



“Guar gum” as an antimicrobial peptide delivery system to be applied to the control of diabetic foot infections

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Dissertation to obtain the Master of Science Degree in

Microbiology

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November 2016

“One change always leaves the way open for the establishment of others.”
Niccolò Machiavelli

Acknowledgments

Firstly, I would like to my sincere gratitude to my advisor Professor Manuela Oliveira, for the continuous support, constant motivation, huge patient and friendship. Her knowledge and guidance was important to me at all times of research, laboratory work and writing of this dissertation.

I would like to thank to Professor Isabel Sá Correia, for consenting me to develop my work in the Faculty of Veterinary Medicine. And also, for her dedication to this Master.

To Professor Luís Tavares and Fernando Bernardo, for welcoming me at the Faculty of Veterinary Medicine.

I would like to thank to Raquel Santos, not only for her knowledge and help with the entire laboratory work, but also for her support and motivation.

To my fellow colleagues and friends, Daniela Freire, Catarina Tibério, Alina Kryshen and Ana Cabo Verde, for their friendship and constant company in laboratory work.

I would like to thank to all my beloved friends, one extensive list, you know who you are! Thank you all for being truly good friends, for always cheering me up and for all the moments of distraction.

Last but not least, I would like to express gratitude to my family: my parents, my brothers and my grandparents, for always supporting me and always encouraged me to want more and to do not give up. Without them I would not be where I am today.

Abstract

Diabetic foot ulcers are a major complication of Diabetes *mellitus*. Several microorganisms can colonize and promote opportunistic infections, being *Staphylococcus aureus* frequently isolated. Antimicrobial peptides have been extensively investigated for new therapies against Gram-positive bacteria, such as nisin, produced by *Lactobacillus lactis*. Guar gum has been tested as delivery system.

Inhibitory potential of nisin against 23 *S. aureus* isolates collected from DFU patients was evaluated. The minimum inhibitory (MIC), bactericidal (MBC), minimum biofilm inhibitory (MBIC) and eradication (MBEC) concentrations were determined for nisin, diluted in HCl and incorporated in guar gum gel. The inhibitory activity of nisin incorporated in guar gum gel throughout 6 months, was tested and positively observed.

All isolates tested are considered susceptible to nisin. For nisin diluted in HCl, mean values for MIC, MBC and MBIC were 90 ± 22.8 $\mu\text{g/mL}$, 495.2 ± 149.9 $\mu\text{g/mL}$ and 150.8 ± 85.5 $\mu\text{g/mL}$, respectively. MBEC values >1000 $\mu\text{g/mL}$ were observed in 65% of isolates. Regarding the nisin incorporated in guar gum gel, mean values for MIC, MBC and MBIC were 180.8 ± 53.9 $\mu\text{g/mL}$, 766.7 ± 272.6 $\mu\text{g/mL}$ and 366.7 ± 140.4 $\mu\text{g/mL}$, respectively. Most isolates (87%) show MBEC values higher than 1000 $\mu\text{g/mL}$. Statistical differences were observed between MIC, MBC and MBIC, while no significant differences were found between MBEC values for the formulations presented.

Results show the importance of nisin as a substitute or complementary therapy to the current antibiotics used for treating DFU infections. This innovative therapeutic strategy shows a promising delivery system for AMP, allowing the development of novel topical therapies as treatments for bacterial skin infections.

Keywords: diabetic foot ulcers, *Staphylococcus aureus*, biofilm, nisin, guar gum, minimum inhibitory concentration

Resumo

As úlceras do pé diabético são a complicação mais severa da diabetes, desenvolvidas por vários microrganismos, sendo *Staphylococcus aureus* frequentemente isolado. Os péptidos antimicrobianos têm sido investigados como novas terapias, como a nisina, produzida por *Lactobacillus lactis*. O Guar gum tem sido investigado como distribuidor para AMP.

O potencial antibacteriano da nisina foi testado contra 23 *S. aureus* isolados de úlceras diabéticas. Tanto para a nisina diluída em HCl como incorporada em guar gum gel, foi determinada a concentração mínima inibitória (CMI) e bactericida (CMB), concentração mínima inibitória de biofilme (CMIB) e de erradicação (CMEB). A actividade inibitória da nisina incorporada no gel ao longo de seis meses foi testada e observada positivamente.

Os isolados foram susceptíveis ao potencial inibitório da nisina. Para a nisina diluída em HCl, os valores médios de CMI, CMB e CMIB foram $90 \pm 22,8$ µg/mL, $495,2 \pm 149,9$ µg/mL e $150,8 \pm 85,5$ µg/mL, respetivamente. Valores de CMEB >1000 µg/mL observou-se em 65% dos isolados. No caso da nisina inserida no gel, os valores médios de CMI, CMB and CMIB foram $180,8 \pm 53,9$ µg/mL, $766,7 \pm 272,6$ µg/mL e $366,7 \pm 140,4$ µg/mL, respetivamente. CMEB na maioria dos isolados (87%) foi superior a 1000 µg/mL. Observaram-se diferenças significativas entre os valores de CMI, CMB e CMIB, oposto aos valores de CMEB entre as fórmulas de nisina apresentadas.

Os resultados mostram-nos a importância da nisina como substituto ou complemento no tratamento das úlceras diabéticas. Esta inovadora estratégia terapêutica mostra-se promissora como novo sistema de distribuição de AMP, permitindo desenvolver novos tratamentos contra infeções bacterianas da pele.

Palavras-chave: úlceras do pé diabético, *Staphylococcus aureus*, biofilme, nisina, guar gum, concentração mínima inibitória.

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List of Abbreviations

Aba	Aminobutyric acid
Abu-S-Aba	B-methyllanthionine
ADI	Acceptable Daily Intake
Ala-S-Ala	Lanthionine
<i>agr</i>	Accessory gene regulator
AMP	Antimicrobial peptides
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
CMB	Concentração Mínima Bactericida
CMEB	Concentração Mínima de Erradicação de Biofilme
CMI	Concentração Mínima Inibitória
CMIB	Concentração Mínima Inibitória de Biofilme
CFU	Colony forming units
DFU	Diabetic foot ulcers
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DM	Diabetes <i>mellitus</i>
DNase	Deoxyribonuclease
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nation
FDA	Food and Drugs Administration
GRAS	Generally Recognized as Safe
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigens
IDF	International Diabetes Federation
IgG	Immunoglobulin G

IU	International Units
<i>luxS</i>	S-ribosylhomocysteinelyase
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistance <i>S. aureus</i>
MSSA	Methicillin Susceptible <i>S. aureus</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBP	Penicillin binding protein
PVD	Peripheral Vascular Disease
PVL	Panton-Valentin leucocidin
QS	Quorum-sensing
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>SCCmec</i>	<i>Staphylococcus</i> cassette chromosome <i>mec</i>
T1D	Type 1 Diabetes <i>mellitus</i>
T2D	Type 2 Diabetes <i>mellitus</i>
TSST-1	Toxic Shock Syndrome toxin-1
WHO	World Health Organization

Chapter 1 | Introduction

1.1. *Diabetes mellitus*

The World Health Organization (WHO) defines *Diabetes mellitus* (DM) as a chronic disease, that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it provides [1]. In both cases, the concentration of sugar in the blood increases, resulting in a group of metabolic disorders characterized by a chronic hyperglycemic condition [2]. DM is a metabolic disorder of heterogenic etiology, characterized by hyperglycemia and alteration of the metabolism of carbohydrates, protein and fat, resulting from defects in insulin secretion and/or action [3]. The effects of DM include long-term damage of various organs, leading to dysfunction and failure, and in absence of effective treatment to death [4]. Diabetes in all its forms imposes unacceptably high human, social and economic costs on both developed and developing countries. Epidemiologically, the International Diabetes Federation (IDF) estimates that diabetes affects 415 million people worldwide, from all age groups. The number of people affected by this disease is set to rise beyond 642 million cases in 2040, which corresponds to 8.8% of the worldwide adult population [5].

Depending on the mechanism that leads to insulin deficiency, diabetes occurs in several forms, including type 1, type 2 and gestational diabetes [5]. However, type 1 and type 2 are the most frequently disorder, being a common global health problem.

In women with uncontrolled diabetes, this disorder can have serious consequences, especially for pregnant, and babies can be born with health complications, called gestational diabetes [5], [6].

The type 1 *Diabetes mellitus* (T1D) or insulin-dependent diabetes is directly related with the damage of the cells that produce insulin on pancreas, due to the chronic destruction of pancreatic β -cells via autoimmune mechanisms, mediated by immune cells such as T lymphocytes and macrophages [7]. The reason why this defense system attack occurs is unknown [5]. Normally, the process of auto-destruction begins months or years before the clinic diagnostic, this way, regardless of the age at which the diagnostic occurs approximately 70 to 90% of β -cells are already destroyed when the first symptoms of hyperglycemia are observed [7]. Epidemiologically, T1D occurs in individuals from any age groups, but particularly in children or young adults, from all racial groups, with equal prevalence in males and females [5], [8]. Today, many studies have described a connection between genetics and T1D development, onset by the triggering of the autoimmune system. Effectively, many genes or genetic regions were found to be associated with its inaction, including the genes that code for the human leukocyte antigens (HLA) or the insulin [8].

Non-insulin-dependent or type 2 *Diabetes mellitus* (T2D) is caused by a combination of impaired insulin secretion by pancreatic β -cells leading to an insufficient production of insulin and the rise of blood sugar concentration and resistance of the peripheral target tissues to insulin, especially of liver and muscle cells [5], [6]. This type is the most common (~90%), affecting mainly adults, but also children and adolescents, being usually related with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity and ethnicity [5], [6]. As mentioned for T1D, the reasons for developing T2D are also still not known, but diverse risk factors have been shown to

play an important role [5]. For example, genetic factors have an important contribution for T2D development, together with environmental factors [6].

Diabetes clinical manifestations include polydipsia, polyuria, blurred vision, weight lost, polyphagia, lack of energy, dry mouth, slow-healing wounds, frequent infections, tingling sensation or numbness in the extremities (hands and feet) and nauseas present in both types of Diabetes [5], [9]. Moreover, these manifestations do not appear all at the same time and may vary between individuals. In T1D, the symptoms are often not severe, but they are sudden and unexpected. In T2D the symptoms are normally mild or absent, rendering T2D a hard disease to diagnose [9].

Both types of Diabetes affect the quality of life of the patients, in terms of health, economics and social. This complex disorder can affect any organ of the body. In long-term, diabetes, often lead to blindness, amputations due to diabetic foot disorders, oral disease (periodontitis), sleep apnea, neuropathy, stroke, heart and blood vessel disease, kidney failure and premature death [6], [8].

1.2. Diabetic foot

Foot infections, are a major concern for the diabetic patients. Diabetic foot ulcers (DFU) and gangrene are common among diabetic patients and may occur in individuals presenting both types of diabetes (T1D and T2D) [10]. Foot ulceration and systemic infections are two of the most frequent complications in diabetes [11], representing major causes of morbidity and mortality among diabetic patients [12], they are of responsible for amputations in 14 to 20% of cases the patients, being observed that 50% of the amputated patients die within 3 years after the produce [13].

Amputations lead to an increased frequency and length of hospitalization, also affecting the patients social and economical status [11], [13]. Life of patient is large modified, affecting your social, economic, medical and familiar routine [14].

Multiple factors are involved in development of foot ulcers and gangrene, being a complex disorder manly promoted by ischemia and diabetic foot ulcer infections [13].

1.2.1. Pathophysiology of diabetic foot ulcers

DFU development is caused by a large range of predisposing factors that alter the normal foot, promoting severe infections that lead to amputations. Without proper treatment foot amputations occurs 15 times more frequently in diabetic patient that in people without this chronic disorder [14], [15].

Firstly, the chronic hyperglycemic stage induces several changes in many systems [13], [14]. The biophysical and biochemical changes lead to development of neuropathy and angiopathy, known

as peripheral vascular disease (PVD), which associated with trauma and deformity of the foot lesion conduces to the development of an ulcer. Later, due to aggravating factors, such as infection, ischemia and neuropathy, lesion extension or necrosis may be observed (Figure 1), leading to the lower limb amputation [13]. Neuropathy, peripheral vascular disease and ulceration are pathologic conditions observed in DFU patients with a prevalence of 23-24%, 9-23% and 5-7%, respectively [14].



Figure 1 - Foot ulcer with large infection [11]. (Adapted from: S. Mazen and M. D. Bader, "Diabetic Foot Infection": American Family Physician).

Neuropathy is the main factor responsible for the progress and evolution of diabetic foot ulcer [13]. All nerves, including motor, sensory and autonomic nervous system are affected particularly in men and patients with more than 60 years of age [14], [15]. The somatic neuropathy, that includes motor and sensitive neuropathies, is related to the loss of sensibility, perception of pressure and temperature and proprioception [14]. Due to this, the protective sensations of injury or trauma are camouflaged or diminished, which can result in foot deformity that leads, in association with other factors, to the development of ulceration [11], [14]. Deformities due to mechanic lesions normally occur in pressure areas, like toes [13], [14].

Automatic neuropathy is responsible for the reduction or total absence of sweat secretion, causing dry skin and fissures [13]. Furthermore, there is an increase in blood flow that causes obstructive arterial disease and a decrease of the perfusion of the capillary network, increased skin temperature and decreasing thermoregulatory capacity, leading to foot hypervascularization [13], [14]. This can result in the Charcot neuroarthropathy, one of the worst consequences of diabetic foot, characterized by the bone and joint destruction and bone remodeling [13], [16].

Many factors conduce to ulceration. Trauma is the main factor, and it can be thermal, mechanical or chemical [13], being promoted by extrinsic and intrinsic factors. The most common factors are extrinsic mechanical traumas, due to loss of protection sensibility, since the trauma caused walking not noticeable, resulting in foot deformities [17].

Intrinsic traumas are associated with malformations related to infections affecting the bones, normally toes, metatarsal heads and calcaneus, leading to osteomyelitis [13], [17].

Ischemia due to atherosclerosis in diabetic patients is observed in 40 to 50% of the cases, normally associated with neuropathy resulting in a red and dry foot [13], [15]. Concerning DFU, 45 to 60% of the ulcers are caused only by neuropathic disorders, approximated 10% are caused only by ischemia and 25 to 45% are promoted by both neuropathic and ischemia disorders [14].

1.2.2. DFU bacteriology

DFU infections occur when the layer of skin is broken and the deep tissues are exposed, allowing the colonization by microorganisms. Then, an infection develops, in which the tissue invasion by the pathogens trigger multiples responses by human organism, such as an inflammatory response with the presence of the typical local signs and purulent secretion together with systemic clinical manifestations [13].

The development of a bacterial infection in DFU depends on the type of pathogen specific characteristics, including if the patient has been subjected to antimicrobial therapy, severity of the infection and also if there are geographic and temporal variations [13]. The classification of infection is based on the severity of infection, which determines the prognosis and the therapeutic strategy [13], [18].

In 2003, the International Working Group on the Diabetic Foot proposed a classification, named PEDIS, which gives a strong importance to the depth of the lesion. According to this classification, the diabetic foot infections can be classified in four subgroups concerning the level of perfusion, sensation loss and infection. In parallel, they can also be classified as uninfected or with mild, moderate or severe infections (Table 1) [13], [19].

Table 1 - PEDIS classification proposed by the International Working Group on the Diabetic Foot [13].

Grade	Severity of infection	Clinical manifestations
1	Uninfected	Absence of signs of purulence or inflammation
2	Mild	Presence of pus, erythema and cellulitis around the ulcer; infection is limited to the skin/subcutaneous tissues
3	Moderate	Infection without no systemic signs; deep tissue abscess, gangrene, involvement of muscle, tendon, joint or bone tissues
4	Severe	Systemic toxicity and/or metabolic instability; fever, chills, vomiting, tachycardia, confusion, leukocytosis, acidosis and severe hyperglycemia

The microorganisms present in DFU vary in the different levels of infection. Generally, mild infections are caused by a single microbial species, being monomicrobial, while moderate and severe, long-term infections are considered polymicrobial [20].

In superficial infections, the first microorganisms to colonize the ulcer are usually aerobic facultative gram-positive bacteria like *Staphylococcus aureus* (*S. aureus*) and group A, B, C and G beta-hemolytic streptococci, being the most common group of bacteria found in the surface of untreated wounds in diabetic foot patients [11], [13].

In moderate and severe infections, ulcers are generally colonized by multiple microorganisms besides staphylococci and streptococci, including *Pseudomonas aeruginosa* (*P. aeruginosa*), *Enterococcus* spp. and *Corynebacterium* spp. [13]. Anaerobic pathogens are also present in mixed infections that occur in ulcers or gangrenes, such as *Prevotella* spp. or *Porphyromonas* spp. [11], [13].

In fact, anaerobic bacteria and enterobacteria are the most common pathogens found in patients with foot ischemia or gangrene. In the case of osteomyelitis, related bacteria are analogous to those found in chronic ulcers, with a high incidence of methicillin – susceptible and –resistant *S. aureus* (MSSA and MRSA) and a low presence of anaerobic bacteria [13].

Antibiotic treatment, duration of therapeutic protocol, regularity and duration of hospital admission increase the complexity of the microbiota found in DFU. MRSA is the most frequent pathogenic microorganism found, being present in almost 50% of the mild infections and in 30% of moderate infections. Moreover, aerobic gram-positive and gram-negative and anaerobic organisms are also present in polymicrobial infections [13], [21]. Usually, the presence of infection by MRSA is a significant indicative of the future amputation of the limb [11], [13].

1.3. *Staphylococcus aureus*

The genus *Staphylococcus* was described for the first time, in 1880, by Sir Alexander Ogston, in samples of purulent discharge from a surgical abscess from a knee joint. Afterwards, in 1884, Anton J. Rosenbach isolated two staphylococci and the differentiation based in the pigmented colonies, gave origin to the name *Staphylococcus aureus* [22], [23].

Taxonomically, the genus belongs to the Staphylococcaceae family, and includes 52 species and 28 subspecies documented to date [24].

S. aureus is an ubiquitous microorganism and the most important pathogenic specie from the genus due to its virulence ability and the capacity to resist to adverse environmental conditions [25]. This bacteria can be found in the environment and in several animal species, including humans, its main reservatory. This species can be frequently found in skin, upper respiratory tract, intestines, perineum and vagina [22], [25].

Being part of the human commensal microbiota, under certain circumstances, *S. aureus* can be responsible for opportunist infections that can develop to chronic, systemic or fatal infections [22].

Patients with DM, with renal disorders and dialysis, with Human Immunodeficiency Virus (HIV), burn wounds, skin lesions and with intravenous catheter are more likely to get an infection by *S. aureus* [22], [25].

1.3.1. General characteristics

S. aureus are Gram-positive cocci, with diameters of 0,5 - 1,5 μm , that can occur singly or form pairs and non-motile clusters and they do not form spores [26].

The microorganism is aerobic facultative, catalase-positive and oxidase-negative, which allows its differentiation from bacteria belonging to the genus *Streptococcus*. Also, it produces coagulase, can resist to high concentration of salt and to adverse environmental conditions including high temperatures [25], [26].

1.3.2. Pathogenic determinants

S. aureus virulence influences its capacity of colonization and pathogenicity, being essential for cellular adhesion, and evasion of the immune host response [25]. These strategies are controlled by several environmental factors that include cell density, nutrients availability and environmental signs, by a mechanism called Quorum-sensing (QS) [27].

The virulence factors, essential for infection, produced by *S. aureus* include a large variety of exoenzymes, exotoxins and β -lactamases, which contribute to their multidrug resistance ability. Some strains are also able to form biofilms, also related with antimicrobial resistance ability and capacity to evade the host immune system [22], [25].

1.3.2.1. Cell wall structures

The Gram-positive cell wall is constituted by peptidoglycan, teichoic acid and proteins [28]. This structure is important for the viability of bacteria, as it allows a mechanic protection against cell rupture and contributes for bacteria pathogenicity [29]. *S. aureus* cell wall structures related with virulence include its capsule, teichoic acids, adhesins and protein A.

The capsule, exopolysaccharide constituted by hexosaminuronic acids, is produced by more than 90% of the strains. It is a structural polysaccharide that involves the cell wall, protecting the pathogen against phagocytosis by polymorphonuclear neutrophils, allowing bacterial tissue invasion and dissemination through the blood flow [25], [30]. This exopolysaccharide also helps the bacteria to

adhere to the host cells or catheters [31]. Eleven capsular types occur in *S. aureus*, being types five and eight related with diseases caused by this microorganism [31].

Teichoic acids are a glycopolymer that participates in complement activation, inhibition of chemotaxis and stimulation of antibody production [22], [32].

Adhesins are present in the microorganism surface, being integrated in the structure of bacterial capsule. They are responsible for the colonization of the host tissues by linking to the chemical receptors of cell host, promoting adhesion and promoting infection development [25], [33].

Finally, protein A is responsible for inhibiting *S. aureus* opsonization and phagocytosis by neutrophils and also for complement activation. This protein impairs the action of the immunoglobulin G (IgG), through the bounding of IgG to the Fc region. This protein is a highly important molecule for pathogenicity of this microorganism, being present in most *S. aureus* pathogenic strains [22], [34].

1.3.2.2. Exoenzymes

S. aureus is able to produce a diversity of extracellular enzymes including coagulase, catalase, deoxyribonuclease (DNase), lipases, proteases and hyaluronidases [35].

Coagulase is an important virulence factor, codified by the gene *coa*. It is responsible for conversion of fibrinogen in fibrin, independently of the presence of blood coagulation factors. It forms a staphylotrombin complex that consists in the binding of the protein to prothrombin. The fibrin is then deposited around the microorganism cell, acting as a protective layer regarding phagocytosis promoting the development of infection [22], [36]. In the laboratory identification of *S. aureus*, the detection of coagulase allowing the identification of *S. aureus* and its differentiation from coagulase-negative species [37].

Catalase consists of a heme protein enzyme that converts the toxic molecules of hydrogen peroxide in oxygen and water molecules, when occurs the phagocyte ingestion of bacterial cells [22], [38].

DNase is a nuclease, an enzyme responsible for the hydrolytic cleavage of the phosphodiester linkages in the DNA, promoting its degradation and helping the infection development [39].

Almost all strains of *S. aureus* are lipolytic. The lipase promotes the hydrolysis of the lipids, converting them into fatty acids and glycerol, facility the bacterial attack through the skin and subcutaneous tissue [30].

Proteases have the ability to cleavage proteins in to amino acids that can be recycled for the synthesis of new proteins. Therefore, proteases have an indirect pathogenic function by providing nutrients for the bacteria [40].

Finally, hyaluronidase is an exoenzyme that cleaves the hyaluronic acid, resulting in unsaturated disaccharides. Acid hyaluronic is the main compound present in the extracellular matrix of human tissues, and its digestion allows the propagation of microorganisms on tissues and infection development [41].

1.3.2.3. Exotoxins

S. aureus produces a several cytotoxins including hemolysins, the Panton-Valentine leucocidin (PVL), enterotoxins and the several toxic shock syndrome toxins, such as toxic shock syndrome toxin-1 (TSST-1) [42].

Hemolysins secreted include alpha (α), beta (β), gama (γ) and delta (δ) toxins. The α -toxin induces the lysis of a large variety of cells, mainly platelets and monocytes. The β -toxin is related with the membrane-damaging of the erythrocytes and γ -hemolysin induces the lysis of enterocytes and leukocytes by a mechanism not truly understood [43].

PVL consists in a two-component cytotoxin (S and F) acting synergistically. This virulent factor induces the lysis of leukocytes and of macrophages by changing the membrane permeability, allowing the entry of calcium and resulting in cell degranulation and tissue necrosis [44].

Staphylococcus enterotoxins are pyrogenic and thermostable exotoxins. This genus produces more than twenty different enterotoxins that can be divided in to eight serological categories: A, B, C, D, E, G, H and I. They are associated with food poisoning, being able to resist to the action of gastric enzymes. Their action mechanism is not fully understood, but they seem to promote the activation of T lymphocytes and antibody presenting cells [22], [45].

TSST-1 is responsible for the toxic shock syndrome, described by Todd and his associates in 1978 as a multisystem disease that leads to failure of several organs. This bacterial toxin is responsible for the massive activation of non-specific T-cell, that acting together with class II major histocompatibility complex molecules, cause a cascade of cytokines release that promote several injuries in major human organs [30], [46].

1.3.2.4. Biofilm and quorum-sensing

Microbial biofilms are essential for *S. aureus* and other microorganisms survival in rigorous environments. Biofilms consist in complex and structured communities of microorganisms adhered to abiotic or biotic surfaces, being enclosed by a matrix of organic polymers [47].

The biofilm is established when the cellular mass cell multiplication is sufficient to aggregate nutrients, residues and other microorganisms, presenting different phenotypes concerning growing yield and gene transcription [48]. The sessile mode of life offers multiple advantages compared to planktonic or free living bacterial cells [49].

Biofilms can be mono or polymicrobial, with diverse interactions and mechanisms [50].

Production and development *Staphylococci* biofilms occurs in sequential steps coordinated by several molecular events, beginning with the colonization or attachment of surfaces followed by bacterial biofilm accumulation, maturation, with a final detachment step (Figure 2) [51].

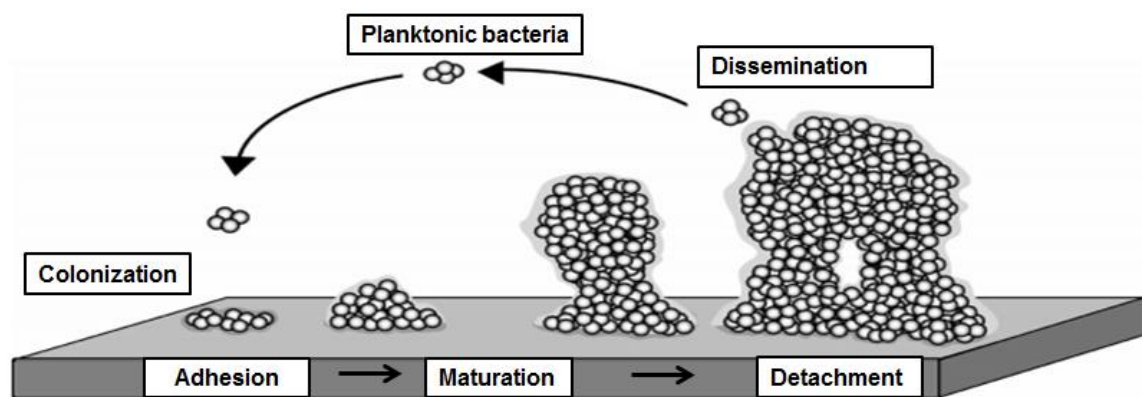


Figure 2 - Biofilm production by *S. aureus* [54] (Original).

The initial step of biofilm formation is the adherence and colonization of surfaces by a low number of planktonic cells [52]. Surface interactions are specific for organic surfaces and non-specific for non-organic surfaces being always a reversible step [52].

Next steps consist in irreversible attachment and the multiplication of bacteria cells and production of the extracellular exopolysaccharide matrix. During multiplication, extracellular polymeric matrix is essential for the development of the biofilm, since it provides a framework for the cells and further promotes the adherence to surfaces. Due to its viscoelastic proprieties, the extracellular exopolysaccharides matrix has intrinsic mechanical stability [53], [54].

Maturation phase is characterized by bacterial growing forming the typical architecture. At this phase, the extracellular polymeric substances are stable and several connections occur between bacterial cells. Besides the biopolysaccharides, this matrix consists of a complex mixture proteins, nucleic acids, peptidoglycan, acid teichoic and lipids [55]. For the biofilm to become mature several molecules are required, including a specific antigen named polysaccharide intercellular antigen, extracellular DNA and a two-component regulator gene, that include a staphylococcus accessory regulator and accessory gene regulator, encoding for a major global regulators implicated in biofilm formation [55], [56].

The last step, detachment, consists in the dispersal of single bacteria or cell clusters that develop the planktonic phenotype and leave the biofilm. This process is related with the capacity of the bacteria to perceive the environmental changes, such as alterations in nutrient availability, oxygen oscillations and increase of toxic products in the environmental [57].

Microbial biofilms structure and evolution depend on several environmental characteristics, specially in biofilms formed by multispecies, such as nutrient availability, cyclic stage, attachment efficiency, physiochemical environmental or/and genotypic elements present in biotic bacteria [51], [58].

Polymicrobial biofilm are extremely relevant in DFU since they play an important role in infection persistence in the chronic wound. In these infections, biofilm allow bacteria to evade the host immune

system, being also very resistant to antimicrobial therapy when compared with the corresponding planktonic bacteria [59].

Quorum-sensing (QS) is defined as a chemical communication through signal molecules (pheromones and autoinducers), activated by the increase in bacteria population, that permits the microorganisms to rule gene expression and to respond collectively to environmental changes [60].

S. aureus has two QS systems. These are the accessory gene regulator (*agr*) and the protein S-ribosylhomocysteinylase (*luxS*), which is found in several Gram-positive and Gram-negative bacteria [61]. The *agr* locus consists of two divergent operons, located on the *S. aureus* chromosome, and is considered to be part of the core genome [62]. In this specie, *agr* is related with the expression of several virulence factors, linked to the control of innate immunity and also playing a significant role in biofilm formation [63].

1.3.2.5. Antimicrobial resistance

Antimicrobials began to be used in 1930 and, soon after, bacteria began to show resistance to their action. In 1959, 80% of the *S. aureus* strains that were able to produce β -lactamase were resistant to β -lactamases, such as penicillinases. These are enzymes that hydrolyze the β -lactam ring of β -lactams antibiotics molecule, inactivating its [25], [64].

Methicillin, a semi-synthetic antibiotic derived from penicillin, was introduced in clinical practice in 1959. However, in 1961, methicillin-resistant *S. aureus* (MRSA) strains were report [42].

In the case the methicillin resistance, the resistance ability is related with the acquisition of a huge transmissible element entitled *Staphylococcus* cassette chromosome *mec* (*SCCmec*), which carries two essential components: the *mec* gene complex, including the *mecA* gene, and the *ccr* gene complex [42]. The *mecA* gene encodes for a transpeptidase, an altered Penicillin Binding Protein (PBP) called PBP2a, which is directly responsible for resistance to methicillin and other β -lactam antibiotics, due to a low affinity of the antibiotic to the bacteria cell wall. The *ccr* gene complex encodes for recombinases responsible for the mobility of *SCCmec* [42], [65].

Eleven different types of the *SCCmec* were already described, classified in types I to XI according to the characteristics of both the *mec* gene complex and the *ccr* gene [42], [66].

The first MRSA isolated belonged to *SCCmec* type I. This strain was found in several hospitals in Europe. For a long period, MRSA infections were confined to hospitalized patients but in the last decade cases of community-associated MRSA infections in healthy individuals were reported [42]. Nosocomial strains usually belong to types I, II and III, producing resistance a large variety of antibiotics, while types IV, V and VI are associated to community acquired strains, being generally resistant only to β -lactam antibiotics [42], [66].

1.4. Antimicrobial peptides

Antimicrobial peptides (AMP) are oligopeptides produced by all living organisms, including animals, plants, bacteria and other. They play an important role as the first line of defense in the innate defense system, impairing the development of most infections [67].

Gramicidin was the first AMP extracted and identified, by Hotchkiss and Dubos in 1940. This AMP was extracted from a *Bacillus* strain from the soil, having a protective role against pneumococcal infections. Researchers also demonstrated that this peptide had an effective role healing in wounds and ulcers. Afterwards, many others AMP were discovered, produced by organisms from all Kingdoms. Today, more than 5 000 AMP have been described, including natural and synthetic AMP [67], [68].

AMP can be classified based on their amino acids composition (anionic and cationic), based on their secondary structure, their target or mechanism of action [69], [70]. Their spectrum activity includes a large variety of pathogenic microorganisms like bacteria, fungi, virus and parasites. The most studied AMP are antibacterial peptides, which are cationic and amphipathic AMP that act by promoting the disintegration of the lipid bilayer of the bacterial cell membranes by simple electrostatic interactions [71].

Antimicrobial peptides have many intrinsic properties which support their future therapeutic application. Plus the large spectrum of activity against several pathogens and even against cancer cells make them an excellent source against diverse infections. AMP are relatively small molecules, easy to synthesize, extract and with a fast action mechanism. Studies have reported that these molecules have an excellent antimicrobial activity against planktonic cells and against mature biofilms. AMP have also been demonstrated to have a low or non-toxic effect on the human body. Therefore, the Food and Drug Administration (FDA) have approved many AMP for clinical use or for other applications, such as food preservation. One example of these AMP is nisin [72], [73].

1.4.1. Nisin

Nisin is a bacteriocin described, in 1928, by Rogers, in England. This polypeptide is produced by the lactic acid bacteria *Lactococcus lactis* subsp. *lactis*. It consists in a lantibiotic composed 34 amino acids and it has a molecular mass of 3354 KDa [74].

The antimicrobial peptide nisin has a large activity spectrum against by several Gram-positive bacteria and also against spore germination [75]. In 1969, nisin was recognized as a safe and legal biological food preservative by the Food and Agriculture Organization of the United Nations (FAO) and the WHO. In 1988, FDA approved the use of nisin as a safe food antimicrobial agent, as it presented low toxicity in animal models [75].

1.4.1.1. General characteristics

This cationic lantibiotic belongs to the class I bacteriocins. Being heat stable, acid tolerant, soluble in acid solutions, odorless, colorless, tasteless, with low toxic, and not promoting nisin resistance dissemination [76].

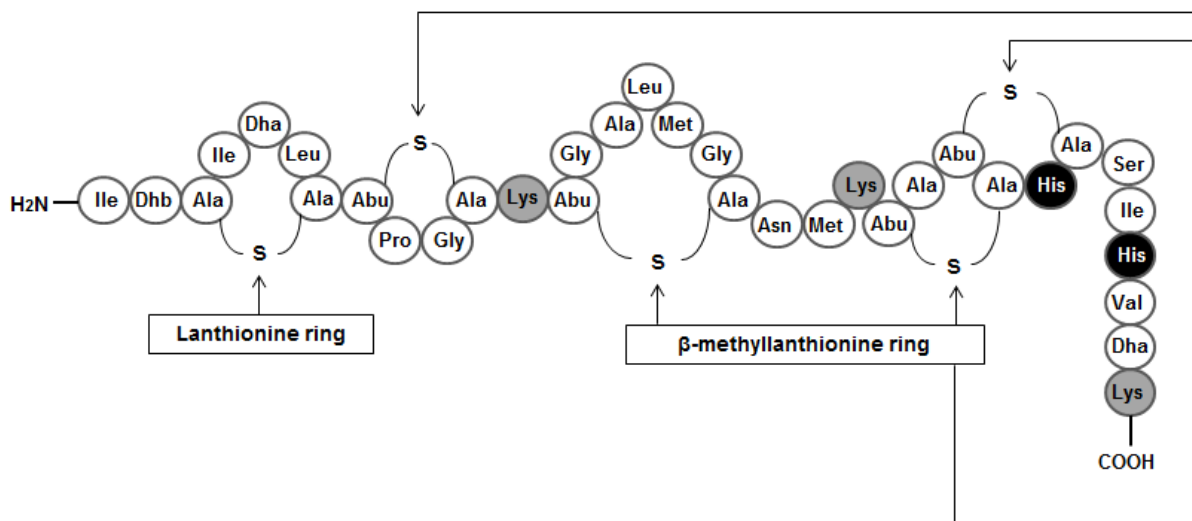


Figure 3 - Nisin 5-ring structure composed by 34 amino acids [77] (Original).

Nisin does not contain aromatic amino acids [74]. The polypeptide can resist to the action of digestive enzymes, such as trypsin and pronase, although being susceptible to chymotrypsin [75].

Natural nisin can occur in different forms, including nisin A, Z, F, Q, U and U2. The first four types (A, Z, F and Q) are produced by *Lactococcus lactis*, while the U and U2 types are produced by *Streptococcus* sp.. However, only nisin A and Z have been properly studied, and nisin A extensively used in food industry [75].

Structure of nisin A and Z are similar. The difference between these types consists only in an alteration of one of the amino acid in the position 27. In this position, the nisin A has a histidine while the other variant, nisin Z, has an asparagine. This structural difference has no effect in their antimicrobial activity, but changes the physical characteristics the solubility and diffusion levels [78].

All variants of nisin present unusual amino acids in their structures including dehydroalanine (Dha), dehydrobutyrine (Dhb), aminobutyric acid (Aba), lanthionine (Ala-S-Ala) and β -methyllanthionine (Abu-S-Ala), and have one lanthionine ring and four β -methyllanthionine rings [75]. This uncommon composition has been related to the molecules essential functional properties of nisin [79].

Nisin is produced after post translational processing of a ribosomal synthesized precursor, on the opposite of other antimicrobials peptides, which are synthesized by complex reactions enzymatic [77]. Lantibiotic nisin is formed as prenisin, which has a leader peptide region and a changeable core peptide sequence with 57 amino acids, which suffers enzymatic modifications [75].

Nisin synthesis occurs in three steps, namely dehydration and cyclization reaction to proceed from the N-terminal to C-terminal direction, and leader peptide digestion. In the step of dehydration, the amino acids serine and threonine present in the prenisin core region are converted through dehydrated to dehydroalanine and dehydrobutyrine, respectively. In the next step, a cyclization reaction occurs with the formation of several thioether bridges between the dehydrated amino acids and the cysteines. The last step consists in a modification of the precursor nisin, in which the prenisin is exported to outside the bacteria and the leader peptide is cleaved off by an extracellular protease, resulting in the active nisin polypeptide with 34 amino acids, liberated as mature nisin [75], [80] .

1.4.1.2. Antimicrobial mechanisms

The antimicrobial nisin does not require a membrane receptor on the Gram-positive bacteria surface as its antimicrobial action occurs through interactions with membrane cells [75].

This lantibiotic is not effective against Gram-negative bacteria and fungi. In these cases the antimicrobial activity is successful when nisin is associated with the other components, such as EDTA [77].

Nisin can inhibit spore formation by Gram-positive bacteria by impairing the occurrence of a link between the Dha residues at the position 5 of nisin and the membrane sulfhydryl groups, essential for many enzymatic reactions responsible for spore germination, preventing its occurrence [81], [82].

The AMP nisin acts against vegetative cells of Gram-positive bacteria through two mechanisms [75]. First, nisin generally interacts with the cytoplasmic membrane. The cationic peptide interacts with the anionic compounds of membrane, such as teichoic acids or phospholipids, occurring a linkage and the consequent pore formation, which allows the passage of small cell compounds and the interruption of transport systems responsible for production of ATP, ultimately promoting cell lysis [77], [81].

On another hand, nisin can promote cell death by pausing the cell wall biosynthesis by interacting with lipid II [77]. The cell wall precursor lipid II is used by nisin as a docking molecule for pore formation, which stays stable due to the linkage of nisin, resulting not only at an increased antimicrobial activity, but at the same time the peptidoglycan is not formed. The N-terminal of peptide nisin binds to the lipid II, while the C-terminal segment is incorporated into the cytoplasmic membrane. There is no biosynthesis of the cell wall together, which together with the pore formation in the cell membrane promotes the increase the membrane permeability, resulting in dissipation of the membrane potential, no production of energy, cells damage and death [83].

1.4.1.3. Applications

Nisin is the only bacteriocin that is widely applied in the food industry, being used as food additive in at least 46 countries [77].

Its antimicrobial properties support its potential as an alternative for antimicrobial therapeutics, necessary in view of the increase in bacterial resistance to traditional antibiotics [84].

Bacteriocins are possible alternative therapeutic for nosocomial infections especially against the most prevalent organisms responsible for skin infection, *S. aureus* and MRSA [85].

Nisin can be a good solution against infections by this bacterium specie [85]. The antimicrobial activity of this peptide was already demonstrated including the application of the natural variant nisin F in the control of *S. aureus* infections in rats. Nisin was also demonstrated to increase the antimicrobial activity of non- β -lactam antibiotics against many MRSA strains [86].

The antimicrobial activity the lantibiotic nisin against a large variety of bacteria was confirmed. This molecule has several physical-chemical properties, including heat stability, solubility, diffusion and protease resistance that render it a potential alternative for therapeutics against bacterial infections [86], [87].

Nisin (E234) is a bacteriocin approved for application in food preservation in the European Union by Directive 95/2/EC, being used as a food additive in cheese, certain sweeteners and desserts. Earlier in 1969, nisin was recognized in the United States as a food preservative by FAO/WHO, and classified as Generally Recognized as Safe (GRAS). It was applied as a food additive in canned products in 1988, to inhibit the growth of *Clostridium botulinum* [88], [89].

In European Union, nisin can be used as starter lactic acid bacteria cultures or added directly to the food product. Lantibiotic nisin has no effect on the gut microbiota and the European Food Safety Authority (EFSA) has established an Acceptable Daily Intake (ADI) of 0.13 mg nisin/kg [88].

In the United States, the Joint Expert Committee on Food Additives recommends a limit for the daily intake of pure nisin of 60 mg for a 70 kg person, while FDA recommends a maximum limit of 10 000 IU/g [89].

1.5. Guar Gum

In spite of nisin antimicrobial properties, their application to the treatment of infected diabetic foot ulcers requires the development of an effective delivery system. Guar gum has been tested as a potential AMP delivery system [90].

Guar gum, also called Guaran, is a galactomannan, a natural polysaccharide, derived from the seeds of the plant *Cyamopsis tetragonoloba*, a member of the *Leguminosae* family [90].

Guar seeds are obtained from the leguminous crop, which have been cultivated for centuries in arid zones with an optimal soil temperature between 25 to 30 °C, mainly in India and Pakistan, where it represents an important food source for humans and animals. The USA, Sudan, China, Australia, South Africa and Brazil also produce guar gum, but in less quantities. India is the leader of guar gum production, 80% of the total guar produced worldwide, followed by Pakistan [90], [91].

1.5.1. General characteristics

The interest of natural polysaccharides, such as guar gum, is emerging due to their potential for application in diverse areas because of its versatile properties and intrinsic characteristics, such as safety, non-toxicity, biodegradability, biocompatibility, low price and easy availability [90].

Guar gum is a hydrophilic polysaccharide derived from the endosperm of the seeds of a leguminous plant. The guar gum percentage in the different parts of the seeds varies as follows: 35 to 42% in the endosperm, 14 to 17% in the hull and 43 to 47% in the germ. As the latter parts of the seed are rich in protein, it is easier to extract the guar gum from the endosperm [92].

The endosperm of the *C. tetragonoloba* seeds consists mainly in galactomannan, which has a high molecular weight. It is composed by a linear chain of galactose and mannose residues, in an approximated proportion of 1:2 [90].

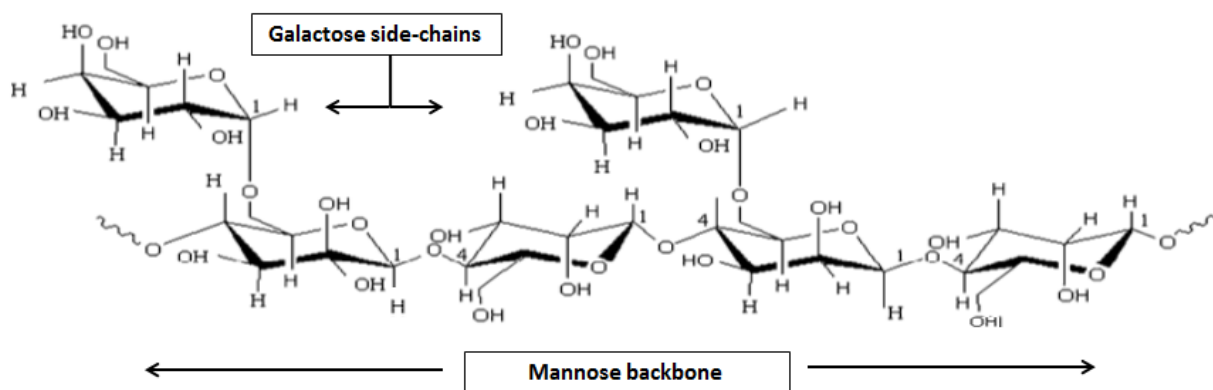


Figure 4 - Structure of guar gum [91]. (Adapted from: D. Mudgil, S. Barak and B. S. Khatkar, "Guar gum: processing, properties and food": Journal of Food Science and Technology).

Guaran is an uncharged molecule, composed essentially by galactomannan but also contains pentosane, phosphorus, protein, pectin, phytic acid, ash and insoluble acid residues. The natural gum structure contains numerous hydroxyl groups, which permit its transformation to create different

derivates that can be used in several industries. It does not contain uronic acid, which usually differentiates this polysaccharide from the great majority of other plant gums and mucilages [90].

Solubility and viscosity proprieties are the most important characteristics of guar gum. Galactomannans present in their composition are usually insoluble in organic solvents, with exception of formamide, but guar gum is soluble in water. Water rapidly hydrates the guar gum allowing it to form colloidal solutions with high viscosity [92]. However, the viscosity of guar gum depends on several factors like temperature, concentration, pH, dispersion, strength of hydrogenated bonds, presence of salt, type and time of agitation [91].

Temperature is the main factor that affects the rate of hydration and viscosity. A temperature range of 25 to 40°C is required for maximum viscosity, but with the increase of temperature the gelling property decreases, because the water molecules become less organized around the galactomannan molecules. Also, pH plays an important role in the gelling mechanism of guar gum. Guar gum solutions are viscous at a wide pH range of about 1.0 to 10.5. At other pH values, the viscosity and gelling stability are lost due to the destruction of the proteins. Finally, the presence of salt in solutions has a peculiar role in the gelling mechanisms, since the salt limits the complete hydration of the natural gum [91], [92].

1.5.2. Applications

The distinctive physical-chemical proprieties of guar gum, mainly the presence of a long chain molecular structure and the abundance of hydroxyl groups in the galactomannan molecule, turn this natural gum a strong candidate to be used as excipient in diverse industries, such as food, cosmetics and pharmaceutical industry, being considered a good drug delivery system for human medicine [92].

The low cost of production and extraction, allied to its non-toxic and biodegradable nature, contribute to the increased interest of researchers in this molecule [90].

Guar gum and its derivatives hydration proprieties make them good substrates to be used in drug incorporation, allowing the control of the drug release and also can be used for inhibiting disorders [92].

Guar gum polysaccharide traits, namely its flexibility to obtain gels with variable viscosity, swelling and film forming capacity, allows its use as colon-specific, antihypertensive, transdermal or oral drug delivery system. Guar gum has also an inherent resistance to low pH, intrinsic gelling properties that allow the control of drug release, intrinsic resistance to the enzymatic activity and microbial degradation in the stomach, allied to its low cytotoxicity allow the guar gum to be used in medical and pharmaceutical industries [90].

Besides being used as a transporter or support material for drugs delivery, natural guaran is a water-soluble fibre that acts as laxative, due to promoting regular bowel movements, and so it can be useful in many diseases like cholera and diarrhea. It also decreases the appetite, since the ingestion

of guar gum creates a bulk forming that increases satisfaction and decreases the desire for eating due to the slow gastric evacuating, allowing its use in obesity prevention [89].

Studies have also revealed that glycosylated guar gum and its sulphated derivate have potential to be applied in prevention of cancer by inhibiting carcinogenic activating enzymes and activating the carcinogen detoxification enzyme glutathione-S-transferase. In fact, its potential in colorectal cancer prevention has been demonstrated [92].

In chronic Diabetes, guar gum can play a role in controlling the blood sugar level. Several studies have been revealed that guar gum, in its natural form or as a hydrolysate, reduces the rise in blood glucose and insulin concentrations after meals. Other studies described guar gum as a useful agent for long treatments of hypercholesterolemia, decreasing the plasma cholesterol levels [93].

As already mentioned, guar gum is a non-toxic natural polysaccharide, biodegradable and eco-friendly. Uncharged natural gum has a low digestibility, which helps to provide hunger and to decrease the glycaemia after meals, being useful in the prevention of DM. In other hand, guar gum can be used as drug delivery system, helping in the therapeutics and treatment of several diseases [93].

Potential applications guar gum in the industry is diverse, with more than 300 applications, in the food industry, agriculture, paper industry, cosmetics, explosives and also in the medical and pharmaceutical industry. FDA regulates the use of gums and has classified the guar gum as GRAS, and has also defined the highest concentrations allowed in many food applications [93], [94].

1.6. Objectives

The main objective of this study was the evaluation of the guar gum gel as a delivery system for the antimicrobial peptide nisin to be applied to the treatment of infected diabetic foot ulcers, using 23 *S. aureus* isolates obtained and characterized in previous studies. This evaluation included:

- Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) of nisin diluted in hydrochloric acid (HCl) and incorporate in guar gum gel against the 23 staphylococci isolates;
- Determination of the Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) of nisin diluted in HCl and incorporated in guar gum gel against biofilms production strains;
- Evaluate the potential of the guar gum gel as a delivery system for AMP for the treatment of infected diabetic foot ulcer;
- Evaluate the inhibitory activity of nisin diluted in guar gum gel over a 6 month period, at different conservation temperatures.

Chapter 2 | Material and Methods

2.1. Bacterial strains

In 2010, between January and July, an epidemiological study conducted in Lisbon analyzed the microbiota of infected diabetic foot ulcers (DFU) in patients with Diabetes *mellitus* (DM). From 49 DFU patients it was possible to collect a total of 54 *Staphylococcus* spp. clinical isolates [95]. All clinical isolates were analyzed based on Pulse Field Gel Electrophoresis (PFGE), which allowed to select a collection of twenty-three (n=23) representative *S. aureus* isolates for further research. Isolates were also characterized regarding their virulence profile, antimicrobial resistance traits and biofilm production ability [96], [97]. These isolates were kept at -80°C, in BPW (buffered peptone water) plus 20 % of glycerol in cryopreservation tubes [96], and used in this study.

The reference strain *S. aureus* ATCC®29213™, was also included in this study as a control strain, being a known biofilm producer.

Table 2 - Phenotypic characterization of the virulence and antimicrobial resistance profile of the 23 staphylococci DFU clinical isolates under study [96], [97].

Isolates code	Collection method	Coagulase	Gelatinase	Biofilm Production	Antimicrobial resistance
A 1.1	Aspirate	+	-	+	Fox, Cip
A 5.2	Aspirate	+	-	+	Cip, Cpt
A 6.3	Aspirate	+	-	+	
B 3.2	Biopsy	+	-	+	
B 3.3	Biopsy	+	-	+	
B 7.3	Biopsy	+	-	+	Fox, Cip, Ery
B 13.1	Biopsy	+	-	+	Fox, Cip, Mem, Ery, Cli, Gen
B 14.2	Biopsy	+	-	+	Fox, Cip, Cpt, Mem
B 23.2	Biopsy	+	-	+	
Z 1.1	Swab	+	-	+	Fox, Cip, Mem
Z 2.2	Swab	+	-	+	Cip, Ery
Z 3.1	Swab	+	-	+	
Z 5.2	Swab	+	-	+	
Z 14.1	Swab	+	-	+	Gen
Z 16.1	Swab	+	-	+	Fox, Cip, Ery
Z 16.2	Swab	+	-	+	
Z 17.2	Swab	+	-	+	
Z 21.1	Swab	+	-	+	Fox, Cip, Ery
Z 21.3	Swab	+	-	+	Fox, Cip, Ery

Z 25.2	Swab	+	-	+
Z 27.2	Swab	+	-	+
Z 27.3	Swab	+	-	+
Z 32.2	Swab	+	-	+

A: aspirate; B: biopsy; Z: swab; +: positive; -: negative; Fox: cefoxitin; Cip: ciprofloxacin; Mem: meropenem; Ery: erythromycin; Cpt: ceftaroline; Cli: clindamycin; Gen: gentamicin.

2.2. Nisin stock and working solutions

Nisin stock solution was prepared using a nisin powder (2.5 % purity, 1000 IU/mg, Sigma-Aldrich®) and hydrochloric acid (HCl) 0.02 M (Merck®). For preparing a nisin stock solution of 1000 µg/mL corresponding to 40 000 IU/mL, 1 g of nisin powder was dissolved in 25 mL of 0.02M HCl. Next, using a 0.22 µm Millipore filter (Frlabo®) the nisin stock solution was sterilized and kept at 4°C, until further use. Serial dilutions of nisin solution were prepared (900, 800, 700, 600, 500, 400, 300, 200, 100, 40, 20, 10 and 5 µg/mL) for following use.

2.3. Guar gum preparation and nisin incorporation

A guar gum gel of 1.5% (w/v) was prepared using guar gum powder (Sigma-Aldrich®) and sterile distilled water. 0.75 g of guar gum were dissolved in 50 mL of sterile distilled water and heat sterilized by autoclave. In a proportion of 1:1, the serial dilutions of nisin were integrated within the gel guar gum, obtaining final gel suspensions of 0.75% (w/v).

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) were determined using the microtiter broth dilution method [98].

Bacterial strains were grown in nonselective Brain Heart Infusion (BHI) agar medium (VWR Chemicals®) at 37°C for 24h. Subsequently, bacterial suspensions were prepared in sterile normal saline (Scharlau®) with approximately 10⁸ CFU/mL using a 0.5 McFarland standard reference. For the MIC and MBC assays, these bacterial suspensions were diluted in BHI broth, at a concentration of approximately 10⁷ CFU/mL.

Various suspensions of nisin were distributed in 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fischer Scientific®), at a volume of 25 µl in the case of nisin in HCl solution, and 50 µl when combined with guar gum gel. Concentrations range from 5 µg/mL to 1000 µg/mL, corresponding to 5 IU to 1000 IU per well, respectively. All wells were inoculated with 150 µl of the 10⁷ CFU/mL bacterial suspensions, except for the wells corresponding to the negative control, that contained only fresh broth medium. Microplates were statically incubated at 37°C for 24 h, and MIC was considered as the lowest concentration of nisin that visually inhibited the microbial growth [98].

Minimum Bactericidal Concentration (MBC) was determined by inoculating 3 µl of the suspensions from the wells where no visible growth was observed on BHI agar plates and incubated at 37°C for 24 h. MBC was considered as the lowest nisin concentration that impaired bacterial growth on to the agar plates.

Experiments were realized for all isolates, including the reference strain, and conducted in triplicate. Independent replicates were performed at least three times in different days.

2.5. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) determination

Minimum Biofilm Inhibitory Concentration (MBIC) was determined using a modified version of the Calgary Biofilm Pin Lid Device [99], applied to the determination of the antimicrobial susceptibility of biofilm bacteria to nisin diluted in HCl and incorporated in guar gum gel.

For broth MBIC and MBEC determinations, bacterial suspensions were prepared in sterile normal saline (Scharlau®) as described before, and diluted in Tryptic Soy Broth (TSB) (VWR Chemicals®) plus 0.25% (w/v) glucose (Merck®) to an approximated concentration of 10⁶ CFU/mL.

Next, except in the negative control well, 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fischer Scientific®) 200 µL of each bacterial suspensions were distributed per well, which contained only fresh TSB plus 0.25% (w/v) glucose. Plates were covered with 96-peg polystyrene lids (Nunc-TSP, Thermo Fisher Scientific®) and incubated at 37°C for 24 h, allowing biofilm formation on pegs. After washing the peg-lids three times in sterile normal saline (Scharlau®) to remove planktonic bacteria, the peg lids were transferred to new microplates. These new microplates contained where nisin suspensions in HCl solution or combined with guar gum gel, with concentration ranging from in 5 µg/mL to 1000 µg/mL, corresponding to 5 IU to 1000 IU per well, respectively. In the same wells were added of 200 µL of Tryptic Soy Broth (TSB) plus 0.25% (w/v) glucose (Merck®). After incubation, the peg lids were removed, and MBIC was considered as the lowest nisin concentration where no viable bacterial growth was visually observed.

Next, Minimum Biofilm Eradication Concentration (MBEC) was determined by directed observation of experimental wells. MBEC quantification was also conducted based in a previously described protocol using Alamar Blue, a redox indicator that yields a colorimetric change in response to metabolic activity [100].

Pegs lids were washed three times in sterile normal saline (Scharlau®) and putted in new microplates that contained only 200 µL of Tryptic Soy Broth (TSB) plus 0.25% (w/v) glucose (Merck®) medium. Afterwards, these plates were placed in an ultrasound bath (Grant MXB14®), at 50 Hz during 15 minutes, to separate the established bacterial biofilm from the peg surface. Next, the peg lids were rejected and the microplates were protected with lids without pegs and incubated at 37°C for 24 h.

After the incubation time, MBEC was read by direct observation of microplates and considered the lowest nisin concentration where no viable microbial growth occurred, and MBEC value quantification was conducted using resazurin (Alamar Blue, Thermo Fisher Scientific®). For this, 5 µL of resazurin were added in all 96 wells and microplates were incubated at 37°C for 1 h. Afterwards, absorbance (A) of each well was determined using a microplate reader (BMG LABTECH®) at 570 nm and 600 nm. MBEC value was defined as the lowest nisin concentration resulting in ≤ 50% of Alamar Blue reduction.

Percent of Alamar Blue reduction was calculated the according to equation 1, where ϵ_{ox} = molar extinction coefficient of Alamar Blue oxidized form ($\epsilon_{ox\lambda_1} = 80.586$ and $\epsilon_{ox\lambda_2} = 117.216$), ϵ_{red} = molar extinction coefficient of Alamar Blue reduced form ($\epsilon_{red\lambda_1} = 155.677$ and $\epsilon_{red\lambda_2} = 14.652$), A = absorbance of test wells, A' = absorbance of negative control well, $\lambda_1 = 570$ nm and $\lambda_2 = 600$ nm [99].

$$\frac{(\epsilon_{ox})\lambda_2 A\lambda_1 - (\epsilon_{ox})\lambda_1 A\lambda_2}{(\epsilon_{red})\lambda_1 A'\lambda_2 - (\epsilon_{red})\lambda_2 A'\lambda_1} \times 100 \quad (1)$$

Experiments were performed for all isolates, including the reference strain, and conducted in triplicate. Independent replicates were performed at least three times at different days.

2.6. Guar gum viability assay

To estimate the effect of storage period and temperature on the inhibitory effect of nisin diluted in guar gum, the supplemented gel was stored at five different temperatures, namely -18, 4, 20, 37 and 44°C, during six months. After 1, 3 and 6 months incubation, its inhibitory ability was tested against two *S. aureus* isolates. At each time, a 3 µL drop of the nisin incorporated guar gum gel was placed on BHI agar plates containing a lawn formed by a culture of the isolate with a approximated 10^7 CFU/mL. BHI agar plates were incubated at 37°C for 24 h, and inhibition halos diameters were measured. Assays were performed in triplicate.

2.7. Statistical Analysis

Statistical analysis was performed using the STATISTICA Data Miner Software, (StaSoft R version 13). Wilcoxon Matched Pairs Tests was applied to determine the significance of the variables under study and a two-tailed p -value < 0.05 was considered to be statistically significant.

Quantitative variables, related with triplicate experiments, were expressed as means \pm standard derivation.

Chapter 3 | Results and Discussion

DFU are common in *Diabetes mellitus* patients. Many factors are involved in their pathophysiology, such as neuropathy, peripheral vascular disease and abnormal foot biomechanics [10], [13]. Infections in DFU patients occur after the colonization of a traumatic injured member by diverse pathogenic bacteria, mainly *S. aureus* [21]. No control of the infections and no adequate treatment may promote several devastating consequences, such as limb amputation, sepsis and even dead [13], [17].

S. aureus is an important human pathogen, responsible for a wide range of both community and nosocomial infections, and recognized as one of the most common bacterial species to be isolated from skin and soft tissue infections, such as DFU [25], [30]. Among *S. aureus* strains, MRSA have been reported as a major cause of antimicrobial resistant related infections worldwide, including in patients with recent history of hospitalization and prolonged therapy. Community cases have also been reported worldwide [42]. According to the European Center for Disease Prevention and Control, Portugal is one of the European countries presenting higher rates of MRSA incidence [101].

Antimicrobial resistant bacteria represent huge clinical, economic and social problems, not only for patients, but for all population, and new therapy must be founded for decrease these problems [21].

In recent years, AMP have been considered new resources as antibacterial agents, due to their extraordinary antimicrobial potential against a large range of bacteria [73]. Nisin is an AMP with inhibitory action against Gram-positive bacteria, including *S. aureus*, and for this reason it has been used for many years as a food additive for control of pathogens [75].

Natural polysaccharide guar gum has been studied extensively due to its intrinsic proprieties that render him an excellent source for diverse applications, especially in pharmaceutical and medical industries, since the natural gums are regarded as safe for humans [90].

In this study, was evaluated the potential of a guar gum gel as a delivery system for nisin, using 23 *S. aureus* strains isolated from DFU.

Gram-positive cocci have been considered a major agent responsible for DFU, with exceptional attention for *S. aureus*, which is the main isolated microorganism, with high rates of MRSA been also detected [96]. In a study conducted by Mottola and collaborators [96] that evaluated a total of 54 staphylococci clinical isolates from diabetic foot ulcers from patients at Lisbon hospital centers, authors observed that 48.7% of the isolates were identified as *S. aureus*, and considered to be MRSA strains these were the isolates used in this study.

3.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

For the MIC value determination a microtiter broth dilution method was used [97]. All DFU isolates, and also the reference strain *S. aureus* ATCC®29213™, were tested against the nisin diluted in HCl and incorporated in the guar gum gel. This test was also used for determining the MBC value.

All 23 *S. aureus* isolates and the reference strain tested were considered susceptible to nisin. The MIC values for the nisin diluted in HCl ranged from 40 to 100 µg/mL, with an average value of 90 ± 22.8 µg/mL. In the case of MBC, values were around 5-fold higher than the MIC ones. The average MBC value, was 495.2 ± 149.9 µg/mL, and only three isolates presented a MBC > 800 µg/mL (Figure 5).

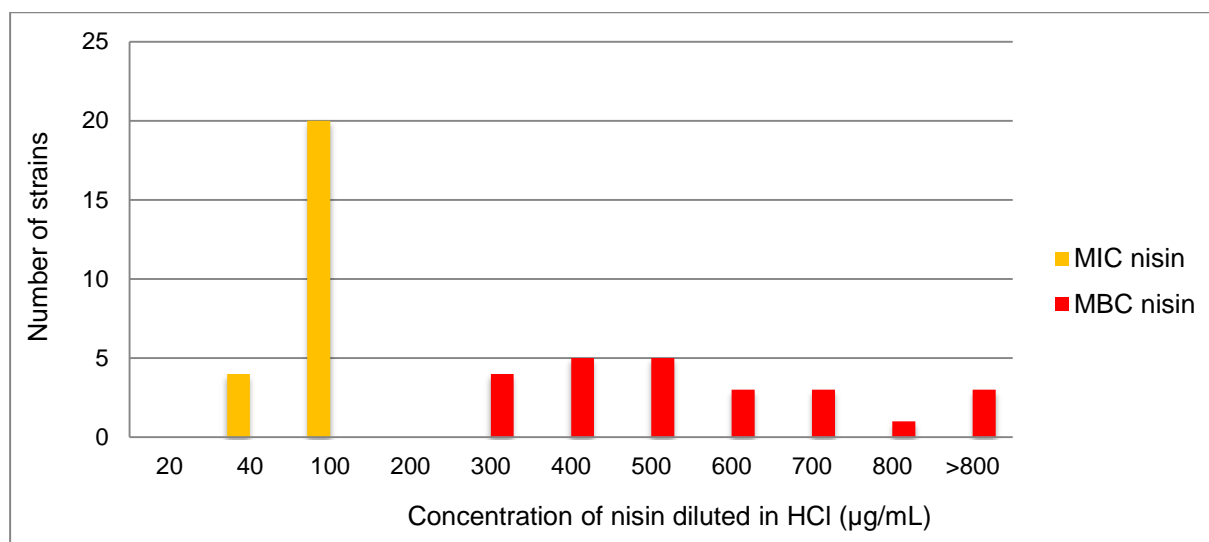


Figure 5 - Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determinations for nisin diluted in HCl against *S. aureus* DFU isolates.

In the case of the nisin incorporated in guar gum gel, all the strains investigated in this study, including the reference strain, were also considered susceptible to nisin. The MIC concentrations ranged from 40 to 300 µg/mL and the average value was 180.8 ± 53.9 µg/mL. The difference between MIC values for the nisin diluted in HCl and incorporated in guar gum gel were significantly different (p -value < 0.05). The same significantly difference was observed for the MBC values. In the case of nisin incorporated in guar gum gel, only three isolates presented a MBC > 1000 µg/mL, and the average MBC value was 766.7 ± 272.6 µg/mL (Figure 6).

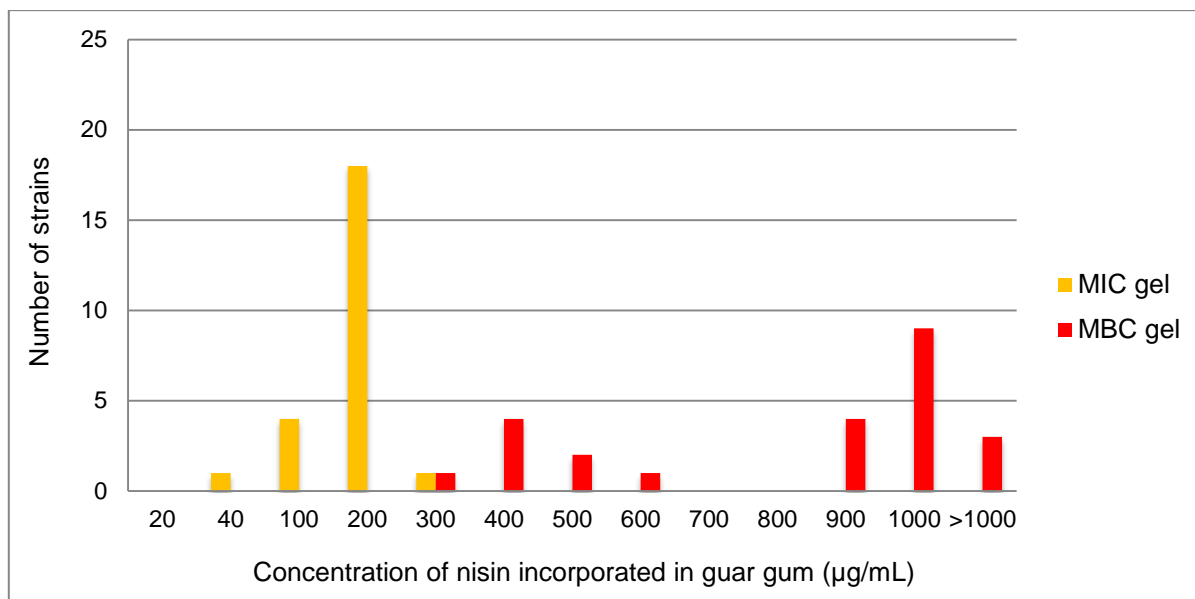


Figure 6 - Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determinations for nisin incorporated in guar gum gel against *S. aureus* DFU isolates.

MIC values, for diluted nisin in HCl indicate a higher antimicrobial activity against planktonic cells, with MIC ≤ 100 $\mu\text{g/mL}$ for majority of isolates tested and MBC 5.5 times higher (Figure 5). French referred that antimicrobial agents are generally classified as bactericidal if the MBC values are no more than four times higher than the MIC values [102]. In our study, the averages of MBC values are higher than that which allowed to conclude that nisin is a bacteriostatic agent against the tested isolates.

Cellular concentration used in this assay, virulence profile of the microorganisms, physiologic and physical characteristics of the assay, such as temperature and time of experience can be conditions factors for the results [103].

S. aureus DFU isolates from this study showed susceptibility to nisin, while diluted in HCl or incorporated in a guar gum gel, showing the potential of this compound as a delivery system for nisin (Figure 5 and Figure 6).

Nisin is an antimicrobial polypeptide, approved by United States FDA as a safe food antimicrobial, having a wide spectrum of inhibitory activity against Gram-positive bacteria [75]. Several researchers have demonstrated that nisin is a promising compound to be applied for the control of bacterial infections, such as respiratory tract infections, gingivitis or *S. aureus* infections in atopic dermatitis [104], [105].

The effect of nisin was evaluated by Felicio and collaborators [106] against *S. aureus* from milk and observed that the use of nisin extend the *lag* phase of bacteria until 8 h. This study indicated that nisin delayed the growth of bacteria, making possible the use of nisin as inhibitor of the adhesion of free cells in a surface.

Okuda and collaborators [107] investigated the effects of diverse bacteriocins on MRSA clinical isolates and demonstrated that nisin showed a higher bactericidal activity against both free-floating and biofilm cells. However, the results observed by Okuda and collaborators were obtained only after end of 4 hours incubation, while our results were obtained after 24 hours. These results seem to suggest that nisin presents bactericidal activity in the first hours of incubation.

The antimicrobial activity of nisin was also assessed in studies using the oral microbiota. In one of these studies, performed by Shin and collaborators, it was observed that nisin inhibited planktonic growth of oral bacteria in lower concentrations (2.5 to 50 µg/mL), that the ones used in our study regarding *S. aureus* DFU isolates [104]. In another study, it was observed that nisin acted against the Gram-positive microorganisms present in oral cavity, but the MIC (until 5000 IU/mL) and MBC values (until 10.000 IU/mL) obtained in this study were higher than the ones obtained in our study [76].

Nisin has a structure that allows amino acids changes, which increase the antimicrobial ability of the polypeptide. This was shown by the research conducted by Field and collaborators that used a novel nisin variant against several pathogenic bacteria, including MRSA strains, demonstrating that the antimicrobial activity in nisin derivatives was higher than in nisin itself. In the referred study, the MIC values obtained for the natural nisin against two MRSA strains (ST528 and ST530) were lower than the ones obtained in this study [108]. All these studies show said that nisin is an optimal agent to be applied against Gram-positive bacteria, including antimicrobial resistant bacteria.

When incorporated in guar gum gel, nisin also were effective against all of the *S. aureus* DFU isolates, which suggests that the guar gum gel permits the diffusion of the nisin polypeptide. As observed for nisin diluted in HCl, the MBC values in this case were higher than the MIC values, and nisin worked as a bacteriostatic agent (Figure 6).

A previous study [109], that used natural polymers for topical delivery of cationic antimicrobial peptides to *Staphylococcus aureus*, showed that incorporation of antimicrobial peptides within gel formulations presents a potential viable for treatment of wound skin. The results from this study also suggest that guar gum gel is a possible delivery system for nisin against *S. aureus* present in polymicrobial DFU [20], [76].

3.2. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) determination

All *S. aureus* DFU isolates under study have the capacity to produce biofilm as determined by a previous study [96] as well as the reference strain *S. aureus* ATCC®29213™.

The method used in this study for MBIC and MBEC determinations consisted in a modified version of the Calgary Biofilm Pin Lid Device [99], in which the two formulations of nisin were tested.

This method was applied to bacteria embedded in a 24 h biofilm, including for each of the DFU isolates and the reference strain *S. aureus* ATCC® 29213™.

Concerning the nisin diluted in HCl, MBIC values ranged from 20 to 300 µg/mL and the average value was 150.8 ± 85.5 µg/mL. In case of MBEC, values observed were higher than the respective MIC. For present nisin diluted in HCl, the MBEC values were >1000 µg/mL for 65% of the isolates tested (n=15) (Figure 7). MBEC values were obtained by visual observation and quantified using an Alamar Blue protocol.

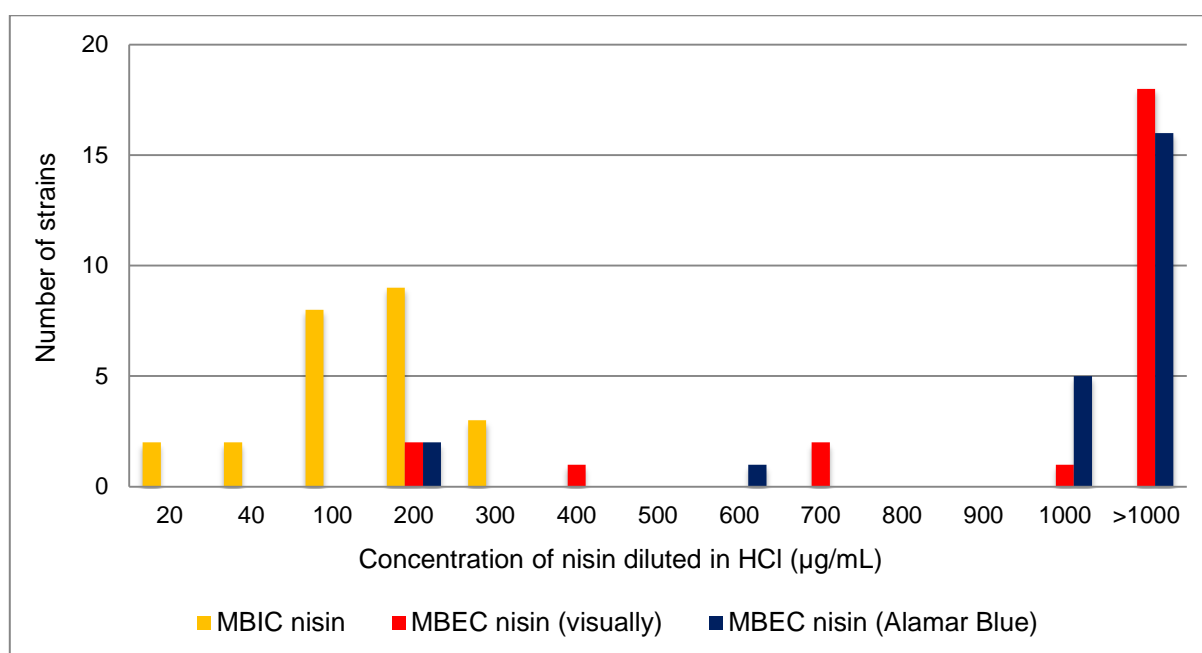


Figure 7 - Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) determinations (µg/mL) for nisin diluted in HCl against *S. aureus* DFU isolates.

In the case of nisin incorporated in guar gum gel, the MBIC values ranged between 100 to 600 µg/mL, and the average value was 366.7 ± 85.5 µg/mL. MBEC values determined by visually observation were higher than the respective MIC, and a large majority of the isolates presented MBEC values > 1000 µg/mL, namely 87% of DFU isolates (n=20) (Figure 8).

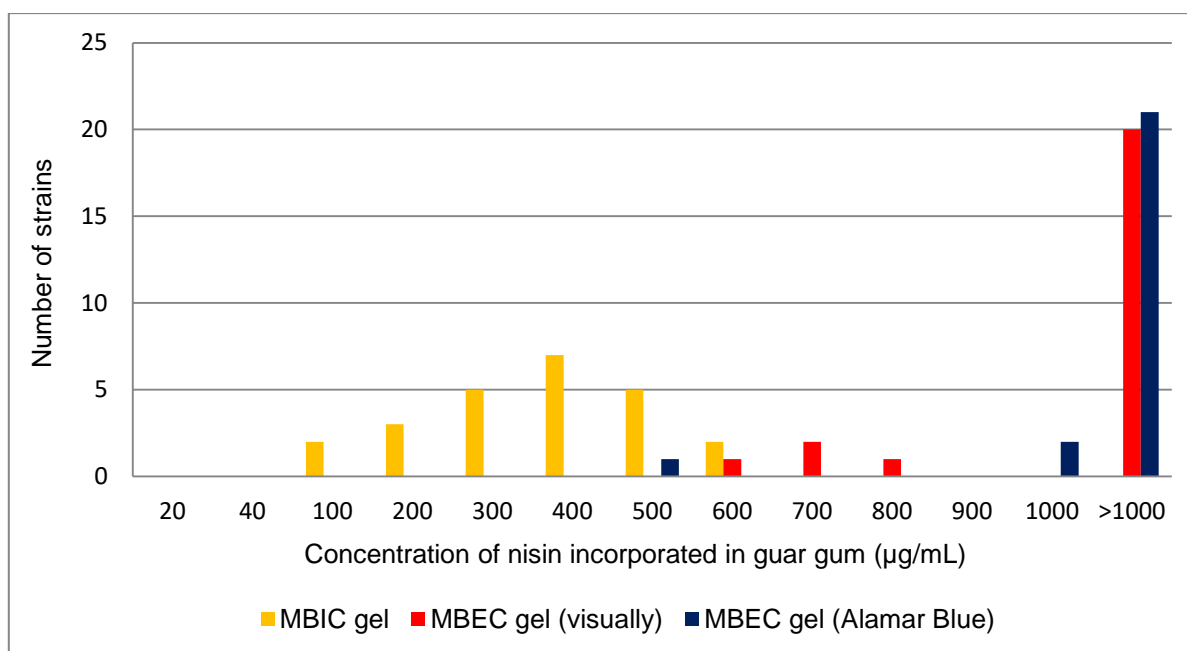


Figure 8 - Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC) determinations (µg/mL) for nisin incorporated in guar gum gel against *S. aureus* DFU isolates.

The comparison between MBIC values from nisin diluted in HCl and incorporated in guar gum gel allowed to observe higher values for the nisin within the guar gum gel, reflecting in a significant difference ($p\text{-value} < 0.05$) between results. However, the inhibitory action of this AMP associated with the guar gum gel was only 2-fold higher than the one from nisin diluted in HCl, demonstrating that this delivery system acts not only in planktonic cells but also in established bacterial biofilms.

Regarding the MBEC it was also observed that values were higher for the nisin incorporated in the guar gum gel, but the difference was not no significant ($p\text{-value} \geq 0.05$) when compared with the values obtained for the nisin in HCl.

MBEC values were higher than MBIC and MBC, as expected since it is more difficult to eliminate established biofilms than planktonic bacteria. In fact, only 13% of performed biofilms were eradicated by the concentrations used in this study. These results can be explained by the fact that assays were performed under ideal conditions, temperature and nutrients and no inhibitors that affect the formation of a biofilm matrix were present [110]. Also, Otto and collaborators [111] stated that the proportion of teichoic acids presents in the *S. aureus* cell wall can be involved in adhesion process and in AMP protection, which may also account for the high MBEC values obtained.

As already mentioned, MBEC values registered by visual observation using microtiter-plate test is one of the most frequently used techniques for quantifying biofilm susceptibility [112]; however, the addition of Alamar Blue allowed the MBEC quantification (Figure 7 and Figure 8). MBEC quantification with Alamar Blue [100] was used for nisin diluted in HCl and for nisin integrated in the guar gum gel. When comparing the MBEC results obtained by both methods, no significant

differences were detected between both MBEC determinations methods, for both nisin formulations ($p\text{-value} \geq 0.05$). This indicates that the visual direct observation of biofilm inhibition offers correct MBEC determinations, avoiding the need for the applications of a very expensive methodology.

However, it is important to refer that Alamar Blue also gives extra information method about cellular viability, unlike other methods, such as visual direct determination, crystal violet or flow cytometry [113].

Results also allowed to continuum that eradication of the *S. aureus* established biofilm is difficult, since only 35% of isolates presented MBEC values below 1000 $\mu\text{g/mL}$ (Figure 7). These results are in agreement with previous studies. Okuda and collaborators [107] used this AMP against MRSA biofilms, obtaining similar results. Emel Mataraci and Sibel Dosler [114] also obtained similar results in the *in vitro* evaluation of diverse antimicrobial cationic peptides, including the polypeptide nisin, against MRSA strains.

DFU bacteriology is normally quite diverse, being usually polymicrobial infections. One study performed by Shin and collaborators [104], that applied nisin against saliva derived multi-species biofilms, showed that nisin has no cytotoxicity to human oral cells and that this AMP retarded the development of multi-species biofilms at concentrations above 1 $\mu\text{g/mL}$. These results suggest that nisin is useful for controlling polymicrobial infections.

Antimicrobial peptides have an important role in inhibiting viability cellular and preventing biofilm matrix formation. According to Balciunas and collaborators [115], nisin targets the cell wall and presents a double action that results in pore formation in the cell wall, resulting in the loss of essential compounds, such as aminoacids, adenosine triphosphate, potassium and other ions, resulting in cell death.

However, biofilm cells are involved in one complex matrix and nisin has difficulties in accessing inside the matrix, especially in established biofilms. According to Nawrocki and collaborators [116], the AMP activity against biofilms can be influenced by several factors that include microbial density, hydrophobicity and amino acids composition.

According to Lewis [117], in biofilm there are differences between the cells in the surface and the ones inside the biofilm matrix. These cells are persistent, having a low metabolism that promotes a resistance to antimicrobial peptides. However, nisin acts against cells promoting pore formation. In fact, results showed that nisin is an excellent AMP because it can act against established biofilms as opposed to other antimicrobials polypeptides, such as lacticin Q, lactoferrin or pexiganan [67], [118].

This activity was also observed for nisin incorporated in guar gum delivery system, which suggests that guar gum gel can be used as a delivery system for nisin against established biofilms present on DFU.

3.3. Guar gum viability assay

A six months assay was performed in order to evaluate the effect of the period and temperature of storage in the inhibitory activity of nisin incorporated in guar gum.

Results showed that nisin maintained its antimicrobial activity in all time periods and all temperatures of storage, although there were variations in the inhibition potential of this AMP (Table 3). Nisin maintained its antimicrobial activity probably due to the physical and chemical characteristics of the guar gum gel formulation, such as its viscosity, stability over a wide range of pH, due its non-ionic nature, and its polymeric nature and functional groups in its structure, and also due to the intrinsic characteristics of the nisin [90], [119], [120].

Table 3 - Diameter of inhibition halos (mm) promoted by nisin incorporated in guar gum gel as evaluated in the guar gum gel viability assay.

Storage Temperature (°C)	Storage period (months)		
	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

It was also observed that the 0.75% (w/v), guar gum gel retained used its viscosity when applied to the human surface skin, which indicates its potential for topical therapeutically administration.

Guar gum polysaccharide is an uncharged natural gum with an exceptional solubility and viscosity, forming a high viscous solution even at low concentration [92]. According to O'Driscoll and collaborators [109], the gel formulations prepared from natural polymers offer new topical delivery systems for wound treatment, allowing direct and continued release of integrated antimicrobial agents, thus ensuring a steady-state concentration of the agent in the wound environment. Also Zhang and collaborators [121] referred that freeze-dried wafer formulations prepared from natural polysaccharides

are new formulas for antibacterial agents delivery, not showing toxicity and immunogenicity problems. In fact, gel formulations seen to be able to promote mucoadhesion, targeting of specific tissues and reduction of the inflammatory response, adding to the many benefits that contribute to wound healing.

As observed in this study, nisin has the capacity for inhibiting the planktonic cells and establishment biofilms at concentrations lower than the established for acceptable daily intake [88], [89]. Nisin is considered Generally Recognized as Safe (GRAS) for oral consumption [88]. Therefore, it can be assumed that the nisin integrated in guar gum gel can be safely and effectively applied topically to clinical patients with DFU.

Chapter 4 | Conclusion

This work represents the preliminary steps towards the development of an innovative therapeutic strategy for the treatment of patients with DFU infected with *S. aureus*. In the future, this strategy can substitute or complement conventional antibiotherapy, and allowing a reduction of the DFU infections and multidrug resistant bacteria dissemination worldwide. Nisin showed the ability to quickly diffuse from the guar gum gel having the capacity to inhibit and eradicate staphylococcal planktonic cells and established biofilms. Furthermore, guar gum gel can represent an alternative, practical and safe delivery system for antimicrobial polypeptides, allowing novel topical therapies for the treatment of several bacterial skin infections.

As a drug carrier system, polysaccharide guar gum gel demonstrates potential to improve hydrophobic nisin and other antimicrobial agents delivery through promoting its enhanced solubility, and increased stability. Besides, antimicrobial nisin and polysaccharide guar gum have been extensively studied, with many studies focusing on the intrinsic physical and chemical properties to be used for diverse applications on medical and pharmaceutical industries.

Bacteria resistance is a major concern worldwide and new treatments are required that presently impairs the treatment of numerous bacterial infections disease, including the ones promoted by MRSA strains. Results show a possible alternative that can contribute to decline the increasing bacterial resistance. Natural polysaccharides are non-toxic, biodegradable, abundantly available, less expensive and their hydrophobic nature can be explored to enhance circulatory stability, enabling a more efficient wound cure [90].

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Supplementary data

6.1. Determinations of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm inhibitory concentration (MBIC), minimum biofilm eradication concentration (MBEC) and minimum biofilm eradication concentration by Alamar Blue quantification (MBEC AB) for nisin diluted in HCl and incorporated in guar gum gel

Table 4 - Medium values of the nisin determination of the different parameters for the 23 *S. aureus* isolates regarding diabetic foot ulcers and the reference strain.

Isolate code	MIC (µg/mL)	MBC (µg/mL)	MBIC (µg/mL)	MBEC (µg/mL)	MBEC AB (µg/mL)
A 1.1	100	600	200	> 1000	> 1000
A 5.2	100	300	100	> 1000	> 1000
A 6.3	100	> 800	300	> 1000	> 1000
B 3.2	100	700	100	> 1000	1000
B 3.3	100	800	100	> 1000	> 1000
B 7.3	100	> 800	100	> 1000	> 1000
B 13.1	40	400	200	> 1000	> 1000
B 14.2	100	500	100	> 1000	> 1000
B 23.2	40	300	20	400	600
Z 1.1	100	700	100	> 1000	> 1000
Z 2.2	40	300	200	> 1000	> 1000
Z 3.1	100	400	200	1000	1000
Z 5.2	100	600	100	700	700
Z 14.1	100	300	300	> 1000	> 1000
Z 16.1	100	700	200	> 1000	> 1000
Z 16.2	100	400	20	200	200
Z 17.2	40	400	40	200	200
Z 21.1	100	500	200	> 1000	> 1000
Z 21.3	100	400	200	700	> 1000
Z 25.2	100	600	40	> 1000	> 1000
Z 27.2	100	500	200	> 1000	1000
Z 27.3	100	> 800	200	> 1000	> 1000
Z 32.2	100	500	100	> 1000	1000
ATCC®29213 TM	100	500	300	> 1000	> 1000

A: aspirate; B: biopsy; Z: swab; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MBIC: minimum biofilm inhibitory concentration; MBEC: minimum biofilm eradication concentration; MBEC AB: minimum biofilm eradication concentration by alamar blue quantification

Table 5 - Medium values of the nisin incorporated in guar gum gel determination of the different parameters for the 23 *S. aureus* isolates regarding diabetic foot ulcers and the reference strain.

Isolate code	MIC (µg/mL)	MBC (µg/mL)	MBIC (µg/mL)	MBEC (µg/mL)	MBEC AB (µg/mL)
A 1.1	200	1000	600	> 1000	> 1000
A 5.2	200	> 1000	400	> 1000	> 1000
A 6.3	100	500	400	> 1000	> 1000
B 3.2	200	1000	400	> 1000	> 1000
B 3.3	200	> 1000	500	> 1000	> 1000
B 7.3	200	900	500	> 1000	> 1000
B 13.1	100	400	300	> 1000	> 1000
B 14.2	200	1000	300	> 1000	> 1000
B 23.2	100	400	100	700	1000
Z 1.1	200	900	400	> 1000	> 1000
Z 2.2	200	500	400	> 1000	> 1000
Z 3.1	200	1000	300	700	> 1000
Z 5.2	200	900	300	> 1000	> 1000
Z 14.1	300	600	600	> 1000	> 1000
Z 16.1	200	900	500	> 1000	> 1000
Z 16.2	200	1000	100	600	1000
Z 17.2	200	400	200	800	500
Z 21.1	200	1000	300	> 1000	> 1000
Z 21.3	200	1000	500	> 1000	> 1000
Z 25.2	100	400	200	> 1000	> 1000
Z 27.2	200	1000	400	> 1000	> 1000
Z 27.3	200	> 1000	400	> 1000	> 1000
Z 32.2	40	300	200	> 1000	> 1000
ATCC® 29213™	200	1000	500	> 1000	> 1000

A: aspirate; B: biopsy; Z: swab; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MBIC: minimum biofilm inhibitory concentration; MBEC: minimum biofilm eradication concentration; MBEC AB: minimum biofilm eradication concentration by alamar blue quantification

6.2. Guar gum viability assay results

Table 6 - Medium values of the diameter of inhibition halos (mm) promoted by nisin integrated in guar gum gel at different temperatures and two different concentrations of nisin.

Storage Temperature (°C)	Storage time (month)					
	1		3		6	
	200 µg/mL	600 µg/mL	200 µg/mL	600 µg/mL	200 µg/mL	600 µg/mL
-18	9.2	12.0	9.2	10.7	13.0	12.3
4	8.2	12.3	10.7	12.0	10.3	13.7
20	8.8	10.3	8.6	10.4	8.2	10.2
37	12.8	15.5	6.8	11.0	9.8	13.5
42	8.0	14.7	7.2	13.8	9.8	11.0