Influence of nisin mutant selection window concentrations in the antimicrobial resistance profile of enterococci from dogs with periodontal disease

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“If you can dream it, you can do it.”

Walt Disney

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Abstract

Periodontal disease (PD) is one of the most frequent inflammatory conditions in dogs. *Enterococcus* spp. not only have been found in the oral cavity of dogs with PD but have also recently been classified as high priority pathogens for drug development by the World Health Organization. Considering the pressing need to introduce new antimicrobial therapeutic protocols to control canine PD, antimicrobial peptides (AMPs), such as nisin, are a promising alternative to antibiotics since resistance and cross resistance has rarely been described. The ultimate purpose of this work is to continue to validate the use of nisin, incorporated in guar gum gel, for the prevention of canine periodontal disease. The influence of dog saliva in the antimicrobial activity of nisin was assessed using the spot-on-lawn assay. In the presence of saliva, 85% (n=17) of the isolates were inhibited by nisin and nisin incorporated in guar gum gel at a concentration of 4.0 mg/mL. The mutant prevention concentration (MPC) is a crucial parameter establishing at which antimicrobial concentration no mutant-colony is recovered when a high-inoculum is applied onto drug supplemented agar plates. The MPC values of 85% (n=17) of the isolates ranged from 16.0 to 24.0 mg/mL and were 15 to 40 times higher than the previously determined MICs. Antimicrobial resistance, MIC and MBC values were found to be higher in the mutant collection.

The results obtained in this study reinforce nisin's potential to treat canine enterococcal-periodontal disease as well as the importance of correct antimicrobial doses to prevent the development of resistant-mutants during therapeutic regimens.

Keywords: Antimicrobial Resistance; *Enterococcus* spp.; MIC; MBC; MPC; Nisin

Resumo

A doença periodontal (DP) é uma das doenças inflamatórias mais comuns em cães. Bactérias pertencentes ao género *Enterococcus* têm sido encontradas na cavidade oral de cães e que recentemente foram classificadas pela WHO como agentes patogénicos de alta prioridade no desenvolvimento de novos compostos antimicrobianos. Assim, é importante introduzir novas terapêuticas antimicrobianas para controlo da DP em cães. Os péptidos antimicrobianos (PAM), como a nisina, são uma alternativa promissora uma vez que são raras as descrições de resistências e resistências cruzadas. O grande objetivo deste trabalho é a continuação da avaliação da aplicação tópica da nisina, incorporada em gel goma de guar, para prevenção da DP nos cães. Desta forma, procedeu-se à avaliação da influência da saliva canina na atividade antimicrobiana da nisina pelo método *spot-on-lawn*. Assim, na presença de saliva, 85% (n=17) dos isolados foram inibidos pela nisina e pela nisina incorporada em gel goma de guar na concentração de 4.0 mg/mL. A concentração que previne a formação de mutantes é um parâmetro fundamental que estabelece a que concentração um agente antimicrobiano inibe a formação de colónias mutantes, quando um inóculo concentrado é aplicado em placas de agar suplementadas com o agente antimicrobiano. Foi possível determinar este valor em 85% (n=17) dos isolados, variando este valor entre 16.0 e 24.0 mg/mL, sendo 15 a 40 vezes superior à CMI de nisina anteriormente determinada para estes isolados. Além disso, verificou-se que, nas colónias mutantes obtidas, a resistência antimicrobiana, os valores de CMI e de CMB foram superiores aos dos isolados originais.

Os resultados obtidos neste estudo reforçam o potencial da nisina no controlo da DP canina, assim como a importância de uma correta dosagem dos compostos antimicrobianos na prevenção do desenvolvimento de mutantes resistentes durante os regimes terapêuticos.

Palavras-chave: Resistência Antimicrobiana; *Enterococcus* spp.; CMI; CMB; CPM; Nisina

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List of Abbreviations

ABC transporter- ATP-Binding Cassette transporter

ALA- alanine

AMP- Antimicrobial Peptide

AMR- Antimicrobial Resistance

ATCC- American Type Culture Collection

CFU- Colony Forming Unit

CLSI- Clinical Laboratory Standards Institute

DNA- Deoxyribonucleic Acid

FDA- Food and Drug Administration

GC- Guanine-Citosine

IE- Infective Endocarditis

IU- International Units

MBC- Minimum Bactericidal Concentration

MDR- Multidrug-Resistant

MIC- Minimum Inhibitory Concentration

MPC- Mutant Prevention Concentration

MRSA- Methicillin-Resistant *Staphylococcus aureus*

MSW- Mutant Selection Window

PD- Periodontal Disease

Rpm- rotations per minute

SD- Standard Deviation

SER- Serine

VRE- Vancomycin-Resistant Enterococci

WHO- World Health Organization

µL- microliter

CHAPTER 1

1.1- Periodontal disease in dogs

1.1.2. Characterization of the dog's oral cavity

The mouth is a unique, open environment, densely populated with genetically diverse bacteria that are influenced by several factors such as the constant presence of saliva, the transient temperature fluctuations and carbon and nitrogen availability (Roberts & Mullany, 2010; Kolenbrander *et al*., 2010). Oral bacteria must be able to adhere to the existent surfaces otherwise they are transported to the digestive tract through the salivary flow (Roberts & Mullany, 2010; Kolenbrander *et al*., 2010). Saliva is a complex exocrine fluid produced by the major and minor salivary glands, being crucial to the maintenance of oral health (Humphrey & Williamson, 2001). It is composed by different electrolytes and proteins whose functions include conservation of tooth integrity, buffering action, lubrication, digestion, food clearance and protection through antibacterial activity (Humphrey & Williamson, 2001). In humans, saliva's antibacterial function has been reported to arise from the presence of immunological particles, like IgG, IgA and IgM, or nonimmunological enzymatic agents, such as mucins, lysozyme and peroxidase (Humphrey & Williamson, 2001).

Nonetheless, the majority of the available literature focuses on human saliva and microbiota, which are considerably different from the canine ones mainly due to their more basic pH (Oh *et al*., 2015; Iacopetti *et al*., 2017). More recently, a study analyzed the salivary proteome of healthy dogs, without evidence of periodontal disease and representing four different breed phylogenies (Torres *et al*., 2018). A total of 2,491 proteins and endogenous peptides were detected in canine saliva. Importantly, seven of the ten most abundant proteins possessed immune functions along with several other proteins identified, which had already been reported to have antimicrobial activity in the human saliva (Torres *et al*., 2018). Additionally, it was also possible to observe variability between breeds since some proteins were not found in all four breed groups (Torres *et al*., 2018).

The microbiota colonizing the mouth can act as a barrier, conferring protection to the host, or they can be pathogenic and lead to diseases following disruptions in the environment (Oh *et al*., 2015). Because the oral microbiome is deeply related to local and systemic disorders, its characterization is fundamental for establishing the adequate treatment for oral diseases (DeBowes *et al*., 2018; Hojo *et al*., 2009; Roberts & Mullany, 2010). Microorganisms found in the oral cavity of dogs have been shown to be significantly different from the ones present in humans (Oh et al., 2015). This is particularly relevant considering that veterinary medical therapeutic protocols are often based on human models (Oliveira *et al.,* 2016).

Anaerobic bacteria isolated from the oral cavity dogs include *Clostridium* spp., *Porphyromonas* spp., *Bacteroides* spp., *Fusobacterium* spp., *Propionibacterium* spp., *Lactobacillus* spp., *Actinomyces* spp., *Bifidobacterium* spp. and *Veillonella* spp.

Regarding aerobic and facultative anaerobic bacteria identified genera include: *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Neisseria*, *Moraxella*, *Acinetobacter*, *Pasteurella*, *Bacillus*, *Pseudomonas*, *Proteus* and *Corynebacterium* (Zambori *et al*., 2012; Davis *et al*., 2013).

Although known for being commensal microorganisms of the mammalian gastrointestinal tract, *Enterococcus* spp. have been frequently isolated from the oral cavity of dogs with PD (Oliveira *et al*., 2016). Furthermore, these bacteria have firmly established themselves as leading infectious agents, highly prone to transmit and acquire resistance determinants (van Harten et al., 2017). Considering that, periodontal disease (PD) is one of the most widespread inflammatory diseases in dogs and may be associated with life-threatening conditions like infective endocarditis (IE), enterococci-pathogenicity assessment and characterization becomes fundamental (Oliveira *et al*., 2016; Semedo-Lemsaddek *et al*., 2016).

1.1.3. PD Pathogenesis

PD is the result of persistent inflammation in the periodontium, that is composed by the supporting structures of the tooth, including the gingiva, periodontal ligament, alveolar bone and cementum (Albuquerque *et al*., 2012). It is, simultaneously, one of the most prevalent inflammatory diseases in dogs, with 80% of them displaying some form of the disease by just the age of two, as well as the most frequently undertreated condition affecting companion animals (Niemiec, 2008a; Albuquerque *et al*., 2012; Oliveira *et al*., 2016). Furthermore, PD underdiagnosis expedites disease progression resulting in numerous local consequences and, ultimately, facilitating bacterial systemic dissemination (Niemiec, 2008a; Albuquerque *et al*., 2012). In fact, severe systemic consequences have been linked to PD, including decreased renal, hepatic, cardiac and pulmonary functions (Niemec, 2008a). Recently, it has also been established an association between bacterial-endocarditis and periodontal disease in dogs (Semedo-Lemsaddek *et al.,*2016). Despite having a multifactorial aetiology, in which characteristics of the environment and host actively intervene in the clinical expression of the disease, formation of a dental plaque is necessary for its onset (Oliveira *et al*., 2016).

Periodontal disease initiates with the formation of an acquired pellicle on the tooth surface followed by bacterial colonization. The salivary pellicle, which permanently covers the surfaces present in the oral cavity, is composed by salivary fluid as well as glycoproteins, lipids and phosphoproteins (Roberts & Mullany, 2010; Mahajan *et al*., 2013). The adherent bacteria establish a series of interactions between them and assemble a dental biofilm.

A biofilm can be defined as an aggregate of genetically distinct microorganisms that adhere to each other and/ or to a surface, being frequently embedded in a self-produced matrix of extracellular polymeric matrix (Flemming *et al*., 2016). Bacteria living within a biofilm display a different behavior when compared to their free-living counterparts, the most striking and clinically relevant being increased

resistance to antimicrobials (Hojo *et al*.,2009). Communication is a key feature for organizations to prosper and bacterial biofilm-communities are no exception. Bacteria residing within a biofilm adopt specific behaviors and the established interspecies communication accounts for the initiation and progression of periodontal disease (Mahajan *et al.,* 2013),

Canine healthy plaque is mainly formed by non-motile, Gram-positive, facultative aerobes, being acknowledged that this early biofilm community presents a characteristic spatio-temporal evolution (figure 2) which accompanies disease progression (Popova *et al*., 2014). Gingivitis corresponds to an inflammation of the gingiva due to plaque accumulation, primarily accompanied by an increase in the number of non-motile Gram-negative rods and anaerobes (Popova *et al*., 2014). When caught early, proper prophylaxis can be implemented and, coupled with consistent home care, this condition can be reversed (Niemec, 2008a; Albuquerque *et al*., 2012). If not, the inflammation will progressively spread to the deeper supporting structures of the tooth, which include the alveolar bone and periodontal ligament (Teng, 2006; Popova *et al*., 2014). Some of the colonizing bacteria secrete metabolic products, such as toxins which elicit complex immune-inflammatory responses from the host that may lead to the destruction of the periodontal tissue, promoting periodontitis (Niemec, 2008b; Albuquerque *et al*., 2012). Therefore, the frontier between gingivitis and periodontitis is determined by the specific host-plaque interactions. Periodontitis is the final and irreversible stage of the disease process and can result in tooth loss. In established PD, high numbers of Gram-negative rods, spirochetes and anaerobes are present in the oral microbiota (Niemec, 2008b).

Adequate treatment significantly contributes to the improvement of the oral health of the patient (Niemiec, 2008b). Prevention and management of PD vary according to the stage and severity of the disease. The main goal in periodontal therapy lies in controlling dental plaque formation to reduce infection and help restore oral health (Niemiec, 2008b). This can be achieved combining professional periodontal supervision with essential home care routine procedures like tooth brushing with dentifrices, topical use of chemical plaque retarding agents or special formulated diets (Niemiec, 2008b). On the other hand, professional treatment comprises several steps, including dental radiographs of the entire mouth, chlorohexidine lavages, supra- and subgingival cleaning, calculus scaling, polishing and sulcal lavage (Niemiec, 2008b).

Consequently, due to its high prevalence and impact upon human and veterinary medicine, characterization of the oral cavity's bacterial inhabitants becomes crucial, not only to assess oral health and impede their systemic spread but also to establish adequate therapeutic protocols (Oliveira *et al*., 2016).

Although recognized as commensal intestinal bacteria, *Enterococcus* spp. have been isolated in the oral cavity of dogs with PD (Oliveira *et al*., 2016). Considering their multidrug-resistant profile and genomic plasticity, that facilitates the acquisition of resistance genes, these opportunistic pathogens can be used as a model for antimicrobial dissemination studies (Oliveira *et al*., 2016).

1.2- *Enterococcus* spp.

Although the animal gastrointestinal tract represents their biggest reservoir, due to their ability to survive under hostile conditions, enterococci are ubiquitously distributed throughout the environment, being isolated from plants, water, soil, sediments and foodstuffs. Moreover, *Enterococcus* spp. persistence enables them to be used as fecal contamination indicators (Lebreton *et al*., 2014). Previously considered to be harmless commensal bacteria, during these last few decades enterococci have risen as serious nosocomial pathogens (Higuita &Huycke, 2018).

Enterococcus genus was introduced for the first time in 1899 by Thiercelin to describe saprophytic gram-positive diplococcus present in the gut and proposed the name “Enterocoque”, emphasizing its origin and morphology (Thiercelin & Jouhaud, 1899; Lebreton *et al*., 2014). Bacteria now belonging to this genus were initially classified as Lancefield group D-streptococci based on the structure of their lipoteichoic acid (Lebreton *et al*., 2014). However, it was not until 1984 that this genus was formally established when Schleifer & Kilpper-Balz, using molecular methods, presented sufficient genetic evidence to allow the reclassification of *Streptococcus faecalis* and *Streptococcus faecium* as *E. faecalis* and *E. faecium* respectively (Lebreton *et al*., 2014).

1.2.1. General characteristics

Enterococci are gram-positive, ovoid or spherical cells with a diameter of 0.6 to 2.5 µm that can arrange themselves in chains or pairs and are not capable of forming spores. Bacteria within this genus are facultative anaerobes, with a low guanine-cytosine (GC) content (<50mol%), both catalase and oxidase negative (Fisher & Phillips, 2009; Guzman *et al*., 2016). Although growing optimally at 35°C, they tolerate temperatures ranging from 10°C to 45°C, due to the lipids and fatty acids present in their membrane. In fact, they can resist heating at 60°C for 30 minutes. These bacteria can grow in the presence of high salt concentrations up to 6.5% NaCl and are able to withstand a wide range of pH values, from 4.8 to 9.6, with 7.5 being the optimum pH. This ability to resist a broad range of pH values is a consequence of membrane durability and impermeability to acidic and basic compounds (Guzman *et al*., 2016; John & Carvalho, 2011). They are homofermentative organisms following the Embden-Meyerhof-Parnas pathway thus converting sugars into lactic acid without the release of gas (Lebreton *et al*., 2014).

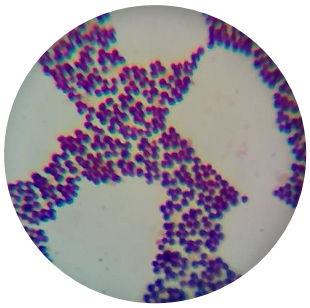


Figure 1- *Enterococcus* spp. Gram coloration (Original).

1.2.2. Enterococci virulence factors

Enterococcus spp., are commensal bacteria, colonizing the gastrointestinal tract of humans as well as other animals (Lebreton *et al.*, 2014; Chajęcka-Wierzchowska *et al.*, 2017). However, enterococci have emerged as serious opportunistic pathogens, able to cause life-threatening infections because of several combined factors: natural resistance to a wide variety of antimicrobials, genomic plasticity and easiness to acquire and transmit resistance genes (Chajęcka-Wierzchowska *et al.*, 2017; Semedo-Lemsaddek, 2018). Furthermore, enterococci possess virulence determinants such as surface factors and secreted molecules which increase its pathogenicity (Chajęcka-Wierzchowska *et al.*, 2017, 2017).

Enterococci can adhere and colonize the host's tissues due to the presence of particular virulence factors: the aggregation substance (As), a collagen-binding protein (Ace), a cell-wall adhesin (EfaA) and a surface-binding protein (Esp). The aggregation substance comprises a range of adhesins that enable cell-to-cell contact in conjugation events as well as binding to the host cells (Chajęcka-Wierzchowska *et al.*, 2017). Moreover, synergistic events with cytolysin can occur, therefore intensifying virulence in these bacteria (Chajęcka-Wierzchowska *et al.*, 2017). The collagen-binding protein is, too, a surface protein encoded by the *ace* gene (Chajęcka-Wierzchowska *et al.*, 2017). It facilitates colonization by attaching to proteins in the extracellular matrix and also, it binds to types I and IV collagen (Chajęcka-Wierzchowska *et al.*, 2017). The *ace* gene has been isolated for *E. faecalis* strains whereas a homologous gene, *acm*, has been found in *E. faecium* strains (Chajęcka-Wierzchowska *et al.*, 2017). The EfaA protein, or endocarditis specific antigen, is a cell-wall adhesin associated to infective endocarditis (Chajęcka-Wierzchowska *et al.*, 2017). Finally, the Enterococcal surface protein (Esp) is the largest one identified so far in this genus and the *esp* gene is located on a pathogenicity island (PAI), among other proteins responsible for antibiotic efflux (Chajęcka-Wierzchowska *et al.*, 2017). The study of this protein has led to the observation of its participation in biofilm formation as well as immune evasion (Chajęcka-Wierzchowska *et al.*, 2017).

Following colonization, secretion of toxic agents takes place, causing further damage on the host. In the case of enterococci, these substances are cytolysin (Cyl), gelatinase (GelE) and hyaluronidase (Hyl) (Chajęcka-Wierzchowska *et al.*, 2017). Cytolysin is a bactericidal exotoxin acting towards gram-negative bacteria and able to damage agents of the host's immune system through hemolysis (Chajęcka-Wierzchowska *et al.*, 2017). Gelatinase is an extracellular hydrolytic metalloendopeptidase, acting upon gelatin, elastin, collagen, hemoglobin and other bioactive peptides (Chajęcka-Wierzchowska *et al.*, 2017). Hyaluronidase has been found in the genome of *E. faecium* strains. This enzyme is responsible for destroying the mucopolysaccharides of the connective tissue (Chajęcka-Wierzchowska *et al.*, 2017).

1.2.3. Enterococcal infections

As previously mentioned, *Enterococcus* spp. are normal residents of the mammalian gastrointestinal tract that do not cause diseases in healthy individuals. However, when the health status of the host is compromised, and its immune system weakened, these bacteria can enter the bloodstream by moving across the intestinal barrier and reaching vital organs, thus being responsible for severe infections (Fisher & Phillips, 2009; Arias & Murray, 2012). These include urinary tract, intra-abdominal, pelvic neonatal and surgical wound infections, bacteremia, endocarditis, meningitis and pneumonia (Higuita &Huycke, 2018).

The shift in the pathogenic potential of enterococci is yet to be fully understood. On the one hand, the continuous medical progress towards more intensive as well as invasive therapeutic protocols has led to the substantial increase in prevalence of these bacteria (Komiyama *et al*., 2016; Higuita &Huycke, 2018). On the other hand, they possess unique traits that account for their persistence in hospital settings, namely their intrinsic resistance to multiple classes of antimicrobials with clinical use, their genomic plasticity and easiness to acquire and transmit resistance genes (Sood, 2018). Enterococci are resilient bacteria that can tolerate and survive hostile conditions for long periods of time, including on environmental surfaces, such as medical equipment (Arias & Murray, 2012). Also, they display unusual resistance to chemical disinfectants like chloride, alcohol and glutaraldehyde. These properties provide enterococci with a selective advantage, allowing them to out-compete the surrounding species, perpetuate and disseminate in medical settings (Garsin *et al*., 2018).

Moreover, contaminated healthcare workers and medical equipment also play an important role in the further dissemination of these potential pathogens amongst hospitalized patients (Arias & Murray, 2012). Under these circumstances, the two most isolated species and therefore the more virulent ones are *Enterococcus faecalis* and *Enterococcus faecium*, both capable of producing biofilms (Arias & Murray, 2012; Lemsaddek & Tenreiro, 2012).

1.2.4. Enterococci antimicrobial resistance profile

Antimicrobial resistance (AMR) is a serious threat to public health worldwide, jeopardizing the effective treatment of an expanding range of infectious diseases (WHO, 2018). Bacteria can be naturally resistant to antimicrobials or, on the contrary, they can become resistant through the acquisition of resistance genes or due to mutations (Munita & Arias, 2016). As mentioned above, enterococci intrinsically resistant to a wide range of antimicrobial compounds. Indeed, all the members of this genus demonstrate an inherent decreased susceptibility to penicillin, ampicillin, semi-synthetic penicillins, cephalosporins, monobactams, polymyxins and lincosamids (Gilmore *et al*., 2013). Also, these microorganisms display a native resistance to clinical concentrations of aminoglycosides, which impedes its application as single antimicrobial agents (Gilmore *et al*., 2013). Despite *E. faecalis* being resistant to quinupristin-dalfopristin, this compound is highly effective against *E. faecium* (Gilmore *et al*., 2013).

In addition, enterococci exhibit a notorious ability for rapidly acquiring resistance to clinically used antimicrobial agents (Gilmore *et al*., 2013). Exchange of genetic material is considered a major force driving bacterial evolution (Raz & Tannenbaum, 2010). In this matter, resistance to glycopeptides represents a bigger challenge, in particular to vancomycin, considering this antimicrobial compound was widely used since it is highly successful in treating infections caused by methicillin-resistant *Staphylococcus aureus* (Gold, 2001). This class of antibiotics inhibits bacterial cell-wall synthesis by binding to the D-ALA-D-ALA termini of the peptidoglycan precursors on the outer membrane (Gilmore *et al*., 2013; Miller *et al*., 2014). Glycopeptide-resistant-enterococci modify the D-ALA-D-ALA termini of these precursors to D-ALA-D-lactate or D-ALA-D-SER (Gilmore *et al*., 2013). In *Enterococcus* spp., resistance to such compounds is mediated by the vancomycin resistance operon, which comprises a response regulator (*vanS-vanR*), a lactate dehydrogenase (*vanH*), a dipeptidase (*vanX*) and a variable ligase, with at least nine-known genes *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN* (Faron *et al*., 2016). The different ligases confer specific levels of resistance, with the most frequently identified genes being *vanA, vanB* and *vanC* (Faron *et al*., 2016). Enterococci harbouring the *vanA* gene exhibit a high degree of resistance to glycopeptides, following the substitution of the termini of the precursors to D-ALA-D-lactate (Faron *et al*., 2016). Also, strains carrying the *vanA* variant can express moderate resistance to teicoplanin due to the presence of *vanZ* gene (Faron *et al*., 2016).

In 2017, the World Health Organization (WHO) published a list of resistant-pathogens for which new antimicrobial compounds are urgently needed (WHO, 2017). Because vancomycin is an effective therapeutic agent against multidrug-resistant gram-positive bacteria, such as MRSA, the greatest concern related to VRE is the risk of transmission of the associated resistant determinants to other pathogens (Faron *et al*., 2016).

1.3- Antimicrobial resistance and alternative compounds

Antibiotics have undoubtedly revolutionized modern medicine, reducing childhood mortality and extending life expectancy by controlling infectious diseases (Blair *et al*., 2014). Nowadays, however, the situation is different. The emergence and dissemination of resistant bacteria, jointly with the lack of development of new antimicrobial drugs, poses one of the biggest challenges to human health. Specifically, the European Commission estimates that in this continent alone each year about 25000 people die with hard-to-treat infections caused by multidrug resistant pathogens, with an associated cost of 1.5 billions (Blair *et al*., 2014). As a result, bacterial infections have become once again a major health concern worldwide, placing a considerable financial and clinical burden on health-cares systems, patients and their families (Ventola, 2015a). Although resistance is a naturally occurring phenomenon, several factors have contributed to the rapid emergence of resistant bacteria, like the misuse and abuse of antibiotics, the extensive utilization in livestock and the lack of financial investment in the development of new antibiotics by pharmaceutical and biotechnological companies, allied with challenging regulatory requirements (Ventola, 2015a).

According with the World Health Organization (WHO), the selective pressure exerted by antimicrobial drugs renders microorganisms capable of not only resisting to previously effective antimicrobial compounds but also allowing them to survive and transmit selected genetic traits to the next generation or to other bacterial species. As a result, antimicrobial resistance is an urgent global threat to human and veterinary medicine, caused by resistant infectious pathogens, requiring extensive immediate action (WHO, 2014).

To address this issue, it is necessary to adopt measures that ensure the continuity of effective prevention and treatment protocols for infectious diseases. These include optimization of therapeutic regimens as well as investment in the research for new antimicrobial compounds (Ventola, 2015b).

1.3.1. Antimicrobial peptides

Considering the continuous emergence of resistant microorganisms, antimicrobial peptides (AMPs) are a promising alternative to conventional antibiotics. These molecules have gained a lot of attention because many organisms naturally produce them for protection, have a broad-spectrum of action and development of resistance is rare (Batoni *et al*., 2011; Kang *et al*., 2014). AMPs produced by bacteria are called bacteriocins and, among these, the ones originated from lactic acid bacteria (LAB) are of particular interest due to their Generally Recognized As Safe (GRAS) status (Balciunas *et al*., 2013). Lantibiotics are post translationally modified bacteriocins that possess unusual amino acids, namely dehydroalanine (DHA), dehydrobutyrine (DHB), lanthionine or methyllanthionine, the latter being

responsible for their specific biological activity (Draper *et al*., 2015; Gharsallaoui *et al*., 2016). Over 25 lantibiotics are known, but the most important and studied one is nisin (Gharsallaoui *et al*., 2016).

1.3.1.1. Nisin

Nisin is the only bacteriocin so far approved as a safe food preservative by the Food and Agriculture Organization and by the World Health Organization (FAO/ WHO) (Gharsallaoui *et al*., 2016). It was discovered in 1928 in fermented milk cultures and began to be commercially marketed in England in 1953 (Shin *et al*., 2016). Since then, nisin not only has consolidated its safety status in the food industry but also its applications have extended to the biomedical field (Shin *et al*., 2016). Specifically, several studies have reported nisin's effectiveness in inhibiting the growth of biofilm-producing pathogens including MRSA and enterococci (Shin *et al*., 2016; Santos *et al*., 2016). Hence, nisin not only acts as a natural preservative against gram-positive foodborne bacteria but also several studies have demonstrated its therapeutic potential.

1.3.1.1.2. General Characteristics

Produced by *Lactococcus lactis*, nisin is a 34-residue cationic, linear peptide that is ribossomally- synthetized. It suffers posttranslational modifications to yield one lanthionine, four β-methyl-lanthionine rings and dehydroalanine and dehydrobutyrine (Tong *et al*.,2014). The antimicrobial effect of nisin is dependent on pH, being more soluble and stable under acidic conditions. Specifically, more basic pH values induce irreversible structural modifications (Gharsallaoui *et al*., 2016). Nisin remains stable at low temperatures, although heating for longs periods of time compromise its effectiveness (Gharsallaoui *et al*., 2016). Furthermore, its thermostability is influenced by the pH. For instance, nisin's antimicrobial activity is maintained when autoclaved at 121°C if the pH is 2, whereas heating at 63°C for half an hour leads to the complete loss of function when pH is 11 (Gharsallaoui *et al*., 2016). Also, nisin is rapidly degraded and inactivated by the proteases present in the digestive system, such as trypsin and pancreatin (Tong *et al*., 2014; Gharsallaoui *et al*., 2016). Various nisin variants have been described, however nisin A and nisin Z are the most commercially available ones (Gharsallaoui *et al*., 2016). One amino acid separates these two variants at position 27: nisin A has an histidine, whereas nisin Z has an asparagine (Gharsallaoui *et al*., 2016). Despite the little differences in many of the biological parameters, nisin Z has been reported to be more soluble in pHs near neutrality than nisin A due to the presence of asparagine, which has a more polar side chain. Nonetheless, the antimicrobial activity remains unaffected by this structural variance (Gharsallaoui *et al*., 2016).

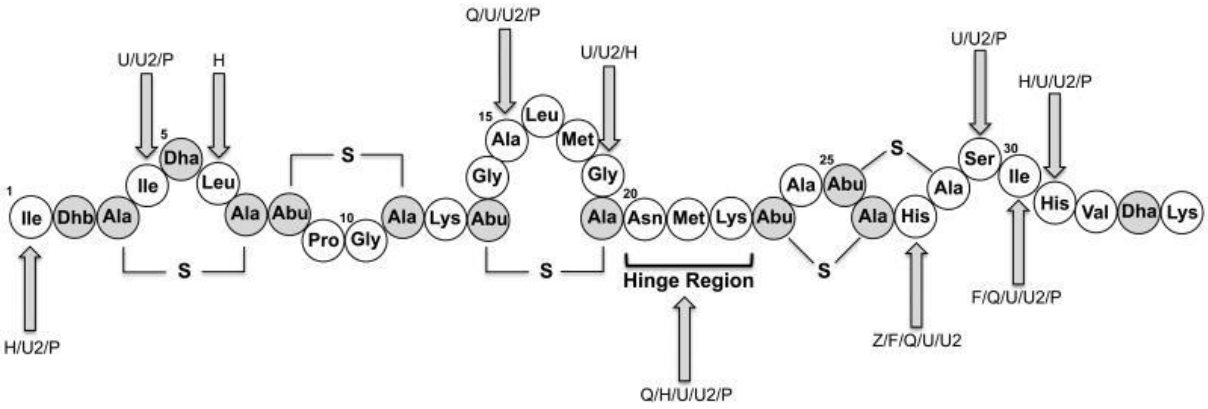


Figure 2- Peptide structure of nisin. Modified amino acids are in grey. Dha, dehydrolalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine. The hinge region is composed of Asparagine-Methionine-Lysine. Arrows indicate the sites of amino acid substitutions for natural variants (Shin *et al*.,2015).

1.3.1.1.3. Mode of action

Like other lantibiotics, nisin has a dual mechanism, acting by pore formation in the membrane of susceptible cells and by preventing cell wall biosynthesis (Bierbaum & Sahl, 2009). Being a cationic molecule, nisin will react with the anionic lipids present in the cytoplasmic membrane. This interaction can take place in two ways: nisin can interact without specificity with anionic lipids and originate pores that lead to membrane depolarization, or it can specifically recognize and bind to lipid II, arresting cell-wall synthesis (Tong *et al*.,2014).

In the first mechanism, nisin will penetrate between the phospholipids, resulting in the formation of pores, which collapse the cell's electrochemical gradient through the efflux of vital cellular molecules, culminating in cell death (Tong *et al*., 2010; Tong *et al*.,2014). The effectiveness of the integration of nisin is dependent on the nature and content of the phospholipids present on the cell-wall membrane, which might explain the different sensitivity of the target bacterial strains (Gharsallaoui *et al*., 2016).

In the second one, nisin can exert its antimicrobial activity by developing a high affinity interaction with lipid II, that is a hydrophobic carrier of the peptidoglycan's (PG) subunits, transporting them from the cytoplasm to the cell wall (Tong *et al*., 2010; Gharsallaoui *et al*., 2016). Thus, upon binding to the lipid II molecule, transglycosylation is prevented by steric hindrance, resulting in the sequestration of the cell-wall precursors and, ultimately, inhibiting peptidoglycan biosynthesis. Afterwards, this complex will insert into the membrane and give rise to pores composed by eight nisin molecules and four lipid II molecules (Tong *et al*.,2014). Similarly, the resulting pores lead to membrane destabilization with the release of vital cytoplasmic components (Gharsallaoui *et al*., 2016). Nonetheless, the lipid II mediated pores are more stable than the ones originated in the absence of this molecule (Tong *et al*.,

2010; Tong *et al*.,2014). Through these mechanisms, nisin not only can lead to cellular lysis by pore formation in the cytoplasmic membrane but also can block peptidoglycan synthesis.

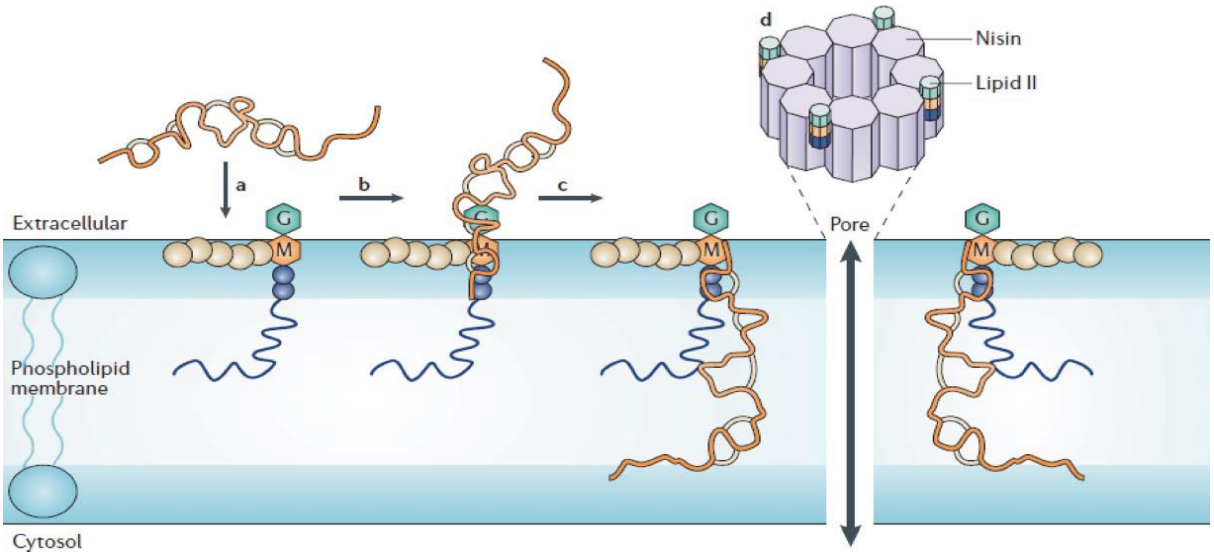


Figure 3- Scheme illustrating nisin's mechanism of action. Once nisin reaches the bacterial plasma membrane binds to lipid II and afterwards a pore is formed. Following the assembly of four nisin: lipid II complexes, four additional nisin molecules are recruited to give rise to the pore complex (Gharsallaoui *et al*., 2016).

1.3.1.1.4. Nisin versus antibiotics

Nisin can be distinguished from conventional antibiotics regarding biosynthesis, toxicity, resistance mechanisms, spectrum of activity and mode of action (Cintas *et al*., 2001; Perez *et al*., 2014). Bacteriocins are proteins gene-encoded and synthesized by ribosomes during the initial stages of bacterial growth whilst antibiotics are secondary metabolites (Cleveland *et al*., 2001).

Nisin acts on a specific bacterial cell wall component, lipid II, and thus has low toxicity for the host when compared to antibiotics, which impair cell function (Cotter *et al*., 2012; Singh *et al*.,2014; Langdon *et al*., 2016). As far as resistance mechanisms, antibiotic resistance happens usually through the acquisition of mobile genetic elements that will act on different sites, by modification of the cellular target, active efflux and chemical modification of the molecule. Regarding bacteriocins, resistance is less frequent due to a combination of factors derived from their intrinsic properties. Specifically, their dual mode of action, as well as their proteinaceous nature, which renders them more susceptible to proteolytic degradation. In addition, bacteriocin producers have a self-immunity mechanism against their own bactericidal compound, ensuring their protection (Draper *et al*., 2015).

1.3.1.1.5. Nisin Resistance

As mentioned earlier, bacteriocins and antibiotics deliver their antimicrobial effect through different mechanisms (Shin *et al*., 2015). Nisin is the most well-known lantibiotic, being used as a preservative in the food industry for over 40 years (Zhou *et al*., 2013; Shin *et al*.,2016). Nevertheless, few cases of nisin resistance have been reported, mainly in gram-positive bacteria (Zhou *et al*., 2013). So far, the mechanisms of resistance to nisin have been studied almost exclusively in foodborne bacteria (Shin *et al*., 2015). Thus, it is important to study and monitor the possible development of resistance in bacteria from medical settings (Shin *et al*., 2015). Some physiological and molecular mechanisms have been suggested/ identified to be responsible for decreasing the effectiveness of nisin, such as cell wall modifications, alterations in the membrane phospholipid composition, enzymatic inactivation of nisin, ABC transporters and two-component systems (Zhou *et al*., 2013).

Occasionally, the same bacteria might present more than one mechanism of resistance (Zhou *et al*., 2013). However, the cellular mechanisms are still poorly understood since resistance has only been observed in laboratory conditions (Shin *et al*., 2016).

Although nisin exerts its action preferentially by binding to lipid II, Kramer and his colleagues hypothesized whether resistance could be related to the different levels of this biomolecule on gram-positive bacteria. Nevertheless, their results suggested that resistance was not determined by lipid II levels, since no correlation with an increase in resistance was observed (Shin *et al*., 2016).

Cell wall changes are regarded as the primary route for bacteriocin resistance. These include thickening and an increase in both hydrophobicity and positive charges (Zhou *et al*., 2013).

Modifications of the phospholipids present in the membrane involve more production of phosphatidylglycerol and less of diphosphatidylglycerol, reduced number of anionic phospholipids and saturated fatty acids, decreased stabilization and fluidity (Zhou *et al*., 2013). Numerous studies have described that certain bacteria produce a neutralizing enzyme, nisinase, which inactivates nisin (Zhou *et al*., 2013; Draper *et al*., 2015). Specifically, this enzyme acts by reducing the C-terminal dehydroalanyl-lysine of nisin to alanyl-lysine (Draper *et al*., 2015).

Also, some strains of *L. lactis* carry a 35kDa nisin resistance protein (NSR), that acts by proteolytic degradation, cleaving the last six amino acids of nisin (Zhou *et al*., 2013; Draper *et al*., 2015). Indeed, the resulting nisin fragment shows less bactericidal activity, reduced affinity for the membrane and lower effectiveness in pore formation (Zhou *et al*., 2013; Draper *et al*., 2015). In fact, its antimicrobial activity is reduced by 100-fold (Shin *et al*., 2015). NSR gene is located onto a 60-kB plasmid, within a five-gene-operon (Khosa *et al*., 2016). The other genes encode a two-component signaling system (NsrRK) and an ABC transporter (NsrFP) (Khosa *et al*., 2016). If expressed simultaneously, then the microorganism becomes fully resistant to nisin (Khosa *et al*., 2016).

Similarly, to what may happen after antibiotic exposure, bacteria may use ABC transporters to remove nisin from the cell envelope (Zhou *et al*., 2013). Besides, two component-systems (TCS) are generally responsible for regulating the expression of ABC transporters involved in lantibiotic resistance (Zhou *et al*., 2013).

Also, lantibiotic producing strains possess self-immunity mechanisms that confer them protection against their bactericidal products, in order to prevent the damaging action on the membrane (Draper *et al*., 2015). Consequently, additional genes encoding for immunity-specific membrane-bound lipoproteins (LanI) or specific ABC transporters (LanFEG), can be present alone or simultaneously (Draper *et al*., 2015; Khosa *et al*., 2016). Although lantibiotics vary in size and activity, the immunity genes appear to be conserved across species (Khosa *et al*., 2016). NisI is the immunity lipoprotein present in the nisin-producing *L. lactis* strains and is composed by 245 amino acid residues (Khosa *et al*., 2016). This sequence contains a signal peptide which, once is recognized and removed, yields a mature protein anchored on the outward membrane leaflet (Khosa *et al*., 2016). This protein binds to nisin, therefore preventing it from reaching the cellular membrane. Moreover, it has been reported that following the addition of nisin to cells containing NisI, cellular clusters begin to form (Khosa *et al*., 2016). As such, nisin no longer binds to lipid II (Khosa *et al*., 2016).

As mentioned previously, antibiotics have been a cornerstone of modern medicine. However, following their implementation in clinical settings, antimicrobial resistance quickly developed (Ventola, 2015a). Take into account the evident tendency for bacterial resistance, strategies to surpass the further development of resistant-pathogens are indispensable (Tong *et al*., 2014). One way to achieve this is by increasing antimicrobial potency, with various studies referring the application of combinatory antimicrobial therapy, coupling antibiotics with bacteriocins (Mathur *et al*., 2017). Moreover, by combining regular antibiotics with antimicrobial peptides, the lifetime of many antibiotics can be prolonged (Tong *et al*., 2014). As a matter of fact, few studies have reported that nisin in combination with other antibiotics is able to enhance their antimicrobial activity, most of the work having been performed on gram-positive pathogens, including *Enterococcus* spp. (Field *et al*., 2016; Shin *et al*., 2016). Besides, it has been shown that nisin coupled with conventional antibiotics is effective against biofilm- producing bacteria (Field *et al*., 2016).

In 2014, a study conducted by Tong and colleagues investigated the effect of nisin together with several antibiotics against *E. faecalis* strains. A total of 18 antibiotics were tested, which included penicillin, ampicillin, gentamycin, vancomycin, polymyxin and ciprofloxacin (Tong *et al*., 2014b). The authors observed that the addition of nisin to the tested antibiotics enhanced their antibacterial and bactericidal action, apart from metronidazole, sulfapyridine and polymyxin (Tong *et al*., 2014b). More specifically, a better synergy was observed when combining nisin with antibiotics that have different bactericidal mechanisms (Tong *et al*., 2014b). Also, it was possible to improve the antibiofilm properties of the antibiotics by adding nisin.

1.3.1.1.6. Nisin in the treatment of periodontal disease

The prevalence of oral diseases such as caries and periodontal disease renders them major public health concerns in both developing and developed countries (Marcenes *et al*., 2013). Oral biofilms are associated with enhanced antimicrobial resistance, further complicating the treatment of PD (Shin *et al*., 2015).

A study conducted by Johnson and colleagues in 1978 first demonstrated nisin's potential as an oral antimicrobial compound. A few years later, another study by Howell and co-workers showed that a mouth rinse wash with nisin reduced plaque formation and gingival inflammation in beagle dogs (Shin *et al*., 2015). Tong and his team further evidenced the bactericidal properties of nisin by demonstrating its ability to inhibit the growth of cariogenic bacteria (Tong *et al*., 2010). Recently, Shin and colleagues also observed that nisin could have anti-biofilm properties without causing cytotoxicity in human oral cells (Shin *et al*., 2015).

Besides the promising potential to treat oral diseases, nisin has also been reported to inhibit emerging pathogens such as opportunistic multidrug resistant *Enterococcus faecalis* strains (Turner *et al*., 2004). Hence, nisin has the potential to be implemented in human and veterinary medical fields as an alternative antimicrobial compound, namely for the treatment of infectious diseases while simultaneously preventing the development and spread of resistance (Tong *et al*., 2014). For nisin to be applied in the oral cavity, it is necessary its incorporation in a stable vehicle of administration, to avoid its degradation or inactivation before reaching the target at effective concentrations (O'Driscoll *et al*., 2013). In this context, hydrophilic polysaccharides like guar gum gel, have raised a lot of interest as oral controlled drug delivery systems due to their stability and non-toxic properties (Prabaharan, 2011).

1.3.1.1.7. Guar Gum Gel as an AMP topical administration vehicle

Recently, pharmaceutical companies have been giving a lot of attention to hydrophilic polysaccharides as potential oral drug delivery systems, because of their suitable and attractive properties, namely stability, biodegradability, biocompatibility, non-toxicity and economic costs (Prabaharan, 2011). In this context, one example is guar gum, a natural occurring water-soluble polysaccharide found in the endosperm of leguminous crop *Cyamopsis tetragonolobus* (Thombare *et al*., 2016).

Guar gum consists of a linear polymer with a D-mannose backbone and D-galactose side chain units, named galactomannan (Thombare *et al*., 2016). Due to the presence of hydroxyl groups in its structure, when added to cold water it yields highly viscous solutions (Mudgil *et al*., 2014; Thombare *et al*., 2016). Its non-ionic and uncharged properties account for its stability over a wide pH range from 1.0

up to 10.5, resulting in a higher resistance to dissociation (Mudgil *et al*., 2014). Temperature is a decisive factor influencing the consistency of this compound, affecting the rate of hydration and maximum viscosity. Generally, guar gum gel solutions prepared at higher temperatures yield less viscosity than when prepared with cold water and allowed to slowly hydrate (Mudgil *et al*., 2014). The thickening, emulsifying, binding and gelling properties, rapid solubility in cold water, broad pH stability and film-forming ability, render guar gum a safe and flexible drug delivery system (Mudgil *et al*., 2014).

Given the current trends of antimicrobial resistance dissemination, it is crucial to determine the appropriate antimicrobial dosages that minimize the likelihood for resistance selection, resulting in the restriction of mutant growth while taking full advantage of nisin potential to treat oral diseases like PD. Indeed, it has been suggested that to restrict the selective enrichment of mutant subpopulations, the mutant prevention concentration (MPC) protocol should be determined in order to establish the drug concentration at which no resistant colonies are recovered (Smith, 2003).

1.3.2. Mutant prevention concentration

The potential emergence of antimicrobial resistance is a major threat to public and animal welfare regarding any antimicrobial protocol under investigation for clinical uses, being extremely important to unveil the mechanisms responsible for resistance development and its environmental persistence. Genetic resistance can arise in two separate ways: either by acquisition of resistance genes through horizontal gene transfer (HGT) or it can be *de novo*, in which case resistance develops gradually, in a stepwise manner generally as a consequence of the accumulation of mutations that decrease susceptibility (Drlica, 2003). As expected, therapeutic protocols that readily enhance mutant subpopulations should facilitate resistance development more quickly than the regimens that suppress mutant formation (Drlica, 2003). Thus, studies focusing on optimization of the antimicrobial concentrations needed to prevent the selection and amplification of resistant mutants are of particular interest (Pasquali & Manfreda, 2007).

In this context, the mutant selection window (MSW) hypothesis, described by Zhao and Drlica postulates that single-step resistant mutant subpopulations, although naturally present, are selectively enriched and amplified when drug concentrations fall within a specific range (Drlica & Zhao, 2007).

The MSW comprises a range of concentrations between the minimal inhibitory concentration (MIC) and (MPC) (Drlica, 2003). MIC is the lowest drug concentration that inhibits the growth of the majority of susceptible individuals (Drlica, 2003). However, this approach only considers the susceptible population, overlooking the subset of resistant cells (Zhao & Drlica, 2008). The mutant prevention concentration is an anti-mutant dosing strategy developed by Dong and his colleagues (1999) which aims to determine the necessary antimicrobial drug concentration that blocks the growth of the least susceptible, first step mutant when a high inoculum is applied, specifically more than 10^{10} cells (Dong

et al., 1999; Drlica, 2003). Selection of this inoculum size takes into account a few considerations: first, sometimes up to 10^{10} cells are found at the infection site; second, it is large enough to ensure the presence of mutant subpopulations; last, it can be difficult to obtain inocula with a higher cell concentration (Drlica, 2003).

The determination of the MPC can be achieved in two ways. In one method, narrow inoculum increments are applied onto various agar plates containing several antimicrobial-concentrations, so that more than 10^{10} cells are tested for a certain drug concentration. This way, it is possible to observe and count the isolated colonies, which decrease progressively with higher drug concentrations (Drlica, 2003). Alternatively, more than 10^{10} cells can be applied on single agar plates of a series containing the antimicrobial drug, each differing by two-fold increments (Drlica, 2003).

The mutant selection window concentration boundaries, MIC and MPC, do not influence the types of mutants selected. As matter of fact, the derived mutants are expected to develop mechanisms that inactivate the antimicrobial drug, like improvement of uptake, efflux or degradation systems (Drlica, 2003). Several MPC values have been determined for many antibiotics mainly against relevant pathogens. In 2004, ciprofloxacin and enrofloxacin MPC values were determined for *Salmonella enterica*. Indeed, MPC values of ciprofloxacin against *S. enterica* serotypes Enteritidis and Typhymurium ranged from 0.25 - 2 and 0.25 - 4 mg/mL whereas for enrofloxacin varied between 0.5 - 4 and 0.5 - 8 mg/mL, respectively (Randall *et al*., 2004). Hansen and colleagues established the MPC of ciprofloxacin and levofloxacin for *Pseudomonas aeruginosa,* respectively 2 and 8 mg/mL (Hansen *et al.*, 2006). In 2014, Mei and collaborators tested the mutant selection window for *Staphylococcus aureus* ATCC 29213 exposed to fosfomycin, in which the MPC value was 0.0576 mg/mL (Mei *et al*., 2014). In the same year Hesje and colleagues, established the MPC of tigecycline for *Streptococcus pneumoniae*, MSSA and MRSA, 16, 2 and 4 mg/mL respectively (Hesje *et al*., 2015). In 2016, the MPC value of daptomycin for *Enterococcus faecalis* clinical isolates was determined, ranging between 2 and 32 mg/mL (Sinel *et al*., 2016). In the same year, the MSW hypothesis was validated of fosfomycin against *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, respectively 0.0576 and 0.1024 mg/mL (Pan *et al.,* 2016).

Aim of this study

The ultimate purpose of this work is to continue to validate the use of the antimicrobial peptide, nisin, incorporated in a topical vehicle of administration, guar gum gel, in the prevention of periodontal disease in dogs as well as the possible derivative systemic complications. In previous studies conducted by the group, not only have the MIC and MBC values of nisin been determined against the bacterial collection of enterococci retrieved from dogs with periodontal PD but also, the potential of guar gum as a topical vehicle of administration has been evaluated. Because nisin is to be applied in the oral environment, it will be important to assess the influence of dog's saliva in nisin antimicrobial activity. For that a spot-on-lawn assay will be applied. For any antimicrobial protocol aiming clinical implementation, it is essential to determine the correct drug dosages that prevent selection of resistant mutants. In this context, the mutant selection window (MSW) of nisin will be established by determining the mutant prevention concentration (MPC). Afterwards, an antimicrobial susceptibility profiling will be performed on the clinical isolates and on the mutants recovered following the MPC protocol, to determine if nisin alters their susceptibility profiles. The disk diffusion method will be used to test 12 different antibiotics and results will be compared with the CLSI standard breakpoints. Finally, the MIC and MBC values of the mutants derived from the MPC protocol will be determined using the broth microdilution method to understand if the pressure of the high nisin concentrations, induced any other changes in the antimicrobial profile.

CHAPTER 2

2. Materials and methods

2.1. Assessing the influence of dog's saliva in nisin antimicrobial activity

2.1.1. Bacterial strains and culture conditions

This work was performed using 20 oral enterococci obtained from dogs diagnosed with periodontal disease, previously phenotypically and genotypically characterized (Tavares, 2014, Oliveira et al, 2016). From these 20 isolates, 17 represent strains belonging to the species *Enterococcus faecalis* and the remaining 3 to *Enterococcus faecium*. Also, one human reference strain was included as control, *Enterococcus faecalis* ATCC 29212. During this study, all isolates were kept at -20°C in a solution of buffered peptone water with 20% of glycerol. When needed they were inoculated onto unspecific enrichment growth medium, namely Brain Heart Infusion (BHI) agar medium (Brain heart infusion broth, VWR Chemicals, ref 84626.0500; Agar, VWR Chemicals, ref 84609.0500) followed by a 24-hour incubation period at 37°C.

2.1.2. Preparation of nisin standard solution and antimicrobial activity tests

Stock solutions of nisin in HCl at 0.02M (Merck, Hydrochloric acid fuming 37%) were prepared from nisin in powder (2.5% purity, 1000 IU/mg, Sigma-Aldrich, St Louis, USA) as described by Santos and collaborators (Santos *et al*., 2016), in order to obtain a solution of 40mg/mL, sterilized by filtration (Frilabo, 0.22µm, ref.FJ25BSCPS002AL10). Nisin stock solutions were stored at 4°C, and serially diluted in sterile water, when required, to yield solutions with nisin concentrations of 0.5, 1.0, 2.0, 4.0 mg/mL.

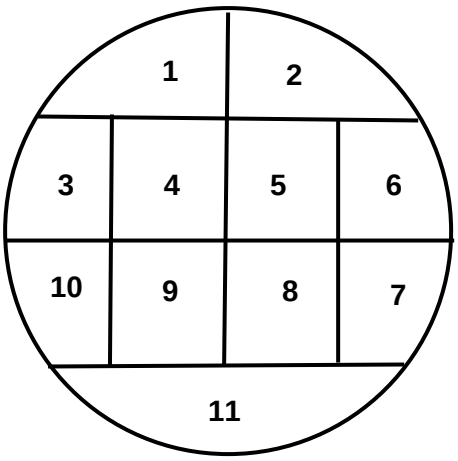
2.1.3. Guar gum gel

A guar gum gel (Sigma-Aldrich, USA) at 1.5% was prepared by dilution in sterile distilled water, followed by sterilization in autoclave and storage at 4°C (Santos *et al.*, 2016). The suspensions of nisin in guar gum gel at 0.5, 1.0, 2.0 and 4.0 mg/mL were performed respecting a proportion of 1:1, homogenised in the vortex and kept at the same temperature as before.

2.1.4. Inhibition potential of nisin in the presence of dog's saliva

The saliva samples used in this study were collected at VetOeiras from healthy dogs that were presented for routine consultations at this clinic. After collection, samples were filtered (Frilabo, 0.22 µm, ref.FJ25BSCPS002AL10) and stored at -20°C. To optimize salivary enzymatic activity, before each assay samples were placed at 37°C for 1 hour (Tong *et al*., 2010).

To evaluate the inhibitory activity of nisin in the presence of saliva, BHI agar plates were inoculated with a lawn of each oral isolate. First, bacterial suspensions were prepared in sterile water with a turbidity of 0.5 McFarland (bioMérieux, ref 70900) which corresponds to approximately 10^8 CFU/mL. Then, these cellular suspensions were diluted (1:10) in sterile water, yielding suspensions of 10^7 CFU/mL. Afterwards, the previously prepared solutions of nisin and of nisin incorporated in guar gum (1:1) were subsequently homogenised using a vortex (maximum speed) and diluted in saliva to yield the following concentrations of nisin: 0.5; 1.0; 2.0 and 4.0mg/mL. Also, saliva, nisin and guar gum supplemented with nisin, at the previously determined MIC concentrations (respectively 0.5 and 1.0 mg/mL) were used as controls for the experiment (Pinheiro, 2016; Trovão, 2017). Afterwards, 10 µL of each solution were spotted onto the BHI plates (representative schemes are illustrated in figures 1 and 2), followed by incubation at 37°C for 24 hours. After incubation, plates were observed for the presence of inhibitory zones, which were measured. All the assays were performed in triplicate, repeated on three independent days and results were averaged.



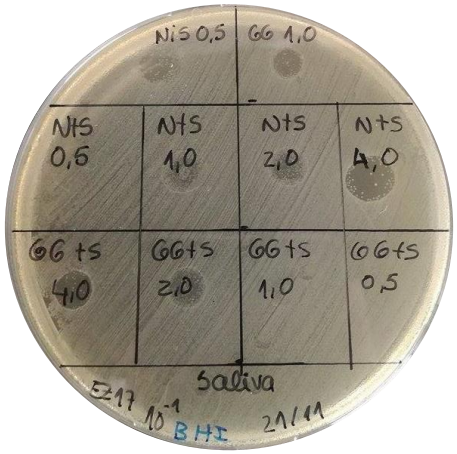


Figure 4 (Left)- Representation of the scheme of a BHI plate used in the spot-on lawn test performed to study the effect of saliva on the antimicrobial activity of nisin and of nisin incorporated in guar gum gel. **1-**Nisin (C=0.5mg/mL); **2-** Nisin+Guar Gum (C=1.0mg/mL); **3-** Nisin+saliva (C=0.5mg/mL); **4-** Nisin+saliva (C=1.0mg/mL); **5-** Nisin+saliva (C=2.0mg/mL); **6-** Nisin+saliva (C=4.0mg/mL); **7-** Nisin+Guar gum+saliva (C=4.0mg/mL); **8-**Nisin+Guar gum+saliva (C=2.0mg/mL); **9-** Nisin+Guar gum+saliva (C=1.0mg/mL); **10-** Nisin+Guar gum+saliva (C=0.5mg/mL); **11-** Saliva.

Figure 5 (Right)- Results from the spot-on-lawn test using the strain EZ17 obtained from the oral cavity of a dog with periodontal disease: **1-** Nis 0.5; **2-** GG 1.0; **3- 4-** N+S 1.0; **5-** N+S 2.0; **6-** N+S 4.0; **7-**GG+S 4.0; **8-** GG+S 2.0; **9-** GG+S 1.0; **10-** GG+S 0.5; **11-** saliva. **Nis/ N** (nisin); **GG** (guar gum); **S** (saliva).

2.2. Determination of the mutant prevention concentration (MPC) and the mutant selection window (MSW)

2.2.1. Bacterial strains

This protocol was performed using the previously mentioned 20 oral enterococci (Tavares, 2014) and the human reference strain *Enterococcus faecalis* ATCC 29212. Isolates were kept at -20°C and inoculated onto BHI agar medium before use, as previously described in 2.1.

2.2.2. Preparation of nisin standard solutions

Stock solutions of nisin in HCl at 0.02M (Merck, Hydrochloric acid fuming 37%) were also prepared as described in 2.1.2 in order to obtain a solution of 40mg/mL.

2.2.3- Determination of the mutant prevention concentration

To determine the mutant prevention concentration of nisin against the isolates under study, a modified version of the protocol described by Sinel and collaborators in 2016 was performed.

2.2.3.1- Protocol Optimization

To determine MPC values, several protocols were tested to achieve suspensions with the necessary bacterial concentration to fulfil this protocol, $\geq 10^{10}$ CFUs/mL (Drlica, 2003). Most protocols available refer to the incubation of cultured bacteria on liquid media for 24 hours, followed by centrifugation and resuspension of the resulting pellet (Randall *et al.,* 2004; Firsov *et al*., 2006; Pasquali & Manfreda, 2007). Nonetheless, no volumes are mentioned and viable counts to confirm bacterial concentration were not performed. Consequently, it was required to test several protocols for this crucial step, performed initially using the human reference strain.

The first protocol tested was described by Sinel *et al* (2016). Briefly, the human reference strain was inoculated in BHI agar and incubated at 37°C for 24 hours. After this period, a bacterial suspension with a turbidity of 6 on the McFarland scale was prepared, corresponding to 10^9 CFU/mL. Then, 500 µL

of this suspension were transferred to 35 mL of Brain Heart Infusion Broth (BHIB) followed by incubation on the orbital incubator (Shel lab, model SSI10) at 180rpm for 18 hours at 37°C.

Afterwards, the suspension was centrifuged at 4000g for 10 minutes and the pellet resuspended in 5 mL of Mueller-Hinton Broth (MHB) (VWR Chemicals, ref 84648.0500). Serial dilutions were performed to quantify the total number of viable cells (CFU/mL). To achieve the necessary concentration (10^{10} CFUs/mL), viable counts should be obtained by plating the 10^{-8} dilution. As no growth was observed beyond the 10^{-6} dilution, it was necessary to test other protocols.

First, a 6 McFarland bacterial suspension was prepared, and 3 mL were inoculated onto 200 mL of BHIB, followed by incubation on the orbital incubator under the same conditions as described before. After the incubation period, the suspension was centrifuged and resuspended in 5mL of MHB.

Second, 500 µL of a bacterial suspension (6 McFarland) were incorporated in 20 mL of BHIB. After centrifugation, the pellet was resuspended in 1 mL of MHB.

Third, subsequent to growth on solid media, cells were harvested using a 10 µL sterile loop, placed on 25 mL of BHIB and incubated with agitation at 180 rpm for 18 hours at 37°C. After 24 hours, the suspension was centrifuged at 4000g for 10 minutes and the pellet resuspended in 100 µL of MHB.

None of these protocols allowed to achieve the desired bacterial concentration (10^{10}). Consequently, another approach was performed based on the work by Credito and collaborators (2010), in which bacteria were grown on rich solid media for 24 hours after which the resulting cells were recovered and directly resuspended in sterile saline (NaCl 0.9%; sodium chloride 0.9%). Subsequently, the cellular content of 2 BHI plates and of 3 BHI plates was resuspended in 1 mL of NaCl 0.9%. However, it was not possible to obtain the required bacterial counts.

Then, the cellular content of 3 BHI agar plates was resuspended in BHIB, placed on 37°C for 20 minutes, and then serial dilutions were subsequently performed. Through this methodology, it was possible to obtain a 10^{9} CFU/mL bacterial suspension. Afterwards, this protocol was validated using a clinical isolate. Strain EZ1 was randomly selected from the collection of oral enterococci clinical isolates, to test the MPC protocol, being observed that it was possible to obtain a 10^{10} CFU/mL bacterial suspension using the clinical isolate.

2.2.3.2 Selected protocol for MPC determination

Each isolate was spread onto three BHI agar plates using sterile 10µL loops and incubated for 24 hours at 37°C. Afterwards, all the bacterial lawn present in the three BHI plates was resuspended in BHIB and further incubated at 37°C for an additional 20 minutes. Specifically, all clinical isolates were resuspended in 450 µL of BHIB except for strains EZ22, EZ25, EZ26, EZ29 and EZ30 which were resuspended in 750 µL of BHIB given their texture. Then, an aliquot of 50 µL of this concentrated

bacterial suspension, with 10^{10} CFU/mL, was inoculated onto solid MH (Mueller-Hinton Agar, VWR Chemicals, ref 84686.0500) plates containing different concentrations of nisin. These MH plates were supplemented with two-fold concentration increments of nisin ranging from 0,25 to 48x the MIC value of 0.5 mg/mL (Pinheiro, 2016). Thus, the MH agar plates series contained 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 24.0 mg/mL of nisin, which were incubated at 37°C for 72 hours and observed daily. The inoculum concentrations were confirmed by performing viable cell counts. Simultaneously, absorbance at 600nm was measured for dilutions 10^{-1} to 10^{-3}.

MPC was defined as the lowest concentration of nisin that prevented the growth of any resistant mutant subpopulations after a 72-hour incubation period. Mutant colonies were isolated and kept at -20°C and - 80°C in a solution of buffered peptone water with 20% glycerol.

It was also possible to establish the mutant selection window (MSW) of nisin for the collection of oral enterococci isolates, a value defined as the antimicrobial concentration ranging between the MIC and MPC values (Zhao & Drlica, 2002; Drilca, 2003).

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility profiling was performed on the clinical isolates and on the mutants recovered by following the MPC protocol, to determine if incubation in the presence of nisin can alter the susceptibility profiles. Using the disk diffusion method, the susceptibility profile regarding, a total of 12 different antibiotics (Table 1) was determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). For that, a bacterial suspension was prepared, from pure cultures, with a turbidity of 0.5 McFarland, which corresponds to 10^8 CFU/mL. Afterwards, the inoculum was evenly spread over the entire surface of a MH agar (Oxoid, CMO337) plate and the disks impregnated with the antimicrobial agent were placed over the surface of the agar plate. The plates were then incubated at 37°C under aerobic conditions for 18 hours or, in the case of vancomycin, 24 hours. After the incubation period, the inhibition zone diameters were measured and compared with the CLSI standard breakpoints. Quality control was performed using the reference strain, *Staphylococcus aureus* ATCC 25293, as indicated by CLSI guidelines.

Table 1- Antimicrobial agents used for antimicrobial susceptibility tests, grouped by target and class.

Target	Antimicrobial Class	Antimicrobial Agent	Symbol	Dose per disk (µg)
Inhibition of cell-wall synthesis	Penicillins	Ampicillin	AMP	10
	β-Lactams	Amoxicillin + clavulanic acid	AMC	30
	Glycopeptides	Vancomycin	VAN	30
	Carbapenems	Imipenem	IPM	10
	Cephalosporins	Cefotaxime	CTX	30
Inhibition of nucleic acid synthesis	Fluoroquinolones	Ciprofloxacin	CIP	5
		Enrofloxacin	ENR	5
Inhibition of protein synthesis	Tetracyclines	Tetracycline	TET	30
		Doxycycline	DTX	30
	Aminoglycosides	Gentamicin	GEN	10/120
		Streptomycin	STR	300

2.4 Determination of the mutants' minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for nisin

Determination of the minimum inhibitory concentration (MIC) was performed on the mutants derived from the MPC protocol using the broth microdilution method, to assess their current susceptibility to nisin. As such and according to the protocol established by Pinheiro optimized to *Enterococcus* spp., the wells of a 96-well microplate (VWR Tissue culture plates, ref. 10062-900) were filled with 20 µL of nisin at different concentrations, apart from the columns designated for the positive and negative controls, according to the scheme illustrated on figure 6 (Pinheiro, 2016). Subsequently, 0.5 McFarland bacterial suspensions were prepared for each mutant, which were then diluted (1:100) in Tryptic Soy Broth (TSB, VWR Chemicals, ref. 84675.0500) in order to achieve 10^6 CFU/mL. Afterwards, 180 µL of the previously prepared bacterial suspensions were placed in each well, except for the negative control column, which was filled with 200 µL of TSB (Figure 6). Consequently, each well contained a volume of 200 µL and a final concentration of nisin of 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mg/mL. The 96-well microplates were incubated at 37 °C for 24 hours, after which bacterial growth was visually assessed in order to determine MIC value. This parameter is defined as the lowest nisin concentration capable of preventing bacterial multiplication *in vitro,* without no visible growth on the well (Jorgensen & Ferraro, 2009).

Subsequently, after the 24-hour incubation period at 37 °C and MIC reading, the minimum bactericidal concentration (MBC) was determined. From each well with no visible growth observed, 5 µL of the bacterial suspension were plated onto TSA (Tryptic Soy Agar, VWR Chemicals, ref.84602.0500), followed by incubation at 37 °C for 24 hours. MBC was defined as the lowest antimicrobial concentration that is needed to inhibit bacterial growth after sub-culture of the suspensions on solid unselective media without any antimicrobial agent (Santos *et al*., 2016). Both the MIC and MBC

assays were performed in triplicate, in independent days, testing as well 10% of replicates to assure results representability.

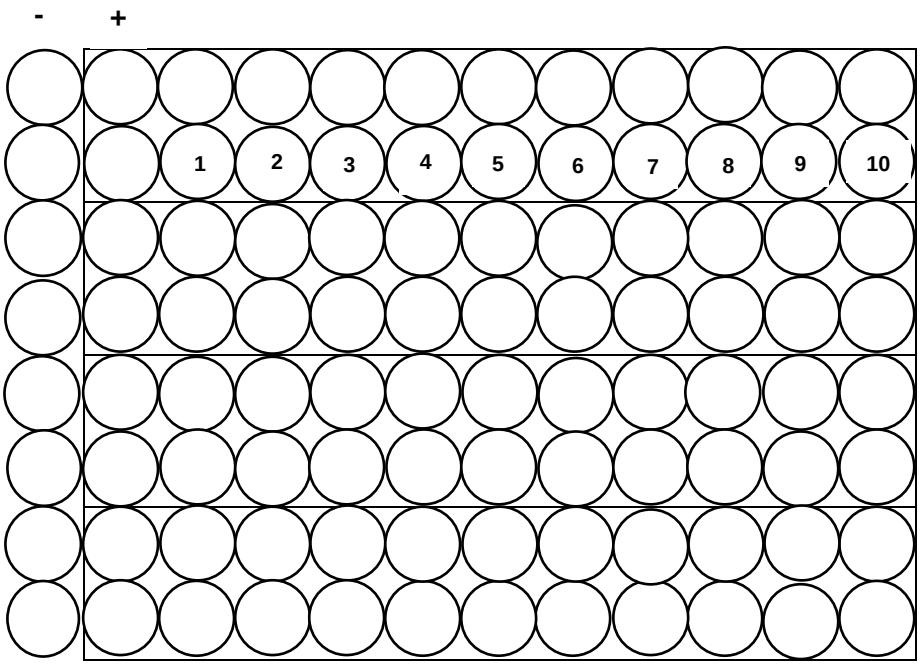


Figure 6- Representative scheme illustrating the distribution of nisin solutions in a 96-well microplate for the determination of the nisin MIC value for the mutants obtained in the MPC protocol (Original). Numbers 1 to 10 refer to the different nisin concentrations present in each well: 1- 4.0 mg/mL; 2- 3.0 mg/mL; 3- 2.5 mg/mL; 4- 2.0 mg/mL; 5- 1.5 mg/mL; 6- 1.0 mg/mL; 7- 0.5 mg/mL; 8- 0.2 mg/mL; 9- 0.1 mg/mL; 10- 0.05 mg/mL. The – and + symbols refer to the negative and positive controls, respectively.

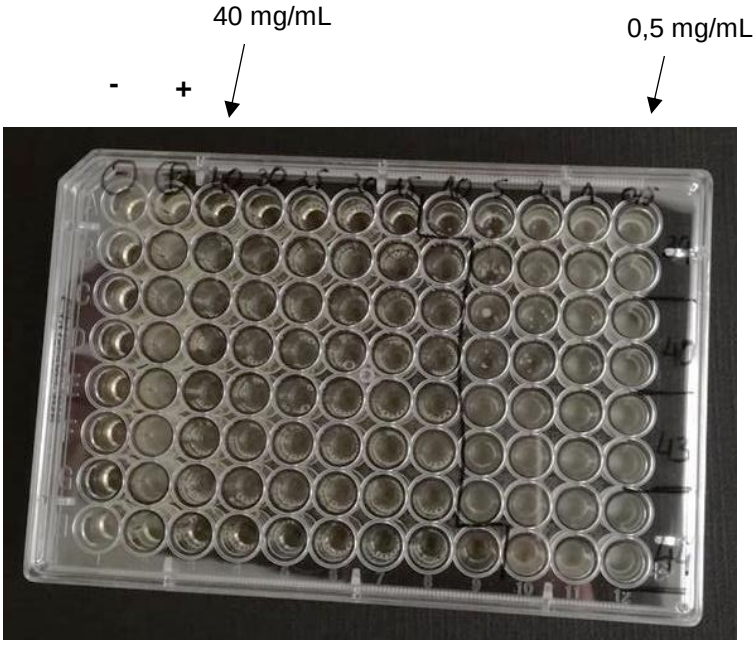


Figure 7- Reading of a 96-well microplate (Original).

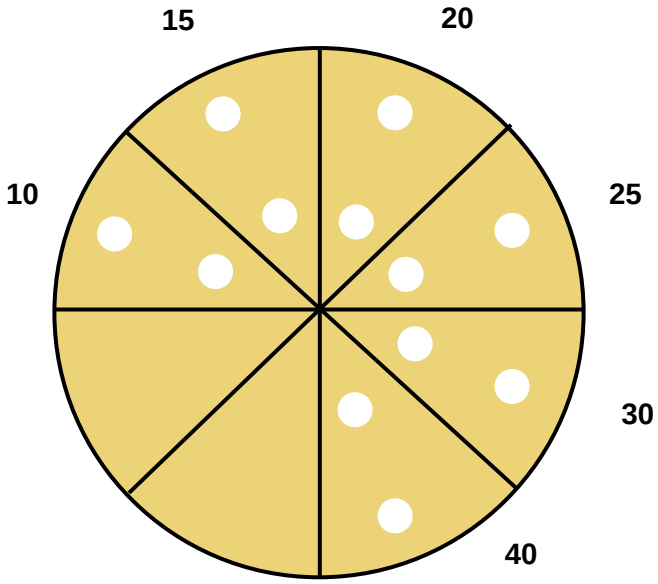


Figure 8- Schematic representation of the inoculation of a TSA plate for determining MBC value of a strain with a MIC value of 10 mg/mL.

Figure 9- Example of a TSA plate with the MBC results for mutants EZ17 and EZ29 (Original).

Statistical analysis

All data, graphs and tables were elaborated using the 2016 version of Microsoft Office Excel software. All quantitative data (concentrations) are expressed as means± SD . Statistical analysis of the MIC and MBC values of the two collections considered in this study was performed by SPSS (IBM SPSS Statistics V25.0). More specifically, a normality test, the Kolmogorov-Smirnov test, was performed for each variable, assuming that for *p*-values higher than 0.05, the variables presented a normal distribution. Afterwards, a Student's T-Test was performed to determine if there was significant statistical difference between the variables (*p*-values below 0.05). A 95% interval of confidence was defined.

Chapter 3

Results and discussion

The emergence of antimicrobial resistance constitutes a threat to public and animal welfare. Thus, for any antimicrobial protocol under investigation aiming at its application for clinical purposes, it becomes necessary to disclose its contribution to the development and maintenance of resistance (Oliveira *et al*., 2015). Periodontal disease is one of the most prevalent inflammatory diseases in dogs, requiring the formation of a biofilm on the surface of the teeth (Niemec, 2008; Oliveira *et al*., 2016). *Enterococcus* spp., natural colonizers of the gastrointestinal tract of a variety of animals, are among the microorganisms encountered in the canine oral cavity (Oliveira *et al*., 2016). Recently, WHO classified Vancomycin Resistant Enterococci (VRE) as high priority pathogens for which there is a pressing need for drug development (WHO, 2017). In this context, antimicrobial peptides such as nisin are naturally occurring biomolecules that have been described to have broad antimicrobial activity as well as low-levels of resistance (Batoni *et al*., 2011; Kang *et al*., 2014). Additionally, there has been growing evidence of nisin's antimicrobial properties against resistant bacteria, as well as biofilm-producing pathogenic bacteria, including enterococci (Shin *et al*., 2016). Nisin has a dual mechanism of action, by forming pores followed by the interaction with the anionic lipids present in the cytoplasmic membrane of susceptible cells and also by preventing cell wall biosynthesis (Tong *et al*., 2010; Tong *et al*., 2014). Considering the potential of nisin application to the oral cavity of dogs, one goal of this work was to determine the influence of saliva in nisin activity, using 20 enterococci isolates recovered from the oral cavity of dogs with periodontal disease, previously characterized (Tavares, 2014). Then, the mutant selection window of nisin was established by determining the mutant prevention concentration. The collection of mutants retrieved following the MPC assay was further studied. Indeed, antimicrobial susceptibility profiling was performed regarding the MPC-derived mutants and the original enterococci collections. Finally, MIC and MBC values of nisin for the mutants were determined.

Canine saliva influence in the antimicrobial activity of nisin

As previously mentioned, the first part of this work consisted in evaluating the influence of dog's saliva in the antimicrobial activity of nisin, whether alone or incorporated in a guar gum gel. The previously determined MIC values for nisin and nisin incorporated in guar gum were, respectively, 0.5 and 1.0 mg/mL, and served as controls in this study (Pinheiro, 2016; Trovão, 2017). Also, these MIC values were used to establish the concentrations of the nisin and nisin supplemented guar gum gel solutions. Indeed, for this protocol two-fold concentration increments of nisin were used, up to 8x the previously determined average MIC value (0.5 mg/ml). As such, the applied concentrations were 0.5, 1.0, 2.0 and 4.0 mg/mL for both nisin and nisin incorporated in guar gum. Despite these solutions being diluted in saliva, the final concentrations remained unaltered.

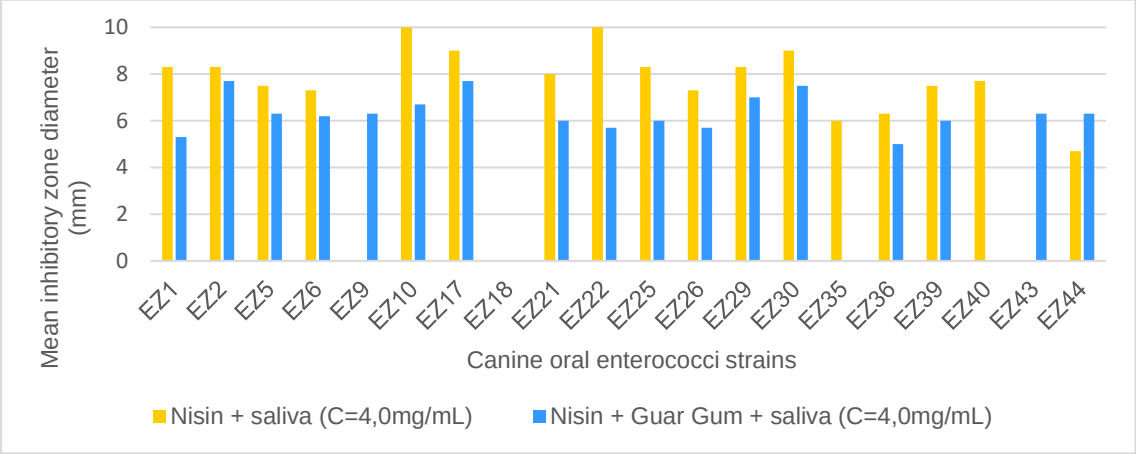


Figure 10- Comparison between the average diameter of the inhibition halos (mm) obtained for each tested enterococci promoted by nisin and nisin incorporated in a guar gum gel, diluted in saliva. E- *Enterococcus*; Z-swab.

According to the results presented in Figure 10, it was possible to observe that when nisin was diluted in saliva at a concentration of 4.0mg/mL, almost all tested strains were inhibited; specifically, 85% (n=17/20) of isolates exhibited inhibition zone diameters with an average diameter of 7.9 mm ±1.3mm. Nisin diluted in saliva had no antimicrobial activity against three isolates (EZ9, EZ18 and EZ 43). When nisin was incorporated in a guar gum gel (proportion of 1:1) at a concentration of 4.0mg/mL and diluted in saliva, once again the majority of the oral enterococci strains under study were inhibited; more precisely, 85% (n=17/20) of the isolates exhibited inhibition zone diameters with an average diameter of 6.3 mm ± 0.75mm. Strains EZ18, EZ35 and EZ40 were not inhibited.

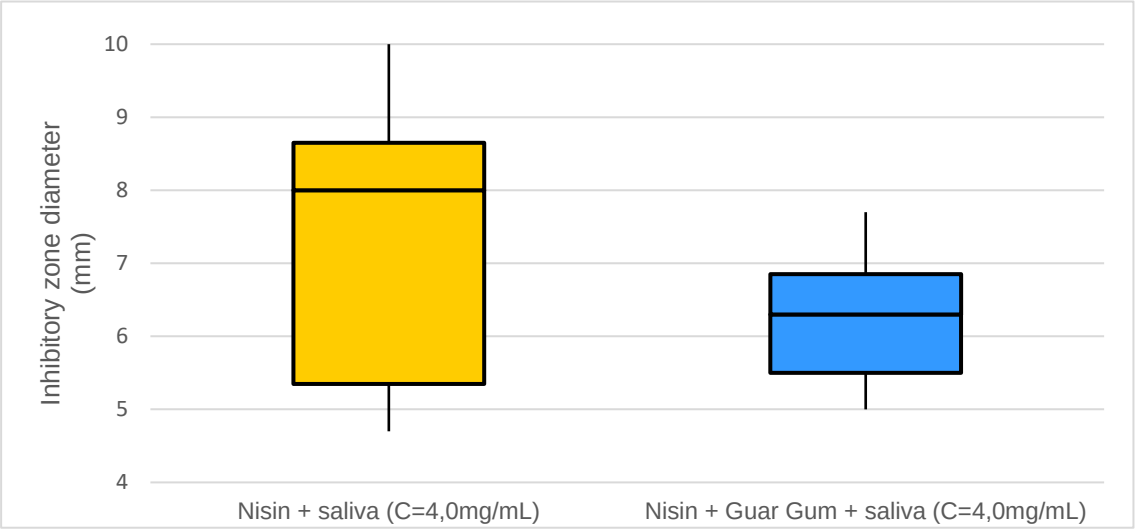


Figure 11- Box-plot of the inhibitory zone diameters (mm) promoted by nisin and by nisin incorporated in guar gum gel upon dilution in saliva.

As observed in Figure 10, the inhibition zone diameters for nisin diluted in saliva ranged between 4.7 and 10 mm. As for nisin incorporated in guar gum gel and diluted in saliva, the inhibition diameters varied between 5.0 and 7.7 mm. Observation of Figure 11 allows to clearly note the differences in the variance between the inhibition diameter sizes distribution for nisin and for nisin incorporated in a guar gum gel, with the latter having a narrower distribution range. These results suggest that the guar gum

gel acts by stabilizing nisin diffusion and promoting more consistent inhibition halos. For instance, the most frequent inhibition diameter size for nisin incorporated in guar gum gel was of 6.3 mm, observed for 5 strains and coinciding with the mean value, whereas for nisin the most frequent inhibition diameter was of 8.3 mm presented by 4 strains, exceeding the mean value (7.9 mm).

These results are important observations, since nisin is being evaluated aiming to be topically applied to the oral cavity of dogs using guar gum gel as a vehicle of administration.

Saliva alone was also tested, as a control, to evaluate if it had any direct influence on the growth of the tested strains. As depicted in Figure 5 (page 34), which illustrates the results obtained for strain EZ17, saliva exerted no effect on bacterial multiplication. Therefore, these results indicate that saliva by itself does not impair enterococci growth. Nevertheless, the addition of saliva to the solutions of nisin and of nisin incorporated in guar gum increased the required concentration for nisin's inhibitory activity.

Saliva biochemical analytes, such as lysozyme, amylase and lactate dehydrogenase, have been regarded as functional biomarkers for periodontal disease in humans (Iacopeti *et al*., 2017). However, in dogs, there are no studies available regarding salivary composition or analysis of the relationship between its biochemical composition and the stage of periodontal disease (Iacopeti *et al*., 2017). In this context, a pilot study from 2017 determined salivary pH and evaluated the presence of lactate dehydrogenase, amylase and lysozyme in the saliva of healthy dogs (Iacopeti *et al*., 2017). These enzymes have been described to have activity against cariogenic bacteria (Tong *et al*., 2010). As such, they may interact with nisin and impair its antimicrobial activity through mechanisms that have not yet been described. According to a pilot proteomic study by Torres, canine saliva is composed by diverse molecules, mainly immunological enzymes, which may also contribute to delaying nisin's antibacterial activity (Torres *et al*., 2018). Furthermore, the study included dogs from different breeds, which presented some variability in relation to some salivary components (Torres *et al*., 2018). Despite the fact that the saliva collected for this study came from different dogs, the various samples were evenly mixed. It is also very important to mention that, according to the same study from 2017, saliva from healthy dogs has a mean pH around 7.9 (Iacopetti *et al*., 2017). Nisin is a small, cationic antimicrobial peptide whose structural stability and antimicrobial activity are largely dependent on pH, being more stable and effective at acidic conditions (Gharsallaoui *et al*., 2016). More specifically, irreversible structural modifications of nisin take place when pH is higher than its isoelectric point (pH>8) (Gharsallaoui *et al*., 2016). Nisin being very sensitive to pH, might explain why its antimicrobial activity was impaired contact.

In conclusion, saliva did not block the antimicrobial effect of nisin against canine PD enterococci, further confirming the potential of this antimicrobial peptide for enterococcal PD control.

Mutant Prevention Concentration and Mutant Selection Window

Improper antimicrobial dosage is considered an important risk factor promoting the development of resistance (Balaje *et al*., 2013). Despite the low resistance rate, a few cases of nisin resistant-bacteria have been reported (Zhou *et al.*, 2014; Draper *et al*., 2015; Shin *et al.,* 2016). More precisely, there has been some evidence that suggests that resistance to nisin derives from mutations (Shin *et al*., 2016). In this context, with the objective of preventing the emergence and amplification of bacteria resistant to nisin due to a future application of this AMP for canine periodontal disease control, the mutant selection window (MSW) for this antimicrobial peptide was determined.

The MSW hypothesis considers *de novo* resistance, which often occurs in a gradual, step-wise manner due to the accumulation of mutations which decrease bacteria susceptibility to an antimicrobial compound (Drlica, 2003). This concept seems to be appropriate in the analysis of antimicrobials for which point mutations are the main driver of bacterial resistance. The MSW is the antimicrobial concentration ranging between the MIC and MPC, lower and upper boundaries respectively. MPC, the upper limit of the window is the concentration needed to inhibit the growth of the least- susceptible single-step mutant (Drilca, 2003; Sinel *et al*., 2016). To establish the MSW of nisin, it was necessary to determine its MPC, since the lower boundary (MIC) has already been previously defined for the collection of enterococci under study (Pinheiro, 2016).

During the optimization of the mutant prevention protocol, it was required to establish a bacterial suspension of 10^{10} CFU/mL. To standardize this concentration, a calibration curve relating the optical density of the bacterial suspension and bacterial count was established. For that, serial dilutions were performed using the initial bacterial suspension, their optical density (OD) measured at 600 nm and respective colony forming units counted (CFUs). Precisely, dilutions 10^{-1} to 10^{-3} were used to measure absorbance and, 100 µL of the 10^{-5} to 10^{-8} dilutions were applied onto BHI agar plates to determine viable counts, which were performed in duplicate for each dilution

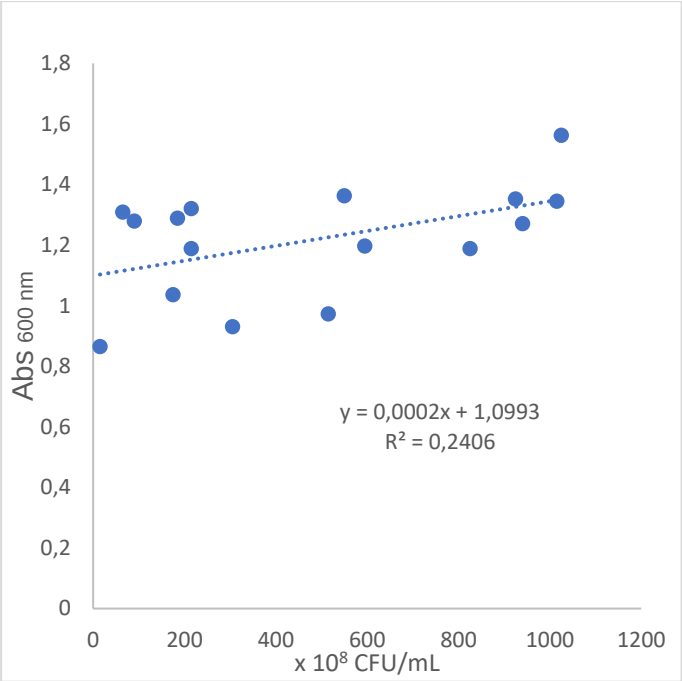


Figure 12- Calibration curve using the absorbance measured at 600 nm for dilution 10^{-2}, which best fitted Lambert-Beer's law, and the CFU per mL of the initial suspension, using the averaged CFU count for each isolate for dilution 10^{-8}. Outliers were eliminated.

To establish this calibration curve (Figure 12), absorbance values of dilution 10^{-2} were plotted against the CFU per mL obtained with the averaged CFU count for each enterococci isolate for dilution 10^{-8}. Although absorbance was measured for the first 3 serial dilutions, the second dilution was chosen for presenting the values that better respect Lambert-Beer's law. This calibration curve means that, when preparing such a concentrated enterococci bacterial suspension (10^{10} CFU/mL), confirmation of the microbial load can be done by: doing a serial dilution of the initial suspension and reading the absorbance at 600nm of the second dilution or by performing viable count of dilution 10^{-8}. This calibration curve is an important analytical reference for future studies requiring high CFU counts, facilitating standardization of the experimental procedure, in particular for enterococci isolates.

Linear regression was used to fit the data, in order to try to establish a relationship between optical density measured at 600 nm (OD) and CFU/mL. After fitting a linear regression model to this data, it was important to determine the fit as well as the respective equation to predict its behavior. In this context, R^2 is the coefficient of determination and expresses how the differences in one variable can be explained by the variation in a second one, varying between 0 and 1. Generally, for the same set of data, a higher R^2 value indicates a better fit, with smaller differences between the data and the fitted values. The resulting coefficient of determination (R^2) is 0.24 suggesting that these variables are poorly correlated.

McFarland Standards serve as references, enabling the standardization of the approximate microbial load in a liquid suspension by comparing the turbidity of the test solution to the McFarland Standard (Lahuerta Zamora & Perez-Garcia, 2012). There are 10 McFarland turbidity standard solutions on the McFarland scale, allowing the preparation of suspensions with up to 10^9 CFU/mL, while remaining within the linear range of absorption to estimate CFU. However, in very concentrated solutions, which is the case of the ones necessary for this study, the linear relationship between absorption and CFU count is of limited scope (Sutton, 2011). Many factors can have contributed to the weak correlation between absorbance and CFU count such as the individual morphological characteristics of each clinical isolate, the low number of strains used (n=20) and also the reduced number of assays performed. Therefore, in order to improve robustness, more strains should be tested and additionally, more assays should be performed to ensure the reproducibility of the results.

It was possible to determine the MPC values for 85% (n=17) of the strains with the exception of strains EZ36, EZ40 and EZ43 (Figure 13). The MPC values for the 17 strains ranged from 16.0 to 24.0 mg/mL whereas for these 3 strains MPC was higher than 24.0 mg/mL. The average MPC value for nisin was 18.0 ± 3.4 mg/mL.

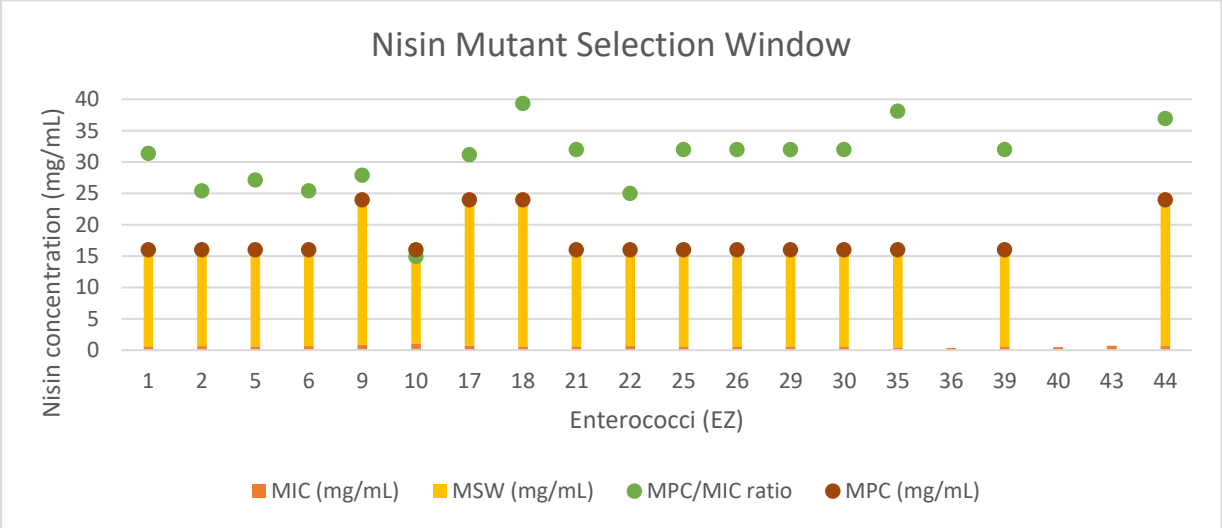


Figure 13- Nisin Mutant Selection Window for the collection of oral-enterococci isolates obtained from dogs with periodontal disease. MIC values were determined previously (Pinheiro, 2016).

In this work it was also possible to establish the MPC/MIC ratio (MSW) of nisin for the majority clinical enterococci isolates under study (Figure 13), which varied between 15 to 39. Indeed, the resulting MPC values were 15 to 40 times higher than the previously determined MIC values (Pinheiro, 2016). The MSW can be defined as a dangerous range of antimicrobial concentrations, which can promote the development of resistant mutants (Blondeau, 2009). As MPC values for nisin varied between 16.0 to 24.0 mg/mL, keeping antimicrobial therapeutic concentrations above these values should prevent the selection and amplification of resistant-mutants.

As mentioned above, isolates EZ36, EZ40 and EZ43 were the only ones with undetermined MPC values (above 24.0 mg/mL). According to their previous phenotypical and genotypical characterization (Tavares, 2014), these isolates share no common virulence traits that might explain why it was not possible to determine their MPC value. Nevertheless, the initial enterococci isolates were retrieved from the oral cavity of dogs with periodontal disease (Tavares, 2014). The oral cavity is inhabited by dense and genetically diverse bacterial populations that are in close contact to one another, which facilitates the exchange of genetic material, including resistance determinants (Roberts & Mullany, 2010; Kolenbrander *et al*., 2010). Furthermore, these genes can remain silent until the bacteria are exposed to certain stresses, which can lead to different phenotypes (Huang & Agrawal, 2016). Because the MPC protocol requires an unusually high amount of inoculum, it might have led to genetic changes. These modifications include the expression of previously silent genes and, consequently, different resistance phenotypes, as the ones observed for mutants EZ36, EZ40 and EZ43. Nevertheless, the presence of nisin resistance determinants was not yet been evaluated on the collection of mutants obtained, which would be important to a better comprehension of these results.

The minimum inhibitory concentration (MIC) is a quantitative measure of antimicrobial susceptibility testing, allowing to determine which antimicrobial concentrations are most effective and adequate to achieve a successful therapeutic outcome (Blondeau, 2009). Specifically, the MIC value establishes the concentration of an antibiotic that is required to inhibit bacterial growth (Zhao & Drlica, 2008). Furthermore, MIC determination involves the standardized use of an inoculum containing 10^6CFU/mL whereas it has been estimated that bacterial load upon infection can reach much higher concentrations, like 10^{10} to 10^{12} microorganisms in the case of human pneumococcal pneumonia (Blondeau, 2009). Consequently, MIC testing requires a microbial load which can be unrepresentative of the bacterial burden existent in an infection site and thus, allowing the survival of mutants resistant to the antimicrobial therapy (Blondeau, 2009). Hence, this dosing strategy fails to consider mutant subpopulations, and resistance can arise during antimicrobial therapeutics (Zhao & Drlica, 2008; Blondeau 2009). Since the mutant prevention concentration (MPC) estimates the antimicrobial concentrations necessary to impair the emergence of mutant pathogens (Zhao & Drlica, 2008), the results obtained in this work denote the importance of determining MPC values to establish precise and effective therapeutic regimens and ultimately limit the emergence and spread of resistances.

So far, MPC determinations have focused only on antibiotics and, from these, only one study was performed using *Enterococcus* spp*.* (Sinel *et al*., 2016), not making possible to directly compare these results obtained with other studies. Nevertheless, because there are antibiotics with a mode of action similar to nisin's, such as daptomycin and vancomycin, certain analogies can be drawn.

Daptomycin (DAP) is a calcium-dependent lipopeptide antibiotic with a strong bactericidal activity against a wide-spectrum of gram-positive bacteria (Steenbergen *et al.,* 2005). It acts by irreversibly altering the bacterial cell membrane, resulting in pore formation and, subsequently, promoting membrane depolarization (Sinel *et al*.,2016). Vancomycin (VA) is a tricyclic, bactericidal glycopeptide, whose molecular weight is 1446 Da (Gupta *et al.*, 2011; Rubinstein & Keynan, 2014). This antibiotic is produced by *Streptococcus orientalis* and is active against gram-positive bacteria

(Rubinstein & Keynan, 2014). Vancomycin inhibits cell-wall biosynthesis by preventing the incorporation of N-acetylmuramic acid (NAM)- and N-acetylglucosamine (NAG)-peptide subunits into the peptidoglycan (Rubinstein & Keynan, 2014). Nowadays, vancomycin is mainly used as a first-line therapeutic alternative to treat MRSA infections in humans (Rybak *et al*., 2008).

In 2014, Fujimura and colleagues determined that the MSW of both vancomycin and daptomycin against MRSA isolates, was 64 times higher than the MIC value (Fujimura *et al*., 2014). More recently, Sinel and collaborators Moreover, the authors were able to establish the MSW for this antibiotic, which varied between 2 and 32 (Sinel *et al*., 2016).

Considering the MSW values established for nisin, they are close to those described in literature for daptomycin and vancomycin. However, it is important to refer that nisin is an antimicrobial peptide with unique physical-chemical properties, such as molecular weight, optimal pH and mode of action. First, not only is nisin a bigger molecule than vancomycin and daptomycin, having a molecular weight of 3500 Da, but also, 8 nisin molecules are needed for the pores to be formed (Gharsallaoui *et al*., 2016).

The final aim of the ongoing project is to develop a nisin product be topically applied to the oral cavity of dogs to control periodontal disease in these animals. In the European Union, nisin use is currently allowed as a food additive (Younes *et al*., 2017). Therefore, it is important to take into consideration the guidelines established by the European Union for food additives. According to the European Food Safety Authority (EFSA), the acceptable daily intake (ADI) estimates the amount of a given substance in foodstuffs (food or drinking water) that can be consumed throughout an individual's lifetime without posing an appreciable health risk to the consumers. ADI determination is normally applied to chemical substances, including food additives and veterinary drugs, and expressed in milligrams of per kilogram of body weight (EFSA, 2018). Nisin had been previously assessed in 2006 by the former EFSA Panel, where an ADI of 0.13 mg of nisin A/kg of body weight was established (Younes *et al*., 2017). Nevertheless, the EFSA panel considered that the new toxicological data made available since then was sufficient to establish a new ADI of 1 mg of nisin A per kg of bodyweight per day, by applying a 200-default uncertainty factor (Younes *et al*., 2017). To estimate the possible toxic impact a few considerations will be taken: the topical application of 1mL of this formulation, containing 18 mg/mL, which is the average MPC value obtained in this study. For medium sized dogs weighting 25 kg the ADI would be around 0.72 mg of nisin A/ kg of bodyweight which is below the established cut-off value (1 mg of nisin A per kg of body weight). Furthermore, the ADI value was determined for a nisin A formulation at 7.5%, thus increasing the safety window for the therapeutic utilization of this antimicrobial peptide.

Antimicrobial susceptibility testing

As stated earlier, antimicrobial resistance is a serious challenge to global health, with high morbidity and mortality rates associated to infections by multidrug-resistant pathogens (Frieri *et al*., 2017). Moreover, the emergence and dissemination of resistance determinants has mainly been attributed to the overuse and misuse of antibiotics (Richardson, 2017). Therefore, for any antimicrobial protocol being investigated it is critical to understand and prevent the development and spread of resistant strains. In this context, the antimicrobial susceptibility pattern of the original enterococci isolates obtained from dogs with periodontal disease was compared with the one from the mutants derived from the MPC protocol. The objective was to assess if incubation in the presence of the antimicrobial peptide nisin influences the susceptibility profiles of the isolates. Susceptibility to ampicillin, amoxicillin, imipenem, vancomycin, cefotaxime, ciprofloxacin, enrofloxacin, tetracycline, doxycycline, gentamicin and streptomycin was tested according to the methodology and breakpoints defined by the Clinical and Laboratory Standards Institute (CLSI, 2017). The selection of the antibiotics for this work was based on their relevance to veterinary medicine as well as public health. Specifically, gentamicin-120 µg and streptomycin-300 µg were selected to detect high-level aminoglycoside resistance in *Enterococcus* spp., whereas imipenem and vancomycin were chosen due to being a public health concern important to monitor (Davido *et al*., 2018).

Regarding the resistance profiles depicted by Tables 2 and 3, it is possible to observe that no isolate was susceptible to all the antibiotics tested, being in fact resistant to more than one antimicrobial. For the original enterococci isolates, resistance levels ranged from 0% (Imipenem; Amoxycillin/clavulanic acid) to 100% (Cefotaxime; Gentamicin-10µg). As for the mutants derived from the MPC protocol, the resistance levels varied between 5.0% (Amoxycillin/clavulanic acid) to 100% (Cefotaxime; Gentamicin-10µg). In both collections the highest resistance levels were observed for the same antimicrobial agents, namely cefotaxime and gentamicin-10µg. These results come as no surprise since enterococci are intrinsically resistant to various antibiotics including cefotaxime, low-level aminoglycosides (Gentamicin-10µg), clindamycin and quinipristin-dalfopristin (Gilmore *et al*., 2013).

Table 2- Antimicrobial susceptibilities and MDR profile of the initial enterococci collection to: ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), vancomycin (VAN, 30 µg), imipenem (IMI, 10 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), tetracycline (TET, 30 µg), doxycycline (DXT, 30 µg), gentamicin (GEN, 10 and 120 µg) and streptomycin (STR, 300 µg). Classification done according to the breakpoints established by CLSI: S- Susceptible; I- Intermediate; R- Resistant. In the case of cefotaxime, because *Enterococcus* are intrinsically resistant, the breakpoint used were the ones established for *Streptococcus* spp. viridans (CLSI, 2017). E- *Enterococcus*; Z- swab.

ISOLATE	AMP 10	AMC 30	VAN 30	IMI 10	CTX 30	CIP 5	ENR 5	TET 30	DXT 30	GEN 10	GEN 120	STR 300	MDR
EZ1	S	S	S	S	R	I	R	R	I	R	S	R	✗
EZ2	S	S	S	S	R	I	I	R	R	R	S	S	✗
EZ5	S	S	S	S	R	R	R	R	R	R	S	R	✓
EZ6	R	S	I	I	R	R	R	R	R	R	S	R	✓
EZ9	S	S	S	S	R	I	R	R	I	R	S	S	✗
EZ10	S	S	S	S	R	R	R	S	S	R	S	R	✗
EZ17	S	S	I	I	R	R	R	R	R	R	S	R	✓
EZ18	S	S	I	S	R	I	I	R	R	R	S	R	✗
EZ21	S	S	S	S	R	I	I	R	R	R	S	S	✗
EZ22	S	S	I	S	R	I	I	R	R	R	S	S	✗
EZ25	S	S	I	S	R	I	R	R	R	R	S	R	✗
EZ26	S	S	I	S	R	I	R	R	R	R	S	R	✗
EZ29	S	S	I	S	R	I	R	R	R	R	S	R	✗
EZ30	S	I	I	I	R	R	R	R	R	R	S	R	✓
EZ35	S	S	S	S	R	R	R	R	R	R	R	R	✓
EZ36	S	S	S	I	R	R	R	R	R	R	R	S	✓
EZ39	R	I	I	S	R	R	R	R	R	R	R	R	✓
EZ40	R	S	S	I	R	R	R	R	R	R	S	R	✓
EZ43	S	S	R	S	R	R	R	R	R	R	S	R	✓
EZ44	S	I	R	I	R	R	R	R	R	R	R	R	✓

Table 3- Antimicrobial susceptibilities and MDR profile of the mutants recovered from the MPC protocol to: ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), vancomycin (VAN, 30 µg), imipenem (IMI, 10 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), tetracycline (TET, 30 µg), doxycycline (DXT, 30 µg), gentamicin (GEN, 10 and 120 µg) and streptomycin (STR, 300 µg). Classification done according to the breakpoints established by CLSI: S- Susceptible; I- Intermediate; R- Resistant. In the case of cefotaxime, because *Enterococcus* are intrinsically resistant, the breakpoints used were the ones established for *Streptococcus* spp. viridans (CLSI, 2017). E- *Enterococcus*; Z- swab.

ISOLATE	AMP 10	AMC 30	VAN 30	IMI 10	CTX 30	CIP 5	ENR 5	TET 30	DXT 30	GEN 10	GENN 120	STR 300	MDR
EZ1	S	S	S	S	R	I	R	R	R	R	S	S	✗
EZ2	S	S	I	S	R	I	R	R	R	R	S	S	✗
EZ5	S	S	I	I	R	R	R	R	R	R	R	R	✓
EZ6	S	S	I	S	R	R	R	R	R	R	S	R	✓
EZ9	S	S	R	I	R	I	R	I	S	R	R	S	✗
EZ10	R	R	S	R	R	R	R	R	I	R	S	R	✓

EZ17	S	I	I	I	R	R	R	R	R	R	R	R	✓
EZ18	R	S	I	S	R	R	R	R	R	R	R	R	✓
EZ21	S	S	I	I	R	R	I	R	R	R	S	S	✗
EZ22	S	S	S	S	R	I	R	R	R	R	S	S	✗
EZ25	S	S	I	S	R	I	R	R	R	R	R	R	✓
EZ26	S	S	R	S	R	I	R	R	I	R	S	R	✗
EZ29	S	S	S	S	R	I	R	R	R	R	S	R	✗
EZ30	S	S	S	S	R	I	I	R	R	R	S	R	✗
EZ35	S	S	S	S	R	R	R	R	R	R	S	R	✓
EZ36	S	S	S	I	R	R	R	R	R	R	S	R	✓
EZ39	S	S	S	S	R	I	R	R	R	R	S	R	✗
EZ40	S	S	S	R	R	R	R	R	R	R	S	R	✓
EZ43	S	S	I	S	R	R	R	R	R	R	S	S	✗
EZ44	R	I	R	R	R	R	R	R	R	R	R	R	✓

Table 4- Comparison of the resistance profile of the two collections of enterococci isolates: group 1 is formed by the original isolates obtained from the oral cavity of dogs with PD **(G1)** and group 2 comprising the mutants derived from the MPC protocol **(G2)**. Classification was based on the CLSI guidelines criteria for *Enterococcus* spp. This table presents the number of isolates resistant to each tested antibiotic and respective percentages: ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), vancomycin (VAN, 30 µg), imipenem (IMI, 10 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), tetracycline (TET, 30 µg), doxycycline (DXT, 30 µg), gentamicin (GEN, 10 and 120 µg) and streptomycin (STR, 300 µg). Because enterococci are intrinsically resistant to cefotaxime there are no breakpoints established for this antibiotic. As such, the breakpoints used were the ones established for *Streptococcus* spp. viridans (CLSI, 2017).

	AMP	**AMC**	**VAN**	**IMI**	**CTX**	**CIP**	**ENR**	**TET**	**DXT**	**GEN 10**	**GEN 120**	**STR 300**
G1 (n=20)	3	0	2	0	20	11	16	19	17	20	4	15
%	15,0	0,0	10,0	0,0	100,0	55,0	80,0	95,0	85,0	100,0	19,0	75,0
G2 (n=20)	3	1	3	3	20	11	18	19	17	20	6	14
%	15,0	5,0	15,0	15,0	100,0	55,0	90,0	95,0	85,0	100,0	30,0	70,0

Despite *Enterococcus* species being intrinsically resistant to cefotaxime, this antimicrobial agent is frequently administered in veterinary medicine. Cefotaxime (CTX) is a third-generation cephalosporin, belonging to the β-lactam family (Mehta, 2015). This bactericidal antibiotic interferes with cell wall biosynthesis via the penicillin binding proteins (PBPs). As shown in Table 4, all the enterococci isolates in both collections exhibited resistance to this antibiotic, as expected. The only exception was EZ2 that exhibited decreased susceptibility, which was unexpected.

Both ampicillin and amoxicillin/clavulanic acid belong to the β-lactams class with a broad-spectrum of activity, having been one of the most successful drugs in treating bacterial infections (Finlay, 2003; Worthington & Melander, 2013; Kaushik *et al*., 2014). β-lactams impair bacterial growth by inactivating the protein-binding proteins (PBPs), which are involved in peptidoglycan biosynthesis (Kristich *et al*., 2018). From the initial enterococci collection, 3 isolates (15.0%) exhibited resistance to ampicillin, namely EZ6, EZ39 and EZ40 and 3 enterococci mutants (15.0%) were resistant to this antimicrobial agent, specifically EZ10, EZ18 and EZ44. As such, from all the initial isolates that were resistant to ampicillin, none of the respective mutants maintained the resistance to this compound.

Regarding amoxicillin/ clavulanic acid , there were no resistant isolates from the initial collection, but there was one resistant mutant, EZ10. Penicillins, namely amoxicillin and ampicillin, have been among the most commonly prescribed antimicrobials in veterinary medicine in Portugal in the years 2010 and 2011, which could facilitate the emergence of more resistant bacteria (Almeida *et al*., 2014). However, the vast majority of the isolates from both collections was susceptible to ampicillin and amoxicillin, with susceptibility of both collections to these antibiotics ranging between 85 to 100%. In fact, similar results had been previously been reported (Lopes *et al*., 2005). In the case of the mutant collection, resistance to ampicillin seems to be associated with multidrug resistance since all of the mutants resistant to this compound were MDR (Tables 2 and 3).

Enrofloxacin is a broad-spectrum bactericidal fluoroquinolone developed in 1980, mainly for veterinary use (Semedo-Lemsaddek *et al*., 2018). In the initial enterococci collection, 80.0% (n=16) of the strains were resistant to enrofloxacin, whereas 90.0% (n=18) of the MPC derived mutants were resistant to this antibiotic. Apart from mutant EZ30, all of the initial resistant isolates remained non-susceptible to enrofloxacin. As for ciprofloxacin, 55.0% (n=11) of the isolates in the original collection and on the mutants collection were resistant to this antibiotic. All of the initial isolates resistant to this antibiotic remained non-susceptible, except for mutants EZ30 and EZ39, which exhibited intermediate resistance. Overall, the results indicate less resistances to ciprofloxacin than to enrofloxacin in both groups of enterococci isolates.

Fluoroquinolones are potent, broad-spectrum synthetic antibiotics which target the DNA (Redgrave *et al*., 2014). Enrofloxacin is available for veterinary use only, while ciprofloxacin is applied to both human and veterinary use (Trouchon & Lefebvre, 2016). Furthermore, in most animals enrofloxacin is metabolized into ciprofloxacin, which is the active form of this antimicrobial compound (Trouchon & Lefebvre, 2016). In dogs, approximately 40% of the enrofloxacin administered is converted into ciprofloxacin, which can also explain the proximity of resistance percentage observed for these two antibiotics (Trouchon & Lefebvre, 2016). Due to their broad-spectrum of action against Gram-positive and Gram-negative bacteria, fluoroquinolones have been frequently used in both human and veterinary medicine, being one of the most important group of antimicrobial drugs available (Redgrave *et al*., 2014). According to the results presented in Table 4, more than half of the enterococci isolates in both collections are resistant to enrofloxacin and ciprofloxacin. Resistance to this class of antimicrobials compromises its utility to treat various infections, including urinary tract infections which are commonly diagnosed in dogs (Weese *et al*., 2011; Kim & Hooper, 2014; Trouchon & Lefebvre, 2016). Moreover,

in 2016, the FDA released a statement forewarning about the potential permanent side effects of fluoroquinolone use. Hence, it becomes crucial to restrict their administration to second-line agents, in order to prevent the further emergence and expansion of resistant mutant subpopulations (Stephan *et al*., 2007).

Resistance to vancomycin constitutes a serious problem to human health, particularly, considering that the presence of VRE strains further complicates the treatment of related infections, resulting in higher morbidity and mortality rates (Lisboa *et al*.,2015; Semedo-Lemsaddek *et al*., 2018). In Europe, vancomycin-resistant enterococci started to arise during early 1990s and were associated to the use of avoparcin, a growth promoter applied in animal farming, which is similar to vancomycin (Cogliani *et al*., 2011). In *Enterococcus* species, resistance to vancomycin is related to the presence of a variable ligase encoded by nine different genes, which confer specific levels of vancomycin-resistance (Faron *et al*., 2016). Nevertheless, despite the high number of genes that have been described as being related to resistance to this antibiotic, the most commonly identified are *vanA, vanB* and *vanC* (Faron *et al*., 2016). Two of the original isolates (10.0%), EZ43 and EZ44, were resistant to vancomycin, whilst three MPC-mutants (15.0%), EZ9, EZ26 and EZ44 exhibited resistance to vancomycin. It was expected that the strains would maintain their resistance to this antimicrobial, but only EZ44 did. Moreover, EZ43 went from being resistant to susceptible, which was not expected. This might be due to a point mutation that originated a different ligase which was present in the initial isolate but absent in the mutant.

Another relevant result is related to imipenem, to which 15.0% (n=3) of the enterococci mutants were resistant, whereas the original strains were all susceptible. Carbapenems, like imipenem, are the most potent class of β-lactams, being used in human medicine as a last resource therapeutic option for severe infections (Papp-Wallace *et al*., 2011). However, the recent appearance of multidrug-resistant (MDR) bacteria is jeopardizing the effectiveness of this class of antimicrobials, with several of the latest studies denoting an increase in resistance to carbapenems (Couchani *et al*., 2011; Livermore *et al*., 2011; Patel & Bonomo, 2011; Papp-Wallace *et al*., 2011). Hence, resistance to carbapenems is of serious importance to antibiotic resistance, which is a current worldwide challenge.

Mutant strains EZ10, EZ40 and EZ44 exhibited resistance to imipenem after being subjected to the nisin stress inherent to the MPC protocol. Furthermore, carbapenems are β-lactams and so, the development of resistance to other antibiotics from this class might promote resistance to these antimicrobial agents, in this case imipenem. Indeed, mutant strains EZ10 and EZ44 were resistant to imipenem as well as to ampicillin and amoxicillin. Even though mutant EZ40 was not resistant to ampicillin, the initial isolate was resistant to this antibiotic, which might have contributed to this result. Carbapenems are regarded as one of the most reliable antimicrobial agents to treat various bacterial infections, and thus resistance to these compounds constitutes a major public health concern (Codjoe & Donkor, 2018).

Tetracyclines, such as tetracycline or doxycycline, are a broad-spectrum family of antibiotics responsible for inhibiting protein synthesis (Chopra & Roberts, 2001). In both collections, 95.0% (n=19) of the isolates demonstrated resistance to tetracycline. Also, isolate EZ9 was initially resistant whilst its mutant had decreased susceptibility. Strain EZ10 went from being susceptible to resistant. As for

doxycycline, 85.0% (n=17) both of the initial and mutant isolates were resistant to this antibiotic. Tetracyclines, were among the most commonly prescribed antibiotics in 2010 and 2011 in veterinary medicine (Almeida *et al*, 2014). Overall, the resistance levels to tetracycline were slightly higher than to doxycycline. Guidelines recommend first the use of first-generation antibiotics to preserve antimicrobial efficacy and considering that tetracycline is a first-generation antibiotic and doxycycline a second-generation tetracycline, this can explain the higher resistance percentages to tetracycline obtained in this study (Fuoco, 2012).

Gentamicin is a widely used bactericidal aminoglycoside, that acts by inhibiting protein synthesis (Chen *et al*., 2014; Kushner *et al*., 2016). According to the results exhibited in Table 4, 100% (n=20) of the original enterococci isolates were resistant to 10 µg of this antibiotic; however, 100% (n=20) of the mutant collection was resistant to this antimicrobial agent. Given the fact that all enterococci are intrinsically resistant to low-doses of aminoglycosides, these results come as no surprise (Chow, 2000). When testing 120 µg of gentamicin, only 4 isolates (20.0%) of the original collection exhibited resistance, while 6 mutants (30.0%) demonstrated resistance, meaning that both collections showed low level resistance to high doses of gentamicin. In the initial collection, isolates EZ35, EZ36, EZ39 and EZ44 exhibited resistance to this antibiotic, yet only mutant EZ44 maintained its resistance. Regarding the mutant collection, resistance was observed in isolates EZ5, EZ9, EZ17, EZ18, EZ25 and EZ44.

Streptomycin is also an aminoglycoside, to which 75.0% (n=15) of the isolates belonging to the initial enterococci collection were resistant. However, only 70.0% (n=14) of the isolates from the mutant collection was resistant to streptomycin, which goes against the overall tendency of an increase in resistance profile of the mutant collection. Despite belonging to the same antimicrobial category, in enterococci strains resistance to gentamicin and streptomycin occurs by different mechanisms, hence the importance of testing both antibiotics (Cetinkaya *et al*., 2000). Resistance to gentamicin is mainly the result of an inactivating enzyme which leads to resistance to other aminoglycosides, like kanamycin and tobramycin (Cetinkaya *et al*., 2000). Regarding streptomycin resistance, it is generally found in enterococci strains that produce streptomycin adenyltransferase (Cetinkaya *et al*., 2000). Moreover, unlike the case of gentamicin, resistance to streptomycin does not influence the susceptibility pattern to other aminoglycosides (Cetinkaya *et al*., 2000). As such, the different enterococcal-resistance mechanisms to these antibiotics might explain the differences observed in the frequency of resistances.

Additionally, enterococci are described to exhibit resistance to high-level aminoglycosides following acquisition of aminoglycoside-modifying-enzymes (AMEs) (Chow, 2000). So far, three major classes of AMEs have been identified: aminoglycoside-acetyltransferase (AAC), aminoglycoside-phosphotransferase (APH) and aminoglycoside nucleotidyltransferase (ANT) (Niu *et al*., 2016). Screening of the associated genes could be performed to determine if the resistances are the result of enzymatic modification. Since resistance to aminoglycosides can be the result of various mechanisms that can simultaneously coexist, other mechanisms can be associated, such as increased efflux (Ramirez & Tolmasky, 2011; Miller *et al*., 2014). In the case of improved efflux, the pumps can recognize different antimicrobial agents (multidrug-efflux pumps) and export more than one aminoglycoside, which could also have contributed to the resistance levels observed for these antibiotics.

In 2012, Magiorakos and collaborators published a study with the objective of harmonizing and standardizing the international nomenclature of the acquired resistance profiles in many relevant pathogens, including *Enterococcus* spp. (Magiorakos *et al*., 2012). According to the definitions purposed, for one enterococci to be considered multidrug resistant (MDR), it has to be resistant to at least one antibiotic in three different antimicrobial categories with different targets, as a consequence of the coexistence several resistance mechanisms in the same microorganism (Magiorakos *et al*., 2012). As such, the initial enterococci and the mutants collections were classified regarding their resistance profiles, results presented in Tables 2 and 3, respectively.

Ten isolates (50.0%) from both collection exhibited a multidrug-resistance profile. Despite both collections having the same number of MDR-isolates, some of the MDR-isolates from the original enterococci collection were no longer so. Specifically, isolates EZ30, EZ39 and EZ43 did not maintain their MDR-classification following the MPC assay, which was not expected. As mentioned before, point mutations in the original enterococci isolates might result in resistances through increased efflux, drug or target modifications. In fact, the pressure deriving from the MPC protocol can have induced changes in the initial resistance and virulence profile. Consequently, the mutants might have different characteristics that are responsible for these results.

These results indicate that the initial enterococci isolates displayed a preliminary high-level resistant profile. These enterococci isolates were retrieved from the oral cavity of dogs with periodontal disease (Tavares, 2014). The mouth being an open environment, densely populated by various bacteria that are in close contact to one another, facilitates the exchange of genetic material, which can be silent until the bacteria are exposed to certain stresses (Roberts & Mullany, 2010; Kolenbrander *et al*., 2010; Huang & Agrawal, 2016). Moreover, dogs are companion animals, that may directly interact with humans, with other dogs and in some cases, even with other animals, such as cats. Due to the dogs' close contact with humans and other domestic animals, they should be regarded as a possible reservoir of virulence genes, promoting de emergence and dissemination of pathogenic bacteria.

Enterococci have been frequently described as good models for antimicrobial studies, because they often act as reservoirs of resistant microorganisms which can infect humans or other animals (Semedo-Lemsaddek *et al*., 2018). Additionally, these bacteria possess several virulence determinants that enhance their pathogenicity, such as biofilm formation (Oliveira *et al.*, 2016). The majority of the oral isolates used in this study produces biofilm, which is a fundamental virulence factor associated with many bacteria responsible for chronic infections (Tavares, 2014; Koo *et al*., 2017). Biofilms are complex microbial structures that confer protection to pathogens and greatly increase their resistance to external compounds, namely antibiotics, as well as protection from the host immune response (Fleming & Rumbaugh, 2017). Also, according to the previous work performed by Tavares (2014), the isolates displayed other virulence factors like proteases (gelatinase, cytolysin) and/or adhesins (enterococcal surface protein, aggregation substance), that promote the establishment and maintenance of the colonization (Oliveira *et al*, 2016).Considering the results obtained in this part of the work, it would be important to confirm the resistances profiles of the enterococci isolates in the two collections by MIC determination. Furthermore, it would also be very relevant to screen for the presence of the genes

responsible for the resistances to vancomycin and imipenem which represent a serious public health threat, contributing to aggravate the treatment of infectious diseases.

Determination of nisin's minimum inhibitory concentration and minimum bactericidal concentration using the collection of mutants

Antimicrobial resistance poses a serious challenge worldwide, compromising current medical care, such as regular surgical procedures (Cock *et al*., 2017). One way to tackle this issue is to reduce the use of antimicrobials as well as improving their administration protocols. To achieve that, it is important to understand at what concentrations are the antimicrobial compounds effective for the microorganism in study. According to the European Committee for Antimicrobial Susceptibility Testing (EUCAST), *in vitro* susceptibility testing is useful for epidemiological studies, in resistance surveillance or prediction of therapeutic outcome (EUCAST, 2003). MIC, the minimum inhibitory concentration, is defined as the minimal concentration needed for a certain antimicrobial compound to prevent bacterial growth, whereas the MBC, the minimum bactericidal concentration, is the lowest concentration of an antimicrobial agent that kills 99.9% of the tested microorganisms (Balouiri *et al*., 2016). Consequently, MIC determination is a standard procedure for susceptibility testing of an antimicrobial agent, reflecting the susceptibility pattern of a certain microorganism (EUCAST, 2003).

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed regarding the mutants derived from the MPC protocol using the broth microdilution method in accordance with the protocol previously established by Pinheiro (2016), to assess their current susceptibility to nisin and compare the results with the ones previously determined for the initial isolates (Pinheiro, 2016).

The initial MIC and MBC values determined by Pinheiro (2016) for the original collection of oral enterococci are illustrated in Figure 14 (Pinheiro, 2016). The lowest and highest MIC values were 0.3 and 1.1 mg/mL, respectively, and the mean MIC value was 0.6± 0.2 mg/mL. The minimum and maximum MBC values were, respectively, 1.5 and 4.0 mg/mL, with the average MBC value being 2.7± 0.7 mg/mL. Considering the results obtained in this study for the mutant collection, also represented in Figure 14, the minimum and maximum MIC values were 0.8 and 5.0 mg/mL, respectively, with the mean MIC value being 2.4± 1.3 mg/mL. As for the MBC values, the lowest and highest were, respectively, 1.5 and 5.0 mg/mL, with the mean MBC value being 3.3± 1.2 mg/mL.

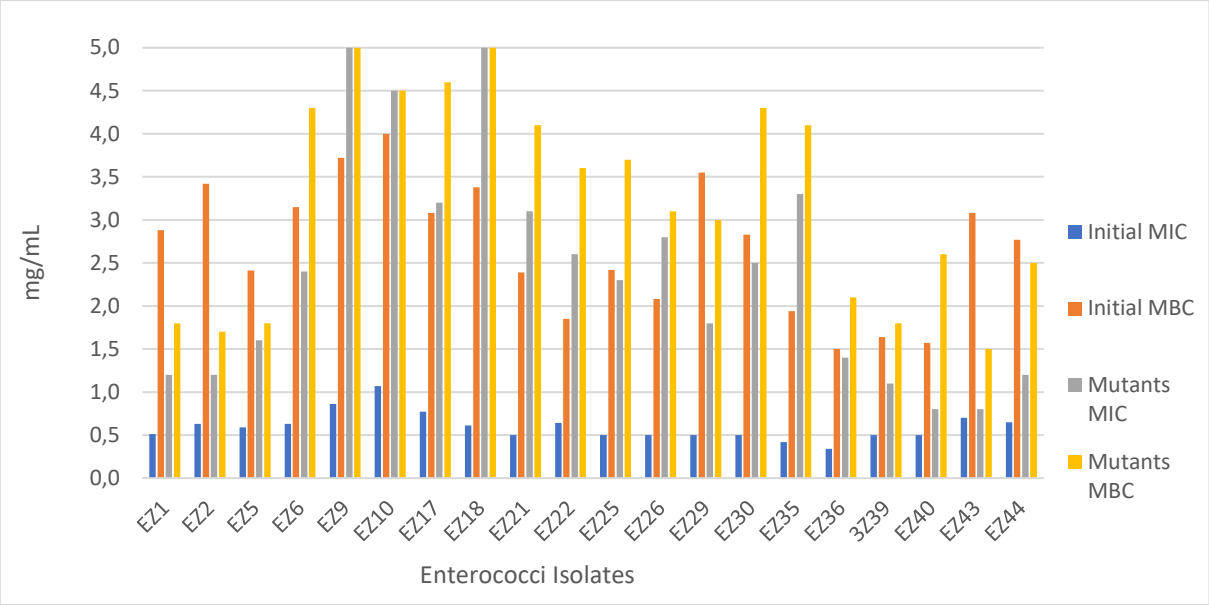


Figure 14 – Comparison between nisin MIC and MBC values (mg/mL) regarding the original collection of oral enterococci determined by Pinheiro (2016), respectively blue and orange, with the MIC and MBC values obtained for the collection of oral-enterococci mutants derived from the MPC protocol, respectively grey and yellow.

As illustrated in Figure 14, it is possible to observe that all of the original enterococci strains had a lower MIC value when compared with those determined in this study for the collection of mutants. Similarly, the majority of the mutants (66,7%) had a higher MBC value, when comparing with those previously established by Pinheiro (2016) for the original isolates. The MIC differences between the mutant and the initial collections ranged from 0.1 to 4.4 mg/mL and the difference average was 1.8± 1.2 mg/mL. Thus, the MIC results obtained for the mutant collection were higher than the MIC values for the collection comprising the enterococci isolates retrieved from the oral cavity of dogs with periodontal disease. In the case of the MBC, it varied from -1.7 and 2.2 mg/mL and the difference average value was 0.6± 1.1 mg/mL, meaning that the MBC results were for the most part higher for the mutants' collection.

To verify the normality of the MIC and MBC values, the Kolmogorov-Smirnov test was performed using IBM SPSS Statistics V25.0, and it was possible to determine that these variables follow a normal distribution because the *p*-values were higher than 0.05. Afterwards, a Student's T-Test for independent variables was performed to determine if there was significant statistical difference (p-value<0.05) between the two variables in this study, MIC and MBC data from the collections. Because the *p*-value was below 0.05, statistical differences were found between the MIC and MBC values of the two collections, further differentiating the mutant collection from the original one. More precisely, when comparing the MIC values of the two collections, the *p*-value was 1.1×10^{-27} and when comparing the MBC values the *p*-value was 0.002. These results indicate that the collection of mutants presented MIC and MBC values significantly different from the ones previously established by Tavares (2014), suggesting that these collections are different.

Table 5- MBC/MIC ratios of the enterococci for the two collections, group1 comprising the original isolates **(G1)** and group 2 with the mutants recovered from the MPC assay **(G2)**, as well as the respective classification of nisin action (Levison & Levison, 2009; Santos *et al*., 2016). The MIC and MBC results of the original collection were previously established by Pinheiro (2016).

	G1		G2	
	MBC/MIC ratio	**Classification**	**MBC/MIC ratio**	**Classification**
EZ1	5.6	Bacteriostatic	1.5	Bactericidal
EZ2	5.4	Bacteriostatic	1.4	Bactericidal
EZ5	4.1	Bacteriostatic	1.1	Bactericidal
EZ6	5.0	Bacteriostatic	1.8	Bactericidal
EZ9	4.3	Bacteriostatic	1.0	Bactericidal
EZ10	3.7	Bactericidal	1.0	Bactericidal
EZ17	4.0	Bactericidal	1.4	Bactericidal
EZ18	5.5	Bacteriostatic	1.0	Bactericidal
EZ21	4.8	Bacteriostatic	1.3	Bactericidal
EZ22	2.9	Bactericidal	1.4	Bactericidal
EZ25	4.8	Bacteriostatic	1.6	Bactericidal
EZ26	4.2	Bacteriostatic	1.1	Bactericidal
EZ29	7.1	Bacteriostatic	1.7	Bactericidal
EZ30	5.7	Bacteriostatic	1.7	Bactericidal
EZ35	4.6	Bacteriostatic	1.2	Bactericidal
EZ36	4.4	Bacteriostatic	1.5	Bactericidal
EZ39	3.3	Bactericidal	1.6	Bactericidal
EZ40	3.1	Bactericidal	3.3	Bactericidal
EZ43	4.4	Bacteriostatic	1.9	Bactericidal
EZ44	4.3	Bacteriostatic	2.1	Bactericidal

A bacteriostatic agent is capable of only inhibiting the growth of bacterial cells, whereas a bactericidal agent kills the microorganism in test (French, 2006). Moreover, bactericidal agents generally have MBC values very close to the MIC, but never 4 times higher than the respective MIC (Levison & Levison, 2009; Santos, 2016). As presented in Table 5, Pinheiro (2016) determined that for the majority of the initial isolates nisin was a bacteriostatic agent (75%). However, in this study the MBC/MIC ratio was always bellow 4, meaning that nisin exerted a bactericidal action in all the mutants (100%).

These results might be explained by the inherent conditions of the MPC protocol. More specifically, as previously described, a high inoculum of the initial isolates was applied onto MH agar plates, containing increasing nisin concentrations and incubated for 72 hours. After the incubation period, the mutants recovered from this protocol presented specific characteristics, such as the higher MIC and MBC values determined in this study. The large increments in the antimicrobial concentration are responsible for the readout transition from bacterial lawn to no growth (Drlica & Zhao, 2007). Consequently, the stress induced by the high nisin concentrations used in the MPC assay may lead to the different characteristics exhibited by the mutant collection, such as the increase of the MIC and MBC values.

Conclusions

Nowadays, antimicrobial resistance is a worldwide concern, compromising not only the medical progresses achieved so far but also the effective control of infectious diseases in both human and veterinary medicine. Periodontal disease is one of the most widespread inflammatory diseases in dogs, which requires the formation of a microbial biofilm in the teeth surface. Bacteria belonging to the *Enterococcus* genus have been found frequently in the canine oral cavity, being related with PD development in dogs. Considering their multidrug resistance (MDR) profile, new safe and efficient antibacterial compounds are needed to control enterococcal PD in dogs. The inhibitory potential of antimicrobial peptides, such as nisin, renders them promising natural alternatives to antibiotics, since resistance and cross-resistance to these antimicrobial agents has rarely been described.

For any antimicrobial protocol aiming clinical implementation, it is essential to determine the correct drug dosages that prevent selection of resistant mutants. Moreover, based on the previous misuse of antibiotics as well as the low-resistance rate observed for antimicrobial peptides, it is crucial to prevent the development of resistance to these compounds. In this context, the mutant selection window (MSW) of nisin was established by determining the mutant prevention concentration (MPC). The MPC values obtained in this work were 15 to 40 times higher than the previously determined MICs, which reinforces the importance of correct antimicrobial doses. It is important to mention that this work is groundbreaking since the MPC value has only been determined to antibiotics so far. As such, in this stage, determination of the MPC value of nisin will allow the establishment of the correct dosages needed to effectively control PD in dogs and, ultimately, prevent resistance development.

Furthermore, the antimicrobial susceptibility testing and determination of MIC and MBC values allowed to do a characterization of the mutants obtained following the MPC assay. In fact, it was possible to observe that not only were the mutants more resistant to the tested antibiotics than the initial isolates but also, that their minimum inhibitory and minimum bactericidal concentrations of nisin regarding this new collection were higher.

Throughout the various assays, it was noticed that some strains consistently exhibited irregular behavior, namely by standing out as exceptions to the overall tendency. More specifically, it was observed that when nisin was diluted in saliva, isolates EZ9, EZ18 and EZ43 were not inhibited at the highest concentration used, 4.0mg/mL, unlike the remaining 17 isolates. When nisin incorporated in guar gum gel was diluted in saliva, isolates EZ18, EZ35 and EZ40 were able to grow at the highest concentration used, 4.0mg/mL, contrary to the rest of the collection of oral enterococci isolates. In the MPC protocol, it was not possible to determine the value of this parameter for 3 isolates: EZ36, EZ40 and EZ43. Regarding the antimicrobial susceptibility testing, in the initial collection, isolates EZ43 and EZ44 were resistant to vancomycin which is of public health concern. As for the mutant collection, not only were mutants EZ9, EZ26 and EZ44 resistant to vancomycin but also, mutants EZ10, EZ40 and EZ44 exhibited resistance to imipenem, another matter of public health importance. For the reasons

pointed above, it would be beneficial to further study these isolates and better disclose their resistance and virulence potential.

A mutant is an organism that presents different characteristics than the wild types due to genetic changes. Even though no genetic assessment was performed, the resistant-isolates recovered following the MPC protocol exhibited differences regarding the previously determined antimicrobial susceptibility patterns as well as MIC and MBC values. Indeed, the pressure resulting from the inherent characteristics of the MPC protocol, might have given rise to important changes regarding the previously established resistance and virulence potential of the initial isolates. Consequently, in the future it would be relevant to perform a putative pathogenicity evaluation by screening for the presence of the same virulence traits as the ones selected by Tavares (2014) and, afterwards, compare the results with the ones obtained for the original enterococci collection.

To conclude, the results from this study reinforce the potential of nisin, incorporated in guar gum gel, to be topically applied to the oral cavity of dogs to control periodontal disease as well as the importance of adequate antimicrobial concentrations in impairing mutant development and dissemination.

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Supplementary Data

Antimicrobial Resistance profile of the initial enterococci isolates

Table 6- Characterization of the virulence profile presented by the initial enterococci isolates, previously determined by Tavares (2014). Legend: - Negative; + Positive; ++ Moderate; +++ Strong; E- *Enterococcus*, Z- swab; Virulence determinants: *ace*—adhesin of collagen from *E. faecalis*, *acm*—adhesin of collagen from *E. faecium*, *agg*-aggregation substance, *cyl*A—cytolysin activator, *ebp*ABC - pili-like from *E. faecalis*, *efaAfs*—cell wall adhesion from *E. faecalis*, *efaAfm*—cell wall adhesion from *E. faecium*, *esp*-cell wall-associated protein, *gel*E-gelatinase, *gls*24- *E. faecalis* stress response regulator.

EZ	*efa(Afm)*	*efa(Afs)*	*acm*	*esp*	*agg*	*cyIA*	*geIE*	*ace*	*gls24*	*ebpA*	*ebpB*	*ebpC*	gelatinase	Hemolisis	Biofilm
1	-	+	-	-	-	-	+	-	+	+	+	+	+	-	++
2	-	+	-	-	-	-	+	-	+	+	+	+	-	-	+++
5	-	+	-	+	+	-	-	+	+	+	+	+	-	+	+++
6	-	+	-	+	+	-	-	+	+	+	+	+	-	+	+++
9	-	+	-	-	-	-	+	-	-	+	+	+	+	-	++
10	-	+	-	-	-	-	+	+	+	+	+	+	-	-	+
17	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+++
18	-	+	-	-	+	-	-	+	+	+	+	+	-	+	+++
21	-	+	-	-	-	+	-	-	+	+	+	+	-	-	+++
22	-	+	-	-	-	+	-	-	+	+	+	+	-	-	+++
25	-	+	-	-	+	+	-	+	+	+	+	+	-	+	+++
26	-	+	-	-	+	+	-	+	+	+	+	+	-	+	++
29	-	+	-	-	-	-	-	-	+	+	+	+	-	-	+++
30	-	+	-	-	-	-	-	-	+	+	+	+	-	-	+++
35	+	-	+	-	-	-	-	-	+	+	+	+	-	-	-
36	+	-	+	-	-	-	-	-	+	+	+	+	-	+	-
39	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+++
40	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
43	-	+	-	-	-	-	+	+	-	+	+	+	+	-	+
44	-	+	-	-	-	-	+	+	-	+	+	+	+	-	++

Nisin MIC results using the collection of mutants

Table 7- MIC values (mg/mL) obtained for the collection of mutants retrieved from the MPC protocol. The fourth round corresponds to the 10% replicates. E- *Enterococcus*, Z- swab

	ROUNDS							
EZ	**1**		**2**		**3**		**4**	
1	2	2,5	0,5	0,5	1	0,5		
2	1,5	1,5	1	1	1	1		
5	2	2	1,5	1,5	1	1,5		
6	2,5	2,5	2,5	2,5	2,5	2		
9	>4	>4	>4	>4	>4	>4		
10	>4	>4	3	4	>4	>4		
17	4	4	4	2,5	2	2	4	3
18	>4	>4	>4	>4	>4	>4		
21	2	1,5	4	3	>4	>4	2	2
22	1	1,5	2,5	3	>4	>4	1,5	1
25	1,5	1,5	2,5	>4	2,5	2,5	1	1,5
26	2	2	1,5	3	4	>4	2,5	2,5
29	1,5	1,5	1,5	1	2,5	2,5		
30	2	2	4	4	2	2,5	1,5	2
35	1,5	1,5	>4	>4	1,5	1,5	>4	>4
36	1	1	0,5	1,5	1	1	2,5	2,5
39	1,5	1	1	1	1	1		
40	1	1	0,5	0,5	1	0,5		
43	1	1	1	1	0,5	0,5		
44	1	0,5	1	1	2	1,5		

Nisin MBC results using the collection of mutants

Table 8- MBC values (mg/mL) obtained for the collection of mutants retrieved from the MPC protocol. The fourth round corresponds to the 10% replicates. E- *Enterococcus*, Z-swab

	ROUNDS							
EZ	**1**		**2**		**3**		**4**	
1	2,5	2,5	1	2,5	1	1,5		
2	2	2	1,5	1,5	1,5	1,5		
5	2	2,5	1,5	1,5	1,5	1,5		
6	2,5	3	>4	>4	>4	>4		
9	>4	>4	>4	>4	>4	>4		
10	>4	>4	3	4	>4	>4		
17	4	>4	4	4	>4	>4	>4	>4
18	>4	>4	>4	>4	>4	>4		
21	>4	2	>4	>4	>4	>4	3	3
22	4	1,5	>4	>4	>4	>4	2	1,5
25	2	4	>4	>4	4	4	2,5	3
26	2	2,5	2	3	>4	>4	2,5	2,5
29	>4	1,5	2,5	2,5	2,5	4		
30	3	3	>4	>4	4	>4	4	>4
35	4	>4	>4	>4	2	2	>4	>4
36	1,5	1,5	0,5	1,5	1	2,5	4	4
39	1,5	>4	1	1	1	1		
40	4	4	2,5	1	1	3		
43	2	2,5	1	1,5	1	1		
44	>4	1	2,5	2,5	2	2		

MIC and MBC differences

Table 9- Differences between MIC and MBC from the two collections used in this work: group 1 comprising the original enterococci isolates (G1) and group 2 with the mutants recovered from the MPC assay (G2). Difference values were obtained by subtracting the results of G1 from the results of G2. The MIC and MBC values for nisin of the original enterococci isolates were obtained by Pinheiro (2016). E- *Enterococcus*, Z-swab.

	G1		G2			
EZ	MIC	MBC	MIC	MBC	MIC_difs	MBC_difs
1	0,5	2,9	1,2	1,8	0,7	-1,1
2	0,6	3,4	1,2	1,7	0,6	-1,7
5	0,6	2,4	1,6	1,8	1,0	-0,6
6	0,6	3,2	2,4	4,3	1,8	1,2
9	0,9	3,7	5	5	4,1	1,3
10	1,1	4,0	4,5	4,5	3,4	0,5
17	0,8	3,1	3,2	4,6	2,4	1,5
18	0,6	3,4	5	5	4,4	1,6
21	0,5	2,4	3,1	4,1	2,6	1,7
22	0,6	1,9	2,6	3,6	2,0	1,8
25	0,5	2,4	2,3	3,7	1,8	1,3
26	0,5	2,1	2,8	3,1	2,3	1,0
29	0,5	3,6	1,8	3	1,3	-0,6
30	0,5	2,8	2,5	4,3	2,0	1,5
35	0,4	1,9	3,3	4,1	2,9	2,2
36	0,3	1,5	1,4	2,1	1,1	0,6
39	0,5	1,6	1,1	1,8	0,6	0,2
40	0,5	1,6	0,8	2,6	0,3	1,0
43	0,7	3,1	0,8	1,5	0,1	-1,6
44	0,7	2,8	1,2	2,5	0,6	-0,3