Carolina de Oliveira Jesus

Degree in Biochemistry

Influence of a nisin-biogel on virulence factors expression by *Staphylococcus aureus* isolates from Diabetic Foot Infections

Dissertation to obtain Master's Degree in Molecular Genetics and Biomedicine

Supervisor:	Maria Manuela Castilho Monteiro de Oliveira, Associate Professor, FMV-ULisboa
Co-supervisor:	Rita Gonçalves Sobral, Assistant Professor, FCT/UNL

Jury:

President:	José Paulo Nunes de Sousa Sampaio, Associate Professor, FCT/UNL
Arguer:	Maria Elisabete Tomé Sousa Silva, Auxiliary Investigator, FMV-ULisboa
Supervisor:	Maria Manuela Castilho Monteiro de Oliveira, Associate Professor, FMV-ULisboa

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Para ti, Magda, porque esta etapa não é só minha, é nossa!

Acknowledgments

This was a long and challenging journey during which I had the opportunity to meet and work with amazing people, who helped me to grow both professionally and personally.

First, I would like to thank my supervisor, Professor Manuela Oliveira, for her guidance, support, friendship and patience. She treated me as a real and mature scientist and tried to make me as independent and autonomous as possible. I would also like to thank all trust placed in me and all the freedom she gave me to pursue my suggestions.

I would also like to thank Professor Luís Tavares for welcoming me to the Faculty of Veterinary Medicine – University of Lisbon.

Next, I would like to thank my co-supervisor, Professor Rita Sobral, for the help, guidance and availability demonstrated during this intense year.

Then, I would like to thank Eva Cunha and Miguel Grilo for all the friendship, all the conversations, all the laughs and all the support they gave me during this year. They always believed in me, in what I was able to do and I will be able to do in the future. They are undoubtedly two people who came into my life and I don't want to let them out, because more than colleagues, we have become friends.

I would also like to thank Cláudia Meirinhos, the person who walked side by side with me during this year. We have always supported ourselves when our results weren't the best. We shared stories, laughs, meals and, above all, we created a beautiful friendship, which I hope will be for life.

Thanks to all the other members of Microbiology and Immunology Laboratory for all the moments and knowledge we shared, I learned a lot with them during this year.

To my parents who have always taught me to do my best to overcome challenges successfully. This was just another challenge, and I think I can check it out. To my sister for all the long talks and laughter, without her, my world would be grayer. To my grandparents for all the wise words of support. I know that for them it is a pride to see their eldest granddaughter finish her studies. I hope I never let them down. Thanks to my family in general for always believe in me, I am so lucky to have such a special and supportive family.

To Pedro for all the support and love, he is the best. Thanks for always believe in me, for all the patience with me (I know that sometimes it must not have been easy), for all the sushi nights that relieved stress, for all the walks, conversations and laughs. Without him, this journey would not have been the same.

Finally, I would like to thank my friends. To "Miúdos Mesmo Mais Giros", what great friends! Thanks for all the adventures, for all the nights, for all the support, for all the fun and for all moments. I know I can always count on them. Cheers to them! To my other friends (they know who they are) that I had the pleasure to meet across the years. Thanks for sharing such special moments with me and for always support and motivate me. I know we will always grow together.

Thanks to all of you,

"Life becomes easier when you have the right people on your side" – Unknown

Resumo

As Úlceras do Pé Diabético (UPDs) constituem um ambiente favorável à colonização por agentes patogénicos oportunistas, sendo o *Staphylococcus aureus* a espécie mais frequentemente isolada a partir das UPDs. Devido à crescente disseminação de estirpes resistentes a antibióticos, incluindo *S. aureus*, os peptídeos antimicrobianos (PAMs) têm sido reconhecidos como candidatos promissores para o tratamento de infeções bacterianas resistentes. Neste estudo o PAM nisina foi combinado com o polissacarídeo natural guar gum com o objetivo de criar um biogel de nisina a ser avaliado como uma nova abordagem terapêutica para as Infeções do Pé Diabético (IPDs). Nas infeções *in vivo*, as bactérias podem ser expostas a concentrações efetivas de agentes antimicrobianos diminuídas, designadas por concentrações subinibitórias (sub-CMIs). As sub-CMIs dos agentes antimicrobianos podem levar a alterações no metabolismo das bactérias, nomeadamente na capacidade de formação de biofilmes e na expressão de genes de virulência. Analisámos os efeitos das sub-CMIs do biogel de nisina em seis isolados clínicos de *S. aureus*, incluindo: (1) na taxa de multiplicação através da determinação da densidade ótica a 600 nm; (2) na expressão de genes de virulência, incluindo os genes que codificam a proteína estafilocócica A (*spA*), a coagulase (*coa*), o fator de aglomeração A (*clfA*), a autolisina (*atl*), a adesina intracelular A (*icaA*), a adesina intracelular D (*icaD*) e o gene regulador acessório I (*agrI*), por RT-PCR quantitativo relativo; (3) na formação de biofilme por uma técnica de microtitulação; (4) na produção de coagulase usando plasma de coelho; e (5) na libertação de SpA usando um ELISA específica. As sub-CMIs do biogel de nisina contribuíram para uma diminuição da multiplicação bacteriana, de uma forma dependente da estirpe e da dose, não influenciando o padrão sigmoidal típico. Observou-se uma diminuição na expressão do mRNA de AgrI, Atl e ClfA, e uma tendência de aumento na expressão do mRNA de SpA, Coa, IcaA e IcaD na presença de sub-CMIs do biogel de nisina. A capacidade dos isolados clínicos de *S. aureus* de formar biofilme mostrou uma tendência para aumentar na presença de biogel de nisina em concentrações correspondentes a 1/4 e 1/8 CMI, enquanto que uma concentração correspondente a 1/2 CMI não teve qualquer efeito na formação do biofilme. O biogel de nisina em sub-CMIs não influenciou relevantemente a produção de coagulase. Relativamente à produção de SpA, o biogel de nisina em sub-CMIs apresentou uma tendência para diminuir a quantidade de SpA produzida de uma forma dependente da dose. Estes resultados sublinham a importância de otimizar as doses do biogel antimicrobiano antes de proceder a ensaios *in vivo*, não só para obter um efeito antibacteriano máximo, mas também para reduzir o risco de sobre-regulação dos fatores de virulência e, consequentemente, obter efeitos indesejáveis no tratamento da infeção.

Palavras-chave: Infeções do Pé Diabético, *Staphylococcus aureus*, Biogel de nisina, Concentrações subinibitórias, Fatores de virulência

Abstract

Diabetic Foot Ulcers (DFUs) constitute a favorable environment for colonization by opportunistic pathogens, and *Staphylococcus aureus* is the most frequent species isolated from DFUs. Due to the increasing dissemination of antibiotic-resistant strains, including *S. aureus*, antimicrobial peptides (AMPs) have been recognized as promising candidates for treating resistant bacterial infections. In this study, the AMP nisin had been combined with the natural polysaccharide guar gum to create nisin-biogel, to be evaluated as a new therapeutic approach to Diabetic Foot Infections (DFIs). In *in vivo* infections, bacteria may be exposed to a decreased effective concentration of antimicrobial agents, referred to as subinhibitory concentrations (sub-MICs). Sub-MICs of antimicrobial agents may lead to changes in bacteria metabolism, namely in biofilm formation ability and virulence genes expression. We analyzed nisin-biogel sub-MICs effects on six different *S. aureus* clinical isolates, including: (1) multiplication rate by determining optical density at 600 nm; (2) virulence gene expression, including of genes encoding for staphylococcal protein A (*spA*), coagulase (*coa*), clumping factor A (*clfA*), autolysin (*atl*), intracellular adhesin A (*icaA*), intracellular adhesin D (*icaD*) and the accessory gene regulator I (*agrI*), by relative quantitative RT-PCR; (3) biofilm formation by a microtiter technique; (4) Coa production using rabbit plasma; and (5) SpA release using a specific ELISA. Nisin-biogel sub-MICs contributed to a decrease in bacteria multiplication in a strain-dependent and dose-dependent manner, not influencing the typical sigmoidal pattern. A decrease on AgrI, Atl and ClfA mRNA expression, and a trend to increase SpA, Coa, IcaA and IcaD mRNA expression were observed in the presence of nisin-biogel at sub-MICs. The biofilm-forming ability of *S. aureus* clinical isolates exhibited a trend to increase in the presence of nisin-biogel at 1/4 and 1/8 MIC, whereas a concentration corresponding to 1/2 MIC had no effect on biofilm formation. Nisin-biogel at sub-MICs did not influence significantly coagulase production. Regarding SpA production, nisin-biogel at sub-MICs exhibited a trend to decrease the amount of SpA produced in a dose-dependent manner. These results highlight the importance of optimizing antimicrobial biogel doses before proceeding to *in vivo* trials, not only to obtain a maximal antibacterial effect but also to reduce the risk for upregulation of virulence factors and, consequently, undesirable effects on the treatment of DFIs.

Key-words: Diabetic Foot Infections, *Staphylococcus aureus*, Nisin-biogel, Subinhibitory concentrations, Virulence-related factors

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List of Abbreviations and Symbols

µg – Microgram

µL – Microliter

β-cells – Beta cells

ADI – Acceptable Daily Intake

AIPs – Auto-Inducing Peptides

AMPs – Antimicrobial Peptides

Bap – Biofilm-associated protein

BHI – Brain Heart Infusion

BPW – Buffered Peptone Water

Bw – Body Weight

CA-MRSA – Community-Acquired Methicillin-Resistant *S. aureus*

cDNA – Complementary Deoxyribonucleic Acid

CFU – Colony-Forming Units

DFIs – Diabetic Foot Infections

DFUs – Diabetic Foot Ulcers

DM – Diabetes *mellitus*

ELISA – Enzyme-Linked Immunosorbent Assay

EPS – Extracellular Polymeric Substract

FAO/WHO – Food and Agriculture Organization/ World Health Organization

Fc – Fragment Crystallizable

Fg – Fibrinogen

Fn – Fibronectin

FnBPs – Fibronectin-Binding Proteins

GD – Gestational Diabetes

HA-MRSA – Hospital-Acquired Methicillin-Resistant *S. aureus*

IDF – International Diabetes Federation

IgG – Immunoglobulin G

IWGDF – International Working Group on Diabetic Foot

LEAs – Lower-Extremity Amputations

MBC – Minimum Bactericidal Concentration

MBEC – Minimum Biofilm Eradication Concentration

MBIC – Minimum Biofilm Inhibitory Concentration

MDR – Multidrug Resistant

mg – Milligram

MIC – Minimum Inhibitory Concentration

mL – Milliliter

MLSA – Multilocus Sequence Type

MRSA – Methicillin-Resistant *S. aureus*

MSCRAMMs – Microbial Surface Component Recognizing Adhesive Matrix Molecules

MSSA – Methicillin-Susceptible *S. aureus*

NICE – National Institute for Health and Care Excellence

NRP – Nisin Resistant Protein

NTC – No Template Control

OD – Optical Density

ORFs – Open Reading Frames

PBP2a – Penicillin-Binding Protein 2a

PCR – Polymerase Chain Reaction

PFGE – Pulse Field Gel Electrophoresis

pg – Picogram

PN – Peripheral Neuropathy

QS – Quorum Sensing

R^2 – Correlation coefficient

RNA – Ribonucleic Acid

-RTC – Minus Reverse Transcriptase Control

RT-qPCR – Quantitative Reverse Transcription Polimerase Chain Reaction

SCC*mec* – Staphylococcal Cromossomal Cassete *mec*

Sub-MICs – Subinhibitory Concentrations

T1DM – Diabetes *mellitus* Type 1

T2DM – Diabetes *mellitus* Type 2

TSB – Trypticase Soy Broth

VRSA – Vancomycin-Resistant *S. aureus*

WHO – World Health Organization

Chapter 1 | Introduction

1.1. Diabetes *mellitus*

Diabetes *mellitus* (DM) is a lifelong metabolic disorder that affects approximately 463 million people worldwide, according to the International Diabetes Federation (IDF), being associated with high mortality rates worldwide. The prevalence of DM has been rapidly rising, being expected to reach 578 million people by 2030 and 700 million by 2045, as a drastic consequence of population aging, urbanization and lifestyle changes. In Portugal, reports from IDF refer that the prevalence of DM is around 14.2% of the population aged between 20 and 79 years, a percentage above the European average (6.3%) (International Diabetes Federation [IDF], 2019; Saeedi et al., 2019).

According to the World Health Organization (WHO), DM is characterized by defects in insulin secretion and/or insulin action that lead to a state of hyperglycemia and, consequently, to fat, carbohydrates and protein metabolism alterations, as well as impaired immunological defenses (Chastain, Klopfenstein, Serezani, & Aronoff, 2019; World Health Organization [WHO], 2019). Processes characterized by the destruction of the pancreas beta cells (β-cells), which are responsible for the synthesis and secretion of insulin, are considered the main pathogenic process involved in the development of DM (Skyler et al., 2017).

The elevated number of DM patients is primarily related to environmental (microbiome, physical activity and dietary factors) and/or genetic factors, which are crucial determinants of β-cell dysfunction and insulin resistance (Skyler et al., 2017). The most common type of DM is Diabetes *mellitus* type 2 (T2DM), affecting 90-95% of diabetic patients. T2DM is characterized by acquired resistance to insulin and/or hyposecretion of insulin. Age, physical inactivity and obesity are the main risk factors that contribute to the development of T2DM, despite the fact that some genetic factors are also implied. This form of DM usually remains undiagnosed for long periods as the development of hyperglycemia is gradual and in the early stages it is not severe enough for the patient to notice symptoms (American Diabetes Association [ADA], 2013; Punthakee, Goldenberg, & Katz, 2018). Diabetes *mellitus* type 1 (T1DM) affects 5-10% of diabetic patients, being a chronic condition in which the pancreas produces little or no insulin, due to autoimmune or to unknown etiology of β-cells destruction. A combination of genetic susceptibility and environmental factors is believed to lead to T1DM, even though the exact causes are still unknown, rendering T1DM unpreventable (ADA, 2013; Punthakee et al., 2018; WHO, 2019). There is also another important type of DM – gestational diabetes (GD) – which starts in pregnancy and can cause many complications not only during pregnancy but also after birth, both for mother and child, increasing the child's risk of developing T2DM in the future. Excessive weight

gain during pregnancy is probably the major risk factor associated with GD (ADA, 2013; Punthakee et al., 2018; WHO, 2019).

The primary diabetes symptoms include thirst, polyuria, weight loss and blurring vision, and patients with chronic hyperglycemia have a higher predisposition to bacterial and fungal infections. These clinical manifestations may differ between patients and may be not simultaneous (ADA, 2013; WHO, 2019). The consequences of uncontrolled DM include long-term damage, dysfunction and failure of diverse organs, mainly associated with vascular system insufficiency and nerve damage, which can increase the risk of premature death. Microvascular complications include neuropathy, nephropathy and retinopathy, while macrovascular complications include cardiovascular disease, stroke and peripheral vascular disease (Chawla, Chawla, & Jaggi, 2016; WHO, 2019). The treatment of DM and the management of DM-related complications are associated with high healthcare costs. As such, an early diagnosis and, consequently, an early and adequate treatment are essential to reduce DM-associated complications and, therefore, the associated costs (Kuzuya, 2000; WHO, 2019).

1.2. Diabetic Foot Ulcers (DFUs)

Diabetic foot ulcers (DFUs) are a serious complication especially common among patients with DM that results in significant morbidity and mortality, being often responsible for lower-extremity amputations (LEAs) (Armstrong, Boulton, & Bus, 2017). The lifetime probability of a DM patient to develop a DFU is 15 to 25%. Approximately 60% of DFUs will progress to Diabetic Foot Infections (DFIs), and 20% of the patients with DFIs will probably undergo a LEA (Grigoropoulou, Eleftheriadou, Jude, & Tentolouris, 2017; Skrepnek, Mills, Lavery, & Armstrong, 2017). The predisposition to develop a DFU is characterized by a triad of neuropathy, ischemia associated with peripheral vascular disease (PAD) and susceptibility to infection. DFUs are mainly divided into three types: neuropathic, neuroischemic and ischemic, being the neuroischemic type the one with a higher prevalence (Pendsey, 2010). Peripheral neuropathy (PN), a deficiency in the normal activities of the nerves in the body that can modify the motor, sensory and autonomic functions, is the major cause that leads to DFUs, with 60% of patients with DFU having PN (Pitocco et al., 2019). The damage of nerve cells is mainly associated with hyperglycemia and insufficient blood flow and, consequently, with the lack of nutrients and oxygen supply to the nerves (Yagihashi, Mizukami, & Sugimoto, 2011).

The International Consensus on the Diabetic Foot by the International Working Group on the Diabetic Foot (IWGDF) defined a DFU as a full-thickness wound below the ankle, infected or uninfected, regardless of its duration (Schaper, Apelqvist, & Bakker, 2003). The PEDIS classification system, developed by the IWGDF to classify DFUs, considers five major categories: perfusion (i.e. ischemia), extent/size, depth/tissue loss, infection and sensation (i.e. neuropathy), which are the most relevant characteristics associated with DFU pathophysiology (Schaper, 2004).

1.3. Diabetic Foot Infections (DFIs)

DFUs are characterized by a severe loss of skin protective barriers creating an opportunity for tissue colonization by opportunistic microorganisms, usually becoming chronic and resulting in patient's morbidity and mortality (Noor, Khan, & Ahmad, 2017). Accordingly, DFIs are complex and multifactor polymicrobial infections in DM patients that can be clinically defined by the presence of inflammation or purulence and then classified by severity, ranging from mild, through moderate to severe (Chastain et al., 2019; Joseph & Lipsky, 2010). DFIs severity is mostly related to host-related factors (neuropathy, immunopathy and angiopathy) and secondarily with pathogen-related issues (density of pathogens, bacterial virulence and bacterial interactions), although both of the aspects are crucial for DFIs development and management (Richard, Lavigne, & Sotto, 2012; Spichler, Hurwitz, Armstrong, & Lipsky, 2015). DFIs can be local and superficial or involve deeper subcutaneous tissues when the protective skin envelope is disrupted, exposing underlying tissues to bacteria, allowing colonization (Uçkay, Aragón-Sánchez, Lew, & Lipsky, 2015). Due to their complexity, the treatment of DFIs requires multidisciplinary care, since there are multiple factors involved in their onset and development, requiring special attention to local (foot) and systemic (metabolic) problems (Liu, Shi, & Sheu, 2012; Uçkay et al., 2015).

1.3.1. Bacteriology of DFIs

Microorganisms involved in DFIs may vary depending on the type of infection and specific patients' situations. In most cases, gram-positive cocci are the first to colonize and infect skin wounds (Spichler et al., 2015). Thus, acute, previously untreated and superficial infected wounds are mainly colonized by aerobic gram-positive bacteria, such as *Staphylococcus aureus*, including both Methicillin-susceptible *S. aureus* (MSSA) and Methicillin-resistant *S. aureus (*MRSA) strains, and beta-hemolytic streptococci. Chronic wounds, especially in DM patients who have recently been subjected to antimicrobial therapy or who have deeper infections, develop a more complex colonizing microbiota, including gram-positive aerobic cocci, gram-negative aerobic bacilli (e.g. *Pseudomonas aeruginosa* and *Escherichia coli*) and anaerobes (e.g. *Clostridium* and *Bacteroides* species) (Jneid, Lavigne, La Scola, & Cassir, 2017; Kwon & Armstrong, 2018; Ramirez-Acuña et al., 2019).

1.4. *Staphylococcus aureus*

1.4.1. Bacterial characterization

S. aureus is both a commensal bacterium associated with skin, skin glands and mucous membranes, and an important human opportunistic pathogen, associated with a wide spectrum of diseases, including endocarditis, bacteraemia, osteomyelitis and skin and soft tissue infections. Regarding DFIs, *S. aureus* is the pathogen most frequently isolated in Occidental countries, alone

or in combination with other pathogens (Dunyach-Remy, Essebe, Sotto, & Lavigne, 2016; Jneid, et al., 2017). *S. aureus* is a gram-positive, facultative aerobic, nonmotile, catalase-positive, coagulase-positive, oxidase-negative and cocci-shape bacterium, belonging to the genus *Staphylococcus* (Plata, Rosato, & Wegrzyn, 2009; Willey, Sherwood, & Woolverton, 2008). Furthermore, the cell wall peptidoglycan structure from *Staphylococcus* contains multiple glycine residues in the cross-bridge, causing lysostaphin susceptibility (Plata et al., 2009).

1.4.2. Bacterial infection

The high pathogenicity of *S. aureus* is largely due to biofilm production, virulence-related genes expression, invasiveness ability and antibiotic resistance profile. To establish persistent infections, *S. aureus* expresses several regulatory and virulence factors, depending on the infection phase – colonization, evasion of host immune system, growth and cell division, or bacterial dissemination (Wang & Muir, 2016). Depending on the infection type, the specific role of a regulatory gene or a virulence determinant in *S. aureus* pathogenesis may differ. Also, virulence factors can act under normal conditions or only take part in bacterial pathogenicity under specific conditions, in both situations allowing pathogens to overcome host immune defenses and cause disease (Jenul & Horswill, 2018). Therefore, understanding *S. aureus* pathogenesis at a molecular level may help in the treatment management of several human diseases, including in the development of novel antimicrobial strategies, since *S. aureus* strains resistant to various antibiotics are increasing (Sayers et al., 2019; Stevenson & Wang, 2014).

1.4.3. Quorum-sensing and Agr regulatory system

Adhesion phase is the first step of a staphylococcal infection and is associated with the lag and early exponential phases of *S. aureus* growth. In this phase occurs the production of cell wall-associated factors that facilitate tissue attachment and evasion of the host immune system. Then, when the late exponential phase of *S. aureus* growth is reached, the bacterial population begins to secrete exoproteins and enzymes and start to down-regulate the cell wall-associated factors, which corresponds to the bacterial invasion phase (Periasamy et al., 2012; Wang & Muir, 2016).

The production of virulence factors and the development of biofilm by *S. aureus* are regulated by Quorum-sensing (QS). In this process, *S. aureus* synthesizes and secretes diffuse molecules, called auto-inducing peptides (AIPs), that work as indicators of the local population density, with the final purpose of controlling virulence factors expression and biofilm formation (Dickschat, 2010; Kong, Vuong, & Otto, 2006). The time period for the expression of pathogenic determinants in *S. aureus* is controlled by global regulators, being observed that one target virulence gene can be under the control of several regulators to ensure the expression of a specific gene only under favorable conditions (Jenul & Horswill, 2018).

The accessory gene regulator (*agr*) of *S. aureus* is a global transcriptional regulator, playing a fundamental regulatory role in *S. aureus* pathogenicity and biofilm dynamics in a time and population density-dependent manner (Novick, 2003; Kim et al., 2017). The signaling circuit encoded by *agr* locus produces and senses the AIPs, and the increase of bacterial density may lead to the accumulation of AIPs to a threshold concentration, inducing the transcription of specific genes by the bacterial population (Jenul & Horswill, 2018; Wang & Muir, 2016).

The *agr* locus is composed of two divergent operons driven by P2 and P3 promotors which encode RNAII and RNAIII, respectively. P2 operon contains 4 Open Reading Frames (ORFs) associated with the translation of 4 Agr proteins involved in autoinducer circuit (AgrA, AgrB, AgrC and AgrD). According to the basis of *agrD* and *agrC* polymorphisms, staphylococcal strains are divided into four major *agr* groups (*agrI-IV*). In each group, all strains produce an AIP that can activate *agr* response in the other members of the same group. Distinct *agr* groups produce and recognize different AIPs (Abdel Haleem, El Nagdy, & Omar, 2015; Novick, 2003). The P3 operon is AgrA-dependent and drives the transcription of RNAIII that has multiple roles. Accordingly, RNAIII is the effector molecule of the *agr* response driving the expression of genes required for exotoxins and exoenzymes secretion and biofilm dismantling, and functions as an mRNA that encodes a δ-toxin, which is a membrane disrupting exoprotein that lyses eukaryotic host cells (Abdel Haleem et al., 2015; Wang & Muir, 2016; Kim et al., 2017).

In addition to *agr*, which is the best studied regulatory system, there are other staphylococcal regulatory genes, such as *saeRS, srrAB, arlRS, sarA*, *rot*, *mgrA* and *sigB,* that control both virulence factor and cytoplasmatic enzymes expression at a transcriptional level (Jenul & Horswill, 2018; Novick, 2003).

1.4.4. Pathogenic determinants

The invasion and colonization of host tissues by *S. aureus* begins with staphylococcal adherence to the host cell, where *S. aureus* expresses several adhesins called microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (Alexander & Hudson, 2001). The fibronectin-binding proteins (FnBPs) are the most important staphylococcal MSCRAMMs mediating *S. aureus* invasion of eukaryotic cells. FnBPs bind to fibronectin (Fn) and promote the interaction between *S. aureus* and host cells integrins, being essential for staphylococcal internalization (Alexander & Hudson, 2001). Clumping factor A (ClfA), encoded by *clfA*, is a MSCRAMM that works as a bacterial cell surface receptor for the blood plasma protein fibrinogen (Fg), and the recruitment of Fg to the bacterial cell surface protects pathogen from neutrophil phagocytosis. It also promotes bacterial attachment to blood clots and traumatized tissue, being an important factor in the onset of many types of infections, as it allows bacterial colonization and biofilm formation (Herman-Bausier et al., 2018). Protein A (SpA), encoded by *spA*, is a MSCRAMM that links covalently to the cell wall peptidoglycan, playing an important role in interfering with host defenses. SpA can bind to the fragment crystallizable (Fc) domain of

immunoglobulin G (IgG) and, consequently, inhibit opsonization and phagocytosis. The conformation of *S. aureus* coated with IgG binded to SpA is not recognized by the Fc receptor in neutrophils, allowing the microorganism to persist in the host cell (Gómez, O'Seaghdha, Magargee, Foster, & Prince, 2006). Coagulase (Coa), encoded by *coa*, is an extracellular protein that stimulates the conversion of Fg to fibrin, causing clots in blood or plasma. This protein binds to prothrombin, forming an active proteolytic complex (Coa-prothrombin) responsible for fibrin polymerization. The formation of the bacterial fibrin shield contributes for *S. aureus* resistance against phagocytosis (Pozzi, Bagnoli, & Rappuoli, 2016; Yanagihara et al., 2006).

When the infection is established and a critical population density is reached, *S. aureus* starts to secrete toxins that disrupt the cytoplasmic membrane of the host cells and interfere with host immune system. Cytolytic toxins secreted by *S. aureus*, including alpha-hemolysin (Hla), beta-hemolysin (Hlb), gamma-hemolysin (Hlg) and Panton-Valentine leucocidin (PVL), are key virulence factors responsible for pore formation in host cells cytoplasmatic membranes, leading to cell lysis and, consequently, to the release of cellular content. Moreover, different cytolytic toxins can act in different cell types. For example, PVL induces lysis of neutrophils, while Hla induces lysis of a wide range of cells, including human keratinocytes, epithelial cells, lymphocytes and erythrocytes and it is unable to lyse neutrophils (Joo et al., 2016; Plata et al., 2009).

Another key point during bacterial growth and, consequently, bacterial infection, is the proper balance between peptidoglycan synthesis and degradation, and peptidoglycan hydrolases are thought to play an important role in cell turnover, cell division, cell separation and antibiotic mediated lysis. Staphylococcal major autolysin (Atl), encoded by *atl*, is a bifunctional cell surface associated peptidoglycan hydrolase composed by two domains, amidase and glucosaminidase, responsible for generating two extracellular lytic enzymes that are attached to *S. aureus* cell surface. Atl functions include staphylococcal attachment to surfaces, bacterial cell wall degradation and cell separation during cell division, lysis mediated biofilm development and secretion of cytoplasmic proteins (Biswas et al., 2006; Porayath et al., 2018; Singh, 2014).

1.4.5. Biofilm formation

Like almost all bacterial species, *S. aureus* has the capacity to form biofilms (Reffuveille, Josse, Vallé, Mongaret, & Gangloff, 2017). Biofilms are a major staphylococcal pathogenic feature and are characterized by the growth of adherent bacterial populations inside a self-produced matrix of extracellular polymeric substances (EPS) that provide architecture and structure to the biofilm, occurring both in biotic and abiotic surfaces. This polymeric matrix sequesters environmental nutrients such as phosphate, nitrogen and carbon, conferring to this sessile mode of life survival advantages when compared with the planktonic mode of life (Dickschat, 2010; Flemming et al., 2016; Reffuveille et al., 2017).

The first step of biofilm formation involves attachment of bacterial cells to a surface, while the second step includes cell multiplication and formation of a mature structure composed by

many cell layers. To complete the biofilm cycle, dispersion of some bacterial cells may occur, allowing the establishment of new sites of infection (Figure 1.1) (Otto, 2019; Reffuveille et al., 2017).

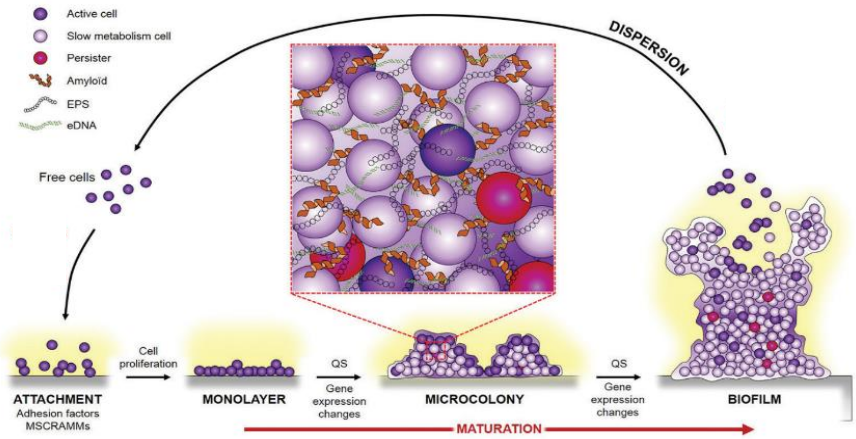


Figure 1.1 | Phases of staphylococcal biofilm formation. Biofilms form by initial surface attachment, followed by cell multiplication and multilayer formation (biofilm maturation). Finally, cell detachment occurs to colonize new sites of infection, leading to the dissemination of infection. Adapted from Ruffuveille et al., 2017.

The intercellular adhesion locus, *icaADBC*, is associated with cell-to-cell adhesion. This locus comprises an N-acetylglucosamine transferase (*icaA* and *icaD*), a polysaccharide intercellular adhesion (PIA) deacetylase (*icaB*), a putative PIA exporter (*icaC*) and a regulatory gene (*icaR*), being responsible for the biosynthesis of the biofilm exopolysaccharide PIA (Maira-Litrán et al., 2002; Otto, 2019; O'Gara, 2007). *icaA* encodes a N-glycosyltransferase that is essential for PIA production, and this IcaA transferase needs IcaD for full activity. After, the nascent PIA chain is exported by IcaC and deacetylated by IcaB to introduce positive charges (Otto, 2019; O'Gara, 2007). In addition to the *ica* operon, there are *icaADBC*-independent biofilm development mechanisms, including biofilm formation mediated by surface adhesion proteins, such as biofilm-associated protein (Bap) (Ghasemian, Peerayeh, Bakhshi, & Mirzaee, 2015; Houston, Rowe, Pozzi, Waters, & O'Gara, 2011).

Biofilm formation only occurs under particular conditions, and biofilm structures allow bacteria survival, withstanding to any type of environmental stress, including lack of nutrients, UV and presence of antimicrobials. In fact, the minimum biofilm inhibitory concentration (MBIC) can be up to 100- to 1000- fold higher than the planktonic bacteria minimum inhibitory concentration (MIC), since biofilms matrix constitutes a barrier against antibiotic penetration and action, contributing to better chances of bacterial survival (Richard et al., 2012; Reffuveille et al., 2017).

1.5. Treatment of DFIs

Currently, the standard method for DFIs treatment consists of surgical debridement followed by wound cleansing with an antiseptic solution and empiric antibiotic therapy (Lipsky et al., 2016). Surgical debridement of DFUs is essential to remove the necrotic and non-viable tissues. During this process, the loss of healthy tissue should be minimized, the foot function preserved and deformities that can lead to the recurrence of DFUs prevented (Amin & Doupis, 2016). Wound cleansing is the removal of surface contaminants, bacteria and/or remnants of previous dressings from the wound surface and its surrounding skin (Moore & Cowman, 2008). Antibiotic therapy is only required in clinically infected wounds. Agents, duration and administration route are established according to the severity of infection. Antibiotic therapy should not be applied to clinically uninfected wounds to prevent infection or promote wound healing, as the overuse of antibiotics increases the incidence of adverse effects, antibiotic resistance and healthcare costs (Kwon & Armstrong, 2018; Lipsky et al., 2016).

Regarding antibiotic treatment of DFIs, causative pathogens, severity of the infection, proven efficacy for the treatment of DFIs, cost and patient's medical history must be considered when choosing the proper antibiotic therapy (Everett & Mathioudakis, 2018). In fact, according to the IWGDF and the National Institute for Health and Care Excellence (NICE), the severity of DFIs is very important to define the correct antibiotic therapy. Thus, oral antibiotics effective against gram-positive bacteria should be applied to the treatment of mild infections, and moderate and severe infections should be managed with antibiotics effective against gram-positive and gram-negative bacteria, including anaerobic microorganisms, which should preferentially be administered by parenteral route (Pitocco et al., 2019). The antibiotic therapy regiment should always include antibiotics active against *Staphylococcus* and *Streptococcus* species, such as cephalexin, clindamycin and amoxicillin-clavulanate. If the patient has risk factors for MRSA infection, an agent active against MRSA should be added, such as linezolid, doxycycline or trimethoprim/sulfamethoxazole. If the infection is severe or if the patient has failed to respond to narrower spectrum therapy, the antibiotic therapy may be extended to vancomycin in combination with a beta-lactam and beta-lactamase inhibitor or a carbapenem (Chastain et al., 2019; Grigoropoulou et al., 2017; John & Sharkey, 2018; Lipsky et al., 2020). Besides select the antibiotic therapy agents and the route of administration, it is also essential to determine the duration of the treatment. Accordingly, patients with mild soft tissue infection are suggested to receive 1 to 2 weeks of therapy, those with moderate soft tissue infection may be treated with 1 to 3 weeks of therapy, and those with severe soft tissue infections are recommended to undergo 2 to 4 weeks of therapy (Chastain et al., 2019).

1.5.1. Antibiotic resistance

S. aureus is known to be a contagious opportunist pathogen that rapidly develops or acquires multiple antibiotic resistance determinants, which is a key factor for the success of *S.*

aureus as a human pathogen. Resistance to antibiotics result from genetic material exchange through different mechanisms such as transformation, transduction and conjugation. Furthermore, chromosomal mutation or inductive expression of a latent chromosomal gene can also be implied in bacterial resistance (Peterson & Kaur, 2018). Broad-spectrum or prolonged antibiotic therapy can predispose DM patients to colonization by and/or infection with antibiotic-resistant strains, such as MRSA (Grigoropoulou et al., 2017).

The cases of antibiotic resistance with more historical importance and with great impact today are penicillin, methicillin and vancomycin. Penicillin was the first antibiotic clinically implemented that contributed to the improvement of the prognosis of patients with staphylococcal infections. However, *S. aureus* resistant to penicillin was first described in 1942. Accordingly, *S. aureus* was capable of destroying penicillin through the expression of a penicillinase (today called β-lactamase), encoded by *blaZ* (gene responsible for penicillin resistance) and synthesized when *S. aureus* is exposed to β-lactam antibiotics (Lowy, 2003). To overcome this issue, the pharmaceutical industry started to develop a semisynthetic antibiotic named methicillin, which is derived from penicillin but resistant to β-lactamase inactivation (Fuda, Fisher, & Mobashery, 2005). However, in 1961, only two years after methicillin became available for clinical use, MRSA was first identified, and has become an important infectious agent, being related to decreased susceptibility to several antibiotics (Brown & Reynolds, 1980; Enright et al., 2002). MRSA is the most common and successful modern pathogen among patients who have been previously hospitalized or who have been recently undergoing antibiotic therapy. Infections caused by MRSA can arise in the community – community-acquired MRSA (CA-MRSA) infections – or can be contracted in medical facilities – hospital-acquired MRSA (HA-MRSA) infections (Chambers, 2001). Methicillin resistance is mediated by *mecA* and MRSA results from a differentiation of MSSA by acquisition of a mobile genetic element denominated staphylococcal cassette chromosome *mec* (SCC*mec*). *mecA* encodes the penicillin-binding protein 2a (PBP2a), that takes over peptidoglycan cross-linking in the presence of β-lactams antibiotics (El-baz, Rizk, Barwa, & Hassan, 2017; Holden et al., 2013; Wielders, Fluit, Brisse, Verhoef, & Schmitz, 2002). Another antibiotic that also started to be used in penicillin-resistant *S. aureus* was vancomycin. Vancomycin is a glycopeptide antibiotic discovered in 1956, which interferes with the production of proteins and with the synthesis of the bacterial cell wall, impeding peptidoglycan cross-linkage (Barna & Williams, 1984). However, the first report of a Japanese patient harboring MRSA intermediately resistant to vancomycin appeared in 1996 (Hiramatsu et al., 1997). In 2002, fully vancomycin-resistant *S. aureus* (VRSA) first appeared in the USA (Chang et al., 2003). In Portugal, the first VRSA strain was isolated from a DM patient in 2013 (Melo-Cristino, Resina, Manuel, Lito, & Ramirez, 2013).

1.6. Novel DFIs therapeutics

Nowadays there is a considerable risk of having no effective antibiotic treatment for many common human diseases, such as DFIs. Unless current trends are reversed, it is estimated that by 2050 infections caused by antibiotic-resistant pathogens will kill more than 10 million people each year worldwide (Chastain et al., 2019). Thus, the discovery, development and implementation of novel antimicrobial agents targeting resistant bacterial strains is crucial to overcome this problem and successfully eradicate staphylococcal DFIs, with a consequent reduction in treatment costs, amputation rate and mortality (Richard et al., 2012). Regarding the administration route, the effectiveness of systemic antibiotics may be compromised by several aspects, including the impaired blood supply, observed in DM patients. Therefore, the local application of antimicrobials agents has been shown to have advantages in relation to the systemic administration (oral and parenteral), leading to higher concentrations in the affected area and limited systemic absorption, reducing side effects and increasing the effectiveness of the treatment (Hurlow, Humphreys, Bowling, & McBain, 2018; Markakis et al., 2018).

1.6.1. Antimicrobial peptides

Antimicrobial peptides (AMPs) are produced by a wide range of organisms, from bacteria to humans. Bacteria produce AMPs to kill other bacteria competing for the same ecological niche, whereas in higher organisms, AMPs protect host against infections, as part of their innate immune response, being considered “endogenous antibiotics”. AMPs spectrum of activity is broad, mainly comprising antibacterial, antiviral, antifungal and antitumor activity, and also exhibit immunomodulatory activity (Fjell, Hiss, Hancock, & Schneider, 2012; Ramirez-Acuña et al., 2019). AMPs are gene-encoded and derive from precursor peptides through proteolytic activation. Most of these endogenous peptides are rapidly mobilized after microbial infection, providing a fast and effective clearance of the pathogen, directly or indirectly by modulating the host defense system (Moual, Thomassin, & Brannon, 2013; Ramirez-Acuña et al., 2019). AMPs are short polypeptides, mostly less than 50 amino acids long, that share a cationic character and an amphipathic structure, being divided into subgroups according to their structure (β-sheet, α-helix, extended and loop peptides). These polypeptides have diverse mechanisms of action, either acting at the membrane level or internally, affecting pathogen’s DNA replication and/or protein synthesis (Ramirez-Acuña et al., 2019; Seo, Won, Kim, Mishig-Ochir, & Lee, 2012).

The AMPs cationic amphipathic structural arrangement allows interaction with the anionic phospholipids, resulting in the accumulation of AMPs on the membrane surface. After electrostatic attraction between AMPs and bacterial surfaces, AMPs are inserted into the phospholipidic membrane, leading to peptide translocation across the membrane and interaction with intracellular targets or leading to pore formation (membranolytic effect) (Kumar, Kizhakkedathu, & Straus, 2018; Seo et al., 2012).

Previous studies have shown that AMPs exhibit broad-spectrum antimicrobial action, with many AMPs being effective against multidrug resistant (MDR) bacteria. Moreover, AMPs possess low propensity for developing resistance, making them promising and strong candidates as alternatives to conventional antibiotics used in the treatment of several infections, including DFIs (Reddy, Yedery, & Aranha, 2004; Seo et al., 2012). However, despite the promising therapeutic properties of AMPs, including broad-spectrum activity, rapid onset of activity and relatively low possibility of resistance emerge, some obstacles to their clinical approval must be considered and evaluated, such as peptide toxicity, peptide/peptide-formulations stability (AMPs are labile, depending on the presence of protease or pH change) and cost of production on a large scale (Seo et al., 2012).

1.6.2. Bacteriocins and nisin

Bacteriocins are a subgroup of AMPs produced by bacteria, and may show bacteriostatic and/or bactericidal activity against other bacteria. Bacteriocins which are small (<5 kDa), heat-stable, enzymatically modified peptides with unusual amino acids, such as lanthionine, 3-methyllanthionine, dehydroalanine and dehydrobutyrine, are named lantibiotics, with nisin belonging to this bacteriocins' subgroup (Bali, Panesar, Bera, & Kennedy, 2016). Lantibiotics are antimicrobial compounds with high potency, low toxicity and capacity to be bioengineered, characteristics that make them strong candidates to overcome antibiotics growing resistance (Field et al., 2019). The introduction of unusual amino acids contributes to the high stability and protection against proteolytic degradation of the lantibiotics. Moreover, posttranslational processing is required for lantibiotics to acquire their active form (Jenssen, Hamill, & Hancock, 2006; Okuda et al., 2013).

Nisin is the most well-known and best-studied lantibiotic, produced by several *Lactococcus lactis* subsp. *lactis* strains, a microorganism that is frequently found in dairy products. Nisin was first commercialized as a food preservative, being recognized as safe by the Food and Agriculture Organization/World Health Organization (FAO/WHO) (Delves-Broughton, 1990; Punyauppa-path & Phumkhachorn, 2015). In recent years, nisin started to be investigated in veterinary and pharmaceutical fields, including in the management of bacterial infections (Shin et al., 2016). This polypeptide presents antimicrobial activity against a broad spectrum of gram-positive bacteria, including *S. aureus*, at minimum inhibitory concentrations (MICs) in the low nanomolar range, but not against gram-negative bacteria, due to their impermeable external membranes. However, when used in combination with outer membrane destabilizing agents, nisin may be also effective against gram-negative bacteria (Jenssen et al., 2006; Punyauppa-path & Phumkhachorn, 2015). Moreover, nisin has an acceptable daily intake (ADI) of 1 mg/kg body weight (bw) per day (Younes et al., 2017).

Nisin is firstly ribosomally synthesized as prenisin, an inactive form of nisin. Prenisin suffers several posttranslational modifications, such as dehydration, cyclization and leader

peptide digestion, resulting in the active nisin with a molecular mass of 3.5 kDa, comprising 34 amino acids and containing five lanthionine rings (Figure 1.2) (Delves-Broughton, 1990; Punyauppa-path & Phumkhachorn, 2015). Nisin A was the first described variant of nisin and in the last few years several naturally occurring and genetically modified variants with unique antimicrobial properties have been discovered and/or bioengineered. Higher efficacy and stability of nisin under different physiological conditions and improved pharmacokinetic properties are promising features of bioengineered nisin variants (Shin et al., 2016).

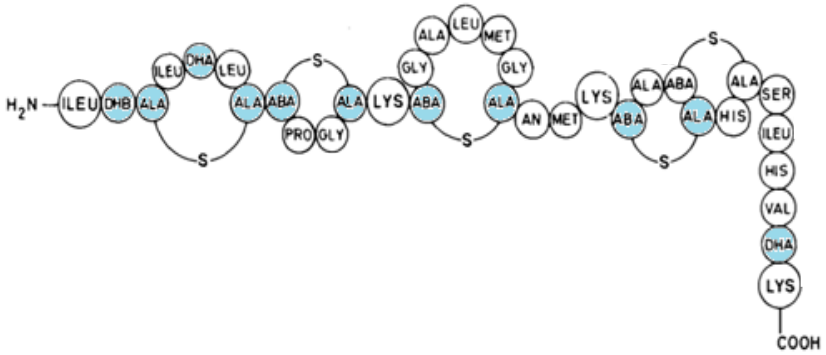


Figure 1.2 | Primary structure of nisin A. Representation of the unusual/modified amino acids: ALA-S-ALA, lanthionine; ABA-S-ALA, 3-methyllanthionine; DHA, dehydroalanine; DHB, dehydrobutyrine; ABA, amino butyric acid. Adapted from Delves-Broughton, 1990.

To kill or inhibit the target cell, nisin must pass through the cell wall by interacting with its anionic components (teichoic acids and phospholipids). Then, nisin interacts with high affinity with lipid II, an essential component for cell wall synthesis that transports the peptidoglycan monomer from bacteria cytoplasm to include it into the rising peptidoglycan in the bacterial cell wall. This interaction blocks the cell wall synthesis. Thus, nisin can kill bacterial cells by interfering with basic energy transduction at the cytoplasmic membrane. Furthermore, nisin can bind to the carbohydrate-pyrophosphate moiety of lipid II by N-terminal, leading to C-terminal insertion into the cell membrane, which culminates in a stable pore formation in the cell membrane, increasing bacterial membrane permeability and, consequently, allowing the diffusion of small compounds (Hsu et al., 2004; Wiedemann, Benz, & Sahl, 2004; Zhao et al., 2016). Briefly, nisin is known to have a dual-mode of action: inhibition of the cell wall synthesis by binding to lipid II (bacteriostatic activity), and pore formation causing the efflux of small molecules such as ATP and amino acids, which rapidly kills bacterial cells (bactericidal activity) (Figure 1.3) (Perez, Perez, & Elegado, 2015). The conventional antibiotic vancomycin also targets lipid II. However, it is believed that nisin interacts with a distinct molecular motif of lipid II, which explains why this peptide is still active against vancomycin-resistant bacteria (Jenssen et al., 2006).

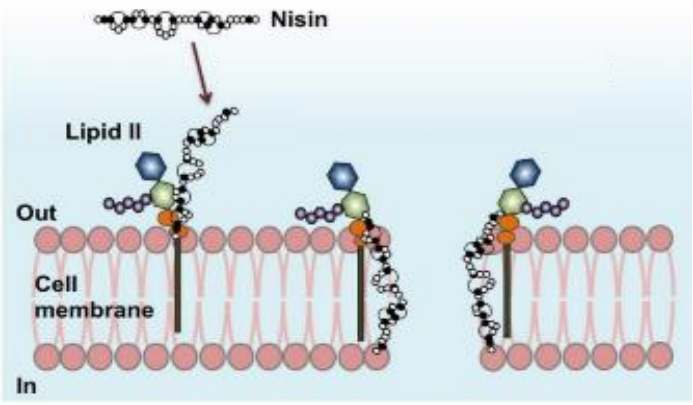


Figure 1.3 | Mechanism of nisin's action. Nisin has a dual mechanism of action on a target cell: (1) inhibition of the cell wall synthesis, and (2) permeabilization of the bacterial cell with consequently pore formation. Adapted from Perez et al., 2015.

Nisin has been proposed as an alternative or complementary method to treat drug-resistant infections, in order to substitute or decrease the use of conventional antibiotics. Previous studies have shown that bacteriocins that form stable pores on the membrane of target cells, such as nisin, are especially effective against MRSA both in planktonic and biofilm state, proving nisin's high potential for treating biofilm-associated infections, such as DFIs. In addition, some studies suggest that the combination of nisin with conventional antibiotics may promote synergic activity, reducing the amount of antibiotics used (Godoy-Santos, Pitts, Stewart, & Mantovani, 2018; Santos et al., 2016; Santos et al., 2019).

Despite its widespread use in food industry, only a few examples of minimal nisin resistance under laboratory conditions were reported (Shin et al., 2016). Accordingly, reported resistance *in vitro* against nisin is mainly associated with mutations that alter membrane and cell wall composition, preventing nisin from binding to lipid II, but it can also be associated with other mechanisms, such as extrusion of nisin through the membrane and inhibition of nisin´s insertion into the bacterial membrane (Kramer, Van Hijum, Knol, Kok, & Kuipers, 2006; Shin et al., 2016). However, a broader use of nisin in clinical and/or veterinary practice could select for resistant strains. Some *S. aureus* strains have been reported to be innately resistant to nisin through diverse mechanisms, such as cell wall modification, biofilm formation, expression of resistant proteins, including nisin resistant protein (NRP) that provides resistance through the proteolytic cleavage of nisin, or through the BraRS-VraDE system that provides resistance against low concentrations of nisin (Field et al., 2019; Kawada-Matsuo et al., 2020). Thus, to prevent potential resistance issues that may impair nisin´s biomedical applications, it is important to have a continuous characterization of specific genetic or protein components and environmental stresses that may contribute to nisin resistance development (Shin et al., 2016).

1.6.3. Guar gum

Different methods for antimicrobials delivery have been widely investigated aiming at increasing antimicrobials clinically efficacy, as they can be inactivated or degraded before achieving their target. Controlled release, triggerability, increased stability, increased effect and reduced toxicity are some of the advantages of combining an antimicrobial with a delivery system. Therefore, the bioavailabity of antimicrobials largely depends on the performance of the delivery system.

In recent years, new topical delivery systems for wound treatment prepared from natural polymers have been investigated. Direct and sustained release of an incorporate antibacterial agent is the most promising feature of these systems, since they guarantee a stationary-state concentration of the antimicrobial agent in the wound environment (Nordström & Malmsten, 2017; O'Driscoll et al., 2013). Moreover, there are several benefits of using natural polysaccharides instead of the synthetic ones, such as their non-toxicity, sustainability, biodegradability, biocompatibility, abundantly availability and cost-effectiveness (Mirhosseini & Amid, 2012).

Guar gum is a natural, uncharged and water-soluble polysaccharide obtained by grinding the endosperm (gum) of the leguminous *Cyamopsis tetragonolobus* (Figure 1.4). The endosperm contains a polymer of D-galactose and D-mannose, called galactomannan. Guar gum is composed by protein (3.5-4.0%), phosphorus (0.06%), ash (1.07%), water soluble polysaccharide (86.50%), water insoluble fraction (7.75%) and alcohol soluble fraction (1.50%) (Thombare, Jha, Mishra, & Siddiqui, 2016). Guar gum is rich in hydroxyl groups and, when added to water, it forms hydrogen bounds, leading to increased viscosity, even at low concentrations. When the concentration of guar gum in water increases, thickening or gelling are induced. Maximum guar gum viscosity can be obtained at pH values of 6 to 9, even though it is stable at pH values from 4 to 10.5. Accordingly, its ability to form a gel or render the solution viscous makes guar gum a strong potential delivery system for AMPs (Mudgil, Barak, & Khatkar, 2014; Prabaharan, 2011; Thombare et al., 2016). This polysaccharide has been widely used in diverse areas, such as food industry, agriculture, textile, paper industry and cosmetic industry (Mudgil et al., 2014). Moreover, guar gum has been largely used for targeted drug delivery, promoting a controlled drug release and availability (Narsaiah, Jha, Wilson, Mandge, & Manikantan, 2014; Prabaharan, 2011).

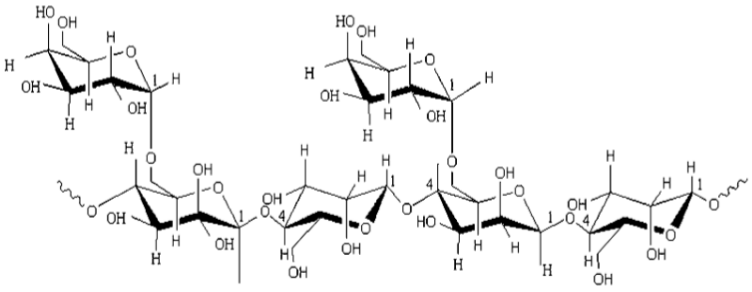


Figure 1.4 | Structure of a guar gum molecule. Guar gum is composed of a linear chain of galactose and mannose residues. Adapted from Mudgil et al., 2014.

1.6.4. Nisin-biogel

Considering all the promising features of nisin and guar gum, a guar gum gel-based delivery system for nisin, nisin-biogel, has been developed by our research group as a substitute for or a complementary strategy to conventional antibiotics used for DFIs treatment (Santos et al., 2016).

According to previous results, *S. aureus* DFIs isolates are susceptible to nisin, including MRSA isolates and MDR isolates resistant to three or more classes of antibiotics (Mottola et al., 2016a; Santos et al., 2016). Nisin has shown high antimicrobial activity against planktonic bacteria, with MIC ≤12.5 μg/mL and minimum bactericidal concentration (MBC) 5.5 times higher, which allowed to conclude that nisin is a bacteriostatic agent against *S. aureus* clinical isolates. Moreover, nisin has the ability to inhibit established biofilms, with minimum biofilm inhibitory concentration (MBIC) ≤ 25 μg/mL. However, established biofilms are more difficult to eradicate, with only 35% of the clinical isolates previously tested having presented minimum biofilm eradication concentration (MBEC) ≤ 125 μg/mL (Santos et al., 2016).

Regarding to nisin-biogel, the inhibitory activity of nisin incorporated in guar gum revealed by its MIC and MBIC values is only two-fold higher than the one from nisin alone, and according to MBC values the eliminatory activity of nisin incorporated in guar gum is less than two-fold higher than the one from nisin alone. As observed for nisin alone, according to MBEC values, established biofilms are more difficult to eliminate, with the nisin-biogel being able to eradicate only 13% of pre-formed biofilms (Santos et al., 2016).

1.7. Subinhibitory concentrations of antimicrobials

One of the fundamental concepts on the antimicrobial dosing and bacterial susceptibility is the determination of antimicrobials MIC value. MIC is defined as the lowest concentration of antimicrobial that inhibits the growth of the majority of the target bacterial population, under controlled *in vitro* conditions, such as growth medium, incubation temperature, duration and inoculum size. However, MIC values may not translate well into an effective concentration *in vivo* (Andersson & Hughes, 2014). In *in vivo* infections, bacteria may be exposed to a decreased effective concentration of antimicrobials, referred to as subinhibitory concentrations (sub-MICs), and recent studies have shown that this may lead to a wide variety of physiological and morphological effects on bacteria and, consequently, can affect infection pathogenesis. These effects include generation of phenotypic variability, selection of resistance and influence of different physiological activities, namely biofilm formation and virulence-related genes expression (Andersson & Hughes, 2014; Otto et al., 2013).

Sub-MICs at the sites of infection may be associated with suboptimal dosing therapy, poor pharmacokinetics, treatment with low-quality drugs and bad patient compliance (Li et al., 2017). In patients with DFIs, poor diffusion of the antimicrobials may result from DM patient's compromised angiopathy and low perfusion, leading to sub-MICs at the site of infection, such that

target bacteria may be only weakly inhibited and not killed (Andersson & Hughes, 2014; Li et al., 2017). Besides patients' and treatment associated factors, the slow transport of antimicrobials within bacteria biofilms may be also associated with the presence of sub-MICs of antimicrobials in *in vivo* infections (Schilcher et al., 2016).

As mentioned before, bacteria that are exposed to sub-MICs of antimicrobials are frequently not killed, being only inhibited in terms of growth. Therefore, the majority of the population survives, resulting in a non-lethal selection that promotes the emergence of a larger range of mutant variants, promoting the spread of resistance (Andersson & Hughes, 2014). Besides promoting bacterial resistance, previous studies have demonstrated that antimicrobials sub-MICs can modulate the virulence of *S. aureus*, by influencing gene expression, biofilm production and QS system, which therefore may impact the outcome of staphylococcal infections, including DFIs (Andersson & Hughes, 2014; Otto et al., 2013).

It is known that many antimicrobials at sub-MICs alter the expression pattern of virulence genes. Depending on the antibiotic class and on the virulence gene, these modifications result in either increased or decreased virulence gene expression, and it is often difficult to identify the underlying physiological mechanism responsible for these changes (Andersson & Hughes, 2014). For example, clindamycin, which is an antibiotic that inhibits bacterial protein synthesis, is known to modulate the production of several exotoxins and adhesins in *S. aureus*, including Coa, Hla, SpA and PVL (Herbert, Barry, & Novick, 2001; Otto et al., 2013; Schilcher et al., 2016). Thus, the efficacy of antibiotic treatment for *S. aureus* infections might depend not only on the bacteriostatic or bactericidal effects of the antibiotic, but also on its influence on virulence factors expression (Otto et al., 2013).

Accordingly, the effect of antimicrobials sub-MICs on virulence genes expression by *S. aureus* is an important consideration in the development of antimicrobial therapy protocols to treat staphylococcal infections, since the modulation of bacterial virulence is considered critical to reach clinical efficacy and control infectious diseases (Davies, Spiegelman, & Yim, 2006; Otto et al., 2013). Therefore, besides allowing the study of their effects on virulence factors, testing antimicrobials at sub-MICs also provides an *in vitro* basis for understanding what may happen during the treatment of a staphylococcal infection *in vivo*, by clarifying the interactions between bacteria and host and helping to define optimal dosages to control the infection pathogenesis (Otto et al., 2013).

1.8. Aim of the study

As the suppression of pathogenic factors has been considered critical for controlling infectious diseases, such as DFIs (Davies et al., 2006; Otto et al., 2013), it was essential to investigate if the presence of sub-MICs of nisin-biogel influences virulence gene expression by *S. aureus,* providing an *in vitro* basis for understanding what may happen during the treatment of a DFI with nisin-biogel, which is essential before proceeding to clinical trials.

With that propose, the first task of this work consisted in determining the growth rate of *S. aureus* clinical isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 under different growth conditions, namely the presence of nisin-biogel sub-MICs, by measuring the absorbance at 600 nm during 24 hours. This task aimed to evaluate how the sub-MICs of nisin-biogel influence bacterial isolates growth kinetics.

Next, it was necessary to determine the expression kinetics of the adhesion-related genes *spA*, *coa* and *clfA*, the major autolysin gene *atl,* the biofilm-related genes *icaA* and *icaD*, and the regulatory gene *agrI* by Quantitative Reverse Transcription Polimerase Chain Reaction (RT-qPCR) assays, using gene encoding gyrase B (*gyrB)* as an internal control and the relative standard curve method to quantify gene transcription fold change. As *S. aureus* pathogenicity could be associated with a wide range of regulatory and virulence genes, that are not all expressed at the same period of *S. aureus* growth (Ruffing et al., 2016), this task was essential to determine the more adequate *S. aureus* clinical isolates growth time for further investigation related with virulence genes expression.

The third task of this study was to evaluate and characterize the effects of nisin-biogel sub-MICs on the expression of *S. aureus* clinical isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 adhesion-related genes *spA*, *coa* and *clfA*, major autolysin gene *atl,* biofilm-related genes *icaA* and *icaD*¸ and regulatory gene *agrI* by RT-qPCR assays, *gyrB* as an internal control and the relative standard curve method to quantify gene transcription fold change. Clindamycin was used as a reference antibiotic to compare the effects of nisin-biogel sub-MICs with those of clindamycin, as it is a conventional antibiotic used in the treatment of DFIs, and clindamycin at sub-MICs influence the expression of virulence factors, including an inhibitory effect on *spA* expression and increasing the levels of *coa* mRNA (Hodille et al., 2017).

Afterwards, the effects of nisin-biogel sub-MICs on the ability of *S. aureus* clinical isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 to form biofilm was evaluated by a microtiter technique, as the inhibition of biofilm formation is known to be an important step on the treatment of bacterial infections, including DFIs (Paharik & Horswill, 2016).

Alterations in virulence determinants mRNA levels in the presence of subinhibitory levels of antimicrobials do not always result in changes in protein synthesis or functional activity, making important to determine whether virulence factors expression levels are associated with mRNA quantification (Hodille et al., 2017). Thus, the fifth task of this study was the phenotypic evaluation of coagulase functional activity and protein A production in the presence of nisin-biogel at sub-MICs by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, using rabbit plasma and a specific ELISA, respectively.

Chapter 2 | Materials and Methods

2.1. Bacterial isolates

S. aureus isolates used in this study were previously obtained from patients with clinically infected DFUs, from samples taken by biopsies (B), swabs (Z) or aspirates (A), according to the current clinical guidelines, and stored in Buffered Peptone Water (BPW) plus 20% (v/v) of glycerol at -80ºC (Mendes et al., 2012). *S. aureus* clinical isolates were previously characterized by Polymerase Chain Reaction (PCR), Pulse Field Gel Electrophoresis (PFGE) and Multilocus Sequence Type (MLST), in order to characterize their virulence profile, antimicrobial resistance traits and capacity to produce biofilms (Mottola et al., 2016a; Mottola et al., 2016b). Therefore, *S. aureus* isolates included in the present work were selected based on their virulence profile, as shown in Table 2.1.

All *S. aureus* DFI isolates shown in Table 2.1 were used in the RT-qPCR optimization assays of this study. To determine the most adequate bacterial growth period for the expression of the different virulence-related genes under study, only isolates A 5.2 and Z 5.2 were used. Finally, isolates underlined in light blue were used to determine the bacterial growth rate under the presence of different nisin-biogel sub-MICs, to determine the effects of nisin-biogel at sub-MICs on virulence genes expression by RT-qPCR, to access the effect of nisin-biogel at sub-MICs on isolate's ability to form biofilm and to quantify the effects of nisin-biogel at sub-MICs on SpA release and Coa production.

The MIC values of nisin-biogel and clindamycin for the *S. aureus* DFI isolates were also previously determined by our group, being of 22.5 µg/mL and 0.033 µg/mL, respectively (Mottola et al., 2016c; Santos et al., 2016).

Table 2.1 | Characterization of *S. aureus* DFI isolates used in the present study regarding methicillin resistance, biofilm production, regulatory genes expression, virulence genes expression and antimicrobial resistance profile (Mottola et al., 2016a; Mottola et al., 2016b).

Isolate	SCC*mec*	*icaA*	*icaD*	*atl*	*agrI*	*agrII*	*clfA*	*coa*	*spA*	Biofilm	ST	CC	AR
A 1.1	+	+	+	+	-	+	+	+	+	+	5	5	Fox, Cip
A 5.2	-	+	+	+	+	-	+	+	+	+	8	5	Cip, Cpt
A 6.3	-	+	+	+	+	-	+	+	+	+	7	7	-
B 1.1	+	+	+	+	+	-	+	+	+	+	22	22	Fox, Cip, Ery
B 3.2	-	+	+	+	-	+	+	+	+	+	582	5	-
B 7.3	+	+	+	+	-	+	+	+	+	+	105	5	-

B 14.2	+	+	+	+	+	-	+	+	+	+	22	22	Fox, Cip, Cpt, Mem
Z 1.1	+	+	+	+	+	-	+	+	+	+	22	22	Fox, Cip, Mem
Z 2.2	-	+	+	+	-	+	+	+	+	+	105	5	Cip, Ery
Z 3.1	-	+	+	+	-	+	-	+	+	+	6	5	-
Z 5.2	-	+	+	+	+	-	+	+	+	+	72	5	-
Z 16.1	+	+	+	+	-	+	+	+	+	+	105	5	Fox, Cip, Ery

A: aspirate; B: biopsy; Z: swab; +: positive; -: negative; MRSA: Methicillin-resistant *Staphylococcus aureus*; MSSA: Methicillin-sensitive *Staphylococcus aureus*; SCC*mec:* staphylococcal chromosome cassette *mec*; *icaA:* gene encoding intracelular adhesin A; *icaD:* gene encoding intracelular adhesin D; *atl:* gene encoding autolysin; *agrI:* accessory gene regulator I; *agrII:* accessory gene regulator II; *clfA:* gene encoding clumping factor A; *coa:* gene encoding coagulase; *spA:* gene encoding staphylococcal protein A; ST: sequence type; CC: clonal complex; AR: antibiotic resistance; Fox: cefoxitin; Cip: ciprofloxacin; Mem: meropenem; Ery: erythromycin; Cpt: ceftaroline.

2.2. Antimicrobial solutions

Nisin solution

To obtain a stock solution of nisin at a concentration of 1000 µg/mL, 1 g of nisin powder (2.5% purity, 1000 IU/mg, Sigma-Aldrich, USA) was dissolved in 25 mL of HCl (0.02M) (Merck, Germany). This solution was sterilized by filtration through a 0.22 µm cellulose acetate membrane filter (Frilabo, USA) and stored at 4ºC during the assays.

Clindamycin solution

To prepare the clindamycin solution, 6.6 mg of clindamycin powder (Sigma-Aldrich, USA) were dissolved in 10 mL of sterile water and filtered using a 0.22 µm cellulose acetate membrane filter. This stock solution was aliquoted and the different aliquots were kept frozen at -80ºC during the study and diluted with sterile water to the final concentration of 0.0165 µg/mL when required.

2.3. Culture media

BHI broth + Guar gum

Brain Heart Infusion (BHI) supplemented with guar gum gel at 0.75% (w/v) was prepared by dissolving 18.5 g of BHI powder (VWR Chemicals, Belgium) and 3.75 g of guar gum (Sigma-Aldrich, USA) in 500 mL of sterile distilled water, and heat sterilized by autoclave. This solution was used in all the assays aiming to access the effect of nisin-biogel at sub-MICs on bacterial growth rate, on virulence-related genes expression by *S. aureus* DFI isolates, on SpA release by

S. aureus DFI isolates and on Coa production by *S. aureus* DFIs isolates, as it already presented the final guar gum concentration used in the nisin-biogel (7.5 mg/mL).

TSB broth + Guar gum

Trypticase Soy Broth (TSB) supplemented with guar gum gel at 0.75% (w/v) was prepared by dissolving 15 g of TSB powder (VWR Chemicals, Belgium) and 3.75 g of guar gum in 500 mL of sterile distilled water, and heat sterilized by autoclave. This solution was used in all the assays aiming to access the effect of nisin-biogel at sub-MICs on the ability of *S. aureus* DFI isolates to form biofilm, as it already presented the final guar gum concentration used in the nisin-biogel (7.5 mg/mL).

2.4. Effects of nisin-biogel at sub-MICs on *S. aureus* DFI isolates growth rate

Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were inoculated in a non-selective BHI agar medium (VWR Chemicals, Belgium) and incubated at 37˚C for 24h. After incubation, bacterial suspensions of 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard in NaCl (Merck, Belgium), and different bacterial suspensions were prepared as follows:

- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth;
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/2 MIC of the nisin-biogel ([nisin] = 11.25 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/4 MIC of the nisin-biogel ([nisin] = 5.625 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/8 MIC of the nisin-biogel ([nisin] = 2.8175 µg/mL).

Then, the wells of a 96-well flat-bottomed polystyrene microtiter plate (Thermo Scientific, US) were inoculated with 200 µL of the negative controls (BHI broth and BHI broth with guar gum at 7.5 mg/mL) or with 200 µL of the different bacterial suspensions previously prepared, and incubated for 24 hours at 37ºC with shaking (150 rpm).

Each different growth condition was evaluated in triplicated wells on three independent assays. During incubation, optical density at 600 nm (OD_{600}) for each well was obtained automatically every hour using the FLUOstar OPTIMA (BMG LABTECH, US) microplate reader. For each *S. aureus* DFI isolate, OD_{600} was calculated by subtracting the average OD_{600} of the three control wells (BHI broth or BHI broth with guar gum at 7.5 mg/mL) from the average of OD_{600} of the three replicates of the sample under evaluation. Final result corresponds to the average of the three independent assays.

2.5. RT-qPCR optimization

Bacterial growth

The 12 selected isolates shown in Table 2.1 were inoculated in a non-selective BHI agar medium and incubated at 37˚C for 24 h. After incubation, bacterial suspensions of 10^8 CFU/mL in NaCl were prepared directly from plate cultures using a 0.5 McFarland standard. Subsequently, bacterial suspensions were diluted in 4.5 mL of fresh BHI broth to achieve a final bacterial concentration of 10^7 CFU/mL, followed by incubation at 37ºC for 4h or at 37ºC for 24h with orbital shaking (180 rpm). After incubation, OD_{600} of each bacterial suspension was measured using a NanoDropTM 2000c spectrophotometer (Thermo Scientific, US) to monitor the bacterial growth rate and to guarantee that the same amount of CFU was used in the subsequent steps. Then, 4h culture suspensions were used for RT-qPCR optimization protocols aiming at evaluating the expression of *gyrB, agrI, spA, coa, clfA* and *atl,* while 24h culture suspensions were used for RT-qPCR optimization protocols aiming at evaluating the expression of *icaA* and *icaD*.

Enzymatic lysis of bacteria

For *in vivo* stabilization of total RNA in bacterial suspension, 1 volume of each bacterial culture, corresponding to 10^8 CFU/mL, was added to 2 volumes of RNAprotect® Bacteria Reagent (Qiagen, Netherlands). After centrifugation at 5000 g for 10 minutes, supernatants were decanted and pellets treated during 30 minutes with 85 µL of Buffer TE containing lysostaphin (Sigma-Aldrich, German), an enzyme that cleaves polyglycine crosslinks in the cellular wall of *Staphylococcus* species, leading to cell lysis. Then, 700 µL of Buffer RLT were added to each tube, followed by the addition of 500 µL of absolute ethanol, as suggested by the manufacturer.

RNA extraction and cDNA synthesis

The total RNA was purified using the Qiagen RNeasy Mini Kit (Qiagen, Netherlands) according to the manufacturer's recommendations, and the optional on-column DNase digestion was performed. RNA was eluted in 50 µL of nuclease-free water, and RNA yield and A260/A280 ratio were assessed using a NanoDrop™ 2000c spectrophotometer. Then, total RNA of each isolate was reverse transcribed with random primers using a Promega Go Script™ Reverse Transcription System (Promega, US), according to the protocol recommended by the manufacturer, optimized for rendering a final volume yield of 40 µL instead of 20 µL, where the total RNA corresponds to 1/5 of the final volume of each reaction. The different heating steps were carried out using a Thermal cycle Mycycler™ (Bio-Rad, USA). To ensure the specificity of cDNA synthesis, a No Template Control (NTC) and one Minus Reverse Transcriptase Control (–RTC) for each isolate's RNA were carried used. The first control aims to guarantee the viability of the Promega Go Script™ Reverse Transcription System, while the second one serves to evaluate for contamination by genomic DNA.

RT-qPCR – Optimization of primers concentration to be used in the amplification of each gene

The resulting cDNA from two different isolates, one with high RNA concentration and one with low RNA concentration, was used as a template for establishing real-time amplification protocols using specific primers synthesized by STABVIDA® for the different genes under study (Table 2.2), in order to determine the optimum primer (forward and reverse) concentration for subsequent RT-qPCR assays.

For the amplification of *gyrB*, a total of 9 different combinations of final primers concentrations (50 nM/50 nM, 50 nM/300 nM, 50 nM/900 nM, 300 nM/50 nM, 300 nM/300nM, 300 nM/900 nM, 900 nM/ 50 nM, 900 nM/300 nM and 900 nM/ 900 nM) were tested, whereas for the other genes under study, a total of 3 different combinations of final primers concentrations (50 nM/50 nM, 300 nM/300 nM and 900 nM/900 nM) were tested.

Table 2.2 | Nucleotide sequence of the primers used in RT-qPCR protocols using 7300 Real Time PCR System.

Gene	Isolates	Sequence (5' → 3')	Product size (bp)	Reference
gyrB	A 6.3 B 14.2	F: GGTGGCGACTTTGATCTAGC R: TTATACAACGGTGGCTGTGC	170	Otto et al., 2013
agrI	Z 5.2 A 5.2	F: CCAGCTATAATTAGTGGTATTAAGTACAGTAAACT R: AGGACGCGCTATCAAACATTTT	106	Francois et al., 2006
spA	Z 5.2 A 5.2	F: TATGCCTAACTTAAATGCTG R: TTGGAGCTTGAGAGTCATTA	119	Otto et al., 2013
atl	A 6.3 B 3.2	F: ACCAAGATCAGTTGCTGCAA R: CGGTATGCGTATTTAGGGAAGT	153	This study
clfA	A 6.3 B 3.2	F: ACCCAGGTTCAGATTCTGGCAGCG R: TCGCTGAGTCGGAATCGCTTGCT	165	Atshan et al., 2013
coa	A 6.3 B 3.2	F: GTAGATTGGGCAATTACATTTTGGAGG R: CGCATCAGCTTTGTTATCCCATGT	117	Kearns et al., 1999
icaA	Z 2.2 B 14.2	F: GAGGTAAAGCCAACGCACTC R: CCTGTTAACCGCACCAAGTTT	151	Atshan et al., 2013
icaD	Z 2.2 B 14.2	F: ACCCAACGCTAAAATCATCG R: GCGAAAATGCCCATAGTTTC	211	Atshan et al., 2013

gyrB: gene encoding gyrase B; *spA:* gene encoding staphylococcal protein A; icaA*:* gene encoding intracelular adhesin A; *icaD:* gene encoding intracelular adhesin D; *coa:* gene encoding coagulase; *clfA:* gene encoding clumping factor A; *atl:* gene encoding autolysin; *agrI:* accessory gene regulator I; F: forward; R: reverse; bp: base pair.

Each RT-qPCR mixture had a final volume of 25 µL, containing 12.5 µL of Power SYBR™ Green PCR Master Mix (Applied Biosystems, USA), 1 µL of cDNA, and a variable volume of each primer (working solution at 10 µM) and of nuclease-free water. A PCR mixture for the controls – RTC for each isolate and NTC was also prepared for each final primer concentration combination.

Samples were tested in duplicate using 96-well plates (Thermo Scientific, US) covered with optical adhesive (Thermo Scientific, US). RT-qPCR amplification was performed in a 7300 Real Time PCR System (Applied Biosystems, USA) using the conditions described on Table 2.3.

Table 2.3 | Steps of the protocol used for RT-qPCR amplification of the genes under study.

Step	Description	Temperature (ºC)	Time
1	Initial uracil-N-glycosylase gene (UNG) activation	50	2 min
2	Initial DNA polymerase activation	95	10 min
3	Melting	95	15 sec
4	Annealing/extending	60	1 min
5	Repeat steps 3 and 4 for 40 cycles		
6	Dissociation curve	95	15 sec
7	Dissociation curve	60	1 min
8	Dissociation curve	95	15 sec
9	Dissociation curve	60	15 sec

Min: minutes; sec: seconds.

RT-qPCR – Calibration curves

To perform *gyrB, coa, clfA, atl, icaA* and *icaD* expression calibration curves, 2 µL of RNA from isolates with higher RNA yield and 260/280 ratio (total of 20 µL) were mixed and reverse transcribed primed with random primers using a Promega Go Script™ Reverse Transcription System, as recommended by the manufacturer. To perform *agrI* and *spA* calibration curves, 4 µL of RNA from isolates that express *agrI* gene (except for isolate B 1.1) were mixed and reverse transcribed. The final volume of the mixture was of 100 µL, including 75 µL of reverse transcriptase mix and 25 µL of RNA and primer mix, where the total RNA corresponds to 1/5 of the final volume of each reaction. cDNA synthesis was performed twice to ensure a final cDNA volume of 200 µL. To guarantee the specificity of cDNA synthesis, a NTC was also carried out.

cDNA was produced using a Thermal cycle Mycycler™, and the synthesized cDNA was used as the standard cDNA and served as the starting point for the 10-fold serial dilutions required to establish calibration curves of the different genes under study. Accordingly, serial dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were made and RT-qPCR amplification of the different genes was performed on a 96-well plate in duplicate, using the non-diluted cDNA and the different diluted cDNA. Each RT-PCR mixture was prepared the same way as mentioned before, and the RT-qPCR program applied was the one previously described in Table 2.3.

2.6. *S. aureus* DFI isolates genes expression kinetics

To further determine the effects of nisin-biogel and clindamycin at sub-MICs on the transcription levels of the different genes under study, it was necessary to first determine the more

adequate bacterial growth period for the expression of the genes. Two different *S. aureus* DFI isolates were used, A 5.2 and Z 5.2, since they express all the genes to be quantified in this study.

Isolates A 5.2 and Z 5.2 were inoculated in a non-selective BHI agar medium at 37˚C for 24h. After incubation, bacterial suspensions of 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard in sterile NaCl. Bacterial suspensions were diluted in 4.5 mL of fresh BHI broth to a final concentration of 10^7 CFU/mL and incubated at 37ºC with orbital shaking (180 rpm). For amplification of *agrI, spA, coa, clfA* and *atl,* aliquots were collected after 2, 3, 4, 5 and 6 hours of incubation for subsequent enzymatic lysis of bacteria, RNA extraction, cDNA synthesis and analysis by RT-qPCR. For *icaA* and *icaD,* aliquots were collect after 8, 24, 32, 48 and 56 hours of incubation for subsequent RNA extraction, cDNA synthesis and analysis by RT-qPCR. RNA extraction, cDNA synthesis and RT-qPCR assays were performed as previously described, and a NTC was also carried out. The relative standard curve method was used to quantify gene transcription, using *gyrB* for normalization. An average ± standard deviation of the fold change obtained for isolates A 5.2 and Z 5.2 was considered for the determination of the expression kinetics of the different genes under study.

2.7. Effects of nisin-biogel at sub-MICs on virulence genes expression by *S. aureus* DFI isolates

S. aureus DFI isolates underlined in light blue mentioned in Table 2.1 were inoculated in a non-selective BHI agar medium and incubated at 37˚C for 24h. After incubation, bacterial suspensions of 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard in NaCl. For each isolate, 5 different growth conditions were considered, and the following bacterial suspensions were prepared:

- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth;
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth plus clindamycin at 1/2 MIC ([clindamycin] = 0.0165 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/2 MIC of the nisin-biogel ([nisin] = 11.25 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/4 MIC of the nisin-biogel ([nisin] = 5.625 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/8 MIC of the nisin-biogel ([nisin] = 2.8175 µg/mL).

All these suspensions were incubated at 37ºC for 4h to *agrI, spA, coa, clfa* and *atl* expression studies, and for 48h to *icaA* and *icaD* expression studies, with orbital shaking (180 rpm).

After incubation, enzymatic lysis of bacteria, RNA extraction and cDNA synthesis were performed, following the protocols mentioned before. To ensure the specificity of cDNA synthesis, a NTC was carried out. The resulting cDNA was used as template for RT-qPCR. RT-qPCR

amplification focused on both the genes of interest (*agrI, spA, coa, clfA, atl, icaA* and *icaD*) and the internal control (*gyrB*), being the expression of the different virulence-related genes quantified relatively to the internal control. The relative standard curve method was used to quantify transcription. More specifically, to determine the effects of nisin-biogel and clindamycin sub-MICs on virulence-related genes expression, the expression levels of the genes under investigation were expressed as fold change of *agrI/gyrB, spA/gyrB, coa/gyrB, clfA/gyrB, atl/gyrB, icaA/gyrB* and *icaD/gyrB* ratios in the presence of antimicrobials (nisin-biogel or clindamycin) relative to *agrI/gyrB, spA/gyrB, coa/gyrB, clfA/gyrB, atl/gyrB, icaA/gyrB* and *icaD/gyrB* ratios, respectively, of the control (no antimicrobial). For each different *S. aureus* DFI isolate and each different incubation condition, two different and independent assays were performed. Final result corresponds to the average of the two independent assays.

2.8. Effect of nisin-biogel at sub-MICs on the biofilm-forming ability of *S. aureus* DFI isolates

To test the influence of nisin-biogel and clindamycin sub-MICs on biofilm formation, a modified version of the protocol described by Santos et al., 2016 was performed. Accordingly, *S. aureus* clinical isolates underlined in light blue mentioned in Table 2.1 were incubated in a non-selective BHI agar medium and incubated at 37˚C for 24h. Then, three to five colonies were collected using a sterile loop, ressuspended in 5 mL of TSB and incubated for 18h at 37ºC. After incubation, the turbidity of bacterial suspension was adjusted to match turbidity comparable to that of 0.5 McFarland standard (10^8 CFU/mL), and a 1:100 dilutions were made in different broth media:

- Fresh TSB supplemented with 0.25% (w/v) glucose (Merck, USA);
- Fresh TSB with guar gum at 7.5 mg/mL supplemented with 0.25% (w/v) glucose plus 1/2 MIC of nisin-biogel ([nisin] = 11.25 µg/mL);
- Fresh TSB with guar gum at 7.5 mg/mL supplemented with 0.25% (w/v) glucose plus 1/4 MIC of nisin-biogel ([nisin] = 5.625 µg/mL);
- Fresh TSB with guar gum at 7.5 mg/mL supplemented with 0.25% (w/v) glucose plus 1/8 MIC of nisin-biogel ([nisin] = 2.8175 µg/mL).

Bacterial suspensions were transferred to a sterile 96-well polystyrene plate (200 µL/well), and plate was incubated at 37ºC for 48h. After incubation, the content of each well was removed, and the wells were carefully washed three times with 180 µL of PBS, pH 7.0. Then, wells were filled with 200 µL of PBS, pH 7.0, and the microtiter plate was covered with the lid and incubated in an ultrasound bath (Grant MXB14) at 50 Hz for 15 min, in order to disperse the biofilm-based bacteria from the microtiter plate. Finally, the optical density of each well was measured at 570 nm (OD_{570}) using the FLUOstar OPTIMA microplate reader.

Positive (bacterial suspensions in broth medium with no antimicrobials) and negative (broth medium only) controls were also included in the assays. For each different *S. aureus* DFI

isolate, each different condition was tested in triplicated wells, and three independent assays were performed.

Results were calculated by subtracting the average OD_{570} of the six blank wells (fresh TSB broth or fresh TSB broth with guar gum at 7.5 mg/mL) from the average of OD_{570} of the three replicates of the sample under evaluation. Final result corresponds to the average of the three independent assays.

2.9. Effect of nisin-biogel at sub-MICs on Coa production by *S. aureus* DFI isolates

S. aureus clinical isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were inoculated in a non-selective BHI agar medium and incubated at 37˚C for 24h. After incubation, bacterial suspensions of 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard in NaCl. For each isolate, 5 different growth conditions were considered, and the following suspensions were prepared:

- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth;
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth plus clindamycin at 1/2 MIC ([clindamycin] = 0.0165 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/2 MIC of the nisin-biogel ([nisin] = 11.25 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/4 MIC of the nisin-biogel ([nisin] = 5.625 µg/mL);
- 500 µL of bacterial suspensions diluted in 4.5 mL of fresh BHI broth with guar gum at 7.5 mg/mL plus 1/8 MIC of the nisin-biogel ([nisin] = 2.8175 µg/mL).

The different bacterial suspensions were incubated for 24h or for 4h at 37ºC. The coagulase test was performed by adding 0.1 mL of each culture to 0.3 mL of rabbit plasma previously rehydrated with sterile water. After gentle mixing, suspensions were incubated at 37ºC and examined every hour during 4h, and after 24h. Results were interpreted qualitatively according to the scale proposed by Sperber & Tatini, 1975, where negative results correspond to no evidence of fibrin formation, positive 1+ to small unorganized clots, positive 2+ to small organized clot, positive 3+ to large organized clot and positive 4+ to the coagulation of the entire content of tube, which is not displaced when tube is inverted. As negative controls, 0.1 mL of BHI broth or 0.1 mL of BHI broth with guar gum at 7.5 mg/mL were added to 0.3 mL of rabbit plasma and incubated without bacteria in the same conditions. For the tests to be valid, control plasma should show no signs of clotting.

2.10. Effect of nisin-biogel at sub-MICs on SpA release by *S. aureus* DFI isolates

Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were inoculated in a non-selective BHI agar medium and incubated at 37˚C for 24 h. After, bacterial suspensions of 10^8 CFU/mL were prepared directly from plate cultures using a 0.5 McFarland standard in NaCl and diluted in 4.5 mL of fresh BHI broth to achieve a final bacterial concentration of 10^7 CFU/mL, followed by incubation at 37ºC for 4h. Then, the following concentrations of clindamycin and nisin-biogel were added to bacterial cultures:

- Clindamycin at 1/2 MIC ([clindamycin] = 0.0165 µg/mL);
- Nisin-biogel at 1/2 MIC ([nisin] = 11.25 µg/mL; [guar gum] = 7.5 mg/mL);
- Nisin-biogel at 1/4 MIC ([nisin] = 5.625 µg/mL; [guar gum] = 7.5 mg/mL);
- Nisin-biogel at 1/8 MIC ([nisin] = 2.8175 µg/mL; [guar gum] = 7.5 mg/mL).

These bacterial cultures were incubated for 18h at 37ºC with shaking (180 rpm) and, after incubation, suspensions were centrifuged at 1500 rpm for 10 min at 4ºC, and supernatant was used to determine SpA level using the SpA ELISA kit (Abcam, UK), as recommended by the manufacturer. Besides adding 100 µL of samples to the 96-well plate, 100 µL of standards with known concentrations of protein A supplied by manufacturer were also added, with the final purpose of construct a standard curve to calculate SpA concentration in samples. The yellow color resulting from the assay was read at 450 nm (excitation filter: 450 nm; emission filter: 620 nm) using the FLUOstar OPTIMA microplate reader. The amount of signal is directly proportional to the level of protein A in the sample, which allowed to calculate the amount of SpA present in samples using the standard curve. Results are ratios of the amount of SpA (pg/mL) in the bacterial cultures incubated with clindamycin or nisin-biogel at sub-MICs to the mean amount of SpA (pg/mL) in the bacterial cultures incubated without antimicrobials, and are expressed as percentages.

2.11. Statistical analysis

Data statistical analysis was carried out using Microsoft Excel 2016®. Quantitative variables are expressed as mean values ± standard deviation. For all datasets, comparisons between treatments and control were performed using two-tailed Student's T-tests. A confidence interval of 95% was considered, and p-values<0.05 indicate statistical significance.

Chapter 3 | Results and Discussion

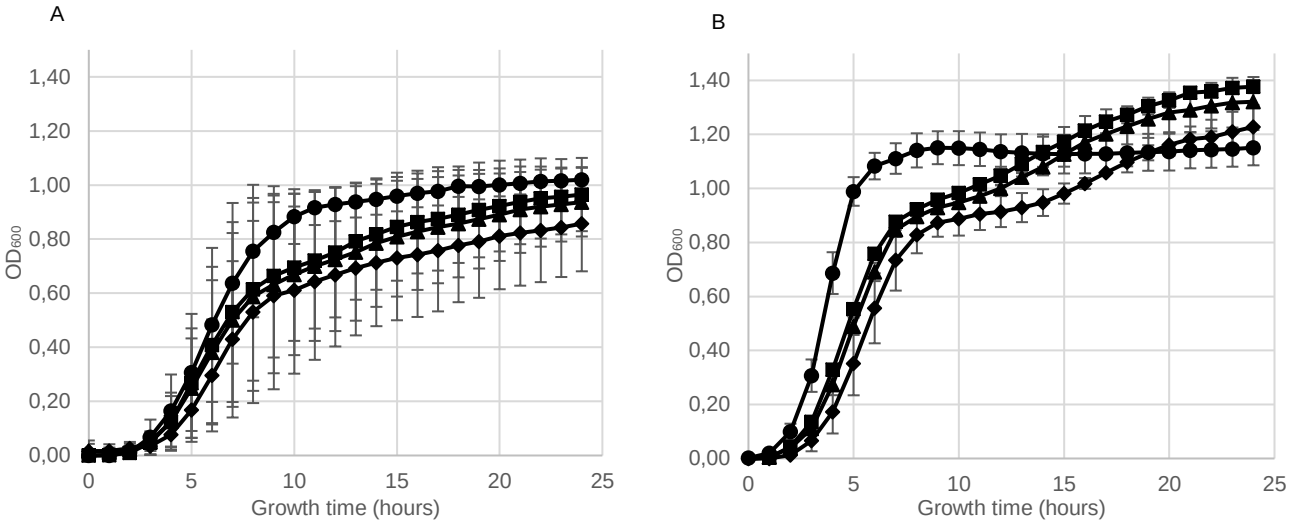
DFUs are one of the major complications associated with DM, and DFUs microenvironmental conditions, such as angiopathy and low perfusion, often prevent antimicrobials to effectively reach the infected DFUs, sometimes leading to sub-MICs at the site of the infection (Andersson & Hughes, 2014; Li et al., 2017).

3.1. Effects of nisin-biogel at sub-MICs on *S. aureus* DFI isolates growth rate

Sub-MICs of antimicrobials may have several effects on bacteria, including altered growth kinetics (Reeks, Champlin, Paulsen, Scruggs, & Lawrence, 2005). Accordingly, it was important to access the effects of nisin-biogel at sub-MICs on the bacterial growth rate, as the reduction or inhibition of bacterial multiplication would slow the progression of infection, allowing the host immune system to clear the pathogen effectively (Herbert et al., 2001; Reeks et al., 2005).

In accordance, previous studies have observed that antimicrobials at sub-MICs decrease or inhibit bacterial growth, with an increase of the lag phase of bacterial growth (Reeks et al., 2005; Zhanel, Hoban, & Harding, 1992). More specifically, Field, O' Connor, Cotter, Ross, & Hill, 2016 showed that nisin at sublethal concentrations slightly increased the lag time of *S. aureus* growth curve.

In the present study, the growth curves of the *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were accessed in the presence or absence of nisin-biogel at 1/2, 1/4 and 1/8 MIC, as shown in Figure 3.1.



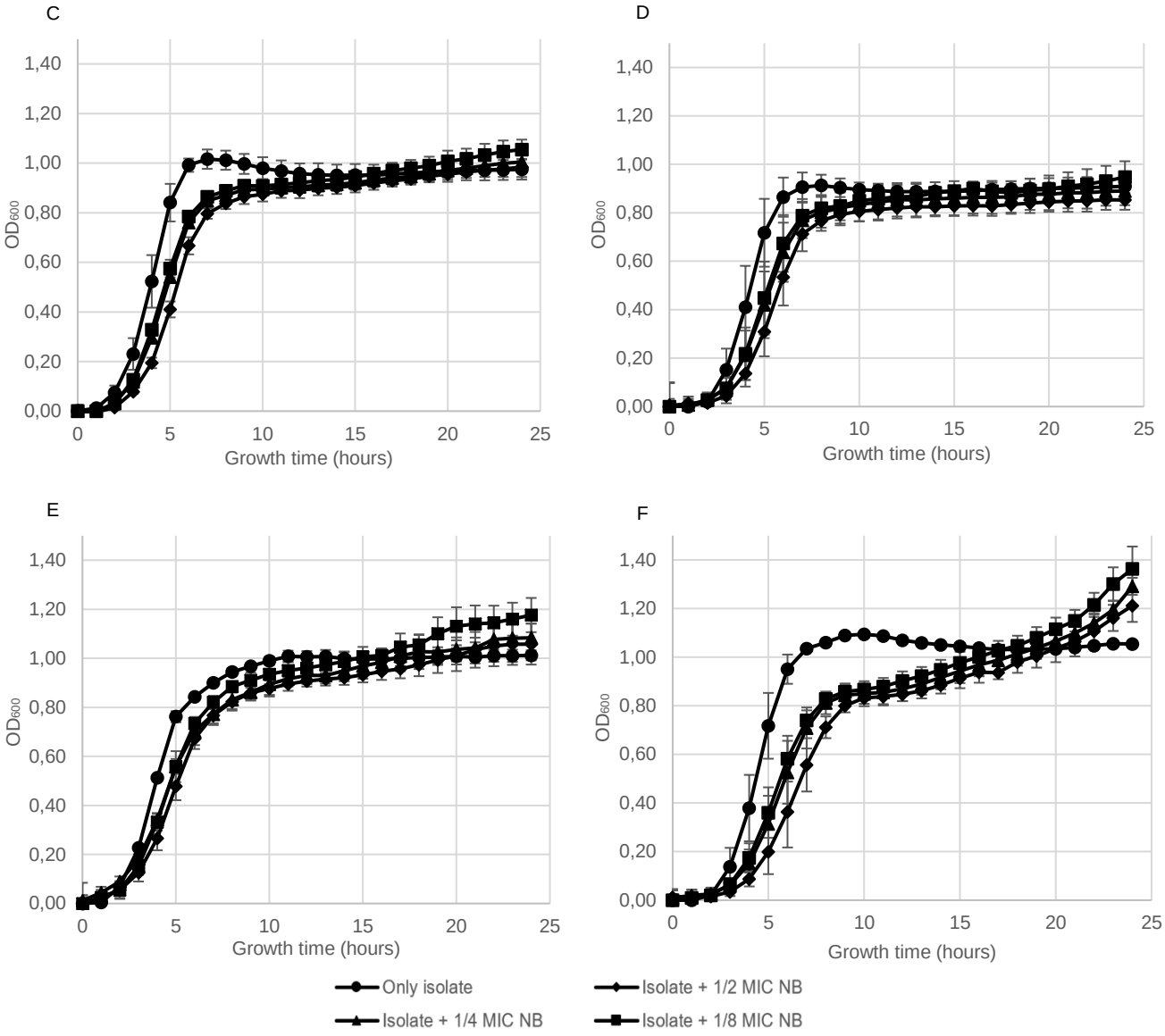


Figure 3.1 | A, B, C, D, E and F: Growth curves obtained for the *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively, when incubated in the presence or absence of nisin-biogel at sub-MICs for 24 hours at 37ºC with shaking (150 rpm). Results are presented as mean values of three independent assays, reflecting bacterial growth for each *S. aureus* DFI isolate under the different conditions tested. OD_{600}: optical density at 600 nm; NB: nisin-biogel; MIC: minimum inhibitory concentration.

Both in the presence or absence of nisin-biogel at sub-MICs, all *S. aureus* DFI isolates growth curves presented the typical sigmoidal pattern with the three phases of bacterial growth curves – lag phase, exponential phase and stationary phase. Nevertheless, different *S. aureus* DFI isolates showed different growth rates, with isolate A 6.3 having the highest growth rate, and isolate A 5.2 having the lowest growth rate.

For all the *S. aureus* DFI isolates under study, nisin-biogel at sub-MICs slowed bacterial growth, delaying the beginning of the exponential growth phase. This reduction in bacterial growth occurred in a dose-dependent manner. Nisin-biogel concentration equivalent to 1/8 MIC was the

one that less affected bacterial growth, while 1/2 MIC was the nisin-biogel concentration that most affected bacterial growth.

In the stationary phase of bacterial growth, for some *S. aureus* DFI isolates, including isolates A 6.3, B 1.1, Z 1.1 and Z 5.2, there was an increase in bacterial growth in the presence of nisin-biogel at sub-MICs when compared to bacterial growth in the absence of nisin-biogel at sub-MICs values. For isolate B 14.2, bacterial growth in the stationary growth phase only increased in the presence of nisin-biogel at 1/8 MIC. Therefore, it can be suggested that *S. aureus* DFI isolates A 6.3, B 1.1, Z 1.1 and Z 5.2 are capable of adapting to the presence of nisin-biogel at sub-MICs, isolate B 14.2 is capable of adapting to nisin-biogel at 1/8 MIC and isolate A 5.2 is not capable of adapting to nisin-biogel at sub-MICs at a later stage of bacterial growth.

3.2. RT-qPCR optimization

RT-qPCR is an efficient and simple technique widely used to quantify mRNA levels and, consequently, commonly used in gene expression studies (Derveaux, Vandesompele, & Hellemans, 2010).

RT-qPCR optimization is crucial to guarantee amplification efficiency. In this study, the first step of RT-qPCR optimization was to determine primers (forward and reverse) optimal concentrations. For *gyrB, agrI, spA, coa, clfA* and *atl,* a concentration of 300 nM both for forward and reverse primers was selected as optimal, whereas for *icaA* and *icaD*, an optimal concentration of 900 nM both for forward and reverse primers was selected, considering cycle threshold (Ct) values. Ct values are inversely related to the amount of starting material, which means that lower Ct values indicate higher amounts of the target gene, while higher Ct values are associated with lower amounts of the target gene (Schmittgen & Livak, 2008) (Table S1, available in Supplementary Data).

As a second step of RT-qPCR optimization, a standard curve and a dissociation curve were obtained for each gene under study, using the 10-fold serial dilutions of the cDNA mix. The standard curve contains information about the performance of RT-qPCR reaction, which can be related to diverse parameters, including slope and correlation coefficient (R^2). R^2 is a measure of reproducibility and ideally $R^2=1$. The slope of the log-linear phase reports amplification reaction efficiency and an efficiency of 100% is equivalent to a slope of -3.32. Ideally, the efficiency should be 100%, meaning that the template doubles after each cycle during exponential amplification. However, a reaction is considered good if it has an efficiency between 90% and 110%, which corresponds to a slope between -3.58 and -3.10 (Taylor, Wakem, Dijkman, Alsarraj, & Nguyen, 2010).

The standard curves obtained for all the genes under study are illustrated in Figure S1, available in Supplementary Data, and parameters values obtained from these standard curves are represented in Table S2, available in Supplementary Data. The graphics and the values of

the different parameters allowed to conclude about the amplification efficiency for all the genes under study, and proceed safely to subsequent gene expression studies, namely *S. aureus* DFI isolates genes expression kinetics and nisin-biogel sub-MICs effects on virulence-related genes expression by *S. aureus* DFI isolates assays. Dissociation curve analysis, following amplification, was used to check RT-qPCR for contaminations, primer dimer artifacts and to ensure reaction specificity, reducing the need for gel electrophoresis (Nolan et al., 2006). All the primers used in this study showed high specificities with a single temperature peak, confirming the absence of non-specific products, and allowing to proceed safely to subsequent gene expression studies.

3.3. *S. aureus* DFI isolates genes expression kinetics

The pathogenicity of *S. aureus* comprises the adherence to and persistence in host tissues and the escape from the host immune system. As such, *S. aureus* expresses a multitude of virulence factors in a coordinated manner, and many of them are under the control of *agr* quorum-sensing system. Virulence factors are not essential for bacterial growth, being only produced at certain phases of bacterial growth or under changing environmental conditions. Thus, gene expression presents a temporal pattern, enabling bacteria to evade host and promote infection (Liu et al., 2018; Pratten, Foster, Chan, Wilson, & Nair, 2001).

According to literature, the evaluation of virulence genes associated with bacterial attachment to the host should be performed in the early exponential *S. aureus* growth phase, when surface proteins are synthesized as a result of their upregulated expression. Oppositely, the evaluation of virulence genes associated with bacterial dissemination, including exotoxins and genes involved in biofilm formation should be performed at a later stage of *S. aureus* growth (Mottola et al., 2016b; Wang & Muir, 2016).

Therefore, to further determine the effects of nisin-biogel sub-MICs on the transcription levels of the different virulence-related genes under study, it was necessary to determine the more adequate bacterial growth period for their expression. For *agrI, spA, coa, clfA* and *atl*, RT-qPCR assays were designed to determine the expression kinetics of the genes within a time frame of 5 hours, by testing aliquots of the bacterial cultures of the *S. aureus* DFI isolates A 5.2 and Z 5.2 obtained at 2, 3, 4, 5 and 6 hours of bacterial incubation (Figure 3.2). For *icaA* and *icaD*, RT-qPCR assays were designed to determine the expression kinetics of the genes within a time frame of 56 hours, by testing aliquots of the bacterial culture of the *S. aureus* clinical isolates A 5.2 and Z 5.2 collected after 8, 24, 32, 48 and 56 hours of incubation (Figure 3.3).

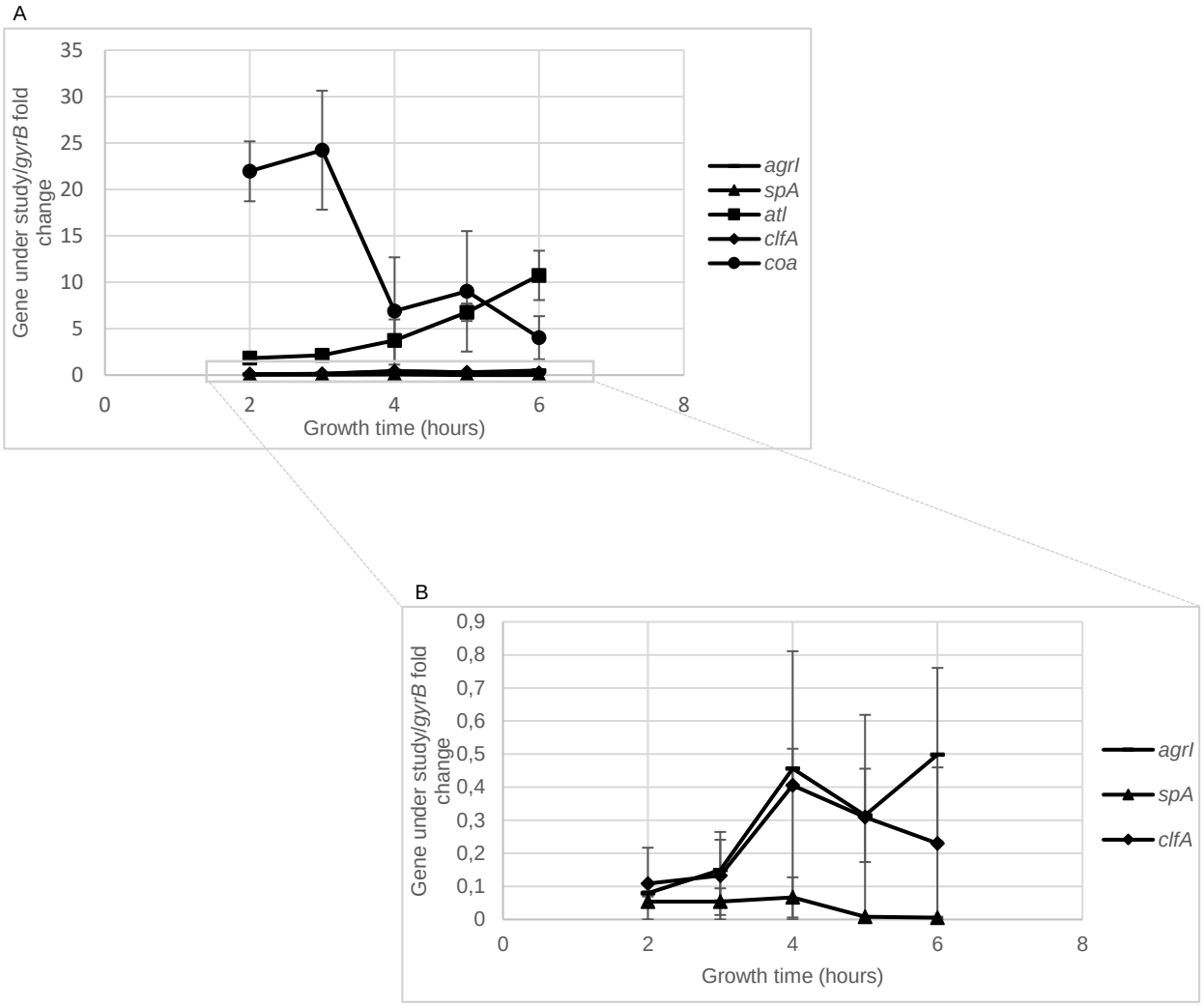


Figure 3.2 | *S. aureus* DFI isolates *agrI, spA, coa, clfA,* and *atl* expression kinetics during a 5 hours' growth period. Results are shown as 'gene under study/*gyrB*' fold changes in the different times of bacterial growth (2, 3, 4, 5 and 6 hours). Results are presented as mean values ± SD (isolates A 5.2 and Z 5.2). Figure 3.2B corresponds to an amplification of part of Figure 3.2A, so the y axis has different scales in the two figures. *agrI*: accessory gene regulator I; *spA:* gene encoding staphylococcal protein A; *atl:* gene encoding autolysin; *clfA:* gene encoding clumping factor A; *coa:* gene encoding coagulase; *gyrB:* gene encoding gyrase B.

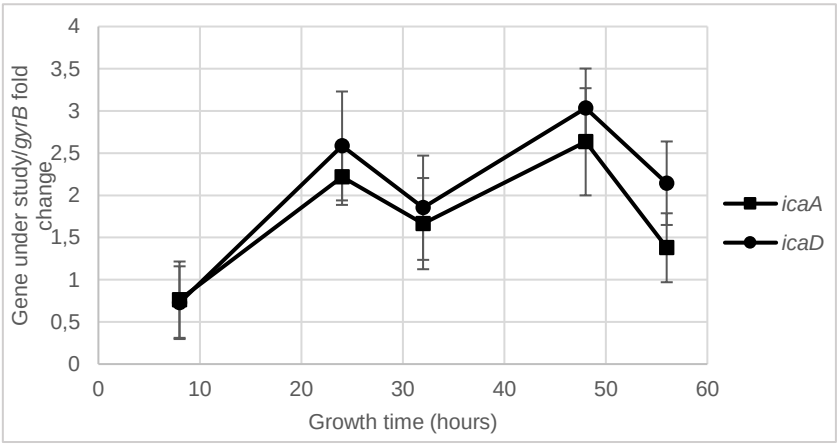


Figure 3.3 | *S. aureus* DFI isolates *icaA* and *icaD* expression kinetics during a 56 hours' growth period. Results are shown as 'gene under study/*gyrB*' fold changes in the different times of growth (8, 24, 32, 48

and 56 hours). Results are presented as mean values ± SD (isolates A 5.2 and Z 5.2). *icaA:* gene encoding intracellular adhesin A; *icaD:* gene encoding intracellular adhesin D; *gyrB:* gene encoding gyrase B.

Agr has the function of regulating several bacterial virulence factors, including the expression of virulence-related genes and the development of staphylococcal biofilms. Accordingly, the *agr* system positively controls the expression of many exotoxins, mainly of those produced after the end of the exponential growth phase, allowing bacteria to spread from the colonization sites to deeper tissues, and negatively controls the transcription of cell wall-associated proteins, mainly synthesized during exponential growth (Pratten et al., 2001).

Regarding biofilm formation, it has been demonstrated that repression of *agr* is necessary for biofilm formation, and *agr* activation is essential for biofilm dissemination (Tan, Li, Jiang, Hu, & Li, 2018).

According to our results, *agrI* expression levels only start to be relevant after 4 hours of *S. aureus* growth, when bacterial adherence to the host tissues already occurred. At this time point, cell-surface proteins start to be down-regulated, whereas virulence genes associated with bacterial dissemination start to be upregulated. Peng, Novick, Kreiswirth, Kornblum, & Schlievert, 1988 showed that *agr* activity is required for post-exponential phase expression of several secreted proteins, which allowed to support our results showing that *agr* mRNA expression reaches its higher expression levels at 6 hours of *S. aureus* growth.

SpA is a microbial surface protein that plays an important role in interfering with host defenses, inhibiting antibody-mediated phagocytosis and, consequently, allowing *S. aureus* to persist in the host cell. Coa, a staphylococcal extracellular protein, also contributes to the persistence of *S. aureus* in the host cell, by reacting with prothrombin in plasma, forming staphylothrombin that can stimulate clotting reaction in the plasma by converting Fg to fibrin, inhibiting host clearance mechanisms (Gómez et al., 2006; Yanagihara et al., 2006). According to our results, genes encoding SpA and Coa are mainly expressed in the early stage of *S. aureus* growth, i.e., before 3-4 hours. For *spA*, the highest expression levels were reached at 4 hours, and for *coa* the maximum expression levels were reached at 3 hours.

Our results are in accordance with those by Vandenesch, Kornblum, & Novick, 1991, and Lebeau, Vandenesch, Greenland, Novick, & Etienne, 1994, which showed that *spA* and *coa* are expressed at the early exponential growth phase. Accordingly, Vandenesch et al., 1991 suggested that *spA* mRNA is synthesized for a brief period early in the exponential phase and then switched off, whereas Lebeau et al., 1994 demonstrated that *coa* mRNA is mainly expressed at the early time point of *S. aureus* growth, followed by a reduction of this transcript at a later time point of bacterial growth.

ClfA is a staphylococcal surface protein that binds to Fg allowing bacteria to colonize traumatized tissue and, later, form biofilms (Farnsworth et al., 2017). Our results suggest that *clfA* is mainly expressed after the 4 hours of *S. aureus* growth, reaching its highest expression levels precisely at 4 hours. Results by Josefsson, Kubica, Mydel, Potempa, & Tarkowski, 2008

previously showed that *clfA* mRNA expression is higher at 6 hours of *S. aureus* growth than at 2 hours. However, they also concluded that *clfA* mRNA expression levels remained high at 24 hours of *S. aureus* growth, so it would be necessary to investigate the expression of *clfA* during a longer time frame to conclude about *clfA* mRNA expression levels in the stationary growth phase.

Atl has a multitude of functions, including staphylococcal attachment, bacterial cell wall degradation and cell separation during division, lysis mediated biofilm development and bacteriolytic activity, contributing to the excretion of cytoplasmatic proteins (Pasztor et al., 2010). In the present study, *atl* expression increased with *S. aureus* growth period, within a time frame period of 5 hours, probably due to the increased growth rate over this time period. This may be associated with the fact that Atl plays an important role in bacterial cell division, which increases throughout the exponential growth phase, and in cytoplasmic proteins excretion, which is more essential in the late exponential phase of *S. aureus* growth, playing an important role in cell multiplication and biofilm formation. Our results are in accordance with those by Oshida, Takano, Sugai, Suginaka, & Matsushita, 1998, that reported an increase in *atl* expression during the exponential growth phase.

Although the optimal expression of the different genes under study occurred at different periods of bacterial growth, all of them revealed a considerable expression at 4 hours' incubation, allowing to select this time point as the more adequate growth time for further evaluation of the effects of nisin-biogel at sub-MICs on *agrI, spA, coa, clfA* and *atl* expression.

One of the most relevant staphylococcal virulence factors is biofilm production, which only occurs when staphylococcal infection is established, conferring bacteria a wide range of adaptive advantages that contribute to their survival and persistence in the host cell, when compared with a planktonic mode of life (Li et al., 2016). As expected, *icaA* and *icaD*, which are involved in *icaADBC*-dependent biofilm formation, are mainly expressed later during *S. aureus* growth, with the maximum expression levels reached at 48 hours, according to our results. As stated by Atshan et al., 2013, *icaA* and *icaD* are mainly upregulated at 24 hours of *S. aureus* growth, opposite to the 48 hours, which suggests that *ica* mRNA expression kinetics may differ between *S. aureus* strains. Furthermore, Patel, Colton, Ebert, & Anderson, 2012, evaluated gene expression during *Staphylococcus epidermidis* biofilm formation and, in accordance with the present study, the expression levels of *icaA* and *icaD* at 48 hours of bacterial growth were the highest ones. Although our study was performed using *S. aureus* isolates, *S. epidermidis* also produces biofilm in an *icaADBC*-dependent manner, allowing us to suggest that the time frame required for the expression of *icaA* and *icaD* might be temporal identical in both species of *Staphylococcus.*

Taking into account our results and part of those in literature, an optimal growth period of 48 hours was selected to further evaluate the effects of nisin-biogel at sub-MICs on *icaA* and *icaD* expression.

3.4. Effects of nisin-biogel at sub-MICs on virulence genes expression by *S. aureus* DFI isolates

The pathogenicity of *Staphylococcus aureus* depends largely on extracellular virulence factors, including on both surface and secreted proteins (Herbert et al., 2001). As such, the multitude of virulence factors contributing to *S. aureus* pathogenesis has stimulated interest in how subinhibitory levels of antimicrobials affect their expression and possibly modulate the outcome of infection (Subrt, Mesak, & Davies, 2011).

Several studies have shown that subinhibitory concentrations of certain antibiotics affect virulence gene expression in *S. aureus*, which may alter the progression of the infection and render antimicrobial therapy unreliable. Thus, determining the effects of antimicrobials sub-MICs on bacterial virulence-related genes expression may provide important information for the rational use of antimicrobials in clinical practice, including in the treatment of DFIs (Dancer, 2008; Reeks, Champlin, Paulsen, Scruggs, & Lawrence, 2005; Subrt et al., 2011).

One of the antibiotics currently used in clinical practice associated with DFIs is clindamycin, which was used in this study as a control for comparing the effects of nisin-biogel on *S. aureus* genes expression, not only to identify the effects that both cause in the transcription levels of virulence, but also to conclude about how promising is nisin-biogel when compared with a conventional antibiotic currently used in DFIs treatment. Clindamycin inhibits bacterial protein synthesis, whereas nisin interacts with lipid II, an essential component for cell wall synthesis, blocking cell wall synthesis. It can also use lipid II as a docking molecule and lead to pore formation in the cell membrane (Perez et al., 2015; Smieja, 1998).

The effects of nisin-biogel and clindamycin at sub-MICs on virulence genes expression by *S. aureus* DFI isolates were investigated using RT-qPCR assays. To determine the effects of nisin-biogel and clindamycin sub-MICs on *agrI, spA, coa, clfA* and *atl* expression, the *S. aureus* clinical isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated at 37ºC for 4 hours in the presence or absence of nisin-biogel and clindamycin at sub-MICs, whereas to determine the effects of nisin-biogel and clindamycin sub-MICs on *icaA* and *icaD* expression, a 48 hours' incubation was performed.

Results showed that nisin-biogel and clindamycin at subinhibitory levels diversely modulate the expression of *agrI, spA, coa, clfA, atl, icaA* and *icaD*. This modulation depended on different variants, including *S. aureus* DFI isolate, virulence-related gene, antimicrobial agent and subinhibitory concentration under study. Moreover, *coa* and *spA* are polymorphic genes (Salehzadeh, Zamani, Keshtkar Langeroudi, & Mirzaie, 2016), which may explain the higher heterogeneity of nisin-biogel and clindamycin at sub-MICs effects on these genes expression by *S. aureus* DFI isolates under study.

3.4.1. Effects of nisin-biogel at sub-MICs on *agrI* expression by *S.aureus* DFI isolates

The effects of nisin-biogel and clindamycin at sub-MICs on *agrI* expression by the different *S. aureus* DFI isolates under study are shown in Figure 3.4, and the overall effects of nisin-biogel and clindamycin at sub-MICs on *agrI* expression are shown in Figure 3.5.

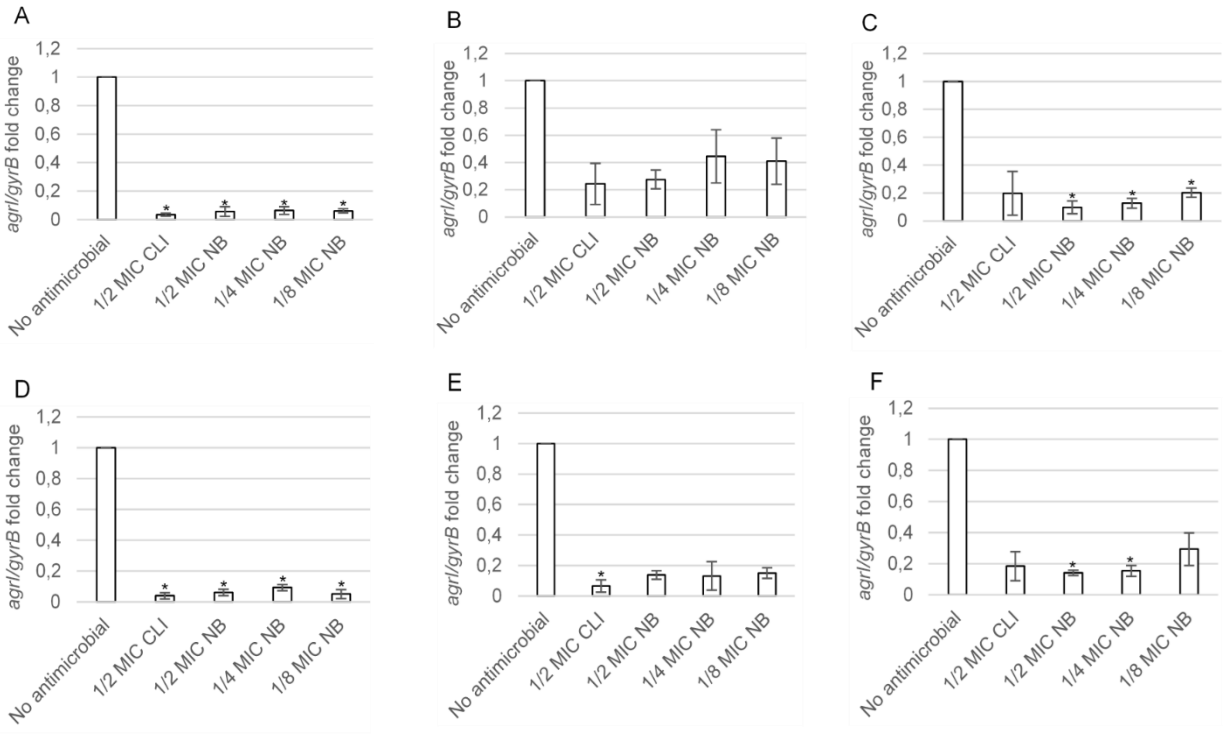


Figure 3.4 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *agrI* mRNA expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *agrI/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *agrI/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *agrI*: accessory gene regulator I; *gyrB:* gene encoding gyrase B.

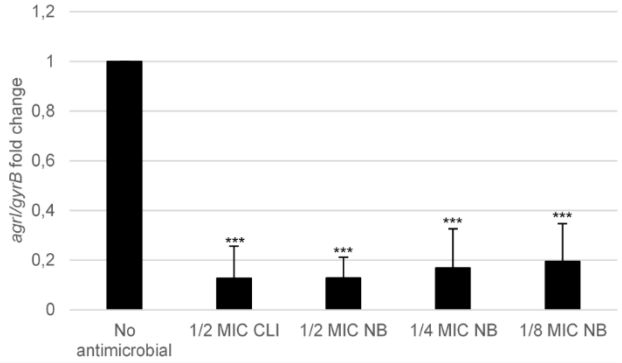


Figure 3.5 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *agrI* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial

cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *agrI/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *agrI/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* DFI isolate). Asterisks indicate statistically significant differences between treatments and control (*** p<0.001). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *agrI*: accessory gene regulator I; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.4, for all *S. aureus* DFI isolates under study, nisin-biogel and clindamycin at sub-MICs decreased *agrI* expression. Nevertheless, the proportion in which the decrease occurred depended on the *S. aureus* DFI isolate, on the antimicrobial agent and on the subinhibitory concentration under study.

Analyzing the overall results demonstrated in Figure 3.5, it can be observed that nisin-biogel and clindamycin at sub-MICs values significantly decreased *agrI* expression, with nisin-biogel at 1/2 MIC being the antimicrobial concentration that reduced *agrI* expression the most, while a nisin-biogel of 1/8 MIC was the one that least reduced this gene expression. When compared with clindamycin, nisin-biogel at 1/2 MIC had a similar effect on *agrI* expression.

In *S. aureus,* the *agr* quorum-sensing system plays a major role in virulence regulation, coordinating the transition from an adherent to an invasive mode, that involves increased production of secreted toxins and down-regulation of surface proteins. This is related to the fact that adhesion proteins are mainly needed at the beginning of the infection when cell density is low and adhesion to host tissue is critical, while toxins and degradative exoenzymes are mainly required when the infection is established, nutrients need to be acquired from the host tissues, and immune evasion factors need to be produced to face host immune system activation (Bronner, Monteil, & Prévost, 2004; Cheung, Wang, Khan, Sturdevant, & Otto, 2011).

Thus, the reduction of *agrI* mRNA expression in the presence of nisin-biogel and clindamycin at sub-MICs observed in the present study could lead to changes in virulence-related factors modulation and, consequently, affect infection pathogenesis.

As such, it can be suggested that nisin-biogel at sub-MICs, by leading to a decrease in the expression of *agrI,* will probably lead to an increase in adhesion genes expression and a decrease on exotoxins genes expression regulated by this quorum-sensing system. Moreover, as the repression of *agr* is necessary for biofilm formation, we also suggest that the presence of nisin-biogel at sub-MICs may lead to excessive biofilm formation.

Cheung et al., 2011 showed that *agr* plays an important role in skin infections, underlining the potential of drugs interfering with *agr* function on attenuate *S. aureus* pathogenesis. Moreover, Abdelnour, Arvidson, Bremell, Rydén, & Tarkowski, 1993, and Bezar, Mashruwala, Boyd, & Stock, 2019 demonstrated that *agr* inhibition leads to a decrease in exoproteins production and an increase in *spA* and *coa* transcripts levels.

3.4.2. Effects of nisin-biogel at sub-MICs on *spA* expression by *S. aureus* DFI isolates

The effects of nisin-biogel and clindamycin at sub-MICs on *spA* expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 are shown in Figure 3.6, and the overall effects of nisin-biogel and clindamycin at sub-MICs on *spA* expression are shown in Figure 3.7.

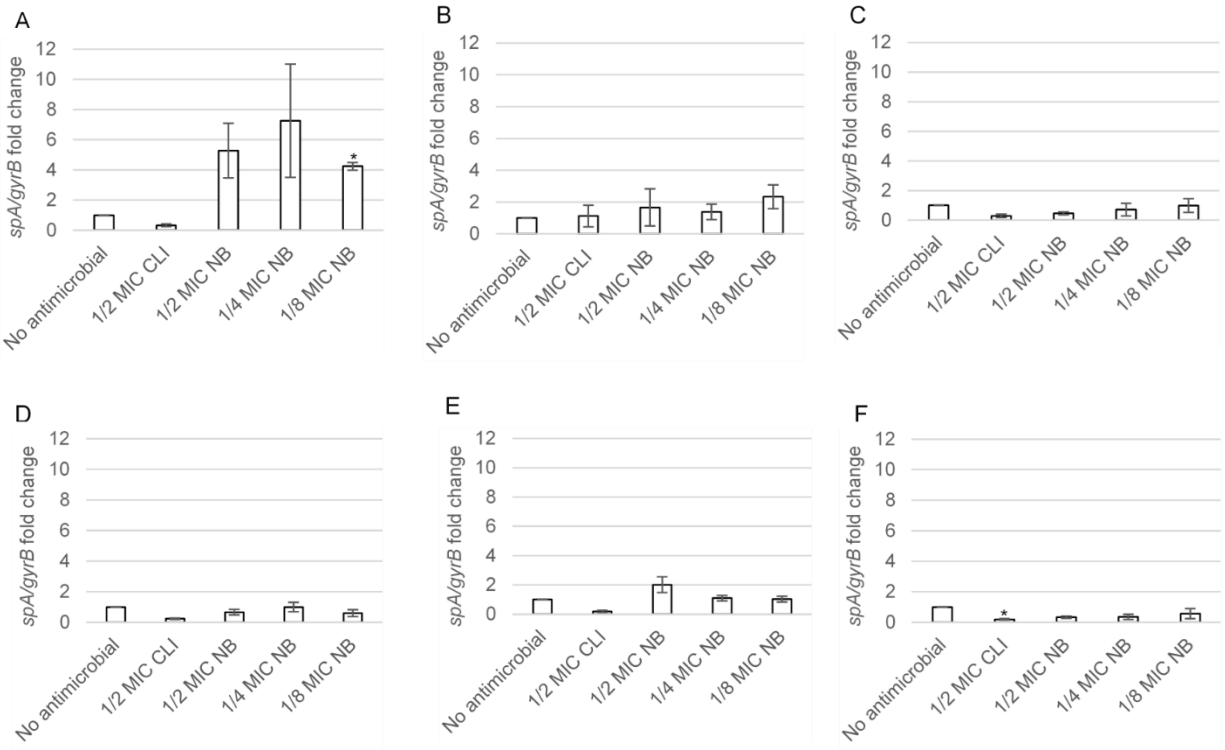


Figure 3.6 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *spA* mRNA expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *spA/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *spA/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *spA*: gene encoding staphylococcal protein A; *gyrB:* gene encoding gyrase B.

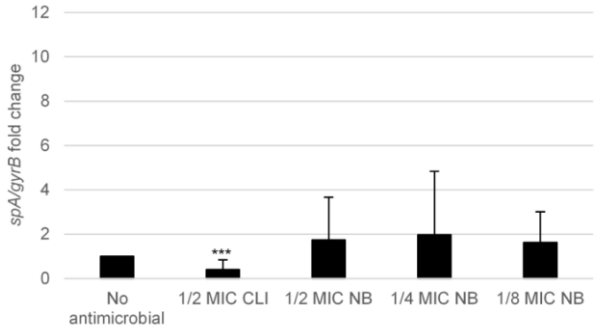


Figure 3.7 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *spA* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and

subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *spA/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *spA/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* clinical isolate). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *spA*: gene encoding staphylococcal protein A; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.6, nisin-biogel and clindamycin at sub-MICs had differential effects on *spA* expression, depending on the *S. aureus* DFI isolate, on the antimicrobial agent and on the subinhibitory concentration under study. For isolates A 5.2, A 6.3 and Z 1.1, the nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC increased *spA* expression, although increase in isolate A 5.2 was much higher than the one observed in isolates A 6.3 and Z 1.1. For isolates B 1.1, B 14.2 and Z 5.2, the nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC decreased or had no effect on *spA* expression. Regarding clindamycin at 1/2 MIC, for all the *S. aureus* DFI isolates under study, except for isolate A 6.3, it contributed to a decrease in *spA* expression. For isolate A 6.3, *spA* expression slightly increased, but in a non-relevant proportion.

Analyzing the overall results represented in Figure 3.7, it can be observed that nisin-biogel at subinhibitory concentrations exhibited a trend to increase *spA* expression, with nisin-biogel at 1/4 MIC being the one that exhibited a higher trend to increase *spA* expression, while nisin-biogel at 1/8 MIC was the one that exhibited a lower trend to increase *spA* expression. Regarding clindamycin at 1/2 MIC, the overall results showed a significant decrease in *spA* expression.

SpA contributes to bacterial evasion from the host immune system, playing an important role at the beginning of an infection, and allowing *S. aureus* to persist in the host cell by inhibiting phagocytosis (Gómez et al., 2006). As such, the presence of subinhibitory levels of nisin-biogel may cause a higher decrease in phagocytosis and, consequently, facilitate *S. aureus* survival in host cell, which can negatively affect infection treatment. On the other hand, clindamycin at 1/2 MIC significantly reduced *spA* expression, possibly contributing to positively affecting infection treatment, since there will be a higher number of free receptor sites for complement C3b and, consequently, a lower inhibition of *S. aureus* opsonization from the host cell. These results are in line with those obtained by Gemmell & O'Dowd, 1983, Herbert et al., 2001 and by Otto et al., 2013, who concluded that subinhibitory concentrations of clindamycin have an inhibitory effect on *spA* expression.

Moreover, as mentioned above, Aneidbdelnour et al., 1993 and Bezar et al., 2019 concluded that the inhibition of *agr* expression leads to an increase in *spA* mRNA expression. This is in line with what we observed in the present study regarding nisin-biogel at sub-MICs, as they provoked a decrease in *agrI* expression by *S. aureus* DFI isolates and, in accordance, exhibited a trend to increase *spA* expression by *S. aureus* clinical isolates. However, regarding clindamycin, results were not so clear, since clindamycin at 1/2 MIC decreased *agrI* and *spA* expression, which may be related to the fact that clindamycin is a protein synthesis inhibitor.

3.4.3. Effects of nisin-biogel at sub-MICs on *coa* expression by *S. aureus* DFI isolates

The effects of nisin-biogel and clindamycin sub-MICs on *coa* expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 are shown in Figure 3.8 and the overall effects of nisin-biogel and clindamycin sub-MICs on *coa* expression are shown in Figure 3.9.

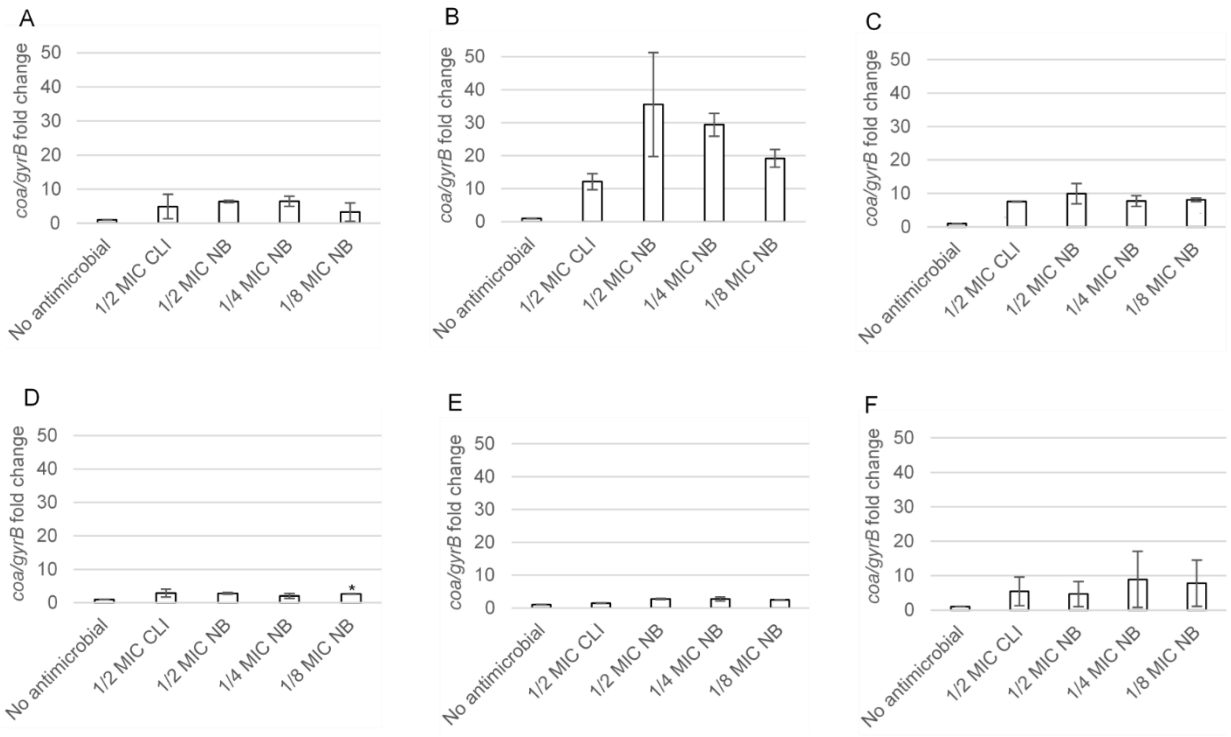


Figure 3.8 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *coa* mRNA expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *coa/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *coa/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *coa:* gene encoding coagulase; *gyrB:* gene encoding gyrase B.

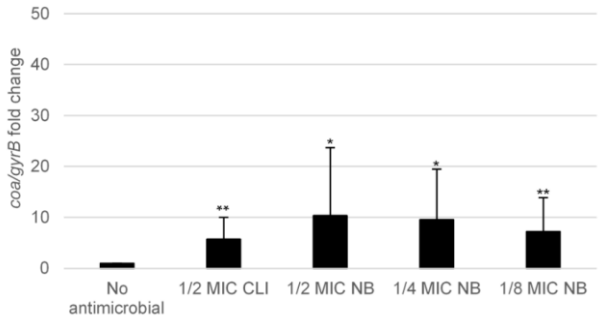


Figure 3.9 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *coa* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial

cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *coa/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *coa/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* clinical isolate). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$; ** $p<0.01$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *coa:* gene encoding coagulase; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.8, nisin-biogel at 1/2, 1/4, and 1/8 MIC and clindamycin at 1/2 MIC increased *coa* expression by all the *S. aureus* DFI isolates under study, although in different proportions. Isolate A 6.3 was the one that showed a higher increase in *coa* expression in the presence of nisin-biogel at subinhibitory levels. For the other *S. aureus* DFI isolates, the increase was less marked, with *coa* expression being between 5-fold to 10-fold higher in the presence of nisin-biogel and clindamycin at sub-MICs than in the absence of antimicrobials.

Analyzing the overall results represented in Figure 3.9, it can be observed that nisin-biogel at subinhibitory concentrations significantly increased *coa* expression in a dose-dependent manner, i.e., nisin-biogel at 1/2 MIC was the one that increased *coa* expression the most, while a nisin-biogel of 1/8 MIC was the one that least increased this gene expression. Regarding *coa* expression in the presence of clindamycin at 1/2 MIC, the overall results show also an upregulation of *coa* expression. These results are in line with those obtained by Blickwede, Wolz, Valentin-Weigand, & Schwarz, 2005 and Herbert et al., 2001, which concluded that subinhibitory levels of clindamycin increased levels of *coa* mRNA.

coa transcription is negatively modulated by *agr* (Wolz, McDevitt, Foster, & Cheung, 1996). As stated above, our results regarding *agrI* mRNA expression showed that nisin-biogel and clindamycin at subinhibitory levels significantly decreased this gene expression. As such, *coa* expression will probably be weaker repressed, which may be related with the increase in *coa* mRNA expression observed in the presence of nisin-biogel and clindamycin at sub-MICs.

Coa contributes to *S. aureus* resistance against phagocytosis, as it promotes modifications of the coagulation cascade (Pozzi et al., 2016). As such, according to our results, the application of subinhibitory levels of nisin-biogel and clindamycin may cause undesirable effects on infection control, negatively influencing the efficiency of host clearance mechanisms against the pathogen and, consequently, allowing it to persist in the host.

3.4.4. Effects of nisin-biogel at sub-MICs on *clfA* expression by *S. aureus* DFI isolates

The effects of nisin-biogel and clindamycin sub-MICs on *clfA* expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 are shown in Figure 3.10, and the overall effects of nisin-biogel and clindamycin sub-MICs on *clfA* expression are shown in Figure 3.11.

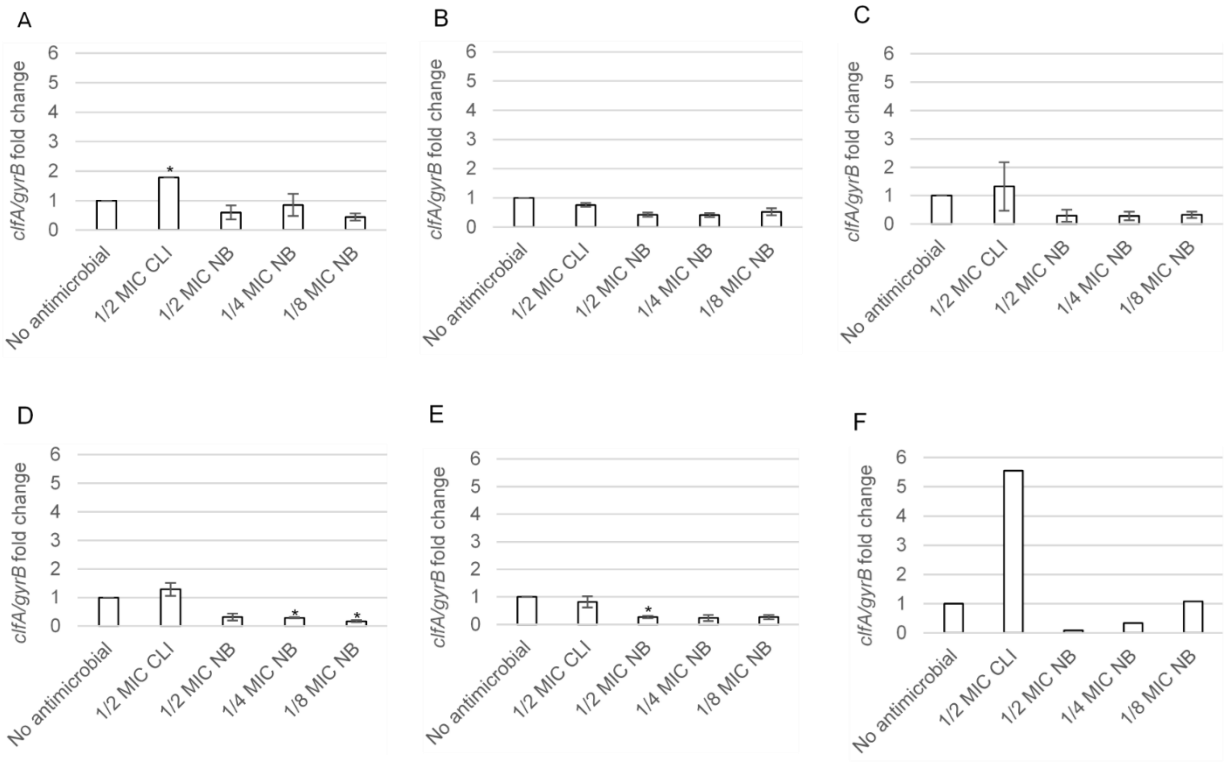


Figure 3.10 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *clfA* mRNA expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *clfA/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *clfA/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments), except for isolate Z 5.2, where only one experiment was performed. Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *clfA:* gene encoding clumping factor A; *gyrB:* gene encoding gyrase B.

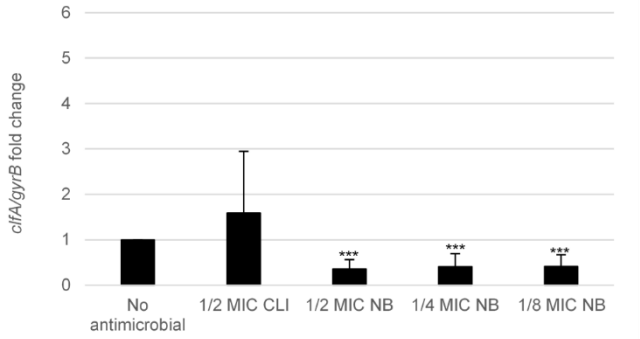


Figure 3.11 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *clfA* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *clfA/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *clfA/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* clinical isolate, except for isolate Z 5.2, where only one experiment was performed). Asterisks indicate statistically significant differences between treatments and control (*** $p<0.001$). NB: nisin-biogel;

CLI: clindamycin; MIC: minimum inhibitory concentration; *clfA:* gene encoding clumping factor A; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.10, nisin-biogel and clindamycin at sub-MICs had differential effects on *clfA* expression, depending on the *S. aureus* DFI isolate, on the antimicrobial agent and on the subinhibitory concentration under study. For isolates A 5.2, A 6.3, B 1.1, B 14.2 and Z 1.1, nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC decreased *clfA* expression, although in different proportions, with isolates B 1.1, B 14.2 and Z 1.1 suffering a severer decrease. For isolate Z 5.2, there was an evident decrease in *clfA* expression in the presence of nisin-biogel at 1/2 and 1/4 MIC, whereas nisin-biogel at 1/8 MIC had no effect on this gene expression. However, for isolate Z 5.2, only one assay was performed, so for the results to be reliable, a second assay will have to be performed in the future.

Regarding clindamycin at 1/2 MIC, results were far more diverse than those obtained for nisin-biogel at sub-MICs. For isolates A 5.2, B 1.1, B 14.2 and Z 5.2, there was an increase in *clfA* expression, and the most significant increase occurred in isolate Z 5.2. For isolates A 6.3 and Z 1.1, *clfA* expression decreased, although in a smaller proportion than in the presence of nisin-biogel at the same concentration. These results showed that, regarding *clfA* expression, nisin-biogel at subinhibitory levels may favorably influence infection treatment when compared with clindamycin at 1/2 MIC, for all the *S. aureus* DFI isolates under study.

Analyzing the overall results represented in Figure 3.11, it can be observed that nisin-biogel at subinhibitory concentrations significantly decreases the expression of *clfA*, with nisin-biogel at 1/2 MIC being the one that decreased *clfA* expression the most, whereas a nisin-biogel of 1/8 MIC was the one that least decreased *clfA* expression. Unlike nisin-biogel at subinhibitory levels, clindamycin at 1/2 MIC exhibited a trend to increase *clfA* expression. Regarding the effects of nisin-biogel at sub-MICs on *clfA* expression, our results are in conformity with those by Zhao et al., 2016, that described that the exposure of *S. aureus* for 1 hour to a nisin concentration equivalent to 1/2 MIC led to a reduction in *clfA* expression.

As ClfA allows bacterial colonization and biofilm formation (Herman-Bausier et al., 2018), nisin-biogel at sub-MICs possibly contributes to decreased *S. aureus* pathogenicity, since it may affect *S. aureus* persistence and dissemination in the host, positively affecting infection treatment. Oppositely, clindamycin at 1/2 MIC may negatively affect infection treatment, contributing to an increase in bacterial attachment to blood clots and traumatized tissues and protection against phagocytosis.

In contrast with *spA* and *coa, clfA* transcription is *agr* independent and it is thought to be positively regulated by SigB, another *S. aureus* regulatory system (Jenul & Horswill, 2018; Wolz et al., 1996). Accordingly, this may be the reason why the decrease in *agr* expression in the presence of nisin-biogel at sub-MICs did not lead to an increase in *clfA* expression as observed regarding *spA* and *coa* mRNA expression.

3.4.5. Effects of nisin-biogel at sub-MICs on *atl* expression by *S. aureus* DFI isolates

The effects of nisin-biogel and clindamycin at sub-MICs on *atl* expression by the different *S. aureus* DFI isolates under study are shown in Figure 3.12, and the overall effects of nisin-biogel and clindamycin at sub-MICs on *atl* expression are shown in Figure 3.13.

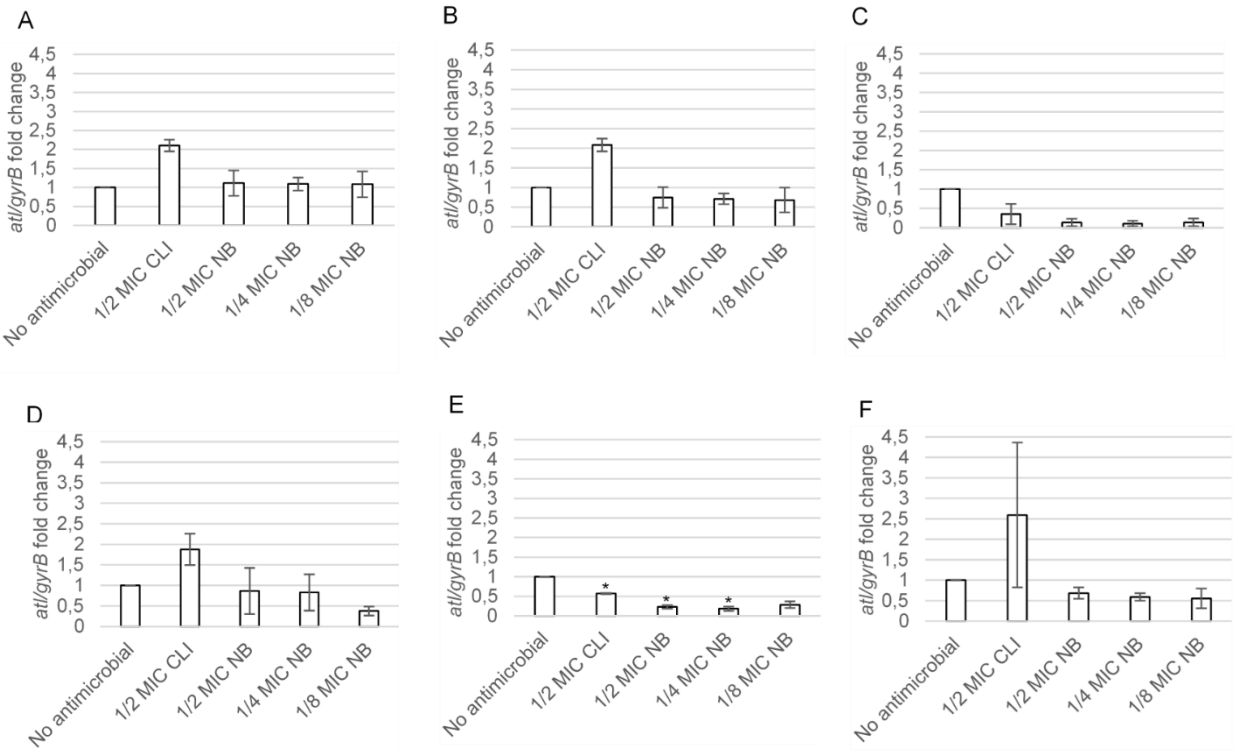


Figure 3.12 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *atl* mRNA expression by *S. aureus* clinical isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *atl/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *atl/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *atl:* gene encoding autolysin; *gyrB:* gene encoding gyrase B.

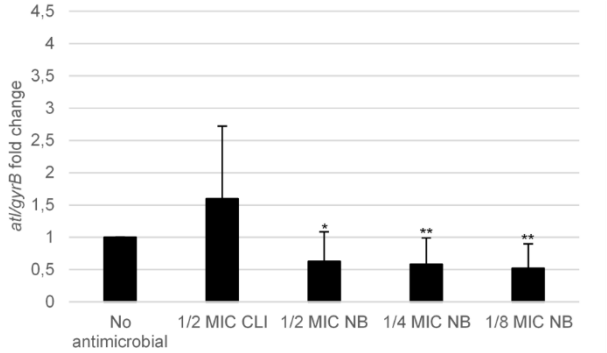


Figure 3.13 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *atl* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial

cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *atl/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *atl/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* clinical isolate). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$; ** $p<0.01$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *atl:* gene encoding autolysin; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.12, the effects of nisin-biogel and clindamycin at sub-MICs depend on the *S. aureus* DFI isolate, on the antimicrobial agent and on the subinhibitory concentration under study. For isolates A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC decreased the expression of *atl*, although in different proportions, with isolates B 1.1 and Z 1.1 suffering a sharper decrease, whereas for isolates A 6.3, B 14.2 and Z 5.2 this decrease was lower. Regarding isolate A 5.2, nisin-biogel at sub-MICs had no effect on *atl* expression. For isolates A 5.2, A 6.3, B 14.2 and Z 5.2 there was an increase in *atl* expression in the presence of clindamycin at 1/2 MIC, whereas for isolates B 1.1 and Z 1.1 *atl* expression decreased, although in a smaller proportion than in the presence of nisin-biogel at the same concentration. These results show that, for all the *S. aureus* DFI isolates under study, nisin-biogel at subinhibitory levels exhibited a trend to favorably influence infection progression when compared with clindamycin at 1/2 MIC.

Analyzing the overall results represented in Figure 3.13, it can be observed that nisin-biogel at subinhibitory concentrations significantly decreased *atl* expression in a dose-dependent manner, with nisin-biogel at 1/2 MIC being the one that least decreased this gene expression, whereas nisin-biogel at 1/8 MIC was the one that most decreased the *atl* expression. Unlike nisin-biogel at subinhibitory levels, clindamycin at 1/2 MIC exhibited a trend to increase *atl* expression, which suggests a better outcome in infection treatment in the presence of nisin-biogel than in the presence of clindamycin at sub-MICs.

Results obtained regarding nisin-biogel are in accordance with those by Zhao et al., 2016, that stated that the exposure of *S. aureus* for 1 hour to a nisin concentration equivalent to 1/2 MIC led to a down-regulation of *atl*. Regarding clindamycin at 1/2 MIC, results obtained are consistent with the study by Schilcher et al., 2016, that showed that subinhibitory levels of clindamycin upregulate the expression of *atl*.

Atl is involved in cell division, staphylococcal attachment to surfaces, biofilm development and secretion of cytoplasmic proteins, contributing to *S. aureus* persistence and dissemination in the host (Houston et al., 2011; Pasztor et al., 2010; Porayath et al., 2018). By decreasing *atl* expression, nisin-biogel at subinhibitory levels may decrease the pathogenic potential of *S. aureus*, since the multiplication of the microorganism becomes less efficient, as well as the staphylococcal invasion and excretion of cytoplasmatic proteins, which are important in biofilm formation.

3.4.6. Effects of nisin-biogel at sub-MICs on *icaA* expression by *S. aureus* DFI isolates

The effects of nisin-biogel and clindamycin sub-MICs on *icaA* expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 are shown in Figure 3.14 and the overall effects of nisin-biogel and clindamycin sub-MICs on *icaA* expression are shown in Figure 3.15.

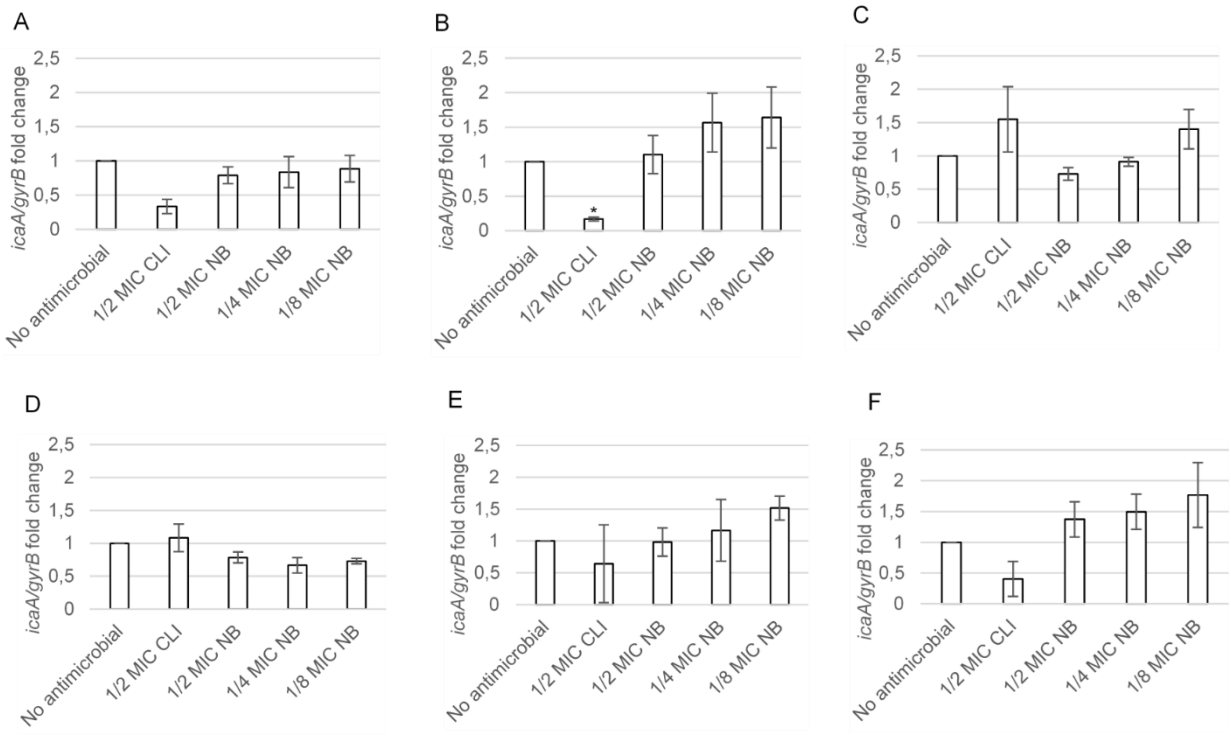


Figure 3.14 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *icaA* mRNA expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with and without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *icaA/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *icaA/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments). Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *icaA:* gene encoding intracellular adhesin A; *gyrB:* gene encoding gyrase B.

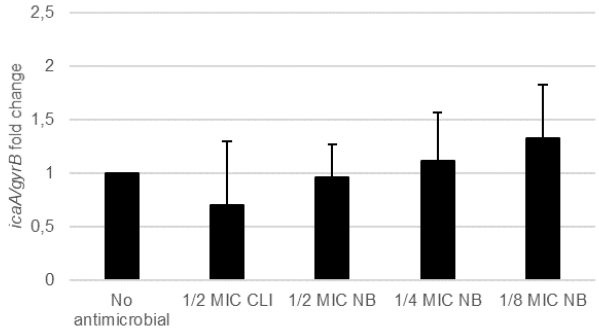


Figure 3.15 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *icaA* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of

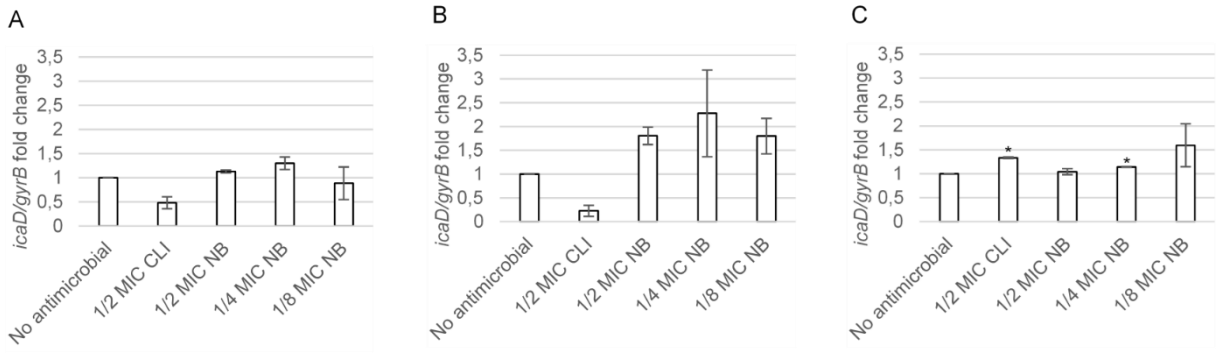
bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *icaA/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *icaA/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* clinical isolate). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *icaA:* gene encoding intracellular adhesin A; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.14, nisin-biogel and clindamycin at sub-MICs had differential effects on *icaA* expression, depending on the *S. aureus* DFI isolate, on the antimicrobial agent and on the subinhibitory concentration under study. For isolates A 5.2 and B 14.2, nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC decreased *icaA* expression, although in different proportions. Oppositely, for isolates A 6.3 and Z 5.2, nisin-biogel at subinhibitory levels increased this gene expression. For isolates B 1.1 and Z 1.1, different concentrations of nisin-biogel had different effects on *icaA* expression. For isolate B 1.1, nisin-biogel at 1/2 and 1/4 MIC reduced *icaA* expression, whereas in the presence of nisin-biogel at 1/8 MIC there was an increase in this gene expression. For isolate Z 1.1, nisin-biogel at 1/4 and 1/8 MIC increased *icaA* expression, while nisin-biogel at 1/2 MIC had no effect on its expression. Regarding the presence of clindamycin at 1/2 MIC, *icaA* expression increased for isolates B 1.1 and B 14.2, and decreased for isolates A 5.2, A 6.3, Z 1.1 and Z 5.2.

Analyzing the overall results represented in Figure 3.15, it can be observed that nisin-biogel at 1/4 and 1/8 MIC exhibited a trend to increase *icaA* expression, nisin-biogel at 1/2 MIC exhibited a slight trend to decrease this gene expression, and clindamycin at 1/2 MIC exhibited a trend to decrease *icaA* expression, in a higher proportion than nisin-biogel at the same concentration.

3.4.7. Effects of nisin-biogel at sub-MICs on *icaD* expression by *S. aureus* DFI isolates

The effects of nisin-biogel and clindamycin sub-MICs on *icaD* expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 are shown in Figure 3.16 and the overall effects of nisin-biogel and clindamycin sub-MICs on *icaD* expression are shown in Figure 3.17.



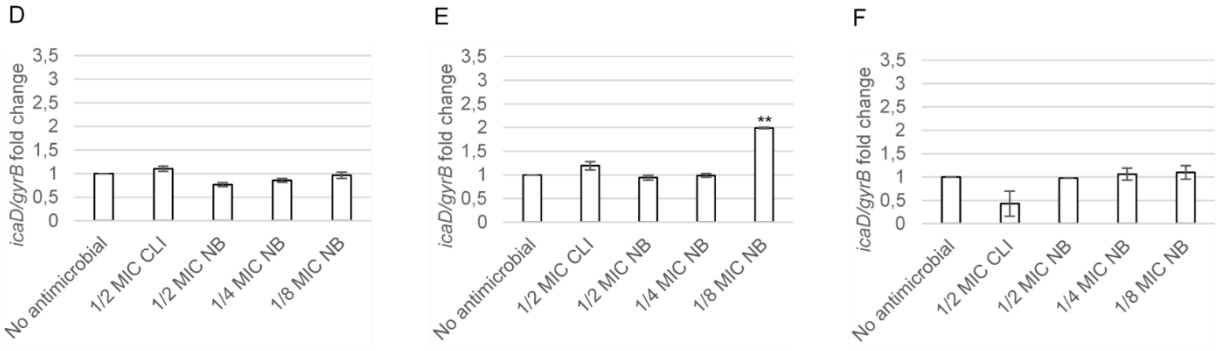


Figure 3.16 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *icaD* mRNA expression by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *icaD/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *icaD/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments). Asterisks indicate significant differences between treatments and control (* $p<0.05$; ** $p<0.01$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *icaD:* gene encoding intracellular adhesin D; *gyrB:* gene encoding gyrase B.

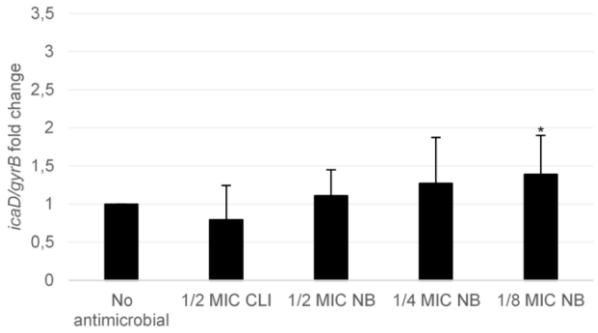


Figure 3.17 | Average effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on *icaD* mRNA expression by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC. Aliquots of bacterial cultures were collected after 4h of incubation and used for total RNA extraction, cDNA synthesis and subsequent RT-qPCR, as described in Materials and Methods section. Results are expressed as n-fold differences in *icaD/gyrB* ratio in the presence of nisin-biogel and clindamycin at sub-MICs relative to *icaD/gyrB* ratio in growth control (no antimicrobial). Values are means ± SD (two repeated different experiments for each *S. aureus* clinical isolate). Asterisks indicate significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration; *icaD:* gene encoding intracellular adhesin D; *gyrB:* gene encoding gyrase B.

As shown in Figure 3.16, nisin-biogel and clindamycin at sub-MICs had differential effects on *icaD* expression, depending on the *S. aureus* DFI isolate, on the antimicrobial agent and on the subinhibitory concentration under study. For isolate A 6.3, nisin-biogel at sub-MICs increased *icaD* expression, whereas for isolate B 14.2, nisin-biogel at sub-MICs decreased this gene expression. For isolates A 5.2, B 1.1, Z 1.1 and Z 5.2, the results differ depending on the subinhibitory level of nisin-biogel under study. For isolate A 5.2, nisin-biogel at 1/2 and 1/4 MIC increased *icaD* expression, whereas nisin-biogel at 1/8 MIC decreased this gene expression. For isolates B 1.1 and Z 5.2, nisin-biogel at 1/2 MIC had no effect on *icaD* expression, while nisin-

biogel at 1/4 and 1/8 MIC increased *icaD* expression. Finally, for isolate Z 1.1, nisin-biogel at 1/2 MIC decreased this gene expression, nisin-biogel at 1/4 had no effect on *icaD* expression, and nisin-biogel at 1/8 MIC significantly increased this gene expression. Regarding the presence of clindamycin at 1/2 MIC, *icaD* expression increased for isolates B 1.1 and Z 1.1 and decreased for isolates A 5.2, A 6.3, B 14.2 and Z 5.2.

Analyzing the overall results represented in Figure 3.17, it can be observed that nisin-biogel at concentrations corresponding to 1/2, 1/4 and 1/8 MIC exhibited a trend to increase *icaD* expression, with nisin-biogel at 1/8 MIC being the one that showed a more significant increase on *icaD* expression. On the other hand, clindamycin at 1/2 MIC exhibited a trend to decrease this gene expression, in a higher proportion than nisin-biogel at the same concentration, possibly leading to a more positive outcome on infection progression when compared with nisin-biogel at subinhibitory levels.

icaA and *icaD* are involved in biofilm formation (Otto, 2019; O'Gara, 2007) and subinhibitory levels of nisin-biogel exhibited a trend to increase these genes expression, which can enhance biofilm formation and, consequently, may lead to therapeutic failure. Moreover, the lower the concentration of nisin-biogel, the higher the trend to increase *icaA* and *icaD* mRNA levels, which reinforces the importance of defining the optimum dosage for the treatment of bacterial infections, aiming at avoiding undesirable effects.

Rachid, Ohlsen, Witte, Hacker, & Ziebuhr, 2000 showed that clindamycin at subinhibitory did not affect *S. epidermidis ica* expression, non-specifying if these effects were observed regarding *icaA* or *icaD*. Our results diverge from these, possibly due to the species, as we studied the effects of clindamycin on *S. aureus* DFI isolates and not on *S. epidermidis*, and due to the fact that we investigated the effects on *icaA* or *icaD* specifically and not on the *ica* locus.

3.5. Effects of nisin-biogel at sub-MICs on the biofilm-forming ability of *S. aureus* DFI isolates

Genotype characterization by RT-qPCR represented a highly sensitive method for the study of nisin-biogel and clindamycin at sub-MICs effects on the *ica* locus, more specifically on *icaA* and *icaD*. However, the phenotypic ability of *S. aureus* DFI isolates to form biofilm can be affected not only by changes in the expression of *ica*, but also by *icaADBC*-independent mechanisms and by environmental conditions. Thus, understanding biofilm assembly and dispersal in response to subinhibitory concentrations of clinically relevant antimicrobials is critical to further optimize antimicrobial treatment strategies of biofilm-associated *S. aureus* infections (Schilcher et al., 2016).

The effects of nisin-biogel and clindamycin at sub-MICs on the ability of *S. aureus* DFI isolates to form biofilm were investigated using a microtiter method. To determine the effects of nisin-biogel and clindamycin sub-MICs on biofilm formation, the *S. aureus* clinical isolates A 5.2,

A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated in the presence or absence of nisin-biogel and clindamycin at sub-MICs in a 96-well plate at 37ºC for 48 hours.

Results obtained show that nisin-biogel and clindamycin at subinhibitory levels diversely modulate biofilm formation by *S. aureus* DFI isolates. This modulation depended on different variants, including *S. aureus* DFI isolate, antimicrobial agent and subinhibitory concentration under study. This is in line with previous studies that showed that subinhibitory concentrations of clindamycin have diverse effects on *S. aureus* biofilm formation, being highly dependent on the strain background (Schilcher et al., 2016).

The effects of nisin-biogel and clindamycin sub-MICs on the ability of *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 to form biofilm are shown in Figure 3.18 and the overall effects of nisin-biogel and clindamycin sub-MICs on biofilm formation are shown in Figure 3.19.

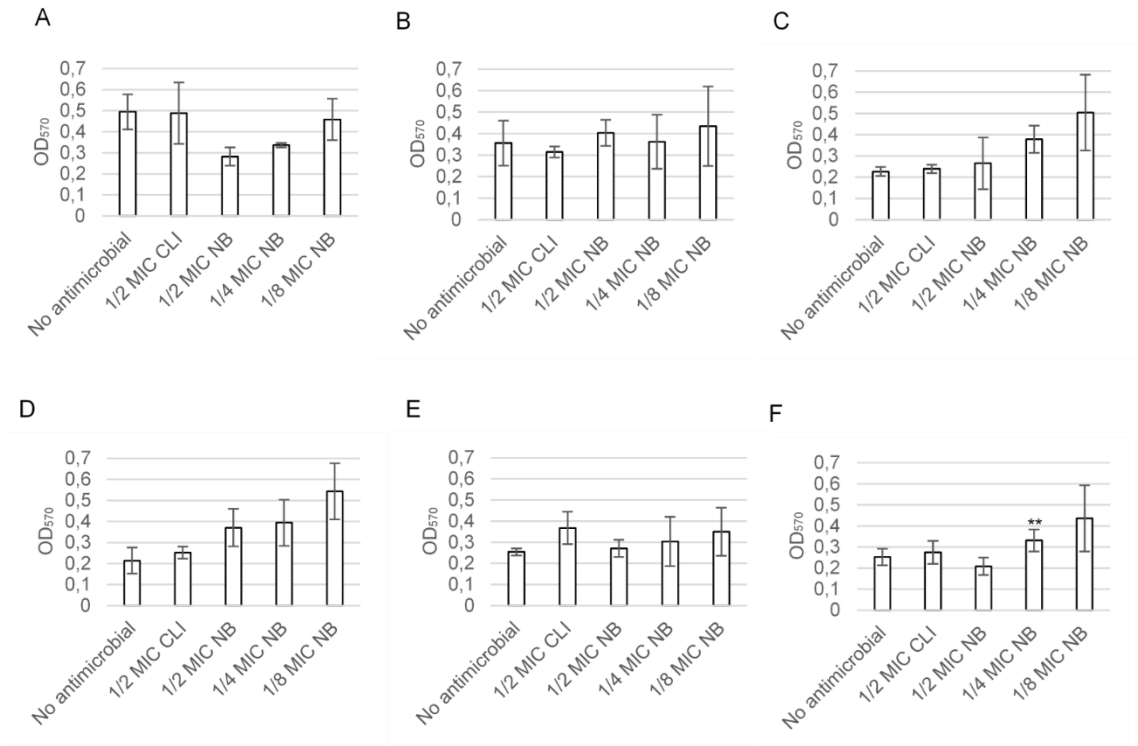


Figure 3.18 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on the ability of *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively, to form biofilm. Isolates were incubated in a microtiter plate with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC for 48h at 37ºC. Then, microtiter plate was incubated in an ultrasound bath to disperse biofilm-based bacteria from the microtiter plate surface and the OD at 570 nm was measured. Values are means ± SD (three repeated different experiments). Asterisks indicate significant differences between treatments and control (** $p<0.01$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration. OD_{570}: optical density at 570 nm.

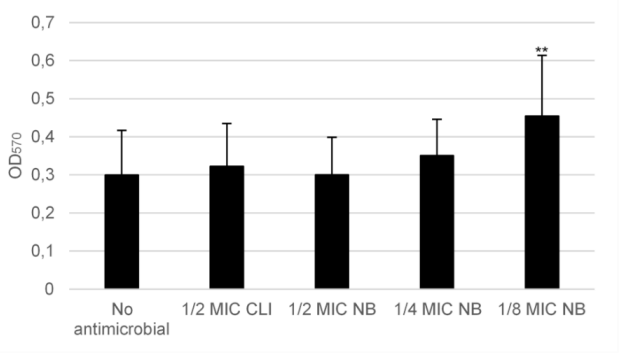


Figure 3.19 | Overall effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on the ability of *S. aureus* DFI isolates to form biofilm. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated in a microtiter plate with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC for 48h at 37ºC. Then, microtiter plate was incubated in an ultrasound bath to disperse biofilm-based bacteria from the microtiter plate surface and the OD at 570 nm was measured. Values are means ± SD (three repeated different experiments for each *S. aureus* clinical isolate). Asterisks indicate significant differences between treatments and control (** $p<0.01$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration. OD_{570}: optical density at 570 nm.

Results shown in Figure 3.18 demonstrate that different *S. aureus* DFI isolates have different responses to sub-MICs of nisin-biogel and clindamycin regarding biofilm formation. For isolate A 5.2, nisin-biogel and clindamycin at subinhibitory levels decrease the ability of this *S. aureus* DFI isolate to form a biofilm, with nisin-biogel at 1/8 MIC being the one that least decreased biofilm formation. Regarding isolate A 6.3, clindamycin at 1/2 MIC decreased its ability to form biofilm, nisin-biogel at 1/4 MIC had no effect on biofilm formation, and nisin-biogel at 1/2 and 1/8 MIC increased biofilm formation by this isolate. For isolates B 1.1 and B 14.2, nisin-biogel and clindamycin at sub-MICs increase isolate's ability to form a biofilm, with the nisin-biogel at 1/8 MIC being the one that most increase biofilm formation. For isolates Z 1.1 and Z 5.2, clindamycin at 1/2 MIC and nisin-biogel at 1/4 and 1/8 MIC increased ability of these *S. aureus* clinical isolates to form a biofilm, whereas nisin-biogel at 1/2 MIC had no effect on biofilm formation by isolate Z 1.1 and decreased ability of *S. aureus* clinical isolate Z 5.2 to form biofilm.

Analyzing the overall effects of nisin-biogel and clindamycin at sub-MICs on the ability of *S. aureus* DFI isolates to form biofilm represented in Figure 3.19, it can be observed that nisin-biogel and clindamycin at 1/2 MIC had no effect on biofilm formation, nisin-biogel at 1/4 MIC exhibit a trend to increase biofilm formation and nisin-biogel at 1/8 MIC significantly increased biofilm formation by *S. aureus* DFI isolates. These results are consistent with the ones regarding the expression of *icaA* and *icaD,* where it was found that nisin-biogel at 1/8 MIC was the concentration of nisin-biogel that increased these genes expression more significantly, as observed regarding the ability of *S. aureus* DFI isolates to form a biofilm. Moreover, as nisin-biogel at sub-MICs led to a decrease in *agrI* expression and *agrI* repression is essential for biofilm formation, it was expected that nisin-biogel at sub-MICs will lead to an increase in the biofilm-forming ability of *S. aureus* DFI isolates.

Biofilms play a major role in the pathogenesis of *S. aureus*, and several studies have shown that sub-MICs of some antimicrobials can affect bacterial biofilm formation *in vitro*, which may have clinical importance (Andre, de Jesus Pimentel-Filho, de Almeida Costa, & Vanetti, 2019; Angelopoulou et al., 2020; Kaplan, 2011). According to our results, the lower the concentration of nisin-biogel, the higher the trend to increase biofilm formation by *S. aureus* DFI isolates, which may be related to the fact that these conditions also contribute to a lower inhibition of bacterial growth, as previously demonstrated by our study, resulting in a higher ability to form a biofilm. Moreover, as stated by other authors, the increase in biofilm formation may be due to the fact that sublethal concentrations of antimicrobials are cell stressors, which can enhance the production of biofilm matrix polymers (Eroshenko, Polyudova, & Korobov, 2017; Kaplan, 2011).

Angelopoulou et al., 2020 observed that nisin at 1/2, 1/4 and 1/8 MIC significantly increased biofilm formation for the *S. aureus* strains under testing, with the concentration of 1/8 MIC being the one that most influenced biofilm formation in most of the strains under study. These results seem to be in line with what we have observed in this study, although we tested nisin-biogel and not nisin alone. However, Andre et al., 2019 reported different effects of subinhibitory concentrations of nisin, associating they with a reduction in biofilm formation, which suggests the need to use a large collection of *S. aureus* DFI isolates for the evaluation of the effects of nisin-biogel at subinhibitory levels on biofilm-forming ability by *S. aureus*, as different strains may present different changes in biofilm formation, according to the strain background.

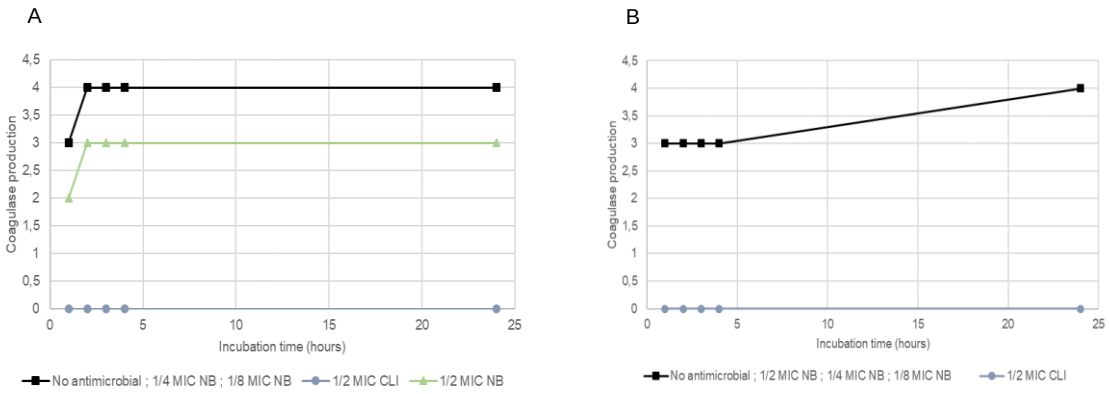
Regarding clindamycin, results by Majidpour et al., 2017 and Schilcher et al., 2016 demonstrated that a concentration corresponding to 1/2 MIC can inhibit or enhance biofilm formation, depending on the *S. aureus* strain under investigation. This is consistent with what we report in the present study, as the effects of clindamycin at 1/2 MIC on biofilm formation depended on the *S. aureus* DFI isolates. In general, our results have shown that clindamycin at 1/2 MIC had no effect on biofilm formation, although the expression of the *icaA* and *icaD* exhibited a trend to decrease when the *S. aureus* DFI isolates were exposed to clindamycin at 1/2 MIC. These results allow us to suggest that the formation of biofilm by these *S. aureus* clinical isolates may not only be associated with *ica*. It may also be associated with other surface adhesion proteins, as is the case of Bap.

Variations in mRNA levels of virulence determinants may occur both by transcriptional modulation or by posttranscriptional mechanisms. Furthermore, alterations in the mRNA levels of virulence determinants in the presence of subinhibitory levels of antimicrobials do not always result in changes in protein synthesis or functional activity (Hodille et al., 2017). Accordingly, we also determined whether coagulase functional activity and SpA expression levels were associated with mRNA quantification.

3.6. Effects of nisin-biogel at sub-MICs on Coa production by *S. aureus* DFI isolates

In the present study, a coagulase test was used to monitor coagulase production by S. aureus DFI isolates in the presence and absence of nisin-biogel and clindamycin at sub-MICs. Results were considered valid if the control plasma showed no signs of clotting in all the observations. Different *S. aureus* DFI isolates showed different coagulase production ability over 4h and after 24h of incubation in the presence or absence of nisin-biogel and clindamycin sub-MICs, as shown in Figure 3.20 and Figure 3.21. Besides depending on the *S. aureus* DFI isolate, the effects of the presence of nisin-biogel and clindamycin at sub-MICs on coagulation depended on the bacterial growth period.

As shown in Figure 3.20, for the isolates incubated during 24 hours in the presence or absence of nisin-biogel and clindamycin at sub-MICs, during the 4h period that coagulase production was monitored on an hourly basis, it was found that clotting in the absence of nisin-biogel sub-MICs was higher than or equal to the one obtained in the presence of nisin-biogel at sub-MICs, except for the isolate B 1.1 in the presence of nisin-biogel at 1/2 MIC and for the isolate Z 1.1 in the presence of nisin-biogel at 1/2 and 1/8 MIC. After 24h of incubation, isolates A 6.3 and B 1.1 showed the same signs of coagulation in the presence or absence of nisin-biogel at sub-MICs, isolates A 5.2 and B 14.2 showed the same signs of coagulation in the presence or absence of nisin-biogel at 1/4 and 1/8 MIC, whereas in the presence of nisin-biogel at 1/2 MIC coagulase production was smaller than in the absence of nisin-biogel, isolate Z 1.1 showed the same signs of coagulation in the presence or absence of nisin-biogel at 1/4 MIC and higher signs of coagulation in the presence of nisin-biogel at 1/2 and 1/8 MIC than in the absence of nisin-biogel, and isolate Z 5.2 showed smaller coagulase production in the presence of nisin-biogel at sub-MICs than in the absence of nisin-biogel. Regarding the effect of clindamycin at 1/2 MIC, there was no signs of coagulation by isolates A 5.2, A 6.3 and Z 5.2 in the presence of the antibiotic, whereas for isolates B 1.1, B 14.2 and Z 1.1, signs of coagulation were equal to or higher than the ones observed in the absence of clindamycin at 1/2 MIC, both during the 4h period that coagulase production was monitored on an hourly basis and after 24h of incubation.



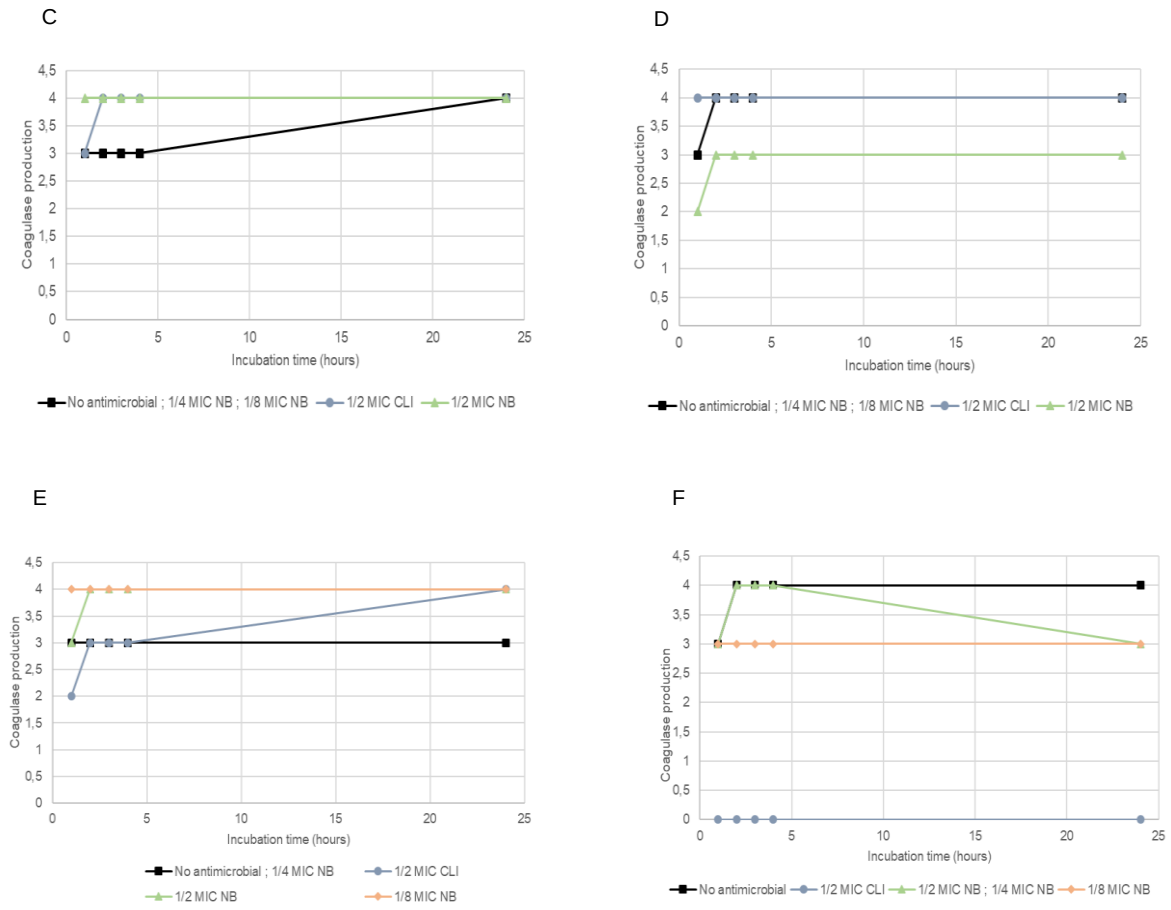


Figure 3.20 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on Coa production by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively, after 24h of incubation with the different conditions under study. Coagulation ability was measured every hour for 4h of incubation and after 24h of incubation. Values are means (two repeated different experiments for each *S. aureus* clinical isolate). The values on the x-axis represent the production of coagulase, where 0 corresponds to no evidence of fibrin formation, 1 corresponds to small unorganized clots, 2 corresponds to small organized clots, 3 corresponds to large organized clots, and 4 corresponds to the coagulation of the entire content of tube. NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration.

As shown in Figure 3.21, for the isolates incubated for 4h in the different conditions under study, results were slightly different. During the 4 hours' period during which coagulase production was monitored on an hourly basis, results were quite heterogeneous among the different *S. aureus* DFI isolates, but in most cases coagulation in the presence of nisin-biogel at sub-MICs was smaller than or similar to the one in the absence of nisin-biogel at sub-MICs. After 24h of incubation, all *S. aureus* clinical isolates, except for isolate B 14.2 in the presence of nisin-biogel at 1/2 MIC, showed the same signs of coagulation in the presence or absence of nisin-biogel at sub-MICs. Regarding the effect of clindamycin at 1/2 MIC, all clinical isolates, except for isolate B 14.2 at 4 hours' time point, showed no signs of coagulation during the 4h period that coagulase production was monitored on an hourly basis. Oppositely, all clinical isolates showed the maximum signs of coagulation after 24h incubation in the presence of this antibiotic.

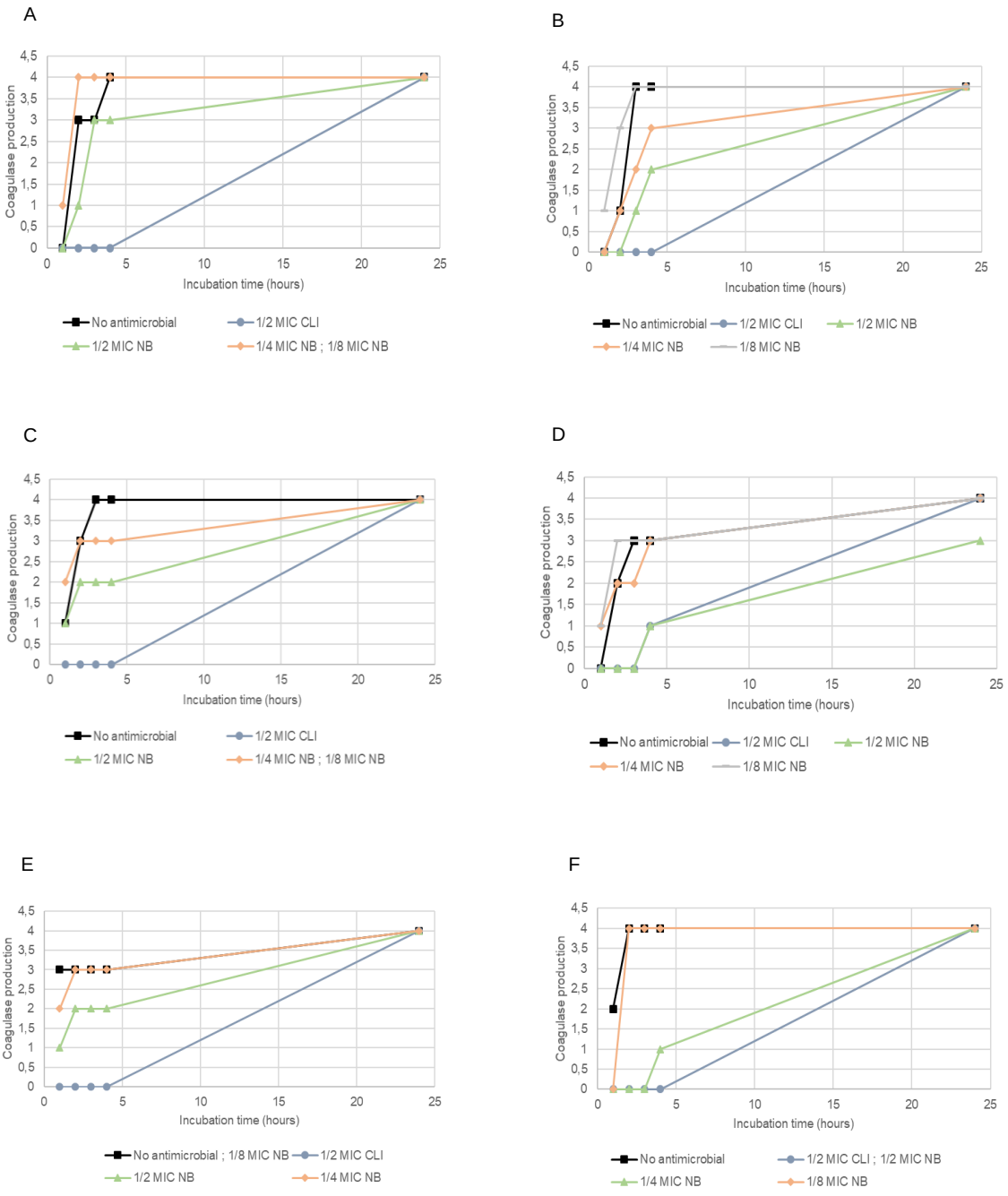


Figure 3.21 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on Coa production by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively, after 4h of incubation with the different conditions under study. Coagulation ability was measured every hour for 4h of incubation and after 24h of incubation. Values are means (two repeated different experiments for each *S. aureus* clinical isolate). The values on the x-axis represent the production of coagulase, where 0 corresponds to no evidence of fibrin formation, 1 corresponds to small unorganized clots, 2 corresponds to small organized clots, 3 corresponds to large organized clots, and 4 corresponds to the coagulation of the entire content of tube. NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration.

Coa promotes modifications of the coagulation cascade and, consequently, plays an antiphagocytic role during host infection. The blockage of Coa activity may reduce the severity of staphylococcal infections, preventing *S. aureus* pathogenesis (McAdow, Missiakas, & Schneewind, 2012). As results have shown, there was a significant increase of *coa* expression in the presence of subinhibitory levels of nisin-biogel and clindamycin, which may impair infection treatment, since host clearance mechanisms against the pathogen can be less efficient, allowing it to remain in the host. However, variations in coagulase mRNA levels induced by subinhibitory levels of antimicrobials may not result in changes in coagulase functional activity (Hodille et al., 2017). When accessing coagulase production, results were different, as most of the *S. aureus* DFI isolates showed a similar coagulase activity in the presence of nisin-biogel and clindamycin at sub-MICs compared to those obtained in the absence of these antimicrobials. Thus, our findings show that *coa* expression variations that occur in the presence of nisin-biogel and clindamycin at sub-MICs are not directly related to coagulation ability, which allows suggesting that increased *coa* mRNA levels may not be directly associated with increased *S. aureus* DFI isolates adherence.

Blickwede et al., 2005 and Herbert et al., 2001 concluded that the increased levels of coagulase mRNA in the presence of clindamycin at 1/2 MIC were probably due to an increase in mRNA stability, since clindamycin at 1/2 MIC induced increased levels of *coa* mRNA, with an associated decrease in coagulase activity. According to these results, it can be suggested that mRNA stability is on the basis of the increased levels of *coa* mRNA that were observed in the present study, not only in the presence of clindamycin at 1/2 MIC but also in the presence of nisin-biogel at sub-MICs. However, some complementary studies would have to be performed to confirm this conclusion.

3.7. Effects of nisin-biogel at sub-MICs on SpA release by *S. aureus* DFI isolates

SpA protein levels in the presence or absence of nisin-biogel and clindamycin at sub-MICs were determined using a specific SpA ELISA.

To determine the amount of SpA (pg/mL) in the bacterial supernatants after incubation in the presence or absence of nisin-biogel and clindamycin at sub-MICs, it was necessary to construct a standard curve using samples with known concentrations supplied by the manufacturer (Figure S2, available in Supplementary Data).

The effects of nisin-biogel and clindamycin at sub-MICs on SpA production relative to growth control are shown in Figure 3.22, whereas the overall effects of nisin-biogel and clindamycin at subinhibitory levels on SpA production relative to growth control are shown in Figure 3.23.

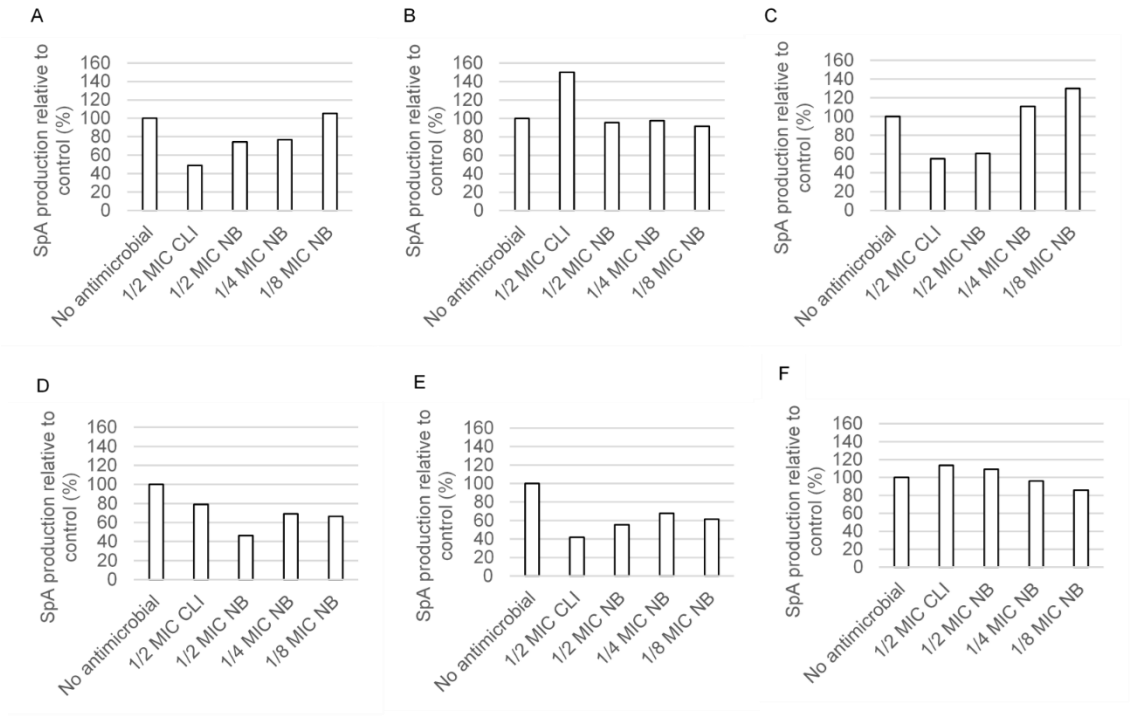


Figure 3.22 | A, B, C, D, E and F: Effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on SpA production by *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, respectively. Isolates were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC, and clindamycin at 1/2 MIC for 18h at 37ºC with orbital shaking (180 rpm). Bacterial supernatants were added to the 96-well plate provided with ELISA kit. After all steps of the assay were performed, optical density at 450 nm was measured and amount of protein A presented in the different bacterial supernatants was calculated using the standard curve. Results are ratios of the amount of SpA (pg/mL) in bacterial supernatants incubated with nisin-biogel and clindamycin at sub-MICs to the amount of SpA (pg/mL) in bacterial supernatants incubated without subinhibitory concentrations of antimicrobials, and are expressed as percentages. NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration. SpA: staphylococcal protein A.

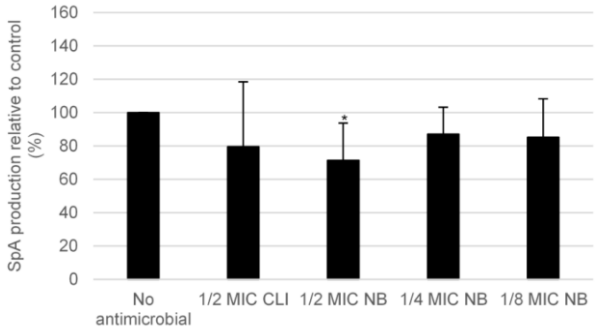


Figure 3.23 | Overall effects of nisin-biogel at 1/2 MIC, 1/4 MIC and 1/8 MIC, and clindamycin at 1/2 MIC on the production of SpA by *S. aureus* DFI isolates. Isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2 were incubated with or without nisin-biogel at 1/2, 1/4 or 1/8 MIC nisin-biogel, and clindamycin at 1/2 MIC for 18h at 37ºC with orbital shaking (180 rpm). Bacterial supernatants were added to the 96-well plate provided with ELISA kit. After all the steps of the assay were performed, optical density at 450 nm was measured and amount of protein A presented in the different bacterial supernatants was calculated using the standard curve. Results are the ratios of the amount of SpA (pg/mL) in bacterial supernatants incubated with nisin-biogel and clindamycin at sub-MICs to the amount of SpA (pg/mL) in bacterial supernatants incubated without subinhibitory concentrations of antimicrobials for all the *S. aureus* DFI isolates under study, and are expressed as percentages. Asterisks indicate statistically significant differences between treatments and control (* $p<0.05$). NB: nisin-biogel; CLI: clindamycin; MIC: minimum inhibitory concentration. SpA: staphylococcal protein A.

For isolate A 5.2, clindamycin at 1/2 MIC and nisin-biogel at 1/2 and 1/4 MIC decreased the amount of SpA, whereas nisin-biogel at 1/8 MIC slightly increased the production of SpA. Relative to isolate A 6.3, clindamycin at 1/2 MIC increased the production of SpA, while nisin-biogel at sub-MICs slightly decreased the amount of SpA, with nisin-biogel at 1/4 MIC being the one that least decreased SpA production. Regarding isolate B 1.1, clindamycin and nisin-biogel at 1/2 MIC decreased the amount of SpA, whereas nisin-biogel at 1/4 and 1/8 MIC increased the production of SpA. For isolates B 14.2 and Z 1.1, nisin-biogel and clindamycin at sub-MICs decreased the production of SpA. Regarding nisin-biogel, a concentration corresponding to 1/4 MIC was the one that least decreased SpA production in both isolates. Finally, isolate Z 5.2 slightly increased SpA production in the presence of clindamycin and nisin-biogel at 1/2 MIC, and slightly decreased the production of SpA in the presence of nisin-biogel at 1/4 and 1/8 MIC.

Different *S. aureus* DFI isolates had different responses regarding SpA production in the presence of clindamycin and nisin-biogel at sub-MICs, suggesting differential effects depending on the *S. aureus* DFI isolate.

Overall results suggested that clindamycin and nisin-biogel at sub-MICs exhibit a trend to decrease SpA production, with nisin-biogel at 1/2 MIC being the only one that significantly decreased SpA production.

SpA is a cell wall-anchored surface protein with a high affinity to the Fc portion of the IgG class of immunoglobulins, protecting *S. aureus* from opsonophagocytic clearance and, consequently, playing a role in the pathogenesis of staphylococcal infections (Kobayashi & DeLeo, 2013). As results have shown, SpA protein levels exhibit a trend to decrease in the presence of nisin-biogel and clindamycin at sub-MICs. Regarding nisin-biogel at sub-MICs, results were not consistent with what happened to SpA mRNA levels, as nisin-biogel at sub-MICs led to an increase in SpA mRNA. This may suggest that the inhibition of virulence expression by nisin-biogel is associated with the blockage of protein translation at the ribosome, and not with the transcription of virulence factor genes. This may be related to bacterial membrane pore formation by nisin, which can lead to the release of molecules essential to translation. Regarding clindamycin, SpA mRNA levels significantly decreased and protein levels exhibited a trend to decrease in the presence of clindamycin at 1/2 MIC of this antibiotic, which is possibly related to the fact that clindamycin is a protein synthesis inhibitor and has an inhibitory effect on the transcription of *spA*. Results regarding clindamycin are in line with a previous report by Otto et al., 2013, which showed that subinhibitory concentrations of clindamycin decreased the levels of SpA mRNA and protein.

3.8. Future perspectives

Results from this experimental work originated new questions that would be interesting to answer in the future. First, it was shown that nisin-biogel at sub-MICs significantly decreased *agrI* expression. As this quorum-sensing system upregulates the expression of exotoxins by *S.*

aureus, it would be interesting to evaluate the effects of nisin-biogel at sub-MICs on exotoxins expression. Second, it was observed that the effects of nisin-biogel on virulence genes expression depended on the *S. aureus* DFI isolate, on the subinhibitory concentration and on the virulence gene under study. As such, it would be interesting to sequence the different *S. aureus* DFI isolates grown under the different conditions tested to determine whether the alterations in virulence genes expression are due or not to different mutations in these genes. Finally, it would be interesting to investigate a longer collection of *S. aureus* DFI isolates, in order to generate results with higher statistical significance.

Chapter 4 | Conclusion

A guar gum gel-based delivery system for nisin, nisin-biogel, was developed aiming to be an alternative treatment for DFIs, as antibiotic resistance is one of the major health concerns of our time. As for all new antimicrobial compounds for clinical application, it is essential to confirm its efficacy and security before proceeding to clinical trials. Accordingly, the effect of antimicrobial agents on bacterial virulence factors has become a major focal point in the study of new antimicrobial alternatives, helping to define proper doses of antimicrobials administration.

S. aureus produces a wide variety of virulence factors, such as adherence and colonization factors, exotoxins and enzymes, and forms biofilms, which contribute to its ability to colonize host tissues and cause diseases, making it difficult to control staphylococcal infections.

The present experiments demonstrated that nisin-biogel at subinhibitory levels affect the growth of *S. aureus* in a strain-dependent and dose-dependent manner, not affecting the typical sigmoid curve of bacterial growth. The different virulence genes under study, including *agrI, spA, coa, clfA, atl, icaA* and *icaD*, are not all expressed in the same phase of *S. aureus* growth, meaning that the assays aiming to access the effects of nisin-biogel on virulence genes expression by *S. aureus* DFI isolates had to be performed at the optimal growth period for each gene expression. Nisin-biogel at sub-MICs differentially affected virulence factors expression, depending on the *S. aureus* DFI isolate, on the antimicrobial agent, on the subinhibitory concentration and on the virulence factor under study. Analyzing the overall effects on the *S. aureus* DFI isolates A 5.2, A 6.3, B 1.1, B 14.2, Z 1.1 and Z 5.2, the expression of some virulence-related genes, such as *agrI, atl* and *clfA*, were found to be repressed by nisin-biogel at sub-MICs, whereas the transcription levels of *spA, coa, icaA* and *icaD* exhibited a trend to increase. Nisin-biogel at 1/2 MIC had no effect on biofilm formation, nisin-biogel at 1/4 MIC exhibited a trend to increase biofilm formation and nisin-biogel at 1/8 MIC significantly increased biofilm formation by *S. aureus* DFI isolates under study. Regarding Coa functional activity and SpA production, nisin-biogel at sub-MICs had no relevant effect on Coa production by *S. aureus* DFI isolates, and exhibited a trend to decrease SpA release by these isolates.

Results highlight the importance of accessing the effects of nisin-biogel sub-MICs at different levels, providing an *in vitro* basis for understanding what happens *in vivo* during a DFI treatment, and reinforce how critical is the proper establishment of antimicrobials doses to be applied in clinical practice.

This study, together with previous studies performed at the Laboratory of Microbiology and Immunology of FMV/CIISA highlights the potential of nisin-biogel to be considered as an alternative antimicrobial therapy for DFIs.

References

Abdel Haleem, R. T., El Nagdy, M. M., Omar, N. S., El-Nahas, M. R., & Badr, R. I. (2015). Identification of Virulence Factors, Accessory Gene Regulator (*agr*) Locus and Antibiotic Resistance Pattern of *Staphylococcus aureus* Isolated from Diabetic Foot Ulcers. *The Egyptian Journal of Medical Microbiology, 24*(4), 41–47.

Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C., & Tarkowski, A. (1993). The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infection and Immunity*, *61*(9), 3879-3885.

Alexander, E. H., & Hudson, M. C. (2001). Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. *Applied Microbiology and Biotechnology*, *56*(3–4), 361–366.

American Diabetes Association. (2013). Diagnosis and classification of Diabetes *mellitus*, ADA Clinical Practice Recommendations. *Diabetes Care*, *36*(Suppl 1), S67-74.

Amin, N., & Doupis, J. (2016). Diabetic foot disease: from the evaluation of the “foot at risk” to the novel diabetic ulcer treatment modalities. *World journal of diabetes*, *7*(7), 153.

Andersson, D. I., & Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology, 12*(7), 465-478.

Andre, C., de Jesus Pimentel-Filho, N., de Almeida Costa, P. M., & Vanetti, M. C. D. (2019). Changes in the composition and architecture of staphylococcal biofilm by nisin. *Brazilian Journal of Microbiology*, *50*(4), 1083-1090.

Angelopoulou, A., Field, D., Pérez-Ibarreche, M., Warda, A. K., Hill, C., & Ross, R. P. (2020). Vancomycin and nisin A are effective against biofilms of multi-drug resistant Staphylococcus aureus isolates from human milk. *Plos one*, *15*(5), e0233284.

Armstrong, D. G., Boulton, A. J., & Bus, S. A. (2017). Diabetic foot ulcers and their recurrence. *New England Journal of Medicine*, *376*(24), 2367-2375.

Atshan, S. S., Shamsudin, M. N., Karunanidhi, A., van Belkum, A., Lung, L. T. T., Sekawi, Z., ... & Abduljaleel, S. A. (2013). Quantitative PCR analysis of genes expressed during biofilm development of methicillin resistant *Staphylococcus aureus* (MRSA). *Infection, genetics and evolution, 18*, 106-112.

Bali, V., Panesar, P. S., Bera, M. B., & Kennedy, J. F. (2016). Bacteriocins: recent trends and potential applications. *Critical reviews in food science and nutrition, 56*(5), 817-834.

Barna, J. C., & Williams, D. H. (1984). The structure and mode of action of glycopeptide antibiotics of the vancomycin group. *Annual Review of Microbiology*, *38*, 339–357.

Bezar, I. F., Mashruwala, A. A., Boyd, J. M., & Stock, A. M. (2019). Drug-like fragments inhibit *agr*-mediated virulence expression in *Staphylococcus aureus*. *Scientific reports*, *9*(1), 1-14.

Biswas, R., Voggu, L., Simon, U. K., Hentschel, P., Thumm, G., & Götz, F. (2006). Activity of the major staphylococcal autolysin Atl. *FEMS microbiology letters*, *259*(2), 260-268.

Blickwede, M., Wolz, C., Valentin-Weigand, P., & Schwarz, S. (2005). Influence of clindamycin on the stability of *coa* and *fnbB* transcripts and adherence properties of *Staphylococcus aureus* Newman. *FEMS microbiology letters*, *252*(1), 73-78.

Bronner, S., Monteil, H., & Prévost, G. (2004). Regulation of virulence determinants in

Staphylococcus aureus: complexity and applications. *FEMS microbiology reviews*, *28*(2), 183-200.

Brown, D. F., & Reynolds, P. E. (1980). Intrinsic resistance to β-lactam antibiotics in *Staphylococcus aureus*. *FEBS letters*, *122*(2), 275-278.

Chambers, H. (2001). The Changing Epidemiology of *Staphylococcus aureus*? *Emerging Infectious Diseases*, *7*(2), 178–182.

Chang, S., Sievert, D. M., Hageman, J. C., Boulton, M. L., Tenover, F. C., Downes, F. P., ... & Cardo, D. (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *New England Journal of Medicine*, *348*(14), 1342-1347.

Chastain, C. A., Klopfenstein, N., Serezani, C. H., & Aronoff, D. M. (2019). A clinical review of diabetic foot infections. *Clinics in podiatric medicine and surgery*, *36*(3), 381-395.

Chawla, A., Chawla, R., & Jaggi, S. (2016). Microvasular and macrovascular complications in Diabetes *mellitus*: Distinct or continuum? *Indian Journal of Endocrinology and Metabolism*, *20*(4), 546–553.

Cheung, G. Y., Wang, R., Khan, B. A., Sturdevant, D. E., & Otto, M. (2011). Role of the accessory gene regulator *agr* in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *Infection and Immunity*, *79*(5), 1927-1935.

Cotter, P. D., Ross, R. P., & Hill, C. (2013). Bacteriocins – a viable alternative to antibiotics? *Nature Reviews Microbiology*, *11*(2), 95–105.

Dancer, S. J. (2008). The effect of antibiotics on methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, *61*(2), 246-253.

Davies, J., Spiegelman, G. B., & Yim, G. (2006). The world of subinhibitory antibiotic concentrations. *Current opinion in microbiology, 9*(5), 445-453.

Delves-Broughton, J. (1990). Nisin and its application as a food preservative. *International Journal of Dairy Technology*, *43*(3), 73–76.

Derveaux, S., Vandesompele, J., & Hellemans, J. (2010). How to do successful gene expression analysis using real-time PCR. *Methods*, *50*(4), 227-230.

Dickschat, J. S. (2010). Quorum-sensingand bacterial biofilms. *Natural Product Reports*, *27*(3), 343–369.

Dunyach-Remy, C., Essebe, C. N., Sotto, A., & Lavigne, J. P. (2016). *Staphylococcus aureus* toxins and diabetic foot ulcers: Role in pathogenesis and interest in diagnosis. *Toxins*, *8*(7), 1–20.

El-baz, R., Rizk, D. E., Barwa, R., & Hassan, R. (2017). Virulence characteristics and molecular relatedness of methicillin resistant *Staphylococcus aureus* harboring different staphylococcal cassette chromosome *mec*. *Microbial Pathogenesis*, 113, 385–395.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences of the United States of America*, *99*(11), 7687–7692.

Eroshenko, D., Polyudova, T., & Korobov, V. (2017). N-acetylcysteine inhibits growth, adhesion and biofilm formation of Gram-positive skin pathogens. *Microbial Pathogenesis*, *105*, 145-152.

Everett, E., & Mathioudakis, N. (2018). Update on management of diabetic foot ulcers. *Annals of the New York Academy of Sciences*, *1411*(1), 153–165.

Field, D., Blake, T., Mathur, H., O'Connor, P. M., Cotter, P. D., Paul Ross, R., & Hill, C. (2019). Bioengineering nisin to overcome the nisin resistance protein. *Molecular microbiology*, *111*(3), 717-731.

Field, D., O'Connor, R., Cotter, P. D., Ross, R. P., & Hill, C. (2016). *In vitro* activities of nisin and nisin derivatives alone and in combination with antibiotics against *Staphylococcus* biofilms. *Frontiers in microbiology*, *7*, 508.

Fjell, C. D., Hiss, J. A., Hancock, R. E. W., & Schneider, G. (2012). Designing antimicrobial peptides: Form follows function. *Nature Reviews Drug Discovery*, *11*(1), 37–51.

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), 563.

Francois, P., Koessler, T., Huyghe, A., Harbarth, S., Bento, M., Lew, D., ... & Schrenzel, J. (2006). Rapid *Staphylococcus aureus agr* type determination by a novel multiplex real-time quantitative PCR assay. *Journal of clinical microbiology, 44*(5), 1892-1895.

Fuda, C. C. S., Fisher, J. F., & Mobashery, S. (2005). β-Lactam resistance in *Staphylococcus aureus*: The adaptive resistance of a plastic genome. *Cellular and Molecular Life Sciences*, *62*(22), 2617–2633.

Gemmell, C. G., & O'Dowd, A. (1983). Regulation of protein A biosynthesis in *Staphylococcus aureus* by certain antibiotics: its effect on phagocytosis by leukocytes. *Journal of Antimicrobial Chemotherapy*, *12*(6), 587-597.

Ghasemian, A., Peerayeh, S. N., Bakhshi, B., & Mirzaee, M. (2015). The microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) genes among clinical isolates of *Staphylococcus aureus* from hospitalized children. *Iranian journal of pathology*, *10*(4), 258.

Godoy-Santos, F., Pitts, B., Stewart, P. S., & Mantovani, H. C. (2018). Nisin penetrates *Staphylococcus aureus* biofilms but shows differences in killing effects against sessile and planktonic cells. *BioRxiv*, 303636.

Gómez, M. I., O'Seaghdha, M., Magargee, M., Foster, T. J., & Prince, A. S. (2006). *Staphylococcus aureus* protein A activates TNFR1 signaling through conserved IgG binding domains. *Journal of Biological* Chemistry, 281(29), 20190–20196.

Grigoropoulou, P., Eleftheriadou, I., Jude, E. B., & Tentolouris, N. (2017). Diabetic foot infections: an update in diagnosis and management. *Current diabetes reports*, *17*(1), 3.

Herbert, S., Barry, P., & Novick, R. P. (2001). Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus. Infection and Immunity*, 69(5), 2996-3003.

Herman-Bausier, P., Labate, C., Towell, A. M., Derclaye, S., Geoghegan, J. A., & Dufrêne, Y. F. (2018). *Staphylococcus aureus* clumping factor A is a force-sensitive molecular switch that activates bacterial adhesion. *Proceedings of the National Academy of Sciences*, *115*(21), 5564-5569.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., & Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *The Journal of antimicrobial chemotherapy, 40*(1), 135-136.

Hodille, E., Rose, W., Diep, B. A., Goutelle, S., Lina, G., & Dumitrescu, O. (2017). The role of antibiotics in modulating virulence in *Staphylococcus aureus*. *Clinical microbiology reviews*, *30*(4), 887-917.

Holden, M. T. G., Hsu, L. Y., Kurt, K., Weinert, L. A., Mather, A. E., Harris, S. R., … Nübel, U.

(2013). A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic. *Genome Research*, *23*(4), 653–664.

Houston, P., Rowe, S. E., Pozzi, C., Waters, E. M., & O'Gara, J. P. (2011). Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. *Infection and Immunity*, *79*(3), 1153-1165.

Hsu, S. T. D., Breukink, E., Tischenko, E., Lutters, M. A. G., De Kruijff, B., Kaptein, R., … Van Nuland, N. A. J. (2004). The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. *Nature Structural and Molecular Biology*, *11*(10), 963–967.

Hurlow, J. J., Humphreys, G. J., Bowling, F. L., & McBain, A. J. (2018). Diabetic foot infection: A critical complication. *International wound journal*, *15*(5), 814-821.

International Diabetes Federation. (2019). *IDF Diabetes* Atlas, 9th Edition. Retrieved from https://www.diabetesatlas.org/en/

Jenssen, H., Hamill, P., & Hancock, R. E. (2006). Peptide antimicrobial agents. *Clinical microbiology reviews*, *19*(3), 491-511.

Jenul, C., & Horswill, A. R. (2018). Regulation of *Staphylococcus aureus* Virulence. *Microbiology Spectrum*, *6*(1).

Jneid, J., Lavigne, J. P., La Scola, B., & Cassir, N. (2017). The diabetic foot microbiota: A review. *Human Microbiome Journal*, *5–6*, 1–6.

John, A. T., & Sharkey, M. (2018). Diabetic foot infections. *Emergency Management of Infectious Diseases*, 228.

Joo, H. S., Chatterjee, S. S., Villaruz, A. E., Dickey, S. W., Tan, V. Y., Chen, Y., ... & Otto, M. (2016). Mechanism of gene regulation by a *Staphylococcus aureus* toxin. *MBio*, *7*(5), e01579-16.

Josefsson, E., Kubica, M., Mydel, P., Potempa, J., & Tarkowski, A. (2008). *In vivo* sortase A and clumping factor A mRNA expression during *Staphylococcus aureus* infection. *Microbial pathogenesis*, *44*(2), 103-110.

Joseph, W. S., & Lipsky, B. A. (2010). Medical therapy of diabetic foot infections. *Journal of Vascular Surgery*, *52*(3), 67S-71S.

Kaplan, J. Á. (2010). Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of dental research*, *89*(3), 205-218.

Kawada-Matsuo, M., Watanabe, A., Arii, K., Oogai, Y., Noguchi, K., Miyawaki, S., ... & Komatsuzawa, H. (2020). *Staphylococcus aureus* Virulence Affected by an Alternative Nisin A Resistance Mechanism. *Applied and environmental microbiology*, *86*(8).

Kearns, A. M., Seiders, P. R., Wheeler, J., Freeman, R., & Steward, M. (1999). Rapid detection of methicillin-resistant staphylococci by multiplex PCR. *Journal of Hospital Infection*, *43*(1), 33-37.

Kim, M. K., Zhao, A., Wang, A., Brown, Z. Z., Muir, T. W., Stone, H. A., & Bassler, B. L. (2017). Surface-attached molecules control *Staphylococcus aureus* quorum-sensingand biofilm development. *Nature microbiology*, *2*(8), 17080.

Kobayashi, S. D., & DeLeo, F. R. (2013). *Staphylococcus aureus* protein A promotes immune suppression. *MBio*, *4*(5).

Kong, K. F., Vuong, C., & Otto, M. (2006). *Staphylococcus* quorum-sensingin biofilm formation and infection. *International Journal of Medical Microbiology*, 296(2-3), 133–139.

Kramer, N. E., Van Hijum, S. A. F. T., Knol, J., Kok, J., & Kuipers, O. P. (2006). Transcriptome analysis reveals mechanisms by which *Lactococcus lactis* acquires nisin resistance. *Antimicrobial Agents and Chemotherapy*, *50*(5), 1753–1761.

Kumar, P., Kizhakkedathu, J. N., & Straus, S. K. (2018). Antimicrobial peptides: Diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. *Biomolecules*, *8*(1), 4.

Kuzuya, T. (2000). Early diagnosis, early treatment and the new diagnostic criteria of Diabetes *mellitus*. *British Journal of Nutrition*, *84*(S2), S177–S181.

Kwon, K. T., & Armstrong, D. G. (2018). Microbiology and antimicrobial therapy for diabetic foot infections. *Infection & chemotherapy*, *50*(1), 11-20.

Lavery, L. A., Armstrong, D. G., Murdoch, D. P., Peters, E. J. G., & Lipsky, B. A. (2007). Validation of the Infectious Diseases Society of America's Diabetic Foot Infection Classification System. *Clinical Infectious Diseases*, *44*(4), 562–565.

Lebeau, C., Vandenesch, F., Greenland, T., Novick, R. P., & Etienne, J. (1994). Coagulase expression in *Staphylococcus aureus* is positively and negatively modulated by an *agr*-dependent mechanism. *Journal of bacteriology*, *176*(17), 5534-5536.

Li, J., Xie, S., Ahmed, S., Wang, F., Gu, Y., Zhang, C., ... & Cheng, G. (2017). Antimicrobial activity and resistance: influencing factors. *Frontiers in pharmacology, 8*, 364.

Li, T., Zhang, L., Han, L. I., Wang, G., Yin, P., Li, Z., ... & Tang, P. (2016). Early application of negative pressure wound therapy to acute wounds contaminated with *Staphylococcus aureus*: an effective approach to preventing biofilm formation. *Experimental and therapeutic medicine*, *11*(3), 769-776.

Lipsky, B. A., Aragón-Sánchez, J., Diggle, M., Embil, J., Kono, S., Lavery, L., … Van Asten, S. (2016). IWGDF guidance on the diagnosis and management of foot infections in persons with diabetes. *Diabetes/Metabolism Research and Reviews*, *32*, 45–74.

Lipsky, B. A., Senneville, É., Abbas, Z. G., Aragón-Sánchez, J., Diggle, M., Embil, J. M., ... & Urbančič-Rovan, V. (2020). Guidelines on the diagnosis and treatment of foot infection in persons with diabetes (IWGDF 2019 update). *Diabetes/Metabolism Research and Reviews, 36*, e3280.

Liu, D., Li, Z., Wang, G., Li, T., Zhang, L., & Tang, P. (2018). Virulence analysis of *Staphylococcus aureus* in a rabbit model of infected full-thickness wound under negative pressure wound therapy. *Antonie van Leeuwenhoek*, *111*(2), 161-170.

Liu, P. Y., Shi, Z. Y., & Sheu, W. H. H. (2012). Diagnosis and treatment of diabetic foot infections. *Journal of Internal Medicine of Taiwan*, *23*(6), 431–441.

Lowy, F. D. (2003). Antimicrobial resistance: The example of *Staphylococcus aureus*. *Journal of Clinical Investigation*, *111*(9), 1265–1273.

Maira-Litrán, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., Goldmann, D. A., & Pier, G. B. (2002). Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity*, *70*(8), 4433–4440.

Majidpour, A., Fathizadeh, S., Afshar, M., Rahbar, M., Boustanshenas, M., Heidarzadeh, M., ... & Moghadam, S. S. (2017). Dose-dependent effects of common antibiotics used to treat *Staphylococcus aureus* on biofilm formation. *Iranian journal of pathology*, *12*(4), 362.

Markakis, K., Faris, A. R., Sharaf, H., Faris, B., Rees, S., & Bowling, F. L. (2018). Local Antibiotic Delivery Systems: Current and Future Applications for Diabetic Foot Infections. *The International Journal of Lower Extremity Wounds*, *17*(1), 14–21.

McAdow, M., Missiakas, D. M., & Schneewind, O. (2012). *Staphylococcus aureus* secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. *Journal of innate Immunity*, *4*(2), 141-148.

Melo-Cristino, J., Resina, C., Manuel, V., Lito, L., & Ramirez, M. (2013). First case of infection with vancomycin-resistant *Staphylococcus aureus* in Europe. *The Lancet, 382*(9888), 205.

Mendes, J. J., Marques-Costa, A., Vilela, C., Neves, J., Candeias, N., Cavaco-Silva, P., & Melo-Cristino, J. (2012). Clinical and bacteriological survey of diabetic foot infections in Lisbon. *Diabetes Research and Clinical Practice*, *95*(1), 153–161.

Mirhosseini, H., & Amid, B. T. (2012). A review study on chemical composition and molecular structure of newly plant gum exudates and seed gums. *Food Research International*, *46*(1), 387–398.

Moore, Z., & Cowman, S. (2008). A systematic review of wound cleansing for pressure ulcers. *Journal of clinical nursing, 17*(15), 1963-1972.

Mottola, C., Matias, C. S., Mendes, J. J., Melo-Cristino, J., Tavares, L., Cavaco-Silva, P., & Oliveira, M. (2016c). Susceptibility patterns of *Staphylococcus aureus* biofilms in diabetic foot infections. *BMC microbiology*, *16*(1), 119.

Mottola, C., Mendes, J. J., Cristino, J. M., Cavaco-Silva, P., Tavares, L., & Oliveira, M. (2016b). Polymicrobial biofilms by diabetic foot clinical isolates. *Folia microbiologica*, *61*(1), 35-43.

Mottola, C., Semedo-Lemsaddek, T., Mendes, J. J., Melo-Cristino, J., Tavares, L., Cavaco-Silva, P., & Oliveira, M. (2016a). Molecular typing, virulence traits and antimicrobial resistance of diabetic foot staphylococci. *Journal of biomedical science*, *23*(1), 33.

Moual, H. Le, Thomassin, J.-L., & Brannon, J. R. (2013). Antimicrobial Peptidesas an Alternative Approach to Treat Bacterial Infections. *Journal of Clinical & Cellular Immunology*.

Mudgil, D., Barak, S., & Khatkar, B. S. (2014). Guar gum: processing, properties and food applications – A Review. *Journal of Food Science and Technology*, *51*(3), 409–418.

Narsaiah, K., Jha, S. N., Wilson, R. A., Mandge, H. M., & Manikantan, M. R. (2014). Optimizing microencapsulation of nisin with sodium alginate and guar gum. *Journal of Food Science and Technology*, *51*(12), 4054–4059.

Nolan, T., Hands, R. E., & Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. *Nature protocols*, *1*(3), 1559-1582.

Noor, S., Khan, R. U., & Ahmad, J. (2017). Understanding Diabetic Foot Infection and its Management. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*, *11*(2), 149–156.

Nordström, R., & Malmsten, M. (2017). Delivery systems for antimicrobial peptides. *Advances in colloid and interface science*, *242*, 17-34.

Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology*, *48*(6), 1429–1449.

O'Driscoll, N. H., Labovitiadi, O., Cushnie, T. P. T., Matthews, K. H., Mercer, D. K., & Lamb, A. J. (2013). Production and evaluation of an antimicrobial peptide-containing wafer formulation for topical application. *Current Microbiology*, *66*(3), 271–278.

O'Gara, J. P. (2007). *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS microbiology letters*, *270*(2), 179-188.

Okuda, K. I., Zendo, T., Sugimoto, S., Iwase, T., Tajima, A., Yamada, S., … Mizunoe, Y. (2013). Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* biofilm. *Antimicrobial*

Agents and Chemotherapy, *57*(11), 5572–5579.

Oshida, T., Takano, M., Sugai, M., Suginaka, H., & Matsushita, T. (1998). Expression analysis of the autolysin gene (*atl*) of *Staphylococcus aureus*. *Microbiology and immunology*, *42*(9), 655-659.

Otto, M. (2019). Staphylococcal biofilms. *Gram-Positive Pathogens*, 699-711.

Otto, M. P., Martin, E., Badiou, C., Lebrun, S., Bes, M., Vandenesch, F., … Dumitrescu, O. (2013). Effects of subinhibitory concentrations of antibiotics factor expression by community-acquired methicillin-*Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, *68*(7), 1524–1532.

Paharik, A. E., & Horswill, A. R. (2016). The staphylococcal biofilm: adhesins, regulation, and host response. *Virulence Mechanisms of Bacterial Pathogens*, 529-566.

Patel, J. D., Colton, E., Ebert, M., & Anderson, J. M. (2012). Gene expression during *S. epidermidis* biofilm formation on biomaterials. *Journal of Biomedical Materials Research Part A*, *100*(11), 2863-2869.

Pasztor, L., Ziebandt, A. K., Nega, M., Schlag, M., Haase, S., Franz-Wachtel, M., ... & Götz, F. (2010). Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. *Journal of Biological Chemistry*, *285*(47), 36794-36803.

Pendsey, S. (2010). Understanding diabetic foot. *International Journal of Diabetes in Developing Countries*, *30*(2), 75.

Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J., & Schlievert, P. M. (1988). Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *Journal of bacteriology*, *170*(9), 4365-4372.

Perez, R., Perez, M. T., & Elegado, F. (2015). Bacteriocins from Lactic Acid Bacteria: A Review of Biosynthesis, Mode of Action, Fermentative Production, Uses, and Prospects. *International Journal of Philippine Science and Technology*, *8*(2), 61–67.

Periasamy, S., Joo, H. S., Duong, A. C., Bach, T. H. L., Tan, V. Y., Chatterjee, S. S., … Otto, M. (2012). How *Staphylococcus aureus* biofilms develop their characteristic structure. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(4), 1281–1286.

Peterson, E., & Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology, 9*, 2928.

Pitocco, D., Spanu, T., Di Leo, M., Vitiello, R., Rizzi, A., Tartaglione, L., … Sanguinetti, M. (2019). Diabetic foot infections: a comprehensive overview. *European Review for Medical and Pharmacological Sciences*, *23*(2 Suppl), 26–37.

Plata, K., Rosato, A. E., & Wegrzyn, G. (2009). *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polonica*, *56*(4), 597–612.

Porayath, C., Suresh, M. K., Biswas, R., Nair, B. G., Mishra, N., & Pal, S. (2018). Autolysin mediated adherence of *Staphylococcus aureus* with Fibronectin, Gelatin and Heparin. *International journal of biological macromolecules*, *110*, 179-184.

Pozzi, C., Bagnoli, F., & Rappuoli, R. (2016). *Staphylococcus aureus* coagulase R domain, a new evasion mechanism and vaccine target. *Journal of Experimental Medicine*, *213*(3).

Prabaharan, M. (2011). Prospective of guar gum and its derivatives as controlled drug delivery

systems. *International Journal of Biological Macromolecules*, *49*(2), 117–124.

Pratten, J., Foster, S. J., Chan, P. F., Wilson, M., & Nair, S. P. (2001). *Staphylococcus aureus* accessory regulators: expression within biofilms and effect on adhesion. *Microbes and infection*, *3*(8), 633-637.

Punthakee, Z., Goldenberg, R., & Katz, P. (2018). *2018 Clinical Practice Guidelines Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome Diabetes Canada Clinical Practice Guidelines Expert Committee*.

Punyauppa-path, S., & Phumkhachorn, P. (2015). Nisin: production and mechanism of antimicrobial action. *International Journal of Current Research and Review*, *7*(2), 47–53.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., & Ziebuhr, W. (2000). Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrobial agents and chemotherapy*, *44*(12), 3357-3363.

Ramirez-Acuña, J. M., Cardenas-Cadena, S. A., Marquez-Salas, P. A., Garza-Veloz, I., Perez-Favila, A., Cid-Baez, M. A., ... & Martinez-Fierro, M. L. (2019). Diabetic Foot Ulcers: Current Advances in Antimicrobial Therapies and Emerging Treatments. *Antibiotics*, *8*(4), 193.

Reddy, K. V. R., Yedery, R. D., & Aranha, C. (2004). Antimicrobial peptides: premises and promises. *International Journal of Antimicrobial Agents*, *24*(6), 536–547.

Reeks, B. Y., Champlin, F. R., Paulsen, D. B., Scruggs, D. W., & Lawrence, M. L. (2005). Effects of sub-minimum inhibitory concentration antibiotic levels and temperature on growth kinetics and outer membrane protein expression in *Mannheimia haemolytica* and *Haemophilus somnus*. *Canadian journal of veterinary research*, *69*(1), 1.

Reffuveille, F., Josse, J., Vallé, Q., Mongaret, C., & Gangloff, S. C. (2017). *Staphylococcus aureus* Biofilms and their Impact on the Medical Field. *The Rise of Virulence and Antibiotic Resistance in Staphylococcus aureus*, (11), 187-212.

Richard, J.-L., Lavigne, J.-P., & Sotto, A. (2012). Diabetes and foot infection: more than double trouble. *Diabetes/Metabolism Research and Reviews*, *28*, 46–53.

Ruffing, U., Abdulla, S., Alabi, A., Akulenko, R., Garrine, M., Germann, A., … Mellmann, A. (2016). Detecting *Staphylococcus aureus* Virulence and Resistance Genes : a Comparison of Whole-Genome Sequencing and DNA Microarray. *Journal of Clinical Microbiology*, *54*(4), 1008–1016.

Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., ... & Shaw, J. E. (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas. *Diabetes research and clinical practice*, *157*, 107843.

Salehzadeh, A., Zamani, H., Langeroudi, M. K., & Mirzaie, A. (2016). Molecular typing of nosocomial *Staphylococcus aureus* strains associated to biofilm based on the coagulase and protein A gene polymorphism. *Iranian journal of basic medical sciences*, *19*(12), 1325.

Santos, R., Gomes, D., Macedo, H., Barros, D., Tibério, C., Veiga, A. S., … Oliveira, M. (2016). Guar gum as a new antimicrobial peptide delivery system against diabetic foot ulcers *Staphylococcus aureus* isolates. *Journal of Medical Microbiology*, *65*(10), 1092–1099.

Santos, R., Ruza, D., Cunha, E., Tavares, L., & Oliveira, M. (2019). Diabetic foot infections: Application of a nisin-biogel to complement the activity of conventional antibiotics and antiseptics against *Staphylococcus aureus* biofilms. *PloS one*, *14*(7).

Sayers, S., Li, L., Ong, E., Deng, S., Fu, G., Lin, Y., … He, Y. (2019). Victors: A web-based

knowledge base of virulence factors in human and animal pathogens. *Nucleic Acids Research*, *47*(D1), D693–D700.

Schaper, N. C. (2004). Diabetic foot ulcer classification system for research purposes: a progress report on criteria for including patients in research studies. *Diabetes/Metabolism Research and Reviews*, *20*(S1), S90–S95.

Schaper, Nicolaas C., Apelqvist, J., & Bakker, K. (2003). The International Consensus and practical guidelines on the management and prevention of the diabetic foot. *Current Diabetes Reports*, *3*(6), 475–479.

Schilcher, K., Andreoni, F., Haunreiter, V. D., Seidl, K., Hasse, B., & Zinkernagel, A. S. (2016). Modulation of *Staphylococcus aureus* biofilm matrix by subinhibitory concentrations of clindamycin. *Antimicrobial agents and chemotherapy*, *60*(10), 5957-5967.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C T method. *Nature protocols*, *3*(6), 1101.

Seo, M. D., Won, H. S., Kim, J. H., Mishig-Ochir, T., & Lee, B. J. (2012). Antimicrobial peptides for therapeutic applications: a review. *Molecules*, *17*(10), 12276-12286.

Shin, J. M., Gwak, J. W., Kamarajan, P., Fenno, J. C., Rickard, A. H., & Kapila, Y. L. (2016). Biomedical applications of nisin. *Journal of Applied Microbiology*, *120*(6), 1449–1465.

Singh, V. K. (2014). High level expression and purification of Atl, the major autolytic protein of *Staphylococcus aureus. International journal of microbiology*, *2014*.

Skrepnek, G. H., Mills, J. L., Lavery, L. A., & Armstrong, D. G. (2017). Health care service and outcomes among an estimated 6.7 million ambulatory care diabetic foot cases in the U.S. *Diabetes Care*, *40*(7), 936–942.

Skyler, J. S., Bakris, G. L., Bonifacio, E., Darsow, T., Eckel, R. H., Groop, L., … Ratner, R. E. (2017). Differentiation of diabetes by pathophysiology, natural history, and prognosis. *Diabetes*, *66*(2), 241–255.

Smieja, M. (1998). Current indications for the use of clindamycin: A critical review. *Canadian Journal of Infectious Diseases*, *9*(1), 22–28.

Sperber, W. Z., & Tatini, S. R. (1975). Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. *Applied microbiology*, *29*(4), 502-505.

Spichler, A., Hurwitz, B. L., Armstrong, D. G., & Lipsky, B. A. (2015). Microbiology of diabetic foot infections: From Louis Pasteur to “crime scene investigation.” *BMC Medicine*, *13*(1).

Stevenson, K. B., & Wang, S.-H. (2014). Understanding the Molecular Pathogenesis of Methicillin-Resistant *Staphylococcus aureus*. *Journal of Infectious Diseases*, *209*(4), 488–490.

Subrt, N., Mesak, L. R., & Davies, J. (2011). Modulation of virulence gene expression by cell wall active antibiotics in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, *66*(5), 979-984.

Tan, L., Li, S. R., Jiang, B., Hu, X. M., & Li, S. (2018). Therapeutic targeting of the *Staphylococcus aureus* accessory gene regulator (*agr*) system. *Frontiers in microbiology*, *9*, 55.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2010). A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. *Methods*, *50*(4), S1-S5.

Thombare, N., Jha, U., Mishra, S., & Siddiqui, M. Z. (2016). Guar gum as a promising starting material for diverse applications: A review. *International Journal of Biological Macromolecules*, *88*, 361–372.

Uçkay, Ilker, Aragón-Sánchez, J., Lew, D., & Lipsky, B. A. (2015). Diabetic foot infections: what have we learned in the last 30 years? *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases*, *40*, 81–91.

Vandenesch, F., Kornblum, J. O. H. N., & Novick, R. P. (1991). A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. *Journal of Bacteriology*, *173*(20), 6313-6320.

Wang, B., & Muir, T. W. (2016). Regulation of Virulence in *Staphylococcus aureus* : Molecular Mechanisms and Remaining Puzzles. *Cell Chemical Biology*, *23*(2), 214–224.

Wiedemann, I., Benz, R., & Sahl, H.-G. (2004). Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. *Journal of Bacteriology*, *186*(10), 3259–3261.

Wielders, C. L. C., Fluit, A. C., Brisse, S., Verhoef, J., & Schmitz, F. J. (2002). *mecA* gene is widely disseminated in *Staphylococcus aureus* population. *Journal of Clinical Microbiology*, *40*(11), 3970–3975.

Willey, J. M., Sherwood, L. M., & Woolverton, C. J. (2008). *Prescott, Harley, and Klein's Microbiology* (7th ed.). New York, NY: McGraw-Hill International Edition.

Wolz, C., McDevitt, D., Foster, T. J., & Cheung, A. L. (1996). Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. *Infection and Immunity*, *64*(8), 3142-3147.

World Health Organization. (2019). *Diabetes*. Retrieved from https://www.who.int/health-topics/diabetes

Yagihashi, S., Mizukami, H., & Sugimoto, K. (2011). Mechanism of diabetic neuropathy: Where are we now and where to go? *Journal of Diabetes Investigation*, *2*(1), 18–32.

Younes, M., Aggett, P., Aguilar, F., Crebelli, R., Dusemund, B., ... & Kuhnle, G. G. (2017). Safety of nisin (E 234) as a food additive in the light of new toxicological data and the proposed extension of use. *EFSA Journal*, *15*(12), e05063.

Zhao, X., Meng, R., Shi, C., Liu, Z., Huang, Y., Zhao, Z., ... & Yu, L. (2016). Analysis of the gene expression profile of *Staphylococcus aureus* treated with nisin. *Food Control*, *59*, 499-506.

Zhanel, G. G., Hoban, D. J., & Harding, G. K. (1992). Subinhibitory antimicrobial concentrations: a review of *in vitro* and *in vivo* data. *Canadian Journal of Infectious Diseases*, *3*.

Supplementary Data

Table S1 | Ct values obtained by RT-qPCR of two different *S. aureus* DFI isolates for the all the genes under study, using different primers concentrations.

Gene	Isolates	$\overline{Ct}$ (50 nM/50 nM)	$\overline{Ct}$ (300 nM/300 nM)	$\overline{Ct}$ (900 nM/900 nM)
gyrB	A 6.3	25.08745	23.1938	23.1988
	B 14.2	17.24855	16.13775	16.21375
spA	Z 5.2	16.0284	15.44055	15.6123
	A 5.2	21.97775	21.3829	22.12485
agrI	Z 5.2	14.41725	14.2744	14.332
	A 5.2	15.3124	15.237	15.2952
icaA	Z 2.2	28.57745	25.9868	24.591
	B 14.2	31.3707	29.40095	27.8006
icaD	Z 2.2	23.10005	22.11145	21.7027
	B 14.2	26.54545	25.63045	25.1496
coa	A 6.3	24.2857	23.8262	23.76115
	B 3.2	25.323	25.11825	25.02935
clfA	A 6.3	30.22555	27.2845	27.7735
	B 3.2	19.69815	19.43015	19.79855
atl	A 6.3	21.91655	19.79345	20.10905
	B 3.2	27.6266	23.62075	23.7182

O1: First assay to primers optimization (4h of bacterial growth); O2: Second assay to primers optimization (4h of bacterial growth); O3: Third assay to primers optimization (24h of bacterial growth); *gyrB:* gene encoding gyrase B; *spA:* gene encoding staphylococcal protein A; *agrI:* accessory gene regulator I; *icaA:* gene encoding intracellular adhesin A; *icaD:* gene encoding intracellular adhesin D; *coa:* gene encoding coagulase; *clfA:* gene encoding clumping factor A; *atl:* gene encoding autolysin; A: aspirate; B: biopsy; Z: swab; nM: nanomolar; Ct: cycle threshold.

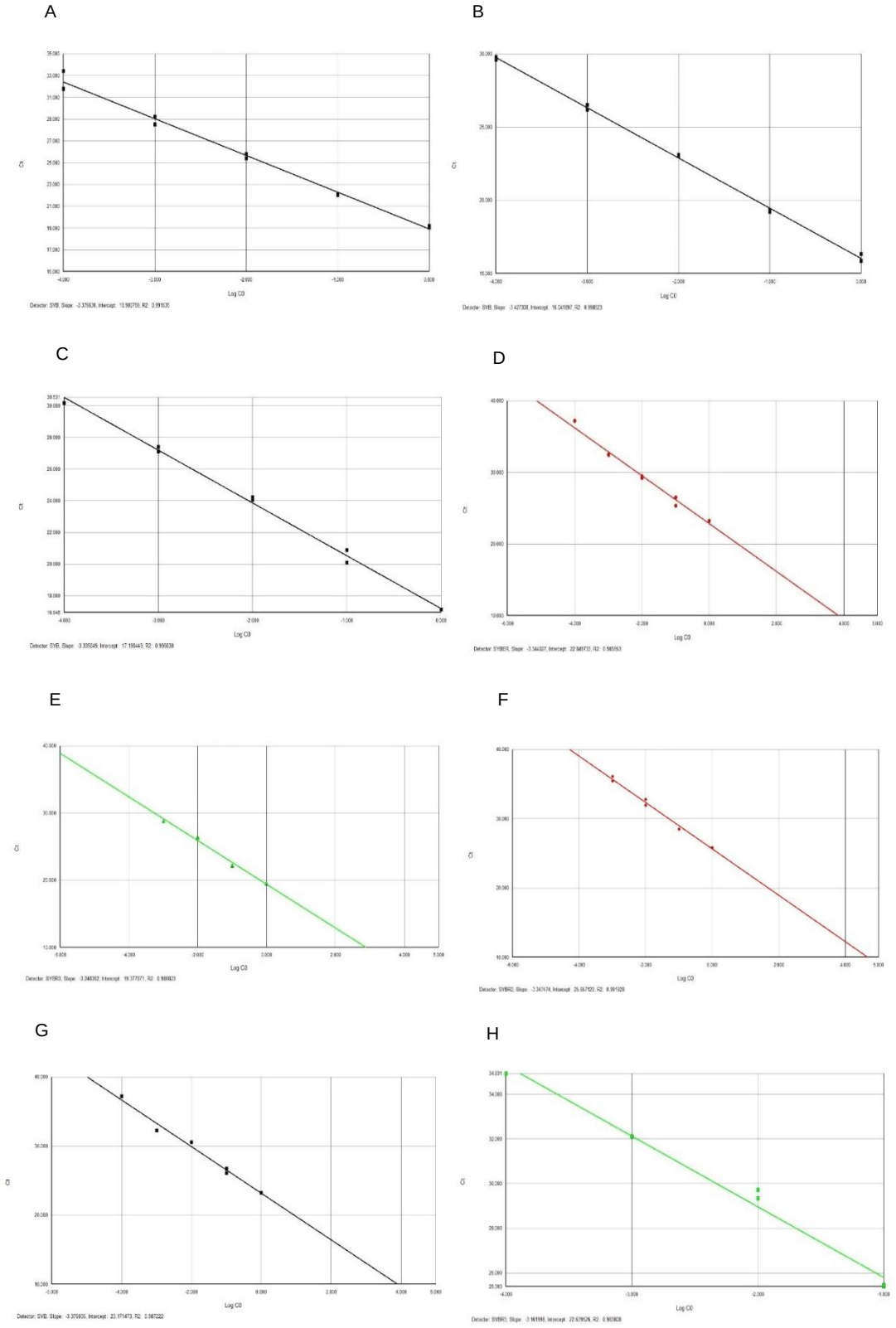


Figure S1 | A, B, C, D, E, F, G and H: Standard curves for *gyrB, agrI, spA, atl, clfA, coa, icaD* and *icaA* established using a dilution series of known concentrations (1, 1:10, 1:100, 1:1000 and 1:10000), respectively.

Table S2 | Standard curves slope, efficiency and correlation coefficient values.

Gene	Slope	Efficiency (%)	Correlation coefficient
gyrB	-3.375630	101.68	0.991535
spA	-3.335049	100.45	0.996630
agrI	-3.427308	103.23	0.998523
atl	-3.238277	97.54	0.997157
icaA	-3.161998	95.24	0.983808
icaD	-3.375035	101.66	0.987222
coa	-3.347474	100.83	0.991928
clfA	-3.248382	97.84	0.986823

gyrB: gene encoding gyrase B; *spA:* gene encoding staphylococcal protein A; *agrI:* accessory gene regulator I; *icaA:* gene encoding intracellular adhesin A; *icaD:* gene encoding intracellular adhesin D; *coa:* gene encoding coagulase; *clfA:* gene encoding clumping factor A; *atl:* gene encoding autolysin.

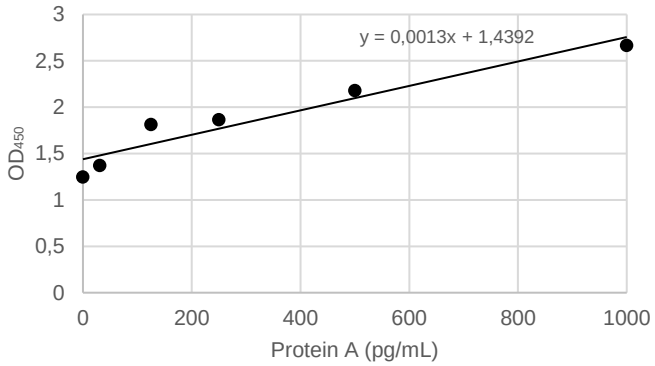


Figure S2 | Standard curve obtained for Protein A using a specific SpA ELISA. This standard curve was used to calculate the amount of SpA (pg/mL) in the bacterial supernatants incubated with or without nisin-biogel and clindamycin at sub-MICs. OD_{450}: optical density at 450 nm.

Abstract accepted for poster presentation at the International Meeting of the Portuguese Society of Genetics

Influence of a nisin-biogel on virulence gene expression by *Staphylococcus aureus* isolates from Diabetic Foot Infections

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Diabetes *mellitus* is a lifelong metabolic disorder that affects millions of people worldwide. This disorder is characterized by defects in insulin secretion and/or insulin action, leading to a state of hyperglycemia that progressively causes dysfunctions in multiple organs and tissues. Diabetic Foot Ulcers (DFUs) are one of the major complications associated with diabetes, often leading to a lower-extremity amputation. DFUs microenvironmental conditions, such as compromised angiopathy and low perfusion, often prevents antibiotherapies to locally reach minimum inhibitory concentrations (MICs), which is a major concern considering DFUs favorable environment for colonization by opportunistic microorganisms. *Staphylococcus aureus* (*S. aureus*) is the pathogen most frequently isolated from DFUs. This bacterium is able to express a broad spectrum of virulence determinants and is presently classified by World Health Organization (WHO) as one of the highest priority pathogens for the development of new therapeutic strategies. Due to the increasing dissemination of antibiotic-resistant strains, antimicrobial peptides (AMPs) have recently been recognized as promising candidates for alternative therapeutics. Nisin is an AMP with demonstrable activity against a broad spectrum of gram-positive bacteria, including *S. aureus*, and had been previously investigated by our group as a new therapeutic approach to Diabetic Foot Infections (DFIs). Guar-gum is the polymeric material proposed as a delivery system for nisin (nisin-biogel), as it avoids its degradation or inactivation. In the present study, we developed sensitive quantitative real-time PCR assays to quantify the expression of *S. aureus* virulence genes. These assays have been used to investigate the effects of subinhibitory concentrations (sub-MICs) of a nisin-biogel on the transcription levels of *S. aureus* virulence genes, allowing to further elucidate on its safe application to DFIs.

Abstract submitted for poster presentation at the XXI SPB – National Congress of Biochemistry

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Diabetic Foot Ulcers (DFUs) are one of the major complications associated with Diabetes *Mellitus* (DM), often leading to lower-extremity amputations (LEAs). DFUs microenvironmental conditions, such as angiopathy and low perfusion, often prevent antibiotics to reach the infected DFUs at minimum inhibitory concentrations (MICs), which is a major concern considering DFUs favorable environment for colonization by opportunistic microorganisms. *Staphylococcus aureus* (*S. aureus*) is the pathogen most frequently isolated from DFUs, and is able to express a broad spectrum of virulence genes, including the accessory regulator gene I (*agrI*), the clumping factor A (*clfA*) and the staphylococcal major autolysin (*atl),* and is presently classified by World Health Organization (WHO) as one of the highest priority pathogens for the development of new therapeutic strategies. Nisin-biogel had been previously investigated by our group as a new therapeutic approach to Diabetic Foot Infections (DFIs). Nisin is an antimicrobial peptide (AMP) with demonstrable activity against a broad spectrum of gram-positive bacteria, including *S. aureus*, and guar-gum is a polymeric material proposed as nisin's delivery system, as it avoids its degradation or inactivation. As in *in vivo* infections bacteria are usually exposed to sub-MICs of antimicrobials, which can lead to a wide variety of physiological and morphological effects on bacteria, in the present study we aimed to evaluate the effects of nisin-biogel subinhibitory concentrations (sub-MICs) on the transcription levels of *agrI, clfA,* and *atl* genes by quantitative real-time PCR assays. Six *S. aureus* DFI isolates were used for evaluating virulence gene expression after exposure to sub-MICs of nisin-biogel. Although different effects were observed, on average, nisin-biogel sub-MICs decreased significantly the expression of *agrI, clfA* and *atl* genes. These findings highlight the importance of accessing the effects of nisin-biogel sub-MICs on virulence expression, providing an *in vitro* basis for understanding what happens *in vivo* during the treatment of a DFI.