UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Obtenção de peptídeos e peptidomiméticos de *Capsicum baccatum* var. *pendulum* (Solanaceae) visando atividade antibiofilme

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PORTO ALEGRE, 2019

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

In international Cotutelle agreement with

UNIVERSITÉ DE RENNES 1

ECOLE DOCTORALE BIOLOGIE SANTE

UMR 6290, CNRS UR1, Institut de Génétique & Développement de Rennes (PU2 UR1)

Peptides and peptidomimetics obtention from *Capsicum baccatum* var. *pendulum* (Solanaceae) aiming to the antibiofilm activity

Thesis presented by **Rafael Gomes Von Borowski** for the obtention of (**PhD.**) **DOCTOR** degree:
Mention: Doutor em Ciências Farmacêuticas by Programa de Pós Graduação em Ciências Farmacêuticas and
Mention: Docteur en Microbiologie, virologie et parasitologie by Ecole doctorale Biologie Santé.

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Porto Alegre, 2019

Thesis presented to the Graduate Program in Pharmaceutical Sciences, at the PhD level of the Faculty of Pharmacy of the Federal University of Rio Grande do Sul in international cotutelle agreement with Université de Rennes 1, Ecole Doctorale Biologie Santé and approved on 21/02/2019 by the examining jury consisting of:

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Von Borowski, Rafael Gomes
Obtenção de peptídeos e peptidomiméticos de Capsicum baccatum var. pendulum (Solanaceae) visando atividade antibiofilme / Rafael Gomes Von Borowski. -- 2019.
131 f.
Orientadores: Simone C. B. Gnoatto, Reynald Gillet.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas, Porto Alegre, BR-RS, 2019.

1. Antibiofilme. 2. Peptídeo. 3. Peptídiomimético. 4. Anti-montagem de matriz. 5. Capsicum. I. Gnoatto, Simone C. B., orient. II. Gillet, Reynald, orient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

This thesis in cotutelle was accomplished through the accord 88887.137539/2017-00 CAPES/COFECUB between the Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil and the Université de Rennes 1 (UR1), Rennes, France.

This project was realized at *Laboratório de Fitoquímica e Síntese Orgânica, Laboratório de Biofilmes e Diversidade Microbiana* (Faculdade de Farmácia, UFRGS), Brazil and *Ribosomes, Bactéries et Stress* (Institut de Génétique et Développement de Rennes, UR1), France.

The *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES, Brazil) and the *Comité Français d'Evaluation de la Coopération Universitaire et Scientifique avec le Brésil* (COFECUB, France) financial supported this study in France. The *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES, Brazil) supported this study in Brazil.

AGRADECIMENTOS

Obrigado aos meus pais e amores Rudi e Nara que acreditam no poder da educação e assim, me transformaram. Eu me sinto honrado por conseguir estudar em um país onde o acesso à educação ainda é um privilégio e fazer ciência é um desafio.

Eu entendo que o mais importante elemento de todo esse processo são, sem dúvida, as pessoas. Foram as pessoas da UFRGS e da Universidade de Rennes1, quem me ensinaram e com quem eu partilhei imensas e inesquecíveis experiências acadêmicas e de vida. Obrigado!

Obrigado em especial a minha orientadora e amiga, a magnânima Simone C. B. Gnoatto e ao formidável professor Alexandre José Macedo por me possibilitarem viver tudo isso. Por me fazerem perceber que foi desse material social e humano que veio o meu maior ganho: a certeza de que os trabalhos em equipe e um ambiente feliz traduzem-se em bons resultados.

Obrigado as exemplares professoras Grace Gosmann e Aline Zimmer pelo suporte e aos dedicados colegas e atenciosos amigos Bianca Leonardi, Bruna Casanova, Igor Fontana, Nádia Mileo, Denise Diedrich, Elenilson Figueiredo e a todos do laboratório LaFiS105. Agradeço ao querido amigo Rafael Schneider pela paciência de ter morado e trabalhado comigo ao mesmo tempo.

Obrigado ao meu orientador, o extraordinário Reynald Gillet por todo o seu conhecimento, entusiasmo e confiança. Obrigado aos colegas e amigos que melhoraram a minha experiência de vida e que contribuíram imensamente com o meu doutoramento: aos inspiradores e inextinguíveis Daniel Thomas e esposa, Emmanuel Giudice e esposa e Sophie Chat, as inestimáveis Charlotte Guyomar (os carangueijos do laboratório RBS), a minha irmã francoportuguesa Lydie Jose Teixiera, suas princesas e família, Marie-Christine Savary, Siou Ku e Awa Diop, as determinadas e dedicadas Fanny Demay, Sylvie Georgeault e Marion Thepaut e aos exímios em suas artes Daniel Boujard, Carlos Blanco, Gwennola Ermel, Renan Goude, Denis Chrétien, Claire Heichette, Romain Gibeaux e Laurence Duchesne.

Quando entramos nessa jornada, entramos com tudo aquilo que somos, com nossas lembranças, inseguranças, alegrias e sonhos. Ao longo dessa trajetória construímos novas histórias, cativamos novos amigos e nos modificamos. Todos vocês fizeram a diferença nessa transformação com suas particularidades, discussões, suporte profissional e pessoal. Vocês são incríveis!

Eu desejo que não nos condenem a sempre transformar ideias em coisas, porque passaremos a ver somente coisas e não mais ideias.

REMERCIEMENTS

Merci à mes parents, mes amours Rudi et Nara, qui croient au pouvoir de l'éducation et m'ont ainsi transformé. Je me sens honoré de pouvoir étudier dans un pays où l'accès à l'éducation est toujours un privilège et où faire de la science est un défi.

Je comprends que l'élément le plus important de tout ce processus concerne, sans doute, les personnes. Ce sont les gens de l'UFRGS et de l'Université de Rennes 1 qui m'ont appris et avec qui j'ai partagé des expériences académiques et de vie immenses et inoubliables. Merci beaucoup !

Je remercie en particulier ma mentor et amie, la magnanime Simone C. B. Gnoatto et le formidable professeur Alexandre José Macedo de m'avoir permis de vivre tout cela. Je les remercie pour m'avoir fait comprendre que c'est de ce contenu social et humain que j'ai reçu le plus grand gain ; la certitude que le travail d'équipe et un environnement heureux se traduisent par de bons résultats.

Merci aux professeurs exemplaires Grace Gosmann et Aline Zimmer pour leur soutien et aux collègues dévoué.e.s et ami.e.s attentifs Bianca Leonardi, Bruna Casanova, Igor Fontana, Nádia Mileo, Denise Diedrich, Elenilson Figueiredo et l'ensemble du laboratoire LaFiS105. Je remercie mon cher ami Rafael Schneider pour la patience d'avoir vécu et travaillé en même temps avec moi.

Je remercie mon mentor, l'extraordinaire Reynald Gillet, pour toutes ses connaissances, son enthousiasme et sa confiance. Merci aux collègue.e.s et ami.e.s qui ont amélioré mon expérience de vie et ont énormément contribué à mon doctorat : aux inspirants et inépuisables Daniel Thomas et son épouse, Emmanuel Giudice et son épouse et Sophie Chat ; L'inestimable Charlotte Guyomar (les crabes du laboratoire RBS), ma soeur franco-portugaise Lydie Jose Teixiera, ses princesses et sa famille, Marie-Christine Savary, Siou Ku et Awa Diop ; les déterminées et dévouées Fanny Demay, Sylvie Georgeault et Marion Thepaut et aux très experts dans leurs arts, Daniel Boujard, Carlos Blanco, Gwennola Ermel, Renan

Goude, Denis Chrétien, Claire Heichette, Romain Gibeaux et Laurence Duchesne.

Lorsque nous commençons ce voyage, nous entrons avec tout ce que nous sommes, nos souvenirs, nos insécurités, nos joies et nos rêves. Tout au long de cette trajectoire, nous avons construit de nouvelles histoires, captivé de nouveaux amis et nous avons changé. Vous avez tous fait une différence dans cette transformation avec vos particularités, vos discussions, votre soutien professionnel et personnel. Vous êtes géniaux!

Je souhaite que personne ne nous condamne à toujours transformer les idées en choses, car nous ne verrons que des choses et non plus des idées.

Tenho medo do silêncio e do vazio do desconhecido e do conformado. A ciência me permite exercer toda a minha inquietude. Nela, podemos ser todos Loucos e Santos (Oscar Wilde). Ela é um ato político, feminista e contrassenso como as pioneiras Bertha Lutz, Marie Curie e tantas outras. A ciência liberta! Ela nos encanta e nos permite ultrapassar fronteiras como se não houvessem países (John Lennon). “Morreram os ditadores, enferrujaram-se as armas, mas não destruíram os sonhos de quem ama ser livre” (Augusto Cury).

J'ai peur du silence et du vide de l'inconnu et du conforme. La science me permet d'exercer toute mon inquiétude. Cela nous permet tous d’être fous et saints (Oscar Wilde). Elle est un acte politique, féministe et contre-sens comme les pionnières Bertha Lutz, Marie Curie et tant d'autres. La science libère ! Cela nous enchante et nous permet de traverser les frontières comme s'il n'y avait pas de pays (John Lennon). "Les dictateurs sont morts, les armes à feu rouillées, mais les rêves de ceux qui aiment être libres n'ont pas été détruits" (Augusto Cury).

Resumo

O biofilme é uma matriz complexa, composta por comunidades de microrganismos envoltos em substâncias poliméricas extracelulares, aderidas a uma superfície biótica (tecidos e órgãos) ou abiótica (cateteres e próteses). O biofilme apresenta vários benefícios às bactérias devido à existência dessa matriz que confere resistência e tolerância aos antibioticos. *Staphylococcus epidermidis* é uma das bactérias com grande relevância clínica devido à sua capacidade de formar biofilmes em dispositivos médicos, tais como, marca-passos, cateteres urinários e próteses. Neste contexto, os peptídeos têm sido propostos como uma alternativa importante, tanto para tratamento de biofilmes, quanto como agentes anti-infecciosos de superfície. Este estudo consiste na identificação de novos peptídeos naturais e sintéticos, derivados da pimenta *Capsicum baccatum* var. *pendulum*, com atividade antibiofilme e no planejamento de seus peptidomiméticos. Por conseguinte, foi selecionado e estudado extensivamente um peptídeo de referência que apresentou a melhor atividade antibiofilme contra *S. epidermidis*. Este peptídeo atua através de um novo mecanismo de ação que descrevemos e chamamos de "anti-montagem de matriz" (AMM). Caracterizamos a atividade antibiofilme desse peptídeo, que age através do novo mecanismo de ação AMM independente da regulação celular, não apresentando citotoxicidade. Esses resultados nos permitiram patentear o peptídeo em questão: Europe nºEP19305205.7. Apresentamos ainda duas revisões, uma abordando a relação estrutura atividade entre peptídeos e a atividade antibiofilme e a outra abordando o uso de peptidomiméticos antibiofilme como uma perspectiva. A estratégia com o planejmaneto de peptidomiméticos é criar pequenas moléculas semelhantes ao peptídeo de referência. Estes peptidomiméticos mantêm as capacidades inerentes ao peptídeo principal, porém são mais resistentes a proteases e / ou mais ativos.

Palavras-chave

Antibiofilme, peptídeo, peptídiomimético, anti-montagem de matriz, *capsicum*.

Résumé

Le biofilm est une matrice complexe, composée de substances polymères extracellulaires enveloppant des communautés de micro-organismes, adhérant à une surface biotique (tissus et organes) ou abiotique (cathéters et prothèses). Le biofilm confère aux bactéries de nombreux avantages en tant que cette matrice qui améliore leur résistance et tolérance aux antibiotiques. *Staphylococcus epidermidis* est l'une des bactéries cliniques importantes en raison de sa capacité à former des biofilms sur des dispositifs médicaux, notamment les stimulateurs cardiaques, les cathéters urinaires et les prothèses. Dans ce contexte, les peptides ont été proposés comme une alternative importante, que ce soit en tant que traitement médicamenteux de biofilms ou en tant qu'agents de surfaces anti-infectieux. Cette étude porte sur l'identification de nouveaux peptides naturels et synthetiques antibiofilm issus du piment *Capsicum baccatum* var. *pendulum* et dans la planification de leurs peptidomimétiques. Un peptide majeur responsable de l'activité antibiofilm contre *S. epidermidis* a été sélectionné et étudié de manière approfondie. Il agit par un nouveau mécanisme d'action que nous nommons « anti-assemblage de la matrice » (AAM). Nous caractérisons l'activité antibiofilm de ce peptide, qui agit par le nouveau mécanisme d'action AMM indépendant de la régulation cellulaire et ne présente pas de cytotoxicité. Ces résultats nous ont permis de breveter ce peptide : Europe n ° EP19305205.7. Nous présentons également deux revues, l'une portant sur la relation de structure et d'activité entre les peptides et l'activité anti-biofilm et l'autre sur l'utilisation des peptidomimétiques anti-biofilm comme perspective. La stratégie des peptidomimétiques consiste à créer de petites molécules similaires au peptide de référence. Ces peptidomimétiques conservent les capacités inhérentes au peptide parent, mais sont plus résistants aux protéases et / ou plus actifs.

Mots clés

Antibiofilm, peptide, peptidomimétique, anti-assemblage de la matrice, *capsicum.*

Abstract

Biofilm is a complex matrix, composed of extracellular polymeric substance wrapping microorganisms communities, adhered to a biotic (tissues and organs) or abiotic (catheters and prostheses) surface. Biofilm confers to bacteria many benefits due to the production of this matrix that improves their resistance and tolerance to antibiotics. *Staphylococcus epidermidis* is an important clinical bacteria, able to form biofilm on medical devices such as pacemakers, urinary catheters and prostheses. In this context, peptides have been proposed as an important alternative as a treatment to biofilms or as anti-infective surface agents. This study focuses on the identification of new antibiofilm natural and synthetic peptides from the *Capsicum baccatum* var. *pendulum* pepper and their peptidomimetic design. As a result, a lead peptide responsible for the antibiofilm activity against *S. epidermidis* was selected and extensively studied. It acts by a new mechanism of action that we call "matrix anti-assembly" (MAA). We characterize the antibiofilm activity of this peptide, which acts through the new mechanism of action AMM independent of cellular regulation, and does not present cytotoxicity. These results allowed us to patent this peptide: Europe No. EP19305205.7. We also present two reviews, one addressing the structure activity relationship between peptides and the antibiofilm activity and the other approaching the use of antibiofilm peptidomimetics as a perspective. The strategy with the design of peptidomimetics is to create small molecules similar to the reference peptide. These peptidomimetics retain the inherent capabilities of the parent peptide, but are more resistant to proteases and / or more active.

Key-words

Antibiofilm, peptide, peptidomimetic, matrix anti-assembly, *capsicum*.

SUMMARY

1. General introduction

This thesis broadly discusses the use of peptides and peptidomimetics as antibiofilm agents. We report the discovery of a novel and strong antibiofilm peptide from *Capsicum baccatum* and evidence a new antibiofilm mechanism of action named “matrix anti-assembly” (MAA).

Biofilm is a complex matrix, composed of extracellular polymeric substance (EPS) wrapping microorganisms communities, usually attached to a biotic (tissues and organs) or abiotic (catheters and prostheses) surface. Micro colonies are the beginning of mature biofilm and its structure and composition can react or be adsorbed with external agents mediating the adhesion to surfaces and also promoting physical protection against the antibiotics or the immune system response (Otto, 2008; Taglialegna *et al*., 2016).

Biofilm formation may worsen infections, since the microorganisms are resilient to most of the treatments available. Besides the recognized and increasing problematic issue of bacterial resistance, biofilm formation represents a rising clinical threat without available drugs (Bjarnsholt *et al*., 2013).

In this context, peptides are an exceptional strategy in drug design and pharmaceutical innovation due to their diverse chemical features, biological activity and biotechnological relevance. Therefore, part of this study is a vast review of the scientific literature (2005-2015) to introduce the universe of peptides and the antibiofilm activity. It proposes a comprehensive assessment of a wide range of peptides, targeting biofilms. It still provides chemical and molecular information and a Structural Activity Relationship perspective in order to delineate minimal requirements for antibiofilm activity and contributing to the development of new antibiofilm agents. In light of this, it was possible to propose a peptide design model (X1-X2-X3-X4-X5-X6X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19-X20) to be tested (Von Borowski *et al.*, 2017), (Chapter 1). This review was also used as an instrument to select the lead peptide used in the experimental part of this work.

Therefore, previous studies with *C. baccatum* led us to the identification of a natural fraction containing different peptides with antibiofilm activity (See 1.2 *Capsicum baccatum* var. *pendulum*). The amino acid sequence composition of these peptides and the most relevant characteristics on antibiofilm peptides raised in our review study, aided in the selection of three peptides to be synthesized. Accordingly, we investigated and identified

a novel antibiofilm peptide named capsicumicine. We demonstrated that capsicumicine prevents methicillin resistant *S. epidermidis* adhesion, biofilm establishment and maintenance. We discovered that its activity is due to a new extracellular mechanism of action called “matrix anti-assembly” (MAA). We evidenced that capsicumicine disturbs the matrix structuration leading to the lost of functionality. Importantly, this peptide is non-antibiotic and non-cytotoxic, providing a new alternative to prevent biofilm infections (Chapter 2).

The consistence of this study drove us to an international patent registration strengthens the relevance and applicability of our research (Chapter 3).

Finally, we propose a molecular simplification and peptidomimetic design of capsicumice, as perspectives. Peptidomimetics are a strategy of molecular improvements to advance some aspects like resistance to proteases or to boost peptide bioactivity. Therefore, we used a scientific review to base ourselves on the best models for this propose. Antimicrobial peptidomimetics have been shown as promising anti-biofilm agents. In other words, these structurally enhanced molecules can mimic the action of peptides but in addition, they suggest improving the characteristic limitations of natural peptides. Thereby, we provide insights into antibiofilm peptidomimetic strategies and molecular targets, and discuss the design of two major peptidomimetics classes: AApeptides (N-acylated-N-aminoethyl-substituted peptides) and peptoids (N-substituted glycine units). In particular, we present details of their structural diversity and discuss the possible improvements that can be implemented in order to develop antibiofilm drug alternatives (Gomes Von Borowski *et al.*, 2018), (Chapter 4).

1.1 *Staphylococcus epidermidis and biofilm*

Bacteria from the genus *Staphylococcus* are gram positive, opportunistic pathogens and include a diverse group of commensals and pathogenic that colonize mammals on the skin or mucous membranes (Méric *et al.*, 2018). Some of the best-known members of this genus, such as *S. aureus* and *S. epidermidis*, are biofilm forming pathogens (Paharik e Horswill, 2016).

Concerning pathogenic biofilms, *S. epidermidis* is the most frequently coagulase negative *Staphylococcus* (CoNS) infection, highly associated to health care bloodstream and medical devices infections (Rogers *et al.*, 2009; Uçkay *et al.*, 2011; Nishizaki *et al.*,

2013). This bacteria correspond to 30% of health care-associated bloodstream infections and is significantly associated to medical devices infections (15-40% of prosthetic valve endocarditis (Lalani et al., 2006; Nishizaki et al., 2013), 30-43% of prosthetic orthopedic devices infections (Teterycz et al., 2010; Otto, 2014; Abad e Haleem, 2018).

Furthermore, *S. epidermidis* is developing antibiotic multi-resistance and while this bacterium expresses many virulence factors, the biofilm formation is the most important mechanism contributing to infection (Otto, 2008; Fey e Olson, 2010; Laverty *et al.*, 2013; Otto, 2013; Otto, 2014). This rapid emergence of antibiotic resistance and tolerance occurs due to various conditions associated to biofilm lifestyle such as the facilitated exchange of genetic material, presence of quorum-sensing, metabolic and growth heterogeneity, persisters and physiological and morphological changes because of general stress conditions (Madeo e Frieri, 2013; Scopel *et al.*, 2013; Travier *et al.*, 2013).

In this sense, *S. epidermidis* biofilm matrix has a fundamental role due to its complex constitution such as exopolysacharides, proteins, extra-DNA (eDNA) and teichoic acids (Figure 1) (Otto, 2008; Paharik e Horswill, 2016). This matrix besides conferring mechanical stability to the biofilm, makes bacteria adhesion irreversible and difficult to treat (Haussler e Fuqua, 2013). Thus, these components are important targets in the search for antibiofilm agents.

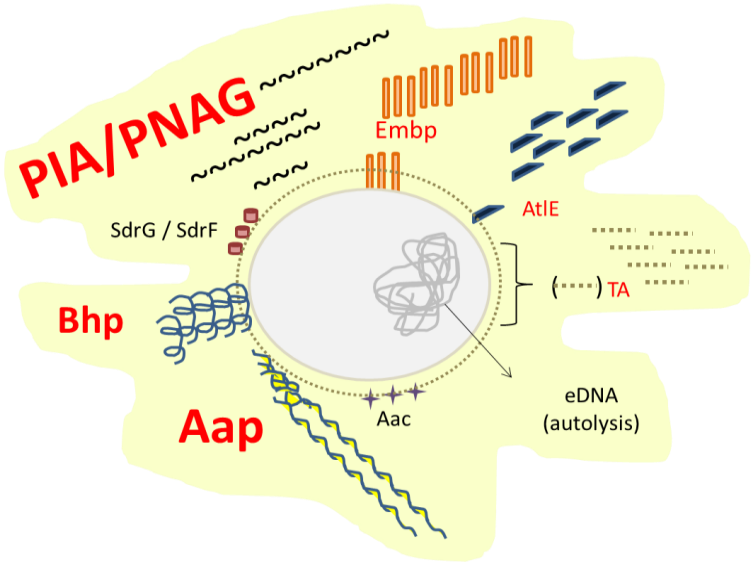


Figure 1. Summary of *Staphylococcus epidermidis* matrix composition. Bacterial cell is illustrated in the center and matrix around in light yellow. The main components of the matrix are named in red and police size is proportional to relevance. PIA /PNAG are polysaccharides, eDNA are extra-cellular DNA, TA are teichoic acids, Aap, Bhp, SdrG/SdrF and Aac are cell wall-

anchored proteins (CWA), AtlE is autholysin, surface associated protein and Embp is extracellular matrix binding protein (Gomes Von Borowski, R., 2018).

1.2 *Capsicum baccatum* var. *pendulum*

Many species of red peppers come from *Capsicum* genus, native from the tropical and humid zones of America and currently cultivated worldwide (Govindarajan, 1986; Menichini *et al.*, 2009; Kim *et al.*, 2014). Some *Capsicum* peppers present a world production of about 19 million tons of fresh fruit from 1.5 million ha (FAOSTAT 2001: FAO Statistical Databases, 2003).

These peppers are popularly known as tili, hot peppers, paprika and red pepper. In particular, *Capsicum baccatum* species are popularly known as “dedo-de moça” pepper (finger pepper) and cambuci, with different colorations and forms. Its cultivation occurs practically in all regions of the country (Brazil) and it is a great example of familiar agriculture (Embrapa, 2002; Ecocrop, 2013).

The *Capsicum baccatum* var. *pendulum* (NCBI: txid40320) plant is characterized by cream flowers with gold/green corolla markings. Typically, fruits are elongated with cream colored seeds (Betemps e Eloi Pinto, 2015), (Figure 2).

The specimen used in this study was obtained from a controlled cultivated area in Turuçu, Rio Grande do Sul (RS), Brazil (latitude: 31º 25 '18 "S, longitude: 52 ° 10 '42 "W, altitude: 30m and area: 286.1 Km^2). A voucher specimen (number P278) was identified and deposited at the Herbarium of Brazilian Government Research Institute EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária, Pelotas, RS, Brazil).

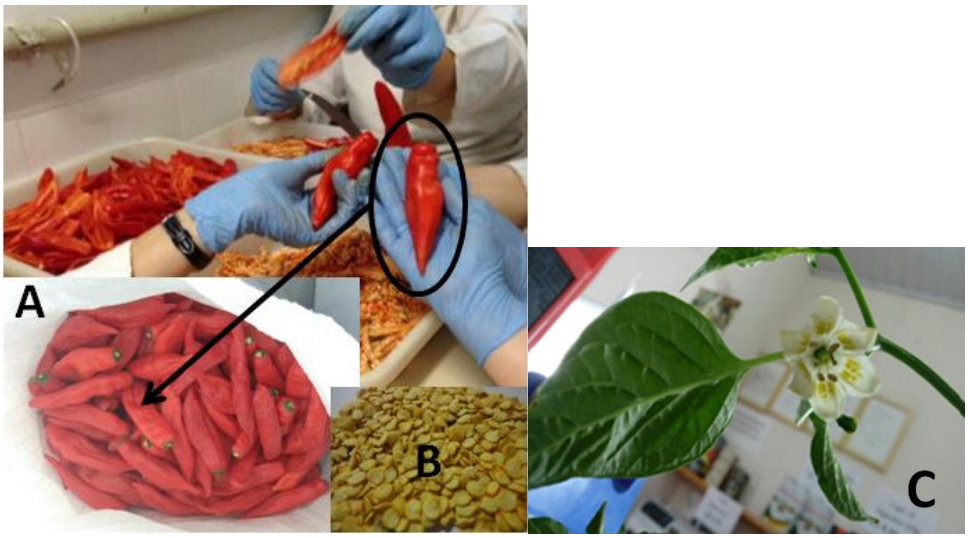
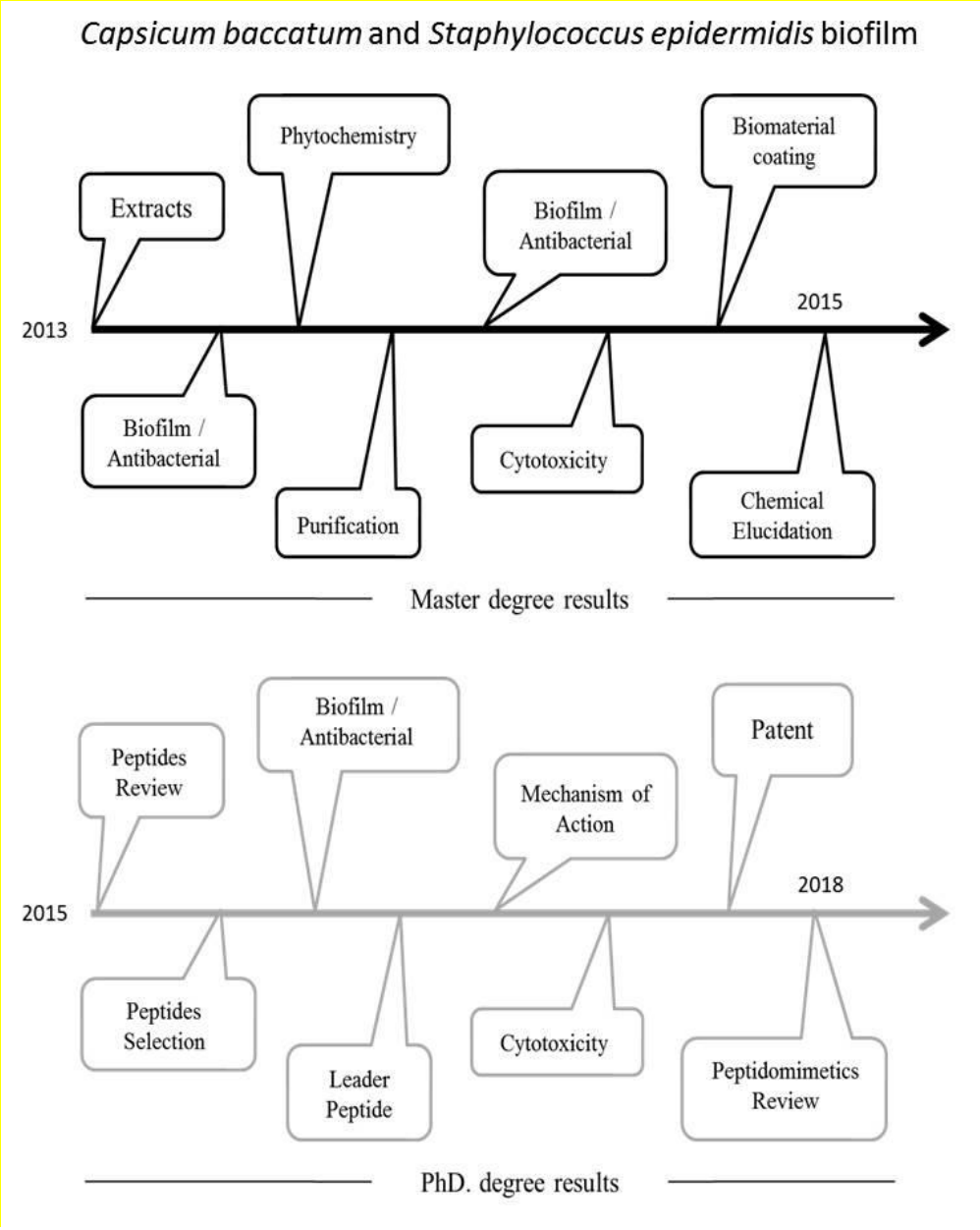


Figure 2. Pictures of dedo-de-moça pepper (*C. baccatum var. pendulum*), author archive; A) fruits with 7 x 1,5cm (length x width), red colored (mature); B) seeds, light yellow colored and C) Flower with white corolla, a pair of yellowish or greenish spots at the base of each wolf of the petals and anthers usually yellowish (one to two flowers per node).

To better guide the readers through this thesis, the history of the studies with *Capsicum baccatum* pepper as well as my scientific course up to now are briefly described (Flow chart 1). Our research group has been developing an important work of chemical and bioactivity characterizations with this species (Zimmer, Aline Rigon *et al.*, 2012; Zimmer, A. R. *et al.*, 2012; Gomes Von Borowski, 2015; Molon, 2016; Leonardi, 2017; Von Borowski *et al.*, 2017). Precisely, we identified and standardized different extracts of *C. baccatum* seeds displaying *S. epidermidis* and *Pseudomonas aeruginosa* antibiofilm activity and evaluated its toxicological profiles *in vitro* and *in vivo*. The most active extract of *C. baccatum* was incorporated in Permanox™ slides by spin-coated technique in order to produce an anti-infective surface. In addition, an *in vivo* toxicological evaluation was performed using the alternative host model of *Galleria mellonella larvae*. The residual aqueous extract from *C. baccatum* seeds (RAqS) was the most active extract, able to inhibit up to 80% and 60% of the *S. epidermidis* and *P. aeruginosa* biofilm, respectively, without inhibiting the planktonic bacterial growth, neither promoting acute toxicity in *G. mellonella*. The surface coating with the RAqS modified the highly hydrophobic feature of Permanox™ slides and prevented the bacterial adhesion and biofilm development. Scanning electron microscopy analysis showed the presence of only small clusters or individual cells without the presence of matrix on the RAqS treated samples, even when RAqS was in solution or as coating. The phytochemical evaluation of RAqS indicated the main presence of amino acids/proteins and tannins (Gomes Von

Borowski *et al.*, 2019). Then, we operated the bioguided fractionation of RAqS, finally leading to a semi-purified active fraction (AF) of peptides. We proceeded with the "AF peptides" characterization and determined its sequence composition and protein origin. After this purification, the "AF peptides" was shown to be more active against *S. epidermidis* than *P. aeruginosa* biofilm formation keeping the the anti-adhesive activity, absence of antibacterial activity and non-cytotoxic effect (Gomes Von Borowski, 2015; Gomes Von Borowski *et al.*, 2019). This original article is in progress.



Flow chart 1. *Capsicum baccatum* and *Staphylococcus epidermidis* biofilm studies. Time line in black displaying a summary of master degree results by Gomes Von Borowski, 2015. Briefly, we obtained the extracts from *C. baccatum* seeds, did the

antibiofilm/antibacterial and phytochemical screenings of theses extracts, finishing with the bio-guided purification of the most active extract. Then, we evaluated the antibiofilm/antibacterial activity of the obtained fractions and evaluated the cytotoxicity profile of the most active fraction (AF). We tested the AF as antiadhesive coating and proceeded with its chemical elucidation. Time line in grey displaying a summary of PhD degree results by Gomes Von Borowski, 2018. Briefly, we published a review about the relationship between peptides and the antibiofilm activity. In sequence, we selected three natural peptides from AF composition to be synthesized. Consequently, we identified a lead antibiofilm peptide (LAP) among theses three tested. We progressed with the elucidation of LAP antibiofilm mechanism of action and cytotoxicity essays. These results led to the register of an international patent (Europe nº EP19305205.7). Finally, we proposed the peptidomimetic design of LAP, as perspectives. To base ourselves on the best models for this proposal we did a review on this subject.

2. Objectives

The objectives of this study were to identify, select and obtain natural and synthetic peptides from *Capsicum baccatum* with significant bacterial antibiofilm activity and to establish their mechanism of action

2.1 Aims of the study

- To identify the amino acid sequence that forms the composition of natural peptides of interest and synthesize them;
- To assay the antibiofilm and the antibacterial activity of selected peptides *in vitro*, using *Staphylococcus epidermidis* ATCC 35984 model, to identify the lead bioactive peptide;
- To evaluate the cytotoxicity profile of the lead peptide;
- To establish the localization/target of the lead peptide in treated bacteria (Intra or extra-cells);
- To analyze the ultrastructural interactions of exposed bacteria;
- To analyze topographic structural interactions of biomaterials exposed to bacteria in the presence/absence of lead peptide;
- To evaluate gene expression variation in the presence/absence of the lead peptide;
- To determine the minor peptide based on the most promising natural peptide;
- To design peptidomimetics based on the lead bioactive peptide.

3. Chapter 1. Literature review : article 1

Chapter 1 consists of a published scientific article, as reference below, which in the full text of this thesis occupies the interval between pages 31-61.

VON BOROWSKI, R. G.; MACEDO, A. J.; GNOATTO, S. C. B. Peptides as a strategy against biofilm-forming microorganisms: Structure-activity relationship perspectives. Eur J Pharm Sci, v. 114, p. 114-137, Nov 2017. ISSN 1879-0720. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/29133243 >.

Abstract

Biofilm forming microorganisms substantially enhance their virulence and drug resistance causing and alternatives are need to combat this health problem. In this context, peptides are an exceptional strategy in drug design and pharmaceutical innovation due to their diverse chemical features, biological activity and biotechnological relevance. Therefore, this study proposes a comprehensive assessment of a wide range of peptides, targeting biofilms. It provides chemical and molecular information and a Structural Activity Relationship perspective in order to delineate minimal requirements for antibiofilm activity and contributing to the development of new antibiofilm agents. In light of this, it was possible to propose a peptide design model (X1-X2-X3-X4-X5-X6X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19-X20) to be tested in the war against resistant microorganisms.

4. Chapter 2. Main article : article 2

Chapter 2 consists of a manuscript in preparation for publication in a scientific periodical, which in the full text of this thesis occupies the interval between pages 65-92.

Capsicumicine, a peptide from Capsicum baccatum pepper displays powerful antibiofilm activity by a novel mechanism of action: matrix anti-assembly

Rafael Gomes Von Borowski, Sophie Chat, Rafael Schneider, Emmanuel Giudice, Simone Cristina Baggio Gnoatto, Alexandre José Macedo, and Reynald Gillet

Abstact

Biofilm forming bacteria are considered an important reservoir of nosocomial infections and antibiotic resistance and tolerance. These bacteria are enclosed in a complex matrix leading to adherence to medical devices and tissues as well as protection against antibiotics and the immune system. Hence, the progress of antibiofilm strategies targeting biofilm matrix is extremely relevant in fighting multi-drug resistant and tolerant bacteria. Plants are constantly submitted to a wide range of pathogens and to defend themselves they have protective factors such as peptides. This peptides are common components of the Capsicum red pepper seeds. Here, we investigated and identified a new antibiofilm peptide named capsicumicine. We demonstrated that capsicumicine prevents methicillin resistant S. epidermidis adhesion, biofilm establishment and maintenance. We discovered that its activity is due to a new extracellular mechanism of action called “matrix anti-assembly” (MAA). We evidenced that capsicumicine disturbs the matrix structuration leading to the lost of functionality. Importantly, this peptide is non-antibiotic and non-cytotoxic, providing a new alternative to prevent biofilm infections.

5. Chapter 3. International patent

All the international patent content is under responsibility of Sociétés d'Accélération du Transfert de Technologies (SATT), Rennes metropole in France.

Europäisches Patentamt | European Patent Office | Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	1000491350	
Application number	EP19305205.7	
File No. to be used for priority declarations	EP19305205	
Date of receipt	20 February 2019	
Your reference	BET 18P4296 APE	
Applicant	UNIVERSITE DE RENNES 1	
Country	FR	
Title	PEPTIDES FOR PREVENTING BIOFILM FORMATION	
Documents submitted	package-data.xml	ep-request.xml
	application-body.xml	ep-request.pdf (5 p.)
	SPECEPO-1.pdf\DV3697_BET18P4296_Filed patent application.pdf (17 p.)	SEQLTXT.txt\DV3697_BET18P4296_Filed sequence listing.txt.txt
	f1002-1.pdf (2 p.)	
Submitted by	EMAIL=pblot@lavoix.eu,CN=Philippe BLOT,O=CABINET LAVOIX,C=FR	
Method of submission	Online	
Date and time receipt generated	20 February 2019, 15:52:53 (CET)	
Official Digest of Submission	A2:99:82:AC:69:98:F0:9F:23:24:B4:C7:CB:98:F1:61:5A:55:74:24	

/INPI, section dépôt/

6. Chapter 4. Perspectives : article 3

Chapter 4 consists of a published scientific article, as reference below, which occupies the interval between pages 97-112.

GOMES VON BOROWSKI, R. et al. Promising Antibiofilm Activity of Peptidomimetics. Front Microbiol, v. 9, p. 2157, 2018. ISSN 1664-302X. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/30271394>.

Abstract

Pathogenic biofilms are a global health care concern, as they can cause extensive antibiotic resistance, morbidity, mortality, and thereby substantial economic loss. Scientific efforts have been made over the past few decades, but so far there is no effective treatment targeting the bacteria in biofilms. Antimicrobial peptidomimetics have been proposed as promising potential anti-biofilm agents. Indeed, these structurally enhanced molecules can mimic the action of peptides but are not susceptible to proteolysis or immunogenicity, the characteristic limitations of natural peptides. Here, we provide insights into antibiofilm peptidomimetic strategies and molecular targets, and discuss the design of two major peptidomimetics classes: AApeptides (N-acylated-N-aminoethyl-substituted peptides) and peptoids (N-substituted glycine units). In particular, we present details of their structural diversity and discuss the possible improvements that can be implemented in order to develop antibiofilm drug alternatives.

6.1 Peptidomimetics design

The molecules proposed in this subchapter have been suppressed because they are part of experiments in progress and will compose a manuscript for publication in a scientific periodical. The complete text of subchapter 6.1 of this thesis occupies the range of pages between pages 113-117.

The peptidomimetics were designed based on experimental results of the lead peptide (capsicumicine) as well as based, discussed and justify on the review article “Promising Antibiofilm Activity of Peptidomimetics” (Gomes Von Borowski *et al.*, 2018).

The synthesis and biological evaluation of these derivatives are in progress as part of the current CAPES-COFECUB project.

6.2 Molecular simplification

The molecules proposed in this subchapter have been suppressed because they are part of experiments in progress and will compose a manuscript for publication in a scientific periodical. The complete text of subchapter 6.2 of this thesis occupies the range of pages between pages 118-120.

To determine antibiofilm minor peptide we proposed a molecular simplification of the lead peptide, capsicumicine. Brief, we synthesized and screened two new peptides from capsicumicine cleavage. The biological evaluation of these derivatives are in progress.

7. General Discussion

In the context of antibiotic resistant bacteria, natural peptides have been increasingly prominent as antimicrobial and antibiofilm agents (Feuillie *et al.*, 2017; Grassi *et al.*, 2017; Von Borowski *et al.*, 2017). During my master degree we identified some natural peptides from *C. bacattum* pepper with promising antibiofilm activity (Gomes Von Borowski, 2015). Those results allowed us to select three antibiofilm peptides for synthesis and biological studies, the mains of this study.

Plants are in constant presence of a variety of pathogens and to defend themselves they have protective factors such as defense peptides and proteins (Castro e Fontes, 2005). This peptides are common chemical components in seeds of the genus *Capsicum*, as well as antimicrobial peptides (AMPs) (Lee *et al.*, 2004; Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2012; Dias *et al.*, 2013; Ribeiro *et al.*, 2013). Some studies suggest that these plant-derived proteins may exhibit anti-adhesive activities (Lengsfeld *et al.*, 2004; Wittschier *et al.*, 2007; Bensch *et al.*, 2011). However, reports on the chemical composition of the species are still scarce, making it necessary to expand studies in this area (Zimmer *et al.*, 2012a).

In this study we identified and characterized capsicumicine, a special antibiofilm peptide (Europe patent nº. EP19305205.7), non-antibiotic, able to prevent the establishment and maintenance of biofilm architecture. It decreases adhesion and cellular aggregation of methicillin resistant *S. epidermidis*.

Our findings regarding the non-antibiotic effect of capsicumicine are in accordance with another study that point to the absence of antibacterial activity of *Capsicum* extracts (Kappel *et al.*, 2008). Likewise, some studies correlate peptide-derived compounds with antiadhesive activity, among them, one that studied extracts of *C. annum* fruits against *Campylobacter jejuni* (Bensch *et al.*, 2011) but none for *C. baccatum*.

In contribution, non-bactericidal peptides are encouraging to find new applications as antivirulence therapy and biomaterials coating, alone or in combination with other drugs to fight against bacteria multiresistance. Therefore, bacterial biofilms are promising targets to control this problem. Thus, the

successful development of antibiofilm compounds will therefore be an important tool to control human biofilm related-infections (Miquel et al., 2016).

We demonstrate the mechanism of action of this peptide, interacting with the biofilm matrix in initial phase (Figure 3), modifying matrix self-assembly (Stewart *et al.*, 2015) and consequently producing loss of functionality. Notably, this mechanism is independently of cell regulation. Importantly, the peptide shows no cytotoxicity to mammalian cells, displaying an important selectivity.

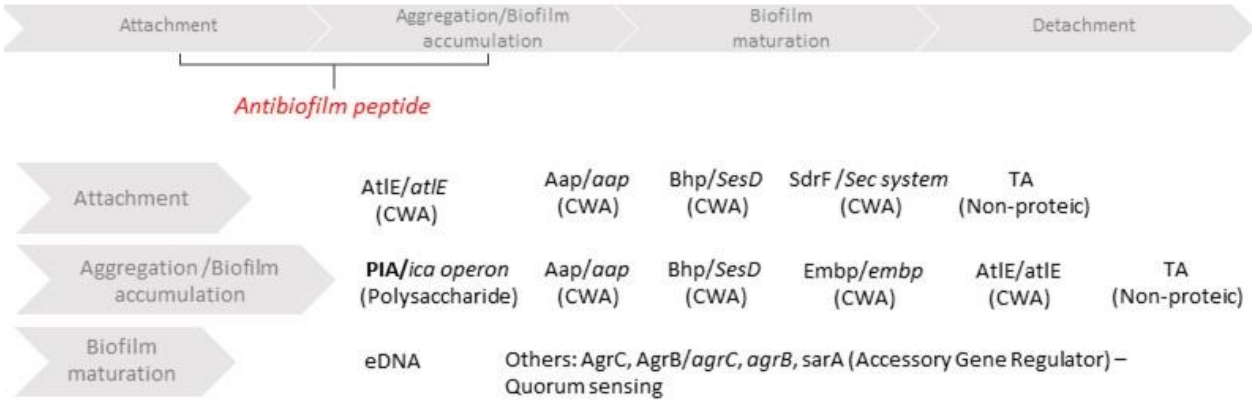


Figure 3. Scheme showing the main stages of biofilm formation in *S. epidermidis*. At each stage, the main compounds/genes involved are briefly described. Highlight in red, the steps involved in the activity of the lead antibiofilm peptide. CWA – Cell wall ancored proteins.

Briefly, we identified the amino acid sequence of peptides that forms the composition of a natural antibiofilm fraction from *C. baccatum* seeds. Then, we selected three of these peptides to be synthesized and thoroughly studied. The results of *in vitro* biological tests indicated the lead peptide capsicumicine. Therefore, bacteria adhesion and aggregation profiles exposed to capsicumicine were defined through topographic structural analysis of plastic coupons exposed to capsicumicine by scanning electron microscopy. It was evidenced that the target of capsicumicine is the extracellular matrix through a set of microscopies (mainly microscopy of fluorescence and ultrastructural analysis of exposed bacteria through transmission electron microscopy). Furthermore, a synthetic model of matrix self-assembly was applied to demonstrate it. Gene expression variation was performed by qRT-PCR in the presence/absence of capsicumicine. The cytotoxicity

of capsicumicine was evaluated using different mammalian lines and multiparameter high-throughput image analysis. Finally, we proposed several peptidomimetic design based on capsicumicine structure after a vast literature review and the currently molecular simplification results in progress (perspectives).

Finally, the reduction in bacterial adhesion and biofilm formation by a pathway that does not involve cell death is a hallmark and contemplates a new concept in antivirulence therapy, aiming to do turn microorganisms more susceptible to antimicrobial agents and to the immune system (Brancatisano *et al.*, 2014). Several non-biocidal strategies have been proposed such as biofilm inactivation as molecular target (Batoni *et al.*, 2016).

8. General Conclusion

Thereby, this study reports the discovery of a novel antibiofilm peptide, capsicumicine (Europe patent nº. EP19305205.7) that significantly prevents biofilm establishment and maintenance. Expressively, capsicumicine decreases adhesion and cellular aggregation of methicillin resistant *S. epidermidis* without antibiotic activity. We discuss a very promising mechanism of action, less susceptible to the development of antimicrobial tolerance. Moreover, the complete absence of cytotoxicity of this peptide with its excellent antibiofilm activity encourages us to keep going the studies through molecular improvements using peptidemimetic strategy.

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