ERIKA LORENA GIRALDO VARGAS

VALIDAÇÃO DO POTENCIAL ANTI-*QUORUM SENSING* DE COMPOSTOS NATURAIS E ANTI-INFLAMATÓRIOS EM BACTÉRIAS GRAM-NEGATIVAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS-BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
G516v
2019

Giraldo Vargas, Erika Lorena, 1992-
Validação do potencial anti-*quorum sensing* de compostos naturais e anti-inflamátorios em bactérias gram-negativas / Erika Lorena Giraldo Vargas. – Viçosa, MG, 2019.
xii, 66 f. : il. (algumas color.) ; 29 cm.

Orientador: Maria Cristina Dantas Vanetti.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Quorum sensing (Microbiologia). 2. Plantas - Composição. 3. Agentes antiinflamatórios: substancias inibitorias.. I. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-Graduação em Microbiologia Agrícola. II. Título.

CDD 22. ed. 571.74

ERIKA LORENA GIRALDO VARGAS

VALIDAÇÃO DO POTENCIAL ANTI-*QUORUM SENSING* DE COMPOSTOS NATURAIS E ANTI-INFLAMATÓRIOS EM BACTÉRIAS GRAM-NEGATIVAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

APROVADA: 29 de março de 2019.

Uelinton Manoel Pinto

José Guilherme Prado Martin

Ramila Cristiane Rodrigues

Maria Emilene Martino Campos-Galvão

Maria Cristina Dantas Vanetti
(Orientadora)

Aos meus pais, Edgar e Gloria,
e às minhas irmãs e sobrinhos,
pelo incentivo e apoio constante.

DEDICO

A menos que modifiquemos nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo".

(Albert Einstein)

AGRADECIMENTOS

À Universidade Federal de Viçosa e ao Departamento de Microbiologia, pela estrutura e por oportunizar a realização desta pesquisa.

Organização dos Estados Americanos (OEA) e Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo apoio financeiro.

Aos meus pais, Edgar e Gloria, pelo amor, pela dedicação e por lutarem comigo ao longo do caminho.

Ás minhas irmãs, Andrea e Leidy, pelo carinho, companheirismo, apoio e ensinamentos.

Aos meus sobrinhos, Mateo e Luciana, por serem minha motivação constante.

A minha avó, Elvia, por seu carinho e, por mesmo cansada, esperar meu retorno para casa sempre.

À minha família, madrinha, padrinho, tias, tios, primas e primos pela torcida.

À professora Maria Cristina Dantas Vanetti, pela oportunidade de trabalhar sob sua impecável orientação, pelos seus ensinamentos e paciência para me guiar neste caminho acadêmico. Estarei profundamente agradecida pela força e acompanhamento que sempre me brindou nos momentos mais difíceis e por estar sempre disposta a ajudar em qualquer momento. OBRIGADA!

Aos meus coorientadores, professores Uelinton e Guilherme pelas sugestões e correções; e aos demais membros da banca, Dras. Emilene e Ramila.

À Claudia, minha irmã, pelo seu carinho, conselhos e apoio constante, por se disponibilizar em qualquer momento para me ajudar e me acompanhar neste caminho.

Ao Felipe, meu amigo, por me acolher e me guiar, pelos ensinamentos constantes e por me motivar sempre neste caminho. Sua luz e alegria tornou este processo mais divertido e interessante.

A Picolé, por seu companheirismo, carinho, momentos de risadas, conhecimento compartilhado e por sempre me brindar com apoio em cada passo.

Ao Leonardo, por seu companheirismo e por compartilhar seu conhecimento e por ajudar em todo momento.

A Cleonice e sua família, por me acolher como uma “filinha” na sua casa e por seu apoio.

A Gabs, pelo seu carinho e sempre me receber com um grande sorriso e abraço no laboratório.

A todos os professores do Departamento de Microbiologia pelos ensinamentos compartilhados;.

Aos funcionários do Departamento de Microbiologia, Letícia, Sandra, Gabriel, José Carlos, Raquel, Paulo, Danilo e Emília pela disponibilidade e ajuda.

Às minhas amigas, por me apoiarem à distância em todo momento, me escutarem e motivarem para continuar no caminho.

Aos amigos do Laboratório de Microbiologia de Alimentos, Ramila, Cleri, Fê, Leandro, Jéssica e Bel pela amizade, apoio e por tornarem minha estadia no Laboratório mais agradável e divertida.

As minhas amigas de republica, pelos momentos de descontração, comidas, conversas e risadas. Minha companheira de "gorduras", Aline, obrigada pelo seu companheirismo, apoio e por me oferecer sua amizade.

Aos amigos do Laboratório de Virologia, Bruna, Flávia, Rafa, Paty, Renan, Jonas e Johan pelo acolhimento e pelos maravilhosos e divertidos momentos no café.

Ao laboratório de Anaeróbios, Sofía, Yasmin e Marlon pela disposição para me ajudar na preparação de material anaeróbio.

Aos meus amigos colombianos, pelos momentos de descontração, conversas, risadas, danças e apoio constante.

Agradecimentos para a empresa Sigma-Aldrich pela contribução com o composto fitol para o desenvolvimento da pesquisa.

A todos que colaboraram direta ou indiretamente para realização dessa pesquisa, que torcem e se alegram com as minhas conquistas;

A todos minha imensa gratidão e carinho!

BIOGRAFIA

ERIKA LORENA GIRALDO VARGAS, filha de Edgar Giraldo Trujillo e Gloria Maria Vargas, nasceu em Ibagué-Tolima, Colombia, em 12 de novembro de 1992. Em setembro de 2015, graduou-se em Biologia pela Universidad del Tolima, Colombia.

Em agosto de 2015 até julho de 2016, foi estudante não vinculada do Programa de Pós-Graduação em Microbiologia Agrícola, na área de Microbiologia de Alimentos, na Universidade Federal de Viçosa. Em março de 2017, ingressou no Programa de Pós-Graduação em Microbiologia Agrícola, com bolsa da Organização dos Estados Americanos (OEA).

SUMÁRIO

RESUMO

VARGAS, Erika Lorena Giraldo, M.Sc., Universidade Federal de Viçosa, março de 2019. **Validação do potencial anti-quorum sensing de compostos naturais e anti-inflamatórios em bactérias Gram-negativas**. Orientadora: Maria Cristina Dantas Vanetti.

Quorum sensing (QS) é um mecanismo de comunicação célula-célula mediado por moléculas sinalizadoras que levam à expressão gênica diferencial em resposta a alta densidade populacional. Numerosas espécies de bactérias utilizam este mecanismo de comunicação intra e intercelular para promoverem as mudanças necessárias para adaptação em ambientes diversos. *Chromobacterium violaceum* é um patógeno humano oportunista que utiliza o *QS* mediado por acil homoserina lactonas (AHLs) para regular fenótipos como a formação de biofilme, produção de cianeto, síntese do pigmento violeta denominado violaceína, entre outros. Esta bactéria regula a produção de violaceína pelo sistema *QS* CviI/CviR, que produz e responde a AHLs de diferentes comprimentos de cadeia acila. Ao contrário do sistema de *QS* por AHLs completo presente em *C. violaceum,* em *Salmonella*, esse sistema é incompleto, devido a ausência da sintase da molécula sinal. Entretanto, *Salmonella* possui a proteína SdiA, homóloga a proteína LuxR, que permite detectar as AHLs produzidas por outras bactérias. Neste trabalho, foi realizada a prospecção *in silico* de moléculas indutoras do mecanismo de *quorum sensing* assim como, de prováveis inibidores (*quorum quenching-QQ*) de *C. violaceum* e testes *in vitro* foram realizados para avaliar o efeito destes compostos sobre a produção de violaceína. Além disso, foi testado o efeito do composto fitol e furanona na interferência de processos regulados por *QS* no metabolismo de *Salmonella.* Testes *in silico* mostraram que as AHLs, compostos de plantas e anti-inflamatórios não esteroides (AINEs) foram capazes de se ligar a proteína CviR de *C. violaceum* com altos escores de afinidade. Além disso, testes *in vitro* mostraram que a maioria dos compostos testados inibiu a produção de violaceína, sendo que, dentre os compostos de plantas, concentração de 600 µg/mL de ácido margárico e ácido palmítico foi mais efetiva para inibir a produção de violaceína por *C. violaceum* ATCC 12472 e mutante CV026. No entanto, dentre os AINEs testados na concentração de 500 µg/mL, somente o cetoprofeno mostrou inibição deste fenótipo nas duas estirpes. O crescimento de *Salmonella* na presença de 0,05 µM de *N*-dodecanoyl-homoserina lactona (C12-HSL), 0,05 µM de furanona ou 600 µg/mL de fitol durante 24 h de incubação em condições

de anaerobiose não foi inibido. Entretanto, na presença de C12-HSL a concentração de tiol livre aumentou em fase exponencial de crescimento com 6 e 7 h de incubação, mas retornou ao mesmo nível observado em células cultivadas na ausência de C12-HSL, quando furanona e fitol foram adicionados na cultura, comparado com o tratamento controle. Os níveis de coenzima NADPH foram significativamente aumentados na presença de C12-HSL, furanona e fitol, enquanto o consumo de glicose foi menor em 6 h de incubação com C12-HSL. A produção de ácidos orgânicos em condições anaeróbias não foi afetada pelas moléculas de *QS* e *QQ*. Os resultados do presente trabalho mostraram a importância do *docking molecular* como uma ferramenta *in silico* válida para prospecção de compostos *QQ* em bactérias. A maioria dos compostos indicados *in silico* como potenciais *QQ* e testados *in vitro* inibiu a produção de violaceína em *C. violaceum,* indicando que podem interferir com o mecanismo de comunicação celular. Além disso, os resultados da influência de moléculas de *QS* e *QQ* sobre alguns processos metabólicos em *Salmonella* revelam a importância de conhecer e compreender os efeitos destes compostos, a fim de encontrar maneiras de reduzir a patogenicidade e, portanto, diminuir o número de surtos de salmonelose registrados em todo o mundo.

ABSTRACT

VARGAS, Erika Lorena Giraldo, M.Sc., Universidade Federal de Viçosa, March, 2019. **Validation of potential anti-quorum sensing of natural and anti-inflammatory compounds in Gram-negative bacteria.** Adviser: Maria Cristina Dantas Vanetti.

Quorum sensing (QS) is a mechanism of cell-cell communication mediated by signal molecules that lead to differential gene expression in response to high population density. Numerous species of bacteria use this mechanism of intra and intercellular communication to promote the changes necessary for adaptation in diverse environments. *Chromobacterium violaceum* is an opportunistic human pathogen that uses the QS mediated by acyl-homoserine lactones (AHLs) to control biofilm formation, cyanide production, synthesis of a violet pigment called violacein, among others. This bacterium regulates the production of violacein by the QS system CviI/CviR, which produces and responds to AHLs of different acyl chain lengths. Unlike the complete AI-1 QS system present in *C. violaceum*, in *Salmonella*, this system is incomplete, because there is no synthesis of the AHL signal molecule. Meanwhile, *Salmonella* has the SdiA protein, homologous to the LuxR protein, which allows the detection of AHLs produced by other bacteria. In this work, a screening of quorum sensing and quorum quenching (QQ) molecules in *C. violaceum* was performed and *in vitro* tests were carried out to evaluate the effect of these compounds on violacein production in this microorganism. In addition, the effect of the phytol and furanone compounds were tested on the interference of QS-regulated processes in *Salmonella* metabolism. The *in silico* tests showed that AHLs, plant compounds and non-steroidal anti-inflammatory drugs (NSAIDs) were able to bind to the CviR protein of *C. violaceum* showing high scores of affinities. In addition, *in vitro* tests showed that most of the tested compounds achieved an inhibition in violacein production, whereas, among the plant compounds, a concentration of 600 μg/mL of margaric and palmitic acid were effective against *C. violaceum* ATCC 12472 and mutant CV026. However, within the NSAIDs tested, only ketoprofen compound showed inhibition of this phenotype in the two strains. Growth of *Salmonella* in the presence of 0.05 μM of *N*-dodecanoyl-homoserine lactone (C12-HSL), 0.05 μM of furanone or 600 μg/mL of phytol for 24 h incubation under anaerobic conditions was not inhibited. In the presence of C12-HSL, free thiol levels increased at 6 and 7 h of incubation but returned to the same level observed in cells cultured in the absence of C12-HSL, when phytol and furanone were added in broth, compared to the control treatment. NADPH coenzyme

levels were significantly increased in the presence of C12-HSL, phytol, and furanone, whereas glucose consumption was lower in 6 h of incubation with C12-HSL. The production of organic acids under anaerobic conditions was not affected by QQ or QS molecules. The results of the present work showed the importance of molecular docking as a valid *in silico* tool for prospecting QQ compounds in bacteria. Most compounds tested *in vitro* inhibited the violacein production in *C. violaceum*, indicating that they may interfere with the mechanism of cellular communication. In addition, the results of the influence of QS and QQ molecules on some physiological processes in *Salmonella*, reveal the importance of knowing and understanding the effects of these compounds, in order to find ways to reduce the pathogenicity and, therefore, to reduce the number of outbreaks of salmonellosis registered in all the world.

CHAPTER 1

Revisão: Mecanismo de comunicação por *quorum sensing* com autoindutor-1 em bactérias gram-negativas

1. REFERENCIAL BIBLIOGRÁFICO

1.1 Mecanismo de comunicação por *quorum sensing* com autoindutor-1 em bactérias gram-negativas

A adaptação de bactérias a um novo ambiente depende da capacidade de detectar e responder a esse ambiente e este comportamento é essencial para sobrevivência e patogênese. Células bacterianas desenvolveram diferentes mecanismos que lhes permitem detectar condições ambientais diversas tais como, pH, osmolaridade, disponibilidade de nutrientes e densidade populacional e alterarem a produção de vários compostos para adaptarem a essas novas condições (SMITH; IGLEWSKI, 2003). Numerosas espécies de bactérias utilizam o mecanismo de comunicação celular denominado de *quorum sensing* (*QS*) para promoverem as mudanças necessárias para adaptação em ambientes diversos. Este processo de sinalização permite a comunicação entre as células bacterianas levando à expressão diferencial de genes em resposta às mudanças na densidade populacional (FUQUA; WINANS; GREENBERG, 1994; FUQUA; PARSEK; GREENBERG, 2001). O mecanismo de *QS* é mediado por moléculas difusíveis denominadas de autoindutores (AIs), os quais são sintetizados intracelularmente, durante todo o crescimento das bactérias e liberadas para o meio circundante. Quando esses AIs atingem concentrações críticas no meio externo, são internalizados e induzem a expressão de genes alvos específicos, resultando na alteração do metabolismo celular. Nas bactérias gram-negativas, os AIs mais estudados são relacionados as acil homoserina lactonas (AHLs), com variações estruturais, tais como diferentes comprimentos de cadeia acil ou a eventual presença de uma função 3-oxo ou 3-hidroxi (GALLOWAY et al., 2011).

Este mecanismo de comunicação por *QS* desempenha função crítica, tanto nas interações bactéria-hospedeiro simbióticas quanto patogênicas (BOYER; WISNIEWSKI-DYÉ, 2009). Diversos patógenos relevantes utilizam esse mecanismo para regular processos associados à virulência, aumentando suas perspectivas de sobrevivência em razão do ataque coordenado contra o hospedeiro só ser feito quando a população bacteriana atingir alta densidade populacional, aumentando a probabilidade de superar as defesas do hospedeiro com sucesso (GESKE et al., 2007; RAINA et al., 2009).

O mecanismo de *QS* foi descrito pela primeira vez na regulação da bioluminescência em *Vibrio fischeri* (NEALSON; PLATT; HASTINGS, 1970). O operon luciferase em *V. fischeri* é regulado por duas proteínas, LuxI, responsável pela produção do autoindutor de *N*-acil-homoserina-lactona (AHL) e LuxR, que é ativada por esse autoindutor para aumentar transcrição do operon da luciferase (ENGEBRECHT; SILVERMAN, 1984). Depois disso, diferentes homólogos do sistema LuxI-LuxR foram identificados em outras bactérias e, em todos esses sistemas, as bactérias produzem um autoindutor que se liga à proteína LuxR e regula a transcrição de vários genes (DAVIES et al., 1998; DE KIEVIT; IGLEWSKI, 2000; PARSEK; GREENBERG, 2000). Estudos demonstraram que *QS* regula uma série de fenótipos bacterianos, incluindo a produção de antibióticos, formação de biofilmes, bioluminescência, diferenciação celular, competência, produção de pigmentos, conjugação, produção de enzimas hidrolíticas extracelulares, esporulação, motilidade em superfícies, produção de toxinas e expressão de genes de virulência (SMITH; FRATAMICO; NOVAK, 2004; WALTERS; SPERANDIO, 2006; KENDALL; RASKO; SPERANDIO, 2007; LIOU et al., 2010).

Embora esse mecanismo de comunicação fosse inicialmente considerado exclusivo para alguns vibriões marinhos por muitos anos, a presença de homólogos de um sistema completo de *QS* (*luxI*/*luxR*) também foi demonstrado em muitas bactérias gram-negativas, capazes de produzir AHLs específicas, incluindo, *Agrobacterium*, *Aeromonas*, *Burkholderia, Chromobacterium*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Hafnia*, *Nitrosomonas*, *Obesumbacterium*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Ralstonia*, *Rhodobacter*, *Rhizobium*, *Serratia* e *Yersinia* (SMITH; IGLEWSKI, 2003).

Entre as bactérias que possuem um sistema *QS* completo, uma das mais estudadas é *Pseudomonas aeruginosa*, um patógeno oportunista reconhecido por produzir fatores de virulência, incluindo exoproteases, sideróforos, exotoxinas e lipases (PARSEK; GREENBERG, 2000). Muitos desses fatores de virulência são regulados pelo mecanismo de *QS* e existem atualmente quatro vias bem conhecidos em *P. aeruginosa*: dois sistemas tipo LuxI/LuxR denominados LasI/LasR e RhlI/RhlR, o sistema quinolona controlado por PqsR e o sistema 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) que funciona sob condições limitantes de fosfato (PAPENFORT; BASSLER, 2016). Nos sistemas tipo LuxI/LuxR, o LasI é uma sintase que produz uma molécula sinal de AHL difusível extracelularmente denominada *N*-(3-oxododecanoil) -L-homoserina lactona (3-oxo-C12-HSL) que é reconhecida pelo regulador transcricional LasR, que direciona a

expressão de vários genes. Da mesma forma, RhlI produz a molécula sinal *N*-butiril-L-homoserina lactona (C4-HSL) que pode ligar-se ao seu regulador de transcrição cognato RhlR. Os reguladores de transcrição LasR e RhlR são ativados quando níveis suficientes de 3-oxo-C12-HSL e C4-HSL estão presentes, resultado da alta densidade populacional e regulam a produção de múltiplos fatores de virulência (LEE et al., 2013).

Outro exemplo de bactéria gram-negativa com o sistema de *QS* completo é *Chromobacterium violaceum,* um patógeno humano oportunista e que utiliza o *QS* mediado por AHL para regular a formação de biofilme, produção de cianeto, síntese do pigmento violeta denominado violaceína, entre outros (DE OCA-MEJÍA et al., 2014). Esta bactéria regula a produção de violaceína pelo sistema *QS* CviI/CviR, que produz e responde a AHLs de diferentes comprimentos de cadeia acil (McCLEAN et al., 1997a; STEINDLER; VENTURI, 2007; SCHUSTER et al., 2013). *C. violaceum* selvagem e mutantes biossensores têm sido utilizados como modelo em diversos estudos relacionados com os sistemas de comunicação bacteriana e inibição do mecanismo de *QS* (*IQS*) por produtos naturais e sintéticos (MUSTHAFA et al., 2010; BURT et al., 2014).

Ao contrário do sistema de *QS* por AI-1 completo presente em bactérias gram-negativas como *P. aeruginosa* e *C. violaceum,* em *Salmonella* e *Escherichia coli* esse sistema é incompleto (WALTERS; SPERANDIO, 2006). Nessas bactérias não foi ainda identificado o gene homólogo à *luxI*, que codifica a sintase do AI-1 e, consequentemente, não há síntese de AHLs. No entanto, um homólogo de LuxR, conhecido como SdiA, está presente e permite a detecção de moléculas-sinais produzidas por outras espécies bacterianas gram-negativas, levando à regulação da expressão gênica (MICHAEL et al., 2001; SMITH; AHMER, 2003; STEENACKERS et al., 2012). Em *Salmonella* e em *E. coli,* alguns fenótipos regulados pelo *QS* foram evidenciados. Wang e Rothfield (1991) sugeriram que a proteína SdiA (supressor da inibição da divisão celular) desempenhou função na regulação dos genes de divisão celular *ftsQAZ* em *E. coli.* Experimentos adicionais mostraram que SdiA reprimiu os genes da ilha de patogenicidade LEE e de motilidade em *E. coli* Enterohemorrágica (EHEC) (KYOKO et al., 2002). Também, em diferentes estirpes de *E. coli*, genes do metabolismo energético, biossíntese de vitamina, transporte, motilidade, reguladores transcricionais e patogênese foram diferencialmente regulados na presença de AHL (HOUDT et al., 2006; DYSZEL et al., 2010; LIOU et al., 2010; SHARMA; BEARSON, 2013). Genes relacionados à patogênese de *E. coli*, como

cbpA, *gadA* e *hdeA* também foram regulados positivamente pela AHL (HOUDT et al., 2006; DYSZEL et al., 2010). Além disso, em *E. coli* não-patogênica, SdiA, em conjunto com o indol, pode regular a formação de biofilme, motilidade e a produção de indol (LEE; JAYARAMAN; WOOD, 2007; LEE et al., 2008).

SdiA em *Salmonella* foi descrito pela primeira vez por Ahmer et al. (1998) quando foi sugerida a ligação entre SdiA e a regulação positiva de 10 genes no plasmídeo de virulência, incluindo o gene *rck*, previamente caracterizado como responsável pelo aumento da resistência e adesão às células epiteliais. Campos-Galvão et al. (2016) e Almeida et al. (2017) mostraram que a formação de biofilme em poliestireno por *Salmonella enterica* sorovar Enteritidis foi estimulada pela C12-HSL após 36 h de incubação em anaerobiose, mas reprimida pela presença concomitante de uma mistura de furanonas, agentes inibidores do *QS*. Foi evidenciado também que genes relacionados à virulência como *hilA, invA* e *invF*, presentes na ilha de patogenicidade PAI-1, e genes envolvidos na formação de biofilme por *Salmonella* Enteritidis foram mais expressos na presença de molécula exógena do sistema *QS* (CAMPOS-GALVÃO et al., 2016). Carneiro (2017) mostrou que o perfil de metabólitos intracelulares de *Salmonella* Enteritidis na presença e na ausência do AI-1 foi diferente nos tempos iniciais de cultivo e esta diferença diminuiu ao longo do período de incubação em anaerobiose. Também foram observadas alterações das concentrações dos metabólitos intracelulares pertencentes às vias dos glicerolipídeos, aminoácidos e nucleotídeos de purina, bem como do consumo de glicose entre células de *Salmonella* Enteritidis cultivadas na presença e na ausência de C12-HSL (CARNEIRO, 2017). O AI-1 C12-HSL alterou o perfil de ácidos graxos e as proteínas relacionadas ao processo de oxidação-redução de células de *Salmonella* nas primeiras horas de cultivo e estas células se apresentaram semelhantes àquelas em final de fase logarítmica e início de fase estacionária que cresceram na ausência deste AI (ALMEIDA et al., 2018).

1.2 Inibidores do mecanismo de *quorum sensing*

O *QS* está implicado em vários eventos relevantes na patogênese bacteriana e, por esse motivo, é provável que os inibidores do *QS* possam ter aplicação terapêutica. Existem diferentes vias de inibição do *QS*, tais como: (1) inibição da síntese do AI; (2) antagonismo do receptor do AI; (3) inibição de alvos de ligação do receptor; (4) sequestro de AIs usando, por exemplo, anticorpos contra AIs; (5) degradação de AI usando anticorpos catalíticos (abzimas) ou enzimas (como lactonases); (6) inibição da

secreção/transporte de AI; e (7) anticorpos que bloqueiam os receptores do AI (DE LAMO MARIN et al., 2007). Portanto, a interrupção deste sistema de comunicação ou a atividade do mecanismo de *QS* em bactérias pode levar à atenuação da virulência microbiana (WHITELEY; LEE; GREENBERG, 1999; DE KIEVIT; IGLEWSKI, 2000; SMITH; IGLEWSKI, 2003).

Compostos naturais e sintéticos com ação anti-*QS* ganharam interesse como agentes potenciais para controlar infecções bacterianas. As plantas superiores consistem em fontes potenciais de antimicrobianos devido à produção de amplo espectro de metabólitos secundários, como compostos fenólicos, flavonoides, alcaloides, terpernoides, poliacetilenos, entre outras classes (ZHANG et al., 2002; VATTEM et al., 2007; SYBIYA VASANTHA PACKIAVATHY et al., 2012). Assim, a busca de inibidores do mecanismo de *QS* que não afetam o crescimento bacteriano, mas agem sobre a virulência, pode auxiliar na problemática da resistência microbiana a antibióticos, uma vez que os mesmos não impõem fortes pressões seletivas para desenvolvimento de resistência em comparação ao uso de antibióticos (KHAN et al., 2009).

As furanonas, compostos produzidos pela alga marinha vermelha *Delisea pulcha*, são amplamente estudadas por funcionarem como antagonistas das AHLs e, portanto, inibir fenótipos regulados pelo *QS* em bactérias (GIVSKOV et al., 1996; CAMPOS-GALVÃO et al., 2016; ALMEIDA et al., 2017). Supõe-se que as furanonas e outros análogos de AHL ligam-se, competitivamente, à proteína receptora análoga à LuxR impedindo a ligação das AHLs e, consequentemente, inibem a ligação do respectivo regulador de transcrição cognato (HENTZER et al., 2003). Givskov et al. (1996) verificaram que furanonas halogenadas interferam no sistema *QS* afetando a motilidade de *Serratia liquefaciens*. O tratamento de *P. aeruginosa* com furanona C-30 a 10 mM, um derivado sintético baseado nas estruturas químicas das furanonas naturais com atividade de interferência de sinais melhorada, reduziu significativamente a produção de fatores de virulência, incluindo exoproteases, piroverdina e quitinase (HENTZER et al., 2003). Além disso, análise de microarranjo de DNA mostrou que 80% dos genes reprimidos pela furanona C-30 também são controlados por *QS* em *P. aeruginosa*, indicando que essa furanona é um inibidor deste mecanismo de comunicação bacteriana (HENTZER et al., 2003). Viana et al. (2009) observaram que furanonas adicionadas ao meio mínimo de sais (MMS) não afetaram o crescimento de *Hafnia alvei* 071. Entretanto, redução significativa ($P < 0,05$) na formação de biofilme foi determinada na presença de 3-metil-2(5H) furanona (MF), 2-metiltetrahidro-3-furanona (MTHF) e 2(5H) -furanona

(F). Também, presença concomitante de uma mistura de furanonas com C12-HSL resultou na inibição da formação de biofilme por *Salmonella* Enteritidis embora não tenha inibido a adesão (CAMPOS-GALVÃO et al., 2016; ALMEIDA et al., 2017).

A atividade anti-*QS* de extratos orgânicos de plantas medicinais tem sido abordada por vários estudos nos últimos anos. Os extratos de plantas podem atuar como inibidores de *QS* devido à semelhança da estrutura química de componentes com as das moléculas sinalizadoras de *QS* e, ou em razão de sua capacidade de degradar os receptores de sinal (VATTEM et al., 2007; AL-HUSSAINI; MAHASNEH, 2009; WU et al., 2014). Extratos de vários produtos naturais, como por exemplo, broto de feijão, camomila, cenoura e alho (RASMUSSEN et al., 2005) e óleos essenciais de várias plantas, como por exemplo lavanda, eucalipto e citrus (SZABÓ et al., 2010) mostraram efeitos anti-*QS*. Alguns extratos de certas variedades de maçãs demonstraram atividade anti-*QS*, provavelmente devido à presença de diferentes polifenóis, como ácidos hidroxicinâmicos, rutina e epicatequina, que atuam como agentes anti-*QS* de forma sinérgica contra *C. violaceum* (FRATIANNI; COPPOLA; NAZZARO, 2011).

Fitol é um composto natural que apresenta uma ampla gama de atividades biológicas, incluindo efeitos anti-inflamatórios, antimicrobianos, antialérgicos, antinociceptivos, imunoestimuladores e antioxidantes (PEJIN et al., 2014). Adicionalmente, o fitol e seus derivados não possuem efeitos inflamatórios ou tóxicos, mesmo em camundongos imunocomprometidos (CHOWDHURY; GHOSH, 2012). Foi demonstrado que o fitol também tem atividade antimicrobiana contra *Mycobacterium tuberculosis* (RAJAB et al., 1997) e *Staphylococcus aureus* (INOUE et al., 2005). Alguns estudos demonstraram o potencial anti-*QS* do fitol por meio de inibições de formação de biofilme, síntese de proteases e alteração da hidrofobicidade em *Serratia marcescens* (SRINIVASAN et al., 2016). Em *P. aeruginosa*, este composto inibiu fenótipos regulados por *QS* em concentrações sub-inibitórias, tais como, a formação de biofilme e motilidade (PEJIN et al., 2014).

O cinamaldeído, principal composto de vários óleos essenciais de plantas e alguns dos seus derivados, pode inibir atividades relacionadas ao *QS*, como a formação de biofilmes (NIU; AFRE; GILBERT, 2006; BRACKMAN et al., 2008). Furocumarinas de origem vegetal inibiram as atividades dos AI-1 e AI-2 em estirpes monitoras do mecanismo de *QS* de *Vibrio harveyi* e também inibiram a formação de biofilmes por agentes patogênicos como *E. coli* enterohemorrágica (EHEC), *S. enterica* sorovar Typhimurium e *P. aeruginosa* (GIRENNAVAR et al., 2008).

Além disso, flavonoides tem sido o foco de pesquisas pela atuação como agentes antioxidantes, anti-inflamatórios e anticancerígenos. Assim, levando em consideração os benefícios para a saúde, os flavanoides como a naringenina, kaempferol, quercetina e apigeneína foram avaliados quanto ao seu potencial anti-*QS* e todos foram capazes de inibir a bioluminescência mediada por AI-1 e AI-2 das estirpes monitoras do mecanismo de *QS* de *V. harveyi* (VIKRAM et al., 2010). Em outro estudo, a quercetina reduziu significativamente os fenótipos dependentes de *QS* em *Klebsiella pneumoniae*, como a formação de biofilmes, a produção de exopolímeros (EPS), a motilidade e a produção de alginato de forma dependente da concentração (GOPU; KOTHANDAPANI; SHETTY, 2015). Também, inibiu significativamente a formação de biofilmes e a produção de fatores de virulência, incluindo piocianina, protease e elastase em *P. aeruginosa* (OUYANG et al., 2016).

Além disso, algumas flavanonas, como a naringenina e a taxifolina, reduziram a produção de piocianina e elastase, as quais são moléculas relacionadas ao *QS* em *P. aeruginosa*, sem afetar o crescimento bacteriano. Esses dois compostos também reduziram a expressão de vários genes que controlam *QS* em *P. aeruginosa*, PAO1. A naringenina também mostrou ser capaz de reduzir, consideravelmente, a produção de mediadores de *QS* tais como *N*-3-oxo-dodecanoil homoserina lactona (3-oxo-C12-AHL) e *N*-butanoil homoserina lactona (C4-AHL) (CHUSRI et al., 2012).

Adicionalmente, substâncias utilizadas na área médica com outra finalidade, como anti-inflamatórios não-esteroides (AINEs), têm sido estudadas em relação à capacidade em inibir o mecanismo de *QS* bacteriano. El-Mowafy et al. (2014) investigaram o potencial anti-*QS* da aspirina, amplamente utilizada como agente antipirético, anti-inflamatório e trombolítico, em *P. aeruginosa* e os resultados demonstraram redução significativa dos sinais do *QS*, como *N*-butiril-homoserina lactona (C4-AHL) e 3-hidroxi-dodecanoil homoserina lactona (3-OH-C12-AHL), além de causar redução significativa na concentração de todos os fatores de virulência testados, como produção de protease total, elastase, hemolisina e piocianina. Os autores também verificaram a interação entre a proteína receptora de AHL deste patógeno, chamada LasR, e a aspirina, utilizando *docking* molecular.

Considerando que alguns genes e fatores de patogenicidade em bactérias têm, reconhecidamente, suas expressões influenciadas pelo *QS* mediado por AI-1, a prospecção de inibidores deste mecanismo de comunicação é de interesse. O *docking* molecular tem sido uma ferramenta comumente usada em estudos de *QS* para sugerir os

locais de ligação entre as proteínas receptoras de sinal e os AIs, bem como na busca de possíveis compostos anti-*QS* de diferentes origens, dentre os quais estão os compostos extraídos de vegetais e algas e os AINEs. Soheili et al. (2015) realizaram uma busca de diferentes AINEs capazes de se ligar às proteínas LasR e PqsE do mecanismo de *QS* de *P. aeruginosa* em comparação com a ligação com 3-oxo-dodecanoil homoserina lactona (3-oxo-C12-AHL) e, a partir dos resultados, sugeriram que piroxicam e meloxicam tem potencial anti-*QS*. Almeida et al. (2018) avaliaram a ligação de 107 compostos de plantas e 73 compostos AINEs com a proteína SdiA de *Salmonella* para inibição do mecanismo *QS* e formação de biofilme. Vários desses, tais como Z-fitol e lonazolac, apresentaram maior afinidade de ligação a SdiA do que AHLs e as furanonas, que são os indutores e inibidores reconhecidos do mecanismo de *QS*, respectivamente. Portanto, esses compostos foram indicados como candidatos potenciais a inibidores de *QS* e sugeridos para testes *in vitro* da inibição do *QS* mediado por AI-1 em *Salmonella* (ALMEIDA et al., 2018).

Esses estudos revelam a importância do mecanismo de *QS* mediado pelo AI-1 na regulação de alguns fenótipos, sendo necessário ampliar as informações a respeito dos processos controlados por essas moléculas. Assim, a partir desses conhecimentos, será possível buscar estratégias de controle de micro-organismos importante na indústria de alimentos e na área médica.

2. REFERÊNCIAS BIBLIOGRÁFICAS

ADONIZIO, A. L. et al. Anti-quorum sensing activity of medicinal plants in southern Florida. **Journal of Ethnopharmacology**, v. 105, n. 3, p. 427–435, 2006.

AL-HUSSAINI, R.; MAHASNEH, A. M. Microbial growth and quorum sensing antagonist activities of herbal plants extracts. **Molecules**, v. 14, n. 9, p. 3425–3435, 2009.

ALMEIDA, F. A. de et al. Acyl homoserine lactone-based quorum sensing stimulates biofilm formation by *Salmonella* Enteritidis in anaerobic conditions. **Archives of Microbiology**, v. 199, n. 3, p. 475–486, 2017a.

ALMEIDA, F. A.; PINTO, U. M.; VANETTI, M. C. D. Novel insights from molecular docking of SdiA from *Salmonella* Enteritidis and *Escherichia coli* with quorum sensing and quorum quenching molecules. **Microbial Pathogenesis**, v. 99, p. 178–190, 2016.

ALMEIDA, F. et al. *N*-dodecanoyl-homoserine lactone influences the levels of thiol and proteins related to oxidation-reduction process in *Salmonella*. **PLoS ONE**, v. 13, n. 10, p. 1–31, 2018a.

ALMEIDA, F. A. et al. Virtual screening of plant compounds and nonsteroidal anti-inflammatory drugs for inhibition of quorum sensing and biofilm formation in *Salmonella*. **Microbial Pathogenesis**, v. 121, p. 369–388, 2018b.

BOYER, M.; WISNIEWSKI-DYÉ, F. Cell-cell signalling in bacteria: Not simply a matter of quorum. **FEMS Microbiology Ecology**, v. 70, n. 1, p. 1–19, 2009.

BRACKMAN, G. et al. Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. **BMC Microbiology**, v. 8, p. 1–14, 2008.

CAMPOS-GALVÃO, M. E. M. et al. Changes in the *Salmonella enterica* Enteritidis phenotypes in presence of acyl homoserine lactone quorum sensing signals. **Journal of Basic Microbiology**, v. 56, n. 5, p. 493–501, 2016.

CARNEIRO, D. G. **INFLUÊNCIA DO MECANISMO DE QUORUM SENSING NO METABOLISMO DE *Salmonella enterica* MINAS GERAIS-BRASIL**. Universidade federal de Viçosa, 2017.

CHOWDHURY, R. R.; GHOSH, S. K. Phytolderived novel isoprenoid immunostimulants. **Frontiers in Immunology**, v. 3, p. 1–11, 2012.

CHUSRI, S.; PHATTHALUNG, P. N.; VORAVUTHIKUNCHAI, S. P. Anti-biofilm activity of *Quercus infectoria* G. Olivier against methicillin-resistant *Staphylococcus aureus*. **Letters in Applied Microbiology**, v. 54, n. 6, p. 511–517, 2012.

DAVIES, D. G. et al. The involvement of cell-to-cell signals in the development of a bacterial biofilm. **Science**, v. 280, p. 295–299, 1998.

DE KIEVIT, T. R.; IGLEWSKI, B. H. Minireview: Bacterial Quorum in pathogenic relationships. **Infection and Immunity**, v. 68, n. 9, p. 4839–4849, 2000.

DE LAMO MARIN, S. et al. Antibody catalyzed hydrolysis of a quorum sensing signal found in gram-negative bacteria. **Bioorganic and Medicinal Chemistry Letters**, v. 17, n. 6, p. 1549–1552, 2007.

DYSZEL, J. L. et al. *E. coli* K-12 and EHEC genes regulated by SdiA. **PLoS ONE**, v. 5, n. 1, p. 1–15, 2010.

ENGEBRECHT, J.; SILVERMAN, M. Identification of genes and gene products necessary for bacterial bioluminescence (lux genes/recombinant DNA/complementation/minicells). **Genetics**, v. 81, p. 4154–4158, 1984.

FRATIANNI, F.; COPPOLA, R.; NAZZARO, F. Phenolic composition and antimicrobial and antiquorum sensing activity of an ethanolic extract of peels from the apple cultivar annurca. **Journal of Medicinal Food**, v. 14, n. 9, p. 957–963, 2011.

FUQUA, C.; PARSEK, M. R.; GREENBERG, E. P. Regulation of gene expression by cell-to-cell communication. **Annual Review of Microbiology**, v. 35, n. 1, p. 439–68, 2001.

FUQUA, C.; WINANS, S.; GREENBERG, E. P. Minireview quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. **Journal of Bacteriology**, v. 176, n. 2, p. 269–275, 1994.

GALLOWAY, W. R. J. D. et al. Quorum sensing in gram-negative bacteria: Small-molecule modulation of AHL and AI-2 quorum sensing pathways. **Chemical Reviews**, v. 111, n. 1, p. 28–67, 2011.

GESKE, G. D. et al. Modulation of bacterial quorum sensing with synthetic ligands: systematic evaluation of *N*-acylated homoserine lactones in multiple species and new insights into their mechanisms of action. **Journal of the American Chemical Society**, v. 129, n. 44, p. 13613–13625, 2007.

GIRENNAVAR, B. et al. Grapefruit juice and its furocoumarins inhibit autoinducer signaling and biofilm formation in bacteria. **International Journal of Food Microbiology**, v. 125, n. 2, p. 204–208, 2008.

GOPU, V.; KOTHANDAPANI, S.; SHETTY, P. H. Quorum quenching activity of *Syzygium cumini* (L.) Skeels and its anthocyanin malvidin against *Klebsiella pneumoniae*. **Microbial Pathogenesis**, v. 79, p. 61–69, 2015.

HENTZER, M. et al. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. **EMBO Journal**, v. 22, n. 15, p. 3803–3815, 2003.

HOUDT, R. Van et al. *N*-acyl- L-homoserine lactone signal interception by E*scherichia coli.* **FEMS Microbiology Letters**, v. 256, p. 83–89, 2006.

INOUE, Y. et al. Biphasic effects of geranylgeraniol, teprenone, and phytol on the growth of *Staphylococcus aureus*. **Antimicrobial Agents and Chemotherapy**, v. 49, n. 5, p. 1770–1774, 2005.

KENDALL, M. M.; RASKO, D. A.; SPERANDIO, V. Global effects of the cell-to-cell signaling molecules autoinducer-2, autoinducer-3, and epinephrine in a *luxS* mutant of enterohemorrhagic *Escherichia coli*. **Infection and Immunity**, v. 75, n. 10, p. 4875–4884, 2007.

KHAN, M. S. A. et al. Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. **Letters in Applied Microbiology**, v. 49, n. 3, p. 354–360, 2009.

KYOKO, K. et al. SdiA, an *Escherichia coli* homolog of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. **Molecular Microbiology**, v. 38, n. 4, p. 805–816, 2002.

LEE, J. et al. Indole cell signaling occurs primarily at low temperatures in *Escherichia coli*. **International Society for Microbial Ecology**, v. 1, p. 1007–1023, 2008.

LEE, J.; JAYARAMAN, A.; WOOD, T. K. Indole is an inter-species biofilm signal mediated by SdiA. **BMC Microbiology**, v. 15, p. 1–15, 2007.

LIOU, L. et al. Chemical sensing in mammalian host-bacterial commensal associations. **Proceedings of the National Academy of Sciences**, v. 107, n. 28, p. 12734–12734, 2010.

McCLEAN, K. H. et al. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. **Microbiology**, v. 143, p. 3703–3711, 1997a.

MICHAEL, B. et al. SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. **Journal of Bacteriology**, v. 183, n. 19, p. 5733–5742, 2001.

NEALSON, K. H.; PLATT, T.; HASTINGS, J. W. Cellular control of the synthesis and activity of the bacterial luminescent. **Microbiology**, v. 104, n. 1, p. 313–322, 1970.

NIU, C.; AFRE, S.; GILBERT, E. S. Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. **Letters in Applied Microbiology**, v. 43, n. 5, p. 489–494, 2006.

OUYANG, J. et al. Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa*. **Journal of Applied Microbiology**, v. 120, n. 4, p. 966–974, 2016.

PARSEK, M. R.; GREENBERG, E. P. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. **Proceedings of the National Academy of Sciences**, v. 97, n. 16, p. 8789–8793, 2000.

PEJIN, B. et al. Anti-quorum sensing activity of selected sponge extracts: A case study

of *Pseudomonas aeruginosa*. **Natural Product Research**, v. 28, n. 24, p. 2330–2333, 2014.

RAINA, S. et al. Microbial quorum sensing: a tool or a target for antimicrobial therapy? **Biotechnology and Applied Biochemistry**, v. 54, n. 2, p. 65–84, 2009.

RAJAB, M. S. et al. Antimycobacterial activity of (E)-phytol and derivatives: a preliminary structure-activity study. **Planta Medica**, v. 64, n. 1, p. 2–4, 1997.

RASMUSSEN, T. B. et al. Screening for quorum-sensing inhibitors (QSI) by use of a

novel genetic system, the QSI selector. **Journal of Bacteriology**, v. 187, n. 5, p. 1799–1814, 2005.

SHARMA, V. K.; BEARSON, S. M. D. Microbial pathogenesis evaluation of the impact of quorum sensing transcriptional regulator SdiA on long-term persistence and fecal shedding of *Escherichia coli* O157 : H7 in weaned calves. **Microbial Pathogenesis**, v. 57, p. 21–26, 2013.

SMITH, J.; FRATAMICO, P.; NOVAK, J. Quorum sensing: a primer for food microbiologists. **Journal of Food Protection**, v. 67, n. 5, p. 1053–1070, 2004.

SMITH, N.; AHMER, B. M. M. Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. **Journal of Applied Microbiology**, v. 185, n. 4, p. 1357–1366, 2003.

SMITH, R. S.; IGLEWSKI, B. H. *P. aeruginosa* quorum-sensing systems and virulence. **Current Opinion in Microbiology**, v. 6, n. 1, p. 56–60, 2003.

SRINIVASAN, R. et al. *Piper betle* and its bioactive metabolite phytol mitigate quorum sensing mediated virulence factors and biofilm of nosocomial pathogen *Serratia marcescens in vitro*. **Journal of Ethnopharmacology**, v. 3, n. 1, p. 70–73, 2016.

STEENACKERS, H. et al. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. **Food Research International**, v. 45, n. 2, p. 502–531, 2012.

STEINDLER, L.; VENTURI, V. Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. **FEMS Microbiology Letters**, v. 266, n. 1, p. 1–9, 2007.

SYBIYA VASANTHA PACKIAVATHY, I. A. et al. Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against Gram negative bacterial pathogens. **Food Research International**, v. 45, n. 1, p. 85–92, 2012.

SZABÓ, M. et al. Inhibition of quorum-sensing signals by essentials oils. **Phytotherapy Research**, v. 24, n. 1, p. 782–786, 2010.

VATTEM, D. A. et al. Dietary phytochemicals as quorum sensing inhibitors. **Fitoterapia**, v. 78, n. 4, p. 302–310, 2007.

VIKRAM, A. et al. Suppression of bacterial cell-cell signaling, biofilm formation and type III secretion system by citrus flavonoids. **Journal of Applied Microbiology**, v. 109, n. 2, p. 515–527, 2010.

WALTERS, M.; SPERANDIO, V. Quorum sensing in *Escherichia coli* and *Salmonella*. **International Journal of Medical Microbiology**, v. 296, n. 2–3, p. 125–131, 2006.

WHITELEY, M.; LEE, K.; GREENBERG, E. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginos*a. **Clinical Infectious Diseases**, v. 96, n. 24, p. 13904–13909, 1999.

WU, D. et al. Sodium houttuyfonate affects production of *N*-acyl homoserine lactone and quorum sensing-regulated genes expression in *Pseudomonas aeruginosa*. **Frontiers in Microbiology**, v. 5, n. 635, p. 1–9, 2014.

ZHANG, J. M. et al. Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. **Meitan Xuebao/Journal of the China Coal Society**, v. 85, n. 6, p. 57–72, 2002.

CHAPTER 2

Plant compounds and nonsteroidal anti-inflammatory drugs regulate quorum sensing in *Chromobacterium violaceum*

Plant compounds and nonsteroidal anti-inflammatory drugs regulate quorum sensing in *Chromobacterium violaceum*

Authors: Erika Lorena Giraldo Vargas[a], Felipe Alves de Almeida[b], Uelinton Manoel Pinto[c], Maria Cristina Dantas Vanetti[a]*

[a]Department of Microbiology, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil;

[b]Department of Nutrition, Universidade Federal de Juiz de Fora (UFJF), Governador Valadares, MG, Brazil;

[c]Food Research Center, Department of Food and Experimental Nutrition, Universidade de São Paulo (USP), São Paulo, SP, Brazil.

*Corresponding author: Department of Microbiology, Universidade Federal de Viçosa, Viçosa, MG, Brazil. E-mail address: mvanetti@ufv.br

Abstract

Chromobacterium violaceum is Gram-negative water and saprophytic soil bacterium but can also infect humans and cause abscesses and bacteremia in immunocompromised individuals. Most strains produce the antibacterial purple pigment violacein which is regulated via the CviI/CviR quorum sensing system via *N*-Acyl-L-homoserine lactones (AHLs). Plant and nonsteroidal anti-inflammatory drugs (NASIDs) compounds have been reported as inhibitors of the quorum sensing mechanism in bacteria and, virtual screening of some of these compounds against CviR protein of *C. violaceum* has been performed in this study. The ability of the evaluated compounds to inhibit violacein production *in vitro* has also been evaluated. In general, results of molecular docking showed that plant compounds, phytol, margaric and palmitic acids, and the NASIDs, dipyrone sodium, ketoprofen and phenylbutazone bound in the structures of CviR protein of *C. violaceum*, and some of them had higher binding affinities than the AHLs and furanone, recognized inducers, and inhibitors of quorum sensing, respectively. When tested *in vitro*, palmitic and margaric acids, at a concentration of 600 µg/mL and ketoprofen at a concentration of 500 µg/mL showed a reduction in the violacein production by strains of *C. violaceum* and this phenotype could be related to the disruption of the quorum sensing system. The results indicate that *in silico* prospection compounds

to inhibit the quorum sensing mechanism may be a good tool to find alternative compounds.

Keywords: Plant compounds, anti-inflammatory drugs, anti-quorum sensing, violacein.

1. Introduction

Quorum sensing is a process of bacterial cell-cell communication in which cells produce, detect, and respond to extracellular signaling molecules called autoinducers. Gram-negative bacteria typically use *N*-acyl-L-homoserine lactone (AHL), an autoinducer-1 (AI-1) that can diffuse into the local environment (Smith and Iglewski, 2003; Boyer and Wisniewski-Dyé, 2009; Galloway *et al.,* 2011). Signal concentration increases with population density and, once the AHLs concentration reaches a threshold level within the cell, these signals bind to their cognate intracellular receptors, the LuxR-type family of transcriptional regulators. The activated LuxR-type receptors alter gene expression levels, including a range of phenotypes such as, sporulation, biofilm formation, motility, bacteriocin and toxin production, conjugation, competence, virulence gene expression, pigment and bioluminescence production, among others (Nealson, Platt and Hastings, 1970; Geske *et al.,* 2007; Raina *et al.,* 2009).

Chromobacterium violaceum, a Gram-negative water and soil bacterium, can infect humans and cause abscesses and bacteremia (Stauff and Bassler, 2011). This bacterium produces the antibacterial purple pigment violacein, synthesized from tryptophan by the products of the *vioABCD* operon, which is regulated by quorum sensing mediated by AI-1 (Stauff and Bassler, 2011). These autoinducers, known as AHLs, are synthesized via the CviI/CviR system, a LuxI/LuxR homolog, where CviI is an AHL synthase, and CviR is a receptor that, when bound to AHLs, modulate the regulation of target genes. CviR is a homodimeric protein, each monomer of which consists of two domains, a ligand-binding domain (LBD) and a DNA-binding domain (DBD) (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). *C. violaceum* ATCC 12472 a wild type strain, produces and responds to AHLs, mainly *N*-(3-hydroxydecanoyl)-DL-homoserine lactone (3-OH-C10-HSL) (Morohoshi *et al.,* 2008) while the strain *C. violaceum* ATCC 31532 produces mainly *N*-hexanoyl-DL-homoserine lactone (C6-HSL). *C. violaceum* CV026, a biomonitor commonly used to evaluate quorum sensing, is a Tn5 mutant strain derived from wild type *C. violaceum* ATCC 31532, unable to produce its own AHL, but retain

the ability to respond to exogenous AHLs (McClean *et al.,* 1992). Wild-type and biosensor mutant strains of *C. violaceum* have been used as a model in diverse studies related to the bacterial communication and quorum sensing inhibition, known as quorum quenching (McClean *et al.,* 1997; Adonizio *et al.,* 2006; Vattem *et al.,* 2007; Ravichandran *et al.,* 2018).

The discovery of the quorum sensing system and its critical role in bacterial survival and virulence has revealed a novel way to attack and attenuate bacterial pathogenicity. The significant advantage of this novel strategy for anti-infective therapy is that it circumvents the problem of antibiotic resistance, which is intimately connected to the use of conventional antibacterial agents, as it specifically interferes with the expression of pathogenic traits rather than to hamper bacterial growth (Singh, Mishra, and Jha 2017; Ravichandran *et al*., 2018). Furanones are compounds produced by the red seaweed *Delisea pulcha*, which have been widely studied for acting as antagonists of AHLs and, therefore, inhibit phenotypes regulated by quorum sensing in bacteria (Givskov *et al*., 1996; Campos-Galvão *et al*., 2016; Almeida *et al*., 2017). Furanones and other AHL analogs are supposed to competitively bind to the LuxR analog receptor protein and prevent binding of the AHLs and, consequently, inhibit binding of the respective cognate transcriptional regulator (Givskov *et al.,* 1996).

Plants compounds and nonsteroidal anti-inflammatory drugs (NSAIDs), substances used in the medical field for other purposes, have been widely screened for compounds that interfere with mechanisms of quorum sensing (Adonizio *et al.,* 2006; El-Mowafy *et al.,* 2014; Singh, Mishra and Jha, 2017). Chaudhari *et al.* (2014) investigated the effect of seed extracts of three different plants on quorum sensing and showed inhibition of the violacein production in *C. violaceum* and reduced motility in *Pseudomonas aeruginosa*, both mechanisms regulated by AHLs. Burt *et al.* (2014) showed that the natural antimicrobial carvacrol inhibited biofilm formation and violacein production in *C. violaceum*.

Taking into account that some genes and pathogenicity factors in bacteria have been known to be influenced by the quorum sensing mediated by AI-1, the prospection of inhibitors of this mechanism of communication is of interest. Molecular docking has been a commonly used tool in quorum sensing studies to suggest binding sites between signal receptor proteins and autoinducers, as well as in the search for possible quorum quenching compounds from different sources. Ravichandran *et al*. (2018) performed a

virtual screening of different compounds in *C. violaceum*, and it was reported that quorum sensing inhibitors (QSIs) and the natural autoinducer showed a high binding affinity for the CviR protein. Also, Almeida *et al*. (2018) performed a virtual screening of 107 plant compounds and 73 NASIDs for inhibition of biofilm formation in *Salmonella* Enteritidis PT4 578 and showed that these compounds bound in, at least, one of three modeled structures of the SdiA protein, an analog of LuxR, acting as possible inhibitors of the mechanism of quorum sensing. Considering the importance of studies focused on the screening of inhibitory compounds of quorum sensing mechanism, the aims of the present study were to perform molecular docking of plant compounds and NASIDs, as well as AHLs and furanones with CviR proteins from strains of *C. violaceum* in order to prospect inhibitors of the mechanism of quorum sensing and to evaluate their activity *in vitro*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used for quorum sensing *in vitro* assays were *C. violaceum* ATCC 12472 and *C. violaceum* CV026. The cultures were stored at -20 °C in Luria Bertani broth (LB, Sigma Aldrich, USA) supplemented with 40% (v/v) of sterilized glycerol. Before each experiment, strains of *C. violaceum* were activated in LB broth for 48 h at 30 °C and, for *C. violaceum* CV026 growth, the LB broth was supplemented with 20 $\mu g.mL^{-1}$ of kanamycin.

2.2. Preparation of compounds solution

The subinhibitory concentrations of the plant compounds, anti-inflammatory drugs, and AHL used in the experiments were evaluated in preliminary studies and based on previously published studies. These compounds were suspended as described in Table 1 and stored at -20 °C.

Table 1. Diluents used to prepare the plant compounds, NSAIDs and AHL and final concentrations used for experiments in the LB broth.

Classification	Compound	Company	Solvent	Final concentration in LB broth
Plant compound	Phytol	Sigma-Aldrich, USA	Methanol	600 µg/mL
	Margaric acid	Sigma-Aldrich, USA	Isopropanol	600 µg/mL
	Palmitic acid	Sigma-Aldrich, USA	Dimethylsulfoxide*	600 µg/mL
NASID	Dipyrone sodium	All Chemistry, Brazil	Sterile water	500 µg/mL
	Ketoprofen	All Chemistry, Brazil	Methanol	500 µg/mL
	Phenylbutazone	Sigma-Aldrich, USA	Acetone	500 µg/mL
AHL	C6-HSL	Sigma-Aldrich, USA	Acetonitrile	10 µM

*Dimethylsulfoxide = DMSO

2.3. Anti-quorum sensing activity by quantification of violacein production of *C. violaceum*

The cultures of *C. violaceum* ATCC 12472 and *C. violaceum* CV026 activated according to item 2.1, were reactivated in 20 mL of LB broth for 18 h at 30 °C. The optical density 600 nm (OD 600 nm) of these cultures were standardized to 0.1 using a spectrophotometer (Thermo Fisher Scientific, Finland) and, an aliquot of 20 µL of this standard inoculum was added in a microtube containing 1 mL of LB broth. Posteriorly, in different microtubes, the plant compounds phytol, margaric acid, and palmitic acid and the NASIDs dipyrone sodium, ketoprofen, and phenylbutazone were added at the final concentration indicated in Table 1. The incubation was at 30 °C for 24 h. It is noteworthy that, for *C. violaceum* CV026 cultivation, C6-HSL was added at a final concentration of 10 µM (Zhu, He and Chu, 2011) (Table 1). After incubation time, growth was evaluated in a spectrophotometer at 600 nm, and violacein production was quantified according to Blooser and Gray (1998). Briefly, the culture was centrifuged at 10,000 x *g* for 10 min, and the supernatant was discarded. The pellet was resuspended in 1 mL of DMSO, mixed vigorously for 30 s in a vortex and centrifuged at 10,000 x *g* for 10 min. An aliquot of 300 µL of supernatant was added in a microplate, and the OD 585 nm was measured by using a spectrophotometer. Percent inhibition of production of violacein was measured

as the ratio of OD585/OD600, which is violacein production per unit of growth (Choo, Rukayadi and Hwang, 2006).

2.4. Comparison of the ligand-binding domain of structures of CviR proteins

The structures of CviR proteins of *C. violaceum* ATCC 12472 (PDB: 3QP6, 3QP8) and *C. violaceum* ATCC 31532 (PDB: 3QP1, 3QP2, 3QP4, 3QP5) were obtained from the RCSB Protein Data Bank database (PDB; http://www.rcsb.org/pdb/home/home.do) (Table 2) and superposed to compare the ligand-binding domain (LBD) by CLC Drug Discovery Workbench 4.0 software.

Table 2. Structures of CviR proteins of *C. violaceum* ATCC 12472 and *C. violaceum* ATCC 31532 obtained from the PDB database.

Bacterial strains	PDB ID	PDB description	Effect of ligand
C. violaceum ATCC 12472	3QP6	CviR bound to C6-HSL	Inhibitor
	3QP8	CviR bound to C10-HSL	Inducer
C. violaceum ATCC 31532	3QP1	CviR bound to C6-HSL	Inducer
	3QP2	CviR bound to C8-HSL	Weak inducer
	3QP4	CviR bound to C10-HSL	Inhibitor
	3QP5	CviR bound to chlorolactone	Inhibitor

Chen *et al*. (2011)

2.5. Molecular docking of CviR proteins of *C. violaceum* with different compounds

The compounds selected for this study were four plant compounds, three NSAIDs, 14 AHLs, seven furanones, and 1-octanoyl-*rac*-glycerol (OCL), totaling 29 compounds (Table 3).

The plant compounds were classified according to their leading functional group, and the classification of NSAIDs was obtained from the KEGG Drug database (http://www.genome.jp/kegg/drug/) (Table 3). The structures of these compounds were obtained from the compound Identifier of the PubChem database (PubChem CID; https://pubchem.ncbi.nlm.nih.gov/https://pubchem.ncbi.nlm.nih.gov/) for molecular docking.

Table 3. Quorum sensing and quorum quenching compounds used for molecular docking with structures of CviR proteins of *C. violaceum* ATCC 12472 and *C. violaceum* ATCC 31532.

Group	Classification	Compound	Pubchem CID
Plant compound	Fatty acid	Margaric acid	10465
		Palmitic acid	985
	Oxygenated diterpene	Z-phytol	6430833
		E-phytol	5280435
NSAID	Propionic acid derivative	Ketoprofen	3825
	Pyrazolone derivative	Dipyrone (Metamizole)	3111
		Phenylbutazone	4781
AHL	Unmodified in 3-oxo and 3-OH	*N*-dodecanoyl-DL-homoserine lactone	11565426
		N-decanoyl-DL-homoserine lactone	11644562
		N-octanoyl-DL-homoserine lactone	3474204
		N-hexanoyl-DL-homoserine lactone	3462373
		N-butyryl-DL-homoserine lactone	443433
	Modified in 3-oxo	*N*-(3-oxododecanoyl)-L-homoserine lactone	3246941
		N-(3-oxodecanoyl)-L-homoserine lactone	10221060
		N-(3-oxooctanoyl)-L-homoserine lactone	127293
		N-(3-oxohexanoyl)-L-homoserine lactone	688505
	Modified in 3-OH	*N*-(3-hydroxydodecanoyl)-DL-homoserine lactone	11507677
		N-(3-hydroxydecanoyl)-DL-homoserine lactone	71353010
		N-(3-hydroxyoctanoyl)-DL-homoserine lactone	11586792
		N-(3-hydroxyhexanoyl)-DL-homoserine lactone	70185030
		N-(3-hydroxybutyryl)-L-homoserine lactone	10330086
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone	10180544
		4-bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone	16127328
		4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	9839657
		5-(bromomethylene)-2(5H)-furanone	9877509
		4-bromo-5-(bromomethylene)-2(5H)-furanone	10131246
	Non-brominated	3-butyl-2(5H)-furanone	11768654
		2,2-dimethyl-3(2H)-furanone	147604
OCL	OCL	1-octanoyl-*rac*-glycerol	3033877

The molecular docking of CviR proteins of *C. violaceum* ATCC 12472 and *C. violaceum* ATCC 31532 (Table 2) was performed with compounds (Table 3) by using the "Dock Ligands" tool of the CLC Drug Discovery Workbench 4.0 software, with 1000 interactions for each compound being performed. These compounds tested were quorum sensing and potential quorum quenching compounds of quorum sensing mechanism mediated by AI-1 (Table 3). The score generated is related to the potential energy change

when the protein and the compound come together based on hydrogen bonds, metal ions, and steric interactions, where scores more negative correspond to higher binding affinities (Almeida, Pinto and Vanetti 2016; Almeida *et al.,* 2018).

Subsequently, molecular docking was performed with the structures linked to natural autoinducers of each strain, 3QP8 (linked to C10-HSL) for *C. violaceum* ATCC 12472 and 3QP2 (linked to C6-HSL) for *C. violaceum* ATCC 31532 with different compounds that showed the best inhibition of violacein production in *C. violaceum* strains, according to analysis performed at 2.3.

2.6. Statistical analyses

Experiments were carried out in three biological replicates. The values of the triplicates were used for the analysis of variance (ANOVA) followed by Tukey's test using the Statistical Analysis System and Genetics Software (Ferreira, 2011). A *p*-value < 0.05 ($p < 0.05$) was considered to be statistically significant.

3. Results and discussion

3.1. Comparison of the ligand-binding domain of CviR proteins

The structures of CviR proteins of *C. violaceum* ATCC 12472 and ATCC 31532 crystallized with different AHLs and chlorolactone were superposed and, in general, binding of the same protein to different ligands alters the conformation of the LBD domain (Figures 1A, 1B, 1C and 1D). Conformational differences of the LBD were also detected between the CviR proteins of *C. violaceum* ATCC 12472 and *C. violaceum* ATCC 31532 (Figures 1E and 1F). Noticeable structural differences at the ligand-binding sites AHL-bound in the LBD domain of the SdiA protein of *Escherichia coli* Enterohemorrhagic, a CivR analog, were observed by Nguyen *et al.* (2015). The comparison of the generated macromolecular structures of SdiA protein of *Salmonella* with their templates showed that the LBD and DBD had slight conformational changes depending upon the presence or absence of AHL, as well as to the size of the AHL carbon chain (Almeida, Pinto and Vanetti, 2016).

Taking into account the changes in protein conformation according to the amino acid sequence and the binding sites of autoinducer molecules with specific amino acids, Chen *et al.* (2011) showed that in *C. violaceum* ATCC 31532, the non-conserved amino

acid M89 is a crucial point for the antagonist or agonist activity of CviR protein when bound to autoinducers. Moreover, a comparison of the C6-HSL, the natural autoinducer of this strain, with C8, C10-HSL, and chlorolactone complexes revealed that these last molecules cause the side chain of M89 to switch to the "antagonist" position of this protein (Chen *et al*. 2011). Thus, depending on the signaling molecule of the quorum sensing mechanism that interacts with the significant amino acids, the conformation of the CviR protein can change and thus act as agonist or antagonist of the bacterial communication system (Chen *et al*. 2011).

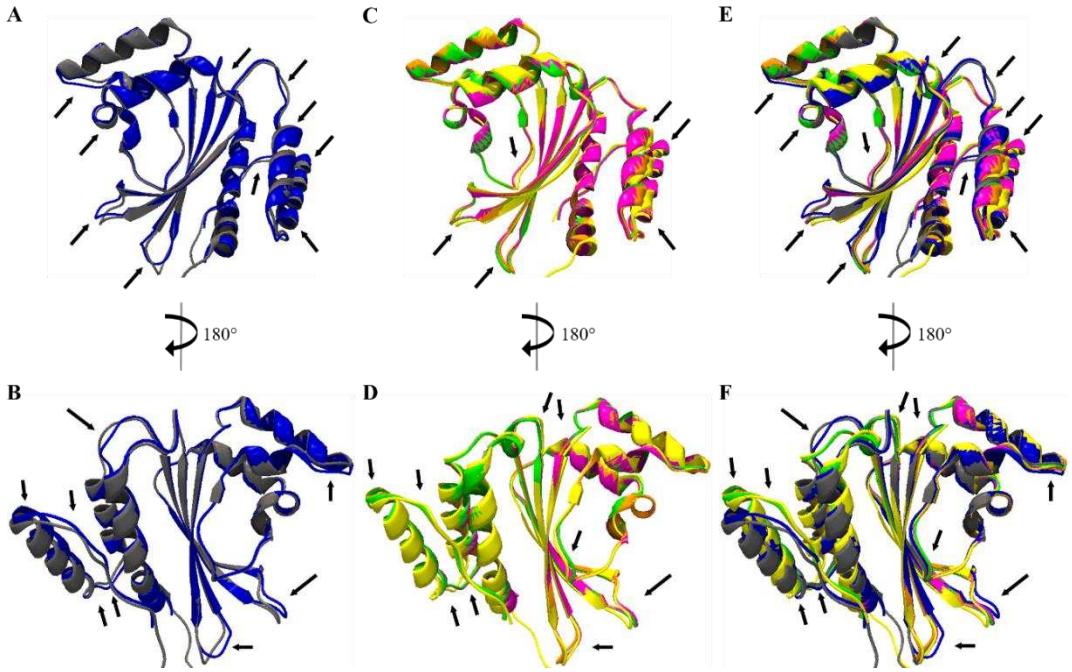

Figure 1. Superposition of LBD structure monomer of CviR protein of *C. violaceum.* **A and B:** 3QP6 (gray) and 3QP8 (blue) structures of *C. violaceum* ATCC 12472; **C and D:** 3QP1 (pink), 3QP2 (orange), 3QP4 (green) and 3QP5 (yellow) structures of *C. violaceum* ATCC 31532; **E and F:** Conformational differences of the LBD between the CviR proteins of *C. violaceum* ATCC 12472 and *C. violaceum* ATCC 31532. Black arrows show conformational changes.

Therefore, considering that these conformational changes may alter binding affinities with ligands as well as the response to the mechanism of quorum sensing, all structures of the CviR proteins of the two strains were used to perform molecular docking with different compounds.

3.2. Molecular docking of structures of CviR proteins of *C. violaceum* with different compounds

Molecular docking of 3QP6 and 3QP8 structures of the CviR protein of *C. violaceum* ATCC 12472 and of 3QP1, 3QP2, 3QP4 and 3QP5 of *C. violaceum* ATCC 31532 showed that AHLs with 12 carbons, with or without 3-oxo and 3-OH modifications presented the highest binding affinities (Tables 4 and 5). The AHLs with 10 carbons with the same modifications also showed high binding affinities for the structures evaluated for these strains (Tables 4 and 5).

It is noteworthy that the *N*-(3-hydroxydecanoyl)-DL-homoserine lactone (3-OH-C10-HSL) showed high binding affinity, reaching a score of -84.80 with 3QP6 structure and -82.24 with the 3QP8 structure of *C. violaceum* ATCC 12472 (Table 4). This AHL is recognized as the most synthesized by *C. violaceum* ATCC 12472 (Morohoshi *et al.,* 2008) and induced violacein production by this strain (Morohoshi *et al.,* 2008; Chen *et al.,* 2011). Besides C10-HSL, C12-HSL was also identified as an inducer of violacein production by *C. violaceum* VIR07, an AHL deficient mutant of *C. violaceum* ATCC 12472 (Morohoshi *et al.,* 2008). Conversely, long-chain acyl AHLs such as C10-HSL and C12-HSL inhibited the production of violacein by *C. violaceum* strain ATCC 31532 (McClean *et al.,* 1997).

Also, AHLs with carbon size chains ranging from four to eight showed lower binding affinities than the AHLs with 10 and 12 carbons. Interestingly, C6-HSL which is an autoinducer naturally synthesized by *C. violaceum* ATCC 31532 (McClean *et al.,* 1997; Chen *et al.,* 2011) had one of the lowest binding affinities with the four structures of CviR protein of this strain (Table 5). These results suggest that C10-AHL and C12-AHL can compete more efficiently for binding to CviR protein than the natural C6-AHL autoinducer and act as inhibitors of violacein production, as demonstrated experimentally by McClean *et al*. (1997). Our results of molecular docking showing that the longer the carbon chain of AHL without 3-oxo and 3-OH modification higher the binding affinity with the CviR protein of *C. violaceum* (Table 4 and 5) may corroborate previous experimental results of the agonist or antagonistic effect by autoinducers molecules and help elucidate the molecular action mechanism.

Table 4. Results of docking molecular of two structures of CviR protein of *C. violaceum* ATCC 12472 with different compounds.

Group	Classification	Molecule	3QP6			3QP8		
			Binding residue	Score	Rank	Binding residue	Score	Rank
AHL	Modified in 3-OH	*N*-(3-hydroxydodecanoyl)-DL-homoserine lactone	Y80, W84, Y88, D97, S155	-89.09	1	Y80, W84, Y88, S155	-88.87	1
AHL	Modified in 3-oxo	*N*-(3-oxododecanoyl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-88.55	2	Y80, W84, Y88, D97, S155	-86.09	2
AHL	Unmodified in 3-oxo and 3-OH	*N*-dodecanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-88.41	3	Y80, W84, D97, S155	-85.88	3
Plant	Oxygenated diterpene	Z-phytol	M135	-81.57	7	Y88, S89, N92	-83.67	4
AHL	Modified in 3-OH	*N*-(3-hydroxydecanoyl)-DL-homoserine lactone	Y80, W84, Y88, D97, S155	-84.80	4	Y80, W84, Y88, S155	-82.24	5
Plant	Oxygenated diterpene	E-phytol	M135	-80.45	8	S89, N92	-81.56	6
AHL	Unmodified in 3-oxo and 3-OH	*N*-decanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-81.97	6	Y80, W84, D97, S155	-81.18	7
AHL	Modified in 3-oxo	*N*-(3-oxodecanoyl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-83.77	5	Y80, W84, Y88, D97, S155	-80.86	8
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-	S155	-77.30	9	Y80, S155	-76.14	9
Plant	Fatty acid	Margaric acid	L85	-77.19	10	Y88, N92, A94	-74.78	10
AHL	Modified in 3-oxo	*N*-(3-oxooctanoyl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-75.68	11	Y80, W84, Y88, D97, S155	-73.99	11
AHL	Unmodified in 3-oxo and 3-OH	*N*-octanoyl-DL-homoserine lactone	Y80, W84, S155	-74.88	12	Y80, W84, D97, S155	-73.83	12
NSAID	Pyrazolone derivative	Dipyrone (metamizole)	W84	-69.34	16	L85	-72.17	13
Plant	Fatty acid	Palmitic acid	Y88, A94	-74.21	13	L85	-71.04	14
NSAID	Propionic acid derivative	Ketoprofen	S155	-73.94	14	Y80, S155	-70.54	15
AHL	Modified in 3-OH	*N*-(3-hydroxyhexanoyl)-DL-homoserine lactone	Y80, W84, Y88, D97, S155	-69.16	17	Y80, W84, Y88, S155	-69.09	16
AHL	Modified in 3-OH	*N*-(3-hydroxyoctanoyl)-DL-homoserine lactone	Y80, Y88, S155	-69.95	15	Y80, Y88, S155	-67.61	17
AHL	Modified in 3-oxo	*N*-(3-oxohexanoyl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-67.53	18	Y80, W84, Y88, D97, S155	-66.52	18
AHL	Unmodified in 3-oxo and 3-OH	*N*-hexanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-67.10	19	Y80, W84, S155	-66.45	19
OCL	OCL	1-octanoyl-*rac*-glycerol	Y80, D97, M135, S155	-61.08	20	Y80, D97, M135, S155	-58.40	20
AHL	Modified in 3-OH	*N*-(3-hydroxybutyryl)-L-homoserine lactone	Y80, W84, D97, S155	-56.96	21	Y80, W84, S155	-57.06	21
AHL	Unmodified in 3-oxo and 3-OH	*N*-butyryl-DL-homoserine lactone	Y80, S155	-51.77	22	Y80, S155	-50.51	22
Furanone	Non-brominated	3-butyl-2(5H)-furanone	W84	-50.57	23	W84	-50.26	23
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-butyl-2(5H)-	-	-	-	S155	-49.12	24
Furanone	Brominated	5-(bromomethylene)-2(5H)-furanone	Y80	-35.29	24	Y80	-34.36	25
Furanone	Brominated	4-bromo-5-(bromomethylene)-2(5H)-furanone	Y80, T140, S155	-34.42	25	W84	-33.58	26
Furanone	Non-brominated	2,2-dimethyl-3(2H)-furanone	Y80	-31.76	26	Y80	-31.12	27
NSAID	Pyrazolone derivative	Phenylbutazone	-	-	-	-	-	-
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone	-	-	-	-	-	-

Molecular docking = Binding affinity scale between CviR protein and the compounds with a color ramp ranging from dark pink (higher affinity) to dark green (lower affinity) and hyphen for no binding.

Table 5. Results of docking molecular of four structures of CviR protein of *C. violaceum* ATCC 31532 with different compounds.

Group	Classification	Molecule	3QP1			3QP2			3QP4			3QP5		
			Binding residue	Score	Rank	Binding residue	Score	Rank	Binding residue	Score	Rank	Binding residue	Score	Rank
AHL	Modified in 3-oxo	*N*-(3-oxododecanoyl)-L-homoserine lactone	Y80, W84, D97, S155	-84.06	1	Y80, W84, Y88, D97, S155	-84.02	1	Y80, W84, Y88, D97, S155	-84.81	3	Y88, S155	-73.52	4
AHL	Unmodified in 3-oxo and 3-OH	*N*-dodecanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-83.12	2	Y80, W84, D97, S155	-82.60	3	Y80, W84, D97, S155	-85.20	1	S155	-74.81	2
AHL	Modified in 3-OH	*N*-(3-hydroxydodecanoyl)-DL-homoserine lactone	Y80, W84, D97, S155	-80.77	3	Y80, W84, S155	-83.36	2	Y80, W84, D97, S155	-85.17	2	W84	-71.97	6
AHL	Modified in 3-OH	*N*-(3-hydroxydecanoyl)-DL-homoserine lactone	Y80, W84, D97, S155	-79.30	4	Y80, W84, D97, S155	-78.56	8	Y80, W84, D97, S155	-80.54	6	Y88, S155	-68.49	10
Plant	Oxygenated diterpene	Z-phytol	V75	-78.35	5	N92	-79.34	5	-	-	-	N77	-74.16	3
AHL	Unmodified in 3-oxo and 3-OH	*N*-decanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-77.75	6	Y80, W84, D97, S155	-78.64	7	Y80, W84, D97, S155	-81.31	5	W84, D97, S155	-70.18	8
AHL	Modified in 3-oxo	*N*-(3-oxodecanoyl)-L-homoserine lactone	Y80, W84, D97, S155	-77.71	7	Y80, W84, Y88, D97, S155	-79.77	4	Y80, W84, Y88, D97, S155	-81.62	4	W84, Y88, D97, S155	-70.31	7
AHL	Modified in 3-oxo	*N*-(3-oxooctanoyl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-74.51	8	Y80, W84, Y88, D97, S155	-72.51	10	Y80, W84, D97, S155	-72.28	11	W84, D97, S155	-63.94	16
Plant	Oxygenated diterpene	E-phytol	V75	-74.40	9	M89	-79.13	6	V75, N77	-76.80	7	L72	-76.70	1
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone	W84	-73.80	10	S155	-74.70	9	W84	-76.67	8	-	-	-
AHL	Unmodified in 3-oxo and 3-OH	*N*-octanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-73.32	11	Y80, W84, D97, S155	-72.19	11	Y80, W84, D97, S155	-72.78	10	W84, D97, S155	-64.34	15
NSAID	Pyrazolone derivative	Dipyrone (metamizole)	W84, Y88	-68.91	12	L85	-70.25	13	L85	-71.22	13	W84	-72.99	5
Plant	Fatty acid	Margaric acid	M135	-68.62	13	W84	-70.49	12	M135	-72.88	9	M89, N92	-69.31	9
NSAID	Propionic acid derivative	Ketoprofen	Y80, S155	-67.70	14	Y80, S155	-67.71	15	Y80, S155	-70.04	14	Y88	-68.43	11
AHL	Modified in 3-OH	*N*-(3-hydroxyhexanoyl)-DL-homoserine lactone	Y80, W84, Y88, D97, S155	-66.86	15	Y80, W84, Y88, S155	-65.81	17	Y80, W84, Y88, D97, S155	-66.47	16	W84, S155	-58.37	19
AHL	Modified in 3-oxo	*N*-(3-oxohexanoyl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-66.50	16	Y80, W84, Y88, D97, S155	-64.73	18	Y80, W84, Y88, D97, S155	-66.20	17	W84, Y88, D97, S155	-58.80	18
AHL	Modified in 3-OH	*N*-(3-hydroxyoctanoyl)-DL-homoserine lactone	W84, Y88, D97	-65.70	17	Y80, Y88, S155	-66.06	16	Y80, Y88, S155	-67.21	15	Y80	-64.36	14
Plant	Fatty acid	Palmitic acid	M135	-65.30	18	M89, N92, A94	-68.38	14	Y88, A94	-71.96	12	N92	-65.16	13

AHL	Unmodified in 3-oxo and 3-OH	*N*-hexanoyl-DL-homoserine lactone	Y80, W84, D97, S155	-64.90	19	Y80, W84, D97, S155	-63.88	19	Y80, W84, D97, S155	-64.88	18	W84, S155	-57.57	20
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone	W84	-59.09	20	-	-	-	S155	-59.07	19	-	-	
OCL	OCL	1-octanoyl-*rac*-glycerol	Y80, M135, S155	-58.10	21	Y80, D97, M135, S155	-58.75	20	L85, Y88	-58.87	20	D97, M135	-59.23	17
AHL	Modified in 3-OH	*N*-(3-hydroxybutyryl)-L-homoserine lactone	Y80, W84, Y88, D97, S155	-56.01	22	Y80, W84, Y88, D97, S155	-54.42	21	Y80, W84, S155	-56.12	21	W84	-50.48	22
Furanone	Brominated	4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	W84	-51.46	23	W84	-50.04	22	S155	-51.63	22	S155	-52.30	21
Furanone	Non-brominated	3-butyl-2(5H)-furanone	W84	-49.35	24	W84	-48.46	24	W84	-49.83	24	-	-	-
AHL	Unmodified in 3-oxo and 3-OH	*N*-butyryl-DL-homoserine lactone	Y80, S155	-49.01	25	Y80	-48.57	23	Y80	-49.90	23	W84	-48.94	23
Furanone	Non-brominated	2,2-dimethyl-3(2H)-furanone	Y80	-35.04	26	-	-	-	Y80	-30.02	26	-	-	-
Furanone	Brominated	5-(bromomethylene)-2(5H)-furanone	Y80	-33.14	27	Y80	-33.24	26	-	-	-	W84	-33.41	25
Furanone	Brominated	4-bromo-5-(bromomethylene)-2(5H)-furanone	S155	-33.02	28	W84	-34.60	25	Y80, S155	-34.35	25	W84	-36.41	24
NSAID	Pyrazolone derivative	Phenylbutazone	-	-	-	-	-	-	-	-	-	S155	-67.29	12

Molecular docking = Binding affinity scale between CviR protein and the compounds with a color ramp ranging from dark pink (higher affinity) to dark green (lower affinity) and hyphen for no binding.

The 4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone was the furanone that showed higher binding affinities with the structures of CviR protein of both strains of *C. violaceum*, except to the 3QP5 structure of *C. violaceum* ATCC 31532. This brominated furanone also showed binding affinities higher than those of AHLs with a carbon size chain ranging from four to six and with or without 3-oxo and 3-OH modification (Tables 4 and 5). This result indicates that this furanone may compete for the binding site of CviR protein and, consequently, inhibit the mechanism of quorum sensing of *C. violaceum* as shown in *in vitro* studies (Oliveira *et al.,* 2016; Ponnusamy *et al.,* 2010). Besides, the two non-brominated furanones evaluated also bound in the CivR protein structures, but with low binding affinities (Tables 4 and 5).

The plant compounds evaluated were able to bind to the structures of CviR protein of two strains of *C. violaceum* and, the phytol isomers such as Z-phytol and E-phytol showed higher binding affinities (Tables 4 and 5). In *C. violaceum* ATCC 31532, phytol isomers showed higher binding affinities with the four structures of CviR protein, with the exception of Z-phytol, which did not bind to the 3QP4 structure (Table 5). These phytol isomers and all furanones evaluated showed higher binding affinities for CviR structures than AHLs with a carbon size chain ranging from six to eight and with or without 3-oxo and 3-OH modification. However, the binding affinities of Z-phytol by the 3QP1 and 3QP2 structures were no higher than AHLs with 12 carbons with or without 3-oxo and 3-OH modification (Tables 4 and 5).

Our studies *in silico* showed that the binding affinity of Z-phytol was also higher than C10-HSL by the 3QP8 structure, which is the CviR protein of *C. violaceum* ATCC 12472 crystallized with this inducer (Chen *et al.,* 2011). This may explain the inhibitory effect of phytol on the mechanism of quorum sensing described by other authors in previous *in vitro* studies (Pejin *et al.,* 2015; Ramathan Srinivasan *et al.,* 2016; Ramanathan Srinivasan, Santhakumari and Ravi 2017). Almeida *et al*. (2018) revealed, by molecular docking of the modeled structures SdiA protein of *Salmonella* Enteritidis PT4 578, that Z-phytol is a good candidate for *in vitro* tests of inhibition of quorum sensing mediated by AI-1, as well as the plant compounds classified as methoxy phenol and fatty acids, such as margaric and palmitic acid. The margaric and palmitic acids had lower binding affinities than those AHLs with carbon size chain ranging from eight to 12 with or without 3-oxo and 3-OH modifications in the CviR protein of two strains of *C. violaceum*, as well as lower than the furanone that most bound in these structures, 4-

bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone (Tables 4 and 5). Molecular docking performed by Priyanka *et al.* (2015) showed that margaric acid (PubChem CID: 10465) had a binding affinity with the structure 3QP5 of *C. violaceum* ATCC 31532 lower than 3-oxo-C6-HSL and higher than palmitic acid (PubChem CID: 985) and phytol (PubChem CID: 6437979). It is noteworthy that the phytol structure used by these authors is different from the structures used in the present study, which were Z-phytol (PubChem CID: 6430833) and E-phytol (PubChem CID: 5280435).

Among the NSAIDs tested, ketoprofen and dipyrone (metamizole) were able to bind in the structures of CivR proteins with lower binding affinities than the AHLs with carbon size chain ranging from 10 to 12 with or without 3-oxo and 3-OH modification and, the 4-bromo-5-(bromomethylene)-3-dodecyl-2(5H)-furanone. These compounds also showed lower binding affinities than phytol isomers and margaric acid. On the other hand, phenylbutazone did not bind to any of the structures of the CviR protein of *C. violaceum* ATCC 12472 evaluated (Table 4). However, it is able to bind only on the 3QP5 structure of CviR protein of *C. violaceum* ATCC 31532 crystallized with chlorolatone, an inhibitor of quorum sensing (Table 5). Molecular docking of the modeled structures of the SdiA protein of *Salmonella* Enteritidis PT4 578 with 73 NSAIDs, including ketoprofen, dipyrone and phenylbutazone showed that all NSAIDs were able to bind to, at least, one of the structure evaluated (Almeida *et al*., 2018). Also, the NSAIDs classified as a pyrazolone derivative, such as phenylbutazone and dipyrone, showed high binding affinities with the SdiA protein.

In general, although the plant compounds and NSAIDs had a lower binding score than some long acyl chain AHLs, they showed good affinity to the structures of CviR protein in *C. violaceum*, which may indicate to be good candidates for *in vitro* tests on inhibition in the violacein production.

3.3. *In vitro* anti-quorum sensing activity of plant compounds and NSAIDs in *C. violaceum*

The anti-quorum sensing activity of plant compounds and NSAIDs, evaluated by inhibition of violacein production, was performed at concentrations that did not interfere with the growth of *C. violaceum* ATCC 12472 and CV026 strains. Thus, OD 600 nm of these strains of *C. violaceum* cultured in the presence of plant compounds and NASIDs

at concentrations indicated in Table 3 were compared to the control and were not statistically significant ($p > 0.05$) (Figures 2 and 3).

3.3.1. Plant compounds

Plant compounds exhibited different effects on the inhibition of violacein production in two strains of *C. violaceum.* The palmitic and margaric acids decreased violacein production by *C. violaceum* ATCC 12472 in 60.3 and 41.4% in comparison to control, respectively ($p < 0.05$); however, the phytol compound showed no effect on the production of violacein in this strain (Figure 2A). The three plant compounds decreased violacein production by *C. violaceum* CV026 ($p < 0.05$) (Figure 2B) and the value search at 33.6%, 15.4% and 15.2% in the presence of phytol, palmitic acid, and margaric acid, respectively.

The results showing that phytol did not affect the inhibition of violacein production in *C. violaceum* ATCC 12472, may be associated with the binding affinity of this compound to CviR protein, which was lower than *N*-dodecanoyl-DL-homoserine lactone, a natural autoinducer (Table 4). In contrast, the inhibition of violacein production by *C. violaceum* CV026, a mutant of *C. violaceum* ATCC 31243, by phytol may be related to the higher binding affinity of this compound than the natural autoinducer, *N*-(3-hydroxyhexanoyl)-DL-homoserine lactone with the CviR protein (Table 5).

The activity of phytol as quorum quenching has been demonstrated in different bacteria (Pejin *et al.,* 2014, 2015; Srinivasan *et al.,* 2016; Srinivasan, Santhakumari and Ravi, 2017). In subinhibitory concentrations of 0.5, 0.25 and 0.125 of MIC (MIC was 19 µg/mL), phytol reduced the biofilm formation by *P. aeruginosa* in the range of 74 to 84.33% (Pejin *et al.,* 2014). This compound also reduced the production of pyocyanin, twitching, and flagella motility on *P. aeruginosa*, phenotypes recognized as regulated by a quorum sensing system (Pejin *et al.,* 2014). Srinivasan *et al.* (2016) showed that phytol interfered with quorum sensing regulated phenotypes in *Serratia marcescens*, as reduced the prodigiosin production and that the ethyl acetate extract of *Piper betle*, containing mainly phytol, at a concentration of 500 µg/mL decreased the prodigiosin production, biofilm formation, swarming motility, production of exopolysaccharide, lipase, and protease. This extract of *P. betle* also reduced biofilm formation, swimming motility, and exopolysaccharide production, as well as, inhibited the quorum sensing mediated

bioluminescence production by *Vibrio harveyi*, without affecting their cell viability (Srinivasan *et al.,* 2016).

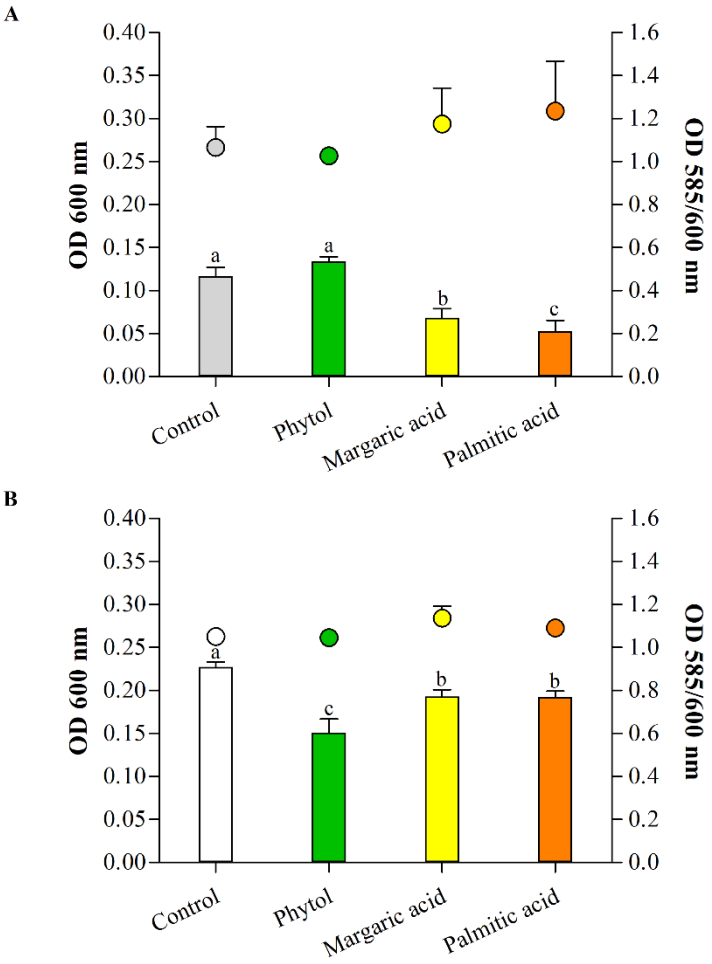

Figure 2. Inhibition of violacein production of *C. violaceum* ATCC 12472 **(A)** and *C. violaceum* CV026 **(B)** in the presence of plant compounds (OD 585/600 nm; bars) and evaluation of growth (OD 600 nm; circle) after 24 h incubation. Control refers to bacterial growth on LB medium. The means of OD 585/600 nm and OD 600 nm followed by different letters differ at 5% probability ($p < 0.05$) by Tukey's test. Where a letter is not shown, no statistical difference among samples was observed.

Phytol exhibits a broad range of biological activities including anti-inflammatory, antiallergic, immunostimulatory, antinociceptive, antimicrobial, antioxidant, anti-quorum sensing and antitumor effects (De Moraes *et al.,* 2014; Pejin *et al.,* 2014, 2015). Phytol is of general occurrence in nature, mainly because it frequently occurs as a component of chlorophyll, is one of the meaningful constituents of plant-derived essential

oils and the presence in human food is mainly restricted to spinach, beans, raw vegetables, and asparagus (Vetter and Lehnert, 2012; Islam *et al.,* 2018).

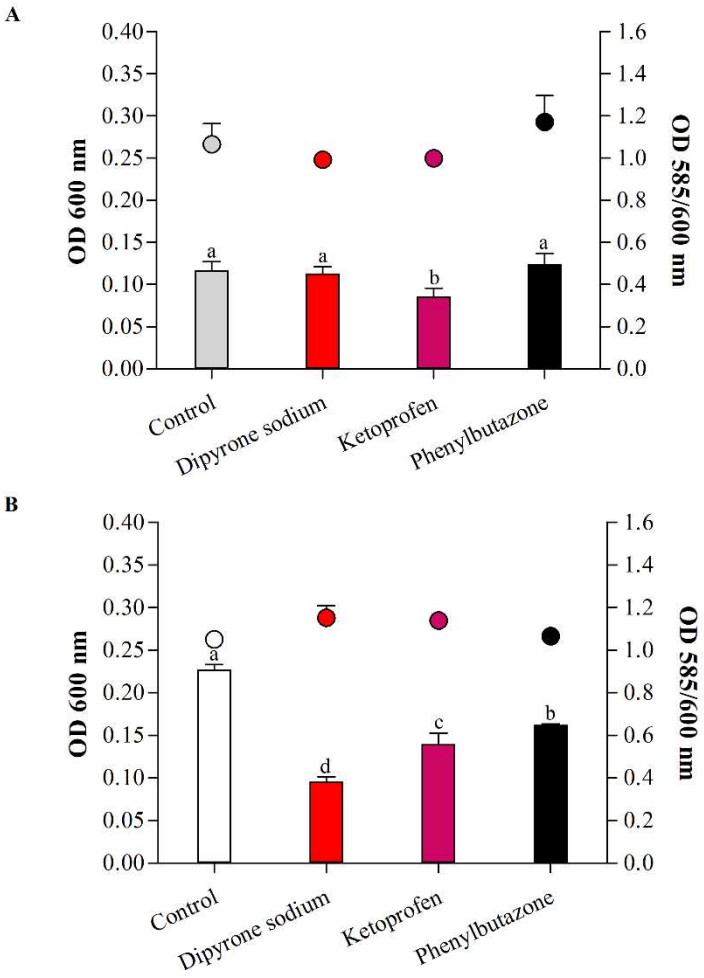

Figure 3. Inhibition of violacein production of *C. violaceum* ATCC 12472 **(A)** and *C. violaceum* CV026 **(B)** in the presence of NSAIDs (OD 585/600 nm; bars) and evaluation of growth (OD 600 nm; circle) after 24 h incubation. Control refers to bacterial growth on LB medium. The means of OD 585/600 nm and OD 600 nm followed by different letters differ at 5% probability ($p < 0.05$) by Tukey's test. Where a letter is not shown, no statistical difference among samples was observed.

The margaric and palmitic acids also inhibited the quorum sensing mechanism in two strains of *C. violaceum*. Fatty acids and their derivatives have been reported to possess antimicrobial properties; specifically, fatty acids containing two double bonds, like linoleic acid, is a more potent bacteriostatic compound (Kabara *et al.,* 1972). In other studies, linoleic acid, oleic acid, palmitic acid, and stearic acid are capable of inhibiting autoinducer-2 activity and consecutively, affecting quorum sensing mechanism (Soni *et al.,* 2008). Marathe and Bundale (2018) evaluated the inhibitory effect of linoleic acid

upon quorum sensing and showed a reducing urease activity and biofilm formation in *Proteus mirabilis* and also, a decreasing of protease and prodigiosin synthesis and biofilm formation in *S. marcescens.* The margaric acid or heptadecanoic acid is a saturated fatty acid and is found as a trace component of the nut and seed oils (Lisa and Holcapek, 2011), as well as fat and milk fat of ruminants, but it does not occur in any vegetable fat or animal at high concentrations. For example, it only comprises 2.2% of the fats from the fruit of the durian species *Durio graveolens* and 0.32% of fats from cocoa butter (Lisa and Holcapek, 2011). On the other hand, the palmitic acid or hexadecanoic acid is a common saturated fatty acid found as the major component of palm oil, but significant amounts of palmitic acid can also be found in meat and dairy products, as well as cocoa butter and olive oil (Carta *et al.,* 2017). The properties as inhibitors of quorum sensing mechanism and the ease of obtaining these compounds in nature makes them an attractive source to be used to combat pathogenic bacteria. This is the first study reported for the effect of anti-quorum sensing mediated by AI-1 by margaric and palmitic acid.

3.3.2. NSAIDs

The NSAIDs showed a low effect on inhibition of violacein production by *C. violaceum* ATCC 12472, and the maximum inhibition registered was 32% obtained by ketoprofen ($p < 0.05$) (Figure 3A). This NSAID is classified as propionic acid derivative, unlike dipyrone and phenylbutazone, that did not inhibit the pigment production and are classified as pyrazolone derivative. On the other hand, the three NSAIDs decreased violacein production by *C. violaceum* CV026 ($p < 0.05$), dipyrone sodium was the compound that most reduced the violacein production with 57.8%, followed by ketoprofen with 38.6% and phenylbutazone reduced in 28.6% (Figure 3B).

The quorum quenching effect of NSAIDs was registered in other bacteria, both in studies *in silico* and *in vitro* (El-Mowafy *et al.,* 2014; Soheili *et al.,* 2015; Almeida *et al.,* 2018). El-Mowafy *et al.* (2014) showed that the aspirin, a widely used NSAID, reduced the concentration of C4-HS and 3-OH-C12-HSL in the supernatant of *P. aeruginosa* culture, as well as reduced production of protease, elastase, hemolysin, and pyocyanin. No studies were found to show the anti-quorum sensing effect of dipyrone, ketoprofen, and phenylbutazone, and this is the first report showing quorum sensing ability inhibitory in a phenotype regulated by quorum sensing in *C. violaceum.*

The results of molecular docking described in this study showed that the dipyrone and ketoprofen bind in all structures evaluated of CviR proteins of *C. violaceum* ATCC 12472 and *C. violaceum* ATCC 31532. The dipyrone showed the highest binding affinities for the CviR protein of *C. violaceum* ATCC 31532 and the more significant inhibition of the quorum sensing of its mutant strain, *C. violaceum* CV026. *In silico*, phenylbutazone was not able to bind to any of the structures of the CviR protein of *C. violaceum* ATCC 12472 and, *in vitro*, did not alter its violacein production. On the other hand, this NSAID bound in one of four structures of the CviR protein of *C. violaceum* ATCC 31532 and regulated the quorum sensing of *C. violaceum* CV026. Other previous studies have shown that the culture conditions, the presence of other substances and the concentrations of the compounds also may interfere in the violacein production by *C. violaceum* (Liu *et al.,* 2013; Choi *et al.,* 2015; Mahmoudi, 2015).

3.4. Visualization of molecular docking of the quorum quenching compounds in *C. violaceum*

Molecular docking of the 3QP8 structure of CviR protein of *C. violaceum* ATCC 12472 showed that palmitic acid, margaric acid, and ketoprofen were accommodated in this structure in a similar fashion to the natural autoinducer 3-OH-C10-HSL (Figures 4A to 4H). The 3-OH-C10-HSL bound in Y80, W84, Y88 and S155 residues of 3QP8 (Figure 4I), margaric acid bound in Y88, N92 and A94 (Figure 4K) and, ketoprofen bound in residues Y80 and S155 (Figure 4L). The palmitic acid bound only to L85 residue (Figure 4J) and showed the highest inhibition of the violacein production in this strain (Figure 2). Margaric acid has the Y88 residue as a common binding site with the 3-OH-C10-HSL in the 3QP8 structure and, ketoprofen the two residues, Y80 and S155. In these results, it can be seen that the compounds bind at near positions in the amino acid sequence, most between 80 and 94 similarly with the natural C10-HSL autoinducer, which may indicate that these compounds may compete for the binding site with the signaling molecule.

Moreover, the molecular docking with the CivR protein of *C. violaceum* ATCC 31532 showed that dipyrone, Z-phytol, palmitic acid, margaric acid, and ketoprofen were accommodated in the 3QP1 structure in a similar fashion to the autoinducer C6-HSL (Figures 5A to 5L). The C6-HSL bound in Y80, W84, D97, and S155 residues of the CivR protein (Figure 5M). These results of molecular docking of C6-HSL produced by

C. violaceum ATCC 31532 are in agreement with Ravichandran *et al.* (2018) which showed the binding of the autoinducer in these same amino acids.

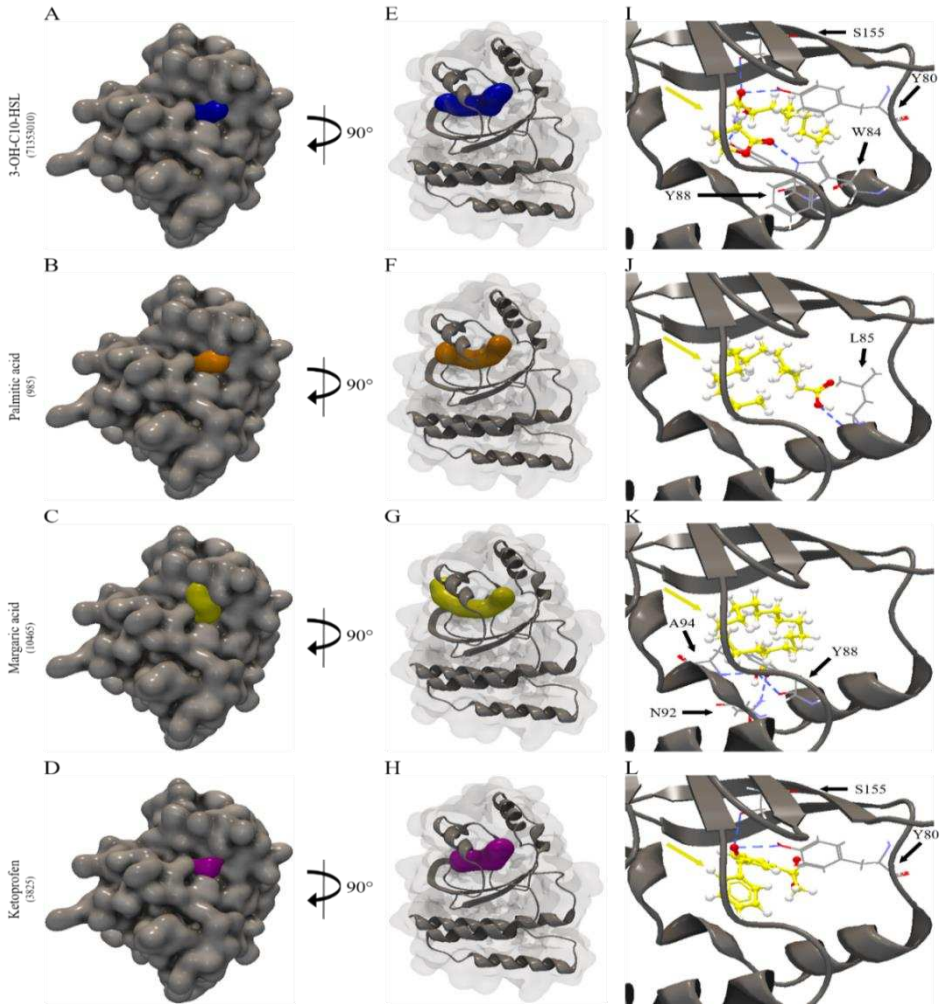

Figure 4. Molecular docking of 3QP8 structure of CviR protein of *C. violaceum* ATCC 12472 with 3-OH-C10-HSL, palmitic acid, margaric acid and ketoprofen. Surface representation of 3QP8 structure of CviR protein of *C. violaceum* ATCC 12472 (A, B, C and D), surface and backbone representations (E, F, G and H) and backbone representation with a hydrogen bond between the amino acid residues and compounds evaluated (I, J, K and L). Gray surface representation, CviR protein; Blue surface representation, 3-OH-C10-HSL; Orange surface representation, palmitic acid; Yellow surface representation, margaric acid; Purple surface representation, ketoprofen; Gray backbone representation, CviR protein; Black arrow indicates the binding site; Yellow arrow, 3-OH-C10-HSL or palmitic acid or margaric acid or ketoprofen; Blue dashed line, hydrogen bond.

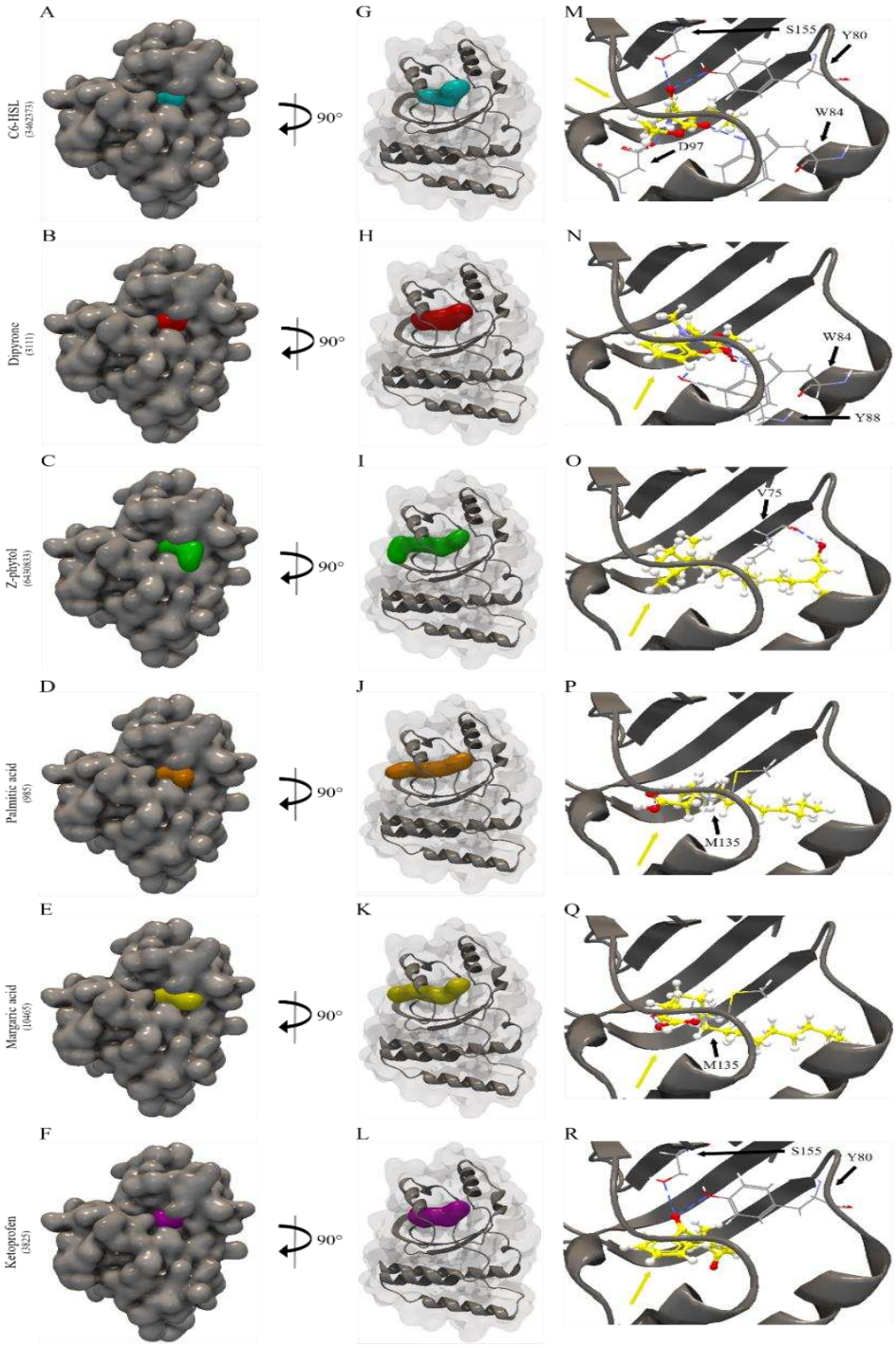

Figure 5. Molecular docking of 3QP1 structure of CviR protein of *C. violaceum* ATCC 31532 with C6-HSL, dipyrone, Z-phytol, palmitic acid, margaric acid and ketoprofen. Surface representation of 3QP1 structure of CviR protein of *C. violaceum* ATCC 31532 (A, B, C, D, E, and F), surface and backbone representations (G, H, I, J, K and L) and backbone representation with hydrogen bond between the amino acid residues and compounds evaluated (M, N, O, P, Q and R). Gray surface representation, CviR protein; Aquamarine surface representation, C6-HSL; Red surface representation, dipyrone; Green surface representation, Z-phytol; Orange surface representation, palmitic acid; Yellow surface representation, margaric acid; Purple surface representation, ketoprofen; Gray backbone representation, CviR protein; Black arrow indicates the binding site; Yellow arrow, C6-HSL or dipyrone or Z-phytol or palmitic acid or margaric acid or ketoprofen; Blue dashed line, hydrogen bond.

The dipyrone was the NSAID that most inhibited the violacein production by *C. violaceum* CV026 (Figure 3) and bound in W84 and Y88 residues (Figure 5N) whereas ketoprofen bound in Y80 and S155 residues in the structure of CivR protein (Figure 5R). On the other hand, the Z-phytol, which was the plant compound that most inhibited the quorum sensing of this strain (Figure 3) bound only to V75 residue (Figure 5O). The palmitic and margaric acids bound in M135 residues (Figure 5P and 5Q). The two NSAIDs compounds which showed a greater reduction in the production of violacein for this strain (Figure 3), presented a binding at amino acid residues that coincide with the binding of the natural C6-HSL autoinducer. These results may also indicate that the compounds compete for the binding site with the signal molecule. Plant compounds bind to different amino acids compared to autoinducer and, palmitic and margaric acid, which showed a small decrease in the production of violacein, bind at a position of the amino acid sequence farthest from that shown by C6-HSL. Probably, the competition of these compounds with the autoinducer in the CviR protein is affected by the binding distance. However, although phytol bound in different amino acid from the auto-inducer, the position is in the C6-HSL binding range, indicating a better compete for binding in the protein.

Conclusion

The plant compounds and all nonsteroidal anti-inflammatory drugs (NSAIDs) evaluated had higher binding affinity in the structures of the CviR proteins, as well as the AHLs and furanones tested, which are the inducers and inhibitors of the quorum sensing mechanism, respectively, in *C. violaceum*. These compounds prospected *in silico* showed inhibition violacein production *in vitro* tests, the phenotype typically regulated by quorum sensing in this bacterium. These results show that the combination of tools *in silico* and *in vitro* tests is a useful methodology for prospecting anti-quorum sensing compounds and then validating these data experimentally so that compounds with an anti-quorum sensing effect can be found in a more effective way to combat pathogens.

References

Adonizio, Allison L., Kelsey Downum, Bradley C. Bennett, and Kalai Mathee. 2006. "Anti-quorum sensing activity of medicinal plants in southern Florida." *Journal of Ethnopharmacology* 105 (3): 427–35. doi:10.1016/j.jep.2005.11.025.

Almeida, Felipe Alves de, Natan de Jesus Pimentel-Filho, Uelinton Manoel Pinto, Hilário Cuquetto Mantovani, Leandro Licursi de Oliveira, and Maria Cristina Dantas Vanetti. 2017. "Acyl homoserine lactone-based quorum sensing stimulates biofilm formation by *Salmonella* Enteritidis in anaerobic conditions." *Archives of Microbiology* 199 (3): 475–86. doi:10.1007/s00203-016-1313-6.

Almeida, Felipe Alves, Uelinton Manoel Pinto, and Maria Cristina Dantas Vanetti. 2016. "Novel insights from molecular docking of SdiA from *Salmonella* Enteritidis and *Escherichia coli* with quorum sensing and quorum quenching molecules." *Microbial Pathogenesis* 99: 178–90. doi:10.1016/j.micpath.2016.08.024.

Almeida, Felipe Alves, Erika Lorena Giraldo Vargas, Deisy Guimarães Carneiro, Uelinton Manoel Pinto, and Maria Cristina Dantas Vanetti. 2018. "Virtual screening of plant compounds and nonsteroidal anti-inflammatory drugs for inhibition of quorum sensing and biofilm formation in *Salmonella*." *Microbial Pathogenesis* 121: 369–88. doi:10.1016/j.micpath.2018.05.014.

Boyer, Mickaël, and Florence Wisniewski-Dyé. 2009. "Cell-cell signaling in bacteria: not simply a matter of quorum." *FEMS Microbiology Ecology* 70 (1): 1–19. doi:10.1111/j.1574-6941.2009.00745.x.

Burt, Sara A., Victoria Ojo-Fakunle, Jenifer Woertman, and Edwin J A Veldhuizen. 2014. "The natural antimicrobial carvacrol inhibits quorum sensing in *Chromobacterium violaceum* and reduces bacterial biofilm formation at sub-lethal concentrations." *PLoS ONE* 9 (4): 1–6. doi:10.1371/journal.pone.0093414.

Campos-Galvão, Maria Emilene Martino, Andrea Oliveira Barros Ribon, Elza Fernandes Araújo, and Maria Cristina Dantas Vanetti. 2016. "Changes in the *Salmonella enterica* Enteritidis phenotypes in presence of acyl homoserine lactone quorum

sensing signals." *Journal of Basic Microbiology* 56 (5): 493–501. doi:10.1002/jobm.201500471.

Carta, Gianfranca, Elisabetta Murru, Sebastiano Banni, and Claudia Manca. 2017. "Palmitic acid: physiological role, metabolism and nutritional implications." *Frontiers in Physiology* 8: 1–14. doi:10.3389/fphys.2017.00902.

Chaudhari, Vimla, Haren Gosai, Shreya Raval, and Vijay Kothari. 2014. "Effect of certain natural products and organic solvents on quorum sensing in *Chromobacterium violaceum.*" *Asian Pacific Journal of Tropical Medicine* 7 (S1): S204–11. doi:10.1016/S1995-7645(14)60233-9.

Chen, Guozhou, Lee Swem, Danielle Swem, Devin Stauff, Colleen O'Loughlin, Philip Jeffrey, Bonnie Bassler, and Frederick Hughson. 2011. "A strategy for antagonizing quorum sensing." *Molecular Cell* 42 (2): 199–209. doi:10.1016/j.molcel.2011.04.003.

Choi, Shiu Hong, and Peter Greenberg. 1991. "The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain." *Proceedings of the National Academy of Sciences of the United States of America* 88 (24): 11115–19. https://www.ncbi.nlm.nih.gov/pubmed/1763027.

Choi, Seong Yeol, Kyoung-hye Yoon, Jin Il Lee, and Robert J Mitchell. 2015. "Violacein: properties and production of a versatile bacterial pigment." *BioMed Research International*, 2015: 1–8. doi:http://dx.doi.org/10.1155/2015/465056 Review.

Choo, J. H., Y. Rukayadi, and J. K. Hwang. 2006. "Inhibition of bacterial quorum sensing by vanilla extract." *Letters in Applied Microbiology* 42 (6): 637–41. doi:10.1111/j.1472-765X.2006.01928.x.

El-Mowafy, Somaia, Khaled Abd El Galil, Shahenda El-Messery, and Mona Shaaban. 2014. "Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*." *Microbial Pathogenesis* 74 (1): 25–32. doi:10.1016/j.micpath.2014.07.008.

Galloway, Warren, James Hodgkinson, Steven Bowden, Martin Welch, and David Spring. 2011. "Quorum sensing in gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways." *Chemical Reviews* 111 (1): 28–67. doi:10.1021/cr100109t.

Geske, Grant D, Jennifer C O Neill, David Miller, Margrith Mattmann, and E Helen. 2007. "Modulation of bacterial quorum sensing with synthetic ligands: systematic evaluation of *N*-acylated homoserine lactones in multiple species and new insights into their mechanisms of action." *Journal of the American Chemical Society* 129 (44): 13613–25. doi:10.1021/ja074135h.Modulation.

Givskov, Michael, Rocky De Nys, Michael Manefield, Lone Gram, Ria Maximilien, Leo Eberl, Soren Molin, Peter D. Steinberg, and Staffan Kjelleberg. 1996. "Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling." *Journal of Bacteriology* 178 (22): 6618–22. doi:10.1128/jb.178.22.6618-6622.1996.

Hanzelka, Brian, and Peter Greenberg. 1995. "Evidence That the *N*-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain." *Journal of Bacteriology* 177 (3): 815–17. https://www.ncbi.nlm.nih.gov/pubmed/7836318.

Islam, Muhammad, Eunüs S Ali, Shaikh J Uddin, Subrata Shaw, Amirul Islam, Iqbal Ahmed, Manik Chandra, et al. 2018. "Phytol: a review of biomedical activities." *Food and Chemical Toxicology Journal* 121: 82–94. doi:10.1016/j.fct.2018.08.032.

Kabara, Jon, Dennis M Swieczkowski, Anthony J Conley, and Joseph P Truant. 1972. "Antimicrobial agents." *Antimicrobial Agents and Chemotherapy* 2 (1): 23–28.

Lisa, Miroslav, and Michal Holcapek. 2011. Triacylglycerols in Nut and Seed Oils. doi:10.1016/B978-0-12-375688-6.10005-2.

Liu, Zhanjun, Weishan Wang, Ying Zhu, Qianhong Gong, Wengong Yu, and Xinzhi Lu. 2013. "Antibiotics at subinhibitory concentrations improve the quorum sensing behavior of *Chromobacterium violaceum*." *Research Letter* 341: 37–44. doi:10.1111/1574-6968.12086.

Mahmoudi, Esmaeil. 2015. “Signaling molecules from *Lactuca sativa L.* induced quorum sensing phenotypes in bacteria.” *Journal of Plant Protection Research* 55 (2): 166–71. doi:10.1515/jppr-2015-0022.

Marathe, Kirti, and Sunita Bundale. 2018. “Influence of linoleic acid on quorum sensing in *Proteus mirabilis* and *Serratia marcescens*” *Biosciences Biotechnology Research Asia* 15: 661–70. http://dx.doi.org/10.13005/bbra/2674.

McClean, Kay H, Michael K Winson, Leigh Fish, Adrian Taylor, Siri Ram Chhabra, Miguel Camara, Mavis Daykin, et al. 1997. “Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones.” *Microbiology* 143: 3703–11. doi:10.1099/00221287-143-12-3703.

Moraes, Josué, Rosimeire N. de Oliveira, Jéssica P. Costa, Antonio L.G. Junior, Damião P. de Sousa, Rivelilson M. Freitas, Silmara M. Allegretti, and Pedro L.S. Pinto. 2014. “Phytol, a diterpene alcohol from chlorophyll, as a drug against neglected tropical disease *Schistosomiasis mansoni*.” *PLoS Neglected Tropical Diseases* 8 (1): 51. doi:10.1371/journal.pntd.0002617.

Morohoshi, Tomohiro, Masashi Kato, Katsumasa Fukamachi, Norihiro Kato, and Tsukasa Ikeda. 2008. “*N*-acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472.” *FEMS Microbiology Letters* 279 (1): 124–30. doi:10.1111/j.1574-6968.2007.01016.x.

Nealson, Kenneth H, Terry Platt, and J Woodland Hastings. 1970. “Cellular control of the synthesis and activity of the bacterial luminescent system.” *Microbiology* 104 (1): 313–22.

Nguyen, Y, Nam X Nguyen, Jamie L Rogers, Jun Liao, John B MacMillan, and Youxing Jiang. 2015. “Structural and mechanistic roles of novel chemical ligands on the SdiA quorum-sensing transcription regulator.” *MBio* 6 (2): 1–10. doi:10.1128/mBio.02429-14.Editor.

Oliveira, Brígida D.Ávila, Adeline Conceição Rodrigues, Bárbara Moreira Inácio Cardoso, Ana Luiza Coeli Cruz Ramos, Michele Corrêa Bertoldi, Jason Guy Taylor, Luciana Rodrigues da Cunha, and Uelinton Manoel Pinto. 2016. "Antioxidant, antimicrobial and anti-quorum sensing activities of *Rubus rosaefolius* phenolic extract." *Industrial Crops and Products* 84: 59–66. doi:10.1016/j.indcrop.2016.01.037.

Pejin, Boris, Ana Ciric, Jasmina Glamoclija, Milos Nikolic, and Marina Sokovic. 2015. "*In vitro* anti-quorum sensing activity of phytol." *Natural Product Research* 29 (4): 374–77. doi:10.1080/14786419.2014.945088.

Pejin, Boris, Aleksandra Talevska, Ana Ciric, Jasmina Glamoclija, Milos Nikolic, Trajce Talevski, and Marina Sokovic. 2014. "Anti-quorum sensing activity of selected sponge extracts: a case study of *Pseudomonas aeruginosa*." *Natural Product Research* 28 (24): 2330–33. doi:10.1080/14786419.2014.934239.

Ponnusamy, Kannan, Diby Paul, Young Kim, and Ji Kweon. 2010. "2(5H)-Furanone: a prospective strategy for biofouling-control in membrane biofilm bacteria by quorum sensing inhibition." *Brazilian Journal of Microbiology* 41 (2): 227–34.

Priyanka, Sharma, J V Priya, S V Rajesh, G Prabhakaran, Shanmugam Gnanendra and Sivaraj Siddha. 2015. "Quorum sensing activity of *Couroupita guianesis* against *Enterobacter aerogens: In silico* studies." *International Journal of Advanced Science and Engineering* 1 (3): 1–6.

Raina, Sheetal, Daniela De Vizio, Mark Odell, Mark Clements, Sophie Vanhulle, and Tajalli Keshavarz. 2009. "Microbial quorum sensing: a tool or a target for antimicrobial therapy?" *Biotechnology and Applied Biochemistry* 54 (2): 65–84. doi:10.1042/BA20090072.

Ravichandran, Vinothkannan, Lin Zhong, Hailong Wang, Guangle Yu, and Youming Zhang. 2018. "Virtual screening and biomolecular interactions of CviR-based quorum sensing inhibitors against *Chromobacterium violaceum* bacterial strains and growth conditions" *Frontiers in Cellular and infection Microbiology* 8: 1–13.

doi:10.3389/fcimb.2018.00292.

Singh, Vijay K., Avinash Mishra, and Bhavanath Jha. 2017. "Anti-quorum sensing and anti-biofilm activity of *Delftia tsuruhatensis* extract by attenuating the quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa*." *Frontiers in Cellular and Infection Microbiology* 7: 1–16. doi:10.3389/fcimb.2017.00337.

Smith, Roger S., and Barbara H. Iglewski. 2003. "*P. aeruginosa* quorum-sensing systems and virulence." *Current Opinion in Microbiology* 6 (1): 56–60. doi:10.1016/S1369-5274(03)00008-0.

Soheili, Vahid, Bibi Sedigheh Fazly Bazzaz, Nooshin Abdollahpour, and Farzin Hadizadeh. 2015. "Investigation of *Pseudomonas aeruginosa* quorum-sensing signaling system for identifying multiple inhibitors using molecular docking and structural analysis methodology." *Microbial Pathogenesis* 89: 73–78. doi:10.1016/j.micpath.2015.08.017.

Soni, Kamlesh A, Palmy Jesudhasan, Martha Cepeda, Kenneth Widmer, G Jayaprakasha, Bhimanagouda Patil, Michael Hume, and Suresh Pillav. 2008. "Identification of ground beef-derived fatty acid inhibitors of autoinducer-2-based cell signaling." *Journal of Food Protection* 71 (10): 134–38.

Srinivasan, Ramanathan, Sivasubramanian Santhakumari, and Arumugam Veera Ravi. 2017. "*In vitro* antibiofilm efficacy of *Piper betle* against quorum sensing mediated biofilm formation of luminescent *Vibrio harveyi*." *Microbial Pathogenesis* 110: 232–39. doi:10.1016/j.micpath.2017.07.001.

Srinivasan, Ramathan, Kannan Rama, Arunachalam Kannappan, Shunmugiah Pandian, and Arumugam Ravi. 2016. "*Piper betle* and its bioactive metabolite phytol mitigates quorum sensing mediated virulence factors and biofilm of nosocomial pathogen *Serratia marcescens in vitro*." *Journal of Ethnopharmacology* 3 (1): 70–73. doi:10.1016/j.jep.2016.10.017.

Stauff, Devin L., and Bonnie L. Bassler. 2011. "Quorum sensing in *Chromobacterium violaceum:* DNA recognition and gene regulation by the CviR receptor." *Journal of Bacteriology* 193 (15): 3871–78. doi:10.1128/JB.05125-11.

Vattem, Dhiraj A., Kathleen Mihalik, Sylvia H. Crixell, and Robert J. McCLean. 2007. "Dietary phytochemicals as quorum sensing inhibitors." *Fitoterapia* 78 (4): 302–10. doi:10.1016/j.fitote.2007.03.009.

Vetter, Walter, and Katja Lehnert. 2012. "Differentiation of refined and virgin edible oils by means of the trans and cis-phytol isomer distribution." *Journal of Agricultural and Food Chemistry* 60: 6103–7.

Zhu, Hongmei, Chao-Che He, and Q. H. Chu. 2011. "Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*." *Letters in Applied Microbiology* 52 (3): 269–74. doi:10.1111/j.1472-765X.2010.02993.x.

CHAPTER 3

Phytol and furanone influences metabolic phenotypes regulated by AI-1 in *Salmonella*

Phytol and furanone influences metabolic phenotypes regulated by AI-1 in *Salmonella*

Authors: Erika Giraldo[1], Felipe Alves de Almeida[2], Leonardo Luís de Freitas[1], Uelinton Manoel Pinto[3], Maria Cristina Dantas Vanetti[1]*

[1] Department of Microbiology, Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil;

[2] Department of Nutrition, Universidade Federal de Juiz de Fora (UFJF), Governador Valadares, MG, Brazil;

[3]Food Research Center, Department of Food and Experimental Nutrition, Universidade de São Paulo (USP), São Paulo, SP, Brazil.

***Corresponding author:** Maria Cristina Dantas Vanetti, Department of Microbiology, Universidade Federal de Viçosa, Viçosa, MG, Brazil. E-mail address: mvanetti@ufv.br

Abstract:

Numerous species of bacteria use an intercellular communication mechanism called quorum sensing (QS) mediated by chemical signals to promote the changes necessary for adaptation in diverse environments. *Salmonella* is a relevant foodborne bacterial pathogen and is unable to synthesize the autoinducer-1 (AI-1) of the QS system, named *N*-acyl-homoserine lactone (AHL), but can recognize AHLs produced by other microorganisms through SdiA protein, a homolog of the LuxR protein. The objectives of the present study were to evaluate the influence of AHL, phytol, and furanone on specific metabolic products of *Salmonella* that could be upon the regulation of the quorum sensing mechanism. *Salmonella* growth was not inhibited in the presence of 0.05 µM of *N*-dodecanoyl-homoserine lactone (C12-HSL), 0.05 µM of furanone or 600 µg/mL of phytol during 24 h of incubation, in anaerobic conditions. In the presence of C12-HSL, the levels of free thiol increased at 6 and 7 h of incubation but return to the same level in cells growing in the absent of C12-HSL when phytol and furanone were added in the culture broth. The levels of NADPH coenzyme were significantly increased in the presence of C12-HSL, phytol, and furanone while the glucose consumption was lower at 6 h of incubation with C12-HSL. The production of organic acids in anaerobic conditions was

not affected by quorum sensing and quorum quenching molecules. These results intend to contribute to the understanding of the effects of AHLs and quorum quenching on the physiology of *Salmonella* and opens the way to continue studies that can better clarify the mechanism of inhibition of these compounds to be applied in the medical or industrial area.

Keywords: Phytol, furanone, quorum sensing, inhibitors, thiol, NADPH, glucose, organic acids.

1. Introduction

Numerous species of bacteria use an intercellular communication mechanism called quorum sensing (QS) to promote the changes necessary for adaptation in diverse environments (Fuqua, Winans, and Greenberg 1994; Fuqua, Parsek, and Greenberg 2001). This mechanism of communication between cells leads to differential expression of genes in response to changes in population density and regulates a range of phenotypes in bacteria, such as, production of virulence factors, biofilm formation, protease and pigment production, swarming motility, bioluminescence, among others (Fuqua, Parsek, and Greenberg 2001; Quécan, Rivera, and Pinto 2018).

Salmonella is the main foodborne bacterial pathogen and is an important cause of gastrointestinal diseases, whose complications can lead to death (Zhao et al. 2008). In this pathogen, the communication by QS can be mediated by three types of autoinducers (AI), called AI-1, AI-2 and AI-3 (Ahmer 2004). The mechanism of AI-1 communication is incomplete in *Salmonella* due to the absence of LuxI or homologs, responsible for the synthesis of the acyl-homoserine lactones signaling molecules (AHLs). However, this pathogen is able to internalize and respond to AHLs synthesized by other bacteria or added to the medium, because it presents a protein known as SdiA, a homolog of the LuxR protein, which allows the detection of AHLs (Michael et al. 2001; Smith and Ahmer 2003; Steenackers et al. 2012; Campos-Galvão et al. 2015).

Few genes whose expression is regulated by SdiA protein in *Salmonella* are recognized. Ahmer et al. (1998) suggested a link between SdiA protein and upregulation of 10 genes in the virulence plasmid, including the previously characterized *rck* gene responsible for the increased resistance and adhesion to epithelial cells. It was also

evidenced that genes related to virulence such as *hilA*, *invA* and *invF* present in the pathogenic island PAI-1, and genes involved in the biofilm formation by *Salmonella* Enteritidis were more expressed in the presence of exogenous C12-HSL (Campos-Galvão et al. 2016; Almeida et al. 2017). Besides, studies have shown the influence of C12-HSL on the abundance of proteins, such as thiol and those related to oxidative stress, and the levels of extracellular organic acids of *Salmonella* Enteritidis (Almeida et al. 2017; Almeida et al. 2018b). Carneiro (2017) observed changes in concentrations of intracellular metabolites belonging to the glycerolipid, amino acid, and purine nucleotides pathways, as well as the glucose uptake between *Salmonella* Enteritidis cells cultured in the presence and absence of C12-HSL.

The number of *Salmonella* serovars isolated from humans and animals, resistant to antibiotics has increased considerably, making it difficult to control with these commercially available antimicrobials (Van et al. 2012). Thus, natural and synthetic quorum quenching compounds have gained interest as potential agents for controlling bacterial infections. The search for quorum quenching molecules with low toxicity and that reduces the virulence of pathogens may aid in the efficacy of antibiotics used in medical therapies.

Different types of compounds extracted from plants and algae can prevent cell communication via AHLs and, consequently, inhibit phenotypes associated with the presence of these inducer molecules (Khan et al. 2009; Oliveira et al. 2016; Campos-Galvão et al. 2016). Furanones are compounds extracted from algae and were the first molecules quorum quenching studied, but are toxic to eukaryotic cells (Givskov et al. 1996). Phytol, a natural compound and one of the major constituents of plant-derived essential oils has been studied for its ability to inhibit quorum sensing in bacteria (Pejin et al. 2014; Srinivasan et al. 2016). Phytol is acyclic diterpene alcohol and has a wide range of biological activities, including anti-inflammatory, antimicrobial, anti-allergic, antinociceptive, immunostimulatory, and antioxidant effects (Pejin et al. 2014).

Moreover, several studies have been developed using the molecular docking tool suggesting binding sites between the signal receptor proteins and AIs, as well as in the search for possible anti-QS compounds from different sources, among which are the extracted compounds of vegetables (Packiavathy et al. 2012; El-Mowafy et al. 2014; Almeida et al. 2018a). Almeida et al. (2018a) perform molecular docking with plant

compounds and nonsteroidal anti-inflammatory drugs (NASIDs) with SdiA of *Salmonella* and observed that many compounds showed higher binding affinities for SdiA than the AHLs; among these compounds tested, the Z-phytol was indicated as good candidate for the *in vitro* inhibition tests of quorum sensing mediated by AI-1 in *Salmonella*.

Considering the importance of the *in silico* results and the need to look for quorum quenching molecules that reduce pathogen virulence, the aims of the present study were validated *in vitro* the influence of AHL, furanone, and phytol on specific metabolic products of *Salmonella* that could be upon the regulation of quorum sensing mechanism.

2. Materials and methods

2.1 Bacterial strain and culture medium

Salmonella enterica serovar Enteritidis phage type 4 (PT4) 578, was isolated from chicken meat and donated by the Oswaldo Cruz Foundation-Fiocruz Rio de Janeiro, Brazil. The culture was maintained in Luria Bertani broth (LB) (tryptone 1%, yeast extract 0.5% and NaCl 0.4%) and 20% (v/v) sterile glycerol at -20 °C.

2.2 Preparation of compounds solution

The commercially available plant compound phytol (PubChem CID: 6430833; Sigma-Aldrich), was dissolved in methanol at stock concentration of 4.36 M. The furanone used in experiments, 4-Bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone (Furanone C-30; CID: 329757998; Sigma-Aldrich) and AHL used was *N*-dodecanoyl-DL-homoserine lactone (C12-HSL; PubChem CID:11565426; Merck, Germany) were suspended in acetonitrile (PubChem CID: 6342; Merck, Germany) at a concentration of 10 mM. The prepared solutions were stored at -20 °C.

2.3 Culture conditions

The inoculum was prepared, according to Almeida et al. (2017) with modifications. Tryptone soy broth (TSB; Sigma-Aldrich, India) was prepared with CO_2 under O_2-free conditions, dispensed in anaerobic bottles and then autoclaved. Before each experiment, cells were cultured two consecutive times in anaerobic bottles containing 10

mL of anaerobic TSB and incubated for 24 h at 37 °C. Then, 0.1 mL of the previously activated inoculum was added to 10 mL of anaerobic TSB and incubated for 4 h at 37 °C. The volume of 1 mL of culture was centrifuged at 12,000 *g* for 10 min at 4 °C (Sorvall, USA), the pellet was washed once with 1 mL of saline 0.85% and the pellet resuspended in 0.85% saline. The optical density of the cells was adjusted to OD_{600nm} = 0.1 (approximately 10^7 CFU/mL), using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.4 Effect of HSL, furanones and phytol on the growth of *Salmonella*

The effect of C12-HSL, phytol, and furanone on the growth *Salmonella* was evaluated in bottles containing 10 mL of anaerobic TSB supplemented with 0.05 µM of C12-HSL, 0.05 µM of furanone and 600 µg/mL of phytol. The media were inoculated with 0.1 mL of the standardized inoculum, and the bottles were incubated at 37 °C for up to 24 h in the anaerobic chamber. In established time points, the OD_{600nm} was determined using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.5 Effect of HSL, furanones and phytol on metabolites produced by *Salmonella*

Aliquots of 300 µL of the standardized culture were inoculated into 30 mL of TSB broth plus 0.05 µM of C12-HSL, 0.05 µM of furanone and 600 µg/mL of phytol, and incubated at 37 °C in anaerobic conditions. Aliquots were collected at times of 6 and 7 h incubation for analyzes of intracellular and extracellular compounds, according to Almeida et al. (2018b).

2.5.1 Preparation of cellular lysate

Cells cultivated in the presence of 0.05 µM C12-HSL, 0.05 µM of furanone and 600 µg/mL of phytol were collected at the different incubation times of treatments and were lysed using the protocol described by Almeida et al. (2018b), with modifications. Initially, the samples were centrifuged at 10,000 *g* for 10 min; the pellet was washed once with 1 mL of PBS and centrifuged at 10,000 *g* for 10 min, finally was resuspended in 500 µL lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 8.0). The suspension was kept on ice for 1 min, vortexed for 1 min and treated with ultrasound (400 W, 20 344 kHz; Sonics & Materials Inc., USA) for 1 min. This cycle was repeated five times. Then,

the lysate was centrifuged at 10,000 *g* for 15 min and the supernatant used for the thiol and NADPH analyzes.

2.5.2 Quantification of free cellular thiol

The quantification of free cellular thiol was performed according to Ellman (1959) and Riddles et al. (1979), with modifications. In a 96-well plate were added 5 µL of 0.4% (w/v) 5,50-dithiobis (2-nitrobenzoic acid) (DTNB or Ellman's reagent; Sigma, USA) in buffer sodium phosphate 0.1 M pH 8.0 containing 1mM EDTA, 250 µL of the buffer sodium phosphate and 25 µL of the cell lysate, obtained as described in 2.5.1, was added. The microplate was incubated at room temperature for 15 min, and absorbance at 412 nm was measured using a spectrophotometer (Thermo Fisher Scientific, Finland). The free cellular thiol was quantified by using cysteine hydrochloride monohydrate (Sigma, USA) as standard at concentrations from 0.0 to 1.5 mM. The obtained equation was as follows: absorbance = (0.9421 x concentration) + 0.0432, with R2 = 0.9936. The results of quantification of free cellular thiol were normalized by OD_{600nm}.

2.5.3 Quantification of nicotinamide adenine dinucleotide phosphate (NADPH) cellular

Quantification of NADPH in the cell lysate, obtained as described in 2.5.1, was done according to Zhang, Yu and Stanton (2000). Briefly, readings were taken on the spectrophotometer based on the measurement of the absorbance of the reduced coenzyme at 340 nm. Standard curve for NADPH was performed with different concentrations of NADPH ranging from 0.02 to 0.1 mM which were added to a 96-well microplate containing a buffer solution (0.1 M Tris–HCl, pH 8.0, 0.01 M EDTA and 0.05% (v/v) Triton X-100) to a final volume of 1 mL. The microplates were incubated at 37 °C for 5 min, and then, the absorbance was measured at 340 nm using a spectrophotometer (Thermo Fisher Scientific, Finland).

2.5.4 Glucose and organic acids quantification in the supernatant

Glucose concentration and organic acids profile in the cell-free supernatant were assessed by high-performance liquid chromatography (HPLC), at 0, 6, and 7 h. An aliquot of 2 mL of the culture was removed, immediately frozen in liquid N_2 and stored at -80 °C until analysis. The sample was centrifuged 12,000 *g* for 10 min, filtered on 13 mm

cellulose nitrate membrane, 22 μm pore, and 1 mL of each sample was analyzed in HPLC (Carneiro 2017). The mobile phase contained 5 mmol/L H_2SO_4 (Sigma-Aldrich, USA), the flow was 0.7 mL/min and the injection of 20 μL. The HPLC was calibrated with the standard glucose curve, prepared at a concentration of 13 mM and the following organic acids were used to calibrate the standard curve: lactic, acetic, succinic, and formic acid. All acids were prepared to a final concentration of 10 mM except acetic acid, which was at 20 mM.

2.6 Statistical analyses

Experiments were carried out in three biological replicates. The values of the triplicates were used for the analysis of variance (ANOVA) followed by Tukey's test using the Statistical Analysis System and Genetics Software (Ferreira, 2011). A *p*-value of < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1 Growth of *Salmonella* in the presence HSL, furanone and phytol

The growth of *Salmonella* Enteritidis PT4 578 was not inhibited in the presence 0.05 μM of C12-HSL, 600 μg/mL of phytol or 0.05 μM of furanone during 24 h of incubation, in anaerobic conditions (Figure 1). The concentration of C12-HSL used in this study is in agreement with previous studies to induce *sdiA* gene expression in *Salmonella*. Michael et al. (2001) showed that depending on the AHL assessed, a concentration of 0.001 μM suffices to induce *sdiA* gene expression in *Salmonella* Typhimurium and confirmed that a 0.1 μM concentration of different AHLs does not negatively affect the growth of this microorganism. A similar result was obtained by Campos-Galvão et al. (2015) and Almeida et al. (2016), when grew *Salmonella* Enteritidis PT4 578 in the presence of 0.05 μM of C12-HSL. Moreover, results showed that low concentrations of furanone do not interfere with the growth of *Salmonella* (Figure 1). Janssens et al. (2008) observed that the growth of *Salmonella* Typhimurium was not influenced by the addition of 50, 60, and 100 μM of different furanones. Other study showed that in the presence of 0.05 μM of furanones the growth of *Salmonella* was not altered (Almeida et al. 2017)

The phytol did not show the inhibitory effect of *Salmonella* growth, even at higher concentrations tested, and the MIC was not established (data not shown). The concentration of 600 µg/mL of phytol used in this study was the same used by Ramanathan et al. (2018) for growing *Acinetobacter baumannii*, and these authors revealed that no MIC was found for this bacteria. In other studies, smaller concentrations of phytol were tested to assess the anti-quorum sensing effect. Pejin et al. (2014) evaluated the effect of phytol on *Pseudomonas aeruginosa* biofilm formation at sub-inhibitory concentrations of 3.901, 1.95, and 0.97 µg/mL, and no interference with growth was detected. Also, Srinivasan et al. (2016) used a concentration of 10 µg/mL of phytol to inhibit a prodigiosin production in *Serratia marcescens* without affecting growth.

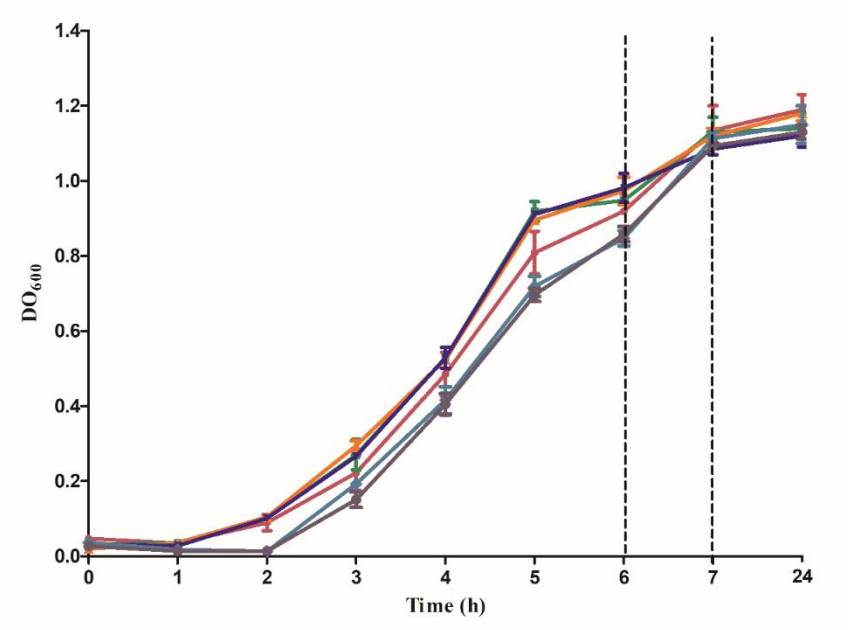

Figure 1. Growth of *Salmonella* Enteritidis PT4 578 in the presence of C12-HSL, furanone, and phytol. *Salmonella* was anaerobically cultivated in TSB at 37 °C for 24 h. Control (—●—), 0.05 µM of C12-HSL (—■—), 0.05 µM of furanone (—◆—), furanone+C12-HSL (—●—), 600 µg/mL of phytol (—▲—) and phytol+C_{12}-HSL (—▼—). The dashed line indicates the time of collection of cells for experiments. Error bars indicate standard error.

3.2 Metabolites produced in the presence of HSL, furanone, and phytol

The free thiol levels were more abundant at the 6 and 7 h incubation, when the quorum sensing C12-HSL autoinducer was added compared to the control ($p > 0.05$) (Figure 2). As demonstrate by Almeida et al. (2018a), the abundance of levels of free cellular thiol increased in the presence of C12-HSL at 7 h incubation in *Salmonella*. Subsequently, these authors observed that at 12 and 36 h of incubation, no differences in thiol levels were observed related to the control treatment, without C12-HSL. The results of the previous study showed that the quorum sensing alters free thiol cellular levels, being suggested by the authors that the resistance to possible oxidative stress can be mediated by the signaling molecule C12-HSL (Almeida et al. 2018a). When the quorum quenching molecules phytol or furanone were added to the media contained C12-HSL the free thiol levels return to value found in the controls treatment (Figure 2). The increased of free thiol cellular by AHL presence and the inhibition of this phenotype by quorum quenching molecules is an important observation to enforce the previous observation of Almeida et al. (2018a) that there is a relationship between thiol proteins and levels of a free cellular thiol with quorum sensing.

The fact that C12-HSL increases the free thiol concentration in cells may suggest that physiological events relevant to survival occur in the bacterial cell. The electrons transfer through the disulfide bond exchange reactions in the cytoplasm recycles essential enzymes such as ribonucleotide reductase, which provides deoxyribonucleotides for DNA replication in all living cells (Nordlund and Reichard 2006). These proteins are important in oxidant environments and can be considered crucial to the maintenance of the cellular redox balance, as well as to anticipate resistance to possible oxidative stress due to excessive production of reactive oxygen/nitrogen species (ROS/RNS) (Jones 2006; Landeta, Boyd, and Beckwith 2018). Changes in the thiol-disulfide redox status of proteins are important both for the reactivation of enzymes, well as oxidative protein folding and stability and, for the control of protein function (Ritz and Beckwith 2001). Oxidation of critical cysteine residues can either activate or inactivate given protein in physiologically significant reactions (Ritz and Beckwith 2001).

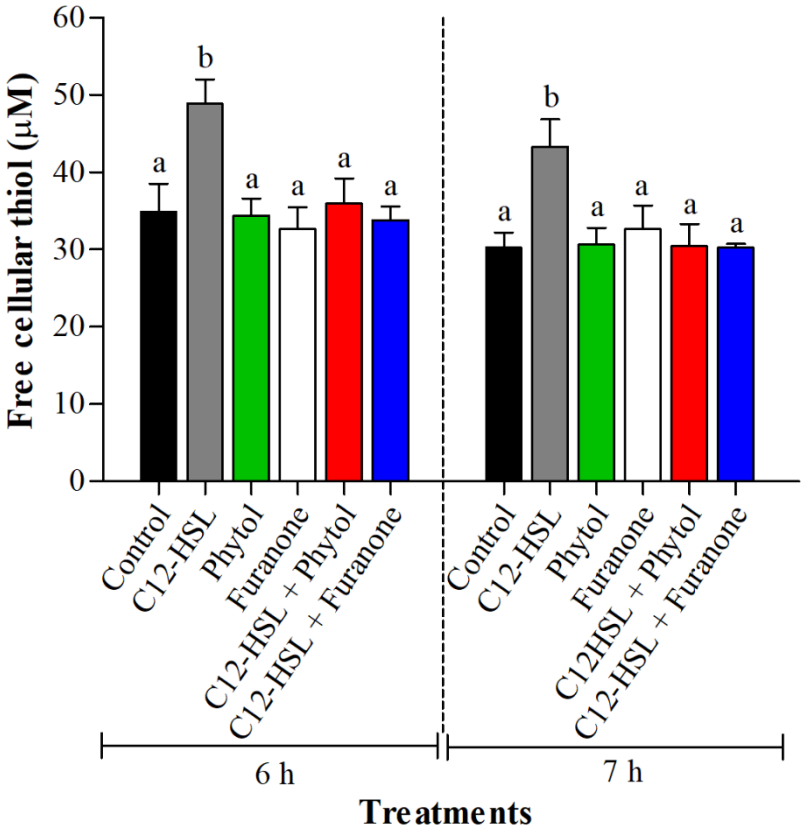

Figure 2. Quantification of free cellular thiol in *Salmonella* Enteritidis PT4 578 anaerobically cultivated in TSB at 37 °C for 6 and 7 h in the presence or absence of C12-HSL and compounds. Averages followed by the same letters do not differ statistically ($p < 0.05$).

However, variations in the abundance of thiol proteins and levels of free cellular thiol due to the growth phase of *Salmonella* and the presence of acyl-homoserine lactone can be related to changes in the structure of the SdiA protein (LuxR homolog) which could alter its ability to bind DNA and, consequently, activate transcription (Almeida et al. 2018a). Contreras-Garcia et al. (2015) evaluated the role of quorum sensing systems in stress tolerance (heat, oxidative stress, heavy metals, and hyperosmolarity) in *P. aeruginosa* and showed that single *lasR* or *rhlR* mutant survival after stress was approximately 3-fold lower than wild-type, while the double mutant was 4.8- to 11.2-fold more sensitive. This study revealed that, in addition to oxidative stress, the LasR and RhlR quorum sensing systems are related to heat, heavy metal, and salt stress (Garcia-

Contreras et al. 2015). These previous results show the importance of proteins with thiol grouping in metabolic processes in bacterial cells for module their homeostasis and, that way, quorum quenching compounds that may interfere with the synthesis of these thiol proteins could be an alternative to help fight pathogens.

The intracellular NADPH concentration did not show significant differences (p> 0.05) between the different treatments with 6 h incubation (data not shown). However, at 7 h incubation, an increase in NADPH levels was observed when cells grew in presence quorum sensing or quorum quenching molecules (Figure 3). The increased of NADPH levels in *Salmonella* in the presence of phytol (Figure 3) should be attributed, based on previous observations of Lee et al. (2016) in *P. aeruginosa*, that was a response to increase of ROS accumulate when treated with phytol. However, the decreased (p > 0.05) of NADPH concentration in the presence of C12-HSL and phytol (Figure 3) might indicate a disruption of the quorum sensing system. Nevertheless, this same phenomenon was not observed in the presence of furanone; a recognize disrupter of quorum sensing (Figure 3). A probable explanation could be related to the lower binding affinity of furanone with the SdiA of *Salmonella*. Almeida et al. (2018b) performed a virtual screening and established that Z-phytol presented higher binding affinities than the AHLs and the furanones to the modeled structures of SdiA proteins of *Salmonella* Enteritidis PT4 578. The 4-Bromo-5-(bromomethylene)-3-hexyl-2(5H)-furanone used in this study presented a rank varying 24 to 32 among 107 compounds evaluated while Z-phytol presented rank varying 1 to 8 (Almeida et al. 2018b). The increase of NADPH promoted by C12-AHL may confer adaptive advantage since this coenzyme is involved in a wide variety of cellular functions, including energy production, metabolism, cell survival and death, transcriptional regulation, and protein modification, and it is also the driving force of most biosynthetic enzymatic reactions, including those responsible for the biosynthesis of all major cell components, such as DNA and lipids (Arnér and Holmgren 2000; Koh et al. 2004; Minard and Mcalister-Henn 2005; Singh et al. 2008).

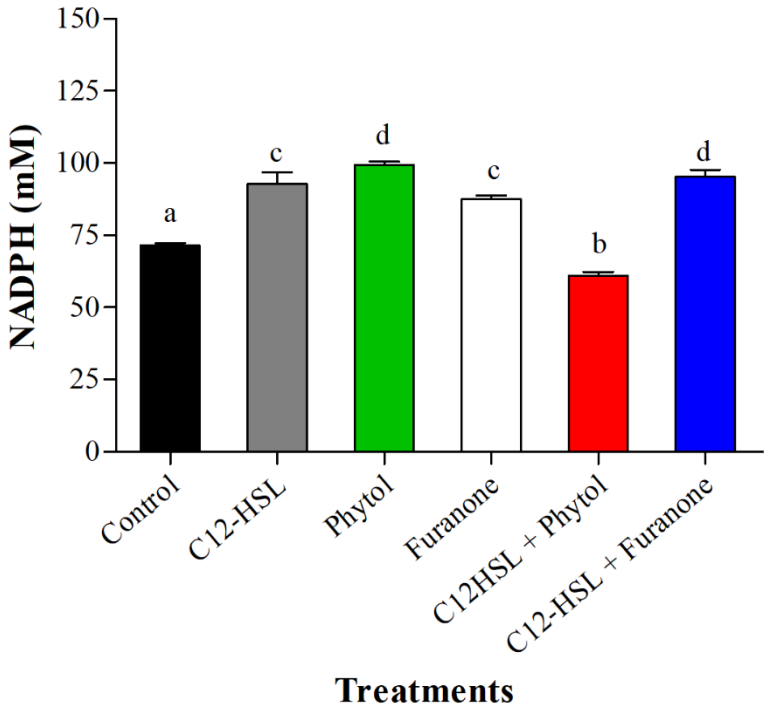

Figure 3. Quantification of NADPH *Salmonella* Enteritidis PT4 578 anaerobically cultivated in TSB at 37 °C for 7 h in the presence or absence of C12-HSL and compounds. Averages followed by the same letters do not differ statistically ($p < 0.05$).

The NADPH-producing enzymes as an activity regulated by a quorum sensing system in *P. aeruginosa* were considered by Contreras-Garcia et al. (2015). Malate dehydrogenase activity was two-fold higher for the wild-type strain than mutants *lasR* and *rhlR*. In contrast, the activities of isocitrate dehydrogenase were not different between the wild-type and mutants. However, these authors showed that the addition of furanone C-30 to the wild-type strain inhibited 50% of the catalase activity in the absence of H_2O_2 and 90% in the presence of 160 mM of H_2O_2. Furanone C-30 slightly increased the activity of isocitrate dehydrogenase in the absence of H_2O_2 (1.18-fold), but in the presence of H_2O_2, activity was inhibited 57% (Garcia-Contreras et al. 2015). These last observations are in agreement with our results, where the addition of furanone to the culture medium without oxidative stress, causes an increase in NADPH levels of *Salmonella.*

3.3 Glucose and organic acids quantification in *Salmonella*

The higher glucose concentration in the extracellular medium in the presence of C12-HSL in the sample corresponding to 6 h of cultivation indicated a lower consumption of this sugar when compared to the control (Table 1). In accordance with the results obtained, Carneiro (2017) showed that the concentration of glucose in the extracellular medium in the presence of C12-HSL in 4 and 6 h of cultivation was 26 and 29% higher when compared to control, indicating that *Salmonella* may have optimized its growth, opting for more energetically favorable metabolic reactions in the presence of the signaling molecule. When evaluating the glucose concentration over time (6 and 7 h) a significant difference was shown, indicating the consumption of this carbohydrate by *Salmonella* compared to the initial time at which the concentration was approximately 15 mM (Table 1).

Considering that organic acids including succinic, lactic, acetic, and formic acids were identified in TSB broth during the growth of *Salmonella* Enteritidis PT4 in anaerobic conditions (Table 1) it could be defined that this pathogen activates the mixed acid pathway fermentation. Microbial fermentation of carbohydrates results in the production of volatile fatty acids, including acetate, propionate, and butyrate, as well as lactate, and lowers the pH of colonic contents (Tellez et al. 1992). Previous study showed that *Salmonella* Typhimurium under anaerobic conditions fermented glucose primarily in acetate, ethanol, and formate, and some lactate and succinate; when acetate is formed, ethanol and/or succinate have to be formed in order to reoxidise the generated reducing equivalents (Driessen, Postma, and Van Dam 1987). The importance of these organic acids in the regulation of bacterial cell metabolism and consequently, in pathogenicity has been considered (Lawhon et al. 2002; Durant and Corrier 2000; Gantois et al. 2006). The quantification of organic acids at 6 and 7 h incubation in the presence of AHL and quorum quenching compounds showed no significant difference ($p > 0.05$) in the levels found (Table 1). In previous studies, C12-HSL altered the pattern of protein expression, especially proteins related to metabolic processes (Almeida et al. 2017; Almeida et al. 2018b) but the levels of extracellular organic acids were not altered at 7 h incubation (Almeida et al. 2017).

Table 1. Concentrations of extracellular organic acids and glucose in *Salmonella.*

6 h						
	Control	**C12-HSL**	**Phytol**	**Furanone**	**C12-HSL+Phytol**	**C12-HSL+Furanone**
Glucose	6.92^{aB}	9.47^{bB}	7.49^{B}	6.61^{B}	7.47^{B}	7.38^{B}
Succinic acid	$3,74^{B}$	3.97^{B}	3.74^{B}	4.13^{B}	3.76^{B}	4.02^{B}
Lactic acid	4.46^{B}	3.89^{B}	3.38^{B}	3.58^{B}	4.08^{B}	3.86^{B}
Acetic acid	12.42^{B}	11.81^{B}	12.30^{B}	11.40^{B}	10.49^{B}	12.54^{B}
Formic acid	12.82^{B}	10.69^{B}	12.63^{B}	12.44^{B}	11.90^{B}	12.51^{B}
7 h						
	Control	**C12-HSL**	**Phytol**	**Furanone**	**C12-HSL+Phytol**	**C12-HSL+Furanone**
Glucose	5.71^{A}	5.61^{A}	4.95^{A}	5.79^{A}	5.38^{A}	5.53^{A}
Succinic acid	5.62^{B}	4.73^{B}	4.98^{B}	4.49^{B}	5.27^{B}	4.18^{B}
Lactic acid	10.99^{B}	5.23^{B}	7.17^{B}	5.98^{B}	7.18^{B}	5.11^{B}
Acetic acid	13.09^{B}	9.86^{B}	10.33^{B}	10.88^{B}	12.26^{B}	9.81^{B}
Formic acid	14.35^{B}	13.17^{B}	13.65^{B}	13.22^{B}	13.51^{B}	11.16^{B}

The comparisons can be drawn between treatments or throughout time. Average followed by different lower case letters in the same line (between treatments) and followed by different capital letters in the columns (throughout time) differs at 5% probability ($p < 0.05$) by Tukey's test. Where a lower case letter is not shown, no statistical difference between control and C12-HSL was observed.

Conclusion

This study revealed that the concentrations of quorum sensing and quorum quenching molecules used did not interfere with the growth of *Salmonella* and allow validation of previous *in silico* study by confirming that phytol acts as a quorum quenching AI-1 regulated phenotypes such as free thiol and NADPH coenzyme concentration, important for modulating cellular homeostasis. However, more studies are needed to determine the specific role of metabolic products of *Salmonella* growing in the presence of AHL and quorum quenching compounds that can be applied in the medical or industrial area.

References

Ahmer, Brian M M. 2004. "MicroReview cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*." *Molecular Microbiology* 52: 933–45. doi:10.1111/j.1365-2958.2004.04054.x.

Almeida, Felipe Alves, Natan de Jesus Pimentel-Filho, Uelinton Manoel Pinto, Hilário Cuquetto Mantovani, Leandro Licursi de Oliveira, and Maria Cristina Dantas Vanetti. 2017. "Acyl homoserine lactone-based quorum sensing stimulates biofilm formation by *Salmonella* Enteritidis in anaerobic conditions." *Archives of Microbiology* 199: 475–86. doi:10.1007/s00203-016-1313-6.

Almeida, Felipe Alves, Natan de Jesus Pimentel-Filho, Lanna Clícia Carrijo, Cláudia Braga Pereira Bento, Maria Cristina Baracat-Pereira, Uelinton Manoel Pinto, Leandro Licursi de Oliveira, and Maria Cristina Dantas Vanetti. 2017. "Acyl homoserine lactone changes the abundance of proteins and the levels of organic acids associated with stationary phase in *Salmonella* Enteritidis." *Microbial Pathogenesis* 102: 148–59. doi:10.1016/j.micpath.2016.11.027.

Almeida, Felipe Alves, Erika Lorena Giraldo Vargas, Deisy Guimarães Carneiro, Uelinton Manoel Pinto, and Maria Cristina Dantas Vanetti. 2018a. "Virtual screening of plant compounds and nonsteroidal anti-inflammatory drugs for inhibition of quorum sensing and biofilm formation in *Salmonella*." *Microbial Pathogenesis* 121: 369–88. doi:10.1016/j.micpath.2018.05.014.

Almeida, Felipe Alves, Deisy Guimarães Carneiro, Tiago Mendez, Osvaldo Barros, Uelinton Manoel Pinto, Leandro Oliveira, and Maria Cristina Dantas Vanetti. 2018b. "*N*-dodecanoyl-homoserine lactone influences the levels of thiol and proteins related to oxidation-reduction process in *Salmonella.*" *PLoS ONE* 13: 1–31. doi:10.1371/journal.pone.0204673.

Arnér, Elias, and Arne Holmgren. 2000. "Physiological functions of thioredoxin and thioredoxin reductase." *European Journal of Biochemistry* 6109: 6102–9.

Campos-Galvão, Maria Emilene Martinho, Tiago Leite, Andrea Oliveira Barros Ribon, Elza Fernandes Araújo, and Maria Cristina Dantas Vanetti. 2015. "A new repertoire of

informations about the quorum sensing system in *Salmonella enterica* serovar Enteritidis PT4." *Genetics and Molecular Research* 14 (2): 4068–84.

Campos-Galvão, Maria Emilene Martino, Andrea Oliveira Barros Ribon, Elza Fernandes Araújo, and Maria Cristina Dantas Vanetti. 2016. "Changes in the *Salmonella enterica* Enteritidis phenotypes in presence of acyl homoserine lactone quorum sensing signals." *Journal of Basic Microbiology* 56: 493–501. doi:10.1002/jobm.201500471.

Carneiro, Deisy Guimarães. 2017. "Influência do mecanismo de quorum sensing no metabolismo de *Salmonella enterica* Minas Gerais-Brasil." Universidade Federal de Viçosa.

Driessen, Martin, P W Postma, and K Van Dam. 1987. "Energetics of glucose uptake in *Salmonella* Typhimurium." *Archives of Biochemistry and Biophysics* 146: 358–61.Igual

Durant, Juliet and Donald Corrier. 2000. "Short-chain volatile fatty acids modulate the expression of the *hilA* and *invF* genes of *Salmonella* Typhimurium." *Journal of Food Protection* 63 (5): 573–78.

El-Mowafy, Somaia, Khaled Abd El Galil, Shahenda El-Messery, and Mona Shaaban. 2014. "Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*." *Microbial Pathogenesis* 74: 25–32. doi:10.1016/j.micpath.2014.07.008.

Fuqua, Clairbone, Stephen Winans, and Peter Greenberg. 1994. "Minireview quorum sensing in bacteria : the *luxR-luxI* family of cell density-responsive transcriptional regulators." *Journal of Bacteriology* 176 (2): 269–75.

Fuqua, Clay, Matthew Parsek, and Peter Greenberg. 2001. "Regulation of gene expression by cell-to-cell communication." *Annual Review of Microbiology* 35: 439–68. doi:10.1146/annurev.genet.35.102401.090913.

Gantois, Ilse, Richard Ducatelle, Frank Pasmans, Freddy Haesebrouck, I Hautefort, A Thompson, and Jay Hinton. 2006. "Butyrate specifically down-regulates *Salmonella* Pathogenicity Island 1 gene expression." *Applied and Environmental Microbiology* 72 (1): 946–49. doi:10.1128/AEM.72.1.946. igual

Garcia-Contreras, Rodolfo, Leslie Nuñez-Lopez, Ricardo Jasso-chávez, Brian Kwan, Javier Belmont, Adrián Rangel-Vega, Toshinari Maeda, and Thomas Wood. 2015. "Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating." *The International Society for Microbial Ecology* 9: 115–25. doi:10.1038/ismej.2014.98.

Givskov, Michael, Rocky De Nys, Michael Manefield, Lone Gram, Ria Maximilien, Leo Eberl, Soren Molin, Peter Steinberg, and Staffan Kjelleberg. 1996. "Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling." *Journal of Bacteriology* 178: 6618–22. doi:10.1128/jb.178.22.6618-6622.1996.

Janssens, Joost, Hans Steenackers, Stijn Robijns, Edith Gellens, Jeremy Levin, Hui Zhao, Kim Hermans. 2008. "Brominated furanones inhibit biofilm formation by *Salmonella enterica* serovar Typhimurium." *Applied and Environmental Microbiology* 74: 6639–48. doi:10.1128/AEM.01262-08.

Jones, Dean P. 2006. "Redefining oxidative stress." *Antioxidants and Redox Signaling* 8: 1865–79.

Khan, Mohd S., Maryam Zahin, S. Hasan, Fohad Mabood Husain, and I. Ahmad. 2009. "Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil." *Letters in Applied Microbiology* 49: 354–60. doi:10.1111/j.1472-765X.2009.02666.x.

Koh, Ho-jin, Su-min Lee, Byung-gap Son, Soh-Hyun Lee, Zae Young, Kyu-Tae Chang, Jeen-woo Park, et al. 2004. "Cytosolic NADP-dependent isocitrate dehydrogenase plays a key role in lipid metabolism." *The Journal of Biological Chemistry* 279: 39968–74. doi:10.1074/jbc.M402260200.

Landeta, Cristina, Dana Boyd, and Jon Beckwith. 2018. "Disulfide bond formation in prokaryotes." *Nature Microbiology* 3; 270-280. doi:10.1038/s41564-017-0106-2.

Lawhon, Sara, Russell Maurer, Mitsu Suyemoto, and Craig Altier. 2002. "Intestinal short-chain fatty acids alter *Salmonella* Typhimurium invasion gene expression and virulence through *barA*/*sirA*." *Molecular Microbiology* 46: 1451–64.

Lee, Wonjong, Eun Rhan Woo, and Dong Gun Lee. 2016. "Phytol has antibacterial property by inducing oxidative stress response in *Pseudomonas aeruginosa*." *Free Radical Research* 50: 1309–18. doi:10.1080/10715762.2016.1241395.

Michael, Bindhu, Jenee Smith, Simon Swift, Fred Heffron, and Brian Ahmer. 2001. "SdiA of *Salmonella* Enterica is a *luxR* homolog that detects mixed microbial communities." *Journal of Bacteriology* 183: 5733–42. doi:10.1128/JB.183.19.5733-5742.2001.

Minard, Karyl I, and Lee Mcalister-Henn. 2005. "Sources of NADPH in yeast vary with carbon source." *The Journal of Biological Chemistry* 280: 39890–96. doi:10.1074/jbc.M509461200.

Nordlund, Par, and Peter Reichard. 2006. "Ribonucleotide reductases." *Annual Review of Biochemistry* 75: 681–706. doi:10.1146/annurev.biochem.75.103004.142443.

Oliveira, Brígida D.Ávila, Adeline Conceição Rodrigues, Bárbara Moreira Inácio Cardoso, Ana Luiza Coeli Cruz Ramos, Michele Corrêa Bertoldi, Jason Guy Taylor, Luciana Rodrigues da Cunha, and Uelinton Manoel Pinto. 2016. "Antioxidant, Antimicrobial and anti-quorum sensing activities of rubus rosaefolius phenolic extract." *Industrial Crops and Products* 84: 59–66. doi:10.1016/j.indcrop.2016.01.037.

Pejin, Boris, Aleksandra Talevska, Ana Ciric, Jasmina Glamoclija, Milos Nikolic, Trajce Talevski, and Marina Sokovic. 2014. "Anti-quorum sensing activity of selected sponge extracts: a case study of *Pseudomonas aeruginosa*." *Natural Product Research* 28: 2330–33. doi:10.1080/14786419.2014.934239.

Quécan, Beatriz Ximena, Liseth Milagros Rivera, and Manoel Uelinton Pinto. 2018. "Bioactive phytochemicals targeting microbial activities mediated by quorum sensing." *Biotechnological Applications of Quorum Sensing Inhibitors*, 397–416.

Ritz, Daniel, and Jon Beckwith. 2001. "Roles of thiol-redox pathways in bacteria." *Annual Review of Microbiology* 55: 21–48.

Singh, Ranji, Joseph Lemire, Ryan J Mailloux, and Vasu D Appanna. 2008. "A novel strategy involved anti-oxidative defense: the conversion of NADH into NADPH by a metabolic network." *PLoS ONE* 3: 1-7. doi:10.1371/journal.pone.0002682.

Smith, Jenee, and Brian Ahmer. 2003. "Detection of other microbial species by *Salmonella*: expression of the SdiA regulon." *Journal of Applied Microbiology* 185: 1357–66. doi:10.1128/JB.185.4.1357.

Srinivasan, Ramathan, Kannan Rama, Arunachalam Kannappan, Shunmugiah Pandian, and Arumugam Ravi. 2016. "*Piper betle* and its bioactive metabolite phytol mitigate quorum sensing mediated virulence factors and biofilm of nosocomial pathogen *Serratia marcescens in vitro*." *Journal of Ethnopharmacology* 3: 70–73. doi:10.1016/j.jep.2016.10.017.

Steenackers, Hans, Kim Hermans, Jos Vanderleyden, and Sigrid Keersmaecker. 2012. "*Salmonella* biofilms: an overview on occurrence, structure, regulation and eradication." *Food Research International* 45: 502–31. doi:10.1016/j.foodres.2011.01.038.

Packiavathy, Sybiya Vasantha, Issac Abraham, Palani Agilandeswari, Khadar Syed Musthafa, Shunmugiah Karutha Pandian, and Arumugam Veera Ravi. 2012. "Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against gram-negative bacterial pathogens." *Food Research International* 45 (1). Elsevier Ltd: 85–92. doi:10.1016/j.foodres.2011.10.022.

Tellez, Guillermo, Donald Corrier, John Deloach, L Jaeger, and Bill Hargis. 1992. "Effect of dietary lactose on cecal morphology, pH, organic acids, and *Salmonella* Enteritidis organ invasion in leghorn chicks." *Poultry Science* 72: 636–42.

Van, Thi, Hoang Nguyen, Peter Smooker, and Peter Coloe. 2012. "The antibiotic resistance characteristics of non-typhoidal *Salmonella enterica* isolated from food-producing animals, retail meat and humans in Southeast Asia." *International Journal of Food Microbiology* 154: 98–106. doi:10.1016/j.ijfoodmicro.2011.12.032.

Zhao, Shanshan, David White, Scott Friedman, Andrea Glenn, Karen Blickenstaff, Sherry L. Ayers, Jason W. Abbott, and Patrick F. Mcdermott. 2008. "Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006" *Applied and Environmental Microbiology* 74: 6656–62. doi:10.1128/AEM.01249-08.