr. 2015.

VERDUGO-FUENTES, A.; GASTÉLUM, G.; ROCHA, J.; DE LA TORRE, M. Multiple and Overlapping Functions of Quorum Sensing Proteins for Cell Specialization in *Bacillus* Species. **Journal of Bacteriology**, v. 202, n. 10, p. 1–14, 27 Apr. 2020.

WANG, M.; ZHU, P.; JIANG, J.; ZHU, H.; TAN, S.; LI, R. Signalling Molecules of Quorum Sensing in Bacteria. **Reviews in Biotechnology and Biochemistry**, v. 1, n. 1, p. 1-5, Feb. 2020.

WHALEN, K. E.; BECKER, J. W.; SCHRECENGOST, A. M.; GAO, Y.; GIANNETTI, N.; HARVEY, E. L. Bacterial alkylquinolone signalling contributes to structuring microbial communities in the ocean. **Microbiome**, v. 7, n. 93, p. 1–17, Jun. 2019.

WHITELEY, M.; DIGGLE, S. P.; GREENBERG, E. P. Bacterial quorum sensing: the progress and promise of an emerging research area. **Nature**, v. 551, n. 7680, p. 313–320, Nov. 2017.

WHO, World Health Organization. **Antibiotic resistance.** World Health Organization, 5 Feb. 2018. Available in: <https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance>. Access on: 9 Dec. 2019.

WHO, World Health Organization. **WHO | Antimicrobial resistance**. Available in: <http://www.who.int/antimicrobial-resistance/en/>. Access on: 14 Dec. 2019.

XIA, Y.; ZHANG, Q.; LI, G.; TU, X.; ZHOU, Y.; HU, X. Biodegradability enhancement of real antibiotic metronidazole wastewater by a modified electrochemical Fenton. **Journal of the Taiwan Institute of Chemical Engineers**, v. 96, p. 256–263, Mar. 2019.

ZAHEER, Z.; KHAN, F. A. K.; SANGSHETTI, J. N.; PATIL, R. H.; LOHAR, K. S. Novel Amalgamation of Phthalazine–Quinolines as Biofilm Inhibitors: One-Pot Synthesis, Biological Evaluation and in Silico ADME Prediction with Favorable Metabolic Fate. **Bioorganic & Medicinal Chemistry Letters**, v. 26, n. 7, p. 1696–1703, Apr. 2016.

ZHAO, K.; DU, L.; LIN, J.; YUAN, Y.; WANG, X.; YUE, B.; WANG, X.; GUO, Y.; CHU, Y.; ZHOU, Y. *Pseudomonas aeruginosa* Quorum-Sensing and Type VI Secretion System Can Direct Interspecific Coexistence During Evolution. **Frontiers in Microbiology**, v. 9, p. 2287, Oct. 2018.

ZUO, R.; GARRISON, A. T.; BASAK, A.; ZHANG, P.; HUIGENS, R. W.; DING, Y. In vitro Antifungal and Antibiofilm Activities of Halogenated Quinoline Analogues against *Candida albicans* and *Cryptococcus neoformans*. **International Journal of Antimicrobial Agents**, v. 48, n. 2, p. 208–211, Aug. 2016.