TÉCNICO LISBOA

Antibiofilm Activity of Antimicrobial Compounds from Different Classes Against Diabetic Foot Staphylococci

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“Messieurs, c’est les microbes qui auront le dernier mot”

(“Gentlemen, it is the microbes that will have the last word”)

Louis Pasteur

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Abstract

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide and its role as a major risk factor for the development of Diabetic Foot Ulcers (DFU) is well established. DFU are vulnerable to opportunistic infections, being that gram-positive bacteria, such as *Staphylococcus aureus*, are the most frequent microorganisms isolated. Due to the emergence of drug resistant bacteria that could impair DFU successful treatment, it is urgent to find new therapeutics protocols that could be an alternative to the current antibiotherapy. This has aroused the interest in antimicrobial peptides (AMPs) and biocides as complementary molecules to the administration of antibiotics.

In this study the antimicrobial potential of chlorhexidine against 23 biofilm-producing strains of *S. aureus* isolated from DFU was tested by determining minimum inhibitory (MIC) and bactericidal (MBC) concentrations. Afterwards, different antimicrobial treatments including combinations of chlorhexidine, nisin incorporated in guar gum gel and antibiotics (clindamycin, gentamycin and vancomycin) were also tested, allowing to evaluate their biofilm inhibitory and eradication action.

Results suggest that chlorhexidine has a good antimicrobial effect even in low concentrations, evidencing a bactericidal effect in most isolates. The treatment that show a higher inhibitory action against biofilms was nisin incorporated in guar gum gel combined with chlorhexidine, followed by these compounds combined with clindamycin. Regarding biofilm eradication assay, overall results were quite similar, being that vancomycin combined with chlorhexidine had the highest eradication effect.

These results highlight the potential of nisin incorporated in guar gum gel and chlorhexidine as a substitute or as complementary compounds to antibiotherapy, for inhibition of *S. aureus* biofilms responsible for Diabetic Foot Infections (DFI).

Key-words: Diabetic Foot Ulcer, Staphylococcus aureus, Chlorhexidine, Nisin, Guar Gum Gel, New therapeutics

Resumo

Diabetes *mellitus* é um grave problema de saúde que se encontra em rápida expansão em todo o mundo, sendo um fator de risco para o desenvolvimento de úlceras de pé diabético (UPD). As UPD são vulneráveis a infeções oportunistas, sendo as bactérias gram-positivas, como *Staphylococcus aureus*, os microrganismos mais frequentemente isolados. Devido ao aparecimento de bactérias resistentes aos antibióticos que podem prejudicar o sucesso dos tratamentos das UPD, é urgente encontrar novos protocolos terapêuticos que possam ser uma alternativa à antibioterapia atual. Esta problemática despertou o interesse nos peptídeos antimicrobianos (AMPs) e biocidas como moléculas complementares a administração de antibióticos.

O potencial antimicrobiano da clorexidina foi testado contra 23 estirpes produtoras de biofilme de *S. aureus* isoladas de UPD, tendo sido determinada a sua concentração mínima inibitória (CMI) e bactericida (CMB). Posteriormente, foram testados diferentes tratamentos incluindo combinações de clorexidina, nisina incorporada em gel de goma guar e antibióticos (clindamicina, gentamicina e vancomicina), através da determinação da ação inibidora e de erradicação de biofilme formados pelas estirpes em estudo.

Os resultados sugerem que a clorexidina tem um bom efeito antimicrobiano mesmo em baixas concentrações, evidenciando um efeito bactericida na maioria dos isolados. A nisina incorporada no gel de goma guar combinado com clorexidina revelou ser o tratamento com melhor ação inibitória, seguido por este mesmo tratamento combinado com clindamicina. Quanto à ação de erradicação de biofilme, os resultados globais foram semelhantes, sendo que a vancomicina combinada com clorexidina teve o melhor efeito.

Estes resultados demonstram o potencial da nisina incorporada no gel de goma guar e da clorexidina como substitutos ou compostos complementares à antibioterapia, principalmente para inibição de biofilme nas infeções do pé diabético (IPD).

Palavras-chave: Ulceras de Pé Diabético, Staphylococcus aureus, Clorexidina, Nisina, Gel de Goma Guar, Novas terapêuticas

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List of Abbreviations

%	Percent
µg	Microgram
µL	Microliter
µm	Micrometer
AMP	Antimicrobialpeptide
ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CA-MRSA	Community-Associated Methicillin-Resistant *Staphylococcus aureus*
CFU	Colony Forming Units
CNS	Coagulase – Negative Staphylococci
DFI	Diabetic Foot Infection
DFU	Diabetic Foot Ulcer
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DM	Diabetes *mellitus*
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Eap	Extracellular Adherence Protein
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FDA	Food and Drug Agency
g	Gram
G+C	Guanine – Cytosine Content
GRAS	Generally Regard As Safe
h	Hour
HA-MRSA	Hospital-Associated Methicillin-Resistant *Staphylococcus aureus*
HCl	Chloridric Acid
Hz	Hertz
IDDM	Insulin-dependent Diabetes *mellitus*
IDSA	Infectious Disease Society of America
IWGDF	The International Working Group on the Diabetic Foot
kDa	Kilodalton
LAB	Lactic Acid Bacteria
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
mL	Milliliter

MLST	Multilocus Sequence Type
Mol%	Mole Percent
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
NaCl	Sodium Chloride
nm	Nanometer
ºC	Celsius Degrees
PBP	Penicillin-Binding Protein
PFGE	Pulse Field Electrophoresis
PFT	Pore-Forming Toxins
PSM	Phenol Soluble Modulins
PVL	Panton-Valentine Loukodicin
RCBD	Randomized Complete Block Design
rRNA	Ribosomal Ribonucleic Acid
S. aureus	Staphylococcus aureus
SAg	Superantigens
SCC*mec*	Staphylococcal Chromosome Cassette *mec*
SSTI	Skin and Soft Tissue Infection
SSTI	Skin and Soft Tissue Infection
TSB	Tryptic Soy Broth
TSS	Toxic Shock Syndrome
WHO	World Health Organization
α	Alfa
β	Beta

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Chapter 1

Introduction

1. Diabetes *mellitus*

It is defined by World Health Organization (WHO) as a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.[1] Diabetes *mellitus* (DM) is probably one of the oldest diseases known to man. It was first reported in Egyptian manuscript about 3000 years ago.[2]

This disease is a serious health problem in rapid expansion worldwide.[3] According to the International Diabetes Federation, the prevalence of diabetes is estimated to be 415 million (8,3% of the world's adult population) globally, being expected to alarmingly rise to 642 million by 2040; which represents a significant rise over a small time period.[4] In Europe, there are around 60 million people with diabetes, from which 10.3% are men and 9.6% are women.[5,6] Every year over half million people die of diabetes.[6]

The number of people with DM has steadily risen over the past few decades, due to population growth, the increase in the average age of the population, the rise in prevalence of diabetes at each age, increasing prevalence of obesity, changes in physical activity levels and patterns of food intake.[7,8,9] It has risen substantially in countries at all income levels, mirroring the global increase in the number of people who are overweight or obese.[7] DM prevalence has risen faster in low- and middle-income countries, where most people with diabetes are between 45 to 64 years of age, than in high-income countries, where diabetic patients are generally older than 64 years of age.[7,8]

DM symptoms are often not severe, or may be absent, and consequently hyperglycemia of sufficient degree can cause pathological and functional changes for a long time before diagnosis.[10] The clinical diagnosis of DM is often prompted by the development of characteristic symptoms such as increased thirst, polydipsia and polyuria, recurrent infections, unexplained weight loss, blurred vision and, in severe cases, ketoacidosis or a non-ketotic hyperosmolar state that may develop and lead to stupor, coma and, in absence of effective treatment, death.[10,11,12] High levels of glycosuria are also usually present.[10]

On the other hand, long-term effects of DM include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints and autonomic neuropathy causing gastrointestinal, genitourinary, cardiovascular diseases and sexual dysfunction.[1,11]

Most cases of diabetes fall into two broad etiopathogenetic categories. In one category, type 1 diabetes and in the other, much more prevalent category, type 2 diabetes.[11]

Type 1 diabetes is a serious, chronic condition that is associated with significant morbidity and mortality.[13] This type is said to account for only a minority of the total burden of diabetes in a population (5-10%), although it is the major type of diabetes in younger age individuals at majority of high-income countries.[11,14] In this case "insulin is required for survival", being previously called insulin-dependent Diabetes *mellitus* (IDDM) or juvenile-onset diabetes.[1] This form of diabetes results from cellular-mediated autoimmune destruction of the β-cells of the pancreas, leading to absolute insulin deficiency.[10,15] In this form of diabetes, the rate of β-cell destruction is quite variable, being rapid in some individuals, mainly in infants and children and slow in others, mainly adults.[11]

Type 2 diabetes is one of the leading causes of premature morbidity and mortality worldwide and accounts for more than 90% of diabetes diagnosed.[11,16] It was previously called non-insulin-dependent Diabetes *mellitus* or adult-onset diabetes and, at least initially and often throughout the patient lifetime, there is no need for insulin treatment to survive.[11,15] Type 2 diabetes is a complex and progressive disease, characterized by various metabolic defects which affect multiple organs.[16] It is intimately associated with improper utilization of insulin, which leads to insulin resistance and usually insulin deficiency, by target cells and peripheral tissues, such as adipose tissue and muscle.[11,14,16] This form of diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to develop any of the classic symptoms of diabetes.[11] Nevertheless, such patients are at increased risk of developing macrovascular (coronary heart disease, peripheral vascular disease and stroke) and microvascular (diabetic nephropathy, neuropathy and retinopathy) complications and they have a greater possibility of developing hypertension, dyslipidemia and obesity.[11,17,18] Ketoacidosis rarely occurs spontaneously in this type of diabetes.[11] The risk of developing this form of diabetes increases with age, obesity, family history of diabetes, prior history of gestational diabetes, impaired glucose tolerance, lack of physical activity and race/ethnicity.[11,15]

2. Foot Complications – Diabetic Foot Ulcer

Foot complications are among the most serious and costly complications of DM, accounting for more hospital admissions than any other of the diabetic complications.[19,20] It occurs in both type 1 and type 2 diabetic patients, showing higher prevalence among males and in patients with more than 60 years old.[21] The major adverse outcomes of foot complications are foot ulcers and amputations.[20] It is estimated that 10% to 25% of the diabetic patients will develop a foot ulcer in their lifetime and that up to 70% of all non-traumatic amputations in the world occur in diabetic patients.[20,22] Diabetic foot ulcers (DFU) are among the most common, several and costly complications of DM progression, being also a clinical marker for limb amputation and death in diabetic patients.[22,23] The annual worldwide incidence of foot ulceration is estimated to be approximately 1% to 4% and its prevalence ranges from 4% to 10%.[21]

According to the International Consensus on the Diabetic Foot, a foot ulcer is defined as a full-thickness wound below the ankle in a diabetic patient, irrespective of duration.[24] DFU frequently result from two or more risk factors, such as duration of diabetes, age, blood glucose levels, blood pressure, peripheral vascular disease, foot deformities, arterial insufficiency, immunosuppression, trauma, impaired resistance to infection, smoking and, frequently, ischemia and neuropathy.[19,23-26]

Neuropathy can be responsible for ulceration due to trauma or excessive pressure in a deformed foot without protective sensibility.[26] Symptoms may vary depending on the type of nerves affected (motor, sensory or autonomic) and nerves location in the body.[27] Diabetic neuropathy is present in almost 60% of patients with diabetes who have foot ulcers and is more prevalent with increasing age and duration of diabetes.[28,29]

Ischemia is caused by peripheral arterial disease.[27] Patients usually complain with pain in the limb as the ischemia progress, caused by poor arterial inflow that decreases blood supply to ulcer area and is associated with reduced oxygenation, nutrition, and ulcer healing.[26,29] The characteristics place the foot and ankle at the risk of ulceration.[29]

The most common regions for ulcer development are toes, followed by the plantar metatarsal and the heel.[30] Moreover, foot ulcers precede approximately 85% of all amputations, including total or partial amputations, performed in diabetic patients.[19,31] This not only contributes dramatically to high morbidity among diabetic patients, but is also associated with severe clinical depression and increased mortality rates.[26] The risk of foot ulceration and limb amputation increases with age and the duration of diabetes.[31]

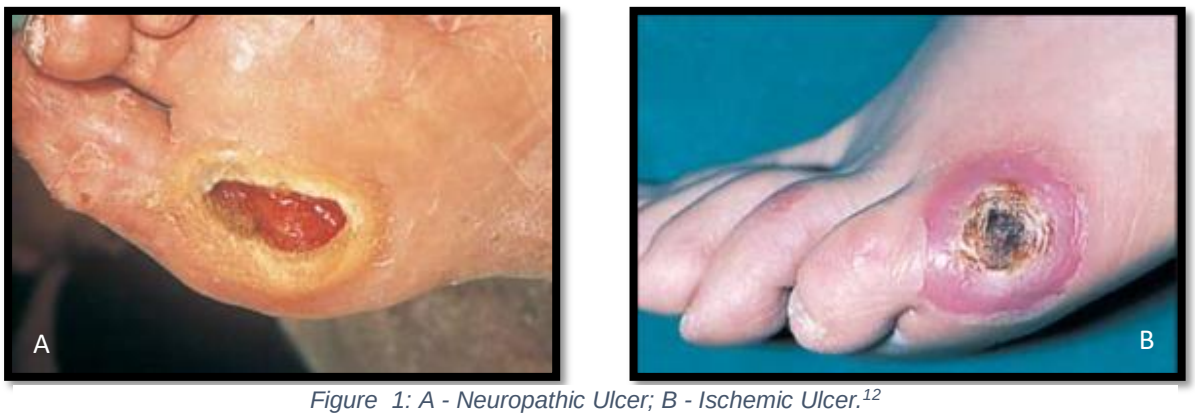

Figure 1: A - Neuropathic Ulcer; B - Ischemic Ulcer.[12]

The anatomy of the foot is the main reason that infection is potentially serious and the structure compartment, tendons, sheaths and neurovascular bundles tend to favor the spread of infection.[30]

2.1. Diabetic Foot Infections

The rise in the prevalence of DM is leading to an increasing problem of infections, especially foot ulcer infections, which are potentially serious.[32,33] With loss of sweat and oil gland function, the diabetic foot becomes dry and keratinized which cracks and fissures more easily.[34] Once the skin is broken typically on the plantar surface, the underlying tissues are exposed to colonization by pathogenic organisms.[33] Even with the best preventive care, 9% of patients will develop a diabetic foot infection (DFI), which increases the risk of amputation.[35] These infections are generally secondary to a skin wound.[36]

Infection is defined by overgrowth of microorganisms within a wound that promotes deleterious inflammation or tissue destruction.[37] It usually begins as a superficial local process with the classic signs and symptoms of inflammation (redness, warmth, pain, tenderness, induration).[33,37] With delay in treatment and impaired body defense mechanisms caused by neutrophil dysfunction and vascular insufficiency, infection can spread to the contiguous subcutaneous tissues and to even deeper structures.[33] DFI is more often the consequence rather than the cause of diabetic foot ulcers.[38]

The International Working Group on the Diabetic Foot (IWGDF) and the Infectious Disease Society of America (IDSA) have out-lined clinical criteria for DFI diagnostic and developed a classification system describing the severity of disease (Table 1).[39] This classification uses the acronym PEDIS, which stands for Perfusion, Extent (size), Depth (tissue loss), Infection and Sensation (neuropathy).[40] In this system are levels of 1 to 4 for each of these factors.[41]

Table 1: PEDIS classification of Diabetic Foot Infection. Adapted from Farzamfar et al 2013.[13]

Clinical Manifestation of Infection	Infection Severity	PEDIS Grade
Wound lacking purulence or any manifestations of inflammation.	Uninfected	1
Presence of purulence or erythema, pain, tenderness, warmth or induration; Cellulitis around the ulcer; Infection is limited to the skin or superficial subcutaneous tissues.	Mild	2
Lymphangitic streaking, spread beneath the superficial fascia, deep-tissue abscess, gangrene and involvement of muscle, tendon, joint or bone.	Moderate	3
Infection with systemic toxicity or metabolic instability (fever, chills, tachycardia, hypotension, confusion vomiting, leukocytosis, acidosis, severe hyperglycemia or azotemia).	Severe	4

Many organisms, alone or in multispecies communities can cause DFI.[40] The most common organisms found in the patients are similar to the ones of non-diabetic patients with skin and soft-tissue infections, namely aerobic gram-positive cocci.[37,42] *Staphylococcus aureus* is the most commonly isolated from these ulcers, either alone or as a component of mixed infections.[37,43]

3. *Staphylococcus* – General Concepts

The genus *Staphylococcus* belongs to the bacterial family Staphylococcaceae.[44] Its name is derived from the Greek words *staphyle* (a bunch of grapes) and *coccus* (grain or berry).[45] The major habitat of staphylococcal species is skin, nose, oral cavity, gastrointestinal tract, feces and are frequently isolated from suppurative processes.[46]

Staphylococci are characterized by being gram-positive spherical bacteria, with 0.5 to 1.5 μm in diameter, that occur in clusters, pairs and occasionally in short chains, which characteristically divide in more than one level, thereby forming irregular clusters like a bunch of grapes.[47,48,49] *Staphylococcus* species are nonmotile, non-spore-forming, facultatively anaerobic, catalase positive, cytochrome oxidase negative and often hemolytics.[46,49] They are resistant to drying and tolerate high concentrations of salt (10% NaCl) when grown on artificial media.[49] The temperatures in which growth is possible varying between 12ºC to 45ºC being the optimal growth temperature at 37ºC.[46] The metabolism is respiratory and fermentative. The cell wall contains peptidoglycan and teichoic acid. A major genotypic

criterion of the members of the genus is a G+C content between 30 to 30mol%, being defined low G+C gram-positive bacteria.[48]

The rRNA hybridization and comparative oligonucleotide analysis of 16S rRNA has demonstrated that staphylococci form a coherent group at the genus level.[50] This genus comprises more than 52 species and 26 subspecies that are separated into two distinct groups based on their ability to clot blood plasma (coagulase reaction).[51] The coagulase-positive staphylococci include the most pathogenic *S. aureus* species and the coagulase-negative staphylococci (CNS) comprises common commensals, although some species can cause infections.[47]

Serious staphylococcal infections often occur when the resistance of the host is low due to hormonal changes, debilitating illness, wounds, treatment with steroids or other drugs that compromise immunity.[49] They can cause different types of infections in a host, including acne, abscesses, sepsis, impetigo, pneumonia, osteomyelitis, carditis, meningitis and arthritis.[49,52]

3.1. ***Staphylococcus aureus***

Staphylococcus aureus was firstly described by Sir Alexander Ogston in 1882 and 2 years later Rosenbach isolated it in a pure culture and suggested the name *Staphylococcus aureus*. Depending on growth conditions and origin, the *S. aureus* colonies pigmentation varies from grey, grey-white with yellowish to orange shades with β-hemolysis in blood agar.[45]

This specie is considered the most important human pathogen among staphylococci, causing a wide range of clinical infections.[47] Although is usually regarded as a transient microorganism in the skin, approximately 50% of the general population are either permanently or intermittently colonized in the nasal mucosa without any pathogenic event.[48,53]

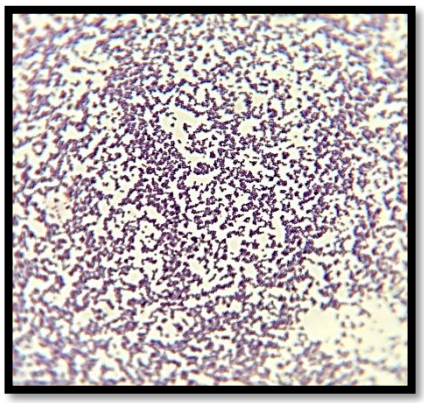

Figure 2: Gram coloration of Staphylococcus aureus isolate (original).

S. aureus can potentially cause some of the most severe hospital-associated and community-acquired infections.[47] However, staphylococcal diseases occur in people whose defensive mechanism have been compromised.[54] In health care facilities, *S. aureus* strains can be transmitted from patient to patient via hand carriage by medical personnel, but also through contaminated objects or through colonized or infected health care personnel, which may act as reservoirs.[48]

The ability to acquire resistance to antibiotics from multiple classes makes *S. aureus* a challenging pathogen to eliminate.[55]

Treatment of *S. aureus* infections before the 1950s involved the administration of penicillin, a β-lactam antibiotic.[56] However, by the late 1950s penicillin-resistant staphylococci were recognized, first in hospitals and subsequently in community, increasing concern.[56,57] Resistant strains typically produced

an enzyme, called a β-lactamase, which inactivated the β-lactam, thus efforts were made to synthesize penicillin derivatives that were resistant to β-lactamase hydrolysis. This was achieved with the synthesis of methicillin, which had the phenol group of penicillin disubstituted with methoxy groups.[56]

Shortly after methicillin was introduced in 1961, methicillin-resistant *Staphylococcus aureus* (MRSA) strains emerged within a year, resulting in increased morbidity, mortality and treatment costs.[55,58] MRSA can be categorized into two major groups, identified as hospital-associated MRSA (HA-MRSA) or as community-associated MRSA (CA-MRSA).[59] Characteristically, MRSA strains are more often multidrug resistant in comparison with methicillin-susceptible *Staphylococcus aureus* (MSSA) strains.[60] In recent years methicillin has not been used, becoming unusable.[58]

Unlike penicillin resistance, which is achieved via the production of enzyme penicillinase, methicillin resistance is due to the presence of an additional penicillin-binding protein (PBP), designated PBP2a, in the cell wall that have a reduced binding affinity to β-lactam antibiotics.[59,61] In the presence of this type of antibiotics, the four native staphylococcal PBPs are inactivated and the reactions conducted by these enzymes aiming at the synthesis of the peptidoglycan chains that constitute the bacterial cell wall are blocked.[60] Since PBP2a is not inhibited by the presence of β-lactams, it is able to promote the peptidoglycan biosynthesis, allowing out the cell wall synthesis and survival of bacteria even in presence of β-lactam antibiotics.[55,60]

PBP2a is encoded by the *mecA* gene, present in a large chromosomal cassette called staphylococcal chromosome cassette *mec* element (SCC*mec*).[59] The expression of this gene is controlled by *mec*I-*mec*RI regulatory genes, which encodes for repressor and inducer proteins, respectively.[59] Due to the presence of *mecA*, MRSA are resistant to nearly all β-lactam antibiotics.[55]

A new divergent *mec*A homologue (*mec*C or *mec*ALGA251) has been recently described. The recent discovery of the SCC*mec* element type XI in *S. aureus* harbors this new *mec*C element, that has been found on the chromosome of MRSA strains of animals and humans.[62,63] The *mec*C gene shares only 70% of sequence similarity to *mec*A gene at the DNA level.[64] This significant difference led to the assumption that the proteins encoded by *mec*C and *mec*A may differ from each other in terms of their structure and function. It was recently demonstrated that PBP2a and the *mec*C-encoded homolog (designated PBP2c by EUCAST) differ in their binding characteristics toward β-lactams, sharing only 63% of identity at the amino acid level. In contrast with PBP2a, PBP2c is thermosensitive, having its activity decreased at 37°C.[62]

3.1.1. Virulence Factors

The pathogenesis of *S. aureus* in DFI corresponds to the physiopathology of skin and soft tissue infection (SSTI), which involves the production of a myriad of virulence factors that allow the organism to enter tissues, attach to host cells and secret exoproteins and toxins.[47,65] Several toxin genes are carried on plasmids and in some cases, genes responsible for pathogenicity reside on both a plasmid and a host chromosome.[54]

The ability of *S. aureus* to cause DFI is defined by numerous virulence factors among which toxins play an important role (participation in colonization, persistence, evasion of the immune system and dissemination).[65]

To facilitate adhesion to host skin tissues *S. aureus* possess microbial surface components recognizing adhesive matrix molecules (MSCRAMM) which interacts with host molecules such as collagen, fibronectin and fibrinogen. They are also involved in host immune evasion.[55]

For break or evading the host immune system, these bacteria secrete toxins that can be pore-forming toxins (PFT), which through pore-forming and pro-inflammatory activities have the ability to lyse host cells.[65] It includes the single-component α-toxin (or α-hemolysin), that destroys erythrocytes and causes skin destruction.[54,65] Is the best characterized and most potent membrane-damaging toxin; phenol soluble modulins (PSM) induce human neutrophil lysis after phagocytosis, a pathogenesis mechanism of great importance for the high toxicity; leukotoxins, namely Panton-Valentine Leukocidin (PVL).[50,65] It consists of two protein components (LukS-PV and LukF-PV) which act together as subunits and form porins on cell membrane of host cells, leading to leakage of cell contents and cell death.[55] PVL is an important factor in necrotizing skin infections, however, their prevalence is extremely diverse, varying between less than 5% and 67% in MSSA.[65]

Extracellular adherence protein (Eap), has a role in tissue invasion. It is a exoprotein which binds to host cell matrix, plasma proteins and endothelial cell adhesion molecule CAM-1. Beyond this, also has immune-modulatory activity.[55] Other factors that participate in tissue invasion are: proteases, which break down proteins; lipases, that break down lipids; hyaluronidase, breaks down hyaluronic acid between cells, allowing for penetration and spread of bacteria; phospholipase C, metalloproteases (elastase), nucleases and staphylokinase cause tissue destruction and thereby, help in bacterial penetration into tissues.[54,55]

Toxinosis is induced by exfoliative toxins A and B.[55] The exfoliatins are serine proteases which selectively recognize and hydrolyze desmosomal proteins in the skin.[55] The prevalence of these toxins ranges from 0.5%–3% in MSSA and around 10% in MRSA.[65]

Enterotoxins also can induce toxinoxis.[55] Enterotoxins are secreted toxins of approximately 20–30 kD that belong to the family of superantigens (SAg). These molecules over-induce cytokine production from both T-lymphocytes and macrophages, causing cell death by apoptosis.[65] Sag includes the Staphylococcal Enterotoxins and Enterotoxins-Like Toxins, which activate T cells, resulting in a high secretion of pro-inflammatory cytokines. This process leads to a chronic inflammatory state in uninfected DFU, inducing a delay or an absence of wound healing; and the Toxic Shock-Syndrome Toxin 1 (TSST-1).[65] Is associated with fever, shock and multisystem involvement of toxic shock syndrome (TSS).[54] TSST-1 is frequently present in Grade 4 DFI.[65]

Other virulence factors produced by *S. aureus* are β-lactamase (breaks down penicilins), catalase (converts hydrogen peroxide into water and oxygen; reduces killing by phagocytosis), coagulase (reacts with prothrombin to form a complex that can cleave fibrinogen and cause the formation of a fibrin clot; fibrin may also be deposited on the surface of staphylococci, which may protect them from destruction

by phagocytic cells; coagulase production is synonymous with invasive pathogenic potential) and DNase (destroys DNA).[54]

3.1.1.1. Biofilms

Growth as a biofilm is a risk factor since, after adhering to tissues, *S. aureus* can evade host defenses and the activity of antibiotics by forming biofilms on host.[61]

In human medicine biofilms have been of great relevance because many pathogenic and non-pathogenic bacteria can grow in such structures as part of their virulence mechanism, allowing the protection against the immune system of the host.[66] Biofilm associated infections represent 80% of nosocomial infections, being *S. aureus* the most frequent isolated species in such cases.[67]

Biofilm is present on biotic or abiotic surfaces.[67] It can be defined as sessile communities of microbial cells irreversibly attached either to a surface, an interface or to each other, which are embedded in a self-produced matrix of extracellular polymeric biomolecules, resulting in an alteration in the phenotype of the organism with respect to growth rate and gene transcription.[68,69] It has been estimated that biofilms can tolerate antimicrobial agents (disinfectants, antibiotics, surphactants) at concentrations of 10-1000 times higher than the ones needed to inactivate genetically equivalent planktonic bacteria.[70]

When conditions favor biofilm formation, *S. aureus* begins by adhering to host cells.[71] Once irreversible adhesion is achieved, the cells divide and start colonizing the tissues.[72] When the local concentration of auto inducers, which are chemical signals, produced by microbial metabolism, reaches a threshold level, it suggest that the microbial population density has reached a minimum, promoting changes in gene expression and behavior, as response.[72,73] This process is known as quorum-sensing.[72] When there is a low cell density, bacteria express protein factors that promote attachment and colonization, however at high cell density, the bacteria repress these traits and initiate secretion of toxins and proteases that are required for dissemination. In *S. aureus* this switch in gene expression is regulated by the Agr quorum-sensing system.[73] The system enables in the autoinduction of the synthesis of the extracellular matrix or exopolysaccharide (composed of polysaccharides, proteins, nucleic acids and lipids) allowing the maturation of biofilm communities which subsequently acquired three-dimensional structure, which module water channels that act as the microcirculation in biofilm colonies.[72] These water channels allow the fluids to flow throughout the biofilm, making the distribution of nutrients and oxygen easier. Moreover, the water channels also enable the removal of metabolic end products.[70]

In the last step of biofilm formation, disruption allows the detachment of single cells or large bacterial clusters from the biofilm, which occurs in the case of good environmental conditions or in the case of biofilm expansion.[67] This process can be caused by the bacteria themselves, which can promote the enzymatic degradation of the biofilm matrix (dissolution of adhesins by proteases and nucleases), by PSMs, that function as surfactants, by quorum sensing or by external forces, such as fluid shear forces, corrosion and human intervention. During detachment of motile microorganisms, cells express genes coding for motility proteins such as pilus and ribosomal proteins.[71] This process may lead to the systemic dissemination of the biofilm.[67]

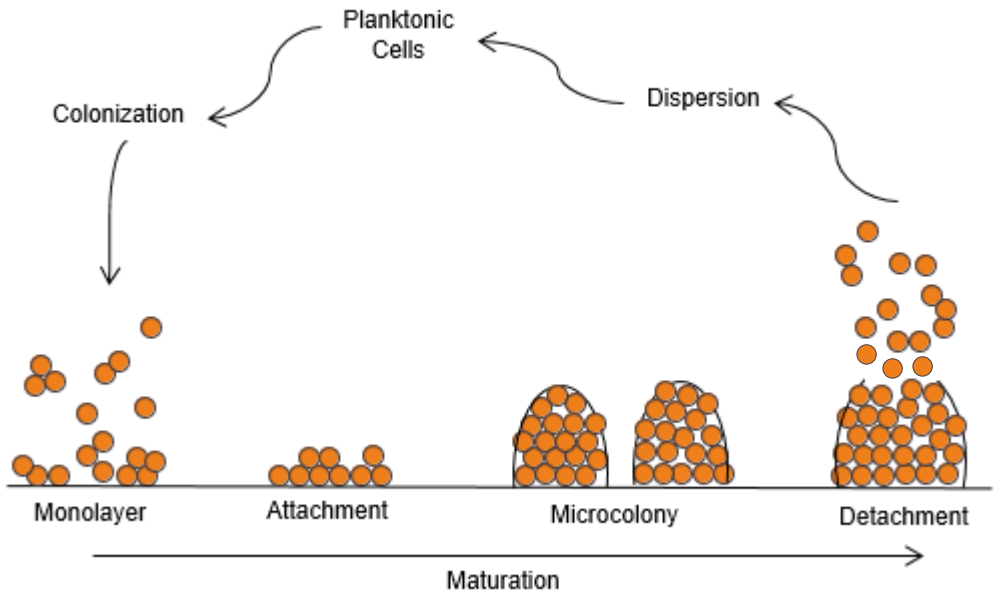

Figure 3: Biofilm cycle by Staphylococcus aureus (original).

The factors that may influence biofilm production by bacteria include the recognition of attachment sites on a surface, nutritional signals, change of environmental pH and temperature, exposure to antibiotics, chemical biocides and host defense mechanisms.[68]

The development of new therapeutic strategies, through a better understanding of biofilms, is necessary and imperative to control the dissemination of such structures which are resistant to the action of the immune system and of antimicrobial drugs.[67]

4. Antibiotics

Diabetic patients may develop many types of foot wounds, which can become infected requiring antibiotic therapy.[40,74] Initial therapy is frequently empirical and should be based on the severity of the infection afterwards, it can be adjusted based on microbiological data, such as recent cultures, gram-stained smears and antimicrobial susceptibility testing.[74]

Several antimicrobial compounds are available for infections treatments, and can be classified based on their type of action, source, spectrum of activity, chemical structure and function.[75]

Regarding the type of action, antibiotics can be bacteriostatic or bactericidal.[75] The ones that kill bacteria, targeting the cell wall or cell membrane, or interfere with essential bacterial enzymes, are referred as bactericidal.[75,76] Those that target protein synthesis, slowing the bacterial multiplication rate, are referred as bacteriostatic.[76]

Antibiotics can be natural or synthetic.[75] Natural antibiotics are produced by a variety of bacteria and fungi, being able to inhibit or killing other microorganisms.[49] Natural antibiotics can often be artificially

modified to improve their efficacy, being then denominated as semisynthetic antibiotics.[49] Synthetic antibiotics are manufactured by chemical procedures, having the ability to inhibit or kill pathogenic microorganisms.[54] They are designed to have even higher efficacy and lower host toxicity.[75]

The susceptibility of individual microorganisms to different compounds varies significantly, due to the range of effectiveness.[49,54] Antibiotics can present a narrow or broad spectrum of activity.[75] The narrow spectrum compounds are effective only against a limited range of pathogens, generally acting only against gram-positive or gram-negative bacteria.[54,75] Broad-spectrum antibiotics act against several groups of pathogens, including both gram-positive and gram-negative bacteria.[54,75]

Considering their structural basis, antibiotics have been classified into β-lactams, combined or not with inhibitors, aminoglycosides, macrolides, quinolones and fluoroquinolones.[75]

Finally, the mechanism of action of an antibiotic is one of the most important factors that influences its choice as a therapeutic agent.[75] Antibiotics can be subdivided into four groups: cell wall synthesis inhibitors, protein synthesis inhibitors, nucleic acid synthesis inhibitors and inhibitors of membrane function.[54,75]

Following guidelines from Lipsky et al (2012), Bader (2008), Chidiac et al (2007), for the management of DFI with different infection severities three antibiotics are usually selected: clindamycin, gentamycin and vancomycin.[33,36,40,77]

4.1. Clindamycin

The lincosamide class was first characterized in the 1960s and includes semisynthetic derivates, clindamycin (most clinically relevant) and pirlimycin.[78] Once the emergence of multidrug-resistant pathogens has become a serious concern, lincosamide use has been revisited.[78]

Clindamycin was developed in 1966 by chemical modification of the naturally occurring lincomycin.[79] It is a bacteriostatic agent, acting by inhibition of protein synthesis at the level of the 50S subunit of the bacterial ribosome.[61,80] Protein synthesis is inhibited primarily in early elongation by interference with the transpeptidation reaction, resulting in a prolonged post-antibiotic effect.[79,80] Clindamycin can decrease toxin production and increase microbial opsonization and phagocytosis even at sub-inhibitory concentrations.[79] Clindamycin is metabolized and excreted by the liver, therefore, in hepatic insufficient individuals, the half-life can be extended twice and doses should be reduced accordingly.[79,81]

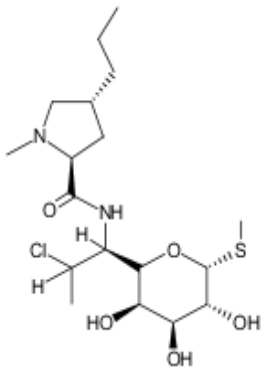

Figure 4: Clindamycin chemical structure (original).

Nowadays, clindamycin is used for the treatment of a broad-spectrum of infections and can be used topically, orally and parenterally.[78,82] Due to its broad-spectrum, excellent tissue penetration and activity against *S. aureus*, clindamycin has been considered an effective choice for the treatment of various skin and soft tissue infections, like DFI.[61,79]

Clindamycin was listed as an alternative for the treatment of mild, moderate and severe diabetic foot infections, although in the last two cases it should be combined with another antibiotic.[79] It has been found that clindamycin can be effective in the treatment of MRSA infections, being community-acquired MRSA infections more susceptible to clindamycin than the hospital-acquired ones.[78]

4.2. Gentamycin

Gentamycin is produced by the actinomycete *Micromonospora purpurea* and belongs to the class of aminoglycosides.[49,54] Although in this class a considerable variation in compounds structure can be observed, all aminoglycosides contain amino sugar bond and a cyclohexane ring.[49,54] Changes in the original structural units of aminoglycosides can be performed either by synthetic or enzymatic mechanimsms.[75]

Gentamycin, has been widely used in medical applications, but due to the progression of pharmaceuticals, its prescription has decreased and is now considered a last choice antibiotic used mainly when other compounds fail.[49,83] Currently, aminoglycosides account for less than 4% of the total of all antibiotics produced and used.[49]

Gentamycin is a broad-spectrum antibiotic with bactericidal activity for some gram-positive bacteria, like *S. aureus*, and can be used in combination with broad-spectrum β-lactams to treat polymicrobial infections.[82-85] It is commonly used for the treatment of moderate and severe DFI and for prophylaxis.[36,85,86]

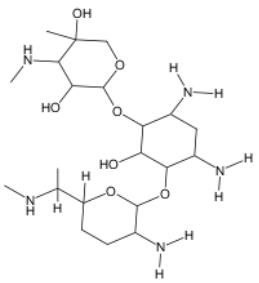

Figure 5: Gentamycin chemical structure (original)

It acts by inhibition of protein synthesis, binding to the 30S ribosomal subunit, causing misreading of t-RNA, leaving the bacteria unable to synthetize proteins vital to its growth.[54,82] Several different steps in protein synthesis can be affected: aminoacyl-tRNA binding, peptide bond formation, mRNA reading and translocation.[54]

Since gentamycin is able to discriminate between prokaryotic and eukaryotic ribosomes, its therapeutic efficacy is high but not as high as the one from cell wall inhibitors.[54] Gentamycin can be effective even when the bacterial load is large and resistance rarely develops during treatment.[83]

4.3. Vancomycin

Glycopeptide antibiotics are actinomycete-derived compounds.[87] The first member of this class was vancomycin, discovered in 1950, derived from *Streptomyces orientalis* (now called *Amycolatopsis orientalis*).[87,88] This antibiotic was found to be bactericidal for *Staphylococcus*, especially against MRSA.[54,89]

Vancomycin was approved as a clinical agent for the treatment of bacterial infections in 1958.[89] However, its toxicity profile and the availability of less toxic alternatives, like β-lactams, made its use rare.[88] It regained prominence after the large-scale emergence and spread of MRSA strains and extensive β-lactams resistance, being nowadays considered a last resort antibiotic against MRSA.[88,90]

Regarding DFI, vancomycin is administrated orally or intravenously and applied in cases of severe infection.[49,54]

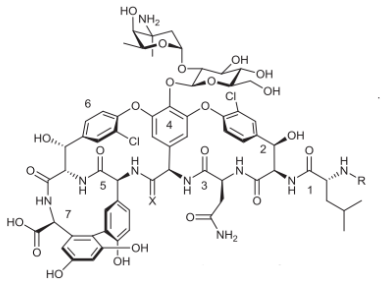

Figure 6: Vancomycin chemical structure.[87]

Vancomycin is composed of a peptide linked to a disaccharide, has a narrow-spectrum and slow bactericidal activity.[54,61] It inhibits cell wall synthesis, having a high therapeutic index because they target structures that are not found in eukaryotic cells.[54] Vancomycin acts by binding tightly to D-alanyl-D-alanine containing peptide at the free carboxyl end.[91] Thus, the synthesis of peptidoglycan is blocked and the membrane-bound lipid intermediates accumulate in the presence of the antibiotic.[91] Vancomycin may also alter the permeability of bacterial cytoplasmic membranes and may selective inhibit RNA synthesis.[82]

Although antibiotics have revolutionized medicine in many respects, unfortunately, the use of these drugs has been accompanied by the rapid appearance of resistant strains, leading the need to search for new therapeutics alternatives.[92]

5. Alternative Therapeutics

Over the years a decrease in microbial susceptibility to existing antimicrobial agents, has been observed both in the hospitals and community settings.[93]

The emergence and dissemination of antibiotic resistance bacteria in DFU patients has led to a lack of response to traditional antimicrobial therapies.[26,94] This biological phenomenon is not recent, being the presence of MRSA and MDR species a major problem.[95,96]

The dramatic spreading of antibiotic-resistant staphylococci is caused by unreasonable usage of antibiotics, especially during long-term therapy with antibiotics belonging to the same group or by their administration without a prior susceptibility assay of the etiological strain responsible for the infection.[97] Since there are very few new antibiotics in the drug development pipeline, it becomes critical to develop alternatives to classical and identify new approaches to treat bacterial infectious diseases.[98]

Biocides and antimicrobial peptides (AMP) are some of the compounds that can be applied as alternatives to classic antibiotic therapeutics or, at least, as complementary therapeutics tools, to treat infectious diseases.

5.1. Biocides

Biocides, a group which includes disinfectants, antiseptics and preservatives, usually refer to any chemical or physical antimicrobial agent that inhibits or inactivates organisms that are harmful to human or animal health.[99-101] They are widely used in hospitals and other healthcare settings, playing an essential role in infection control and in the preventing of infectious organisms transmissions.[99] They are produced both in liquid and powder forms, in ready-to-use formulations, or as concentrates, and are applied using a variety of techniques.[102]

Unlike antibiotics, which target a specific physiological process in the bacteria, most biocides act in more than one target site, which renders resistance development leads to a less common.[103,104] A particular biocide may thus inactivate (or sometimes inhibit) more than one type of microorganism.[105] However, at low or sub-inhibitory concentrations, the action of a biocide may be reduced to a single target site.[103]

Because biocides antimicrobial activity may vary, other terms may be more specific, including "-static," referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and "-cidal," referring to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal).[106]

5.1.1 Chlorhexidine

Chlorhexidine is an antiseptic, which destroys or inhibits the growth of microorganisms present in or on living tissue.[106] It was developed by Imperial Chemical Industries (Manchester, UK) in the 1950s and since then has been used worldwide as a topical antiseptic solution.[107,108] Chlorhexidine is included on the World Health Organization's List of Essential Medicine, which represents the minimum list of medicines required for a basic health-care system by listing the most effective, safe and cost-effective medicines for priority conditions.[109]

Chlorhexidine is a synthetic cationic biguanide with two symmetrical 4-cholorophenyl rings and two biguanide groups connected by a central hexamethylene chain (Figure 7).[110] It has broad activity spectrum against gram-positive and gram-negative bacteria (having an increased affinity for the cell wall of gram-positive organisms), facultative anaerobes and aerobes, yeasts, fungi and some lipid-enveloped viruses.[107,108]

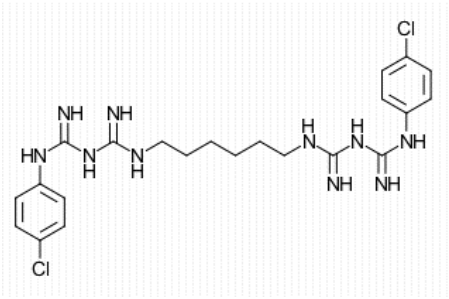

Figure 7: Chlorhexidine chemical structure (original)

The antimicrobial effect of chlorhexidine involves the attraction and adsorption of cationic molecules to the cell surface of microorganisms, being its activity pH and concentration dependent.[106,111]

At low concentrations, chlorhexidine affects membrane integrity.[108] It penetrates and disrupts the bacterial cytoplasmic membrane, leading to an alteration of the bacterial cell osmotic equilibrium and leakage of potassium and phosphorous, resulting in a bacteriostatic effect.[107,108] At higher concentrations, chlorhexidine exerts a bactericidal action. It enters the cytoplasm through the damaged cytoplasmic membrane, forming irreversible precipitates with intracellular adenosine triphosphate and

nucleic acids, resulting in cell death.[107,112] In fact, prolonged exposure increases the bactericidal effect for most bacteria.[107] The uptake of chlorhexidine by bacteria is extremely rapid, with a maximum effect occurring within 15 to 30 seconds.[106,113] In contrast with other antiseptic agents, the residual antimicrobial activity of chlorhexidine is not affected by the presence of body fluids or blood.[114]

Chlorhexidine has also shown some ability to inhibit microorganism's adherence to surfaces, thereby preventing the growth and development of biofilms.[110]

Chlorhexidine is commercially available at a variety of concentrations (0.5%–4%) and formulations (with and without isopropyl alcohol or ethanol).[108] It can be used in children and adults therapeutics since it has provided an excellent record of safety and efficacy for applications as diverse as skin and hand disinfection, vaginal antisepsis, treatment of gingivitis, body washes to pre-vent neonatal sepsis, cosmetics (additive to creams, toothpaste, deodorants and antiperspirants) and pharmaceutical products (preservative in eyedrops, active substance in wound dressings and antiseptic mouthwashes).[108,115]

Daily skin cleansing with the antiseptic agent chlorhexidine has been used to control outbreaks of *S. aureus* infections, having an important role in prevention and control measures during MRSA outbreaks.[108,116,117] Additionally, chlorhexidine has been demonstrated to be effective against MRSA responsible for recurrent skin and soft-tissue infections, supporting its inclusion as the active ingredient in several wound dressings used to treat DFI.[117,118]

5.2. Antimicrobial Peptides

Antimicrobial peptides (AMPs, also known as host defense peptides or HDPs), are a diverse class of molecules that function as a first line of defense against microbial threats, inducing both pro- and anti-inflammatory signals.[98,119,120] So far, there are listed 2901 examples of AMPs originated from all kingdoms of life, such as bacteria, archaea and eukaryotes (including plants, animals, fungi and protists).[120,121]

In general AMPs consist of molecules with 7-100 amino-acid residues.[122] These peptides lack any specific consensus of amino-acid sequences that are associated with biological activity, but most of them maintain certain common features, such as containing positive charge, relatively hydrophobic and amphipathic structure.[123]

Based on their amino-acid composition, size, and conformational structures, AMPs can be divided in four categories (Table 2).[120,123]

Table 2: AMPs division based on their conformational structures. Adapted from Steckbeck et al 2014 and Andersson et al 2016.[119,120]

Family	Description
Alpha	Helical structures
Beta	β-strands
Alphabeta	Both α-helical and β-strands structures in the same 3D fold
Non-alphabeta	Neither α-helical nor β-strands

They are multifunctional molecules with antimicrobial activity against bacteria (gram-positive and gram-negative), fungi, viruses and protozoan parasites.[98,124] Some AMPs also exhibit antitoxic activity, neutralizing bacterial toxins.[95] Additionally, AMPs are able to prevent biofilm formation and act on pre-formed biofilms, supporting their potential as alternatives to currently available DFI therapeutic agents.[96,98]

The mechanisms of action of AMPs are diverse and in some cases AMP specific.[98] The antibacterial properties are associated with two interrelated characteristics of peptides: their net charge and their propensity to be amphipathic. Both features facilitate their interaction with the negatively charged components of the bacterial envelope and with the negatively charged phospholipids of the bacterial membrane.[120]

The fact that AMPs can have complex, multi-target mechanisms that can be distinct from antibiotics, may avoid the resistance development.[119]

5.2.1. Nisin

Bacterial AMPs are called bacteriocins, being produced by both gram-positive and gram-negative bacteria.[125] Bacteriocins are peptides or proteins that show bacteriostatic and/or bactericidal activity against other bacteria, not affecting the producing strain.[126,127] As a group, bacteriocins are heterogeneous and can be classified based on their molecular weight, bacterial spectrum, chemical structure and mode of action (Table 2).[125,127]

Table 3: Classes of bacteriocins produced by gram-positive bacteria. Adapted from Jozala et al 2015 and Abts et al 2011.[125,128]

Class	Characteristics
I (lantibiotics)	Thermostable low-molecular peptides (<5 kDa), 19-38 amino acid residues; posttranslational modification; divided into two subclasses: type A (linear molecules) and type B (globular molecules).
II	Thermostable low molecular peptides (<10 kDa); 30-60 amino acid residues; do not undergo posttranslational modification.
III	Thermolabile bacteriocins of high molecular weight (>30 kDa); complex in nature of activity and protein structure; mechanism of action distinct from others bacteriocins (bacteriolysins).

Class I bacteriocins are also called lantibiotics because they contain the posttranslational modified amino acids lanthionine and methyllanthionine, being also the major group within this class.[124,129,130] This class includes nisin, one of the best characterized lantibioic produced by Lactic Acid Bacteria (LAB).[130,131] It is produced by *Lactococcus lactis* subsp. *lactis* strains.[131] Its biosynthesis pathway requires the expression of at least 11 gene products and its major polypeptide is Nisin A.[132,133]

This bacteriocin was first commercialized in England in 1953 and has been approved for use in over 48 countries.[133] It was considered safe for use in foods in 1969 by the Joint Food and Agriculture Organization from World Health Organization (FAO/WHO) Expert Committee on Food Additives.[125] In 1983, this bacteriocin was added to the European list of food additive under the number E234 and, in 1988, it was approved by the US Food and Drug Agency (FDA) as generally regarded as safe (GRAS) for use in pasteurized products and processed cheeses to inhibit the growth of *Clostridium botulinum*.[125,127,133]

Nisin is a cationic polypeptide that is synthesized in the ribosome and is composed by 34 amino acids (3,5 kDa).[124,131] Its amino acids composition is rarely found in nature, which consist in one lanthionine, four β-methyllanthionine, one Dhb (dehydrobutyrine) and two Dha (dehydroalanine) residues.[124,125,131] Nisin solubility, stability, and biological activity are highly dependent on pH, temperature and the nature of the substrate**.** Nisin solubility and stability increase with acidity, rendering it to be almost insoluble under neutral or alkaline conditions.[125] This might be due to the fact that Dha and Dhb are susceptible to modifications by nucleophiles that are present at high pH.[131]

The spectrum of action of nisin includes a range of gram-positive bacteria and spore germination, but it has little or no activity against gram-negative bacteria, fungi or viruses.[124,125] It exerts two mechanisms of action: interfering with cell wall synthesis and pore formation.[125]

Since this bacteriocin is positively charged with hydrophobic regions, it may develop electrostatic interactions with the negatively charged phosphate group from the cell membrane.[125] At this point, lipid II (essential membrane-anchored cell-wall precursor and also the target for therapeutic antibiotics such as vancomycin) serves as a docking molecule.[126,128,133] This leads to a bacteriostatic effect due to the masking of lipid II and the inhibition of cell wall synthesis.[126,134] Posteriorly, the binding of nisin to lipid II induces the integration of nisin in the membrane, resulting in formation of pores (Figure 8).[128] These membrane pores have 2-2.5 nm in diameter, allowing small and essential molecules (like K^+, ATP and amino acids) to leak from the cell, resulting in the disruption of the barrier function and, consequently, in the dissipation of the membrane potential.[125] Finally, it results in the abrupt arrest of all cellular processes and in cell death (bactericidal effect).[125,126] The fact that amino acids at the N-terminal domain of nisin binds to an invariable region of lipid II, helps to avoid resistance development by microorganisms.[133]

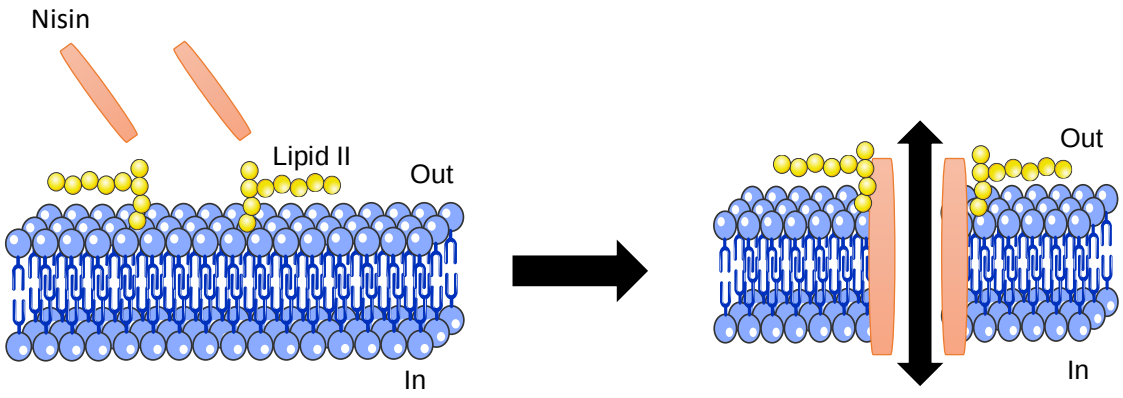

Figure 8: Mode of action of nisin on a cell target and posterior binding to lipid II, permeabilizing the membrane which leads to pore formation (original).

Nisin has shown promising activity towards multi-drug resistant staphylococci clinical isolates, MRSA and also against biofilms.[128,135]

5.3. Guar Gum Gel

Natural polysaccharides, which are obtained from a biological origin, are now recognized by their potentially influence in the rate and/or extent of absorption of a drug.[136,137]

Polysaccharides are complex polymers comprising multiple monosaccharides units interlinked with glyosidic linkages to form a large, branched or unbranched chain.[136] They can have different origins, such as algae, plants, bacteria and animal.[138] Pharmaceuticals industries preferred this kind of polysaccharides as a drug delivery system over the synthetic, mainly because of their safety, non-toxicity, biodegradability, biocompatibility, abundant availability in nature, ecofriendly and economical costs.[98,139]

In the context of wound healing, a delivery system of antimicrobial compounds should accomplish the following characteristics: maintain its bioactivity through protection from proteolysis in the wound bed; increasing its bioavailability by preventing rapid dilution in wound fluid and systemic uptake; and distribution and release within the wound at a physiologically relevant rate and duration.[140] Most of natural polysaccharides used in food, pharmaceutical and cosmetic industries are regarded as safe for humans.[136]

The applications of natural polysaccharides have expanded over the years due to their versatility, being able to be used as thickeners, suspending agents, moisturizers, emulsifiers, emollients and as wound-healing agents.[136]

In recent years, a considerable attention has been focused on hydrophilic polysaccharides.[138] Gums have a large industrial application due to their ability to form gels, forming viscous solutions or stabilizing the emulsion systems.[141]

Natural gums are polysaccharides consisting of multiple sugar units linked together to create large molecules.[142] Thus, these molecules show tremendous variation in the length of the linear chain, branching characteristics and molecular weight.[136] Most of gums are produced by higher plants, as part of their protection mechanisms against mechanical or microbial injury.[136,141] The different available gums can be classified based on the source (plant exudate, seed, microbial or marine), charge (non-ionic seed or anionic), semi synthetic nisin (starch derivates or cellulose derivates), shape (linear or branched) and monomeric units in the chemical structure (homoglycans, diheteroglycans, tri-heteroglycans, tetra-heteroglycans or penta-heteroglycans).[136,143]

Guar gum is a natural non-ionic, water soluble polysaccharide obtained from the ground endosperm of the seed of the leguminous crop *Cyamopsis tetragonolobus*.[98,136,143] It grows in arid zones of west and north-west India, Pakistan, Sudan and parts of USA.[136] Guar gum comprises high molecular weight polysaccharides, composed by galactomannanas consisting of linear chains of (1→4)-β-d-mannopyranosyl units with α-D-galactopyranosyl units attached by (1→6) linkages.[138,143] Water is the most important solvent for galactomannans, forming hydrogen bonds that confer a high viscosity to the solution even at low concentrations.[98,136]

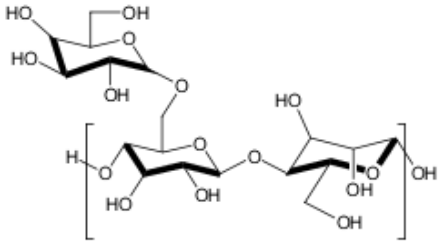

Figure 9: Guar Gum chemical structure (original)

Due to its thickening, emulsifying, gelling and binding properties, quick solubility in cold water, wide pH stability and film forming ability, guar gum is used in pharmaceuticals formulations, having also application as a versatile system for the delivery of bioactive agents.[98,138]

The prevalence of infectious diseases caused by *S. aureus*, including DFI, and their rapid ability of these strains to become resistance to various antibiotics, has led to the search for new therapeutic compounds. For this, the development of *in vitro* protocols to test the efficacy of new compounds or of new therapeutic protocols is mandatory.

Chapter 2

Material and Methods

6. Objective

The first aim of the present study was to evaluate the inhibitory potential of the biocide chlorhexidine against *S. aureus* isolates obtained from DFU. The next step was to evaluate its inclusion as a complementary step in common DFI therapeutic protocols, by determining the efficacy of combined use of chlorhexidine, antibiotics (clindamycin, gentamycin and vancomycin) and an antimicrobial peptide incorporated in a delivery system against the *S. aureus* isolates. This experiment allowed us to assess the inhibitory and eradication effect that each compound or combination of compounds has against biofilm-based isolates.

7. Bacterial Strains

Isolates under study were obtained in a previous epidemiological survey regarding DFU infections, conducted at 4 clinical centers in Lisbon from January to June 2010.[3] A total of 53 *Staphylococcus* spp were collected and isolated from samples obtained from 49 DFU patients. From this collection, 23 representative biofilm-producing *S. aureus* isolates were then selected, based on Pulse Field Gel Electrophoresis (PFGE) and Multilocus Sequence Type (MLST) analysis.[43] In addition to these 23 isolates, a reference strain, *S. aureus* ATCC 29313, a known biofilm producer was also included as a control strain.

Regarding the antimicrobial resistance profile of the strains, it was characterized by the determination of Minimal Inhibitory Concentration (MIC) for ten antibiotics and multiplex PCR for detection of genes: *mecA* and its homologous *mecC, erma, ermB, ermC, blaZ, msrA, aac-aph, tetK, tetL, tetM, tetO* and *norA*.[43] It was observed that 35% (n=8) of the isolates were resistant to cefoxitin and carriers of the *mecA* gene, thus being classified as MRSA (Table 4, in supplementary data). Moreover, 30% (n=7) were considered to be multidrug resistant, since were resistant to three or more antimicrobials belonging to different antibiotic classes.[2,3] Isolates were also previously characterized regarding their phenotypic virulence profile, including the presence of exoenzymes such as coagulase, hemolysins, gelatinase, DNase, lipase, biofilm production and virulence genes, such as: *agrI*, *agrII*, *agrIII* and *agrIV*, *bap, icaA, icaD, atl, pls, clfa, spa, coa, tst* and *pvl* (Table 5, in supplementary data).[144,145]

Isolates were stored at -20ºC in buffered peptone water plus 20% of glycerol during the period of this study. When necessary, strains were grown in a nonselective Brain Heart Infusion (BHI) agar medium (VWR Chemicals, Belgium) at 37ºC for 24h.

8. Minimum Inhibitory Concentration (MIC) for Chlorhexidine

After incubation, bacterial suspensions were performed for each isolate in 5 mL of sterile normal saline (NaCl) (Merck, Germany) and their concentration were standardized visually using a 0.5 McFarland standard (BioMérieux, France), which corresponds to approximately 10^8 CFU/mL.

Afterwards, bacterial suspensions were diluted in 9 mL of BHI broth (VWR Chemicals, Belgium) to obtain a suspension with a concentration of 10^7 CFU/mL.

MIC were determined using the broth microdilution method, using 96-well flat-bottomed polystyrene microtiter plates (VWR, Belgium).[96,146] The set of chlorhexidine (AGA, Portugal) concentrations tested (obtained by diluting chlorhexidine at 4% (AGA, Portugal) in sterile water) was as follows: $1x10^{-4}$, $5x10^{-4}$, $1x10^{-3}$, $5x10^{-3}$, $1x10^{-2}$ and $5x10^{-2}$ %. These concentrations correspond, respectively, to 1, 5, 10, 50, 100 and 500 µg/mL.

In all wells were distributed 25 µL of chlorhexidine solution, except for the negative control, that only contained broth medium. Afterwards, 150 µL of the 10^7 CFU/mL bacterial suspension was also placed in each well (Figure 10). For that reason, the final concentrations of chlorhexidine in the wells were: 0.143, 0.714, 1.429, 7.143, 14.286, 71.429 µg/mL.

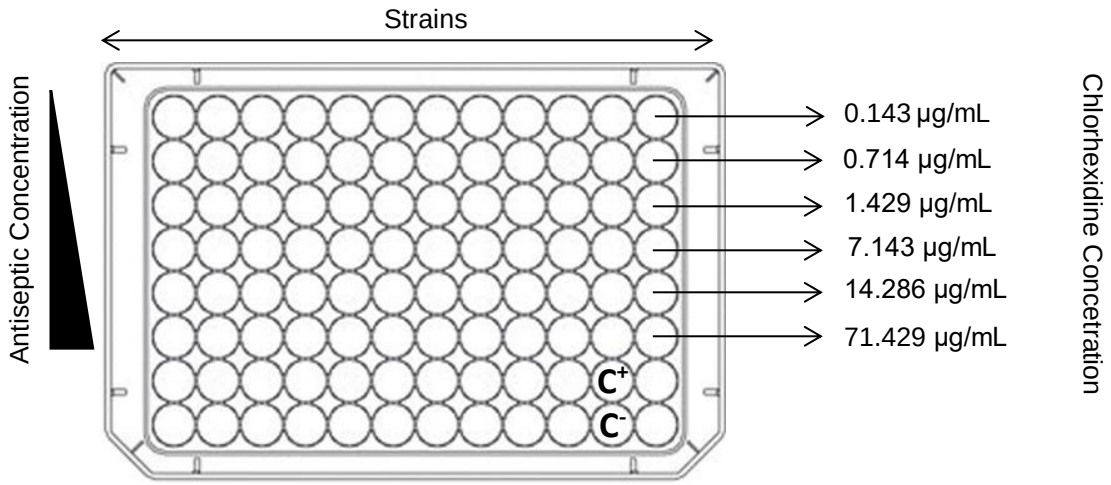

Figure 10: Scheme used for MIC protocol. C^+: positive control; C^-: negative control.

8.1. Minimum Bactericidal Concentration (MBC) for Chlorhexidine

MBC assessment was carried out after MIC determination, by inoculating 3 µL of the suspensions from the wells where there was no visible growth on Brain Heart Infusion (BHI) agar plates, followed by incubation at 37ºC for 24h. MBC was determined as the lowest chlorhexidine concentration at which no colonies were observed.[96] Trials were held in duplicate and independent replicates were performed at least three times in different days.

9. Preparation of Compounds Tested

9.1. Chlorhexidine

Concentration of chlorhexidine used in this assay was the mean value of all strains obtained in the MIC assay (Table 6, in supplementary data). Therefore, the concentration used was $6x10^{-4}$ %, which correspond to 6 µg/mL in the well.

9.2. Nisin Incorporated in Guar Gum Gel

Concentrations of nisin, guar gum gel and nisin incorporated in guar gum gel used in this study were the ones previously described by Santos et al.[96]

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). This solution was filtered using a 0.22 µm Millipore filter (VWR, Belgium) and stored at 4 ºC. The stock solution was then diluted with sterile water to a concentration of 45 µg/mL.

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 gel in a proportion of 1:1. For this, 1.8 mL of the stock solution of nisin was diluted in 38.2 mL of sterile distilled water, which was added to the 40 mL of guar gum gel. Thus, there was obtained a final gel of 0,75% (w/v) at 22.5 µg/mL, corresponding to the MIC previously determined.[96]

9.3. Antibiotics

The antibiotics used in this assay where Clindamycin, Gentamycin and Vancomycin. MIC concentrations used in this study were previously described by Mottola et al. 2016.[43] MIC values obtained for each antibiotic were as follows: for Clindamycin, 0.033 µg/mL; for Gentamycin, 0.238 µg/mL; and for Vancomycin, 0.531 µg/mL.

In order to obtain these concentrations, stock solutions were performed, using 6.6 mg of Clindamycin, 4.76 mg of Gentamycin and 10.62 mg of Vancomycin. Respectively each one of the antibiotics weighted were diluted in 10 mL of sterile water and filtered using a 0.22 µm Millipore filter (VWR, Belgium)., allowing to obtain stocks solutions of 0.66 mg/mL, 4.76 mg/mL and 1.062 mg/mL.

From the stock solutions, serial dilutions (1:10) were performed to obtain the finals concentrations of: 0.66 µg/mL (0.033 µg/mL in the well) for Clindamycin; 4.76 µg/mL (0.238 µg/mL in the well) for Gentamycin; and 10.62 µg/mL (0.531 µg/mL in the well) for Vancomycin.

10. Combined Protocol

A modified version of the Calgary Biofilm Pin Lid Device was used to determine the antimicrobial susceptibility of bacteria embedded in a 24h biofilm in order to evaluate which of the compounds or combination of compounds has a best inhibitory and/or eradication effect.[96,147] For this assay, bacterial suspensions were prepared as previously described for the MIC protocol. Afterwards, bacterial suspensions were diluted in Tryptic Soy Broth (TSB) (VWR Chemicals, Belgium) medium supplemented with 0.25% (w/v) glucose (Merck, USA), to a concentration of 10^6 CFU/mL. Then, 200µL of the bacterial suspensions were distributed in a 96-well flat-bottomed polystyrene microtiter plate (Nunc, Thermo

Fisher Scientific, Denmark), covered with 96-peg polystyrene lids (Nunc, Thermo Fisher Scientific, Denmark) and statically incubated for 24h at 37ºC, to allow biofilm formation on the pegs.

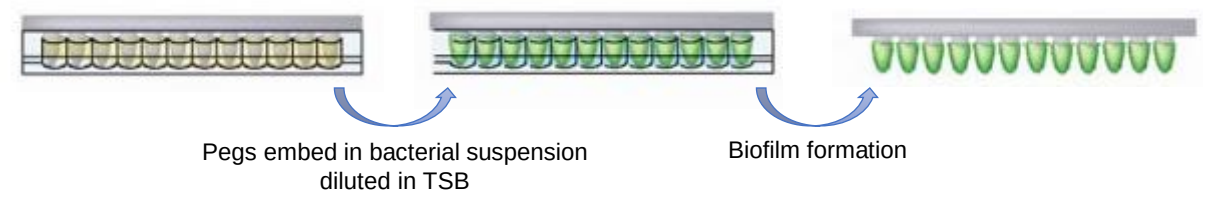

Figure 11: Biofilm formation in the peg lids. Adapted from Harrison et al 2005.[148]

During the period of 24h biofilm formed in the peg lids, was rinsed periodically, at intervals of 8h, in different combinations of antiseptic, antibiotics and antimicrobial peptide solutions. These steps were performed in 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fisher Scientific, Denmark) at room temperature. The assays were performed by placing the lids three times in 0.9% NaCl (Merck, Germany) for 30 seconds, to remove planktonic bacteria; one time in chlorhexidine during 15 seconds; one time in nisin incorporated in guar gum gel for 3 minutes; and a final drying step, in an empty microplate during 30 minutes.

After this drying step, peg lids were placed on microplates containing 10 µL of antibiotic (Clindamycin, Gentamycin or Vancomycin) plus 190 µL of TSB (VWR Chemicals, Belgium) medium supplemented with 0.25% (w/v) glucose medium. Then, the microplates were incubated at 37ºC during 8h, until the next rinsing step. A total of three cycles were performed.

The inhibitory action of different combinations between the compounds was tested in the formed biofilms, as shown in Figure 12.

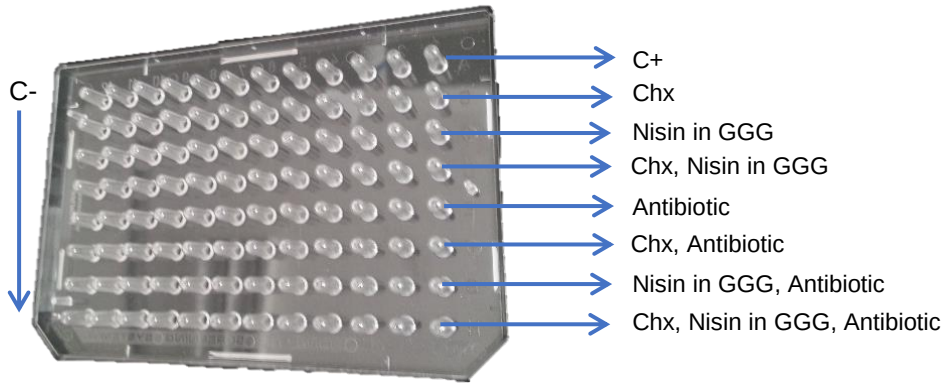

Figure 12: Scheme of the different combinations to which the biofilm formed in the peg lids was subjected. C+: Positive Control; C-: Negative Control; Chx: Chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel; Antibiotic: Clindamycin or Gentamycin or Vancomycin.

The inhibitory effect of compounds was determined by removing the peg lids and determining the absorbance values of the suspensions in the 96 well-plate using a microplate reader (BGM LABTECH, Germany).

Pegs lids that were removed for inhibitory action determination were rinsed three more times in 0.9% NaCl, placed in new microplates containing 200 µL of TSB medium supplemented with 0.25% (w/v) glucose (Merck, USA) and incubated in an ultrasound bath (Grant MXB14, England), at 50Hz for 15 minutes, in order to disperse the biofilm-based bacteria from the peg surface. Afterwards, pegs lids were discarded and microplates were covered with normal lids and incubated for 24h at 37 ºC. The eradication effect was determined using the same protocol applied for the inhibitory action.

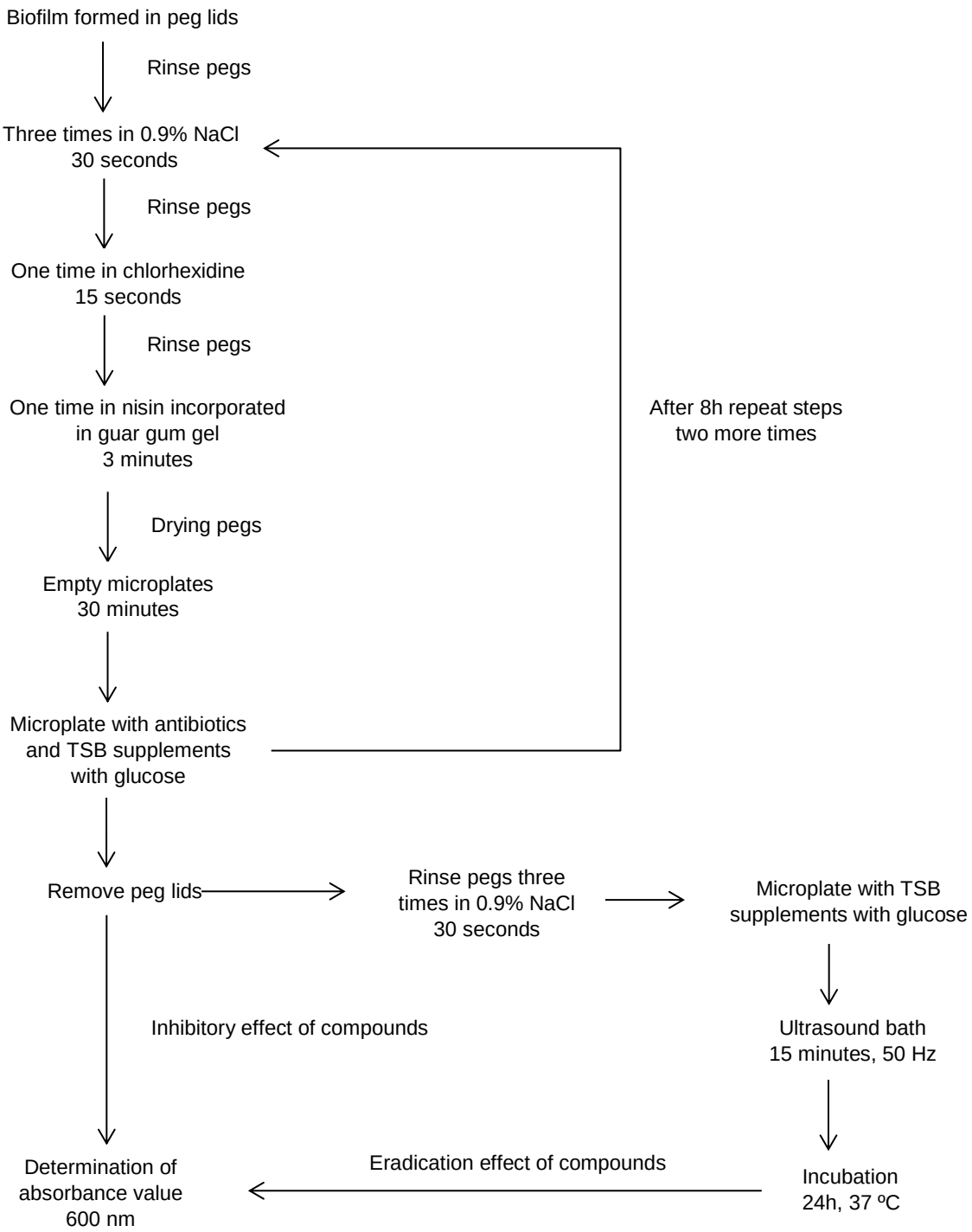

Figure 13: Flow chart of the combined protocol.

11. Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics™ V20 Software for Windows.

Minimum and maximum, mean and standard deviation values were determined for all quantitative variables. Differences between MIC and MBC values were evaluated using the T-test. Correlation between MIC, MBC and antibiotic resistance (previously determined by Mottola, et al. 2016)[43] were determined through Pearson's correlation coefficient. Analysis of variance (ANOVA) for Randomized Complete Block Design (RCBD) was used for evaluating biofilm inhibition and eradication absorbance results, in order to determine which is the most effective combination of compounds.

A two-tailed *p*-value ≤ 0.05 was considered to be statistically significant in all applied tests.

Chapter 3

Results and Discussion

12. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Chlorhexidine

To evaluate the susceptibility to chlorhexidine, MIC and MBC methods are the most used.[149] MIC can be defined as the lowest concentration of the antimicrobial agent that prevents visible growth of a microorganism, while MBC, is the lowest chlorhexidine concentration at which colonies were observed, after the inoculation of wells without visible growth (Figure 14).[96,150]

Figure 14: MIC determined for strains B13.1, B14.2, B23.2, Z1.1, Z2.2 and Z3.1. B: MBC determination for strains B13.1 and B14.2.

In Figure 15, MIC and MBC are represented as a histogram, which is a graphical representation of frequency distribution for each test dilution (µg/mL) of chlorhexidine, showing a normal distribution. Is possible to observed that 50% of the strains had a MIC of 7 µg/mL and 83,3% had a MBC value of 9,8 µg/mL.

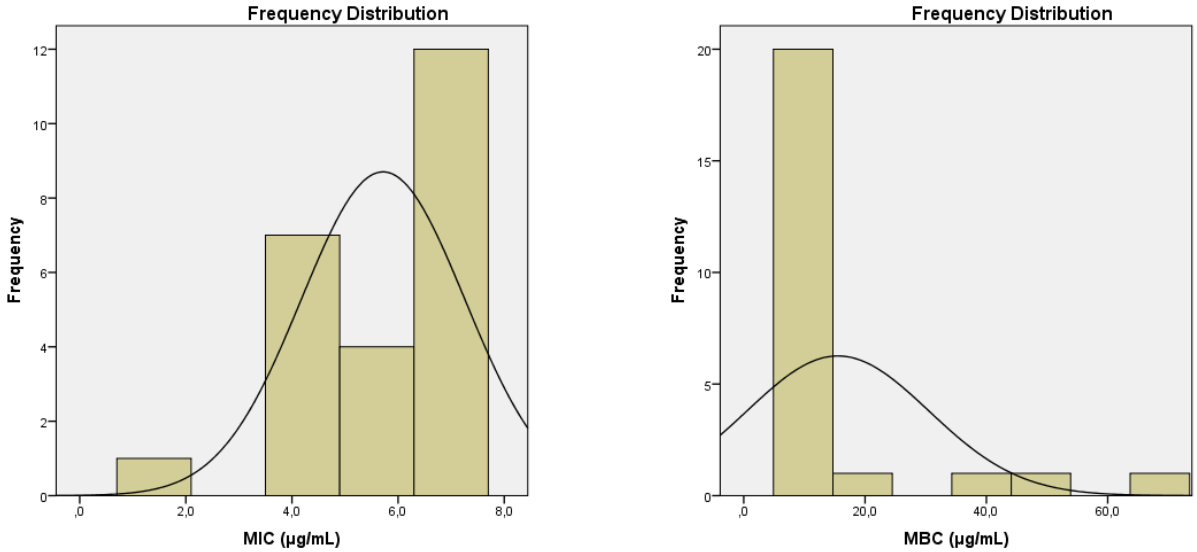

Figure 15: MIC and MBC Frequency Distribution.

MIC and MBC are statistically different (p-value<0.05), as determined through a paired sample T-test (Table 7). Meaning that concentration values of MIC and MBC are deafferents, being MBC values higher.

Table 7: Paired sample T-test for MIC and MBC assays.

Paired Samples Test								
	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
MBC (µg/mL) - MIC (µg/mL)	9.8000	14.7989	3.0208	3.5510	16.0490	3.244	23	.004

The mean values of absorbance obtained for each strain regarding MIC and MBC are present in Table 8. For MIC, mean values were 5.7±1.5 µg/mL, with a minimum value of 1.4 µg/mL and a maximum of 7.0. Regarding MBC values, they were higher, with a mean value of 15.5±14.9 µg/mL, with a minimum of 9.8 µg/mL and a maximum of 68.8 µg/mL.

An antimicrobial agent can be classified as bactericidal if the MBC is no more than four times de MIC value.[96] In this case, MBC values where 2.72-fold higher than MIC, therefore chlorhexidine can be considered bactericidal for 20 strains (including *S. aureus* ATCC 23213) and bacteriostatic for strains A6.3, B7.3, Z12.2.

Using a breakpoint, which is based in MIC, is possible to categorize microorganisms as susceptible (S), which is associated with a greater probability of therapeutic success, and resistant (R), with values above the breakpoint, is related with the probability of therapeutic failure. For isolates classified as intermediate (I), the therapeutic effect is uncertain.[150]

According to the studies conducted by Horner et al 2012, Aykan et al 2013, Schlett et al 2014 and Morrissey et al 2014 is possible to propose a MIC breakpoint. For values less than 8 µg/ml strains are considered susceptible, 8 to 64 µg/ml are low-level resistant and higher than 512 µg/ml are high-level resistance.[117,149,151-153] As results have shown, *S. aureus* strains and MRSA under study are all susceptible to chlorhexidine.

Regarding MBC, diverse values were obtained. Vali et al 2016 described values between 0.94-60 µg/ml, Acton 2011 between 16-32 µg/ml and Liu et al 2016, 32 µg/ml for MRSA. Values obtained in this assay are within these ranges, with exception for isolate B7.3.[154-156]

The fact that isolate B7.3 has high values for MIC and MBC, can be related to the fact of being a MRSA and MDR strain. Furthermore, harbor the antibiotic resistance gene *norA* which presence is related to increased tolerance to disinfectants agents, such as chlorhexidine.[156]

Table 8: MIC and MBC absorbance values of chlorhexidine.

Strains	MIC (µg/mL)	MBC (µg/mL)	
A 1.1	5.6	9.8	Bactericidal
A 5.2	4.2	9.8	Bactericidal
A 6.3	4.2	39.2	Bacteriostatic
B 3.2	5.6	9.8	Bactericidal
B 3.3	5.6	9.8	Bactericidal
B 7.3	7.0	68.6	Bacteriostatic
B 13.1	7.0	9.8	Bactericidal
B 14.2	5.6	9.8	Bactericidal
Z 1.1	7.0	19.6	Bactericidal
Z 2.2	7.0	9.8	Bactericidal
Z 3.1	7.0	9.8	Bactericidal
Z 5.2	4.2	9.8	Bactericidal
Z 12.2	1.4	9.8	Bacteriostatic
Z 14.1	4.2	9.8	Bactericidal
Z 16.1	4.2	9.8	Bactericidal
Z 17.2	4.2	9.8	Bactericidal
Z 21.1	7.0	9.8	Bactericidal
Z 21.3	7.0	9.8	Bactericidal
Z 23.2	4.2	9.8	Bactericidal
Z 25.2	7.0	9.8	Bactericidal
Z 27.2	7.0	9.8	Bactericidal
Z 27.3	7.0	49.0	Bacteriostatic
Z 32.2	7.0	9.8	Bactericidal
ATCC 23213	7.0	9.8	Bactericidal
Mean	5.7	15.5	
Minimum	1.4	9.8	
Maximum	7.0	68.6	
Std. Deviation	1.5	14.9	

Correlations between MIC, MBC and antibiotic resistance were evaluated by Pearson correlation, being observed that the linear relationship between these variables are weak (Table 9). The MIC and MBC values are not dependent of the strains antibiotic resistance profile, which means that regardless of whether bacterial strains have resistance to one or more antibiotics, it would not affect MIC or MBC.

Table 9: Pearson correlation between MIC, MBC and Antibiotic Resistance.

Correlations

		MIC (µg/mL)	MBC (µg/mL)	Antibiotic Resistance
MIC (µg/mL)	Pearson Correlation	1	.177	.208
	Sig. (2-tailed)		.409	.330
	N	24	24	24
MBC (µg/mL)	Pearson Correlation	.177	1	.035
	Sig. (2-tailed)	.409		.870
	N	24	24	24
Antibiotic Resistance	Pearson Correlation	.208	.035	1
	Sig. (2-tailed)	.330	.870	
	N	24	24	24

13. Biofilm Inhibition and Eradication

Microbial biofilms have received a lot of attention recently, as this growth mode may be a key factor in persistent or chronic infections.[68] Bacterial growth as a biofilm structure almost always leads to an increase in resistance to antimicrobial agents when compared with cultures grown as suspensions (planktonic), with up to 1000-fold decrease in susceptibility.[157] Therefore, therapeutic protocols against biofilm-related infections should be considered in order to improve management strategies of these infections.[72]

To determine which were the treatments under study with a higher biofilm inhibitory and eradication effect an ANOVA RCBD was performed. This type of analysis is used for estimating the effect of multiple treatments.[158] For this statistical analysis, each one of the strains was considered a block, since the strains under study are similar to one another, that was subject to all the treatments. Each block is composed by one strain and its replicates. This process is called blocking and its purpose is to reduce as much variability as possible to make differences between treatments more evident.[158]

In order to compare the action of the different treatments, absorbance values were determined for each strain. The turbidity measurement of microbial cultures is a widely used method to determine the number of microorganisms in a culture.[159]

Absorbance mean values obtained for the assays are presented in Table 10 and 11 (in supplementary data), both for the inhibitory and eradication assays.

13.1. Biofilm Inhibition

Regarding the evaluation of the inhibitory action of the antimicrobials study, it was observed that statistical differences (p-value≤0.05) exist between the independent variables (block and antimicrobials) and the dependent variable (absorbance), indicating that absorbance values vary among blocks and antimicrobials (Table 10). The R Square associated is 0.858, meaning that the model explains most of variability found between variables.

Table 10: Variance between dependent and independent variables regarding the inhibitory effect of antimicrobials.

Tests of Between-Subjects Effects

Dependent Variable: Absorbance

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10.565[a]	38	.278	54.806	.000
Intercept	68.309	1	68.309	13465.416	.000
Block	1.000	23	.043	8.569	.000
Antimicrobials	9.565	15	.638	125.703	.000
Error	1.750	345	.005		
Total	80.624	384			
Corrected Total	12.315	383			

a. R Squared = .858 (Adjusted R Squared = .842)

After statistical analysis was obtained Table 11 (supplementary data). It is a table of multiple comparations between antimicrobials showing significative differences, mean differences, standard error and confidence intervals.

For a better perception of the absorbance values, in Table 12 are represented the averages of the mean absorbance values obtained regarding the inhibition effect of antimicrobials in descending order, and in Table 13 (in supplementary data) are presented the absorbance values obtained for each strain.

Regarding antimicrobials applied alone, were observed that for antibiotics, clindamycin was the compound with higher value of absorbance, being close to the value of positive control and having no significative differences (p-value>0.05), followed by gentamycin and then vancomycin. The three antibiotics had close absorbance values, meaning that their inhibitory effect against the biofilm of the strains under study were similar, although significative differences between them (p-value<0.05) were found.

When chlorhexidine is applied alone, its inhibitory effect against biofilm producing strains is very similar to the antibiotics, as no significative differences were observed between these antimicrobials (p-value > 0.05). Taking this in account, chlorhexidine can be a good alternative to antibiotics applications.

Nisin incorporated in guar gum gel showed inhibitory results higher than chlorhexidine and antibiotics, being observed significative differences between these antimicrobials (p-value<0.05). These results demonstrated that nisin incorporated in guar gum gel had a good inhibitory effect against bacterial biofilm, being able to be an alternative to classic therapeutic for DFI. Besides that, Okuda et al 2013 studies indicated that pore formation leading to ATP efflux is important for the activity against biofilm cells. Suggesting that bacteriocins that form stable pores on biofilm cells are highly potent for the treatment of MRSA biofilm infections.[126]

Concerning dual application of antimicrobials with chlorhexidine, were observed that a sharp decrease in absorbance values is not observed for antibiotics combined with chlorhexidine, though significative differences were found at a statistical level (p-value<0.05). The fact that absorbance values were similar can be related with the low chlorhexidine concentration used and the short incubation period.

The lowest absorbance values were obtained for the dual application of nisin incorporated in guar gum gel and chlorhexidine, and there were significative differences regarding other antimicrobials that were combined with chlorhexidine (p-value<0.05). Through the analysis of absorbance values is possible to observed differences. It should be noted that the inhibitory effect of chlorhexidine increased when combined with nisin incorporated in guar gum gel. The synergetic effect can be related with both compounds acting in the bacteria membrane.[126] Since this combination of antimicrobials had the best inhibitory effect against bacterial biofilm, is a hypothesis to be studied in order to substitute the use of antibiotics in DFI.

Another dual application performed was the combination of antibiotics with nisin incorporated in guar gum gel. When clindamycin is combined with nisin incorporated in guar gum gel, absorbance values reduced for more than half comparing with the absorbance values of the antibiotic when applied alone. The same were observed for gentamycin and vancomycin.

Regarding clindamycin and gentamycin, this synergetic effect can be due to their mode of action. Both antibiotics inhibit protein synthesis, with clindamycin binding to the 50S subunit of the bacterial ribosome and gentamycin to the 30S subunit, affecting bacterial multiplication. When also adding nisin incorporated in guar gum gel, bacteria not only will have difficulties in multiplicate, but pores will also be formed in its membrane leading to cell death.

In case of vancomycin, the synergetic action can be because this antibiotic and nisin are members of two different classes of antimicrobial agents that both target the essential cell wall precursor lipid II. The molecular mechanism of action of both antibiotics is very different but starts with the noncovalent binding to lipid II. This molecular basis of mechanisms may explain how nisin acts in synergy with vancomycin, also several studies have demonstrated synergistic relationships between conventional antibiotics and lantibiotics.[137,160] For example, nisin displayed synergistic activity with ramoplanin and other non-β-lactam antibiotics against many strains of MRSA.[137]

The application of three antimicrobials combined had also a good inhibitory effect against bacterial biofilm. From the three antibiotics, vancomycin was the only that when combined with nisin incorporated

in guar gum and chlorhexidine, absorbance values were higher than the antibiotic combine only with nisin incorporated in guar gum gel. However, there are not significative differences between these two treatments, as well as between antimicrobials including the simultaneous applications of other antibiotics and nisin incorporated in guar gum gel.

The combinations of antimicrobials involving clindamycin or gentamycin combined with nisin incorporated in guar gum gel and chlorhexidine, had the lowest values following dual combination of nisin incorporated in guar gum gel and chlorhexidine, having no significative differences between combination of antimicrobials (p-value>0.05). Meaning that the use of antibiotics in a combination of antimicrobials involving nisin incorporated in guar gum gel and chlorhexidine did not had an increased in the inhibitory effect against the bacterial biofilm.

Table 12: Means of absorbance values for each biofilm inhibitory antimicrobial and respective standard deviation. Chx: : Chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel Abs: absorbance; SD: standard deviation

	Abs	**±SD**
Positive Control	0.654	0.057
Clindamycin	0.626	0.076
Chx	0.599	0.058
Gentamycin	0.580	0.063
Vancomycin	0.563	0.060
Gentamycin, Chx	0.553	0.080
Clindamycin, Chx	0.546	0.175
Vancomycin, Chx	0.480	0.166
Vancomycin, Nisin in GGG, Chx	0.298	0.060
Vancomycin, Nisin in GGG	0.287	0.087
Gentamycin, Nisin in GGG	0.282	0.068
Clindamycin, Nisin in GGG	0.270	0.072
Nisin in GGG	0.264	0.056
Gentamycin, Nisin in GGG, Chx	0.255	0.061
Clindamycin, Nisin in GGG, Chx	0.252	0.066
Nisin in GGG, Chx	0.242	0.054
Negative Control	0.101	

The fact that combination with nisin had the higher inhibitory effects can be related with the ability of nisin to rapidly diffuse in the guar gum gel, increasing it antimicrobial activity.[96] Furthermore, Dosler et al 2011 found that nisin can enhance the activity of antibiotics, such as vancomycin, even when it is used at low concentrations.[160]

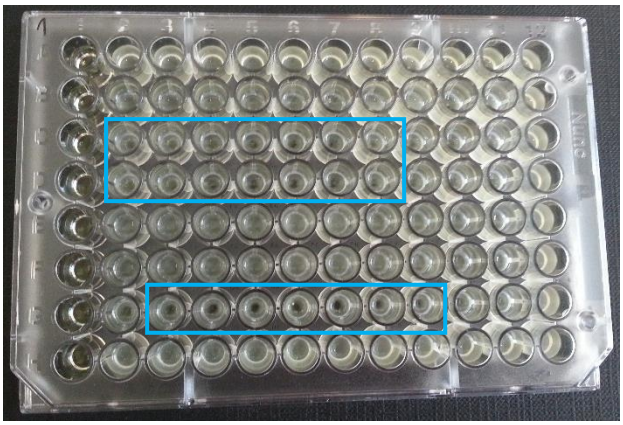

Figure 16: Result obtained in the Inhibitory assay of antimicrobials against biofilm. In blue are signalize the wells which biofilm in peg lids were inhibited (original).

13.2. Biofilm Eradication

As previously observed regarding the inhibitory action of antimicrobials, there were significative differences (p-value ≤ 0.05) between the independent variables (block and antimicrobials) and dependent variable (absorbance). However, the R Squared value obtained was lower (0.499), showing that due to the similarity in absorbance values it was not possible to detect high variability between antimicrobials (Table 14).

Table 14: Variance between dependent and independent variables regarding the eradication effect of antimicrobials.

Tests of Between-Subjects Effects					
Dependent Variable: Absorbance					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.160[a]	38	.031	9.036	.000
Intercept	137.653	1	137.653	40755.728	.000
Block	.588	23	.026	7.567	.000
Antimicrobials	.572	15	.038	11.288	.000
Error	1.165	345	.003		
Total	139.978	384			
Corrected Total	2.325	383			
a. R Squared = .499 (Adjusted R Squared = .444)					

In Table 15 are represented the average of the mean absorbance values obtained in the biofilm eradication assay, in Table 16 (supplementary data) are presented the absorbance values obtained for

each one of the strains and in Table 17 (supplementary data) is presented the statistical analysis of the results.

In overview it was observed that in the biofilm eradication analysis the absorbance values were significantly higher and similar between them.

Table 15: Means absorbance values for biofilm eradication and respective standard deviation regarding eradication action of treatments. Chx: chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel Abs: absorbance; SD: standard deviation

	Abs	**±SD**
Positive Control	0.688	0.045
Gentamycin, Nisin in GGG, Chx	0.652	0.052
Chx	0.633	0.048
Vancomycin, Nisin in GGG, Chx	0.620	0.063
Clindamycin	0.614	0.063
Gentamycin, Nisin in GGG	0.613	0.045
Gentamycin	0.604	0.050
Vancomycin, Nisin in GGG	0.603	0.041
Nisin in GGG	0.595	0.052
Nisin in GGG, Chx	0.591	0.052
Vancomycin	0.587	0.050
Gentamycin, Chx	0.586	0.053
Clindamycin, Nisin in GGG	0.565	0.064
Clindamycin, Nisin in GGG, Chx	0.552	0.061
Clindamycin, Chx	0.543	0.134
Vancomycin, Chx	0.534	0.136
Negative Control	0.101	

Regarding the eradication effect of antimicrobials without combinations, it was observed that chlorhexidine had de highest absorbance value and vancomycin the lowest. However, there were not significative differences (p-value>0.05) between vancomycin, clindamycin, gentamycin and nisin incorporate in guar gum gel, meaning that these antimicrobials had a similar effect of eradication over the bacterial biofilm.

Concerning antibiotics, the low eradication effect of gentamycin can be related with the fact that aminoglycoside effectiveness relies heavily on *S. aureus* growth phase and extra bacterial factors, including the availability of oxygen and the pH in the surrounding environment.[161] Regarding clindamycin, the low eradication effect against the bacterial biofilm can be due to the presence of *erm* genes, which mediate target site modifications that leads to a reduced susceptibility to this class of antibiotics.[163] In turn, low absorbance values of eradication for vancomycin can be related to the presence of the accessory gene regulator (*agr*) of *S. aureus.* In fact, the presence of *agr* types I and II is associated with evolution towards reduced vancomycin susceptibility; *agr* type II polymorphism is

associated with vancomycin therapeutic failures and reduced bacterial killing due to diminished autolysis; and decreased *agr* function promotes organism survival especially in the hospital environment.[162]

Biofilm protection against chlorhexidine may be due to reduced penetration in the biofilm matrix.[118] For a higher eradication effect, a much longer time of contact between chlorhexidine and biofilm-formed bacteria than that for planktonic cells may be required.[164] Another factor that can interfere with chlorhexidine action is the existence of bacterial biofilms at different developmental stages.[111]

Okuda et al 2013 observed that nisin has eradication activity against MRSA organized in biofilm and Santos et al 2016 suggested that nisin incorporated in guar gum is able to inhibit established biofilms of *S. aureus*.[96,126] In our study nisin incorporated in guar gum gel demonstrated some inhibitory action against the strains tested but not eradication, even when combined with other antimicrobials.

Regarding combinations of antimicrobials, were observed that combinations involving vancomycin and chlorhexidine, and clindamycin and chlorhexidine, had the highest eradication effect against biofilm, having no significative differences between them (p-value>0.05). These results, demonstrated that combining these antimicrobials increased the eradication effect.

Figure17: Result obtained after sonication of pegs and incubation for 24h at 37ºC for the eradication assay of antimicrobials against biofilm. As can be observed there were growth in all wells (original).

Bacteria within biofilm are more persistent due to reduced growth rate and resistance gene expression as compared to planktonic bacteria, being difficult to eradicate.[165] As our results showed, for a higher eradication effect the best option is to combined antimicrobials, which could help to reduce the concentration of antibiotic used in therapeutic protocols.

Chapter 4

Conclusion

Diabetes *mellitus* is a major worldwide health problem, being observed that one of its most severe complications is the development of DFU which can subsequently infected.[26,167] DFI are usually polymicrobial, being promoted by several bacterial genera, principally gram-positive bacteria, being *Staphylococcus aureus* the most common specie isolated from these ulcers.[96]

Due to the rapid emergence of resistant bacterial strains, novel therapeutic protocols for DFU management are extremely urgent.[26,167] Biocides and AMP have been proposed as alternatives to antibiotics or as complementary therapeutics tools.

Chlorhexidine is a widely used antiseptic agent that has excellent antimicrobial activity.[114] One of the aim of the present study was to determine the MIC and MBC values of chlorhexidine against *S. aureus* isolates from DFU. The mean MIC and MBC values obtained were below the one established for wound cleansing, which is 0.05%.[168,169] These results demonstrated that even at low concentrations chlorhexidine demonstrates an inhibitory effect, having a bactericidal effect over the *S. aureus* strains under study. Since these effective concentrations are low, their application will avoid side effects, such as skin irritation and allergies.[168]

The bacterial biofilm mode of growth is another major responsible for the healing impediment of DFU.[96] It has been estimated that biofilms can tolerate antimicrobial agents at concentrations 10 to1000-times higher than the ones needed to inactivate genetically equivalent planktonic bacteria.[70] Since biofilms have a significant impact on public health, there is a major need to search for new antibiofilm agents.[170]

Antimicrobials tested in this study aiming at inhibiting biofilm formation showed promising results. As observed in Figure 18, antimicrobials combinations that include nisin incorporated in guar gum gel and chlorhexidine showed the higher inhibitory effects. Like chlorhexidine, nisin concentrations required to inhibit biofilm cells were below nisin acceptable daily intake, even when incorporated in guar gum gel.[96]

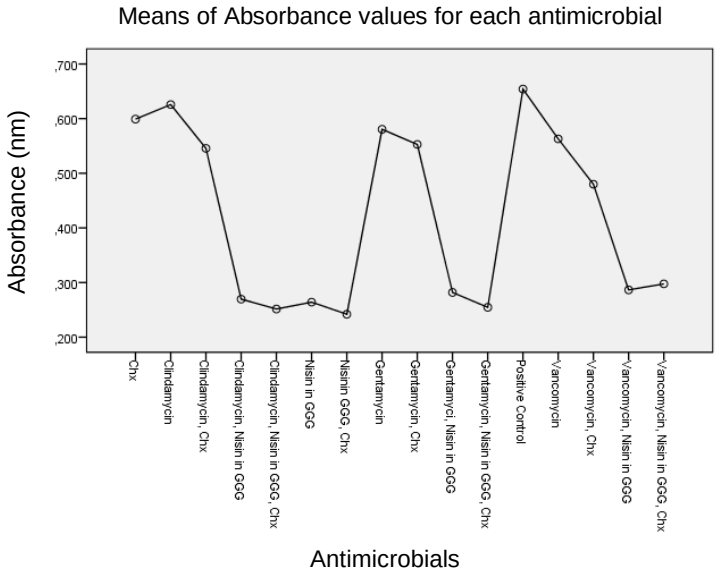

Figure 18: Summary of absorbance values obtained for each antimicrobial regarding inhibitory action. Chx: chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel.

These antimicrobials could be applied as a complement to antibiotics, allowing to reduce their dose.

Bacteria embedded within a biofilm are difficult to eradicate due to a wide variation of nutrient gradients that slow or arrest bacterial growth, protein synthesis and other physiologic activities.[171] Although nisin incorporated in guar gum gel and chlorhexidine presented an inhibitory effect against bacterial biofilms, the same was not observed in the eradication assays. As observed in Figure 19, the absorbance values were higher. In order to achieve a better eradication effect, a good option would be to use higher antimicrobial concentrations.

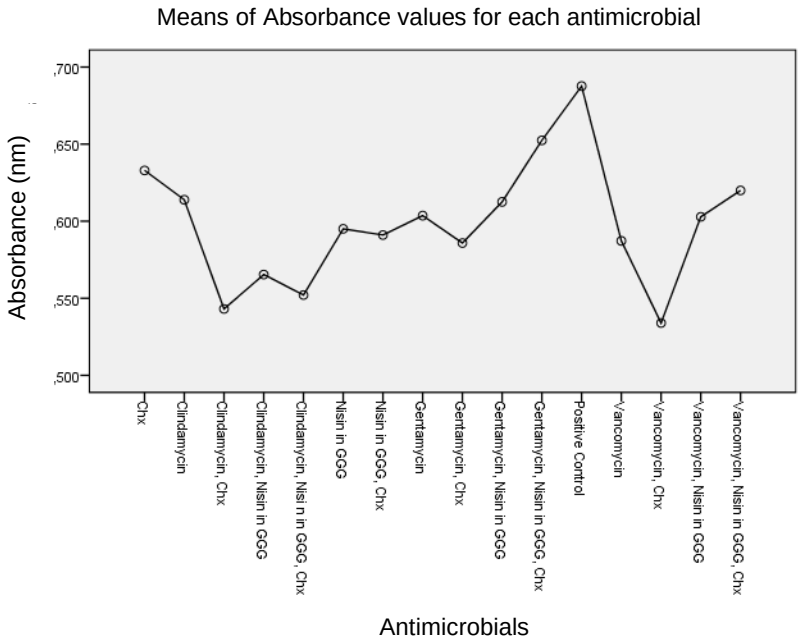

Figure 19: Summary of absorbance values obtained for each antimicrobial regarding eradication action. Chx: Chlorhexidine; GGG: Nisin incorporated in guar gum gel.

Overall, results suggest that nisin incorporated in guar gum and chlorhexidine have a good inhibitory effect against *S. aureus* isolates from DFU. This can be a new therapeutic alternative, or a complement to antibiotherapy, with the advantage that there are currently no resistances described to these compounds.

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Supplementary Data

15. Bacterial Strains

Table 4: Antibiotic resistance genes and MIC for antibiotics previously described by Mottola et al 2016.[43]

Isolate	*mecA*	*mecC*	*ermA*	*ermB*	*ermC*	*blaZ*	*msrA*	*aac-aph*	*tetK*	*tetL*	*tetM*	*tetO*	*norA*	FOX	CPT	CIP	CLI	DOX	ERY	GEN	LZD	MEM	VAN
A 1.1	+	-	+	-	-	-	-	+	-	-	-	-	+	R	S	R	S	S	S	S	S	I	S
A 5.2	-	-	-	-	-	-	-	-	-	-	-	-	+	S	R	R	S	S	S	S	S	S	S
A 6.3	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
B 3.2	-	-	-	-	+	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
B 3.3	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
B 7.3	+	-	+	-	-	-	-	-	-	-	-	-	+	R	S	R	S	S	R	S	S	S	S
B 13.1	+	-	-	-	-	+	-	-	-	-	+	-	+	R	S	R	R	S	R	R	S	R	S
B 14.2	+	-	-	-	+	-	-	-	-	-	-	-	-	R	R	R	S	S	R	S	S	I	S
Z 1.1	+	-	-	-	+	-	-	-	-	-	-	-	-	R	S	R	S	S	R	S	S	R	S
Z 2.2	-	-	+	-	-	-	-	-	-	-	-	-	+	S	S	R	S	S	R	S	S	S	S
Z 3.1	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
Z 5.2	-	-	-	-	-	+	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
Z 12.2	-	-	-	-	-	-	-	+	+	-	-	-	+	S	S	I	S	S	S	R	S	S	S
Z 14.1	-	-	-	-	-	+	-	+	+	-	-	-	+	S	S	S	S	S	S	R	S	S	S
Z 16.1	+	-	+	-	-	-	-	-	-	-	-	-	+	R	S	R	S	S	R	S	S	S	S
Z 17.2	-	-	-	-	-	-	-	-	+	-	-	-	-	S	S	S	S	S	S	S	S	S	S
Z 21.1	+	-	+	-	-	-	-	-	-	-	-	-	+	R	S	R	S	S	R	S	S	S	S
Z 21.3	+	-	+	-	-	-	-	-	-	-	-	-	+	R	S	R	S	S	R	S	S	S	S
Z 23.2	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	S	S	S	S	S
Z 25.2	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
Z 27.2	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
Z 27.3	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S
Z 32.2	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	S	S	S	S	S

A: Aspirate; B: Biopsy; Z: Swab; α: Alfa; β: Beta; +: positive; -: negative; *mec*A and *mec*C: Oxacillin resistance; *erm*A, *erm*B, *erm*C and *msr*A: Erythromycin resistance; *bla*Z: Penicillin resistance; aac-aph: Gentamycin resistance; *tet*K, *tet*L, *tet*M and *tet*O: Teracycline resistance; *nor*A: Ciprofloxacin resistance; FOX: cefoxitin; CPT: Ceftaroline; CIP: Ciprofloxacin; CLI: Clindamycin; DOX: Doxycycline; ERY: Erythromycin; GEN: Gentamycin; LZD: Linezolid; MEM: Meropenem; VAN: Vancomycin; R: resistant; S: susceotible; I: intermediate

Table 5: Characterization of isolates regarding virulence factors and biofilm production as previously described by Mottola et al 2016b and Mottola et al 2015.[144,145]

Isolate	Haemolysis	Lipase	DNase	Gelatinase	Coagulase	Biofilm	*agr*	*bap*	*icaA*	*icaD*	*atl*	*pls*	*clfa*	*spa*	*coa*	*tst*	*pvl*
A 1.1	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
A 5.2	β	-	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
A 6.3	β	+	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
B 3.2	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
B 3.3	β	-	+	-	+	+	II	-	+	+	+	-	-	+	+	-	-
B 7.3	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
B 13.1	β	-	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
B 14.2	β	+	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
Z 1.1	β	+	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
Z 2.2	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
Z 3.1	β	-	+	-	+	+	I	-	+	+	+	-	-	+	+	-	-
Z 5.2	β	+	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
Z 12.2	-	-	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
Z 14.1	α	-	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
Z 16.1	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
Z 17.2	-	-	+	-	+	+	-	-	+	+	+	-	+	+	+	-	-
Z 21.1	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
Z 21.3	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
Z 23.2	β	+	+	-	+	+	I	-	+	+	+	-	+	+	+	-	-
Z 25.2	β	+	+	-	+	+	II	-	+	+	+	-	+	+	+	-	-
Z 27.2	β	+	+	-	+	+	I	-	+	+	+	-	-	+	+	-	-
Z 27.3	β	+	+	-	+	+	I	-	+	+	+	-	-	+	+	-	-
Z 32.2	α	+	+	-	+	+	II	-	+	+	+	-	+	+	+	+	-

A: Aspirate; B: Biopsy; Z: Swab; α: Alfa; β: Beta; +: positive; -: negative; *agr*: Accessory regulators genes; I: Group one; II: Group two*; bap*: Biofilm associated protein gene; *ica*A and *ica*D: Biofilm formation adhesin genes; *atl*: Autolysin; *pls*: Plasmin sensitive; *clfa*: Clumping gene; *spa*: Protein A gene; *coa*: Coagulase gene; *tst*: Toxic shock syndrome toxin 1 gene; *pvl*: Panton-valentine leucocidin.

16. Preparation of Compounds Tested - Chlorhexidine

Table 6: MIC values of chlorhexidine obtained for each strain.

Strains	MIC values (%)														Mean (%)
A 1.1	0.005	0.001	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.001	0.001	0.001	0.001	0.0036
A 5.2	0.005	0.005	0.005	0.005	0.005	0.001	0.001	0.001	0.001						0.0032
A 6.3	0.005	0.005	0.005	0.005	0.005	0.001	0.001	0.001	0.001						0.0032
B 3.2	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.001	0.001	0.001					0.0044
B 3.3	0.005	0.005	0.005	0.001	0.001	0.005									0.0037
B 7.3	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005					0.0050
B 13.1	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005							0.0050
B 14.2	0.005	0.005	0.005	0.005	0.005	0.001	0.001	0.005	0.005						0.0041
Z 1.1	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005			0.0050
Z 2.2	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005			0.0050
Z 3.1	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005			0.0050
Z 5.2	0.005	0.001	0.001	0.005	0.001										0.0026
Z 12.2	0.001	0.001	0.001	0.001											0.0010
Z 14.1	0.005	0.001	0.001	0.001	0.005										0.0026
Z 16.1	0.005	0.005	0.001	0.005	0.001	0.001									0.0030
Z 17.2	0.005	0.005	0.005	0.001	0.001										0.0034
Z 21.1	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005					0.0050
Z 21.3	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005					0.0050
Z 23.2	0.001	0.001	0.005	0.005	0.001	0.005	0.001	0.001							0.0025
Z 25.2	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005							0.0050
Z 27.2	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005							0.0050
Z 27.3	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005							0.0050
Z 32.2	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005							0.0050
ATCC 23213	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005							0.0050
Total Mean															0.0041
Minimum: 0.001			**Maximum:** 0.0050			**Standard Deviation:** 0.0011								**[Well] =** 0.0006 % = 6 µg/mL	

17. Biofilm Inhibition

Table 11: Table of multiple comparisons for inhibitory action of antimicrobials against bacterial biofilm. Chx: Chlorhexidine. GGG: Nisin incorporated in guar gum gel; Std. Error: Standard error; Sig: Significance.

Multiple Comparisons

Dependent Variable: Absorbance

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Chx	Clindamycin	-.02663	.020561	.196	-.06707	.01382
	Clindamycin, Chx	.05346*	.020561	.010	.01302	.09390
	Clindamycin, Nisin in GGG	.32950*	.020561	.000	.28906	.36994
	Clindamycin, Nisin in GGG, Chx	.34758*	.020561	.000	.30714	.38802
	Nisin in GGG, Chx	.35712*	.020561	.000	.31668	.39757
	Gentamycin	.01867	.020561	.365	-.02177	.05911
	Gentamycin, Chx	.04633*	.020561	.025	.00589	.08677
	Gentamycin, Nisin in GGG	.31717*	.020561	.000	.27673	.35761
	Gentamycin, Nisin in GGG, Chx	.34446*	.020561	.000	.30402	.38490
	Positive Control	-.05500*	.020561	.008	-.09544	-.01456
	Vancomycin	.03608	.020561	.080	-.00436	.07652
	Vancomycin, Chx	.11921*	.020561	.000	.07877	.15965
	Vancomycin, Nisin in GGG	.31250*	.020561	.000	.27206	.35294
	Vancomycin, Nisin in GGG, Chx	.30158*	.020561	.000	.26114	.34202
	Nisin in GGG	.33500*	.020561	.000	.29456	.37544
Clindamycin	Chx	.02663	.020561	.196	-.01382	.06707
	Clindamycin, Chx	.08008*	.020561	.000	.03964	.12052
	Clindamycin, Nisin in GGG	.35613*	.020561	.000	.31568	.39657
	Clindamycin, Nisin in GGG, Chx	.37421*	.020561	.000	.33377	.41465
	Nisin in GGG, Chx	.38375*	.020561	.000	.34331	.42419
	Gentamycin	.04529*	.020561	.028	.00485	.08573
	Gentamycin, Chx	.07296*	.020561	.000	.03252	.11340
	Gentamycin, Nisin in GGG	.34379*	.020561	.000	.30335	.38423
	Gentamycin, Nisin in GGG, Chx	.37108*	.020561	.000	.33064	.41152
	Positive Control	-.02838	.020561	.168	-.06882	.01207
	Vancomycin	.06271*	.020561	.002	.02227	.10315
	Vancomycin, Chx	.14583*	.020561	.000	.10539	.18627
	Vancomycin, Nisin in GGG	.33912*	.020561	.000	.29868	.37957
	Vancomycin, Nisin in GGG, Chx	.32821*	.020561	.000	.28777	.36865

	Nisin in GGG	.36163*	.020561	.000	.32118	.40207
Clindamycin, Chx	Chx	-.05346*	.020561	.010	-.09390	-.01302
	Clindamycin	-.08008*	.020561	.000	-.12052	-.03964
	Clindamycin, Nisin in GGG	.27604*	.020561	.000	.23560	.31648
	Clindamycin, Nisin in GGG, Chx	.29413*	.020561	.000	.25368	.33457
	Nisin in GGG, Chx	.30367*	.020561	.000	.26323	.34411
	Gentamycin	-.03479	.020561	.092	-.07523	.00565
	Gentamycin, Chx	-.00712	.020561	.729	-.04757	.03332
	Gentamycin, Nisin in GGG	.26371*	.020561	.000	.22327	.30415
	Gentamycin, Nisin in GGG, Chx	.29100*	.020561	.000	.25056	.33144
	Positive Control	-.10846*	.020561	.000	-.14890	-.06802
	Vancomycin	-.01737	.020561	.399	-.05782	.02307
	Vancomycin, Chx	.06575*	.020561	.002	.02531	.10619
	Vancomycin, Nisin in GGG	.25904*	.020561	.000	.21860	.29948
	Vancomycin, Nisin in GGG, Chx	.24813*	.020561	.000	.20768	.28857
	Nisin in GGG	.28154*	.020561	.000	.24110	.32198
Clindamycin, Nisin in GGG	Chx	-.32950*	.020561	.000	-.36994	-.28906
	Clindamycin	-.35613*	.020561	.000	-.39657	-.31568
	Clindamycin, Chx	-.27604*	.020561	.000	-.31648	-.23560
	Clindamycin, Nisin in GGG, Chx	.01808	.020561	.380	-.02236	.05852
	Nisin in GGG, Chx	.02762	.020561	.180	-.01282	.06807
	Gentamycin	-.31083*	.020561	.000	-.35127	-.27039
	Gentamycin, Chx	-.28317*	.020561	.000	-.32361	-.24273
	Gentamycin, Nisin in GGG	-.01233	.020561	.549	-.05277	.02811
	Gentamycin, Nisin in GGG, Chx	.01496	.020561	.467	-.02548	.05540
	Positive Control	-.38450*	.020561	.000	-.42494	-.34406
	Vancomycin	-.29342*	.020561	.000	-.33386	-.25298
	Vancomycin, Chx	-.21029*	.020561	.000	-.25073	-.16985
	Vancomycin, Nisin in GGG	-.01700	.020561	.409	-.05744	.02344
	Vancomycin, Nisin in GGG, Chx	-.02792	.020561	.175	-.06836	.01252
	Nisin in GGG	.00550	.020561	.789	-.03494	.04594
Clindamycin, Nisin in GGG, Chx	Chx	-.34758*	.020561	.000	-.38802	-.30714
	Clindamycin	-.37421*	.020561	.000	-.41465	-.33377
	Clindamycin, Chx	-.29413*	.020561	.000	-.33457	-.25368
	Clindamycin, Nisin in GGG	-.01808	.020561	.380	-.05852	.02236
	Nisin in GGG, Chx	.00954	.020561	.643	-.03090	.04998
	Gentamycin	-.32892*	.020561	.000	-.36936	-.28848
	Gentamycin, Chx	-.30125*	.020561	.000	-.34169	-.26081
	Gentamycin, Nisin in GGG	-.03042	.020561	.140	-.07086	.01002

	Gentamycin, Nisin in GGG, Chx	-.00313	.020561	.879	-.04357	.03732
	Positive Control	-.40258*	.020561	.000	-.44302	-.36214
	Vancomycin	-.31150*	.020561	.000	-.35194	-.27106
	Vancomycin, Chx	-.22838*	.020561	.000	-.26882	-.18793
	Vancomycin, Nisin in GGG	-.03508	.020561	.089	-.07552	.00536
	Vancomycin, Nisin in GGG, Chx	-.04600*	.020561	.026	-.08644	-.00556
	Nisin in GGG	-.01258	.020561	.541	-.05302	.02786
Nisin in GGG	Chx	-.33500*	.020561	.000	-.37544	-.29456
	Clindamycin	-.36163*	.020561	.000	-.40207	-.32118
	Clindamycin, Chx	-.28154*	.020561	.000	-.32198	-.24110
	Clindamycin, Nisin in GGG	-.00550	.020561	.789	-.04594	.03494
	Clindamycin, Nisin in GGG, Chx	.01258	.020561	.541	-.02786	.05302
	Nisin in GGG, Chx	.02213	.020561	.283	-.01832	.06257
	Gentamycin	-.31633*	.020561	.000	-.35677	-.27589
	Gentamycin, Chx	-.28867*	.020561	.000	-.32911	-.24823
	Gentamycin, Nisin in GGG	-.01783	.020561	.386	-.05827	.02261
	Gentamycin, Nisin in GGG, Chx	.00946	.020561	.646	-.03098	.04990
	Positive Control	-.39000*	.020561	.000	-.43044	-.34956
	Vancomycin	-.29892*	.020561	.000	-.33936	-.25848
	Vancomycin, Chx	-.21579*	.020561	.000	-.25623	-.17535
	Vancomycin, Nisin in GGG	-.02250	.020561	.275	-.06294	.01794
	Vancomycin, Nisin in GGG, Chx	-.03342	.020561	.105	-.07386	.00702
Nisin in GGG, Chx	Chx	-.35712*	.020561	.000	-.39757	-.31668
	Clindamycin	-.38375*	.020561	.000	-.42419	-.34331
	Clindamycin, Chx	-.30367*	.020561	.000	-.34411	-.26323
	Clindamycin, Nisin in GGG	-.02762	.020561	.180	-.06807	.01282
	Clindamycin, Nisin in GGG, Chx	-.00954	.020561	.643	-.04998	.03090
	Gentamycin	-.33846*	.020561	.000	-.37890	-.29802
	Gentamycin, Chx	-.31079*	.020561	.000	-.35123	-.27035
	Gentamycin, Nisin in GGG	-.03996	.020561	.053	-.08040	.00048
	Gentamycin, Nisin in GGG, Chx	-.01267	.020561	.538	-.05311	.02777
	Positive Control	-.41213*	.020561	.000	-.45257	-.37168
	Vancomycin	-.32104*	.020561	.000	-.36148	-.28060
	Vancomycin, Chx	-.23792*	.020561	.000	-.27836	-.19748
	Vancomycin, Nisin in GGG	-.04463*	.020561	.031	-.08507	-.00418
	Vancomycin, Nisin in GGG. Chx	-.05554*	.020561	.007	-.09598	-.01510
	Nisin in GGG	-.02213	.020561	.283	-.06257	.01832
Gentamycin	Chx	-.01867	.020561	.365	-.05911	.02177

	Clindamycin	-.04529*	.020561	.028	-.08573	-.00485
	Clindamycin, Chx	.03479	.020561	.092	-.00565	.07523
	Clindamycin, Nisin in GGG	.31083*	.020561	.000	.27039	.35127
	Clindamycin, Nisin in GGG, Chx	.32892*	.020561	.000	.28848	.36936
	Nisin in GGG, Chx	.33846*	.020561	.000	.29802	.37890
	Gentamycin, Chx	.02767	.020561	.179	-.01277	.06811
	Gentamycin, Nisin in GGG	.29850*	.020561	.000	.25806	.33894
	Gentamycin, Nisin in GGG, Chx	.32579*	.020561	.000	.28535	.36623
	Positive Control	-.07367*	.020561	.000	-.11411	-.03323
	Vancomycin	.01742	.020561	.398	-.02302	.05786
	Vancomycin, Chx	.10054*	.020561	.000	.06010	.14098
	Vancomycin, Nisin in GGG	.29383*	.020561	.000	.25339	.33427
	Vancomycin, Nisin in GGG, Chx	.28292*	.020561	.000	.24248	.32336
	Nisin in GGG	.31633*	.020561	.000	.27589	.35677
Gentamycin, Chx	Chx	-.04633*	.020561	.025	-.08677	-.00589
	Clindamycin	-.07296*	.020561	.000	-.11340	-.03252
	Clindamycin, Chx	.00712	.020561	.729	-.03332	.04757
	Clindamycin, Nisin in GGG	.28317*	.020561	.000	.24273	.32361
	Clindamycin, Nisin in GGG, Chx	.30125*	.020561	.000	.26081	.34169
	Nisin in GGG, Chx	.31079*	.020561	.000	.27035	.35123
	Gentamycin	-.02767	.020561	.179	-.06811	.01277
	Gentamycin, Nisin in GGG	.27083*	.020561	.000	.23039	.31127
	Gentamycin, Nisin in GGG, Chx	.29812*	.020561	.000	.25768	.33857
	Positive Control	-.10133*	.020561	.000	-.14177	-.06089
	Vancomycin	-.01025	.020561	.618	-.05069	.03019
	Vancomycin, Chx	.07287*	.020561	.000	.03243	.11332
	Vancomycin, Nisin in GGG	.26617*	.020561	.000	.22573	.30661
	Vancomycin, Nisin in GGG, Chx	.25525*	.020561	.000	.21481	.29569
	Nisin in GGG	.28867*	.020561	.000	.24823	.32911
Gentamycin, Nisin in GGG	Chx	-.31717*	.020561	.000	-.35761	-.27673
	Clindamycin	-.34379*	.020561	.000	-.38423	-.30335
	Clindamycin, Chx	-.26371*	.020561	.000	-.30415	-.22327
	Clindamycin, Nisin in GGG	.01233	.020561	.549	-.02811	.05277
	Clindamycin, Nisin in GGG, Chx	.03042	.020561	.140	-.01002	.07086
	Nisin in GGG, Chx	.03996	.020561	.053	-.00048	.08040
	Gentamycin	-.29850*	.020561	.000	-.33894	-.25806
	Gentamycin, Chx	-.27083*	.020561	.000	-.31127	-.23039
	Gentamycin, Nisin in GGG, Chx	.02729	.020561	.185	-.01315	.06773

	Positive Control	-.37217*	.020561	.000	-.41261	-.33173
	Vancomycin	-.28108*	.020561	.000	-.32152	-.24064
	Vancomycin, Chx	-.19796*	.020561	.000	-.23840	-.15752
	Vancomycin, Nisin in GGG	-.00467	.020561	.821	-.04511	.03577
	Vancomycin, Nisin in GGG, Chx	-.01558	.020561	.449	-.05602	.02486
	Nisin in GGG	.01783	.020561	.386	-.02261	.05827
Gentamycin, Nisin in GGG, Chx	Chx	-.34446*	.020561	.000	-.38490	-.30402
	Clindamycin	-.37108*	.020561	.000	-.41152	-.33064
	Clindamycin, Chx	-.29100*	.020561	.000	-.33144	-.25056
	Clindamycin, Nisin in GGG	-.01496	.020561	.467	-.05540	.02548
	Clindamycin, Nisin in GGG, Chx	.00313	.020561	.879	-.03732	.04357
	Nisin in GGG, Chx	.01267	.020561	.538	-.02777	.05311
	Gentamycin	-.32579*	.020561	.000	-.36623	-.28535
	Gentamycin, Chx	-.29812*	.020561	.000	-.33857	-.25768
	Gentamycin, Nisin in GGG	-.02729	.020561	.185	-.06773	.01315
	Positive Control	-.39946*	.020561	.000	-.43990	-.35902
	Vancomycin	-.30837*	.020561	.000	-.34882	-.26793
	Vancomycin, Chx	-.22525*	.020561	.000	-.26569	-.18481
	Vancomycin, Nisin in GGG	-.03196	.020561	.121	-.07240	.00848
	Vancomycin, Nisin in GGG, Chx	-.04287*	.020561	.038	-.08332	-.00243
	Nisin in GGG	-.00946	.020561	.646	-.04990	.03098
Positive Control	Chx	.05500*	.020561	.008	.01456	.09544
	Clindamycin	.02838	.020561	.168	-.01207	.06882
	Clindamycin, Chx	.10846*	.020561	.000	.06802	.14890
	Clindamycin, Nisin in GGG	.38450*	.020561	.000	.34406	.42494
	Clindamycin, Nisin in GGG, Chx	.40258*	.020561	.000	.36214	.44302
	Nisin in GGG, Chx	.41213*	.020561	.000	.37168	.45257
	Gentamycin	.07367*	.020561	.000	.03323	.11411
	Gentamycin, Chx	.10133*	.020561	.000	.06089	.14177
	Gentamycin, Nisin in GGG	.37217*	.020561	.000	.33173	.41261
	Gentamycin, Nisin in GGG, Chx	.39946*	.020561	.000	.35902	.43990
	Vancomycin	.09108*	.020561	.000	.05064	.13152
	Vancomycin, Chx	.17421*	.020561	.000	.13377	.21465
	Vancomycin, Nisin in GGG	.36750*	.020561	.000	.32706	.40794
	Vancomycin, Nisin in GGG, Chx	.35658*	.020561	.000	.31614	.39702
	Nisin in GGG	.39000*	.020561	.000	.34956	.43044
Vancomycin	Chx	-.03608	.020561	.080	-.07652	.00436
	Clindamycin	-.06271*	.020561	.002	-.10315	-.02227
	Clindamycin, Chx	.01737	.020561	.399	-.02307	.05782
	Clindamycin, Nisin in GGG	.29342*	.020561	.000	.25298	.33386

	Clindamycin, Nisin in GGG, Chx	.31150*	.020561	.000	.27106	.35194
	Nisin in GGG, Chx	.32104*	.020561	.000	.28060	.36148
	Gentamycin	-.01742	.020561	.398	-.05786	.02302
	Gentamycin, Chx	.01025	.020561	.618	-.03019	.05069
	Gentamycin, Nisin in GGG	.28108*	.020561	.000	.24064	.32152
	Gentamycin, Nisin in GGG, Chx	.30837*	.020561	.000	.26793	.34882
	Positive Control	-.09108*	.020561	.000	-.13152	-.05064
	Vancomycin, Chx	.08312*	.020561	.000	.04268	.12357
	Vancomycin, Nisin in GGG	.27642*	.020561	.000	.23598	.31686
	Vancomycin, Nisin in GGG, Chx	.26550*	.020561	.000	.22506	.30594
	Nisin in GGG	.29892*	.020561	.000	.25848	.33936
Vancomycin, Chx	Chx	-.11921*	.020561	.000	-.15965	-.07877
	Clindamycin	-.14583*	.020561	.000	-.18627	-.10539
	Clindamycin, Chx	-.06575*	.020561	.002	-.10619	-.02531
	Clindamycin, Nisin in GGG	.21029*	.020561	.000	.16985	.25073
	Clindamycin, Nisin in GGG, Chx	.22838*	.020561	.000	.18793	.26882
	Nisin in GGG, Chx	.23792*	.020561	.000	.19748	.27836
	Gentamycin	-.10054*	.020561	.000	-.14098	-.06010
	Gentamycin, Chx	-.07287*	.020561	.000	-.11332	-.03243
	Gentamycin, Nisin in GGG	.19796*	.020561	.000	.15752	.23840
	Gentamycin, Nisin in GGG, Chx	.22525*	.020561	.000	.18481	.26569
	Positive Control	-.17421*	.020561	.000	-.21465	-.13377
	Vancomycin	-.08312*	.020561	.000	-.12357	-.04268
	Vancomycin, Nisin in GGG	.19329*	.020561	.000	.15285	.23373
	Vancomycin, Nisin in GGG, Chx	.18238*	.020561	.000	.14193	.22282
	Nisin in GGG	.21579*	.020561	.000	.17535	.25623
Vancomycin, Nisin in GGG, Chx	Chx	-.30158*	.020561	.000	-.34202	-.26114
	Clindamycin	-.32821*	.020561	.000	-.36865	-.28777
	Clindamycin, Chx	-.24813*	.020561	.000	-.28857	-.20768
	Clindamycin, Nisin in GGG	.02792	.020561	.175	-.01252	.06836
	Clindamycin, Nisin in GGG, Chx	.04600*	.020561	.026	.00556	.08644
	Nisin in GGG, Chx	.05554*	.020561	.007	.01510	.09598
	Gentamycin	-.28292*	.020561	.000	-.32336	-.24248
	Gentamycin, Chx	-.25525*	.020561	.000	-.29569	-.21481
	Gentamycin, Nisin in GGG	.01558	.020561	.449	-.02486	.05602
	Gentamycin, Nisin in GGG, Chx	.04287*	.020561	.038	.00243	.08332
	Positive Control	-.35658*	.020561	.000	-.39702	-.31614
	Vancomycin	-.26550*	.020561	.000	-.30594	-.22506

	Vancomycin, Chx	-.18238*	.020561	.000	-.22282	-.14193
	Vancomycin, Nisin in GGG	.01092	.020561	.596	-.02952	.05136
	Nisin in GGG	.03342	.020561	.105	-.00702	.07386
Vancomycin, Nisin in GGG, Chx	Chx	-.31250*	.020561	.000	-.35294	-.27206
	Clindamycin	-.33912*	.020561	.000	-.37957	-.29868
	Clindamycin, Chx	-.25904*	.020561	.000	-.29948	-.21860
	Clindamycin, Nisin in GGG	.01700	.020561	.409	-.02344	.05744
	Clindamycin, Nisin in GGG, Chx	.03508	.020561	.089	-.00536	.07552
	Nisin in GGG, Chx	.04463*	.020561	.031	.00418	.08507
	Gentamycin	-.29383*	.020561	.000	-.33427	-.25339
	Gentamycin, Chx	-.26617*	.020561	.000	-.30661	-.22573
	Gentamycin, Nisin in GGG	.00467	.020561	.821	-.03577	.04511
	Gentamycin, Nisin in GGG, Chx	.03196	.020561	.121	-.00848	.07240
	Positive Control	-.36750*	.020561	.000	-.40794	-.32706
	Vancomycin	-.27642*	.020561	.000	-.31686	-.23598
	Vancomycin, Chx	-.19329*	.020561	.000	-.23373	-.15285
	Vancomycin, Nisin in GGG, Chx	-.01092	.020561	.596	-.05136	.02952
	Nisin in GGG	.02250	.020561	.275	-.01794	.06294

Based on observed means.

The error term is Mean Square(Error) = .005.

*. The mean difference is significant at the 0.05 level.

Table 13: Absorbance obtained in the assays aiming to determine the inhibitory effect of antimicrobials against biofilm. C+: Positive control; C-: Negative control; Chx: Chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel; SD: Standard deviation.

	C+	Chx	Nisin in GGG	Nisin in GGG, Chx	Gentamycin	Gentamycin, Chx	Genta, Nisin in GGG	Gentamycin, Nisin in GGG, Chx	Clindamycin	Clindamycin, Chx	Clindamycin, Nisin in GGG	Clindamycin, Nisin in GGG, Chx	Vancomycin	Vancomacyn, Chx	Vancomycin, Nisin in GGG	Vancomycin, Nisin in GGG, Chx
A 1.1	0.585	0.587	0.293	0.270	0.531	0.538	0.358	0.202	0.596	0.560	0.207	0.253	0.509	0.360	0.174	0.249
A 5.2	0.624	0.572	0.292	0.252	0.571	0.572	0.291	0.221	0.545	0.506	0.216	0.219	0.498	0.447	0.179	0.235
A 6.3	0.722	0.613	0.388	0.308	0.686	0.626	0.385	0.261	0.769	0.765	0.411	0.393	0.597	0.608	0.321	0.271
B 3.2	0.658	0.595	0.312	0.276	0.539	0.570	0.278	0.211	0.601	0.562	0.284	0.206	0.583	0.504	0.356	0.280
B 3.3	0.661	0.696	0.311	0.297	0.589	0.637	0.367	0.191	0.611	0.517	0.235	0.207	0.631	0.514	0.229	0.237
B 7.3	0.576	0.571	0.228	0.222	0.503	0.550	0.254	0.212	0.548	0.526	0.193	0.201	0.497	0.529	0.196	0.286
B 13.1	0.663	0.602	0.223	0.192	0.574	0.525	0.205	0.326	0.621	0.686	0.392	0.333	0.564	0.522	0.364	0.339
B 14.2	0.666	0.594	0.201	0.183	0.632	0.589	0.210	0.264	0.668	0.620	0.248	0.268	0.540	0.591	0.276	0.265
Z 1.1	0.681	0.668	0.252	0.177	0.648	0.594	0.175	0.254	0.595	0.562	0.221	0.235	0.607	0.557	0.190	0.250
Z 2.2	0.594	0.588	0.211	0.183	0.521	0.501	0.222	0.206	0.591	0.518	0.204	0.208	0.501	0.508	0.254	0.194
Z 3.1	0.687	0.704	0.292	0.312	0.729	0.726	0.363	0.314	0.651	0.745	0.306	0.291	0.647	0.630	0.308	0.302
Z 5.2	0.660	0.568	0.231	0.223	0.570	0.605	0.332	0.379	0.580	0.543	0.384	0.327	0.576	0.623	0.382	0.383
Z 12.2	0.724	0.452	0.253	0.257	0.649	0.552	0.336	0.270	0.570	0.201	0.177	0.112	0.520	0.085	0.235	0.318
Z 14.1	0.667	0.542	0.224	0.168	0.533	0.523	0.211	0.234	0.617	0.143	0.268	0.208	0.601	0.081	0.287	0.324
Z 16.1	0.629	0.532	0.173	0.173	0.557	0.279	0.208	0.183	0.577	0.082	0.257	0.204	0.571	0.076	0.302	0.340
Z 17.2	0.660	0.548	0.257	0.251	0.566	0.528	0.315	0.223	0.622	0.553	0.281	0.300	0.581	0.537	0.339	0.247
Z 21.1	0.604	0.591	0.289	0.282	0.543	0.544	0.355	0.197	0.593	0.602	0.188	0.183	0.578	0.557	0.205	0.220
Z 21.3	0.675	0.610	0.268	0.263	0.542	0.516	0.308	0.262	0.666	0.595	0.260	0.260	0.559	0.520	0.269	0.266
Z 23.2	0.667	0.574	0.126	0.119	0.560	0.558	0.133	0.156	0.704	0.600	0.313	0.240	0.611	0.527	0.355	0.369
Z 25.2	0.495	0.560	0.262	0.238	0.443	0.457	0.249	0.210	0.505	0.557	0.238	0.267	0.367	0.458	0.203	0.327
Z 27.2	0.706	0.630	0.294	0.293	0.582	0.537	0.285	0.324	0.613	0.643	0.293	0.309	0.596	0.549	0.431	0.422
Z 27.3	0.752	0.681	0.316	0.313	0.657	0.615	0.325	0.341	0.767	0.773	0.396	0.325	0.611	0.594	0.465	0.389
Z 32.2	0.727	0.679	0.333	0.278	0.608	0.601	0.318	0.345	0.826	0.681	0.331	0.333	0.546	0.635	0.399	0.365
ATCC 29213	0.615	0.621	0.309	0.277	0.597	0.523	0.283	0.325	0.581	0.555	0.167	0.154	0.621	0.505	0.159	0.262
Average	0.654	0.599	0.264	0.242	0.580	0.553	0.282	0.255	0.626	0.546	0.270	0.252	0.563	0.480	0.287	0.298
±SD	0.057	0.058	0.056	0.054	0.063	0.080	0.068	0.061	0.076	0.175	0.072	0.066	0.060	0.166	0.087	0.060

18. Biofilm Eradication

Table 16: Absorbance obtained in the assays aiming to determine the eradication effect of antimicrobials against biofilm. C+: Positive control; C-: Negative control; Chx: Chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel; SD: Standard deviation.

	C+	Chx	Nisin in GGG	Nisin in GGG, Chx	Gentamycin	Gentamycin, Chx	Genta, Nisin in GGG	Gentamycin, Nisin in GGG, Chx	Clindamycin	Clindamycin, Chx	Clindamycin, Nisin in GGG	Clindamycin, Nisin in GGG, Chx	Vancomycin	Vancomacyn, Chx	Vancomycin, Nisin in GGG	Vancomycin, Nisin in GGG, Chx
A 1.1	0.637	0.600	0.541	0.530	0.562	0.547	0.565	0.587	0.570	0.579	0.548	0.561	0.573	0.604	0.569	0.583
A 5.2	0.629	0.612	0.564	0.582	0.565	0.568	0.620	0.638	0.581	0.570	0.602	0.539	0.585	0.572	0.558	0.546
A 6.3	0.711	0.659	0.647	0.620	0.625	0.619	0.651	0.688	0.644	0.633	0.610	0.588	0.598	0.613	0.611	0.613
B 3.2	0.648	0.534	0.502	0.514	0.504	0.527	0.554	0.613	0.613	0.571	0.588	0.569	0.567	0.587	0.607	0.636
B 3.3	0.673	0.689	0.536	0.594	0.622	0.562	0.623	0.671	0.526	0.518	0.489	0.468	0.522	0.511	0.538	0.560
B 7.3	0.680	0.623	0.588	0.584	0.569	0.579	0.612	0.632	0.578	0.557	0.540	0.524	0.552	0.566	0.634	0.682
B 13.1	0.676	0.605	0.562	0.535	0.642	0.586	0.551	0.609	0.659	0.643	0.613	0.610	0.684	0.590	0.636	0.604
B 14.2	0.686	0.637	0.614	0.621	0.659	0.585	0.646	0.585	0.633	0.667	0.574	0.555	0.553	0.588	0.617	0.594
Z 1.1	0.733	0.694	0.676	0.675	0.683	0.678	0.695	0.674	0.711	0.671	0.660	0.673	0.666	0.667	0.675	0.683
Z 2.2	0.639	0.556	0.538	0.544	0.558	0.540	0.570	0.607	0.585	0.555	0.568	0.617	0.550	0.576	0.578	0.523
Z 3.1	0.714	0.698	0.685	0.692	0.641	0.592	0.657	0.682	0.545	0.569	0.582	0.549	0.559	0.556	0.570	0.618
Z 5.2	0.688	0.673	0.669	0.650	0.632	0.639	0.643	0.671	0.666	0.614	0.617	0.589	0.608	0.612	0.606	0.655
Z 12.2	0.782	0.670	0.593	0.542	0.598	0.640	0.614	0.733	0.425	0.193	0.360	0.390	0.660	0.190	0.693	0.730
Z 14.1	0.662	0.625	0.582	0.553	0.564	0.585	0.606	0.669	0.641	0.236	0.510	0.537	0.547	0.209	0.570	0.611
Z 16.1	0.687	0.639	0.580	0.578	0.596	0.432	0.622	0.623	0.662	0.216	0.602	0.640	0.611	0.200	0.634	0.657
Z 17.2	0.636	0.538	0.522	0.528	0.511	0.511	0.524	0.583	0.582	0.550	0.543	0.519	0.514	0.540	0.570	0.570
Z 21.1	0.685	0.598	0.595	0.581	0.578	0.582	0.622	0.641	0.619	0.532	0.531	0.503	0.512	0.497	0.550	0.518
Z 21.3	0.641	0.611	0.566	0.556	0.560	0.551	0.540	0.580	0.591	0.542	0.550	0.476	0.555	0.527	0.568	0.572
Z 23.2	0.685	0.686	0.586	0.559	0.648	0.623	0.611	0.621	0.608	0.595	0.543	0.583	0.570	0.572	0.583	0.565
Z 25.2	0.692	0.604	0.581	0.651	0.648	0.625	0.592	0.657	0.599	0.533	0.488	0.576	0.582	0.533	0.556	0.567
Z 27.2	0.698	0.616	0.593	0.563	0.563	0.577	0.592	0.682	0.652	0.604	0.556	0.490	0.584	0.635	0.634	0.670
Z 27.3	0.806	0.692	0.646	0.647	0.679	0.639	0.678	0.756	0.720	0.658	0.630	0.600	0.631	0.632	0.642	0.695
Z 32.2	0.755	0.656	0.637	0.667	0.657	0.634	0.660	0.764	0.684	0.627	0.599	0.546	0.686	0.637	0.647	0.688
ATCC 29213	0.661	0.676	0.678	0.619	0.623	0.639	0.653	0.693	0.639	0.601	0.667	0.548	0.624	0.601	0.622	0.739
Average	0.688	0.633	0.595	0.591	0.604	0.586	0.613	0.652	0.614	0.543	0.565	0.552	0.587	0.534	0.603	0.620
±SD	0.045	0.048	0.052	0.052	0.050	0.053	0.045	0.052	0.063	0.134	0.064	0.061	0.050	0.136	0.041	0.063

Table 27: Multiple comparisons of eradication effect of antimicrobials against the bacterial biofilm. Chx:Chlorhexidine; GGG: guar gum ge; Std. Error: Standard error; Sig: Significance.

Multiple Comparisons

Dependent Variable: Absorbance

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Chx	Clindamycin	.01908	.016777	.256	-.01391	.05208
	Clindamycin, Chx	.08988*	.016777	.000	.05688	.12287
	Clindamycin, Nisin in GGG	.06754*	.016777	.000	.03454	.10054
	Clindamycin, Nisin in GGG, Chx	.08088*	.016777	.000	.04788	.11387
	Nisin in GGG	.03792*	.016777	.024	.00492	.07091
	Gentamycin	.02933	.016777	.081	-.00366	.06233
	Gentamycin, Chx	-.01950	.016777	.246	-.05250	.01350
	Positive Control	-.05471*	.016777	.001	-.08771	-.02171
	Vancomycin	.04575*	.016777	.007	.01275	.07875
	Vancomycin, Chx	.09900*	.016777	.000	.06600	.13200
	Vancomycin, Nisin in GGG	.03012	.016777	.073	-.00287	.06312
	Vancomycin, Nisin in GGG, Chx	.01300	.016777	.439	-.02000	.04600
	Nisin in GGG, Chx	.04192*	.016777	.013	.00892	.07491
	Gentamycin, Chx	.04713*	.016777	.005	.01413	.08012
	Gentamycin, Nisin in GGG	.02042	.016777	.224	-.01258	.05341
Clindamycin	Chlorhexidine	-.01908	.016777	.256	-.05208	.01391
	Clindamycin, Chx	.07079*	.016777	.000	.03779	.10379
	Clindamycin, Nisin in GGG	.04846*	.016777	.004	.01546	.08146
	Clindamycin, Nisin in GGG, Chx	.06179*	.016777	.000	.02879	.09479
	Nisin in GGG	.01883	.016777	.262	-.01416	.05183
	Gentamycin	.01025	.016777	.542	-.02275	.04325
	Gentamycin, Chx	-.03858*	.016777	.022	-.07158	-.00559
	Positive Control	-.07379*	.016777	.000	-.10679	-.04079
	Vancomycin	.02667	.016777	.113	-.00633	.05966
	Vancomycin, Chx	.07992*	.016777	.000	.04692	.11291
	Vancomycin, Nisin in GGG	.01104	.016777	.511	-.02196	.04404
	Vancomycin, Nisin in GGG, Chx	-.00608	.016777	.717	-.03908	.02691
	Nisin in GGG, Chx	.02283	.016777	.174	-.01016	.05583
	Gentamycin, Chx	.02804	.016777	.096	-.00496	.06104
	Gentamycin, Nisin in GGG	.00133	.016777	.937	-.03166	.03433
Clindamycin, Chx	Chlorhexidine	-.08988*	.016777	.000	-.12287	-.05688
	Clindamycin	-.07079*	.016777	.000	-.10379	-.03779
	Clindamycin, Nisin in GGG	-.02233	.016777	.184	-.05533	.01066

	Clindamycin, Nisin in GGG, Chx	-.00900	.016777	.592	-.04200	.02400
	Nisin in GGG	-.05196*	.016777	.002	-.08496	-.01896
	Gentamycin	-.06054*	.016777	.000	-.09354	-.02754
	Gentamycin, Chx	-.10938*	.016777	.000	-.14237	-.07638
	Positive Control	-.14458*	.016777	.000	-.17758	-.11159
	Vancomycin	-.04413*	.016777	.009	-.07712	-.01113
	Vancomycin, Chx	.00912	.016777	.587	-.02387	.04212
	Vancomycin, Nisin in GGG	-.05975*	.016777	.000	-.09275	-.02675
	Vancomycin, Nisin in GGG, Chx	-.07688*	.016777	.000	-.10987	-.04388
	Nisin in GGG, Chx	-.04796*	.016777	.005	-.08096	-.01496
	Gentamycin, Chx	-.04275*	.016777	.011	-.07575	-.00975
	Gentamycin, Nisin in GGG	-.06946*	.016777	.000	-.10246	-.03646
Clindamycin, Nisin in GGG	Chlorhexidine	-.06754*	.016777	.000	-.10054	-.03454
	Clindamycin	-.04846*	.016777	.004	-.08146	-.01546
	Clindamycin, Chx	.02233	.016777	.184	-.01066	.05533
	Clindamycin, Nisin in GGG, Chx	.01333	.016777	.427	-.01966	.04633
	Nisin in GGG	-.02962	.016777	.078	-.06262	.00337
	Gentamycin	-.03821*	.016777	.023	-.07121	-.00521
	Gentamycin, Chx	-.08704*	.016777	.000	-.12004	-.05404
	Positive Control	-.12225*	.016777	.000	-.15525	-.08925
	Vancomycin	-.02179	.016777	.195	-.05479	.01121
	Vancomycin, Chx	.03146	.016777	.062	-.00154	.06446
	Vancomycin, Nisin in GGG	-.03742*	.016777	.026	-.07041	-.00442
	Vancomycin, Nisin in GGG, Chx	-.05454*	.016777	.001	-.08754	-.02154
	Nisin in GGG, Chx	-.02563	.016777	.128	-.05862	.00737
	Gentamycin, Chx	-.02042	.016777	.224	-.05341	.01258
	Gentamycin, Nisin in GGG	-.04713*	.016777	.005	-.08012	-.01413
Clindamycin, nisin in GGG, Chx	Chlorhexidine	-.08088*	.016777	.000	-.11387	-.04788
	Clindamycin	-.06179*	.016777	.000	-.09479	-.02879
	Clindamycin, Chx	.00900	.016777	.592	-.02400	.04200
	Clindamycin, Nisin in GGG	-.01333	.016777	.427	-.04633	.01966
	Nisin in GGG	-.04296*	.016777	.011	-.07596	-.00996
	Gentamycin	-.05154*	.016777	.002	-.08454	-.01854
	Gentamycin, Chx	-.10037*	.016777	.000	-.13337	-.06738
	Positive Control	-.13558*	.016777	.000	-.16858	-.10259
	Vancomycin	-.03513*	.016777	.037	-.06812	-.00213
	Vancomycin, Chx	.01812	.016777	.281	-.01487	.05112
	Vancomycin, Nisin in GGG	-.05075*	.016777	.003	-.08375	-.01775
	Vancomycin, Nisin in GGG, Chx	-.06788*	.016777	.000	-.10087	-.03488
	Nisin in GGG, Chx	-.03896*	.016777	.021	-.07196	-.00596

	Gentamycin, Chx	-.03375*	.016777	.045	-.06675	-.00075
	Gentamycin, Nisin in GGG	-.06046*	.016777	.000	-.09346	-.02746
Nisin in GGG	Chlorhexidine	-.03792*	.016777	.024	-.07091	-.00492
	Clindamycin	-.01883	.016777	.262	-.05183	.01416
	Clindamycin, Chx	.05196*	.016777	.002	.01896	.08496
	Clindamycin, Nisin in GGG	.02962	.016777	.078	-.00337	.06262
	Clindamycin, Nisin in GGG, Chx	.04296*	.016777	.011	.00996	.07596
	Gentamycin	-.00858	.016777	.609	-.04158	.02441
	Gentamycin, Chx	-.05742*	.016777	.001	-.09041	-.02442
	Positive Control	-.09263*	.016777	.000	-.12562	-.05963
	Vancomycin	.00783	.016777	.641	-.02516	.04083
	Vancomycin, Chx	.06108*	.016777	.000	.02809	.09408
	Vancomycin, Nisin in GGG	-.00779	.016777	.643	-.04079	.02521
	Vancomycin, Nisin in GGG, Chx	-.02492	.016777	.138	-.05791	.00808
	Nisin in GGG, Chx	.00400	.016777	.812	-.02900	.03700
	Gentamycin, Chx	.00921	.016777	.583	-.02379	.04221
	Gentamycin, Nisin in GGG	-.01750	.016777	.298	-.05050	.01550
Nisin in GGG, Chx	Chlorhexidine	-.04192*	.016777	.013	-.07491	-.00892
	Clindamycin	-.02283	.016777	.174	-.05583	.01016
	Clindamycin, Chx	.04796*	.016777	.005	.01496	.08096
	Clindamycin, Nisin in GGG	.02563	.016777	.128	-.00737	.05862
	Clindamycin, Nisin in GGG, Chx	.03896*	.016777	.021	.00596	.07196
	Nisin in GGG	-.00400	.016777	.812	-.03700	.02900
	Gentamycin	-.01258	.016777	.454	-.04558	.02041
	Gentamycin, Chx	-.06142*	.016777	.000	-.09441	-.02842
	Positive Control	-.09662*	.016777	.000	-.12962	-.06363
	Vancomycin	.00383	.016777	.819	-.02916	.03683
	Vancomycin, Chx	.05708*	.016777	.001	.02409	.09008
	Vancomycin, Nisin in GGG	-.01179	.016777	.483	-.04479	.02121
	Vancomycin, Nisin in GGG, Chx	-.02892	.016777	.086	-.06191	.00408
	Gentamycin, Chx	.00521	.016777	.756	-.02779	.03821
	Gentamycin, Nisin in GGG	-.02150	.016777	.201	-.05450	.01150
Gentamycin	Chlorhexidine	-.02933	.016777	.081	-.06233	.00366
	Clindamycin	-.01025	.016777	.542	-.04325	.02275
	Clindamycin, Chx	.06054*	.016777	.000	.02754	.09354
	Clindamycin, Nisin in GGG	.03821*	.016777	.023	.00521	.07121
	Clindamycin, Nisin in GGG, Chx	.05154*	.016777	.002	.01854	.08454
	Nisin in GGG	.00858	.016777	.609	-.02441	.04158
	Gentamycin, Chx	-.04883*	.016777	.004	-.08183	-.01584
	Positive Control	-.08404*	.016777	.000	-.11704	-.05104

	Vancomycin	.01642	.016777	.328	-.01658	.04941
	Vancomycin, Chx	.06967*	.016777	.000	.03667	.10266
	Vancomycin, Nisin in GGG	.00079	.016777	.962	-.03221	.03379
	Vancomycin, Nisin in GGG, Chx	-.01633	.016777	.331	-.04933	.01666
	Nisin in GGG, Chx	.01258	.016777	.454	-.02041	.04558
	Gentamycin, Chx	.01779	.016777	.290	-.01521	.05079
	Gentamycin, Nisin in GGG	-.00892	.016777	.595	-.04191	.02408
	Chlorhexidine	-.04713*	.016777	.005	-.08012	-.01413
	Clindamycin	-.02804	.016777	.096	-.06104	.00496
	Clindamycin, Chx	.04275*	.016777	.011	.00975	.07575
	Clindamycin, Nisin in GGG	.02042	.016777	.224	-.01258	.05341
	Clindamycin, Nisin in GGG, Chx	.03375*	.016777	.045	.00075	.06675
	Nisin in GGG	-.00921	.016777	.583	-.04221	.02379
	Gentamycin	-.01779	.016777	.290	-.05079	.01521
Gentamycin, Chx	Gentamycin, Chx	-.06663*	.016777	.000	-.09962	-.03363
	Positive Control	-.10183*	.016777	.000	-.13483	-.06884
	Vancomycin	-.00138	.016777	.935	-.03437	.03162
	Vancomycin, Chx	.05187*	.016777	.002	.01888	.08487
	Vancomycin, Nisin in GGG	-.01700	.016777	.312	-.05000	.01600
	Vancomycin, Nisin in GGG, Chx	-.03413*	.016777	.043	-.06712	-.00113
	Nisin in GGG, Chx	-.00521	.016777	.756	-.03821	.02779
	Gentamycin, Nisin in GGG	-.02671	.016777	.112	-.05971	.00629
	Chlorhexidine	.05471*	.016777	.001	.02171	.08771
	Clindamycin	.07379*	.016777	.000	.04079	.10679
	Clindamycin, Chx	.14458*	.016777	.000	.11159	.17758
	Clindamycin, Nisin in GGG	.12225*	.016777	.000	.08925	.15525
	Clindamycin, Nisin in GGG, Chx	.13558*	.016777	.000	.10259	.16858
	Nisin in GGG	.09263*	.016777	.000	.05963	.12562
	Gentamycin	.08404*	.016777	.000	.05104	.11704
Positive Control	Gentamycin, Chx	.03521*	.016777	.037	.00221	.06821
	Vancomycin	.10046*	.016777	.000	.06746	.13346
	Vancomycin, Chx	.15371*	.016777	.000	.12071	.18671
	Vancomycin, Nisin in GGG	.08483*	.016777	.000	.05184	.11783
	Vancomycin, Nisin in GGG, Chx	.06771*	.016777	.000	.03471	.10071
	Nisin in GGG, Chx	.09662*	.016777	.000	.06363	.12962
	Gentamycin, Chx	.10183*	.016777	.000	.06884	.13483
	Gentamycin, Nisin in GGG	.07512*	.016777	.000	.04213	.10812
	Chlorhexidine	-.04575*	.016777	.007	-.07875	-.01275
Vancomycin	Clindamycin	-.02667	.016777	.113	-.05966	.00633
	Clindamycin, Chx	.04413*	.016777	.009	.01113	.07712

	Clindamycin, Nisin in GGG	.02179	.016777	.195	-.01121	.05479
	Clindamycin, Nisin in GGG, Chx	.03513*	.016777	.037	.00213	.06812
	Nisin in GGG	-.00783	.016777	.641	-.04083	.02516
	Gentamycin	-.01642	.016777	.328	-.04941	.01658
	Gentamycin, Chx	-.06525*	.016777	.000	-.09825	-.03225
	Positive Control	-.10046*	.016777	.000	-.13346	-.06746
	Vancomycin, Chx	.05325*	.016777	.002	.02025	.08625
	Vancomycin, Nisin in GGG	-.01563	.016777	.352	-.04862	.01737
	Vancomycin, Nisin in GGG, Chx	-.03275	.016777	.052	-.06575	.00025
	Nisin in GGG, Chx	-.00383	.016777	.819	-.03683	.02916
	Gentamycin, Chx	.00138	.016777	.935	-.03162	.03437
	Gentamycin, Nisin in GGG	-.02533	.016777	.132	-.05833	.00766
Vancomycin, Nisin in GGG	Chlorhexidine	-.03012	.016777	.073	-.06312	.00287
	Clindamycin	-.01104	.016777	.511	-.04404	.02196
	Clindamycin, Chx	.05975*	.016777	.000	.02675	.09275
	Clindamycin, Nisin in GGG	.03742*	.016777	.026	.00442	.07041
	Clindamycin, Nisin in GGG, Chx	.05075*	.016777	.003	.01775	.08375
	Nisin in GGG	.00779	.016777	.643	-.02521	.04079
	Gentamycin	-.00079	.016777	.962	-.03379	.03221
	Gentamycin, Chx	-.04962*	.016777	.003	-.08262	-.01663
	Positive Control	-.08483*	.016777	.000	-.11783	-.05184
	Vancomycin	.01563	.016777	.352	-.01737	.04862
	Vancomycin, Chx	.06887*	.016777	.000	.03588	.10187
	Vancomycin, Nisin in GGG, Chx	-.01712	.016777	.308	-.05012	.01587
	Nisin in GGG, Chx	.01179	.016777	.483	-.02121	.04479
	Gentamycin, Chx	.01700	.016777	.312	-.01600	.05000
	Gentamycin, Nisin in GGG	-.00971	.016777	.563	-.04271	.02329
Vancomycin, Nisin in GGG, Chx	Chlorhexidine	-.01300	.016777	.439	-.04600	.02000
	Clindamycin	.00608	.016777	.717	-.02691	.03908
	Clindamycin, Chx	.07688*	.016777	.000	.04388	.10987
	Clindamycin, Nisin in GGG	.05454*	.016777	.001	.02154	.08754
	Clindamycin, Nisin in GGG, Chx	.06788*	.016777	.000	.03488	.10087
	Nisin in GGG	.02492	.016777	.138	-.00808	.05791
	Gentamycin	.01633	.016777	.331	-.01666	.04933
	Gentamycin, Chx	-.03250	.016777	.054	-.06550	.00050
	Positive Control	-.06771*	.016777	.000	-.10071	-.03471
	Vancomycin	.03275	.016777	.052	-.00025	.06575
	Vancomycin, Chx	.08600*	.016777	.000	.05300	.11900
	Vancomycin, Nisin in GGG	.01712	.016777	.308	-.01587	.05012
	Nisin in GGG, Chx	.02892	.016777	.086	-.00408	.06191

	Gentamycin, Chx	.03413*	.016777	.043	.00113	.06712
	Gentamycin, Nisin in GGG	.00742	.016777	.659	-.02558	.04041
Gentamycin, Nisin in GGG	Chlorhexidine	-.02042	.016777	.224	-.05341	.01258
	Clindamycin	-.00133	.016777	.937	-.03433	.03166
	Clindamycin, Chx	.06946*	.016777	.000	.03646	.10246
	Clindamycin, Nisin in GGG	.04713*	.016777	.005	.01413	.08012
	Clindamycin, Nisin in GGG, Chx	.06046*	.016777	.000	.02746	.09346
	Nisin in GGG	.01750	.016777	.298	-.01550	.05050
	Gentamycin	.00892	.016777	.595	-.02408	.04191
	Gentamycin, Chx	-.03992*	.016777	.018	-.07291	-.00692
	Positive Control	-.07512*	.016777	.000	-.10812	-.04213
	Vancomycin	.02533	.016777	.132	-.00766	.05833
	Vancomycin, Chx	.07858*	.016777	.000	.04559	.11158
	Vancomycin, Nisin in GGG	.00971	.016777	.563	-.02329	.04271
	Vancomycin, Nisin in GGG, Chx	-.00742	.016777	.659	-.04041	.02558
	Nisin in GGG, Chx	.02150	.016777	.201	-.01150	.05450
	Gentamycin, Chx	.02671	.016777	.112	-.00629	.05971
Gentamycin, Nisin in GGG, Chx	Chlorhexidine	.01950	.016777	.246	-.01350	.05250
	Clindamycin	.03858*	.016777	.022	.00559	.07158
	Clindamycin, Chx	.10938*	.016777	.000	.07638	.14237
	Clindamycin, Nisin in GGG	.08704*	.016777	.000	.05404	.12004
	Clindamycin, Nisin in GGG, Chx	.10037*	.016777	.000	.06738	.13337
	Nisin in GGG	.05742*	.016777	.001	.02442	.09041
	Gentamycin	.04883*	.016777	.004	.01584	.08183
	Positive Control	-.03521*	.016777	.037	-.06821	-.00221
	Vancomycin	.06525*	.016777	.000	.03225	.09825
	Vancomycin, Chx	.11850*	.016777	.000	.08550	.15150
	Vancomycin, Nisin in GGG	.04962*	.016777	.003	.01663	.08262
	Vancomycin, Nisin in GGG, Chx	.03250	.016777	.054	-.00050	.06550
	Nisin in GGG, Chx	.06142*	.016777	.000	.02842	.09441
	Gentamycin, Chx	.06663*	.016777	.000	.03363	.09962
	Gentamycin, Nisin in GGG	.03992*	.016777	.018	.00692	.07291
Vancomycin+Chlorhexidine	Chlorhexidine	-.09900*	.016777	.000	-.13200	-.06600
	Clindamycin	-.07992*	.016777	.000	-.11291	-.04692
	Clindamycin, Chx	-.00912	.016777	.587	-.04212	.02387
	Clindamycin, Nisin in GGG	-.03146	.016777	.062	-.06446	.00154
	Clindamycin, Nisin in GGG, Chx	-.01812	.016777	.281	-.05112	.01487
	Nisin in GGG	-.06108*	.016777	.000	-.09408	-.02809
	Gentamycin	-.06967*	.016777	.000	-.10266	-.03667

	Gentamycin, Chx	-.11850*	.016777	.000	-.15150	-.08550
	Positive Control	-.15371*	.016777	.000	-.18671	-.12071
	Vancomycin	-.05325*	.016777	.002	-.08625	-.02025
	Vancomycin, Nisin in GGG	-.06887*	.016777	.000	-.10187	-.03588
	Vancomycin, Nisin in GGG, Chx	-.08600*	.016777	.000	-.11900	-.05300
	Nisin in GGG, Chx	-.05708*	.016777	.001	-.09008	-.02409
	Gentamycin, Chx	-.05187*	.016777	.002	-.08487	-.01888
	Gentamycin, Nisin in GGG	-.07858*	.016777	.000	-.11158	-.04559

Based on observed means.

The error term is Mean Square(Error) = .003.

*. The mean difference is significant at the 0.05 level.

19. Accepted Abstracts

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Alternatives to conventional antibiotics: chlorhexidine and nisin inhibitory activities against diabetic foot ulcer staphylococci

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Keywords: Chlorhexidine; Diabetic Foot Ulcer; Nisin; *Staphylococcus aureus*

Diabetes *mellitus* (DM) is a chronic disease that affects more than 422 million people worldwide, with 15 to 25% of patients developing diabetic foot ulcers (DFU) in their lifetime. Around half of these ulcers become clinically infected, usually by opportunistic pathogens, being *Staphylococcus aureus* the most frequent [1]. The presence of antibiotic resistant *S. aureus* strains is a major problem in DFU treatment. Therefore, it is utterly important to define new strategies to control these infections, based on antimicrobial compounds that represent an alternative to conventional antibiotics, such as chlorhexidine and nisin.

Chlorhexidine is a broad-spectrum antiseptic, active against bacteria, fungi and some enveloped viruses. Despite its potential, the increasing use in hand hygiene and patient washing raises concern regarding development of acquired bacterial resistance. Nisin is an antimicrobial peptide produced by *Lactococcus lactis* that is mainly active against Gram-positive bacteria. Nisin has been used for pathogen control in food products and differs from conventional antibiotics regarding its synthesis, toxicity, resistance mechanisms and mode of action. As the exposure to sub-lethal antimicrobial concentrations may enhance resistance towards these biocidal compounds, it is crucial to determine their minimum inhibitory (MIC) and bactericidal concentration (MBC) values against selected pathogens. This work aimed to evaluate chlorhexidine and nisin antibacterial activity against 23 *S. aureus* strains isolated at Lisbon medical centres from infected foot ulcers of DM patients, including both multidrug-resistant and MRSA strains (22 and 35%, respectively)[2]. Isolates *in vitro* susceptibility to chlorhexidine and nisin was assessed using standard microdilution assays.

All strains, including those with relevant antibiotic resistance profiles, presented susceptibility to these compounds. Mean MIC values were 6±2 and 90.0±22.8 µg/mL, and mean MBC values were 15±16 and 495.2±149.9 µg/mL, for chlorhexidine and nisin, respectively.

Results support the potential use of these compounds in clinically infected DFU. They also provide a valuable contribution for the establishment of effective antimicrobial protocols, as the application of these

inhibitory compounds may ultimately contribute to the reduction of conventional antibiotic administration to these patients and to the dissemination of resistant strains.

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[1]J.J. Mendes et al. Clinical and bacteriological survey of diabetic foot infections in Lisbon. Diabetes Res Clin Pract,2012,95:153-161.

[2]C. Mottola et al. Molecular typing, virulence traits and antimicrobial resistance of diabetic foot Staphylococci. J Biomed Sci,2016,23:33.

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Chlorhexidine *in vitro* activity against diabetic foot ulcer isolates

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Diabetes *mellitus* (DM) is a chronic disease that affects more than 422 million people worldwide. In recent decades, the prevalence of DM has increased, and consequently the incidence of DM-associated foot ulcers is also higher. Around half of these ulcers become clinically infected, usually by opportunistic pathogens, being *Staphylococcus aureus* the most frequently isolated pathogen [1]. Staphylococci, particularly *S. aureus*, have been described as the most virulent pathogens in infected foot ulcers, presenting a correlation between specific virulence genotypic markers and ulcer outcome. In addition, these bacteria are recognized for their ability to develop resistance to different antibiotic classes, and infections caused by *S. aureus* strains, particularly by methicillin-resistant *S. aureus* (MRSA), are reaching epidemic proportions globally.

Considering that the presence of antibiotic resistant *S. aureus* pathogens is a key problem in the treatment of DM infected foot ulcers, it is utterly important to define new strategies to control these infections, using alternative biocidal compounds. Chlorhexidine gluconate is a water soluble broad-spectrum antiseptic. It is a cationic biguanide that binds to the negatively charged bacterial cell wall, affecting membrane integrity and altering its osmotic equilibrium. Since it binds strongly to the proteins present in the skin and mucosa, it has a persistent antiseptic effect. Chlorhexidine is most active against Gram-positive bacteria, but also has activity against Gram-negative bacteria, anaerobes, fungi and some enveloped viruses. Despite its potential, the increasing use of chlorhexidine for hand hygiene, skin antisepsis and patient washing, raises concern regarding development of acquired bacterial resistance [2].

As the exposure to sub-lethal antimicrobial concentrations may enhance resistance towards chlorhexidine, it is crucial to determine its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against selected pathogens. This work aimed to evaluate chlorhexidine antibacterial activity against 23 *S. aureus* strains isolated at Lisbon medical centres from patients diagnosed with DM infected foot ulcers. The collection under study includes both multidrug-resistant and MRSA strains (22% and 35%, respectively) [3].

Isolates *in vitro* susceptibility to chlorhexidine was assessed using standard microbroth dilution assays and all strains, including those with the highest rates of antibiotic resistance, presented susceptibility to this compound. Chlorhexidine MIC values ranged from $1.4x10^{-3}$ to $7.1x10^{-3}$ mg/ml with an average value of $6x10^{-3} \pm 2x10^{-3}$ mg/ml and MBC values ranged from $4.7x10^{-3}$ to $71.4x10^{-3}$ mg/ml with an

average value of $15x10^{-3} \pm 16x10^{-3}$ mg/ml. Chlorhexidine showed bactericidal activity against the vast majority of the isolates (91%).

These results support the use of chlorhexidine as a skin antiseptic for patients presenting infected diabetic foot ulcers. They also provide a valuable contribution for the establishment of effective antisepsis protocols using chlorhexidine, to be applied in medical centres in order to reduce additional selection pressure in DM foot ulcer pathogens, ultimately contributing for the reduction of conventional antibiotic administration to these patients and dissemination of resistance strains.

This work was supported by the Interdisciplinary Centre of Research in Animal Health (CIISA), Faculty of Veterinary Medicine, University of Lisbon (FMV/UL) (Project UID/CVT/00276/2013).

Keywords: Chlorhexidine; Diabetic Foot Ulcer; *Staphylococcus aureus*

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