BRUNA MARIA MAGRO PEREIRA

ATIVIDADE DA PROTEÍNA GP16-43 DO *Ralstonia virus phiAP1* NA INIBIÇÃO E DEGRADAÇÃO DE BIOFILME BACTERIANO

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*.

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(Orientadora)

Dedico

A toda minha família

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SUMÁRIO

RESUMO

PEREIRA, Bruna Maria Magro, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Atividade da proteína Gp16-43 de *Ralstonia virus phiAP1* na inibição e degradação de biofilme bacteriano.** Orientadora: Poliane Alfenas-Zerbini.

Os vírus que infectam bactérias (bacteriófagos) são os organismos mais abundantes do planeta, e desempenham importantes funções para a manutenção do ecossistema. Nos últimos anos, o uso de vírus como ferramenta de controle biológico tem ganhado bastante atenção, devido, principalmente, à dificuldade de se isolar novas drogas antimicrobianas e à seleção de bactérias resistentes às drogas já existentes. Devido a esse potencial como agentes de controle biológico, as descobertas sobre esses vírus têm aumentado o que amplia as perspectivas de controle de doenças. O biofilme bacteriano representa um importante fator de virulência em bactérias patogênicas. A matriz de exopolímeros presente no biofilme confere limitação à penetração de agentes antimicrobianos e aumenta a tolerância às defesas do hospedeiro, dificultando o controle de bactérias patogênicas produtoras de biofilme. Os bacteriófagos codificam proteínas associadas à lise celular com interessante aplicação no controle desses microrganismos. As despolimerases virais são uma classe de proteínas que desempenham um papel importante no processo de infecção do vírus em seu hospedeiro, atuando na matriz exopolissacarídea do biofilme bacteriano para permitir uma infecção viral eficiente. Desta forma, tem sido apontada como sendo um interessante agente de inibição e/ou degradação do biofilme de bactérias patogênicas. Este estudo traz a caracterização da atividade de uma nova despolimerase associada ao vírion (Gp16-43) codificada pelo vírus *Ralstonia virus phiAP1*. Gp16-43 foi eficiente para inibir a formação do biofilme de *E. coli*, *R. pseudosolanacearum* e *S. aureus*. Baixas concentrações de proteínas (250-500 µg/mL) já foram capazes de reduzir a formação de biofilme dessas bactérias. A proteína Gp16-43 foi capaz de degradar o biofilme formado de *E. coli* e *R.*

pseudosolanacearum, sendo mais eficiente na concentração de 1375 µg/mL, mas não no biofilme de *S. aureus*. Com base nesses resultados, a Gp16-43, uma nova exopolissacarídeo-despolimerase derivada de vírus, representa uma nova estratégia de grande potencial biotecnológico para prevenir e degradar a formação de biofilme de microrganismos Gram-positivos e Gram-negativos.

ABSTRACT

PEREIRA, Bruna Maria Magro, M.Sc., Universidade Federal de Viçosa, February, 2019. **The activity of *Ralstonia virus phiAP1* Gp16-43 protein in the inhibition and degradation of bacterial biofilm.** Adviser: Poliane Alfenas-Zerbini.

Viruses that infect bacteria (bacteriophages) are the most abundant organisms on the planet and play important roles in maintaining the ecosystem. In recent years, the use of viruses as a biological control tool has gained much attention, mainly due to the difficulty of isolating new antimicrobial drugs and the selection of existing drug-resistant bacteria. Because of their potential as biological control agents, discoveries about these viruses have increased, which broadens the prospects for disease control. Bacterial biofilm represents an important virulence factor in pathogenic bacteria. The exopolymer matrix present in biofilm confers antimicrobial penetration limitation and increases tolerance to host defenses, making it difficult to control biofilm-producing pathogenic bacteria. Bacteriophages encode proteins associated with cell lysis with interesting applications in the control of these microorganisms. Viral depolymerases are a class of proteins that play an important role in the process of virus infection in their host, acting on the exopolysaccharide matrix of the bacterial biofilm to enable efficient viral infection. Thus, it has been pointed out as an interesting agent of biofilm inhibition and/or degradation of pathogenic bacteria. This study characterizes the activity of a new virion-associated depolymerase (Gp16-43) encoded by the *Ralstonia virus phiAP1* virus. Gp16-43 was efficient to inhibit the biofilm formation of *E. coli*, *R. pseudosolanacearum* and *S. aureus*. Low protein concentrations (250-500 µg / mL) have already been able to reduce the biofilm formation of these bacteria. The protein Gp16-43 was able to degrade the biofilm formed from *E. coli* and *R. pseudosolanacearum*, being more efficient in the concentration of 1375 µg / mL, but not in the *S. aureus* biofilm. Based on these results, Gp16-43, a new virus-derived

exopolysaccharide depolymerase, represents a novel strategy of great biotechnological potential to prevent and degrade biofilm formation of Gram-positive and Gram-negative microorganisms.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

Nos últimos anos, o estudo de vírus que infectam micro-organismos tem ganhado grande interesse tendo em vista o seu potencial biotecnológico de aplicação para o controle de micro-organismos patogênicos ou que causam prejuízos à saúde, indústria e agricultura. A introdução da prática de fagoterapia não é recente e existem relatos da sua utilização anterior aos anos 1920 (ABEDON et al., 2011; HADDAD KASHANI et al., 2017; KUTTER et al., 2010). Contudo, com a descoberta da penicilina nos anos 1940 por Alexander Fleming, a antibioticoterapia dominou o cenário de controle de patógenos. Entretanto, os crescentes relatos de bactérias patogênicas resistentes a múltiplas drogas possibilitou o ressurgimento da fagoterapia como uma estratégia efetiva para o controle destes patógenos emergentes (HADDAD KASHANI et al., 2017; PIRES et al., 2016).

Existem várias propostas de utilização da fagoterapia, a mais antiga é o uso dos coquetéis de vírus que se multiplicam exclusivamente pelo ciclo lítico (ABEDON et al., 2011; KUTTER et al., 2010). Esses coquetéis são específicos restringindo sua ação aos casos de infecções ou contaminações por bactérias hospedeiras desses vírus, podendo caracterizar em uma vantagem do uso desse tipo de terapia. Uma desvantagem dessa terapia é a biodisponibilidade, ou seja, a concentração de vírus necessária para inibir o crescimento da bactéria alvo que chega ao seu local de ação, denominado concentração inibitória fracional (CIF). Esses desafios são os principais responsáveis pela dificuldade da difusão da fagoterapia (ABEDON et al., 2011; COOPER; KOONJAN; NILSSON, 2018; HADDAD KASHANI et al., 2017; KUTTER et al., 2010).

Recentemente, a utilização das proteínas virais responsáveis pela lise celular, como as despolimerases e hidrolases, vem sendo amplamente estudadas, tendo em vista seu potencial no controle biológico de patógenos de animais e de plantas (FISCHETTI, 2005; HADDAD KASHANI et al., 2017; LOVE et al., 2018; OLIVEIRA; SÃO-JOSÉ; AZEREDO, 2018). WU *et al.*, (2019) demostrou a aplicação de uma despolimerase derivada do vírus SH-KP152226 no controle de formação e na degradação do biofilme do isolado tipo capsular K47 de *K. pneumonae* multidroga-resistente e a atividade de despolimerização de sua cápsula. Em outro estudo, CHEN *et al*., (2018) utilizou uma despolimerase derivada do fago *P. multocida* T7-like phage vB_PmuP_PHB02 para combater a infecção sistêmica em camundongos de *Pasteurella multocida*, mostrando significativos resultados na sobrevida dos camundongos após administração continuada por 5 dias da proteína. Esses trabalhos, assim como muitos outros, demostram o potencial biotecnológico de proteínas derivadas de fagos.

A estratégia de controle que utiliza proteínas de origem viral é baseada na obtenção de um produto capaz de atuar contra micro-organismos que não respondem as terapias tradicionais com os antimicrobianos atualmente disponíveis. Outra característica interessante dessas proteínas virais é a sua ação sobre o biofilme, que é um mecanismo importante para a patogenicidade de diversas bactérias (HARPER et al., 2014 ; LOVE et al., 2018; OLIVEIRA; SÃO-JOSÉ; AZEREDO, 2018).

O processo de formação do biofilme se inicia com a adesão de células planctônicas a uma superfície abiótica ou biótica (STEPANOVIC´ et al. 2007; DONLAN, 2002; SATPATHY et al. 2016). O biofilme é um agregado de células aderidas a uma superfície embebidas em substâncias extracelulares poliméricas (EPS). Os exopolímeros da matriz do biofilme sintetizados pelas células microbianas variam muito em sua composição e arquitetura estando sujeitos a determinantes fisiológicos,

incluindo substratos e metabólitos. O EPS pode variar em propriedades químicas e físicas, mas é composto principalmente de polissacarídeos que pode representar 50% a 90% do carbono orgânico total de biofilmes (SUTHERLAND, 2001a).

Alguns desses polissacarídeos são neutros ou polianiônicos, como é o caso do EPS de bactérias gram-negativas. A presença de ácidos urônicos (como os ácidos D-glucurônico, D-galacturônico e manurônico) ou ceto-piruvatos confere a propriedade aniônica. Essa propriedade é importante porque permite a associação de cátions di-valentes, como cálcio e magnésio, que se mostraram reticulados com as cadeias poliméricas e fornecem maior força de ligação em um biofilme desenvolvido. No caso de algumas bactérias gram-positivas, como os estafilococos, a composição química do EPS pode ser bastante diferente e pode ser primariamente catiônica. Muito poucos EPS podem até ser policatiônicos como exemplificado pelo polímero adesivo obtido a partir de estirpes de *Staphylococcus epidermidis* associadas a biofilmes (MACK et al., 1996; SUTHERLAND, 2001b; DONLAN, 2002).

A composição e estrutura dos polissacarídeos determina a conformação primária do biofilme, além disso, a estrutura secundária frequentemente assume a estrutura de hélices agregadas. As ligações de polissacarídeos 1,4-β e 1,3-β conferem maior rigidez ao esqueleto estrutural, como no caso da xantana, já as ligações 1,2-α e 1,6-α formam estruturas mais flexíveis, encontradas em dextranos. Na maioria dos ambientes naturais e experimentais, o EPS será encontrado nas configurações ordenadas que são encontradas em temperaturas mais baixas e na presença de sais. Os polissacarídeos são essencialmente cadeias moleculares muito longas e finas com massa molecular da ordem de 0,5-2,0 $x10^6$ Da, mas podem associar-se de várias maneiras diferentes. Em várias preparações, os polissacarídeos foram visualizados como uma fita ligada à superfície bacteriana e formando uma rede complexa ao redor da célula.

Após a adesão, genes relacionados com a formação do biofilme passarão a ser expressos, como por exemplo, moléculas sinalizadoras de *Quorum sensing*, exopolissacarídeos e outros componentes que irão originar uma matriz polissacarídica extracelular (SUTHERLAND, 2001ª; BRANDA et al., 2005; SIMÕES; SIMÕES; VIEIRA, 2010). Concluída a aderência inicia-se o processo de crescimento celular e maturação do biofilme.

O biofilme confere características importantes para a comunidade microbiana, como resistência a antimicrobianos, variações de temperaturas e agentes sanitizantes, tornando difícil seu controle (COSTERTON; STEWART; GREENBERG, 1999; SATPATHY et al., 2016; WANG et al., 2018).

A primeira barreira que os vírus encontram ao infectar bactérias é o polissacarídeo estrutural na superfície bacteriana, que atua como uma barreira física para os vírus(YAN; MAO; XIE, 2013). Neste contexto, as proteínas codificadas pelos vírus atuam por diferentes mecanismos enzimáticos em diferentes etapas do processo de infecção viral. Esses vírus possuem em sua estrutura da fibra de cauda proteínas que irão atuar no mecanismo de invasão à célula microbiana (HARPER et al., 2014).

As despolimerases virais, comumente presentes na estrutura do vírus que infectam bactérias, atuam no processo de invasão do vírus em seu hospedeiro por meio da degradação de exopolissacarídeos, os principais constituintes da cápsula bacteriana e do biofilme (PIRES et al., 2016; SANTOS et al., 2018). As despolimerases são compostas estruturalmente por proteínas fibrosas com topologia de β-hélices paralelas composta de cadeias β ortogonais paralelas ao longo do eixo. Esta forma saliente alarga o local ativo da enzima para reconhecer e ligar sequências específicas ocultadas dentro dos polissacáridos da superfície celular (YAN; MAO; XIE, 2013; LATKA et al., 2017). Esse arranjo estrutural confere às despolimerases uma alta estabilidade, conferindo

resistência a altas temperaturas, proteases e detergentes à temperatura ambiente. Essa característica corresponde às condições extremas ao quais essas proteínas estão expostas nos mais diversos ambientes, como aqueles com presença de proteases e condições desnaturantes (LATKA et al., 2017; YAN; MAO; XIE, 2013).

As despolimerases se dividem em dois grupos principais, dependendo da atividade enzimática ao degradar os polissacarídeos na superfície de células bacterianas: hidrolases e liases. As hidrolases degradam o peptidoglicano, os polissacarídeos capsulares ou as cadeias laterais do antígeno O do LPS, ao catalisar por hidrólise a clivagem da ligação glicosil-oxigênio na ligação glicosídica. As liases, por sua vez, utilizam o mecanismo de β-eliminação para introduzir uma dupla ligação entre o C4 e C5 do ácido urônico não redutor após a clivagem da ligação glicosídica entre um monossacarídeo e o C4 do ácido urônico (SUTHERLAND, 1999).

HERNANDEZ-MORALES (2018) demonstraram a atividade de uma despolimerase associada à fibra de cauda de um vírus que infecta uma estirpe de *Acinetobacter baumannii* multirresistente. Foi mostrado que esta proteína atua degradando a cápsula de isolados bacterianos testados e também tem ação exo-polidespolimerase sendo capaz de remover o biofilme desses patógenos, apresentando, desta forma, interessante aplicação para o controle destes micro-organismos patogênicos.

As endolisinas de origem viral é um grupo de proteínas atualmente bem estudado que têm sua ação lítica na parede celular das bactérias, especificamente no peptideoglicano (LOVE et al., 2018). Estas proteínas apresentam dois sítios catalíticos, o domínio N-terminal que é enzimaticamente ativo e atua quebrando as ligações entre os açúcares N-acetilglucosamina e ácido N-acetilmurâmico e o domínio C-terminal que se liga à parede celular. Esses domínios atuam de forma modular (apenas em bactérias

Gram-positivas) ou globular (em bactérias Gram-positivas e Gram-negativas), sendo as globulares de maior interesse para aplicações biotecnológicas (FISCHETTI, 2005; LOVE et al., 2018; OLIVEIRA; SÃO-JOSÉ; AZEREDO, 2018).

Diversas endolisinas derivadas de vírus que infectam bactérias vêm sendo descritas e suas aplicações demonstradas não apenas contra a bactéria que o vírus infecta, mas em diversas outras bactérias, incluindo as patogênicas multirresistentes (HADDAD KASHANI et al., 2017). LAI *et al.*, (2011) descreveram a atividade antibacteriana de uma endolisina, denominada *LysAB2*, derivada do vírus lítico *phiAB2* que infecta *A. baumanni*. Neste estudo, foi testada a atividade de *LysAB2* em bactérias Gram-negativas e Gram-positivas e, em cepas de bactérias multirresistentes, mostrando que essa proteína apresenta efetividade lítica tanto para bactérias Gram-negativas quanto para Gram-positivas.

Além disso, a ação destas endolisinas sobre a formação de biofilme também vem sendo estudada. LINDEN *et al.*, (2014) avaliaram a atividade da endolisina *PlyGRCS* contra cepas de *Staphylococcus aureus* resistentes à meticilina e observaram que a endolisina apresentou ação efetiva contra o crescimento e formação de biofilme. OLSEN *et al.*, (2018) demonstraram a atividade antibacteriana e sobre a formação do biofilme uma endolisina associada a uma despolimerase contra cepas de *S. aureus*.

Outra classe de proteínas expressas por vírus e de interesse biotecnológico são as holinas. As holinas são proteínas hidrofóbicas expressas pelo cassete de lise dos vírus que infectam bactérias formando oligômeros que se ancoram na membrana citoplasmática dessa bactéria causando alterações na permeabilidade da membrana, podendo levar à morte da célula independente de endolisina (SANTOS et al., 2018; SONG et al., 2016; WHITE et al., 2011).

SONG *et al.*, (2016) analisaram a atividade da holina HolGH15 do vírus *Staphylococcus aureus* phage GH15 e verificaram que essa proteína apresenta atividade antimicrobiana em uma ampla variedade de micro-organismos como *S. aureus, Listeria monocytogenes, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae* e *E. coli*. SHI *et al.*, (2012) mostraram que a atividade da endolisina LySMP foi ampliada quando combinada com uma holina HolSMP, ambas originadas do mesmo vírus *S. suis* serotype 2 (SS2), revelando uma nova estratégia de utilização de proteínas derivadas de vírus para o controle de bactérias de interesse.

Recentemente, XAVIER *et al.*, (2018) identificaram e caracterizaram um novo vírus que infecta *Ralstonia* spp. O vírus classificado no gênero *Phikmvvirus* e inicialmente nomeado como *"Ralstonia virus phiAP1" (phiAP1)* é um vírus dsDNA, exclusivamente lítico, caudado, com um capsídeo icosaédrico de aproximadamente 60 nm de diâmetro e uma cauda curta não-contrátil, com uma ampla gama de hospedeiros e uma forte atividade bacteriolítica. A análise genômica do vírus mostrou que esse isolado viral possui atividade de EPS-despolimerase, além de codificar duas putativas hidrolases de peptídeoglicano associada ao vírus (VAPGHs). Uma análise do cassete de lise deste isolado mostrou que o vírus codifica quatro proteínas envolvidas na lise celular. Uma holina, (ORF50), uma endolisina (ORF51), uma i-espanina (ORF52) e uma O-espanina (ORF53). Além disso, também foram identificadas outras duas ORFs putativas, ORF 43 e ORF 47, que codificam proteínas das fibras de cauda curta e longa, respectivamente, que teriam atividade despolimerase (XAVIER et al., 2018).

Ralstonia spp. são patógenos bacterianos de plantas de grande interesse econômico que pertencem a um complexo de espécies formado pelas espécies *Ralstonia solanacearum, R. pseudosolanacearum e R. syzygii* (HAYWARD, 1991; FEGAN e PRIOR, 2005; SAFNI *et al.*, 2014). Esse complexo de espécie infecta mais

de 200 espécies de plantas dentro de mais de 50 famílias botânicas (SAFNI et al., 2014; XAVIER et al., 2018).

São bactérias Gram-negativas, não-fermentativas, encontradas no solo com distribuição global, o qual constitui o seu habitat natural (SALANOUBAT et al., 2002; XAVIER et al., 2018). Seu mecanismo de patogenicidade depende de diversos genes relacionados com a virulência, como genes *hpr* hidrolases, exopolissacarídeos (EPS), lipopolissacarídeos (LPS), lectinas, proteínas relacionadas com aderência, entre outras (SALANOUBAT et al., 2002; SCHELL, 2000).

Um importante mecanismo de patogenicidade é a formação do biofilme no xilema da planta hospedeira, que obstrui o fluxo de água e nutrientes para a parte superior da planta levando a sua morte por murcha (GENIN, 2010; SCHELL, 2000).

A alta variabilidade genética do patógeno, ampla gama de hospedeiros e sobrevivência da bactéria por longos períodos no solo, torna a utilização de métodos de controle tradicionais ineficaz. Neste contexto, a utilização de proteínas bacteriolíticas produzidas por vírus pode ser uma estratégia de controle alternativa interessante. Desta forma, este trabalho teve como objetivos realizar a caracterização da atividade de uma EPS-Despolimerase derivada do vírus phiAP1 e analisar sua atividade no controle e degradação do biofilme de diferentes espécies bacterianas.

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CAPÍTULO 2

The activity of *Ralstonia virus phiAP1* Gp16-43 protein in the inhibition and degradation of bacterial biofilm

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The activity of *Ralstonia virus phiAP1* Gp16-43 protein in the inhibition and degradation of bacterial biofilm

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Abstract

Proteins associated with virus lysis are promising agents for the control of pathogenic microorganisms. Viral depolymerases are a class of proteins that play an important role in the process of phage infection in their host. Depolymerases act on the exopolysaccharide matrix of the bacterial biofilm, being an interesting agent of inhibition and degradation of the biofilm of pathogenic bacteria. In the present study, a depolymerase (Gp16-43) derived from the phage *Ralstonia virus phiAP1* was cloned and expressed in *E. coli* and characterized. Gp16-43 protein was able to inhibit the formation of *E. coli* and *S. aureus* biofilm at low protein concentrations (250 and 500 µg / mL). The protein Gp16-43 degraded the biofilm formed by *E. coli* and *Ralstonia pseudosolanacearum*, being more efficient at a concentration of 1375 µg / mL, although this effect was not observed in the biofilm formed by *S. aureus*. Based on these results, Gp16-43 appears to be a phage-derived depolymerase that represents a new potential strategy for prevent and degrade biofilm formation of Gram-positive and Gram-negative microorganisms.

Keywords: Phage, depolymerase, biofilm, exopolysacharide

Introduction

Bacteriophage proteins (phages) are promising agents for the control of pathogenic microorganisms, including multiresistant bacteria (HARPER et al., 2014; LAI et al., 2011). Several studies have been conducted with these proteins to control different bacterial pathogens such as S. aureus, E. coli and Salmonella spp (Lai et al. 2011; Wang et al. 2017; Olsen et al. 2018). Such phage lytic proteins, such as depolymerase, endolysins, and virus-associated peptidoglycan hydrolases, are proposed as potential antibacterial and anti-biofilm agents. (SÃO-JOSÉ, 2018).

In recent years, phage proteins have been shown to inhibit biofilm formation and the ability to degrade biofilms already formed. For instance, the tail tubular proteins B of the phage KP32 are polysaccharide depolymerase (PD) that shows a high inhibitory activity on biofilm formation of *Enterobacter cloacae, Enterococcus faecalis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains (BRZOZOWSKA et al., 2018). The phage vB_SepiS-phiIPLA7 that infect *Staphylococcus epidermidis* encodes a PD that can be used to prevent biofilm formation of *S. epidermidis* and *S. aureus* strains as well as in the degradation of *S. aureus* biofilm (GUTIÉRREZ, D. et al., 2015; GUTIÉRREZ et al., 2017).

These depolymerases are classified as: hydrolases, lyases and lypases. The hydrolases are enzymes that catalyze the hydrolysis of glycosidic bonds, such as rhamnosidases (hydrolyze the outer membrane of Gram-negative bacteria), sialidases and dextranases. Lyases cleave (1, 4) glycosidic bonds by a β-elimination mechanism. This class of enzymes comprises three groups of phages depolymerases: hyaluronate, alginate, and pectin/pectate lyases. Finally, the lypases or triacylglycerol hydrolases, that acts on the carboxyl ester bonds of triacylglycerols to liberate organic acids and

glycerol. This classification was according to the specific activity of the enzyme in degrading the polysaccharides or polysaccharide derivatives (PIRES et al., 2016).

The biofilm is a complex aggregate of structured microbial communities attached to a surface and embedded in an extracellular polymeric substance (EPS) that can be composed of polysaccharides, proteins, nucleic acids, and lipids(COSTERTON; STEWART; GREENBERG, 1999; FLEMMING; WINGENDER, 2010). The biofilm represents an important virulence factor, confering limitation to penetration of antimicrobial agents and increased tolerance to host defenses (BRIDIER et al., 2015; OLSEN et al., 2018). Thus, the search for alternative strategies for eradication and prevention of biofilm formation is very relevant to develop new tools to control pathogenic microorganisms.

Ralstonia spp. are bacterial pathogens of plants of great economic interest belonging to a heterogeneous complex of species: *Ralstonia solanacearum* (*R. solanacearum*), *Ralstonia pseudosolanacearum* and *Ralstonia syzygii* (HAYWARD, 1991; FEGAN and PRIOR, 2005; SAFNI et al., 2014). This species complex infects more than 200 plant species within more than 50 botanical families, showing its relevance as phytopathogens of interest to the scientific community in finding ways to combat them (SAFNI et al., 2014; XAVIER et al ., 2018). Thus, due to the high pathogenicity and the high economic loss that *Ralstonia* spp. cause, the search for strategies to control this plant pathogen is extremely important.

XAVIER et al. (2018) isolated and characterized a new virus that infects *Ralstonia* ssp., *Ralstonia virus phiAP1* (phiAP1). This virus has a lysis cassette that encodes four putative proteins: Holina (ORF 50), endolysin (ORF 51), o-Spaniard (ORF 52) and 1-Spaniard (ORF 53). In addition its genome encodes two putative tail fibers, one short (ORF 43) and one long (ORF 47). In his work, Xavier demonstrated the broad

spectrum of Ralstonia hosts that this virus possesses, and found that by forming the lysis plate, this virus produces a characteristic halo of EPS depolymerase activity. This activity was attributed by the author to the depolymerase activity of putative virus-encoded tail fibers in function prediction analyzes.

The ORF 43 of phiAP1 encodes a putative short tail fiber protein of approximately 16 kDa, named Gp16-43. Gp16-43 has a putative exopolysaccharide-depolymerase activity that can be exploited for the use of in inhibition and/or degradation of biofilm. Thus, the aim of this study was to evaluate the activity of putative protein of phiAP1Gp 16-43 on formation and degradation biofilm of Gram-negative bacteria (*E. coli* and *R. pseudosolanasearum*) and Gram-positive bacteria (*S. aureus*).

Material and Methods

Bacterial strains, plasmids and culture conditions.

E. coli strains DH5α and BL21 (DE3) were used for gene cloning and protein expression of the ORF 43. The strains *of E. coli* ATCC25922, *S. aureus* ATCC25923 and *R. pseudosolanacearum* GMI1000 were used for to evaluate of the antibacterial activity, inhibitory activity of the biofilm formation and degradation of formed biofilm of the putative protein. *E. coli* strains (DH5α and BL21) were cultured in Luria-Bertani broth (LB) supplemented with ampicillin (100 μg/ml) at 37 °C, at 250 rpm. *E. coli* ATCC25922 was cultured in Luria-Bertani broth (LB) at 37 °C, at 250 rpm. *S. aureus* ATCC25923 was grown in Trypticase Soy Broth (TSB) at 37 °C, at 250 rpm. *R. pseudosolanacearum* was grown in Casamino acid-Peptone-Glucose (CPG) broth

containing casein (1 g/L), peptone (10 g/L) and glucose (5 g/L) at 28 °C, at 250 rpm. All strains were growth for 24 h.

Standardization of the inoculum

Aliquot of 100 µL of the stock culture of *E. coli*, *S. aureus* and *R. pseudosolanacearum* were activated and reactivated in 10 mL of LB, TSB and CPG, respectively. *E coli* and *S. aureus* was incubated at 37 °C and *R. pseudosolanacearum* at 28 °C for 24 h, in a shaker at 250 rpm. Then, 1 mL of each culture was pelleted in a centrifuge at 10,000 *g* for 5 minutes and the pellet formed was washed twice in 1 mL of 10 mM PBS, pH 7.2. The inoculum was standardized to OD 0.10 (10^7 CFU.mL^{-1}) using PBS as diluent in a spectrophotometer at 600 nm (Thermo Fisher Scientific, Finland).

DNA manipulation techniques and cloning procedures

DNA fragments corresponding to the ORF 43 (short tail fiber) gene were obtained from PCR amplification of PHIAP virus genomic DNA fragments with the use of primers Depol_ORF_43_F: TTTGGATCCATGGAAGTACCGAGC and Depol_ORF_43_R: CCCAAGCTTTCATGATCCCTCCTC. and restriction enzymes BamHI and HindIII by cleavage. The amplification product was cleaved using restriction enzymes BamHI and HindIII and cloned in pRSET-A (Invitrogen, California-USA) vector. *E. coli* DH5α and BL21 were transformed using standard technique (SAMBROOK et al., 2000). Plasmid DNA from the transformants was extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison-USA). The clones were sequenced (Macrogen, Seoul-Republic of Korea) to confirm the cloning.

Expression and purification of Gp16-43

The expression of Gp16-43 protein was conducted according to Obeso et al. (2008) with following modifications: cells induced with 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) at 37 ° C for 5 hours at 250 rpm. After induction the culture was centrifuged at 5,000 *g* for 10 minutes at 4 °C.

The pellet was lysed in lysis buffer (Tris-HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 8.0), at the concentration of 20 mL per 200 mL of induced, with 500 µL of lysozyme (10 mg/mL) in five sonication cycles (400 W, 20 KHz; Sonics & Materials Inc., USA) interspersed with vortexing. The cell lysate was centrifuged at 14,000 g for 5 minutes and the supernatant was discarded. Thereafter lysis buffer was added with 1% Triton X-100, the pellet was suspended by sonication and centrifuged. For removal of the Triton residues, one wash of the protein extract was performed with buffer N ($NaHCO_3$ 100 mM, pH 9.0), suspending the protein extract by sonication. After the final centrifugation, the final protein extract was suspended in 1 mL of buffer N with sodium lauryl sulfate 1% (SDS).

Purification was performed according to OLSEN et al. (2018) by affinity chromatography with Ni-NTA manual column eluted with buffer N + SDS + Imidazole (250 mM). The protein was dialyzed in renaturing buffer (Tris-HC 10mM, Nacl 100 mM, EDTA 1mM, glycerol 10%, DTT 1mM, PMSF 1mM) and again dialyzed in 10mM PBS ph 7.2 buffer for 24 hours and sterilized by filtration. The collected fractions were run on SDS-PAGE Gel and protein quantification was done by Bradford assay (BRADFORD 1976). Protein identity was confirmed by mass spectrometry (MALDI-TOF / TOF) and compared to the putative sequence deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Determination of Minimum Inhibitory Concentration (MIC)

Cultures of *E. coli*, *S. aureus* and *R. pseudosolanacearum* were standardized as described before and inoculated into polystyrene microplates 96 wells containing 2X LB or 2X TSB broth plus different concentrations of protein (250, 500, 100 and 2000 µg/mL). Wells with bacterial cultures without protein addition and only with bacterial culture and PBS were used as controls. The culture was maintained for 24 h at 37 °C and bacterial growth was evaluated by spectrophotometry at OD 600 nm.

Inhibition test of biofilm

Biofilm formation by *E. coli* was performed in LB broth (with 1% glucose), *S. aureus* biofilm was formed using TSB broth and *R. pseudosolanacearum* was formad using CPG broth (whit 1% glucose). The microplates were assembled as described for MIC determination with different protein concentrations. Wells with bacterial cultures without protein addition and only with bacterial culture and PBS were used as controls. The microplates were incubated at 37 °C and the absorbance at OD 600 nm read after 24 h. The microplates were stained with violet crystal 0.1 % and the wells washed gently, for three consecutive times, with 10 mM PBS. The plates were dried at 60 °C for 30 minutes. The violet crystal retained was removed by adding 95 % (v/v) ethanol and the absorbance was measured by spectrophotometer at OD 590 nm. The results were expressed by relation between the absorbance of the crystal violet extract at 590 nm and growth of bacteria at 600 nm (VIANA et al., 2009).

Planktonic cell colonies were counted to evaluate the action of Gp16-43 protein on planktonic growth. For this purpose, 100 µl of bacterial suspension was diluted in

PBS buffer collected from biofilm inhibition plates. Dilutions 10^{-3} to 10^{-6} were plated by the drop plate method on Plate Count Ágar (PCA). The plates were incubated at 37 °C for *E. coli* and *S. aureus* and at 28 °C for *R. pseudosolanacearum*, for 8-12 hours. Colony counts were expressed as CFU/mL.

Biofilm degradation assay

E. coli was growth in LB broth (with 1 % glucose), *S. aureus* in TSB broth and *R. pseudosolanacearum* in CPG broth with 1 % glucose for 24 h to form biofilm in 96 well microplates. The microplates of *E. coli* and *S. aureus* were incubated at 37 ºC and *R. pseudosolanacearum* at 28 ºC for 24 h. Then, the culture medium was removed, and protein diluted in PBS (1375-5500 µg/mL) was added. The microplates were incubated at 30 °C for 2 hours. Sodium metaperiodate ($NaIO_4$) was used as a positive control of biofilm degradation. The violet crystal retained was removed by adding 95 % (v/v) ethanol and the absorbance was measured by spectrophotometer at OD 590 nm (VIANA et al., 2009).

Statistics

Experiments were conducted in three biological replicates. Statistical analyses were performed using Minitab statistical software 17.0. All data were subjected to analysis of variance (ANOVA) followed Tukey's test to determine the existence of differences between the treatments and controls. A significance level of 0.05 was adopted.

Results

Cloning, gene expression and protein analysis

After extracted and purified a band of about 16 kDa corresponding with the molecular weight of Gp16-43 was observed in gel of SDS_PAGE (Figure 1). The protein sequence was confirmed by mass spectrometry and a total of four peptide fragments were generated (Figure 2). A coverage at 30 % and an identity at 93.94 % of the complete sequence of the putative protein (putative short tail fiber *Ralstonia virus phiAp1*) deposited in the GenBank database was obtained (https://www.ncbi.nlm.nih.gov/genbank/). Observing the expected size band according to the genome prediction and protein sequencing allows us to conclude that the protein of interest has been expressed and will be used for activity assays.

Gp16-43 is able to inhibit the formation of *E. coli, S. aureus* and *R. pseudosolanacearum* biofilms

The *E. coli* biofilm formation inhibition assay showed that the lower concentration of Gp 16-43 protein (250 µg / mL) was able to inhibit the biofilm when compared to the control. (Figure 3A).

Interestingly, high concentration of protein inhibited the biofilm formation but no statistical differences were observed between the concentrations of proteins tested in this study. In addition, we tested the influence of protein concentrations on the growth of *E. coli* planktonic cells. As observed in figure 3B, only the concentration of 1000 µg/mL interfered in growth compared to the control.

. As observed in *S. aureus* the low concentrations tested (250 µg/mL and 500 µg/mL) inhibited biofilm formation but did not affect growth. It was observed that the

concentrations of 1000 µg/mL and 2000 µg/mL decreased the planktonic growth of this bacterium (Fig. 4B).

The protein Gp 16-43 showed excellent efficiency in inhibition of *R. pseudosolanacearum* biofilm at all concentrations tested (Fig. 5A). Unlike the other bacteria tested, all concentrations influenced the growth of planktonic cells of *R. pseudosolanacearum*, when compared to the control (Fig. 5B). Protein Gp 16-43 showed better efficiency for *R. pseudosolanacearum*, which was expected since this protein is derived from a virus that infects *Raltonia* spp. These results indicate that the Gp16-43 protein has activity in inhibiting the formation of biofilm of Gram-negative and Gram-positive bacteria.

The minimum inhibitory concentration was not obtained from this work. The results obtained on the growth of planktonic cells we can suggest that the enzymatic mechanism of Gp16-43 is not related to cell death but to the degradation of sugar present in the exopolysaccharide matrix of the biofilm. Further testing should be conducted to determine the target protein action.

Gp16-43 degrades biofilms

The action of protein in degrading the biofilm of the strains *E. coli*, *S. aureus* and *R. pseudosolanacearum* was evaluated with different concentrations of Gp16-43 for 2 h at 30 °C. The concentrations of 1375 µg / mL and 5500 µg / mL were able to degrade *E. coli* biofilm when compared to NaIO4. The protein concentration of 2750 µg / mL showed no statistical difference with NaIO4. (Figure 6A). Unlike *E. coli*, all protein concentrations tested in *S. aureus* were not able to degrade biofilm when compared to control, as well as NaIO4. (Figure 6B).

All protein concentrations tested were able to efficiently remove the biofilm of *R. pseudosolanacearum* (Figure 6C), showing the great potential of biotechnology Gp16-43 protein to remove the biofilm *Ralstonia* spp.

Discussion

Over the last few years, the study of phage lytic proteins for the control of pathogenic microorganisms has attracted great interest (PIRES et al., 2016; SANTOS et al., 2018). These proteins have activity in formation and degradation of biofilm (SÃO-JOSÉ, 2018). In this study, the Gp16-43 was purified and used in biofilm inhibition and degradation assays of *E. coli*, *S. aureus* and *R. pseudosolanacearum*. The inhibition of biofilm formation was observed in all bacteria tested by Gp16-43. This protein was recently characterized as a depolymerase (XAVIER et al., 2018). GUO et al. (2017) identified and characterized a depolymerase (Dpo42) derived from *E. coli* Phage vB_EcoM_ECOO78 capable of inhibiting the formation of the biofilm of *E. coli* strains. Thus, the depolymerases can to degrade exopolysaccharide produced by bacteria preventing the formation of the initial structure of the biofilm, but the complete mechanism remains unknown.

Depolymerases are a common constituent of the tail structure of phages that act as an adjuvant for phage infection promoting the degradation of polymers present on the surface capsular polysaccharides, or exopolysaccharide present in bacterial biofilms (YAN; MAO; XIE, 2013). The results of the present study showed that Gp16-43 has activity against the formed biofilm of *E. coli* and *R. pseudosolanacearum* but did not degrade *S. aureus* biofilm. The composition of the biofilm is different for each species of bacteria. Thus, a depolymerase can degrade EPS produced by one species may not degrade the EPS produced by other bacteria (HARPER et al., 2014).

CORNELISSEN et al. (2011) evaluated the ability of the *Pseudomonas putida* Phage φ15 to degrade the biofilm of *P. putida* strains, attributing its activity to the action of the tail-associated depolymerase. In literature mentions that biofilms produced by strains of *Staphylococcus* spp. containing a significant amount of exopolysaccharides (FERREIRA et al., 2015). However, ONICIUC et al. (2016) have shown that the biomass formed by *S. aureus* was removed in greater proportion when proteinase K (60-70%) was used compared to $NaIO_4$ (20-49%). These results suggest that proteins are important constituents of the mature biofilm of *S. aureus*. Thus the present study did not observe significant degradation of the biofilm of *S. aureus* possibly due to the composition of this biofilm. Interestingly, a depolymerase (Dpo7) derived from *Staphylococcus epidermidis* Phage vB_SepiS-phiIPLA7, showed activity on the *S. aureus* 15981 biofilm but not activity on *S. aureus* V329 biofilm (Gutiérrez et al. 2014). These authors suggest that this difference in activity of Dpo7 is attributed the composition of the S. *aureus* strain V329 biofilm that is predominantly protein. In addition, Gp16-43 is a depolymerase in which it is predicted to degrade exopolysaccharide, however the mode of action of this protein remains unclarified.

Conclusion

The findings of this study showed that the depolymerase Gp16-43 has activity in inhibiting the formation of biofilms of *E. coli* and *S. aureus* as well as to degrade the *E. coli* and *R. pseudosolanacearum* biofilm but not *S. aureus*. Thus, the perspectives are to elucidate the mechanism of action of Gp16-43 in inhibiting the formation and degradation of bacteria biofilms. In addition, to evaluate the spectrum of action in other pathogenic microorganisms and to check the optimal conditions of activity such as temperature and pH.

Acknowledgments

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Figure Legends

Fig 1 Purification of the recombinant exopolysaccharide depolymerase Gp16-43 from *E. coli* BL21 pRSETA-*gp16-43*. L: Standard molecular weight marker in kDa (Low Range Molecular Weight SDS-PAGE Standards, BioRad); 1- Total protein extract obtained from the extraction of *E. coli* BL21 pRSETA-*gp16-43*. 2- Fraction eluted from nickel affinity chromatography containing purified Gp16-43. Band indicated with a red arrow was identified by mass spectrometry. 3- Total protein extract obtained from the extraction *E. coli* BL21 pRSETA. 4- Fraction eluted from nickel affinity chromatography containing purified empty vector.

Fig 2. Alignment of proteins obtained from the Mascot software. Matched peptides shown in bold red**.**

Fig. 3 Activity of Gp16-43 against biofilms *E. coli* ATCC25922 and on planktonic cell growth. A- Inhibition of biofilm on different concentrations of Gp16-43 protein (250-2000 µg/mL). Absorbance of crystal violet was read at OD 590 nm, and biofilm formation in LB with 1% glucose, values expressed for the ratio 590/600 nm. B- Number of colony forming units of *E. coli* planktonic cells on different concentrations of Gp16-43 protein (250-2000 µg/mL) expressed as $CFU.ml^{-1}$. (ANOVA; $P < 0.05$).

Fig. 4 Activity of Gp16-43 against biofilms *S. aureus* ATCC25923 static model and on planktonic cell growth. A- Inhibition of biofilm on different concentrations of Gp16-43 protein (250-2000 µg/mL). Absorbance of crystal violet was read at OD 590

nm, and biofilm formation in TSB medium, values expressed for the ratio 590/600 nm. B- Number of colony forming units of *S. aureus* planktonic cells on different concentrations of Gp16-43 protein (250-2000 µg/mL) expressed as $CFU.ml^{-1}$. (ANOVA; $P < 0.05$).

Fig. 5 Activity of Gp16-43 against biofilms *R. pseudosolanacearum* GMI1000 static model and on planktonic cell growth. A- Inhibition of biofilm on different concentrations of Gp16-43 protein (250-2000 µg/mL). Absorbance of crystal violet was read at OD 590 nm, and biofilm formation in TSB medium, values expressed for the ratio 590/600 nm. B- Number of colony forming units of *R. pseudosolanacearum* planktonic cells on different concentrations of Gp16-43 protein (250-2000 µg/mL) expressed as $CFU.ml^{-1}$. (ANOVA; $P < 0.05$).

Fig. 6 Degradation of the biofilm of different bacterial species by the Gp16-43 protein. A- Degradation of *E. coli* ATCC 25922 biofilm; B- Degradation of *S. aureus* ATCC 25923 biofilm; C- Degradation of *R. pseudosolanacearum* GMI1000 biofilm. Total attached biomass was measured by crystal violet staining after treatment and expressed as A590 nm. (ANOVA; $P < 0.05$).

Figures

Figure 1

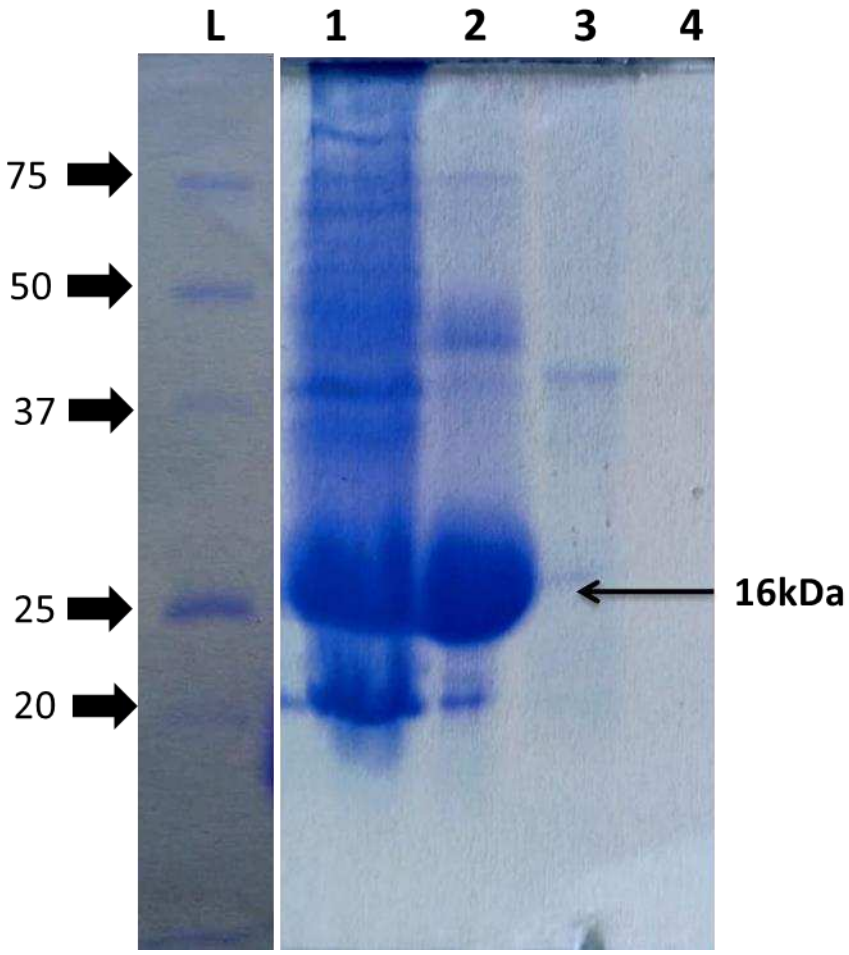

Figure 2

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  1 MEVPSTLTSR DEQVLRLQDA VAKLPQVECR VRNYFAPGVY CREMTIPAGV
 51 VAVGARHKTE HLSILVQGHV HITTDDGTVE VHAPATFLSK AGAKRACFAV
101 TEAIITTIHP TDETDLDKLC ELLTDTTSDR LIGGSNNVQL QLSGRAEEGS
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Figure 3

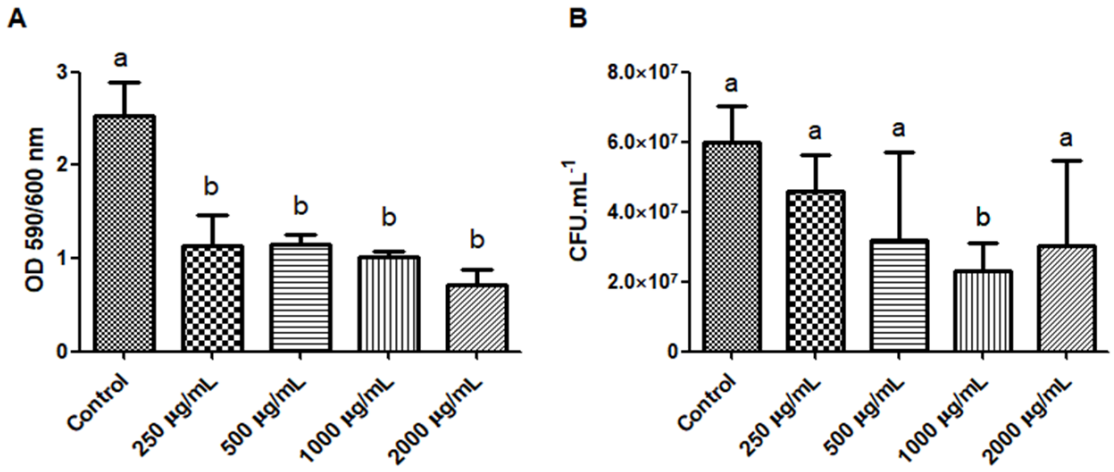


Figure 4

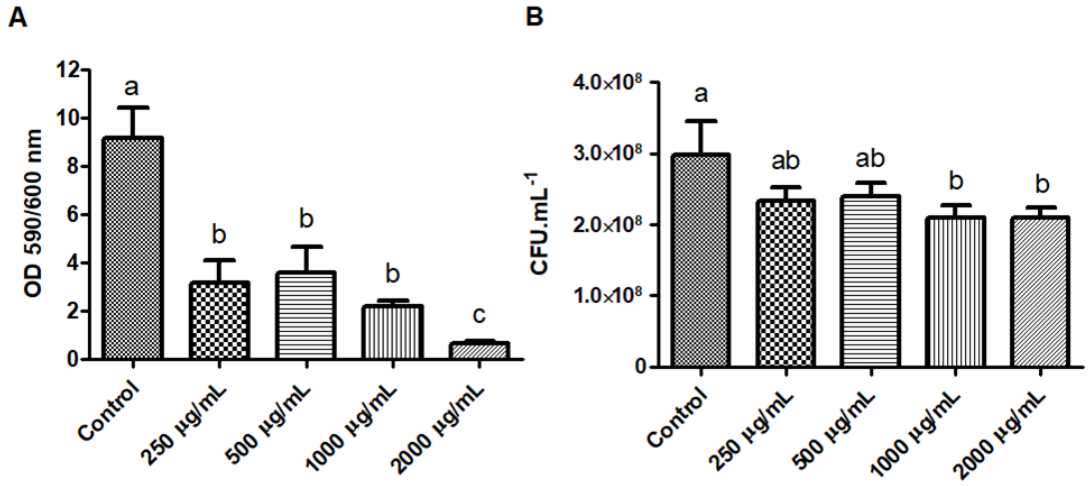


Figure 5

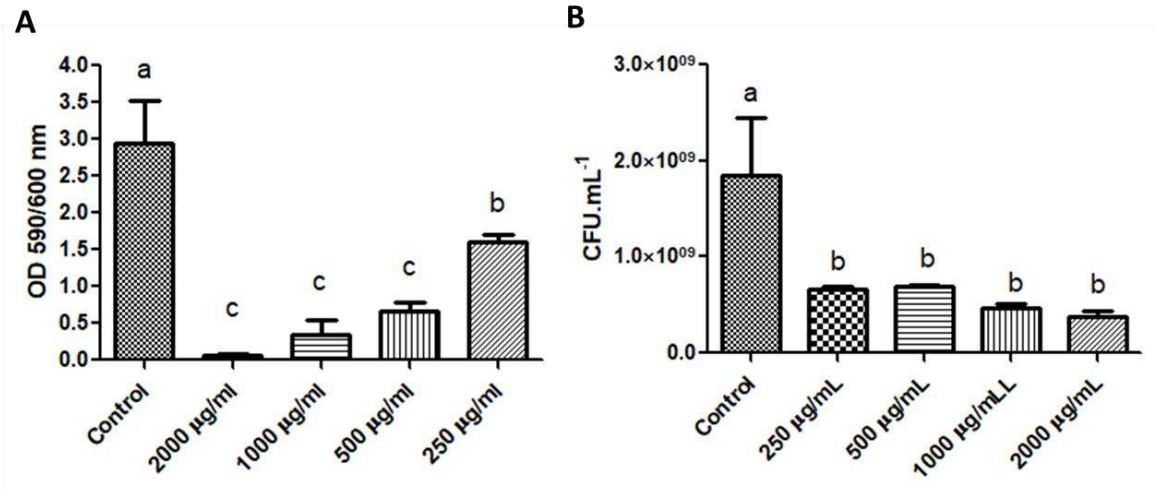
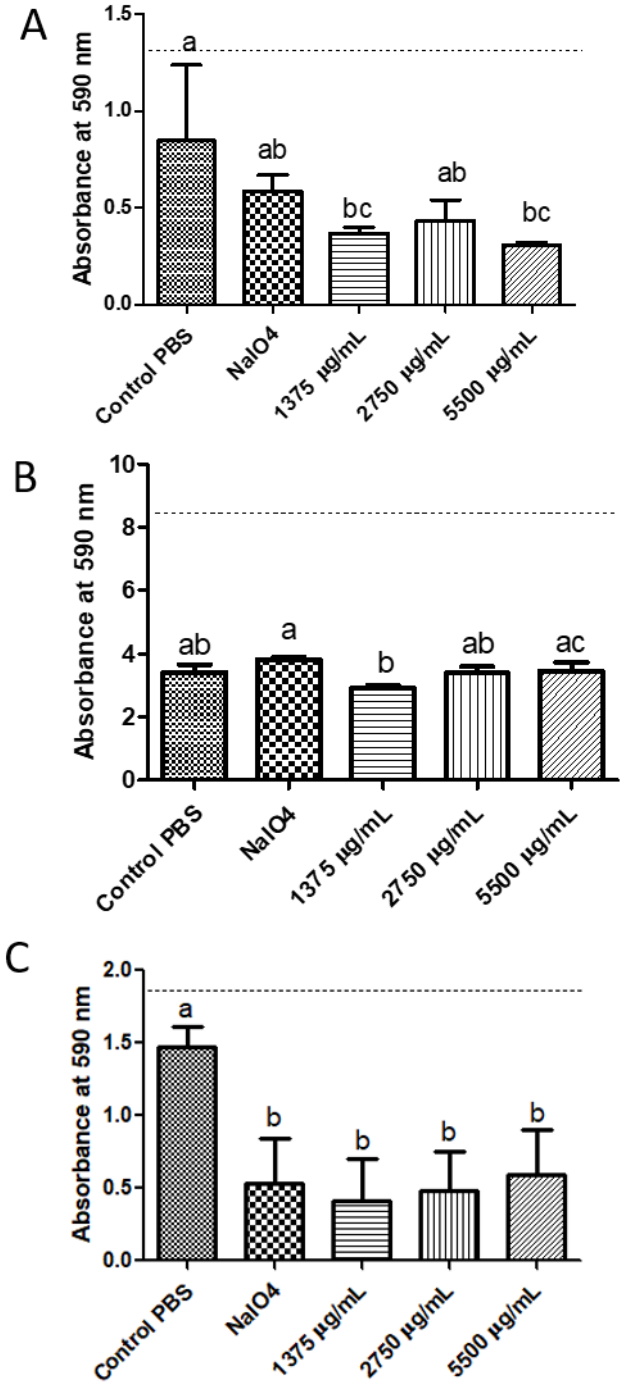

A
OD 590/600 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
c
c
c
b
Control
2000 µg/ml
1000 µg/ml
500 µg/ml
250 µg/ml
B
CFU.mL-1
3.0×10^09
2.0×10^09
1.0×10^09
0.0
a
b
b
b
b
Control
250 µg/mL
500 µg/mL
1000 µg/mLL
2000 µg/mL

Figure 6


A
Absorbance at 590 nm
1.5
1.0
0.5
0.0
a
ab
bc
ab
bc
Control PBS
NaIO4
1375 µg/mL
2750 µg/mL
5500 µg/mL
B
Absorbance at 590 nm
10
8
6
4
2
0
ab
a
b
ab
ac
Control PBS
NaIO4
1375 µg/mL
2750 µg/mL
5500 µg/mL
C
Absorbance at 590 nm
2.0
1.5
1.0
0.5
0.0
a
b
b
b
b
Control PBS
NaIO4
1375 µg/mL
2750 µg/mL
5500 µg/mL