Unveiling the role of nisin on resistance development by Diabetic Foot staphylococci: mutant selection window and horizontal gene transfer

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“It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change.” **– Charles Darwin**

Preface

The work presented in this thesis was performed at Laboratory of Veterinary Bacteriology of Faculty of Veterinary Medicine (Lisbon, Portugal), during the period October – October 2019, under the supervision of Prof. Doc. Manuela de Oliveira. The thesis was co-supervised at Instituto Superior Técnico by Prof. Leonilde Moreira.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Lisbon University.

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Abstract

The most prevalent microorganism in diabetic foot infections (DFI) is *Staphylococcus aureus*, an important pathogen due to its frequent antibiotic multi-resistant profile. As such, it is mandatory to develop alternative compounds for DFI treatment. The antimicrobial peptide nisin is considered a promising alternative because it has been showed to be effective against S. aureus DFI isolates and due to its use in food industry for 90 years. However, correct drug therapeutic doses must be established before instituting a new DFI therapeutic protocol based on nisin, to avoid the selection and amplification of resistant mutants.

The mutant selection window (MSW) of nisin was determined for 24 DFI *S. aureus* isolates. MSW ranged from 11.25-360 μg/mL for two isolates, from 11.25-540 μg/mL for three isolates and from 11.25-720 μg/mL for one isolate. It was not possible to determine the MSW for the remaining 18 isolates since they were able to grow at the highest nisin concentration tested (720 μg/mL).

Results are in accordance with the previously determined MSW for vancomycin regarding *S. aureus* isolates which is relevant since the action mode of these antimicrobials is similar.

To understand if nisin could potentiate the transfer of resistant genes from *Enterococcus* to the clinical *S. aureus* isolates, a protocol aiming to prompt the horizontal gene transfer of *vanA* between these bacterial species was performed. In the presence of nisin sub-MIC values no transconjugants were obtained, indicating that nisin sub-MIC values do not promote *vanA* transfer, supporting nisin future application to DFI treatment.

Keywords: Diabetic Foot Infection; *S. aureus*; Nisin; Mutant Selection Window; Horizontal Gene Transfer.

Resumo

O microrganismo mais prevalente nas infeções do pé diabético (IPD) é o *Staphylococcus aureus*, um agente patogénico importante devido ao seu perfil multirresistente a antibióticos. É assim, necessário o desenvolvimento de compostos alternativos para o tratamento das IPD. A nisina, um péptido antimicrobiano, é considerada uma alternativa promissora devido ao seu uso na indústria de alimentos há 90 anos e por ser eficaz contra isolados de *S. aureus* de IPD. No entanto, para evitar a seleção de mutantes resistentes, têm que ser consideradas doses terapêuticas corretas da nisina.

A janela para seleção de mutantes (JSM) da nisina foi determinada para 24 isolados *S. aureus* de IDF. As JSM variaram de 11,25-360 µg/mL para dois isolados, de 11,25-540 µg/mL para três isolados e de 11,25-720 µg/mL para um isolado. Não foi possível determinar a JSM para os restantes 18 isolados, pois estes foram capazes de crescer na maior concentração de nisina testada (720 µg/mL).

Os resultados estão de acordo com a JSM previamente determinada para a vancomicina em relação a isolados *S. aureus*, sendo relevante, pois o modo de ação destes antimicrobianos é semelhante. Para entender se a nisina poderia potenciar a transferência de genes de resistência, como *vanA* (provoca resistência à vancomicina), foi realizado um protocolo de transferência horizontal de genes de *Enterococcus* para os isolados *S. aureus*. Na presença de valores de sub-MIC de nisina, não foram obtidos transconjugados, indicando que esta concentração não promove a transferência de *vanA*, apoiando a futura aplicação de nisina no tratamento de IPD.

Palavras-chaves: Infeções do Pé Diabético; *S. aureus;* Nisina; Janela para Seleção de Mutantes; Transferência Horizontal de Genes

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ACME - Arginine-Catabolic Mobile Element

AMP - Antimicrobial Peptide

BHI - Brain Heart Infusion agar

BHIB - Brain Heart Infusion broth

CA-MRSA - Community Acquired Methicillin Resistant *Staphylococcus aureus*

CC – Clonal Complex

CDC - Center for Disease Control and Prevention

DFI – Diabetic Foot Infection

DFU - Diabetic Foot Ulcer

DM - Diabetes *mellitus*

EFSA – European Food Safety Authority

GI - Gastrointestinal

GlcNAc - N-acetylglucosamine

HA-MRSA – Hospital Acquired Methicillin Resistant *Staphylococcus aureus*

HGT - Horizontal Gene Transfer

MBIC - Minimum Biofilm Inhibitory Concentration

MGE - Mobile Genetic Elements

MIC - Minimum Inhibitory Concentration

MHA - Mueller Hinton agar

MLST - Multilocus Sequence Typing

MMR - Methyl-directed Mismatch

MPC - Mutant Prevention Concentration

MRSA - Methicillin Resistant *Staphylococcus aureus*

MSA - Mannitol Salt agar

MSSA - Methicillin Susceptible *Staphylococcus aureus*

MSW - Mutant Selection Window

MurNAc – N-acetylmuramic acid

PBP2a - Penicillin-Binding Protein 2a

PCR - Polymerase Chain Reaction

PVL - Panton–Valentine Leukocidin

TSA - Tryptic Soy agar

TSB - Tryptic Soy broth

VISA - Vancomycin-Intermediate *Staphylococcus aureus*

VRE - Vancomycin Resistant *Enterococcus*

VRSA - Vancomycin-Resistant *Staphylococcus aureus*

WHO – World Health Organization

Chapter 1 | Introduction

1.1. Diabetes *mellitus*

In healthy individuals, insulin is produced by pancreas in parallel to the glucose ingested, leading to the absorption of glucose by the blood cells. Diabetes *mellitus* (DM) is a chronic metabolic disease in which the insulin concentration produced is not enough to promote sugar absorption or it is not efficiently used, leading to an increase of blood glucose concentration.

There are two main types of diabetes, type I and type II. In type I diabetes, the pancreas does not produce enough insulin and the patients need to obtain a higher concentration through injection, while in type II diabetes, the blood cells are resistant to insulin, not using it efficiently. In both cases, the increase in blood glucose concentrations leads to several damages mainly at the blood vessels level ("What is Diabetes?," 2018).

The increase in diabetes incidence may represent a major health problem since this disease has numerous consequences, including: the damage of small blood vessels in the eye, that ultimately may lead to blindness; the damage of small blood vessels in the kidney that can lead to renal failure; and the clogging of major blood vessels that can lead to myocardial infarction or stroke. Diabetes can also affect the limbs health due to hyperglycemia and neuropathy, which together with decreased blood flow can be responsible for, in worst-case scenarios, the amputation of the damaged site ("WHO | Diabetes programme," 2018).

According to the World Health Organization (WHO), in 2015 422 million adults suffered from diabetes, which was directly responsible for 1.6 million deaths worldwide ("WHO | Diabetes programme," 2018). These numbers are expected to increase, being foreseen that, considering only the United States, the prevalence of diabetic patients will reach more than 54.9 million individuals between 2015 and 2030 (Rowley, Bezold, Arikan, Byrne, & Krohe, 2017). In Portugal, reports refer that in 2017 there were about 1.065 million people with diabetes in our country, a number which is expected to rise to 1.1147 million individuals by 2045 ("IDF diabetes atlas - Home," 2017).

1.2. Diabetic Foot Infection

1.2.1. Characterization and epidemiology of diabetic foot ulcer

One of the major complications of diabetes is the formation of foot ulcers, which have an incidence of 25% in diabetic patients (Singh, David, & Benjamin, 2005). Around 60% of patients with diabetic foot ulcers (DFU) develop infections (diabetic foot infection - DFI) and 20% of these infections may lead to lower limb amputation, being observed that mortality of patients after amputation ranges from 13% to 40% (Singh et al., 2005; Skrepnek, Mills, Lavery, & Armstrong, 2017). In a five-year study conducted in Portugal, it was observed that 45.6% of patients with DFUs have died, while 44.8% of the remaining population under study was subjected to amputation (Garrido, Couto De Carvalho, & Carvalho, 2016).

The ulcers can result from several factors, including the peripheral neuropathy due to the damage of the nerves from the peripheral nervous system (Hobizal & Wukich, 2012). There are different types of neuropathy: sensory, motor and autonomic. Sensory neuropathy causes lack (or low) sensitivity to pain, pressure and temperature, which can lead to burns, cuts and wounds promoted by the use of

tight shoes, that remain unnoticed by the patient. Motor neuropathy causes the deterioration of foot muscles, leading to their deformation and the development of “hammer” or “claw” toes, and also to the alteration of the normal pressure that the foot exerts on the floor. Autonomic neuropathy affects the oil gland function, rendering the foot skin dry and promoting the appearance of cuts and fissures (Blanes et al., 2011; Farzamfar et al., 2013; Hobizal & Wukich, 2012).

Vascular deficiencies such as macro and microangiopathy are also important factors in the formation of ulcers, as they are responsible for a deficient blood circulation in the affected area (Blanes et al., 2011; Farzamfar et al., 2013). In some cases, the increase in vasoconstriction can also induce ischemia, promoting nerve damage, tissue hypoxia and deficient blood circulation, frequently leading to ulcer formation. These ischemic related ulcers have the tendency to gangrene (Farzamfar et al., 2013).

Another factor that influences the formation of ulcers is the impaired immune system that diabetic patients usually present. It has been demonstrated that hyperglycemia causes morphologic differences in macrophages and impairs leukocyte response (Blanes et al., 2011; Farzamfar et al., 2013; Hobizal & Wukich, 2012).

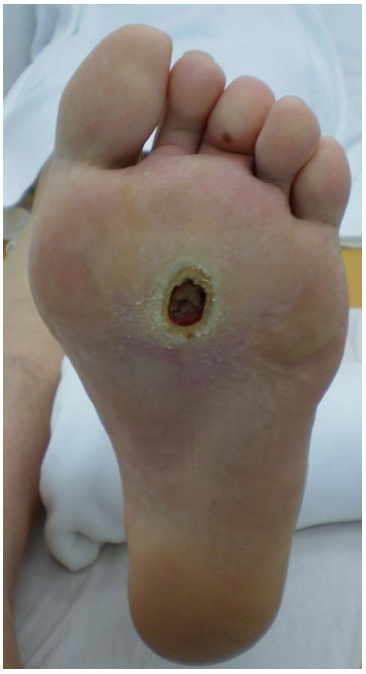

Figure 1 - Neuropathic diabetic foot ulcer. From: Wikipedia

The treatment protocol and prognosis of the foot disease are directly linked to the severity of the neuropathy, ischemia and ulcer characteristics (Farzamfar et al., 2013). Also different patients can present different outcomes depending on their region or social status (Blanes et al., 2011; Game, 2016). Therefore, to uniformize information and to facilitate the ulcer clinical approach, the implementation of a classification or scoring system for DFI evaluation is important. There are several classifications and scores available, although no universal consensus about none.

The different systems are used by three main areas: clinical care, research and clinical audit (Game, 2016). The International Working Group on the Diabetic Foot proposed the PEDIS classification system for DFU, which focus on the categorization of different populations of patients with DFU for research purposes (Schaper, 2004). According to this classification, ulcers are evaluated regarding five main characteristics, each one classified in different grades: perfusion, extent/size, depth/tissue loss, presence of infection and sensation (Table 1) (Farzamfar et al., 2013; Schaper, 2004).

Table 1 - Diabetic Foot Infection - PEDIS Classification. Adapted from Farzamfar et al. 2013 and Schaper, 2004 (Farzamfar et al., 2013; Schaper, 2004).

PEDIS Grade	Perfusion	Depth	Infection severity	Sensation
1	No signs of peripheral arterial disease with no symptoms	Superficial ulcer	No signs of infection	No loss of protective sensation
2	Symptoms and/or signs of peripheral arterial disease	Ulcer penetration into skin structures (could involve muscle or tendon)	Infection of skin or superficial subcutaneous tissues	No loss of protective sensation
3	Critical limb ischemia	Penetration into the foot deeper layers (could involve bone and/or join)	Moderate infection which could involve deep tissues	-
4	-	-	Foot infection involving systemic inflammatory response syndrome	-

1.2.2. Pathogenic agents frequently related to diabetic foot infections

When an ulcer is formed the probability of infection is high, mainly due to the impairment of the immune system induced by DM (Hobizal & Wukich, 2012). DFI are characterized by their polymicrobial feature as referred by several studies, which are also in accordance regarding the identification of the related microorganisms (Abdulrazak, Ibrahim Bitar, Ayesh Al-Shamali, & Ahmed Mobasher, 2005; Blanes et al., 2011; Citron, Goldstein, Merriam, Lipsky, & Abramson, 2007; Hobizal & Wukich, 2012). Nevertheless, these studies point out that differences in the stage/severity of the ulcer, presence of infection, sample collection technique, previous treatments and geographical localization may have an impact on the differences observed in DFI microorganisms prevalence (Abdulrazak et al., 2005; Blanes et al., 2011; Citron et al., 2007; Hobizal & Wukich, 2012). In a study

conducted in Portugal in patients with DFI, researchers obtained an average of 3.0±1.4 organisms per sample (J.J. Mendes et al., 2011). The most prevalent genus was *Staphylococcus*, with *Staphylococcus aureus* being the most frequent isolated species (51%), which is in accordance with previous studies (Abdulrazak et al., 2005; Blanes et al., 2011; Citron et al., 2007; Hobizal & Wukich, 2012; J.J. Mendes et al., 2011).

Recent ulcers are usually monomicrobial, being *S. aureus* the first colonizer followed by coagulase negative staphylococci and enterococci, both facultative anaerobic gram-positive cocci. *Pseudomonas aeruginosa* can be found in moderate infections, in association with the previously mentioned bacteria, and it is the most prevalent gram-negative species in DFU. Anaerobes are generally present in severe infections (Abdulrazak et al., 2005; Blanes et al., 2011; Citron et al., 2007; Hobizal & Wukich, 2012).

1.2.2.1. *Staphylococcus aureus*

1.2.2.1.1. Characterization

Staphylococcus aureus is a gram-positive, facultative anaerobic, oxidase negative, catalase and coagulase positive coccus (Medvedova & Valik, 2012). Its first characterization was made by Sir Alexander Ogston in 1882, but these species was only isolated in 1884 by Anton J. Rosenbach (Medvedova & Valik, 2012; Miljković-Selimović, Dinić, Orlović, & Babić, 2015). *S. aureus* is a frequent commensal colonizer of human mucosa and skin, being present in 20% of the individuals; it can also be found in animals and in the environment (Bimali, Shrestha, Tuladhar, & Lekhak, 2015; Miljković-Selimović et al., 2015). These bacteria are an opportunistic pathogen responsible for several infections, from minor ones, like folliculitis, to severe infections like endocarditis and osteomyelitis. In fact, if present in the bloodstream they are able to infect all organs (Bimali et al., 2015; Medvedova & Valik, 2012; Miljković-Selimović et al., 2015). According to the Center for Disease Control and Prevention (CDC) there are five factors that can promote *S. aureus* transmission (five C's), Crowding, skin-to-skin Contact, Compromised skin integrity, Contaminated objects and surfaces, and deficiency of Cleanliness (DeLeo, Otto, Kreiswirth, & Chambers, 2010). The pathogenic profile presented by *S. aureus* is related with its high virulence and ability to survive in different conditions (Miljković-Selimović et al., 2015). *S. aureus* expresses several virulence factors, including toxins, surface factors and enzymes, which allows it to evade and modulate the immune system of the hosts, being also involved in food poisoning cases (Medvedova & Valik, 2012; Miljković-Selimović et al., 2015). The ability of *S. aureus* to acquire mobile genetic elements (MGE), like arginine-catabolic mobile element (ACME) and Panton–Valentine leukocidin (PVL), contributes, as well, to the high virulence of this bacteria, since ACME promotes a better adaptation to the human skin environment and the PVL appears to be involved in the lysis of the hosts neutrophils (DeLeo et al., 2010; Turner et al., 2019). Additionally, infections caused by *S. aureus* become more difficult to treat over the years owing to is frequent multidrug resistant profile (Kraus & Peschel, 2008). This high genetic adaptability of *S. aureus* allows the dissemination of strains increasingly adapted to the host (human and animal) and the environment, revealing the importance of these bacteria to the one health concept (Turner et al., 2019).

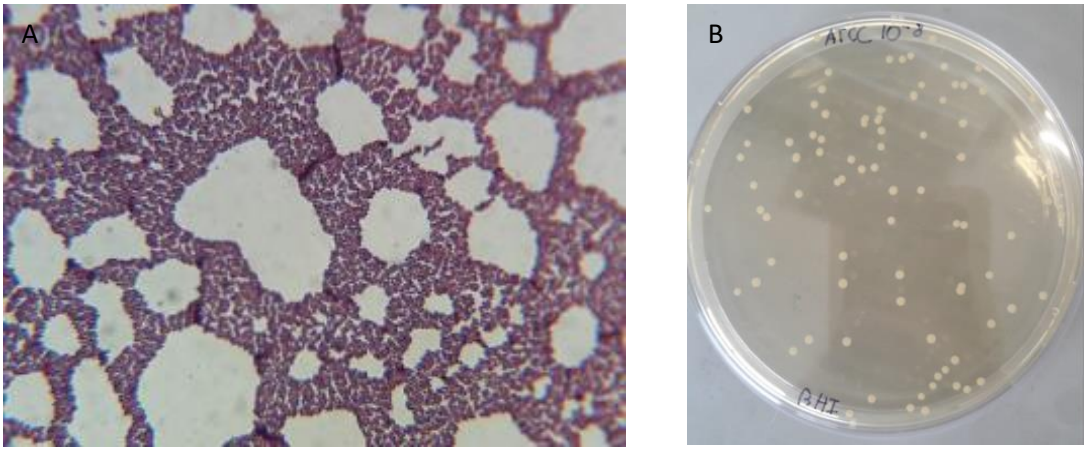

Figure 2 – A- Optical microscopy image of Staphylococcus aureus after Gram staining (1000x). B- Colonies of S. aureus ATCC 29213 in Brain Heart Infusion Agar (BHI) medium (originals).

1.2.2.2. Biofilm and quorum sensing

Generally, infectious diseases are responsible for a mortality rate of 20% worldwide being also estimated that 80% of human infections are related to bacterial biofilms (Salwiczek et al., 2014).
In biofilm formation, planktonic or free-floating cells begin to adhere to a surface (biotic or abiotic) in a reversible way (Malik, Mohammad, & Ahmad, 2013; Richard, Sotto, & Lavigne, 2011). After, as result of the proliferation and adherence of other bacteria, a microcolony is formed and becomes irreversibly attached. The communication between microorganisms is achieved through quorum-sensing, a mechanism essential to biofilm development (Davis, Martinez, & Kirsner, 2006; Malik et al., 2013; Richard et al., 2011). Due to the secretion of chemical signals, bacteria have information about cell density, which leads to the formation of an extracellular polymer matrix when the population reaches a required threshold, after which the biofilm stabilizes (Davis et al., 2006; Richard, Lavigne, & Sotto, 2012). The cells on the biofilm surface can switch to planktonic state and re-colonize other locals (Salwiczek et al., 2014; Schierle, De la Garza, Mustoe, & Galiano, 2009). The different phases of a biofilm formation are demonstrated in figure 3.

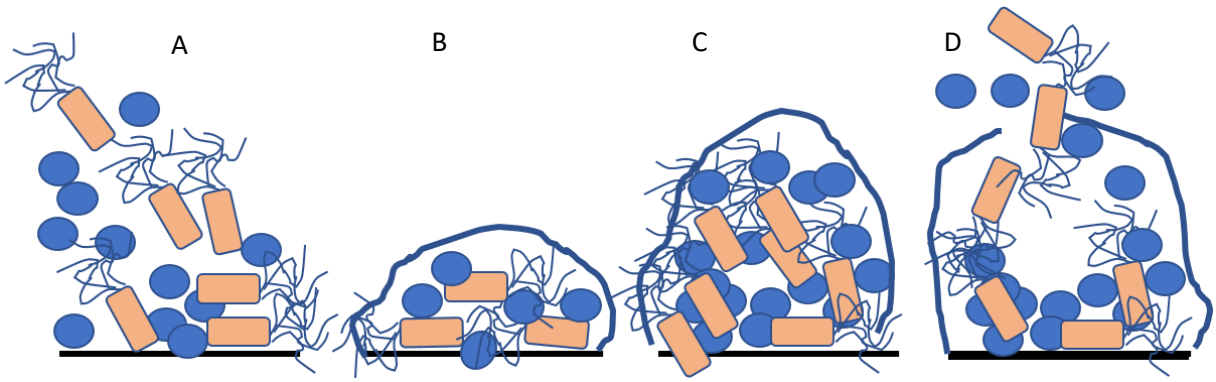

Figure 3 – Biofilm formation. A- Planktonic cells adhering to a surface. B- Formation of a microcolony. C- Mature biofilm formed. D- Dispersion of cells that can re-colonize other locals. (original)

The association between biofilms and chronic wounds, such as DFI, has already been described, being observed that the presence of biofilm in a wound can inhibit reepithelization (Schierle et al., 2009). Moreover, the antibiotic concentration required to inhibit biofilm formation, the minimum biofilm inhibitory concentration (MBIC), can be 100 to 1000 times higher than the minimum inhibitory concentration (MIC) for their planktonic counterparts, which was also confirmed in a study conducted by our research team using isolates from patients with DFI (Mottola, Matias, et al., 2016; Richard et al., 2012).

There are several reasons for the increased resistance of bacterial biofilms, including: the physical and chemical barriers formed by the exopolysaccharide layer hampering antibiotic diffusion; the differences in the environment inside the biofilm affecting antimicrobial efficacy; the polymicrobial feature of the biofilm rendering antimicrobial susceptibility patterns variable and heterogeneous; the proximity of cells which facilitates the transference of antibiotic resistant genes between bacteria, promoting horizontal gene transfer (HGT); and the low growth rate of bacteria that compose the lower layers of the biofilms influencing the activity of antimicrobial compounds (Richard et al., 2012).

The probability for the development of biofilms in a DFU is high since most of the bacterial colonizers of these wounds are biofilm producers. A study conducted in India, using 255 bacterial isolates from patients with DFU, revealed that 70% of the isolates were biofilm producers (Malik et al., 2013). Biofilms are believed to be related with the major complications of DFI, being also responsible for the impairment of the applied treatments (Davis et al., 2006).

In addition to being one of the most frequent pathogens in DFI, as already mentioned, *S. aureus* is the most frequent biofilm producer bacterial species associated with chronic infections (Malik et al., 2013; Mottola, Mendes, et al., 2016; Santos, Veiga, Tavares, Castanho, & Oliveira, 2016). In fact, in another study conducted by our research team, *S. aureus* isolates from DFI were all classified as biofilm producers, being the majority able to produce biofilms after a 24h period (Mottola, Mendes, et al., 2016).

1.2.3. Antibiotic Therapies

When a diabetic foot ulcer becomes infected, an empirical antibiotherapy protocol is usually applied to avoid the progression of the infection, since the characteristics of the diabetic patients and the ulcer itself promote the rapid evolution of the infection (Richard et al., 2011). The administration of empirical antibiotherapy to patient with ulcers that are not clinically infected is not recommended since there are no evidences that it can contribute to a better ulcer prognosis and could promote the emergence of antimicrobial resistant strains (Davis et al., 2006; Lipsky, 2004; Richard et al., 2011).

Empirical antibiotherapy is established following the detection of clinical signs of infection such as atypical coloration, odor, tissue granulation, irregular wound edges, impaired sensitivity at the wound site and/or delay of the healing process (Lipsky, Berendt, et al., 2012). There are certain aspects that have to be considered before establishing an empirical antibiotic therapy protocol, including which antibiotic is going to be used, the period of the treatment and the route of administration that is usually oral or parenteral. In particular cases, a topical administration can also be considered. These

decisions are intrinsically linked to the severity of the infection, the most prevalent bacterial species and their usual resistance profile, the duration and clinical presentation of the ulcer, the history of the patient (like allergies and previous antibiotic treatments) and the treatment cost (Lipsky, 2004; Richard et al., 2011). If necessary, treatment has to be revised after antibiotic susceptibility testing of the pathogens present, aiming to confirm if the empirical treatment established is the most adequate one (Abbas, Uçkay, & Lipsky, 2015; Bader, 2008; Lipsky, Berendt, et al., 2012; Société de Pathologie Infectieuse de Langue Française, 2007).

Mild and some moderate infections can be treated with oral antibiotics with activity spectrum against aerobic and facultative anaerobic Gram-positive cocci, while severe infections usually require parenteral therapy with broad spectrum antibiotics, since anaerobic and Gram-negative organisms are typically present in such infections, as previous referred (Kosinski & Lipsky, 2010; Lipsky, Berendt, et al., 2012).

When recommended, the topical administration is applied to mild infections; however, the application of topical antibiotics to DFI is a controversial subject (Abbas et al., 2015; Lipsky, Kuss, Edmonds, Reyzelman, & Sigal, 2012; Markakis et al., 2018). Some authors do not advise this administration route, since in DFI the vascularization is usually compromised, which may contribute for the topical antibiotic to not reach the infection at the required concentration not being able to eliminate all the bacteria present and therefore, promoting the increase of antibiotic resistance (Davis et al., 2006; Pereira, Moura, Carvalho, & Empadinhas, 2017). However, other authors recommend topical antimicrobials for DFI treatment, claiming that this type of administration would allow the compound to maintain a higher concentration at the infection site than a systemic administration which, due to poor circulation, may not allow the antibiotic to achieve the necessary concentration at the infection site. These authors also claim that the topical application could even decrease the toxic effect and systemic absorption of the antibiotic (Abbas et al., 2015; Lipsky & Hoey, 2009; Lipsky, Kuss, et al., 2012; Markakis et al., 2018). Some of these authors also defend that infections promoted by polymicrobial biofilms would require higher antibiotic concentrations, which would be toxic if administrated via systemic route (Lipsky & Hoey, 2009; Markakis et al., 2018). Nevertheless, no topical antimicrobial is accredited for the treatment of DFI and topical administration should not be used as the only treatment option, being a good alternative in specific circumstances (Abbas et al., 2015; Davis et al., 2006; Lipsky, 2004; Lipsky, Kuss, et al., 2012; Richard et al., 2012).

Previous studies refer that DFI *S. aureus* and their biofilms are more susceptible to three antibiotics, namely gentamicin, clindamycin and vancomycin, being the last two more effective against planktonic cells and biofilm inhibition while gentamicin is able to eradicate and inhibit staphylococci biofilms (Adkison et al., 2016; Bader, 2008; Lipsky, Berendt, et al., 2012; Mottola, Matias, et al., 2016). However, no antibiotic or association of antibiotics is defined as preferential for DFI treatment (Abbas et al., 2015; Lipsky, 2004; Richard et al., 2012).

Gentamicin was first isolated from *Micromonospora* and belongs to aminoglycosides antibiotic class (Chen, Chen, Wu, & Chen, 2014). Aminoglycosides bind to the 30S ribosomal subunit of bacteria, changing its conformation and consequently inhibiting protein synthesis (Chen et al., 2014; Ullah & Ali, 2017). Gentamicin has a broad spectrum of action, being effective against aerobic and facultative

anaerobic Gram-negative and Gram-positive bacteria (Lipsky, Kuss, et al., 2012). The most usual mechanism of resistance to aminoglycosides is their enzymatic modification by enzymes generally encoded in plasmids, which can be transferred between bacteria (Sparo, Delpech, & Allende, 2018). Gentamicin can be applied through parental, systemic and topical routes (Chen et al., 2014). The topical application of a gentamicin - collagen sponge on DFI was already evaluated, concluding that this treatment, together with the systemic administration of an antibiotic, could improve the prognosis of these type of infections (Lipsky, Kuss, et al., 2012).

Clindamycin is an antibiotic that belongs to the lincosamide class of antibiotics, being obtained by chemical modification of lincomycin, a natural compound (Smieja, 1998). Lincosamide acts by binding to the 50S subunit of the bacterial 23S rRNA, inhibiting protein synthesis (Morar, Bhullar, Hughes, Junop, & Wright, 2009). Clindamycin has a broad spectrum of action, including Gram-positive aerobes and Gram–positive and Gram–negative anaerobes. It has been frequently used to treat infections by Methicillin Resistant *S. aureus* (MRSA) (Addy & Martin, 2005; Morar et al., 2009; Smieja, 1998). With the increased clinical application of this antibiotic, reported resistances have also increased. The most frequent clindamycin resistance mechanism is target site modification due to *erm* genes expression (Mottola, Matias, et al., 2016). Clindamycin can be delivered through parenteral and oral route. Topical application of clindamycin in infected wounds is not usual (Lipsky, 2004).

Vancomycin belongs to glycopeptide antibiotic class and is produced by *Actinomycetes*. Vancomycin was the first glycopeptide described, being produced by *Streptomyces orientalis* (now called *Amycolatopsis orientalis*) (Butler, Hansford, Blaskovich, Halai, & Cooper, 2014). This antibiotic presents some level of nephrotoxicity and started to be more used in the clinical setting after the emergence of MRSA infection cases, being also active against *Clostridium difficile* (Butler et al., 2014; Sujatha & Praharaj, 2012). Vancomycin targets the cell wall precursors, promoting the transformation of the carboxyl terminal of lipid II, D-alanyl-D-alanine, to D-alanyl-D-lactate (Breukink & Kruijff, 2006). The parenteral administration of vancomycin is the most frequent protocol used in DFI treatment (Bader, 2008; Kosinski & Lipsky, 2010). Some studies about the use of bone autographs impregnated with vancomycin in DFI were already performed, with positive results (Markakis et al., 2018).

1.2.3.1. *Staphylococcus aureus* resistance to vancomycin

One of the biggest concerns about the treatment of *S. aureus* infections is the resistance ability of this bacterial species to antibiotics action. When in 1960 its resistance to penicillin started to increase due to the production of β-lactamases (which inactivate the β-lactams antibiotics), the semi-synthetic penicillin, methicillin, begun to be used to treat penicillin resistance infections. The spread use of methicillin lead to the emergence of new resistant strains, such as MRSA, which became a major health problem in hospitals in 1980. Methicillin resistance is due to the acquisition of the *mecA* gene that codifies for the penicillin-binding protein 2a (PBP2a), which has decreased affinity to methicillin (Mottola, Matias, et al., 2016; Stapleton & Taylor, 2002; Weigel et al., 2003).

MRSA is intrinsically linked to DFI since the first two MRSA strains identified were isolated from DFI patients, being a major concern in the treatment of these infections (Citron et al., 2007). In fact, a

study conducted by our research team, revealed that 48.7% of the *S. aureus* isolates from DFI patients were MRSA (Mottola, Semedo-Lemsaddek, et al., 2016).

Until around 1990, MRSA infections were linked to hospital settings. These infections affected mostly individuals associated with risk factors such as previous admission to hospital or addition to intravenous drugs (DeLeo et al., 2010; Glaser et al., 2016; Grundmann et al., 2014; Mediavilla, Chen, Mathema, & Kreiswirth, 2012). The hospital acquired MRSA strains (HA-MRSA) usually present multiple antimicrobial resistances and correspond to a restricted number of well adapted clones (Glaser et al., 2016; Mediavilla et al., 2012). Around the mid-1990s, infections in healthy individuals without risk factors associated or previously hospitalization caused by MRSA started to increase (Glaser et al., 2016). The strains causing these infections belong to genetically different lineages and are usually more virulent than the HA-MRSA ones, being classified as community acquired MRSA (CA-MRSA) (DeLeo et al., 2010; Glaser et al., 2016; Mediavilla et al., 2012; Turner et al., 2019). The CA-MRSA strains spread rapidly and become endemic in several countries, overcoming the HA-MRSA in healthcare settings (DeLeo et al., 2010; McDougal et al., 2010; Mediavilla et al., 2012; Turner et al., 2019). Over the years an overlapping of the two types of clonal lineages began to be observed (Grundmann et al., 2014; Mediavilla et al., 2012; Turner et al., 2019).

Since MRSA strains are commonly resistant to other classes of β-lactam antibiotics, one of the alternatives found for the treatment of infections caused by methicillin resistant *S. aureus* was vancomycin (Sujatha & Praharaj, 2012; Weigel et al., 2003). Due to the emergence of methicillin-resistant strains and other beta-lactams, the use of vancomycin spread in the clinical settings, potentiating the emergence of resistant strains (Sujatha & Praharaj, 2012). In 1988, the first vancomycin resistant *Enterococcus faecium* strain was reported. *S. aureus*, decreased susceptibility to vancomycin, VISA (vancomycin-intermediate *S. aureus)*, was described in 1997 and in 2002 the first vancomycin-resistant *S. aureus* was isolated from a patient in Michigan (Weigel et al., 2003). In 2017, *Staphylococcus aureus* methicillin and vancomycin resistant, and *Enterococcus faecium* vancomycin resistant were characterized by WHO as high priority pathogens in terms of antimicrobial resistance, rendering the development of new antibiotics against those strains a priority (Tacconelli, Carrara, Savoldi, Kattula, & Burkert, 2017).

The first, and to our knowledge the only, VRSA detected in Europe was isolated in Portugal in 2013, from a DFI in a patient with an amputated toe (Friães et al., 2015). This was probably related to the fact that Portugal is one of the European countries with higher rates of MRSA in hospitals (Mottola, Semedo-Lemsaddek, et al., 2016).

1.2.3.1.1. Horizontal Gene Transfer

The frequency of antibiotic resistant strains has increased in the last decades, mainly due to the selective pressure that bacteria are subjected to when their hosts are under antimicrobial treatment. When an isolate of a strain susceptible to an antibiotic is able to multiply, it is denominated a resistant mutant, which is selected between the wild-type strains. In fact, it is observed that the rate of mutants increases with prolongated antibiotic treatments (Giraud, Matic, Radman, Fons, & Taddei, 2002).

Resistance develops mostly through point mutations or due to the acquisition of genetic resistant determinants. The genetic variability of pathogens is mainly found in the accessory genome, which consist mostly of mobile genetic elements acquired through horizontal genetic transfer (HGT). Pathogenicity islands, chromosomal cassettes, transposons and plasmids are part of the genetic material that can be transferred between different bacteria, often belonging to different generas (Turner et al., 2019).

Methyl-directed mismatch (MMR) is a system that can recognize and repair mismatches, not allowing recombination between genetic material from different bacteria. HGT is not a frequent event and usually occurs when MMR is defective, enhancing the probability for mutations and the recombination between DNA from different bacterial species (Dzidic & Bedeković, 2003).

There are three types of gene transfer mechanisms in bacteria: transformation, which is the uptake of free DNA by a recipient cell; conjugation, that involves cell to cell contact before DNA transfer; and transduction, in which DNA transfer is mediated by a bacteriophage (bacterial virus) (Dzidic & Bedeković, 2003; Madigan, Martinko, Stahl, & Clark, 2012).

It is through conjugation with enterococci followed by *vanA* transfer, that *S. aureus* manages to become resistant to vancomycin. When the bacteria is resistant to vancomycin, the cell wall construction is not impaired by the presence of this antibiotic (Weigel et al., 2003). Resistance to vancomycin, as previously described, was first reported in *Enterococcus faecium* and is associated with the *Tn1546* transposon, which carries the *vanA* operon (Palmer, Kos, & Gilmore, 2010; Weigel et al., 2003). Transfer of the *vanA* gene from *Enterococcus faecalis* to *S. aureus* was described in 1992, ten years prior to the isolation of the first clinical VRSA (Noble, Virani, & Cree, 1992; Soju et al., 2003). Researchers believe that VRSA develop due to single and independent acquisitions of *Enterococcus Tn1546* transposon by MRSA from the clonal complex 5 (CC5) (Kos et al., 2012). Clonal complexes are defined through multilocus sequence typing (MLST), a molecular technique that is based on the sequencing of seven housekeeping genes. This technique allows the organization of strains into sequence types (clones that have the same allelic profile of the seven housekeeping genes), with the clonal complexes being the set of sequence types that vary within one of the seven loci (Enright, Day, Davies, Peacock, & Spratt, 2000). VISA strains are almost always CC5 and this clonal cluster is involved in the early acquisition of methicillin resistance as well as resistance to other antibiotics (King, Kulhankova, Stach, Vu, & Salgado-Pabón, 2016; Kos et al., 2012). In fact, CC5 has an increased predisposition to horizontal resistance gene acquisition, which is probably responsible for these strains being frequently hospital associated MRSA infections (Albrecht et al., 2014; King et al., 2016; Kos et al., 2012; McDougal et al., 2010). For horizontal gene transfer to occur it is necessary that the donor and the recipient bacteria are in contact, coexisting in a population and in an environment that promotes the transfer and establishment of the resistant genes (Kos et al., 2012). Almost all VRSA isolated so far were obtained from patients with DFU (Gardete & Tomasz, 2014; Kos et al., 2012; J.J. Mendes et al., 2011).

The first VRSA strain was isolated from a DFI patient in Europe, more specifically in Portugal, and belonged to CC5, being once again observed that this lineage seems to be predisposed to acquire the transposon *Tn1546* from vancomycin resistant *Enterococcus* (VRE) (Friães et al., 2015). The *S.*

aureus collection isolated from DFU patients used in this study (J.J. Mendes et al., 2011) was previously characterized in terms of clonality, allowing to cluster the majority of the isolates in the CC5, which raised some concerns about new cases of VRSA dissemination (Mottola, Semedo-Lemsaddek, et al., 2016).

1.2.3.1.1.1. pSK41-like Plasmid

The pSK41-like plasmid is a class of conjugative staphylococci plasmids, like pSK41, pGO1 and pLW1043, that can integrate multiple Mobile Genetic Elements (MGE) and which contain a highly conserved group of 15 *tra* genes, located in a transfer associated region (Liu, Kwong, Jensen, Brzoska, & Firth, 2013; McDougal et al., 2010; Zhu, Clark, & Patel, 2013). Multiple antimicrobial resistances have already been described in these plasmids, including vancomycin resistance (Kwong, Ramsay, Jensen, & Firth, 2017; McDougal et al., 2010).

In 2013, Zhu et al. associated the transfer of the transposon *Tn1546,* that contains the *vanA* operon*,* from *Enterococcus* to *S. aureus* to the need of the recipient strain to transport the pSK41-like plasmid (Zhu et al., 2013). Also, previous studies already related the transfer of the *vanA* operon with the presence of the pSK41 plasmid (Palmer et al., 2010). The transfer system of the Inc18-type plasmid, carrier of the *Tn1546*, and the pSK41-like belong to the same superfamily of macromolecular transport mechanisms, the type IV secretion system (Zhu et al., 2013). The type IV secretion system promotes the transfer of intracellular material between cells, indicating that one of the reasons that facilitate the transfer of the *vanA* operon is the presence of these two plasmids (Wallden, Rivera-Calzada, & Waksman, 2010; Zhu et al., 2013). However, the mechanism used in this transconjugation is not fully understood (Zhu et al., 2013).

A study conducted in 2013, showed that the presence of chronic wounds for over two years in patients was a statistically significant risk factor for the isolation of pSK41-positive *S. aureus*; the same study concluded that the colonization with pSK41-positive *S. aureus* was rare (Tosh et al., 2013).

1.3. Novel Diabetic Foot Infection Therapeutics

The treatments currently applied to foot ulcers are expensive and could be therapeutically more overall effective, rendering the development of effective treatments a challenge to the scientific community. New alternative methods and treatments are being developed around the world, aiming to reduce treatments cost, amputation rate and mortality (Gottrup & Apelqvist, 2012).

1.3.1. Alternative Treatments

Other treatments, methods and devices for DFU management are emerging, one of which being the treatment of DFU with stem cells. Stem cells, in addition of being capable to differentiate into several different types of cells, can produce cytokines, which can promote wound healing. This type of therapeutics has been demonstrated to be effective towards DFU; however, although there are no

consensus yet concerning the type of stem cells to be used and their delivery route, authors suggests that autologous stem cells administrated topically through hydrogel could be a possibility (Lopes et al., 2018).

Bacteriophages are another potential therapy targeting DFU infections. Bacteriophages are virus that only infect prokaryotic cells, and the aim of bacteriophage therapy consists in using lytic bacteriophages to eliminate or reduce pathogenic bacteria (João J. Mendes et al., 2014; Santos, Veiga, et al., 2016). In a study conducted in Portugal, a bacteriophage suspension was applied in infected cutaneous wounds after debridement, being observed that bacteriophage treatment decreased the presence of pathogens and increased wound healing. However, studies are still ongoing aiming of validate phage therapy (João J. Mendes et al., 2013).

Probiotics can also be used as therapeutic agents against DFI. Probiotics are defined by the Food and Agriculture Organization and the World Health Organization as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Santos, Veiga, et al., 2016). Probiotics can act by direct modification of the microbial populations by competition or by modulation of host immune system, among other mechanisms (Piewngam et al., 2018; Santos, Veiga, et al., 2016). Lactic acid bacteria already showed good results when applied to DFU infections. For example, *Lactobacillus* species already showed the ability to eliminate pathogens, including resistant bacteria, such as MRSA (Santos, Veiga, et al., 2016). Another study conducted in 2018, found that the introduction of probiotics belong to *Bacillus* genus in the human nutrition promote the eradication of *S. aureus* from the intestine and nostrils by blocking bacterial quorum sensing (Piewngam et al., 2018).

Other alternative DFI treatments being studied include several different types of wound dressings, neuropeptides, microRNAs, oxidative stress control, natural products, like honey, and maggots for debridement. However, these therapies still need to be validated as safe and/or beneficial (Dhall et al., 2014; Pereira et al., 2017).

1.3.2. Antimicrobial Peptides

The antimicrobial peptides (AMPs) are oligopeptides naturally produced by prokaryotes and eukaryotes, being part of their initial immune response against several microorganisms, including virus, bacteria and fungi, additionally they can also act against cancer cells (Bahar & Ren, 2013; Mahlapuu, Håkansson, Ringstad, & Björn, 2016; Zhang & Gallo, 2016). The AMPs are usually cationic peptides, have a small size (between 10 to 50 amino acids), are hydrophobic and some are amphipathic (Hale & Hancock, 2007; Zhang & Gallo, 2016). These molecules have different features according to the producer organisms: ribosomal synthesized AMPs are produced by all types of organisms, while non-ribossomal AMPs are synthesized mostly by bacteria (Mahlapuu et al., 2016; Zhang & Gallo, 2016). AMPs produced by bacteria are denominated bacteriocins (Hale & Hancock, 2007; Hassan, Kjos, Nes, Diep, & Lotfipour, 2012). The first bacteriocin detected was extracted from a soil *Bacillus* strain in 1939 and later identified as gramicidin. It was showed that this AMP contributed for controlling pneumococci infections in mice and could be used for topical applications to help ulcers and wounds healing (Bahar & Ren, 2013; Dubos, 1939a, 1939b).

AMPs are potentially good antimicrobial compounds because they can act against both Gram-positive and Gram-negative bacteria (Salwiczek et al., 2014). Cationic AMPs are charged with positive amino acids that establish electrostatic interactions with negatively charged microbial membranes, causing the disruption of the lipidic structure (Mahlapuu et al., 2016; Salwiczek et al., 2014). This ability to link to hydrophobic, like lipids, and hydrophilic components, like phospholipid groups, are due to the AMP amphipathic feature (Bahar & Ren, 2013). Since, the mammalian cells membrane constitution significantly differs from the bacterial membrane, this difference provides protection to the eukaryotic cell, resulting, in lower toxicity levels of the AMP under clinical use (Mahlapuu et al., 2016; Omardien, Brul, & Zaat, 2016). Additionally, there are some evidences that some AMPs do not only act on the membrane, but also have the ability to pass through it and block the DNA, RNA and protein synthesis, leading to the cell death (Omardien et al., 2016; Zhang & Gallo, 2016). Some of these peptides have, as well, the ability to inhibit biofilm formation and eradicate established biofilms (Santos, Veiga, et al., 2016; Zhang & Gallo, 2016). Besides their antimicrobial potential, AMPs also have an immunomodulatory effect in the host, which include the stimulation of chemotaxis and promotion of the immune cell production and differentiation, among others (Mahlapuu et al., 2016; Santos, Veiga, et al., 2016; Zhang & Gallo, 2016).

Antimicrobial peptides have low affinity targets unlike antibiotics, which usually have one high affinity target, which contributes for the development of resistant strains. Since AMPs mostly cause membrane disruption through physical interaction, it makes more difficult to the bacteria surpass this attack since the membrane is a highly conserved constituent (Mahlapuu et al., 2016). However some resistances, mostly *in vitro*, have already been described, due to the change of the membrane charge, pump-efflux systems, synthesis of AMP inhibitors and alteration of the intracellular targets (Bahar & Ren, 2013; Hassan et al., 2012; Salwiczek et al., 2014).

There are already some experiments regarding the clinical application of AMPs. Polymyxins were approved for clinical used in 1950 and some others AMPs are under development for their use in the clinical area (Mahlapuu et al., 2016). Pexiganan, a synthetic AMP, analog from a peptide extracted of a frog skin, achieved the phase III in two clinical trials, as topical antimicrobial, for DFI treatment (Flamm et al., 2015; Mahlapuu et al., 2016). However, AMPs have some limitations regarding their clinical approve, due to their low metabolic stability. In fact, they are easily degraded when applied systemically (Mahlapuu et al., 2016).

1.3.3. Nisin

Nisin is one of the better described AMPs, being a natural compound produced and ribossomally synthesized by *Lactococcus lactis* subsp. *lactis* (Abee & Delves-Broughton, 2003; Hassan et al., 2012; Mitchell, Truscott, Dickman, Ward, & Tabor, 2018; Nissen-Meyer & Nes, 1997). It has 34 amino acids, several of which unusual, and a positive overall charge (Abee & Delves-Broughton, 2003; Breukink & Kruijff, 2006; Hassan et al., 2012). Nisin possesses in its constitution five lanthionine rings. Between the first three rings (A, B and C) and the lasts two there is a hinge region, contributing for the flexibility of the peptide (Breukink & Kruijff, 2006; Mitchell et al., 2018; Prince et al., 2016). Nisin is classified as

lantibiotics (small peptides containing post-translational modifications and rings containing thioether structures) or bacteriocins class I (Abee & Delves-Broughton, 2003; Hassan et al., 2012). This cationic AMP was first discovered in 1928 and approved for use in food industry, as a safe food additive, by the Food and Agriculture Organization and by the World Health Organization in 1969. In 1988 nisin was approved in the United States by the Food and Drug Administration, being used for the control of microorganisms in processed cheese, due to its effectiveness against pathogens and its low toxicity to hosts (Shin et al., 2016). In fact, the European Food Safety Authority determined the acceptable daily intake of nisin to be of 1 mg/kg body weight (Younes et al., 2017). In 2016, its use as a biopreservative in foods was expanded to around 50 countries, being the only bacteriocin with widespread application in this industry (Shin et al., 2016; Vaithiyanathan, Ethiraj, C, & V, 2012). There are several variants of nisin, being nisin A and nisin Z the most frequent, having similar antimicrobial properties since they differ in only one amino acid. However, nisin Z is more soluble in neutral pH and has a higher diffusion rate (Shin et al., 2016).

As previously referred, nisin is a cationic AMP, being an amphipathic peptide, with an hydrophobic N-terminal and an hydrophilic C-terminal (Gough et al., 2017). Nisin can act in two independent forms, producing pores on the bacterial membrane and blocking the cell wall synthesis (Field, Cotter, Hill, & Ross, 2015; Hassan et al., 2012). The inhibition of the cell wall synthesis is accomplished by the sequestration of lipid II, which is a peptidoglycan subunit linked via a pyrophosphate to the polyisoprenoid membrane anchor (Field et al., 2015; Hale & Hancock, 2007; Hassan et al., 2012; Shin et al., 2016). In fact, lipid II is formed in the cytoplasmic side of the cell membrane by the assembly of the cell wall subunits, a polymer alternated between N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) and for a pentapeptide attached to the carboxyl group of the MurNAc, (Breukink & Kruijff, 2006; Hasper et al., 2006; Hsu et al., 2004).

Pore formation in the bacterial membranes is also improved through the bonding of nisin and lipid II, although nisin may disturb the membrane independently of lipid II presence (Field et al., 2015; Prince et al., 2016; Shin et al., 2016). The lanthionine rings A and B of nisin form a structure that binds to the pyrophosphate of lipid II, using it as a docking molecule; then, the D and E rings are inserted in the target membrane to form the pore structure (Breukink & Kruijff, 2006; Mitchell et al., 2018). By the end, the pore complex is formed by four molecules of lipid II and eight molecules of nisin (Breukink & Kruijff, 2006; Hasper et al., 2006).

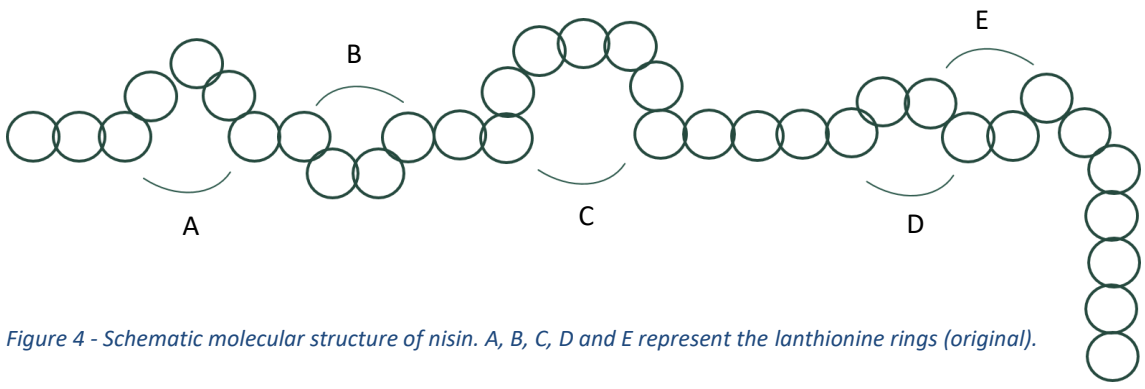

Figure 4 - Schematic molecular structure of nisin. A, B, C, D and E represent the lanthionine rings (original).

The bacterial resistance to nisin is not, for now, comparable to antibiotic resistance, since nisin acts by two distinct modes and the principal interaction is with the pyrophosphate group of the lipid II, which is a highly conserved portion of the molecule (Medeiros-Silva, Jekhmane, Breukink, & Weingarth, 2019). However, there are already some descriptions available regarding the development of *in vitro* resistance to nisin, which can be related to modifications in cell wall, preventing the binding of nisin; alterations in the phospholipids of the cell membrane that result in an increased net positive charge, which could prevent the binding of this cationic AMP; and the production of nisinase, an enzyme that can neutralize nisin activity (Zhou, Fang, Tian, & Lu, 2013). However, these mechanisms are not well characterized, since the majority of the reported resistances were described in *in vitro* conditions (Shin et al., 2016).

Nisin is a promising product for biomedical applications since it is effective against a wide range of Gram-positive bacteria at low nanomolar to millimolar concentrations (Field et al., 2015; Lagedroste, Reiners, Smits, & Schmitt, 2019; Zhou et al., 2013). It is also effective against some Gram-negative strains but these are mostly susceptible when their outer membrane is fragile (Begde et al., 2011; Vaithiyanathan et al., 2012). Nisin can also present antimicrobial activity against antibiotic resistant strains, such as MRSA, VRE and VRSA (Field et al., 2015; Lagedroste et al., 2019; Mitchell et al., 2018). Bacterial biofilms are also susceptible to this AMP, pointing out for its potential use against biofilm-related infections (Cunha et al., 2018; Santos, Gomes, et al., 2016). Besides its antimicrobial activity, nisin also presents immunomodulatory effects on hosts (Begde et al., 2011; Shin et al., 2016).

Although administration of nisin does not appear to be a risk to human health, there are difficulties in its clinical application (Bernbom et al., 2006; Mitchell et al., 2018). This cationic AMP is not able to reach the final gastrointestinal (GI) tract, since the α-chymotrypsin and trypsin enzymes produced by pancreas rapidly cause its proteolytic degradation in the small intestine (Bernbom et al., 2006; Gough et al., 2017, 2018; Tong, Ni, & Ling, 2014). Therefore, the topical application of nisin is more suitable to avoid its proteolytic degradation (Boakes & Wadman, 2008).

In a study performed by our research team, a biogel formed by guar gum, a natural polysaccharide, supplemented with nisin was tested against *S. aureus* DFI isolates, being observed that, in addition to the good diffusion of nisin in the gel, this supplemented gel also promoted the inhibition and eradication of planktonic cells and established biofilms. These results also promote the use of nisin in DFI treatment, as its incorporation in a guar-gum gel allows its topical application which may increase its local efficacy (Santos, Gomes, et al., 2016).

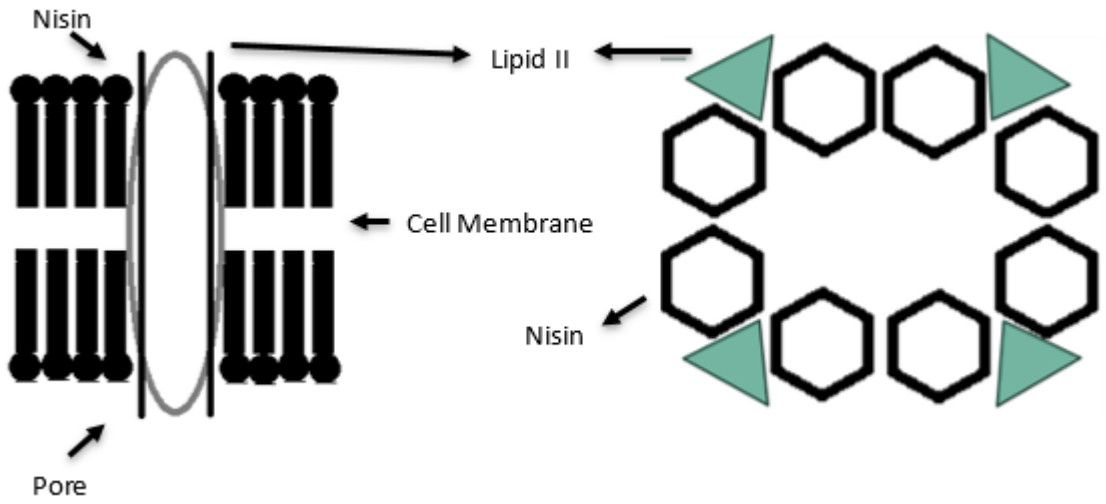

Figure 5 - Nisin mode of action and the schematic interaction with the lipid II (original).

1.4. Mutant Selection Window (MSW)

1.4.1. Definition and practical importance

As previously referred, in the last decades antimicrobial resistance has been a growing problem as demonstrated by the increment in reported resistances. To prevent resistance development, among other precautions, it is important that antimicrobial compounds are prescribed at appropriate doses and concentrations (Nakase, Nakaminami, Toda, & Noguchi, 2017). When a dose of an antibiotic only eliminates susceptible bacteria, that dose will promote the selection of bacteria resistant to that antibiotic. If a higher dose is applied, the emergence of these resistant bacteria can be suppressed. To avoid a selective mutant environment, Zhao and Drlica proposed the mutant selection window (MSW) concept (Zhao & Drlica, 2002), referring to an antibiotic concentration range that has as lower limit the minimum inhibitory concentration (MIC) and as the higher limit the mutant prevention concentration (MPC) (Cairns & Payne, 2008). The MIC is the lowest concentration of an antimicrobial that inhibits the growth of the majority of the susceptible cells, while the MPC is the concentration that inhibits the growth of the least susceptible mutant (Drlica, 2003). These are usually single-step mutants, being difficult for a cell to multiply in the presence of antibiotic concentrations above MPC values, which would require the simultaneous occurrence of two or more mutations, which is a rare event (Zhao & Drlica, 2002).

To determine MPC values, bacterial response to concentrations representing two-fold increases of the MIC values must be tested. To determine MSW, a 10^{10} CFU/mL bacterial suspension is incubated with different concentrations of the antimicrobial. This concentration was selected based on the fact that the usual bacterial concentration found in infections is around 10^5 CFU/mL per gram of tissue, fivefold lower, which guarantees that the antimicrobial concentration obtained will be able to eliminate all the bacteria present in *in vivo* infections (Abbas et al., 2015; Drlica & Zhao, 2007). In antimicrobial concentrations closer to the MIC value a bacterial lawn is expected to be formed, while in the highest concentrations, any resistant mutants isolate are expected to develop (Drlica & Zhao, 2007).

These determinations may allow to control drug resistance dissemination since if, the drug concentration applied in therapeutic protocols remains above the MPC, the emergence of mutants resistant to these antimicrobials is prevented (Cairns & Payne, 2008; Drlica & Zhao, 2007).

1.5. Objective

This work had two different tasks with a common objective: Evaluate the role of nisin in the emergence of new resistant strains. The first task was to determine the MPC of nisin regarding 24 *S. aureus* isolates from patients with DFI in order of the future establishment of the right therapeutic dosage to avoid the development of resistant mutants.

The second task was to understand if the presence of nisin may potentiate the transference of resistant genes, from *Enterococcus* to *S. aureus,* in particular of *vanA*, which is one of the genes that confers resistance to vancomycin.

Chapter 2 | Materials and Methods

2.1. Bacterial Isolates

A collection of 23 *S. aureus* isolates was used in this study. These isolates were previously collected from patients with DFI (J.J. Mendes et al., 2011) and further selected and characterized in terms of clonality, antimicrobial resistance and virulence profiles (Mottola, Semedo-Lemsaddek, et al., 2016). Additionally, the reference strain *S. aureus* ATCC 29213 was also included in this study as a control. Each isolate was maintained at - 20 ºC in buffered peptone water with 20% of glycerol during this study.

Table 2 – Sampling method and isolates characterization in terms of the presence of the mecA gene, clonal complex allocation and antimicrobial resistance profile. Fox – cefoxitin; Cip – ciprofloxacin; Mem – meropenem; Ery – erythromycin; Cpt – ceftaroline; Cli – clindamycin; Gen – gentamicin.

Isolate code	Sampling Method	*mecA*	Clonal Complex	Antimicrobial resistance profile
A.1.1	Aspiration	+	5	Fox, Cip
A.5.2	Aspiration	-	8	Cip, Cpt
A.6.3	Aspiration	-	7	-
B.3.2	Biopsy	-	5	-
B.3.3	Biopsy	-	5	-
B.7.3	Biopsy	+	5	-
B.13.1	Biopsy	+	5	Fox, Cip, Mem, Ery, Cli, Gen
B.14.2	Biopsy	+	22	Fox, Cip, Cpt, Mem
Z.1.1	Swab	+	22	Fox, Cip, Mem
Z.2.2	Swab	-	5	Cip, Ery
Z.3.1	Swab	-	5	-
Z.5.2	Swab	-	5	-
Z.12.2	Swab	-	5	Gen
Z.14.1	Swab	-	5	Gen
Z.16.1	Swab	+	5	Fox, Cip, Ery
Z.17.2	Swab	-	30	-
Z.21.1	Swab	+	5	Fox, Cip, Ery
Z.21.3	Swab	+	5	Fox, Cip, Ery
Z.23.2	Swab	-	45	-
Z.25.2	Swab	-	182	-
Z.27.2	Swab	-	5	-
Z.27.3	Swab	-	5	-
Z.32.2	Swab	-	5	-

2.2. Nisin solution

The nisin (ref N5764; Sigma-Aldrich, USA) used has a purity of 2.5% (1000 IU/mg). To obtain a stock solution of 1000 µg/mL, 1 g were dissolved in 25 mL of 0.02M HCl (Merck, Germany). After dilution, nisin was filtered with a 0.22 µm filters (Frilabo, USA) and stored at 4ºC.

2.3. Determination of the Mutant Prevention Concentration

A modified version of the protocol elaborated by Sinel et al. in 2016 was used to determine the MPC of nisin regarding the 24 *S. aureus* DFI isolates under study (Sinel, Jaussaud, Auzou, Giard, & Cattoir, 2016).

Each isolate was inoculated in Brain Heart Infusion agar (BHI) (Brain heart infusion broth, VWR Chemicals, ref 84626.0500; Agar, VWR Chemicals, ref 84609.0500), and after a 24h incubation at 37ºC, a suspension of 0.5 MacFarland ($1x10^8$ CFU/mL) was performed and used to inoculate two plates. After a 24h incubation at 37ºC, the bacterial lawn was collected from the two plates and resuspended in 1mL of Brain Heart Infusion broth (BHIB) to achieve a bacterial suspension with a concentration of 10^{10} CFU/mL. In order to confirm the concentration values, serial dilutions of the suspensions 10^0 to 10^{-8} were performed, after witch, 100 µL of the dilutions 10^{-7} and 10^{-8} were inoculated in BHI agar and incubated for 24h at 37ºC, for viable cell count.

Afterwards, 50 µL from the original suspension, were inoculated in Mueller Hinton agar (MHA) (Mueller-Hinton Agar, OXOID, ref CM0337) supplemented with the following nisin concentrations: 5.63 µg/mL, 11.25 µg/mL, 22.5 µg/mL, 45 µg/mL, 90 µg/mL, 180 µg/mL, 360 µg/mL and 720 µg/mL. These concentrations were selected considering a two-fold increase of the MIC value (11.25 µg/mL) that was previously determined (Santos, Gomes, et al., 2016). A sub MIC value was also included (5.63 µg/mL). Finally, plates were incubated for 72h at 37ºC for MPC determination.

The MPC corresponded to the minimum concentration of nisin that prevented the growth of resistant mutants after the incubation period. For each isolate, the mutants grown at the concentration below the MPC of nisin were isolated and stored at -20°C and - 80°C in a solution of buffered peptone water with 20% glycerol (Peptone water buffered, VWR Chemicals, ref 84600.0500; Glycerine 87%, VWR, ref 24385.295). The MPC values of nisin were determined in two different and independent rounds.

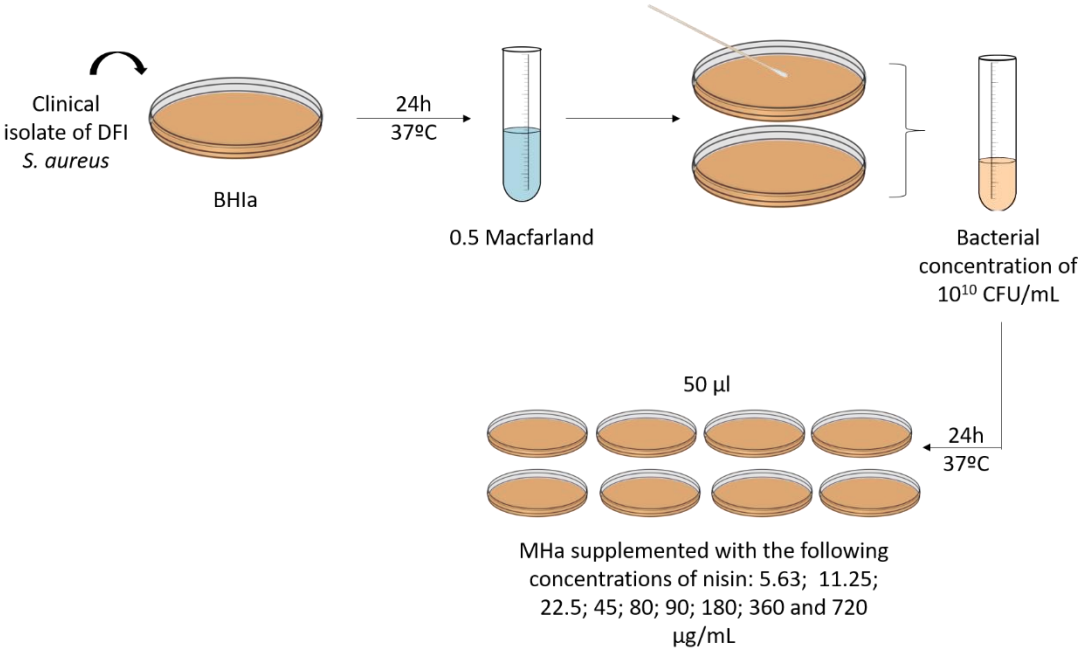

Figure 6 - Schematic representation of the workflow of the determination of the mutant prevention concentration protocol (original). BHIa – Brain Hearth Infusion agar; DFI – Diabetic Foot Infection; CFU/mL – Colony Forming Unit per milliliter; µg/mL – Microgram per milliliter

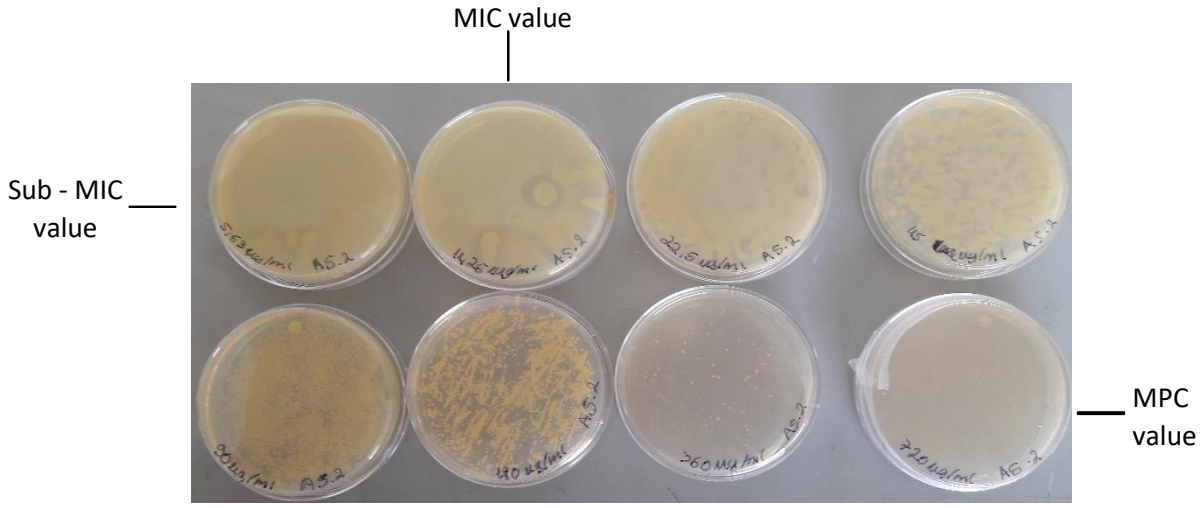

Figure 7 – MSW test results for S. aureus isolate A.5.2. It is possible to observe a bacterial lawn in lower concentrations of the AMP and isolated colonies in the highest concentrations (original).

2.4. Horizontal Gene Transfer

2.4.1. DNA extraction

DNA extraction was performed based on the protocol described by Mottola (Mottola, Semedo-Lemsaddek, et al., 2016).

All isolates were inoculated in BHI agar for 24h at 37ºC. Four to five bacterial colonies were collected using a sterile loop and resuspended in 100 µL of TBE buffer (0.9 M Tris-Borate, 0.01 M EDTA, pH 8.3 – Omega, ref. AC10078) supplemented with 0.1% Tween 20 (Merck-Schuehardt, ref. 8.22184.0500)

solution. After homogenization, the solution was incubated for seven minutes at 97ºC and centrifuged at 15000 rpm for 5 minutes (Hermle Labortechnik). The supernatant was collected for PCR screening.

2.4.2. Multiplex PCR for *vanA* detection

Before the Horizontal Gene Transfer protocol, it was necessary to confirm the absence of *vanA* gene in the 24 *S. aureus* isolates, using a multiplex PCR (Ramos-Trujillo, Pérez-Roth, Méndez-Alvarez, & Claverie-Martín, 2003).
Two pairs of primers, targeting *vanA* and *mecA* were used in this PCR, synthesized by STABVIDA®.

Table 3 - Primers used in the multiplex PCR. The first sequence of each gene corresponds to the forward primer and the second to the reverse primer.

Target gene	Primer Sequence	Product size (bp)	Reference
vanA	GGG AAA ACG ACA ATT GC GTA CAA TGC GGC CGT TA	732	(Ramos-Trujillo et al., 2003)
mecA	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162	(Mottola, Matias, et al., 2016)

The PCR mixture had a final volume of 28.5 µL, 10 µL of the Supreme NZYTaq 2x Green Master Mix (Nzytech®) consisting in 1x reaction buffer (50 mM Tris – HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP), 0.29 µL (0.5 uM) of the *vanA* primer, 0.23 µL (0.4 uM) of the MecA primer, 16.88 µL of PCR-grade water and 5 µL (170 ng/µL) DNA template.
PCR amplification was completed in a MyCycler Thermal Cycler (BioRad®) using the following conditions: initial denaturation at 94°C for 4 min; 10 cycles involving denaturation at 94°C for 30s, annealing at 64°C for 30s and elongation at 72°C for 45s; 25 cycles involving denaturation at 94°C for 30s, annealing at 50 °C for 45s and elongation at 72°C for 2min, and a final extension step at 72°C for 10min.
An electrophoresis gel was performed to perceive the amplified products, using a 1.5% agarose gel (Nzytech, ref. MB14402) and a buffer stained with GreenSafe (Nzytech®) at 90V for 45 min. A molecular weight marker, NZYDNA ladder VI (Nzytech®) was also included. Results were visualized by transillumination (ChemiDoc XRS+, Bio-rad).
Two positive control strains, *Staphylococcus aureus* 01-00694 (*mecA* positive) and *Enterococcus faecium* CCUG 36804 (*vanA* positive), were included in each PCR amplification protocol, as well as a negative control, with no DNA.

2.4.3. Induction of rifampicin resistant isolates

To allow the selection of the *vanA* transconjugants mutants in the mating experiments, all the 23 clinical *S. aureus* isolates and the control strain under study were inoculated in TSA (Tryptic Soy Agar, VWR Chemicals, ref.84602.0500) supplemented with increasing concentrations of rifampicin

(Rifampicin, PanReac AppliChem, ref A2220,0001) (1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL, 32 µg/mL, 64 µg/mL, 128 µg/mL and 256 µg/mL). To confirm isolates resistance ability, the resistant mutants were inoculated two times in TSA supplemented with rifampicin at 256 µg/mL.
The resistant mutants were stored at -20°C and -80°C in a solution of buffered peptone water with 20% glycerol.

2.4.4. Horizontal Gene Transfer protocol

To test if nisin selective pressure induces horizontal gene transfer, a protocol adapted from Niederhäusern in 2011 was performed (Niederhäusern et al., 2011). Mating experiments were performed in three rounds, using the VRE rifampicin susceptible (Van^r Rif^s) *Enterococcus faecium* (*E. faecium)* CCUG 36804 strain as a donor of the *vanA* gene and as recipients all the 24 *S. aureus* isolates, obtained in the previous task, which were resistant to rifampicin and susceptible to vancomycin (Van^s Rif^r).
After performing a 0.5 MacFarland suspension for each isolate, 500 µL of the donor and 500 µL of one of the recipients were added to 5 mL of TSB (Tryptic Soy broth, VWR Chemicals, ref. 84675.0500) and incubated at 35ºC for 18h.
After incubation, 1 mL of the bacterial suspension was added to 5 mL of TSB and further incubated for 6h at 37ºC. Afterwards, 2 mL of each suspension were inoculated in TSA and incubated for 5h at 37ºC on a shaker, to promote mating. Then, the plates were incubated at 37ºC for 24h. The bacterial suspension that remained at the surface of the agar plates was removed and inoculated in 5 mL of TSB. After an incubation period of 12h at 37ºC, 100 µL of the solution was inoculated in MSA (Mannitol Salt agar, PanReac AppliChem, ref 413783.1210) supplemented with 64 µg/mL of rifampicin and 8 µg/mL of vancomycin (Vancomycin hydrochloride, Abcam, ref. ab141224) to select the transconjugants. If mating occurred, recombinant isolates that developed on these plates should be resistant to rifampicin and vancomycin. The transconjugants were stored at -20°C and - 80°C in a solution of buffered peptone water with 20% glycerol and a PCR analysis was performed to confirm the presence of the *vanA* gene.
The second mating round was performed in the presence of nisin, with all the media used being supplemented with nisin at sub-MIC (5.63 µg/mL) concentration. The third mating round was performed in the presence of vancomycin. Since the MIC value of vancomycin for all the isolates was previous determined (Mottola, Matias, et al., 2016), being found to present an average value of 0.55 µg/mL, all the media used were supplemented with vancomycin at sub-MIC concentration of 0.28 µg/mL.

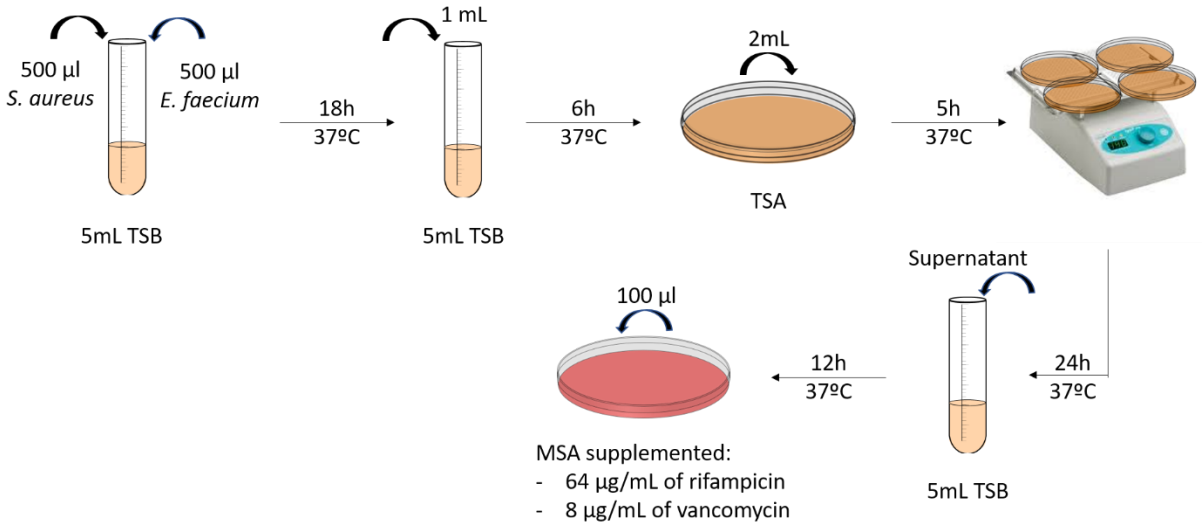

Figure 8 - Schematic representation of the workflow of the horizontal gene transfer protocol (original). TSB – Tryptic Soy Broth; MSA – Mannitol Salt agar

2.4.4.1. PCR for pSK41-like plasmid detection

To evaluate the presence of the pSK41-like plasmid in the 23 clinical isolates under study, a PCR protocol was performed, using a pair of primers targeting the *traE* gene synthesized by STABVIDA® (Albrecht et al., 2014; Zhu et al., 2013).

Table 4 - Primers used in the PCR. The first sequence of each gene corresponds to the forward primer and the second to the reverse primer.

Target gene	Primer Sequence	Product size (bp)	Reference
traE	ACA AAT GCG TAC TAC AGA CCC TAA ACG A GCC CTG CTG TTG CTG TAT CCA TAT T	317	(Albrecht et al., 2014)

The PCR mixture had a final volume of 50 µL, 10 µL of the Supreme NZYTaq 2x Green Master Mix (Nzytech®) consisting in 1x reaction buffer (50 mM Tris – HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP), 0.4 µL (0.4 uM) of *traE* primer and 39.2 µL of PCR-grade water and 5 µL (170 ng/µL) DNA template.

PCR amplification was completed in a MyCycler Thermal Cycler (BioRad®) using the following conditions: initial denaturation at 94°C for 2 min; 30 cycles involving denaturation at 95°C for 15s, annealing at 53°C for 90s and elongation at 72°C for 90s, and a final extension step at 72°C for 7min.

A positive control strain, *Staphylococcus aureus* RN4220 (pGO1 positive), gently provided by Dr. Alex O'Neill, from University of Leeds, was included in the PCR amplification protocol, as well as a negative control, with no DNA (Caryl & O'Neill, 2009).

An electrophoresis gel was performed to perceive the amplified products, using a 1.5% agarose gel (Nzytech, ref. MB14402) and a buffer stained with GreenSafe (Nzytech®) at 90V for 45 min. A molecular weight marker, NZYDNA ladder VII (Nzytech®) was also included. Results were visualized by transillumination (ChemiDoc XRS+, Bio-rad).

Chapter 3 | Results and Discussion

3.1. Mutant Selection Window

Antibiotic resistance is a worldwide concerning problem. Nowadays the antibiotic concentrations established in the therapeutic protocols for *in vivo* administration, have as reference the MIC determination. However, the clinical application of antimicrobial doses based on MIC values, could exert a selective pressure on bacteria, allowing the selection of resistant mutants (Drlica & Zhao, 2007).

Although there are not many reports of bacterial resistance to nisin, the fact that some cases have already been reported shows the importance of determining the MSW in order to establish proper therapeutic concentrations to be applied at the clinical settings and to avoid promoting resistance.

MPC determination was performed for all the 23 *S. aureus* isolates and for the reference strain *S. aureus* ATCC 29213. To our knowledge, the determination of the MPC of nisin regarding *S. aureus* was not previously performed.

The MPC values ranged from 360 µg/mL to >720 µg/mL. The distribution of the MPC values obtained in the two rounds is shown in table 5, being observed that nisin MPC average values was of 360 µg/mL for 8.33% of the isolates (n=2), of 540 µg/mL for 12.5% of the isolates (n=3) and of 720 µg/mL for 4.17% (n=1) of the isolates. MPC value could not be determined regarding 18 isolates (75%), since they were able to grow in the presence of the highest concentration of nisin tested (720 µg/mL).

A higher concentration of nisin could not be tested since the commercial nisin used in this study can only be concentrated until 1000 µg/mL.

Table 5 – Nisin MPC values for the 24 S. aureus isolates under study.

Isolates	MPC values of 1º round (µg/mL)	MPC values of 2º round (µg/mL)	MPC average values
A1.1	720	>720	>720
A5.2	720	>720	>720
A6.3	>720	>720	>720
B3.2	>720	>720	>720
B3.3	>720	720	>720
B7.3	720	>720	>720
B13.1	720	720	720
B14.2	720	>720	>720
Z1.1	720	>720	>720
Z2.2	720	>720	>720
Z3.1	>720	>720	>720
Z5.2	720	>720	>720
Z12.2	360	720	540
Z14.1	>720	>720	>720
Z16.1	>720	>720	>720
Z17.2	360	360	360
Z21.1	>720	720	>720
Z21.3	>720	>720	>720
Z23.2	720	360	540
Z25.2	720	360	540
Z27.2	>720	>720	>720
Z27.3	>720	>720	>720
Z32.2	360	360	360
ATCC 29213	>720	>720	>720

Our results are in accordance with a previous study that determined the vancomycin MPC_{80} value for 855 *S. aureus* clinical isolates, which was 64 times higher than the MIC_{80} (Fujimura, Nakano, & Watanabe, 2014). Vancomycin and nisin have similar modes of action since they both act on lipid II, although through different mechanisms (Hasper et al., 2006). Vancomycin inhibits the cell wall synthesis by binding to the sequence of the C-terminal D-ala-D-ala of the lipid II, while on the other hand, the lanthionine rings of nisin bind to the pyrophosphate of lipid II (figure 9), using it as a docking molecule to form pores on the target membranes (Breukink & Kruijff, 2006; Hasper et al., 2006).

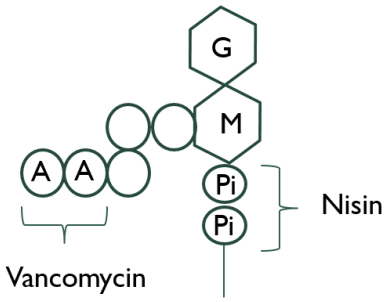

Figure 9 - Partial schematic figure of the structure of lipid II and the binding sites of vancomycin and nisin. (adapted from (Breukink & Kruijff, 2006)

Nisin MPC values regarding most isolates was superior to 720 µg/mL. It cannot be stated, yet, that this dose can be applied *in vivo* or that it will not be toxic for diabetic patients presenting infected ulcers. In a study performed in 2008, nisin was applied to the nipple and mammary areola of four women with clinical signs of mastitis infection by *S. aureus* (Fernández, Delgado, Herrero, Maldonado, & Rodríguez, 2008). The values of nisin applied in the previous referred study were based on the study performed on the toxicity of nisin published in 2006 by the European Food Safety Authority (EFSA), which determined the toxicity related to the oral administration of nisin. The acceptable daily intake of nisin determined by EFSA was of 0.13 mg/kg body weight. Since the nipples presented infected fissures (infected wound) and no signs of toxicity were observed after the application of nisin, the EFSA acceptable daily intake was also considered in this study for comparison purposes. EFSA recently updated the acceptable daily intake of nisin to 1 mg/kg body weight (Younes et al., 2017), which means that a person with medium weight (65 kg) can ingest a maximum of 65 mg of nisin per day. As the MPC average concentration of nisin determined in this study was of 0.72 mg/mL (720 µg/mL), if 2 ml of a biogel supplemented with nisin at this concentration were applied to DFI 3 times a day, this would correspond to the application of 4 mg of nisin to the wound, which is 16 times below the acceptable daily intake for a medium weight individual. However, since the final objective of the project where this work is included is to apply the nisin topically to the infected diabetic ulcers, cytotoxicity studies still need to be performed.

The emergence of mutants resistant to this AMP can be prevented if the administration doses remains above the MPC value, being the recommended dose determined in this study probably safe, since the acceptable daily intake of nisin is above the MPC value.

3.2. Horizontal Gene Transfer

The emergence of VRSA is a current problem, since vancomycin is often a last resort antibiotic applied in the treatment of several types of infections promoted by resistant bacteria, including DFI (Bader, 2008; Butler et al., 2014; Hsu et al., 2004). It is known that the rate of resistant mutants increases with prolonged antimicrobial treatments (Giraud et al., 2002). For this reason, and considering that nisin binds to the same molecule that vancomycin, it is important to understand if a

new therapeutic protocol based on nisin would promote the transfer of resistant genes, in particular of *vanA* (Breukink & Kruijff, 2006; Giraud et al., 2002).

Initially, a multiplex PCR confirmed the absence of the *vanA* gene in all the *S. aureus* isolates under study, being possible to use the 24 clinical isolates as recipients to evaluate the occurrence of horizontal gene transfer (figure 10).

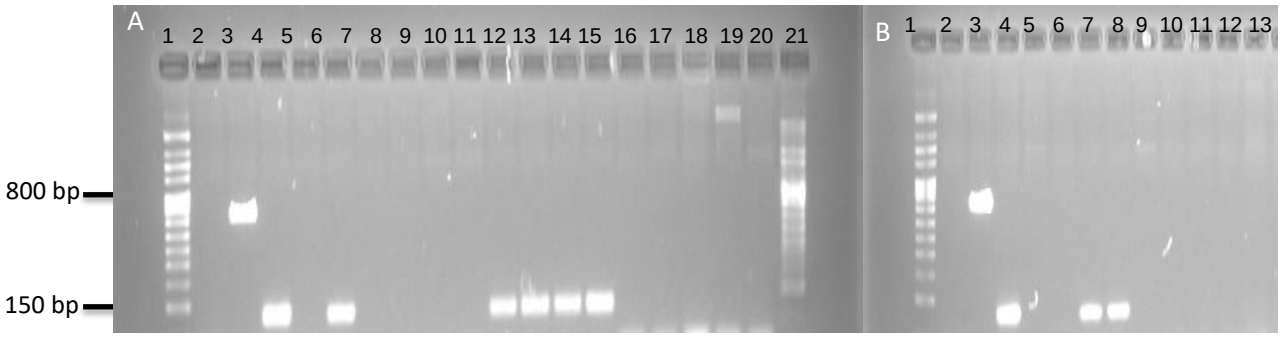

Figure 10 - Electrophoresis results of a multiplex PCR reaction for determining the presence of the vanA gene in the isolates under study. Image A: Lane 1 and 20 ladder VI (Nzytech®). Lane 2 negative control; Lane 3 vanA positive control Enterococcus faecalis CCUG 36804; Lane 4 mecA positive control S. aureus 01-00694; Lane 5 S. aureus ATCC 29213; Lane 6 to 19 the S. aureus DFI clinical isolates under study in the following order: A.1.1, A.5.2, A.6.3, B.3.2, B.3.3, B.7.3, B.13.1, B.14.2, Z.1.1, Z.2.2, Z.3.1, Z.5.2, Z.12.2, Z.14.1. Image B: Lane 1 ladder VI (Nzytech®). Lane 2 negative control; Lane 3 vanA positive control; Lane 4 the mecA positive control; Lane 5 to 13 the S. aureus DFI clinical isolates under study in the following order: Z.16.1, Z.17.2, Z.21.1, Z.21.3, Z.23.2, Z.25.2, Z.27.2, Z.27.3, Z.32.2.

The horizontal gene transfer protocol was performed, using the 23 *S. aureus* clinical isolates and the reference strain as potential recipients and the *E. faecium* CCUG 36804 as the donor of the *vanA* gene. PCR analysis was performed regarding all isolates recovered from the media used to select the possible transconjugants. A band matching the *vanA* positive control was obtained from the mating between the recipient *S. aureus* Z5.2 and *E. faecium* CCUG 36804 (figure 11).

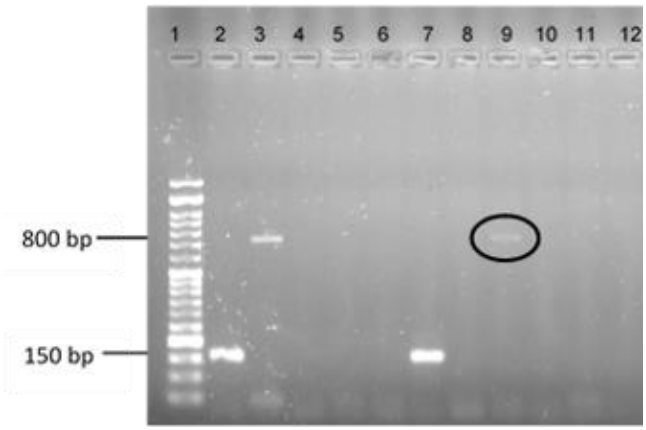

Figure 11 - Electrophoresis results of a multiplex PCR reaction for determining the presence of the vanA gene in the isolates under study. Lane 1 ladder VI (Nzytech®). Lane 2 mecA positive control S. aureus 01-00694; Lane 3 vanA positive control Enterococcus faecalis CCUG 36804; Lane 4 negative control; Lane 5 S. aureus ATCC 29213; Lane 6 to 20 the S. aureus DFI clinical isolates under study in the following order: A6.3, B14.2, Z2.2, Z5.2, Z17.2, Z.27.2, Z32.2.

This clinical isolate is a methicillin susceptible *S. aureus* (MSSA) and belongs to the Clonal Complex 5, as the majority of the clinical isolates under study (69.5%) (Mottola, Semedo-Lemsaddek, et al., 2016). Clones belonging to the CC5 are the predominant cause of HA-MRSA infections, being also present in community. Additionally, the majority of the VRSA strains reported so far belong to the clonal complex 5 (King et al., 2016; Rossi et al., 2014).

Since the pSK41 plasmid has been described as required for the transfer of the *vanA* gene from enterococci to staphylococci, a PCR analysis was performed regarding all the clinical *S. aureus* isolates to evaluate the presence of this plasmid. pSK41 was already detected in multiple strains, including CA-MRSA (ex. CC8) and HA-MRSA (ex. CC5) (Albrecht et al., 2014; McDougal et al., 2010). Surprisingly, in our collection all the isolates were negative for pSK41-plasmid, even *S. aureus* Z5.2 (figure 12).

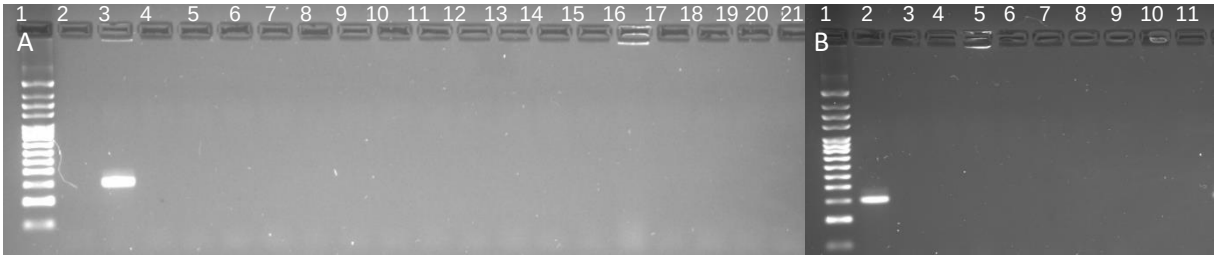

Figure 12 - Electrophoresis results of a multiplex PCR reaction for determining the presence of the psK41 plasmid in the isolates under study. Image A: Lane 1 ladder VII (Nzytech®). Lane 2 negative control; Lane 3 pSK41 positive control S. aureus RN4220 (pGO1 positive); Lane 4 S. aureus ATCC 29213; Lane 5 to 20 the S. aureus DFI clinical isolates under study in the following order: A.1.1, A.5.2, A.6.3, B.3.2, B.3.3, B.7.3, B.13.1, B.14.2, Z.1.1, Z.2.2, Z.3.1, Z.5.2, Z.12.2, Z.14.1, Z.16.1, Z.17.2. Image B: Lane 1 ladder VII (Nzytech®). Lane 2 pSK41 positive control S. aureus RN4220 (pGO1 positive); Lane 3 to 11 the S. aureus DFI clinical isolates under study in the following order: Z.21.1, Z.21.3, Z.23.2, Z.25.2, Z.27.2, Z.27.3, Z.32.2.

Another interesting fact is the methicillin susceptible profile of transconjugant *S. aureus* Z5.2, since almost all the VRSA reported are also MRSA (Friães et al., 2015; Kohler, Vaishampayan, & Grohmann, 2018). The association between the emergence of VRSA with MRSA is probably due to the fact that treatment with vancomycin is only recommended when semi-synthetic penicillin fail, which indicates the presence of methicillin-resistant mutants at the site of infection when the new vancomycin-based antibiotherapy is started. Results from this study seems to indicate that the MSSA strains also have the ability to acquire other resistant determinants besides *mecA*.

In 2012, in Brazil, two different clinical *S. aureus* isolates obtained from blood samples of one patient were found to be resistant to vancomycin. Researchers believe that both of these isolates resulted from the mating of enterococci with two *S. aureus* isolates which presented different characteristics: one was susceptible to methicillin and the other was a MRSA without a pSK41 plasmid; the MSSA belonged to CC5 and the MRSA to CC8 (Panesso et al., 2015; Rossi et al., 2014). The researchers found that both VRSA presented a 55,7 bp plasmid denominated pBRZ01, which is not related with

the pSK41 plasmid. This plasmid is a rearranged *Tn1546*-like element and holds an insertion region flaking the *vanA* gene cluster, which could be responsible for providing mobility to pBRZ01 (Rossi et al., 2014). Therefore, it would be interesting to evaluate the role of PBRZ01 in gene transference between the isolates under study.

The fact that strains that belong to CC5 are repeatedly acquiring resistance to vancomycin is probably related with some predisposition of these strains to horizontal gene transfer (Kos et al., 2012). In our study, the acquisition of *vanA* was accomplished by a MSSA that belongs to CC5 but does not present pSK41, which supports the hypotheses that other plasmids could be related with the transfer of the *vanA* gene. This phenomenon may contribute for the increasing virulence of the *S. aureus* strains found in Portugal at the hospital settings, since CC5 is the second most predominant clone in Portuguese hospitals; as such, the transfer of resistant elements can be more widespread than expected (Semedo-Lemsaddek et al., 2015). Although these strains were considered to be specific of the hospital settings, they have also been found in the community, which raises more concerns about these bacteria, since they seem to have a genetic or biological predisposition to acquire resistance factors (Friães et al., 2015; King et al., 2016). Additionally, the fact that MSSA can also be involved in horizontal gene transfer increases the range of possible events.

Despite the highlight that has been given to the role of the pSK41 plasmid in the horizontal gene transfer events between enterococci and *S. aureus*, it could have a lower relevance than researchers believe, since other plasmids, like pBRZ01, appear to have the ability to acquire resistance determinants such as the *vanA* gene. It is important to understand the mechanisms of resistance genes transference, since infections, in particular the ones promoted by biofilms, present perfect conditions for the transfer of resistance determinants. It is also important to highlight that the transfer only occur in 4.16% (n=1) of the clinicals isolates under study, which is also relevant, since if bacteria is susceptible to first line antibiotics, the use of last resort antibiotics decrease, not promoting the emergence of new resistances to the last resort antibiotics.

It was not possible to obtain a *vanA* positive transconjugant in the second mating round (with selective pressure of nisin) and in the third mating round (with selective pressure of vancomycin).

The presence of nisin at a sub-MIC value of 5.63 µg/mL apparently does not promote the transfer of the *vanA* gene which is an important characteristic to support the future clinical application of this AMP for DFI treatment.

The presence of vancomycin at a sub-MIC value (0.55 µg/mL) apparently does not enhance selectively enhance the transfer of the *vanA* gene, which is a surprising result, since antibiotics at low levels, like sub-MIC values appears to promote the emergence of resistant bacteria (Wistrand-Yuen et al., 2018). This result was not expected but it may be related to the fact that the acquisition of resistance genes has a fitness cost for bacteria due to the fact that several genes must be activated to acquire and maintain resistance factors (Hernando-Amado, Sanz-García, Blanco, & Martínez, 2017).

It is important to refer that the donor-recipient ratio used in this study was of 1:1, which could have influenced the low rate of transconjugants obtained.

The HGT is a concerning problem in our days, mainly between *S. aureus* and enterococci, since these microorganisms were classified by WHO as high priority pathogens due to the ability to acquire new

resistance factors (Tacconelli et al., 2017). For that reason it is important that the mechanisms under these processes are understood.

To our knowledge this was the first time that nisin ability to induce transferability of resistance genes was evaluated, being interesting to observe that nisin at sub-MIC values does not seem to induce *vanA* gene transfer.

The characteristics of the microenvironment of diabetic foot infections may prompt the acquisition of resistant determinants, including vancomycin resistance, as the majority of VRSA isolated so far were obtained from patients with DFI (Kos et al., 2012). Diabetic patients with these types of infections are often hospitalized and under antibiotherapy, which could promote the emergence of new resistant strains, becoming important and worrying vehicles for the dissemination of resistant isolates in and out of the hospital setting (Kos et al., 2012; Mottola, Semedo-Lemsaddek, et al., 2016).

3.3. Future Perspectives

The results from this thesis originated new questions that would be interesting to answer.

First, to confirm if the band that matched the *vanA* positive control in the PCR analysis is in fact the gene that confers resistance to vancomycin, sequencing is going to be performed.

It would also be interesting to understand if the MIC values previously determined for several antibiotics regarding the clinical *S. aureus* under study (Mottola, Matias, et al., 2016), changed for the mutant isolates obtained in both the mutant selection window and horizontal gene transfer protocols.

Finally, it would be interesting to perform the horizontal gene transfer protocol using a biofilm-model.

Chapter 4 | Conclusion

The development of a new antimicrobial compound for clinical application depends on several previous studies to confirm its efficacy and security. Over the years, nisin has been shown to be effective against MRSA, VRSA and *S. aureus* DFI strains.

Considering the usual multi-resistant profile of *S. aureus,* it is important to prevent the emergence of new resistance strains, which can be accomplish with the application of proper doses and concentrations of antimicrobials. Taking this into account, determination of the MSW becomes essential. Since the MIC value of nisin regarding the *S. aureus* isolates under study was already established, the MPC value of nisin was determined in this work. Most of the clinical *S. aureus* isolates from DFI presented an MPC 64 times higher than the MIC value, which is in accordance with the previously determined MPC of vancomycin for *S. aureus*. Since vancomycin and nisin have a similar action mode as they act by binding to the same molecule, lipid II, these results are relevant.

Determination of the MSW for nisin regarding the DFI *S. aureus* isolates will allow the future establishment of the right therapeutic dosage in order to avoid the development of resistant mutants. The fact that the higher dose of nisin used in this study is below the acceptable daily intake dose establish by EFSA is a good predictor that this high dosage can be used *in vivo*, mainly because this nisin dose was used in a study were this AMP was applied to an infected wound producing no toxic effects on the patients.

A second task also aiming for the prevention of the emergence of new antimicrobial resistant strains was performed. Vancomycin is a last resort antibiotic and the dissemination of vancomycin resistant strains is increasing. The vancomycin resistance in *S. aureus* is acquired through conjugation with enterococci, with the acquisition of the *vanA* gene. As vancomycin is usually applied to DFI treatment and has a mode of action similar to nisin, as previously mentioned, it is important to understand if nisin could potentiate the transfer of resistant genes, including vancomycin resistance genes. A horizontal gene transfer protocol was performed in the presence of nisin sub-MIC values, mimicking selective pressure conditions. No transconjugant was recovered, indicating that nisin at this concentration does not promote the transfer of *vanA*. However a transconjugant was obtained from the mating with the clinical isolate *S. aureus* Z5.2, an MSSA which belong to CC5, and the reference strain *E. faecium* CCUG 36804. These are important characteristics since CC5 is the second most predominant clone of *S. aureus* in Portuguese hospitals, and almost all reported VRSA until now belong to this clonal complex. Since the majority of VRSA described until now are also MRSA, the fact that *S. aureus* Z5.2 is an MSSA suggests that a broader range of staphylococci can acquire resistance to vancomycin and become a VRSA strain.

This study together with previous studies performed at the Laboratory of Microbiology and Immunology of FMV/CIISA, highlights the potential clinical use of nisin as an antimicrobial for DFI treatment.

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