UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

TESE DE DOUTORADO

AVALIAÇÃO DE LECTINA SOLÚVEL EM ÁGUA DE SEMENTES DE *Moringa oleifera* (WSMoL) NO CONTROLE DA BIOCORROSÃO

MAIARA CELINE DE MOURA

RECIFE
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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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RECIFE
2016

Catalogação na fonte
Elaine Barroso
CRB 1728

Moura, Maiara Celine de
Avaliação de lectina solúvel em água de sementes de *Moringa oleifera* (WSMoL) no controle da biocorrosão / Maiara Celine de Moura- Recife: O Autor, 2016.

123 folhas: il., fig., tab.
Orientadora: Luana Cassandra Breitenbach Barroso Coelho
Coorientadora: Patrícia M.G. Paiva
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Bioquímica e fisiologia, 2016.
Inclui referências

1. Lectinas 2. Bactérias 3. Moringa oleifera I. Coelho, Luana Cassandra Breitenbach Barroso (orientadora) II. Paiva, Patrícia M. G. (coorientadora) III. Título

572.6 CDD (22.ed.) UFPE/CB-2017-197

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Data: 29/07/2016

Aos meus pais.

AGRADECIMENTOS

A Deus pela vida, pela saúde, pelo amor incondicional e por permanecer ao meu lado em todos os lugares e situações, difíceis ou não, sempre me guiando.

Aos meus pais, “painho” Valter e “mainha” Nadja, por absolutamente tudo que me ensinaram, principalmente a superar desafios e em ser responsável. Amor, atenção e educação nunca faltaram na minha vida graças a eles. Valorizo cada minuto que tenho com vocês dois.

À minha irmã Uiara ou “Lala” por ser um exemplo de perseverança e carinho. E ao seu esposo, Sérgio, por fazê-la feliz e por participar da nossa família. Amo muito os dois.

Ao meu mais novo irmão, João Pedro, que em um pouco mais de um ano de convivência trouxe mais luz e amor para nossa casa. O tamanho da felicidade que existe em meu coração desde que você chegou é imensurável. Eu amo você.

À Flávia, esposa do meu pai e mãe do meu irmão, por ter entrado na nossa família para trazer mais felicidade e leveza. Seu cuidado e carinho com meu pai e João é algo que tenho como exemplo. Muito obrigada!

Aos meus tios simplesmente maravilhosos e carinhosos, Sebastiana e Bezerra, pelo grande afeto, pelo amor, pelas orações e por todo o cuidado a mim dedicado.

A toda minha família pelos encontros, pelas brincadeiras, pela amizade, presença e confiança.

À Professora Luana, minha orientadora, pela oportunidade, dedicação, energia positiva, apoio e pelos ensinamentos transmitidos, que foram e serão fundamentais para o meu crescimento como pesquisadora.

À Professora Patrícia pela co-orientação, apoio, alegria e torcida.

Ao Professor Alexandre Macedo, pela colaboração e por me receber em seu laboratório na UFRGS de braços abertos. A experiência foi incrível. Muito obrigada. Estendo esse agradecimento a todos que compõem o LabDIM pelo carinho, pela hospitalidade e por todos os momentos juntos. Em especial, a Danielle Trentin, que foi minha parceira de bancada.

A Paulo César, meu namorado, por acreditar e incentivar os meus sonhos, por estar presente no meu dia a dia e, principalmente, por ser tão especial para mim.

À Raphaella, minha amiga e, mais que isso, uma irmã. Anos convivendo juntas, compartilhando todos os momentos. Confiança, carinho e amor é a base da nossa amizade. Ela representa um grupo maior, as Amdrcryf´s, tempos de escola e muitas saudades.

À Mayara, pela amizade, pelo carinho, pelas risadas e pelo amor. Hoje estou extremamente grata por ser madrinha da Maria Clara que já chega ao mundo. Estou muito feliz! Muito obrigada!

À Mércia, Verônica, Isis, Giliane, Taíse e Priscila, as “pimentinhas”, por participarem da minha vida da forma mais alegre e mais descontraída. Vocês foram um presente que May me entregou em 2013. Amo vocês.

A Emmanuel que conheci quando entrei para o laboratório de Glicoproteínas, em 2008, ainda inexperiente e desastrada. Agradeço seus ensinamentos indispensáveis para o meu crescimento pessoal e científico e, mais que isso, agradeço pela sua amizade. Seu carinho, sua presença e sua ajuda me fizeram te admirar ainda mais. És para mim um exemplo de amigo, filho, marido e pai. Inabelle e Isabella são e serão as pessoas mais felizes e completas do mundo graças a tua dedicação e amor. Eu amo você Mano.

A Thiago por ser tão amigo, tão especial, tão prestativo. São tantas qualidades que é impossível listar todas aqui. Sua presença é maravilhosa, nossos momentos juntos com Emmanuel, Lidiane e Nataly são os melhores que podem existir. Amo você. Amo nossa amizade. Não quero perdê-la nunca. Acrescento aqui meus agradecimentos à Lidiane e Nataly, minhas amigas do coração, por tudo que representam para mim. Muita garra, determinação e perseverança. Admiro muito as duas. Amo muito as duas.

A todos que compõem o Laboratório de Glicoproteínas, pelo convívio, pela troca de experiências e pela torcida, em especial, Thamara, Marília, Cynarha, Ana Patrícia, Kézia, Thâmarah, Felipe, Leonardo, Lívia, Igor, Afonso e Bernardo.

A Fundação de Ámparo a Ciência e Tecnologia do Estado de Pernambuco (FACEPE) pelo suporte financeiro e a todos os órgãos de fomento. À FACEPE agradeço também a bolsa concedida através do Auxílio Mobilidade Discente (AMD).

“Ama-se mais o que se conquista com esforço”
Benjamin Disraeli

RESUMO

Bactérias são organismos procariontes capazes de se adaptar aos mais variados tipos de habitat; podem crescer e aderir a várias superfícies, formando comunidades complexas conhecidas como biofilmes. O desenvolvimento de biofilme, na maioria dos casos, resulta em riscos para a saúde em ambientes clínicos, devido a elevada patogênese, e causa perdas econômicas em diferentes setores da indústria, sendo a principal causa da biocorrosão. Estratégias de controle dos biofilmes envolve o uso de antibióticos e biocidas; entretanto, bactérias dentro de biofilmes são mais resistentes a vários tipos de tratamentos. Diversos mecanismos de resistência bacteriana aos antimicrobianos sintéticos são descritos. Pesquisas têm sido desenvolvidas com o objetivo de identificar compostos naturais capazes de agir frente a microrganismos planctônicos e em biofilmes visando aplicações médicas e industriais. As lectinas são proteínas que se ligam especificamente a carboidratos e podem ter atividades biológicas importantes, podendo atuar como agentes antibacterianos e antibiofilme. Este trabalho avaliou a atividade antibacteriana da lectina solúvel em água de sementes de *Moringa oleifera* (WSMoL) contra bactérias que podem estar envolvidas na biocorrosão e infecções humanas (*Bacillus* sp., *Bacillus cereus*, *Bacillus pumillus*, *Bacillus megaterium*, *Micrococcus* sp., *Pseudomonas* sp., *Pseudomonas fluorescens*, *Pseudomonas stutzeri* e *Serratia marcescens*) investigando seu efeito no crescimento, sobrevivência e permeabilidade celular. Em adição, também estudamos a formação de biofilme por *S. marcescens* e *Bacillus* sp. (ambas bactérias sensíveis a WSMoL) e avaliamos o efeito de WSMoL na formação de biofilme e sobre biofilmes pré-estabelecidos para ambas as espécies. Um protótipo de revestimento de superfícies com WSMoL por técnica de imersão (*dipping coating*) e a viabilidade das células dentro de biofilmes desenvolvidos sob a superfície revestida com a lectina também foram determinados. WSMoL inibiu o crescimento bacteriano, induziu a aglutinação e promoveu o extravasamento de proteínas de todas as espécies bacterianas avaliadas. Efeito bactericida foi detectado contra *Bacillus* sp., *B. pumillus*, *B. megaterium*, *P. fluorescens* e *S. marcescens*. Microscopia de fluorescência de células de *S. marcescens* mostrou que WSMoL causou danos a sua estrutura. A lectina foi capaz de impedir a formação de biofilmes de *S. marcescens* e *Bacillus* sp., principalmente para *Bacillus* sp. a 41,6 e 20,8 µg/mL; o mecanismo de antiformação pode estar associado com a perda da viabilidade celular. WSMoL não destruiu a matriz de biofilmes pré-estabelecidos de *S. marcescens*, entretanto, as células aderidas apresentaram-se presas e danificadas em estruturas envolvidas pela lectina. Sobre biofilmes de *Bacillus* sp., a lectina a 208 µg/mL drasticamente reduziu o número de células aderidas a superfície. WSMoL espontaneamente aderiu a superfície de vidro produzindo uma superfície revestida com a área de 116 µg/cm^2, sendo capaz de alterar a viabilidade de células de *S. marcescens* que aderiram a essa superfície e prevenir a adesão de células de *Bacillus* sp., impedindo a formação de biofilme, sem interferir na viabilidade. Esse trabalho demonstra o potencial de WSMoL como uma ferramenta no controle de biofilmes formados por bactérias corrosivas e patogênicas, que representam um desafio em ambientes clínicos e industriais.

Palavras-chave: Bactéria; biofilmes; adesão; agentes antimicrobianos; proteínas.

ABSTRACT

Bacteria are prokaryotic organisms able to adapting to the most varied types of habitat; can grow and adhere to many surfaces, forming complex communities known as biofilms. Biofilm development, in most cases, results in health risks in clinical environments and causes economic losses in different industries sectors, being the main cause of biocorrosion. Control strategies of biofilms involves the use of antibiotics and biocides; however, bacteria whitin biofilms are more resistant to various treatments. Various mechanisms of bacterial resistance to antimicrobial synthetic compounds are described. Researches have been developed in order to identify natural compounds able to act against planktonic microorganisms and inside biofilms for medical and industrial applications. Lectins are proteins that bind specifically to carbohydrates and may have important biological activities, acting as antibacterial and antibiofilm agents. This work evaluated the antibacterial activity of a water-soluble *Moringa oleifera* seed lectin (WSMoL) against bacteria that could be involved in biocorrosion and human infections (*Bacillus* sp., *Bacillus cereus*, *Bacillus pumillus*, *Bacillus megaterium*, *Micrococcus* sp., *Pseudomonas* sp., *Pseudomonas fluorescens*, *Pseudomonas stutzeri* and *Serratia marcescens*) by evaluating its effect on growth, survival and cell permeability. In addition, we also studied the formation of biofilm by *S. marcescens* and *Bacillus* sp. (both bacteria sensitive to WSMoL) and evaluated the effect of WSMoL on biofilm formation and under established biofilms for both strains. A prototype of WSMoL-coated surfaces by dipping coating and the viability of cells within biofilm developed under the coated-surface were also determinated. WSMoL inhibited the bacterial growth, induced agglutination and promoted the leakage of proteins from cells of all strains. Bactericidal effect was detected against *Bacillus* sp., *B. pumillus*, *B. megaterium*, *P. fluorescens* and *S. marcescens*. Fluorescence microscopy of *S. marcescens* cells showed that WSMoL caused damage to its structure. The lectin was able to impair biofilms formation of *S. marcescens* and *Bacillus* sp., mainly for *Bacillus* sp. at 41.6 and 20.8 μg/mL; the antibiofilm mechanism can be associated with the lost of cell viability. WSMoL did not destroy preformed *S. marcescens* biofilms; despite the fact that attached cells presented trapped and damaged in hollow structures made by lectin. On *Bacillus* sp. biofilm, the lectin at 208 μg/mL drastically reduced the number of attached cells on surface. WSMoL spontaneously adhere on glass surface producing a lectin-coated area at 116 μg/cm^2 that was able to alter the viability of *S. marcescens* cells, which adhered to the surface and preventing *Bacillus* sp. attachment, impairing biofilm development, without interfering in cell viability. This work propose the use of WSMoL as a potential tool to control biofilms formed by corrosive and pathogenic bacteria, which pose a challenge in clinical and industrial settings.

Keywords: Bacteria; biofilms; adhesion; antimicrobial agents; proteins.

LISTA DE FIGURAS

FUNDAMENTAÇÃO TEÓRICA

ARTIGO 1

ARTIGO 2

ARTIGO 3

ARTIGO 4

LISTA DE TABELAS

FUNDAMENTAÇÃO TEÓRICA

ARTIGO 1

ARTIGO 2

ARTIGO 4

LISTA DE ABREVIATURAS

Acil HSL	Acil homoserina lactona
AH/ HA	Atividade hemaglutinante (no inglês, *hemagglutinating activity*)
AHH	Enzima aril hidrocarboneto hidroxilase (do inglês *aryl hydrocarbon hydroxylase*)
AIs	Sinais autoindutores (do inglês *autoinducers signals*)
ApulSL	Lectina de sementes de *Apuleia leiocarpa*
BHI	Meio de infusão de cérebro e coração (do inglês *Brain-Heart Infusion*)
BRS/ SRB	Bactérias redutoras de sulfato (do inglês *sulfate-reducing bacteria*)
BSL	Lectina de alga vermelha marinha *Bryothamnion seaforthii*
BTL	Lectina de alga vermelha marinha *Bryothamnion triquetrum*
BVL	Lectina de sementes de *Bauhinia variegata*
CCL	Lectina de sementes de *Cajanus cajan*
CMA/ MAC	Concentração mínima aglutinante (no inglês, *minimal agglutinating concentration*)
CMI/ MIC	Concentração mínima inibitória (no inglês, *minimal inhibitory concentration*)
cMoL	Lectina coagulante de sementes de *M. oleifera*
Con A	Lectina de sementes de *Canavalia ensiformis*
ConM	Lectina de sementes de *Canavalia maritima*
DNA	Ácido desoxirribonucléico
EPS	Substância polimérica extracelular (do inglês *extracellular polymeric substance*)
GlcNAc	*N*-acetilglicosamina
MBC	Concentração mínima bactericida (do inglês *minimal bactericide concentration*)
MH	Meio Caldo Mueller Hinton (do inglês *Mueller Hinton Broth*)
MoL	Lectina de sementes de *M. oleifera*
MSCRAMMs	Do inglês *microbial surface component recognizing adhesive matrix molecules*
NA	Meio Ágar Nutriente (do inglês *Nutrient Agar*)

NACE	do inglês *National Association of Corrosion Engineers*
NB	Meio Caldo Nutriente (do inglês *Nutrient Broth*)
PHA	Lectina de sementes de *Phaseolus vulgaris*
PNA	Lectina de sementes de *Arachis hypogaeae*
PSA	Lectina de sementes de *Pisum sativum*
QS	Do inglês *quorum sensing*
QSI	Compostos inibidores do sistema QS
rBVL-I	Lectina recombinante de sementes de *Bauhinia variegata*
SEM	Microscopia eletrônica de Varredura (do inglês *Scanning electron microscopy*)
TFA	Lectina de sementes de *Trigonella foenumgraecum*
THPS	Sulfato de tetrakis (hidroximetil) fosfônico
TTML	Lectina de sementes de *Tinospora tomentosa*
UFC/ CFU	Unidades formadoras de colônias (do inglês *colony forming units*)
VML	Lectina de sementes de *Vatairea macrocarpa*
WGA	Lectina de sementes de *Triticum aestivum*
WSMoL	Lectina solúvel em água de sementes de *M. oleifera*

SUMÁRIO

1 INTRODUÇÃO

Os microrganismos são capazes de aderir a muitas superfícies formando comunidades complexas conhecidas como biofilmes, agregados celulares imersos em uma matriz polimérica composta principalmente por DNA extracelular, proteínas e polissacarídeos - substâncias poliméricas extracelulares (EPS) (SUTHERLAND, 2001; ABDEL-AZIZ & AERON, 2014). Apesar de quase todos os microrganismos produzirem biofilmes, os biofilmes bacterianos predominam em praticamente todo o ecossistema (SUTHERLAND, 2001; DONLAN *et al*., 2005). Os biofilmes bacterianos podem se formar em superfícies bióticas ou abióticas, líquidas ou sólidas, exibem distintos fenótipos em relação a transcrição de determinados genes e são, geralmente, resistentes aos processos convencionais de desinfecção (ABDEL-AZIZ & AERON, 2014).

Os biofilmes bacterianos podem se desenvolver em diferentes setores industriais, causando a biocorrosão, bem como podem ser um problema de grande relevância no setor médico, devido à maior resistência e tolerância das células microbianas quando crescidas em forma de biofilmes (ACOSTA-DIAZ *et al*., 2011). A biocorrosão corresponde à deterioração de estruturas metálicas causada, direta ou indiretamente, pela atividade metabólica de microrganismos em biofilmes (RAJASEKAR *et al*., 2011). As bactérias redutoras de sulfato (BRS) têm sido descritas como o grupo mais atuante (RAJASEKAR *et al*., 2007a); contudo, estudos recentes têm evidenciado a presença de outros grupos bacterianos envolvidos na biocorrosão, incluindo bactérias patogênicas para humanos e outros animais (RAJASEKAR *et al*., 2011; LENG *et al*., 2015; LÓPEZ *et al*., 2015). A formação de biofilmes em filtros, dutos de estocagens e transporte de derivados de petróleo é responsável por sérios problemas econômicos e ambientais nas indústrias petrolíferas, sendo estimado que 40% de toda corrosão interna associada aos dutos são causadas por bactérias (RAJASEKAR *et al*., 2007a; 2011; MOURA *et al*., 2013). Outro problema associado à presença de biofilmes nas indústrias corresponde à contaminação dos combustíveis através da degradação dos hidrocarbonetos e presença de metabólitos (RAJASEKAR & TING, 2010).

Biocidas (cloro, ozônio, brometo, etc.), revestimentos anti-incrustantes (tintas, silicones, resinas, etc.) e inibidores de corrosão (aminas, ácidos graxos, ésteres de fosfato, etc.) são amplamente utilizados, geralmente combinados, em indústrias petrolíferas a fim de prevenir o processo biocorrosivo (VIDELA & HERRERA, 2005). Entretanto, o uso de biocidas está associado ao desenvolvimento da resistência bacteriana (ACOSTA-DÍAZ *et al*., 2011). Além

disso, estudos têm demonstrado que alguns revestimentos anti-incrustantes e inibidores de corrosão podem ser utilizados por algumas espécies bacterianas como fonte nutricional, reduzindo assim, sua capacidade anticorrosiva e causando um aumento do crescimento microbiano e, consequentemente, da taxa de biocorrosão (RAJASEKAR *et al*., 2007a, 2007b). Em adição, são tóxicos e podem causar sérios problemas ambientais (VIDELA & HERRERA, 2005; ZUO, 2007).

Compostos de origem vegetal têm sido descritos como agentes antibacteriano e antibiofilme (TRENTIN *et al*., 2014; VASCONCELOS *et al*., 2014; HUSAIN *et al*., 2015). As lectinas constituem um grupo complexo de proteínas e/ou glicoproteínas de origem não-imune que possuem, pelo menos, um domínio não catalítico que se liga especificamente e reversivelmente a monossacarídeos, oligossacarídeos, polissacarídeos e glicoconjugados (PEUMANS & VAN DAMME, 1995; DIAS *et al*., 2015). São encontradas em diversos grupos de plantas e têm sido isoladas a partir de diferentes tecidos vegetais (LANNOO & VAN DAMME, 2014). A capacidade de interagir com carboidratos permite a atuação das lectinas em diversos processos biológicos, incluindo sua ação antibacteriana (atividade bacteriostática e bactericida), bem como no impedimento do desenvolvimento de biofilmes bacterianos (CAVALCANTE *et al*., 2011, 2013; KLAFKE *et al*., 2013; SAHA *et al*., 2014).

Moringa oleifera (Moringaceae), planta nativa do Nordeste da Índia, tem sido objeto de várias pesquisas devido às suas propriedades industriais e medicinais (GHEBREMICHAEL *et al*., 2005; SUAREZ *et al*., 2005). Suas sementes contêm a lectina solúvel em água, WSMoL, que possui propriedades coagulante, inseticida e antibacteriana (COELHO *et al*., 2009; FERREIRA *et al*., 2011; AGRA-NETO *et al*., 2014). Com base nessas considerações, o objetivo desse trabalho foi primeiramente realizar um estudo acerca dos grupos bacterianos envolvidos no processo de biocorrosão, que foi em seguida publicado na forma de revisão. Posteriormente, investigamos o efeito antibacteriano de WSMoL frente a bactérias planctônicas que podem estar envolvidas no processo corrosivo; realizamos o estudo do biofilme formado pelas duas espécies mais sensíveis ao tratamento com a lectina (*Serratia marcescens* e *Bacillus* sp.), através de técnicas de microscopia; avaliamos o potencial antibiofilme de WSMoL (antiformação e erradicação) *in vitro*; e desenvolvemos um protótipo de superfície revestida com WSMoL capaz de impedir a adesão bacteriana. Finalmente, uma segunda revisão foi escrita tendo como principal objetivo abordar os compostos de origem vegetal já estudados frente a biofilmes bacterianos.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 ASPECTOS GERAIS DOS BIOFILMES BACTERIANOS

2.1.1 Definição e estrutura dos biofilmes

Biofilme microbiano é uma comunidade bem organizada de microrganismos que apresentam uma grande variedade de interações, incluindo a partilha de carbono e comunicações interespécies (MACEDO *et al*., 2005; KOKARE *et al*., 2009). Esse agregado de células pode ser constituído por uma única espécie ou múltiplas espécies; entretanto, em condições naturais, biofilmes formados por uma única espécie são relativamente raros (SUTHERLAND, 2001; JEFFERSON, 2004). Em biofilmes multiespécies, há uma maior complexidade geral da mistura macromolecular presente (SUTHERLAND, 2001). Várias espécies de microrganismos são capazes de formar biofilmes, no entanto, bactérias são encontradas em maior quantidade, compreendendo a cerca de 98% (SUTHERLAND, 2001; DONLAN *et al*., 2005).

Normalmente, biofilmes maduros têm uma estrutura altamente diferenciada (semelhantes a cogumelos ou colunas), formados por uma estruturada matriz polimérica hidratada (LYNCH *et al*., 2002; PARSEK & GREENBERG, 2005). A matriz polimérica, formada principalmente por EPS (substâncias poliméricas extracelulares), é produzida pelas próprias células microbianas que formam o biofilme (FLEMMING & WINGENDER, 2010). Dentro da matriz existem canais de circulação de água e nutrientes (FLEMMING & WINGENDER, 2010); nesses canais também existe a troca efetiva de substratos interespécies, através da distribuição de produtos metabólicos, facilitando a utilização de certos substratos por bactérias metabolicamente distintas, normalmente para obtenção de energia (KOKARE *et al*., 2009). Na maioria dos biofilmes, a matriz compreende mais que 90%, enquanto que os microrganismos são responsáveis por menos que 10% da massa seca (SUTHERLAND, 2001; FLEMMING & WINGENDER, 2010). A água é o principal componente da matriz e a EPS é constituída por um conglomerado de diferentes tipos de biopolímeros (polissacarídeos, lipídeos, proteínas e DNA extracelular), que promovem a estabilidade mecânica dos biofilmes, medeiam a sua adesão às superfícies e formam uma rede tridimensional de polímeros coesos que interconecta e imobiliza as células transientes dos biofilmes (SUTHERLAND, 2001; FLEMMING & WINGENDER, 2010) (Tabela 1). Materiais não celulares tais como cristais

minerais, partículas corrosivas, argila, sedimentos ou células sanguíneas podem também ser encontrados na matriz (DONLAN, 2002).

Tabela 1. Composição geral da matriz produzida pelas células bacterianas que compõem o biofilme.

Componentes	Subunidades/ precursores
Água	-
Polissacarídeos	Monossacarídeos
	Ácidos urônicos
	Amino açúcares
Proteínas (polipeptídeos)	Aminoácidos
Ácidos nucléicos	Nucleotídeos
Fosfolipídios	Ácidos graxos
	Glicerol
	Fosfato
	Etanolamina
	Serina
	Colina
Substâncias húmicas	Compostos fenólicos
	Açúcares simples
	Aminoácidos

Fonte: WINGENDER *et al*. (1999).

A matriz é responsável por conferir proteção contra fatores de estresse ambientais, como radiação, estresse hídrico ou atrito; uma condição totalmente diferente do estado planctônico das células microbianas (MACEDO *et al*., 2005; FLEMMING & WINGENDER, 2010). Em adição, algumas evidências sugerem que o EPS pode estar envolvido na tolerância de biofilmes aos agentes antimicrobianos, como compostos desinfetantes ou antibióticos, e é responsável por sequestrar íons, cátions e toxinas (KOKARE *et al*., 2009). Essas características fazem os biofilmes a forma de vida preferida de microrganismos que vivem em habitats extremos (MACEDO *et al*., 2005).

2.1.2 Formação e desenvolvimento dos biofilmes

A formação do biofilme é um processo dinâmico que envolve contínuas mudanças na estrutura e composição das comunidades microbianas (JEFFERSON, 2004). Estudos indicam que o processo de construção de um biofilme se dá a partir de interações entre as células bacterianas que compõem o biofilme inicial, através da produção de sinalizadores intracelulares, conhecidos por moléculas *quorum sensing* (QS) ou sinais autoindutores (O´TOOLE *et al*., 2000; PARSEK & GREENBERG, 2005). Esses fatores microbianos são constantemente liberados pelas bactérias na fase posterior à formação de agregados e no início da formação do biofilme e, quando presentes em uma concentração crítica, induzem a expressão de genes específicos que regulam várias funções como motilidade, virulência, produção de EPS e, por fim, a formação de biofilmes (LYNCH *et al*., 2002; JEFFERSON, 2004). Por exemplo, quando células de *Staphylococcus epidermidis* entram em contato com a superfície sólida, as células esféricas formam um apêndice que facilita o processo de adesão (KODJIKIAN *et al*., 2003). Segundo Jefferson (2004), bactérias patogênicas produzem um impressionante conjunto de adesinas, conhecidas como MSCRAMMs do inglês "*microbial surface component recognizing adhesive matrix molecules*" responsáveis por se ligar a proteínas do hospedeiro humano, tais como fibronectina, fibrinogênio, vitronectina e elastina, que formam um filme condicionante; uma vez que o biofilme esteja formado, as proteínas não são mais necessárias tendo sua expressão inibida. Vários sistemas de detecção de QS são conhecidos; o sistema acil homoserina lactona (bactérias gram-negativas) e o sistema de sinalização baseado em peptídeos (bactérias gram-positivas) constituem os dois sistemas QS atualmente mais estudados (JEFFERSON, 2004; PARSEK & GREENBERG, 2005).

A adesão bacteriana compreende o primeiro passo para a formação do biofilme e ocorre normalmente na interface sólido-líquido de uma superfície sólida biótica ou abiótica (DUNNE, 2002; JEFFERSON, 2004). Várias caraterísticas da superfície sólida, tal como a rugosidade, hidrofobicidade e presença de um filme condicionante de biopolímeros orgânicos são importantes para o processo de adesão (DONLAN, 2002; KOKARE *et al*., 2009). A velocidade do líquido e outras características do meio aquoso (níveis de pH e nutrientes, força iônica e temperatura) também influenciam a taxa de adesão microbiana a um substrato (DONLAN, 2002; KOKARE *et al*., 2009). Quanto maior a velocidade linear, mais rápida a associação de células na superfície, até o ponto que se torne tão elevada que as forças de cisalhamento desprendam as células aderidas a superfície (DONLAN, 2002). Estudos demonstraram que um

aumento da concentração de nutrientes e temperatura estão correlacionados com o aumento do número de células bacterianas aderidas (DONLAN, 2002).

Nas superfícies abióticas, foco desse trabalho, a adesão primária ou reversível ocorre mediada por interações físico-químicas não específicas (forças hidrodinâmicas, interações eletrostáticas, forças de Van der Waals e interações hidrofóbicas), por meio dos quais as células planctônicas aderem à superfície aleatoriamente (movimento Browniano ou força gravitacional) ou de modo dirigido via quimiotaxia e motilidade através de flagelos e pili (PAVITRA & DOBLE, 2008; TRENTIN *et al*., 2013a). Em adição, a adesão primária pode ocorrer de forma direta ou através de um filme condicionante (Figura 1), processo conhecido por bioacumulação, do inglês "*biofouling*", que ocorre devido ao acúmulo de material orgânico ou inorgânico na superfície de um material (BEECH *et al*., 2005). Esse filme pode ser formado por proteínas como albumina, imunoglobulina, fibrinogênio e fibronectina, quando a superfície em questão é um biomaterial (dispositivo médico interno), ou macromoléculas orgânicas ou inorgânicas presentes no meio líquido, quando a superfície é uma tubulação de transporte de petróleo ou gás, por exemplo (O`TOOLE *et al*., 2000; TRENTIN *et al*., 2013a). É importante observar que as bactérias aeróbicas e anaeróbicas facultativas possuem a característica de serem colonizadoras iniciais, aderindo às superfícies e produzindo EPS, criando um ambiente favorável à adesão de outros microrganismos anaeróbicos em fases posteriores (MARANGONI *et al*., 2013).

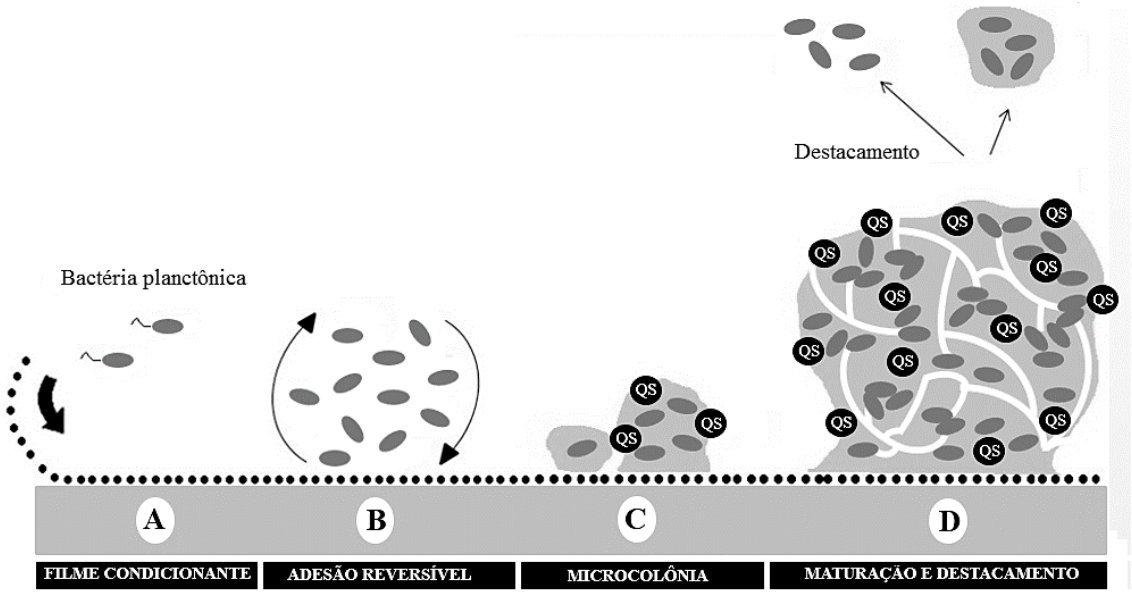
Figura 1. Adesão bacteriana primária direta e via filme condicionante em uma superfície sólida. O filme condicionante é formado pela deposição de materiais orgânicos ou inorgânicos na superfície do material.

Fonte: A autora (2016).

Na adesão secundária ou irreversível, os microrganismos já começam a produzir EPS, a qual pode se complexar com materiais presentes na superfície, fortalecendo assim, as ligações entre as células e/ou agregados e a superfície, formando microcolônias (STOODLEY *et al*., 2002; BOARI, 2009). Nesse momento não existe motilidade (BOARI, 2009). A maturação do biofilme ocorre na sequência com o aumento da densidade populacional e pela elevada produção de EPS, aumentando a espessura do biofilme e a estabilidade da colônia; o aumento

da população acontece tanto por divisão celular quanto por adesão de novas células planctônicas (STOODLEY *et al*., 2002). Sob determinadas situações, por exemplo, quando o ambiente não se encontra mais favorável ou devido a uma programação celular para virulência, ocorre o destacamento de células planctônicas ou até grupos de células unidas pelo EPS, podendo colonizar um outro local (HALL-STOODLEY & STOODLEY, 2005; TRENTIN *et al*., 2013a) (Figura 2).

Figura 2. Estágios de formação de um biofilme numa superfície sólida. (A) Formação de um filme condicionante por biopolímeros orgânicos presentes no meio. (B) Aderência reversível de células planctônicas na superfície condicionada. (C) Aderência irreversível das células, produção de exopolissacarídeos e formação de microcolônias. (D) Biofilme maduro com um aumento da densidade e complexidade, com canais de circulação em evidência; nesse momento, pode haver desprendimento de células planctônicas e agregados celulares, com o objetivo de colonizar outras áreas. Moléculas *Quorum sensing* (QS) são responsáveis pela comunicação célula-célula dentro do biofilme.



Fonte: A autora (2016).

2.2 BIOCORROSÃO E BACTÉRIAS CORROSIVAS

A biocorrosão é um processo que ocorre sob influência de microrganismos presentes em biofilmes (DAVEY & O'TOLLE, 2000). Embora existam exemplos de corrosão atribuídos a fungos e algas, a biocorrosão se processa quase que exclusivamente na presença de bactérias, devido a sua fácil adaptação a extremos de temperatura, pH e pressão (MORAES, 2009). Os microrganismos participam de forma ativa na dissolução do metal, acelerando ou induzindo a corrosão, mas sem modificar a natureza eletroquímica característica desse processo (COETSER & CLOETE, 2005). Tem-se assim, uma região anódica desenvolvendo um processo de oxidação que leva à dissolução do metal (corrosão) e simultaneamente a redução de algum

componente do meio através da reação catódica (CHARRET, 2011). De acordo com Gentil (2007), a ação dos microrganismos nos processos de corrosão pode ocorrer de acordo por um ou mais dos seguintes fatores: I- Atuação direta na velocidade das reações anódicas e catódicas; II- Redução da resistência de películas protetoras existentes nas superfícies metálicas pela ação dos produtos do metabolismo microbiano; III- Geração de meios corrosivos devido à formação de ácidos. IV- Formação de tubérculos que possibilitam o aparecimento de pilhas de aeração diferencial.

Os microrganismos associados à biocorrosão estão amplamente distribuídos na natureza e nos sistemas industriais (água doce e do mar, sistemas de tratamentos de líquidos e tanques de armazenamento e transporte de compostos derivados de petróleo, por exemplo) e podem influenciar na propagação de todos os tipos de corrosão metálica (SHI *et al*., 2011). As bactérias relacionadas à biocorrosão são divididas em grupos que se diferenciam quanto ao metabólito produzido e, consequentemente, a sua atuação no processo corrosivo, como apresentamos na Tabela 2.

Tabela 2. Grupos de bactérias corrosivas associadas à biocorrosão.

Grupos bacterianos corrosivos	Atuação na biocorrosão
Produtoras de EPS (aeróbicas e anaeróbicas facultativas)	Colonizadoras iniciais no processo de formação do biofilme, responsáveis pelo consumo de oxigênio (O_2), tornando o interior do biofilme anaeróbico.
Produtoras de ácido (aeróbicas)	A partir do metabolismo fermentativo de materiais orgânicos produzem ácidos orgânicos de cadeias curtas, essenciais no metabolismo das BRS, além de diminuir o pH do meio.
Oxidantes de enxofre (aeróbicas e anaeróbicas facultativas)	Oxidam o enxofre (S) ou compostos de enxofre a sulfato (SO_4^{-2}), tendo o ácido sulfúrico (H_2SO_4) como produto final, responsável pela acidificação do ambiente e corrosão.
Oxidantes de ferro (aeróbicas)	Na presença de O_2, oxidam o íon ferroso (Fe^{2+}) a íon férrico (Fe^{3+}) liberando energia e depositando o hidróxido férrico ($Fe(OH)^3$) (insolúvel) na superfície, formando tubérculos e promovendo regiões com

	diferentes níveis de oxigênio, o que leva a ocorrência de corrosão por aeração diferencial.
Redutoras de sulfato (BRS) (anaeróbicas)	Reduzem o SO_4^{-2} a sulfeto de hidrogênio (H_2S), um gás tóxico extremamente corrosivo e que também atua na acidificação do petróleo e seus derivados.

Fonte: A autora (2016).

As BRS são responsáveis por grande parte dos danos causados pela corrosão microbiana nas indústrias, sendo este o grupo mais abordado pelos trabalhos voltados à biocorrosão (RAJASEKAR *et al*., 2007a, 2007b). Entretanto, é importante observar que a presença desses microrganismos não é obrigatória para que a biocorrosão ocorra (NERIA-GONZÁLEZ *et al*., 2006; RAJASEKAR *et al*., 2011). Diversas bactérias patogênicas para humanos e animais podem atuar na corrosão (NERIA-GONZÁLEZ *et al*., 2006; RAJASEKAR *et al*., 2011; MOURA *et al*., 2013). Rajasekar *et al*. (2010) isolaram 11 espécies bacterianas (*Serratia marcescens*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Klebsiella oxytoca*, *Bacillus* sp., *Bacillus subtilis*, *B. cereus*, *B. litoralis*, *B. pumilus*, *B. carboniphilus* e *B. megaterium*) a partir de produtos de corrosão de dutos de transporte de diesel e nafta e demonstraram que o consórcio formado por essas bactérias estava associado com a corrosão localizada do metal.

As bactérias presentes nas estruturas de armazenamento e transporte das indústrias petrolíferas utilizam os hidrocarbonetos dos derivados de petróleo como principal fonte nutricional (RAJASEKAR *et al*., 2007b; RAJASEKAR & TING, 2010); além disso, inibidores de corrosão amplamente utilizados como medida anticorrosiva (como explicaremos no tópico "Estratégias de controle de biofilmes") são totalmente degradados e metabolizados (RAJASEKAR *et al*., 2007a, 2007b). Esses mecanismos propiciam a aceleração do crescimento bacteriano (MARUTHAMUTHU *et al*., 2005; RAJASEKAR *et al*., 2007a, 2007b). Estudos conduzidos com bactérias dos gêneros *Pseudomonas*, *Bacillus*, *Gallionella*, *Siderocapsa*, *Thiobacillus*, *Thiospira*, *Sulfolobus* e *Vibrio*, encontradas em tubulações de nafta, indicaram uma relação direta de crescimento entre a taxa de corrosão microbiana e a degradação desse composto (RAJASEKAR *et al*., 2005). Alguns estudos demonstraram que *S. marcescens* e *Bacillus* sp. produzem uma enzima conhecida como aril hidrocarboneto hidroxilase (AHH), responsável pela degradação dos hidrocarbonetos aromáticos (mecanismo que acelera o crescimento bacteriano), além de atuar na corrosão devido à acidez gerada (RAJASEKAR *et al*., 2005; 2011; SIVAKUMAR *et al*., 2010). Além disso, Rajasekar *et al*. (2011), observaram

que *Bacillus* sp. era capaz de acelerar a corrosão através da conversão dos elementos do aço carbono API 5LX em óxidos metálicos (MnO_2 e FeO_3) como produtos de corrosão.

2.3 IMPORTÂNCIA E IMPACTO ECONÔMICO DE BIOFILMES ASSOCIADOS ÀS INDÚSTRIAS PETROLÍFERAS

O crescimento não desejado dos biofilmes tem um impacto negativo em diversas atividades e representam perdas significativas para diversos setores industriais (petroquímica, naval, química, meios de transporte, comunicação) (XAVIER *et al*., 2005; CHARRET, 2011). Um estudo conduzido pela *National Association of Corrosion Engineers* (NACE), em 2002, estimou que 3% do produto interno bruto dos Estados Unidos da América (EUA) anualmente (aproximadamente US$ 400 bilhões) são destinados aos custos associados à corrosão (MOTHÉ, 2010). No Brasil, os gastos podem chegar a cerca de U$S 10 bilhões segundo Mothé (2010). Dentre as principais áreas industriais afetadas pela biocorrosão, destacam-se as indústrias petroquímica e petrolífera em seus diversos sistemas, tais como: circuitos de resfriamento, tanques de armazenamento, tubulações de usos diversos, membrana de osmose reversa, linhas de injeção de água, entre outros (VIDELA, 2003). Os sistemas de tubulações de transporte são as estruturas mais afetadas (KOCH *et al*., 2001); dentre os produtos mais transportados destacam-se os hidrocarbonetos, gasosos ou líquidos, incluindo gás natural, petróleo bruto, produtos de alta pressão de vapor, tal como o gás propano e, produtos refinados, tais como, gasolina ou querosene de aviação, entre outros (MOTHÉ, 2010).

Sabe-se que 40% da corrosão das tubulações na indústria petrolífera são atribuídos à biocorrosão e que esta provoca estragos na ordem dos 100 milhões de dólares para monitoração, substituição e manutenção de dutos de petróleo em operação todos os anos nos EUA (RAJASEKAR *et al*., 2007b; MOTHÉ, 2010). As perdas causadas pela biocorrosão nas indústrias petrolíferas podem ser diretas, quando associadas à manutenção e substituição de peças, e indiretas, quando relacionadas, por exemplo, à paralisação de produção e contaminação de produtos (GENTIL, 2007). A comunidade microbiana altera a composição dos combustíveis, através da degradação dos hidrocarbonetos além de liberar seus metabólitos no meio, diminuindo sua qualidade (RAJASEKAR & TING, 2010). Nas tubulações, os biofilmes formados podem causar desde a redução na velocidade de escoamento dos fluidos, decorrente do processo de incrustação nas paredes dos dutos, até desgaste de tanques de armazenamento e dutos de transporte que podem resultar em microvazamentos causando contaminação de água

e de solos, podendo inclusive atingir os lençóis freáticos (GENTIL, 2007; HANSSON, 2010). Em adição, incêndios e explosões estão entre problemas relacionados aos vazamentos (MOTHÉ, 2010). Apesar da fiscalização dos órgãos competentes e de toda rigidez legal frente ao exercício das atividades que caracterizem crimes ambientais, os derrames ainda continuam ocorrendo com considerável frequência no mundo (MOURA & URTIGA, 2011). Em 2007, a indústria de petróleo no Brasil revelou custo de cerca de R$ 120 mil por metro cúbico vazado (NOVAS FERRAMENTAS, 2007). Além disso, vazamento de produtos como gases, petróleo ou multifásicos provocam desastres ambientais previstos como crime pela lei brasileira de crimes ambientais (9605, de 13/02/1998), que pune tais ocorrências com pesadas multas e prisão dos responsáveis (ANTUNES, 2011).

2.4 ESTRATÉGIAS DE CONTROLE DE BIOFILMES

2.4.1 Processos físicos e químicos

A detecção e monitoramento da biocorrosão são formas de prevenção e de controle e os investimentos para este fim têm aumentado nos últimos tempos devido ao aumento dos prejuízos causados nos diversos setores industriais (VIDELA & HERRERA, 2005). Nas indústrias, a maioria dos biofilmes estão associados aos *foulings* abióticos, ou seja, surgem após a formação de um filme condicionante (VIDELA, 2002). Sendo assim, o estudo do controle e prevenção da biocorrosão não pode ser focado apenas na atividade e crescimento de microrganismos, mas também, nas propriedades físico-químicas na interface metal/solução (VIDELA, 2002).

Os métodos implementados para prevenir a biocorrosão atuam inibindo o crescimento ou atividade metabólica dos microrganismos e modificando o ambiente em que ocorre o processo corrosivo, a fim de evitar a adaptação destas bactérias (VIDELA, 2002). Métodos físicos (procedimentos de limpeza) e químicos (sanitização com a utilização de biocidas e revestimentos anti-incrustantes - *antifouling* - como tintas e inibidores de corrosão) são utilizados geralmente combinados visando à otimização do procedimento (VIDELA, 2002; VIDELA & HERRERA, 2005). A utilização desses métodos tem o propósito de diminuir ou erradicar a exposição do metal à ação da biocorrosão, seja através da eliminação direta dos microrganismos ou redução do efeito dos seus metabólitos sobre o material (VIDELA, 2003). Entretanto, estratégias de limpeza e monitoramento ineficientes dos sistemas e falta de

profissionais especializados na área de corrosão microbiológica proporcionam o aumento de prejuízos decorrentes da corrosão (VIDELA, 2003).

O método físico compreende uma limpeza mecânica que corresponde a qualquer método capaz de promover a remoção física do material depositado na superfície e inclui o uso de escovação, jatos d'agua, entre outros (VIDELA, 2003). Por outro lado, a limpeza química resume-se no uso de substâncias químicas diversas, sendo utilizada após a limpeza mecânica (VIDELA, 2002, 2005). Segundo Videla (2003) a utilização de biocidas se destaca dentre os métodos de controle efetivamente mais usados. Os biocidas (cloro, ozônio, bromo, aldeídos, acroleína e sulfato de tetrakis (hidroximetil)fosfônico -THPS), são um grupo heterogêneo de agentes químicos amplamente utilizados nas indústrias de petróleo para a proteção interna de oleodutos, gasodutos, na área de refino, na injeção de água, nas recuperações secundárias, nos fluidos de perfuração e na produção propriamente dita do petróleo, para controle de microrganismos planctônicos e principalmente para geradores de biofilmes, possuindo de forma geral atividade bactericida, fungicida e algicida (MAINIER & SILVA, 2004; ACOSTA-DÍAZ *et al*., 2011). Contudo, alguns biocidas podem não possuir atividade frente a determinados tipos de microrganismos dentro de um mesmo grupo, devido ao desenvolvimento da resistência bacteriana (ACOSTA-DÍAZ *et al*., 2011).

Os revestimentos protetores ou anti-incrustantes (silicones, resinas, tintas, etc.) e inibidores de corrosão (aminas, ácidos graxos, ésteres de fosfato, nitrato de amônio, etc.) formam uma película protetora na interface metal/solução, que tem como objetivo o impedimento da formação do filme condicionante e a posterior aderência de bactérias (RAJASEKAR *et al*., 2007a; RAVIKUMAR *et al*., 2012). Particularmente, os inibidores de corrosão são bastante utilizados nos sistemas industriais de petróleo e gás, em pequenas proporções, requerendo atualmente, restrições ambientais para a sua aplicação devido à elevada toxicidade (KOCH *et al*., 2001; RAJASEKAR *et al*., 2007a, 2007b; ZUO, 2007). Ainda, pesquisas demonstraram que seu uso está associado ao aumento do crescimento bacteriano, visto que são utilizados como fonte nutricional por algumas espécies de bactérias, perdendo, dessa forma, a sua capacidade de proteção (MARUTHAMUTHU *et al*., 2005; RAJASEKAR *et al*., 2007a). Além disso, os ácidos produzidos pelos microrganismos podem contribuir para a degradação desses recobrimentos, assim como os gases que são formados durante o metabolismo microbiano (GALVÃO, 2008). Esses gases podem difundir-se, gerando pressão em regiões que apresentam descontinuidades, como inclusões e vazios, ocasionando a formação de bolhas, que levam à ruptura da proteção (GALVÃO, 2008). Um estudo conduzido por

Rajasekar *et al*. (2008) demonstrou que a utilização de um inibidor de corrosão comercial (amino e ácido carboxílico), em sistema de diesel-água na presença de *S. marcescens*, causou severa corrosão na superfície do metal API 5L-X60, indicando que a bactéria foi capaz de utilizar esse inibidor de corrosão como fonte de nutrientes.

2.4.2 Plantas como fonte de compostos antibacterianos e antibiofilmes

As plantas possuem uma variedade de compostos biologicamente ativos, produzidos através de um processo natural de crescimento e desenvolvimento ou em resposta a ataque de patógenos ou estresse (TRENTIN *et al*., 2013b). O potencial antibacteriano e, mais recentemente, a atividade antibiofilme desses produtos, têm sido muito estudados (TRENTIN *et al*., 2014; VASCONCELOS *et al*., 2014; HUSAIN *et al*., 2015; LIMA *et al*., 2015). Dentre as principais classes de antimicrobianos investigados provenientes de plantas encontram-se os grupos dos (a) polifenóis, (b) terpenoides e óleos essenciais, (c) alcaloides, (d) lectinas e polipetídeos e (e) poliacetilenos (COWAN, 1999; AIYEGORO & OKOH, 2009).

As estratégias de combate aos biofilmes com a utilização de compostos de plantas são divididas em dois segmentos: (a) a inibição da formação de biofilmes e (b) a erradicação de biofilmes já formados (TRENTIN *et al*., 2013a). A inibição da formação de biofilmes, ou atividade de antiformação, se dá a partir do uso de compostos bacteriostáticos e/ou bactericidas, ou através da utilização de moléculas que impedem a adesão bacteriana à superfície ou que atuem no bloqueio da sinalização QS (MARTIN *et al*., 2008). Os dois últimos mecanismos (antiadesão e antiQS) são considerados novos conceitos terapêuticos por não atuarem na destruição celular, apenas no impedimendo do desenvolvimento de biofilmes, visando assim, dificultar o rápido desenvolvimento de resistência bacteriana; além disso, devido à permanência das células no estado planctônico, os agentes antimicrobianos são utilizados em baixas concentrações (RASKO & SPERANDIO, 2010). Taninos, estruturas complexas de proantocianidinas, isolados de *Pityrocarpa moniliformis* inibiram completamente a formação de biofilme de *Staphylococcus epidermidis* nas concentrações 0,125, 0,25, 0,5, 1, 2 e 4 mg/mL sem afetar a viabilidade do microrganismo, visto que não foi observado inibição do crescimento bacteriano (TRENTIN *et al*., 2015). Similarmente, extratos de raiz de *Rubus ulmifolius* reduziram a capacidade de formação de biofilme por células de *S. aureus* quando usados na faixa de concentração 50-200 µg/mL, enquanto que concentrações elevadas (530-1040 µg/mL) foram necessárias para impedir o crescimento bacteriano (QUAVE *et al*., 2012).

A ação antibacteriana de extratos e produtos isolados de plantas (inibição do crescimento bacteriano e morte celular) limita o número de células bacterianas hábeis para formar biofilmes, podendo também ser considerada um mecanismo de antiformação (MILLEZI, 2012). Extrato de casca do caule de *Anadenanthera colubrina* inibiu a formação de biofilme de *P. aeruginosa* através da ação bacteriostática e os autores destacaram que a presença de taninos pode estar relacionada com essa atividade; em adição, microscopia eletrônica de transmissão revelaram danos na membrana bacteriana (TRENTIN *et al*.,2013b).

Por outro lado, alguns produtos vegetais bioativos (antissépticos ou antimicrobianos), quando em contato com as células bacterianas, atuam como moléculas antiaderentes (BAZAKA *et al*., 2010). Esses compostos, através da adsorção ou imobilização, podem ser utilizados no recobrimento de superfícies abióticas acarretando no impedimento da adesão primária de células bacterianas e, consequentemente, da formação de biofilmes; entretanto, esse mecanismo apresenta o desafio da manutenção da atividade biológica do composto após retenção na superfície e o tempo de uso (BAZAKA *et al*., 2010; TRENTIN *et al*., 2013a, 2015). Segundo Trentin *et al*. (2015), proantocianidinas de *P. moniliformis* têm a capacidade de se aderir em superfícies hidrofóbicas (permanox) e hidrofílicas (vidro) quando utilizado na faixa de concentração 4-0,125 mg/mL, produzindo uma película compatível ao tecido humano que impede posterior adesão de *S. epidermidis*.

A interferência na comunicação intercelular, normalmente ocorre após a adesão primária, através do uso de compostos inibidores do sistema QS (QSI), que competem com o receptor das moléculas QS ou degradam esses compostos, bloqueando a comunicação celular que é essencial para o desenvolvimento e formação dos biofilmes (MARTIN *et al*., 2008). Husain *et al*. (2015) verificaram que o óleo de *Mentha piperita* em concentrações subinibitórias apresentou propriedades *anti*-QS através da inibição do pigmento violaceína em *Chromobacterium violaceum* sem alterar o crescimento microbiano; adicionalmente, foi observado a inibição de fatores de virulência regulados por QS em *P. aeruginosa* (elastase, protease, pigmento piocianina, quitinase, EPS e motilidade celular) após o tratamento com o óleo nas concentrações entre 0,375-3 % (v/v) e a inibição da formação do biofilme. Resultados similares foram observados por Sarkar *et al*. (2014), que utilizaram concentrações subinibitórias do extrato metanólico de *Sclerocarya birrea* contra células de *P. aeruginosa* e correlacionaram o efeito antibiofilme do extrato com a inibição de alguns fatores regulados via QS, tais como a motilidade, produção de protease e do pigmento pioverdina.

A erradicação de biofilmes já formados inclui a atuação de moléculas capazes de desintegrar a EPS, destruindo a estrutura tridimensional do biofilme e liberando células livres (BOLES & HORSWILL, 2011). Polifenóis de trifala (mistura de ervas ayurvédicas) e chá verde erradicaram o biofilme de *Enterococcus faecalis* de 3 e 6 semanas formado na superfície do dente humano (PRABHAKAR *et al*., 2010). Proantocianidinas apresentam atividade de antiformação (bloqueando a adesão primária) e erradicação frente a biofilmes de *Streptococcus mutans* em modelos de hidroxiapatita e em dentes humanos (DAGLIA *et al*., 2010). Oliveira *et al*. (2010) demonstraram que soluções de óleos essenciais de *Cymbopogon citratus* e *C. nardus*, combinados ou não, após 60min de contato, foram capazes de reduzir o número de células do biofilme de *Listeria monocytogenes* aderidas à superfície do aço inoxidável.

2.4.2.1 Lectinas de plantas: potenciais agentes antibacterianos

Lectinas são proteínas ou glicoproteínas que ligam reversivelmente a mono, oligo ou polissacarídeos com alta especificidade, sem alterar sua estrutura, sendo capazes de induzir a aglutinação de diferentes tipos de células ao interagirem com glicoconjugados presentes na superfície celular (PEUMANS & VAN DAMME, 1995; CORREIA *et al*., 2008). A seletividade de ligação de lectinas com os carboidratos ocorre por meios de sítios de reconhecimento através de pontes de hidrogênio, interações de van der waals e interações hidrofóbicas (SHARON & LIS, 2002; PAIVA *et al*., 2010).

Essas moléculas apresentam uma ampla distribuição na natureza e têm sido isoladas de plantas (SAHA *et al*., 2014), microrganismos (SCHNEIDER *et al*., 2015) e animais (OLAYEMI *et al*., 2015). Nas plantas têm sido isoladas a partir de diferentes tecidos, como frutos (WEARNE *et al*., 2013), sementes (CARVALHO *et al*., 2015), folhas (RAJA *et al*., 2016), flores (ITO, 1986), cascas (NUNES *et al*., 2015), rizomas (SANTANA *et al*., 2012), cerne (SAHA *et al*., 2014) e raízes (WANG & NG, 2006). Apresentam vários papéis fisiológicos importantes, dentre os quais se destacam a sua atuação na ativação de enzimas (KESTWAL *et al*., 2007), no mecanismo de nodulação em leguminosas (WANG *et al*., 2012) e na defesa contra microrganismos e insetos (FERREIRA *et al*., 2011; OLIVEIRA *et al*., 2011).

As lectinas de origem vegetal possuem diversas atividades biológicas e representam ferramentas valiosas em diferentes aplicações biotecnológicas (CORREIA *et al*., 2008). Dentre as várias atividades das lectinas, destacam-se (a) modulação de células do sistema imune de mamíferos (SOUZA *et al*., 2013), (b) atividade anti-inflamatória (SILVA *et al*., 2010), (c)

antitumoral (QUIROGA *et al*., 2015), (d) antifúngica (CULVERHOUSE *et al*., 2012), (e) antibacteriana (CARVALHO *et al*., 2015) e (f) antiviral (YONGTING *et al*., 2007). Adicionalmente, lectinas de plantas têm sido aplicadas na inibição da proliferação de diferentes linhagens de células cancerosas humanas (DEEPA & PRIYA, 2012), na detecção e separação de glicoconjugados (NAPOLEÃO *et al*., 2013) e na determinação de tipos sanguíneos e diagnósticos de processos de desenvolvimento, diferenciação e transformação neoplásica (LIU *et al*., 2010).

Existem vários relatos da ação antibacteriana de lectinas vegetais frente a microrganismos Gram-positivos e Gram-negativos (GOMES *et al*., 2013; SAHA *et al*., 2014; CARVALHO *et al*., 2015; XU *et al*., 2015). A lectina isolada a partir de folhas de *Schinus terebinthifolius* apresentou atividade contra *Escherichia coli*, *K. pneumoniae*, *P. aeruginosa*, *Proteus mirabilis*, *S. aureus* e *Salmonella enteritidis*, com valores de concentração mínima inibitória (CMI) de 0,45 a 28,75 µg/mL (GOMES *et al*., 2013). Recentemente, Xu *et al*. (2015) isolaram uma lectina do brócolis (*Brassica oleraceae italica* ***x*** *alboglabra*), BL, capaz de inibir o crescimento bacteriano de *P. aeruginosa*, *Shigella dysenteriae*, *S. aureus*, *E. coli* e *H. pylori* com CMI na faixa de 143,95-486,33 µg/mL. Carvalho *et al*., (2015) detectaram a ação bacteriostática e bactericida da lectina de sementes de *Apuleia leiocarpa* (ApulSL) contra espécies gram-positivas e gram-negativas, apresentando maior efetividade contra três diferentes variedades da espécie *Xanthomonas campestris*.

Bactérias gram-positivas e gram-negativas apresentam carboidratos expostos na superfície celular que podem estar covalentemente ligados ou não, como ácidos teicóicos glicosilados, ou na constituição de cápsulas polissacarídicas (UTSUNOMIYA *et al*., 2000; SAHA *et al*., 2014). As lectinas têm a capacidade de reconhecer de forma específica determinadas porções desses carboidratos e esse reconhecimento pode ocasionar a indução da aglutinação bacteriana, através da formação de complexos com os glicoconjugados microbianos, promovendo a imobilização, inibição do crescimento ou destruição das células dos microrganismos (SHARON & GALLAGHER, 2007; COSTA *et al*., 2010; SAHA *et al*., 2014). Outro mecanismo proposto envolve a atuação de lectinas na formação de canais na membrana da célula acarretando no extravazamento do conteúdo celular (TALAS-OGRAS *et al*., 2005). As interações entre lectinas e células bacterianas ocorrem através de ligações covalentes ou não covalentes, dependendo do peso molecular dos oligômeros e das subunidades das lectinas (RITTIDACH *et al*., 2007). Estudos conduzidos com a lectina isolada da goiaba (*Psidium guajava*) mostraram sua capacidade de se ligar às células de *E. coli*, aglutinando-as e

impedindo sua adesão na parede intestinal e posterior infecção; os resultados indicaram que os sítios de ligação a carboidratos estão envolvidos no mecanismo de adesão celular, confirmando a interação lectina-bactéria (COUTIÑO-RODRÍGUEZ *et al*., 2001). Lectina do cerne de *Myracrodruon urundeuva* apresentou atividade aglutinante sobre *Bacillus subtilis* (Concentração mínima aglutinante – CMA de 4,68 µg/mL), *Corynebacterium callunae* (CMA de 4,68 µg/mL), *S. aureus* (CMA de 2,34 µg/mL), *E. faecalis* (CMA de 4,68 µg/mL), *E. coli* (CMA de 9,37 µg/mL), *K. pneumoniae* (CMA de 9,37 µg/mL) e *P. aeruginosa* (CMA de 9,37 µg/mL) (SÁ *et al*., 2009). De acordo com Costa *et al*. (2010), a habilidade de lectinas de aglutinar bactérias resulta em menor dispersão da massa microbiana, que acarreta na diminuição da quantidade necessária de biocida a ser utilizada em um determinado local.

Os polissacarídeos presentes na superfície celular bacteriana medeiam interações entre célula-célula e célula-superfície que são essenciais para a formação e estabilização de biofilmes microbianos (ISLAM *et al*., 2008; ABDEL-AZIZ & AERON, 2014). Segundo alguns autores, o mecanismo antibiofilme de lectinas pode ocorrer devido à bivalência dessas moléculas, possuindo sítios de ligação a carboidratos, proteínas e/ ou outros principais constituintes dos biofilmes e, através desse processo, interromper a polimerização desses componentes (HASAN *et al*., 2014); em adição estudos revelam que as lectinas podem atuar na interrupção da sinalização via QS, culminando na alteração da expressão de genes essenciais à formação de biofilmes (CAVALCANTE *et al*., 2011, 2013). De acordo com Cavalcante *et al*. (2011) a lectina purificada de sementes de *Canavalia maritima* (ConM) foi capaz de inibir o crescimento de células planctônicas e a formação de biofilmes de *S. mutans*; posteriormente, em 2013, os mesmos pesquisadores verificaram que essa atividade pode ser atribuída não só à ação direta nas células, mas também à habilidade da lectina em inibir a expressão de alguns genes relacionados a formação do biofilme e virulência, como o *gtfb* (fator de virulência e essencial para o processo de formação do biofilme), *gbpB* e *spaP* (codificadores de adesinas importantes para o estabelecimento do biofilme) e *brpA* (codificador de proteínas importantes na resposta ao estresse). Outros trabalhos relatam que a lectina de *Solanum tuberosum* inibiu a formação de biofilmes de *P. aeruginosa*, sendo atribuída essa atividade à ligação da lectina aos resíduos de *N*-acetilglicosamina (GlcNAc) (HASAN *et al*., 2014), e a lectina específica para glicose/manose de sementes de *Trigonella foenumgraecum* (TFA), em concentrações subinibitórias, impediu a adesão e posterior formação de biofilmes de *S. mutans* (ISLAM *et al*., 2008). Abaixo, na Tabela 3, temos alguns exemplos de lectinas isoladas de plantas com potencial antibacteriano.

Tabela 3. Lectinas vegetais com ação antibacteriana e/ ou antibiofilme.

Lectina	Origem	Atividade
TTML	*Tinospora tomentosa*	Ação bacteriostática e bactericida contra *Salmonella typhi*
WSMoL	*M. oleifera*	Ação bacteriostática contra *S. aureus* e *E. coli*; efeito bactericida apenas contra *S. aureus*
ApulSL	*Apuleia leiocarpa*	Ação bacteriostática e bactericida contra *Xanthomonas campestris*
BVL/ rBVL-I* (*recombinante)	*Bauhinia variegata*	Inibição da adesão inicial das células e antiformação de biofilmes de *S. mutans*, *S. sanguis* e *S. sobrinus*
BTL	*Bryothamnion triquetrum*	Inibição da adesão inicial das células e antiformação de biofilmes de *S. mutans*, *S. sobrinus*, *S. sanguis*, *S. mitis* e *S. oralis*
BSL	*Bryothamnion seaforthii*	
Con A	*Canavalia ensiformis*	Ação bacteriostática, inibição da adesão inicial das células e antiformação de biofilmes de *S. mutans*
PSA	*Pisum sativum*	
TFA	*Trigonella foenumgraecum*	
ConM	*Canavalia maritima*	Ação bacteriostática, inibição da adesão inicial das células, antiformação de biofilmes e interrupção da sinalização via QS de *S. mutans*

Fonte: TEIXEIRA *et al*. (2007); ISLAM *et al*. (2008); FERREIRA *et al*. (2011); CAVALCANTE *et al*. (2011, 2013); KLAFKE *et al*. (2013); SAHA *et al*. (2014); CARVALHO *et al*. (2015).

2.5 *Moringa oleifera*

Moringa oleifera (Figura 3A), pertencente à família das Moringaceae, é uma planta nativa do nordeste da Índia e está amplamente distribuída em vários países (BEZERRA *et al*., 2004; KARADI *et al*., 2006; SANTOS *et al*., 2015). De acordo com Souza & Lorenzi (2008), a moringa é uma planta rústica, de rápido crescimento e produtora de frutos comestíveis. Cresce rapidamente e está extremamente adaptada às condições de seca, sendo capaz de sobreviver em solos pobres e com baixo teor de umidade (MCCONNACHIE *et al*., 1999). Constitui um

vegetal muito importante com diferentes propriedades, sendo útil para uma vasta gama de aplicações industriais, tais como na produção de óleos, alimentos e condimentos, e medicinais, na produção de fitoterápicos (MAKKAR & BECKER, 1997; SANTOS *et al*., 2015).

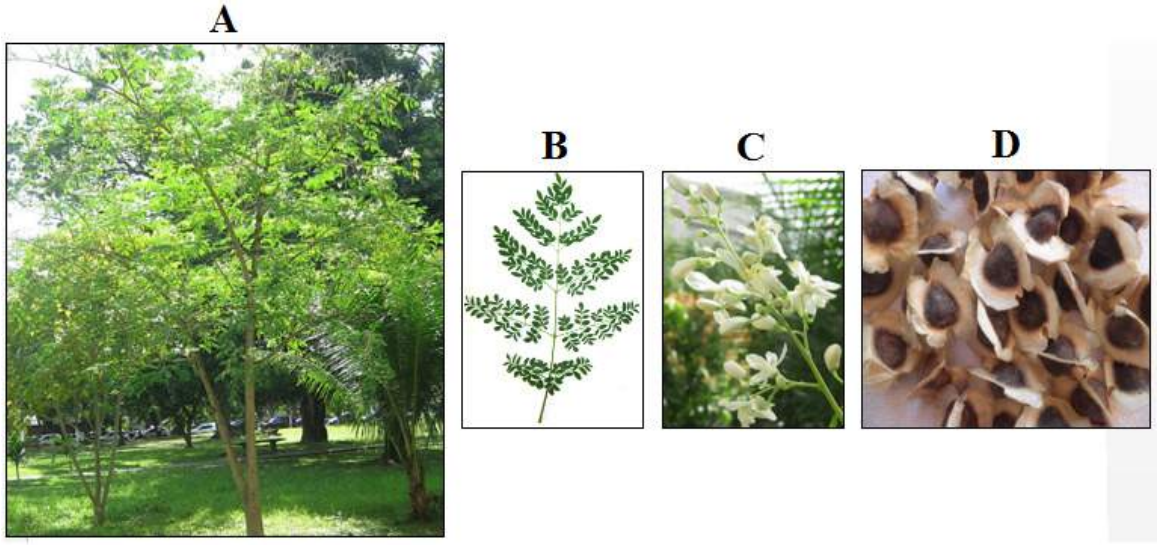
Os tecidos de *M. oleifera* são tradicionalmente utilizados na medicina popular para tratar doenças em países como Guinea, La Reunión, Madagascar, Guiana e Burma (KARADI *et al*., 2006; SANTOS *et al*., 2015). É também consumida no sudoeste da Ásia, onde se acredita que apresente efeitos benéficos sobre a visão (LIU *et al*., 2007). Suas folhas (Figura 3B) são ricas em β-caroteno, proteínas, vitamina C, cálcio e potássio e apresentam atividade antioxidante, hipotensiva, hipoglicemiante, hipocolesterolêmica e contra o vírus herpes *simplex* tipo 1 (FAIZI *et al*., 1995; DILLARD & GERMAN, 2000; LIPIPUN *et al*., 2003; SIDDHURAJU & BECKER, 2003; LAKO *et al*., 2007). Em adição, apresentam efeitos hipolipidêmico e anti-ateroscleróticos com potencial terapêutico para a prevenção de doenças cardiovasculares (CHUMARK *et al*., 2008). Segundo El Sohaimy *et al*. (2015), extrato metanólico e etanólico de folhas apresentam atividade antimicrobiana contra fungos e bactérias patogênicas.

Suas raízes são dotadas de atividade antiurolitíaca e ação citotóxica contra células de leucemia (HL-60 e CEM) e melanoma (COSTA-LOTUFO *et al*., 2005; KARADI *et al*., 2006). A casca do tronco apresenta atividade hipoglicemiante (KAR *et al*., 2003). Suas flores (Figura 3C) são utilizadas para o tratamento de ascite, reumatismo, picadas venenosas e estimulantes cardíacos (ANWAR *et al*., 2003; 2007). São ricas em antioxidantes, como α- e γ-tocoferol, e contêm aminoácidos, sacarose, D-glicose, cera, além de serem ricas em íons potássio e cálcio (RUCKMANI *et al*., 1998; SÁNCHEZ-MACHADO *et al*., 2006; SANTOS *et al*., 2015); contêm ainda inibidor de tripsina, compostos com atividade inseticida sobre cupins *Nasutitermes corniger* e larvas de *Aedes aegypti* e com ação antibacteriana frente bactérias patogênicas (PONTUAL, 2008; MOURA *et al*., 2011; PONTUAL *et al*., 2012a; 2014). De acordo com Pontual *et al*. (2012b), as flores de *M. oleifera* possuem também propriedades caseinolíticas e coagulante de leite e, ainda, segundo Rocha-Filho *et al*. (2015) possuem ação moluscicida contra embriões e adultos da espécie *Biomphalaria glabrata*.

As sementes de moringa (Figura 3D) possuem de 35 a 45% de óleo e são amplamente utilizadas como coagulantes naturais na purificação de água para consumo humano em alguns países em desenvolvimento (ABDULKARIM *et al*., 2005; SANTOS *et al*., 2005; ANWAR *et al*., 2007; UZAMA *et al*., 2011). O óleo da semente é composto principalmente por ácido oleico (> 73%), sendo bastante utilizado na indústria de cosméticos e como matéria-prima para a produção de biodiesel (MOFIJUR *et al*., 2014). Resultados obtidos por Ghebremichael *et al*.

(2005) confirmaram que proteínas são os principais componentes coagulantes em extratos de sementes. Estudos mostram que as sementes apresentam atividade hipotensiva, antioxidante, antibacteriana, inseticida, antitumoral e antifúngica contra dermatófitos, em condições *in vivo* (FAIZI *et al*., 1995; GUEVARA *et al*., 1999; BROIN *et al*., 2002; GHEBREMICHAEL *et al*., 2005; SANTOS *et al*., 2005; CHUANG *et al*., 2007; FERREIRA *et al*., 2011; OLIVEIRA *et al*., 2011; PAIVA *et al*., 2011).

Figura 3. Aspectos de *Moringa oleifera*. A: árvore; B: folhas; C: flores; D: sementes.



Fonte: A autora (2016).

Três diferentes lectinas já foram identificadas nas suas sementes: 1- MoL (do inglês *M. oleifera* lectin) (KATRE *et al.,* 2008); 2- cMoL (do inglês coagulant *M. oleifera* lectin) (SANTOS *et al*., 2009) e; 3- WSMoL (do inglês water-soluble *M. oleifera* lectin) (SANTOS *et al.,* 2005). MoL é uma lectina catiônica formada por subunidades de 7,1 kDa unidas por ligação dissulfeto; foi isolada por Katre *et al*. (2008) através de cromatografia em DEAE-Celulose e CM-Sephadex. Extremos valores de pH e temperatura não alteraram a sua atividade hemaglutinante (AH) que foi inibida pela adição de tiroglobulina, fetuina e holotransferina (KATRE *et al.,* 2008).

O procedimento do isolamento de cMoL foi definido por Santos *et al*. (2009) através de cromatografia de gel de guar. Essa lectina foi ativa entre pH 4,0 e 9,0 e permaneceu com atividade após aquecimento à 100 °C durante 7 horas; possui natureza catiônica e constitui uma proteína monomérica com peso aproximado de 26,5 kDa (SANTOS *et al.*, 2009). cMoL apresentou a propriedade coagulante e habilidade de se ligar à ácidos húmicos (SANTOS *et al*., 2009). A atividade hemaglutinante de cMoL é inibida por vários carboidratos (glicose, rafinose,

lactose, arabinose, trealose, ramnose e galactose), exceto frutose (SANTOS *et al*., 2009). Atividade inseticida foi observada contra larvas e pupas de *Ephestia kuehniella*, causado por desordens nutricionais e retardo no desenvolvimento (OLIVEIRA *et al*., 2011); além disso, apresentou ação frente a operários de *N. corniger* (PAIVA *et al*., 2011).

A lectina WSMoL foi detectada em extratos aquosos de sementes de *M. oleifera* por Santos *et al*. (2005). Posteriormente, Coelho *et al*. (2009) estabeleceram o protocolo do isolamento de WSMoL através da cromatografia de coluna de quitina. É uma proteína aniônica com massa molecular de 60kDa composta por arranjos oligoméricos com subunidades de 5 kDa e foi ativa em uma ampla faixa de pH (4,5 a 9,5) e temperatura (100°C) quando aquecida durante 5 horas (ROLIM *et al*., 2011; MOURA *et al*., 2016). Essa lectina reconhece D(+)-frutose e *N*-acetilglicosamina, uma vez que a presença desses carboidratos inibiu a AH (ROLIM *et al*., 2011). WSMoL não apresentou genotoxicidade de acordo com os testes de Ames, Kado e DNA plasmidial (ROLIM *et al*., 2011) e foi ativa contra as bactérias patogênicas *S. aureus* e *E. coli* e microrganismos provenientes da água natural eutrofizada (FERREIRA *et al*., 2011). Recentemente, um trabalho desenvolvido por Freiras *et al*. (2016) mostrou que WSMoL foi capaz de remover metais da água e que essa atividade bloqueia a capacidade da lectina de se ligar com carboidratos dos eritrócitos, além disso, não apresentou atividade antibacteriana frente *E. coli*, *Salmonella enterica* serovar Enteritidis, indicando que o sítio de interação com carboidratos da superfície bacteriana estava envolvido na capacidade de remoção dos metais. Essa lectina apresentou efeitos deletérios contra *Aedes aegypti*, promovendo mortalidade de larvas no quarto estágio larval (L_4), onde se observou alterações morfológicas no trato digestivo (COELHO *et al*., 2009). Posteriormente, Agra-Neto *et al*. (2014) demonstraram que essa lectina pode causar uma desregulação dos processos digestivos das larvas, através de estimulação da atividade enzimática das proteases, tripsinas e α -amilases. Atividade ovicida e efeito estimulatório sobre a oviposição de *A. aegypti* também foram observados para WSMoL em laboratório e em condições de campo simulado (SANTOS *et al*., 2012; 2014). Atividade termiticida também foi observado para WSMoL contra operários e soldados da espécie *N. corniger* (PAIVA *et al*., 2011) e, adicionalmente, a lectina foi capaz de promover mortalidade (39-43%) e redução do peso (27-54%) de larvas da espécie *Callosobruchus maculatus* de forma dose-dependente, se apresentando resistente à degradação de proteolítica pelas enzimas do intestino das larvas (MOURA *et al*., 2012).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar o efeito antibacteriano de WSMoL sobre o crescimento, sobrevivência e permeabilidade celular de bactérias patogênicas e corrosivas, avaliar o potencial antibiofilme de WSMoL (antiformação e erradicação) sobre *Serratia marcescens* e *Bacillus* sp. e desenvolver um protótipo de revestimento de superfície com a lectina *in vitro*.

3.2 OBJETIVOS ESPECÍFICOS

- Isolar a lectina WSMoL através de protocolo previamente estabelecido.
- Determinar valores de concentração mínima inibitória (CMI), concentração mínima bactericida (CMB) e concentração mínima aglutinante (CMA) para *Bacillus* sp., *Bacillus cereus*, *Bacillus pumillus*, *Bacillus megaterium*, *Micrococcus* sp., *Pseudomonas* sp., *Pseudomonas stutzeri*, *Pseudomonas fluorescens* e *Serratia marcencens*.
- Determinar a curva de crescimento e o extravasamento de proteínas citoplasmáticas de células bacterianas expostas ou não a WSMoL.
- Avaliar danos à permeabilidade celular de *S. marcescens* após tratamento com a lectina.
- Estabelecer protocolo de formação do biofilme *in vitro* produzido por *S. marcescens* e *Bacillus* sp.
- Avaliar o potencial antibiofilme de WSMoL (antiformação e erradicação) *in vitro* frente biofilmes de *S. marcescens* e *Bacillus* sp.
- Desenvolver um protótipo de revestimento da lectina por imersão (*dip coating*) em superfície hidrofílica (vidro)
- Avaliar a formação de biofilmes de *S. marcescens* e *Bacillus* sp. em superfície de vidro revestidas com WSMoL.

4 ARTIGO 1

An Outline to Corrosive Bacteria

Artigo de Revisão publicado como capítulo do livro: **Microbial Pathogens and Strategies for Combating them: Science, Technology and Education**

(Méndez-Vilas, A. (ed.). Badajoz: Formatex Research Center, p. 11-22, 2013).

Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.)

An Outline to Corrosive Bacteria

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The metallic corrosion is a spontaneous process that causes damage in almost all sectors of human activity. Among the most affected structures stand out the pipes for oil transportation. The biocorrosion occurs due to the fixation of bacteria, release of metabolites and formation of biofilms that induce or accelerate the corrosion process. Among the groups of bacteria involved in the corrosion process are included: I- EPS-producing bacteria, II- acid-producing bacteria, III- sulfur-oxidizing bacteria; IV- iron-precipitating bacteria and V- sulfate-reducing bacteria (SRB). The main methods used in industry to prevent the spread of corrosive bacteria include biocides, protective coatings (antifouling) and corrosion inhibitors. This article provides an overview of biofilm formation process and biocorrosion discussing the main groups of corrosive bacteria, as well as the current methods and alternative techniques to control these environmentally harmful processes. In addition, the review throws the idea that plant compounds may be employed to control biocorrosion.

Keywords corrosion; biocorrosion; biofilms, corrosive bacteria; corrosion inhibitors; natural products

1. Introduction

Corrosion is the deterioration of a material, especially metal, as a result of chemical or electrochemical reactions on the metal/solution interface [1, 2]. The electrochemical process is the most common in nature and consists of oxidation-reduction irreversible reactions which results in the formation of a corrosion cell [3]. The metallic corrosion is a spontaneous process (AG <0) which consists of the inverse of metallurgical processes, where the processed metals revert to their natural state of lower free energy, i.e. chemical compounds or minerals [1]. The metallic corrosion represents an economic burden for many industry sectors [4, 5]. According to the World Corrosion Organization [6], the annual cost of corrosion is greater than 3% of global GDP (Gross Domestic Product); however, governments and industries pay little attention to corrosion, except in high-risk areas, like aircrafts and pipelines.

The corrosion influenced by microorganisms is called biocorrosion, presenting the same electrochemical nature of the traditional corrosion [7, 2]. The attachment of bacteria, release of metabolites and formation of biofilms change the electrochemical conditions at the metal/solution interface inducing or accelerating the corrosion process [8-10]. The bioaccumulation, accumulation of organic material on the metal surface, results in the formation of biofilms, which is a basic characteristic of the process of microbial influenced corrosion [11, 12]. The unwanted growth of biofilms has negative impact on various activities and represents significant losses for industries [13]. It is estimated that about 20% of all damages caused by corrosion are influenced by microorganisms; corrosion is the main cause of problems in the pipes of oil industry, affecting the costs of production and storage [10, 14].

The groups of bacteria involved directly and indirectly in the process of biocorrosion are: I- exopolysaccharide or extracellular polymeric substances (EPS)-producing bacteria; II- acid-producing bacteria, III- sulfur-oxidizing bacteria; IV- iron-precipitating bacteria; V- sulfate-reducing bacteria (SRB) [15]. The major responsibility for biocorrosion is attributed to SRB, since there is H_2S release during its metabolism, a reactive, toxic and corrosive agent [16, 17]. The SRB cause serious damage to the petrochemical industry resulting from the formation of biofilms on the metal surface [18, 19].

The methods used to prevent biocorrosion act by inhibiting the growth or metabolic activity of the microorganisms and changing the environment in which the corrosion process occurs [20]. Among the main methods used in the industries are included: I- physical processes [7, 20]; II- biocides [21], III- protective coatings [20] and; IV- corrosion inhibitors [22]. These methods are used generally combined in order to decrease or eradicate the metal exposure to the action of biocorrosion [20, 21, 23]. However, the use of biocides is associated with development of bacterial resistance [24]. Moreover, antifouling coatings and corrosion inhibitors can have their effectiveness diminished due to the metabolization by certain bacteria species [22, 25], in addition to being highly toxic [14].

Plant compounds have been described as potential tools to control the growth of several microorganisms involved in biocorrosion [26-35]. This review suggests the idea that these compounds may be employed in biocorrosion control. Also, the current scenario of corrosion influenced by microorganisms, the major groups of corrosive bacteria, and the methods used to control biocorrosion are discussed.

2. Corrosion

Corrosion is a spontaneous deterioration process of a material, metallic or non-metallic (plastic, concrete and ceramics), by chemical or electrochemical action of the environment, allied or not to mechanical strains [1, 2]. These reactions

occur on the surface of separation between the material and corrosive medium resulting in undesirable changes, such as wear, chemical variations or structural changes, making the material unsuitable for use [1, 36].

The chemical process occurs without water, corresponding to the direct attack of a chemical agent on the material without electron transfers [1]. The electrochemical process consists of spontaneous and irreversible reaction, being most common in nature, necessarily involving the presence of water and electrons transfer [3].

The metal corrosion is the transformation of a metal or metal alloy by its interaction with a particular means of exposure resulting in the formation of corrosion products and the release of energy, i.e. consisting of the natural tendency of processed metals to revert to their native state (AG <0), as chemical or mineral compounds of lower free energy (Figure 1) [1].

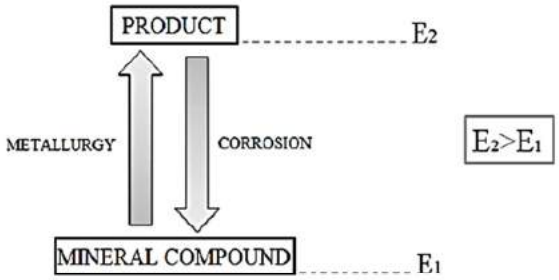

Fig. 1 Transformation of a mineral substance into metal products as performed in the metallurgical industries which occurs with absorption of energy ($E_2>E_1$). On the other hand, the corrosion corresponds to the inverse of metallurgical process and thus occurs spontaneously with decreasing energy ($E_2<E_1$).

The electrochemical process of metal corrosion occurs due to the difference in chemical potential between metal and environment, involving the reaction of these materials with non-metallic substances present in the medium (O_2, H_2S, CO_2, etc.), i.e. there is the metal oxidation (anodic reaction) (Figure 2A) and the reduction of an oxidizing agent (cathodic reaction) (Figure 2B) leading to the formation of a corrosion cell [1, 3].

A $A \rightarrow A^{n+} + ne$

B $B^{n+} + ne \rightarrow B$

Fig. 2 Electrochemical process of corrosion. (A) Anodic reaction representing the oxidation of a metal by a non-metallic substance (eg. O_2, H_2S or CO_2) releasing a number (n) of electrons (e). (B) Cathodic reaction where an oxidizing agent is reduced.

2.1. Economic importance of metallic corrosion

The metallic corrosion can have a major impact on the economy of each country, since high financial costs are required to avoid this process; in 2010, costs related to corrosion were the equivalent of US$ 500 billion for the United States and US$ 76 billion for Brazil [5, 37]. Economic losses caused by corrosion have been the subject of several studies [37].

The wear of storage tanks and pipelines for oil caused by corrosion may result in micro-leaks causing environmental contamination [5, 37]. In addition, leakage of products such as gas, oil or multiphase cause environmental disasters predicted as crime by the Brazilian law of environmental crimes.

2.2. Biocorrosion, bioaccumulation and biofilms

The biocorrosion is the deterioration of a metal influenced by microorganisms that influence corrosion by modifying the electrochemical conditions on the metal/solution interface [2, 7, 38, 39]. This change occurs by the attachment of bacteria to the metal through the release of metabolites on the material surface or biofilm formation that influence the anodic and cathodic reactions, creating conditions for corrosion [8-10].

The process of bioaccumulation or biofouling, which occurs due to accumulation of organic material on the metal surface including cellular debris, EPS and microorganisms, results in the formation of biofilms [12]. According to Videla [23], most of the microorganisms studied so far are related to the process of bioaccumulation and biocorrosion.

The biofilm that occurs after the adsorption of organic and inorganic molecules on the material surface is formed by adherent sessile bacteria that will grow and produce EPS. The latter, once formed, allow new reactions taking place or that the existing kinetic reactions being altered, accelerating the metal wear [8, 40]. In biofilms, microbial populations exhibit functional interdependence and collectively promote microbial activity that would not be likely to occur for only one of the species of composing the biofilm [41, 42].

The formation of a biofilm comprises the following stages: I - formation of a film, through the adsorption of organic and inorganic molecules on the metal, which modifies the load distribution on the metallic surface and, also serves as a nutritional source for bacteria, facilitating the adherence of free-floating microorganisms present in the liquid; II - adhesion and multiplication of aerobic bacteria forming microcolonies; III - production of EPS by some sessile bacteria; IV - colonization by aerobic free-floating microbial cells, that will consume the oxygen by respiration, creating a local anaerobic environment in the biofilm as required by strict anaerobic bacteria and ; V - increase of biofilm thickness, which may favour the shedding of the outer layers [8, 43-46].

The EPS produced by the bacteria adhered to the biofilm capture essential ions to their growth, they are used as a means of attachment, protect bacteria against biocides interfere with the mechanisms of corrosion by favoring creation of differential aeration areas, besides serving as a nutritional source in case of low nutrient availability [12]. The process of corrosion by differential aeration occurs due to uneven distribution of the biofilm on the metal with aerated regions (surrounding the biofilm) and non-aerated regions (below the biofilm) [1]. The biofilm formation on the metal surface is a basic feature of microbial corrosion process and its presence decreases the oxygen content, reaching levels of almost total anaerobiosis [11].

It is known that 40% of pipe corrosion for oil industry is attributed to microbiological corrosion and it causes havoc from the order of US$100 million in production, transportation and storage of oil every year in the U.S. [14, 47]. Pipe biofilms can cause from the reduction in the fluid velocity due to the process of incrustation on the duct walls to the lost through leaks of products generated by corrosion, with consequent impacts on the environment [5].

Environmental problems are associated with biocorrosion including the use of biocides (disinfectants or antiseptics), antimicrobial substances or preparations capable of preventing, inhibiting or eliminating microorganisms, and the use of antifouling coatings (antifouling) and corrosion inhibitors, which are toxic and often cause pollution [7]. Among the most widely used biocides there are chlorine, ozone, bromine, aldehydes, acrolein and phosphonic tetrakis sulfate (hydroxymethyl) (THPS) [21, 48, 49].

3. Corrosive bacteria

Bacteria are simpler prokaryotic organisms from the structural point of view but complex from the biochemical and metabolic point of view, allowing the adaptation to the most varied types of habitat [15]. These microorganisms live and reproduce in pH between 0.5 and 13, temperatures ranging from -12 °C to 110 °C and under pressures of up to 1400 bar [15]. About 98% of the microorganisms making up biofilms are bacteria [50].

Most bacteria related to the corrosion process are part of the sulfur cycle in nature [51]. This cycle consists of microorganisms capable of metabolizing sulfur compounds in two ways: I- chemo-autotrophic: bacteria use electrons of inorganic compounds (H_2S, S and Fe^{2+}) as an energy source, using the CO_2 as their main carbon source [52], and II- chemo-heterotrophic: bacteria utilize electrons specifically from hydrogen atoms of organic compounds as energy source. This category of microorganisms can utilize various compounds as final acceptors of the respiratory chain such as nitrate, nitrite, sulfate, Fe^{3+}, sulfur, fumarate and pyruvate [53].

According to Gentil [1], the action of microorganisms in the processes of corrosion can occur through one or more of the following factors: A- direct influence on the speed of anodic and cathodic reactions; B- modification on the resistance of films existing on the metal surfaces caused by products of microbial metabolism; C- formation of corrosive media due to the acids generation. D- formation of tubers that enable the emergence of differential aeration cells, E- combined action of bacteria.

The EPS-producing bacteria, acid-producing bacteria, sulfur oxidizing bacteria, iron precipitating bacteria and sulfate reducing bacteria (SRB) are involved in the process of biocorrosion [15]. The latter group is responsible for much of the damage caused by microbial corrosion, thus most studies related to biocorrosion is focused on the SRB [22]. The microorganisms associated with biocorrosion are widely distributed (freshwater, sea water, industrial systems and storage tanks, among others) and may influence the spread of all types of metal corrosion [2]. Table 1 shows some bacteria causing corrosion as well as their damage and Figure 3 summarizes the biocorrosion process promoted by some types of corrosive bacteria which are discussed below.

Table 1 Bacteria associated with biocorrosion and their respective local of isolation and damaging actions.

Species	Local of isolation	Damaging actions
Serratia marcescens	• Products of corrosion of diesel and naphtha pipelines.	• Degrades petroleum products due to the release of the enzyme aryl hydrocarbon hydroxylase (AHH) that acts on the corrosion of metals.
Gallionella sp.	• Products of corrosion of petroleum and naphtha pipelines; • Metal surfaces immersed in fresh water and sea water.	• Promotes biomineralization due to deposition of iron hydroxides on the metal surface, modifying the electrochemical processes at the interface metal/solution, inducing corrosion.
Pseudomonas sp.	• Products of corrosion of diesel pipelines; • Metal surfaces immersed in fresh water and sea water.	• Production of EPS that favors the formation of biofilm.
Bacillus sp.	• Products of corrosion of diesel pipelines; • Metal surfaces immersed in fresh water and sea water.	• Degrades petroleum products due to the release of the enzyme aryl hydrocarbon hydroxylase (AHH) that acts on the corrosion of metals; • Production of EPS that favors the formation of biofilm.
Thiobacillus sp.	• Products of corrosion of naphtha pipelines.	• Promotes biomineralization due to deposition of iron hydroxides on the metal surface, modifying the electrochemical processes at the interface metal/solution, inducing corrosion; • Production of sulfuric acid, which increases the corrosion process.
Sulfolobus sp.	• Products of corrosion of naphtha pipelines.	• Promotes biomineralization due to deposition of iron hydroxides on the metal surface, modifying the electrochemical processes at the interface metal/solution, inducing corrosion; • Production of sulfuric acid, which increases the corrosion process; • They may also oxidize sulfur.

References [2, 14, 54-59]

3.1. EPS-producing bacteria

EPS-producing bacteria are initial colonizers due to aerobic and facultative anaerobic metabolism, with the ability of EPS excretion [60]. They alter electrochemical characteristics of metal surface creating a favourable environment for adhesion by other microorganisms, thus stimulating biofilm formation and favouring biocorrosion [11, 61-63]. Due to the aerobic metabolism, they provide ideal conditions for anaerobic bacteria by consuming the oxygen over time [63]. EPS, formed by proteins, polysaccharides, lipids and nucleic acids, protects microbial cells against metal ions, biocidal action and helps to trap other microbial species, thereby contributing to increased thickness of biofilm [12, 64]. *Pseudomonas* is the main genus of EPS producer [60, 65].

3.2. Acid-producing bacteria

Aerobic bacteria are able to produce short-chain organic acids such as acetic, formic, lactic, propionic and butyric acids as products of their metabolism from the fermentative metabolism of organic materials [66]. They are also initial colonizers due to aerobic metabolism [67]. These microorganisms are present in a variety of environments, including gas stands and oils [68]. Organic acids serve as substrates for the SRB, accelerating the corrosion process, besides reducing the pH of the medium [66, 69, 70]. Furthermore, the large amount of organic acid produced acts by metal depolarization starting the local corrosive process [67].

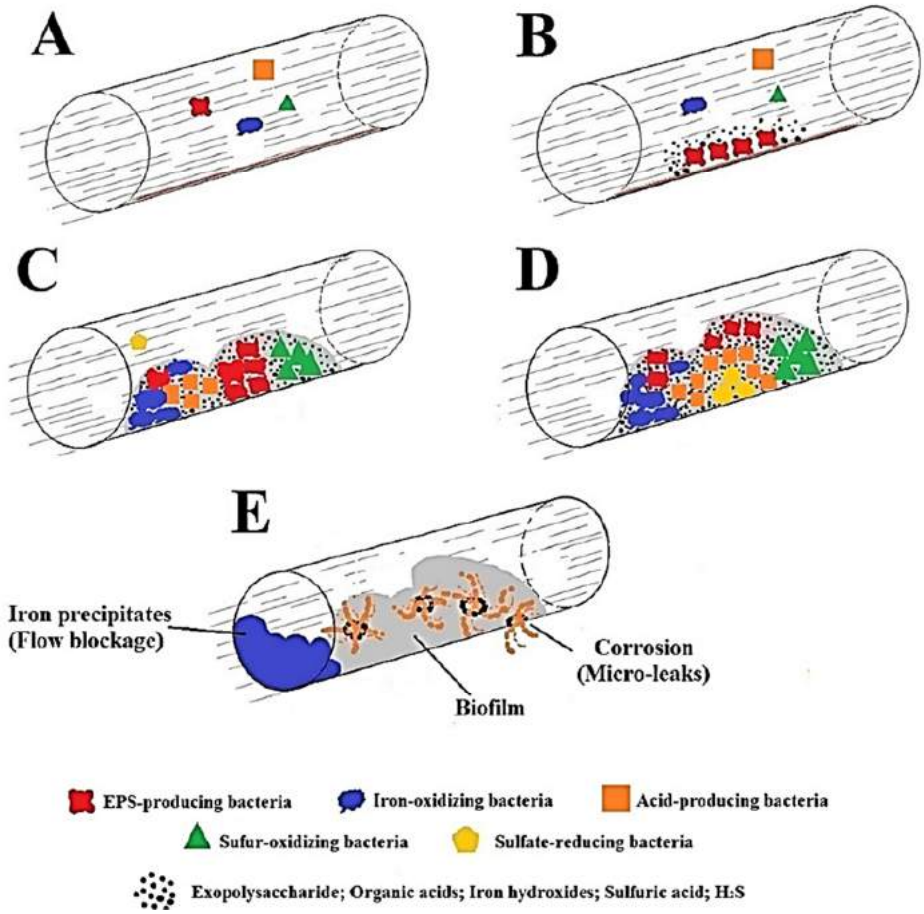

Fig. 3 Biocorrosion process promoted by some types of corrosive bacteria. (A) Aerobic corrosive bacteria from freshwater, sea water, industrial systems or storage tanks reach out the pipeline, that have a conditioning organic and inorganic film on the surface. (B) EPS-producing bacteria are attached to pipeline walls and produce exopolysaccharides, which creates a favourable environment for adhesion by other microorganisms. (C) Adhesion of other groups of corrosive bacteria to pipeline walls, which will release their metabolites, developing into a microcolony through cell division, consuming oxygen available. Action of iron oxidizing bacteria results in large accumulation of ferric precipitation leading to blockage in pipeline; sulfuric acid released by sulfur oxidizing bacteria promotes the acidification of the environment. (D) The low oxygen concentration and organic acids released by acid-producing bacteria favour attach and development of sulfate-reducing bacteria producing hydrogen sulfide (H_2S) accelerating corrosion process and reducing the pH. (E) A corroded pipeline partially blocked by iron precipitates with micro-leaks and containing bacterial biofilm.

3.3. Sulfur-oxidizing bacteria

The sulfur oxidizing bacteria are aerobic and facultative anaerobic microorganisms which obtain the energy necessary for growth from the oxidation of inorganic sulfur compounds such as sulfide, sulfite, thiosulfate and, in some cases the sulfur [56]. Oxidative metabolism results in the production of sulfuric acid which promotes the environment acidification; this high acidity provides great aggressiveness to the environment [71].

Regarding sources of carbon and energy, sulfur oxidizing bacteria may be divided into four groups [56]: I- mandatory chemolithotrophic: grows from CO_2 and inorganic compounds, II- facultative chemolithotrophic: may grow chemolithotrophically (CO_2 and inorganic compounds), heterotrophically (organic compounds as energy and carbon source) or mixotrophically (using both paths simultaneously); III- chemolithoheterotrophic: the energy is generated from the oxidation of inorganic sulfur, but they are unable to fix CO_2; IV- n chemorganoheterotrophic: oxidize inorganic sulfur compounds but do not derive the energy from this reaction.

This group encompasses many genera, being the *Acidithiobacillus* species the most studied; they are gram-negative, rod-shaped, strict aerobic and use CO_2 as the sole carbon source [72]. This group also include bacterial species from the genera *Sulfolobus*, *Thiomicrospira*, *Beggiatoa*, *Acidithiobacillus*, and *Thiothrix* as well as the species *Thiosphaera pantotropha* and *Paracoccus denitrificans* [56].

3.4. Iron-oxidizing bacteria

Iron oxidizing bacteria are aerobic microorganisms, belonging to a large and diverse group, that get energy necessary for their metabolism from iron oxidation [23, 52]. Consequently, there is the formation of iron hydroxides that generally form insoluble precipitate on the surfaces, promoting regions with different oxygen levels [73, 74]. They are characteristically difficult to be isolated and cultured in the laboratory, being widely found in water from rivers, lakes and oil production [23]. They have mostly a locomotor sheath and their presence can be detected by a large accumulation of ferric precipitated as corrosion product [23]. This accumulation or inorganic fouling leads to problems to industrial equipment such as blockages in oil pipelines [23, 52]. Among the most common iron bacteria are highlighted: the species *Thiobacillus ferrooxidans* and the genera *Crenothrix*, *Gallionella*, *Leptothrix* and *Spherotillus*.

3.5. Sulfate-reducing bacteria (SRB)

The SRB form a morphological- and phylogenetically heterogenous group that includes bacteria and restricted anaerobic archaebacteria, although some species have significant tolerance to oxygen [75, 76]. They are mainly gram-negative bacteria, mesophilic and some thermophilic generally spore-forming [77]. These microorganisms are capable of oxidizing various organic compounds of low molecular weight, including mono- or dicarboxylic aliphatic acids, alcohols, hydrocarbons and aromatic compounds, using sulfate ion or other sulfur compounds (thiosulfate, sulfite, etc.) as electron acceptors [36, 76, 78]. Acetate, lactate, pyruvate and ethanol are among the most commonly used substrates by SRB [78]. The stimulation of SRB growth is due to the existing anaerobic conditions in the biofilm explained by the deposition of corrosion products combined with microorganisms and, during the secondary of oil recovery, where there is injection of sea water, rich in sulfate [18, 19, 79, 80].

The ability to utilize sulfate as the final electron acceptor is a reduction process restricted to SRB, whose H_2S formed in the assimilative reduction of sulfate is immediately converted to organic sulfur, while H_2S is secreted in the dissimilative reduction and acts as oxidizing agent to metabolize the organic matter. Large amounts of biogenic hydrogen sulfide can be produced (above 1,100 kg day^{-1}) [80]. Although also being chemically produced by the dissolution of rock's metal sulfides in the reservoir, most of the H_2S formed in pipelines originates from the metabolic activity of sulfate-reducing bacteria [81].

Considering the numerous economic losses related to metabolic activity of SRB in the petrochemical industry, much research has been directed towards prevention and control of corrosion caused by this group of bacteria [56]. These studies are directed to the use of metabolic inhibitors such as molybdate, nitrate and nitrite, and application of biocides, which help the control of metabolic activity of SRB and subsequent inhibition of biogenic H_2S production [56, 82-84].

Several mechanisms contribute to contain the formation process of biogenic H_2S by using metabolic inhibitors: I- competition between SRB and heterotrophic bacteria reducer of nitrite or nitrate by ordinary electron donors, resulting in competitive SRB exclusion [85]; II- increased redox potential due to the presence of intermediaries of nitrate reduction (nitrous oxide and nitric oxide), since the biological production of H_2S occurs only at low redox potential (below -100 mV) [86]; III- Change of energy metabolism of some SRB, reducing nitrate instead of sulfate [18]; IV- sulfide oxidizing bacteria and nitrate or nitrite reducing use the nitrate or nitrite to re-oxidizing H_2S, resulting in the H_2S removal [87]; V- inhibition of the dissimilatory sulfite reductase by nitrite that performs final enzymatic step via sulfate reduction by SRB [86].

The use of metabolic inhibitors widely used in oil industry has been associated with increased risk of metal corrosion [18, 48, 84, 85]; explained by the formation of polysulfide, inorganic sulfur (S (aq)) and thiosulfate through simultaneous oxidation of sulfide by sulfide oxidizing bacteria and nitrate reduction by nitrate-reducing bacteria [48].

Some research has been conducted to study the mechanisms responsible for biofilm formation and biocorrosion processes by SRB [88, 89]. SRB are responsible for much of the damage caused by microbial corrosion [14, 51, 90, 91]. Another economic impact on the oil industry is related to the acidification of oil and gas by H_2S [47, 79, 92, 93]; furthermore, this gas is toxic to humans [94]. These bacteria have been found in storage tanks and transport of oil and gas; their presence is associated with problems of biofouling and biocorrosion [14]. The genera *Desulfovibrio* and *Desulfotomaculum* are the most abundant among SRB associated with biofilms and corrosion in pipelines of petrochemical industries [19, 95, 96].

4. Biocorrosion control

The biocorrosion reaches different industries, especially petrochemical and oil areas in their various systems, such as: cooling circuits, storage tanks, water injection lines, among others [23]. The detection and monitoring of biocorrosion are ways to prevent and control this type of corrosion and investments for this purpose have increased in recent times due to the increasing damage caused in various industries [21]. In most industrial systems, bioincrustations are associated with abiotic fouling [20]. Thus, the study on the control and prevention of biocorrosion cannot be focused only on the activity and growth of microorganisms, but on the physicochemical properties of the metal/solution interface [20].

The methods employed to prevent biocorrosion act by inhibiting the growth or metabolic activity of microorganisms changing the environment in which the corrosion process occurs in order to avoid the adaptation of these bacteria [20]. Physical (cleaning procedures) and chemical methods (sanitization through the use of biocides and antifouling coatings such as inks or corrosion inhibitors) are used generally combined in order to improve the procedure [20, 21]. These methods intend to reduce or eliminate metal exposure to the action of biocorrosion, either by direct elimination of microorganisms or reduced effect of their metabolites on the material [23]. However, inefficient cleaning and monitoring strategies for systems and lack of skilled professionals in the area of microbiological corrosion provide increased corrosion losses [23, 97]. Below is Table 2 of the main control methods of biocorrosion used worldwide, as well as the action and problems associated with use.

Table 2 Methods employed to control biocorrosion and their respective mode of action and associated problems.

Method	Examples	Mode of action	Associated problems
Physical	• Washing with brushes; • Injection of air or gas; • Manual cleaning.	Removes physical deposits.	• Abrasive method that may damage protective films; • Not effective in removing thick biofilms.
Biocides	• Chlorine; • Ozone; • Bromine; • Aldehydes; • Acrolein; • THPS	Prevention, inhibition or elimination of microorganisms.	• High toxicity.
Protective or antifouling coatings	• Silicones; • Resins; • Antifouling paints.	Formation of a protective film.	• They are used as nutritional source by some bacteria. • High toxicity.
Corrosion inhibitors	• Amines; • Fatty acids; • Phosphate esters; • Ammonium nitrate.	Formation of a protective film.	• They are metabolized by some bacteria, causing increased microbial growth and increased rate of corrosion; • High toxicity.

References [7, 20-22, 25, 49, 54, 98]

The physical method comprises mechanical cleaning which matches any method capable of promoting physical removal of the material deposited on the surface and includes the use of brushing, water jets, among others [23]. Furthermore, the chemical cleaning is summarized in the use of various chemical substances, being used after mechanical cleaning [7, 20]. According to Videla [23], biocides stands among the most commonly used effective control methods. Biocides, a heterogeneous group of agents, are widely used in the petroleum industry for internal protection of oil and gas pipelines in the refining area, water injection, secondary recoveries, drilling fluids and the oil production itself for control of planktonic microorganisms and mainly for biofilm generators, possessing antibacterial, antifungal and algaecide activities [24]. However, some biocides may not have activity against certain types of microorganisms within the same group, due to the development of bacterial resistance [24].

Protective or antifouling coatings and corrosion inhibitors form a protective film adsorbed on the metal/solution interface [22, 98]. They are widely used in industrial systems, however their use is associated with increased bacterial growth, since they are nutrient source for some bacteria species [22, 25] in addition of being highly toxic to humans and other non-target organisms [22, 34]. The continuous use of these inhibitors can result in reversible (temporary) or irreversible (permanent) damages to organs such as kidneys and liver, or disrupt a set of biochemical processes [34]. In this scenario, the use of these compounds has been limited on the basis of environmental regulations [34].

Natural products are pointed as corrosion inhibitors, and among them plant compounds have attracted interest since they are inexpensive and ecologically acceptable [29, 30, 32, 34, 35, 99, 100]. Tannins, organic acids, alkaloids, pigments and proteins from plants are known to inhibit metal corrosion [34, 101]. Extracts of *Zenthoxylum alatum* were active on the corrosion of carbon steel in phosphoric acid [29]. Li et al. [32] investigated the inhibitory effect of berberine extracted from *Coptis chinensis* in soft steel which was active against corrosion in 1 M sulfuric acid. Molecules present in aqueous extract for Fenugreek leaves were spontaneously adsorbed on mild steel surface and were capable of inhibiting corrosion on steel in a dose-dependent manner in the presence of HCl and H_2SO_4 [102]. Aqueous extract of *Rosmarinus officinalis* leaves [99], *Lawsonia inermis* leaves [30], *Allium sativum* [8], *Chamaemelum mixtum* [100], *Cymbopogon proximus* [100], *Nigella sativa* [100] and *Phaseolus vulgaris* [100] inhibited metal corrosion. The adhesive protein from the marine mussel *Mytilus edulis* and the bovine serum albumin (BSA) were both adsorbed on

carbon steel and were able to inhibit corrosion [103]. BSA addition in buffered saline significantly decreased the corrosion rate of titanium [104] and molybdenum [105].

Plant products have showed antibacterial activity and the studies have focused in the use of extracts or isolated compounds to combat human-pathogenic and/or phytopathogenic bacteria [26, 27, 106, 107]. As noted earlier, several of these bacteria species are involved in biocorrosion arising from natural biofilms that develop in industrial facilities [23, 108].

Serratia marcescens, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Bacillus sp.*, *B. subtilis*, *B. cereus*, *B. pumilus* and *B. megaterium* were isolated from diesel and naphtha-transporting pipelines located in the northwest and southwest regions in India; the association with localized corrosion of the pipeline steel in the presence of these consortia was corroborated [108]. A joint project of different european aircraft manufacturers confirmed the involvement of isolates from genera *Micrococcus*, *Enterococcus*, *Staphylococcus* and *Bacillus* in strong corrosion damage in aluminium alloy, commonly used in aircraft construction [109]. These bacteria may create a microacidic environment (acid producing bacteria), which favors the development of other bacteria, or produce EPS, favoring the formation of biofilm (EPS-producing bacteria) [108, 109]. The arguments presented here point to the importance of investigating plant compounds as potential agents in control of biocorrosion (Table 3).

Table 3 Plant compounds active against pathogenic bacteria that are involved in biocorrosion.

Plant product	Bacteria
Protein preparations of *Moringa oleifera* flowers (MoE and MoPFT)	• *B. subtilis* • *S. aureus* • *E. faecalis*
Seed lectin from *Eugenia uniflora* (EuniSL)	• *S. Aureus* • *P. aeruginosa* • *Klebsiella* sp. • *B. subtilis*, • *Enterococcus* sp.
Volatile oil of black pepper (*Piper nigrum*)	• *M. luteus* • *B. subtilis* • *S. marcescens* • *E. faecalis*
Volatile oil of oregano (*Origanum vulgare*)	• *M. luteus* • *B. subtilis* • *S. marcescens* • *E. faecalis*
Root of liquorice (*Glycyrrhiza glabra*)	• *B. cereus* • *B. megaterium* • *B. subtilis* • *E. faecalis* • *K. pneumoniae* • *M. luteus* • *P. aeruginosa* • *S. aureus*
Extract of pomegranate peel (*Punica granatum*)	• *P. stutzeri*

References [27, 110-113].

5. Conclusion

Agents currently used to control biocorrosion are strongly toxic and may induce the development of bacterial resistance. In this sense, natural compounds such as extracts and products isolated from plants comprise promising alternatives due to its large spectrum of biological properties, low environmental risk and low cost. This review, motivated by an ecological awareness and based on the success of plant compounds as anticorrosive and antimicrobial agents intends to stimulate the search for new agents able to avoid biocorrosion in an environmental friendly way.

Acknowledgements The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research grants and fellowships (PMGP and LCBBC). We are also grateful to the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. MCM would like to thank FACEPE for graduate scholarship. EVP would like to thank CAPES and FACEPE for post-doctoral fellowship.

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5 ARTIGO 2

Water-soluble *Moringa oleifera* lectin interferes with growth, survival and cell permeability of corrosive and pathogenic bacteria

Artigo publicado no periódico: **Journal of Applied Microbiology**

(Volume: 119; Issue: 3; p. 666-676, 2015)

Fator de Impacto: 2,479

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Water-soluble *Moringa oleifera* lectin interferes with growth, survival and cell permeability of corrosive and pathogenic bacteria

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Keywords
antibacterial activity, biocorrosion, lectin, *Moringa oleifera*, seeds, *Serratia marcescens*.

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2015/0752: received 16 April 2015, revised 3 June 2015 and accepted 5 June 2015

doi:10.1111/jam.12882

Abstract

Aims: This work evaluated the antibacterial activity of a water-soluble *Moringa oleifera* seed lectin (WSMoL) by evaluating its effect on growth, survival and cell permeability of *Bacillus* sp., *Bacillus cereus*, *Bacillus pumillus*, *Bacillus megaterium*, *Micrococcus* sp., *Pseudomonas* sp., *Pseudomonas fluorescens*, *Pseudomonas stutzeri* and *Serratia marcescens*. In addition, the effect of lectin on membrane integrity of most sensitive species was also evaluated. All the tested bacteria are able to cause biocorrosion and some are also responsible for human infections.

Methods and Results: WSMoL inhibited the bacterial growth, induced agglutination and promoted the leakage of proteins from cells of all strains. Bactericidal effect was detected against *Bacillus* sp., *B. pumillus*, *B. megaterium*, *Ps. fluorescens* and *Ser. marcescens*. The bacteriostatic effect of lectin was evident with only 6 h of incubation. Fluorescence microscopy of *Ser. marcescens* showed that WSMoL caused loss of cell integrity and indicated an anti-biofilm activity of the lectin.

Conclusions: WSMoL was active against the bacteria by inhibiting growth and affecting cell permeability. The lectin also interfered with membrane integrity of *Ser. marcescens*, the most sensitive species.

Significance and Impact of the Study: The study indicates that WSMoL was active against bacteria that cause serious problems in both industrial and health sectors. Also, the study contributes for the 'state-of-art' on antibacterial mechanisms of lectins.

Introduction

Microbiologically influenced corrosion (or biocorrosion) corresponds to the deterioration of metallic structures caused directly or indirectly by the metabolic activity of micro-organisms in biofilms, which are complex clusters of microbial cells constituted mostly by bacteria (O'Toole *et al.* 2000; Rajasekar *et al.* 2007b; Harimawan *et al.* 2011). The bacteria release metabolites to attach on the metal surface, which modify the electrochemical conditions at the metal/solution interface favouring the corrosion (Moura *et al.* 2013). The biofilm formation in some regions along the metal surface facilitates or accelerates the biocorrosion because it creates an oxygen gradient leading to corrosion by differential aeration (Gentil 2007).

The microbial contamination of stored hydrocarbons is responsible for serious problems in oil industries due to the formation of biofilms in pipelines and filters, resulting in corrosion of the equipments (Harimawan *et al.* 2011; Moura *et al.* 2013). The corrosion is the main component affecting the operation and maintenance costs of petroleum industry pipelines and it has been estimated that 40% of all internal pipeline corrosion in petroleum

industry is caused by micro-organisms (Rajasekar *et al.* 2007c, 2011).

Several Gram-positive and Gram-negative bacteria can be involved in a biocorrosion process (Harimawan *et al.* 2011; Rajasekar *et al.* 2011). Studies have concluded that the major bacteria involved in biocorrosion in petroleum pipelines are the group of anaerobic sulphate-reducing bacteria (SRB) (Rajasekar *et al.* 2007a). However, it is not imperative the presence of SRB in abundance in the microbial communities capable of promoting biocorrosion (Neria-González *et al.* 2006; Rajasekar *et al.* 2011). Bacteria from the genera *Pseudomonas*, *Bacillus*, *Gallionella*, *Siderocapsa*, *Thiobacillus*, *Thiospira*, *Sulfolobus* and *Vibrio* were detected in a naphtha pipeline and studies clearly indicated a relation between the breakdown of naphtha and the enhance of microbial corrosion rate (Rajasekar *et al.* 2005). Rajasekar *et al.* (2010) isolated 11 bacterial species (*Serratia marcescens*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Klebsiella oxytoca*, *Bacillus* sp., *Bacillus subtilis*, *Bacillus cereus*, *Bacillus litoralis*, *Bacillus pumilus*, *Bacillus carboniphilus* and *Bacillus megaterium*) from diesel and naphtha-transporting pipelines and demonstrated that the bacterial consortia composed by these species was associated with localized corrosion of the pipeline steel.

Cleaning procedures and use of biocides (chlorine, ozone, bromine, etc.), antifouling coatings (anti incrustations paints, silicones, resins) and corrosion inhibitors (amines, fatty acids, phosphate esters, etc.) are employed, generally combined, to prevent biocorrosion process (Videla 2002; Videla and Herrera 2005; Moura *et al.* 2013). However, there is an increasing level of bacterial resistance to these biocides (Acosta-Díaz *et al.* 2011). It has been also reported that some antifouling coatings and corrosion inhibitors can be utilized by some bacteria as a nutritional source, which reduces their effectiveness and causes an increase in microbial growth and, consequently, of the biocorrosion rate (Maruthamuthu *et al.* 2005; Rajasekar *et al.* 2007a,b).

Some corrosive bacteria are also pathogenic to humans and animals. *Bacillus cereus* is commonly present in food and causes digestive disturbances usually with mild symptoms; however, some strains are more virulent causing severe intoxications known as emetic and diarrheic syndromes (López *et al.* 2015). *Serratia marcescens* is an important pathogen associated with nosocomial infections, being usually resistant to several antibiotics such as amoxicillin and the most of β-lactam antibiotics. It can be found, for example, in bronchoscopes and medication vials and was already isolated from cerebrospinal fluid as well as in sputum and other secretions (Liou *et al.* 2014; Leng *et al.* 2015). *Pseudomonas fluorescens* has great importance in aquaculture as it is a pathogen for a wide range of farmed fishes (Zhou *et al.* 2015).

Lectins are carbohydrate-binding proteins widely found in plant tissues and that have shown antibacterial activity, with both bacteriostatic and bactericidal effects (Sá *et al.* 2009; Costa *et al.* 2010; Gomes *et al.* 2013; Ramos *et al.* 2014). The antibacterial effect of lectins may involve the interaction with carbohydrates and glycoconjugates in the cell wall of bacteria (Paiva *et al.* 2010).

Moringa oleifera Lam. (Moringaceae) is a plant native from India and has been the subject of several researches due to its industrial and medicinal properties (Ghebremichael *et al.* 2005; Suarez *et al.* 2005). Its seeds contain the so-called water-soluble *M. oleifera* lectin (WSMoL), which showed coagulant and insecticidal properties (Coelho *et al.* 2009; Ferreira *et al.* 2011; Santos *et al.* 2012). In addition, WSMoL showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* and caused reduction in microbial population density in eutrophic natural water (Ferreira *et al.* 2011).

This work evaluated the effect of WSMoL on the growth and survival of bacteria that are reported to be associated with biocorrosion and are also pathogenic. It was also evaluated the ability of WSMoL in agglutinating the bacterial cells and promote the leakage of intracellular proteins. In addition, the effects of WSMoL on membrane integrity of the most sensitive species were evaluated by confocal fluorescence microscopy.

Materials and methods

Plant material

Seeds of *M. oleifera* (known as 'moringa' in Portuguese, 'árbol del ben' in Spanish and horseradish tree in English) were collected in Recife City, State of Pernambuco, northeastern Brazil. A voucher specimen is deposited under number 73,345 at the herbarium Dárdano de Andrade Lima from the *Instituto Agronômico de Pernambuco* (IPA) at Recife, Brazil. Plant collection was performed with authorization (number 36301-2) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from the Brazilian Ministry of Environment.

Lectin isolation

WSMoL was isolated according to the procedure described by Coelho *et al.* (2009). Quiescent seeds of *M. oleifera* were dried at 28°C, milled to a fine powder and then homogenized with distilled water (in proportion of 10%, w/v) in a magnetic stirrer (3000 g, 16 h). Following homogenization, the mixture was filtered through cotton gauze and centrifuged at 3000 ***g*** for 15 min. Next, the supernatant was treated with ammonium sulphate at

60% saturation (Green and Hughes 1955). The precipitated protein fraction was collected by centrifugation (3000 *g*, 15 min, 4°C) dissolved in water and submitted to dialysis (3·5 kDa cut-off membrane) against 0·15 mol l^{-1} NaCl, for 6 h at 4°C. The dialysed 0–60 fraction (50 mg of proteins) was then loaded onto a chitin column (7·5 × 1·5 cm) equilibrated with 0·15 mol l^{-1} NaCl (0·3 ml min^{-1} flow rate). After extensive washing, the adsorbed protein (WSMoL) was eluted with 1·0 mol l^{-1} acetic acid and dialysed (3·5 kDa cut-off membrane) against distilled water by 6 h at 4°C for eluent elimination.

Protein concentration

The protein concentration was estimated with the method described by Lowry *et al.* (1951) using a standard curve of bovine serum albumin (31·25–500 μg ml^{-1}).

Hemagglutinating activity

Hemagglutinating activity was determined in microtitre plates (Kartell S.P.A., Noviglio, Italy) according to Paiva and Coelho (1992). In a row of the plate, seed extract, 0–60 fraction or WSMoL sample was serially two-fold diluted in 0·15 mol l^{-1} NaCl (50 μl in each well) prior to addition of 2·5% (v/v) suspension of rabbit erythrocytes (50 μl in each well). The hemagglutinating activity was quantified as the reciprocal of the highest dilution of sample that promoted hemagglutination. Specific hemagglutinating activity was defined as the ratio between the hemagglutinating activity and protein concentration (mg ml^{-1}). Inhibitory assay was performed by incubation (15 min) of lectin sample with 200 mmol l^{-1} D(+)-fructose solution in 0·15 mol l^{-1} NaCl before addition of erythrocyte suspension.

Determination of minimal inhibitory (MIC) and bactericide (MBC) concentrations

The bacterial strains *Bacillus* sp. (UFPEDA 189), *B. cereus* (ATCC 11778), *Bacillus pumillus* (ATCC 14884), *B. megaterium* (ATCC 14945), *Micrococcus* sp. (UFPEDA 100) and *Pseudomonas* sp. (UFPEDA 416) were provided by the culture collections from *Departamento de Antibióticos* from the *Universidade Federal de Pernambuco*, which belongs to the World Data Centre for Microorganisms (WDCM 114). The strains *Ps. stutzeri* (ATCC 31258), *Ps. fluorescens* (WDCM 0015) and *Ser. marcescens* (ATCC 14756) were provided by the *Fundação Oswaldo Cruz* (FIOCRUZ), Rio de Janeiro, Brazil. Stationary cultures were maintained in Nutrient Agar (NA) and stored at 4°C. The bacteria used in the assays were cultured in Nutrient Broth (NB) overnight at 37°C under permanent shaking. Next, the culture concentrations were adjusted turbidimetrically to 10^5–10^6 colony forming units (CFU) per ml.

Broth microdilution assay for MIC determination was performed in 96-well microplates. Firstly, 100 μl of NB were dispensed in each well of the plate. In each row, WSMoL (668 μg ml^{-1}) was two-fold serially diluted in the culture medium from the third well and next 20 μl of the overnight bacterial culture was added to each well. The first well contained only the culture medium (negative control) and the second well contained medium and micro-organism (100% growth control). The plates were incubated at 37°C for 24 h and assays were made in triplicate. After incubation, the optical density at 490nm (OD_{490}) was measured using a microplate reader. MIC was determined as the lowest WSMoL concentration able to promote a reduction ≥50% in the optical density relative to the 100% growth control (Amsterdam 1996).

Minimal bactericide concentration (MBC) was determined from the MIC assays. Aliquots from the wells corresponding to WSMoL concentrations ≥MIC were inoculated in petri plates containing NA and incubated for 24 h at 37°C. The MBC corresponded to the lowest lectin concentration able to reduce the bacterial growth in 99·9%.

Bacterial agglutination assay

The ability of WSMoL to agglutinate the bacterial cells was investigated by determination of the minimal agglutinating concentration (MAC) according to Sá *et al.* (2009). The overnight bacterial cultures obtained as described above were diluted at a ratio of 1 : 100 with NB. Agglutination assay was performed in V-bottom microtitre plates. In a plate row, WSMoL (100 μl; 2·19 mg ml^{-1}) was two-fold diluted in 0·15 mol l^{-1} NaCl and then aliquots (100 μl) of bacterial suspension was added in each well. Negative control contained only micro-organism and 0·15 mol l^{-1} NaCl. After overnight incubation of plates at 37°C, MAC was determined as the lowest concentration of WSMoL able to promote visual agglutination of bacteria.

Bacterial agglutination assay was also performed in presence of D(+)-fructose. The lectin (100 μl) was two-fold serially diluted in wells containing an equal volume of monosaccharide solution (200 mmol l^{-1}) in 0·15 mol l^{-1} NaCl. After incubation at 28°C for 30 min, 100 μl of micro-organism culture was added to each well and the bacterial agglutination was evaluated as described above.

Bacterial growth curves in absence and presence of WSMoL

The bacteria sensitive to WSMoL had their growth curve (6 h) established in the absence and presence of lectin. The assays were performed in sterile microtitre plates according to the method described by Gaidamashvili and Van Staden (2002). To each plate well, it was added 20 μl of bacteria culture (in NB) in the exponential growth phase plus 100 μl of WSMoL (in NB) at concentrations corresponding to MIC and 2 × MIC. Negative controls (without lectin) were carried out. The plates were kept at 37°C and the optical density at 600nm was determined every hour until 6 h.

In the cases which cell precipitation was detected, the contents of plate wells were collected and centrifuged (9000 *g*; 10 min; 25°C). The precipitates (resuspended into distilled autoclaved water) and the supernatants were smeared in petri plates containing NA medium. The growth of colonies was evaluated after 24 h.

Evaluation of protein leakage from bacteria cells treated with WSMoL

Protein leakage from bacterial cells was evaluated according to the method of Ogundare (2006). Bacteria culture (100 μl, 10^5–10^6 CFU ml^{-1}) was incubated with WSMoL (900 μl) at MIC and 2 × MIC in a shaking incubator at 37°C. After 24 h the cell suspensions were centrifuged (300 *g*; 10 min, 25°C) and the supernatant was evaluated for protein concentration according to Lowry *et al.* (1951). The amount of leaked protein was calculated by subtracting the protein content in culture medium at the end of assay by the content at time zero. The assays were performed in quadruplicate.

Evaluation of *Serratia marcescens* membrane disruption by WSMoL using fluorescence confocal microscopy

Cells of *Ser. marcescens* exposed or not to WSMoL were stained using the LIVE/DEAD *Bac*Light kit for microscopy (Molecular Probes, Inc., Eugene, OR) to evaluate the loss of bacterial membrane integrity. The samples evaluated were: (i) bacterial cells (10^5–10^6 CFU ml^{-1}) exposed to WSMoL at MIC and 2 × MIC for 24 h at 37°C; (ii) bacterial cells not exposed to the lectin, corresponding to the negative control; (iii) bacterial cells exposed to isopropyl alcohol (700 ml l^{-1}) for 1 h, corresponding to positive control of cell death. Firstly, the cellular material was collected by centrifugation and prepared according to the manufacturer's instructions. Double staining with propidium iodide (PI) and SYTO®9 was performed by incubating bacterial samples with 1·5 mmol l^{-1} PI and 250·5 nmol l^{-1} SYTO 9 at 28°C for 15 min. Next, the samples were observed using a confocal microscope (Leica SPII AOBS, Leica Microsystems, Wetzaler, Germany) at excitation/emission wavelengths of 480/500nm for SYTO 9 and 490/635nm for PI. The images collected were analysed with LITE 2.0 software (Chem-Table Software, Moscow, Russia).

Statistical analysis

All experiments were repeated at least three times. The results are represented as means ± SD. All data were compared using Student's *t*-test and a *P*-value lower than 0·05 was considered statistically significant.

Results

WSMoL (7·18 mg of protein) was isolated through affinity chromatography on chitin column with specific hemagglutinating activity (1,427) higher than obtained for the extract (42) and the precipitated protein fraction (122·5). Hemagglutinating activity of WSMoL was inhibited by D(+)-fructose. These data corroborate with those reported by Coelho *et al.* (2009).

The minimal inhibitory (MIC), bactericidal (MBC) and agglutinating (MAC) concentrations of WSMoL for the tested bacteria are shown in Table 1. WSMoL inhibited the growth of all tested bacteria with highest activity against *Bacillus* sp., *B. pumillus*, *Ps. stutzeri* and *Ser. marcescens*. In assays with *B. cereus* and *Bacillus sp.* there was a precipitation of cells at the bottom of microplate wells, which increased with the lectin concentration, interfering with the reading of optical density. In this sense, we can assume that the MIC value is lower than

Table 1 Minimal inhibitory (MIC), minimal bactericidal (MBC) and minimal agglutinating (MAC) concentrations of water-soluble *Moringa oleifera* seed lectin (WSMoL)

	WSMoL		
Corrosive bacteria	MIC	MBC	MAC
Bacillus sp. (+)	<10·4	167	0·002
Bacillus cereus (+)	<334	ND	0·0005
Bacillus pumillus (+)	10·4	83·5	4·3
Bacillus megaterium (+)	20·8	83·5	4·3
Micrococcus sp. (+)	41·7	ND	4·3
Pseudomonas sp. (−)	167	ND	17
Pseudomonas stutzeri (−)	10·4	ND	8·5
Pseudomonas fluorescens (−)	20·8	20·8	0·03
Serratia marcescens (−)	5·2	5·2	0·03

MIC, MBC and MAC expressed as μg ml^{-1} of WSMoL. Lectin initial concentration, MIC assay = 0·334 mg ml^{-1}; MAC assay = 1·095 mg ml^{-1}.

the concentration corresponding to the last well of row where there was a reduction of 50% in optical density.

The lectin showed bactericidal effect only against *Bacillus* sp., *B. pumillus*, *B. megaterium*, *Ps. fluorescens* and *Ser. marcescens*. WSMoL acted as a bactericidal drug (MBC/MIC ratio of one) on *Ps. fluorescens* and *Ser. marcescens* and worked as a bacteriostatic drug against *B. pumillus*, *B. megaterium*, and *Bacillus* sp. (MBC/MIC ratio higher than two) as well as against *B. cereus*, *Micrococcus* sp., *Pseudomonas* sp. and *Ps. stutzeri* (no bactericidal effect). The bacterium most sensitive to the lectin was *Ser. marcescens*. WSMoL was able to agglutinate all bacterial strains (Table 1), with MAC values ranging from 0·0005 $\mu g\ ml^{-1}$ (*B. cereus*) to 17·0 $\mu g\ ml^{-1}$ (*Pseudomonas* sp.). The agglutination promoted by WSMoL was abolished by the monosaccharide fructose.

The growth curves of the micro-organisms during 6 h can be seen in Fig. 1 and reveal that, although we used the MIC for 24 h as reference, WSMoL was able to inhibit the growth of *B. pumillus*, *B. megaterium*, *Micrococcus* sp., *Pseudomonas* sp., *Ps. stutzeri*, *Ps. fluorescens* and *Ser. marcescens* already in few hours.

The growth curves could not be established for *B. cereus* and *Bacillus* sp. Then, the material from each well was centrifuged and the pellets (containing the clusters of coagulated bacteria) were inoculated in petri plates containing NA; it was observed bacterial growth for both species, In the case of the supernatants, it was detected bacterial growth of *Bacillus* sp. (MIC and 2 × MIC) but not of *B. cereus* treated with 2 × MIC.

For determination of leakage of intracellular proteins, bacteria were incubated with WSMoL for 24 h. Protein concentration in the culture medium was higher in all assays using WSMoL in comparison with controls (Fig. 2). For all the other bacteria, it was observed that the leakage of proteins increased with the WSMoL concentration (Fig. 2b–i).

Confocal microscopy of *Ser. marcescens* cells treated with different concentrations of WSMoL (MIC and 2 × MIC) for 24 h revealed the loss of wall/membrane integrity at both concentrations tested, as observed by the increase in PI positive cells (Fig. 3a,b). The number of viable cells, labelled with SYTO 9 fluorescent probe, was inversely proportional to the increase in the number of PI stained cells and to the WSMoL concentration. In the samples treated with 2 × MIC, most of cells were unviable, as demonstrated by the increase in PI^+ signal (Fig. 3b). Negative control cells (Fig. 3c) presented strong green fluorescent signal, whereas all the cells in the positive control (Fig. 3d) showed strong fluorescent signal at red channel, due to wall permeabilization and membrane damage induced by isopropyl alcohol treatment. Confocal microscopy also revealed different patterns of cell aggregation in negative control when compared with WSMoL-treated bacteria. Control cells formed large cell aggregates (Fig. 3c), indicative of bacterial biofilm formation. On the other hand, lectin-treated cells were visualized as small-dispersed individual clusters (Fig. 3a,b).

Discussion

Currently, the prevention of biocorrosion in industrial systems is facing many difficulties such as the toxicity to nontarget organisms of traditional methods, the bacterial resistance development, and the ability of some microorganisms in using the corrosion inhibitors as nutrient sources (Maruthamuthu *et al.* 2005; Rajasekar *et al.* 2007a,b; Acosta-Díaz *et al.* 2011; Singh *et al.* 2012). This scenario stimulates the search for new alternatives for controlling the biocorrosion process. In this regard, in this work we evaluated the effects of WSMoL, an antibacterial lectin, on corrosive bacteria commonly associated with the formation of biofilms on metallic surfaces. WSMoL showed bacteriostatic effect on all bacteria tested and was bactericide for most of them. The fact that some of the tested corrosive bacteria – such as *B. cereus*, *Ps. fluorescens* and *Ser. marcescens*—are also pathogenic to humans and animals broadens the relevance of WSMoL as an antibacterial agent.

Lectins from plants have shown antibacterial activity against a wide variety of bacteria. Study conducted by Gomes *et al.* (2013) showed that lectin from *Schinus terebinthifolius* leaf was active against *E. coli*, *Klebsiella pneumoniae*, *Ps. aeruginosa*, *Proteus mirabilis*, *Staph. aureus* and *Salmonella enteritidis* with MIC values ranging from 0·45 to 115 $\mu g\ ml^{-1}$. The *Eugenia uniflora* seed lectin demonstrated a remarkable antibacterial activity, strongly inhibiting the growth of *Staph. aureus*, *Ps. aeruginosa* and *Klebsiella* sp. with MIC of 1·5 $\mu g\ ml^{-1}$ and moderately inhibiting the growth of *B. subtilis*, *Streptococcus* sp. and *E. coli* with MIC of 16·5 $\mu g\ ml^{-1}$ (Oliveira *et al.* 2008). According to Ferreira *et al.* (2011), WSMoL also showed antibacterial activity against *Staph. aureus* and *E. coli* with MIC of 7·8 $\mu g\ ml^{-1}$ and 250 $\mu g\ ml^{-1}$, respectively, and bactericidal effect only against *Staph. aureus* (MBC of 300 $\mu g\ ml^{-1}$).

WSMoL was also able to agglutinate bacterial cells and the abolition of this phenomenon after addition of monosaccharide fructose indicates that the carbohydrate-binding site of lectin was involved in the agglutination. Thus, the different MAC values were probably due to differences in the carbohydrates present in the cell surfaces of the bacteria tested. The agglutination by lectins promotes immobilization of the bacterial cells and occurs by noncovalent interactions (Rittidach *et al.* 2007; Costa *et al.* 2010). Lectin from the heartwood of *Myracrodruon*

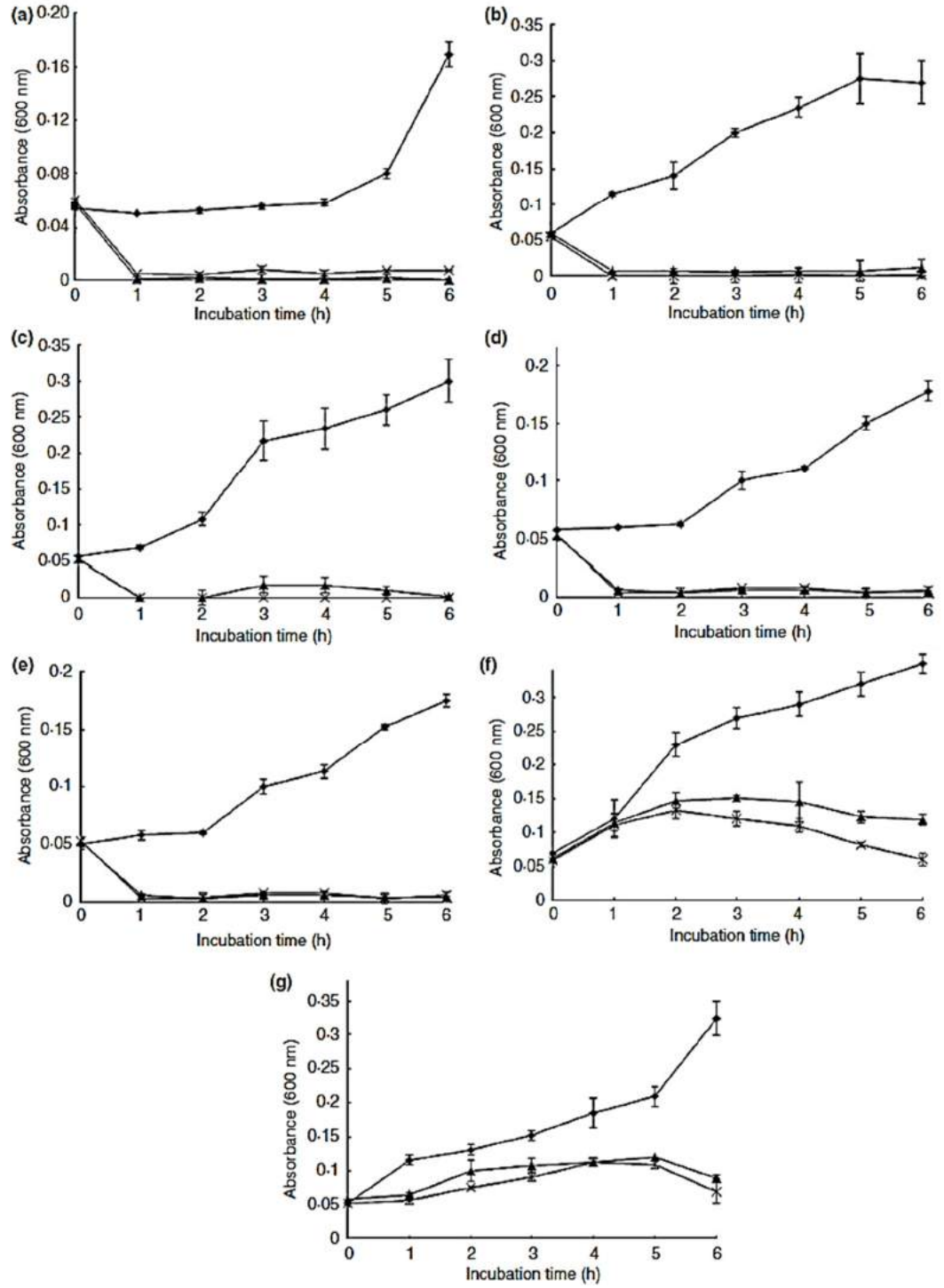

Figure 1 Growth curves of bacterial cells in absence and presence of water-soluble *Moringa oleifera* seed lectin (WSMoL). (a) *Bacillus pumillus*, (b) *Bacillus megaterium*, (c) *Pseudomonas* sp., (d) *Pseudomonas fluorescens*, (e) *Serratia marcescens*, (f) *Micrococcus* sp. and (g) *Pseudomonas stutzeri* growth under lectin effect. Lectin was added to the culture medium to obtain the minimal inhibitory concentrations (MIC) (–▲–) and 2xMIC (–✶–). Negative controls (without WSMoL;–◆–) were carried out for each bacteria using bacterial suspensions in NB. Data were expressed as the mean ± standard deviation (SD) of four determinations.

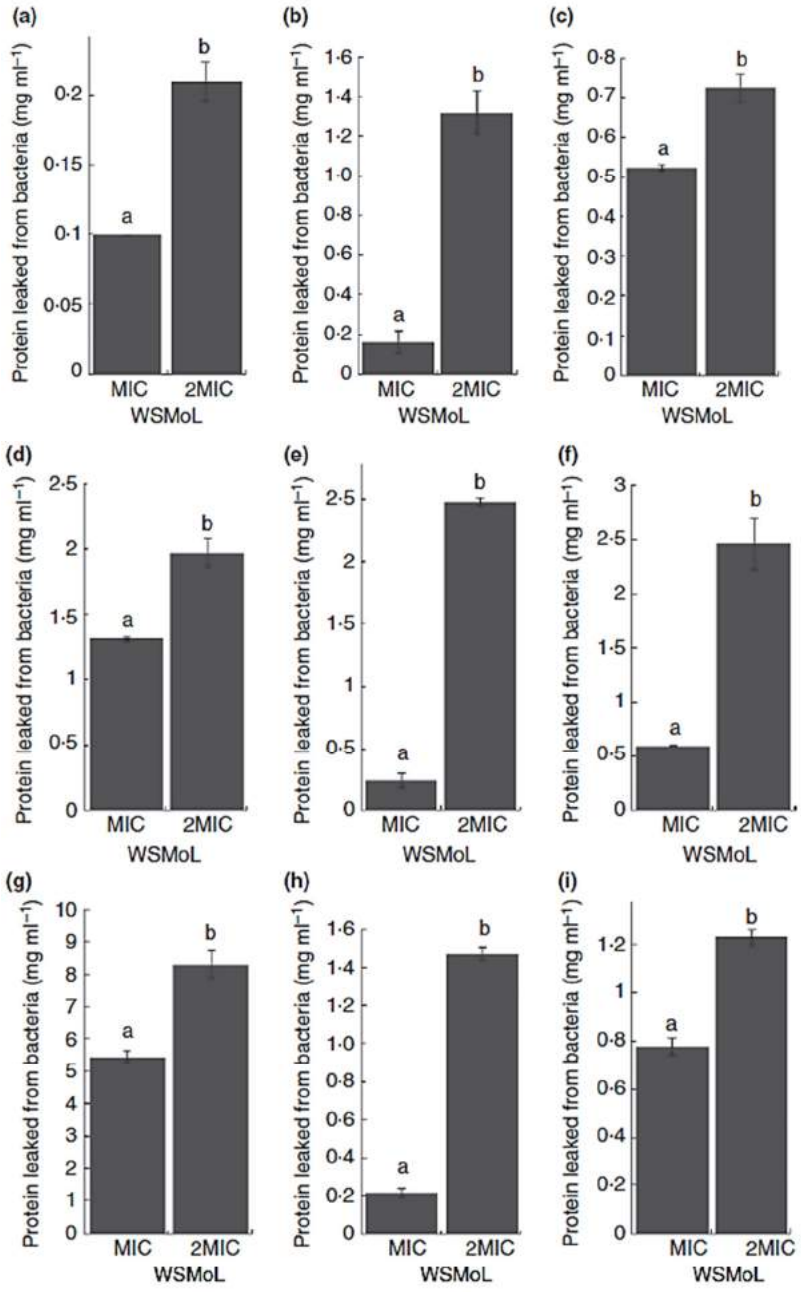

Figure 2 Leakage of proteins from (a) *Bacillus* sp., (b) *Bacillus cereus*, (c) *Bacillus pumillus*, (d) *Bacillus megaterium*, (e) *Micrococcus* sp., (f) *Pseudomonas* sp., (g) *Pseudomonas fluorescens*, (h) *Pseudomonas stutzeri* and (i) *Serratia marcescens* cells exposed to water-soluble *Moringa oleifera* seed lectin (WSMoL). Bacteria cultures (in NB), in the exponential growth phase, were incubated with WSMoL (minimal inhibitory concentrations, MIC and 2 × MIC) for 24 h at 37°C and then the protein content in culture medium was determined. There was no protein leakage in controls. Assays were performed in four replicates. Different letters indicate significant differences between treatments ($P < 0.05$).

urundeuva showed agglutinating activity on *B. subtilis* (MAC: 4·68 μg ml^{-1}), *Corynebacterium callunae* (4·68 μg ml^{-1}), *Staph. aureus* (2·34 μg ml^{-1}), *Enterococcus faecalis* (4·68 μg ml^{-1}), *E. coli* (9·37 μg ml^{-1}), *Kl. pneumoniae* (9·37 μg ml^{-1}) and *Ps. aeruginosa* (9·37 μg ml^{-1}) (Sá *et al.* 2009). In addition, lectins from *Phthirusa pyrifolia* leaf and from the shrimp *Fenneropenaeus chinensis* were able to agglutinate bacteria cells (Sun *et al.* 2008; Costa *et al.* 2010). The ability of lectins to agglutinate bacteria results in concentration of microbial mass, which may implicate in the use of lower amount of a biocide to target a high number of cells (Costa *et al.* 2010).

As WSMoL showed bacteriostatic activity against all tested bacteria, we evaluated its effect on growth of the micro-organisms during 6 h. At the first hour of incubation with the lectin, the optical densities in the cultures of *B. pumillus*, *B. megaterium*, *Pseudomonas* sp., *Ps. fluo-*

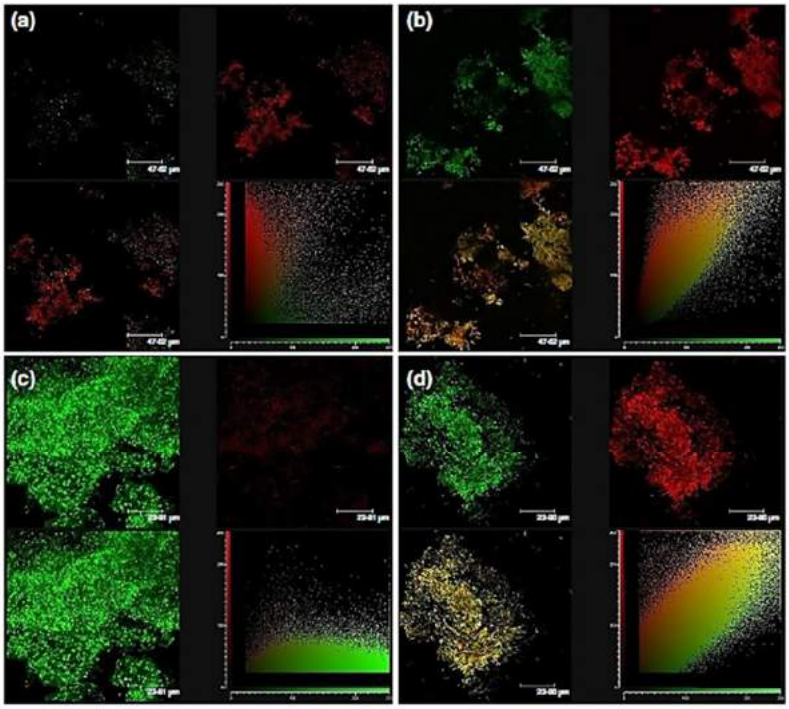

Figure 3 Confocal microscopy of the effects of water-soluble *Moringa oleifera* seed lectin (WSMoL) on *Serratia marcescens* membrane integrity. (a) Cells treated with WSMoL at minimal inhibitory concentrations (MIC). (b) Cells treated with WSMoL at 2 × MIC. (c) Negative control cells. (d) Cells treated with isopropyl alcohol (positive control). The viable cells stained with SYTO 9 were green fluorescent (1) and cells with damaged membranes were marked with IP and appeared red fluorescent (2). The overlay of the green and red fluorescences appears as yellowish signal (3) and represents the gradual permeabilization of bacterial wall/membrane allowing the displacement of SYTO 9 by propidium iodide labelling in damage bacteria.

rescens and *Ser. marcescens* decreased in comparison with time zero, indicating that the lectin not only impaired the colony growth but also caused cell death. Interestingly, for *Ps. fluorescens* and *Ser. marcescens* the optical density remained near zero along the next 5 h, which was expected, as the MIC value determined was equal to the MBC. For *B. pumillus* and *B. megaterium*, the results suggest that the lectin, although was mainly bacteriostatic to these bacteria, is able to maintain the amount of viable cells in a very low level when present at concentrations ≥MIC and lower than the MBC. WSMoL was less effective in inhibiting the growth of *Micrococcus* sp. and *Ps. stutzeri*, bacteria to which it did not show bactericidal effect. In these two cases, the inhibitory effect was dose-dependent and, although there was no colony growth in the first 5 h, the lectin did not reduce the number of viable cells as there was no decrease in the optical density regarding time zero. The ability of WSMoL in promoting sedimentation of bacterial cells was also reported by Ferreira *et al.* (2011) using *Staph. aureus* and *E. coli*.

Our results showed that the growth curves could not be established for *B. cereus* and *Bacillus* sp. because of the clustering of bacterial cells caused by WSMoL, the same problem observed in assay for determination of MIC. The results obtained after smearing of the clusters and supernatants in solid culture medium reveal that the lectin promoted aggregation and removed cells from the suspension but did not kill them. This is in agreement with the high MBC (167 μg ml^{-1}) and low MAC (0·002 μg ml^{-1}) determined for *Bacillus* sp. As WSMoL did not show bactericidal effect for *B. cereus*, the results indicates that, in this concentration, the lectin was able to coagulate all bacterial cells.

One of the mechanisms of action proposed for lectins with antibacterial effect included the ability in forming pores in the cell membrane causing leakage of cellular content (Talas-Ogras *et al.* 2005). In this sense, we evaluated whether the incubation of bacteria with WSMoL would result in the leakage of intracellular proteins. The results suggested that the lectin treatment, at least, increased the cell permeability allowing the passage of proteins. It is important to note that this effect was observed even for those bacteria in which WSMoL did not showed bactericidal effect (*B. cereus*, *Micrococcus* sp., *Pseudomonas* sp. and *Ps. stutzeri*). An N-acetyl-D-glucosamine specific lectin isolated from *Araucaria angustifolia* seeds showed antibacterial activity and electron microscopy revealed that it promoted morphological changes, including formation pores in the bacterial membrane (Santi-Gadelha *et al.* 2006).

WSMoL demonstrated highest efficiency against the bacterium *Ser. marcescens*. This species is a bacterium also pathogenic to humans, frequently associated with hospital-acquired infections, and is able to adhere to solid surfaces including catheters (Hejazi and Falkiner 1997). *Serratia marcescens* also degrades petroleum products due

to the release of the enzyme aryl hydrocarbon hydroxylase (AHH) that acts on the corrosion of metals, and also reduces the effects of corrosion inhibitors used in industries (Rajasekar *et al.* 2007a, 2011). To better understanding the effects induced by WSMoL on this species, we analysed the occurrence of damage integrity of bacterial cell wall and membrane as well as changes in the protein expression profile.

Confocal microscopy analysis confirms that the bactericidal effects of WSMoL involves the loss of cell wall integrity. Additionally, we also observed that the biofilm formation pattern in the control cells was different from those found in treated cells, suggesting that lectin treatment disturbed the bacterial biofilm. The anti-biofilm activity of lectins has been already reported (Hasan *et al.* 2014; Vasconcelos *et al.* 2014; Klein *et al.* 2015). A recent study demonstrated the anti-biofilm activity of a C-type lectin from *Bothrops jararacussu* venom on staphylococcal biofilms (Klein *et al.* 2015). According to these authors the lectin could interact with biofilm carbohydrates via carbohydrate-binding domain, disturbing the biofilm formation during bacterial growth. An alternative mechanism could involve disturbance via unknown domain(s) present in the lectin. The possibility of lectin acts directly on biofilm is particularly interesting as biofilms are important factor of protection that enable bacteria to survival in unfavourable conditions (Klein *et al.* 2015).

Natural antimicrobial compounds can interact and increase the permeability of bacterial membranes as part of their killing mechanism (Mangoni *et al.* 2004; Wu *et al.* 2003). The antimicrobial peptide temporin L isolated from skin secretions of the frog *Rana temporaria* showed the ability to increase the permeability of *E. coli* inner membrane in a dose-dependent manner without destroying the cell integrity; in high concentrations, it is observed the leakage of large molecules resulting in cell death (Mangoni *et al.* 2004). A study conducted by Wu *et al.* (2003) demonstrated that the pulmonary collectins, surfactant proteins A and D from mice inhibit the proliferation of *E. coli* by increasing the permeability of the microbial cell membrane. Essential oils and plant extracts also showed the ability to induce morphological changes in bacteria cells (Cox *et al.* 2000; Nohynek *et al.* 2006; Azerêdo *et al.* 2012). According to Azerêdo *et al.* (2012), essential oils from *Origanum vulgare* and *Rosmarinus officinalis*, either alone or in combination at subinhibitory concentrations, were able to cause membrane damage of *Listeria monocytogenes* after 15 min of incubation.

In summary, this study indicates that WSMoL was active against corrosive and pathogenic bacteria. The lectin showed bacteriostatic and bactericide effects and the mechanism of action may involve changes in the cell permeability leading to leakage of cytoplasmic proteins. In addition, incubation of *Ser. marcescens* cells with WSMoL resulted in damage to integrity of cell wall and membrane.

Acknowledgements

The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research grants and fellowships (PMGP and LCBBC). We are also grateful to the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Brazilian Ministry for Science, Technology and Innovation (MCTI) for financial support. MCM thank FACEPE for graduate scholarship. MCC would like to thank FACEPE for post-doctoral fellowship.

Conflict of Interest

The authors declare no conflict of interest.

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6 ARTIGO 3

Multi-effect of Moringa oleifera lectin against Serratia marcescens and Bacillus sp.: antibacterial, antibiofilm and anti-adhesive properties

Artigo submetido ao periódico: **Journal of Applied Microbiology**

Fator de Impacto: 2,479

Multi-effect of *Moringa oleifera* lectin against *Serratia marcescens* and *Bacillus* sp.: antibacterial, antibiofilm and anti-adhesive properties

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Running headline: Antibiofilm properties of *M. oleifera* lectin

Abstract

Aims: This study aimed to evaluate the potential of *Moringa oleifera* seed lectin, WSMoL, in inhibiting biofilm formation and eradicating preformed biofilms of *Serratia marcescens* and *Bacillus* sp. In addition, a prototype of WSMoL-coated surface obtained by dip coating was evaluated as anti-adhesive surface.

Methods and Results: WSMoL was able to inhibit biofilm formation by *S. marcescens* at concentrations lower than 2.6 μg/mL and showed antibiotic effect at higher concentrations, avoiding biofilm formation. For *Bacillus* sp., the lectin showed antibiotic effect at all concentrations. Confocal microscopy images evidenced that the action of WSMoL is associated with loss of cell viability. WSMoL did not disrupt preformed *S. marcescens* biofilms but was able to penetrate them and kill the cells. On the other hand, the lectin caused a reduction in the number of cells in *Bacillus* sp. biofilm treated with it. WSMoL spontaneously adhered on glass leading to a production of a lectin-coated surface (116 $\mu g/cm^2$) that possesses a more hydrophilic character than non-coated surface. Confocal laser scanning microscopy indicated that WSMoL is able to control biofilm formation even when immobilized on a surface, decreasing viability of *S. marcescens* and avoiding adherence of *Bacillus* sp. cells.

Conclusion: WSMoL prevented biofilm development by *S. marcescens* and *Bacillus* sp. and the antibiofilm effect is also observed when the lectin is immobilized on a glass surface.

Significance and Impact of Study: Taking together, our results provide support to the potential use of WSMoL for controlling biofilm formation by pathogenic and corrosive bacteria.

Keywords: antibiofilm activity; corrosive bacteria; *Moringa oleifera*; anti-adhesive coating, *Serratia marcescens*; *Bacillus* sp.

Introduction

Biofilms are complex microbial communities attached to a tissue or an abiotic surface and composed by microcolonies embedded in a matrix of self-produced polymeric substances (Rendueles *et al*. 2013). These communities represent the predominant mode of growth for bacteria in most of natural, industrial and clinical environments and are associated with high tolerance to exogenous stress and treatments with antibiotics and biocides (Hall-Stoodley and Stoodley 2005; Rendueles *et al*. 2013).

Biofilm formation is related with chronic and nosocomial infections in humans (Hoiby *et al*. 2011; Rabin *et al*. 2015) as well as is a major problem in almost all industrial sectors, causing biocorrosion and contamination of different materials (Rajasekar *et al*. 2007). This scenario has stimulated efforts aiming to identify novel substances with antibiofilm activity (Trentin *et al*. 2013; Vasconcelos *et al*. 2014; Rabin *et al*. 2015).

Serratia marcescens is an opportunistic enteric pathogen, responsible for a significant proportion of nosocomial infections, such as in the urinary tract (Murdoch *et al*. 2011). The genus *Bacillus* is composed by saprophyte bacteria, which have been associated with mild and severe human infections (Mugoyela and Mwambete 2010). Additionally, these bacteria may form biofilms on industrial steel and are involved in biocorrosion and degradation of petroleum products (Rajasekar *et al*. 2007).

Lectins are carbohydrate-binding proteins widely distributed in plants (Saha *et al*. 2011). The interactions between lectins and sugars play important roles in several biological processes and mediate a broad range of biological activities, including antibacterial and antibiofilm activities (Klafke *et al*. 2013; Dias *et al*. 2015). Several studies have reported that these proteins are able to affect bacterial growth and viability as well as to disrupt the bacterial intracellular signaling (quorum sensing) involved in the formation of biofilms (Islam *et al*. 2009; Saha *et al*. 2011; Cavalcante *et al*. 2013).

Moringa oleifera (Moringaceae) is a highly valued plant with industrial and medical importance, cultivated in the tropics and subtropics (Mbikay 2012). The water-soluble lectin isolated from *M. oleifera* seeds (WSMoL) showed coagulant, insecticidal and antibacterial properties (Coelho *et al*. 2009; Agra-Neto *et al*. 2014) and did not show genotoxicity *in vitro* (Rolim *et al*. 2011). Our research group reported the activity of WSMoL on growth, survival and cell permeability of pathogenic bacteria which could be also associated with biocorrosion process (*Bacillus* sp., *Bacillus cereus*, *Bacillus pumilus*, *Bacillus megaterium*, *Micrococcus* sp.,

Pseudomonas sp., *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *S. marcescens*) (Moura *et al*. 2015). It was also demonstrated that the WSMoL action against *S. marcescens* (the most sensitive species) involves the impairment of bacterial cell wall/membrane integrity.

In the present study, we evaluated the potential of WSMoL in inhibiting biofilm formation by *S. marcescens* and *Bacillus* sp. as well as its action on preformed biofilms of these bacteria. The structure and viability of bacterial cells in untreated and lectin-treated biofilms were also evaluated by microscopy. In addition, it was developed a prototype of a *Green*-coated surface by the coating with WSMoL lectin in order to protect materials against microbial biofilm development.

Materials and Methods

Plant material

Seeds of *M. oleifera* were collected in Recife, Pernambuco, northeastern Brazil, under the authorization (license number 38690) of the regulatory organ *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from Brazilian Ministry of Environment. Taxonomic identification (voucher number 73345) was confirmed at the herbarium Dárdano de Andrade Lima from the *Instituto Agronômico de Pernambuco* (IPA) located at Recife.

Lectin isolation

WSMoL was isolated according to the procedure reported by Coelho *et al*. (2009). The seeds were dried at 28 °C and powdered using a blender. The powder was homogenized with distilled water (in proportion of 10%, w/v) under constant agitation for 16 h at 25ºC. The aqueous extract was obtained after filtration with cotton gauze and centrifugation (3000 *g*, 15 min, 4°C). Then, the extract was treated with ammonium sulfate at 60% saturation (Green and Hughes 1955) during 4 h at 28ºC and the precipitated fraction was collected by centrifugation (3000 *g*, 15 min, 4°C), ressuspended and dialyzed against distilled water (4 h) and 0.15 M NaCl (4 h). The dialyzed fraction (50 mg of proteins) was then loaded onto a chitin column (7.5 × 1.5 cm) previously equilibrated (0.3 mL/min) with 0.15 M NaCl. WSMoL was eluted with 1.0 M acetic acid and then dialysed against distilled water (6 h, 4°C) for eluent elimination, providing a yield of 3.4 mg per column.

Determination of protein concentration and hemagglutinating activity

The protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin as standard (31.25-500 µg/mL). The hemagglutinating activity (HA) was evaluated aiming to monitor the carbohydrate-binding ability of WSMoL as described previously (Paiva and Coelho 1992). In the HA assay, the lectin (50 µL) was two-fold diluted in 0.15 M NaCl in 96-well microplates (Corning Inc., USA) and then it was added in each well 50 µL of a 2.5% (v/v) suspension of glutaraldehyde-treated rabbit erythrocytes (collected with authorization of the Ethics Committee on Animal Experimentation from the *Universidade Federal de Pernambuco*; process 23076.033782/2015-70). After 45 min, the HA was recorded as the reciprocal of the highest sample dilution that promoted hemagglutination (Paiva and Coelho 1992). Specific HA corresponded to a ratio between the HA and the amount of protein (mg/mL). HA inhibitory assay was performed by replacing the 0.15 M NaCl by a 200 mM D(+)-fructose solution.

Bacterial growth and biofilm formation

Bacillus sp. (UFPEDA 189) was provided by the Culture Collection of the *Departamento de Antibióticos* (WDCM 114) from the *Universidade Federal de Pernambuco*. *Serratia marcescens* (ATCC 14756) was provided by the *Fundação Oswaldo Cruz* (Rio de Janeiro, Brazil) and is also deposited in the culture mentioned above. Stock cultures were maintained at −80°C in sterile nonfat milk powder with 10% (v/v) glycerol. The bacteria were cultured in Mueller Hinton Agar (MHA) overnight at 37°C for *Bacillus* sp. and 28°C for *S. marcescens* and then the colonies were suspended in sterile saline solution (0.9 % NaCl) to obtain a suspension equivalent to 10^8 colony forming units (CFU) per mL.

Bacterial growth was assessed by the difference between optical density at 600 ηm (OD_{600}) at the end and at the beginning of incubation time and biofilm formation was evaluated by the crystal violet assay according to Trentin *et al*. (2011), in sterile 96-well polystyrene flat-bottom microplates (Costar 3599; Corning Inc., USA). Each well received 80 µL of the bacterial suspension, 80 µL of Milli-Q water and 40 µL of Mueller Hinton Broth (MHB). Optical density at 600 ηm (OD_{600}) was read at this moment (time zero) using a microplate reader and the microplate was incubated at 37°C during 24 h. After this period, the OD_{600} was read again in

order to verify bacterial growth. The content of each well was removed and the wells were washed three times with sterile 0.9 % NaCl. The biofilms (remaining attached bacteria) were heat-fixed at 60°C for 60 min and stained with 0.4 % (w/v) crystal violet for 15 min at 25ºC. The wells were washed with water in order to remove the unbound crystal violet and the dye that bound to the biofilm was solubilized with absolute ethanol (15 min) and absorbance was measured at 570 ηm. All experiments were performed in triplicate.

Effect of WSMoL on biofilm formation

To evaluate the ability of WSMoL to impair biofilm formation, the protocol described in the previous section was used in the assays in absence and presence of the lectin. Initially, WSMoL solutions were prepared in Milli-Q water aiming to achieve final concentrations corresponding to 4×MIC, 2×MIC, MIC, ½MIC, ¼MIC, ⅛MIC; 1/16 MIC in the microplate wells. The MIC_{50} values are 5.2 and 10.4 µg/mL for *S. marcescens* and *Bacillus* sp., respectively, as determined by Moura *et al*. (2015). The lectin solutions were subjected to filtration through 0.20 µm syringe filters (Chromafil® Xtra PVDF). In the assays, 80 µL of bacterial suspension, 80 µL of WSMoL solution and 40 µL of MHB were added to each well. For the control of growth and biofilm formation, WSMoL was replaced by sterile water. Gentamicin and rifampicin (Sigma-Aldrich - 8 µg/mL) were used as antibiotic controls in assays with *S. marcescens* and *Bacillus* sp., respectively. The microplate was incubated for 24 h at 37ºC and the biofilms formed were fixed and stained as described in section 2.4.

Scanning electron microscopy (SEM)

A piece of Permanox^{TM} slide (a solvent-resistant plastic) was put in each well of 96-well microtiter plates and the assay was performed as described in section 2.5. After incubation, the slides were fixed in 2.5% glutaraldehyde, washed with 100 mM cacodylate buffer pH 7.2 and dehydrated in increasing concentrations of acetone. The slides were then dried by the CO_2 critical point technique (CPD 030 Balzers, Liechtenstein), fixed on aluminum stubs, covered with gold film and examined in a JEOL JSM-6060 scanning electron microscope. Three-dimensional images of the structure of the biofilms were obtained. A control containing only lectin at 41.6 µg/mL and medium, in absence of bacteria, was also visualized in order to observe the adherence of WSMoL to the slides.

Confocal laser scanning microscopy (CLSM)

Biofilm formation by *S. marcescens* and *Bacillus* sp. and cell viability were evaluated by confocal microscopy. Bacterial cells were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, USA) according to the manufacturer instructions. In this staining, the SYTO-9 labels cells with damaged or intact membranes (green fluorescence), whereas the propidium iodide penetrates only in the cells with damaged membranes (red fluorescence), reducing the fluorescence of SYTO-9. Images were obtained using an OlympusIX81 confocal microscope and UPLSAPO 60X W NA:1.20 objective and were overlaid using Olympus FV 1000 software.

Biofilm eradication assay

Serratia marcescens and *Bacillus* sp. 24 h-old biofilms were obtained as described in section 2.4 and then incubated with 80 µL of sterile water, 40 µL of MHB and 80 µL of the lectin at 20×MIC, 10×MIC, 5×MIC, 2.5×MIC, MIC, ½MIC or ¼MIC, which corresponded to concentrations ranging from 1.3–104 µg/mL for *S. marcescens* and 2.6–208 µg/mL for *Bacillus* sp. For the untreated control, biofilms were incubated with sterile water, instead of lectin, while for antibiotic control, biofilms were treated with gentamicin or rifampicin (8 µg/mL) for *S. marcescens* and *Bacillus* sp., respectively. After 24 h at 37°C, the contents were removed and the wells were washed three times with sterile 0.9 % (w/v) NaCl. The remained biofilms were stained with crystal violet or visualized by SEM as described in sections 2.4 and 2.6, respectively.

Green-coated surfaces: Preparation, characterization and evaluation of biofilm formation on WSMoL-coated surface

After verify the antibiofilm activity of WSMoL free in solution, we assessed its ability to prevent biofilm formation when immobilized on a hydrophilic surface, according to the procedure described by Trentin *et al.* (2015) with modifications. To obtain this *Green*-coating, a sterile glass slide was dip coated with WSMoL (diluted in water) during 12 h, followed by heat treatment (50°C, 60 min) to promote the annealing of protein film to the surface. The

lectin-coated surface was produced in order to achieve 116 µg/cm^2 of WSMoL while untreated control corresponded to immersion of the glass slide in sterile water. The surfaces were characterized through measurements of glycerol contact angle, which were carried out using the sessile drop technique. The drop images were captured and analyzed by a Theta Lite Optical Tensiometer (Attension). The reported glycerol contact angles are means of at least five measurements performed on different areas of each sample. Biofilms of *S. marcescens* and *Bacillus* sp. were challenged to grown on uncoated and WSMoL-coated (116 µg/cm^2) cover glass-bottom dishes (Glass Bottom Dish 35, 0/10mm, 4 compartments, TC) under the conditions described in section 2.4 and were analyzed by CLSM after staining with the LIVE/DEAD BacLight Bacterial Viability Kit as described in section 2.7.

Statistical analysis

Experiments were carried out in three independent experiments in triplicate. The data were expressed as the mean or the percent mean ± standard deviation (SD) and statistical differences were determined using Tukey test; a p value <0.05 was considered statistically significant.

Results

WSMoL suppressed *S. marcescens* biofilm formation when used at low concentrations (0.325, 0.65 and 1.3 µg/mL) without interfering with bacterial growth (Figure 1A), acting as an antibiofilm compound. On the other hand, it demonstrated antibiotic effect against *S. marcescens* cells when used at concentrations from 2.6 µg/mL and thus no biofilm was formed. Gentamicin (antibiotic control) strongly impaired the *S. marcescens* growth and avoided biofilm formation similarly to WSMoL at 10.4 and 20.8 µg/mL.

SEM images of untreated control showed that the *S. marcescens* biofilm completely covered the surface (Figure 2A) while images of cells treated with lectin (5.2, 10.4 and 20.8 µg/mL) revealed the presence of few clusters of bacteria composed by malformed and elongated cells (Figure 2B-D). Corroborating with these results, images obtained with confocal microscopy demonstrated that the biofilm in untreated control exhibited a bright green fluorescence indicative of intact viable cells (Figure 2E), while biofilms formed in the presence

of WSMoL showed a gradual change of green fluorescence to red signal (cells with damaged membrane), according to the increase of lectin concentration (Figure 2F-H).

For *Bacillus* sp., the lectin acted as an antibiotic drug in all concentrations tested (Figure 1B). In treatments with the lectin at 20.8 and 41.6 µg/mL, it was observed a remarkable suppression of bacterial growth, similarly to the antibiotic control rifampicin, resulting in absence of biofilm formation due to low number of viable cells.

SEM images of untreated control showed a biofilm formed by large cells clusters (Figure 3A) while cells treated with WSMoL (10.4, 20.8 and 41.6 µg/mL) formed few flat aggregates or even isolated cells adhere, some of them with altered morphology including multiseptation (Figure 3B-D). Although the crystal violet assay revealed absence of biofilm (bacteria and matrix) in treatments using WSMoL at 41.6 µg/mL, it was noted the presence of a material adhered in the permanox surface in the SEM images (Figure 3D). We confirmed that this material corresponds to the lectin since WSMoL was able to self-adhere on permanox surface (Figure 3E). The analysis by confocal microscopy evidenced a higher number of cells stained with red in presence of WSMoL (Figure 3G-I) than in the control (Figure 3F), indicating that the lectin damaged the integrity of *Bacillus* sp. membrane, which is in accordance with the changes in bacterial structure observed by SEM.

In the biofilm eradication assay, biofilms of *S. marcescens* were not disrupted after treatment with lectin at concentrations ranging from 1.3 to 104 µg/mL, in comparison with untreated control (Figure 4A). In addition, it was observed an increase in crystal violet absorbance in treatments using concentrations higher than 13 µg/mL. SEM images of untreated control showed a flat biofilm covering the surface of permanox (Figure 4B), while images of biofilm treated with the highest concentration of WSMoL (104 µg/mL) displayed hollow structures wrapped by a material that probably consists of lectin (Figure 4C). The increase in crystal violet absorbance may be due to the adherence of lectin to the biofilm.

Oppositely, the results observed in eradication assay with *Bacillus* sp., showed that WSMoL (at 52, 104 and 208 µg/mL) reduced the biofilm layer present on polystyrene plates in a significant manner when compared with untreated biofilm (Figure 5A). In SEM analysis, the untreated biofilm (Figure 5B) presented a large number of cell clusters and presence of exopolymeric matrix. In contrast, a drastic reduction in the number of adherent cells can be visualized when *Bacillus* sp. biofilms were exposed to 208 µg/mL of WSMoL (Figure 5C); in addition, it can be seen the lectin covering the remaining attached cells.

The hydrophobicity of the WSMoL-coated surface (116 µg/cm^2) and the uncoated surface was analyzed by measures of contact angle. Figure 6A shows that the surface containg 116 µg/cm^2 of lectin presented higher glycerol contact angle than that obtained for the uncoated surface, indicating the more hydrophilic character of lectin surface. As observed in Figure 6B, WSMoL-coated surfaces allowed *S. marcescens* biofilm formation, however cells present no viability. In contrast, WSMoL-coated surface strongly prevent *Bacillus* sp. bacterial attachment and biofilm formation, without interfering in cell viability (Figure 6C).

Discussion

The lectin WSMoL is an anionic protein with a molecular mass of 60 kDa composed by oligomeric arrangements of 5 kDa subunits (Moura *et al*. 2016). WSMoL possess 70% homology with the coagulant proteins from *M. oleifera* seeds M02.1 and M02.2 and is active in a broad pH range (4.5–9.5) and active after heating at 100ºC (Coelho *et al*. 2009; Rolim *et al*. 2011). This lectin showed the ability to impair planktonic growth and survival of corrosive and pathogenic bacteria, including *S. marcescens* and *Bacillus* sp. (Moura *et al*. 2015). This activity, together with reports about antibiofilm activity of other lectins, stimulated us to investigate the action of WSMoL on formation and eradication of *S. marcescens* and *Bacillus* sp. biofilms.

The processes of biofilm formation and stabilization involve cell-cell and cell-surface interactions, which are mediated by macromolecules such as proteins and polysaccharides present on the bacterial cell surface (Vu *et al*. 2009; Abdel-Aziz and Aeron 2014). Bacteriostatic and bactericidal effects of lectins occur due to the ability of these proteins in interacting specifically with glycoconjugates and polysaccharides present in cell wall (Paiva *et al*. 2010; Klafke *et al*. 2013). Damages caused by bioactive compounds on cell wall and membrane account for the impairment of biofilm development (Quave *et al*. 2008; Klafke *et al*. 2013; Trentin *et al*. 2013; Hasan *et al*. 2014). In addition, the interactions between bacterial cells and lectins may also be linked to the inhibitory effect on biofilm formation by preventing the interactions between bacterial polysaccharides and surfaces as well as between other bacterial cells. Other mechanisms cited in studies that report antibiofilm activity of lectins involve their binding to constituents of the biofilm exopolymeric matrix, interrupting its polymerization and as anti-*quorum sensing* molecules, preventing the expression of genes required for biofilm

development and for other virulence factors (Cavalcante *et al*. 2013; Klafke *et al*. 2013; Hasan *et al*. 2014).

According to the results described herein, it can be proposed that WSMoL act as an antibiotic compound, decreasing *S. marcescens* and *Bacillus* sp. viability and limiting the number of cells capable of forming biofilms. For *Bacillus* sp., this property was observed in all concentrations evaluated. Vasconcelos *et al*. (2014) described that the lectin from *Vatairea macrocarpa* seeds inhibits growth and reduces cell viability and biofilm formation of *Staphylococcus aureus* and *Staphylococcus epidermidis*. The antibiofilm properties of some plant extracts and essential oils are also due to reduction of viable cells (Gursoy *et al*. 2009; Trentin *et al*. 2013).

The potential of WSMoL in eradicating preformed biofilms was also investigated in this work. The result obtained indicates that WSMoL is not able to remove preformed biofilms of *S. marcescens* but it penetrated inside them and interacted with the cells reducing their viability. On the other hand, for *Bacillus* sp. biofilms, the lectin was able to destroy the biofilm which was covering the polystyrene plates. The different effects observed in the eradication biofilm assays with *S. marcescens* (gram-negative) and *Bacillus* sp. (gram-positive) may be due to differences in the carbohydrate patterns at the cell surface of these bacteria.

The eradication of established biofilms normally involves the destruction of the structure by disintegration of the exopolymeric matrix (Boles and Horswill 2011). Studies have demonstrated that several compounds from plants can act in this process, including in synergism with antibiotics (Nostro *et al*. 2009; Artini *et al*. 2012; Kavanaugh and Ribbeck 2012; Quave *et al*. 2012). As an example, the treatment of preformed *S. mutans* biofilms with the lectin from *Trigonella foenumgraecum* seeds at 200 mg/mL altered the morphology of the cells, causing invaginations, and destroyed the matrix (Islam *et al*. 2009). According to these authors, the eradication property of this lectin occurs due to the interference with the arrangement and structural integrity of the biofilm after binding to the bacterial cell surface molecules.

The emergence of multiresistant bacteria is associated with the use of high concentrations of disinfectants and antibiotics normally used to treat biofilm; thus, the most important treatment strategy consists in the preventing biofilm development (Glinel *et al*. 2012). The use of natural compounds that can impair cell adhesion is a promising tool for reduction of bacterial colonization on several surfaces due their biocompatibility and nontoxicity (Glinel *et al*. 2012). In fact, several studies have proposing the use of natural agents to modify surfaces of medical devices as well as different surfaces in industrial sectors, in order

to prevent the formation of bacterial biofilms (Oguzie *et al*. 2013; Fletcher *et al*. 2014; Trentin *et al*. 2015). In this sense, we evaluated the potential of WSMoL to prevent biofilm formation through the production of *Green*-coated surfaces. Glass surfaces, which possess hydrophilic property similarly as stainless steel used in medical devices and in various industrial materials, were coated with WSMoL.

The lectin spontaneously adhered on glass forming a *Green*-coating surface that interfered on *S. marcescens* cells viability and blocked *Bacillus* sp. adherence, preventing the biofilm development. These results indicated that WSMoL is able to control biofilm of these bacterial species, acting on viability or suppressing adhesion, when immobilized on glass surface. In this context, proanthocyanidins isolated from *Pityrocarpa moniliformis* leaves demonstrated capacity to spontaneously adhering to hydrophobic and hydrophilic surfaces when used in the concentration range from 0.125 to 4.0 mg/mL, preventing the adhesion of *S. epidermidis* cells (Trentin *et al*. 2015). Additionally, surfaces coated with sub-inhibitory concentrations of natural products, such as capsaicin and zosteric acid, isolated from pepper and *Zostera marina*, respectively, were shown to prevent the attachment and further biofilm formation of *P. putida* (Xu *et al*. 2005).

In conclusion, this study shows that WSMoL was able to control biofilm formation by *S. marcescens* at sub-inhibitory concentrations and acted as an antibiotic compound avoiding biofilm formation by *Bacillus* sp. The lectin was also able to disrupt preformed biofilms of *Bacillus* sp. and to penetrate *S. marcescens* biofilms, killing the cells inside them. Antibiofilm action was detected for the lectin immobilized on a hydrophilic surface, indicating its potential application to cover surfaces in medical and industrial devices.

Acknowledgments

The authors express their gratitude to the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for research grants (472546/2012-0; 443150/2014-1) and fellowships (PMGP, AJM and LCBBC). They are also grateful to the *Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco* (FACEPE; APQ-0318-2.08/13; APQ-0108-2.08/14), *Fundação de Amparo à Ciência e Tecnologia do Estado do Rio Grande do Sul* (FAPERGS 1871-25511/13-4), and the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES; AUXPE 1454/2013) for financial support and fellowship (DST PNPD/CAPES). MCM would like to thank FACEPE for graduate scholarship (IBPG-0185-

2.08/12) and financial support for mobility (AMD-0010-2.00/15). We are grateful to technical assistance from the CMM/UFRGS in the microscopies.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure captions

Figure 1. Effect of WSMoL on biofilm formation and bacterial growth by *Serratia marcescens* and *Bacillus* sp. Dose response effects on biofilm formation and bacterial growth of *S. marcescens* (A) and *Bacillus* sp. (B) were evaluated by crystal violet assay and OD_{600}, respectively. The values obtained for untreated control corresponded to 100% biofilm formation or bacterial growth. Gentamicin and rifampicin were used as antibiotic controls. Different letters indicate significant differences ($p<0.05$) between treatments. Lowercase letters were used for comparison of biofilm formation and upper case letters for comparison of growth.

Figure 2. Effect of WSMoL on bacterial growth and biofilm formation by *Serratia marcescens*. SEM and confocal scanning electron microscopy images of biofilms formed in the presence of water (untreated control) (A; E) and WSMoL at 5.2 (B; F), 10.4 (C; G) and 20.8 (D; H). The bars indicate 50 µm in the main figure and 1 µm in the inserts. Viable cells are stained with SYTO 9 (green) and damaged cells are marked with propidium iodide (red).

Figure 3. Effect of WSMoL on bacterial growth and biofilm formation by *Bacillus* sp. SEM and confocal scanning electron microscopy images of biofilms formed in the presence of water (untreated control) (A; F) and WSMoL at 10.4 (B; G), 20.8 (C; H) and 41.6 (D; I). The last image (E) corresponds to the permanox surface exposed to WSMoL (41.6 µg/mL) and medium, without bacteria. The bars indicate 50 µm in the main figure and 1 µm in the inserts. Viable cells are stained with SYTO 9 (green) and damaged cells are marked with propidium iodide (red).

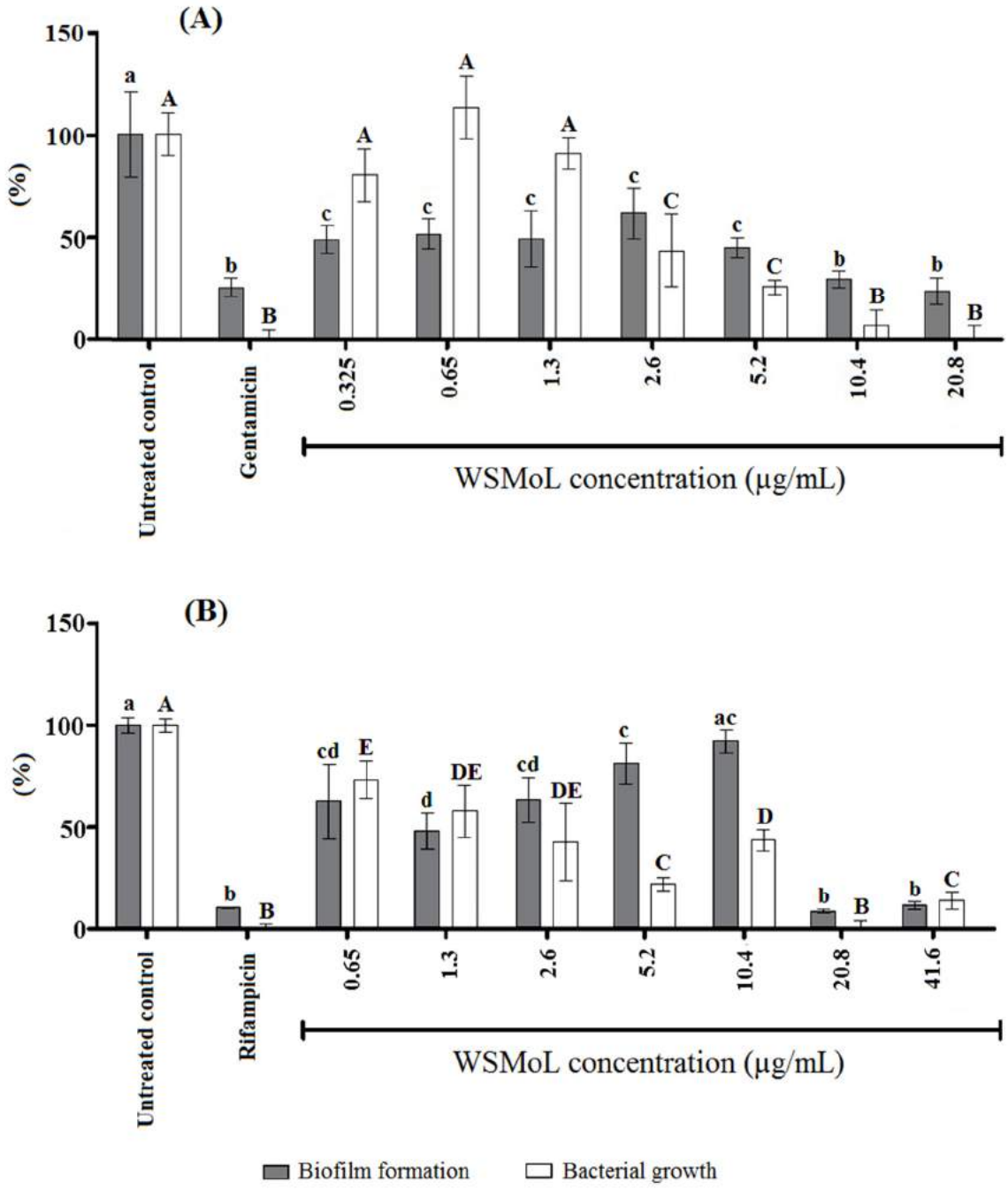
Figure 4. Effect of WSMoL on preformed biofilm of *Serratia marcescens*. (A) Dose response effect showing the percentage of remaining biofilm biomass after WSMoL treatments. The values obtained for untreated biofilm (untreated control) corresponded to 100% of biofilm biomass. Gentamicin was used as antibiotic control. SEM images of untreated biofilm (B) and

biofilms treated with WSMoL at 104 µg/mL (C). Different letters indicate significant differences ($p<0.05$) between treatments.

Figure 5. Effect of WSMoL on preformed biofilm of *Bacillus* sp. (A) Dose response effect showing the percentage of remaining biofilm biomass after WSMoL treatments. The values obtained for untreated biofilm (untreated control) corresponded to 100% of biofilm biomass. Rifampicin was used as antibiotic control. SEM images of untreated biofilm (B) and biofilms treated with WSMoL at 208 µg/mL (C). Different letters indicate significant differences ($p<0.05$) between treatments.

Figure 6. WSMoL-coated surface. (A) Glycerol contact angles for WSMoL-coated glass surfaces (116 µg/cm^2) and an uncoated surface. (*) indicate significant difference ($p < 0.05$) in comparison to the uncoated samples. Confocal microscopy images showing biofilm formation by *S. marcescens* (B) and *Bacillus* sp. (C) on control surfaces (uncoated) and WSMoL-coated surfaces (116 µg/cm^2). Viable cells are stained with SYTO 9 (green) and damaged cells are marked with propidium iodide (red).

Figure 1


(A)
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a
A
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b
B
b
B
Untreated control
Gentamicin
0.325
0.65
1.3
2.6
5.2
10.4
20.8
WSMoL concentration (µg/mL)
(B)
150
100
50
0
(%)
a
A
b
B
cd
E
d
DE
cd
DE
c
C
ac
D
b
B
b
C
Untreated control
Rifampicin
0.65
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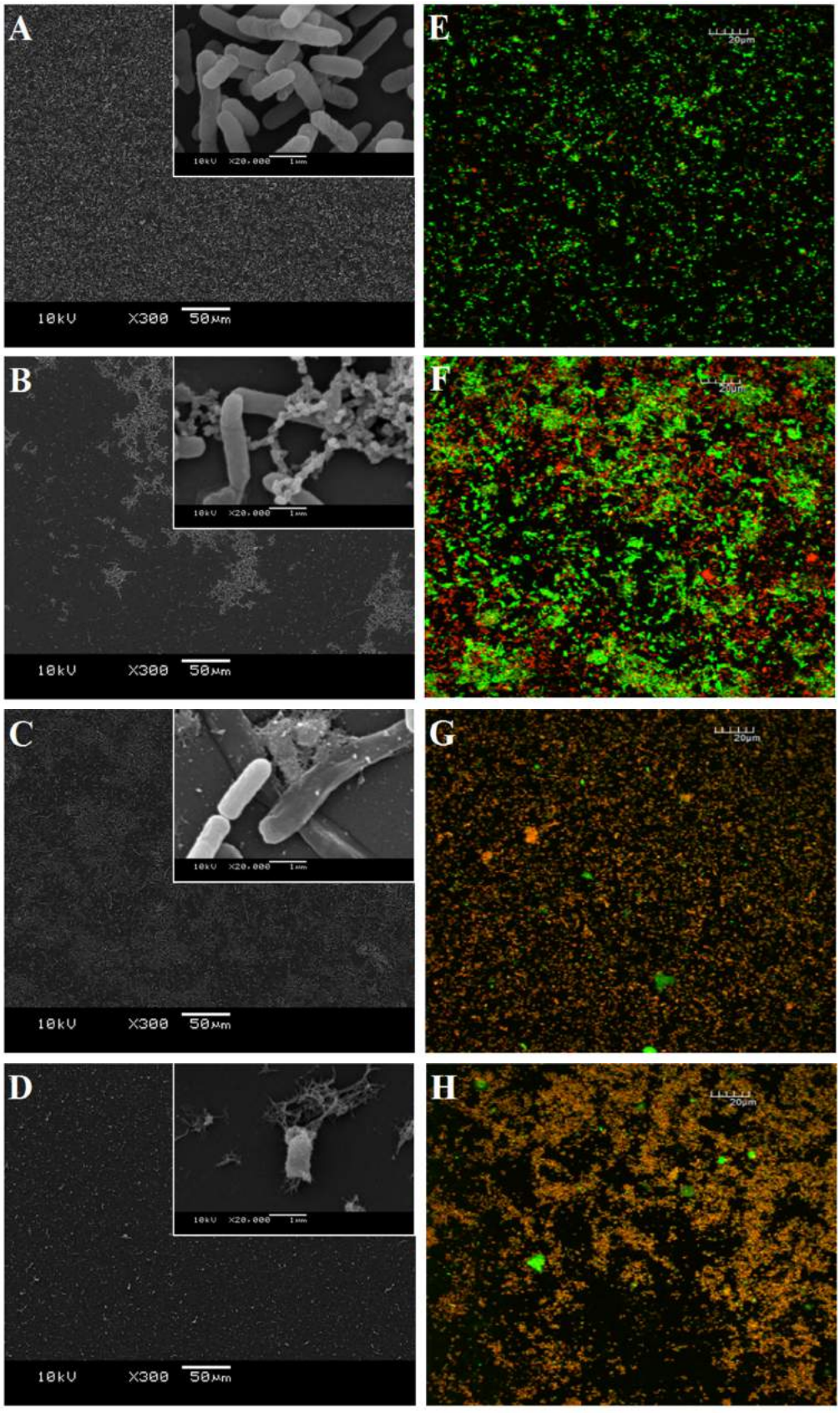
5.2
10.4
20.8
41.6
WSMoL concentration (µg/mL)
Biofilm formation
Bacterial growth

Figure 2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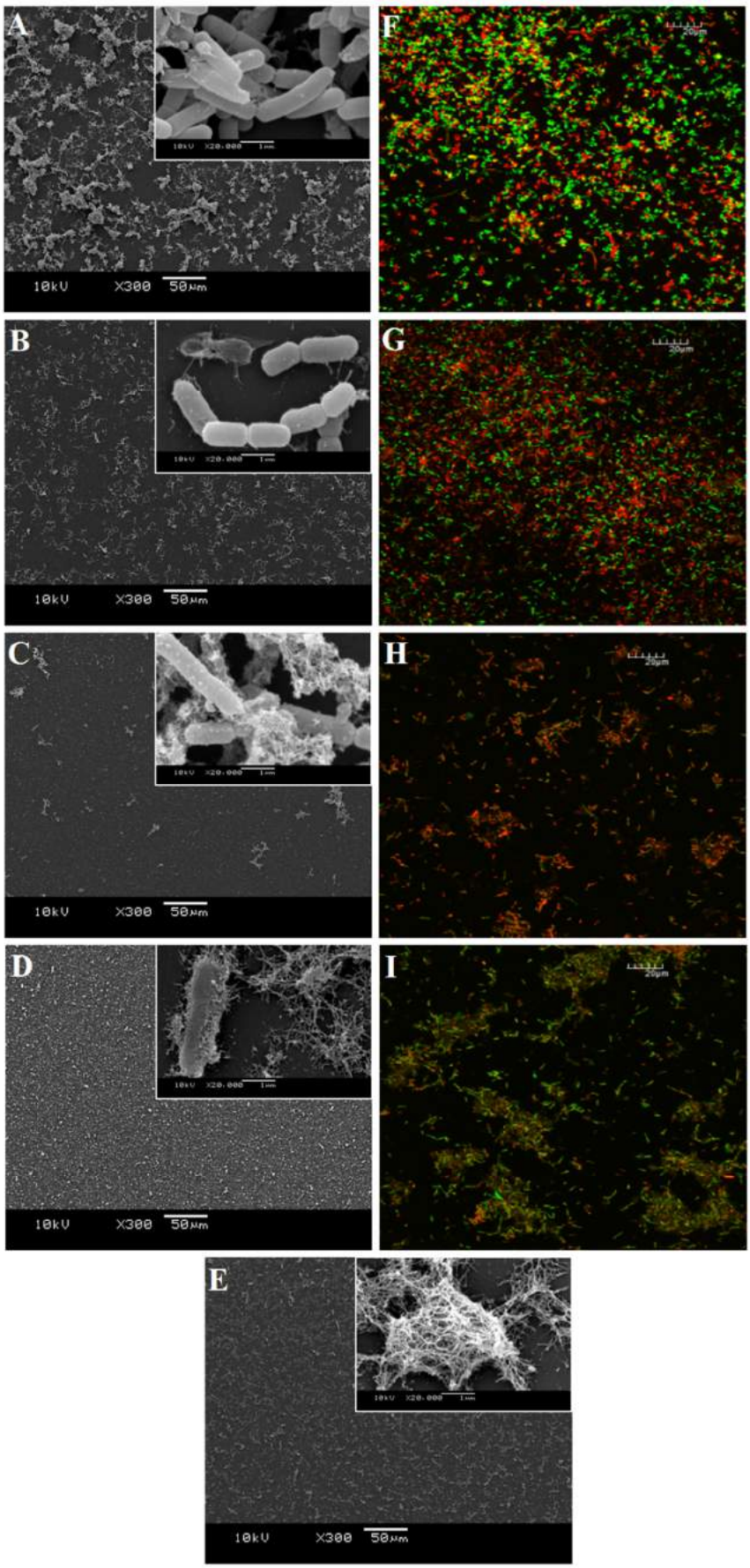
D
10kV X300 50µm
H

Figure 3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10kV X300 50µm

Figure 4

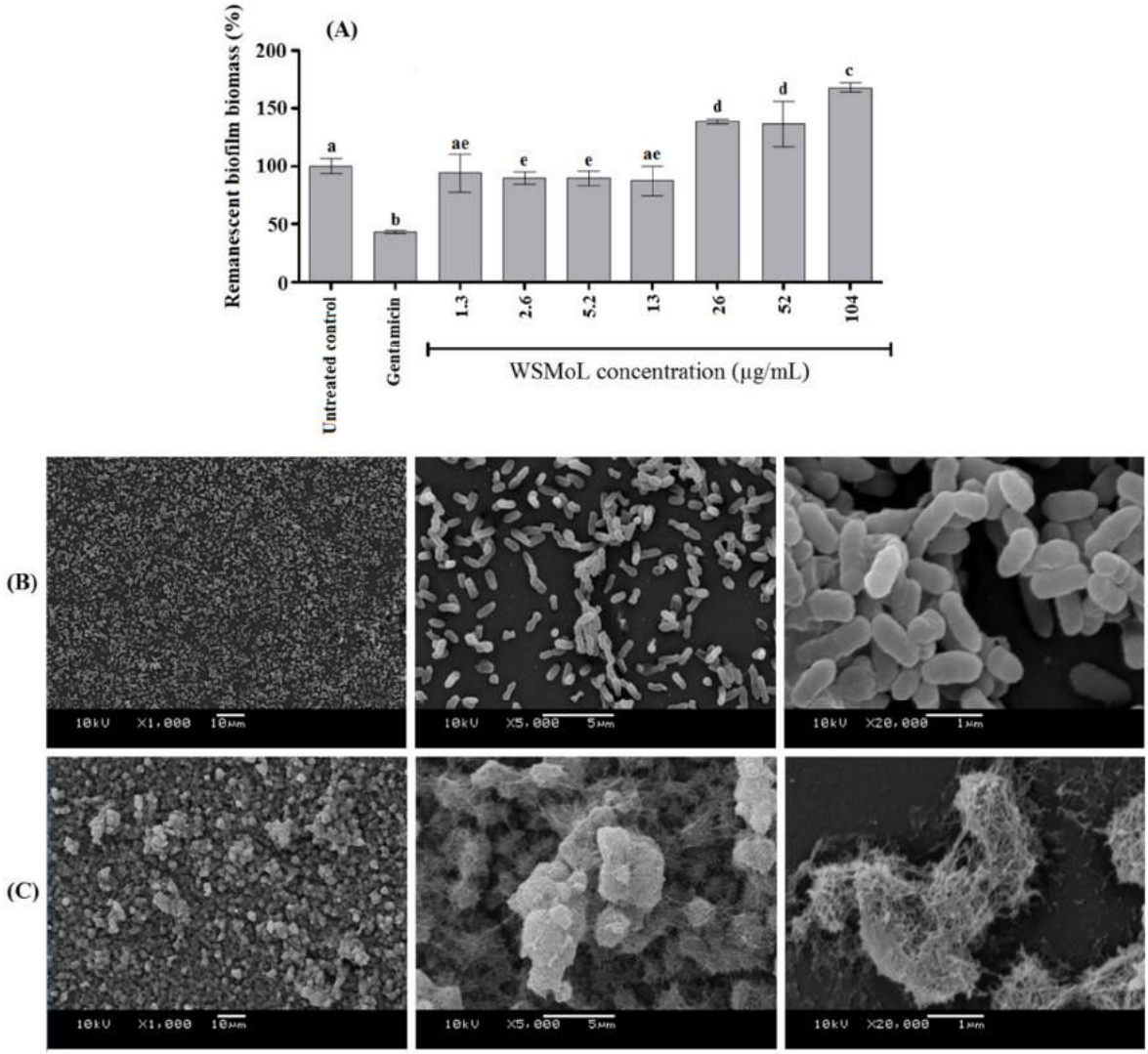

(A)
Remanescent biofilm biomass (%)
200
150
100
50
0
a
b
ae
e
e
ae
d
d
c
Untreated control
Gentamicin
1.3
2.6
5.2
13
26
52
104
WSMoL concentration (µg/mL)
(B)
(C)
10kV X1,000 10µm
10kV X5,000 5µm
10kV X20,000 1µm

Figure 5

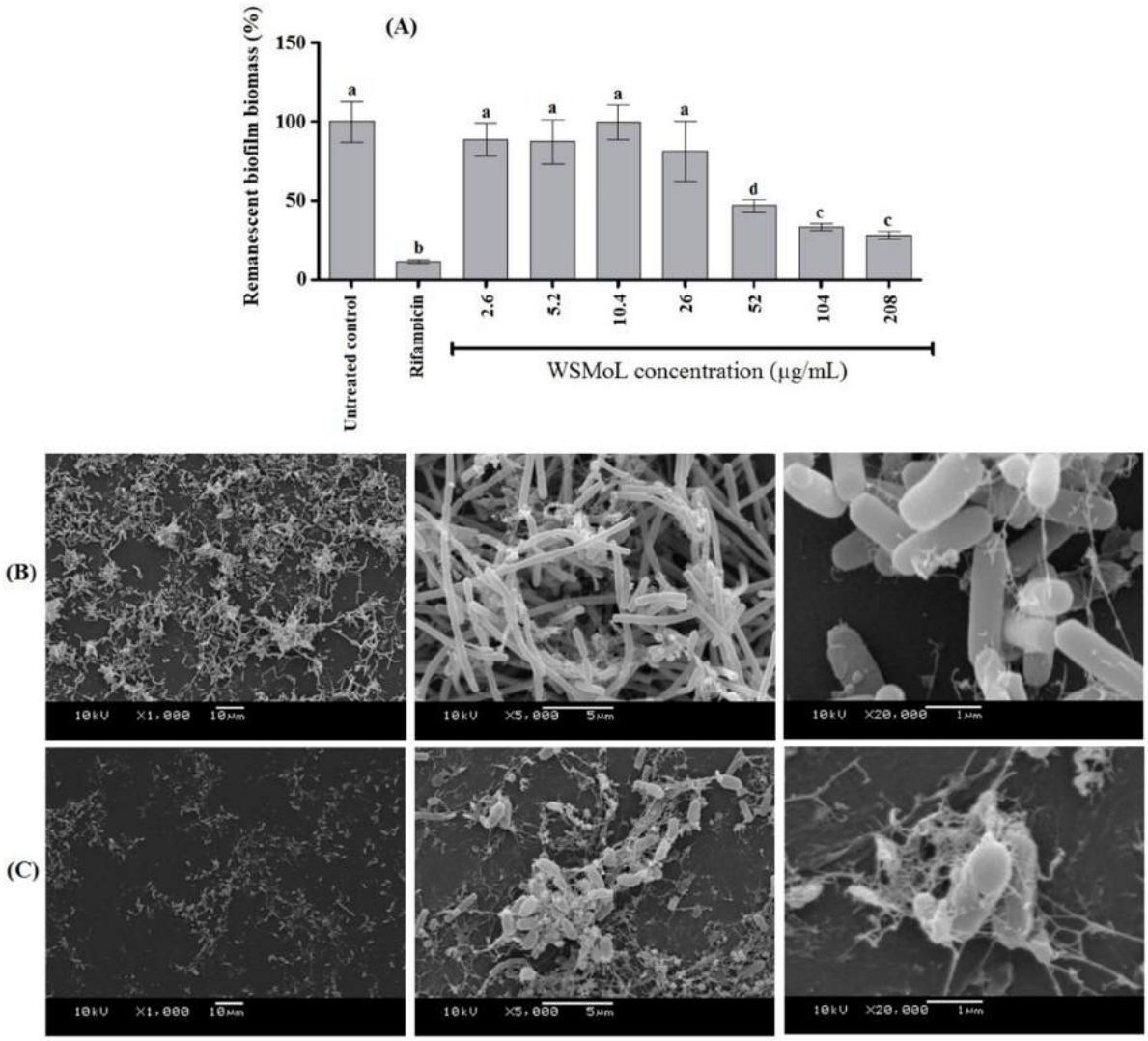
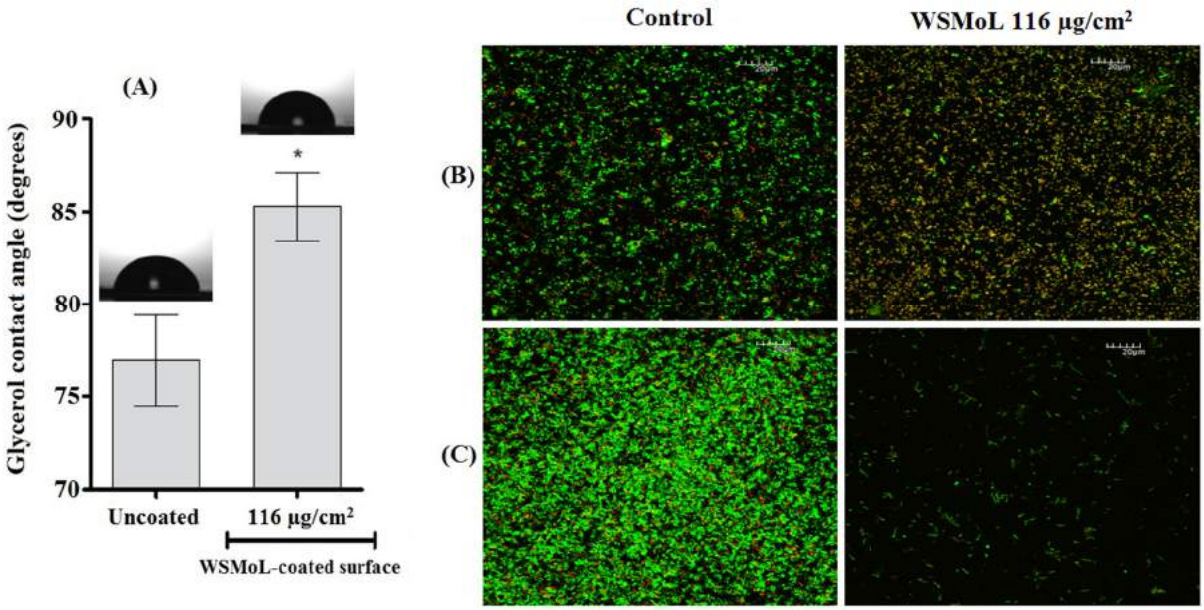

(A)
Remanescent biofilm biomass (%)
150
100
50
0
a
b
a
a
a
a
d
c
c
Untreated control
Rifampicin
2.6
5.2
10.4
26
52
104
208
WSMoL concentration (µg/mL)
(B)
(C)
10kV X1,000 10µm
10kV X5,000 5µm
10kV X20,000 1µm

Figure 6


(A)
Glycerol contact angle (degrees)
90
85
80
75
70
*
Uncoated
116 µg/cm²
WSMoL-coated surface
Control
WSMoL 116 µg/cm²
(B)
(C)

7 ARTIGO 4

Bacterial Biofilms: The Structure, Development and Potential of Plant Compounds for Alternative Control

Artigo de Revisão aceito para publicação como capítulo do livro: **Advances in Bactericides Research**

(Nova Science Publishers, Inc. NOVA, 2017).

Chapter

BACTERIAL BIOFILMS: THE STRUCTURE, DEVELOPMENT AND POTENTIAL OF PLANT COMPOUNDS FOR ALTERNATIVE CONTROL

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ABSTRACT

Bacterial biofilms may develop in natural and artificial habitats, including clinical and industrial environments, resulting in risks to human health and causing economic losses. Biofilms are typically more resistant to antibiotics and conventional methods of disinfection, in comparison with planktonic cells. Research has been focused on the identification of alternative antibiofilm agents for treating biofilm-associated infections and minimizing damages caused by biofilms in food and fuel industries. In this way, plant-derived molecules have been evaluated for their potential application for reducing bacterial colonization and biofilm development in different surfaces. This review deals with the structural and developmental aspects of bacterial biofilms; the problems associated with biofilm formation and current control strategies utilized in clinical and industrial sectors; and the advances regarding the use of compounds derived from plants to control the formation of biofilms at the different stages of development. The mechanisms of action of these compounds are also discussed.

1. INTRODUCTION

Bacterial biofilms are structured communities of cells enclosed in a self-produced polysaccharide matrix (Abdel-Aziz and Aeron, 2014). These communities develop structures that provide an optimal environment for the exchange of genetic material between cells and protection against several environmental conditions (such as UV exposure, dehydration, salinity) as well as against the host immunological system (Donlan, 2002). Given that almost all microorganisms can produce biofilms, bacterial biofilms are predominant and constitute a highly relevant problem in the medical and industrial sectors (Costerton et al., 1995; Donlan and Costerton, 2002; McLandsborough et al., 2006).

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Biofilms may form on biotic (living) and abiotic (inert) surfaces and the conversion from planktonic form to biofilm comprise a complex regulatory network, which involves the participation of QS molecules that are largely known as extracellular signals (Parsek and Greenberg, 2005; Abdel-Aziz and Aeron, 2014). The formation of bacterial biofilms starts with the bacterial adhesion, which is followed by bacterial growth and biofilm expansion (Kokare et al., 2009). Mostly, the adhesion of cells will occur on surfaces that are rougher, more hydrophobic and coated by a conditioning surface (Donlan, 2002; Kokare et al., 2009).

In comparison with their planktonic counterparts, the microorganisms in biofilms are usually less sensitive to antibiotics and biocides (Van Houdt and Michiels, 2010; Abdel-Aziz and Aeron, 2014). This fact complicates the management of chronic infections caused by biofilms in humans – mainly device-associated infections – which limits therapeutic options (Di Martino et al., 2002). In industrial sectors, such as food and petroleum industries, the presence of biofilms causes food spoilage and biocorrosion, resulting in several food-borne infections and leakage/degradation of stored hydrocarbon fuels, respectively (Guiamet and Saravia, 2005; Rajasekar et al., 2007a,b; Brooks and Flint, 2008; Kokare et al., 2009).

The use of natural compounds has shown efficacy in vitro as alternative strategies to disrupt biofilm formation or destroy preformed biofilms. For example, plant-derived essential oils, lectins, polypeptides, tannins, and polyphenols have been described as antibacterial and antibiofilm agents (Quave et al., 2012; Husain et al., 2015; Moura et al., 2015; Tan and Lim, 2015). The antibiofilm activity involves the ability of the molecule to impair the biofilm formation process through antibacterial effect (bacteriostatic and bactericidal action), preventing cell adhesion on surface and inhibiting QS signals (Martin et al., 2008; Trentin et al., 2013a).

This review deals with the main mechanisms of formation and development of bacterial biofilms, their association with damages in medical and industrial sectors, and the current start-of-art on the use of plant compounds as strategies to combat biofilms.

2. MICROBIAL BIOFILMS: DEFINITION AND STRUCTURE

Microbial biofilm comprehend an efficient community of cooperating cells that present various interactions (Macedo et al., 2005; Kokare et al., 2009). This aggregate of cells may be composed of a single species but, in natural conditions, most biofilms are composed of several species, which contributes to the interspecies interactions and result in high complexity of the macromolecular mixture present (Sutherland, 2001; Jefferson, 2004). Several microbial species are capable of forming biofilms but bacterial biofilms predominate in practically the entire ecosystem (Costerton et al., 1995). According to Abdel-Aziz and Aeron (2014), almost all bacteria exist in biofilms in contrast with the planktonic state.

A mature biofilm has a highly differentiated structure, being called as mushroom- and pillar-like structure, constituted by a hydrated polymeric matrix (Watnick and Kolter, 2000; Parsek and Greenberg, 2005; Abdel-Aziz and Aeron, 2014). This matrix is composed mainly by extracellular polymeric substances (EPS) and is produced by biofilm microbial cells themselves (Flemming and Wingender, 2010). Within this matrix there are channels for the circulation of nutrient and water (Flemming and Wingender, 2010). These channels also provide interspecies bacterial exchange or sharing of different metabolic substrates in biofilm (Kokare et al., 2009). Normally, the EPS comprises a large part by mass of biofilm when compared microbial cells (Flemming and Wingender, 2010). The matrix also confers

to the biofilm protection against environmental stress factors such as radiation, water stress, or grazing (Macedo et al., 2005; Flemming and Wingender, 2010; Hoiby et al., 2011).

Water is the major component of the EPS, which is also made by a conglomeration of different biopolymers (polysaccharides, lipids, proteins and extracellular DNA). These components enhances the stability of biofilms and keep interconnected cells (Sutherland, 2001; Flemming and Wingender, 2010). According to Flemming and Wingender (2010), bacterial polysaccharides are the most abundant components among the EPS biopolymers and mediate most of the cell-cell and cell-surface interactions required for biofilm formation and stabilization. Noncellular materials such as mineral crystals, corrosion particles, clay or silt particles as well as blood components (in case of bacterial biofilms present within the human body) may also be found in the biofilm matrix (Donlan, 2002). In addition, some evidences suggest that the EPS may also be involved in tolerance of biofilms to antimicrobial agents, like disinfectant and antibiotics, and is responsible to sequester metal ions, cations and toxins (Jefferson, 2004; Kokare et al., 2009).

In a biofilm, the microenvironment results from a heterogeneous community and can be highly variable, since the individual cells express different genes and follow different developmental pathways (Jefferson, 2004; Abdel-Aziz and Aeron, 2014). A flexible genetic adaptation – mutations and recombination of genes, acquisition of new genetic material, and regulation of gene expression – allows the microbial survival in the biofilm form in environments where conditions can change rapidly (Jefferson, 2004). Thus, if a biofilm is removed or their environmental conditions radically change, all cells can quickly adapt to the new habitat or convert to the planktonic mode of growth if necessary (Jefferson, 2004; Ito et al., 2009).

Genetic studies have demonstrated that there is heterogeneity in the gene expression profiles of the individual cells even within a single-species biofilm, resulting in specialized functions of the cells (Jefferson, 2004; Yarwood et al., 2004; Ito et al., 2009). Inside this community, some bacterial species may evolve on the transcription of genes required for EPS synthesis in response to certain environmental stimuli; for example, the iron deprivation and osmotic stress induce the expression of genes encoding proteins that synthetize EPS in Staphylococci and Enterococci (Baldassarri et al., 2001; Jefferson, 2004) and elevated glucose levels in the bloodstream induce the EPS formation as a mechanism of glucose storage for *Pseudomonas* genus and *Vibrio cholerae* and *Escherichia coli* species (Jefferson, 2004; O´Toole et al., 2000).

3. BIOFILM DEVELOPMENT

The biofilm formation is a dynamic process that involves continuous changes in structure and composition of the communities (Jefferson, 2004). It occurs through the attachment and detachment of cells that interact among themselves, through intercellular signaling involving QS molecules (or autoinducer signals, AIs), which correspond to small molecules that are secreted by biofilm cells (O´Toole et al., 2000; Parsek and Greenberg, 2005). These QS factors are constitutively released by bacteria from the start of biofilm formation and, when present at a critical concentration, induce the expression of particular genes that regulate motility, virulence, and production of EPS, among other functions (Lynch et al., 2002; Jefferson, 2004; Rutherford and Bassler, 2012).

There are evidences that a sensing system triggers QS signaling immediately after the interaction of microbial cells to a solid surface, leading to an early gene expression pattern necessary to biofilm formation (Kuchma and O'Toole, 2000; Jefferson, 2004; O´Toole et al., 2000). For example, when

Staphylococcus epidermidis cells make contact with a solid surface, the spherical cells form a leg-like appendage that facilitates the process of attachment (Kodjikian et al., 2003). According to Jefferson (2004), pathogenic bacteria produce an impressive array of surface adhesins (referred as MSCRAMMs: microbial surface component recognizing adhesive matrix molecules) in order to adhere and form biofilms in the human body. These MSCRAMMs are able to bind to host extracellular matrix proteins such as fibronectin, fibrinogen, vitronectin, and elastin, forming a conditioning film (Figure 1A). Once the development of the biofilm has passed this stage, these adhesins are no longer needed and their expression is inhibited, as well as it inhibits the production of fimbriae and flagella. In a biomaterial (internal medical device, for example), this conditioning film may be formed by proteins such as albumin, immunoglobulin, fibrinogen and fibronectin; when the surface in question is an oil transport pipe or gas, for example, inorganic or organic macromolecules present in the liquid medium may form this film (O'Toole et al., 2000; Rochford et al., 2012; Trentin et al., 2013a).

Several QS systems are known, being the most studied system the acyl-homoserine lactone (acyl-HSL) for many gram-negative bacteria and the peptide-based signaling systems of many gram-positive bacteria (Jefferson, 2004; Parsek and Greenberg, 2005). Studies have shown that the QS signaling is also activated when a bacterial population reaches a threshold density level, leading to alteration of gene expression by a cell density-dependent mechanism (Lynch et al., 2002).

Bacterial primary adhesion (Figure 1B) comprises the first step to form a mature biofilm and involves the reversible attachment of planktonic bacteria to a surface (Hoiby et al., 2011). It usually occurs in the solid-liquid interface of an abiotic (inert materials) or biotic (living tissue or cells) solid surface (Donlan, 2002). This primary adhesion may occur directly or through the formation of the conditioning film previously discussed. In this last case, the process is known as bioaccumulation or biofouling, which leads to alteration in the surface properties of the substratum and allows microorganisms to adhere to the surface (Abdel-Aziz and Aeron, 2014).

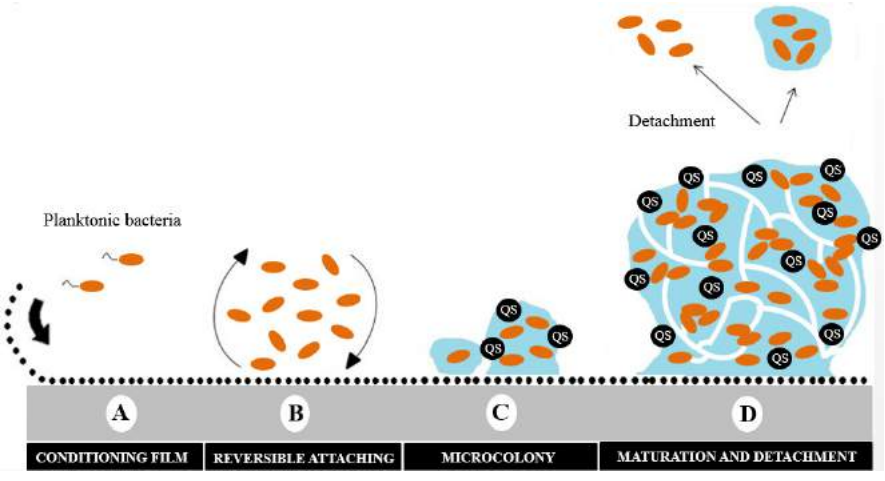

Figure 1. Schematic representation of biofilm development on a solid surface. Conditioning film formation by organic biopolymers from the medium (A). Reversible adherence of planktonic bacteria in a conditioned surface, which represents a nutrient-rich environment that stimulates cell congregation (B). Irreversible attachment of cells, exopolysaccharides production and microcolony formation (C). Mature biofilm with an increase of overall density and complexity, with water channels in evidence (D); at this time, there can be detachment of planktonic cells and biofilm aggregates, which are able to colonize other areas. Quorum sensing (QS) molecules are responsible for cell-to-cell communication into the biofilm.

The direct primary adhesion to abiotic surfaces is mediated by non-specific physicochemical interactions (hydrodynamic forces, electrostatic interactions, Van der Waals forces and hydrophobic

interactions) and the planktonic cells adhere to a surface randomly (Brownian motion and gravitational force) or in a directed way via chemotaxis, flagella motility and pili (Pavitra and Doble, 2008; Trentin et al., 2013a). The adherence to biotic surfaces is accompanied by molecular interactions mediated by lectins or adhesins (Dunne, 2002). It is important to note that the facultative anaerobic bacteria have the characteristic of being early colonizers, adhering to surfaces and producing the EPS, creating then a favorable environment for anaerobic microorganisms in the later stages (Marangoni et al., 2013).

The next step is the secondary adhesion (Figure 1C), which corresponds to an irreversible binding to the surface. In this stage, the microorganisms are beginning to produce the EPS, which can complex with materials present on the surface strengthening the links between the cells and/or aggregates and the surface, forming microcolonies (Stoodley et al., 2002; Hoiby et al., 2011). At this moment, there is no motility and the cells start to communicate through QS signals (Kuchma and O'Toole, 2000).

The biofilm maturation occurs in response to increasing population density and high EPS production, which increases the biofilm thickness and the stability of the colony; the population growth takes place through both cell division and adhesion of new planktonic cells (Stoodley et al., 2002). Once formed the mature biofilm (Figure 1D), cells or small portions of the biofilm may detach and disperse as a result of nutrient depletion, QS signaling or shearing of biofilm aggregates because of flow effects (Donlan, 2002; Hoiby et al., 2011); this process provides a mechanism of cell migration from heavily colonized areas to habitats more supportive to growth (Donlan, 2002; Hall-Stoodley and Stoodley, 2005; Trentin et al., 2013a). Also, the cells that have been shed may revert quickly to the planktonic stage (Donlan, 2002).

Some characteristics of the solid surface, such as roughness (surface area is higher), hydrophobic regions and presence of conditioning films, may increase the probability of bacterial adhesion (Donlan, 2002; Kokare et al., 2009). The velocity of the liquid as well as the pH, nutrient levels, ionic strength and temperature may interfere with the microbial attachment to a surface (Donlan, 2002; Kokare et al., 2009). Furthermore, cell characteristics such as the surface hydrophobicity and the presence of fimbriae and flagella influence the attachment of these cells to solid surfaces (Watnick and Kolter, 2000). According to Donlan (2002), motile cells attach in greater number and even against the liquid flow and cell surfaces that contain a high proportion of hydrophobic amino acid residues attach more easily.

4. IMPORTANCE OF THE BIOFILMS

4.1. Medical Importance: Biofilms and Pathogenesis

According Abdel-Aziz and Aeron (2014), approximately 65% of infections disease are related with bacterial biofilms and gram-positive and gram-negative bacteria have been associated with these biofilms. The bacterial adherence can occur on biotic (teeth, heart valves, lungs and middle ear) and abiotic (intravenous catheters, prostheses and stents) surfaces (Di Martino et al., 2002; Tacconelli et al., 2009; Palmer et al., 2010). The site of bacterial colonization usually triggers the site of infection development (Di Martino et al., 2002). Some pathologies associated to the detachment of biofilm parts or cells are summarized in Table 1.

Some characteristics of biofilms are important for the infectious process, such as: detachment of biofilm aggregates, which are transported through the bloodstream and may colonize new areas resulting in chronic infections, persistent inflammation and tissue damage; and resistance of biofilm bacteria against antibiotics and phagocytosis by the adaptive host immune mechanism (Donlan, 2002; Jefferson,

2004; Hoiby et al., 2011). According to Hoiby et al. (2011), the minimal inhibitory and minimal bactericidal concentrations of antibiotics for biofilm growing bacteria may be up to 100-1000 fold higher than that for planktonic bacteria.

Coating of medical devices surface with one or more antibiotics or entrapping of these agents within the device material is the most often approach used to obtain devices with lasting antimicrobial effects with broad spectra (Donelli and Francolini, 2001; Zilberman and Elsner, 2008). However, studies demonstrated that the extensive use of antibiotics can destroy the host microbiota, leading to prevalence of opportunistic pathogens, as well as can favor the multiplication of multiresistant microbial strains to these drugs (Saleem et al., 2013; Abdel-Aziz and Aeron, 2014).

Table 1. Examples of different sources of contamination by detached cells or aggregates from bacterial biofilms, new colonized areas and associated pathologies

Contamination sources	Bacteria species involved	Colonization areas/ Associated pathologies	References
Biofilms of oral cavity (gingival and dental plaques); potable water distribution systems.	*Helicobacter pylori*	Gastro-enteric diseases: gastritis, gastric lymphoma of mucosa- associated lymphoid tissue	Gebara et al. (2004) Santiago et al. (2015)
Soil, air and potable water distribution systems; some domestic and wild animals	Nontuberculous mycobacteria (NTM)	Lung disease, cervical lymphadenitis	Falkinham (2015)
Potable water distribution systems, plumbing fixtures and heating, ventilating and air-conditioning equipments	*Legionella pneumophila*	Respiratory diseases: Legionnaires' disease (pneumonia)	Parr et al. (2015)
Patient's own microbiota, hands of health-care personnel during catheter insertion or manipulation of the collection system	*Bacillus sp.; Escherichia coli; Enterococcus sp.; Klebsiella pneumoniae; Lactobacillus plantarum; Proteus sp.; Pseudomonas aeruginosa; Staphylococcus aureus; Serratia sp.; Staphylococcus epidermidis; Streptococcus sp.*	Several devices-associated infections	Boumis et al. (2010) Magill et al. (2014)
Devices-associated infections (Central venous catheter; prosthetic heart valves; contact lenses; intrauterine devices; urinary catheters)		Devices-related bloodstream infections	
Bloodstream, primarily via oropharynx, gastrointestinal and genitourinary tracts and cardiovascular system	*Enterococcus sp. Staphylococcus sp. Streptococcus sp.*	Endocarditis in native valves with endothelium damaged	Boumis et al. (2010) Freedman (2012)
Normal inhabitants of oral cavity	*Porphyromonas gingivalis; Fusobacterium nucleatum*	Periodontitis, oral squamous cell carcinoma disease	Gallimidi et al. (2015)

Resistance mechanisms aim to prevent the antibiotic binding to its target and may involve: target modification by mutation; target modification by specialized enzymatic changes; target substitution; antibiotic modification or destruction; antibiotic efflux; and restricted permeability to antibiotics (Levy and Marshall, 2004; Lewis, 2007). For example, biofilms of *Pseudomonas aeruginosa* demonstrated resistance to beta-lactam and colistin antibiotics due to the production of increased amounts of beta-lactamase and production of a modified lipopolysaccharide which makes the bacteria resistance to colistin, respectively (Johansen et al., 2008; Herrmann et al., 2010). In addition, the phagocytes that attempt an assault against a biofilm usually cause more damage to the surrounding tissues than to the biofilm (Jefferson, 2004). Thus, the development of a resilient biofilm usually results in a chronic infection and biofilm-related infections usually respond to an antibiotic therapy only after weeks or even months, being frequent and unfortunately needed the surgical removal of the infected tissue or the replacement of the medical device (Jefferson, 2004; Anderson and O´Toole, 2008).

After treatment, the antibiotic could kill most biofilm and planktonic cells but usually there are persister cells that remain alive (Lewis, 2007). These persister cells, which are contained in the biofilm, can survive both to the antibiotic treatment and the host immune system and then repopulate the biofilm when the concentration of antibiotic reduces, which will shed off new planktonic cells leading to the relapsing of biofilm infection (Jesaitis et al., 2003; Lewis, 2007). It is important to note that in a biofilm, due to the close proximity, resistance mechanisms can be transferred among the bacterial cells through horizontal exchanges of genetic material (Cvitkovitch et al., 2003; Lewis, 2007). Also, drug tolerance mechanisms that originate the persister cells are present in all bacteria (Lewis, 2007).

4.2. Biofilm Industrial Problems

4.2.1. Biofilms and Food Industry

Biofilms may contain spoilage and pathogenic bacteria that cause problems in many branches of the food industry, increasing post-processing contamination and risk to public health (Shi and Zhu, 2009; Van Houdt and Michiels, 2010). In addition, the presence of biofilms in the equipments and structures of food industries may result in corrosion, mechanical blockage in fluid handling systems, and reduction of heat transfer, resulting in economic losses (Chmlelewskl and Frank, 2003; Brooks and Flint, 2008).

Cleaning processes, chemical disinfectants and physical methods are normally used to remove and prevent biofilms in food-processing surfaces (Van Houdt and Michiels, 2010). The cleaning processes remove product residues through alkali-based and acid-based cleaning or application of mechanical force, such as by brushing and scrubbing (Parkar et al., 2004; Antoniou and Frank, 2005). However, although efficient, the cleaning process can result in the transfer of bacteria or biofilm pieces (Chmlelewskl and Frank, 2003; Van Houdt and Michiels, 2010).

The chemical disinfectants commonly used in food industry are halogens, acids, peroxygens and quaternary ammonium compounds, which can be used in combination with detergents and enzymes to enhance the disinfection efficiency (Kokare et al., 2009; Van Houdt and Michiels, 2010). Sanitation with short intervals is usually proposed as an effective approach to prevent or limit the deposition of organic material that induces bacterial attachment and consequent biofilm maturation (Lindsay et al., 2005; Van Houdt and Michiels, 2010). These short intervals are needed because several studies indicated that the antimicrobial efficacy of various sanitizers used in food industry is higher for planktonic cells than for biofilm-associated cells (Guiamet and Saravia, 2005; Moretro et al., 2012).

A study conducted by Joseph et al. (2001) with isolates of Salmonella sp. on three surfaces commonly found in food industries showed that biofilm cells had a greater resistance to hypochlorite

and iodophor when compared to their planktonic counterparts. Biofilms of Listeria monocytogenes were also more resistant to cleaning agents and disinfectants (trisodium phosphate, chlorine, ozone, hydrogen peroxide, peracetic acid and quaternary ammonium compounds) in comparison with planktonic cells (Van Houdt and Michiels, 2010). Chlorine concentrations as high as 1000 ppm are necessary for a substantial reduction of L. monocytogenes and *Staphylococcus xylosus* biofilms while only a 10 ppm concentration is effective against planktonic cells; this has been linked to a hindered penetration of the chlorine into the biofilm (Norwood and Gilmour, 2000; Van Houdt and Michiels, 2010). The slow or incomplete penetration of the biocide into the biofilms can be partly explained by a limited diffusion in the EPS matrix or by neutralization of the active compound in the outermost regions of the matrix (Van Houdt and Michiels, 2010).

In addition, the ability of microbial cells to sense the biocide presence and then activate responses against it is more effective in biofilm cells than planktonic cells. The exposure to a biocide may result in induction of EPS synthesis and proliferation of the biofilm matrix (Szomolay et al., 2005; Van Houdt and Michiels, 2010). Furthermore, although the disinfectants are not well-studied as the antibiotics, it is known that bacteria can employ the same major resistance strategies against these both groups of antibacterial chemicals (Chapman, 2003).

Physical treatments (ionizing radiation, ultrasounds and electric fields) are used as an alternative for the chemical disinfectants or to enhance the efficiency of biocides (Birkenhauer and Neethirajan, 2015). However, the applicability of physical methods requires high costs for implementation and also have stricter regulatory guidelines to be used (Van Houdt and Michiels, 2010; Birkenhauer and Neethirajan, 2015). Thermal disinfection (pasteurization and canning processes) is the most common physical method used in food industry and have the objective to denature microbial enzymes and kill some microorganisms; however, food products still contain viable spores (Birkenhauer and Neethirajan, 2015).

4.2.2. Biofilm and Fuel Industry

There are several reports on the tampering of stored hydrocarbon fuels due to microbial action and on the formation of bacterial biofilms in steel pipelines that are commonly used in the transport of petroleum derivatives in all the world; these problems lead to reduction of fuel quality and blocking of pipelines and filters, respectively (Guiamet and Saravia, 2005; Rajasekar et al., 2007a,b; Samimi, 2012). Bacterial biofilms are also responsible for microbiologically induced corrosion (MIC) in petroleum industry, damaging different industrial steel surfaces and affecting the operation and maintenance costs (Guiamet and Saravia, 2005; Rajasekar et al., 2008). It has been estimated that 40% of all pipeline corrosion in the oil and gas industries can be attributed to MIC (Rajasekar et al., 2011).

The entry of microorganisms in pipelines occurs through the injection of freshwater or seawater in onshore and offshore fields, respectively, during the oil production (Okoro et al., 2014). In pipelines, nutrients like seawater sulfate and organic oil mix (alkanes, aromatics and volatile fatty acids) and high temperatures yield ideal conditions for microbial growth (Okoro et al., 2014). Additionally, degraded petroleum hydrocarbons can be good nutrients for bacteria, increasing the microbial proliferation and biofilm formation on the steel (Rajasekar et al., 2011). A direct relationship between microbial growth, corrosion rate and degradation of naphtha was indicated in studies on biofilms composed by *Pseudomonas*, *Bacillus*, *Gallionella*, *Siderocapsa*, *Thiobacillus*, *Thiospira*, *Sulfolobus* and *Vibrio* isolated from naphtha pipes (Rajasekar et al., 2005). Strains of *Bacillus* sp. and *Serratia marcescens* are able to degrade hydrocarbons in diesel fuel due to secretion of the enzyme aryl hydrocarbon hydroxylase (Rajasekar et al., 2011).

The major bacteria usually involved with MIC in petroleum production systems are the anaerobic sulfate-reducing bacteria (SRB). However, recent studies demonstrated that the SRB are not present in great abundance in microbial communities responsible for MIC, which can be explained by the high flow velocity in pipelines that creates a uniform oxygen distribution leading to a suppression of the SRB growth (Jan-Roblero et al., 2004, 2008). In this way, aerobic bacteria are being evaluated for involvement in MIC of petroleum pipelines. Rajasekar et al. (2010) showed that a microbial community composed by *S. marcescens*, *Klebsiella oxytoca*, *P. aeruginosa*, *P. stutzeri*, *Bacillus* sp., *B. subtilis*, *B. cereus*, *B. pumilus* and *B. megaterium* was associated with corrosion of diesel and naphtha-transporting pipelines.

Similarly to that was described for food industries, the prevention and treatment of MIC in fuel industries are mainly based on avoiding or minimizing the development of biofilms. The control methods include application of protective coatings, corrosion inhibitors, and anodic or cathodic protectors; use of corrosion resistant metals; and the utilization of cleaning process by mechanical procedures and sanitization with biocides (Akpan and Solomon, 2014; Liu et al., 2016).

The protective coatings (silicones, resins, paints, etc.) and corrosion inhibitors (amines, oxyalkylated amines, fatty acids, dimer, trimer acids, naphtheneic acid, phosphate esters and dodecyl benzene sulphonic acids) form a protective film on the metal/solution interface, preventing the formation of the conditioning film and the later adherence of bacteria (Rajasekar et al., 2007a). Particularly, the corrosion inhibitors are widely used in petroleum industry, requiring environmental restrictions for application due to their high toxicity (Rajasekar et al., 2007a). These products are often subject to microbial degradation and then lose their protection power against corrosion (Maruthamuthu et al., 2005). A study conducted by Rajasekar et al. (2008) demonstrated that the use of a commercial corrosion inhibitor (amine and carboxylic acid) in diesel-water systems with the presence of *S. marcescens* strains resulted in a severe pitting type of corrosion indicating that the bacteria were able to use the corrosion inhibitor as a carbon source. For this reason, many protective coatings currently used contain biocides (Zuo, 2007).

The use of biocides stands out among the control methods (Videla, 2003). A heterogeneous group of chemicals (glutaraldehyde, sodium hypochlorite, formaldehyde and quaternary ammonium compounds) are widely used as biocides in the oil industry to control planktonic microorganisms and biofilms establishment in pipelines, refining area, water injection, secondary recovery, and drilling fluids, for example; these biocides may also have fungicide and algaecide activities (Acosta-Díaz et al., 2011). However, some of these biocides may be not active against certain types of microorganisms due to the development of bacterial resistance (Acosta-Díaz et al., 2011). Also, they are inherently toxic and frequently difficult to degrade, persisting in the natural environment and then causing a very negative impact (Guiamet and Saravia, 2005). Environmental concern has led to legislation which encourages the replacement of toxic biocides by antimicrobial agents that are more readily degradable, compatible with system operation and less toxic to the environment (Zuo, 2007).

5. PLANT-DERIVED PRODUCTS FOR BIOFILM CONTROL

Several researches have been directed on strategies to control bacterial biofilms and many of these studies have focused on the identification of alternative and natural compounds for replacing synthetic control agents (Kuzma et al., 2007; Bazargani and Rohloff, 2016). The most of these alternative compounds have been obtained from plants that possess antimicrobial properties (Coenye et al., 2012;

Pompilio et al., 2012; Bazargani and Rohloff, 2016). Plant extracts, essential oils and other biologically active compounds isolated from leaves, flowers, stems and roots have been investigated for antibiofilm activity against a variety of microorganisms (Husain et al., 2015; Trentin et al., 2015; Bazargani and Rohloff, 2016).

The plant compounds with antibiofilm activity can act by inhibition of biofilm formation through different ways or eradication of biofilms already formed (Figure 2). Some examples of different antibiofilm mechanisms of plant-derived compounds are presented in Table 2. Inhibition of biofilm formation may occur due to bacteriostatic and/or bactericidal effects, blocking of bacterial adhesion to surface or by molecules that inhibit QS signaling (Martin et al., 2008).

Bacteriostatic and bactericide compounds can act as biofilm inhibitors by limiting the number of bacterial cells able to form biofilms (Kuzma et al., 2007; Quave et al., 2008; Trentin et al., 2013b). Stem bark extract of *Anadenanthera colubrina* inhibited the biofilm formation of *P. aeruginosa* due to its bacteriostatic effect and the authors pointed out that the presence of tannins may be related to this activity; in addition, transmission electron microscopy revealed damage to membrane and the presence of vacuoles in bacterial cells (Trentin et al., 2013b).

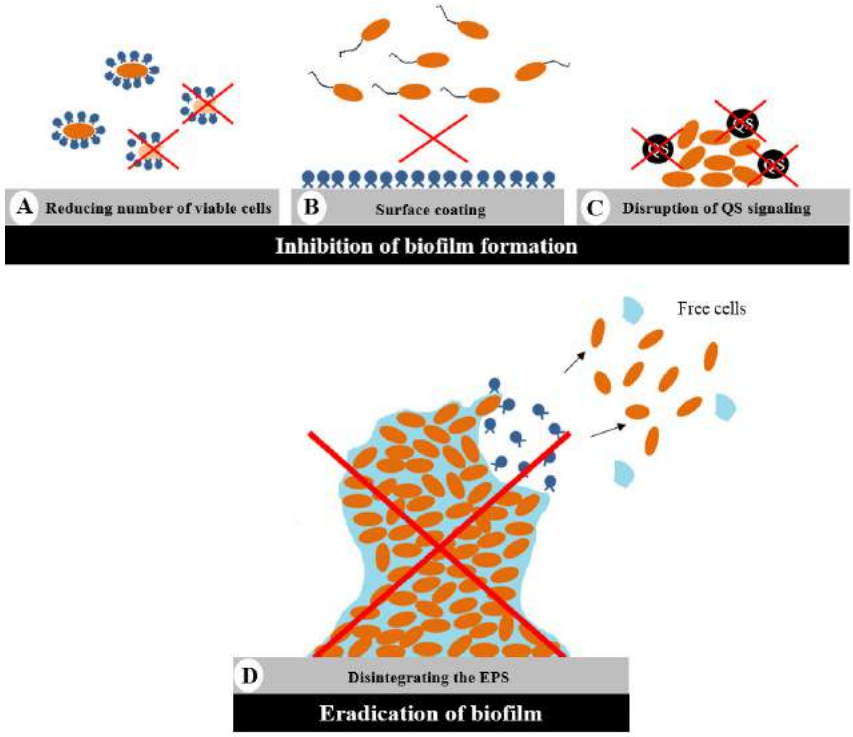

Figure 2. Strategies to combat bacterial biofilms. Antibiofilm molecules can inhibit the biofilm formation by bacteriostatic and bactericide effects, which reduce the number of viable cells able to form biofilm (A). Coating of surfaces may block bacterial adhesion (B). Antibiofilm compounds may exert inhibitory effects on quorum sensing (QS) signals (C). Mature biofilm can be destroyed by molecules that are able to penetrate the biofilm matrix and cause its disintegration, releasing free cells (D).

Compounds with anti-adhesion property and inhibitory effect on QS signals are considered as new therapeutic concepts, because they did not promote death and then a rapid development of bacterial resistance is hindered and antimicrobial agents can be used in combination at low concentrations (Rasko and Sperandio, 2010). Tannins isolated from *Pityrocarpa moniliformis* leaves completely inhibited biofilm formation of *S. epidermidis* at concentrations ranging from 0.125 to 4.0 mg ml^{-1} without affecting the viability of the microorganism, since it was not observed inhibition of bacterial growth

(Trentin et al., 2015). Similarly, *Rubus ulmifolius* root extracts reduced biofilm formation by *Staphylococcus aureus* when used at the concentration range 50-200 µg ml^{-1}, while higher concentrations (530-1040 mg ml^{-1}) were necessary to prevent bacterial growth (Quave et al., 2012).

Table 2. Plant-derived antibiofilm agents against bacteria

Bacteria	Plant (preparation/ compound)	Antibiofilm mechanism	References
Listeria monocytogenes	*Rosmarinus officinalis* (extract)	Anti-adhesion, eradication and effect on metabolic (respiratory) activity in preformed biofilms	Sandasi et al. (2010)
	Melaleuca alternifolia (extract)	Eradication and effect on metabolic (respiratory) activity in biofilms preformed	
Staphylococcus aureus	*Coriandrum sativum* (essential oil)	Anti-adhesion and eradication	Bazargani & Rohloff (2016)
	Pimpinella anisum (essential oil)	Anti-adhesion and eradication	
	Salvia sclarea (diterpenoids: salvipisone and aethiopinone)	Antibiofilm activity probably due to decrease in the number of viable cells and changes in biofilm morphology	Kúzma et al. (2007)
Escherichia coli	*Mentha piperita* (essential oil)	Anti-adhesion and eradication	Bazargani & Rohloff (2016)
	Coriandrum sativum (essential oil)	Eradication	
Staphylococcus epidermidis	*Nigella sativa* (thymoquinone)	Anti-adhesion, eradication and effect on oxidative metabolism in preformed biofilms	Chaieb et al. (2011)
	Vatairea macrocarpa (lectin)	Antibiofilm activity probably due to decrease in the number of viable cells in the biofilm	Vasconcelos et al. (2014)
Propionibacterium acnes	*Epimedium brevicornum* (icariin)	Eradication	Coenye et al. (2012)
	Polygonum cuspidatum (resveratrol)	Eradication	
Bacteria	Plant (preparation/ compound)	Antibiofilm mechanism	References
Streptococcus mutans	*Morus alba* (1-deoxynoijirimycin)	Anti-adhesion, reduction of a bacterial EPS secretion and agglutination of cells	Islam et al. (2008)
	Trigonella foenumgraecum (lectin)	Anti-adhesion, reduction of a bacterial EPS secretion, alteration of biofilm morphology and eradication	Islam et al. (2009)

	Bauhinia variegata (lectin)	Anti-adhesion	Klafke et al. (2013)
Pseudomonas aeruginosa	*Anadenanthera colubrina* (extract)	Anti-adhesion, decrease in the number of viable cells in the biofilm and alteration of biofilm morphology	Trentin et al. (2014)
	Cuminum cyminum (methyl eugenol)	Antibiofilm activity due to the presence of quorum sensing (QS) inhibitors able to inhibit acyl homoserine lactone, resulting in interference in biofilm formation and EPS production.	Packiavathy et al. (2012)
	Solanum tuberosum (lectin)	Antibiofilm activity attributed to affinity to GlcNAc polymers presented in peptidoglycan layer of bacterial cell wall	Hasan et al. (2014)
Chromobacterium violaceum	*Cuminum cyminum* (methyl eugenol)	Antibiofilm activity due the presence of QS inhibitors able to inhibited violacein pigment	Packiavathy et al. (2012)

Some plant compounds can act as anti-adhesion molecules when in contact with bacterial cells (Bazaka et al., 2010). These compounds, adsorbed or immobilized, can be used to form surface coatings (known as Green-coatings) for prevention of primary adhesion of bacterial cells and thus the formation of biofilms; however, the main challenge in this application is the maintenance of the biological activity of the compound after retention on the surface and during a reasonable period of time (Bazaka et al., 2010; Trentin et al., 2013a, 2015). Adhesion of *Helicobacter pylori* cells on the surface of human stomach was strongly affected after pre-treatment of bacterial cells with root extract of *Pelargonium sidoides* (no bacteriostatic and/or bactericidal effect against *H. pylori*); anti-adhesion effect was observed in a dose-dependent manner within the concentration range of 0.001 to 10 mg ml^{-1} (Wittschier et al., 2007). Trentin et al. (2015) showed that proanthocyanidins from *P. moniliformis* leaves were able to adhere to hydrophobic (permanox) and hydrophilic (glass) surfaces, when used in the concentration range from 0.125 to 4.0 mg ml^{-1}, producing a film compatible with human tissue that prevents the adhesion of *S. epidermidis* cells. A glucose/mannose specific lectin isolated from *Trigonella foenumgraecum* seeds, at sub-inhibitory concentrations, prevented the adhesion and subsequent biofilm formation of *Sreptococcus mutans*, (Islam et al., 2009). A lectin purified from tubers of *Solanum tuberosum* inhibited formation of *P. aeruginosa* biofilm after 24h and this activity was attributed to the ability of lectin to bind *N*-acetylglucosamine residues (GlcNAc) (Hasan et al., 2014).

The presence of some compounds within plant extracts, including proteins, could provide a conditioning film promoting microbial adhesion and enhancing biofilm development (Sandasi et al., 2010; Samoilova et al., 2014). For example, low concentrations of black tea and aqueous extracts prepared according to popular medicine from *Arctostaphylos uvaursi*, *Vaccinium vitis-idaea*, *Tilia cordata*, *Betula pendula* and *Zea mays* stimulated biofilm formation by *E. coli* (Samoilova et al., 2014).

Compounds that interfere with QS signaling system are able to damage the development and formation of biofilms. Husain et al. (2015) found that the oil of *Mentha piperita* leaves, at sub-inhibitory concentrations, showed anti-QS properties by inhibiting the violacein pigment in *Chromobacterium violaceum*, without affecting microbial growth. In addition, it was observed inhibition of virulence factors regulated by QS in *P. aeruginosa* (elastase, protease, pyocyanin pigment, and chitinase) after

treatment with this same oil in concentrations ranging from 0.375 to 3% (v/v). Sarkar et al. (2014) found that sub-inhibitory concentrations of ethanolic extract from stem bark of *Sclerocarya birrea* showed antibiofilm property against *P. aeruginosa* cells and inhibited some QS factors, such as protease production and pioverdin pigment.

Cavalcante et al. (2011, 2013) showed that the lectin from *Canavalia maritima* seeds (ConM) was able to inhibit the growth of planktonic cells and biofilm formation of *S. mutans*; the authors found that this activity can be attributed not only to a direct action of the lectin on the cells but also to alteration in the expression of some genes related to biofilm formation and virulence, such as gtfb (virulence factor essential to the formation biofilm process), gbpB and spaP (coders of important adhesins for the establishment of biofilm) and brpA (coders of important proteins in the stress response). The antibiofilm mechanism of lectins probably involves the binding of these proteins to polysaccharides on the bacteria cell wall preventing cell attachment and/or interruption of the polymerization of EPS components (Klafke et al., 2013; Hasan et al., 2014). Other mechanism involves the interaction with animal cell surface saccharides blocking the bacterial adhesion and then enhancing the clearance of bacteria by the host immune system (Donlan, 2002).

Molecules that are capable to disintegrate the EPS and destroy the three-dimensional structure of biofilms are useful for eradication of biofilms already formed (Boles and Horswill, 2011). Polyphenols from Triphala (a mixture of ayurvedic herbs) and green tea eradicated *Enterococcus faecalis* biofilms formed on the surface of human tooth (Prabhakar et al., 2010). Oliveira et al. (2010) showed that solutions of essential oils from *Cymbopogon citratus* and *C. nardus* were able to reduce the number of *L. monocytogenes* biofilm cells attached to stainless steel surface.

CONCLUSION

Almost all microorganisms are able to form a complex community named biofilm, which are related with medical and industrial concerns. Prolonged application of antimicrobial agents are responsible to the developed of resistant and persister cells. The most promising molecules to control the formation and eradication of bacterial biofilm are originated from plants and shows various mode of action, explained by different characteristics of these compounds.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico (CNPq) under grants 308640/2015-2 and 306809/2015-0; Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) under grant IBPG-0185-2.08/12.

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8 CONCLUSÕES

- WSMoL inibiu o crescimento, induziu a aglutinação e promoveu o extravasamento de proteínas de bactérias corrosivas e patogênicas avaliadas (*Bacillus* sp., *B. cereus*, *B. pumillus*, *B. megaterium*, *Micrococcus* sp., *Pseudomonas* sp., *P. stutzeri*, *P. fluorescens* e *S. marcencens*).
- Efeito bactericida foi observado contra *Bacillus* sp., *B. pumillus*, *B. megaterium*, *P. fluorescens* e *S. marcescens*.
- Imagens de microscopia de fluorescência revelaram que WSMoL foi capaz de causar danos a nível celular de *S. marcescens*.
- *S. marcescens* e *Bacillus* sp. foram capazes de formar biofilme em superfície de polisestireno após 24h a à 37ºC detectado através da técnica do cristal violeta.
- WSMoL apresentou efeito antibiofilme *in vitro* frente *S. marcescens* nas menores concentrações testadas e ação antibiótica sobre células de *Bacillus* sp. em todas as concentrações avaliadas.
- Imagens de microscopia eletrônica de varredura e de fluorescência detectaram que essa atividade pode estar relacionada com a perda da viabilidade celular de ambas as estirpes.
- Biofilmes pré-formados de *S. marcescens* não foram destruídos após a adição da lectina, embora as células apresentassem danos estruturais. Por outro lado, a lectina foi capaz de erradicar biofilmes de *Bacillus* sp., principalmente quando usada na concentração 208 µg/mL.
- A superfície revestida com a lectina (WSMoL-*coating*) altera a viabilidade de células de *S. marcescens* que aderiram a superfície e impede a adesão de células de *Bacilus* sp., impedindo a formação de biofilme, sem interferir na viabilidade celular.
- Embora existam relatos prévios sobre o potencial antibacteriano de WSMoL, os resultados aqui relatados são de grande importância desde que fornecem suporte para a aplicação da lectina como composto antibiótico e antibiofilme contra bactérias patogênicas e corrosivas e, adicionalmente, propõe o uso da lectina como revestimento anti-aderente.

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