CLERIANE ANDRE

EFFECT OF BIOENGINEERED NISIN ON *Staphylococcus aureus* CELLS AND BIOFILM

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 22 de fevereiro de 2019.

A meu pai Cleres Andre

In memorian

DEDICO.

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BIOGRAFIA

CLERIANE ANDRE, filha de Cleres Andre e Zenadir Maria de Oliveira Andre, nasceu em Colatina, Espírito Santo no dia 11 de outubro de 1988.

Em fevereiro de 2013, graduou-se em Bacharel em Nutrição pela Faculdade Católica Salesiana do Espirito Santo, tornando-se bacharel em fevereiro de 2013.

Em março de 2013, ingressou no Programa de Mestrado em Microbiologia Agrícola, na Universidade Federal de Viçosa, concentrando seus estudos na área de Microbiologia de Alimentos, tendo recebido o título de *Magister Scientiae* em Microbiologia Agrícola no dia 26 de maio de 2015.

Em março de 2015, iniciou o curso de Doutorado em Microbiologia Agrícola na Universidade Federal de Viçosa, concentrando seus estudos na mesma área. Participou do Programa Doutorado Sanduíche no Exterior/CAPES no TEAGASC, Irlanda, de abril de 2017 a dezembro de 2017. Submeteu-se a defesa de tese em 22 de fevereiro de 2019.

SUMÁRIO

RESUMO

ANDRE, Cleriane, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Efeito de nisina bioengenherada sobre células e biofilmes de *Staphylococcus aureus***. Orientadora: Maria Cristina Dantas Vanetti. Coorientadores: Natan de Jesus Pimentel-Filho e José Guilherme Prado Martin

Lantibiótico é um grupo de peptídeos que possuem estruturas de anel intramolecular, contendo resíduos de lantionina e metilantionina com atividade antimicrobiana. Nisina é o lantibiótico mais estudado e usado como conservante de alimentos em todo o mundo, sendo sua estrutura molecular composta por cinco anéis de lantionina (A, B, C, D e E). Este peptídeo é caracterizado por um modo duplo de ação, consistindo da inibição da biossíntese da parede celular e a formação de poros na membrana celular bacteriana, devido a sua capacidade de interagir com a molécula alvo, o lipídio II. Em razão da estrutura da nisina e da sua natureza codificada por genes, técnicas de bioengenharia podem ser usadas para gerar novos derivados, com atividade antimicrobiana melhorada. Neste estudo, nisina A e três derivados denominados de N20P, S29A e M21V foram caracterizados, *in silico*, quanto à estrutura molecular e o efeito antimicrobiano foi validado por métodos experimentais sobre células de *S. aureus*. Posteriormente, a atividade antimicrobiana de nisina A e de seus derivados foi avaliada contra o biofilme de *S. aureus* pela detecção do conteúdo de biomassa e atividade metabólica, utilizando o teste de cristal violeta e 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), respectivamente. Os resultados obtidos por análises *in silico* mostraram que as mutações pontuais causaram modificações estéricas na molécula de nisina, deixando-a mais plana e possivelmente, facilitando melhor ajuste dos anéis C, D e E, envolvidos na formação de poros. Os ensaios de extravasamento de conteúdo celular mostraram maior perda de NADPH e ATP em um curto período de tempo a partir de células tratadas com nisina. Modificações na membrana citoplasmática foram observadas por análise de microscopia de força atômica. Alterações na posição 21 da molécula de nisina podem resultar em um peptídeo com maior atividade e maior interação com moléculas de membrana, facilitando a formação de poros e promovendo maior perda de ATP intracelular. Os derivados N20P, S29A e M21V de nisina A apresentaram maior capacidade de inativar o biofilme de *S. aureus*, tanto pela remoção de células, quanto

pela inativação das células sésseis. A influência dos genes *dltA* e *mprF*, que estão envolvidos no mecanismo de resistência a peptídeos catiônicos, como a nisina, também foi avaliada usando os mutantes de *S. aureus* Δ*dltA* e Δ*mprF*. A deleção dos genes *dltA* e *mprF* não alterou a capacidade dessas linhagens de formar biofilme, entretanto, ficaram mais sensíveis à ação das bacteriocinas. Os resultados mostraram o aumento do potencial antibiofilme da nisina modificada e sugerem que a melhoria, por técnicas de bioengenharia, pode ser uma estratégia para produzir um antimicrobiano mais efetivo para controlar o crescimento e remover o biofilme de *S. aureus*

ABSTRACT

ANDRE, Cleriane, D.Sc., Universidade Federal de Viçosa, February, 2019. **Effect of bioengineered nisin on *Staphylococcus aureus* cells and biofilm**. Adviser: Maria Cristina Dantas Vanetti. Co-advisers: Natan de Jesus Pimentel-Filho and José Guilherme Prado Martin

Lantibiotics are a group of peptides that have intramolecular ring structures containing lanthionine and methylantionine residues with antimicrobial activity. Nisin is the most studied lantibiotic, used as a food preservative all around the world, and its structure is composed of five lanthionine rings (A, B, C, D and E). This peptide is characterized by a dual mode of action, consisting of the inhibition of cell wall biosynthesis and the formation of pores in the bacterial cell membrane, due to its ability to interact with the target molecule, lipid II. Due its structure and its gene encoding nature, nisin can be used by bioengineering to generate new derivatives with improved antimicrobial activity. In this study, the molecular structure of nisin A and three derivatives N20P, S29A and M21V were characterized *in silico* and the antimicrobial effect on *S. aureus* cells was validated by experimental methods. Sequentially, the antimicrobial activity of nisin A and its derivatives was evaluated against the *S. aureus* biofilm by detection of the biomass content and metabolic activity using the violet crystal test and 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), respectively. The results obtained by *in silico* analysis showed that the point mutations caused steric alterations in the nisin molecule, become it more planar, and possibly facilitating the molecular interactions of the C, D and E rings involved in the formation of pores. Cell leakage assays showed greater loss of NADPH and ATP over a short period of time from nisin treated cells. Modifications in the cytoplasmic membrane were observed by analysis of atomic force microscopy. Changes in position 21 of the nisin molecule may result in a peptide with higher activity and greater interaction with membrane molecules, facilitating the formation of pores and promoting greater loss of intracellular ATP. The N20P, S29A and M21V derivatives of nisin A presented greater capacity to inactivate the *S. aureus* biofilm, both by the removal of cells and inactivation of the sessile cells. The influence of the *dltA* and *mprF* genes, which are involved in the cationic peptide resistance mechanism, such as nisin, was also evaluated using the *S. aureus ΔdltA* and *ΔmprF* mutants. The deletion of the *dltA* and

mprF genes did not alter the ability of these strains to form a biofilm, but it made the action of more sensitive to bacteriocins. The results showed the increased antibiofilm potential of modified nisin and suggest that improvement by bioengineering techniques may be a strategy to produce a better antimicrobial to control growth and to remove *S. aureus* biofilm.

INTRODUÇÃO GERAL

Bacteriocinas são peptídeos ou proteínas com atividade antimicrobiana, sintetizadas ribossomicamente por bactérias. Nisina, a mais conhecida e amplamente estudada bacteriocina, pertence a classe I (lantibiotico). Este grupo de bacteriocinas tem sua atividade antimicrobiana atribuída à interação com os lipídios aniônicos na membrana citoplasmática das células bacterianas, mais especificamente o lipídio II, ocasionando rompimento de membrana, formação de poros, efusão de íons, dissipação da força próton motora, hidrólise de ATP e, consequentemente, perda de viabilidade e lise celular. Nisina é composta por 34 aminoácidos, com estrutura composta por cinco anéis de lantionina (A, B, C, D e E) e sua principal aplicação é como conservante de alimentos. Entretanto, a ampliação de seu uso é de grande interesse e vêm sendo alvo de pesquisas em todo o mundo, tanto para indústria de alimentos como para a área médica, como por exemplo, sua atividade antibiofilme.

Recentemente, estratégias de bioengenharia tem sido exploradas para melhorar a atividade de nisina, desenvolvendo moléculas derivadas com maior atividade antimicrobiana. Esta estratégia tem obtido sucesso e, dependendo do ponto da mutação e do aminoácido substituído dentro da molécula, pode-se conseguir aumento da atividade antimicrobiana. Entretanto, essas moléculas melhoradas, derivadas de nisina, requerem avaliação em relação ao seu potencial antimicrobiano. Mais recentemente, estas moléculas também vêm sendo testadas sobre biofilmes microbianos, principalmente naqueles formados por patógenos. Entretanto, o mecanismo pelo qual os variantes de nisina tem a atividade aumentada e como atuam na célula bacteriana, ainda não foi esclarecido.

S. aureus é um patógeno de grande importância em diferentes setores, como na área médica, por causar infecções oportunísticas e, na indústria de alimentos, em função da sua capacidade de produzir toxinas. Além do frequente envolvimento em casos e surtos de intoxicação alimentar, *S. aureus* é capaz de aderir e formar biofilmes em diferentes superfícies bióticas e abióticas, resultando em sérios problemas de contaminação e perdas econômicas em razão da deterioração de alimentos, danos nos equipamentos e impacto na saúde humana. Além de formar biofilmes, *S. aureus* possui mecanismos refinados para resistir à ação de peptídeos antimicrobianos catiônicos, como nisina. Entre esses mecanismos, a alteração da expressão gênica para

modificação da composição química do envoltório celular, que resulta na alteração de cargas da superfície da célula, é uma estratégia de resistência da célula. Existem evidências de que esse mecanismo de resistência está relacionado também com a formação de biofilmes.

Diante disso, o objetivo desse trabalho foi o de avaliar a atividade dos derivados de nisina N20P, S29A e M21V sobre as células de *S. aureus* e as possíveis modificações estruturais ocasionadas pelas modificações. Objetivou-se ainda, avaliar o efeito desses peptídeos sobre biofilmes de estirpes mutantes de *S. aureus* nos genes *dltA* e *mprF*, envolvidos no mecanismo de resistência a peptídeos catiônicos.

CHAPTER 1: Mini review: Nisin and its derivates: antimicrobial and antibiofilm potential

Bacteriocins are antimicrobials peptides or proteins ribosomally synthesized produced by bacteria that display bacteriostatic and/or bactericidal activity against other related bacteria (COTTER; ROSS; HILL, 2013; OKUDA *et al.*, 2013). Bacteriocins are a heterogeneous group and are usually classified into peptides that undergo significant post-translational modifications (class I) and unmodified (class II) (COTTER; ROSS; HILL, 2013). The best known and extensively studied bacteriocin class I (lantibiotic) is nisin, an antimicrobial peptide produced by *Lactococcus lactis* (DEEGAN *et al.*, 2006). Nisin is used in food preservation and recognized by the Food and Drug Administration (FDA) since 1989, as GRAS (Generally Recognized as Safe) substance for use in food.

The antibacterial activity of nisin is attributed to interaction with the anionic lipids in the cytoplasmic membrane of bacterial cells, occasioning in membrane disruption, pore formation, ion efflux, dissipation of protomotive force, ATP hydrolysis and, consequently, loss of viability and cell lysis (BREUKINK; KRUIJFF, 2006; ENNAHAR *et al.*, 2000; LANNE *et al.*, 2019; TONG; NI; LING, 2014). The mechanism of action of nisin occurs when nisin binds to lipid II causing damage to cells. In the structure of the nisin-lipid II complex, the N-terminal region of nisin anchor to the pyrophosphate moiety of lipid II, sequestering the lipid II and thereby inhibits cell wall biosynthesis. Moreover, its C-terminal region facilitates pore formation that leads efflux of intracellular metabolites and disruption of membrane potential (BREUKINK *et al.*, 2003; BREUKINK; KRUIJFF, 2006; GE *et al.*, 2016).

Nisin is composed of 34 amino acids and, the structure of this peptide is defined by the presence of non-standard residues including lanthionine and/or methyl lanthionine, which are promoted by a series of enzyme-mediated post-translational modifications (FIELD *et al.*, 2015). The Figure 1 shows in more details, the process of nisin synthesis. Nisin has a pentacyclic structure with one lanthionine residue (ring A) and four β-methyl lanthionine residues (B, C, D and E rings) (ROSS; MORGAN; HILL, 2002). Nisin is active over a broad pH range, relatively amphipathic and small molecule (3,353 Da), cationic, and effective at low concentrations (CHANDRAPATI; O'SULLIVAN, 1998). The activity of nisin in bacterial cell wall is possible due to the presence of two-structural domains, located at the N- and C-terminal, respectively (HSU *et al.*, 2004). The N-terminal domain, containing three post-translationally incorporated (β-methyl) lanthionine rings (rings A, B and C), is linked to the C-

terminal rings (rings D and E) by a flexible region, or hinge, consisting of three amino acids (Asn20–Met21–Lys22) (BAUER; DICKS, 2005; FIELD *et al.*, 2008). It has been established that the A, B and C rings form a cage that facilitates binding of the pyrophosphate moiety of lipid II, thus interfering with cell wall synthesis (HSU *et al.*, 2004; MULHOLLAND *et al.*, 2016). This binding enhances the ability of the C-terminal segment, containing rings D and E, to form pores in the cell membrane, resulting in the rapid efflux of ions and cytoplasmic solutes (BAUER; DICKS, 2005; FIELD *et al.*, 2008).

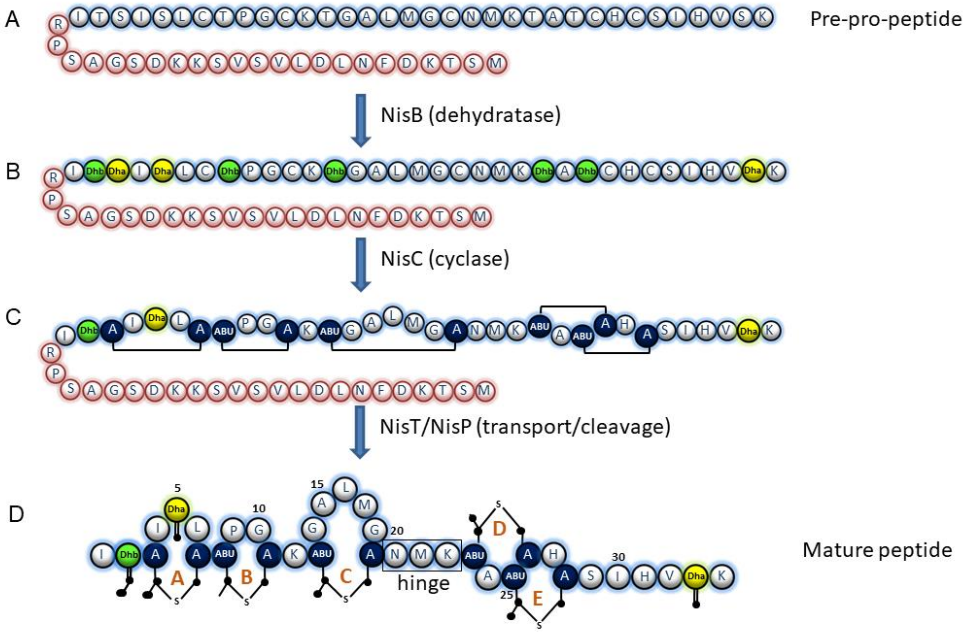

Figure 1. Post translational processing of nisin A. First, nisin is synthesized as a biologically inactive pre-peptide consisting of an N-terminal leader peptide attached to the C-terminal pro-peptide. B. Then, serine and threonine residues are dehydrated by NisB, forming dehydroalanine (Dha) and dehydributyrine (Dhb). C. NisC then couples nearby cysteine residues and the Dha and Dhb to form lanthionine (lan) and methylanthionine (MeLan) rings. D. modified nisin with the leader peptide still attached is subsequently transported via the dedicated ABC-type transporter NisT. Only after proteolytic cleavage of the N-terminal leader sequence, mediated by the extracellular serine protease NisP is the mature bioactive nisin peptide released (FIELD *et al.*, 2015).

Antimicrobial potential of bioengineering nisin

In view of its antimicrobial activity and the search for more natural alternatives for food preservation, nisin has been widely studied. Due its commercial success, knowledge about its genetic structure, mode of action and chemical properties, as well as the research for other practical applications (FIELD *et al.*, 2015), as therapeutic potential (COTTER; ROSS; HILL, 2013) and antibiofilm activity has increased (DAVISON *et al*., 2010; PIMENTEL-FILHO *et al.*, 2014; SHARAHI *et al.,* 2019; WANG *et al.*, 2017).

Bioengineering strategies has been promising and caused an improvement in physicochemical properties of nisin, including enhancements in solubility and stability, allowing greater diffusion through complex polymers (FIELD *et al.*, 2015). There is also an increasing number of engineered peptides that exhibit enhanced functionalities (activity and / or stability) (COTTER; ROSS; HILL, 2013). Genetic manipulation promotes modifications at specific points of the nisin molecule generating improved derivatives that should be evaluated related their antimicrobial activity (FIELD *et al.*, 2015). Some nisin mutations, created using random mutagenesis, showed enhanced activity against Gram-positive bacteria (FIELD *et al.*, 2008) and Gram-negative pathogens (FIELD *et al.*, 2012). Important to highlight that, the point of the molecule where substitution or amino acid incorporation occurs and the characteristic of the amino acid added, may directly interfere with the activity of the variants.

Activity studies with three of these nisin A derivatives (M21V, K22T and N20P) revealed that M21V has enhanced specific activity against *Staphylococcus aureus* and *Listeria monocytogenes*, that K22T has enhanced specific activity on *Streptococcus agalactiae* ATCC 13813 and *S. aureus* ST528 (MRSA) and that N20P has enhanced activity against *S. aureus* ST528, but reduced activity against *S. agalactiae* (FIELD *et al.*, 2008). Another study by Field *et al.* (2010) showed that nisin M21V was consistently twofold more potent that nisin A against all three pathogens methicillin-resistant *S. aureus* (MRSA).

The activity of bioengineered nisin derivatives has already been evaluated in complex matrices as agar and carrageenan-based matrices. Rouse *et al.* (2012) studied two variants of nisin, which contain the residues Serine, Valine and Alanine (SVA) and Asparagine, Alanine and Lysine (NAK), respectively, into the hinge region.

Carrageenan is a polysaccharide extracted from seaweed, which is used in food as a gelling agent. When carrageenan was employed instead of agar in well assays, using *L. monocytogenes* UCC35 as indicator, NAK and SVA produced significantly larger zones of inhibition than those generated by nisin A in this matrix. This result demonstrate that the enhanced trait enables the peptides to drastically outperform nisin A with respect to controlling *L. monocytogenes* in commercially produced chocolate milk that contains carrageenan as a stabilizer.

Campion *et al.* (2017) studied the enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7 and demonstrated that sub-inhibitory concentrations of nisin variants and carvacrol caused complete inactivation of *E. coli* O157:H7 in apple juice within 3 h at room temperature compared to that of the equivalent nisin A combination. And, in this same work, activity test with the nisin derivative S29A, presented two-fold greater specific activity than nisin A against *C. sakazakii* and *E. coli* O157:H7. Nisin and its derivatives have been shown to be efficient against a range of bacteria and other application as antibiofilm activity has also been researched. Critically, unlike antibiotics, the ribosomally synthesised nature of bacteriocins renders them one of the most amenable agents to manipulate or bioengineer to target specific pathogens and biofilm formers (MATHUR *et al.,* 2018).

Potential antibiofilm action

Biofilms can be defined as aggregates of microbial cells that have the ability to adhere to surfaces, multiply and embed themselves in a viscous matrix composed of extracellular polymeric substances (EPS) produced by them (FLEMMING; NEU; WOZNIAK, 2007; SIMÕES; SIMÕES; VIEIRA, 2010; SREY; JAHID; HA, 2013). In biofilm, bacteria are more resistant to unfavourable environmental conditions, including stresses caused by stimulation of the external environment (FLEMMING; NEU; WOZNIAK, 2007; FLEMMING; WINGENDER, 2010), a major concern has been antimicrobial resistance, because they are hardly diffused in the biofilm matrix (PARSEK; FUQUA, 2004; SAÁ IBUSQUIZA; HERRERA; CABO, 2011).

The steps governing biofilm formation sequentially include surface preconditioning by macromolecules present in the liquid or intentionally coated on the surface; transport of planktonic cells to the surface; attractive and repulsive forces

involved bacterial adhesion to surfaces, including van der Waals forces, electrostatic forces between surface and microorganisms, ionic bonds, hydrophobic interactions; irreversible adhesion when attractive forces are greater than the forces of repulsion; production of cell-cell signalling molecules and transport of substrates into the biofilm; metabolism of the substrate by the cells attached to the biofilm and transport of products out of the biofilm, accompanied by cell growth, replication and EPS production; release of biofilm microorganisms by disaggregation of the biofilm (BRYERS; RATNER, 2004; ZOTTOLA; SASAHARA, 1994). Despite the application of modern technologies and safety concepts in the food industry, there are still many problems related to contamination of food by foodborne pathogens from biofilms (MARTÍNEZ *et al.*, 2008).

Alternatives to increasing conservation and food safety involving the use of microorganisms and /or their products for food conservation has been a common practice in the history of mankind (ROSS; MORGAN; HILL, 2002). Lactic acid and other by products of the metabolism of lactic acid bacteria, including hydrogen peroxide, diacetyl, acetoin, reutericicline, antifungal peptides and bacteriocins act as bioconservants by altering the intrinsic properties of foods and inhibit spoilage microorganisms (DEEGAN *et al.*, 2006; MAGNUSSON; SCHNÜRER, 2001). Ribosomal synthesized peptides with antimicrobial properties are produced by many living organisms, from prokaryotes to higher eukaryotes (PAPAGIANNI, 2003) and have aroused interest as inhibitors of important microorganisms in food. Nisin is the most studied bacteriocin and has been investigated for its potential as antibiofilm agent.

Leriche *et al.* (1999) examined the survival of biofilm cells and planktonic cells of *L. monocytogenes* in the presence of a nisin producer strain and saw a reduction of, at least, two log cycles in pathogen number. Nel *et al.* (2002) compared the effect of bacteriocins on planktonic and sessile cells of *Oenococcus oeni* and found that the bacteriocin pediocin was much more effective against biofilms, whereas planktonic cells were more sensitive to nisin.

Nisin at a concentration of 250 IU/ mL was sufficient to reduce by 99% the number of planktonic *S. aureus* cells and in biofilms on polypropylene surfaces (CABO *et al.*, 2009). On a stainless steel surface, the reuterine, bacteriocin produced by some strains of *Lactobacillus reuteri*, showed bactericidal effect on *E. coli* and

Listeria innocua biofilms after 30 seconds of exposure (EL-ZINEY; JAKOBSEN, 2009). In the latter study, the synergistic effect on both microorganisms was observed when reuterine was combined with sodium hypochlorite or nisin. Sadowska *et al.* (2010) found that metabolites of lactobacilli similar to bacteriocins were able to suppress the formation of biofilms and to induce ultrastructural changes in *S. aureus* and *Staphylococcus epidermidis*, which caused cellular inactivation.

Davison *et al.* (2010) found antimicrobial action of chlorine, glutaraldehyde and nisin within the cell groups in *S. epidermidis* biofilms and reported that nisin reached the inside of the biofilm faster than the other antimicrobial agents, resulting in a rapid and uniform loss of cell fluorescence, however did not affect the structure of the biofilm. Nostro *et al.* (2010) incorporated different concentrations of nisin in ethylene vinyl acetate (EVA) films to evaluate the effect on the biofilm formation capacity of *L. monocytogenes*, *S. aureus* and *S. epidermidis* and the results showed that nisin was effective in reducing formation of biofilms on the surface, being more effective against *S. epidermidis* than against *L. monocytogenes* and *S. aureus*. Inactivation of *S. aureus* sessile cells increased notably when bacteriocin enterocin AS-48 was used in the concentration 50 mg/l combined with benzalkonium chloride, triclosan or polyhexamethylene biguanide hydrochloride in polystyrene microplates (CABALLERO *et al.*, 2013). Pimentel-Filho *et al.* (2014) evaluated sub-MIC concentrations of bacteriocins bovine HC5 and nisin in the conditioning of polystyrene coupons and found that these bacteriocins may reduce *S. aureus* adhesion, probably due to changes in the hydrophobicity of bacterial cell and polystyrene surfaces. It was shown that nisin and bovicin HC5 also had an effect on the transcription of certain genes in *S. aureus*, as *clfB*, *fnbA* and *icaD*, which are involved in biofilm formation. Due the high antimicrobial potential and more recently antibiofilms action, improved nisin derivatives has also been applied in biofilm studies

Recent studies reported the activity of nisin and bioengineered variants against other *Staphylococcus* biofilm. Indeed, the nisin variant I4V was particularly effective at inhibiting the formation of *Staphylococcus pseudintermedius* DSM21284 biofilm and decreasing the biomass of established biofilm (FIELD *et al.*, 2015). Smith *et al.* (2016) assess the activity of nisin derivative against *L. monocytogenes* biofilm, nisin M21A (0.1 µg/ml) alone or in combination with cinnamaldehyde (35 µg/ml) or citric acid (175 µg/ml) showed significantly better than combinations involving nisin A. All

combinations of M21A with either citric acid or cinnamaldehyde eradicated the *L. monocytogenes* biofilm, in relation to biofilm control (SMITH *et al.*, 2016). Bioengineered nisin has been shown to have higher activity than nisin A and more studies need to be developed with different groups of bacteria to show their potential of these new molecules.

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CHAPTER 2 - Modeling of bioengineered nisin and its effects on *Staphylococcus aureus* cell membrane

Title: Modeling of bioengineered nisin and its effects on *S. aureus* cell membrane

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Abstract

Lantibiotics are group of modified peptides that contain intramolecular ring structures, introduced through the thioether containing lanthionine and methyllanthionine residues which has antimicrobial activity. Nisin is the most studied lantibiotic and it is used as a food preservative around the world. This peptide is characterized by a dual mode of action inhibiting cell wall biosynthesis and forming a pore in the bacterial membrane, by its ability to interact with a target molecule, lipid II. Due the structure of nisin and its the gene-encoded nature it can be used to gene-based bioengineering to generate novel derivatives, with improved antimicrobial activity. In this study the effect of three nisin A derivatives with the mutations N20P, S29A and M21V against *S. aureus* cells, using *in silico* methodologies and validating with experimental methods. Analyzes *in silico* showed that the point mutations caused steric modifications in the nisin molecule, leaving it more planar and possibly facilitating a better fit of the rings C, D and E, which are involved with the formation of pores. Cell contents extravasation assays showed a greater loss of NADPH and ATP in a short period of time after exposition to nisin. The modifications in the topography of cells analysis of atomic force microscopy, was more intense when treated with bioengineered nisins, these modifications suggest a higher pore formation. Modifications in position 21 of nisin resulted in a peptide with greater antimicrobial activity and membrane alterations.

Keyword: antimicrobial peptide, nisin, lantibiotic, bacteriocin,

2.1 Introduction

Lantibiotics are a group of antimicrobial, gene-encoded, ribosomally synthesized derived peptides that have attracted widespread scientific attention in recent years, not only as promising safe and natural food additives but also, as potential chemotherapeutic agents (Field *et al.*, 2008; Cotter *et al.*, 2013). Nisin is the lantibiotic most studied and the mechanism of action involves disruption of cytoplasmic membranes by forming pores, inhibition of cell wall synthesis and deregulation of bacterial cell division (Lanne *et al*., 2019; Wiedemann *et al.*, 2001).

Nisin is a 34-amino acid polypeptide, produced by *Lactococcus lactis* strains, that is active over a broad pH range, relatively amphipathic and small (3353 Da), cationic, and effective at low concentrations (Chandrapati and J. O'Sullivan, 1998). The activity of nisin in bacterial cell wall is possible due to the presence of two-structural domains, located at the N- and C-terminal, respectively (Hsu *et al.*, 2004). The N-terminal domain, containing three post-translationally incorporated (b-methyl) lanthionine rings (rings A, B and C), is linked to the C-terminal rings (rings D and E) by a flexible region, or hinge, consisting of three amino acids (Asn20–Met21–Lys22) (Bauer and Dicks, 2005a; Field *et al.*, 2008). It has been established that the A, B and C rings form a cage that facilitates binding of the pyrophosphate moiety of lipid II, thus interfering with cell wall synthesis (Hsu *et al.*, 2004; Bauer and Dicks, 2005a). This binding enhances the ability of the C-terminal segment, containing rings D and E, to form pores in the cell membrane, resulting in the rapid efflux of ions and cytoplasmic solutes (Bauer and Dicks, 2005b; Field *et al.*, 2008).

Bioengineering strategies has been promising and caused an improvement in physicochemical properties of nisin, including enhancements in solubility and stability, allowing greater diffusion through complex polymers (Field *et al.*, 2015). Genetic manipulation promotes modifications at specific points of the nisin molecule generating improved derivatives that should be evaluated related their antimicrobial activity (Field *et al.*, 2015a). Important to highlight that the point of the molecule where substitution or amino acid incorporation occurs may directly interfere with the activity of the variants due to new characteristics of the incorporated amino acid. Wiedemann *et al.* (2001) generating mutants with change of a hydrophobic valine in

the position 32 by a positive charged residue of Lys or negative residue of Glu and did not have a significant increase in nisin activity. In the other hand, a strong reduction of the activity of nisin in the liposome assay by almost two orders of magnitude was observed when a Lys residue was introduced in the place of a Met in the position 17 (M17K) into ring C of the peptide demonstrating that a charged residue is not tolerable in the central segment of the molecule hindering the formation of the pore. However, *in vivo*, the M17K peptide lost only 50% of its activity against *Mariniluteicoccus flavus*. Other nisin variants such as N20K and M21K displayed enhanced activity against Gram-negative bacteria including *Shigella*, *Pseudomonas* and *Salmonella* spp. (Yuan *et al.*, 2004). While the variant K22T displayed enhanced activity against *Streptococcus agalactiae*, a human and bovine pathogen (Field *et al.*, 2012). The introduction of aromatic residues at any position in the hinge had a negative impact on nisin bioactivity (Field *et al.*, 2008). In fact, variants nisin depending on the mutation point may display a significant increase in activity, but little is known about the effect of this enhancement of the molecule on the microbial cell.

In this work, the molecular effects of three amino acids changes in nisin named N20P, M21V and S29A were modeling and the hypothesis of effects in pore formation derivate of the models were validate. Nisin N20P had a asparagine replace by proline at position 20, whereas nisin M21V had a methionine replace by a valine at position 21 and both forms have been showed increased activity on Gram-positive bacteria (Field *et al.*, 2008; Field *et al.*, 2015). Nisin derivate S29A had a serine replace by an alanine at position 29, hinge region E and this derivate was more potent than nisin A against Gram-positive and Gram-negative bacteria (Field *et al.*, 2012).

2.2 Materials and Methods

2.2.1 Bacterial strains and culture conditions

The bacterial strains used in the study are shown in Table 1. Strains of *L. lactis* mutants for the production of modified nisin were obtained from the bank of nisin derivatives (Field *et al.*, 2008) belong to University College Cork and Teagasc Moorepark, Ireland.

Wild type nisin A and nisin derivatives purification were carried out as described by Field *et al*. (2010, 2015b) and Campion *et al*. (2017). Nisin and nisin derivatives producing *L. lactis* strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30 °C. Chloramphenicol at 10 μg mL^{-1} was used for the growth of *L. lactis* mutants. *S. aureus* strain was grown in Tryptic Soy Broth (TSB) (Sigma, India) and incubated for approximately 18 h at 37 °C. After growth, cells were centrifuged at 2500 *g* for 10 min, washed twice in phosphate buffered saline (PBS) and resuspended in TSB broth.

Table 1 Strains used in this study

Strains	Characteristics	Reference
L. lactis NZ9800 pCI372*nisA*	Wild type nisin A producer	(FIELD *et al.*, 2008)
L. lactis NZ9800 pCI372*nisA*::N20P	Strain N20P producer	(FIELD *et al.*, 2008)
L. lactis NZ9800 pCI372*nisA*::S29A	Strain S29A producer	(FIELD *et al.*, 2012)
L. lactis NZ9800 pCI372*nisA*::M21V	Strain M21V producer	(FIELD *et al.*, 2010)
S. aureus 113 WT - ATCC 35556	Model virulence studies	(PESCHEL *et al.*, 1999)

2.2.2 *In silico* analysis

The structural data of the nisin-lipid II complex with code 1WCO was imported from the Protein Data Bank (PDB) (Berman *et al.*, 2000). The molecule available in the PDB is nisin Z. The PDB file was loaded and the molecule of nisin were mutated by manual adjustment of the rotamer conformations and optimization of conformations using UCSF Chimera software, Version 1.2 (Pettersen *et al.*, 2004). First, the complex 1WCO containing nisin Z molecule was aligned using the CLUSTAL Omega Version 1.2.4 (https://www.ebi.ac.uk/Tools/msa/clustalo) with the sequence of nisin A. The distinct amino acids were substituted in order to constructed the nisin A molecule using the UCSF Chimera software. Once that nisin A structure was obtained, the other alterations corresponding to each mutations were also made and optimized. At position 20 the amino acid N (asparagine) was replaced by the P (proline), generating the N20P derivative. At position 29 the amino acid S (serine) was replaced by A (alanine) and at position 21 the amino acid M (methionine) was replaced by V (valine).

The atomic distances were calculated among the mutation sites of the variant nisins and lipid II using The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC (http://www.pymol.org/). All structures were visualized and figures were prepared in PyMOL software either.

Analysis of hydrophobicity, physicochemical proprieties and contact surface was also performed to nisinA and nisin variants through of measurement of the parameters LogP, LogD and the geometry of the molecules calculated by MarvinSketch software version 16.6.20.0 (https://chemaxon.com/products/marvin).

2.2.3 Analysis of NADPH and ATP

To determine intracellular content leakage, *S. aureus* cells grown in BHI at 37 °C for 24 h were harvested by centrifugation at 10,000 *g* for 10 min. The cell pellet was washed with PBS twice and re-suspended using BHI to a final optical density (OD) of 0.2 at 600 nm measured with a spectrophotometer (Thermo Fisher Scientific, Finland). The BHI broth was incubated at 37 °C and absorbance was monitored until OD 0.5. Then, peptides were added at a final concentration of 0.625 µM (12.8 µg/mL). This concentration represents 1/4 of the MIC of the peptide more effective. Controls were performed with untreated cells.

During incubation, aliquots of 2 mL were collected in times 30 min, 1 and 2 h for analysis of NADPH and ATP leakage. The aliquots were centrifuged at 2,500 *g* at 4 ºC for 10 min. The supernatant was filtered on membranes with 0.22 µm pores (Millipore®, Merck, Darmstadt, Germany). In order to measure the concentrations of NADPH, 200 µL of the filtrate were added to 96-wells microplate and checked by absorbance at 340 nm using a spectrophotometer (Thermo Fisher Scientific, Finland) (Zhang *et al.*, 2000) The standard curve was obtained using concentrations of NADPH (Sigma-Aldrich, USA) ranging from 0.007 to 0.5 mM diluted in medium BHI and the R^2 obtained was 0.973.

The ATP analysis was performed using the Kit Bac Titer-Glo™ Microbial Cell Viability Assay Reagent (Promega, Madison, Wisconsin, USA). The volume 50 µL of the filtrate was mixture with 50 µL of Bac Titer-Glo™ Reagent and added in opaque 96-wells microplate. The microplate was incubated at room temperature for 15 min in the dark. The ATP concentration leakage was determined using a fluorescence detector (SpectraMax M5, Molecular Device, Sunnyvale, California, USA) at 560 nm.

Standard curve was done using standard ATP (Promega, Madison, Wisconsin, USA) ranging from 1 pM to 100 µM diluted in medium BHI. The standard curve obtained showed R^2 of 0.995. The experiments were performed two times. Statistical analyses were performed using Minitab statistical software 17.0. Tukey's test was used to determine the existence of differences between the treatments in each time. A significance level of 0.05 was adopted.

2.2.4 Atomic force microscopy (AFM)

S. aureus cells were prepared as described previously, briefly, peptides were added at a final concentration of 0.625 µM (12.8 µg/mL) and the cells on BHI broth was incubated at 37 °C. Then, 1 mL aliquots were collected at 1 h, centrifuged at 10,000 *g* for 10 min, washed five times with 0.85 % saline, and resuspended in 0.85 % saline. The cells were spread onto previously sterilized glass slides (1 cm×1 cm). Changes in the *S. aureus* cell envelope were observed by AFM (NT-MDT, N Ntegra Prima, Moscow, Russia).

2.3 Results and discussion

2.3.1 Modeling and *in silico* analysiss

The nisin molecule consists of 34 amino acids (Table 2) stabilized by five thioester rings (A, B, C, D, E) and acts by two mechanisms of action including inhibition of peptidoglycan biosynthesis and membrane pore formation (Cotter *et al*., 2005). The key point for the process to occur is its binding to lipid II, the biosynthetic precursor of the bacterial cell wall (Mitchell *et al*., 2018). The A, B and C rings form a cage that facilitates binding of the pyrophosphate moiety of lipid II (Field *et al*., 2015a). The structural role of ring C and the "hinge region" is not completely understood, but are clearly important for the activity of nisin and the flexibility in this region is a key factor (Mitchell *et al*., 2018). So, right after binding to lipid II, the C-terminal moiety, nisin comprising the D and E rings, inserts into the bacterial membrane to form the pore structure (Hasper *et al*., 2004).

Genetic manipulation of strains of *L. lactis* that results in modification of nisin at specific points generated improved derivatives, however the mechanism by which

this occurs is still unknown. In order to help understand the potential molecular mechanisms associated to increase of activity of modified nisin, the structure models of nisin-lipid II complexes were proposed and measurements between the mutation point and the lipid II linker were performed. All mutations were made from position 20 and, in the nisin N20P, the mutation point was localized 9.9 Ä, whereas the nisin S29A this alteration was at 30.4 Ä and M21V was at 11.5 Ä distance of lipid II (Figure 1). The N20P and M21V nisin molecule modelled in this study have the point of mutation in the flexible region of the molecule, whereas in the nisin S29A, the point of mutation is localized in the hinge region E. The distance between the mutation where the amino acid substitution occurred and the lipid II binding was supposed to be not interfering with the increased activity of the nisin derivatives, mainly because of the three derivatives studied here, M21V was the one that presented greater activity (100%) than nisin A against *S. aureus*, but its mutation is far from the point of interaction with lipid II.

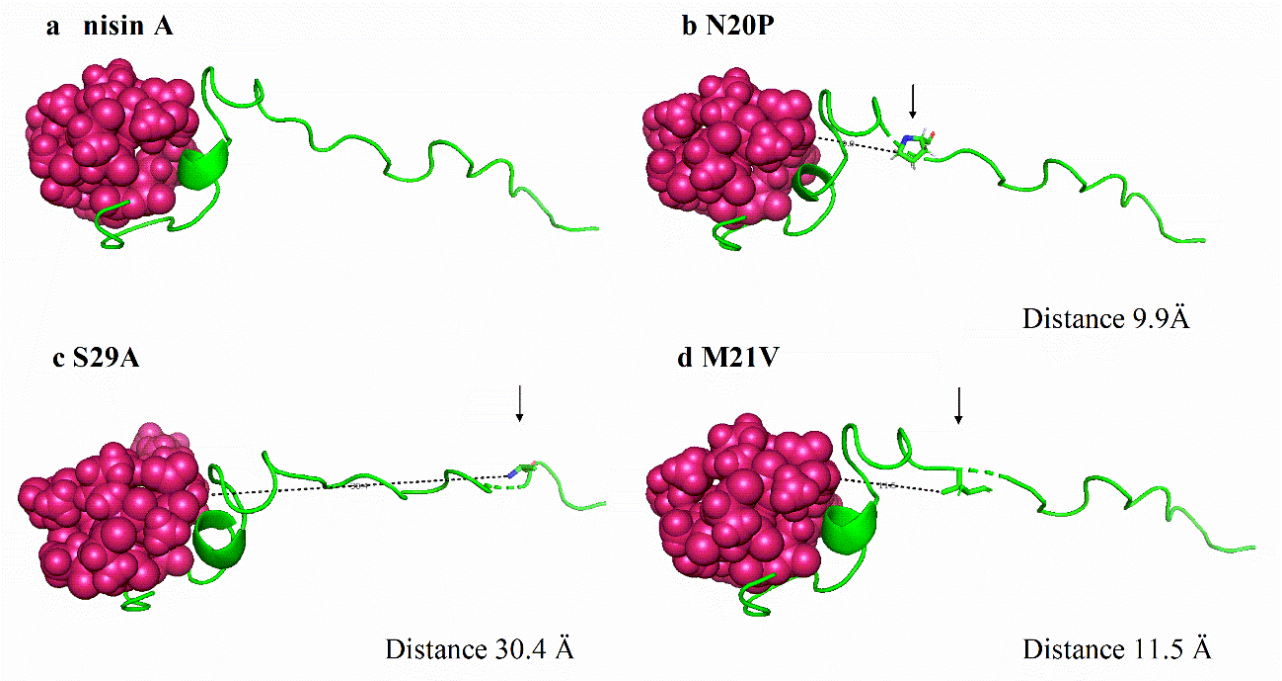

Figure 1: The measurement of the distance from mutation points to lipid II in predicted structure models. a) Nisin A, b) N20P, c) S29A and d) M21V. Nisin molecule displayed in green and lipid II in magenta. The mutation points are showed as color sticks (carbon - green, nitrogen - blue, oxygen - red) and they are also signalled by arrows.

Other inherent factors of the molecule may lead to improved antimicrobial activity. Ge *et al.* (2016) studied the improving both nisin induction and antimicrobial activity and observed that modifications at that point of the molecule may result in increased activity, but it depends on the amino acid which has been changed. Most substitutions at position 21 improved induction activities by 11 to 17% except M21H with reduced induction activity of 29% and M21W with induction activity of 94% compared with nisin A (Ge *et al.*, 2016).

Another issue to be observed is the steric and physicochemical characteristics of those amino acids that have been replaced and, in the three modified nisin evaluated, the common point is that they are all hydrophobic. The results of Figure 2 show that these amino acid relocations generate small steric modifications of the nisin structure of N20P (Figure 2b) and S29A (Figure 2c) when compared with nisin A, however, the M21V derivative (Figure 2d) showed a higher conformational modification resulting from the exchange of Methionine by valine. The structure of the molecule of N20P and S29A remained similar to that of nisin A, while the modification of M21V presented a steric modification because was a removal of a branch in the surface of molecule (circled in the Figure 2). Nisin binds with high selectivity to lipid II, a key biosynthetic precursor of the bacterial cell wall, with the resulting 8:4 nisin:lipid II complex leading to the formation of pores in the bacterial membrane (Mitchell *et al.*, 2018). Structural analysis of nisin indicated that the hydrophilic groups of the peptide interact with the phospholipid headgroups, and the hydrophobic side chains are immersed in the hydrophobic core of the membrane (Wiedemann *et al.*, 2001; Mulholland *et al.*, 2016). The interaction of the molecule with the ligand depends on functional groups and complementarity of surface and this steric modification presented by the molecule M21V (Figure 2d) may have become the structure more planar, enabling a better complementarity of the molecules during the pore formation. This modification can also improve the interaction of the nisin molecule with the constituents of the plasma membrane.

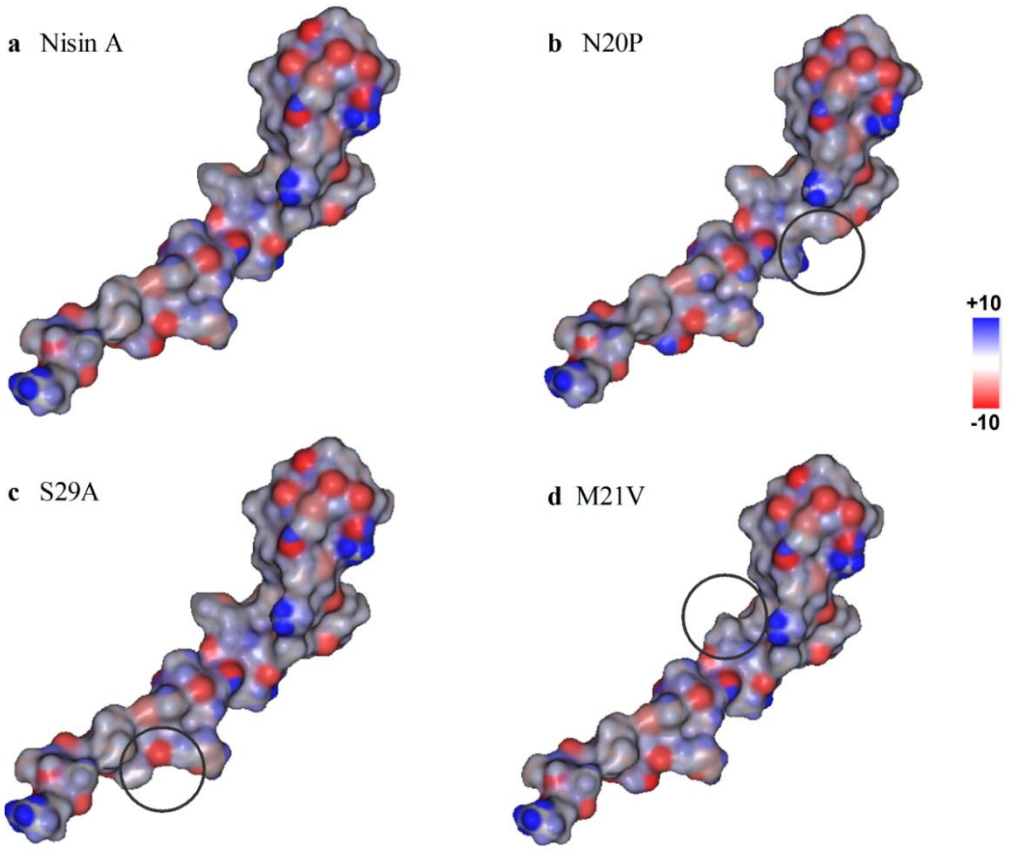

Figure 2: Changes in charges and conformations of the nisin A molecule after mutations. a) Nisin A, b) N20P, c) S29A and d) M21V. The point of the mutation with the charge changes is indicated by a circle. Color variations for blue shows positive charges, and color variations for red indicate negative charges. Gray colors indicate neutral charges.

The contact surface together with the hydrophobic forces are fundamental for interactions between molecules to occur (Tareste *et al.*, 2007). After observing the modification in M21V steric groups, hydrophobicity parameter measurements, Log P of non ionic species and Log D were calculated (Table 2), the results showed that the substitution of amino acids become the molecule more apolar observed by increase in

the logP and logD values. In this case, M21V remained with polarity characteristics similar to nisin A, while N20P and S29A became more apolar. Contact surface was also calculated for different nisin derivative (Table 2) and, although the polarity characteristics remain similar to nisin A, M21V had a reduction in its contact surface (Table 2) probably due to the increase flatness of the molecule.

Table 2 Polarity and steric characteristics of nisin variants measured by the parameters of LogP, LogD and molecular surface area in pH 7.2 .

Peptides	**Log P**	**LogD**	**Surface area***
Nisin A	-12.97	-15.86	5080.96
N20P	-11.15	-14.14	5071.14
S29A	-11.92	-14.81	5059.54
M21V	-12.73	-15.63	5050.55

*pH 7.2

In silico analyzes indicated modifications in the structure of the mutated nisins. If the molecule becomes more planar, it will probably have a better fit of molecule with binder generating important change that may be related to the pore in the *S. aureus* membrane. In order to confirm the hypotheses raised by *in silico* analyzes such as higher efficiency to produce pores by M21V, the quantification of the extravasation of cellular contents represented by NADH and ATP were performed.

2.3.2 Leakage of NADPH and ATP

The pore formation on cellular membrane by nisin that results in the leakage of intercellular content was confirmed by NADPH and ATP leakage (Figure 3). The concentration at which the *S. aureus* cell was exposed was fixed, represent 1/4 of the MIC of the bacteriocin more effective (M21V). This concentration was necessary to be able to compare and observe the possible changes in *S. aureus* cell membrane by nisins.

The concentration of NADPH and ATP in the untreated control and nisin A treatment did not present statistical differences at all times analysed. This result does not mean that nisin A does not lead to leakage but may indicate that the concentration used may not have been sufficient for the process to occur. At 30 min of cultivation, a high concentration of extracellular NADPH was observed in the treatments with nisin

derivates in comparison to the untreated control (Figure 3a), but no statistical difference was observed for ATP (Figure 3b).

The N20P and S29A did not cause an increase in NADPH and ATP extracellular concentration when compared to nisin A, over two hours of incubation, indicating that pore formation in the membrane occurs similarly between these peptides. Interestingly, *S. aureus* treated with M21V demonstrated considerable leakage of NADPH in the time 1 and 2 h in comparison to the N20P and S29A. This result confirms the hypothesis that this nisin derivative may be leading to larger and consequent pores leading to greater leakage of intracellular content as predicted in the *in silico* analysis of the structure planarity of the molecule.

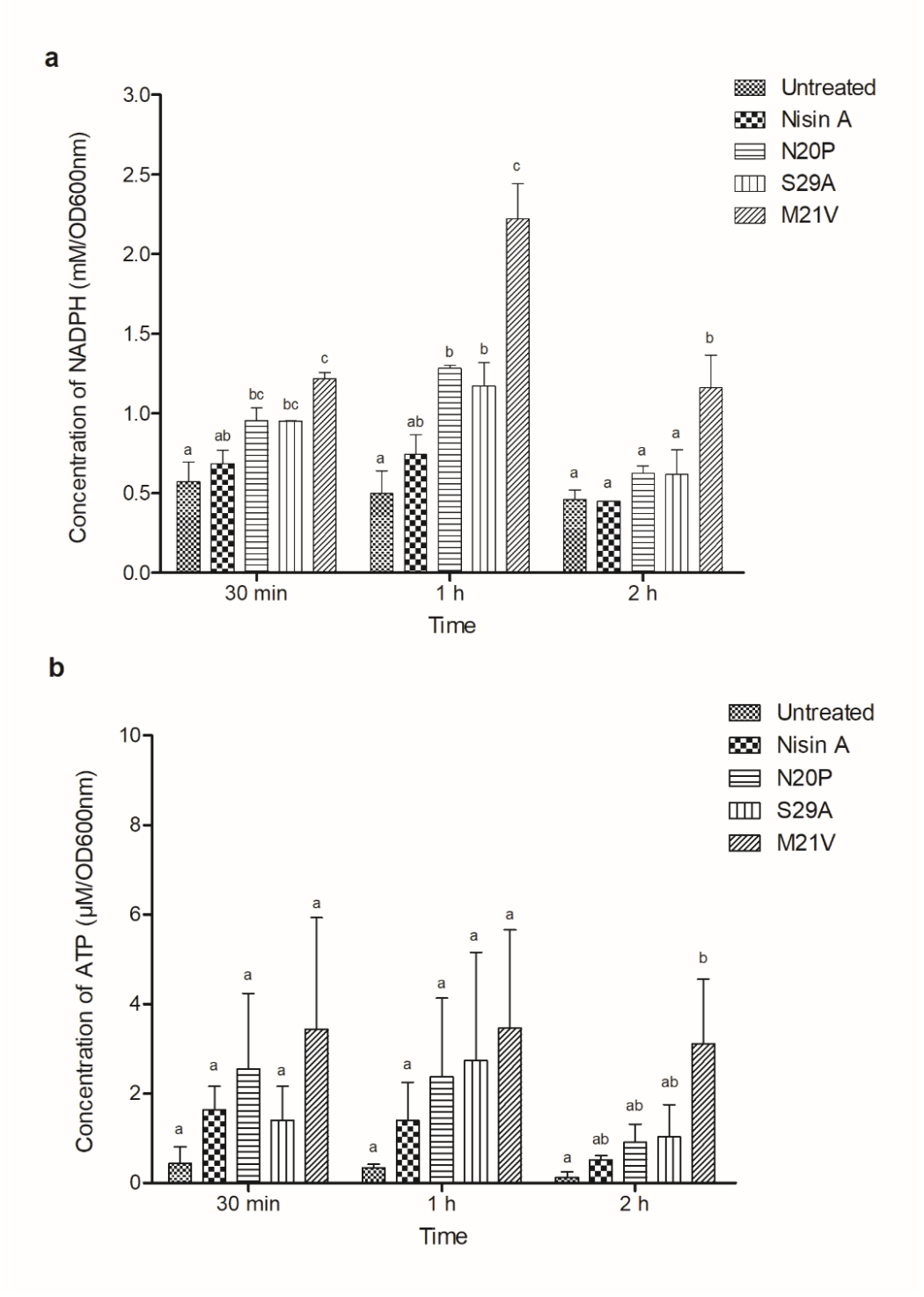

Figure 3: Quantification of extracellular NADPH and ATP levels in *S. aureus* 113 treated with nisin variants. A) NADPH. B) ATP. The cells were incubated over two hours at 37 °C in the presence of 0.625 µM (12.8 µg/mL) of nisin A and each variant.

2.3.3 Atomic force microscopy

The cellular morphology of untreated *S. aureus* cells during the logarithmic growth phase in BHI broth exhibited a coccoid form and a compact and slightly homogeneous surface topography and no obvious damage was observed by atomic force microscopy (Figure 4). In contrast, the cells treated with peptides showed different modifications in cell surface structure, with the topographic profiles presenting variation that was slightly more irregular in the presence of nisin A (Figure 4B) and derivatives (Figure 4 C-E). Cells treated with nisin N20P and S29A presented major alterations in the cell wall topography when compared to nisin A (Figure 4C and 4D) and those in presence of nisin M21V demonstrated considerable decrease in volume, visualized by cell surface irregularity, with higher roughness and depressions (Figure 4E). These modifications on cellular topography were associate with loss of turgidity due the formation of pores in the cell membrane and extravasations of intracellular contents. Similar results of alteration of cell surface topography due to the use of bacteriocin that act to form pores were observed by other authors. Prudêncio *et al.* (2016) observed this effect when the *Salmonella* Typhimurium cells were treated with of bovicin HC5 (100AU/mL) and EDTA (1.6mM). In resume, our results showed that the nisin A derivatives cause major changes in the cell wall, being that the M21V is the most effective.

In conclusion, *in silico* analysis revealed that the nisin molecule after the amino acid replace at position 21 became more planar, planar molecules usually have better fit with others, since it improves the hydrophobic interaction between them, although the modification led to a smaller contact surface after removal of an arm from the molecule. It is important to say that the more atoms that are close to each other the better the interaction with other molecules, the exit of a branch after the exchange of methionine with valine, can increase the number of atoms that approaches one of the others, because the hydrophobic interactions depend on that number of atoms. The experimental results showed that this modification, M21V, led to a higher extravasation of the cellular content and greater changes in the surface of the membrane. Our findings help to clarify why some point changes may cause increased nisin activity.

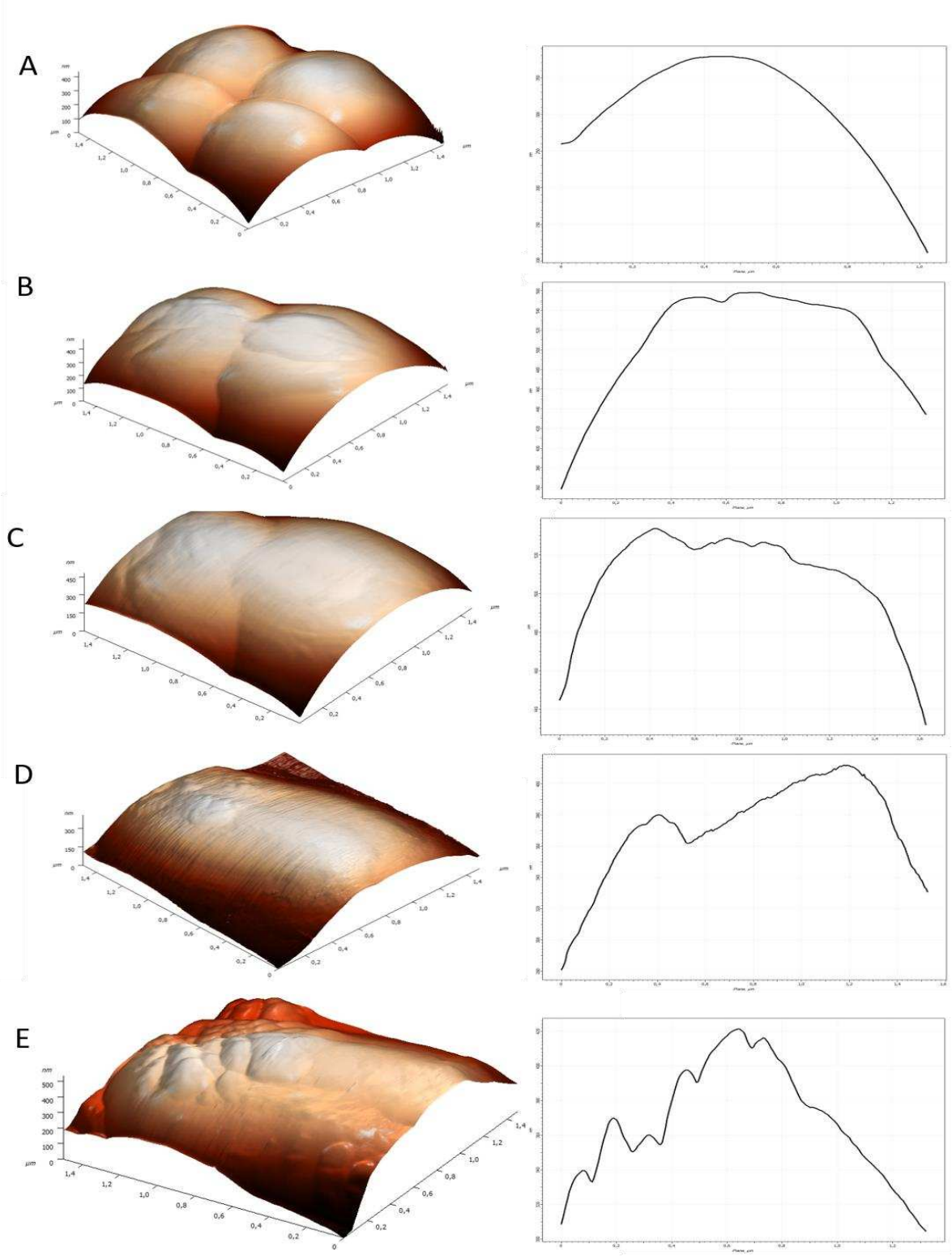

Figure 4: Atomic force microscopy (AFM) images of *S. aureus* cells. A) Untreated B) nisin A, C) N20P, D) S29A and E) M21V. Incubated by 1 h at 37 °C in the presence of 0.625 µM (12.8 µg/mL).

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CHAPTER 3 Activity of nisin and nisin derivatives against *Staphylococcus* biofilm

Activity of nisin and nisin derivatives against *Staphylococcus* biofilm

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Abstract

The emergence of multi-resistant drug pathogens has necessitated the search for novel or improved alternatives to control microbial growth. For this purpose, the bioengineering of antimicrobials is a strategy that has been successfully explored in recent years. Nisin is a bacteriocin that is widely used as a preservative in the food industry and, more recently, has been improved for a wider use, including as an antibiofilm agent. Nisin exhibits strong antibacterial activity against many Gram-positive bacteria, including pathogens such as *Staphylococcus aureus.* In this present study, the activity of wild-type nisin A and the nisin bioengineering derivatives N20P, S29A and M21V, was assessed against *S. aureus* biofilms. Biofilm integrity and metabolic activity were determined using crystal violet and XTT reduction tests, respectively. The bioengineered nisin peptides had a greater ability to inactivate *S. aureus* biofilms, both by removing cells or by killing sessile cells. The influence of *dltA* and *mprF*, i.e., genes that contribute to the resistance of *S. aureus* to cationic peptides such as nisin, was also evaluated using isogenic *S. aureus* mutant strains. Deletion of genes *dltA* and *mprF* do not altered the ability of these strains to form biofilm but mutants' sessile cells were more sensitive to the action of the bacteriocin. The results showed the enhanced antibiofilm potential of modified nisin peptides and highlight the merits of employing bioengineering-based approaches to generate antimicrobials to better control the growth and removal of *S. aureus* biofilms.

Keywords: biofilm, bacterial resistance, antimicrobial peptide, nisin, lantibiotic, bacteriocin, staphylococci

3.1 Introduction

Staphylococcus aureus is an opportunistic pathogen of importance in medicine and within the food chain (Di Ciccio *et al.*, 2015; Srey; Jahid; Ha, 2013). It is also recognized as a potent biofilm former (Otto, 2008; Vaishampuan *et al.*, 2018; Zapotoczna *et al.*, 2018). *S. aureus* can adhere to, and form biofilm on, different biotic and abiotic surfaces, resulting in serious contamination problems and economic losses due to food spoilage, equipment damage and impact on public health (Di Ciccio *et al.*, 2015; Jahid; Ha, 2012; Zottola; Sasahara, 1994). Biofilms are three-dimensional cellular aggregates characterized by the presence of self-produced polymeric matrix substances that conferring protection of adherent cells against antimicrobial agents (Costerton, 1999; Flemming; Wingender, 2010). Biofilms play a key role in the survival of bacterial species across a variety of environments by providing resistance to antimicrobial agents (Wu *et al.*, 2015).

Antimicrobial peptides, such as bacteriocins, have been widely studied because of their frequently broad bactericidal spectrum, potential applications in a variety of foods, as well as their ability, alone or in association with other antimicrobial compounds, to inactivate bacterial biofilms of relevance to food or medicine (Cabo *et al.*, 2009; Davison *et al.,* 2010; Field *et al.*, 2015; Mataraci; Dosler, 2012; Saá Ibusquiza et al., 2011).

The most studied and extensively used bacteriocin is the food preservative nisin A (nisin). Nisin is produced by *Lactococcus lactis*, and has been recognized by the Food and Drug Administration (FDA) since 1989 as GRAS (Generally Recognized as Safe) (Yang *et al.*, 2014). Nisin acts on the bacterial cell envelope by binding with lipid II and undecaprenyl pyrophosphate to inhibit cell wall formation and by forming pores that allow the extravasation of the cellular content and the loss of proton motive force in target cells (Bierbaum and Sahl, 2009; Cotter *et al.*, 2013; Lanne *et al.*, 2019). Nisin also inhibits bacterial cell division (Lanne *et al.*, 2019). Although the potential effect of nisin on bacterial biofilm has been demonstrated (Davison *et al.*, 2010; Field *et al.*, 2015; Okuda *et al.*, 2013; Pimentel-Filho *et al.*, 2014), its ability to remove sessile cells is low (Davison *et al.*, 2010).

Bioengineering strategies have provided a promising means of enhancing the physicochemical properties of nisin, including through enhanced specific

antimicrobial activity, improving solubility and stability and permitting better diffusion through complex polymers (Field *et al.*, 2015). Indeed, screening of large collections of nisin derivatives has resulted in the identification of variant peptides with highly bactericidal effect against both sessile and planktonic forms of pathogens (Field *et al.*, 2008; Field *et al.*, 2015; Campion *et al.*, 2017). Among the enhanced nisin derivatives are nisin N20P, M21V and S29A (Field *et al.*, 2012). The first two peptides were created by substitution of the amino acid residue in the flexible central 'hinge' region of nisin A, i.e., nisin N20P contains an asparagine rather than a proline at position 20 and, in nisin M21V, a methionine has been replaced by a valine at position 21, with both peptides exhibiting improved activity against *S. aureus* (Field *et al.*, 2008; Field *et al.*, 2015). The nisin derivative S29A contains an alanine instead of a serine at position 29 (i.e., within hinge region E near the C-terminus of the peptide) and is more potent than nisin A against a range of Gram-positive and Gram-negative bacteria (Field *et al.*, 2012).

S. aureus possesses a number of features that contribute to its ability to resist the action of antimicrobial peptides. These include altering the anionic charge of its surface by modification of teichoic acids with D-alanyl groups, as well as possessing positively charged phospholipids in its membrane (Zapotoczna *et al.*, 2018). These modifications contribute to making the *S. aureus* cell envelope more positively charged, thus repelling cationic antimicrobials peptides (Cheung *et al.*, 2018). The inactivation of the *dltA* and *mprF* genes, which are responsible for these modifications, result in increased susceptibility to antimicrobial peptides (Joo *et al.*, 2016). In this study, the efficacy of nisin A and the three bioengineered derivatives, N20P, M21V and, S29A, against biofilms formed by a wild type and Δ*dltA* and Δ*mprF* strains of *S. aureus* is investigated.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains used in the study are shown in Table 1. Strains of *L. lactis* capable of producing bionegineered nisin peptides have been described previously (Field *et al.*, 2008, 2012). Nisin and nisin derivative producing *L. lactis* strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30 °C

(where necessary in the presence of chloramphenicol at 10 μg mL^{-1}). *S. aureus* strains were grown in Tryptic Soy Broth (TSB) (Sigma, India) and incubated for approximately, 18 h at 37 °C. After growth, cells were centrifuged at 2,500 *g* for 10 min, washed twice in PBS and resuspended in TSB broth.

Table 1 Strains used in this study

Strains	**Characteristics**	**Reference**
L. lactis NZ9800 pCI372nisA	Wild type nisin A producer	(FIELD *et al.*, 2008)
L. lactis NZ9800 pCI372*nisA*::N20P	Strain N20P producer	(FIELD *et al.*, 2008)
L. lactis NZ9800 pCI372*nisA*::M21V	Strain M21V producer	(FIELD *et al.*, 2010)
L. lactis NZ9800 pCI372*nisA*::S29A	Strain S29A producer	(FIELD *et al.*, 2012)
S. aureus 113 WT - ATCC 35556	Model virulence studies	(PESCHEL *et al.*, 1999)
S. aureus 113 Δ*dltA*	Inactivation of the *dltA*	(PESCHEL *et al.*, 1999)
S. aureus 113 Δ*mprF*	Inactivation of the *mprF*	(PESCHEL *et al.*, 2001)

3.2.2 Nisin purification

Nisin purification was carried out as described by Field *et al.* (2010, 2015b) and Campion *et al.* (2017). Stock solution of peptides (1000 mg/mL) in sterile sodium phosphate buffer (10 mM, pH 7.2) were stored at -20 ° C until use, at which point they were diluted in culture medium to the appropriate concentration.

3.2.3 Minimal inhibitory concentration assays

The Minimal Inhibitory Concentration (MIC) of nisin and nisin derivatives were determined as described by Field *et al.* (2015). Briefly, each well of 96 well microtitre plates were pre-treated with 200 μL of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (PBS/BSA) (Sigma, USA) and incubated at 37 °C for 30 min. The wells were washed with 200 μL PBS and allowed to air-dry. Then, 200 μL of TSB medium supplemented with starting concentration of 10 μM of nisin A and nisin derivatives and 2-fold serial dilutions of each peptide were distributed on wells. The strains of *S. aureus* were centrifuged, washed with 10 mM PBS at pH 7.4 and diluted until search at OD_{600} of ~0.5 (approximately 5×10^5 cfu/mL).

Absorbance was measured at 600 nm using a microtiter plate reader (Biotek, Germany) and the nisin MIC was taken as the lowest concentration of peptide at which growth was inhibited after 18 h incubation at 37 °C.

3.2.4 Biofilm formation

S. aureus strains were tested for biofilm formation following growth in TSB and BHI (Brain Heart Infusion) (Himedia, India) and were classified as biofilm forming as described by Stepanovic *et al.* (2007). In brief, the average OD (Optical density) values and negative control with only culture medium were calculated for three strains. Then, the break point value, ODc (Optical density of control) was established. The ODc was defined as three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3×SD of negative control). Lastly, the OD value of a tested strain was expressed as the average OD value of the strain, reduced by ODc value (OD=average OD of a strain −ODc). The ODc value was calculated for each microtiter plate separately. After calculations, the strains were classified as biofilm producer (-), weak biofilm producer (+), moderate biofilm producer (++) and strong biofilm producer (+++), based in the calculated of OD values: OD <ODc = no biofilm producer; ODc < OD ≤ 2xODc = weak biofilm producer; 2x ODc <OD ≤ 4xODc = moderate biofilm producer; 4xODc <OD = strong biofilm (Stepanovic *et al.*, 2007).

S. aureus biofilms were formed for 24 h in microtiter plate wells, washed once with PBS then and treated with nisin and nisin derivatives, which were added to the microtiter plate wells at 1X, 2X, 4X, 8X and 16X MIC. After incubation for 24 h, at 37 °C, the culture supernatant was discarded, and the surface-attached cells were stained with 200 µL of 0.1% (w/v) crystal violet for 30 min. Then, the crystal violet was removed and the plates were washed three times with water. After air drying for 15 min at 37 °C, the attached cells were determined at 590 nm with the microtiter plate reader (Biotek, Germany) by addition of 200 µL of 95% (v/v) ethanol. The average of the data obtained in triplicate and repeated three times was determined by non-linear dose response analysis. The percentage removal of cells was calculated considering: (% biofilm reduction) = 100 – [(OD590nm of peptide treated cells / OD590nm of control) * 100] and R-squared greater than 0.8 was adopted.

The viability of the biofilm cells was evaluated after incubation for 24 h, at 37 °C. For this, biofilms exposed to peptides were gently washed once with PBS, then 100 µL of a solution containing 500 mg XTT/L (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma, USA) with of 10 mM menadione (Sigma, USA) was added to each well. Microtitre plates were incubated for 2 h at 37 °C in the dark. Absorbance was measured at 492 nm using a microtiter plate reader. The tests were performed in duplicate, repeated three times and, results were evaluated by non-linear dose response analysis. The percentage of dead cells was calculated as follows: (% Death of biofilm cells) = 100 – [(OD492nm of peptide treated cells / OD492nm of control) * 100] and R-squared greater than 0.8 was adopted. All analyses and graphs were made using the software GraphPad Prism 5.

3.2.5 Statistical analysis

The impact of nisin on OD (590 nm, 492 nm) were tested by regression analysis, verifying the fitting of polynomial models for the OD (dependent variable) as a function of different nisin A and derivates concentrations (explanatory variable). The concentrations were chosen based on the MIC of the peptide for each strain. The best regression model was selected based on *p*-value significance and adjusted R-squared. Thus, the estimated concentration that resulted in the lowest OD was selected for subsequent analyses. All analyses were performed using R software.

3.2.6 Confocal microscopy

For confocal microscopic analysis, biofilms of *S. aureus* were formed in µ-Slide 8 Well (Ibidi, Germany) in TSB broth in the presence and absence of nisin at a concentration defined by the regression analysis, i.e., the best concentration of peptides capable of inactivating the biofilm. The biofilm was formed in the same way as described previously. Then, the µ-Slide 8 Well were rinsed twice by immersion in PBS (0.2 M; pH 7.2) and stained using a Live/Dead BacLight bacterial viability kit (Life Technologies, USA). After incubation in the dark for 15 min, the residual stain was removed. The images were captured by a LSM 5 exciter confocal microscope (Zeiss, Germany) with a Plan-Apochromat 100x/1.40 Oil M27 lens and images were acquired using the Zeiss LSM image software (Zeiss, Germany). Images were acquired using

two separate confocal channel (one for Syto 9 and one for PI) with pinhole adjusted to 1 (confocal pinhole).

3.3 Results

3.3.1 Minimal inhibitory concentration (MIC) of nisin A and nisin derivatives against *S. aureus*

The MICs of nisin A and derivatives thereof against *S. aureus* 113 WT, *S. aureus* 113 Δ*dltA* and *S. aureus* 113 Δ*mprF* were determined. The changes introduced into the N20P and S29A derivatives of nisin A did not result in an altered MIC, relative to nisin A, against *S. aureus* WT (Table 2). However, the nisin M21V peptide was twofold more effective than nisin A against *S. aureus* WT and mutants thereof (Table 2). As expected, mutation of *dltA* sensitised *S. aureus* to the action of nisin A and its derivatives, resulting in MIC values between two and four times lower. Mutation of *mprF* less dramatically sensitized *S. aureus* to the action of nisin and its derivatives (Table 2).

Table 2 Minimal inhibitory concentration of nisin A and nisin derivates against on *S. aureus* WT and mutants.

Strains	Nisin A	Nisin N20P	Nisin S29A	Nisin M21V
	μM (μg/mL)	μM (μg/mL)	μM (μg/mL)	μM (μg/mL)
S. aureus 113 WT	2.5 (8.33)	2.5 (8.33)	2.5 (8.33)	1.25 (4.16)
S. aureus 113 Δ*dltA*	1.25 (4.16)	1.25 (4.16)	0.625 (2.08)	0.625 (2.08)
S. aureus 113 Δ*mprF*	2.5 (8.33)	1.25 (4.16)	1.25 (4.16)	1.25 (4.16)

3.3.2 Biofilm assays

Prior to the treatment of biofilm with the variant peptides, the strains were tested for their ability to form biofilm in TSB and BHI and, after 24 h, all the strains were classified as strongly biofilm producers (Table 3). Even though the strains formed biofilms under both conditions, TSB medium resulted in a higher OD 590 nm and, thus, it was used in subsequent experiments. Although the deletion of *dltA* and *mprF* genes impacted on the OD 590 nm of the corresponding strains, the mutant strains were still classified as strong biofilm producers (Table 3).

Table 3 Production of *S. aureus* biofilm in different culture media

	WT	Δ*dltA*	Δ*mprF*
		OD 590nm	
TSB	+++ (3.59)	+++ (2.45)	+++ (2.85)
BHI	+++ (2.07)	+++ (1.38)	+++ (1.35)

Break point to TSB: OD ≥ 1.01 and break point to BHI: OD ≥ 1.02 (The strains were classified as strong biofilm producer +++)

Biofilms of *S. aureus* WT and the *S. aureus* mutants Δ*dltA* and Δ*mprF* pre-formed on 96-plate were treated with 1x, 2x, 4x, 8x, 16x MIC of nisin A and its derivatives for 24 h. Then, biofilms were assessed by two colorimetric techniques, violet crystal to measure residual biofilm biomass and XTT to quantity the metabolic activity of the biofilm cells treated with nisin and derivatives. The type exposure-response relationship was plotted for easier interpretation. As the concentrations used were constantly doubled, data relative to MIC concentrations for each peptide were transformed in Log (Figure 1).

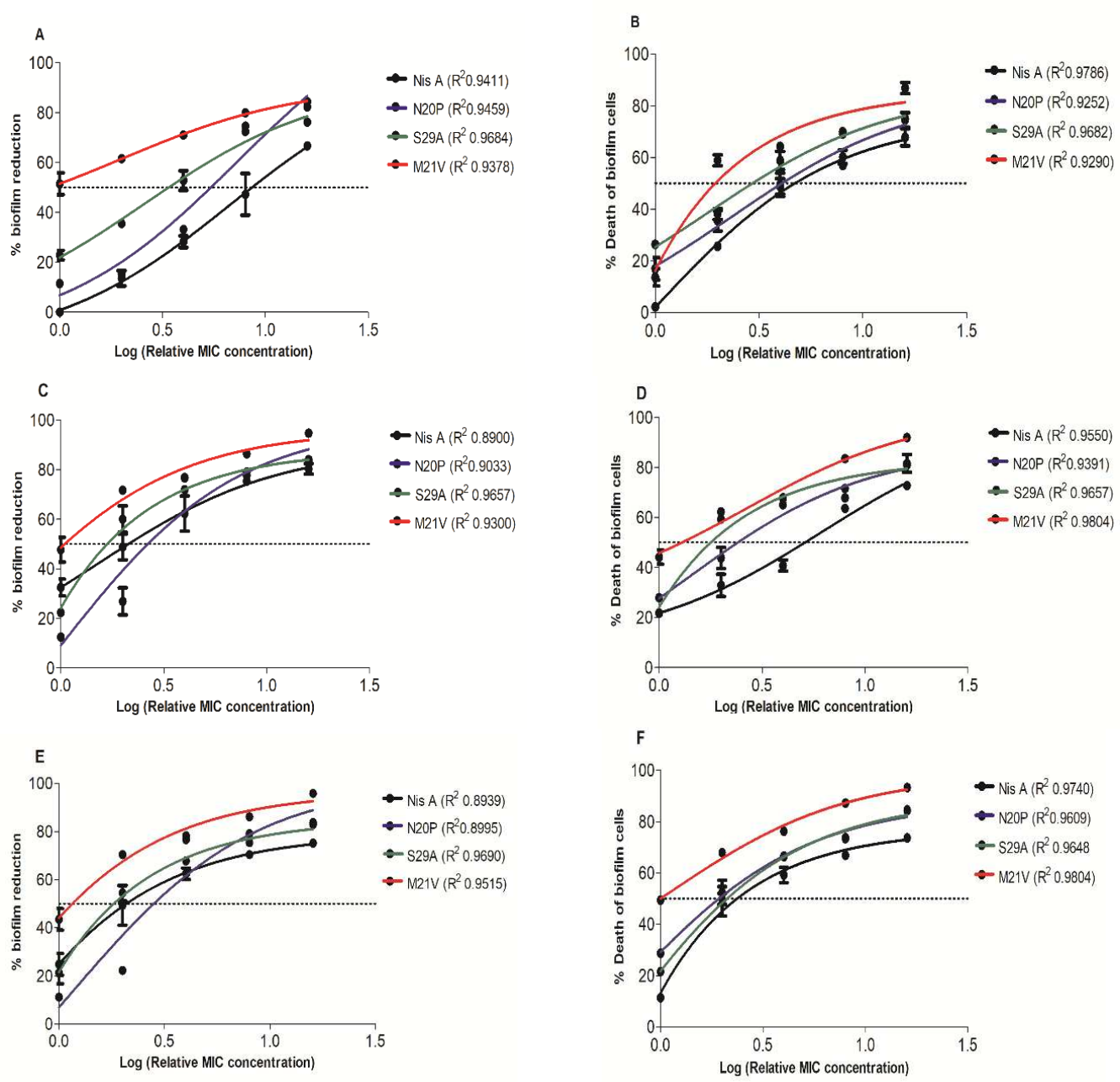

Figure 1: Effect of nisin A and nisin derivate against of *S. aureus* biofilm. Reduction biofilm of A) *S. aureus* 113 WT, C) *S. aureus* 113 Δ*dltA* E) *S. aureus* 113 Δ*mprF*, and viability of biofilm cells of B) *S. aureus* 113 WT, D) *S. aureus* 113 Δ*dltA* and F) *S. aureus* 113 Δ*mprF* formed in microtiter well for 24h. Biofilms were treated with MIC, 2XMIC, 4XMIC, 8XMIC, 16XMIC of nisin A, N20P, S29A and M21V by 24 hours at 37°C followed by measurement of the adhesion by Crystal violet using microtiter plate reader (OD 590 nm) and by the XTT (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) assay measured using a microtiter plate reader (OD 492 nm). The dashed line indicates 50% of reduction or death of biofilm cells.

From this analysis, it is apparent that a higher concentration of nisin A (41.4 µM) was needed to remove *S. aureus* WT cells from biofilms compared with assays

involving *S. aureus* mutants and/or the bioengineered nisin N20P (34.7 µM), S29A (29.3 µM) and M21V (13.7 µM) (Table 4). It is thus apparent that the nisin derivatives are more effective than nisin A with respect to removing biofilms. The M21V peptide was most effective in all cases and the concentration required to inactivate biofilms cells (OD 492 nm) was similar to that necessary to remove attached cells (OD 590 nm).

Table 4 Regression analysis to determine the best concentration of each peptide for removal (OD 590 nm) or inactivate (OD 492 nm) the biofilm of *S. aureus* WT and mutants.

Strains	Peptides	Concentrations (µM)	
		OD 590 nm	OD 492 nm
S.aureus WT	Nisin A	41.4	30.9
	N20P	34.7	31.4
	S29A	29.3	29.0
	M21V	13.7	15.0
S.aureus Δ*dltA*	Nisin A	14.2	16.1
	N20P	14.1	15.0
	S29A	6.9	7.0
	M21V	7.0	7.2
S.aureus Δ*mprF*	Nisin A	28.0	28.3
	N20P	14.2	14.5
	S29A	14.1	14.4
	M21V	14.1	13.9

3.3.3 Confocal microscopy

To further evaluate the impact of nisin treatments, the previous experiment was repeated to allow visualization of the treated biofilms using confocal microscopy in combination with the Live/Dead BacLight viability kit, which facilitates differentiation between metabolic active and dead cells (Figure 2). The concentrations used for each peptide were indicated by the regression analysis (Table 4). The nisin derivatives N20P, S29A and M21V (Figure 2C, 2D and 2E respectively) showed higher removal and inactivation of *S. aureus* WT biofilms compared to Nisin A-treated biofilm (Figure 2B). Minor fluorescence signals were observed in biofilm treated with nisin M21V (Figure 2E) corroborating with previous results.

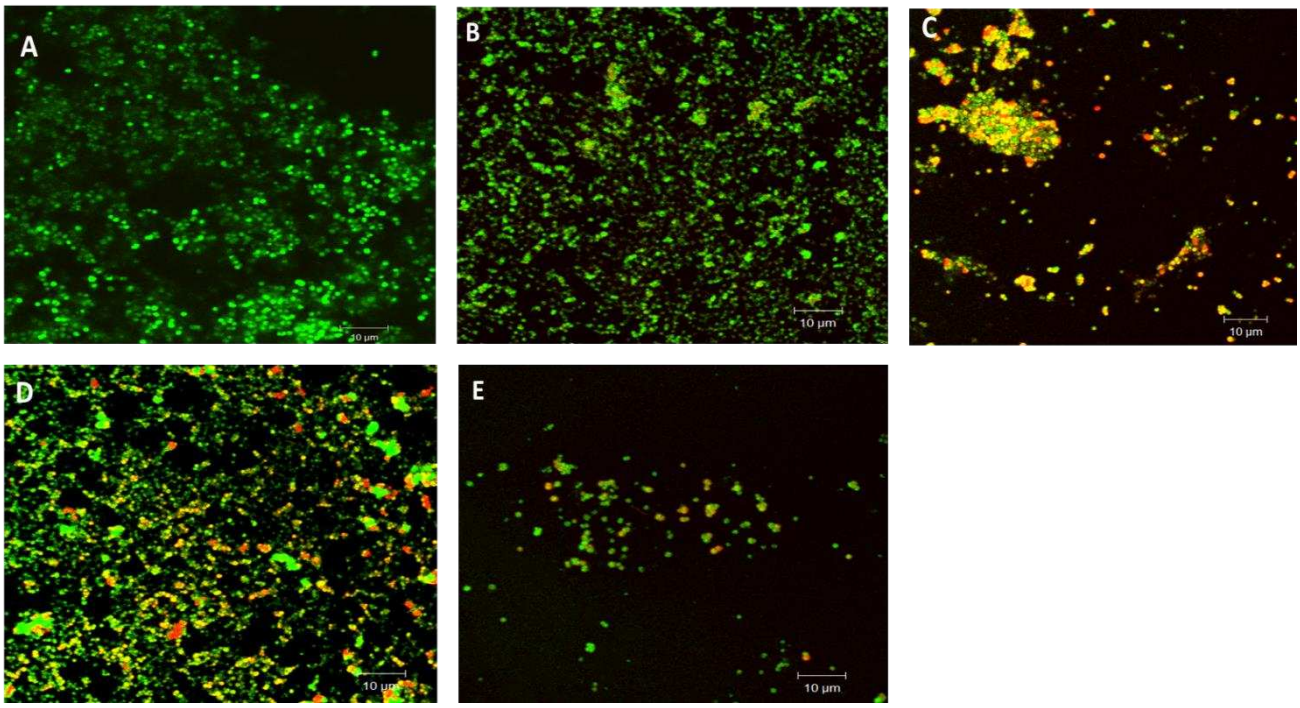

Figure 2: Live/Dead images of *S. aureus* 113 WT biofilm after treatment with nisin A and nisin derivates. Biofilm formed in µ Slides for 24 h. Antimicrobial peptides were incubated with biofilm for 24 h at 37 °C. After washing, the living and dead cells in the biofilm were stained by SYTO9 (green) and PI (red), respectively. A) *S. aureus* 113 WT (untreated) B) *S. aureus* 113 WT treated with (41.4 µM) nisin A (WT). C) *S. aureus* 113 WT treated with (34.7µM) nisin N20P D) *S. aureus* 113 WT treated with (29.3µM) nisin S29A E) *S. aureus* 113 WT treated with (13.7µM) nisin M21V.

3.4 Discussion

S. aureus is a pathogen of great importance in public health and, due to the continuing emergence of multi-drug resistance-related problems, the search for alternative therapies has become ever more important (Li; Webster, 2018). Nisin has been the focus of several studies relating to its potential as an antimicrobial agent beyond its existing use as a food preservative and the previous application of bioengineering techniques resulted in an increase in its antibacterial activity against numerous pathogens, including *S. aureus* (Cotter *et al*., 2013; Field *et al.*, 2008).

Although bioengineering has the potential to enhance antimicrobials, in this instance, not all modifications resulted in enhanced activities as evidenced by the fact that the MICs of the N20P and S29A peptides did not differ from nisin A against *S.*

aureus WT. Field *et al.* (2008) emphasized that the improvement of nisin depends on the point of the molecule where the mutation occurred and the type of amino acid that was replaced. In fact, Field *et al.* (2012) noted that, despite the large collection of nisin derivatives which have been generated by bioengineering, relatively few have been found to consistently exhibit enhanced activity against pathogenic microorganisms, with strain or species-specific enhancements also having been reported.

S. aureus 113 Δ*dltA* showed greater sensitivity to nisin and the nisin derivatives used in this study than *S. aureus* WT. However, deletion of *mprF* did not sensitize the strain to nisin A but did increase sensitivity to N20P, S29A and M21V. The MprF protein participates in the biosynthesis of a positively charged cell membrane lipid lysyl-phosphatidylglycerol (Lys-PG), which reduces the negative net charge of the cellular membrane (Joo *et al.*, 2015). The *mprF*-deficient mutants, according Peschel *et al.* (2001), had increased susceptibility to antimicrobial cationic peptides such as defensins and protegrins, most likely due to an increase in the net negative charge of the bacterial cell membrane, which presumably leads to the accumulation of membrane-damaging peptides on the cell surface. Similar result was found by Nilsson *et al.* (2016) with mutants of *dltA* that presented increased susceptibility to gentamicin. The *dlt* operon that includes d-alanine into teichoic acids, contributes to neutralizing the negative net charge of the staphylococcal cell wall (Peschel *et al.*, 1999).

Formation of biofilm is frequently associated with virulence. The mutants Δ*dltA* and Δ*mprF* continue to be classified as strong biofilm producers even though OD 590 nm values were up to 35% less than those observed in biofilm formed by *S. aureus* WT. This contrasts with the results of Bao *et al.* (2012), who found that *mprF* deletion in *Enterococcus faecalis* resulted in 42% increased biofilm formation compared to the wild type. Furthermore, *Streptococcus mutans* Δ*dltA* exhibited no defect in biofilm formation, but yet, was eight times less tolerant to gentamicin than the corresponding wild type when growing as a biofilm. In contrast, planktonic susceptibility of the Δ*dltA* mutant was only slightly increased compared to wild type (Nilsson *et al.*, 2016). It is thus apparent that the consequences of deletion of these genes across different taxa is variable.

Regarding the activity of nisin derivatives against biofilms, it was noted that the M21V peptide removed around 80% of the biofilm among the tested strains and exhibited the highest bactericidal activity against biofilm of the set of peptides

evaluated. It has also been reported that nisin I4V displays superior activity in reducing biofilm of *Staphylococcus pseudintermediums* compared to nisin A (Field *et al.,* 2015).

It was apparent that the remaining cells within the biofilm showed metabolic activity after 24 h incubation when 16x MIC of nisin A and derivatives were used. According to Davison *et al.* (2010), nisin is able to penetrate deeply into biofilms, and causes a rapid and uniform loss of green fluorescence from all parts of a *Staphylococcus epidermidis* biofilm, indicating loss of viability. Indeed, nisin accessed the interior of biofilm cell clusters faster than the other smaller compounds under examination, including a quaternary ammonium compound and chlorine. It should be considered that nonspecific interactions between bacteriocin molecules and the components of the biofilm matrix and/or the physiological state of the biofilm cells may affect bacteriocin action (Okuda *et al.*, 2013). The inhibitory effect of nisin on *S. aureus* biofilm are considered by other studies (Davison *et al*., 2010; Field *et al.*, 2016; Pimentel-Fiho *et al.*, 2014) but, the mechanisms underlying the bacteriocin resistance of biofilm cells remain unclear.

The two colorimetric techniques used to confirm results, i.e., crystal violet and XTT, provide complementary insights. Alonso *et al.* (2016) and Field *et al.* (2016) suggested that biomass production and metabolic activity must be assessed in parallel, as these methods are used to measure different properties of biofilms, and this was confirmed in our study. Confocal laser microscopy was another technique used to access the effect of bacteriocins on biofilms, and using this approach it was possible to visualize the effectiveness of nisin derivatives against biofilms of *S. aureus*.

In conclusion, our findings demonstrated that nisin derivatives, in addition to exerting enhanced antimicrobial activity, also exert activity against *S. aureus* biofilms. The genes *mprF* and *dltA*, related to mechanisms of resistance to cationic peptides, do not seem to interfere in the biofilm formation of *S. aureus*. However, the deletion of these genes made the biofilms more susceptible to the action of nisin action, and especially to enhanced nisin derivatives. These results support other studies showing that nisin can be improved with a view to its application in both the food industry and the medical field as a means to control biofilms.

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CONCLUSÕES GERAIS

Por análise *in silico*, pode-se concluir que a substituição do aminoácido na posição 21 na molécula de nisina tornou-a mais planar e provavelmente favoreceu as interações hidrofóbicas com o lipídeo II. O maior extravasamento de NADPH e ATP e maiores mudanças na superfície da membrana após exposição à nisina confirmou que o derivado M21V tem maior potencial antimicrobiano. Os resultados obtidos ajudam a esclarecer por que algumas mudanças pontuais podem causar aumento da atividade da nisina.

Derivados de nisina, além de exercer atividade antimicrobiana aumentada, também exerceram atividade contra biofilmes de *S. aureus* e mutantes nos genes *mprF* e *dltA,* relacionados a mecanismos de resistência a peptídeos catiônicos, formam biofilmes mais suscetíveis à ação da nisina e, derivados. Os resultados obtidos confirmam que estratégias de bioengenharia, que tem sido explorada para melhorar a atividade de nisina, podem resultar em moléculas derivadas com maior atividade antimicrobiana. Esta estratégia tem obtido sucesso e, dependendo do ponto da mutação e do aminoácido substituído dentro da molécula, pode-se conseguir aumento da atividade antimicrobiana de nisina.