UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

NOVEL THERAPEUTIC STRATEGIES FOR THE MANAGEMENT OF DIABETIC FOOT INFECTIONS: THE EVALUATION OF SELECTED ANTIMICROBIAL PEPTIDES AGAINST CLINICALLY ISOLATED BACTERIAL PATHOGENS

TÂNIA RAQUEL MARTINS DOS SANTOS

Orientadora: Professora Doutora Maria Manuela Castilho Monteiro de Oliveira

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade Ciências Biológicas e Biomédicas

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Para a minha mãe.

Espero que estejas certa.

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Abstract

Diabetic foot infections (DFIs) are a frequent complication of Diabetes *mellitus*. These ulcers are prone to be colonized by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, including multidrug resistant and biofilm-producing strains, possibly leading to DFI chronicity and amputation. New therapeutic strategies for DFI management are urgent and the antimicrobial peptides (AMPs) nisin and pexiganan are potential candidates. This project aimed to evaluate the activity of these AMPs, incorporated in a guar gum biogel, against selected DFI clinical isolates.

Firstly, nisin's activity against a collection of *S. aureus* DFI clinical isolates was determined. Results showed that nisin was able to inhibit and eradicate *S. aureus* planktonic and biofilm cells at concentrations below its acceptable daily intake. When incorporated in the biogel, nisin kept its antimicrobial activity. This work also evaluated the potential of nisin to complement the activity of conventional antiseptics and antibiotics against established biofilms formed by these isolates. An *in vitro* antimicrobial schematic protocol was developed to mimetize DFI management guidelines. Fifteen antimicrobial combinations, including nisin-biogel, chlorhexidine, clindamycin, gentamicin and vancomycin, were tested. Results showed that the higher levels of biofilm inhibitory effects were presented by therapeutic combinations that included the nisin-biogel formulation.

Nisin-biogel ideal storage conditions and cytotoxicity were also evaluated. Results demonstrate that if stored at temperatures between -20 and 22ºC, nisin-biogel is able to maintain its antimicrobial activity up to 24 months. Moreover, after 24 h of exposition, the nisin-biogel presented no significant levels of toxicity regarding the human keratinocytes under study. Lastly, to cover the complex microbiota present in DFIs, a combination of AMPs with different action spectra was developed, based on the simultaneous incorporation of nisin and pexiganan in the biogel. The activity of this dual-AMPs formulation was tested against two *S. aureus* and *P. aeruginosa* strains isolated from the same DFI. Acting together, these AMPs were able to diffuse from the biogel and inhibit and eradicate biofilms formed by these DFI isolates.

The effectiveness of AMPs, particularly nisin and pexiganan, as novel antimicrobial strategies for the management of DFIs is still an unknown territory that merits investigation. *In vitro* biofilm models are the basis of preliminary research; however, they underrepresent the complex microbiota present in DFIs and their interaction with the immune system and skin cells constituents. Further research is necessary to understand the AMPs full potential regarding the clinical management of biofilm-related diseases, such as DFIs.

Key words: Antimicrobial peptide; Biofilm; Diabetic foot infection; Nisin; *Staphylococcus aureus*.

Resumo

As infecções do pé diabético (IPDs) são uma complicação frequente da Diabetes *mellitus*. Estas úlceras tendem a ser colonizadas por *Staphylococcus aureus* e *Pseudomonas aeruginosa*, incluindo estirpes multirresistentes e produtoras de biofilme, possivelmente causando cronicidade da IPD e amputação. É urgente criar novas estratégias para o tratamento das IPD e os péptidos antimicrobianos (PAMs) nisina e pexiganan são potenciais candidatos. Este projecto avaliou a actividade destes PAM, incorporados num biogel de goma de guar, contra isolados de IPD.

Primariamente, foi determinada a actividade da nisina contra uma colecção de *S. aureus* isolados de IPD. Os resultados mostraram que a nisina é capaz de inibir e erradicar *S. aureus* na forma planctónica e de biofilme a concentrações abaixo da dose diária recomendada. Quando incorporada no biogel, a nisina manteve a sua actividade. Foi ainda avaliado o potencial da nisina para complementar a actividade de antissépticos e antibióticos convencionais contra biofilmes formados por estes isolados. Foi criado um protocolo que simula *in vitro* o tratamento convencional das IPDs. Foram testadas 15 combinações de antimicrobianos, incluindo biogel de nisina, clorohexidina, clindamicina, gentamicina e vancomicina. Os resultados mostraram que o maior efeito inibidor de biofilmes pertencia a combinações que incluam o biogel de nisina.

Foram também avaliadas as condições de armazenamento ideais para o biogel de nisina e a sua citotoxicidade. Quando armazenado a temperaturas entre -20 e 22ºC, o biogel de nisina manteve a sua actividade antimicrobiana durante pelo menos 24 meses. Adicionalmente, após exposição durante 24 horas, o biogel de nisina não apresentou níveis significativos de toxicidade relativamente aos queratinócitos humanos em estudo. Por último, para abranger a complexa microbiota presente nas IPDs, foi avaliada uma combinação de PAMs com diferentes espectros de acção, baseada na incorporação simultânea de nisina e pexiganan no biogel. A actividade desta formulação foi testada contra duas estirpes de *S. aureus* e *P. aeruginosa* isoladas da mesma IPD. Conjuntamente, estes PAMs foram capazes de se difundir do biogel e inibir e erradicar biofilmes formados por estes isolados.

A eficácia dos PAMs como novas estratégias para o tratamento das IPD é ainda uma área desconhecida. Os modelos *in vitro* de biofilmes são a base da investigação; contudo, não representam a microbiota presente nas IPD nem a sua interacção com o sistema imunitário e outros constituintes celulares. É essencial continuar a investigar para compreender o potencial dos PAMs na terapêutica de doenças onde haja formação de biofilmes, como é o caso das IPDs.
Palavras chave: Péptido antimicrobiano; Biofilme; Infecção do pé diabético; Nisina; *Staphylococcus aureus*.

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List of Abbreviations and Symbols

A	Aspirate
AB	Alamar Blue
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
B	Biopsy
BHI	Brain Heart Infusion
CFU	Colony Forming Units
Chx	Chlorhexidine
Cli	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon Dioxide
DFI	Diabetic Foot Infection
DFU	Diabetic Foot Ulcer
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediamine Tetraacetic Acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
Gen	Gentamicin
HCl	Hydrochloric acid
HEKa	Human Epidermal Keratinocyte adult
IU	International Units
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MHCA	Mueller Hinton Cation Adjusted
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Type
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride

NBG	Nisin-Biogel
OD	Optical Density
PFGE	Pulsed-Field Gel Electrophoresis
RCBD	Randomized Complete Block Designs
S	Swab
TSB	Tryptic Soy Broth
WHMNG	Wound Healing and Management Node Group
WHO	World Health Organization
w/v	Weight/Volume
USA	United States of America
Van	Vancomycin

Chapter 1

1. Bibliographic review and objectives

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1.1 Bacterial biofilms in diabetic foot ulcers – Potential alternative therapeutics

1.1.1 Abstract

Diabetes *mellitus* is a major health problem that affects approximately 171 million people globally. One of its most severe complications is the development of diabetic foot ulcers (DFUs). Ischemic and neurophatic lesions are of major importance for DFU onset; however, it is the infection by multidrug-resistant and biofilm-producing microorganisms, along with local microenvironmental conditions unfavorable to antibiotics action that ultimately cause infection chronicity and lower limbs amputation.

Novel therapeutic protocols for DFU management are extremely urgent. Bacteriophages, probiotics and antimicrobial peptides (AMPs) have recently been proposed as alternatives to currently available antibiotics. Bacteriophages are viruses that specifically infect and multiply within bacterial cells. Their ability to diffuse through polymeric matrixes makes them particularly efficient to eradicate biofilm-based bacteria. Promising results were also observed with probiotic therapy. Probiotics are well-characterized strains with the ability to compete with pathogenic microorganisms and modulate the host immune response. AMPs are molecules produced by living organisms as part of their innate immune response. Unlike conventional antibiotics, AMP also act as immunomodulators and resistance to AMPs was rarely observed, supporting their potential as therapeutic agents.

These innovative therapeutic strategies may in the future substitute or complement antibiotherapy, ultimately contributing for the decrease in multidrug-resistant bacteria dissemination.

1.1.2 Introduction

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide. It is estimated that there are 171 million diabetic patients worldwide and this number is expected to double by the year 2030 (Hadaegh et al. 2009). Diabetic foot ulcers (DFUs) are one of the most frequent complications of diabetes, resulting from a complex interaction of factors, namely ischemia and neuropathy (Jeffcoate and Harding 2003).

Neuropathy, which is characterized by modifications in sensitive and autonomic functions, causes ulceration due to trauma or excessive pressure in a deformed foot without protective sensibility. Autonomic neuropathy causes dryness of the skin by decreasing sweating, and therefore the vulnerability of the skin to break down increases. Once the protective layer of skin is damaged, deep tissues are exposed to bacterial colonization (Vuorisalo et al. 2009). Diabetes-associated ischemia is caused by peripheral arterial disease. Poor arterial inflow decreases blood supply to ulcer area and is associated with reduced oxygenation, nutrition, and ulcer healing (Vuorisalo et al. 2009).

These ulcers are frequently colonized by pathogenic bacteria and infection is facilitated by immunological deficits related to diabetes (Geerlings and Hoepelman 1999), rapidly progressing to deeper tissues, increasing the presence of necrotic tissue, rendering amputation inevitable (Lipsky et al. 2004). In fact, diabetic patients frequently require minor or major amputations of the lower limbs (15-27%) (Jeffcoate and Harding 2003), which not only contribute dramatically to high

morbidity among diabetic patients, but is also associated with severe clinical depression and increased mortality rates (Ismail et al. 2007).

Although ischemic and neuropathic changes have the initial role in DFU pathophysiology, in the majority of cases it is the infection by multidrug-resistant microorganisms and the unfavorable microenvironmental conditions to the action of antibiotics that leads to amputation (Lipsky et al. 2004).

Diabetes-associated foot ulcer infections are predominantly polymicrobial and several bacterial genera can be part of the DFU microbiota, namely *Staphylococcus*, *Pseudomonas*, *Streptococcus*, *Enterococcus*, *Corynebacterium*, *Acinetobacter*, *Prevotella*, *Porphyromonas*, and members of the family *Enterobacteriaceae*. The predominant Gram-positive and Gram-negative species present in DFU are *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively (Mendes et al. 2014; Banu et al. 2015; Spichler et al. 2015).

There is, to date, little understanding of the ecology of such chronic infections, but bacterial biofilms seem to play a major role (James et al. 2008). These are ubiquitous and complex structures consisting of an interactive community of polymicrobial cells embedded in a self-produced extracellular matrix of hydrated polymeric substances, such as proteins, polysaccharides, nucleic acids and others, irreversibly attached to the biological surface of the ulcer. These characteristics make them recalcitrant to the action of most antibiotics and also resistant to the innate immune system (Dickschat 2010).

Therapy of biofilm-based infections generally requires local surgical procedures as well as antibiotic administration. However, in infected DFUs, because of deficient vascularization, antibiotics frequently reach the local ulcer microenvironment only at subtherapeutic concentrations (Lipsky et al. 2004). Even when topically applied, antibiotics rarely reach bacteria that reside within mature biofilms at therapeutic concentrations (Lipsky et al. 2008).

Biofilm formation is a major mechanism of adaptation that is able to protect bacteria from antibiotics, due to several physiological traits. Firstly, biofilm spatial structure provides a protective coat against antimicrobial compounds. Secondly, in most cases, biofilms are polymicrobial, formed by complex mixtures of different species. It was proposed that, in such biofilms, the chemical interactions that occur between polymeric substances produced from different bacterial strains may lead to a more viscous matrix, impairing the contact between the bacterial cell wall and the antibiotic. Lastly, the production of degradative enzymes by different pathogenic species can act synergistically against antimicrobial compounds. These biofilm features are responsible for a reduced diffusion of the antibiotic within the biofilm matrix (Burmølle et al. 2006; Bridier et al. 2011).

In addition, patients suffering from DFUs face the emergence and dissemination of antibiotic resistant bacteria, which is not a recent biological phenomenon. Seventy years ago, after the discovery of penicillin and the beginning of the antibiotic era, Alexander Fleming noticed the emergence of bacterial strains resistant to penicillin. Indeed, resistance began to appear in target microorganisms, including *S. aureus* isolates from hospitals, a few years after the introduction of penicillin into medical practice (Wenzel 2004). Fleming described the occurrence of antibiotic resistance and warned the scientific and medical community of this phenomenon in his Nobel Prize lecture in 1945 (Fleming 1945).

Several causes can explain the emergence and dissemination of antibiotic resistance. Firstly, the overuse and, most importantly, the misuse of antibiotics in different but interconnected areas, like human and veterinary medicine, agriculture and animal production. Secondly, the effects of antibiotic compounds in the environment are not yet completely described and understood. Finally, antibiotic compounds are stable and static chemical substances that are used to fight living and evolving bacterial cells (Levy and Marshall 2004). Microorganisms, namely bacteria, are ubiquitous and interact with all other living beings. Considering that nature is a highly complex system supported by extremely dynamic interactions and exchanges between all its elements, the emergence and evolution of bacterial populations able to resist against antibiotic substances is not surprising. In fact, over the last decades microbiologists have demonstrated the influence that antibiotics exert upon bacterial populations. Previously seen as miracle drugs, capable of virtually eradicating all species of bacteria, antibiotics are now seen as substances with limited antimicrobial capacity and multifaceted proprieties. These compounds have the ability to induce or inhibit different bacterial responses and to influence bacterial virulence and survival strategies (Hoffman et al. 2005; Kaplan 2011).

As mentioned above, biofilm formation is a well-known virulence factor of some bacterial strains that, along with many other advantages, confers them a protective layer against adverse elements. Recently, it was demonstrated that some antibiotics are able to induce this adaptative strategy. In 2005, when Hoffman et al. were testing the efficacy of aminoglycosides, a widely exploited antibacterial therapeutic agent, against biofilm-forming bacteria, they observed an unexpected bacterial response. Aminoglycosides not only did not eliminate the *P. aeruginosa* strain in study, but also stimulated their ability to form biofilm. In fact, they demonstrated that aminoglycosides interact with the *P. aeruginosa* aminoglycoside response regulator gene, *arr*, which encodes for an inner-membrane phosphodiesterase essential to the regulation of cyclic di-guanosine monophosphate levels, which represents a bacterial second messenger that regulates cell surface adherence (Hoffman et al. 2005). Later on, Kaplan et al. (2011) also reported that in

Escherichia coli, not only sub inhibitory antibiotic concentrations but also disinfectants such as chlorhexidine are responsible for the induction of biofilm formation. From their work, one can conclude that, for some bacterial strains, biofilm formation can be a specific defensive reaction to the presence of antibiotics.

Despite all the evidences showing that biofilms provide advantages to microorganisms, namely enhanced resistance towards environmental stresses, including the presence of antimicrobial compounds, many antibiotics that are currently in use were developed, tested and regulated using *in vitro* tests against planktonic bacteria.

It is known that microbial cells growing within a biofilm are physiologically distinct from planktonic cells of the same strain. The overall resistance level in biofilms is distinct from the one observed at a cellular level (Stewart and Costerton 2001). As a consequence, the antimicrobial concentration required to inhibit biofilms can be up to hundreds or even a thousand times higher than the corresponding concentration necessary to eliminate free-living bacterial cells (Ceri et al. 1999). Such phenomena cannot be overlooked in the development of novel strategies to combat infectious diseases.

Taking into account that biofilm formation is a threatening characteristic of the microbiome that colonizes diabetic foot wounds, it is not unexpected that in the past few decades a major problem in treating DFU infections has been the increasing rate of isolation of antibiotic resistant pathogens. This is the case of methicillin-resistant *S. aureus* (MRSA), and, to a lesser degree, glycopeptide-intermediate *S. aureus*, vancomycin-resistant enterococci, extended-spectrum β-lactamase- or carbapenamase–producing gram-negative bacilli and highly resistant strains of *P. aeruginosa.* In fact, the infection by polymicrobial communities of multidrug-resistant bacteria is an important cause of DFU healing impediment (Dang et al. 2003; Tascini et al. 2006; Kandemir et al. 2007; Stanaway et al. 2007; Richard et al. 2008; Spichler et al. 2015; Lipsky et al. 2012).

The rates of isolation of these multidrug-resistant pathogens vary widely among geographical area and treatment center. However, the increasing incidence of multidrug-resistant microorganisms together with the incapacity of antibiotics to act on resistant and biofilm-producing bacteria at therapeutical concentrations emphasizes the importance of developing new treatment strategies to effectively eradicate these infections.

Considering that biofilms were only described by the scientific community by the end of the 20th century, it is comprehensible that research on biofilms is still an expanding area (Costerton et al. 1995). The lack of understanding of the mechanisms behind the biofilm mode of life has impaired the development of antimicrobial compounds that specifically operate on biofilm polymicrobial communities (Costerton et al. 1995). However, in recent years, the increased failure

in infectious diseases therapeutic protocols and the dissemination of antibiotic resistance has demonstrated the importance of developing such substances and several novel therapeutic strategies, namely bacteriophages, probiotics and antimicrobial peptides (AMPs), are recently been explored and proposed as potential alternatives to eradicate bacterial biofilms in DFUs.

1.1.3 Bacteriophages

Bacteriophages were discovered almost a century ago by two independent microbiologists, Twork in 1915 in the United Kingdom and D'Herelle in 1917 in France. D'Herelle named these bacteria-eating entities as bacteriophages and explored them as antibacterial agents (Twork 1915; D'Herelle 1919).

Bacteriophages are bacteria-specific viruses that infect and multiply within bacterial cells. In contrast to lysogenic bacteriophages, the replication of lytic bacteriophages and release of the newly formed virus particles always involves lysis of the host bacterial cell. Bacteriophage therapy is the use of lytic bacteriophages to reduce or eliminate pathogenic bacteria (Sulakvelidze and Kutter 2004). Lytic bacteriophages seem to be efficient therapeutical agents in biofilm microenvironment due to several particular characteristics: specificity and efficiency in lysing pathogenic bacteria; absence of pathogenicity to man and animals; efficiency over bacteria organized in polymeric matrixes, namely biofilms; action in microaerophilic environments with high bacterial load; and rapid and economical accessible production capability (Sillankorva et al. 2004; Njoroge and Sperandio 2009).

Bacteriophage therapy has become a broadly relevant technology for veterinary, agricultural and food microbiological applications; however, the treatment of human infections with bacteriophage-based protocols attracts the greatest interest (Kutter et al. 2010).

Bacteriophages are viruses that specifically infect prokaryotic cells. In fact, the prokaryotic biochemical machinery that enables the interaction between bacteriophages and bacterial cells has particular characteristics that are not present in eukaryotic cells. For instance, the outer membrane receptors of bacterial cells, with which bacteriophage capsid coat or molecular appendages first connect with the purpose of being anchored on the bacterial cell wall, as well as the polymerases required for the bacteriophage genome replication, are specific of prokaryotic bacterial cells and are structurally and functionally different from those presented by eukaryotic cells (Sulakvelidze and Kutter 2004). For that reason, bacteriophages can only directly interact and infect bacterial cells, and not eukaryotic cells. The bacterio-specificity feature allows

classifying bacteriophages as ‘safe’ for use in eukaryotic organisms, namely plants and animals, including humans.

The use of bacteriophages as antibacterial agents for suppurative infections began shortly after their discovery with Bruynoghe’s and Maisin’s application for treating *S. aureus* skin infections (Bruynoghe and Maisin 1921). However, following the discovery and general application of antibiotics, interest in the therapeutic uses of bacteriophages waned. Recently, the increase in antibiotic-resistant bacterial strains has reinvigorated enthusiasm about these bacteria-specific viruses (Chopra et al. 1997). This interest is particularly true in cases in which bacteriophages can be applied topically, as is the case of DFUs.

Recently, a topically delivered bacteriophage suspension was tested for its antimicrobial activity and wound healing capability against ulcers chronically infected with *S. aureus*, *P. aeruginosa* and *Acinetobacter baumannii*. In this study, conducted by Mendes et al. in 2013, the bacteriophage suspension was applied in debrided infected cutaneous wounds and microbiologic, histological and planimetric parameters were evaluated. It was shown that the bacteriophage treatment successfully decreased bacterial colony counts and improved wound healing, as indicated by smaller epithelial and dermal gaps. The bacteriophage therapy protocol developed was proven to be an effective methodology in the treatment of two animal models of Diabetes *mellitus*, rodents and porcines (Mendes et al. 2013).

The same bacteriophage suspension also demonstrated *in vitro* activity against both planktonic cells and established biofilms. Using metabolic activity as a measure of cell viability, it was observed that bacteriophage treatment significantly increased cell impairment within biofilms. Moreover, bacteriophage exposure repeated every four hours caused a further decrease in cell activity (Mendes et al. 2014).

There is still much to unravel regarding bacteriophage therapy. For instance, not all phages would be suitable for clinical application. More information is required, namely detailed studies of potentially useful phages with respect to their interaction with target bacteria and their genetic content. Nonetheless, despite the paucity of experimental data regarding bacteriophage therapy in DFUs, a consensus appears to have emerged on the feasibility of this potential alternative to treat biofilm-infected DFUs.

1.1.4 Probiotics

The increasing global antimicrobial drug resistance problem led to an urge in researching alternatives to drug therapies, making the concept of bacteriotherapy more interesting and

pertinent than ever. Bacteriotherapy is a promising alternative approach to fight infections by employing harmless bacteria to displace pathogenic microorganisms (Leone et al. 2012).

The concept of 'probiotic' arose in 1907 from a hypothesis proposed by Noble Prize-winning Ilya Mechnikov. At the turn of the 20th century, Mechnikov noticed that peasant populations in Bulgaria had increased average life spans in comparison with wealthier European populations (Mechnikov, 1908). He also observed that yogurt and other fermented milk products were a substantial part of their diets and described the beneficial effects of the 'Bulgarian *bacillus'* present in those foods (Kingsley and Gregor, 2007; Azizpour et al. 2009). These healthy bacteria, later classified *Lactobacillus bulgaricus*, helped digestion, impaired the putrefactive effects of gastrointestinal metabolism, and contributed to the improvement of the immune system (Kingsley and Gregor, 2007).

Mechnikov was not the only one to notice the health benefits of lactic acid bacteria. A few years before, in 1899, another important discovery was made at the Pasteur Institute, in Paris. Henri Tissier demonstrated that children suffering from diarrhea had a low number of bacteria characterized by a peculiar Y-shaped morphology. On the other hand, these "bifid" bacteria were abundant in the gut flora of healthy breast-fed infants. Moreover, Tissier demonstrated that the administration of these Y-shaped bacteria, later classified *Bifidobacterium*, to patients with diarrhea allowed them to re-establish a healthy intestinal microbiome (Tissier 1906).

The definition of probiotic as well as their characteristics have evolved in the last century and nowadays probiotics are defined by Food and Agriculture Organization (FAO) and World Health Organization (WHO) as: 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2002). Probiotics are either a single strain or a mixture of commensal microorganisms with the ability to outcompete pathogenic bacteria through several mechanisms of action. The two most common are direct modification of the microbial populations and modulation of host immune system (FAO/WHO 2002).

Direct modification of the microbiome includes competition with pathogenic bacteria for adhesion to epithelial receptor, production of antimicrobial substances like acids, hydrogen peroxide and bacteriocins, and inhibition of toxic substances produced by pathogens. Immunomodulation includes strengthening of host immune response, promotion of anti-inflammatory action and enhancement of the wound healing process, by stimulating the accumulation of inflammatory cells like lymphocytes, macrophages and polymorphonuclear cells in the site of wound (Oelschlaeger 2010).

As one would expect, not all commensal bacteria are suitable to be used as a probiotic. The screening and selection of probiotics includes a rigorous evaluation of the probiotic candidate strain in order to determine whether it fulfills all the required criteria.

Firstly, it is important to assess its safety. An evaluation that includes strain identification and typing, antimicrobial resistance profiling and determination of virulence and pathogenic properties, including metabolic activities associated with toxic compounds production, is mandatory (Sanders et al. 2010). Secondly, it is relevant to determine its technological potential. It is essential for a probiotic strain to be genetically stable and bacteriophage-resistant. Also, it must present viability during processing and storage and be adequate for large-scale production (Conway 1996). Thirdly, it is required to establish its physiological properties. To survive the host inner environment, which is rather complex and hostile, a probiotic strain must possess specific characteristics such as gastric acid and bile tolerance and mucosal surface adhesion stability (Tuomola et al. 2001). Lastly, the functional properties must be evaluated. Validated and documented health effects are mandatory, namely antagonistic activity towards pathogens, immunomodulatory activity and anticarcinogenic properties. Some probiotic strains are also able to interfere with the host cholesterol and lactose metabolism, preventing the damages by its metabolites (Donovan et al. 2012).

Probiotics have already been exploited for prevention as well as treatment of a number of health disorders, including irritable bowel syndrome, hypersensitivity such as food allergies, hypercholesterolemia, renal failure, gastritis and gut infection, parasitic infections, urogenital infections, colorectal cancer and dental disorders (Hickson 2013; Singh et al. 2013). Since the putative probiotic mechanisms of action should be the same in the peripheral wounds as they are in other parts of the body, these can be considered as a potential DFU treatment alternative.

Lactic acid bacteria, in particular *Lactobacillus* and *Bifidobacterium* species, have been extensively used as probiotic strains. The genus *Lactobacillus* is formed by ubiquitous and usually harmless bacteria. In animals, including humans, they are present in the gastrointestinal and genitourinary tracts where they act as health promoters (Salminen et al. 1996). The genus *Bifidobacterium* includes anaerobic bacteria that produce acetic and lactic acid without release of carbon dioxide. *Bifidobacterium* is the third most abundant genus in the complex microbiome of the human intestinal tract where it exerts beneficial functions of paramount importance (Finegold et al. 1983). However, other species of bacteria, and even some fungi, also present probiotic properties, such as *Enterococcus faecium*, *Bacillus cereus*, *E. coli* strain Nissle, *Propionibacterium freudenreichii, Propionibacterium acnes* and the yeasts *Saccharomyces cerevisiae* and *Saccharomyces boulardii* (Psomas et al. 2001; Endres et al. 2011; Franz et al. 2011).

Lactic acid bacteria commonly produce antimicrobial substances with effect against gastric and intestinal pathogens and compete for cell surface and mucin binding sites (Ljungh and Wadström 2006). Recent studies have demonstrated the efficacy of lactic acid bacteria-based therapy for DFU infections control. A study on effectiveness of bacteriotherapy using *Lactobacillus plantarum* on infected chronic DFUs demonstrated that topical application of this bacterial culture induced debridement, granulation tissue formation and total healing in half of the diabetic patients treated (Valdéz et al. 2005; Peral et al. 2010). *Lactobacillus fermentum* also showed promising applications in treating DFU infections. When co-incubated *in vitro* with *S. aureus and P. aeruginosa*, *L. fermentum* reduced the cytotoxicity and biofilm formation ability of several pathogenic strains (Varma et al. 2011).

Additional studies have suggested that *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus acidophilus* and *Lactococcus lactis* are also promising probiotics with the ability to naturally eliminate pathogenic microorganisms, including clinical MRSA isolates (Sikorska and Smoragiewicz 2013).

In the last years, probiotics have been widely studied and all these recent data point out the beneficial effects of probiotics to human and animal health. Naturally, no probiotic strain will provide all the proposed benefits. However, one can no longer ignore the emergence of probiotics as a novel approach to fight multidrug-resistant and biofilm-producing bacteria commonly present in DFUs.

1.1.5 Antimicrobial peptides

Antimicrobial peptides are major components of the host innate immune system that act as endogenous antibiotics (Zasloff 2002; Hancock and Sahl 2006). These multifunctional molecules are produced by living organisms from all kingdoms, including bacteria, fungi, plants, insects and vertebrates, as part of their defense strategy against pathogens. Most AMPs act as the first defense barrier against dissemination of a wide spectrum of microorganisms, such as bacteria, fungi, viruses and protozoan parasites (Hancock and Sahl 2006).

In addition to their antimicrobial activity, AMPs serve as modulators of the immune system and even show antitoxic activity, since they neutralize bacterial toxins, including lipopolysaccharide lipid A (Kirikae et al. 1998; Rosenfeld et al. 2006). Some AMPs are also able to prevent biofilm formation and act on pre-formed biofilms (Overhage et al. 2008).

The majority of AMPs are polypeptides with ten to forty amino acid residues; however, some can have up to a hundred. AMP are amphipathic molecules, with two regions in their

structure, a polar or hydrophilic region and a nonpolar or hydrophobic region. Due to the presence of multiple lysine, arginine and histidine residues, the polar region of AMP is cationically charged. On the other hand, hydrophobicity derives from the abundant presence of hydrophobic amino acids, such as tryptophan, tyrosine and phenylalanine (Hou et al. 2010; Baltzer and Brown 2011).

The distinctive physical-chemical properties of AMPs are what confers them their potential as antimicrobial compounds. It has been generally accepted that AMPs exert their bactericidal activity through electrostatic interactions with the negatively charged bacterial cytoplasmic membrane followed by permeabilization of the membrane, which causes cell lysis. Membrane permeabilization can occur through pore formation in the lipid membrane, membrane dissolution, narrowing of the membrane bilayer or lipid-peptide domain formation (Gaspar et al. 2013). The AMPs amphipathic structure, namely their cationic and hydrophobic regions, interacts with the negatively charged phospholipids present in the surface of the microorganisms' cytoplasmic membranes. Bacterial membranes are rich in lipids such as phosphatidilglycerol and cardiolipin, whereas host cells have eukaryotic membranes that are rich in phosphatidylcholine, cholesterol and sphingomyelin (Wimley 2010).

It is the difference in the lipids that constitute the membranes of bacteria and host cells that allows AMP to selectively target the microbial cells over mammal cells and confers them the criterion of safety to be use in eukaryotic organisms, including humans.

Additionally to their role as membrane disruptors, several studies have also suggested alternative targets for AMPs. In fact, it was proven that some AMPs are able to translocate into the cytoplasm of pathogens and attack intracellular targets. This way, AMPs impair essential bacterial metabolic processes, including nucleic acids synthesis and cell-wall assembly (Subbalakshmi et al. 1998; Brogen 2005; Schneider et al. 2010). AMPs can present multiple and simultaneous mechanisms of action, including both membrane permeabilization and intracellular effects. This property is probably the reason why they present antimicrobial activity against such a wide range of pathogens.

Regarding their immunological functions, AMPs are also known as host-defense peptides (Lai and Gallo 2009; Nijnik and Hancock 2009; Bowdish et al. 2005; Bowdish et al. 2006). By interacting with a variety of host cell receptors, AMP promote the recruitment of leukocytes to the site of infection through direct chemotactic activity and stimulation of chemokine production by leukocytes, epithelial cells and other cell types (Davidson et al. 2004; Nijnik et al. 2009). Finally, some AMPs also play a role in angiogenesis and wound healing (Heilborn et al. 2003; Koczulla et al. 2003).

The production of AMPs is not limited to multicellular organisms; bacteria can also synthesize AMPs that are active against other bacteria. These AMPs of bacterial origin include non-ribosomally synthesized peptides such as gramicidins and ribosomally synthesized peptides such as bacteriocins, and have been used for years (Hancock and Chapple 1999; Cotter et al. 2005). Gramicidin S is a cyclic decapeptide produced by *Bacillus aneurinolyticus* and has been used as a topical antimicrobial agent against Gram-positive bacteria since 1946 (Gause 1946). Nisin is a bacteriocin produced by *L. lactis* that acts primarily against Gram-positive bacteria and has been used safely as a food preservative for over fifty years (Cleveland et al. 2001).

Several studies have analyzed the *in vitro* activity of different AMPs against DFUs clinical isolates. In 2013, Okuda et al. evaluated the antimicrobial activity and mode of action of three bacteriocins, nisin A, lacticin Q and nukacin ISK-1, against a clinically isolated and biofilm-producing MRSA strain. Nukacin ISK-1, produced by *Staphylococcus warneri*, presented only bacteriostatic effects. However, both nisin A and lacticin Q, produced by *L. lactis*, showed bactericidal efficacy against planktonic and biofilm cells (Okuda et al. 2013). Synthetic cationic antimicrobial peptides, namely NP101 and NP108 also showed *in vitro* activity against bacterial species commonly associated with DFU infections, such as *S. aureus* and *P. aeruginosa*, as demonstrated by O'Driscoll et al. in 2013. These results suggest that bacteriocins that act on biofilm-producer cells are highly suitable for the treatment of DFUs infections.

However, there are some limitations in the use of AMPs as a clinical alternative for antibiotics. Apparently, bacteria resistance to AMP is rare, in opposition to what is observed towards classic antibiotics (Yeaman and Yount 2003). This characteristic of AMPs is likely to be related to the ionic interaction between the positively charged AMPs and the negatively charged bacteria membrane. Since these interactions are not dependent of specific protein binding sites, in order to develop resistance to AMPs, bacteria would have to change the basic structure, namely the lipid bilayer, of its cytoplasmic membrane (Wimley and Hristova 2011). Moreover, attachment of the AMPs with the bacterial membrane and consequent cell lysis happens in such a short period of time, rendering the possibility to develop AMPs resistance quite scarce (Fernebro 2011). However, there are reports of distinct species of bacteria which present resistance towards AMP. The mechanisms of resistance include degradation of AMPs through secretion of proteases; removal of AMPs from their site of action via efflux pumps; production of inhibitors that bind to AMPs and prevent them from reaching their target; and modulation of AMPs gene expression (Otto 2009; Guilhelmelli et al. 2013; Nawrocki et al. 2014).

Another obstacle to the successful implementation of AMPs as an alternative to conventional antibiotics is the production costs. AMPs discovery and development is time

consuming, reaching up to ten years, and can cost millions of dollars. In fact, production costs are estimated to be approximately fifty to four hundred American dollars per gram of amino acid (Marr et al. 2006).

Even so, AMPs are still a promising alternative to antibiotics. A possible solution to reduce costs associated with AMPs production is the reduction of the peptide size, maintaining its antimicrobial activity (Seo et al. 2012). Moreover, AMPs exhibit physiological and functional advantages over other molecules that make them so attractive to be used in clinical practice. For instance, physiological concentrations of AMPs *in vivo* are much lower than the minimal inhibitory concentration required for its antimicrobial activity *in vitro* (Lai and Gallo 2009). In fact, AMP are antimicrobial agents with a broad-spectrum activity displayed at micromolar concentrations, usually in the one to fifty μg/mL range (Diamond et al. 2009). A plausible justification for this fact may be the synergistic effect that some AMP possess, which enhances their antimicrobial activity *in vivo* (Cassone and Otvos 2010).

For all these reasons, the development of AMPs-based therapies to eliminate microbial pathogens, such as those present in DFU infections, is extremely promising and deserves further exploration.

1.1.6 Conclusive remarks

The severity of diabetic foot infections and the economic burden associated with its prevention, treatment and control, have compelled scientists and clinicians to invest substantial time and effort in not only understanding how these mechanisms work, but also how they can interfere with them.

As mentioned before, a major factor responsible for healing impediment of DFUs are infections by multidrug-resistant or biofilm-producing bacteria. Dissemination of these strains, coupled with disinvestment in new antibiotics development, calls for increasing research to find new approaches to prevent and control these pathogens. In this chapter, the potentialities of bacteriophage viruses, probiotic strains and antimicrobial peptides as novel strategies for management of DFUs were reviewed. Several studies, conducted by independent research teams, have demonstrated promising results, both *in vitro* as *in vivo*, regarding their competence to eradicate the pathogenic microorganisms present in DFUs. However, further investigation is required, so that in the future these strategies could be applied in clinical practice alongside with conventional therapeutics.

1.2 Are antimicrobial peptides the answer for diabetic foot infection management?

1.2.1 Abstract

Diabetes *mellitus* is a serious health problem that has shown an increasing prevalence in the last decades, affecting more than 422 million people globally nowadays. As a consequence of multiple pathophysiological factors, namely neuropathy, vasculopathy and immunopathy, the lifetime risk for diabetic patients of developing a foot ulcer can be as high as 25%. Approximately half of these ulcers can become clinically infected, usually by opportunistic pathogens, including both aerobic and anaerobic bacteria and yeasts. Due to local micro-environmental conditions unfavorable to wound healing, infected ulcers may result in purulent discharge, intense inflammation and progressive tissue damage.

Several bacteria are related with diabetic foot infections (DFIs), mainly *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Enterobacteriaceae*, *Pseudomonas* spp., *Acinetobacter* spp. and *Peptoniphilus* spp. These species have the ability to express numerous virulence factors that are putatively involved in their pathogenicity, including quorum-sensing molecules and biofilm structures. Moreover, DFI pathogens are known for their antibiotic resistance profile. The increasing prevalence of multidrug resistant isolates, formation of biofilms and inadequate wound healing found in DFIs may impair the successful outcome of conventional anti-infectious therapeutics in these patients. In fact, foot gangrene subsequent to a non-healing DFI is nowadays the leading cause of non-traumatic lower limb amputations.

Antimicrobial peptides have emerged as a potential strategy to be used in combination with or as an alternative to conventional antibiotherapy in the management of chronic DFIs. AMPs are amphipathic molecules containing cationic and hydrophobic amino acid residues, enabling them to form non-specific interactions with the negatively charged bacterial membranes. There are several studies available regarding the activity of these small peptides, providing information on their antimicrobial spectrum, mechanisms of action and biological effects in wound healing.

Nisin and pexiganan are two of the most promising AMPs for application against antibiotic resistant bacteria. Both nisin and pexiganan are able to disrupt prokaryotic membranes, inducing a fast killing of bacteria. Nisin binds to the peptidoglycan precursor lipid II, inhibiting cell wall synthesis and promoting pore formation on bacterial cytoplasmic membranes; on the other hand, pexiganan exerts its antibacterial effect via toroidal pore formation. The multiple mechanisms of action, the quick onset of activity and the low specificity in terms of molecular targets decreases the tendency of bacteria to develop resistance towards AMPs.

Given the increasing prevalence of antibiotic resistant pathogens and, consequently, the failure of antibiotic-exclusive therapeutics in DFIs treatment, combinations involving AMPs and antibiotics may be a potential treatment alternative in a near future.

1.2.2 Diabetic foot infection

Diabetes *mellitus* is a chronic disease that affects more than 422 million people worldwide and which prevalence is expected to double by 2030 (WHO 2016). Diabetic patients have a predisposition to develop vascular, neurological and immunological diseases, being peripheral neuropathy and lower extremity arterial disease the main factors responsible for the onset of diabetic foot ulceration (Armstrong et al. 2011). Secondary to multiple pathophysiological factors, including diabetes-associated immunopathy, diabetic patients are unable to establish a normal inflammatory response against microbial pathogens, and diabetic foot infection following ulceration of the protective skin is a common and devastating complication presented by these patients (Hobizal and Wukich 2012).

Diabetic foot ulcers represent one of the most severe complications of diabetes, affecting up to a quarter of diabetic patients, being expected that during their lifetime, approximately half of these ulcers will become clinically infected (Hobizal and Wukich 2012).

Although ischemic and neuropathic lesions promote the DFU onset, it is the infection by pathogenic microorganisms along with the local microenvironmental conditions unfavorable to antibiotics action that are ultimately responsible for DFI recalcitrance (Armstrong et al. 2011; Lipsky et al. 2016). Chronically infected DFUs, characterized by severe inflammation and progressive tissue damage with the involvement of bacterial biofilms, are often resistant to antibiotherapy and can evolve to gangrene. As a result, DFIs are the most common diabetic complications requiring hospitalization and the worldwide leading cause of non-traumatic lower extremity amputation (Lipsky et al. 2016). In fact, it is estimated that more than 60% of non-traumatic lower limb amputations occur in diabetic patients (Kosinski and Lipsky 2010; Yazdanpanah et al. 2015), with these patients presenting a lower limb amputation rate of 15 times higher than patients without diabetes (Yazdanpanah et al. 2015).

1.2.3 Associated microbiota

Diabetes-associated foot infections are caused by a polymicrobial community of pathogens. While Gram-positive bacteria, including *Staphylococcus* spp., *Streptococcus* spp.,

Enterococcus spp. and *Corynebacterium* spp. tend to predominate in acute DFIs, the microbiota of chronic DFIs is mainly constituted by Gram-negative bacteria, such as *Pseudomonas* spp., *Proteus* spp., *Acinetobacter* spp. and *Klebsiella* spp., followed by anaerobes, namely *Peptoniphilus* spp. and *Bacteroides* spp. (Lipsky et al. 2012; Mendes et al. 2012; Banu et al. 2015). Despite the variety of pathogens associated to DFIs, epidemiological studies report a clear predominance of *S. aureus* and *P. aeruginosa* as the main Gram-positive and Gram-negative bacteria, respectively, present in these infections (Mendes et al. 2012; Banu et al. 2015).

The microorganisms from the microbiota of DFIs are frequently characterized as resistant to the standard antibiotics prescribed within general clinical practice (Mendes et al. 2012). Both *S. aureus* and *P. aeruginosa* are well-known for their increased resistance to most conventional antibiotic agents, and the infections caused by antibiotic-resistant strains represent a serious threat to public health (Hancock and Speert 2000; Lowy 2003; Chambers and DeLeo 2009; Chatterjee et al. 2016). Diabetic patients are a particular high-risk group, since the morbidity and mortality of patients with DFIs caused by resistant strains are significantly higher than those caused by non-resistant strains (Tascini 2018).

Both *S. aureus* and *P. aeruginosa* are also known for their ability to produce several virulence factors, namely protein and carbohydrate adhesins, exotoxins, exoenzymes and proteins involved in immune system evasion. The interaction of pathogens within the DFI polymicrobial biofilms favors the expression of quorum-sensing molecules, hemolysins, collagenases, proteases and short-chain fatty acids, responsible for inflammation and wound healing impeding, ultimately leading to DFI chronicity (Citron et al. 2007; Hauser 2011; Oogai et al. 2011; Jenkins et al. 2015).

Staphylococci, particularly *S. aureus*, are perhaps the most virulent pathogens in DFIs, presenting a correlation between specific virulence genotypic markers and ulcer outcome (Sotto, et al. 2008). The overall burden of staphylococcal disease, particularly the one caused by MRSA strains, is increasing in many countries (Mottola, Semedo-Lemsaddek, et al. 2016; Akhi et al. 2017). Portugal presents one of the highest prevalence of Diabetes *mellitus*-associated lower limb amputations (Carinci et al. 2016) and MRSA skin and soft tissue infections in Europe (Moet et al. 2007). Among hospitalized diabetic patients, the prevalence of MRSA in DFIs can range from 15 to 30% (Hobizal and Wukich 2012). *S. aureus* infections, particularly those affecting diabetic patients, are associated with severe consequences, since they can evolve from minor skin and soft tissue infections to extremely serious systemic diseases, such as endocarditis, septicemia and osteomyelitis (Jenkins et al. 2015).

1.2.4 Biofilm mode of growth

DFIs are predominantly polymicrobial and their microorganisms can exhibit different modes of growth. DFI bacterial cells can be present in a non-adherent planktonic form, or they can form sessile microbial communities, irreversibly attached to surfaces, encaged within a self-produced matrix of extracellular polymeric substances, called biofilms (Dickschat 2010; Banu et al. 2015).

In the DFI environment, the majority of bacterial cells are naturally organized in biofilms (Banu et al. 2015; Mottola, Mendes, et al. 2016). This biofilm-forming ability is an important virulence factor presented by these pathogens and has been associated with resilient chronic foot wound infections that respond unsuccessfully to antibiotic therapy (James et al. 2008; Banu et al. 2015). Bacteria within biofilms are sheltered from numerous stressful conditions and the increased resistance to conventional antibiotics along with the recurrence presented by DFIs is a direct consequence of the multiple resistance mechanisms that biofilm-related bacteria possess (Batoni et al. 2016).

The deleterious effect of the biofilm mode of microbial growth on wound healing has been known for decades (James et al. 2008). These slime-enclosed aggregates of bacteria are characterized for being a very hostile environment for an efficient immune system response, as well as for antimicrobial agents penetration and diffusion (Hall and Mah 2017). Moreover, biofilm-based bacterial cells are physiologically distinct from non-adherent planktonic cells. Their growth rate is reduced and the quorum-sensing signaling system enables biofilm cells to activate specific genetic determinants of antibiotic tolerance and resistance (Dickschat 2010; Hall and Mah 2017), which can increase antibiotic resistance by up to 1000 fold (Stewart and Costerton 2001). Acting in concert, these mechanisms are responsible for the emergence of antibiotic-resistant strains and for biofilm recalcitrance, which is a major issue in the re-occurrence and delayed healing of infected chronic wounds, such as those presented by diabetic patients (Burmølle et al. 2006; Lipsky et al. 2016).

1.2.5 Inhibitory potential of antimicrobial peptides

Over the last decades, AMPs have attracted considerable interest as a new class of antimicrobial agents (Strempel et al. 2015; Pletzer et al. 2016; Mahlapuu et al. 2016). Considering the dissemination of bacterial resistance and the failure of conventional antibiotic-based therapies amongst diabetic patients, it is crucial to develop alternative treatment strategies, and AMPs are

570 emerging as potential new weapons against these chronically infected wounds (Strempel et al.
571 2015, Pletzer al. 2016; Mahlapuu et al. 2016).

572 Since DFIs are caused by a diverse community of biofilm-producing bacteria, when
573 managing these persistent infected wounds it is essential to use antimicrobial agents whose
574 spectrum of activity covers both planktonic bacteria and sessile polymicrobial communities
575 present in the DFI environments (Lipsky et al. 2016). For that reason, the development of new
576 therapeutic strategies, namely the ones based on AMPs administration, which by their own or in
577 a combination with other antimicrobial agents may target different elements of the DFI microbiota,
578 might prove to be successful in the treatment and management of these infections.

579 AMPs are part of the innate immune defense system of virtually all living organisms,
580 including bacteria, protozoan, fungi, plants, insects and animals (Bahar and Ren 2013; Mahlapuu
581 et al. 2016). These peptides are characterized by a low molecular weight, since they usually have
582 less than one hundred amino acid residues; a cationic character, due to the high amount of
583 positively charged residues; and an amphipathic structure, resulting from the presence of
584 hydrophobic and hydrophilic regions in opposite sides of these molecules (Shai 1999; Wu et al.
585 1999; Aoki and Ueda 2013).

586 Considering their polypeptide backbone, AMPs are commonly classified based on their
587 structural characteristics, including linear, α-helical and β-hairpin-like structures (Zasloff 2002).
588 Linear AMPs include indolicin and PR-39 from mammals (Agerberth et al. 1991; Selsted et al.
589 1992) and type-A lantibiotics such as nisin from lactic acid bacteria (McAuliffe et al. 2001); AMPs
590 with an α-helical structure include magainins from frogs (Bevins and Zasloff 1990), cecropins from
591 insects and mammals (Lee et al. 1989) and cathelicidins from mammals (Bals et al. 1998; Dürr et
592 al. 2006); and the β-hairpin-like AMPs include polyphemusin and tachyplesin from crabs (Powers
593 et al. 2006; Imura et al. 2007) and α- and β-defensins from humans (Ganz et al. 1985) (Dhople et
594 al. 2006).

595 Since the isolation of the first AMP, gramicidin, from a soil *Bacillus* strain by Dubos in 1939,
596 AMPs have received much attention as a potential class of antimicrobial agents (Dubos 1939),
597 and to date, almost six thousand AMPs have already been discovered or synthesized (Zhao et al.
598 2013). AMPs have been shown to function as the first line of defense against several pathogenic
599 organisms, with demonstrated antimicrobial efficacy against Gram-positive and Gram-negative
600 bacteria (Bahar and Ren 2013), anaerobic bacteria (Arzese et al. 2003), fungi (Delattin et al. 2017)
601 and even viruses (Hsieh and Hartshorn 2016).

In addition to their direct antimicrobial activity, these small cationic peptides are multifunctional components of the innate immunity of their hosts also playing an important role in inflammation, immune activation and wound healing (Bahar and Ren 2013; Mahlapuu et al. 2016).

AMPs can act as effector molecules of the immune defense mechanism, with several studies describing their ability to modulate the host's inflammatory response (Gaspar et al. 2013). Some AMPs are able to impede the lipopolysaccharide-induced cytokine release by macrophages, reducing the inflammation that develops during an infection by Gram-negative bacteria (Zhang et al. 1999). Other AMPs are able to stimulate the inflammatory response by inducing the release of cytokines and growth factors; recruitment of neutrophils and macrophages and antigen presentation; and migration and proliferation of endothelial cells, fibroblasts and keratinocytes (Bowdish et al. 2005; Lai and Gallo 2009). Moreover, some AMPs also play a role during the late phase of wound healing by acting on granulation tissue formation via stimulation of extracellular matrix biosynthesis, collagen production, neovascularization and angiogenesis (Mangoni et al. 2016). AMPs involvement in tissue remodeling have also been observed and occurs through modulation of the extracellular matrix and stimulation of myofibroblasts differentiation (Mangoni et al. 2016).

1.2.6 Antimicrobial peptides mechanisms of action

The mechanisms of action presented by AMPs are surprisingly diverse and different from those presented by conventional antibiotics (Friedrich et al. 2000; Aoki and Ueda 2013). There are three major targets of AMPs in bacterial cells: the cell wall, including the outer membrane and the peptidoglycan layer; the plasma membrane; and the cytoplasmic components (Mahlapuu et al. 2016). Despite their ability to penetrate the bacterial cells and repress intracellular processes, namely protein and nucleic-acids synthesis, protein folding and enzymatic activity (Brogden 2005), it is well established that AMPs main mechanism of action is the disruption of microbial cell membranes (Mahlapuu et al. 2016; Bechinger and Gorr 2017). Regardless of the differences in peptide sequence and structure, the majority of AMPs are highly cationic owing to the presence of a cluster of cationic amino acid residues (Shai 1999; Wu et al. 1999; Aoki and Ueda 2013). Due to the highly content of negatively charged phospholipids, bacterial cell membranes are naturally attracted, through electrostatic forces, to cationic AMPs; on the contrary, eukaryotic cellular membranes, containing predominantly neutral phospholipids, tend to be unaffected by these small peptides. Moreover, the presence of cholesterol molecules in eukaryotic lipidic membranes also favors their resistance against AMPs disruption (Gottler and Ramamoorthy 2009).

Bacterial membrane disruption by AMPs can occur through diverse mechanisms, including pore formation in the lipid bilayer (barrel stave and toroidal pore models), membrane dissolution (carpet model), membrane thinning/thickening, lipid-peptide domain formation (micellization model), non-lytic membrane depolarization and electroporation (Nguyen et al. 2011; Gaspar et al. 2013).

As previously mentioned, the formation of surface-attached and matrix-protected microbial biofilms and the slow growth rate and reduced metabolic activity presented by biofilm-encased bacterial cells are directly related to bacterial resistance towards antibiotics and innate immune system molecules (Burmølle et al. 2006; James et al. 2008). On the other hand, AMPs mainly exert their antibacterial activity by disrupting and permeating cell membranes, i. e, they present a mechanism of action that is independent of the bacterial metabolic state (Nguyen, Haney and Vogel 2011, Mahlapuu, et al. 2016, Bechinger and Gorr 2017). Considering that membrane integrity is essential for bacterial survival, this feature allows AMPs to be effective against metabolic active and dormant microbial cells, both co-existing in the polymicrobial environment of mature biofilms (Strempel et al. 2015; Pletzer et al. 2016).

Due to their mechanism of action AMPs generally induce a fast-killing-kinetics of bacterial cells. They are able to interact with the microbial cells and exert their activity in a short time frame, inducing a rapid bacterial death and decreasing the probability of resistance development (Fernebro 2011).

1.2.7 Antimicrobial peptides resistance

AMPs play a key role on host immunity by being one of its most old and efficient defense mechanisms. Possibly due to their different modes of action, bacteria have still not developed highly effective resistance mechanisms, such as those that impair the action of many therapeutic antibiotics (Peschel and Sahl 2006). In fact, while conventional antibiotics usually present a single defined primary target and a single mode of action, acting on specific components of the microbial cells to which they have a high affinity, AMPs molecules exert multiple antimicrobial activities, aiming at less specific cellular targets and affecting numerous biological functions (Yeaman and Yount 2003; Wang et al. 2016).

While rarely observed, there are reports of resistance towards AMPs by bacterial pathogens. Resistance occurs through several mechanisms, namely proteolytic cleavage of AMPs due to the release of extracellular proteases, AMP-specific binding and extrusion via efflux pumps and alteration of the bacterial surface, specifically regarding surface molecules charges which

contribute to decrease their affinity with AMPs. Neverthless, AMPs resistance is limited and significantly reduced when compared to conventional antibiotics (Yeaman and Yount 2003; Park et al. 2011).

The multiple modes of action presented by these peptides and the targeting of vital bacterial structures, such as the cytoplasmic membrane, are amongst the main reasons impairing the bacterial development of stable and competent AMPs resistance mechanisms (Yeaman and Yount 2003; Fernebro 2011; Park et al. 2011; Jorge et al. 2017). Also, as the mechanisms responsible for AMPs resistance are diverse and different from antibiotic resistance mechanisms (Park et al. 2011), cross-resistance between antibiotics and AMPs is rare, as demonstrated in a recent study by Lázár and colleagues that showed that antibiotic-resistant *E. coli* strains present high susceptibility towards AMPs. These results support the hypothesis of the use of AMPs in combination with currently used antibiotics in order to control the emergence of multidrug-resistant bacteria (Lázár et al. 2018).

1.2.8 Antimicrobial peptides in the diabetic foot infection management

The biomedical properties of AMPs support their potential as a new therapeutic approach to manage antibiotic-resistant infections, including DFIs. An acceptable antimicrobial agent to be used in DFI management should present activity against the broad-spectrum of bacteria in the DFI environment, limited toxicity in order to avoid serious adverse effects and low risk of resistance development. The growing interest in AMPs is not only due to the above-mentioned characteristics, but also to their immunomodulatory properties (Batoni et al. 2016; Mahlapuu et al. 2016). Also, many studies have demonstrated the antimicrobial activity of these molecules against both Gram-positive and Gram-negative bacteria and their ability to interfere with different stages of the biofilm growth mode (Park et al. 2011; Batoni et al. 2016; Pletzer et al. 2016). Among the AMPs with potential to be applied in DFI treatment, nisin and pexiganan are two of the most promising ones.

Nisin is a class I bacteriocin, produced by *L. lactis*, and one of the most widely studied AMPs (Abts et al. 2011; Zhu et al. 2017). In 1969, this small polypeptide was considered safe for use as a food preservative by the FAO and WHO. Later, in 1983, nisin was added to the European list of food additives under the code E234 and five years later it was also approved by the United States Food and Drug Administration (FDA) as “Generally Regarded As Safe” for use in pasteurized products and processed cheeses to inhibit the growth of *Clostridium botulinum* and *Listeria monocytogenes* (Jozala et al. 2015). The safety and efficacy of nisin as a food preservative

have resulted in its widespread use throughout the world. Nowadays, nisin is used in over 48 countries (Jozala et al. 2015).

Nisin is a ribosomally synthesized, linear polypeptide containing 34 amino acid residues and with a molecular weight of 3500 Da. For presenting the unusual amino acid lanthionine in its structure, nisin is classified as a lantibiotic (Hansen 1994; McAuliffe et al. 2001). Besides lanthionine and methyl-lanthionine, dehydroalanine and dehydrobutyrine, amino acids that are rarely found in nature, are also present on nisin's sequence and can be responsible for its antimicrobial activity and biophysical properties such as thermostability and solubility (McAuliffe et al. 2001). The cationic nature of nisin is mainly due to the presence of lysine and histidine amino acid residues, while its amphipathicity is due to the presence of hydrophobic and hydrophilic amino acid residues at the N-terminal and C-terminal regions, respectively (McAuliffe et al. 2001). Nisin biophysical properties are pH-dependent, presenting an increased solubility and stability under acidic conditions. In neutral or alkaline environments nisin tends to lose its efficiency (McAuliffe et al. 2001).

Nisin has been shown to present a strong antimicrobial activity against a broad spectrum of Gram-positive bacteria and stable resistance is rarely reported (Zhu et al. 2017). In fact, the long-term use of nisin in food industry does not seem to have prompted significant bacterial resistance towards this AMP (Bechinger and Gorr 2017). Nisin's spectrum of activity includes a wide range of Gram-positive bacteria, such as Staphylococci, Streptococci, Enterococci, Bacilli and Micrococci (Arauz et al. 2009; Jozala et al. 2015; Zhu et al. 2017). This peptide exerts its antimicrobial activity through a dual mode of action: inhibition of cell wall synthesis and pore formation in the bacterial cytoplasmic membrane. Both mechanisms result from its interaction with the membrane-anchored peptidoglycan precursor lipid II, which is simultaneously used as a target and a pore constituent. Pore formation by nisin binding to lipid II molecules leads to efflux of cellular constituents, ultimately resulting in microbial death (Wiedemann et al. 2001).

Nisin has also demonstrated ability to inhibit and kill biofilm-associated *S. aureus*, including some antibiotic resistant strains, isolated from infected diabetic foot ulcers (Santos et al. 2016). However, the use of nisin as mono-therapeutic option to treat DFI can be limited. Indeed, the activity of nisin against Gram-negative organisms is much lower than its activity against peptidoglycan-rich Gram-positive bacteria (Breukink and Kruijff 1999; Li et al. 2018). A possible reason for this constraint is the fact that lipid II is predominantly located at the inner membrane of Gram-negative cells and their considerably impermeable outer membrane impedes nisin from reaching these molecules (Li et al. 2018). In order to overcome this limitation, nisin could be

combined with a different AMP whose spectrum of action includes Gram-negative bacteria, such as pexiganan.

Pexiganan is a synthetic 22 amino acids residues peptide, analogue of magainin, co-discovered in 1987 by Zasloff (Zasloff 1987) and Giovannini and colleagues (Giovannini et al. 1987). These scientists found out that this cationic small peptide, present in the skin secretion of the South African clawed frog *Xenopus laevis*, was directly related to its ability to resist microbial infections (Giovannini et al. 1987; Zasloff 1987). Magainin is a water soluble polypeptide, containing 23 amino acid residues and a molecular mass of 2500 Da (Giovannini et al. 1987) and has a broad-spectrum antimicrobial activity against various species of bacteria, fungi and protozoa (Zasloff et al. 1988). Despite its well-known antimicrobial properties, magainin high non-specific toxicity makes its therapeutic application difficult. For that reason, its structure and activity have been widely studied and modifications have been introduced in order to reduce its toxicity towards animal cells and improve the antimicrobial activity of the related synthetic AMP, pexiganan (Zhu et al. 2017). More specifically, single amino acid modifications were introduced with the aim of increasing the electrostatic attraction between this AMP and the negatively charged bacterial membranes (Gottler and Ramamoorthy 2009). Substitutions between the amino acid residues glycine and alanine increased the stability of the pexiganan α-helical structure, leading to an increased antimicrobial activity (Chen et al. 1988).

It is believed that pexiganan exerts its antibacterial effect by disturbing the permeability of the bacterial cell membranes via toroidal pore formation. Specifically, pexiganan binds to the negatively charged bacterial lipid bilayers and forms an antiparallel dimer of amphipathic α-helices (Gottler and Ramamoorthy 2009). The toroidal pore mechanism is characterized by the bending of the cellular membrane, resulting in the formation of pores whose surface is formed by the lipid head groups (Gottler and Ramamoorthy 2009).

Pexiganan presents activity against a wide range of bacterial species. In a study conducted by Ge and colleagues, this AMP demonstrated an excellent *in vitro* activity against numerous bacterial species, including Gram-positive and Gram-negative aerobes and anaerobes isolated from diabetic patients with infected DFUs (Ge, Macdonald, Henry, et al. 1999). Pexiganan's activity against DFI isolates, namely *Staphylococcus* spp. including *S. aureus*, *Streptococcus* spp., *Enterococcus* spp., *Pseudomonas* spp. including *P. aeruginosa*, *Stenotrophomonas* spp., *Acinetobacter* spp., *Citrobacter* spp., *Bacteroides* spp., *Peptoniphilus* spp. and *Clostridium* spp. prompted its potential as a novel antimicrobial agent with promising therapeutic applications (Ge, Macdonald, Henry, et al. 1999; Ge, Macdonald, Holroyd, et al. 1999). Additionally, Ge and collegues also reported that the repeated contact with subinhibitory pexiganan concentrations did

not generate resistant mutants and that cross-resistance with commonly used antibiotics, such as beta-lactams, quinolones, macrolides and lincosamides, was not observed (Ge, Macdonald, Holroyd, et al. 1999).

Pexiganan was the first AMP to be considered for commercial development aiming DFI treatment, and several clinical trials involving patients with infected DFU were conducted to evaluate its therapeutic potential (Gordon and Romanowski 2005; Mangoni et al. 2016). Regardless of excellent *in vitro* results, clinical trials results were not satisfactory. Pexiganan did not meet the primary clinical endpoint, since it did not produce any significant improvement in wound closure when compared to the topical placebo. Neither met the secondary endpoint of demonstrating a higher rate of bacterial eradication. Following these results, FDA approval was denied (Dipexium Pharmaceuticals 2017).

1.2.9 Conclusion

The prevalence of Diabetes *mellitus* and DFIs related complications have drastically increased globally (WHO 2016). Due to the high incidence of multidrug-resistant microorganisms in DFIs and the ineffectiveness of conventional antibiotic-based therapies, diabetic patients are at increased risk of developing the severe consequences of recalcitrant DFIs, namely wound inflammation, infection chronicity, foot gangrene, ultimately leading to lower-limb amputation (Hobizal and Wukich 2012; Lipsky et al. 2016). The emergence and dissemination of multidrug-resistant pathogens is a major global medical challenge, and diabetic patients therapeutics is no exception (Lipsky et al. 2016). Indeed, the biofilm forming ability and the antibiotic-resistance profile presented by numerous DFI isolates are accountable for the frightening scenario faced by these patients (Mendes et al. 2012; Mottola, Mendes, et al. 2016).

Over the last decades, AMPs have emerged as a potential new answer to solve this problematic situation (Strempel et al. 2015; Pletzer et al. 2016) and there are high expectations regarding the future of these peptides as alternative antimicrobial agents. In addition to their demonstrated antimicrobial activity against a wide range of pathogenic bacteria, these molecules are also able to modulate the host inflammatory response (Bahar and Ren 2013; Mahlapuu et al. 2016).

Nisin and pexiganan are two of the most promising AMPs for application in the management of DFIs. These AMPs are amongst the most studied ones and are under research as potential therapeutics against DFI pathogens, including *S. aureus* and *P. aeruginosa* (Brumfitt et al. 2002; Field, O' Connor, et al. 2016; Field, Seisling, et al. 2016; Flamm et al. 2016; Santos et

al. 2016; van Staden et al. 2016). However, previous studies suggest that these peptides present some limitations that need to be overcome. The development of combined therapeutics involving different antimicrobial agents may be one possible solution to surpass the limitations of pexiganan to act on DFIs *in vivo* (Dipexium Pharmaceuticals 2017) and the reduced activity of nisin against Gram-negative bacteria (Breukink and Kruijff 1999; Li et al. 2018).

AMPs can be used as antimicrobial agents alone or in combination with conventional antibiotics or other AMPs with different mechanisms and activity spectrum, in order to promote additive or synergistic effects (Pletzer et al. 2016). Indeed, it is well established that synergistic interactions between antimicrobial molecules could decrease antimicrobial resistance and toxicity, improving their therapeutic potential (Pletzer et al. 2016). The consensus among the scientific community is that AMPs exert their activity mostly through disruption of bacterial membranes (Gaspar et al. 2013; Mahlapuu et al. 2016; Bechinger and Gorr 2017). Microbial loss of membrane integrity promotes the entrance into the cell of antimicrobial agents, which makes AMPs efficient molecules to be used together with conventional antibiotics that have intracellular targets (Grassi et al. 2017). In the literature there are numerous reports regarding the synergistic and additive effect of combinations between AMPs, such as nisin and pexiganan, and other antibacterial agents, reflecting their predisposition to be used as adjuvants of conventional antibiotic therapies (Garbacz et al. 2017; Jorge et al. 2017).

The promising results obtained in the studies developed so far (Field, O' Connor, et al. 2016; Field, Seisling, et al. 2016) point out for the importance of further investigations regarding the use of AMPs against microbial pathogens, such as those present in DFIs. In conclusion, this chapter reinforces the need for a paradigm shift in antimicrobial treatment strategies by highlighting the potential use of AMPs as novel therapeutic weapons against antibiotic-resistant and biofilm-forming pathogens.

1.3 Objectives and thesis outline

Taking into consideration the major potential of antimicrobial peptides (AMPs) as novel therapeutic compounds against antibiotic-resistant pathogens, this work aimed to evaluate the potential of selected AMPs for the treatment of diabetic foot infections (DFIs). The development of a new antibacterial strategy for the management of recalcitrant DFIs requires the careful selection of an appropriate antimicrobial compound, the development of an effective delivery system and the screening of its possible cytotoxic effects. For that reason, in order to accomplish the aim of this work, a multidisciplinary approach was carefully designed to cover these topics. The experimental work was divided in four main parts, that can be summarized as follows:

- To determine the antimicrobial activity of nisin against a collection of *Staphylococcus aureus* isolated from DFIs and to develop an efficient delivery system for this AMP (Chapter 2);
- To study the potential of nisin to complement the activity of conventional antiseptics and antibiotics regularly used in the management of DFIs (Chapter 3);
- To determine the ideal storage conditions for the nisin-biogel regarding time and temperature and to evaluate its cytotoxic potential against epidermal keratinocytes (Chapter 4);
- To evaluate the potential of nisin to complement the activity of pexiganan against two selected *S. aureus* and *P. aeruginosa* strains co-isolated from the same DFI (Chapter 5).

To properly address and discuss the above-mentioned objectives, this thesis was divided into six chapters. The first chapter consists of a detailed state of the art review and includes two chapters published in international scientific books. Chapters 2 and 3 correspond to scientific papers already published in international peer reviewed journals, chapters 4 and 5 correspond to scientific papers under consideration for publication in international peer reviewed journals. Finally, chapter 6 integrates the results presented in the previous chapters, aiming at a global discussion and conclusion.

Chapter 2

2. Guar gum as a new antimicrobial peptide delivery system against diabetic foot ulcers *Staphylococcus aureus* isolates

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Adapted from:

Santos R, Gomes D, Macedo H, Barros D, Tibério C, Veiga AS, Tavares L, Castanho M, Oliveira M. 2016. Guar gum as a new antimicrobial peptide delivery system against diabetic foot ulcers *Staphylococcus aureus* isolates. J Med Microbiol. 65:1–8. Doi: https://doi.org/10.1099/jmm.0.000329.

2.1 Abstract

Diabetic patients frequently develop diabetic foot ulcer (DFU), particularly vulnerable to *Staphylococcus aureus* opportunistic infections. It is urgent to find new treatments for bacterial infections. The antimicrobial peptide (AMP) nisin is a potential candidate, mainly due to its broad spectrum of action against pathogens. Considering that AMPs can be degraded or inactivated before reaching their target at therapeutic concentrations, it is mandatory to establish effective AMPs delivery systems, being the natural polysaccharide guar gum one of the most promising.

We analyzed the antimicrobial potential of nisin against 23 *S. aureus* DFUs biofilm-producing isolates. Minimum inhibitory (MIC), bactericidal (MBC), biofilm inhibitory (MBIC) and biofilm eradication (MBEC) concentrations were determined for nisin diluted in HCl and incorporated in guar gum gel. Statistical analysis was performed using the Wilcoxon Matched Pairs Test.

Nisin was effective against all isolates, including some multidrug-resistance clinical isolates, independently of being or not incorporated in guar gum.

While differences between MIC, MBC and MBIC values were observed for HCl- and guar gum nisin, no significant differences were found between MBEC values. Inhibitory activity of both systems seems to differ only 2-fold, which does not compromise guar gum gel efficiency as a delivery system.

Our results highlight the nisin potential as a substitute or complementary therapy to current antibiotics used for treating DFU infections, extremely relevant considering the increase in multidrug-resistant bacteria. The guar gum gel represents an alternative, practical and safe delivery system for AMPs, allowing the development of novel topical therapies as treatments for bacterial skin infections.

2.2 Introduction

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide. Recently, the WHO Global report on diabetes demonstrated that the number of adults suffering from diabetes has almost quadrupled since 1980 to 422 million people. This dramatic rise is largely due to the rise in type 2 diabetes and factors driving it include overweight and obesity (Roglic 2016). Diabetic foot ulcers (DFUs) are one of the most frequent complications of diabetes, resulting from a complex interaction of several pathophysiological factors. Although ischemic and neuropathic lesions have the initial role in DFU onset (Jeffcoate and Harding, 2003; Vuorisalo et al. 2009; Armstrong et al. 2011), it is the infection by pathogenic microorganisms along with local microenvironmental conditions unfavorable to antibiotics action, that ultimately cause infection chronicity and lower limbs amputation (Lipsky et al. 2004; Richard et al. 2011).

Diabetes-associated foot ulcer infections are usually polymicrobial and several bacterial genera can be part of its microbiota, mainly gram-positive bacteria, being *S. aureus* the most predominant species (Mendes et al. 2014; Mottola, Mendes, et al. 2016). *S. aureus* is a commensal bacterium known to colonize the human skin and mucosal surfaces. Colonized individuals are at increased risk for developing *S. aureus* infections, which range from minor skin and soft tissue infections to severe diseases, such as endocarditis, septicaemia and osteomyelitis (Jenkins et al. 2015).

These bacteria have the ability to produce several virulence factors, being biofilm formation one of the most important. These are ubiquitous and complex structures consisting of an interactive community of polymicrobial cells embedded in a self-produced extracellular matrix of

hydrated polymeric substances, such as proteins, polysaccharides, nucleic acids and others, irreversibly attached to biological surfaces (Dickschat 2010). Due to inefficient diffusion or sequestering of the agent within the biofilm matrix, biofilm-based bacteria are recalcitrant to the action of most antibiotics and also more resistant to the innate immune system (An et al. 2016; Stewart and Costerton 2001). Moreover, in the past few decades a major problem in treating DFU infections is the presence of antibiotic resistant pathogens, particularly Methicillin-Resistant *S. aureus* (MRSA) (Stanaway et al. 2007; Akhi et al. 2016; Dang et al. 2003; Mottola, Semedo-Lemsaddek, et al. 2016). The rates of isolation of these multidrug-resistant pathogens vary widely among geographical area and treatment center (Kandemir et al. 2007; Richard et al. 2008). However, the increasing incidence of multidrug-resistant microorganisms, together with the incapacity of antibiotics to act on resistant and biofilm-producing bacteria at therapeutical concentrations, emphasizes the importance of developing new treatment strategies to effectively eradicate these infections.

Antimicrobial peptides (AMPs) are molecules produced by the vast majority of living organisms as part of their innate immune response against a broad range of pathogens (Zasloff 2002; Hancock and Sahl 2006; Lewis 2013) and, unlike conventional antibiotics, AMP can also act as modulators of the immune system (Kirikae et al. 1998; Rosenfeld et al. 2006; Batoni et al. 2016).Additionally, some authors suggest that AMPs are able to prevent biofilm formation and act on pre-formed biofilms (Overhage et al. 2008; Strempel et al. 2015), supporting their potential as alternatives to currently available DFUs therapeutic agents (Mohammad et al. 2015). One of the best studied and characterized AMP is nisin (Abts et al. 2011). It belongs to the class I bacteriocins, also known as lantibiotics. These are small peptides containing unusual amino acids such as lanthionine and L-methyllanthionine and a number of dehydrated amino acid residues (McAuliffe et al. 2001). Nisin is produced by *Lactococcus lactis*, acts principally against Gram-positive bacteria and has been used as a food preservative for over sixty years (Cleveland et al. 2001; Gharsallaoui et al. 2016).

Despite all their advantages, AMP successful delivery represents a challenge, since they can be degraded or inactivated before reaching their target at therapeutic concentrations (O'Driscoll et al. 2013). Natural polysaccharides have been considered as promising drug delivery systems by the pharmaceutical industries, mainly because of their non-toxicity, biodegradability, biocompatibility, abundant availability in nature and economical costs (Reddy et al. 2011). Guar gum is a natural polysaccharide obtained from the endosperm of the leguminous crop *Cyamopsis tetragonolobus* and consists of a linear polymer of d-galactose and d-mannose, called galactomannan (Thombare et al. 2016). This hydroxyl group rich polymer when added to water

forms hydrogen bonds that confer a significant viscosity to the solution. Due to its thickening, emulsifying, gelling and binding properties, quick solubility in cold water, wide pH stability and film forming ability, it finds application as a safe and versatile system for delivery of bioactive agents (Reddy et al. 2011; Thombare et al. 2016).

The present study was designed not only to determine the antimicrobial activity of nisin against both planktonic and biofilm-based *S. aureus* diabetic foot clinical isolates collected in Lisbon Medical Centers, but also to evaluate the efficiency of the peptide incorporated in a guar gum gel to be used as a delivery system for this AMP.

2.3 Materials and methods

2.3.1 Bacterial isolates

In a previous epidemiological survey regarding DFUs infectious microbiota conducted from January to July 2010, a total of 54 S*taphylococus* spp. clinical isolates were collected from 49 DFU patients (Mendes et al. 2012). All isolates were characterized regarding clonality, antimicrobial resistance and virulence profiles. Based on macrorestriction analysis by pulsed-field gel electrophoresis and multilocus sequence typing, 23 representative *S. aureus* strains were selected (Mottola, Semedo-Lemsaddek, et al. 2016). All the 23 strains were the object of the current study. Additionally, a reference strain, *S. aureus* ATCC 29213, a known biofilm producer, was also included as a control strain. As a result, the number of strains analyzed in this work is 24.

2.3.2 Antimicrobial peptide preparation and guar gum incorporation

A nisin stock solution (1000 µg/mL, corresponding to 40 000 IU/mL) was obtained by dissolving 1 g of nisin powder (2.5% purity, 1000 IU/mg, Sigma-Aldrich, USA) in 25 mL of HCl (0.02 M) (Merck, Germany). The nisin stock solution was filtered using a 0.22 µm Millipore filter (Frilabo, Portugal) and stored at 4°C. A set of dilutions of nisin were prepared, corresponding to the following concentrations: 900, 800, 700, 600, 500, 400, 300, 200, 100, 40, 20, 10 and 5 µg/mL.

A guar gum gel of 1.5% (w/v) was prepared by dissolving 0.75 g of guar gum (Sigma-Aldrich, USA) in 50 mL of sterile distilled water, and heat sterilized by autoclave. The set of dilutions of nisin were incorporated within the gel in a proportion of 1:1, obtaining a final gel of 0.75% (w/v).

2.3.3 Minimum inhibitory concentration and minimum bactericidal concentration determination

MIC value of nisin was determined by microtiter broth dilution method (Wiegand et al. 2008).

Strains were grown in a non-selective brain heart infusion (BHI) agar medium (VWR Chemicals, Belgium) at 37 °C for 24 h. Bacterial suspensions with approximately 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard (BioMérieux, France) in sterile normal saline (Scharlau, Spain). For MIC and MBC assays, bacterial suspensions were diluted in fresh BHI broth (VWR Chemicals, Belgium) to a concentration of ≈10^7 CFU/mL.

The set of concentrations of nisin, diluted in HCl or incorporated in the guar gum gel, ranging from 5 µg/mL (5 IU per well) to 1000 µg/mL (1000 IU per well), were distributed in 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fisher Scientific, Denmark). All the wells, except for the negative control (with only broth medium), were inoculated with 150 µL of the 10^7 CFU/mL bacterial suspensions. Microplates were statically incubated for 24 h at 37 °C and MIC was determined as the lowest concentration of nisin that visually inhibited the microbial growth.

MBC value was determined by inoculating a 3 µL dot of the suspension from the wells where no bacterial growth was observed on BHI agar plates that were incubated at 37 °C for 24 h. MBC was determined as the lowest nisin concentration at which no colonies were observed. Experiments were conducted in triplicate and independent replicates were performed at least three times in different days. For each strain, nine results were obtained and analyzed.

2.3.4 Minimum biofilm inhibitory concentration and minimum biofilm eradication concentration determination

A modified version of the Calgary Biofilm Pin Lid Device (Ceri et al. 1999) was used to determine the antimicrobial susceptibility of bacteria embedded in a 24 h biofilm.

For MBIC and MBEC assays, bacterial suspensions prepared as described before were diluted in fresh Tryptic Soy Broth (TSB) (VWR Chemicals, Belgium) + 0.25% (w/v) glucose (Merck, USA) medium to a concentration of ≈10^6 CFU/mL.

Briefly, 200 µL of the ≈10^6 CFU/mL bacterial suspensions were distributed in 96-well flat-bottomed polystyrene microtiter plates, covered with 96-peg polystyrene lids (Nunc-TSP, Thermo

Fisher Scientific, Denmark) and statically incubated for 24 h at 37°C, to allow biofilm formation on pegs. Peg lids were then rinsed three times in sterile normal saline to remove planktonic bacteria and placed on new microplates containing the set of nisin concentrations, diluted in HCl or incorporated in the guar gum gel, with concentrations ranging from 5 µg/mL (5 IU per well) to 1000 µg/mL (1000 IU per well), and 200 µL of fresh TSB + 0.25% glucose medium. Microplates were incubated for 24 h at 37 °C, without shaking. After incubation, peg lids were removed and the MBIC value was determined as the lowest nisin concentration that visually inhibited the microbial growth.

Subsequently, in order to determine the MBEC value, peg lids were rinsed three times in sterile normal saline, placed in new microplates containing only 200 µL of fresh TSB + 0.25% (w/v) glucose medium and incubated in a ultrasound bath (Grant MXB14, England), at 50 Hz during 15 min in order to disperse the biofilm-based bacteria from the peg surface. Afterwards, peg lids were discarded and microplates were covered with normal lids and incubated for 24 h at 37 °C.

Next, MBEC was determined through direct observation of experimental wells and MBEC value was defined as the lowest nisin concentration that visually eliminate the microbial growth. Aditionally, MBEC quantification was also conducted according with a previously described protocol using Alamar Blue, a redox indicator that yields a colorimetric change in response to metabolic activity (Pettit et al. 2005). Briefly, 5 µl of resazurin (Alamar Blue, Thermo Fisher Scientific, Spain) were added in each well and microplates were incubated for 1 h at 37°C. Absorbance values at 570 nm and 600 nm were then recorded using a microplate reader (BMG LABTECH, Germany).

Percent of Alamar Blue reduction was calculated using the following formula (Pettit et al. 2005):

$$\frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2}{(\varepsilon_{red})\lambda_1 A'\lambda_2 - (\varepsilon_{red})\lambda_2 A'\lambda_1} \times 100$$

where ε_{ox} = molar extinction coefficient of Alamar Blue oxidized form ($\varepsilon_{ox}\lambda_1$ = 80.586 and $\varepsilon_{ox}\lambda_2$ = 117.216), ε_{red} = molar extinction coefficient of Alamar Blue reduced form ($\varepsilon_{red}\lambda_1$ = 155.677 and $\varepsilon_{red}\lambda_2$ = 14.652), A = absorbance of test wells, A' = absorbance of negative control well, λ_1 = 570 nm and λ_2 = 600 nm.

MBEC value was defined as the lowest nisin concentration resulting in ≥ 50% of Alamar Blue reduction. Experiments were conducted in triplicate and independent replicates were

performed at least three times in different days. For each strain, nine results were obtained and analysed.

2.3.5 Guar gum gel viability assay

The nisin-incorporated guar gum gel was stored at different temperatures (-18, 4, 20, 37 and 44°C) during six months. Its efficacy as a delivery system was tested at three different time points (1, 3 and 6 months) by placing a 3 µL drop of the nisin-incorporated guar gum gel on BHI agar plates with a lawn culture executed using 10^7 CFU/mL bacterial suspensions. Plates were incubated at 37 °C for 24 h and inhibition halos diameters were measured.

2.3.6 Statistical analysis

Qualitative variables (presence/absence of growth) were expressed as percentages, and quantitative variables (concentrations) are expressed as means ± standard deviation. Data analysis was performed using STATISTICA Data Miner software, version 13. Significance of the study variables was tested using Wilcoxon Matched Pairs Tests. A two-tailed *p*-value < 0.05 was considered to be statistically significant.

2.4 Results

2.4.1 Minimum inhibitory concentration and minimum bactericidal concentration

MIC and MBC values are presented in Table 1 and summarized in Figure 1.

All isolates, including the reference strain *S. aureus* ATCC 29213 were considered susceptible to nisin. MIC values for nisin diluted in HCl ranged from 40 to 100 µg/mL, with an average value of 90 ± 22.8 µg/mL. When incorporated in guar gum gel, nisin MIC concentrations were significantly different (*p*-value < 0.05) and ranged from 40 to 300 µg/mL. The average value was 180.8 ± 53.9 µg/mL - Table 1 and Figure 1 a, b.

MBC values were approximately 5-fold higher than the MIC ones. For nisin diluted in HCl, the average MBC value was 495.2 ± 149.9 µg/mL, and only three isolates presented a MBC >800 µg/mL. For nisin incorporated in guar gum gel, MBC were also significantly different (*p*-value <

0.05) with the average MBC being 766.7 ± 272.6 µg/mL, and only three isolates presenting a MBC >1000 µg/mL - Table 1 and Figures 1 a, b.

2.4.2 Minimum biofilm inhibitory concentration and minimum biofilm eradication concentration

MBIC and MBEC values are presented in Table 1 and summarized in Figure 1.

Considering nisin diluted in HCl, MBIC values ranged from 20 to 300 µg/mL and the average value was 150.8 ± 85.5 µg/mL. When delivered through guar gum gel, nisin MBIC concentrations were significantly different (p-value < 0.05) and ranged from 100 to 600 µg/mL. The average value was 366.7 ± 140.4 µg/mL - Table 1 and Figure 1 c, d.

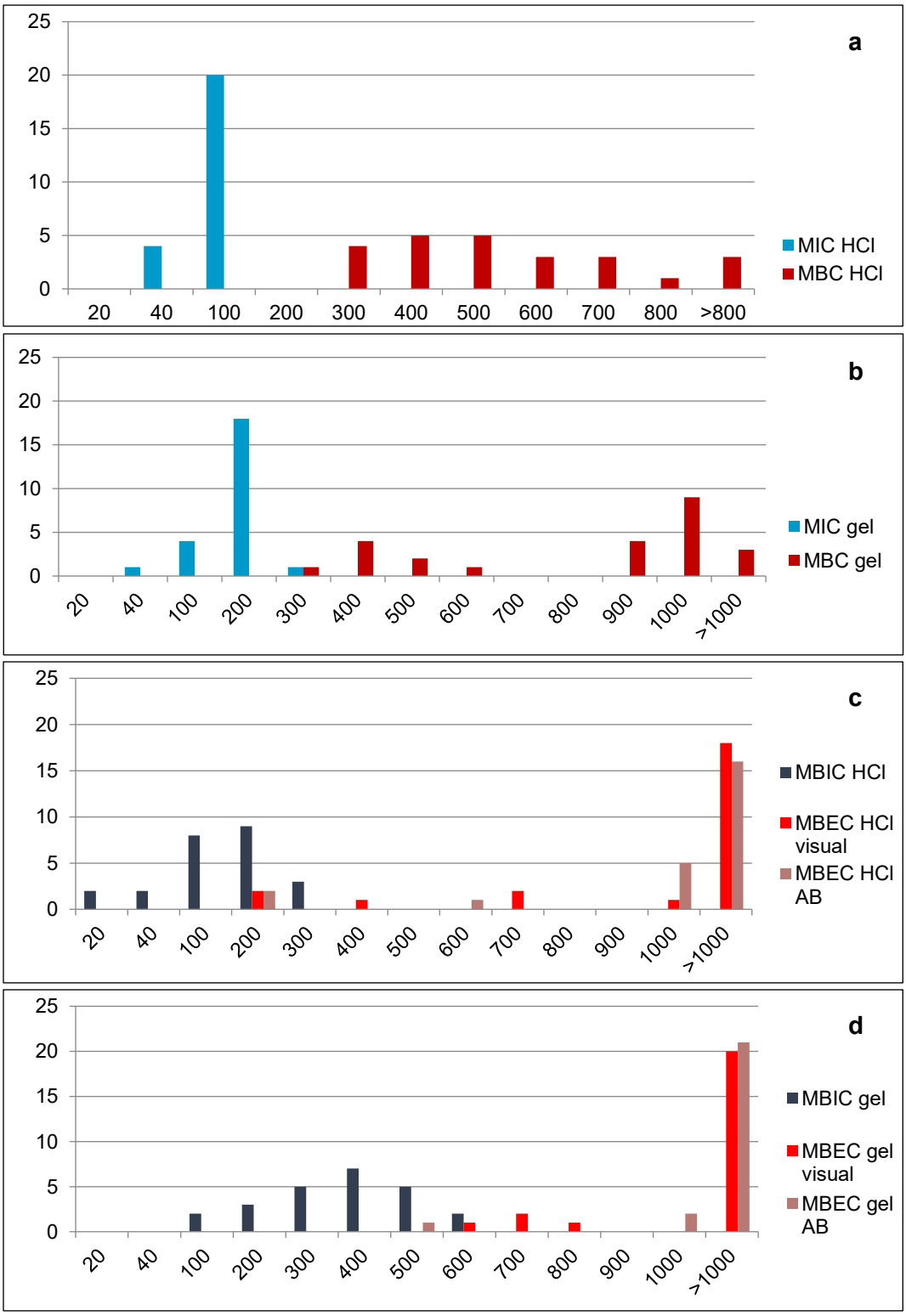
MBEC values were higher than the respective MBIC. No significant differences (p-value ≥0.05) were observed between the nisin diluted in HCl and the nisin impregnated in the guar gum gel. The majority of isolates presented MBEC values >1000 µg/mL, namely 65% (n=15) for nisin diluted in HCl and 87% (n=20) for nisin impregnated in guar gum gel - Table 1 and Figure 1 c, d.

In the MBEC assay, before adding the Alamar Blue to the wells, cell growth was visually evaluated and MBEC values were registered, for nisin diluted in HCl and for nisin incorporated in the guar gum gel. When compared to the MBEC values obtained after quantification using the Alamar Blue reduction formula (Pettit et al. 2005), no significant differences were observed between results from both MBEC determination methods, neither for nisin diluted in HCl nor for nisin incorporated in guar gum gel (p-value ≥0.05).

Table 1 – Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Minimum Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration determinations for nisin diluted in HCl and incorporated in guar gum against *Staphylococcus aureus* diabetic foot ulcer isolates.

			Nisin - HCl					Nisin – Guar gum				
Strain ID	**Strains characterization**		**MIC (µg/ml)**	**MBC (µg/ml)**	**MBIC (µg/ml)**	**MBEC Visual (µg/ml)**	**MBEC AB (µg/ml)**	**MIC (µg/ml)**	**MBC (µg/ml)**	**MBIC (µg/ml)**	**MBEC Visual (µg/ml)**	**MBEC AB (µg/ml)**
A 1.1	MRSA		100	600	200	>1000	>1000	200	1000	600	>1000	>1000
A 5.2			100	300	100	>1000	>1000	200	>1000	400	>1000	>1000
A 6.3			100	>800	300	>1000	>1000	100	500	400	>1000	>1000
B 3.2			100	700	100	>1000	1000	200	1000	400	>1000	>1000
B 3.3			100	800	100	>1000	>1000	200	>1000	500	>1000	>1000
B 7.3	MRSA	MDR	100	>800	100	>1000	>1000	200	900	500	>1000	>1000
B 13.1	MRSA	MDR	40	400	200	>1000	>1000	100	400	300	>1000	>1000
B 14.2	MRSA		100	500	100	>1000	>1000	200	1000	300	>1000	>1000
B 23.2			40	300	20	400	600	100	400	100	700	1000
S 1.1	MRSA		100	700	100	>1000	>1000	200	900	400	>1000	>1000
S 2.2			40	300	200	>1000	>1000	200	500	400	>1000	>1000
S 3.1			100	400	200	1000	1000	200	1000	300	700	>1000
S 5.2			100	600	100	700	1000	200	900	300	>1000	>1000
S 14.1			100	300	300	>1000	>1000	300	600	600	>1000	>1000
S 16.1	MRSA	MDR	100	700	200	>1000	>1000	200	900	500	>1000	>1000
S 16.2			100	400	20	200	200	200	1000	100	600	1000
S 17.2			40	400	40	200	200	200	400	200	800	500
S 21.1	MRSA	MDR	100	500	200	>1000	>1000	200	1000	300	>1000	>1000
S 21.3	MRSA	MDR	100	400	200	700	>1000	200	1000	500	>1000	>1000
S 25.2			100	600	40	>1000	>1000	100	400	200	>1000	>1000
S 27.2			100	500	200	>1000	1000	200	1000	400	>1000	>1000
S 27.3			100	>800	200	>1000	>1000	200	>1000	400	>1000	>1000
S 32.2			100	500	100	>1000	1000	40	300	200	>1000	>1000
ATCC 29213			100	500	300	>1000	>1000	200	1000	500	>1000	>1000

A – Aspirate; AB – Alamar Blue; ATCC – American Type Culture Collection; B – Biopsy; HCl – Hydrogen Chloride; ID – Identification; MBC – Minimum Bactericidal Concentration; MBEC – Minimum Biofilm Eradication Concentration; MBIC – Minimum Biofilm Inhibitory Concentration; MDR – Multidrug Resistant; MIC – Minimum Inhibitory Concentration; MRSA – Methicillin-Resistant *Staphylococcus aureus*; S – Swab.



1117 **Figure 1 – Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Minimum**
1118 **Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration determinations**
1119 **(µg/mL) for nisin diluted in HCl – Figure 1 a, c – and incorporated in guar gum – Figure 1 b, d –**

against *Staphylococcus aureus* diabetic foot ulcer isolates. The y-axis represents the number of isolates and the x-axis the nisin concentrations tested.

AB – Alamar Blue; HCl – Hydrogen Chloride; MBC - Minimum Bactericidal Concentration; MBEC – Minimum Biofilm Eradication Concentration; MBIC – Minimum Biofilm Inhibitory Concentration; MIC – Minimum Inhibitory Concentration.

2.4.3 Guar gum gel viability assay

The effect of temperature and storage period on the antimicrobial activity of nisin incorporated in the guar gum gel was investigated using the agar diffusion method. Results revealed that nisin kept its activity in all temperatures tested, from -18 to 44 °C, during six months (Table 2).

Table 2 – Guar gum gel viability assay: diameters of inhibition halos (mm) promoted by nisin incorporated in guar gum gel on brain hearth infusion agar plates with 10^7 CFU/mL bacterial lawn cultures.

	Stored time (months)		
T (ºC)	**1**	**3**	**6**
-18	10.6 ± 2.7	9.9 ± 1.8	12.7 ± 3.8
4	10.3 ± 4.0	11.6 ± 2.4	12.8 ± 2.1
20	9.6 ± 1.4	9.5 ± 1.3	9.2 ± 2.8
37	14.2 ± 2.1	8.9 ± 2.4	13.0 ± 3.5
44	11.3 ± 4.6	10.1 ± 4.1	10.2 ± 2.9

CFU – Colony Forming Units; T – Temperature

2.5 Discussion

Multiple factors are involved in diabetic foot ulceration, namely neuropathy, abnormal foot biomechanics and peripheral arterial disease (Jeffcoat and Harding 2003; Vuorisalo 2009). Infection occurs following the traumatic injury with introduction of pathogenic bacteria, mainly *S. aureus* (Mendes et al. 2014; Mottola, Semedo-Lemsaddek, et al. 2016). Failure to recognize and control the infectious process may have devastating consequences, such as limb amputation, sepsis and even death (Lipsky et al. 2004).

According to the European Center for Disease Prevention and Control, MRSA has been the most important cause of antimicrobial resistant healthcare-associated infections worldwide and Portugal is one of the European countries presenting higher rates of MRSA incidence (ECDC, 2015).

All *S. aureus* DFU isolates under analysis were previously characterized regarding their antimicrobial resistance profile (Mottola, Semedo-Lemsaddek, et al. 2016), being observed that 35% (n=8) were resistant to cefoxitin and carriers of the *mecA* gene, thus being classified as MRSA (CLSI, 2013). Moreover, 22% (n=5) were considered to be multidrug resistant, since were resistant to three or more antimicrobials belonging to different antibiotic classes (Magiorakos and Srinivasan 2012).

The biofilm mode of growth of the infecting organisms is another major contributor to the healing impediment of DFUs since biofilm-based bacteria can resist to antibiotic concentrations 10-10000 times higher than those needed to kill planktonic cells (Kaplan, 2011). Besides their antimicrobial resistant nature, all *S. aureus* strains evaluated in this study were able to create, at adequate conditions, a stable biofilm matrix in less than 24 h (Mottola, Mendes, et al. 2016).

Considering the overall clinical and economical burden caused by such virulent strains, it is of utmost importance to identify, develop or redesign effective alternative treatment regiments for DFUs. In recent years, AMPs have attracted great interest in their potential use as new antibacterial agents mainly due to their high antibacterial activity and low AMPs resistance development (Kirikae et al. 1998; Zasloff, 2002; Hancock and Sahl 2006; Rosenfeld et al. 2006).

Nisin is one of these peptides, being produced by *L. lactis* and possessing antimicrobial activity against a broad range of Gram-positive bacteria, including *S. aureus* strains. For that reason, it is regularly used for the control of pathogens in food products (Cleveland et al. 2001). In fact, nisin (E234) is authorized for food preservation in the European Union by Directive 95/2/EC on food additives and its acceptable daily intake is 0.13 mg/kg body weight (EFSA, 2006).

Here, we set out to evaluate for the first time the ability of nisin to control a range of *S. aureus* DFU isolates when incorporated in guar gum, a natural galactomannan polymer, with the ultimate aim of identifying its efficacy as a topical delivery system for AMPs.

As results have shown, susceptibility to nisin was a characteristic of all *S. aureus* DFU clinical isolates studied. It is important to refer that this group of bacteria includes, among others, eight MRSA isolates, being five of them also resistant to three or more antibiotic classes (Mottola, Semedo-Lemsaddek, et al. 2016).

Nisin presented high levels of antimicrobial activity towards planktonic bacteria, with MIC ≤100 μg/mL and MBC 5.5 times higher. Since antimicrobial agents are usually classified as

bactericidal if the MBC is no more than four times the MIC (French, 2006), our results showed that nisin is a bacteriostatic agent against *S. aureus* strains. However, since the MBC value is similar to the limit value used to classify an antimicrobial agent as bacteriostatic, its bactericidal potential cannot be disregarded and nisin should be considered a valued AMP to kill free-floating bacteria.

When applied to biofilm cells, nisin MBIC values were ≤300 µg/mL. Established biofilms were more difficult to eradicate and only 35% of isolates presented MBEC values ≤1000 µg/mL. These results are in agreement with some previous studies that have already analyzed the *in vitro* activity of this AMP against biofilm-producing *S. aureus* strains (Okuda et al. 2013). It is also important to refer that MBEC values were determined using two approaches, namely by MBEC quantification according to the percentage of Alamar Blue reduction that depends on bacterial cells metabolic viability (Pettit et al. 2005) and by the visual direct observation of microbial growth. No statistically significant differences were observed between these two approaches, suggesting that the visual direct observation of biofilm inhibition provides accurate MBEC determinations, avoiding the need for the application of a very expensive methodology. However, visual determinations should not be applied to rigorous cell metabolic activity determination Also, the natural polysaccharide guar gum displayed a very good efficacy as a delivery system for this peptide. In fact, nisin kept its antimicrobial activity towards *S. aureus* DFU strains when incorporated in the guar gum gel, with all strains presenting susceptibility to this AMP-delivery system combination. As observed in the MIC and MBIC determinations, the inhibitory activity of this AMP incorporated in guar gum was only 2-fold higher than the one from nisin diluted in HCl, proving that this delivery system acts not only in free-living cells but also in established biofilms. Similarly, MBC values of nisin incorporated in guar gum were less than 2-fold higher than those from nisin alone. As predicted, sessile bacteria were consistently more difficult to eliminate and only 13% of preformed biofilms were eradicated by the concentrations used in this study.

Furthermore, nisin-incorporated in guar gum maintained its antimicrobial activity when stored in a broad range of temperatures for a minimum of six months, which is probably due to the physical and chemical characteristics of the guar gum gel formulation (Reddy et al. 2011; Thombare et al. 2016). Besides its storage characteristics, the 0.75% (w/v) guar gum gel keeps its viscosity when applied in the human surface skin (data not shown), which shows its potential for topical therapeutical administration. Also, its eventual clinical application is strengthened by the fact that nisin minimum concentrations required to inhibit and eradicate planktonic cells and to inhibit biofilm cells are below nisin's acceptable daily intake, either when the peptide is diluted in HCl or incorporated in the guar gum gel. Moreover, it is important to refer that the Directive 95/2/EC on nisin (EFSA, 2006) was established for oral consumption. Considering that we are developing

a gellified delivery system for topical application, we assume that the nisin-incorporated guar gum gel can be safely and effectively applied to clinical patients suffering from DFUs.

In conclusion, results suggest that nisin has the ability to rapidly diffuse in the guar gum polymer and to inhibit and eradicate staphylococcal planktonic cells and established biofilms. This innovative therapeutic strategy may in the future substitute or complement antibiotherapy, ultimately contributing for the decrease in multidrug resistant bacteria dissemination. The use of guar gum gel as a delivery system for antimicrobial compounds can lead to the development of novel topical therapies for the treatment of generalized bacterial skin infections, particularly those promoted by pathogenic bacteria with reduced susceptibility to current antibiotic agents.

Chapter 3

3. Diabetic foot infections – Application of a nisin-biogel to complement the activity of conventional antibiotics and antiseptics against *Staphylococcus aureus* biofilms

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Adapted from:

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3.1 Abstract

Background: Diabetic foot infections (DFIs) are a frequent complication of Diabetes *mellitus* and a major cause of nontraumatic limb amputations. The Gram-positive bacterium *Staphylococcus aureus,* known for its resilient biofilms and antibiotic resistant profile, is the most frequent DFI pathogen. It is urgent to develop innovative treatments for these infections, being the antimicrobial peptide (AMP) nisin a potential candidate. We have previously proposed the use of a guar gum biogel as a delivery system for nisin. Here, we evaluated the potential of the nisin-biogel to enhance the efficacy of conventional antibiotics and antiseptics against DFIs *S. aureus* clinical isolates.

Methods: A collection of 23 *S. aureus* strains isolated from DFI patients, including multidrug- and methicillin-resistant strains, was used. The antimicrobial activity of the nisin-biogel was tested alone and in different combinations with the antiseptic chlorhexidine and the antibiotics clindamycin, gentamicin and vancomycin. Isolates' *in vitro* susceptibility to the different protocols was assessed using broth microdilution methods in order to determine their ability to inhibit and/or eradicate established *S. aureus* biofilms. Antimicrobials were added to the 96-well plates every 8 h to simulate a typical DFI treatment protocol. Statistical analysis was conducted using RCBD ANOVA in SPSS.

Results: The nisin-biogel showed a high antibacterial activity against biofilms formed by DFI *S. aureus*. The combined protocol using nisin-biogel and chlorhexidine presented the highest efficacy in biofilm formation inhibition, significantly higher (p-value < 0.05) than the ones presented by the antibiotics-based protocols tested. Regarding biofilm eradication, there were no significant differences (p-value > 0.05) between the activity of the combination nisin-biogel plus chlorhexidine and the conventional antibiotic-based protocols.

Conclusions: Results provide a valuable contribution for the development of complementary strategies to conventional antibiotics protocols. A combined protocol including chlorhexidine and nisin-biogel could be potentially applied in medical centres, contributing for the reduction of antibiotic administration, selection pressure on DFI pathogens and resistance strains dissemination.

3.2 Introduction

Diabetes *mellitus* (DM) is a chronic disease that affects more than 422 million people worldwide. Moreover, in the recent decades, the prevalence of DM has increased from 4.7% in 1980 to 8.5% in 2014 (WHO 2016). As a consequence, DM-associated foot ulcers prevalence has also increased (Lipsky et al. 2012). These ulcers result from consequence of a complex interaction of several pathophysiological factors, mainly neuropathy, vasculopathy and immunopathy (Armstrong et al. 2011), being observed that approximately 15 to 25% of patients with DM develop DFUs in their lifetime (Hobizal and Wukich 2012).

Around half of diabetic foot ulcers (DFUs) become clinically infected, usually by opportunistic pathogens (Mendes et al. 2012). DFIs are a frequent and complex problem that causes severe morbidity, including distress, and reduced physical and psychological quality of life. DFI treatment requires wound care, antimicrobial therapy, and often surgical procedures (Lipsky

et al. 2012). As a result, DFIs are the most common diabetic complication requiring hospitalization and the world's leading cause of nontraumatic lower extremity amputation (Lipsky et al. 2016).

DFIs are caused by a polymicrobial community of pathogens, mainly formed by Gram-positive bacteria, with *S. aureus* being the most prevalent species (Dang et al. 2003; Hobizal and Wukich 2012; Mendes et al. 2012). This commensal bacterium is known to asymptomatically colonize the human skin and mucosal surfaces, being permanently present in 20 to 30% of the population, while other 30% are transient carriers (Kluytmans et al. 1997).

S. aureus is recognized for its ability to develop resistance to different antibiotic classes and infections caused by antibiotic resistant *S. aureus* strains are globally reaching epidemic proportions (Chambers and DeLeo 2009). In fact, a key problem in DFI treatment is the increasing incidence of antibiotic resistant pathogens, particularly Methicillin-Resistant *S. aureus* (MRSA) (Mottola, Semedo-Lemsaddek, et al. 2016; Akhi et al. 2017). Among hospitalized patients, the prevalence of MRSA in DFIs can range from 15 to 30% (Hobizal and Wukich 2012).

Another important *S. aureus* virulence factor responsible for antibiotic therapeutic failure in DFIs is the formation of biofilms (Dickschat 2010). These slime-enclosed aggregates of sessile bacteria are embedded within a self-produced matrix of extracellular polymeric substances and irreversibly attached to surfaces (Vert et al. 2012). Due to ineffective diffusion or sequestering of antimicrobial agents within the biofilm, these bacterial communities demonstrate great resistance to most antibacterial agents as well as to host defenses (Malik et al. 2013).

Currently, the treatment of infected DFUs consists of surgical debridement followed by wound cleansing with an antiseptic solution and antibiotics administration (Lipsky et al. 2016). A wide variety of antiseptics is available, being chlorhexidine one of the most frequently used in DFIs Lipsky et al. 2014). It is widely used worldwide for skin antisepsis and daily skin cleansing with chlorhexidine has been used to control *S. aureus* infections, including MRSA outbreaks (Schlett et al. 2014). Additionally, chlorhexidine has also shown some ability to inhibit microorganism's adherence to surfaces, thereby preventing the growth and development of biofilms (Bonez et al. 2013; Touzel et al. 2016).

Antibiotics administration for DFIs treatment can be performed oral or intravenously, depending on the severity of infection. According to the guidelines for the medical management of DFI from Lipsky et al. (2012, 2016), Chidiac et al. (2007), Bader (2008), and Duarte and Gonçalves (2011), the antibiotics of choice for mild, moderate and severe DFI are, respectively, clindamycin (450 mg, 8/8h, oral), gentamicin (5 mg/kg, 24/24h, intravenous) and vancomycin (30 mg/kg, 12/12h, intravenous).

Clindamycin has been considered a first line choice for the treatment of various skin and soft tissue infections, like DFIs. It can also be used for the treatment of moderate and severe DFI, but in such cases it should be combined with other antibiotics from different classes (Chidiac et al. 2007; Bader 2008; Lipsky et al. 2012), Gentamicin is commonly used for the prophylaxis and treatment of moderate and severe DFIs (Chidiac et al. 2007; Duarte and Gonçalves 2011), while vancomycin use is reserved for cases of severe infection, being considered a last resource antibiotic against MRSA infections (Binda et al. 2014).

As the DFI treatments available are often ineffective (Lipsky and Hoey 2009), new therapeutic strategies for DFI treatment are urgent and the application of topical AMPs may be a useful complement or alternative to conventional treatments. These molecules are produced by living organisms as part of their immune response against pathogens (Hancock and Sahl 2006), can act as modulators of the immune system (Rosenfeld et al. 2006), and are able to prevent biofilm formation and act on pre-formed biofilms (Batoni et al. 2016; Santos et al. 2016), supporting their potential as DFIs therapeutic agents.

Nisin is an AMP produced by *Lactococcus lactis*, whose spectrum of activity includes a wide range of Gram-positive bacteria, including *S. aureus* (Santos et al. 2016; Zhu et al. 2017). In 1969, this bacteriocin was considered safe for use as a food preservative by the Food and Agriculture Organization and World Health Organization, being also approved by the US Food and Drug Administration in 1988. Nowadays, it is used in over 48 countries (Santos et al. 2015).

Considering that AMPs can be degraded or inactivated before reaching their target at therapeutic concentrations (O'Driscoll et al. 2013), it is mandatory to establish effective AMP delivery systems, with the natural polysaccharide guar gum being one of the most promising (Santos et al. 2016). A previous work conducted by our team demonstrated that a biogel formed by nisin incorporated in guar gum not only presented a high level of antimicrobial activity against planktonic *S. aureus* DFI isolates, but most importantly, it was able to inhibit and eradicate biofilm-based bacteria, including those formed by MRSA and multidrug resistant clinical strains (Santos et al. 2016).

Although AMPs represent a potential novel strategy for DFIs treatment, conventional antibiotics remain the standard therapeutic protocols and cannot be fully replaced at the present. Considering that AMPs can be used in combination with antibiotics (Mataraci and Dosler 2012), this work aimed at evaluating the potential of the previously developed nisin-biogel (Santos et al. 2016) in enhancing the efficacy of DFI treatment based on conventional antibiotics and antiseptics, using *S. aureus* clinical isolates as bacterial models, and an innovative protocol to simulate *in vitro* the application of currently accepted DFI therapeutic protocols.

3.3 Materials and methods

3.3.1 Bacterial strains

Isolates were obtained in a previous epidemiological survey regarding DFU infections, conducted at 4 clinical centers in Lisbon from January to June 2010 (Mendes et al. 2012). A total of 53 staphylococci were collected from 49 DFU patients, from which 23 representative biofilm-producing *S. aureus* isolates were selected, based on pulse field gel electrophoresis (PFGE) and multilocus sequence type (MLST) profiling (Mottola, Semedo-Lemsaddek, et al. 2016). In addition, a biofilm-producing reference strain, *S. aureus* ATCC 29213, was also included in this study as a control strain.

The antimicrobial resistance profile of these strains was previously characterized through determination of the minimal inhibitory concentration for ten antibiotics and by multiplex polymerase chain reaction for detection of the following genes: *mecA, mecC, erma, ermB, ermC, blaZ, msrA, aac-aph, tetK, tetL, tetM, tetO* and *norA*. It was observed that 35% (n=8) of the isolates were MRSA and 30% (n=7) were considered to be multidrug resistant (Mottola, Semedo-Lemsaddek, et al. 2016). All of these strains (n=23) were classified as biofilm-producers (Mottola, Mendes, et al. 2016).

Isolates were stored at -80 °C in buffered peptone water supplemented with 20% (v/v) of glycerol.

3.3.2 Chlorhexidine minimum inhibitory concentration and minimum bactericidal concentration

Strains were grown in a non-selective brain heart infusion (BHI) agar medium (VWR, Belgium) at 37 °C for 24 h. Bacterial suspensions of approximately 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard (bioMèrieux, France) in sterile normal saline (Scharlau, Spain). Afterwards, bacterial suspensions were diluted in fresh BHI broth to a concentration of 10^7 CFU/mL.

A stock solution of chlorhexidine at 4% (w/v) (AGA, Portugal) was filtered using a 0.22 µm cellulose acetate membrane filter (VWR, Belgium) and diluted in sterile water to obtain a set of solutions with concentrations ranging from 0.15 to 70 µg/mL. Solutions were stored protected from the light at 22 °C until use.

The set of chlorhexidine solutions were distributed in 96-well flat-bottomed polystyrene microtitre plates (Nunc; Thermo Fisher Scientific, Denmark). All the wells, except for the negative control (with broth medium only), were inoculated with 150 µL of the 10^7 CFU/mL bacterial suspensions. Microplates were incubated statically for 24 h at 37°C, and minimum inhibitory concentration (MIC) was determined as the lowest concentration of chlorhexidine that visually inhibited bacterial growth (CLSI 2015).

Minimum bactericidal concentration (MBC) value was determined by inoculating on BHI agar plates 3 µL of the suspensions from the wells where no bacterial growth was observed. Plates were incubated at 37 °C for 24 h and MBC was determined as the lowest chlorhexidine concentration from which no bacterial colonies were observed (CLSI 1999).

Experiments were conducted in triplicate, and independent replicates were performed at least three times in different days.

3.3.3 Antimicrobial solutions

A stock solution of nisin (1000 µg/mL) was obtained by dissolving 1 g of nisin powder (2.5% purity Sigma-Aldrich, USA) in 25 mL of HCl (0.02 M) (Merck, Germany), filtered using a 0.22 µm cellulose acetate membrane filter and stored at 4°C. The stock solution was then diluted with sterile water to a concentration of 45 µg/mL.

A guar gum gel 1.5% (w/v) was prepared by dissolving 0.6 g of guar gum (Sigma-Aldrich, USA) in 40 mL of sterile distilled water and heat sterilized by autoclave. The solution of nisin was incorporated within the guar gum gel in a proportion of 1:1, obtaining a final 0.75% (w/v) biogel with 22.5 µg/mL of nisin.

Regarding antibiotics solutions, 6.6, 4.76 and 10.62 mg of Clindamycin (Cayman, USA), Gentamicin (PanReac AppliChem, USA) and Vancomycin (PanReac AppliChem, USA), respectively, were dissolved in 10 mL of sterile water and filtered through a 0.22 µm cellulose acetate membrane filter. Stock solutions were kept frozen at -80 °C and diluted to the final concentrations of 0.033 µg/mL for clindamycin, 0.238 µg/mL for gentamicin and 0.531 µg/mL for vancomycin, prior to utilization.

3.3.4 *In vitro* evaluation of the inhibitory action of combined antimicrobial

An innovative *in vitro* protocol (Figure 2) was designed to mimic currently accepted DFI therapeutic protocols, aiming at evaluating the combined action of the antiseptic chlorhexidine,

the AMP nisin and the antibiotics clindamycin, gentamicin and vancomycin against the DFI staphylococci under study.

Strains were grown in a non-selective BHI agar medium at 37 °C for 24 h. Bacterial suspensions of approximately 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard in sterile normal saline and then diluted in Tryptic Soy Broth (TSB) (VWR, Belgium) medium supplemented with 0.25% (w/v) glucose (Merck, USA), to a concentration of 10^6 CFU/mL. A 200 µL volume of each bacterial suspension was distributed in a 96-well flat-bottomed polystyrene microtiter plate, covered with 96-peg polystyrene lid (Nunc, Thermo Fisher Scientific, Denmark) and incubated statically for 24 h at 37°C, to allow biofilm formation on the pegs surface. After establishment of *S. aureus* biofilms, the peg lid was rinsed periodically using different combinations of antiseptic, nisin, and antibiotics solutions, in order to evaluate the inhibitory potential of fifteen different combinations of antimicrobials, as follows: Chlorhexidine (Chx), nisin-biogel (NBG), nisin-biogel plus chlorhexidine (NBG+Chx), clindamycin (Cli), clindamycin plus chlorhexidine (Cli+Chx), clindamycin plus nisin-biogel (Cli+NBG), clindamycin plus chlorhexidine plus nisin-biogel (Cli+Chx+NBG), gentamicin (Gen), gentamicin plus chlorhexidine (Gen+Chx), gentamicin plus nisin-biogel (Gen+NBG), gentamicin plus chlorhexidine plus nisin-biogel (Gen+Chx+NBG), vancomycin (Van), vancomycin plus chlorhexidine (Van+Chx), vancomycin plus nisin-biogel (Van+NBG) and vancomycin plus chlorhexidine plus nisin-biogel (Van+Chx+NBG).

Positive (bacterial suspensions in broth medium with no antimicrobials) and negative (broth medium only) controls were also included in the assays.

The concentration of antimicrobials used corresponded to the MIC values obtained both in this experiment and in previous studies (Table 3).

First, biofilm-covered peg lids were rinsed three times in 0.9% NaCl (w/v) for 15 s, to remove planktonic bacteria; then placed in chlorhexidine (6 µg/mL) during 15 s; then placed in the nisin-biogel (22.5 µg/mL) for 3 min; and finally incubated in an empty microplate during 30 min to allow the biogel to dry. Afterwards, peg lids were placed in 96-well flat-bottomed polystyrene microtiter plates containing fresh TSB + 0.25% glucose medium supplemented with the antibiotics clindamycin (0.033 µg/mL), gentamicin (0.238 µg/mL) or vancomycin (0.531 µg/mL). Microplates were incubated at 37 °C during 8 h, after which the protocol cycle was repeated. A total of three cycles were performed, corresponding to a 24 h period.

When a treatment combination did not include chlorhexidine or nisin-biogel, the peg lid was placed in an empty microplate during the corresponding incubation period. When a treatment combination did not include antibiotics, the peg lid was placed in non-supplemented TSB broth.

The inhibitory effect of the antimicrobials was determined by removing the peg lids and determining the optical density (OD) at 600 nm of the suspensions in the 96 well-plate using a microplate reader (BGM LABTECH, Germany). Then, the peg lids where rinsed three more times in 0.9% NaCl, placed in new microplates containing only 200 µL of fresh TSB + 0.25% glucose medium and incubated in an ultrasound bath (Grant MXB14, England), at 50 Hz for 15 min, in order to disperse the biofilm-based bacteria from the pegs surface. Afterwards, peg lids were discarded and microplates were covered with normal lids and incubated for 24 h at 37 °C to allow the growth of surviving bacterial cells. The biofilm eradication effect was determined through measurement of the OD at 600 nm of these overnight suspensions.

Experiments were conducted in triplicate, and independent replicates were performed at least three times in different days.

Table 3 – Minimum inhibitory concentration values of the antimicrobial solutions chlorhexidine, nisin-biogel, clindamycin, gentamicin and vancomycin regarding the diabetic foot infection S*taphylococcus aureus* isolates under study.

Class	**Antimicrobial**	**MIC** (µg/mL)	**Reference**
Antiseptic	Chlorhexidine	6	Santos et al. 2019
Antimicrobial Peptide	Nisin-biogel	22.5	Santos et al. 2016
Antibiotic	Clindamycin	0.033	Mottola, Matias, et al., 2016
	Gentamicin	0.238	
	Vancomycin	0.531	

MIC – Minimum Inhibitory Concentration

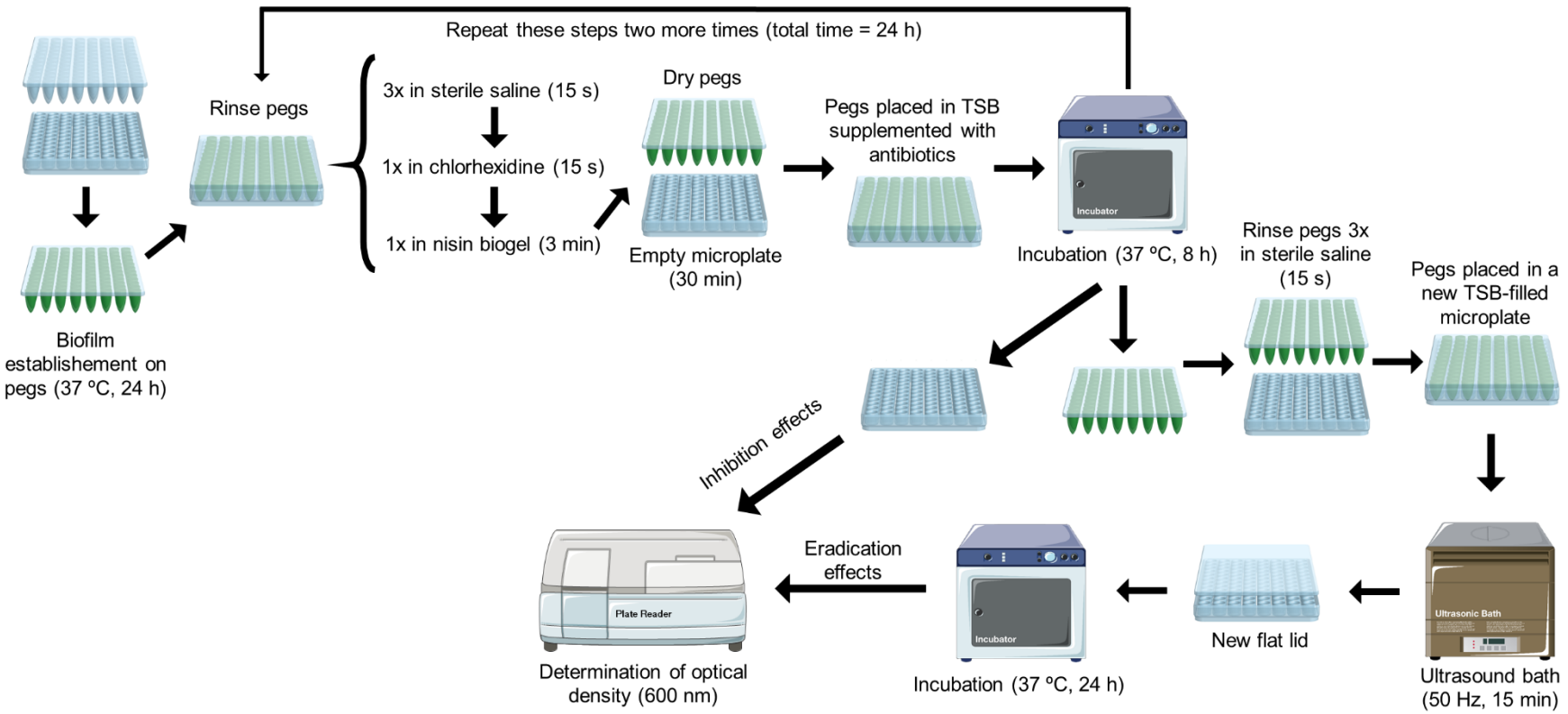

Figure 2 – Schematic representation of the protocol developed to study the susceptibility of diabetic foot infection *Staphylococcus aureus* biofilms to different antimicrobial compounds combinations.

The schematic representation shows the treatment combination when all three antimicrobials, chlorhexidine, nisin guar gum gel and antibiotics, are applied. TSB – Tryptic Soy Broth

3.3.5 Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics V20 Software for Windows. Minimum, maximum, mean and standard deviation values were determined for all quantitative variables. Differences between MIC and MBC values were evaluated using the T-test.

Analysis of variance (ANOVA) for Randomized Complete Block Design (RCBD) was used to analyze the variables studied and post-hoc comparisons were assessed using Least Significant Differences tests. The OD results obtained in the biofilm inhibition and eradication assays were evaluated in order to determine the most effective combination of antimicrobial compounds. Each combination was considered a different treatment and all the *S. aureus* strains (each strain acting as a block) were exposed to all the different treatments. A two-tailed *p*-value ≤ 0.05 was considered to be statistically significant in all the applied tests.

3.4 Results

3.4.1 Chlorhexidine minimum inhibitory concentration and minimum bactericidal concentration values

Chlorhexidine MIC and MBC values are presented in Table 4. MIC values ranged from 1.4 to 7.0 µg/mL, with an average value of 5.7±1.5 µg/mL; MBC values ranged from 9.8 to 68.8 µg/mL, with an average value of 15.5±14.9 µg/mL. MIC and MBC are statistically different (*p* value = 0.004), as determined through a paired sample T-test.

Antimicrobial agents are classified as bactericidal if the MBC value is no more than four times higher than their MIC value (French 2006). Chlorhexidine mean MBC was 2.72-fold higher than the mean MIC; therefore, chlorhexidine can be considered as a bactericidal agent against the *S. aureus* strains used in this study.

Table 4 – Chlorhexidine minimum inhibitory concentration and minimum bactericidal concentration values regarding S*taphylococcus aureus* diabetic foot infection strains.

Strain (n = 24)		**MIC** (µg/mL)	**MBC** (µg/mL)
A 1.1	MRSA	5.6	9.8
A 5.2		4.2	9.8
A 6.3		4.2	39.2
B 3.2		5.6	9.8
B 3.3		5.6	9.8
B 7.3	MRSA; MDR	7.0	68.6
B 13.1	MRSA; MDR	7.0	9.8
B 14.2	MRSA; MDR	5.6	9.8
S 1.1	MRSA; MDR	7.0	19.6
S 2.2		7.0	9.8
S 3.1		7.0	9.8
S 5.2		4.2	9.8
S 12.2		1.4	9.8
S 14.1		4.2	9.8
S 16.1	MRSA; MDR	4.2	9.8
S 17.2		4.2	9.8
S 21.1	MRSA; MDR	7.0	9.8
S 21.3	MRSA; MDR	7.0	9.8
S 23.2		4.2	9.8
S 25.2		7.0	9.8
S 27.2		7.0	9.8
S 27.3		7.0	49.0
S 32.2		7.0	9.8
ATCC 29213		7.0	9.8
Mean		5.7	15.5
Minimum		1.4	9.8
Maximum		7.0	68.6
Std. Dev.		1.5	14.9

A – Aspirate; ATCC – American Type Culture Collection; B – Biopsy; MBC – Minimum Bactericidal Concentration; MDR – Multidrug Resistant; MIC – Minimum Inhibitory Concentration; MRSA – Methicillin-Resistant *Staphylococcus aureus*; S – Swab; Std. Dev. – Standard Deviation.

3.4.2 *In vitro* evaluation of the inhibitory action of combined antimicrobials

Growth rates were approximately the same between all strains under study. Considering that bacterial suspensions OD values are directly related to their biomass, the OD of each suspension after incubation with the different antimicrobial combinations was measured to compare their efficacy and to determine which antimicrobial combinations exhibited the higher biofilm inhibition and eradication levels.

First, inhibitory activity of the individual antimicrobial compounds alone was evaluated. Results showed that the nisin-biogel presented the highest level of biofilm inhibition, followed by the antibiotics vancomycin and gentamicin (Figure 3). Clindamycin had the lowest biofilm-inhibitory effect and no significant differences were detected between the OD of the suspension incubated with this antibiotic and the positive control (Table 5). When chlorhexidine was applied alone, its inhibitory activity against the biofilm-producing *S. aureus* strains was very similar to the inhibitory activity presented by the different antibiotics, as no significant differences were observed between results (p-value > 0.05) (Table 5). Regarding the inhibitory action of the antimicrobial combinations tested, the higher inhibitory effect was presented by the combined application of chlorhexidine and nisin-biogel. Furthermore, when combined with the biogel, all antibiotics presented a significantly higher (p-value < 0.05) antibiofilm ability (Figure 3, Table 5). No relevant differences were detected between the antibiotic resistant and the antibiotic susceptible strains under study. Treatment combinations that included nisin-biogel were the most effective regarding biofilm inhibition for all isolates tested (Table 6).

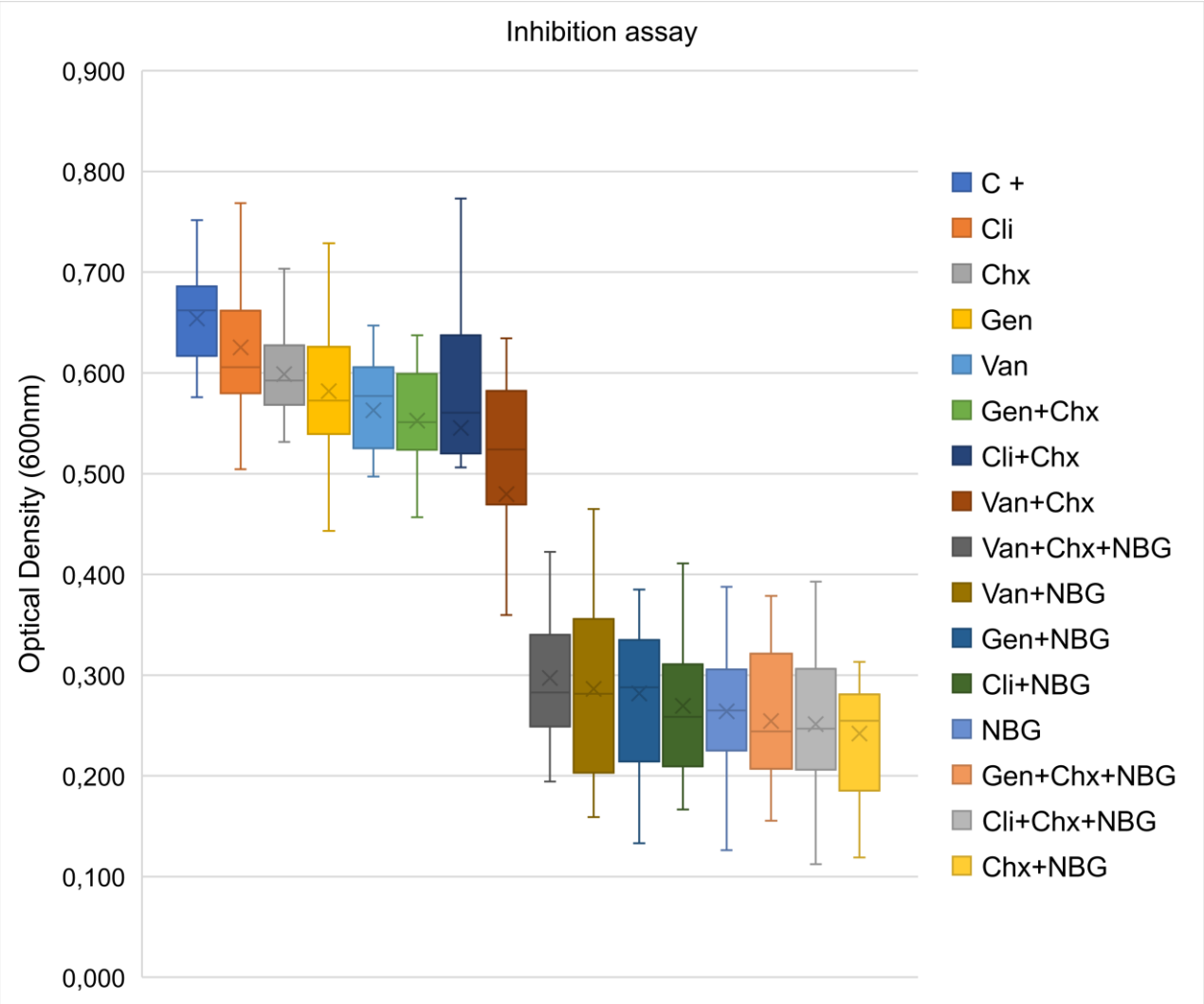

Figure 3 – Inhibitory activity of antimicrobial compounds, alone or in combination, against biofilms formed by diabetic foot infection *Staphylococcus aureus* isolates.

C + – Positive control; Chx – Chlorohexidine (6 µg/mL); Cli – Clindamycin (0.033 µg/mL); Gen – Gentamicin (0.238 µg/mL); NBG – Nisin-biogel (22.5 µg/mL); Van – Vancomycin (0.531 µg/mL).

The means (x) and standard deviations of three independent determinations are presented. The negative control mean optical density value was 0.101.

Table 5 – Inhibitory activity of different antimicrobial compounds combinations against diabetic foot infection *Staphylococcus aureus* biofilms.

A / B		C +	Chx	NBG	Chx + NBG	Cli	Cli + Chx	Cli + NBG	Cli + Chx + NBG	Gen	Gen + Chx	Gen + NBG	Gen + Chx + NBG	Van	Van + Chx	Van + NBG	Van + Chx + NBG
C+	**A – B**	0.0551	0.3900	0.4122	0.0286	0.1086	0.3846	0.4027	0.0721	0.1014	0.3722	0.3997	0.0912	0.1744	0.3676	0.3568
	***p*-value**	0.008	< 0.001	< 0.001	0.164	< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Chx			0.3349	0.3570	- 0.0264	0.0534	0.3294	0.3475	0.0169	0.0462	0.3170	0.3445	0.0360	0.1192	0.3124	0.3016
			< 0.001	< 0.001	0.199	0.010	< 0.001	< 0.001	0.409	0.025	< 0.001	< 0.001	0.081	< 0.001	< 0.001	< 0.001
NBG				0.0221	- 0.3614	- 0.2814	- 0.0054	0.0126	- 0.3179	- 0.2886	- 0.0178	0.0096	- 0.2988	- 0.2156	- 0.0224	- 0.0332
				0.283	< 0.001	< 0.001	0.791	0.539	< 0.001	< 0.001	0.385	0.640	< 0.001	< 0.001	0.276	0.107
Chx + NBG					- 0.3835	- 0.3035	- 0.0275	- 0.0094	- 0.3400	- 0.3107	- 0.0400	- 0.0124	- 0.3210	- 0.2378	- 0.0445	- 0.0553
					< 0.001	< 0.001	0.181	0.645	< 0.001	< 0.001	0.053	0.544	< 0.001	< 0.001	0.031	0.007
Cli						0.0799	0.3559	0.3740	0.0434	0.0727	0.3435	0.3710	0.0625	0.1457	0.3389	0.3281
						< 0.001	< 0.001	< 0.001	0.035	< 0.001	< 0.001	< 0.001	0.003	< 0.001	< 0.001	< 0.001
Cli + Chx							0.2759	0.2940	- 0.0364	- 0.0071	0.2635	0.2910	- 0.0174	0.0657	0.2590	0.2481
							< 0.001	< 0.001	0.077	0.727	< 0.001	< 0.001	0.397	0.002	< 0.001	< 0.001
Cli + NBG								0.0180	- 0.3124	- 0.2831	- 0.0124	0.0150	- 0.2934	- 0.2102	- 0.0169	- 0.0278
								0.380	< 0.001	< 0.001	0.546	0.464	< 0.001	< 0.001	0.410	0.177
Cli + Chx + NBG									- 0.3305	- 0.3012	- 0.0305	- 0.0030	- 0.3115	- 0.2283	- 0.0350	- 0.0458
									< 0.001	< 0.001	0.139	0.884	< 0.001	< 0.001	0.089	0.026
Gen										0.0293	0.3000	0.3275	0.0190	0.1022	0.2955	0.2846
										0.155	< 0.001	< 0.001	0.355	< 0.001	< 0.001	< 0.001
Gen + Chx											0.2707	0.2982	- 0.0102	0.0729	0.2661	0.2553
											< 0.001	< 0.001	0.618	< 0.001	< 0.001	< 0.001
Gen + NBG												0.0275	- 0.2810	- 0.1977	- 0.0045	- 0.0153
												0.182	< 0.001	< 0.001	0.825	0.455
Gen + Chx + NBG													- 0.3085	- 0.2253	- 0.0320	- 0.0428
													< 0.001	< 0.001	0.120	0.038
Van														0.0832	0.2764	0.2656
														< 0.001	< 0.001	< 0.001
Van + Chx															0.1932	0.1824
															< 0.001	< 0.001
Van + NBG																- 0.0108
																0.599

Differences (A – B) between the optical density means presented by each treatment combination were assessed using Fisher's least significant differences test. Significant differences ($p \leq 0.05$) are highlighted (grey box). Chx – Chlorohexidine (6 µg/mL); Cli – Clindamycin (0.033 µg/mL); Gen – Gentamicin (0.238 µg/mL); NBG – Nisin-biogel (22.5 µg/mL); Van – Vancomycin (0.531 µg/mL).

Table 6 – Inhibitory activity of antimicrobial compounds, alone or in combination, against biofilms formed by diabetic foot infection *Staphylococcus aureus* isolates.

Strain (n=24)	C +	Chx	NBG	Chx + NBG	Cli	Cli + Chx	Cli + NBG	Cli + Chx + NBG	Gen	Gen + Chx	Gen + NBG	Gen + Chx + NBG	Van	Van + Chx	Van + NBG	Van + Chx + NBG
A 1.1	0.585	0.587	0.293	0.270	0.596	0.560	0.207	0.253	0.531	0.538	0.358	0.202	0.509	0.360	0.174	0.249
A 5.2	0.624	0.572	0.292	0.252	0.545	0.506	0.216	0.219	0.571	0.572	0.291	0.221	0.498	0.447	0.179	0.235
A 6.3	0.722	0.613	0.388	0.308	0.769	0.765	0.411	0.393	0.686	0.626	0.385	0.261	0.597	0.608	0.321	0.271
B 3.2	0.658	0.595	0.312	0.276	0.601	0.562	0.284	0.206	0.539	0.570	0.278	0.211	0.583	0.504	0.356	0.280
B 3.3	0.661	0.696	0.311	0.297	0.611	0.517	0.235	0.207	0.589	0.637	0.367	0.191	0.631	0.514	0.229	0.237
B 7.3	0.576	0.571	0.228	0.222	0.548	0.526	0.193	0.201	0.503	0.550	0.254	0.212	0.497	0.529	0.196	0.286
B 13.1	0.663	0.602	0.223	0.192	0.621	0.686	0.392	0.333	0.574	0.525	0.205	0.326	0.564	0.522	0.364	0.339
B 14.2	0.666	0.594	0.201	0.183	0.668	0.620	0.248	0.268	0.632	0.589	0.210	0.264	0.540	0.591	0.276	0.265
S 1.1	0.681	0.668	0.252	0.177	0.595	0.562	0.221	0.235	0.648	0.594	0.175	0.254	0.607	0.557	0.190	0.250
S 2.2	0.594	0.588	0.211	0.183	0.591	0.518	0.204	0.208	0.521	0.501	0.222	0.206	0.501	0.508	0.254	0.194
S 3.1	0.687	0.704	0.292	0.312	0.651	0.745	0.306	0.291	0.729	0.726	0.363	0.314	0.647	0.630	0.308	0.302
S 5.2	0.660	0.568	0.231	0.223	0.580	0.543	0.384	0.327	0.570	0.605	0.332	0.379	0.576	0.623	0.382	0.383
S 12.2	0.724	0.452	0.253	0.257	0.570	0.201	0.177	0.112	0.649	0.552	0.336	0.270	0.520	0.085	0.235	0.318
S 14.1	0.667	0.542	0.224	0.168	0.617	0.143	0.268	0.208	0.533	0.523	0.211	0.234	0.601	0.081	0.287	0.324
S 16.1	0.629	0.532	0.173	0.173	0.577	0.082	0.257	0.204	0.557	0.279	0.208	0.183	0.571	0.076	0.302	0.340
S 17.2	0.660	0.548	0.257	0.251	0.622	0.553	0.281	0.300	0.566	0.528	0.315	0.223	0.581	0.537	0.339	0.247
S 21.1	0.604	0.591	0.289	0.282	0.593	0.602	0.188	0.183	0.543	0.544	0.355	0.197	0.578	0.557	0.205	0.220
S 21.3	0.675	0.610	0.268	0.263	0.666	0.595	0.260	0.260	0.542	0.516	0.308	0.262	0.559	0.520	0.269	0.266
S 23.2	0.667	0.574	0.126	0.119	0.704	0.600	0.313	0.240	0.600	0.558	0.133	0.156	0.611	0.527	0.355	0.369
S 25.2	0.495	0.560	0.262	0.238	0.505	0.557	0.238	0.267	0.443	0.457	0.249	0.210	0.367	0.458	0.203	0.327
S 27.2	0.706	0.630	0.294	0.293	0.613	0.643	0.293	0.309	0.582	0.537	0.285	0.324	0.596	0.549	0.431	0.422
S 27.3	0.752	0.681	0.316	0.313	0.767	0.773	0.396	0.325	0.657	0.615	0.325	0.341	0.611	0.594	0.465	0.389
S 32.2	0.727	0.679	0.333	0.278	0.826	0.681	0.331	0.333	0.608	0.601	0.318	0.345	0.546	0.635	0.399	0.365
ATCC 29213	0.615	0.621	0.309	0.277	0.581	0.555	0.167	0.154	0.597	0.523	0.283	0.325	0.621	0.505	0.159	0.262
Mean	**0.654**	**0.599**	**0.264**	**0.242**	**0.625**	**0.545**	**0.269**	**0.251**	**0.582**	**0.553**	**0.282**	**0.254**	**0.563**	**0.480**	**0.286**	**0.297**
Std. Dev.	0.057	0.058	0.057	0.054	0.076	0.175	0.072	0.066	0.063	0.080	0.068	0.061	0.060	0.166	0.087	0.060

Optical density values presented in the table were measured at 600 nm. The means and standard deviations of three independent determinations are presented. The negative control mean optical density value was 0.101.

A – aspirate; ATCC – American Type Culture Collection; B – biopsy; C + – Positive Control; Chx – Chlorohexidine (6 µg/mL); Cli – Clindamycin (0.033 µg/mL); Gen – Gentamicin (0.238 µg/mL); NBG – Nisin-biogel (22.5 µg/mL); S – Swab; Std. Dev. – Standard Deviation; Van – Vancomycin (0.531 µg/mL).

Concerning the biofilm eradication assay, the OD values obtained after the application of the different antimicrobial compounds presented an uniform distribution and were significantly higher than those observed in the biofilm inhibition assay (Figure 4, Table 7). For individual compounds, the lowest OD values, which correspond to the highest eradication effect, were obtained after incubation with vancomycin, followed by incubation with nisin-biogel, gentamicin and clindamycin. There were no relevant differences between results, as all antimicrobial compounds presented a similar eradication effect of *S. aureus* biofilms. As observed in the biofilm inhibition results, no relevant differences were detected between antibiotic resistant and antibiotic susceptible strains under study (Table 8).

Regarding biofilm eradication, results suggest that chlorhexidine and nisin-biogel increased the eradication potential of the other compounds, as the highest effects were presented by the following combinations: vancomycin plus chlorhexidine, clindamycin plus chlorhexidine, clindamycin plus chlorhexidine plus nisin-biogel and clindamycin plus nisin-biogel.

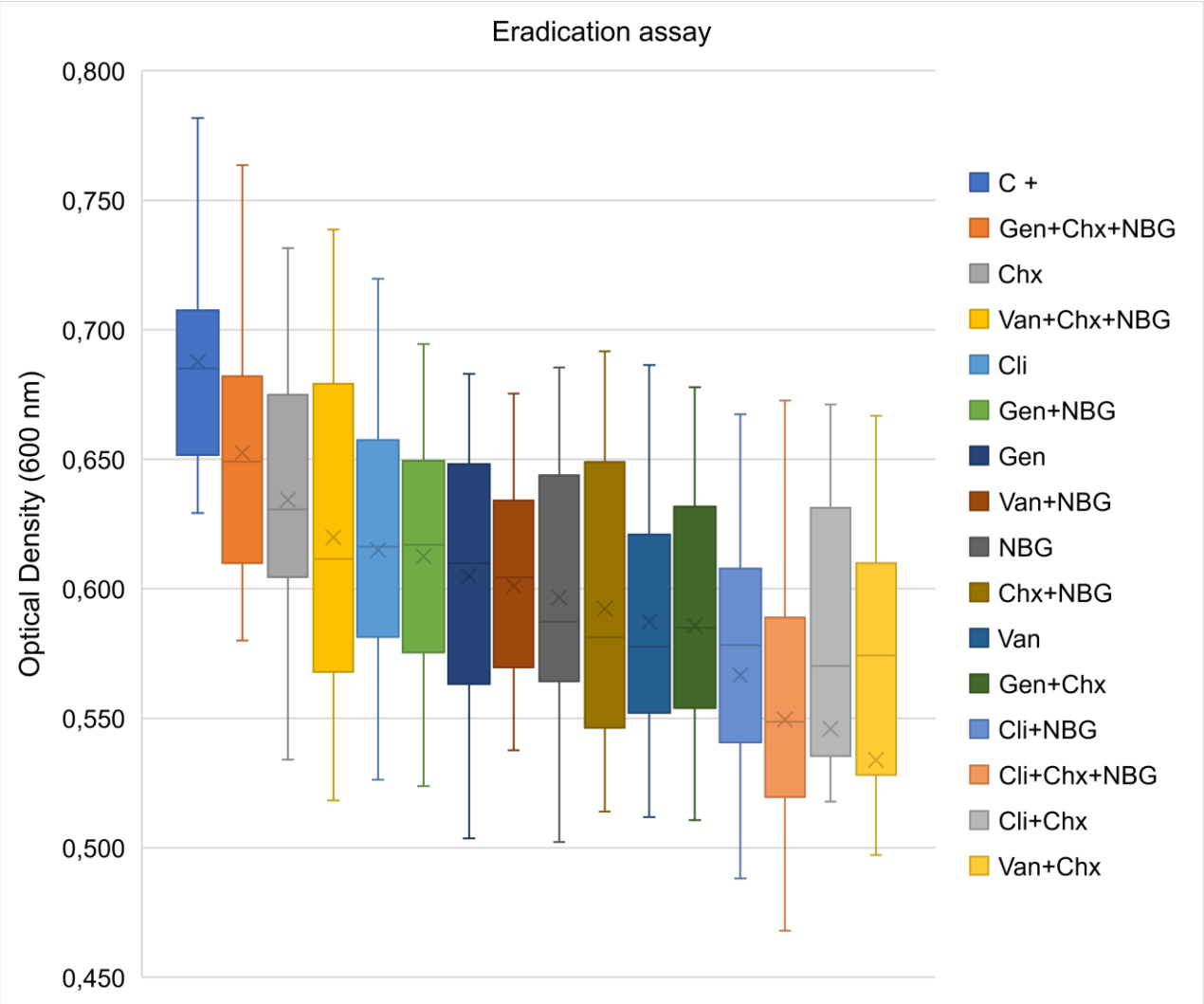

Figure 4 – Eradication activity of antimicrobial compounds, alone or in combination, against biofilms formed by diabetic foot infection *Staphylococcus aureus* isolates.

C + – Positive control; Chx – Chlorohexidine (6 µg/mL); Cli – Clindamycin (0.033 µg/mL); Gen – Gentamicin (0.238 µg/mL); NBG – Nisin-biogel (22.5 µg/mL); Van – Vancomycin (0.531 µg/mL).

The means (x) and standard deviations of three independent determinations are presented. The negative control mean optical density value was 0.101.

Table 7 – Eradication activity of different antimicrobial compounds combinations against diabetic foot infection *Staphylococcus aureus* biofilms.

A / B		C +	Chx	NBG	Chx + NBG	Cli	Cli + Chx	Cli + NBG	Cli + Chx + NBG	Gen	Gen + Chx	Gen + NBG	Gen + Chx + NBG	Van	Van + Chx	Van + NBG	Van + Chx + NBG
C+	**A – B**	0.0534	0.0910	0.0953	0.0726	0.1416	0.1210	0.1281	0.0827	0.1019	0.0751	0.0351	0.1004	0.1537	0.0867	0.0678
	***p*-value**	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.036	< 0.001	< 0.001	< 0.001	< 0.001
Chx			0.0376	0.0419	0.0192	0.0882	0.0676	0.0847	0.0293	0.0485	0.0217	- 0.0182	0.0470	0.1003	0.0333	0.0144
			0.025	0.013	0.250	< 0.001	< 0.001	< 0.001	0.080	0.004	0.194	0.277	0.005	< 0.001	0.047	0.389
NBG				0.0043	- 0.0183	0.0506	0.0299	0.0471	- 0.0082	0.0109	- 0.0158	- 0.0558	0.0094	0.0627	- 0.0043	- 0.0231
				0.796	0.274	0.003	0.074	0.005	0.623	0.515	0.344	0.001	0.573	< 0.001	0.797	0.167
Chx + NBG					- 0.0226	0.0463	0.0256	0.0428	- 0.0125	0.0065	- 0.0201	- 0.0601	0.0051	0.0584	- 0.0086	- 0.0275
					0.176	0.006	0.126	0.011	0.453	0.694	0.229	0.001	0.760	0.001	0.606	0.101
Cli						0.0689	0.0483	0.0654	0.0100	0.0292	0.0024	- 0.0375	0.0277	0.0810	0.0140	- 0.0048
						< 0.001	0.004	< 0.001	0.547	0.081	0.882	0.026	0.098	< 0.001	0.402	0.772
Cli + Chx							- 0.0206	- 0.0034	- 0.0588	- 0.0397	- 0.0664	- 0.1064	- 0.0412	0.0120	- 0.0549	- 0.0738
							0.218	0.835	< 0.001	0.018	< 0.001	< 0.001	0.014	0.470	0.001	< 0.001
Cli + NBG								0.0171	- 0.0382	- 0.0190	- 0.0458	- 0.0858	- 0.0205	0.0327	- 0.0342	- 0.0531
								0.305	0.023	0.255	0.006	< 0.001	0.220	0.051	0.041	0.002
Cli + Chx + NBG									- 0.0553	- 0.0362	- 0.0630	- 0.1029	- 0.0377	0.0155	- 0.0514	- 0.0703
									0.001	0.031	< 0.001	< 0.001	0.025	0.352	0.002	< 0.001
Gen										0.0191	- 0.0076	- 0.0476	0.0176	0.0709	0.0039	- 0.0149
										0.253	0.649	0.005	0.292	< 0.001	0.814	0.372
Gen + Chx											- 0.0267	- 0.0667	- 0.0014	0.0518	- 0.0152	- 0.0340
											0.111	< 0.001	0.929	0.002	0.364	0.042
Gen + NBG												- 0.0399	0.0252	0.0785	0.0115	- 0.0073
												0.017	0.132	< 0.001	0.490	0.662
Gen + Chx + NBG													0.0652	0.1185	0.0515	0.0326
													< 0.001	< 0.001	0.002	0.052
Van														0.0533	- 0.0137	- 0.0326
														0.002	0.412	0.052
Van + Chx															- 0.0670	- 0.0859
															< 0.001	< 0.001
Van + NBG																- 0.0188
																0.260

Differences (A – B) between the optical density means presented by each treatment combination were assessed using Fisher's least significant differences test. Significant differences ($p \leq 0.05$) are highlighted (grey box). Chx – Chlorohexidine (6 µg/mL); Cli – Clindamycin (0.033 µg/mL); Gen – Gentamicin (0.238 µg/mL); NBG – Nisin-biogel (22.5 µg/mL); Van – Vancomycin (0.531 µg/mL).

Table 8 – Eradication activity of antimicrobial compounds, alone or in combination, against biofilms formed by diabetic foot infection *Staphylococcus aureus* isolates.

Strain (n=24)	C +	Chx	NBG	Chx + NBG	Cli	Cli + Chx	Cli + NBG	Cli + Chx + NBG	Gen	Gen + Chx	Gen + NBG	Gen + Chx + NBG	Van	Van + Chx	Van + NBG	Van + Chx + NBG
A 1.1	0.637	0.600	0.541	0.530	0.570	0.606	0.548	0.561	0.562	0.547	0.565	0.587	0.573	0.604	0.569	0.583
A 5.2	0.629	0.612	0.564	0.582	0.581	0.570	0.602	0.539	0.565	0.568	0.620	0.638	0.585	0.572	0.558	0.546
A 6.3	0.711	0.659	0.647	0.620	0.644	0.633	0.610	0.588	0.625	0.619	0.651	0.688	0.598	0.613	0.611	0.613
B 3.2	0.648	0.534	0.502	0.514	0.613	0.571	0.588	0.569	0.504	0.527	0.554	0.613	0.567	0.587	0.607	0.636
B 3.3	0.673	0.689	0.569	0.594	0.526	0.518	0.489	0.468	0.622	0.562	0.623	0.671	0.522	0.511	0.538	0.560
B 7.3	0.680	0.623	0.588	0.584	0.578	0.557	0.540	0.524	0.569	0.579	0.612	0.632	0.552	0.566	0.634	0.682
B 13.1	0.676	0.605	0.562	0.535	0.659	0.657	0.613	0.610	0.642	0.586	0.551	0.609	0.684	0.590	0.636	0.604
B 14.2	0.686	0.637	0.614	0.653	0.633	0.667	0.574	0.555	0.659	0.585	0.646	0.585	0.553	0.588	0.617	0.594
S 1.1	0.733	0.694	0.676	0.675	0.711	0.671	0.660	0.673	0.683	0.678	0.695	0.674	0.666	0.667	0.675	0.683
S 2.2	0.639	0.556	0.538	0.544	0.585	0.555	0.568	0.617	0.558	0.540	0.570	0.607	0.550	0.576	0.578	0.523
S 3.1	0.714	0.732	0.685	0.692	0.570	0.569	0.582	0.549	0.673	0.592	0.657	0.682	0.559	0.556	0.570	0.618
S 5.2	0.688	0.673	0.669	0.650	0.666	0.644	0.617	0.589	0.632	0.639	0.643	0.671	0.608	0.612	0.606	0.655
S 12.2	0.782	0.670	0.593	0.542	0.425	0.193	0.360	0.390	0.598	0.640	0.614	0.733	0.660	0.190	0.603	0.730
S 14.1	0.662	0.625	0.582	0.553	0.641	0.236	0.510	0.537	0.564	0.585	0.606	0.669	0.547	0.209	0.570	0.611
S 16.1	0.687	0.639	0.584	0.578	0.662	0.216	0.602	0.640	0.596	0.432	0.622	0.623	0.611	0.200	0.634	0.657
S 17.2	0.636	0.538	0.522	0.528	0.582	0.550	0.543	0.519	0.511	0.511	0.524	0.583	0.514	0.540	0.570	0.570
S 21.1	0.685	0.598	0.595	0.581	0.619	0.532	0.531	0.503	0.578	0.582	0.622	0.641	0.512	0.497	0.595	0.518
S 21.3	0.641	0.611	0.566	0.556	0.591	0.542	0.550	0.476	0.560	0.551	0.540	0.580	0.555	0.527	0.568	0.572
S 23.2	0.685	0.686	0.586	0.559	0.608	0.595	0.543	0.522	0.648	0.623	0.611	0.621	0.570	0.572	0.583	0.565
S 25.2	0.692	0.604	0.581	0.651	0.599	0.533	0.488	0.576	0.648	0.625	0.592	0.657	0.582	0.533	0.556	0.567
S 27.2	0.698	0.616	0.593	0.563	0.652	0.604	0.585	0.490	0.563	0.577	0.592	0.682	0.584	0.635	0.634	0.670
S 27.3	0.806	0.692	0.646	0.647	0.720	0.658	0.630	0.600	0.679	0.639	0.678	0.756	0.631	0.632	0.642	0.695
S 32.2	0.755	0.656	0.637	0.667	0.684	0.627	0.599	0.546	0.657	0.634	0.660	0.764	0.686	0.637	0.647	0.688
ATCC 29213	0.661	0.676	0.678	0.619	0.639	0.601	0.667	0.548	0.623	0.639	0.653	0.693	0.624	0.601	0.622	0.739
Mean	**0.688**	**0.634**	**0.597**	**0.592**	**0.615**	**0.546**	**0.567**	**0.549**	**0.605**	**0.586**	**0.612**	**0.652**	**0.587**	**0.534**	**0.601**	**0.620**
Std. Dev.	0.045	0.051	0.051	0.053	0.062	0.136	0.065	0.061	0.051	0.053	0.045	0.052	0.050	0.136	0.035	0.063

Optical density values presented in the table were measured at 600 nm. The means and standard deviations of three independent determinations are presented. The negative control mean optical density value was 0.101.

A **–** aspirate; ATCC **–** American Type Culture Collection; B **–** biopsy; C + **–** Positive Control; Chx **–** Chlorohexidine (6 µg/mL); Cli **–** Clindamycin (0.033 µg/mL); Gen **–** Gentamicin (0.238 µg/mL); NBG **–** Nisin-biogel (22.5 µg/mL); S **–** Swab; Std. Dev. **–** Standard Deviation; Van **–** Vancomycin (0.531 µg/mL).

3.5 Discussion

Diabetes *mellitus* is a serious public health problem, being one of four priority noncommunicable diseases (WHO 2016). Foot skin ulceration is one of the most frequent and costly complications of diabetes, being frequently infected by pathogenic microorganisms (Lipsky et al. 2016).

Diabetic foot infections have a multifactorial etiology, being *S. aureus* the most prevalent pathogen isolated from these wounds (Hobizal and Wukich 2012; Mendes et al. 2012). The emergence of antibiotic resistant and biofilm-forming *S. aureus* strains, together with the impairment of conventional antibiotic-based DFI therapeutics, emphasis the importance of developing novel therapeutic protocols for DFI management. This work analyzed the potential of the antiseptic chlorhexidine and the AMP nisin to be applied together with conventional antibiotics in DFI treatment.

Chlorhexidine is a widely used antiseptic agent with high antimicrobial activity (Milstone et al. 2008). Chlorhexidine MIC and MBC values obtained showed that chlorhexidine presented inhibitory and eradication action against the *S. aureus* strains under study at concentrations below 0.05% (500 µg/mL), the concentration established for wound cleansing (Main 2008; WHMNG 2017).

The higher chlorhexidine MIC and MBC values regarding isolate B7.3 can be related to the fact of it being a MRSA and MDR strain. This strain harbors the antibiotic resistance gene *norA* (Motolla, Matias, et al. 2016), which presence is associated with increased resistance to antiseptic agents such as chlorhexidine Liu et al. 2015). Nonetheless, previous studies suggest that daily chlorhexidine bathing can reduce the acquisition of MRSA in intensive care unit patients (Climo et al. 2009). In fact, chlorhexidine antimicrobial effects are persistent, mainly due to its ability to strongly bind to proteins present in the skin and mucosal surfaces (Lim and Kam 2008). The uptake of chlorhexidine by bacteria is extremely rapid, with a maximum effect occurring within 15 to 30 seconds (McDonnell and Russel 1999) and, in contrast with other antiseptic agents, the residual antimicrobial activity of chlorhexidine is not affected by the presence of body fluids or blood (Huang et al. 2016). Thus, chlorhexidine can be recommended for DFI wound cleansing.

The bacterial biofilm mode of growth is a major cause for the failure of conventional DFI antibiotherapy. It has been estimated that biofilm-based bacteria can tolerate antimicrobial agents at concentrations 10 to 1000-times higher than their genetically equivalent planktonic forms (Kaplan 2011). Since biofilms have a significant impact on public health, there is an urgent need for antibiofilm agents. Previous studies (Okuda et al. 2013) suggest that nisin's ability to form

stable pores on prokaryotic membranes also occurs in biofilm-based bacteria, thus explaining its potent activity against *S. aureus* biofilms. Moreover, other studies reported an increase of the antimicrobial activity of antibiotics when combined with nisin (Mataraci and Dosler 2012). Given that resistance to AMPs that target lipid II, such as nisin, does not develop easily (Yeaman and Yount 2003), therapeutic protocols based on the combined administration of nisin with antibiotics may be an innovative strategy to control drug-resistant infections, such as DFIs.

This study evaluated the influence of chlorhexidine and the nisin-biogel in the inhibitory efficacy of conventional antibiotics against established biofilms formed by *S. aureus* DFI strains. As results demonstrate, individual antimicrobial compounds did not allow the complete elimination of the microorganisms, and the combination of different compounds resulted in an enhanced inhibitory efficacy against DFI pathogens.

Regarding biofilm inhibition, the combined action of the nisin-biogel and chlorhexidine showed the higher inhibitory effects. As observed for chlorhexidine, the nisin concentration required to inhibit biofilm cells was below its acceptable daily intake (1 mg/kg body weight) (EFSA 2017).

Results also showed that clindamycin and gentamicin biofilm inhibitory effects increased when combined with nisin. Both nisin and chlorhexidine exert their antimicrobial effect by disrupting the bacterial membrane (Milstone et al. 2008; Wiedemann et al. 2011), while clindamycin and gentamicin are antibiotics that inhibit protein synthesis. The application of nisin will allow the formation of stable pores in the bacterial membrane, allowing the antibiotic penetration to the bacterial cytoplasm, thus enabling them to act on bacterial ribosomes. Vancomycin biofilm inhibitory effects also increased when combined with this AMP. Although vancomycin and nisin are members of two different classes of antimicrobial agents, both target the essential cell wall precursor lipid II, blocking the cell wall biosynthesis (Kohanski et al. 2010). These results are in agreement with previous studies that demonstrated synergistic relationships between conventional antibiotics and lantibiotics, such as nisin (Mataraci and Dosler 2012).

Bacteria embedded within biofilms are more persistent and difficult to eradicate (Kaplan 2011), due to inefficient diffusion or sequestering of antibiotics within the biofilm matrix and also because biofilm-based bacterial cells tend to reduce their growth rate, protein synthesis and other physiologic activities, usually targeted by conventional antibiotic (LaPlante and Mermel 2009). In fact, the low eradication effect observed for gentamicin can be related with the fact that aminoglycosides effectiveness relies heavily on bacterial growth phase and extra bacterial factors, such as oxygen availability, not maintained in the biofilm microenvironment (Henry-Stanley et al. 2014).

A previous study conducted by our team demonstrated the capability of nisin to eradicate established *S. aureus* biofilms, even when incorporated in a guar gum gel (Santos et al. 2016; Okuda et al. 2013). The combination of different antimicrobial compounds allowed the higher eradication effects. Combinations of chlorhexidine plus antibiotics, nisin plus antibiotics, or even chlorhexidine plus nisin plus antibiotics, presented a higher eradication efficacy against DFI *S. aureus* strains than antibiotics alone. Also, since the nisin-biogel and chlorhexidine have a strong inhibitory and eradication effect against DFI *S. aureus* biofilms, these antimicrobial compounds could complement conventional antibiotherapy, enhancing antibiotics activity and possibly allowing to reduce the burden of antibiotic-resistant infections. Therefore, therapeutic protocols that include a first step of wound debridement, followed by antiseptic cleansing, AMP topical application and oral or systemic administration of antibiotics may represent the best approach to treat chronically infected skin ulcers and deserve further investigation aiming at their application to diabetic patients.

Chapter 4

4. Influence of storage on the antimicrobial and cytotoxic activities of a nisin-biogel with potential to be applied to diabetic foot infections treatment

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4.1 Abstract

Introduction: *Staphylococcus aureus* is the most prevalent pathogen in diabetic foot infections (DFIs). *S. aureus* is also known for being resistant to most antibiotics commonly used in clinical practice. It is urgent to develop new approaches to control this pathogen and antimicrobial peptides (AMPs) are emerging as potential new therapeutics for the management of DFIs.

Aim: This study evaluated the influence of storage conditions on the antimicrobial and cytotoxic activities of nisin, an AMP with demonstrated activity towards *S. aureus* DFI strains.

Methodology: Nisin was incorporated within a guar gum biogel and stored for 24 months. The effects of four storage temperatures (-20, 4, 22 and 37ºC) and two delivery systems (sterile water and guar gum biogel) on nisin's activity were analyzed. Additionally, the cytotoxic potential of nisin and of the nisin-biogel, either freshly prepared or after 24 months of storage at 4ºC, was also evaluated, using a human keratinocyte cell line.

Results: We demonstrate that when stored at temperatures below 22ºC, nisin's antimicrobial activity is not significantly influenced by the duration of storage or delivery system. Regarding cytotoxicity, nisin suspensions under study presented no significant levels of cytotoxicity on human keratinocyte cells. Also, no significant differences were observed between nisin suspensions freshly prepared and stored at 4ºC for 24 months.

Conclusion: The nisin-biogel can be considered a good candidate to be used as an alternative or complement for conventional antibiotherapies. Further research is necessary in order to evaluate its full potential in the management of DFIs.

4.2 Introduction

Antibiotic resistance is a serious threat to public health and infections caused by antibiotic-resistant strains are increasingly being reported worldwide (Chambers and DeLeo 2009). Antimicrobial peptides (AMPs) are emerging as novel therapeutic approaches to overcome the challenges raised by the spreading of antibiotic-resistant bacteria. This diverse group of small peptides can be found in all living organisms as part of their innate immune system and may be used as an alternative to conventional antibiotics (Mahlapuu et al. 2016). Besides their direct antimicrobial activity against pathogens, AMPs also play a key role in the modulation of the immune system (Lai and Gallo 2009). Moreover, due to their action mechanisms, bacteria are less likely to develop resistance towards AMPs compared to conventional antibiotics (Yeaman and Yount 2003; Park et al. 2011).

Lantibiotics are a class of AMPs that contain the aminoacids lanthionine or methyllanthionine, being produced by Gram-positive bacteria to prevent the multiplication of other microorganisms (McAuliffe et al. 2001). Nisin, a type A lantibiotic, is the most well studied and characterized AMP. This small cationic peptide is produced by *Lactococcus lactis* and approved by the Food and Drug Administration, the European Food Safety Authority, the Food and Agriculture Organization and the World Health Organization as a safe additive. Over the past decades, nisin has made a significant impact in the food industry as a natural biopreservative for use in processed cheeses and heat-treated meat products (FAO/WHO 2013; Shin et al. 2016;

EFSA 2017). Nisin's most recently established acceptable daily intake dose is of 1 mg/kg body weight (EFSA 2017).

Nisin's potent antimicrobial activity against a wide range of pathogens has prompted research towards its application in biomedical fields. Several studies have already demonstrated that the antimicrobial action of nisin also includes clinical isolates (Shin et al. 2016). Particularly, a recent study conducted by our team has shown that nisin is able to inhibit and eradicate planktonic and biofilm-organized *Staphylococcus aureus* strains isolated from clinically infected diabetic foot ulcers, including methicillin-resistant and multidrug-resistant strains. Nisin was tested alone and incorporated within a guar gum biogel, to evaluate its efficiency as a delivery system for this AMP (Santos et al. 2016), and the promising results obtained supported nisin's application for the management of diabetic foot infections (DFIs).

In order to confirm the inhibitory ability and safety of the nisin-biogel formulation as a novel antimicrobial topical therapy, it is mandatory to evaluate the optimal environmental conditions for its storage, especially in terms of time and temperature, and its cellular toxicity potential. The study hereby presented was designed to evaluate nisin's antimicrobial activity against *S. aureus* DFI isolates after storage at different temperatures during a 24 months period, and to investigate nisin's cytotoxic activity using a culture of human epidermal keratinocytes.

4.3 Materials and methods

4.3.1 Bacterial isolates

This study included four *S. aureus* isolates obtained from clinical swab samples collected by the Levine method from infected foot ulcers of hospitalized and ambulatory patients with Diabetes *mellitus* (Mendes et al. 2012). Isolates virulence and antibiotic resistance profile was previously characterized (Mottola, Semedo-Lemsaddek 2016), as well as their biofilm-forming ability (Mottola, Mendes 2016) and nisin's susceptibility profile (Santos et al. 2016).

4.3.2 Antimicrobial peptides solutions

A nisin stock solution (1000 µg/mL) was prepared by dissolving 1 g of nisin powder (2.5% purity, Sigma-Aldrich, USA) in 25 mL of HCl (0.02 M) (Merck, Germany), filtered using a 0.22 µm cellulose acetate membrane filter (VWR, Belgium) and stored at -20°C.

A guar gum biogel of 1.5% (w/v) was prepared by dissolving 0.75 g of guar gum (Sigma-Aldrich, USA) in 50 mL of deionized sterile water, followed by sterilization by autoclave. Nisin was incorporated within this biogel in a proportion of 1:1, in order to obtain a final 0.75% (w/v) biogel.

A set of nisin solutions, either diluted in water or incorporated within the biogel, with final concentrations of 6.25, 25 and 50 µg/mL, was prepared and stored at four different temperatures (-20, 4, 22 and 37°C) during a period of 24 months.

4.3.3 Storage assay

Evaluation of storage influence on the antimicrobial activity of the nisin-biogel was performed using a spot-on-lawn assay. Briefly, the four *S. aureus* strains used in this study were cultured in a non-selective brain-heart infusion (BHI) agar medium (VWR, Belgium) at 37ºC for 24 h. Afterwards, bacterial suspensions at approximately 10^7 CFU/mL were prepared in fresh BHI broth. Confluent bacterial lawns were produced by evenly spreading the 10^7 CFU/mL bacterial suspensions onto BHI agar plates using sterile cotton swabs. Then, plates were dried for 10 min before the application of a 3 µL dot of each nisin suspension to be tested. Plates were incubated at 37ºC for 24 h to allow bacterial growth before measurement of inhibition halos. Assays were performed in triplicate and repeated every 3 months, for 24 months.

4.3.4 Cytotoxicity assay

For evaluating the cytotoxic potential of the nisin-biogel, cryopreserved normal adult Human primary adherent Epidermal Keratinocytes (HEKa) (PCS-200-011, ATCC, USA) were cultured in Dermal Cell Basal Medium (PCS-200-030, ATCC, USA) supplemented with the Keratinocyte Growth Kit (PCS-200-040, ATCC, USA) in 75 cm^2 cell culture flasks (Nunc; Thermo Fisher Scientific, Denmark), incubated at 37ºC in a humidified atmosphere of 5% CO_2. Upon reaching a confluence of approximately 80%, cells were harvested using trypsin-EDTA (0.25%, Gibco; Thermo Fisher Scientific, Denmark) and viable cells were quantified after a 1:10 dilution in trypan blue (0.4%, Sigma-Aldrich, USA) using a Neubauer haemocytometer.

For *in vitro* citoxicity assays, HEKa cells were seeded at a density of 10 000 cells per well in flat bottom polystyrene 96-well microplates (Nunc; Thermo Fisher Scientific, Denmark) and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 48 h. Afterwards, growth medium was removed and HEKa cells were exposed to 12 different suspensions of nisin, that varied in terms of concentration, delivery system and storage duration, as described in Table 9. Testing

wells were filled with 180 µL of growth medium plus 20 µL of the nisin suspensions under evaluation. As a positive control, cells were treated with doxorubicin hydrochloride (4 µM; Medac, Germany). Solvent (0.02 M HCl) and delivery system (0.75% guar gum biogel) controls were also included in the assay.

After a 24 h incubation at 37ºC in a humidified atmosphere of 5% CO_2, *in vitro* cell viability was determined using the MTT Cell Proliferation Assay Kit (ab211091, Abcam, UK). Briefly, growth medium was removed from all wells, and 50 µL of growth medium and 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were added into each well. Cells were then incubated at 37ºC for 3 h, after which 150 µL of MTT solvent was added into each well. Microplates were wrapped in foil and agitated on an orbital shaker for 15 min at room temperature. Cell viability was evaluated using a microplate reader (BGM LABTECH, Germany) to measure absorbance at a wavelength of 584 nm. Growth medium without cells was set as the blank control. Cell viability was expressed as a percentage relative to the untreated control (growth medium plus HEKa cells), which was set as being 100% viable. Assays were performed in triplicate.

Table 9 – Characteristics of the nisin suspensions tested in the cytotoxicity assays.

Nisin concentration (µg/mL)	Delivery system	Storage conditions
6.25	Sterile water	Freshly prepared
		Stored at 4ºC for 24 months
	Guar gum biogel	Freshly prepared
		Stored at 4ºC for 24 months
25	Sterile water	Freshly prepared
		Stored at 4ºC for 24 months
	Guar gum biogel	Freshly prepared
		Stored at 4ºC for 24 months
50	Sterile water	Freshly prepared
		Stored at 4ºC for 24 months
	Guar gum biogel	Freshly prepared
		Stored at 4ºC for 24 months

4.3.5 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 Software for Windows. For storage assays, differences between delivery systems were evaluated using the T-test. Differences between storage temperatures were determined by analysis of variance using the one-way ANOVA followed by Tukey's post-test. Finally, the influence of storage duration on nisin's activity was analyzed using linear regression.

For cytotoxicity assays, the optical density values presented by the suspensions under study were evaluated by analysis of variance using the one-way ANOVA followed by Dunnett's post-test. A two-tailed *p*-value < 0.05 was considered to be statistically significant in all the applied tests.

4.4 Results

4.4.1 Evaluation of storage assays

Results regarding the influence of different storage conditions on the nisin-biogel antimicrobial activity against DFI staphylococci are summarized in Figure 5

For both delivery systems under study, the inhibition halos diameters were directly proportional to the nisin's concentration used in each assay. At a concentration of 6.25 µg/mL, no significant differences in nisin's antimicrobial activity were observed between the AMP suspension in the two delivery systems under study when stored at 4, 22 or 37ºC. In contrast, when stored at -20ºC, the nisin-biogel presented an antimicrobial activity significantly lower (*p*-value = 0.0029; difference between means = 0.5938 ± 0.1649 mm) than nisin diluted in sterile water (Figure 5 a, d). At a concentration of 25 µg/mL, no significant differences were detected in nisin's antimicrobial activity between the AMP suspension in the two delivery systems under study when stored at 22 and 37ºC. However, when stored at -20 and 4ºC, the nisin-biogel exhibited an antimicrobial activity significantly lower (*p*-value < 0.0001; difference between means = 1.063 ± 0.1875 mm and *p*-value = 0.0007; difference between means = 0.8125 ± 0.1875 mm, respectively) than nisin diluted in sterile water (Figure 5 b, e). Similar results were observed for the highest concentration of nisin analyzed, 50 µg/mL, with no significant differences between nisin suspensions in the two delivery systems when stored at 22 and 37ºC, and a significantly lower antimicrobial activity presented by the biogel delivery system when stored at -20 and 4ºC (*p*-value < 0.0001; difference between

means = 1.219 ± 0.1826 mm and *p*-value < 0.0001; difference between means = 1.188 ± 0.1628 mm, respectively) (Figure 5 c, f).

Regarding storage temperatures, no significant differences were observed between nisin's antimicrobial activity when stored at -20, 4 and 22ºC for the two delivery systems and all concentrations of nisin analyzed. However, when stored at 37ºC, nisin's inhibition halos were significantly smaller (*p*-value < 0.05). In fact, at a concentration of 6.25 µg/mL, the mean difference between the inhibition halos produced by nisin stored at 37ºC and by nisin stored at lower temperatures was > 1.938 mm for nisin-biogel and > 1.469 mm for nisin diluted in sterile water (Figure 5 a, d); this difference increased for > 4.188 mm for nisin-biogel and for > 4.250 mm for nisin diluted in sterile water at a concentration of 25 µg/mL (Figure 5 b, e), and for > 4.719 mm for nisin-biogel and for > 4.750 mm for nisin diluted in sterile water at a concentration of 50 µg/mL (Figure 5 c, f).

Regarding the duration of storage, a linear regression analysis showed that for all the nisin's concentrations and delivery systems under study stored at -20, 4, and 22ºC, the storage period does not influence significantly (*p*-value > 0.05) nisin's antimicrobial activity against the DFI staphylococci under study. However, when stored at 37ºC, the storage period significantly influences nisin's activity (*p*-value < 0.05). The longer the storage duration, the lower the antimicrobial activity exhibited by nisin. In fact, for all the suspensions under study, nisin did not maintain its activity for more than 12 months when stored at 37ºC (Figure 5).

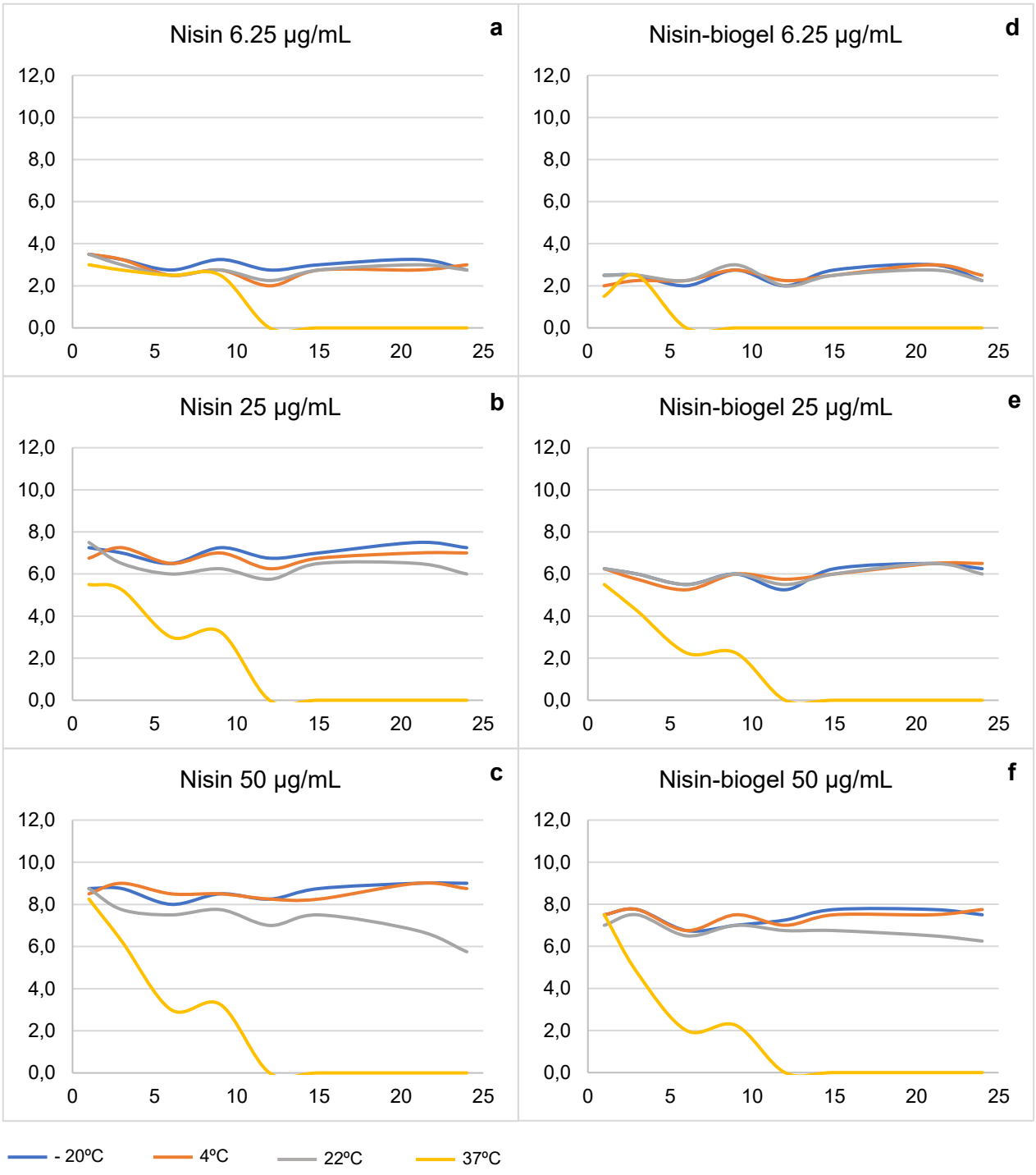

Figure 5 – Influence of storage conditions in terms of temperature, time and delivery system used, on nisin antimicrobial activity against the diabetic foot infection staphylococci under study.

The x-axis represents the duration of storage (months) and the y-axis represents the diameter of the inhibition halos (mm).

4.4.2 Evaluation of nisin cytotoxicity

The cytotoxic effects of the nisin suspensions tested on human keratinocyte cells are presented in Figure 6. As the nisin stock solution was using 0.02 M HCl, this HCl solution was used in the cytotoxicity assay as a solvent control and all the cytotoxicity results regarding the nisin suspensions under study were compared to this control.

Results show that the absorbance value presented by the solvent control was slightly different from the one presented by the untreated control (p-value = 0.0068; difference between means = 0.0306 ± 0.0060) and significantly different from the one presented by the positive control (p-value < 0.0001; difference between means = 0.0600 ± 0.0040).

However, regarding the nisin suspensions tested, no significant differences (p-value > 0.05) were observed between their cytotoxicity results and the ones from the solvent control. Also, no significant differences (p-value > 0.05) were observed between the cytotoxicity of the nisin suspensions freshly prepared and of the ones stored at 4ºC for 24 months. Similar results were presented by the analysis of nisin cytotoxicity when incorporated in the delivery systems under study, with no significant differences (p-value > 0.05) being detected between the absorbance values presented by nisin suspensions in sterile water and by nisin suspensions in the guar gum biogel.

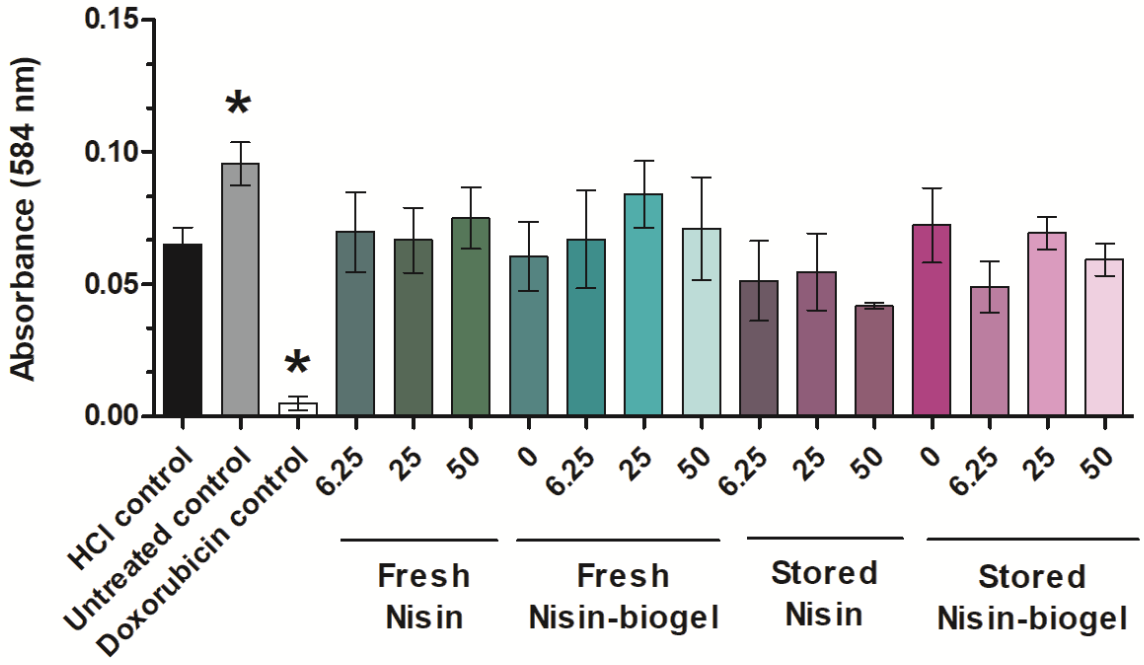

Figure 6 – Cytotoxicity of nisin suspensions under study regarding adult human epidermal keratinocyte (HEKa) cells.

Comparisons between treatments and HCl control was done by analysis of variance with the level of significance set at * *p*-value < 0.05. Concentrations of nisin are expressed in µg/mL.

4.5 Discussion

During the last decades, AMPs have gained an increasing interest as novel potential alternatives for the treatment of a vast array of clinical conditions, particularly those caused by antibiotic resistant microorganisms. Nisin is a well-known AMP with recognized activity towards gram-positive bacteria, being used as food preservative for over 50 years and 48 countries (Jozala et al. 2015). However, despite its demonstrated antimicrobial activity against pathogenic bacteria, including *Bacillus*, *Clostridium*, *Listeria* and *Streptococcus*, nisin is only used as a food preservative and has currently no therapeutic use (EFSA 2006; EFSA 2017).

Since 2015, our team has been studying the activity of nisin against bacterial isolates collected from infected diabetic foot ulcers, focusing on the potential topical administration of this peptide. For this reason, nisin's antimicrobial potential has been evaluated by incorporating this AMP within a guar gum gel, a natural polysaccharide which upon dilution in water forms a gellified formulation suitable for skin application. In spite of both nisin and guar gum being considered safe

for human administration (EFSA 2017), the cytotoxic potential of their combined use was still unknown. The study hereby presented determined the most suitable conditions for the storage of the nisin-biogel and evaluated its potential toxic effects regarding human keratinocyte cells.

Nisin was incorporated in the guar gum gel and stored at four different temperatures during 24 months. Results obtained demonstrated that the biogel delivery system allows nisin to maintain its antimicrobial activity against DFI staphylococci when stored at a wide range of temperatures, namely between -20 and 22ºC. Having in mind that a storage temperature of -20ºC implies a thawing step prior to the application of the nisin-biogel, our recommendation for diabetic patients' daily utilization is that the supplemented biogel should be stored at 4ºC, the temperature of a conventional domestic fridge.

An adequate antimicrobial compound for topical administration must present low cytotoxic effects on human skin cells. In this study, HEKa cells were exposed to nisin and to nisin-biogel and their cytotoxicity was evaluated using the MTT cell viability assay, which provides a simple and accurate method to quantify cell viability. The assay is based on the conversion of water soluble MTT compound to an insoluble formazan product, being observed that only viable cells with active metabolism, specifically mitochondrial respiration, can convert MTT into formazan. Therefore, the measured absorbance is proportional to the number of metabolic active cells (van Meerloo et al. 2011).

Studies available on the cytotoxicity of nisin regarding keratinocyte cells are scarce, being observed that results depend on cell type. Kamarajan and colleagues (2015) showed that nisin ZP, a naturally occurring variant of nisin, does not induce apoptosis in human oral keratinocytes. Shin et al. (2015) reported that human cells present in the oral cavity, mainly gingival fibroblasts, are unaffected by exposure to nisin at anti-biofilm concentrations, showing no signs of apoptotic changes. Moreover, subacute toxicity studies in rats demonstrated that repetitive intravaginal application of nisin induced no morphological changes in vaginal epithelial cells. Additionally, this study by Aranha et al (2004) described no histopathological abnormalities in vaginal tissue or any changes in blood and serum biochemical profiles (Aranha et al. 2004). However, a previous study by Murinda and colleagues (2003) indicated that some bacteriocins, including nisin, can present toxicity regarding colonic and kidney epithelial cells in a dose-dependent manner, and Kamarajan et al. (2015) also reported an induced apoptosis dose-dependent in human umbilical vein endothelial cells after exposure to nisin ZP.

Our work evaluated the viability of HEKa cells after exposure to three different concentrations of nisin after incorporation in two different delivery systems, as well as the influence of storage at 4ºC for 24 months on nisin suspensions cytotoxic potential. Results from all the

suspensions under study were compared to a 0.02 M solution of HCl, the nisin solvent, being observed that while the HCl control presented slight, but significant, cytotoxicity regarding HEKa cells, no significant differences were observed between the cytotoxicity results from the HCl control and the nisin suspensions tested. Therefore, we can conclude that the cytotoxicity presented by these suspensions is due to the HCl solvent and not by the nisin peptide itself. Further research is necessary to develop strategies to prevent and minimize the toxicity presented by HCl regarding human keratinocyte cells.

Cytotoxicity assay results also demonstrate that the guar gum biogel is a safe delivery system for this peptide, since no significant differences were observed between nisin suspensions diluted in sterile water and those incorporated within the biogel. Additionally, regarding storage duration, results demonstrate that nisin suspensions stored at 4ºC for 24 months presented cytotoxicity levels similar to freshly prepared nisin.

Overall, the data presented in this study shows that, at concentrations up to 50 µg/mL, nisin can be safely administered to human keratinocyte cells. Moreover, the guar gum biogel has proven to be a safe and effective delivery system for this peptide. In conclusion, the work hereby presented supports the potential use of the nisin-biogel as a new therapeutic approach in the management of chronic DFIs.

Chapter 5

5. The combined action of the antimicrobial peptides nisin and pexiganan against biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa* diabetic foot ulcer isolates

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Adapted from:

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5.1 Abstract

Introduction: *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the main pathogens present in diabetic foot infections (DFIs). Their antibiotic resistance and biofilm-producing ability renders these infections extremely recalcitrant to conventional antibiotherapy. Antimicrobial peptides (AMPs), namely pexiganan and nisin, are promising alternative therapeutic strategies.

Methods: The antimicrobial activity of these peptides was evaluated against planktonic co-cultures and established polymicrobial biofilms formed by S. aureus and P. aeruginosa clinical isolates. Their antimicrobial activity was also tested after incorporation in a guar gum biogel.

Results: Pexiganan's concentration required to inhibit and eradicate both planktonic and biofilm-based bacteria was substantially reduced when combined with nisin. Also, the biogel constitutes an efficient delivery system for these AMPs, allowing them to diffuse and reach biofilm embedded bacteria at effective concentrations.

Conclusions: Considering their antimicrobial activity against multidrug resistant and biofilm-forming pathogens, the combined use of nisin and pexiganan may represent a potential therapeutic solution to manage recalcitrant DFIs.

5.2 Introduction

The prevalence of Diabetes *mellitus* is increasing, affecting now more than 422 million people worldwide (WHO 2016). Diabetic patients are prompt to develop foot ulcers, which can become infected by polymicrobial biofilms (Lipsky et al. 2016). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the predominant Gram-positive and Gram-negative pathogens, respectively, present in DFIs (Mendes et al. 2012), and are known for their resistance profile towards commonly used antibiotic agents (Hancock and Speert 2000; Chambers and DeLeo 2009).

The spread of multidrug-resistant bacterial strains, along with the ineffectiveness of antibiotics to eradicate biofilm-based infections, has instigate the development of alternative treatment strategies, including the administration of AMPs.

Pexiganan is a synthetic peptide that presents a broad-spectrum of action and acts by disrupting the bacterial cell membrane through toroidal-type pore formation (Gottler and Ramamoorthy 2009). In 1998, two phase III clinical trials evaluated pexiganan’s wound healing and clinical cure potential among DFI patients (Lamb and Wiseman 1998). Pexiganan’s promising results encouraged the investigation towards its commercial use. However, in 2017, a placebo-controlled phase III clinical trial of a pexiganan cream applied to DFI patients failed to demonstrate a clear advantage of this AMP over the topical placebo control (Dipexium Pharmaceuticals 2017).

Considering that combinations of antimicrobial molecules often allow to reduce their individual effective concentrations and expand their action range (Pletzer et al. 2016), a possible solution to overcome pexiganan’s limitations may be its combination with antimicrobial agents that exhibit different mechanisms of action, such as nisin, one of the best studied and characterized

AMPs. Nisin is produced by *Lactococcus lactis*, acts principally against Gram-positive bacteria and has been used as a food preservative for over 60 years (Gharsalloui et al. 2016). Nisin's antimicrobial activity results from its interaction with the bacterial cell wall precursor lipid II inhibiting its incorporation into the peptidoglycan network. Additionally, nisin also uses the lipid II as a docking molecule for subsequent pore formation (Christ et al. 2007).

In this study, a dual-species biofilm incorporating *P. aeruginosa* and *S. aureus* strains co-isolated from the same diabetic foot ulcer was established *in vitro*, and used to evaluate the antimicrobial ability of pexiganan combined with nisin. Both AMPs were delivered to the biofilm micro-environment through a guar gum biogel previously developed by our team (Santos et al. 2016).

5.3 Materials and methods

5.3.1 Bacterial isolates

The *S. aureus* S25.2 and *P. aeruginosa* S25.1 strains used in this study were co-isolated from a DFI (Mendes et al. 2012). Two biofilm-producing reference strains*, S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853, were also used as control strains.

5.3.2 Antimicrobial peptides solutions

A stock solution of nisin (1000 µg/mL) was obtained by dissolving nisin (2.5% purity, Sigma-Aldrich, USA) in 0.02 M HCl (Merck, Germany). A stock solution of pexiganan (2048 µg/mL) was prepared by dissolving pexiganan (>95% purity, Innovagen, Sweden) in deionized sterile water.

5.3.3 Guar gum biogel preparation

The guar gum biogel was prepared as previously described (Santos et al. 2016) and the AMPs dilutions were incorporated within this biogel in a proportion of 1:1.

5.3.4 Pexiganan minimum inhibitory concentration, minimum bactericidal concentration, minimum biofilm inhibitory concentration and minimum biofilm eradication concentration assays

S. aureus and *P. aeruginosa* DFI strains were incubated in Mueller-Hinton Cation-Adjusted (MH-CA) agar medium (Becton, Dickinson and Company, USA) at 37°C during 24 h. Afterwards, bacterial suspensions were prepared as previously described (Santos et al. 2016) and diluted in fresh MH-CA broth to obtain 10^7 CFU/mL suspensions for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays and of 10^6 CFU/mL for minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) assays. Dual-microbial suspensions containing equal concentrations of each pathogen were also prepared. For MBIC and MBEC assays, biofilms were formed on hydroxyapatite-coated pegs on the lid of a 96-well microplate (MBEC Biofilm Inoculator; Innovotech, Canada).

Pexiganan was tested diluted in water or incorporated within the biogel, in concentrations ranging from 1 to 256 µg/mL.

MIC, MBC, MBIC and MBEC determinations were performed as previously described (Santos et al. 2016). Three independent replicates were performed. Data obtained were analyzed and results were expressed as mode values.

5.3.5 Antimicrobial activity of a dual-AMP solution

Nisin's antimicrobial activity against the two DFI isolates was determined previously (Santos et al. 2016). Nisin presented no antimicrobial effect towards the *P. aeruginosa* strain (data not published); however, it presented inhibitory and eradication activity against planktonic and biofilm-based *S. aureus* DFI isolates. Mean MIC values obtained were of 12.5 µg/mL for nisin diluted in water and of 22.5 µg/mL for nisin incorporated within the biogel (Santos et al. 2016).

For the dual-AMPs assays, pexiganan solutions, either in deionized sterile water or within the biogel, were supplemented with nisin at MIC values.

Broth microdilution assays for MIC, MBC, MBIC and MBEC determination were performed as described. Three independent replicates were performed. Data obtained were analyzed and results were expressed as mode values.

5.4 Results

5.4.1 Pexiganan minimum inhibitory concentration, minimum bactericidal concentration, minimum biofilm inhibitory concentration and minimum biofilm eradication concentration assays

Regarding the dual-species suspensions (Figure 7 c, f), pexiganan presented a MIC value two-fold higher than the one obtained for mono-species suspensions (Figure 7 a, b, d, e). Pexiganan MBC value was 2- to 4-fold higher than the MIC value for all mono-species suspensions (Figure 7 a, b, d, e). For dual-species suspensions, the pexiganan MIC and MBC values were similar (Figure 7 c, f). Regarding biofilm-based cells, pexiganan was more effective against *S. aureus* biofilms (Figure 7 a, d), than against *P. aeruginosa* biofilms (Figure 7 b, e). Polymicrobial biofilms formed by the two DFI isolates (Figure 7 f) were less susceptible to pexiganan than those formed by the reference strains (Figure 7 c). When incorporated within the biogel, pexiganan kept its anti-planktonic and anti-biofilm activity. MIC, MBC, MBIC and MBEC values of pexiganan incorporated within the biogel were only 2- to 4- fold higher than the values presented by pexiganan diluted in water (Figure 7 g, h, i).

5.4.2 Antimicrobial activity of a dual-AMP solution

Regarding *S. aureus* mono-microbial suspensions and biofilms, when combined with 12,5 µg/mL of nisin, pexiganan MIC, MBC, MBIC and MBEC values were below 1 µg/mL, the lowest pexiganan concentration used in these assays (Figure 7 a, d). When incorporated within the biogel, nisin also contributed to reduce pexiganan concentration values up to 8-fold (Figure 7 g).

Regarding *P. aeruginosa* strains, combination with nisin only promoted the decrease of pexiganan's MBC and MBIC values (Figure 7 b, e, h).

Nisin's potential to complement pexiganan's antimicrobial activity was also observed in dual-suspensions and dual-biofilms, since nisin allowed to reduce pexiganan's effective concentrations by 2- to 4-fold (Figure 7 c, f, i).

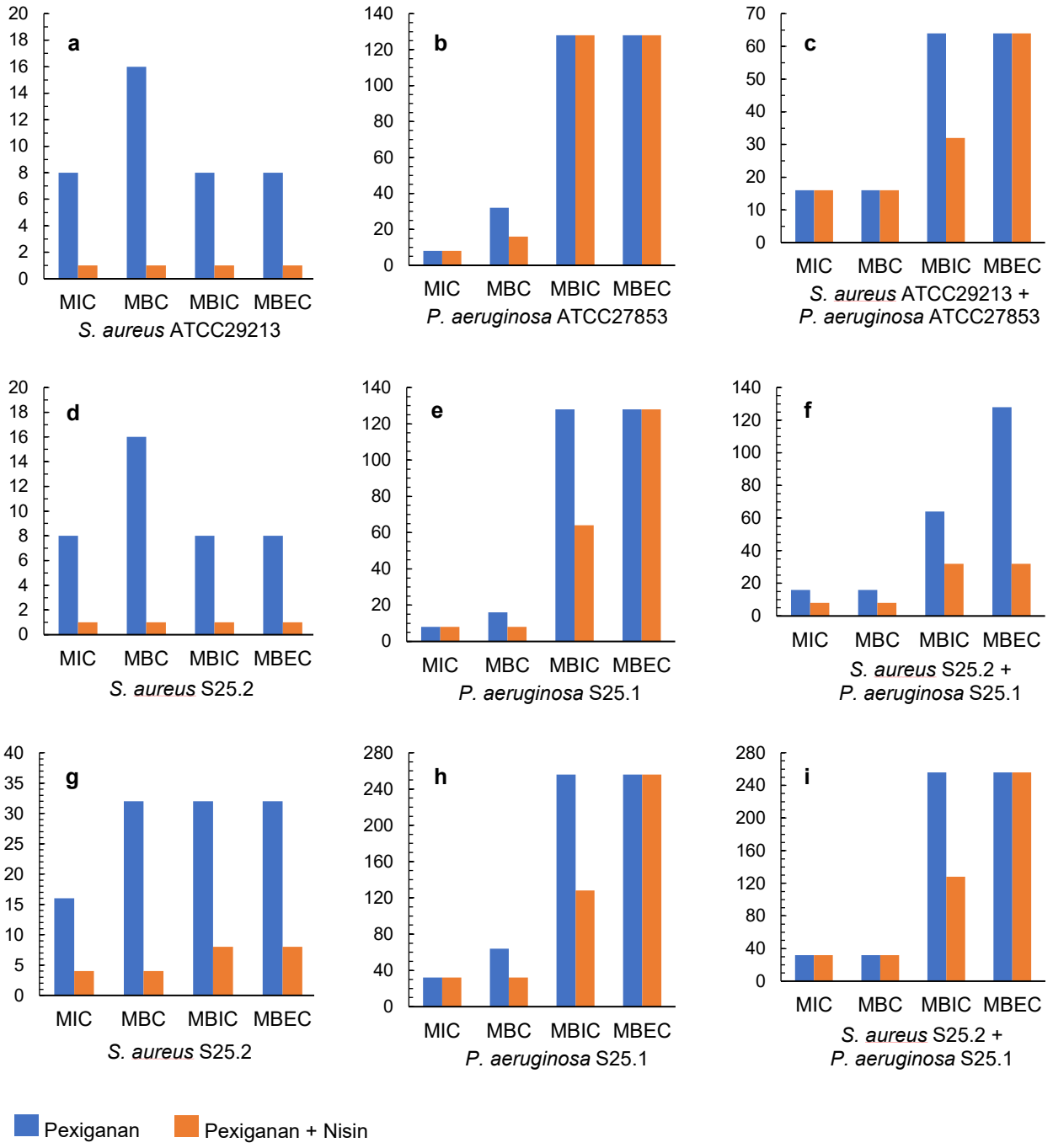

Figure 7 – Minimum inhibitory concentration, minimum bactericidal concentration, minimum biofilm inhibitory concentration and minimum biofilm eradication concentration for pexiganan and pexiganan plus nisin solutions diluted in deionized sterile water (a, b, c, d, e, f) or incorporated within the guar gum biogel (g, h, i), regarding *Staphylococcus aureus* and *Pseudomonas aeruginosa* clinical isolates and reference strains under study.

(**a**) Mono-suspension of *S. aureus* ATCC 29213. The bar at 1 µg/mL represents the smallest concentration of pexiganan tested at which no visible growth was observed; (**b**) Mono-suspension of *P. aeruginosa* ATCC 27853; (**c**) Dual-suspension of *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853; (**d**) Mono-suspension of *S. aureus* S25.2. The bar at 1 µg/mL represents the smallest concentration of pexiganan tested at which no visible growth was observed; (**e**) Mono-suspension of *P. aeruginosa* S25.1; (**f**) Dual-suspension of *S. aureus* S25.2 and *P. aeruginosa* S25.1; (**g**) Mono-suspension of *S. aureus* S25.2; (**h**) Mono-suspension of *P. aeruginosa* S25.1; (**i**) Dual-suspension of *S. aureus* S25.2 and *P. aeruginosa* S25.1.
The y-axis represents the concentration of pexiganan (µg/mL).
ATCC – American Type Culture Collection; MBC – Minimum Bactericidal Concentration; MBEC – Minimum Biofilm Eradication Concentration; MBIC – Minimum Biofilm Inhibitory Concentration, MIC – Minimum Inhibitory Concentration; S – Swab.

5.5 Discussion

Currently, DFIs management includes debridement and antibiotherapy (Lipsky et al. 2016; Mendes et al. 2012). However, the emergence of antibiotic resistant strains (Hancock and Speert 2000; Chambers and DeLeo 2009) and their propensity to form recalcitrant biofilms, render this approach often unsuccessful (Lipsky et al. 2016).

To date, pexiganan is the only AMP to undergo a phase III clinical trial regarding the treatment of DFIs (Dipexium Pharmaceuticals 2017) and nisin is one of the most established AMPs, being safely used in the food industry for over 60 years (Gharsallaoui et al. 2016). Considering the results of pexiganan's latest clinical trial, particularly its failure to promote bacteria eradication (Dipexium Pharmaceuticals 2017), this study evaluated the potential of nisin at MIC values to enhance pexiganan's antimicrobial activity against selected DFI pathogens.

The combination with nisin allowed to reduce the concentration of pexiganan required to inhibit and eradicate the DFI isolates, either in their planktonic or biofilm states. This effect was more noticeable on *S. aureus* mono-cultures than on *P. aeruginosa* ones, which is probably related with nisin's mode of action. Upon binding to lipid II, nisin inhibits cell wall biosynthesis and promotes the formation of pores in bacterial membranes, leading to cytoplasmic constituents' efflux and cell death (Christ et al. 2007). Considering that lipid II is mainly located at the inner membrane, the outer membrane of Gram-negative bacteria may prevent nisin from reaching lipid II molecules, rendering Gram-positive bacteria more susceptible to nisin than Gram-negative ones (Li et al. 2018).

The ability of nisin to complement pexiganan's anti-biofilm activity favors their combined use for the therapeutic of recalcitrant DFIs. Results also confirmed the guar gum biogel potential as a delivery system for these AMPs, since pexiganan's MIC, MBC, MBIC and MBEC values were only 2- to 4-fold higher when these peptides where incorporated within the biogel.

Biofilm suppression requires inhibition of the initial planktonic population, prevention of bacterial adhesion to surfaces, prevention of biofilm formation and maturation, and disruption of established biofilms. The dual-AMP biogel demonstrated a higher ability to inhibit biofilm formation than to eradicate pre-existing biofilms. For that reason, the potential of this anti-biofilm formulation might be enhanced if used immediately or shortly after DFI wound debridement.

AMPs can act in synergy with conventional antibiotics, particularly when they exhibit different action mechanisms (Park et al. 2011). For that reason, peptides such as nisin and pexiganan, known to disrupt the bacterial membrane, might be good adjuvants for antibiotics that target intracellular pathways. Therefore, this novel dual-AMP biogel may be used in a multifactorial approach towards DFI treatment.

Chapter 6

6. General discussion and future perspectives

Diabetic foot ulceration followed by infection is one of the most common complications of Diabetes *mellitus* (Lipsky et al. 2016; WHO 2016). These infections are a major health care problem and have a large impact in terms of patients' morbidity and mortality, being the number one cause of hospitalization and nontraumatic lower limbs amputation in diabetic patients (Vuorisalo et al. 2009; Lipsky et al. 2016).

These infections are usually polymicrobial and the bacterial species *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the main Gram-positive and Gram-negative pathogens, respectively, associated with diabetic foot infections (DFIs) (Mendes et al. 2012; Murali et al. 2014). Besides expressing numerous virulence factors (Hauser 2011; Oogai et al. 2011; Jenkins et al. 2015), these bacteria also demonstrate high resistance ability towards most antibiotic agents. Infections caused by *S. aureus* and *P. aeruginosa* drug-resistant strains are increasingly being reported worldwide, representing a serious threat to public health (Hancock and Speert 2000; Chambers and DeLeo 2009; Chatterjee et al. 2016).

In the DFI environment, most bacteria are naturally organized as biofilms, which represent bacteria consortia related to persistent and chronic infections that respond transiently to antibiotic therapy (James 2008). This feature is a direct consequence of the multiple resistance mechanisms that biofilm-structures present, including inefficient diffusion and sequestering of antimicrobial agents within the biofilm matrix (Batoni 2016). The ulcer micro-environmental pathophysiological conditions unsuitable to wound healing (Vuorisalo et al. 2009), together with the presence of multidrug resistant pathogens (Mendes et al. 2012) able to form persistent biofilms (Mottola, Mendes, et al. 2016) render the treatment of chronic skin infections particularly challenging.

Novel therapeutic approaches are needed to successfully treat DFIs and the fact that antimicrobial peptides (AMPs) may be used to control resistant bacteria has prompted research on these molecules as potential alternatives to traditional therapeutics. These peptides may act through several mechanisms of action; they can penetrate the bacterial cells and repress

intracellular processes, namely protein and nuclei-acid synthesis, protein folding and enzymatic activity, or act on the bacterial cell wall and plasma membrane (Mahlapuu et al. 2016).

Biofilm-based bacterial cells are physiologically distinct from non-adherent planktonic ones. Their growth rate can be reduced and the quorum-sensing signaling systems enable biofilm cells to activate specific genetic determinants related to antibiotic resistance (Dickschat 2010; Hall and Mah 2017). Despite their numerous modes of action, in the majority of cases AMPs exert their antibacterial activity by disrupting cell membranes through several different mechanisms, namely pore formation in the lipid bilayer (barrel stave and toroidal pore models), membrane dissolution (carpet model), membrane thinning/thickening, lipid-peptide domain formation, non-lytic membrane depolarization and electroporation (Nguyen et al. 2011; Gaspar et al. 2013). Independently of the metabolic state of the bacteria, membrane integrity is essential for survival. For that reason, AMPs have the potential to kill not only metabolically active microbes, but also slow growing cells and persister cells often found in bacterial biofilms (Strempel et al. 2015; Batoni et al. 2016).

In patients with Diabetes *mellitus*, vascular complications are quite frequent and foot tissues can become ischemic due to peripheral arterial disease (Armstrong et al. 2011). This vascular insufficiency is one of the major healing impediments of DFIs because it impairs the systemically administered antibiotic compounds from reaching the wound environment at effective therapeutic concentrations (Lipsky et al. 2016). Considering the problems associated with systemic therapies, treating DFIs with topical antimicrobials has potential benefits. Topical administration of AMPs has the advantage of avoiding the adverse effects of systemic therapeutics, allowing the use of agents that cannot be administered systemically (Lipsky and Hoey 2009), and promoting an increased concentration of antibacterial molecules at target site (Lipsky et al. 2008; Dumville et al. 2017).

Within this context, the present work aimed to contribute for the development of an innovative and promising antimicrobial therapy for topical administration to DFIs. With that purpose, a multidisciplinary strategy involving several complemental objectives was carefully designed. The present work comprises six chapters with distinct objectives that altogether contributed to achieve the main goal of this project. The first chapter covers the state of the art and explains the challenges and prospects of DFIs antimicrobial therapies available and the potential role that AMPs might play in suppressing the limitations presented by conventional antibiotics. The following chapters cover specific goals and are focused on the main achievements and implications of these findings. Specifically, the second chapter comprises the determination of the antimicrobial activity of nisin against a collection of *S. aureus* isolated from DFIs and the

development of an efficient delivery system for this AMP. The third chapter covers the study of the potential of nisin to complement the activity of conventional antiseptics and antibiotics regularly used in the management of DFIs. The fourth chapter comprises the determination of the ideal storage conditions for the nisin-biogel regarding time and temperature and the evaluation of its cytotoxic potential against epidermal keratinocytes. The fifth chapter is focused on the determination of the potential of nisin to complement the activity of pexiganan against two *S. aureus* and *P. aeruginosa* strains co-isolated from the same DFI. Finally, this sixth chapter includes a general discussion of the results obtained in the previous chapters and their potential impact in the management of DFIs.

During the first task of this project, the antimicrobial activity of nisin, a well characterized AMP used as a food preservative (Cleveland et al. 2001), against a collection of *S. aureus* DFI clinical isolates was evaluated. The *S. aureus* collection under study included 8 (34.8%) MRSA isolates, being 7 (30.4%) of them also multidrug resistant (Mottola, Semedo-Lemsaddek, et al. 2016), as they were resistant to three or more antibiotics from different classes (Magiorakos and Srinivasan, 2012). Additionally, all the *S. aureus* strains evaluated in this study were able to establish, at adequate conditions, biofilms in less than 24 hours (Mottola, Mendes, et al. 2016).

The biofilm mode of growth is a major virulence factor presented by bacteria, being observed that these slime-enclosed aggregates of bacteria are characterized by forming a micro-environment very hostile for antimicrobial agents penetration and diffusion (Hall and Mah 2017). Matrix-encased bacteria can survive antibiotic concentrations up to 1000 times higher than those required to kill free-living bacterial cells (Kaplan 2011). Having that in mind, the high concentration of nisin required to eradicate established *S. aureus* biofilms determined in chapter 2 was expected. However, the low concentration of nisin required to inhibit the formation of *S. aureus* biofilms, including those formed by multidrug resistant strains was not expected. Nisin was able not only to inhibit and eradicate *S. aureus* planktonic cells, but also to inhibit *S. aureus* biofilms at concentrations below its acceptable daily intake (EFSA 2017).

Afterwards, the project aimed to develop a delivery system for this AMP, and a gellified formulation which allowed the incorporation of nisin was developed. In order to be adequate for topical administration to diabetic foot ulcers, that formulation must meet numerous criteria. It should be biocompatible and non-toxic for living tissues, water soluble and present a thick and viscous consistency to allow its adherence to skin epithelium. The guar gum gel selected not only meets the requisites (Reddy et al. 2011; Thombare et al. 2016), but also presents additional characteristics that favors its use as a delivery system for nisin. This polysaccharide polymer is highly abundant in nature and can be produced at economical costs (Reddy et al. 2011),

conditions quite important when considering its mass production. When incorporated within this guar gum biogel, nisin kept its activity against planktonic and biofilm-encased *S. aureus*, with all DFI clinical isolates under study presenting susceptibility towards this formulation. Results obtained during this task showed that the polysaccharide biogel allowed nisin's diffusion and antimicrobial activity maintenance, strengthening its potential as a novel topical therapeutic formulation against infected diabetic foot ulcers.

Currently, the standard wound care for DFIs consists of surgical debridement followed by wound cleansing with an antiseptic solution and topical, oral or intravenous administration of antibiotics, depending on the severity of infection (Lipsky et al. 2016). Antiseptic agents frequently used for wound cleansing include chlorhexidine and povidone iodine, which can be applied to intact skin and some open wounds to inhibit or kill microorganisms (Dumville et al. 2017). Antiseptics tend to possess a broad spectrum of action, multiple cell targets and residual antimicrobial activity (Dumville et al. 2017). Antibiotics usually have a spectrum of action narrower than antiseptics and tend to act on specific cell targets (Dumville et al. 2017). Several antibiotic agents have been used in the management of DFIs, including penicillins (amoxicillin/clavulanate, ampicillin/sulbactam), cephalosporins (cephalexin, cefoxitin), lincosamides (clindamycin), fluoroquinolones (lexofloxacin, ciprofloxacin), carbapenems (imipenem/cilastatin), glycopeptides (vancomycin) and aminoglycosides (gentamicin) (Lipsky et al. 2016).

Unfortunately, even when proper therapeutic protocols are established, the resolution of a DFI is often unreachable. Many factors can be associated with ulcers poor healing, including severe inflammation, progressive tissue damage and the presence of pathogenic microorganisms and their ability to form recalcitrant biofilms (Lipsky et al. 2016). Conventional antibiotherapies are often unsuccessful and about 20% of moderate or severe DFIs evolve to gangrene, leading to some level of limb amputation (Armstrong et al. 2017).

Considering the failure of antibiotics-based therapies, the work described in chapter 3 was dedicated to testing the potential of nisin to enhance the activity of conventional antiseptics and antibiotics against established biofilms formed by *S. aureus* clinical isolates. To achieve this goal, an antimicrobial schematic protocol aiming at mimetizing the management guidelines for DFI performed in standard clinical practice was established *in vitro.*

According to the guidelines for the medical management of DFI from Lipsky et al. 2016, Chidiac et al. 2007, Bader 2008, and Duarte and Gonçalves 2011, the antibiotics of choice for mild, moderate and severe DFI are clindamycin, gentamicin and vancomycin, respectively. Regarding cleansing of infected ulcers, chlorhexidine is the most widely used antiseptic (Dumville et. 2017). The work presented in chapter 3 consisted of testing fifteen different antimicrobial

combinations, including chlorhexidine, nisin-biogel, clindamycin, gentamicin and vancomycin, against biofilms formed by *S. aureus* DFIs clinical isolates.

Chlorhexidine is a synthetic cationic biguanide molecule that binds to the negatively charged bacterial cell walls and presents a broad activity spectrum against Gram-positive and Gram-negative bacteria, yeasts, fungi and some lipid-enveloped viruses (Lim and Kam 2008; Milstone et al. 2008). Chlorhexidine is able to eradicate or inhibit the growth of microorganisms present in living tissues. At low concentrations, it affects the prokaryotic membrane integrity, by penetrating and disrupting the bacterial cytoplasmic membrane, which leads to an alteration of the bacterial cell osmotic equilibrium and leakage of potassium and phosphorous, resulting in a bacteriostatic effect (Milstone et al. 2008). At higher concentrations, chlorhexidine exerts a bactericidal action after entering the cytoplasm through the damaged cytoplasmic membrane and forming irreversible precipitates with intracellular adenosine triphosphate and nucleic acids, which results in cell death (Lim and Kam 2008).

Nisin is a small peptide that presents two mechanisms of action. Nisin uses the cell wall precursor lipid II simultaneously as a target and as a pore constituent. By binding to lipid II molecules, nisin inhibits their incorporation into the peptidoglycan wall, thereby affecting the microbial growth; on another end, the formation of stable pores leads to cytoplasm efflux and cell death (Wiedemann et al. 2001; Christ et al. 2007).

Clindamycin belongs to the lincosamide class of antibiotics and can be administered topically, orally and parenterally (Morar et al. 2009). It presents a broad-spectrum activity and excellent tissue penetration (Chidiac et al. 2007; Bader 2008; Lipsky et al. 2016). Gentamicin is an aminoglycoside with bactericidal activity against some Gram-positive bacteria, including *S. aureus*, and can be used in combination with broad-spectrum β lactams to treat polymicrobial infections (Chen et al. 2014; Garraghan and Fallon 2015). Clindamycin binds to the 50S subunit of the bacterial ribosome and gentamicin to the 30S subunit, for that reason both antibiotics are able to impair bacterial multiplication (Kohanski et al. 2010). Vancomycin is a glycopeptide that targets the cell wall precursor lipid II, blocking the peptidoglycan network biosynthesis (Kohanski et al. 2010). Vancomycin possesses bactericidal activity against staphylococci, including MRSA strains (Sujatha and Praharaj 2012). It was approved for clinical use in 1958; however, due to its high toxicity levels and low resistance rates, vancomycin is reserved for unique conditions, such as severe infections caused by multidrug resistant pathogens (Binda et al. 2014).

Results presented in chapter 3 show that all the antimicrobial associations tested that included the nisin-biogel presented biofilm inhibitory effects significantly higher than those that did not include the nisin-biogel. Nisin's ability to form pores on prokaryotic membranes may provide

the desirable conditions for antibiotics penetration into the bacterial cytoplasm space, enabling them to act upon the bacterial intracellular machinery. However, regarding the eradication assay, the effect of the antimicrobial combinations that included the nisin-biogel was not significantly higher than the one of those that did not include this formulation. The inefficient diffusion and sequestering of antibiotic compounds within the biofilm matrix together with the reduced growth rate, protein synthesis and other metabolical activities presented by biofilm-encased cells render these cells particularly difficult to erradicate.

Biofilm resolution can be achieved through different mechanisms, including the inhibition and eradication of the initial planktonic population, prevention of bacterial adhesion to surfaces, inhibition of biofilm formation and matrix maturation and disruption of established biofilms (Park et al. 2011; Strempel et al. 2015; Batoni et al. 2016; Pletzer et al. 2016). Considering that associations involving the nisin-biogel formulation presented better results at inhibiting biofilm formation than at eradicating established biofilms, the nisin-biogel seems to be most suitable for application immediately or shortly after debridement of infected wounds, as a preventive measure for DFI development in diabetic patients.

The development of a novel antimicrobial formulation aiming at being topically administered to DFI also comprises the evaluation of its optimal storage conditions. Chapter 4 includes the investigation regarding the influence of storage during 24 months at different temperatures on the nisin-biogel inhibitory activity and cytotoxic potential.

Results demonstrate that when stored at 37ºC, the duration of storage significantly influences nisin's and nisin-biogel's antimicrobial activity against DFI staphylococci. However, when stored at -20, 4 and 22ºC, the duration of storage does not have any significant influence on the inhibitory activity of nisin's and of the nisin-biogel's, which supports the use of the guar gum biogel as an adequate delivery system for this AMP, as it enables the maintenance of nisin's activity even when stored at a wide range of temperatures. Considering that a storage temperature of -20ºC implies a thawing step prior to every utilization, our recommendation for diabetic patients' daily utilization is the storing of nisin-biogel at 4ºC, the temperature presented by conventional domestic fridges.

An acceptable topical antimicrobial agent to be used in DFI treatment must show activity against the broad spectrum of bacteria present in the ulcer environment without causing significant damage to the host cells (Batoni et al. 2016; Mahlapuu et al. 2016; Dumville 2017). Chapter 4 describes the work conducted to evaluate the cytotoxic potential of nisin and of the nisin-biogel, either freshly prepared or after a 24 months storage at 4ºC, regarding human epidermal keratinocyte cells.

Results obtained in the cytotoxicity assays show that nisin suspensions under study, either diluted in sterile water or incorporated within the guar gum biogel, did not present significant levels of cytotoxicity on human keratinocyte cells. Moreover, no significant differences were observed between the cytotoxic activity of nisin suspensions freshly prepared and stored at 4ºC for 24 months.

In conclusion, data presented in this chapter shows that at concentrations up to 50 µg/mL nisin's cytotoxicity is not significantly affected by a 24 months storage, neither by the delivery system used. Moreover, it was once again proven that the guar gum biogel is a safe and effective delivery system for the administration of this antimicrobial peptide to infected diabetic foot ulcers.

The microbiota present in the DFI microenvironment is complex, with different stages of wound infection comprising different bacterial strains. Since nisin is mainly active against Gram-positive bacteria, to inhibit the spectrum of pathogens present in DFIs an association of AMPs with different action spectra may be required. In chapter 5, the evaluation of the inhibition potential of a biogel guar gum supplemented with a combination of nisin and pexiganan is presented.

Pexiganan is a synthetic AMP with a wide spectrum of action that includes both Gram-positive and Gram-negative bacteria (Flamm et al. 2016). Upon binding to the lipid bilayer, pexiganan molecules form antiparallel dimers of amphipathic helices and exert their antimicrobial effect through toroidal-type pore formation (Gottler and Ramamoorthy 2009). Both nisin and pexiganan act directly towards bacteria lipidic membranes. As previously described for nisin, the mode of action of pexiganan is independent of the metabolic stage of the bacterial cells (Gottler and Ramamoorthy 2009; Wiedemann et al. 2011), which allows it to be effective against active and dormant cells that co-exist in mature biofilms (Pletzer et al. 2016).

Chapter 5 covers the investigation conducted in order to determine the potential of nisin to complement pexiganan's activity against planktonic and biofilm-organized *S. aureus* and *P. aeruginosa* strains co-isolated from an infected diabetic foot ulcer. Both AMPs were tested diluted in deionized water and incorporated within the guar gum biogel. Results showed that acting together, these AMPs were able to diffuse from the biogel polymer and inhibit and eradicate biofilms formed by the *S. aureus* and *P. aeruginosa* strains, so this dual-AMPs formulation has the potential to be a novel therapeutic in the treatment of DFIs. AMPs can act in synergy with conventional antibiotics, particularly when they exhibit different action mechanisms (Park et al. 2011; Pletzer et al. 2016). For that reason, peptides such as nisin and pexiganan, known to disrupt the bacterial membrane, might be good adjuvants for antibiotics that target bacterial intracellular pathways. The fact that pexiganan and nisin are peptides with relevant differences regarding their amino acid sequences and 3D structures (McAuliffe et al., 2001; Gottler and Ramamoorthy, 2009),

points out for the importance of conducting further research in order to establish the guar gum biogel full potential as a delivery system for bioactive molecules.

Understanding the biomedical properties of AMPs might be regarded as a key advance towards the establishement of new therapeutic approaches to manage antibiotic-resistant infections. The potential of AMPs for the management of DFIs goes far beyond their biocidal effect. The growing interest in AMPs is also due to their established anti-inflammatory and immunomodulatory properties (Batoni et al. 2016; Mahlapuu et al. 2016). In addition to their direct antimicrobial activity, numerous AMPs are capable to modulate the host's innate immune response, recruiting defense cells at the site of infection and prompting their activity (Batoni et al. 2016). The effectiveness of these AMPs, particularly of nisin and pexiganan, regarding the polymicrobial biofilms present in DFI wounds *in vivo* still an unknown territory that merits exploration. *In vitro* biofilm models are the foundation of preliminary basic research and preclinical investigation. However, they underrepresent the complex microbiota present in DFIs, the microenvironmental singular characteristics and the interaction between the human immune system, skin cells constituents and bacterial cells. Further research is necessary in order to determine AMPs full potential regarding the clinical management of biofilm-related diseases, such as DFIs.

Due to the emergence of antibiotic resistant strains, the current landscape of antimicrobial therapy is facing a profound transformation. Microbiology research needs to adapt to a rapidly changing scenario to effectively translate novel concepts into efficient and sustainable therapeutic options. This work opened up a new perspective in DFI management as it contributed for the validation of a novel AMPs-biogel formulation with significant activity against biofilms formed by DFI clinical isolates. The advent of innovative therapies, such as those based on the topic administration of AMPs, may revolutionize the conventional treatment paradigm in current infections disease practice.

Chapter 7

7. References

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