Activity and distribution of the antimicrobial peptides nisin and pexiganan in a Diabetic Foot Infection 3D model

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Thesis to obtain the Master of Science Degree in

Microbiology

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December 2018

“*The only way to do great work, is to love what you do. If you haven’t found it yet, keep looking. Don’t settle.*”

Steve Jobs

Acknowledgments

Initially, I would like to express my sincere gratitude and consideration to my supervisor Professor Manuela Oliveira, for the teaching opportunities that I had but also for her patience, guidance and knowledge, during the laboratory work and during the writing of my dissertation. Additionally, I also thank for her confidence, motivation and friendship.

From the Microbiology and Immunology Laboratory in the Faculty of Veterinary Medicine of the Lisbon University, I would like to express my gratitude to Professor Luis Tavares for allowing me to perform my dissertation in the laboratory.

From the Microbiology and Immunology Laboratory in the Faculty of Veterinary Medicine of the Lisbon University, I would like to thank to Doctor Eva Cunha and Doctor Miguel Grilo for the constant support, motivation and company during my dissertation. I would like to also thank to Doctor Carla Carneiro for her help during the experimental work. I also thank to Doctor Raquel Santos for her confidence on my work.

From the Laboratory of Pathology of the Faculty of Veterinary Medicine of the Lisbon University, I would like to initially express my gratitude to Doctor Conceição Peleteiro, responsible for the laboratory and in particular, to Doctor Solange Reis and Doctor Sandra Carvalho for the laboratorial support during the histochemical analysis performed during the experimental work.

From Instituto de Tecnologia Química e Biológica António Xavier (ITQB) of the Universidade Nova de Lisboa, I would like to thank to Doctor Américo Duarte for the idea to create the peg-lid in a 3D printer that would be used in the experimental work.

From Instituto Superior Técnico, I initially would like to thank to Professor Jorge Leitão for the cooperation between both universities for the peg-lid creation. In particular, I would like to express my gratitude to Doctor Pedro Rego for the peg-lid creation, being a highly important part of this work.

From Instituto de Medicina Molecular, I would like to express my gratitude to Doctor Salomé Veiga for her knowledge and support during the laboratorial work. I would like to also thank to Doctor Susana Dias for her constant availability, knowledge and support during the experimental work.

From Instituto Superior Técnico, I would like to express my gratitude to Professor Leonilde Moreira for her availability during this dissertation.

To my friends, namely Joana Moreira da Silva and Marta Mota, I would like to thank for their motivation and friendship during this dissertation.

Finally, to my Family, I would like to express my gratitude for the constant patient, motivation and support during my thesis, since without this highly important part of my life, it was not possible to conclude it and be the person that I am today.

To all, I am, and I will be always grateful.

This work was financially supported by the project CIISA 29 - Effect of a nisin-biogel on the Diabetic Foot Infections microenvironment and by the project PTDC/SAU-INF/28466/2017 - AMPfoot - Polyphasic validation of antimicrobial peptides as alternative treatment for diabetic foot infections.

Abstract

Diabetic Foot Ulcers (DFU) constitute one of the major and devastating complications of Diabetes *mellitus*. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the most important pathogenic agents isolated from infected DFU and their increased resistance to traditional antibiotic-based treatments prompts the development of new therapeutic alternatives, with antimicrobial peptides (AMP) being a promising strategy.

The inhibitory efficacy of two AMP, pexiganan and nisin, in individual and in dual solutions was assessed against bacterial isolates obtained from infected DFU through Minimum Inhibitory (MIC), Minimum Bactericidal (MBC), Minimum Biofilm Inhibitory (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) determinations using microtiter plate assays.

Additionally, a three-dimensional (3D) collagen model was developed aiming at mimicking the ulcer microenvironment, to evaluate AMP and bacterial diffusion ability and the inhibitory potential of a guar gum biogel, supplemented with several combinations of nisin, pexiganan and the antibiotic (AB) gentamicin.

Results suggest that pexiganan has an effective bacteriostatic inhibitory action against planktonic cells, with promising results against biofilm structures. The combination of nisin plus pexiganan is more effective than the single solutions in the eradication one biofilm-producer strain as well as- in the inhibition and eradication of the dual bacterial suspensions.

The assays using the collagen 3D model allowed to observe bacterial diffusion across all the areas of the model as well as AMP diffusion. However, gentamicin was also tested, and its diffusion was not observed. The guar gum gel supplemented with the antimicrobials individually and in combination (including the AB) allowed to observe that the guar gum gel supplemented with nisin plus pexiganan was able to eradicate the one of the isolates present in the model. Several combinations including AMP, AB and AMP plus AB presented an inhibitory activity against another isolate, but none of them allowed its eradication from the model, being required further studies in order to develop new antimicrobial alternatives.

In conclusion, the dual AMP biogel constitute a promising alternative or complement to antibiotic-based therapy, for topical application in diabetic foot infection (DFI) treatment.

Key-words: antimicrobial peptides; collagen model; diabetic foot ulcer; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; treatments.

Resumo

A úlcera de pé diabético (UPD) está descrita como uma das principais e mais significativas complicações de Diabetes *mellitus*. *Staphylococcus aureus* e *Pseudomonas aeruginosa* são duas das espécies bacterianas patogénicas mais importantes e isoladas a partir de UPD infetadas e o aumento da sua resistência ao tratamento convencional, nomeadamente a antibióticos, requer o desenvolvimento de novas alternativas terapêuticas, nomeadamente péptidos antimicrobianos (PA) que constituem uma estratégia promissora.

A eficácia de dois AMP, pexiganan e nisina, foi testada individualmente e em combinação contra isolados bacterianos obtidos de UDP infetadas, através da determinação de Concentrações Mínima Inibitória (CMI), Mínima Bactericida (CMB), Mínima Inibitória de Biofilme (CMIB) e Mínima de Erradicação de Biofilme (CMEB) em placas de 96 poços.

Adicionalmente, um modelo tridimensional (3D) de colagénio foi desenvolvido com o objetivo de mimetizar o ambiente de úlcera, para avaliar a capacidade de difusão dos PA e das bactérias e o potencial inibitório do gel goma de guar suplementado com várias combinações de nisina, pexiganan e o antibiótico (AB) gentamicina.

Os resultados sugerem que o pexiganan tem uma atividade inibitória eficaz como agente bacteriostático contra células plantónicas, com resultados promissores contra biofilmes. A combinação de nisina com pexiganan é mais eficaz do que suspensões individuais na erradicação de biofilmes produzidos por um dos isolados bem como na inibição e erradicação das suspensões bacterianas mistas.

Os ensaios baseados no modelo 3D de colagénio permitiram observar difusão bacteriana em todas as áreas do modelo bem como a difusão dos PA. No entanto, a gentamicina foi também testada e a sua difusão não foi observada. O gel goma de guar suplementado com compostos antimicrobianos foi testado individualmente e em combinação (incluindo o AB) permitindo observar que o gel goma de guar suplementado com nisina e pexiganan permitiu erradicar um isolado presente no modelo. Várias combinações que incluíram PA, AB e PA combinado com AB apresentaram atividade inibitória contra outro isolado, mas nenhuma delas permitiu a sua erradicação, sendo necessário estudos adicionais para o desenvolvimento de novas alternativas com propriedades antimicrobianas.

Para concluir, o gel goma de guar suplementado com nisina e pexiganan é muito promissor, constituindo uma alternativa ou de complementação ao tratamento convencional, para aplicação tópica, no tratamento de úlceras de pé diabético infetadas.

Palavras-Chave: modelo de colagénio; péptidos antimicrobianos; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; tratamentos; úlceras de pé diabético.

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List of Abbreviations

World Health Organization	WHO
Diabetic Foot Ulcer	DFU
Peripheral Arterial Disease	PAD
Ankle-brachial index	ABI
Diabetic Foot Infections	DFI
Millimeter	mm
Methicillin-Resistant *Staphylococcus aureus*	MRSA
Autoinducers Peptides	AIP
N-acyl-homoserine lactone	AHL
Autoinducers-2	AI-2
Infectious Disease Society of America	IDSA
Acronym of Perfusion, Extent, Depth, Infection and Sensation	PEDIS
Extracellular matrix	ECM
Matrix metalloproteinases	MMP
Total-contact cast	TCC
Extended-Spectrum beta-lactamases	ESBL
Clindamycin	CLI
Penicillin-Binding-Proteins	PBPs
Vancomycin	VAN
Vancomycin-Resistant Enterococci	VRE
Gentamicin	GEN
Minimum Inhibitory Concentration	MIC
Minimum Biofilm Inhibitory Concentration	MBIC
Minimum Biofilm Eradication Concentration	MBEC
Platelet-Derived Growth Factor	PDGF
Platelet-Rich Plasma	PRP
Granulocyte Colony Stimulating Factor	GCFS
Negative-Pressure Wound Therapy	NPWT
Bacteriophage Therapy	BT
basic Fibroblast Growth Factor	bFGF
Epidermal Growth Factor	EGF
Antimicrobial Peptides	AMP
Two-dimensional	2D
Three-dimensional	3D
Minimum Bactericidal Concentration	MBC
Micrograms	µg
Milliliters	mL
Institute of Molecular Medicine	IMM
Milligrams	mg
Molar	M
Hydrochloric acid	HCl
Micrometers	µm
Mueller-Hinton agar	MH
Colony-Forming Unit	CFU
Sodium chloride	NaCl
Mueller-Hinton Cation Adjusted broth	MHCA
Microliters	µl
Brain Heart Infusion	BHI
Tryptic Soy Broth	TSB
Tryptic Soy Agar	TSA
Simulated Wound Fluid	SWF
Fetal Bovine Serum	FBS
Peptone Water	PW
Sodium Hydroxide	NaOH
Phosphate-Buffered Saline	PBS
Centimeters	cm

Lipopolysaccharides	LPS
Glycine	Gly
Proline	Pro
Hydroxyproline	Hyp
Normal	N
Acceptably Daily Intake	ADI

CHAPTER 1

Introduction

1.1. Diabetes *mellitus*

Diabetes *mellitus* is a major health problem and continues to increase significantly worldwide. It is estimated that in 2030, there will be 439 million people suffering from this disease (Shaw *et al*., 2010).

According to World Health Organization (WHO), Diabetes *mellitus* is a chronic disease that occurs when fluctuations of glucose concentrations in the blood are observed, due to the mal function of the pancreas, an organ that is responsible for the insulin production. Therefore, this disease occurs when the pancreas does not produce enough insulin, or, when the insulin produced is not used properly by the body. Consequently, it accumulates in the body, leading to the increase of glucose concentration in the blood and ultimately, to the damage of other organs, such as the heart (WHO, 2016).

There are three types of diabetes: type I that is characterized by a deficiency of insulin production (previously referred as insulin-dependent or childhood-onset diabetes), requiring daily administration of insulin for patients survival; type II that is characterized by the ineffective use of insulin produced by the body (being previously known as non-insulin-dependent or adult-onset diabetes), which is linked to physical inactivity and excess body weight; and type III or gestational diabetes, that can appear during pregnancy, being characterized by higher levels of glucose in the blood, but below the levels required for diagnosing a patient as diabetic (WHO, 2016).

1.2. Diabetic Foot Ulcer (DFU)

1.2.1. DFU development

One of the most frequent complications of diabetes is the development of diabetic foot ulcer (DFU) (Price *et al*., 2016). This condition is mainly associated to diabetes type II, being present in 90% of the affected individuals (Kasiewicz & Whitehead, 2017). In fact, people with diabetes can develop a diabetic foot, which occurs in the lower limb (foot) due to several factors, namely neuropathy, peripheral arterial disease (PAD) (Alexiadou & Doupis, 2012; Peters & Lipsky, 2013; Price *et al*., 2016) and foot traumas (Alexiadou & Doupis, 2012; Santos *et al*., 2016).

Neuropathy can be divided in three categories, namely, sensory, motor and autonomic neuropathy. Sensory neuropathy is mainly characterized by the loss of protective sensibility (International Best Practice Guidelines, 2013). Ulceration develops due to the loss of protective sensitivity, being a result of metabolic modifications. These changes lead to nerve damage, affecting the peripheral sensation, and ultimately, causing the appearance of an ulcer, particularly in areas subjected to high pressure (Jeffcoate & Harding, 2003; Vuorisalo *et al*., 2009).

Autonomic neuropathy is related with the peripheral nerve function, affecting the distribution of the blood in the arteriolar vessels of the foot (Jeffcoate & Harding, 2003). Therefore, it is associated with the dryness of the skin due to a decrease in sweat, interfering with the skin integrity (Figure 1).

Consequently, this can cause the development of callus and fissures on the foot (Vuorisalo *et al*., 2009, International Best Practice Guidelines, 2013).

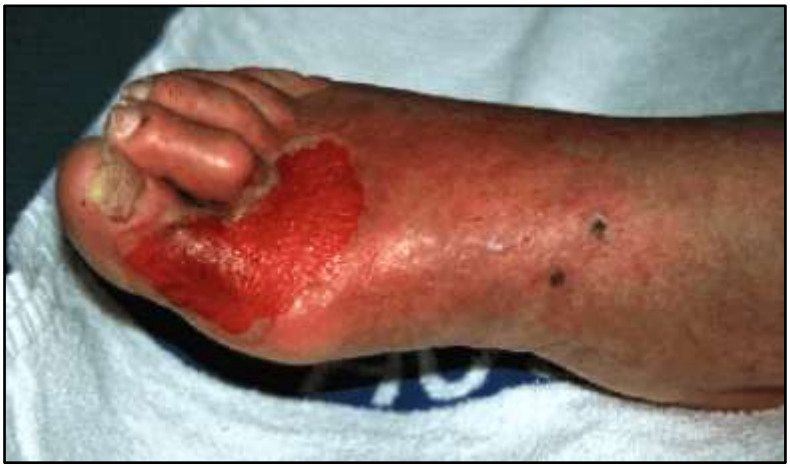


Figure 1. Superficial diabetic foot ulcer (Grade 1). Reproduced with permission from McCulloch DK. Patient education: Foot care in Diabetes *mellitus* (Beyond the Basics). In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. (Accessed on [17-01-2018].) Copyright © 2018 UpToDate, Inc. For more information visit www.uptodate.com.

Finally, motor neuropathy is related with modifications that can occur on the foot structure, since the muscles involved on the normal functionality of the foot are affected. Therefore, this results in abnormal pressure on the foot, leading to ulceration (Figure 2) (International Best Practice Guidelines, 2013).

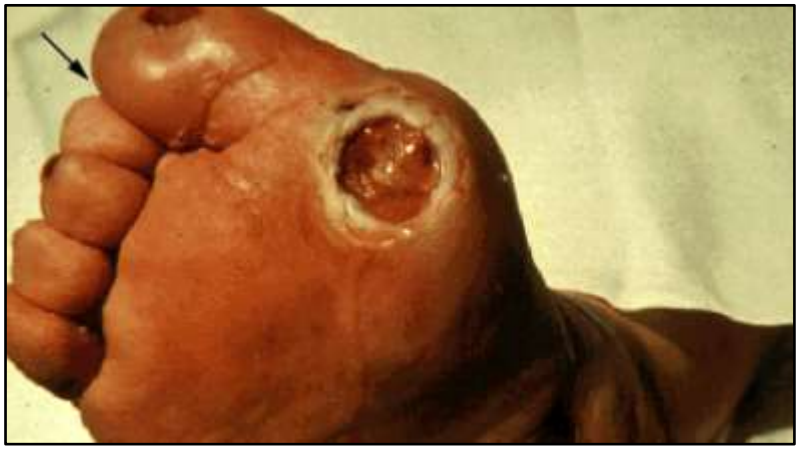


Figure 2. Full thickness diabetic foot ulcer and claw toe (Grade 2). Reproduced with permission from McCulloch DK. Patient education: Foot care in Diabetes *mellitus* (Beyond the Basics). In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. (Accessed on [17-01-2018].) Copyright © 2018 UpToDate, Inc. For more information visit www.uptodate.com.

Ischaemia is a result of PAD (Vuorisalo *et al*., 2009; Price *et al*., 2016), being characterized by a decreased ability of arterial inflow as well as oxygenation and nutrition to the affected area. Consequently, the wound healing process is delayed and, ultimately, can lead to limb loss (Vuorisalo *et al*., 2009; Brownrigg *et al*., 2013).

PAD diagnosis is performed through the ankle-brachial index (ABI), that is calculated based on the ratio between systolic pressures from arm and foot. The normal range values for ABI is between 0.9 and 1.3. Therefore, the presence of PAD is detected by the absence of the peripheral pulses or if ABI is lower than 0.9, being an inexpensive method to detect PAD severity (Lipsky *et al*., 2004; Peters & Lipsky, 2013; Amin & Doupis, 2016).

Therefore, the decreased consciousness of pain, neuropathy and ischaemia observed in diabetic patients, increases significantly the probability of DFU development (International Best Practice Guidelines, 2013).

Approximately 10 to 15% of DFU do not heal and remain active, with the potential of becoming chronically infected, originating Diabetic Foot Infections (DFI). This chronically infected DFU is defined as nonhealing ulcer, being characterized by an extended process of inflammation and progressive tissue damage with the presence of bacterial biofilms associated infections (Alexiadou & Doupis, 2012; Price *et al*., 2016).

There are multiple risk factors associated with the development of DFU, such as gender, since it is more prevalent in males; a duration period for diabetes longer than 10 years; advanced age, as approximately 3% of people with more than 60 years are affected by chronic wounds such as DFU and inappropriate self-care habits of the foot, aiming at controlling its microbiota (Lipsky & Hoey, 2009; Yazdanpanah *et al*., 2015).

For several years, research has been performed to characterize the human skin, namely its microbiota (Hannigan & Grice, 2013; Pereira *et al*., 2017). Information regarding skin microbiota composition was very useful and important because it allows to describe and characterize the natural resident species of the skin microbiota, which are involved in several physiological functions of the host (Human Microbiome Project Consortium, 2013; Pereira *et al*., 2017). One of the most important ones is its role as a physical barrier between the external environment and the body (Hannigan & Grice, 2013; Pereira *et al*., 2017). However, when the skin barrier is broken (for example, due to a wound), it can potentially cause serious problems, such as DFI development (Human Microbiome Project Consortium, 2013; Pereira *et al*., 2017).

Skin microbiota is generally composed by four main phyla, namely Actinobacteria (Hannigan & Grice, 2013; Pereira *et al*., 2017), Proteobacteria (Gram-negative) (Hannigan & Grice, 2013; Lipsky *et al*., 2013), Firmicutes (Gram-positive) (Hannigan & Grice, 2013; Lipsky *et al*., 2013; Pereira *et al*., 2017) and Bacteroidetes (Hannigan & Grice, 2013). Moreover, the three dominant genera associated to skin microbiota are *Staphylococcus*, *Propionibacterium* and *Corynebacterium*. *Streptococcus* and *Pseudomonas* can also be found but less frequently. The abundance of the different genera depends on the area of the skin as well as the related microenvironment (Pereira *et al*., 2017).

Considering skin bacterial composition, if an infection is present it is vital that it is rapidly diagnosed (Human Microbiome Project Consortium, 2013; Hannigan & Grice, 2013; Pereira *et al*., 2017). In fact, bacterial infection is also very important for DFU chronicity, besides neuropathy and ischaemia (Peters & Lipsky, 2013; Amin & Doupis, 2016). Therefore, a proper therapeutic protocol should aim all these conditions (Hannigan & Grice, 2013; Pereira *et al*., 2017). A DFU infection or DFI is characterized by clinical evidences of invasion and multiplication of microorganisms that can be

accompanied by tissue destruction and/or host inflammatory response (Peters & Lipsky, 2013; Lipsky *et al*., 2016). To diagnose a DFI, several symptoms should be considered, namely, redness, temperature, pain, edema, loss of function and the presence of pus. However, it is necessary to take into account that there are signs that could be masked by other factors, such as the presence of neuropathy or ischemia (PAD), complicating the diagnosis (Bader, 2008; Peters & Lipsky, 2013; Amin & Doupis, 2016).

Osteomyelitis is a clinical situation that could also appear due to a severe infection in the bone, generally by contiguous spread from a chronic ulcer. It can be classified as chronic or acute. This situation increases the risk of amputation and the duration of antibiotic treatment (Duarte & Gonçalves, 2011; Peters & Lipsky, 2013).

1.2.2. DFU Bacteriology and Infection development

1.2.2.1. Polymicrobial infections

Foot wounds constitute one of the most serious complications related with diabetes (World Health Organization, 2016). The microbiological composition of the wound depends on the severity of the infection and the wound site (Peters & Lipsky, 2013). In general, the first microorganisms to colonize DFU are Gram-positive bacteria that reach the wound through skin damage, whereas the Gram-negative bacteria colonize chronic wounds (Mendes *et al*., 2014; Lipsky *et al*., 2016).

Infections linked to DFU are normally classified as polymicrobial involving a combination of Gram-positive and Gram-negative bacteria (Peters & Lipsky, 2013; Lipsky *et al*., 2016; Mottola *et al*., 2016). These infections could be promoted by several bacterial genera, such as *Staphylococcus, Pseudomonas, Corynebacterium, Acinetobacter, Streptococcus, Enterococcus, Prevotella, Porphyromonas* and by some species of the *Enterobacteriaceae* family. It is important to emphasize that the most predominant species found in the wounds are *Staphylococcus aureus* and *Pseudomonas aeruginosa*, belonging, respectively to Gram-positive and Gram-negative bacterial groups (Mendes *et al*., 2012; Banu *et al*., 2015; Spichler *et al*., 2015; Santos *et al*., 2016). However, the group of microorganisms present in DFI vary according to the type of infection (Ki & Rotstein, 2008; Peters & Lipsky, 2013; Lipsky *et al*., 2016). In mild infections, the presence of aerobic Gram-positive cocci, specifically *S. aureus* and additionally streptococci (beta-hemolytic) is observed whereas in chronic infections or in previously treated patient's, Gram-negative bacilli, such as Enterobacteriaceae and *P. aeruginosa*, can be present among other bacterial species. Other bacterial families that can be found include Bacteroidaceae, Corynebacteriaceae, Enterococcaceae, Peptostreptococcaceae and Prevotellaceae (Ki & Rotstein, 2008; Price *et al*., 2009; Peters & Lipsky, 2013).

Therefore, some of the bacterial species of the natural microbiota are involved in chronic wounds infection, including in DFI. Being the skin a barrier that protects the organism from the external environment, in the presence of a breach in this organ, the entrance of pathogenic bacteria can occur (Hannigan & Grice, 2013; Pereira *et al*., 2017). However, commensal bacteria can also infiltrate into deeper layers of the skin and cause infection (Ki & Rotstein, 2008). In diabetic patients,

this situation is even worse since the immune response of the host is compromised (Hannigan & Grice, 2013; Pereira *et al*., 2017).

The therapeutic administered to DFI patients is frequently ineffective (Peters & Lipsky, 2013), because the infection can be caused by multiresistant microorganisms (defined as resistant to three or more antibacterial drugs from different classes) (Baltzer & Brown, 2011), including multiresistant *S. aureus* strains (Mendes *et al*., 2012; Field *et al*., 2016).

Due to the high importance of *S. aureus* in DFI development, this bacterial species will be characterized with more detail. It is a commensal microorganism of the human skin that can also cause several diseases, such as bacteremia, if the conditions are appropriate. Consequently, it is also considered a major human pathogen (Field *et al*., 2016). They are characterized as Gram-positive, coccus-shaped bacteria, with approximately 1 millimeter (mm) in diameter, facultative anaerobes, catalase positive and coagulase positive (Figure 3) (Goering *et al*., 2013a).

Some strains of this species can be classified as Methicillin-Resistant *Staphylococcus aureus* (MRSA) being often isolated from diabetic patients who recently have undergone antibiotic therapy (Peters & Lipsky, 2013; Lipsky *et al*., 2016). MRSA-related diabetic foot infections are difficult to treat, especially since the local microenvironmental conditions in the diabetic foot complicate even more the antibiotics action. Ultimately, this situation can lead to infection chronicity and limb amputation (Richard *et al*., 2011; Santos *et al*., 2016).

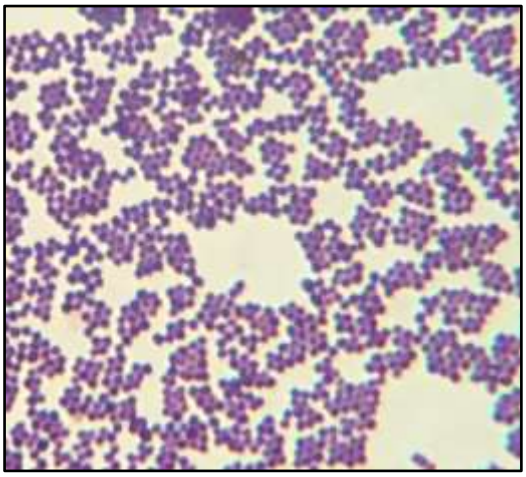


Figure 3. *Staphylococcus aureus* (Original, 1000x, 2018).

1.2.2.2. Biofilms

The infection with biofilm-producing bacteria is the main cause for healing impediment of DFI due to several characteristics conferred by this structure. Biofilm is defined as a bacterial community surrounded by a matrix of extracellular polymeric substances (such as polysaccharides, nucleic acids, teichoic acids among others) that is produced by a first group of cells that irreversibly attach to a biotic or abiotic substratum (Donlan & Costerton, 2002; Arciola *et al*., 2012; Banu *et al*., 2015). Additionally, these structures are ubiquitous in nature (Dickschat, 2010; Arciola *et al*., 2012).

The process of biofilm formation involves four main stages (Arciola *et al*., 2012; Salwiczek *et al*., 2014). Initially, a first group of cells attaches to a substrate. Next, cell aggregation and

accumulation of bacterial cells occurs (forming several layers). In the final steps, the biofilm becomes mature and finally, the detachment of the mature cells occurs, given origin to planktonic cells with the ability to form new biofilm structures elsewhere, initiating a new cycle (Figure 4) (Arciola *et al*., 2012; Salwiczek *et al*., 2014).

The formation of this structure requires bacterial communication that is based, in general, in chemical "language" (Dickschat, 2010). The chemical compounds or signal molecules used by several bacteria are different, namely among bacteria with the same Gram classification (Gram-positive and Gram-negative) and between Gram-positive and Gram-negative bacteria. Gram-positive bacteria, such as *S. aureus*, generally use autoinducers peptides (AIP), whereas Gram-negative bacteria, such as *P. aeruginosa*, use N-acyl-homoserine lactones (AHL). The communication between Gram-positive and Gram-negative bacteria is based in autoinducers-2 (AI-2), since these molecules are common among these bacterial groups (Keller & Surette, 2006; Dickschat, 2010).

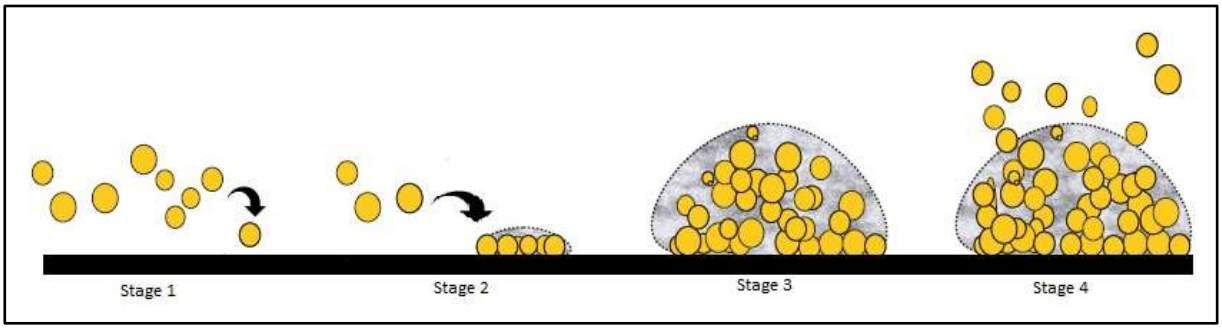


Figure 4. Stages of biofilm development. Adapted from Salwiczek *et al*., 2014.

Biofilm structure increases bacterial resistance to antibiotics, which difficults infection treatment (Donlan & Costerton, 2002; Banu *et al*., 2015) and also increases the resistance to the host innate immune system (Arciola *et al*., 2012; Santos *et al*., 2016). Additionally, it presents other characteristics that provides advantages to the bacteria involved in this structure, such as mechanical stability and protection against the environmental stressful conditions, including higher tolerance to a wide range of temperatures or pH (Dickschat, 2010). Therefore, it is urgent and necessary to find new alternative approaches to antibiotherapy to prevent and control biofilm-related infections, including DFI (Moual *et al*., 2013).

1.3. Classification systems for DFI

For the administration of the proper DFI treatment to patients, it is essential to perform the correct classification of the infected ulcers. To achieve this purpose, several authors tried to create a consensus classification for these ulcers. However, until the present, there is no agreement about a universal classification. One of the most commonly cited diabetic wound classification system is the one created by Meggitt in 1976 and complemented by Wagner in 1981 (Lavery *et al*., 1996).

In the current days, for clinical practice, there are three main classification systems, namely, the Meggitt-Wagner classification system (Alexiadou & Doupis, 2012); the University of Texas Health

Science Center San Antonio system and IDSA (Infectious Diseases Society of America) in combination with PEDIS classifications (Hobizal & Wukich, 2012; Peters & Lipsky, 2013).

Meggitt-Wagner classification system includes six wound grades, based on the wound depth (Table 1). However, it is limited since ischemia and infection are not recognized in more than one grade (Lavery *et al*., 1996; Hobizal & Wukich, 2012).

Table 1. Meggitt-Wagner classification system of diabetic foot infections. From: Classification of Diabetic Foot Wounds (Lavery *et al*., 1996), Diabetic foot infections: current concept review (Hobizal & Wukich, 2012), Management of Diabetic Foot Ulcers (Alexiadou & Doupis, 2012).

Grade 0	Intact skin (Pre-ulcerative area without open lesion)
Grade 1	Superficial ulcer (partial or full thickness)
Grade 2	Deep ulcer to tendon, capsule and bone
Grade 3	Deep ulcer with abscess, osteomyelitis or join sepsis
Grade 4	Localized gangrene
Grade 5	Global foot gangrene

The classification system developed by the University of Texas Health Science Center San Antonio (Table 2) is divided in four grades according to the evaluation of the wound and the presence of infection, ischemia or of both factors (Hobizal & Wukich, 2012).

Table 2. University of Texas Health Science Center San Antonio classification system of diabetic foot infections. From: Diabetic foot infections: current concept review (Hobizal & Wukich, 2012).

	Grade 0	**Grade 1**	**Grade 2**	**Grade 3**
Stage A	No open lesion	Superficial wound	Tendon/Capsule	Bone/Joint
Stage B	With infection	With infection	With infection	With infection
Stage C	Ischemic	Ischemic	Ischemic	Ischemic
Stage D	Infection/Ischemia	Infection/Ischemia	Infection/Ischemia	Infection/Ischemia

IDSA, proposed by the Infectious Disease Society of America, and PEDIS, proposed by the International Working Group on the Diabetic Foot, are other classification systems that were developed aiming at facilitating the evaluation of the severity of infection (Peters & Lipsky, 2013; Hobizal & Wukich, 2012). IDSA developed a scheme with four progressive infection levels, namely uninfected, mild, moderate and severe that describe the severity of the disease, allowing the determination of the presence and extension of the infection. Regarding PEDIS, it represents an acronym of five features that should be considered in the DFU evaluation, namely perfusion (arterial blood supply), extent (size), depth (tissue loss), infection and sensation. This classification system, through the attribution of the grades 1, 2, 3 and 4, corresponds to the infection levels of IDSA classification system, respectively, being based in the evaluation of the wound considering the presence of erythema, systemic toxicity and absence or presence of metabolic alterations (Hobizal & Wukich, 2012; Peters & Lipsky, 2013; Amin & Doupis, 2016).

Nevertheless, different classification systems can be used for the DFU classification with the aim to achieve the best evaluation possible and, consequently, the appropriate treatment (Hobizal & Wukich, 2012).

1.4. Treatments

There are four stages in a normal wound healing process, involving different types of cells (Falanga, 2005). The process occurs in cascade, starting by Coagulation (phase 1), followed by Inflammation (phase 2). In the first phase, the main type of cells involved are platelets that are essential for coagulation, required for the maintenance of hemostasis and to provide temporary wound protection (Falanga, 2005; Gale, 2011). Afterwards, neutrophils and monocytes are recruited to act at the inflammation site. The phase 2 involves the recruitment of macrophages, that help in wound debridement. Besides this, neutrophils and macrophages are able to secrete growth factors that help in the wound healing process. The two last phases are respectively Migration/Proliferation (phase 3) and Remodeling (phase 4). In phase 3, keratinocytes, fibroblasts and endothelial cells are involved in wound contraction and consequently in wound closure. These types of cells are required for several processes, namely formation and deposition of extracellular matrix (ECM) proteins such as collagen which provides initial support, angiogenesis, among others. Finally, phase 4 constitutes the final step of this process that includes the action of myofibroblasts necessary for wound closure and, finally, the formation of scar, ECM degradation promoted by a type of matrix metalloproteinases (MMP), such as collagenase, regulating the cell movement in the wound healing process and, consequently, the contraction movement of the wound also occurs through the contraction and tensile strength. It is necessary to emphasize that this process is dependent on the location of the wound as well as on the presence of infection, which can difficult the healing process (Falanga, 2005; Kasiewick & Whitehead, 2017).

However, the wound healing process can be impaired, as observed in DFI (Falanga, 2005; Kasiewick & Whitehead, 2017). In these patients, it becomes more complicated, requiring different treatment approaches (Falanga, 2005).

1.4.1. Current treatments

Nowadays, in the clinical practice, the treatments that are applied to DFI include antibiotherapy and surgical procedures (Mendes *et al*., 2014; Lipsky, 2016).

The gold standard method for DFI treatment involves surgical debridement of the wound, management of infection, revascularization procedures and off-loading of the ulcer (Alexiadou & Doupis, 2012; Amin & Doupis, 2016). Wound debridement involves the removal of non-viable and necrotic tissues and it should be performed in all chronic wounds. Consequently, the healing process is prompt by the production of healthy granulation tissue. Wound debridement can be achieved through several processes, namely, surgical (using scalpels blades, to remove damaged and dead tissue), enzymatic (through the use of several enzymes to degrade necrotic tissue), biological (applying sterile maggots, that digest unhealthy tissue and decrease the bacterial load present) (Alexiadou & Doupis, 2012; Amin & Doupis, 2016) and by autolysis (through the use of dressings

providing a moist environment, being highly selective for the damaged area, avoiding the surrounding skin tissue) (Alexiadou & Doupis, 2012). Other benefits that wounds debridement provides is the reduction of internal pressure at the affected area as well a more complete observation of the underlying tissues. Furthermore, it helps the discharges at the area and it improves the results from conventional treatments, namely dressings that could be applied in further stages (International Best Practice Guidelines, 2013).

Pressure offloading of the ulcer is performed to alleviate abnormal pressure at the affected zone (ulcer), aiming to promote wound healing. This method is especially important in plantar ulcers, because previous studies demonstrated that elevated pressure on the foot, among other factors such as foot deformities, contribute to the development of this type of ulcers in patients with diabetes. Several approaches can be used, including total-contact cast (TCC), short leg walkers, half shoes and felted foam dressings (Alexiadou & Doupis, 2012; Amin & Doupis, 2016). Specifying the several options for offloading, the TCC is based on plaster, that is molded to acquire an identical pressure distribution at the foot. The advantages of this method are the relatively low costs and efficacy in wound healing. However, it also has disadvantages that could limit their use, such as the time and specialized technicians required for their correct application; skin irritations and the daily access to the wound becomes impossible, requiring that it must be changed, at least, weekly to a proper wound healing. The short leg walkers and half shoes are another form of devices that are available, being easily applied and accepted by the patient as well as presenting a reasonable cost. The easiness with which the patient can remove the device represents a disadvantage relatively to the pressure needed for a correct wound healing, comparing with TCC. Felted foam dressings could also be used to reduce the pressure through a felt-foam pad, allowing the access to the ulcer for wound assessment and treatment (Alexiadou & Doupis, 2012; Amin & Doupis, 2016). Among these alternatives, the best method is the nonremovable TCC, that is indicated for ulcers located at the midfoot or forefoot, being a good option to an effective offloading of these areas (Alexiadou & Doupis, 2012).

Regarding the administration of antibiotics to treat DFI, it can occur through oral or parenteral routes (Price *et al*., 2016). The empirical treatment should consider several factors, namely the severity of the infection, the most likely microorganisms that may be present in the wound as well as the resistance mechanisms that these microorganisms can present (Kosinsky & Lipsky, 2010).

Considering the factors previously referred and the wound classification, several antibiotics or a combination of them are administrated. It is necessary to take in consideration microorganisms that have known resistance mechanisms, namely MRSA or Extended-Spectrum beta-lactamases (ESBL) producing strains. Therefore, when the wound is classified as acute and with mild infection, in patients who have not undergone antibiotic therapy, aerobic Gram-positive cocci are the most likely microorganisms present. Relatively to infections classified as soft-tissues mild infections, they could be treated orally through the administration of one antibiotic or of a combination of drugs/compounds, such as clindamycin or the combination of amoxicillin and clavulanate. For infections classified as chronic, that could be moderate or severe, the use of broad-spectrum antibiotics is recommended, especially in patients who did not respond to previous treatments (Bader, 2008; Kosinsky & Lipsky, 2010).

Clindamycin (CLI) is an antibiotic that belongs to the lincosamides class, acting through the inhibition of peptides bond formation (Magiorakos *et al*., 2012; Goering *et al*., 2013b). Consequently, it can inhibit protein synthesis through the connection to the 50S ribosomal subunit, being more effective against infections caused by Gram-positive bacteria (Kosinsky & Lipsky, 2010; Goering *et al*., 2013b). These antibiotics can be administrated by three routes: oral, intravenous or intramuscular, being the oral route the most frequently used. The resistance mechanism to this class include modification of the drug/compound target (Goering *et al*., 2013b). This antibiotic is used as an option to treat infections with potential of cross-resistance as well as the possibility of resistance to other antibiotics (Bader, 2008).

Amoxicillin in combination with clavulanate is a good option to treat polymicrobial infections (Bader, 2008). It is used against *S. aureus* and streptococci as well as against Gram-negative bacilli and obligate anaerobes (Kosinsky & Lipsky, 2010).

Amoxicillin is a beta-lactam antibiotic, a class that includes penicillin (Magiorakos *et al*., 2012; Goering *et al*., 2013b), namely, semi-synthetic penicillin, that acts through the inhibition of cell wall synthesis (specifically, through the connection of the beta-lactam ring to penicillin-binding-proteins (PBPs)) and is active against Gram-positive and Gram-negative bacteria. Relatively to clavulanate, in combination with amoxicillin, it can inhibit the activity of the majority of beta-lactamases produced, being a beta-lactamase inhibitor compound. Therefore, it allows amoxicillin to inhibit cells that produce this type of enzymes (Goering *et al*., 2013b).

However, to treat soft-tissue infections classified as moderate to severe, another antibiotic therapeutic protocol is required, including broad-spectrum drugs. This therapy includes beta-lactam or beta-lactamase inhibitor compounds, such as ampicillin in combination with sulbactam, that have shown to be effective against *S. aureus*, streptococci, Gram-negative bacilli, obligate anaerobes and polymicrobial infections (Bader, 2008; Kosinsky & Lipsky, 2010).

Ampicillin belongs to beta-lactam antibiotics class (Kosinsky & Lipsky, 2010), specifically to penicillin class (Magiorakos *et al*., 2012; Goering *et al*., 2013b), being classified in this group as semi-synthetic penicillin. This antibiotic is active against Gram-positive and Gram-negative bacteria and acts through the inhibition of the cell wall synthesis, namely through the connection to PBPs (Goering *et al*., 2013b). Moreover, sulbactam is a compound that acts as beta-lactamases inhibitor (Kosinsky & Lipsky, 2010).

Additionally, moderate to severe infections therapeutics also comprises carbapenems, such as ertapenem, and vancomycin, specific for ESBL and MRSA strains, respectively (Bader, 2008; Kosinsky & Lipsky, 2010). Carbapenems are also classified as beta-lactams (Goering *et al*., 2013b). One example is ertapenem, being one of the most recent antibiotics. However, is not active against enterococci, MRSA or *P. aeruginosa* (Bader, 2008; Kosinsky & Lipsky, 2010).

Vancomycin (VAN) is used to treat DFI specifically infected with MRSA strains, being administrated by parenteral route (Kosinsky & Lipsky, 2010; Mottola *et al*., 2016). This antibiotic belongs to the glycopeptide class (Magiorakos *et al*., 2012; Goering *et al*., 2013b) and acts through the inhibition of cell wall synthesis of Gram-positive microorganisms at an earlier stage than beta-

lactams. The antibiotic binds to the terminal D-alanine-D-alanine at the end of the peptide chain, preventing the production of the cell wall structure (Goering *et al*., 2013b).

There are several resistance mechanisms associated to this class, namely, natural resistance resulting from the fact that this antibiotic is composed by large molecules that have difficulty to pass through Gram-negative cells membrane; target modification; and acquisition of resistance genes from other bacterial species, such as of transposable elements. In the case of *S. aureus*, the resistance could be acquired by mutation or by transfer from resistant enterococci, including, the acquisition of the *vanA* gene from vancomycin-resistant enterococci (VRE) (Goering *et al*., 2013b).

The lack of activity against Gram-negative and anaerobic microorganisms requires that therapeutic protocols include the combination of this antibiotic with another agent to treat polymicrobial infections. One strategy used for patients with a suspicious infection involving MRSA strains could present three possible options: combination of vancomycin with beta-lactams antibiotics or with a beta-lactamase inhibitor compound. Another possibility is the combination of vancomycin with carbapenems (Kosinsky & Lipsky, 2010; Goering *et al*., 2013b). However, the administration of vancomycin should be performed with specially attention because this antibiotic is associated with nephrotoxicity. Additionally, the risk of developing nephrotoxicity increases when a combination of vancomycin with antibiotics from aminoglycosides class, that include nephrotoxic antibiotics, is administrated. Moreover, in previous studies, vancomycin was tested against biofilms present in DFI, being observed that it was ineffective (Kosinsky & Lipsky, 2010).

Gentamicin (GEN), belonging to the aminoglycosides class (Magiorakos *et al*., 2012; Goering *et al*., 2013b), acts as a protein synthesis inhibitor, through binding to specific proteins present in the 30S ribosomal subunit (Goering *et al*., 2013b). This antibiotic is not effective against anaerobes or streptococci, but is active against Gram-negative bacilli, such as *P. aeruginosa*, and Gram-positive bacteria, such as staphylococci (Duarte & Gonçalves, 2011; Goering *et al*., 2013b). There are several resistances mechanisms described for aminoglycosides: modification of the 30S ribosomal target protein, alteration of the cell wall permeability and impairment of the transport across the cytoplasmic membrane. However, the main resistance mechanism occurs through the production of enzymes that can modify the antibiotic structure and, consequently, its action (Goering *et al*., 2013b).

A study performed by Mottola *et al*. in 2016, has shown that three of the antibiotics previously mentioned, namely vancomycin, gentamicin and clindamycin, were effective against DFI isolates. MIC (Minimum Inhibitory Concentration), MBIC (Minimum Biofilm Inhibitory Concentration) and MBEC (Minimum Biofilm Eradication Concentration) concentrations regarding *S. aureus* DFI isolates were determined with the aim to study the efficacy of the antibiotics against planktonic and biofilm-forming cells. Results from this study have shown that, between the three antibiotics described, only gentamicin was effective in inhibiting and eradicating biofilms. Since this antibiotic could inhibit biofilm production by MRSA isolates and, additionally, eradicate biofilm production, it was considered the most potent agent tested. Relatively to clindamycin and vancomycin, they were effective against planktonic cells and in promoting the inhibition of biofilm production by most of the isolates under study. Despite this, these antibiotics did not present the ability to eradicate biofilms, not being considered the more suitable to treat DFI caused by biofilm-producing *S. aureus* (Mottola *et al*., 2016).

To treat infections located at the bone or joints, it is important to consider the regimens previously described and adapt them to the patient situation, according to the clinical signs observed (Bader, 2008). Also, the time required for the treatment administration tends to increase according to the severity of the wound and the type of infection associated. For mild degree soft tissue infections, the duration of the treatment is between one to two weeks. However, in moderate degree infections, the treatment lasts between two to four weeks depending of the patient response observed. In these cases, the therapeutics administration by oral or parenterally route is usually performed in the beginning of the treatment and finally, by oral route. For severe infections, the therapy administration starts through the parenteral route, followed by oral via, and the whole treatment takes between two to four weeks, depending on the patient's response. The therapy used to treat osteomyelitis could take between 2 to more than 3 months, and the administration route may vary according to the clinical signs observed (Bader, 2008).

Nevertheless, an increased presence of antimicrobial resistance bacteria in these wounds has been described, rendering antibiotic-based treatment protocols ineffective (Lipsky, 2016).

1.4.2. New therapeutic approaches

As previously mentioned, the increased dissemination of resistant bacteria demands the development of alternatives to antibiotics (Mendes *et al*., 2014; Lipsky, 2016). Some of the alternative therapeutics under study include bacteriophage therapy (Mendes *et al*., 2014); synthetic and natural biocides (Zainol *et al*., 2013; Tadeu *et al*., 2013); teixobactin (Ling *et al*., 2015; Lipsky, 2016); dressings; growth factors, such as PDGF-beta (becaplermin), Platelet-Rich Plasma (PRP), Granulocyte Colony Stimulating Factor (GCFS) and related products; Bioengineered Skin Substitutes; Extracellular Matrix Proteins (Hyaff®; collagen); MMP modulators (Dermax); Negative-Pressure Wound Therapy (NPWT) and Hyperbaric Oxygen Therapy (Alexiadou & Doupis, 2012).

Bacteriophages were discovered 100 years ago and were used as alternatives to antibiotics (Lipsky, 2016). Bacteriophage therapy (BT) is a method that is based in the use of lytic virus that eliminate or reduce the load of pathogenic bacteria at the infection site (Mendes *et al*., 2014). According to Abedon (2010), the development of such treatments includes several stages such as bacteriophage isolation and consequently evaluation of the antimicrobial activity against specific bacterial strains; characterization of the bacteriophage and detection of undesirable traits; performing *in vitro* evaluation of posology and dosage protocols; pre-clinic trials in animals to test the levels of efficacy and toxicity and, finally, human trials (Abedon, 2010; Mendes *et al*., 2014).

Another potential protocol to treat *S. aureus* and *P. aeruginosa* DFU infections include the application of synthetic (L-mesitran®, iodopovidone and chlorhexidine) or natural (natural honey) biocides (Zainol *et al*., 2013). Previous studies have shown that these compounds have a high antibacterial potential against DFU isolates, being considered as possible alternative treatments (Tadeu *et al*., 2013).

Teixobactin is a promising agent because it acts as a cell wall inhibitor through a unique mechanism, being especially important against Gram-positive bacteria (Ling *et al*., 2015; Lipsky,

2016). Its mode of action is based on the connection of this agent to motifs of the cell wall lipid II and lipid III, with the important characteristic of being highly conserved. These regions located in the lipids are linked to the synthesis of peptidoglycan and teichoic acids, respectively, being considered their precursors. Therefore, the inhibition of these precursors has as main consequence the inhibition of the cell wall formation (Ling *et al*., 2015).

Dressings are widely used because they can contain and preserve a certain type of environment, required for a proper wound healing process. Ulcers tend to heal more rapidly and are often less complicated by infection when they are surrounded by a moist environment, except when gangrene is involved. A wound's exudate presents cytokines, platelets, white blood cells, growth factors, MMPs, and other enzymes. Many of these factors contribute to the healing process through fibroblast and keratinocyte proliferation pathway and angiogenesis (Falanga, 2005; Alexiadou & Doupis, 2012), while others, namely bacterial toxins, contribute for the inhibition of the healing process (Alexiadou & Doupis, 2012).

The ideal dressing should present several characteristics, such as be sterile, allow the gaseous exchange as well as the removal of the excess of exudate and toxic components; maintenance of a moisty environment at the ulcer area; impairment of the colonization of microorganisms; easy to remove and finally, present an affordable cost (Alexiadou & Doupis, 2012; Lipsky *et al*., 2016). However, it is important to refer that these treatments were so far only applied to wounds and not to DFI, being necessary more studies to evaluate its efficacy in DFI treatment (Alexiadou & Doupis, 2012).

Collagen-based biomaterials are also an option to considerer regarding dressings-based treatments (Fleck & Simman, 2010; Walters & Stegemann, 2014). Some of the advantages of this polymer include biocompatibility, being non-toxic and very well-described, specifically, at structure, physical, chemical, biological and immunological levels (Ruszczak & Friess, 2003).

In medical applications, this type of biomaterials allows the stimulus and recruitment of cells, such as macrophages and fibroblasts, as well as provides moisture and absorption properties that are essential for the wound healing process (Fleck & Simman, 2010; Walters & Stegemann, 2014). It is described that products in which collagen keeps the native structure present more advantages, namely, in the angiogenesis process rendering the process more effective and consequently, contributing for the wound healing process (Fleck & Simman, 2010).

Growth factors englobe several products that could be used in the treatment of DFI, namely becaplermin (PDGF-beta), Platelet-Rich Plasma (PRP), Granulocyte Colony Stimulating Factor (GCFS) and related products. Becaplermin, a recombinant Platelet-Derived Growth Factor (PDGF), is constituted by two B chains due to the incorporation of the gene codding for the B-chain of human PDGF in *Saccharomyces cerevisiae*, being denominated as PDGF-beta. It was developed to be topically applied, in gel form, to treat non-infected DFU. This treatment is used as a complementary treatment, being applied once per day, along with debridement that occurs on a weekly basis (Papanas & Maltezos, 2010; Alexiadou & Doupis, 2012). Previous studies demonstrated that this product is a promising alternative. However, the increased incidence of cancer in patients submitted to this treatment points out for the need of further studies to evaluate the associated benefits and risks

(Alexiadou & Doupis, 2012). PRP, an autologous product extracted from patient's plasma, is another possible treatment protocol. It is constituted by a high platelet concentration in fibrin clot, which facilitates its application to the ulcer area. After the application of the fibrin clot, absorption occurs within days to weeks, facilitating the wound healing process (Yang *et al*., 2011; Alexiadou & Doupis, 2012). Despite this product's potential, it is necessary to perform further studies to evaluate the beneficial effects of this method when applied to DFU during the healing process (Alexiadou & Doupis, 2012).

There is no agreement about the results of the administration of GCFS applied subcutaneously to patients with infected foot ulcers. Nevertheless, there are related products, specifically basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) which were already demonstrated to be effective to the healing process. bFGF is recognized by its efficacy, namely contributing to the formation of granulation tissue and normal healing. Despite this, support data has been difficult to obtain (Alexiadou & Doupis, 2012). EGF is considered another alternative. To promote the wound healing process, it acts on epithelial cells, fibroblasts and smooth muscle cells. Evidences of the advantage of this treatment to DFU healing are also limited, requiring further studies (Tsang *et al*., 2003; Alexiadou & Doupis, 2012).

Based on matrices, bioengineered skin substitutes or tissue-engineered skin substitutes have showed potential to be applied to DFU treatment. They can be classified according to the type of matrice, being allogenic cell-containing, autologous cell-containing and acellular matrices. Allogenic cell-containing and autologous cell-containing are characterized by the presence of living cells, namely fibroblasts or keratinocytes, in a matrix. Alternatively, acellular matrices do not contain cells and the mode of action is based on the release of growth factors to stimulate the formation of new blood vessels and consequently, wound healing. In the future, this treatment constitutes a promising therapeutic protocol to be applied along with the standard treatment, namely for the management of noninfected DFU, being necessary further studies to confirm its potential (Alexiadou & Doupis, 2012).

Extracellular Matrix Proteins, such as Hyaff® (Fidia Farmaceutici, Abano Terme, Italy) and collagen, constitute another possibility for DFI treatment. Hyaff® is a semi-synthetic ester of hyaluronic acid, which mode of action is based on the facilitation of the growth and movement of fibroblasts, as well as of hydration control (Caravaggi *et al*., 2003; Alexiadou & Doupis, 2012). Collagen, as adjunctive therapy in DFU management, constitute another alternative. Obtained from several sources, it can induce the production of endogenous collagen and promote platelet adhesion and aggregation. However, studies to support the use of this product are still limited (Veves *et al*., 2002; Alexiadou & Doupis, 2012).

MMP modulators or matrix metalloproteinases (MMP) constitute another possible treatment (Alexiadou & Doupis, 2012). MMP are responsible for the regulation of extracellular matrix components (Falanga, 2005; Alexiadou & Doupis, 2012). In a normal wound healing, the extracellular matrix suffers alterations, but there is a balance between the construction and destruction of the matrix. In contrast, in chronic wounds, the observation of a high expression of MMP-2 in fibroblasts and endothelium is allegedly related with the destruction of the matrix. As opposite, a downregulation of MMP-2 expression is understood as an improvement of the healing process (Alexiadou & Doupis,

2012). One example of this product is DerMax® (Tyco Healthcare Group Lp, North Haven, CT, USA), that is a dressing that contains metal ions, inhibiting reactive oxygen species, and citric acid, that acts as scavenger of superoxide anions. This product is applied topically and is associated with a decreased expression of MMP-2 by fibroblasts and endothelial cells. More studies are also necessary to establish the role of this dressing in diabetic ulcers (Karim *et al*., 2006; Alexiadou & Doupis, 2012).

Negative-Pressure Wound Therapy (NPWT), indicated for complex diabetic foot wounds, constitutes another possibility (Alexiadou & Doupis, 2012). This method is based on the use of intermittent or continuous sub atmospheric pressure, that passes through a special pump with vacuum-assisted closure, leaving the wound surface moist (Alexiadou & Doupis, 2012; Amin & Doupis, 2016). Experimental results suggest that NPWT is capable of optimizing blood flow, decreasing tissue edema and removing several bacteria and products from the wound area (Xie *et al*., 2010; Alexiadou & Doupis, 2012). Despite its potential, it is not indicated in cases where the patient has an active bleeding ulcer. Additionally, this treatment should be performed after debridement and continued until the formation of normal granulation tissue at the ulcer surface (Alexiadou & Doupis, 2012).

Hyperbaric Oxygen Therapy constitutes another possibility. It is based on the use of high levels of oxygen allowing to accelerate the wound healing process in patients with diabetes. In fact, fibroblasts, endothelial cells and keratinocytes replicate at increased rates at high concentrations of oxygen. Furthermore, when leukocytes are supplied with oxygen, their bacteria killing potential is enhanced (Broussard, 2004; Alexiadou & Doupis, 2012). The application of this treatment occurs in a chamber, in which the patient is breathing oxygen at 100%, in an intermittent form, with the atmospheric pressure increased among 2 to 3 atmospheres, during 1 to 2h. The full treatment includes between 30 to 40 sessions and promotes a significant reduction of the ulcer area (Kessler *et al*., 2003; Alexiadou & Doupis, 2012) and also a reduction of the risk of major amputation (Faglia *et al*., 1996). This type of treatment could be applied to patients which do not respond to traditional treatment after considering the adverse effects or as an auxiliary therapy for patients with soft-tissue foot infections and osteomyelitis (Tiaka *et al*., 2011).

Another option are probiotics, that are defined as foodstuff containing live microorganisms in adequate amounts to provide benefits to human health (Santos *et al*., 2016; FAO, 2017).

Probiotics can include a mixture of different microorganisms or a single strain, for example of *Lactobacillus* or *Bifidobacteria*. They prompt the elimination of pathogenic bacteria and promote wound healing, having two main action mechanisms, namely direct modification of the microbiome and immune system modulation. The direct modification of the microbiome can occur through competition with pathogenic bacteria, for nutrient resources and adhesion to epithelial receptors, inhibition by production of antimicrobial substances (for example, hydrogen peroxide and bacteriocins) and degradation of toxic substances produced by pathogenic bacteria. The modulation of the immune system has two effects, namely the enhancement of host immune system and consequently of the wound healing process and the promotion of the anti-inflammatory response, through the accumulation of inflammatory cells, such as macrophages and lymphocytes. Therefore, probiotics

could be used in prevention and treatment of these infections (Oelschlaeger, 2010; Sekhar *et al*., 2014; Santos *et al*., 2016).

1.5. Antimicrobial Peptides

The increased resistance of bacteria to conventional therapeutics demonstrates that is urgent to find new alternatives, such as Antimicrobial Peptides (AMP). Due to their properties, namely the decreased resistance presented by bacteria to these molecules in comparison with antibiotics, AMP have been under study, already showing to be a promise therapeutic approach for DFI (Yeaman & Yount, 2003; Gottler & Ramamoorthy, 2009; Santos *et al*., 2016).

AMP are natural molecules produced by the innate immune system with inhibitory properties against invading pathogenic organisms. They are produced by all living organisms, such as animals (vertebrates and invertebrates), plants, bacteria and fungi (Baltzer *&* Brown, 2011; Reddy *et al*., 2004; Straus *et al*., 2006). These peptides present a small size (containing less than 50 amino acid residues), being positively charged (classified as cationic peptides, with an excess of lysine and arginine residues when compared with acidic residues) and with approximately 50% of hydrophobic amino acids (Hancock, 2001).

AMP have been demonstrated to have a large action spectrum, including Gram-positive and Gram-negative bacteria, viruses, fungi and parasites (Hancock, 2001; Moual *et al*., 2013). The two main action mechanisms are divided into bactericidal activity and immunomodulatory effect, being described that AMP mainly presents bactericidal effect (Gottler & Ramamoorthy, 2009; Moual *et al*., 2013). The bactericidal activity of AMPs is generally described through the electrostatic interaction of the AMP (cationic peptides) with the bacterial cytoplasmic membrane (the lipid bilayer is negatively charged), promoting the killing of bacteria (Hancock, 2001; Gottler & Ramamoorthy, 2009; Moual *et al*., 2013). However, other mechanisms regarding the bactericidal effect were described for the disruption of the bacterial membrane which includes membrane depolarisation, creation of micelles that leads to bacterial cell lysis, degradation of the cell walls or alteration of the bilayer membrane, particularly of lipids interfering with bacterial viability (Gottler & Ramamoorthy, 2009; Baltzer *&* Brown, 2011; Moual *et al*., 2013).

Besides AMP potential in DFI treatment (Cotter *et al*., 2013) against *S. aureus* (Cotter *et al*., 2013; Santos *et al*., 2016) and *P. aeruginosa*, namely in combination with antibiotics (Cotter *et al*., 2013), they could be also used against other pathogenic bacterial species such as *Clostridium difficile* and *Propionibacterium acnes* (Cotter *et al*., 2013). Nevertheless, besides the antifungal and antiparasitic activities as previously mentioned, also demonstrates anti-viral (specific to some virus) activity and additionally, synergy with antibiotics as well as with antifungal compounds. Moreover, there are also AMP that present activity as anti-cancer agents as well as synergy with anti-cancer compounds (Hancock, 2000).

1.5.1. Nisin

Produced by Gram-positive bacteria, namely *Lactococcus lactis*, nisin is composed by 34 amino acids, being classified in the bacteriocins group (specifically in class I, also known as lantibiotics) (Shin *et al*., 2015; Field *et al*., 2016). Due to its properties, it has been applied as a food preservative, but it also has potential to be applied in clinical area (Moual *et al*., 2013; Shin *et al*., 2015). Being an AMP, some of its properties include high stability, low toxicity, broad action spectrum, allowing nisin to prevent and control multi-resistant bacteria, namely biofilm-forming strains, such as *S. aureus* strains, including Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Okuda *et al*., 2013; Field *et al*., 2016).

Nisin differs from antibiotics in its mode of action, synthesis, toxicity and resistance mechanisms (The EFSA Journal, 2006; Okuda *et al*., 2013). This AMP acts through the connection with lipid II, inhibiting the cell wall synthesis (bacteriostatic effect) or through the formation of pores, killing the bacteria (bactericidal effect) (Breukink & Kruijff, 2006; Okuda *et al*., 2013; Kramer *et al*., 2004).

Moreover, nisin could have several applications, besides the potential to be applied in DFI treatment (Santos *et al*., 2016). It can be applied to oral health for the prevention of oral diseases due to the antimicrobial activity of nisin; cancer treatments, since these peptides presented cytotoxic effects in cancer cells; and modulation of the immune system of the host through the similar properties presented between AMP (produced by bacteria) and host-defense peptides molecules produced by the host immune system (Shin *et al*., 2015).

1.5.2. Pexiganan

Other antimicrobial agent, that is also classified as AMP and has been under study is pexiganan or MSI-78, an analog from magainin. This AMP potential to be used topically in the treatment of DFI was already tested (Ge *et al*., 1999; Moual *et al*., 2013).

Magainin-2 was discovered in 1987, in the skin of the frog *Xenopus laevis*. Several studies were performed using maganin-2 and discovered that, for the preservation of its antimicrobial activity, this peptide could not be changed, or at least, the minimum length of the peptide had to be maintained (Gottler & Ramamoorthy, 2009).

Pexiganan is a synthetic peptide that was constructed based on magainin-2. Some characteristics of this peptide helped to its synthesis and action mode, namely, a length of 22 amino acids allowing it to be easily synthetized *in vitro*, being positively charged (Gottler & Ramamoorthy, 2009; Flamm *et al*., 2015); in the presence of a lipidic membrane, it acquires a coil structure due the establishment of electrostatic interactions when associated to the bacterial membrane surface, namely an α-helical structure, characteristic of the naturally produced magainin isolated from frogs. The electrostatic interactions allow the pore formation on the bacterial membrane, the main action mode of this AMP. It presents antimicrobial activity against a wide range of bacteria, namely Gram-positive and

Gram-negative (Gopinath *et al*., 2005). It is important to refer that its inhibitory effect against *S. aureus*, a bacterial species with known resistance mechanisms, has already been demonstrated. These previous results allowed to considerer pexiganan as a promising molecule for DFI treatment. Furthermore, this peptide is also able to act in a synergistic mode with antibiotics (such as β-lactams) against *P. aeruginosa* and *S. aureus* isolates among others, in specific conditions such as in the bloodstream (Gottler & Ramamoorthy, 2009).

1.5.3. Antimicrobial potential of combination of AMPs

Several studies have demonstrated that the efficacy of individual lantibiotics (Cavera *et al*., 2015; Shin *et al*., 2015) can be enhanced by their combination with other AMP (Cavera *et al*., 2015; Grassi *et al*., 2017). The combination of antimicrobial agents can present three different forms, namely by synergistic, additivity or antagonistic interactions. Respectively, the effect of the combination of two antimicrobial agents could demonstrate a stronger, equal or a weaker inhibitory effect (Worthington & Melander, 2013; Yu *et al*., 2016). The impact that these combinations have on bacteria resistance include the alteration of the biofilm formation process and the prevention or delay of resistance development (Worthington & Melander, 2013; Field *et al*., 2016; Yu *et al*., 2016; Grassi *et al*., 2017).

Also, it is known that AMP and antibiotics have been combined in a synergistic form, in order to increase their action against bacterial species (Field *et al*., 2016).

1.5.4. AMP Delivery Systems - Guar Gum

In spite of the potential AMP including nisin, for application in infections control, their inhibitory action can be affected by their inhibition or degradation before it reaches the target zone at therapeutic concentrations. Therefore, to guarantee its successful action, it is necessary to find a proper delivery system (O'Driscoll *et al*., 2013).

The characteristics of natural polysaccharides make them a promising delivery system for the topical application of AMP to DFI, including being biodegradable, non-toxicity, inert, low cost and abundant in nature (Reddy *et al*., 2011).

Guar gum, classified as a natural polysaccharide, is obtained from *Cyamopsis tetragonolobus* (a leguminous crop), specifically from seed gums (Reddy *et al*., 2011; Thombare *et al*., 2016). It is constituted by galactose and mannose residues (also denominated galactomannan) linked by glycosidic bridges. It is soluble in water but insoluble in organic solvents (Reddy *et al*., 2011; Mudgil *et al*., 2014; Thombare *et al*., 2016). In water, a rearrangement of guar gum structure occurs, allowing to produce a viscous jellified matrix. Besides, it is also nonionic, a property that provides consistency and stability in a wide range of pH values (Thombare *et al*., 2016).

1.6. Three-dimensional model for diabetic foot infection

Several *in vitro* studies have been performed to evaluate new possibilities for DFI treatment. These studies allow an improved understanding of the product in study, being essential before *in vivo* studies. For DFI, the studies performed so far were, generally, based in microtiter plates protocols used for evaluating the effect of a potential treatment in test, including of nisin incorporated in a guar gum gel, in a two-dimensional model (2D) (Santos *et al*., 2016). Therefore, it is necessary to evaluate the characteristics of the same product in a representative three-dimensional model (3D) aiming at mimicking the *in vivo* conditions. This model will allow to mimetize the environment conditions present in DFI, allowing to evaluate bacteria dissemination and treatments efficacy in deeper tissues. For the construction of 3D models, one material that can be used is collagen (Werthén *et al*., 2010; Price *et al*., 2016). Nevertheless, the increased resistance demonstrated by bacteria to conventional antibiotics therapeutic enhance the urgency to find new alternatives, being AMP one possible alternative, such is the case of pexiganan (Gottler & Ramamoorthy, 2009).

In this work, the first aim was to evaluate the antimicrobial activity of pexiganan and of a dual AMP solution containing pexiganan and nisin regarding two DFI strains using a 96-well plate assay.

The main aim of this work was to establish an *in vitro* collagen-based three-dimensional DFI model, to study the diffusion of bacteria; the diffusion of the guar gum gel supplemented with antimicrobials, namely nisin, pexiganan and gentamicin through the model; and, finally, the evaluation of the inhibitory activity of the guar gum gel supplemented with the antimicrobials, alone and in combination against selected DFI isolates present in the 3D DFI model.

This model allowed to study the AMP efficacy in *in vitro* conditions that better mimetize *in vivo* conditions, representing a further step in the evaluation of the therapeutic potential of these AMP to be applied as an alternative or as a complementary therapy to antibiotherapy in DFI treatment.

CHAPTER 2

Materials and Methods

Initially, the antimicrobial activity of pexiganan was tested through the determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) values. Regarding the dual AMP combination, only MBIC and MBEC values were determined since MIC and MBC were previously obtained (Manuela Oliveira, personal communication).

Moreover, the establishment of a three-dimensional (3D) diabetic foot infection (DFI) model as a representation of a diabetic foot ulcer (DFU) was the main aim of this work, allowing to perform several diffusion assays, including the evaluation of the diffusion of bacteria; the diffusion of antimicrobial peptides (AMP) and antibiotic (AB); and, finally, the evaluation of the inhibitory activity of the antimicrobial compounds against selected DFI isolates.

2.1. Antimicrobial Activity of Pexiganan

2.1.1. Bacterial isolates

To evaluate the antimicrobial activity of pexiganan, two DFI clinical isolates that belong to a collection previously obtained from DFU samples (Mendes *et al*., 2012) were used as well as two reference strains. These isolates were already characterized (Mendes *et al*., 2012; Mottola *et al*., 2015; Mottola *et al*., 2016). The susceptibility of the bacterial strains to pexiganan was evaluated individually and in combination, aiming at the determination of MIC, MBC, MBIC and MBEC values.

2.1.2. Antimicrobial Peptides

Pexiganan solution

A stock solution of pexiganan (Innovagen, Sweden) was used, and provided by Castanho's Laboratory at the Institute of Molecular Medicine (IMM), in Lisbon.

Nisin solution

A stock solution of nisin (Sigma-Aldrich, USA) was prepared and stored at 4ºC until further use (Santos *et al*., 2016).

2.1.3. Determination of the Antimicrobial Activity of Pexiganan

2.1.3.1. MIC and MBC determination

The bacterial strains were previously inoculated in Mueller-Hinton agar (MH, Oxoid; England) and incubated. For the MIC and MBC protocol (Santos *et al*., 2016), a 0.5 McFarland inoculum in sodium chloride was prepared for each bacterial strain. A dilution (1:10) was performed in Mueller-Hinton Cation Adjusted broth (MHCA; Becton, Dickinson and Company, USA), and a dual species suspension was also produced.

Regarding the inoculation of the microtiter plate used for MIC determination, several concentrations of pexiganan were tested. Afterwards, inoculum in MHCA were added to each well; the negative control (represented by C- in the first column) and the positive control (represented by C+ in the third column) were filled, being then the plate posteriorly incubated.

Following incubation, the determination of MIC, that is defined as the lowest concentration of antimicrobial that promotes the inhibition of the bacterial visible growth (Wiegand *et al*., 2008), was visually performed for each strain (Santos *et al*., 2016).

Subsequently, MBC protocol was performed, being defined as the lowest antimicrobial concentration that does not allow colony development in agar plates (French, 2006). Experiments were performed in triplicate, on different days.

2.1.3.2. MBIC and MBEC determination

The bacterial strains were previously inoculated in MH and incubated. For the determination of pexiganan MBIC and MBEC (Santos *et al*., 2016), a 0.5 McFarland bacterial inoculum in NaCl. Serial dilutions (1:10) were performed in MHCA, and a dual species suspension was also produced.

Regarding the inoculation of the microtiter plate used for MBIC determination, each microtiter plate (MBEC Biofilm Inoculator, Innovotech; Canada) was filled with the bacterial suspension and covered with a 96 peg-lid (MBEC Biofilm Inoculator, Innovotech; Canada), being then incubated. After incubation, the peg-lid was rinsed three times in microtiter plates (Nunc Thermo Scientific, Roskilde, Denmark), being then placed on a new microtiter plate (COSTAR 3879; USA) to evaluate the inhibitory activity of the peptide pexiganan.

The microtiter plate was filled with inoculum and pexiganan at several concentrations, being then incubated. After incubation, MBIC determination was defined as the lowest concentration in which there was no bacterial visible growth (LaPlante *et al*., 2009).

For MBEC determination, the peg-lid was rinsed three times after which the peg-lid was placed on a new microtiter plate (COSTAR 3879; USA). This microtiter plate was incubated on an ultrasonic bath (Grand MXB14, England), in order to promote the release of bacteria from the peg-lid surface (LaPlante *et al*., 2009). Afterwards, the peg-lid was discarded, and the microtiter plate was incubated. After incubation, MBEC was determined using the same method as used for MBIC determination. Experiments were performed in triplicate, on different days.

2.2. Determination of the Antimicrobial Activity of a dual AMP

For these assays, the same bacterial strains and AMP were used (see 2.1.1. and 2.1.2.).

2.2.1. MIC and MBC determination

The AMP concentrations to be tested in this assay were based in the MIC and MBC values of nisin against one of the selected strains previously determined by Santos *et al*. (2016).

For the MIC and MBC protocol (Santos *et al*., 2016), performed only for one bacterial species, a 0.5 McFarland inoculum was prepared in NaCl. A dilution (1:10) was performed in MHCA.

Regarding the preparation of the microtiter plate (COSTAR 3879; USA), several concentrations of nisin were tested (Santos *et al*., 2016). Then, inoculum in MHCA was added to each well and both positive and negative controls were also filled, being then the plate incubated.

Following incubation, the determination of MIC, defined as the lowest concentration of antimicrobial that promotes the inhibition of the bacterial visible growth (Wiegand *et al*., 2008) was performed. For MBC protocol, after incubation, it was defined as the lowest concentration that does not allow colony development (French, 2006).

2.2.2. MBIC and MBEC determination

The protocol used was similar to the one previously described for pexiganan in 2.1.3.2. However, in this protocol, besides pexiganan filling in the microtiter plate, also nisin were added to each well at the previously determined MIC concentration. Experiments were performed in triplicate, on different days.

Statistical Analysis

For the evaluation of MIC, MBC, MBIC and MBEC, the average and standard deviation of the results obtained were determined using Microsoft Office Excel 2016.

2.3. Collagen DFI 3D Model

2.3.1. Bacterial isolates

Two DFI clinical isolates were also used to evaluate their diffusion ability in a collagen 3D model as well as the inhibitory potential of antimicrobial compounds applied to this model.

2.3.2. Antimicrobial Peptides preparation

In another study conducted by our research team, the inhibitory activity of nisin incorporated in a guar gum gel against DFI isolates was evaluated, through the determination of its MIC, MBC, MBIC and MBEC values (Santos *et al*., 2016). Based on the results obtained by Santos *et al*. (2016), the concentration of nisin used in this study was based on the MBEC value previously determined.

Nisin solution

The stock solution of nisin was prepared as previously referred in 2.1.2.

Guar gum gel

A guar gum gel of 1.5% (w/v) was prepared. Before the assays, dilutions of nisin, pexiganan and antibiotic were incorporated within the gel in a proportion of 1:1.

Pexiganan

The stock solution of pexiganan were prepared as previously referred in 2.1.2.

2.3.3. Antibiotic preparation

A stock solution of gentamicin (ITW Reagents; Italy) was previously prepared according to the manufacturer PanReac Appli Chem. and kept at -80ºC until use.

2.3.4. Establishment of calibration curves for the AMP and Antibiotic

For evaluation of the results from the AMP and antibiotic diffusion ability assays in the collagen 3D model, it was necessary to establish calibration curves with the aim to quantify the diffusion of AMP and the antibiotic through the model.

2.3.4.1. Calibration curve for Nisin incorporated in a guar gum gel

Initially, bacterial strain was inoculated in Brain Heart Infusion medium (BHI, VWR Chemicals, Belgium) and incubated. After incubation, a 0.5 McFarland inoculum was prepared in sodium chloride. Serial dilutions (1:10) were performed in Tryptic Soy Broth (TSB, VWR Chemicals, India), and inoculated in Tryptic Soy Agar medium (TSA, VWR Chemicals, Belgium) in order to produce a bacterial lawn. Then, several nisin solutions incorporated in a guar gum gel were spotted in the agar

surface, after which the plates were incubated, with posterior observation and measurement of inhibition halos.

All assays were performed in duplicate.

2.3.4.2. Calibration curve for Pexiganan incorporated in a guar gum gel

Regarding the establishment of a calibration curve for pexiganan incorporated in a guar gum gel, the protocol used was based on the previously described in 2.3.4.1. Nevertheless, some modifications were performed namely, it was used other bacterial isolate and another medium.

2.3.4.3. Calibration curve for Gentamicin incorporated in a guar gum gel

The establishment of the calibration curve for gentamicin (GEN) incorporated in a guar gum gel was performed according to the protocol previously described in 2.3.4.2. All assays were performed in duplicate.

2.3.5. Establishment of a Collagen DFI 3D Model

2.3.5.1. Composition of the collagen suspension

For the establishment of the DFI 3D model, a collagen suspension were prepared using Collagen I High Concentration from rat tail (Corning, US), cold Simulated Wound Fluid (SWF), composed by 50% of fetal bovine serum (FBS; biowest; France) plus 50% of peptone water (PW; Biokar Diagnostics; France), acetic acid at 0.1% (Sigma-Aldrich; USA) and sodium hydroxide at 0.1M (NaOH, Merck; Germany), with a final pH of 7.5 (Macherey-Nagel; Germany) (Price *et al*., 2016).

2.3.5.2. Optimization of collagen polymerization

The system used to reproduce the collagen ulcer model was composed by a 6 well-plate (Corning; Falcon, USA), in which an insert (High Density translucent PET Membrane, 6 well 3.0 µm pore size; Corning, Falcon; USA) was placed in the wells. A volume of the collagen suspension prepared as described in 2.3.5.1. was placed in the insert, and afterwards a peg-lid, previously washed and sterilized was placed on the plate, followed by incubation to allow collagen polymerization (Price *et al*., 2016).

After incubation, the collagen did not polymerize, originating a loosely and slime consistence solution with a pH of 7.0. To improve the consistency of the collagen model, other modifications were also tested, namely different volumes and concentrations of NaOH used in the collagen solution were tested, aiming at further improving collagen polymerization.

2.3.5.3. Nisin biogel activity and distribution in a collagen DFI 3D Model

The diffusion pf the AMP, of the bacteria and of the AMP plus bacteria in a DFI 3D model was evaluated using the optimized protocol.

Regarding the evaluation of AMP and bacteria diffusion, the ulcers models were produced as further described. A collagen suspension was made using cold SWF, NaOH and Collagen I High Concentration from rat tail.

A volume of the previously prepared collagen suspension was placed in a 6-well plate with an insert (Price *et al*., 2016); then a peg-lid was washed and sterilized, being then placed over the plate. Afterwards, the 6-well plate was incubated in order to allow collagen polymerization.

Evaluation of the Nisin biogel diffusion

To evaluate AMP diffusion within the model, after polymerization, a volume of SWF were added to the well (Price *et al*., 2016). Additionally, a solution of nisin incorporated in a guar gum gel was added, followed by incubation. After incubation, AMP quantification was performed in both liquid and solid phases (Price *et al*., 2016), as follows.

For the quantification of AMP present in the liquid phase, the SWF present in the well were placed in a TSA plate containing a bacterial lawn, posteriorly incubated. The experiments were performed in duplicate.

The quantification of AMP present in the solid phase was performed after sectioning the collagen model that allowed obtaining three distinct areas in the model.

Afterwards, each area was placed in falcons, to which a volume of collagenase (500 µg/mL in PBS; Merck, Germany) solution was added, followed by incubation (Price *et al*., 2016). Then, each suspension was centrifuged (HERMILE Z383K) to obtain the supernatant (Werthén *et al*., 2010). Afterwards, a volume of the supernatant from each falcon was placed in a TSA plate containing a bacterial lawn. Finally, the plates were incubated, after which the presence of inhibition halos was evaluated. The experiments were performed in duplicate.

A second assay was performed, by adding the nisin biogel three times, following an identical protocol previously described. After the incubation period, the diffusion of the AMP was evaluated in the liquid and solid phases, as described in 2.3.5.3.

Evaluation of bacterial diffusion

To assess bacterial diffusion throughout the 3D model, after the polymerization process, a bacterial suspension in SWF was added to the model. Then the plate was incubated, after which, bacterial quantification was performed in the liquid and solid phases of the collagen model.

For the bacterial quantification in the liquid phase, a volume of the inoculated SWF was removed from the well, and 10-fold serial dilutions were performed. Then, a volume of the bacterial suspension correspondent to each serial dilution was inoculated in TSA medium in duplicate. After incubation, bacterial colonies were quantified. The experiments were performed in duplicate for each serial dilution.

The bacterial quantification in the solid phase was performed after the sectioning of the collagen model as previously described in 2.3.5.3. Afterwards, for pellet evaluation, each area was placed in falcons and processed as described in 2.3.5.3. For bacterial quantification, a volume of the resuspended pellet was 10-fold serial diluted. Then, each bacterial dilution was inoculated in TSA plates in duplicate. After incubation, bacterial colonies were quantified. The experiments were performed in duplicate.

Evaluation of the inhibitory activity of nisin biogel using a 3D DFI model

In the first assay, after polymerization of the collagen model as previously described in 2.3.5.3., a bacterial suspension was added to the model after which the plate was incubated to allow bacteria diffusion. Then, a solution of nisin incorporated in a guar gum gel was added to the insert, followed by incubation.

A second assay was performed, aiming at adjusting the periods of nisin application. First, a bacterial suspension was prepared as described in 2.3.5.3. and placed on the collagen ulcer model after polymerization. Then, the 6-well plate was incubated. After incubation, a solution of nisin incorporated in a guar gum gel was added to the insert, following incubation. After incubation, a second solution of nisin incorporated in a guar gum gel were added to the insert, after removing a volume of the inoculated SWF present in the well, that was used for bacterial quantification. The 6-well plate was posteriorly incubated. This process was repeated one more time. Both experiments were performed in duplicate and bacterial quantification was performed as described in 2.3.5.3.

2.3.5.4. Histochemical Evaluation of the Nisin Biogel inhibitory activity in the DFI 3D Model

Bacteria and nisin biogel diffusion were also evaluated in the collagen 3D model by histochemical analysis. These protocols were performed with the collaboration of the Laboratory of Pathology of the Faculty of Veterinary Medicine of the Lisbon University.

The histochemical protocol was divided into 7 steps, namely Fixation, Trimming, Pre-embedding, Embedding, Sectioning, Staining and Mounting (Slaoui & Fiette, 2014)

2.3.5.5. Pexiganan biogel activity and distribution in a collagen DFI 3D Model

In these assays, the bacterial strains used were the same as previously referred in 2.3.1. Regarding AMP preparation, it was similar as previously described in 2.1.2. and 2.3.2.

The protocols used were also similar to those previously described for the evaluation of the inhibitory activity of the nisin biogel by using the collagen DFI 3D Model (2.3.5.3.), with some adjustments.

Evaluation of the Pexiganan biogel diffusion

An assay was performed in order to evaluate the diffusion of pexiganan incorporated in a guar gum gel within the collagen model, according to the previously described protocol in 2.3.5.3. After polymerization, a volume of SWF was added to the well. Additionally, a solution of pexiganan incorporated in a guar gum gel was added. After incubation, AMP quantification was performed in a bacterial lawn, for both liquid and solid phases, as previously described in 2.3.5.3.

Evaluation of bacterial diffusion

The evaluation of another bacterial species throughout the collagen 3D model was also performed, being based in the protocol previously described in 2.3.5.3.

Evaluation of the inhibitory activity of pexiganan biogel using a 3D DFI model

To evaluate the inhibitory activity of a pexiganan biogel using a 3D DFI model, two assays were performed.

In the first assay, after polymerization of the collagen model performed as previously described in 2.3.5.3., a bacterial suspension in SWF was added to the model after which the plate was incubated to allow bacteria diffusion. After incubation, a volume of pexiganan incorporated in a guar gum gel was placed on the collagen model. After incubation, bacterial quantification was performed as described in 2.3.5.3.

In the second assay, for each bacterial strain, a bacterial suspension in SWF was prepared and added to the collagen model after polymerization, as previously described in 2.3.5.3. Then, the 6-well plate was incubated. Afterwards, a volume of pexiganan incorporated in a guar gum gel was added to each insert. After incubation, a volume of pexiganan incorporated in a guar gum gel were added to the insert, after removing a volume of the inoculated SWF present in the well, that was used for bacterial quantification (performed as previously described in 2.3.5.3.). The 6-well plate was

posteriorly incubated. This process was repeated one more time. Finally, bacterial quantification was performed as previously described in 2.3.5.3. The experiments were performed in duplicate.

2.3.5.6. Histochemical Evaluation of the pexiganan biogel inhibitory activity in the DFI 3D Model

Diffusion of bacteria and of the pexiganan biogel in the DFI 3D model diffusion were evaluated as previously mentioned in 2.3.5.4.

2.3.5.7. Dual AMP biogel activity and distribution in a collagen DFI 3D Model

The bacterial strains used in this assay were the same as previously referred in 2.3.1. Regarding AMP preparation, namely of nisin and pexiganan incorporated in a guar gum gel, it was similar to the previously described in 2.1.2. and 2.3.2.

The protocols used were also similar to those previously described for the evaluation of the inhibitory activity of the nisin biogel by using the collagen DFI 3D Model (2.3.5.3.), with some adjustments.

Evaluation of bacterial diffusion

Considering that the diffusion of both bacterial isolates was previously evaluated individually throughout the 3D model, an assay was performed in order to assess their simultaneous diffusion. Therefore, based on the protocol described in 2.3.5.3., to produce the dual species suspension, a 0.5 McFarland initial inoculum was prepared for each bacterial strain. Afterwards, serial dilutions (1:10) were performed to obtain a mixed bacterial suspension in SWF. Then, the plate was incubated. After incubation, bacterial quantification was performed in the liquid and solid phases of the collagen model, as previously described in 2.3.5.3. The experiments were performed in duplicate.

Evaluation of the inhibitory activity of a dual AMP biogel using a 3D DFI model

The evaluation of the inhibitory activity of a dual AMP guar gum biogel, namely of nisin and pexiganan incorporated in a guar gum gel, using a 3D DFI model, was performed. First, after polymerization, a dual species suspension was prepared as described in 2.3.5.7. and placed on the collagen ulcer model. After incubation, a solution of a dual AMP guar gum gel including nisin and pexiganan was added to the insert, following incubation. Afterwards, another solution of the dual AMP guar gum gel was added to the insert, after removing a volume of the inoculated SWF present in the well, which was used for bacterial quantification (performed as previously described in 2.3.5.3.). The 6-well plate was posteriorly incubated. This process was repeated one more time. Finally, bacterial

quantification was performed as previously described in 2.3.5.3. The experiments were performed in duplicate.

2.3.5.8. Gentamicin biogel activity and distribution in a collagen DFI 3D Model

The bacterial strains used were the same as previously mentioned in 2.3.1. and the antibiotic preparation was performed as previously described in 2.3.3.

The protocols performed were also similar to those previously described (in 2.3.5.3.), with some adjustments.

Evaluation of the Antibiotic biogel diffusion

An assay was performed in order to evaluate antibiotic diffusion within the model, namely of gentamicin incorporated in a guar gum gel, tested independently with each bacterial species bacterial lawns, using the protocol previously described in 2.3.5.3. Therefore, after polymerization, a volume of SWF was added to the well. Additionally, a solution of gentamicin incorporated in a guar gum gel was added to the insert, followed by incubation.

After incubation, gentamicin quantification was performed in both liquid and solid phases as previously described in 2.3.5.3. The experiments were performed in duplicate.

Evaluation of the inhibitory activity of gentamicin biogel using a 3D DFI model

The evaluation of the inhibitory activity of a gentamicin biogel using a 3D DFI model was based on two assays.

In the first assay, gentamicin biogel inhibitory activity was evaluated for each bacterial strain, as previously described in 2.3.5.3. and 2.3.5.5.

In the second assay, the gentamicin biogel was applied three times for each bacterial strain tested individually (2.3.5.3. and 2.3.5.5.), as well as for the dual species combination (2.3.5.7.).

For both assays, bacterial quantification was performed as previously described in 2.3.5.3. The experiments were performed in duplicate.

2.3.5.9. Triple biogel supplemented with Nisin, Pexiganan and Gentamicin activity and distribution in a collagen DFI 3D model

The bacterial strains used were the same as previously referred in 2.3.1.; the AMP preparation was similar to the previously described in 2.1.2. and 2.3.2.; regarding antibiotic preparation, it was similar to the previously described in 2.3.3.

The protocols used were also similar to those previously described for the evaluation of the inhibitory activity of the nisin biogel by using a collagen DFI 3D Model (2.3.5.3.), with some adjustments.

Evaluation of the inhibitory activity of a triple guar gum biogel supplemented with Nisin, Pexiganan and Gentamicin

For the evaluation of the inhibitory activity of the triple guar gum biogel supplemented with Nisin, Pexiganan and Gentamicin in a 3D DFI model, a dual species suspension was prepared as described in 2.3.5.7. and placed on the collagen ulcer model after polymerization. Then, the 6-well plate was incubated. After incubation, a solution of a triple guar gum biogel supplemented with nisin, pexiganan and gentamicin were added to the insert, following incubation. Afterwards, another solution of the same supplemented gel was added to the insert, after removing a volume of the inoculated SWF present in the well, which was used for bacterial quantification (performed as previously described in 2.3.5.3.). The 6-well plate was posteriorly incubated. This process was repeated one more time. Finally, bacterial quantification was performed as previously described in 2.3.5.3. The experiments were performed in duplicate.

2.3.5.10. Histochemical Evaluation of the triple biogel supplemented with Nisin, Pexiganan and Gentamicin inhibitory activity in the DFI 3D Model

The evaluation of bacteria and of the triple biogel supplemented with Nisin, Pexiganan and Gentamicin diffusion occurred as previously described in 2.3.5.4.

Statistical Analysis

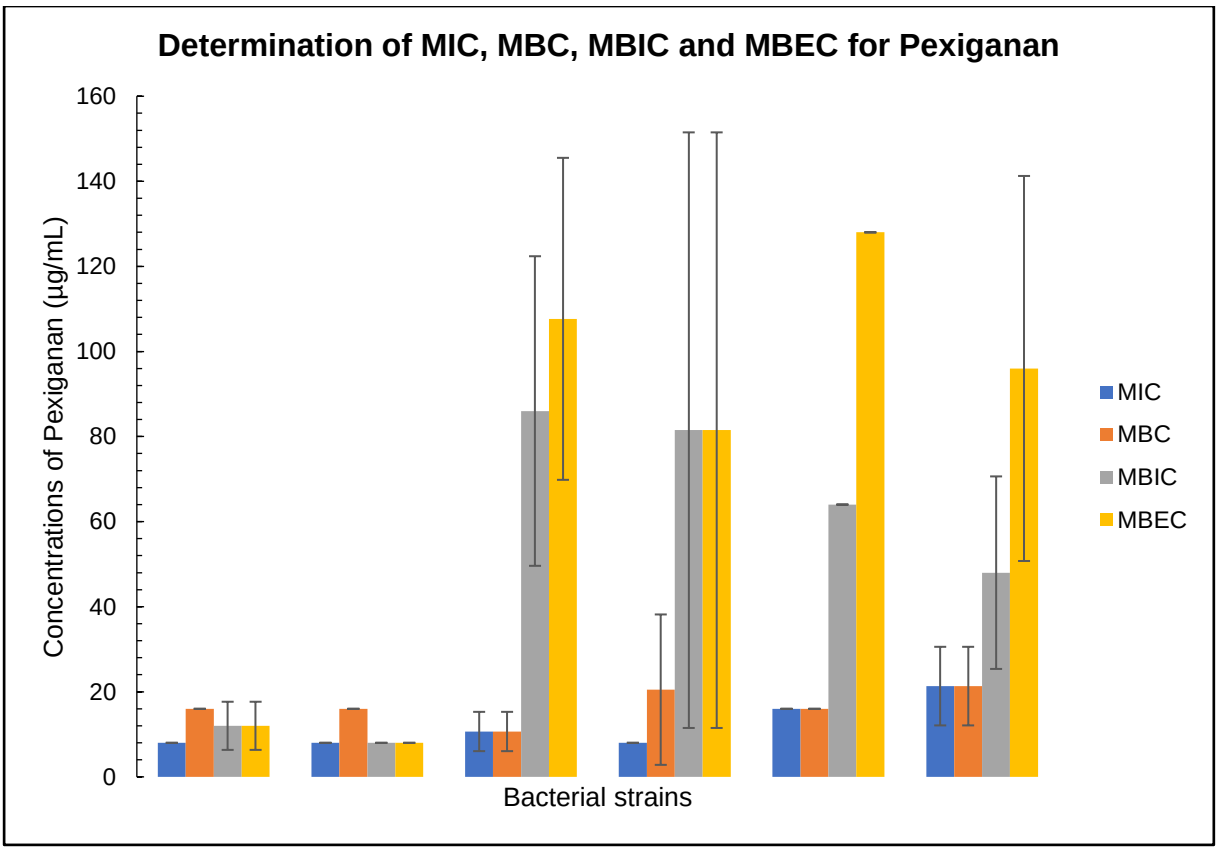
The average and standard deviation of the results obtained were determined using Microsoft Office Excel 2016.

CHAPTER 3

Results

3.1. Antimicrobial Activity of Pexiganan

The MIC and MBC values for pexiganan against the planktonic bacterial species strains under study are presented in Graphic 1, as well as the MBIC and MBEC values determined for biofilm cultures. The isolates were tested individually and in dual combinations.



Graphic 1. MIC, MBC, MBIC and MBEC results (in average) for pexiganan against DFU isolates and bacterial dual species combinations. **Legend**. **MIC**: Minimum Inhibitory Concentration; **MBC**: Minimum Bactericidal Concentration; **MBIC**: Minimum Biofilm Inhibitory Concentration; **MBEC**: Minimum Biofilm Eradication Concentration.

3.1.1. MIC and MBC

The MIC and MBC determination (Graphic 1) allowed to evaluate the effect of pexiganan in planktonic suspensions from the tested bacterial strains.

For the strains tested individually, MIC determination has shown that for the two reference strains, pexiganan had an average MIC value of 8 ± 0 µg/mL. Relatively to the remaining strains obtained from swabs samples collected from DFU patients, the average MIC value for pexiganan was of 8 ± 0 µg/mL for one isolate and of 11 ± 5 µg/mL for another isolate. When evaluating the MIC values for the dual suspensions, the average MIC value for the dual suspensions prepared with the reference strains was higher than for the dual suspension prepared with the clinical isolates, being respectively, 21 ± 9 µg/mL and 16 ± 0 µg/mL.

Regarding MBC determination, for one bacterial species, pexiganan had an average MBC value of 16 ± 0 µg/mL for the reference strain and for the clinical isolate. Relatively to another bacterial species, namely for the reference strain and for the clinical isolate, the average pexiganan MBC value were, respectively, 21 ± 18 µg/mL and 11 ± 5 µg/mL. Results obtained for the combination of the strains were similar to the ones previously obtained for MIC, namely 21 ± 9 µg/mL and 16 ± 0 µg/mL for the dual suspensions composed by the reference strains and by the clinical isolates, respectively.

3.1.2. MBIC and MBEC

The MBIC and MBEC determination allowed observing the effect of pexiganan in the biofilm structure by the bacterial species used in this study (Graphic 1).

The average MBIC values varied among the two clinical isolates and the reference strains, being of 8 ± 0 µg/mL for the reference strain of one bacterial species, 12 ± 6 µg/mL for one clinical isolate, 82 ± 70 µg/mL for another reference strain and 86 ± 36 µg/mL for another clinical isolate. The concentration required to inhibit the dual species biofilms was of 48 ± 23 µg/mL and 64 ± 0 µg/mL, respectively for the dual biofilms formed by the reference strains and for those formed by clinical isolates.

Regarding pexiganan MBEC, the average concentration value for one reference strain was 8 ± 0 µg/mL, for one clinical isolate was 12 ± 6 µg/mL, for another reference strain was 82 ± 70 µg/mL and for other clinical isolate, it was 108 ± 38 µg/mL. For the dual species biofilms, the average concentrations obtained were 96 ± 45 µg/mL for the reference strains and 128 ± 0 µg/mL for the clinical isolates, respectively.

3.2. Antimicrobial Activity of a dual AMP

3.2.1. MIC and MBC

Initially, it was necessary to determine nisin MIC and MBC values to evaluate its effect against planktonic suspensions of the selected bacterial species strains (clinical isolate and reference strain). The results are presented in Table 3.

Table 3. MIC and MBC values for nisin against one bacterial species strains.

Nisin concentrations in MHCA broth		
Bacterial Strains	**MIC (µg/mL)**	**MBC (µg/mL)**
Clinical isolate	5	12.5
Reference strain	12.5	50
Legend. MHCA: Mueller Hinton Cation Adjusted; **MIC:** Minimum Inhibitory Concentration; **MBC:** Minimum Bactericidal Concentration.		

Considering the similarity of the results obtained in this assay (Table 3) with those obtained for the same bacterial strains by Santos *et al*. in 2016, the selected concentration was used in the assays regarding the combined action of nisin plus pexiganan.

3.2.2. MBIC and MBEC

The MBIC and MBEC values for pexiganan combined with nisin against biofilm cultures of bacterial strains are presented in Table 4.

Table 4. MBIC and MBEC results (in average) for pexiganan plus nisin against DFU isolates and bacterial dual species combinations.

Determination of MBIC and MBEC for Pexiganan plus Nisin		
Isolates	**MBIC (µg/mL)**	**MBEC (µg/mL)**
Clinical isolate	1±0 plus 12.5	1±0 plus 12.5
Reference strain	1±0 plus 12.5	1±0 plus 12.5
Clinical isolate	75±49 plus 12.5	96±55 plus 12.5
Reference strain	107±37 plus 12.5	128±0 plus 12.5
Dual species combination by clinical isolates	64±55 plus 12.5	64±55 plus 12.5
Dual species combination by reference strains	43±18 plus 12.5	96±55 plus 12.5
Legend. **MBIC**: Minimum Biofilm Inhibitory Concentration; **MBEC**: Minimum Biofilm Eradication Concentration.		

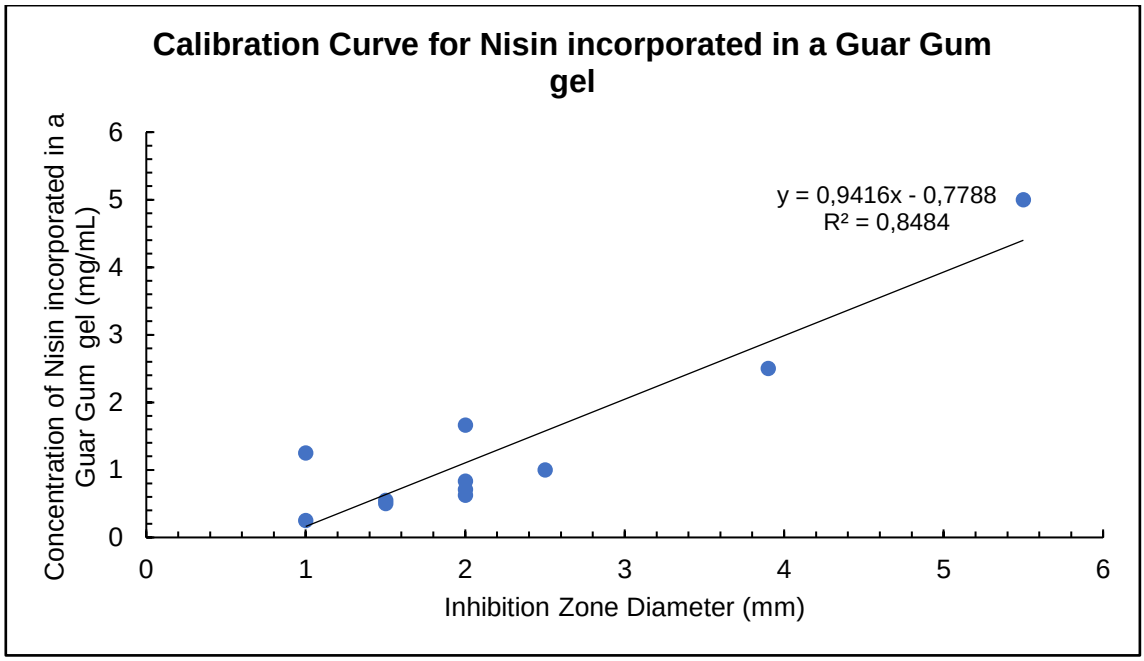
The MBIC and MBEC determination of pexiganan plus nisin (Table 4) allowed observing an eradication effect of one of the bacterial strains. However, inhibitory and eradication potential was also observed against another bacterial strains as well as against both dual biofilms formed by clinical isolates and for those formed by reference strains.

3.3. Collagen DFI 3D Model

3.3.1. Calibration Curves

3.3.1.1. Calibration Curve for Nisin incorporated in a guar gum gel

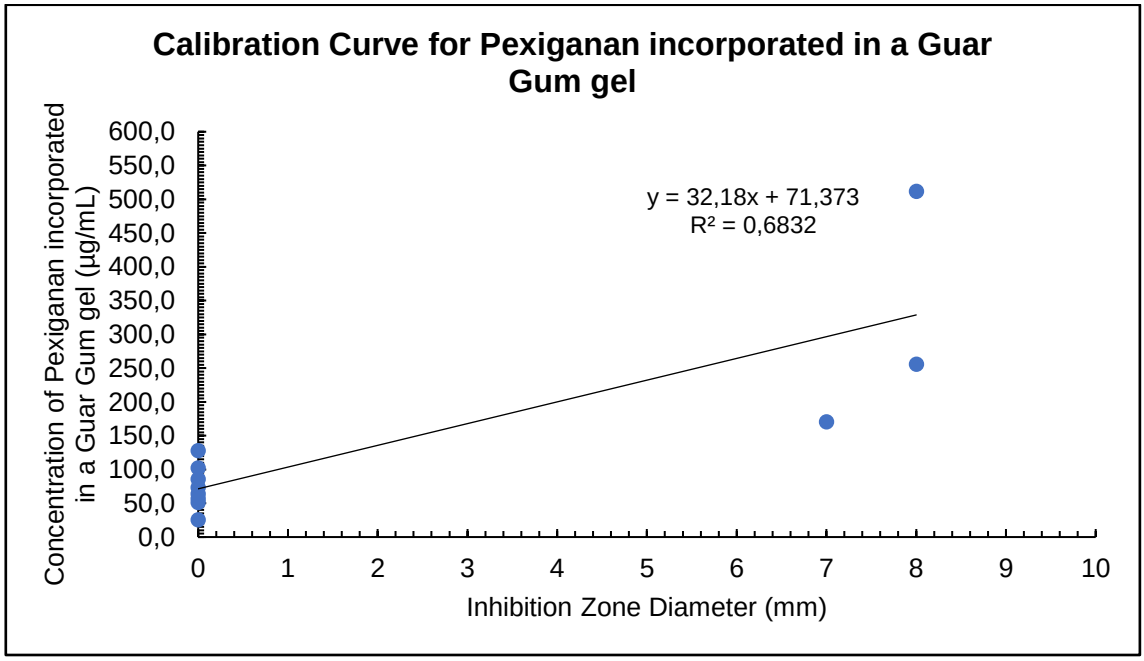
A calibration curve relating the concentration of nisin incorporated in a guar gum gel and the related inhibition zone diameters was established, to allow evaluating AMP diffusion in the DFI 3D model (Graphic 2).



Graphic 2. Calibration Curve for the determination of the concentration of Nisin incorporated in a Guar Gum gel based on its inhibitory activity against a clinical isolate (results in average).

3.3.1.2. Calibration Curve for Pexiganan incorporated in a guar gum gel

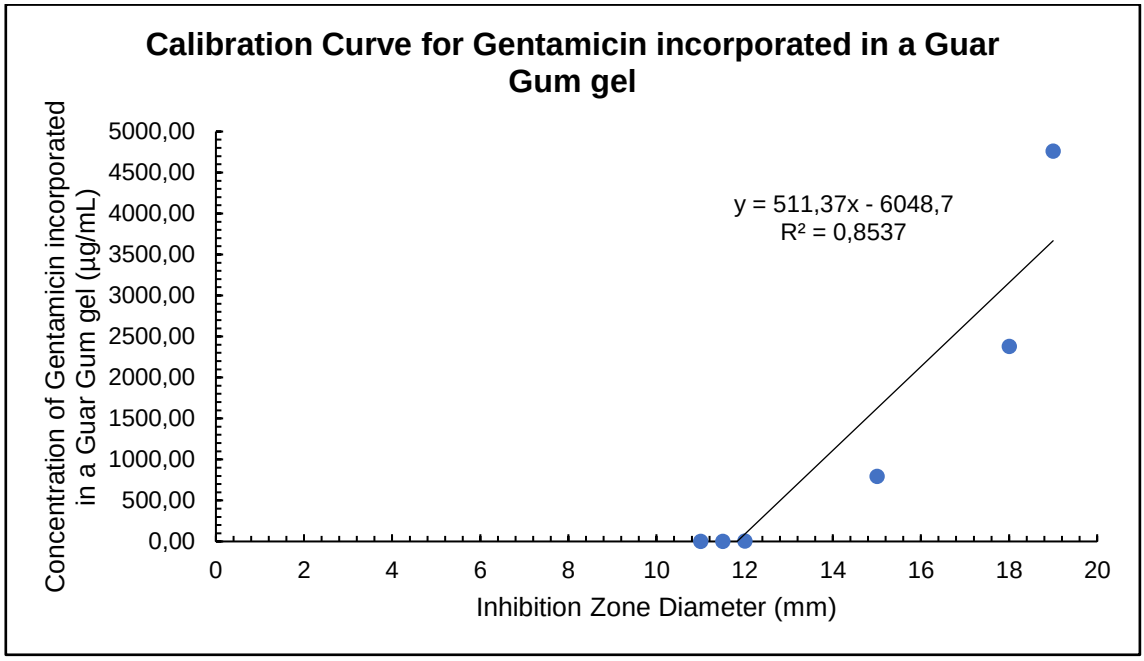
A calibration curve relating the concentration of pexiganan incorporated in a guar gum gel and the diameter of the inhibition zone produced was also established (Graphic 3).



Graphic 3. Calibration Curve for the determination of the concentration of Pexiganan incorporated in a Guar Gum gel based on its inhibitory activity against a clinical isolate (results in average).

3.3.1.3. Calibration Curve for Gentamicin incorporated in a guar gum gel

For the antibiotic diffusion evaluation in the DFI 3D model, a calibration curve correlating the concentration of gentamicin and the diameter of the inhibition zone was established (Graphic 4).



Graphic 4. Calibration Curve for the determination of the concentration of Gentamicin incorporated in a Guar Gum gel based on its inhibitory activity against a clinical isolate (results in average).

3.3.2. Nisin biogel activity and distribution in the DFI 3D Model

3.3.2.1. Evaluation of the Nisin biogel diffusion

Regarding the AMP diffusion evaluation, two assays were performed. The results are presented in Table 5.

Table 5. Evaluation of nisin incorporated in a guar gum gel diffusion (average results for the liquid and solid phases).

Diffusion of Nisin incorporated in a Guar Gum gel					
Assay	**AMP Quantification**	**Liquid Phase**	**Solid Phase**		
			Area 1	**Area 2**	**Area 3**
First	**Ø halos**	2.0 ± 0	2.8 ± 0.35	2.0 ± 0	0
	[AMP] (mg/mL)	1.10	1.85	1.10	0
Second	**Ø halos**	9 ± 6.363	8.0 ± 0.707	5.5 ± 0.707	2.50 ± 0.707
	[AMP] (mg/mL)	7.70	6.75	4.4	1.57

Legend. **Inhibition Zone Diameter**: Ø halos (diameter in mm with standard deviation); **AMP:** Antimicrobial Peptide; **[AMP]:** AMP Concentration in liquid and solid phases of the collagen model; **Areas of the model:** Area 1; Area 2; Area 3.

Results confirm the diffusion of nisin incorporated in a guar gum gel throughout the collagen model (Table 5), namely on Areas 1 and 2, since in Area 3 was not possible to quantify AMP concentration.

Regarding the second assay, it was also possible to confirm the diffusion of nisin incorporated in a guar gum gel in all areas of the collagen model (Table 5).

Considering the liquid phase of each assay (Table 5), it allowed to observe AMP diffusion from the insert to the well.

3.3.2.2. Evaluation of bacterial diffusion

Regarding the evaluation of the diffusion of the bacterial strain, the results are presented in Table 6.

Table 6. Evaluation of bacterial diffusion (average results).

Bacterial Diffusion (CFU/mL)			
Liquid Phase	**Solid Phase**		
	Area 1	**Area 2**	**Area 3**
120	2.3×10^7	3.1×10^7	3.1×10^7
Legend. **Areas of the model:** Area 1; Area 2; Area 3.			

Observing the average results of bacteria diffusion evaluation (Table 6), it was possible to observe that bacteria diffused from the insert to the well and diffused across all areas of the collagen 3D model. In the liquid phase it was also possible to observe bacteria, demonstrating diffusion from the insert to the well (Table 6).

3.3.2.3. Evaluation of the inhibitory activity of a nisin biogel using a DFI 3D model

The inhibitory activity of a nisin biogel against a clinical isolate was tested in two assays. The average results are presented in Table 7.

Table 7. Evaluation of the AMP inhibitory potential for a clinical isolate using a DFI 3D model (results in average according to the performed assays).

Evaluation of nisin biogel inhibitory potential using a DFI 3D model (CFU/mL)					
Assay	**Before AMP addition**	**Liquid Phase**	**Solid Phase**		
			Area 1	**Area 2**	**Area 3**
First	4.2×10^8	3.7×10^7	8.3×10^5	1.7×10^6	5.1×10^6
Second	4.2×10^8	2.9×10^6	2.6×10^4	9.2×10^4	1.9×10^5
Legend. **AMP:** Antimicrobial Peptide; **Areas of the model:** Area 1; Area 2; Area 3.					

Regarding the evaluation of the AMP inhibitory potential using a DFI 3D model, the bacterial diffusion occurred across the collagen 3D model (Table 7). However, in first assay, the bacterial concentration increased from Area 1 (8.3×10^5 CFU/mL) to Area 2 (1.7×10^6 CFU/mL), stabilizing in Area 3 (5.1×10^6 CFU/mL).

The same was observed in the second assay when the AMP was added to the model three times (Table 7). Nevertheless, it is important to refer that in this assay, bacterial counts were always lower than in the first assay, as follows: 2.6×10^4 CFU/mL (Area 1); 9.2×10^5 CFU/mL (Area 2); and 1.9×10^5 CFU/mL (Area 3).

Considering the liquid phase of each assay, it was possible to observe a high bacterial concentration, which demonstrated diffusion from the insert to the well (Table 7).

3.3.2.4. Histochemical Analysis of Nisin biogel inhibitory activity in the collagen 3D DFI model

Regarding histochemical analysis, the results (Figure 5) allowed to observe the clinical isolate in the three areas of the collagen model with the tested protocols.

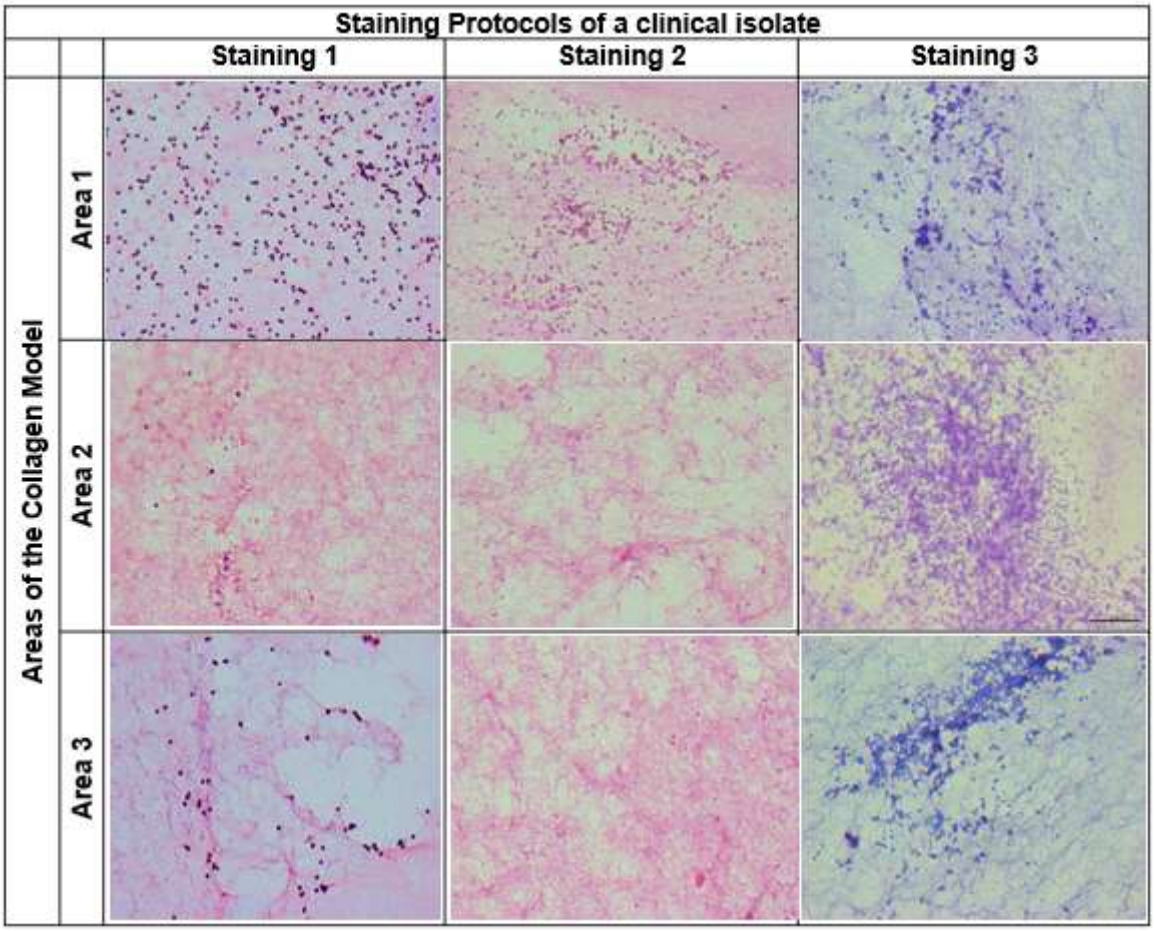


Figure 5. Evaluation of a clinical isolate diffusion in the collagen 3D model through histochemical analysis. **Legend**. **Areas of the collagen model**: Area 1, Area 2, Area 3 (Original, 1000x).

3.3.3. Pexiganan biogel activity and distribution in a collagen DFI 3D Model

3.3.3.1. Evaluation of the Pexiganan biogel diffusion

Regarding the evaluation of pexiganan diffusion in the ulcer 3D model, one assay was performed for pexiganan incorporated in a guar gum gel (Table 8).

Table 8. Evaluation of pexiganan incorporated in a guar gum gel diffusion (average results for the liquid and solid phases).

Diffusion of Pexiganan incorporated in a Guar Gum gel				
AMP Quantification	**Liquid Phase**	**Solid Phase**		
		Area 1	**Area 2**	**Area 3**
Ø halos	0	4.0 ± 0	3.5 ± 0.71	0
[AMP] (µg/mL)	0	200.09	184.0	0

Legend. **Inhibition Zone Diameter**: Ø halos (diameter in mm with standard deviation); **AMP:** Antimicrobial Peptide; **[AMP]:** AMP Concentration in liquid and solid phases of the collagen model; **Areas of the model:** Area 1; Area 2; Area 3.

Results confirmed the diffusion of pexiganan incorporated in a guar gum gel across the collagen 3D model until Area 3. However, in the liquid phase, no inhibition halos were observed, suggesting that pexiganan does not diffused to this phase (Table 8).

3.3.3.2. Evaluation of bacterial diffusion

Regarding the bacterial diffusion evaluation of a clinical isolate, the results are presented in Table 9.

Table 9. Evaluation of bacterial diffusion (average results).

Bacterial Diffusion (CFU/mL)			
Liquid Phase	**Solid Phase**		
	Area 1	**Area 2**	**Area 3**
1.1×10^9	2.0×10^8	1.0×10^{10}	5.7×10^9

Legend. **Areas of the model:** Area 1; Area 2; Area 3.

Observing the results (Table 9), it was possible to observe that this bacterial strain diffused across the collagen 3D model, being the bacterial concentration higher in Area 2. Nevertheless, in the liquid phase, it was also possible to observe a high bacterial concentration.

3.3.3.3. Evaluation of the inhibitory activity of a pexiganan biogel using a DFI 3D model

The inhibitory activity of a pexiganan biogel against the clinical isolates was tested in two assays. The average results are presented in Table 10.

Table 10. Evaluation of the AMP inhibitory potential for the clinical isolates using a DFI 3D model (results in average according to the performed assays).

Evaluation of a pexiganan biogel inhibitory potential using a DFI 3D model (CFU/mL)						
Assays	**Bacterial Strains**	**Before AMP Addition**	**Liquid Phase**	**Solid Phase**		
				Area 1	**Area 2**	**Area 3**
First	Clinical isolate	1.0×10^9	1.9×10^9	2.8×10^8	1.6×10^8	3.2×10^8
Second	Clinical isolate	2.2×10^8	1.1×10^8	7.5×10^6	1.3×10^7	1.5×10^6
	Clinical isolate	5.0×10^8	Uncountable	1.7×10^8	9.0×10^7	3.3×10^8
Legend. **AMP:** Antimicrobial Peptide; **Areas of the model:** Area 1, Area 2, Area 3; **Uncountable**: plates without possibility of counting/number of colonies superior to 300.						

The evaluation of pexiganan incorporated in a guar gum gel inhibitory potential using a DFI 3D model demonstrated bacterial diffusion across the collagen 3D model (Table 10) for both assays. In the first assay, the bacterial concentration was similar among the three areas of the collagen model, as follows: Area 1 (2.8×10^8 CFU/mL); Area 2 (1.6×10^8 CFU/mL) and Area 3 (3.2×10^8 CFU/mL).

Regarding the second assay (Table 10), for one isolate, the bacterial counts decreased from Area 1 (1.7×10^8 CFU/mL) to Area 2 (9.0×10^7 CFU/mL), increasing in Area 3 (3.3×10^8 CFU/mL), in comparison with the first assay. Nevertheless, for the other isolate, the bacterial concentration decreased in the three areas in comparison with the bacterial concentration before the AMP addition, as follows: Area 1 (7.5×10^6 CFU/mL); Area 2 (1.3×10^7 CFU/mL) and Area 3 (1.5×10^6 CFU/mL).

Considering the liquid phase of each assay (Table 10), it allowed to observe a high bacterial concentration, demonstrating diffusion from the insert to the well.

3.3.3.4. Histochemical Evaluation of the pexiganan biogel inhibitory activity in the DFI 3D Model

Analyzing the results of histochemical analysis (Figure 6), the staining protocols allowed the observation of the clinical isolate in the three areas of the model as well as biofilm production.

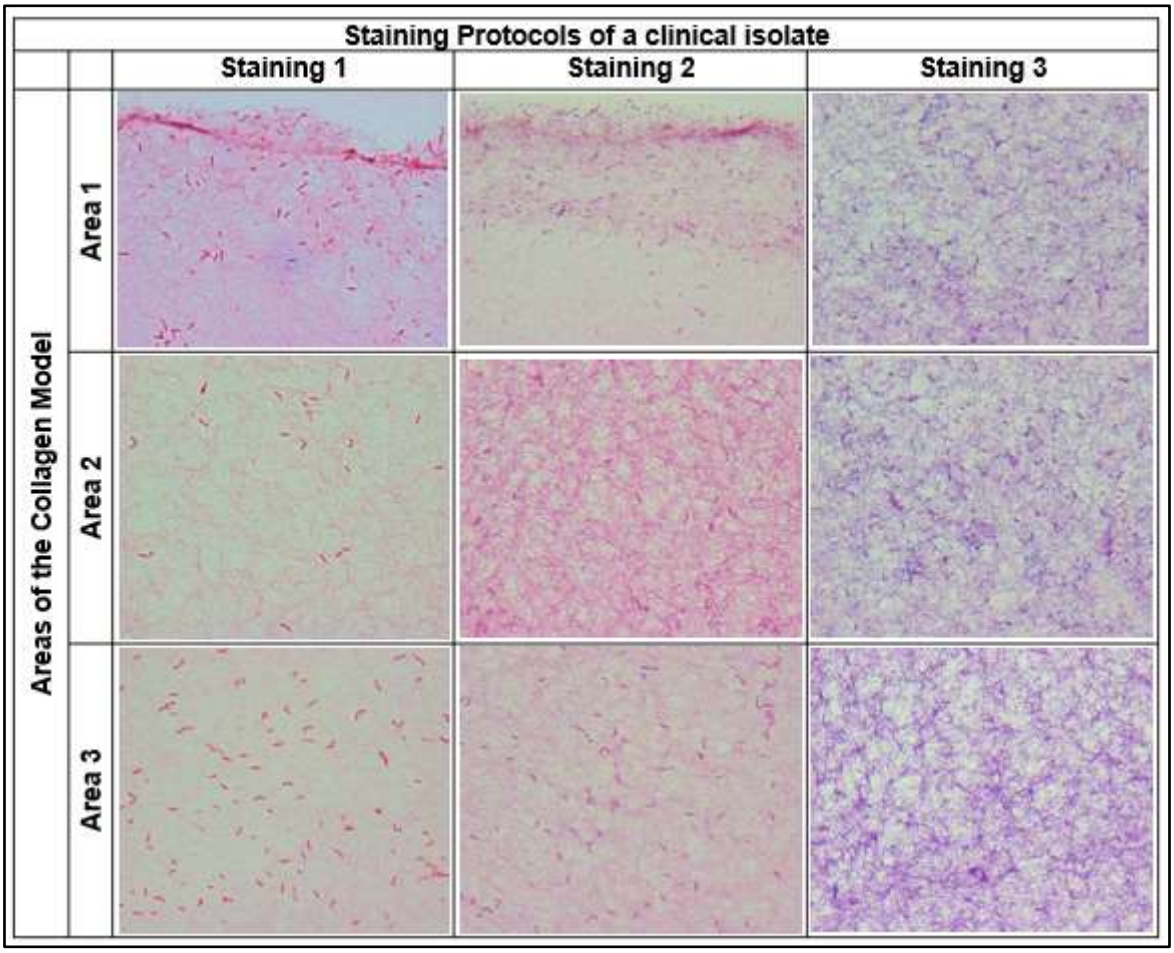


Figure 6. Evaluation of a clinical isolate diffusion in the collagen 3D model through histochemical analysis. **Legend**. **Areas of the collagen model**: Area 1, Area 2, Area 3. (Original, 1000x).

3.3.4. Dual AMP biogel activity and distribution in a collagen DFI 3D model

3.3.4.1. Evaluation of dual species diffusion

Regarding the dual bacterial diffusion evaluation of the clinical isolates, the results are presented in Table 11.

Table 11. Evaluation of a dual inoculum composed by clinical isolates diffusion (results in average).

Evaluation of the dual species diffusion (CFU/mL)					
Bacterial Strains		**Liquid Phase**	**Solid Phase**		
			Area 1	**Area 2**	**Area 3**
Dual inoculum	Clinical isolate	3.7×10^7	5.5×10^7	5.0×10^7	2.8×10^9
	Clinical isolate	2.3×10^8	3.5×10^7	5.5×10^7	2.0×10^9
Legend. **Areas of the model:** Area 1, Area 2, Area 3.					

Analyzing the results presented in Table 11, it was possible to observe that diffusion has occurred across both phases of the collagen model, as both bacterial species were present across all areas of the model with similar bacterial concentrations.

3.3.4.2. Evaluation of the inhibitory activity of a dual AMP biogel using a DFI 3D model

The results from the evaluation of the inhibitory activity of a dual AMP biogel composed by nisin plus pexiganan incorporated in a guar gum gel against the clinical isolates are presented in Table 12.

Table 12. Evaluation of the dual AMP inhibitory potential regarding a dual inoculum of clinical isolates using a DFI 3D model (results in average).

Evaluation of the dual AMP biogel inhibitory potential using a DFI 3D model (CFU/mL)						
Bacterial Strains		**Before dual AMP addition**	**Liquid Phase**	**Solid Phase**		
				Area 1	**Area 2**	**Area 3**
Dual inoculum	Clinical isolate	5.2×10^7	0	0	0	0
	Clinical isolate	3.0×10^8	1.3×10^9	3.6×10^7	1.1×10^8	1.1×10^8
Legend. **AMP:** Antimicrobial Peptide; **Areas of the model:** Area 1, Area 2, Area 3.						

Analyzing the results presented in Table 12, the dual AMP presented a high impact on one of the clinical isolates. In fact, this AMP combination allowed the eradication of this isolate in the collagen model. Nevertheless, the other clinical isolate diffused across the three areas of the collagen model and although the AMPs presented some inhibitory activity against this strain, it was not high enough to eradicate it.

3.3.5. Gentamicin biogel inhibitory activity and distribution in a collagen DFI 3D Model

The inhibitory activity of the antibiotic biogel, namely of gentamicin incorporated in a guar gum gel, was evaluated against the clinical isolates tested individually and combined in a dual bacterial inoculum, being performed two assays.

3.3.5.1. Evaluation of the Antibiotic diffusion

One assay was performed for the evaluation of antibiotic diffusion, which results are presented in Table 13.

Table 13. Evaluation of gentamicin incorporated in a guar gum gel (average results for the liquid and solid phases).

Diffusion of Gentamicin incorporated in a Guar Gum gel				
AB Quantification	**Liquid Phase**	**Solid Phase**		
		Area 1	**Area 2**	**Area 3**
Ø halos	0	0	0	0
[AB] (µg/mL)	0	0	0	0

Legend. **AB**: Antibiotic; **Inhibition Zone Diameter**: Ø halos (diameter in mm with standard deviation); **Areas of the model:** Area 1, Area 2, Area 3.

No inhibition halos were observed when testing the presence of gentamicin in the liquid and solid phases of the collagen models (Table 13). Therefore, these results suggested that gentamicin does not diffuse across the collagen model.

3.3.5.2. Evaluation of the inhibitory activity of a gentamicin biogel using a DFI 3D model

The inhibitory activity of a gentamicin biogel against the clinical isolates was determined using two assays, which results are presented in Table 14.

Table 14. Evaluation of the AB inhibitory potential for the clinical isolates (individually and in a dual inoculum) using a DFI 3D model (results in average according to the performed assays).

Evaluation of gentamicin biogel inhibitory potential using a DFI 3D model (CFU/mL)							
Assay	**Bacterial Strains**		**Before AB addition**	**Liquid Phase**	**Solid Phase**		
					Area 1	**Area 2**	**Area 3**
First	Clinical isolate		8.0×10^7	2.6×10^8	6.6×10^7	1.4×10^8	7.0×10^7
	Clinical isolate		6.5×10^9	6.0×10^9	1.4×10^8	1.7×10^8	5.6×10^8
Second	Clinical isolate		1.6×10^8	1.0×10^8	8.7×10^6	1.2×10^7	3.6×10^7
	Clinical isolate		7.0×10^8	4.5×10^8	8.5×10^7	8.8×10^7	7.8×10^7
	Dual inoculum	Clinical isolate	8.5×10^6	1.1×10^7	7.5×10^6	4.1×10^7	1.5×10^7
		Clinical isolate	6.2×10^8	1.1×10^9	5.2×10^7	3.0×10^8	Uncountable

Legend. **AB**: Antibiotic; **Areas of the model:** Area 1, Area 2, Area 3; **Uncountable**: plates without possibility of counting/number of colonies superior to 300.

Considering the evaluation of the AB inhibitory potential using a DFI 3D model, the bacterial diffusion occurred across the collagen 3D model (Table 14). In the first assay, the bacterial concentration of a clinical isolate increased from Area 1 (6.6×10^7 CFU/mL) to Area 2 (1.4×10^8 CFU/mL), decreasing in Area 3 (7.0×10^7 CFU/mL). For another clinical isolate, the bacterial

concentration among the three areas presented some variability, as follows: 1.4 × 10^8 CFU/mL (Area 1); 1.7 × 10^8 CFU/mL (Area 2) and 5.6 × 10^8 CFU/mL (Area 3).

In the second assay, the results were similar (Table 14) The gentamicin biogel presented a higher inhibitory activity against one clinical isolate in comparison with the other one without promoting its complete eradication. In fact, the first isolate concentration presented a ten-fold decrease in Area 1.

Concerning the dual inoculum and comparing both isolates (Table 14), it was also observed that gentamicin incorporated in a guar gum gel does not present an effective inhibitory activity against these two isolates.

Regarding the liquid phase of both assays (Table 14), it was possible to observe a high bacterial concentration of both isolates as well as in the dual inoculum, demonstrating that diffusion occurred between the insert and the well.

3.3.6. Triple biogel supplemented with Nisin, Pexiganan and Gentamicin activity and distribution in a collagen DFI 3D model

3.3.6.1. Evaluation of the inhibitory activity of a biogel with multiple combination of AMP plus Antibiotic using a DFI 3D model

The inhibitory activity of a biogel containing nisin, pexiganan and gentamicin was determined using the clinical isolates (Table 15).

Table 15. Evaluation of a triple guar gum biogel supplemented with Nisin, Pexiganan and Gentamicin inhibitory potential for a dual inoculum of clinical isolates using a DFI 3D model (results in average).

Evaluation of a triple guar gum biogel supplemented with Nisin, Pexiganan and Gentamicin inhibitory potential using a DFI 3D model (CFU/mL)							
Assay	**Bacterial Strains**		**Before antimicrobial addition**	**Liquid Phase**	**Solid Phase**		
					Area 1	**Area 2**	**Area 3**
	Dual inoculum	Clinical isolate	3.0 × 10^7	5.0 × 10^6	1.9 × 10^6	8.5 × 10^5	6.0 × 10^6
		Clinical isolate	1.7 × 10^9	1.4 × 10^9	2.5 × 10^8	1.8 × 10^8	1.8 × 10^8
Legend. **Areas of the model:** Area 1, Area 2, Area 3.							

Analyzing the results presented in Table 15, it was possible to observe, a higher decrease of bacterial concentration of one of the clinical isolates in comparison with the other one. Bacterial diffusion occurred across the collagen model, with bacterial concentrations decreasing ten to twenty-fold.

Regarding one of the isolates, the bacterial concentration was maintained throughout the model, with a ten-fold decrease in all areas, being also in higher concentration in the liquid phase (Table 15).

Therefore, these results confirmed that both bacterial species selected for this study are able to diffuse across the collagen 3D model and demonstrated that the triple combination of nisin plus pexiganan plus gentamicin incorporated in a guar gum gel did not allow the complete inhibition of the dual inoculum.

3.3.6.2. Histochemical Evaluation of the inhibitory activity of the biogel with Nisin plus Pexiganan plus Gentamicin in the DFI 3D Model

Regarding histochemical analysis results (Figure 7), it was possible to observe the clinical isolates diffusion in the three areas of the model as well as biofilm production using the protocols.

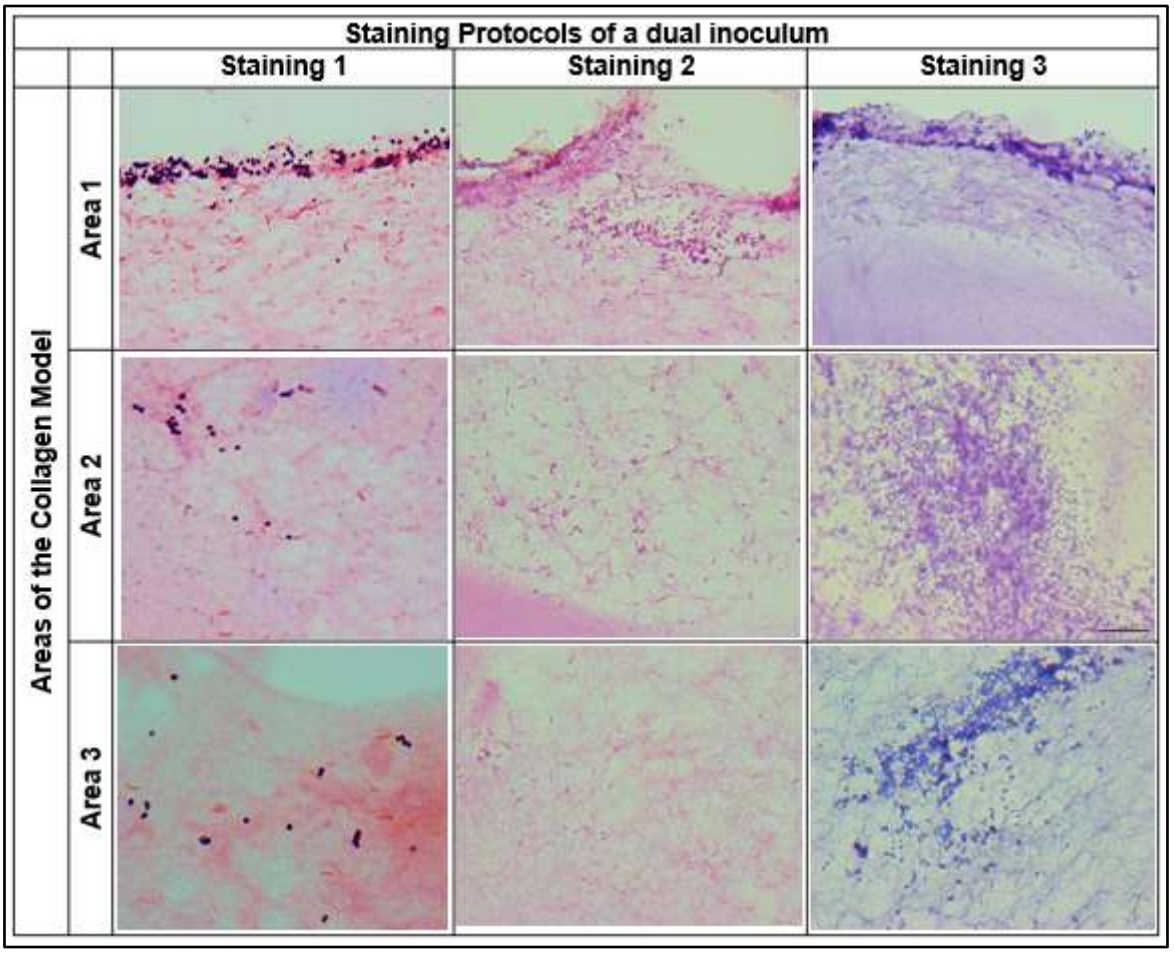


Figure 7. Evaluation of a dual inoculum of clinical isolates diffusion in the collagen 3D model through histochemical analysis. **Legend**. **Areas of the collagen model:** Area 1, Area 2, Area 3 (Original, 1000x).

CHAPTER 4

Discussion

Considering the increased resistance to conventional therapeutics, it is required to find new alternatives for DFI treatment (Mendes *et al*., 2014; Santos *et al*., 2016), such as antimicrobial peptides (AMP) (Andersson *et al*., 2016; Kumar *et al*., 2018), that present an inhibitory potential against a wide range of microorganisms (Cotter *et al*., 2013), allowing infections control (Hancock, 2000; Shin *et al*., 2015), being also associated to the enhancement of the wound healing process (Hancock, 2000).

4.1. Antimicrobial Activity of the antimicrobial peptides Pexiganan and Nisin

The increased resistance to conventional antibiotics, demonstrated by some DFI bacterial strains due to several mechanisms including biofilm formation, demands new alternatives for DFI treatment (Mendes *et al*., 2014; Santos *et al*., 2016), such as antimicrobial peptides (AMP) (Andersson *et al*., 2016; Kumar *et al*., 2018).

Considering AMP inhibitory potential (Kumar *et al*., 2018), it is necessary to refer that its action mode presents several steps (Melo *et al*., 2009; Andersson *et al*., 2016). Initially, it occurs an electrostatic interaction between the AMP and the bacterial cell surface, namely with the teichoic acids of the Gram-positive bacterial membrane and the lipopolysaccharides (LPS) components of the Gram-negative bacterial membrane (Guilhelmelli *et al*., 2013; Andersson *et al*., 2016). Then, an accumulation of AMP concentration in the bacterial surface is required to reach a threshold level that triggers an antibacterial action (Melo *et al*., 2009; Andersson *et al*., 2016; Kumar *et al*., 2018). Afterwards, a rearrange occurs in the peptide structure, namely in the amphipathic region, allowing the positively charged molecules to interact with the negatively charged phospholipids (Zasloff, 2002; Baltzer & Brown, 2011; Kumar *et al*., 2018). Finally, the negatively internal charge of the bacteria cell leads to the AMP uptake, since the internal environment presents higher concentrations of negative charges in comparison with the exterior (Melo *et al*., 2009).

The AMP pexiganan (Ge *et al*., 1999) is one possible alternative to conventional antibiotics. It exhibits a broad activity range, namely against Gram-negative and Gram-positive bacteria (Gopinath *et al*., 2005; Gottler & Ramamoorthy, 2009). Until now, the development of resistance against pexiganan was not described, rendering this AMP a very promising molecule to be applied in the treatment of infected diabetic foot ulcer (DFU) (Gottler & Ramamoorthy, 2009).

Pexiganan inhibitory action occurs through the formation of toroidal pores in the cytoplasmic membrane (Gopinath *et al*., 2005; Gottler & Ramamoorthy, 2009). Being an amphipathic peptide, this AMP aligns with the bacterial membrane surface, followed by the interaction between the bacterial membrane (phospholipids) and the AMP, causing a curvature on the lipid bilayer, allowing AMP intake (Hancock, 2001; Gottler & Ramamoorthy, 2009; Kumar *et al*., 2018).

In this study, the inhibitory action of pexiganan was tested against two bacterial strains, belonging to a collection of DFI isolates (Mendes *et al*., 2012).

Comparing the Minimum Inhibitory Concentration (MIC) values of pexiganan against the clinical isolates, it was possible to observe that the MIC value for one of the isolates was higher in comparison with the other one. The isolates tested belong to two different bacterial groups, namely

Gram-negative and Gram-positive bacteria (Schmidtchen *et al*., 2014). Considering the cell wall properties, the peptidoglycan layer is considered an essential component of the cell wall (Goering *et al*., 2013c), and, in the case of Gram-positive bacteria, its thickness is superior when compared with Gram-negative bacteria. Regarding its composition in teichoic acids, it is also higher, conferring negatively charge properties as well as membrane support (Guilhelmelli *et al*., 2013; Schmidtchen *et al*., 2014; Percy & Gründling, 2014). Nevertheless, in the case of Gram-negative bacteria, an outer membrane is also present, being negatively charged due to the presence of LPS, contributing for the structural integrity and, consequently, for bacteria protection (Beverigde, 1999; Raetz & Whitfield, 2002; Erridge *et al*., 2002; Andersson *et al*., 2016). Considering the pexiganan mode of action, the MIC value for pexiganan obtained in this study against a specific bacterial species could be due to an increase of the Lipid A concentration in the outer membrane that contributes for an increase in the density of the membrane structure, diminishing its permeability (Erridge *et al*., 2002). Additionally, the higher pexiganan MIC value could be due to the chemical composition of Lipid A, in which the addition of compounds (amines) may result in a mask effect of the negative charge of this lipid (Guilhelmelli *et al*., 2013). Regarding the other bacterial species, the presence of teichoic acids in the peptidoglycan layer allow the interaction between the AMP and the bacterial membrane (Guilhelmelli *et al*., 2013), which could have allowed a higher pexiganan inhibitory action.

Nevertheless, for the two strains, the results obtained for pexiganan MIC were in the range of 8–16 µg/mL (Ge *et al*., 1999), demonstrating its inhibitory potential against the planktonic forms. Considering toxicity, several studies had determined that a pexiganan concentration of at least 250 µg/mL was required to induce 100% of hemolysis in human cells (Gottler & Ramamoorthy, 2009). Considering our MIC values for pexiganan, namely the range of 8-11 µg/mL for both strains tested individually and the range 16–21 µg/mL for the dual species suspensions, no toxic effects are expected.

Regarding the dual species suspensions, the pexiganan MIC values obtained were higher than those obtained for the single suspensions, which was expected since it is described that monocultures of these bacterial species in planktonic form compared with their combination in a co-culture requires lower concentrations of an antimicrobial compound (DeLeon *et al*., 2014).

Comparing pexiganan MIC results of the dual species suspension formed by the reference strains and the one formed by the clinical isolates, the suspension composed by the reference strains revealed higher resistance.

Relatively to pexiganan Minimum Bactericidal Concentration (MBC) values, the results obtained for individual suspensions of one bacterial species were similar, being approximately two times higher than the MIC ones. For one reference strain, the result obtained was higher to those previously obtained for the remaining strains, being approximately three times higher than the MIC values. Considering that the bactericidal effect is only achieved when MBC is not more than four times higher than the MIC value (French, 2006), results demonstrate that pexiganan acts as a bactericidal agent against both strains. However, for one clinical isolate, the MBC value obtained was equal to the MIC one, which means that the AMP inhibitory and bactericidal effect for this strain occurs at the same concentration, which was also observed for both dual species suspensions. These results were not

expected, since clinical isolates usually present higher resistance ability in comparison with reference strains (Hotterbeekx *et al*., 2017).

One of the best described bacterial resistance mechanisms is biofilm production, being necessary higher concentrations of an AMP or antibiotic, namely 10 to 1000 times higher, to eradicate cells that are organized in biofilms when compared with planktonic cells (Spoering & Lewis, 2001; Olson *et al*., 2002; Jefferson *et al*., 2005; Field *et al*., 2016).

Concerning Minimum Biofilm Inhibitory Concentration (MBIC) values, higher concentrations of pexiganan were obtained against a bacterial species in comparison with other one . The biofilm structure provides mechanical stability and protection against environmental stressful conditions (Dickschat, 2010), increasing bacterial resistance to antimicrobial compounds (Arciola *et al*., 2012; Guilhelmelli *et al*., 2013; Banu *et al*., 2015), impairing its action (Hotterbeekx *et al*., 2017). In the case of *P. aeruginosa*, the presence of alginate in the biofilm may constitute an additional protection mode against AMP action, since alginate is not only able to mimetize the bacterial membrane in the interaction with the AMP but also prevents AMP diffusion and consequently, its action (Guilhelmelli *et al*., 2013). Therefore, this ability could increase the bacterial resistance of this bacterial species in comparison with another one to pexiganan action mode.

Nevertheless, pexiganan MBIC values against the clinical isolates were higher, which could be explained since in planktonic form, these isolates demonstrated higher resistance (Hotterbeekx *et al*., 2017). Therefore, with the biofilm production, the resistance increased, as expected.

Comparing a reference strain with the clinical isolate of the same bacterial species , the same pexiganan MIC value was observed; however, pexiganan MBIC value for the clinical isolate was two times higher than the one obtained for the reference strain. These results demonstrate that the inhibition of planktonic cells and biofilm structures produced by the reference strain occurs at the same AMP concentration, what was not expected considering biofilm properties (Guilhelmelli *et al*., 2013; Banu *et al*., 2015). Nevertheless, it is described that AMPs could act in biofilm structures with equal or higher MIC values, promoting the inhibition of the biofilm (Batoni *et al*., 2016). Therefore, our results suggest that pexiganan, until now with unknown resistant mechanisms (Gottler & Ramamoorthy, 2009), could constitute a promising antimicrobial compound that is not only able to inhibit its planktonic cells but also its biofilm structures.

For the dual bacterial suspensions, MBIC values were also significantly higher for the suspension composed by the clinical isolates, being in accordance to what was previously described (Hotterbeekx *et al*., 2017) as well as the additional protection provided by the biofilm (Guilhelmelli *et al*., 2013; Banu *et al*., 2015).

Considering pexiganan Minimum Biofilm Eradication Concentration (MBEC) results, for a reference strain, the MIC, MBIC and MBEC values were similar, demonstrating that both inhibitory effect of planktonic cells and of cells organized in a biofilm structure as well as biofilm eradication occurs with the same AMP concentration. Regarding the clinical isolate, MBIC and MBEC concentrations were similar, as also observed regarding other reference strain, demonstrating that the inhibition and eradication of the biofilm structure occurs at the same AMP concentration. Since this particular clinical isolate is classified as a weak biofilm-producer strain (Mottola *et al*., 2015) and

considering pexiganan action mode, it could possibly bind to the extracellular matrix of the biofilm, impairing its establishment as well as its maintenance (Gottler & Ramamoorthy, 2009; Batoni *et al*., 2016). However, for the other clinical isolate, MBEC values were higher, being in accordance to what was previously referred, regarding biofilm structure characteristics and clinical isolate higher resistance (Guilhelmelli *et al*., 2013; Hotterbeekx *et al*., 2017).

For the dual bacterial suspensions, the pexiganan MBEC values were higher for the suspension composed by the clinical isolates in comparison with the one formed by the reference strains as expected, considering the higher resistance of the clinical isolates and biofilm production (Guilhelmelli *et al*., 2013; Hotterbeekx *et al*., 2017). In conclusion, pexiganan constitutes a promising AMP for DFI treatment. Nevertheless, considering the number of isolates tested as well as the associated standard deviation, more isolates should be tested in order to confirm these results.

It has been described that the combination of AMPs could enhance their inhibitory potential (Cavera *et al*., 2015; Grassi *et al*., 2017). Consequently, the antimicrobial activity of a combination of pexiganan plus nisin was evaluated.

Nisin is an AMP that is mainly active against Gram-positive bacteria (Moual *et al*., 2013) and its antimicrobial activity was already demonstrated regarding DFI isolates (Santos *et al*., 2016). It acts through the interaction with lipid II, inhibiting the cell wall synthesis (bacteriostatic effect) or through the formation of pores, killing the bacteria (bactericidal effect) (Breukink & Kruijff, 2006; Okuda *et al*., 2013; Kumar *et al*., 2018). As previously described for this bacterial group, the peptidoglycan presents teichoic acids in its composition, conferring negative charge to the bacterial membrane (Guilhelmelli *et al*., 2013), allowing the interaction between nisin and lipid II, leading to conformational alterations of the cytoplasmic membrane and to pore formation (Breukink & Kruijff, 2006; Zhou *et al*., 2014).

Regarding MIC determination, although it was already performed in a previous work (Santos *et al*., 2016), the broth medium used in those assays was different. As in this study Mueller-Hinton Cation Adjusted broth (MHCA) was used for determining pexiganan MIC, MBC, MBIC and MBEC values, it was necessary to determine the MIC values of nisin against the bacterial strains suspended in this broth medium. Nisin MIC values against MHCA bacterial suspensions were similar to those obtained for Brain Heart Infusion broth (BHI) (Santos *et al*., 2016), allowing to adapt the previously determined concentrations to this work, in order to perform the evaluation of the inhibitory potential of a dual suspension of pexiganan and nisin.

The pexiganan plus nisin MBIC and MBEC values obtained for a bacterial species strains were lower in comparison with the ones obtained with only pexiganan, suggesting that for these bacterial strains, the presence of nisin at MIC value decreases the concentration of pexiganan required for the inhibition and eradication of biofilms produced by the clinical isolate as well as the reference strain. Therefore, results suggest that the combination of these AMPs presents a stronger inhibitory effect (Worthington & Melander, 2013; Yu *et al*., 2016) when compared with a single AMP suspension. These results are consistent with the fact that both nisin and pexiganan act against Gram-positive bacteria (Gottler & Ramamoorthy, 2009; Moual *et al*., 2013).

Regarding another bacterial species suspensions, the pexiganan plus nisin MBIC value for the clinical isolate was inferior to the one obtained with only pexiganan, demonstrating that this dual AMP

solution was more effective. As nisin does not inhibit Gram-negative bacteria (Moual *et al*., 2013), results were expected to be similar to the ones obtained with a single AMP suspension, which may suggest a synergistic effect among pexiganan and nisin (Worthington & Melander, 2013; Yu *et al*., 2016). A synergistic interaction between two antimicrobial compounds exhibits a stronger effect of the bacterial strains, and could be evaluated through pharmacodynamics assays, relating the drug dosage and the bacterial growth or dead (Yu *et al*., 2016). However, for the reference strain, MBIC and MBEC values were higher for the dual AMP solution concentrations, what was not expected since clinical isolates should present higher resistance (Hotterbeekx *et al*., 2017).

Nevertheless, MBIC and MBEC results for the dual species combinations suggest that the presence of nisin could influence pexiganan biofilm inhibition and eradication potential, which could be explained by the action range of each AMP (Gottler & Ramamoorthy, 2009; Moual *et al*., 2013) as well as the resistance mechanisms associated (Guilhelmelli *et al*., 2013; Hotterbeekx *et al*., 2017).

The production of biofilm provides protection and stability for the bacterial species involved (Banu *et al*., 2015). Additionally, it is known that the stability of polymicrobial biofilms depends on the communication mechanisms developed between the different bacterial species, namely through quorum-sensing systems. These systems are based on the production of signaling molecules at a threshold level of cell density that leads to the coordination of the behavior of the bacteria in the community (Keller & Surette, 2006; Peters *et al*., 2012). In this case, the dual suspensions are composed by two bacterial species and the communication among these two different groups can occur through autoinducers-2 (AI-2), molecules that are common among them due to the conservation of the *lux*S gene (Keller & Surette, 2006).

In conclusion, the dual AMP solution was more effective in the eradication of the biofilm structure produced by one bacterial species strains as well as in the inhibition and eradication of the biofilm structure produced by the dual bacterial suspensions, confirming its potential as an alternative therapeutic to conventional antibiotic for the treatment of biofilm related infections, as frequently observed in DFI.

4.2. Collagen DFI 3D Model

The development of a three-dimensional (3D) representation of an ulcer (Price *et al*., 2016) was considered important to evaluate several parameters that are directly related with the success of diabetic foot infection (DFI) treatment, such as the diffusion of bacteria, antimicrobial peptides (AMP) and antibiotics (AB). Therefore, a 3D ulcer model aiming at better mimetizing the *in vivo* conditions of a diabetic foot ulcer (DFU) was developed using collagen. These assays were performed using AMP at their Minimum Biofilm Eradication Concentration (MBEC) value, aiming at guaranteeing biofilm eradication, which would be essential for DFI treatment (Arciola *et al*., 2012; Banu *et al*., 2015; Jneid *et al*., 2017), while for antibiotics it was used their Minimum Inhibitory Concentration (MIC) value, as it is the one used in clinical practice (Wiegand *et al*. 2008).

The 3D model was established using collagen, as it is the most abundant protein (about 25%) in the human body, being present in connective tissue, bones and skin. Additionally, it is considered a major natural and structural protein of the organs, being also involved in several steps of the wound healing process, such as the stimulation of cellular migration and the development of new tissue at the wound site (Ruszczak & Friess, 2003; Fleck & Simman, 2010; Gorgieva & Kokol, 2011).

From the 31 types of collagen that are distributed by several classes, collagen type I constitutes the most abundant class in the human body, belonging to the fibril-forming collagen class (Cooper & Hausman, 2013). This type of collagen is composed by two identical polypeptides chains, with a third polypeptide chain that presents a different sequence (Walters & Stegemann, 2014).

The formation of collagen occurs inside fibroblast cells through several stages (Fleck & Simman, 2010; Gorgieva & Kokol, 2011). At the early stages, occurs the formation of a repeated amino acid sequence with around 1000 amino acids, namely the sequence Glycine (Gly)-X-Y, in which Gly is the repetitive unit (being present in every third position of the sequence), while X and Y can vary; in general, X corresponds to proline (Pro) and Y corresponds to hydroxyproline (Hyp). Regarding the function of each amino acid in the final structure of collagen, Gly allows the compaction of the three polypeptide chains and posterior rotation of the helical form whereas Pro and Hyp allow the stabilization of the helical conformations. Through inter and intra-molecular bonds that contribute for the stabilization of the molecule, three polypeptide chains are formed and undergo some rearrangements in order to form microfibrils (Gorgieva & Kokol, 2011; Cooper & Hausman, 2013; Walters & Stegemann, 2014).

After the secretion of the previous structures, it occurs the association into fibrils, which are stabilized through covalent cross-links. These structures can undergo new rearrangements and be assembled into fibers (Fleck & Simman, 2010; Gorgieva & Kokol, 2011; Cooper & Hausman, 2013). These types of structures contribute for the tensile strength of the skin, which is required for the support of specialized skin structures, including organs (Fleck & Simman, 2010).

During this work, the optimization of the polymerization of the collagen for the 3D model was considered an important step since it could influence the diffusion of the AMP, antibiotic and bacteria throughout the model. Several parameters must be considered in the collagen polymerization process, namely pH, temperature and rate of polymerization (Antoine *et al*., 2014; Walters & Stegemann,

2014). It is described that collagen is maintained solubilized at low temperature and low pH values which prevent the polymerization process, whereas high temperature and high pH values promote the aggregation and the creation of crosslinks bonds between the molecules, allowing the formation of a hydrogel structure (Walters & Stegemann, 2014). For the induction of fibrils formation, several neutralizing agents can be used, such as sodium hydroxide (NaOH), so that during the polymerization process the hydrogels can be converted into more solid structures (Walters & Stegemann, 2014). Therefore, to obtain a proper consistency of the collagen 3D model, several adjustments had to be made regarding the available protocols.

Regarding the protocol described by Price *et al*. (2016), it was necessary to increase the incubation period for polymerization, allowing to obtain a more consistent collagen 3D model. Also, consistence was improved by adjusting the solution pH to 7.5, which was achieved by eliminating the addition of acetic acid at 0.02N from the final suspension of collagen and by adjusting NaOH concentration and volume.

It is important to refer that other strategies could be used to improve the consistency of the collagen model namely the use of chemical agents, such as carbodiimides (Chattopadhyay & Raines, 2014; Walters & Stegemann, 2014; Davidenko *et al*., 2015). Carbodiimides are used as chemical cross-links to improve the structural stability of biomaterials, such as collagen (Everaerts *et al*., 2007; Chattopadhyay & Raines, 2014; Davidenko *et al*., 2015).

The improved 3D collagen DFU model was then used to evaluate the distribution and the inhibitory activity of a guar gum gel supplemented with antimicrobial compounds, individually or in several combinations. In the first assay, the diffusion of a guar gum biogel supplemented with nisin was evaluated. Results showed that AMP diffusion occurred throughout all areas of the collagen 3D model except in Area 3, which could be due to the collagen polymerization that promotes the formation of a cross-linked matrix of fibrils (Werthén *et al*., 2010; Antoine *et al*., 2014), and, consequently, could impair AMP diffusion.

A second assay was performed by applying the supplemented guar gum gel three times to the 3D ulcer model. AMP diffusion occurred across the three areas of the model, but the concentration of AMP that reached Area 3 was lower in comparison with Areas 1 and 2, as expected.

The presence of AMP in deeper layers of the collagen 3D model confirms the potential of the nisin biogel to be applied *in vivo* for DFI treatment. Moreover, the concentration of AMP that reached Area 3 in the second assay was superior to the MIC values previously determined by Santos *et al*. (2016) for nisin incorporated in a guar gum gel.

The final aim of the development of this gel is its topical application to infected mucosa. The topical application of nisin was already demonstrated to be effective in reducing mastitis signs as well as staphylococci concentration in breast milk from women treated with this AMP (Fernández *et al*., 2008; Shin *et al*., 2015).

At the concentration used in the collagen model, the application of this supplemented guar gum gel can be considered to have a low toxic potential. In fact, considering the acceptably daily intake (ADI) of 0 to 2 mg/kg defined by WHO and FAO in 2013, the nisin concentration obtained could be administrated to an average person of 60 kg without toxic effects. It is important to refer that this

ADI value was determined for the oral administration of nisin (WHO & FAO, 2013), but they can be extrapolated for its topical application to DFI, considering that the absorption by digestive tract mucosa is similar (Santos *et al*., 2016). Moreover, the widely application of nisin as food preservative suggests that it could be safely used not only in food industry as well as in the clinical setting (Fernández *et al*., 2008; Shin *et al*., 2015). Therefore, the application of nisin incorporated in a guar gum gel with the aim of topical application to DFI could be considered safe and effective for DFI patients (Santos *et al*., 2016).

Considering the evaluation of a bacterial species diffusion, bacterial diffusion was observed across the three areas of the collagen model, increasing from Area 1 to Area 2 and stabilizing in Area 3. Characterized as a non-motile species (Baird-Parker, 1972), it was not expected that it was able to diffuse across all areas of the collagen model, which may have been influenced by the polymerization process (Antoine *et al*., 2014).

Afterwards, the model was used to evaluate the inhibitory activity of nisin by mimicking its application to infected DFU with a bacterial species. This effect was tested using two protocols. Some variability was found across the 3D model, which could be due to the diffusion ability of bacteria, to the AMP action and to the properties of collagen matrice (Antoine *et al*., 2014). In general, a lower concentration of bacteria was detected in the three areas of the collagen model when the AMP was applied three times. These results were expected, since a high AMP concentration was present in Area 1, where it was four times higher in comparison with the first assay.

Another assay was performed aiming at the evaluation of pexiganan and bacterial species distribution throughout the model and of the inhibitory activity of this AMP. Regarding pexiganan diffusion, it was possible to observe the presence of this AMP until Area 3 of the collagen model, similarly to what was previously mentioned for nisin biogel assay (Antoine *et al*., 2014).

Regarding the evaluation of the clinical isolate diffusion, bacteria were able to spread across the three areas of the collagen model, and their concentration increased from Area 1 to Area 2 and stabilized in Area 3. This bacterial species presents a flagellum, being motile, which may increase its diffusion ability (Goering *et al*., 2013a). However, although both bacterial species presented similar diffusion ability, the polymerization process could have influenced their diffusion (Antoine *et al*., 2014).

Considering the inhibitory ability of pexiganan in relation to the clinical isolate, a single addition of pexiganan did not allow the eradication of this isolate from the model. Pexiganan is described as an AMP with an inhibitory activity against Gram-positive and Gram-negative bacteria. However, as previously referred, the cell wall composition of Gram-negative bacteria is more complex in comparison with Gram-positive bacteria, which may have influenced pexiganan action (Gottler & Ramamoorthy, 2009; Goering *et al*., 2013c; Schmidtchen *et al*., 2014), together with the presence of an increased Lipid A concentration (Erridge *et al*., 2002) or with modifications on Lipid A (Guilhelmelli *et al*., 2013).

The inhibitory potential of pexiganan against both isolates was also evaluated (independently). Results suggested that pexiganan incorporated in a guar gum gel presented a higher inhibitory activity against a clinical isolate in comparison with the other one. This may be due to not only to the fact that the interaction between bacteria and the AMP depends on the bacterial groups, since in Gram-positive

bacteria occurs through teichoic acids and in Gram-negative bacteria occurs through LPS present in the outer membrane, but also due to differences in the biofilm production mechanisms. In a bacterial species, biofilm production involves the presence of alginate, which impairs the AMP action as previously referred (Guilhelmelli *et al*., 2013). Additionally, pexiganan may not have been able to correctly align with the bacterial membrane surface and then promote a rearrangement between the bacterial membrane (phospholipids) and the AMP, not allowing its intake and consequently the formation of pores in the cytoplasmic membrane (Hancock, 2001; Gottler & Ramamoorthy, 2009).

Afterwards, a guar gum simultaneously supplemented with nisin and pexiganan was prepared, aiming at producing a biogel with increased antimicrobial potential. This double gel was tested against the two bacterial species under study after evaluating the simultaneous diffusion of both isolates throughout the model, being observed that both species diffused across the collagen model as previously described.

The dual AMP gel was added to the polymicrobial DFI model for three times, being observed that it presented an effective inhibitory action against a clinical isolate, promoting the eradication of this strain in all areas of the model, which allowed to confirm the higher inhibitory effect of this dual AMP biogel (Worthington & Melander, 2013; Yu *et al*., 2016). However, the other clinical isolate was not eradicated from the ulcer model, as it remained in the three areas of the collagen model. This may be due to the fact that Gram-negative bacteria are only inhibited by pexiganan, while Gram-positive bacteria are inhibited by both pexiganan and nisin (Ge *et al*., 1999; Santos *et al*., 2016). Additionally, this bacterial species presents a higher survival ability which could be due to its ability to produce toxins that can also inhibit other bacterial species (Nair *et al*., 2014; Hotterbeekx *et al*., 2017), as well as its cell wall properties and biofilm production ability, as previously referred (Guilhelmelli *et al*., 2013).

Therefore, a final assay was performed, with the further incorporation of an antibiotic in the guar gum gel aiming at promoting the eradication of one of the isolates from the model. Gentamicin was the antibiotic chosen for this assay, as it constitutes a promising treatment for topical application to diabetic foot infections treatment (Lipsky *et al*., 2012).

Gentamicin MIC values were previously determined by our research team (Mottola *et al*., 2016), in which clindamycin and vancomycin were also tested. However, clindamycin is mainly administered orally, intravenously or intramuscularly, being the oral route the most frequently used (Goering *et al*., 2013b) and vancomycin is administered through the parenteral route (Kosinsky & Lipsky, 2010).

First, the distribution of a gentamicin biogel in the collagen DFI 3D model was evaluated, being observed that gentamicin did not diffuse throughout the collagen model, which may have been influenced by the polymerization process of the collagen matrice (Antoine *et al*., 2014).

The inhibitory activity of gentamicin incorporated in a guar gum gel was also evaluated and did not present a high antibacterial effect, particularly against the aimed isolate. This antibiotic acts through the inhibition of protein synthesis (Goering *et al*., 2013b), being active against Gram-negative and Gram-positive bacteria (Duarte & Gonçalves, 2011; Goering *et al*., 2013b). However, this bacterial species resistance to aminoglycosides was already described and related to the presence of the outer

membrane, since it presents low permeability acting as a selective barrier (Breidenstein *et al*., 2011), which could have impaired its action.

When the gel supplemented with gentamicin was applied three times, the results were similar to the previously described for the first assay and also to the obtained against a dual inoculum.

Finally, a guar gum biogel supplemented with Nisin, Pexiganan and Gentamicin was prepared, aiming at evaluating its distribution in the collagen DFI 3D model, as well as its inhibitory potential against a polymicrobial ulcer. In this assay, it was possible to observe a lower concentration of one of the isolates in comparison with the other one in all areas of the model, demonstrating the inhibitory potential of this combination against Gram-positive bacteria (Ceri *et al*., 1999; Ge *et al*., 1999; Lipsky *et al*., 2012; Mottola *et al*., 2016; Santos *et al*., 2016). However, regarding a bacterial species, the inhibitory activity of the multiple supplementation of the biogel with the two AMP plus the Antibiotic did not present relevant improvements, since it did not allow the inhibition of this bacterial species.

Although several studies have demonstrated that the combination of AMP with antibiotics promote an enhanced action (Grassi *et al*., 2017), the combination of nisin plus pexiganan plus gentamicin was not studied until now. Results similar to the ones obtained with the dual AMP biogel were expected; however, since the eradication of both bacterial species was not observed, the results suggested that the addition of gentamicin could have had an antagonist impact on the triple guar gum biogel (Yu *et al*., 2016), since a lower inhibitory activity was observed as it did not eradicate none of the isolates from the collagen model. Therefore, other antibiotics must be evaluated in further work.

The collagen 3D models from all assays were also evaluated by histochemical analysis, aiming at confirming the diffusion of bacteria throughout the model. The results allowed to confirm the results obtained in the bacterial quantification process.

This work represents a novelty regarding the studies performed by Werthén *et al*., in 2010 and by Price *et al*. in 2016, since it was not only based on the evaluation of bacterial diffusion across the collagen model but also focused on the study of new potential alternatives to conventional DFI treatments. Werthén *et al*., in 2010, were able to create a collagen model as a wound representation and study the biofilm production as well as *S. aureus* and *P. aeruginosa* tolerance against selected antibiotics. Through histochemical analysis, they confirmed the presence of biofilm from both bacterial species and resistance of *P. aeruginosa* biofilms against the antibiotics tested (Werthén *et al*., 2010), which is in accordance with our results.

Regarding the study performed by Price *et al*. in 2016, the authors were also able to create a collagen model to study biofilm production and also an alternative delivery system for antibiotics to be used in DFI treatment, namely calcium sulfide beads. Histochemical analysis showed biofilm production by *S. aureus* and *P. aeruginosa* and the calcium sulfide beads combined with tobramycin and gentamicin tested allowed eradicating *P. aeruginosa*. However, the incubation periods were different, not allowing a direct comparison with our work. In Price *et al*. (2016), the loaded beads were placed in the model after a 24h to 72h incubation for biofilm formation, and the evaluation of their inhibitory activity was performed after an incubation period of 72h. Therefore, the increased incubations periods for biofilm formation as well as for antibacterial beads could have been responsible for the broader antimicrobial activity observed by Price *et al*. (2016).

4.3. Conclusions

This work was able to demonstrate that the AMP pexiganan can effectively inhibit planktonic cells (Ge *et al*., 1999) and demonstrates potential against biofilms produced by two DFI isolates, being a promising agent to be used in the treatment of infected DFU as alternative or complement to conventional antibiotics (Gottler & Ramamoorthy, 2009).

The obtained results allowed observing that this AMP maintained its inhibitory potential. Moreover, our results were in accordance with previous studies, which stated that the concentration of an AMP or antibiotic required to inhibit biofilm is superior to the concentration required for the inhibition of planktonic cells (Spoering & Lewis, 2001). Considering that MBC results were approximately two to three times higher than the MIC ones for all isolates except one, and considering the dual suspensions results, it was possible to conclude that pexiganan is an effective bactericidal agent.

Regarding pexiganan MBIC and MBEC determinations, it is known that biofilm structures provide protection and stability for the bacterial species involved, which difficults the penetration of the AMP, being necessary higher concentrations of this agent to eradicate biofilms (Spoering & Lewis, 2001; Arciola *et al*., 2012; Banu *et al*., 2015). Therefore, the results demonstrated that pexiganan is more effective as a biofilm inhibitor compound than as a biofilm eradicator, but with promising results against biofilm eradication.

Results also suggest that the combination of nisin and pexiganan could be effective against infected DFU with both Gram-positive and Gram-negative strains. Relatively to Gram-positive bacteria, the results suggested an enhanced activity of this dual AMP solution (Worthington & Melander, 2013; Yu *et al*., 2016) as observed for one of the clinical isolates tested.

As no resistance mechanisms against pexiganan were described until now (Gottler & Ramamoorthy, 2009), it is possible to conclude that this AMP constitutes a promising alternative to conventional antibiotics. Nonetheless, since the number of isolates was low and considering the standard deviation associated, more tests should be performed.

The 3D representation of an ulcer is an important step in order to obtain a better understanding of the bacteria diffusion in the DFU environment *in vivo* (Werthén *et al*., 2010; Price *et al*., 2016), aiming at the development and testing of new alternatives to conventional treatments (Mendes *et al*., 2014; Lipsky, 2016). In this work, the study of the inhibitory potential of a guar gum gel supplemented with several antimicrobial combinations, including the AMP nisin and pexiganan and the antibiotic gentamicin, was evaluated using a 3D collagen model. In spite of the ability of the supplemented biogel to eradicate one of the bacterial species present in the 3D collagen ulcer model, further studies are required to develop new strategies for the other bacterial species and biofilm eradication.

It is important to refer that a 0.8% pexiganan acetate cream (Locilex®) has already been subjected to clinical trials aiming at its clinical application. In spite of the promising results, it failed in Phase 3 since it did not present a superior effect in comparison with the conventional oral antibiotics used for DFI treatment (Gomes *et al*., 2017). Our results suggest that the further supplementation of this cream with a complementary AMP, such as nisin, may allow to increase its inhibitory potential.

Finally, the 3D representation of an ulcer allowed to understand the bacterial diffusion as well as the AMP and antibiotic diffusion *in vitro*, constituting an important tool aiming at the development of innovative DFI treatment strategies.

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Supplementary Data

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17 January 2018

Diana Gomes

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Dear Dr. Gomes,

Figure(s):	Full thickness diabetic foot ulcer and claw toe [64087] Superficial diabetic foot ulcer [52333]
Topic:	McCulloch DK. Patient education: Foot care in diabetes mellitus (Beyond the Basics).

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