Infective Endocarditis due to Periodontal Disease in dogs: the potential of nisin as a new preventive approach

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ABSTRACT

Infective endocarditis is a severe and difficult to treat disease in small animals and the dissemination of bacteria from the oral cavity of individuals with periodontal disease is considered a risk factor for its development. *Enterococcus* spp. are frequently isolated from the oral cavity of animals with these affections, thus being necessary to develop new therapeutic protocols.

Minimum inhibitory and eradication concentrations of nisin to planktonic bacteria were determined using the broth microdilution method and the minimum biofilm inhibitory and eradication concentrations were determined using a modified version of the Calgary Biofilm Pin Lid Device. Also, the potential of a toothpaste as an administration vehicle of the peptide was evaluated.

MIC values varied between 0.5 and 10 mg/mL and MBC values varied between 5 and more than 40 mg/mL while MBIC values ranged from 1 to 10 mg/mL and MBEC values ranged from 2 to more than 40 mg/mL. Nisin presented bactericide activity against 65% of the strains and bacteriostatic against the other 35%. The toothpaste has potential to be an effective administration vehicle of nisin.

In the future, nisin can be administered to dogs using toothpaste as a delivery vehicle contributing to prevent and treat periodontal disease as well as avert other systemic diseases, such as infective endocarditis. Once dogs are considered models of PD in humans, these results can be extrapolated to human medicine.

Key words: Periodontal Disease, Nisin, Infective Endocarditis, *Enterococcus* spp.

INTRODUCTION

PERIODONTAL DISEASE

Periodontal disease (PD) refers to a group of inflammatory diseases in the periodontium caused by bacterial plaque, being the most diagnosed and less treated health problem in small animals: with only two years of age, 80% of dogs present some form of this disease.[1–3] It is a progressive disease that begins with gingivitis and, if untreated, can develop to periodontitis, depending on the virulence of the bacteria involved combined with the host response.[2,4]

Gingivitis is the initial phase of PD, being characterized by the inflammation of the gingiva, which may be reversible through thorough dental prophylaxis and home care.[1,2] It begins when bacteria from the oral cavity adhere to teeth and form the dental plaque that is a biofilm of oral anaerobic and aerobic bacteria enveloped in a matrix of saliva, glycoproteins and extracellular polysaccharides produced by the microorganisms involved.[1,2,4] These microorganisms can reach the gap between the gingiva and teeth or alveolar bone and secrete toxins and other metabolic products that invade tissues and cause inflammation in gingival and periodontal tissues, promoting tissue damage, resulting in gingivitis.[1] Besides directly stimulating inflammation, bacterial metabolites also induce an inflammatory response from the animal's defense system, including the release of leucocytes and the production of cytokines.[1,2] These enzymes increase the inflammation of the gingiva and periodontal tissues, so frequently it is the host response that is responsible for damaging the periodontal tissues.[1]

As inflammation increases and the destruction of the periodontal ligament and alveolar bone occurs, gingivitis can evolve to periodontitis, resulting in irreversible histopathological changes such as gingival recession, periodontal pocket formation and eventually tooth loss.[1,2,5] It is during this phase that bacteria can access the circulating blood as the inflammation that allows the immune system to attack the bacterial invaders also allows bacteria to gain access to the host's body causing systemic diseases, such as renal, hepatic and cardiac diseases, including infective endocarditis (IE).[1,2] In fact, oral bacteria have been isolated from the mitral and aortic heart valves of dogs with IE.[6]

The dogs' oral microbiota is similar to those of humans. The differences between dogs' and humans' oral cavities are the higher number of anaerobic bacteria isolated from humans and the prevalence of enterobacteria in dogs, including *Klebsiella*, *Escherichia*, *Citrobacter* and *Enterobacter*. *Enterococcus* is also frequently isolated.[7,8]

INFECTIVE ENDOCARDITIS (IE)

IE is a severe disease caused by bacterial invasion of the endothelium of heart valves or endocardium. Its prevalence in small animals is low, ranging from 0.09% to 6.6%, but it is certainly underestimated because of how difficult it is to diagnose. Nevertheless, is a major disease, due to its severe consequences and high morbidity and mortality rates.[9,10] In dogs, this disease is established when three characteristics are simultaneously

present: predisposed risk, bacteremia (which can be promoted by PD) and bacterial virulence.[9–11] The endothelial surface of the heart and its valves are naturally resistant to microbial invasion, but they become susceptible if damaged. Mechanical or inflammatory lesions can promote bacterial colonization and invasion of the endothelium, triggering IE.[10]

Disease evolution may promote acute congestive heart failure, thromboembolic disease and arrhythmias.[9]

Enterococci are frequently related to valve endocarditis in humans, specifically *Enterococcus faecalis*[12], being frequently present in the oral cavity of dogs. Their role in PD driven IE in dogs remained unclear until recently, when a 2016 study demonstrated this association.[9]

IE treatment requires the systematic administration of broad-spectrum antibiotics for 8 to 12 weeks, having poor prognosis especially when the bacteria involved are multiresistent.[10] Antibiotics most frequently utilized are β-lactams and quinolones, but others like aminoglycosides, glycopeptides and tetracyclines are also used.[9,10]

Antimicrobial peptides

Since antibiotics were introduced into human medicine, they have had an enormous impact on treatment of infectious diseases.[13] However, antibiotic resistance has always been a concern due to the short generation time and genetic plasticity of bacteria that allows these microorganisms to easily adapt to new conditions.[14] Two factors have accelerated the development of resistance: accumulation of mutations over time and the absence of the development of new classes of antibiotics.[15] Thus, there is an urgent need to discover novel classes of antimicrobial compounds.[16]

Antimicrobial peptides (AMP) with bactericidal or bacteriostatic activity are a promising alternative to conventional antibiotics. Antimicrobial small peptides emerged as a promising class of antimicrobial agents that have the potential to become an alternative to conventional antibiotics for treating infectious diseases, impairing the emergence of antimicrobial resistance.[13–15,17]

The main group of antimicrobial peptides corresponds to cationic peptides. They are usually between 12 to 50 aminoacids long and present a net positive charge due to higher quantities of lysine and arginine and about 50% of hydrophobic aminoacids. They possess disulphide bridges that allow them to fold into three-dimensional amphiphilic structures in which the positive and hydrophilic domains are well separated from the hydrophobic domains. This distance is maintained when in contact with cell membranes because of their negative charge, and also because of their hydrophilic head groups and hydrophobic cores.[13,14,18] Virtually all organisms produced AMP that directly kill microorganisms and also recruit and prompt the action of other elements of host immunity.[13,14]

The cytoplasmic membrane is the main site of action of these peptides. The electrostatic interaction with the anionic surface of the bacterial cell membrane, facilitates the approach of the peptide and induces its insertion into the membrane to cause several defects such as formation of pores, phase separation, promotion of non-lamellar lipid structure or disruption of the membrane bilayer.[13,14,18] AMP have broad spectrum activity against bacteria, parasites, fungi and virus with envelop and act faster than conventional antibiotics.[13] Because they kill bacteria quickly by physically disrupting the cell membrane, peptide antibiotics may not face the rapid emergence of resistance.[15]

Nisin

Nisin is an example of a cationic peptide. It acts against many foodborne and spoilage bacteria, having the ability to adsorb to various surfaces being currently approved as a food preservative in more than 50 countries around the world.[14,19,20]

Nisin is a 34-residue cationic and amphiphilic antimicrobial peptide, produced by *Lactococcus lactis* and it has activity against vegetative cells and spores of Gram-positive bacteria, including some multidrug-resistant strains.[17,19,21–23] It is classified as a class I bacteriocin, meaning that nisin is a peptide produced by bacteria able to inhibit or even kill other bacteria. Nisin can also be classified as a type A lantibiotic. Lantibiotics are gene-encoded peptides, synthesized by the ribosome and quorum-sensing controlled, that contain unusual aminoacids such as lanthionine or methyllanthionine and the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid.[19,22–24]

Nisin acts by binding to Lipid II to form pores in the membrane and interfere with cell wall biosynthesis, leading to bacterial death.[25]

Nisin vs conventional antibiotics

Nisin differs from conventional antibiotics in its synthesis pathways, toxicity, resistance mechanisms, activity spectrum and mode of action.[22]

Conventional antibiotics are synthesized as secondary metabolites, while bacteriocins are synthesized by ribosomes during the primary phase of bacterial growth and secreted out of the cell.[19,24,25]

In terms of toxicity, it has been shown that antibiotics impair cell function and mitochondrial physiology in humans and animals and high doses may induce cytotoxic effects and even cell death.[26,27] Some classes of antibiotics have limited use due to their nephrotoxicity and ototoxicity.[28] Antibiotics should be selectively toxic, meaning they should kill pathogens without damaging the host cells; however, antibiotics may present several adverse side effects.[29] Nisin presents low toxicity as it has low hemolytic activity and targets specifically Lipid II that is only present in the bacteria cell wall.[25]

Regarding resistance mechanisms, pathogens become resistant to antibiotics due to acquirement

of genetically transferable determinants that can affect different sites, by modification of a drug target molecular bypass, active efflux and chemical modification of the compound.[30,31] In the case of bacteriocins, resistance to them is less common than to conventional antibiotics due to their speed and double mode of action, as well as their proteinaceous nature that makes them more easily degraded by proteolytic enzymes, thus lessening the chances of target strains developing any resistance machinery.[18,32] Nonetheless, it has been described; some species became resistant by changing their cell wall or membrane phospholipid composition. They can also have enzymes that inactivate nisin or transporters that remove it from the cell.[33]

The main difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to bacterial strains related or similar to the producing species, while antibiotics have a wider activity spectrum and do not show any preferential effect on strains closely related to the producers.[24]

Also, bacteriocins are naturally tolerant to higher thermal stress and are more active at a wider pH range than conventional antibiotics.[32]

Their mode of action is different as well. Antibiotics target specific cellular activities such as synthesis of DNA, protein, or cell wall, while AMP target the lipopolysaccharide layer of cell membrane, which is universal in microorganisms, forming pores in the membrane. They also inhibit cell wall synthesis by docking onto some specific molecules.[19,34,35]

MATERIALS AND METHODS

This study aimed to develop and evaluate a novel experimental approach to be applied to the control of PD in dogs, aiming at the prevention of IE following PD. Enterococci are natural inhabitants of the oral cavity of both human and animals.[36] They are opportunistic pathogens, recognized as the leading cause of nosocomial infections and frequently related to valve endocarditis in humans. It has long been established that PD is associated to IE in humans, but until recently there was no confirmed association in small animals. A 2016 study confirmed this association in dogs and the enterococci role in PD driven IE.[9] IE is clearly an important and potentially life-threatening condition, often underestimated by health professionals and the general public.[1,37] As *E. faecalis* has both intrinsic and acquired resistance, being amongst the major antibiotic-resistant bacterial groups at present, having the ability to quickly acquire and disseminate antibiotic resistance genes,[38] it is important to develop new strategies to prevent enterococci PD and its consequences, including PD-driven IE.

Enterococci isolates from a previous study[9,39] were used as bacterial models to evaluate the efficacy of a prevention protocol based on incorporation of the AMP nisin in a veterinary toothpaste, to be used as a delivery system with the advantage of acting directly at the primary site of bacterial dissemination. Therefore, the inhibitory activity of nisin was evaluated against 46 enterococci clinical isolates from dogs diagnosed with PD and IE, including planktonic and biofilm producing strains, as well as the potential of the toothpaste, C.E.T.® Enzymatic Toothpaste for Dogs and Cats, provided by Virbac, as an effective delivery system for this AMP.

Two human reference strains, *Enterococcus faecalis* OG1-10 (PD) and *Enterococcus faecalis* V583 (bacteremia) were also included in this study as control strains.

NISIN STOCK AND WORKING SOLUTIONS

Nisin stock solutions were prepared from dry powder at a concentration of 40 mg/mL as described by Tong et al.[23] Briefly, 500 mg of nisin from *Lactococcus lactis* (Sigma®) were diluted in 12.5 mL of 0.02 M HCl (Merck®) to obtain a stock solution of 40 mg/mL. This solution was filtered (Frilabo, 0.20 µm, ref. FJ25BSCPS002AL01) and serial dilutions were prepared in distilled sterile water: 35, 30, 25, 20, 15, 10, 5, 2, 1 mg/mL. These solutions were kept at 4ºC.

TOOTHPASTE

The veterinary toothpaste to be used as an oral administration vehicle in this study is from Virbac (C.E.T.® Enzymatic Toothpaste for Dogs and Cats). It contains water, glycerine, sorbitol, hydrogenated starch hydrolysates, silica, sodium triphosphate (anti-tartar agent), carboxypolyethylene; powdered egg and pork liver (aroma), enzymes (subtilisin protease and glucose oxidase), sodium chloride, potassium sorbate, sodium benzoate, sodium citrate, dititanium trioxide, sodium phosphate and calcium chloride.

NISIN SUSCEPTIBILITY SCREENING

Susceptibility tests were conducted to evaluate the inhibitory potential of nisin against the isolates under study. A spot-on-lawn protocol was performed by testing 3 µL of several 2-fold dilutions of nisin ranging from 0.312 mg/mL to 40 mg/mL, prepared from the stock solution, applied in TSA (tryptic soy agar) plates with a lawn of a 0.5 McFarland suspension, corresponding to 10^8 CFU/mL, of the reference strain *E. faecalis* OG1-10 in sterile saline that were evenly spread across the surface with a swab. After a 24h incubation at 37ºC, the presence or absence of bacterial growth around the nisin drop was observed. *Listeria monocytogenes* CECT935 was used as positive control with nisin concentrations ranging from 5 to 40 mg/mL.

MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

Isolates' MIC values for the antimicrobial peptide nisin were determined using the broth microdilution technique as described by the Clinical and Laboratory Standards Institute[40] (CLSI). Briefly, 20µL of working solutions with different concentrations of nisin (0.5, 1, 2, 5, 10, 15, 20, 25, 30, 35 and 40 mg/mL) plus 180µL of a 0.5 McFarland

bacterial suspension diluted 1:100, which corresponds to 10^6 CFU/mL, were distributed in the wells of a 96-well microplate (VWR® Tissue culture plates, ref. 10062-900). Negative (only TSB – tryptic soy broth) and positive (only inoculum) controls were also included. After incubation at 37°C for 24h, the MIC value was defined as the lowest concentration of AMP to inhibit bacterial growth, as detected by direct observation. Experiments were performed in triplicate, in independent assays.

To determine the Minimum Bactericidal Concentration (MBC), 5µL of the suspension of every well with no visible growth were plated into TSA and incubated at 37°C for 24h. The MBC was defined as the lowest concentration of antimicrobial that inhibited bacterial growth after sub-culture on antibiotic free media.

Minimum Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration

A modified version of the Calgary Biofilm Pin Lid Device (CBPD)[41] was used to evaluate the antimicrobial susceptibility of bacteria embedded in a 48 hours biofilm, in order to determine the nisin Minimum Biofilm Inhibitory Concentration (MBIC) and the Minimum Biofilm Eradication Concentration (MBEC).

MBIC and MBEC determinations were performed as described by Tremblay et al. (2014)[42] with some modifications. Briefly, 200µL of a 0.5 McFarland standard bacterial suspension diluted 1:100 in TSB, which corresponds to 10^6 CFU/mL, were deposited in a 96-well microplate (Nunc™, Thermo Scientific), covered with a peg lid (Nunc™ Immuno TSP Lids, Thermo Scientific™) and statically incubated for 48h at 37°C. After incubation, the pegs were washed three times in sterile distilled water and transferred to a 96-well plate containing 180µl of TSB and 20µL of the desired nisin concentration. The plate was incubated for 24h at 37°C. The MBIC was determined at this step being defined as the lowest concentration of AMP to inhibit bacterial growth, as detected by direct observation of the broth media in the wells. The pegs were then washed three times in sterile distilled water and transferred to a 96-well plate containing 200µL of fresh TSB. The plates were sealed and incubated in an ultrasonic bath (Gramt, Ultrasonic Bath, MXB14) for 15 minutes at high frequency (50-60 Hz). Afterwards, the peg lid was substituted by a conventional one and the plate was incubated at 37°C for 24h. After incubation, OD_{600} was measured using a microtiter plate reader (BMG Labtech, FLUOstar OPTIMA). The MBEC, defined as minimum biofilm eradication concentration, was the lowest concentration of the antimicrobial agent nisin to promote a bacterial suspension with an OD_{600}<0.100.

Potential of a toothpaste to be used as an oral administration vehicle to nisin

The potential of a veterinary toothpaste (C.E.T.® Enzymatic Toothpaste for Dogs and Cats, Virbac) as an oral administration vehicle for nisin to dogs was evaluated *in vitro* using a well diffusion assay.

For each reference strain, *E. faecalis* OG1-10 and *E. faecalis* V583, two dilutions (10^{-1} and 10^{-2} CFU/mL), were prepared from a bacterial suspension with turbidity equal to 0.5 McFarland (10^8 CFU/mL) and evenly spread in TSA plates. Wells were prepared using the largest end of a 200µL tip (Ø = 0.6 cm) and filled with approximately 50µL of toothpaste plus 20µL of nisin in different concentrations (5, 10, 20, 30 and 40 mg/mL). The plates were incubated for 24h at 37°C, and inhibition halos were observed and measured.

Inhibitory potential of nisin incorporated in toothpaste in tooth brushing period

To evaluate the inhibitory efficacy of nisin incorporated in toothpaste during the brushing period, first a 0.5 McFarland bacterial suspension, corresponding to 10^8 CFU/mL, was prepared and serially diluted (10^{-3}, 10^{-4} and 10^{-5}). Then, 500µL of each microbial suspension were mixed with 500µL of toothpaste with two different concentrations of nisin incorporated (0, 25 or 40 mg/mL). All preparations were agitated for 2 to 3 minutes and 100µL were plated in TSA and incubated at 37°C for 24h. After incubation colonies were counted and the percentual decrease of bacterial growth was determined by comparison with the positive control, which consisted of 100µL of each dilution plated in TSA and incubated at 37°C for 24h.

Results and discussion

Nisin susceptibility screening

First it was necessary to determine if nisin had inhibitory activity against enterococci and select the proper nisin concentrations to be applied in the following determinations (MIC, MBC, MBIC, MBEC).

E. faecalis OG1-10 was used to evaluate susceptibility of enterococci to nisin, while *Listeria monocytogenes* CECT935 was used as control for positive inhibition. For both species, the best bacterial concentration that allowed to obtain an evenly spread growth across the surface of the agar medium was to use a swab of 0.5 McFarland suspension as lawn, which corresponds to approximately 10^8 CFU/mL. *L. monocytogenes* CECT935 was susceptible to all nisin concentrations used, which ranged from 5 mg/mL to 40 mg/mL. *E. faecalis* OG1-10 was inhibited by the 0.625 mg/mL to 40 mg/mL nisin solutions, but not by the 0.312 mg/mL solution. Initially, *E. faecalis* OG1-10 was only tested with 5 to 40 mg/mL but once it was susceptible to all these concentrations, lower nisin concentrations were tested in order to find which nisin concentration did not present a growth inhibitory effect, which turned out to be somewhere between 0.312 mg/mL and 0.625 mg/mL. Thus, the lowest nisin concentration selected for MIC testing was 0.2 mg/mL.

The susceptibility of these isolates was confirmed, as it was possible to observe inhibitory zones as well as an increase in their diameter as the

nisin concentration increased, which was expected since nisin is recognized by its activity against Gram-positive bacteria. [23]

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Determination of the MIC, based on antimicrobial activity against planktonic cells, is the standard assay for susceptibility testing.[43] Susceptibility testing is indicated for any organism responsible for an infectious process that requires antimicrobial chemotherapy, if it cannot be reliably predicted from the organism's identity. Identification can also be used for the in vitro investigation of peptides as potential antimicrobial agents.[40,44] Recurring to a broth microdilution technique, a collection of forty-six PD and IE *Enterococcus* spp. isolates obtained from dogs diagnosed with PD and IE was analyzed and the MIC and MBC values determined, in order to measure quantitatively the *in vitro* activity of the antimicrobial agent nisin against these isolates.[40] The main advantages of this protocol are the reproducibility and low cost of reagents.[44]

At first nisin concentrations ranging from 0.2 to 20 mg/mL were tested, but results showed that no strain was susceptible to a 0.2 mg/mL concentration and that some isolates were still resistant to 20 mg/mL; so concentration values tested were adjusted to range from 0.5 to 40 mg/mL.

The MIC and MBC values for nisin against the tested strains ranged respectively from 0.5 to 10 mg/mL and from 5 to >40 mg/ml. From the 46 strains evaluated, 80% (37/46) showed MIC values of 5 mg/mL, 9% (4/46) of 10 mg/mL, 7% (3/46) of 2 mg/mL and 2% (1/46) of 1 mg/mL and 0.5 mg/mL. As for MBC, 11% (5/46) had MBC values superior to the highest value tested (40 mg/mL), 7% (3/46) had MBC values of 40 mg/mL, 13% (6/46) of 35 mg/mL and 10 mg/mL, 37% (17/46) of 20 mg/mL, 9% (4/46) of 15 mg/mL and 11% (5/46) of 5 mg/mL (figure 1).

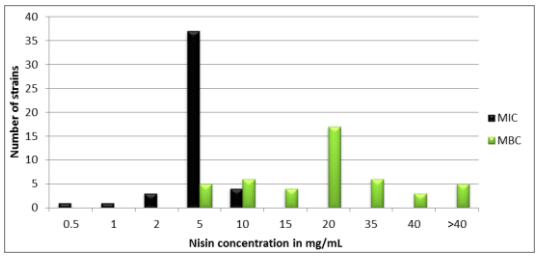

Figure 1 - Distribution of MIC and MBC values

For bactericidal drugs, the MBC value is usually similar to or not more than fourfold higher than the MIC.[45] Considering this definition, it can be concluded that nisin has bactericidal activity against 65% (30/46) of the tested isolates and bacteriostatic activity against the remaining 35% (16/46).

Strain EZC4c showed the lower resistance to nisin, with MIC and MBC values of 0.5 mg/mL and 5 mg/mL, respectively, while *E. faecalis* EZB4a and EZB4c showed the lower susceptibility to nisin with MIC and MBC values of 10 mg/mL and >40 mg/mL, respectively. These discrepancies may be explained by the virulence factors present in the difference strains: EZC4c presents none of the virulence factors screened for in a previous study[39], while EZB4c presents seven (*gelE*, *ace*, *gls24*, *efaAfs*, *ebpA, ebpB*, *ebpC*) and EZB4a has five (*gelE*, *efaAfs*, *ebpA*, *ebpB*, *ebpC*) out of twelve. Curiously, these three strains were isolated from the same dog that had gingivitis.

MIC of nisin against several different genus, including enterococci, have been reported in other studies.[22,23,38,46] MIC and MBC values for enterococci were different between these studies and lower than the values obtained during this study. However, these values cannot be directly compared because the tested conditions were very different, namely inoculum and nisin preparations, as well as different ratios of nisin/inoculum (higher volumes of nisin to less volume of inoculum).

Minimum Biofilm Inhibitory Concentration and Minimum Biofilm Eradication Concentration

A factor that also contributes to bacterial virulence is biofilm production. Cells that grow in biofilms are physiologically different from planktonic cells of the same bacteria and are usually less susceptible to antibiotics.[47] To test biofilms' susceptibility a modified version of the Calgary Biofilm Pin Lid Device was used. This assay is a high throughput screening assay used to determine the efficacy of antimicrobials against biofilms of a variety of microorganisms and is not prone to leakage nor contamination since it is manipulated in a laminar flow cabinet. Several protocols were tested to determine MBIC and MBEC values and a modified version of the protocol described by Tremblay et al. (2014)[42] was chosen due to its reproducibility of results and lower costs.

From the 46 clinical isolates, 43 are biofilm producers, including 44% (19/43) of strong, 35% (15/43) of moderate and 21% (9/43) of weak producers.[39] It seems that there is no evident relation between the strength of the biofilm and the resistance to nisin, as it was observed that neither strong biofilm producers correspond to the higher MBIC and MBEC values nor the weak biofilm producers correspond to the lower values.

The MBIC and MBEC values ranged from 1 to 10 mg/mL and 2 to >40 mg/mL, respectively. From the 43 strains evaluated, 5% (2/43) represented MBIC values of 10 mg/mL and 1 mg/mL, 81% (35/43) of 5 mg/mL and 9% (4/43) of 2 mg/mL. Regarding MBEC values, 5% (2/43) showed MBEC values superior to the highest value tested, 5% (2/43) have MBEC values of 30 mg/mL and 2 mg/mL, 7% (3/43)of 20 mg/mL, 9% (4/43) of 15 mg/mL, 30% (13/43) of 10 mg/mL and 40% (17/43) of 5 mg/mL (figure 2).

MBIC and MBEC values were not determined for three strains (EZB28a, EZB28d and EZB29c) because they are not able to produce biofilm.[39]

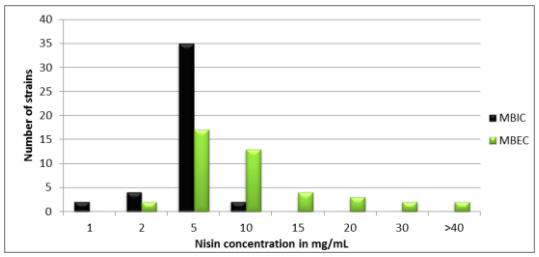

Figure 2 - Distribution of MBIC and MBEC values

Once again, EZC4c is the most susceptible strain along with EZC4a among the 43 biofilm producers, which may be explained by their lack of virulence factors. The most resistant strains are *E. faecium* EZC6a and *E. faecalis* EZB15d that have two (*acm* and *efaAfm*) and seven (*agg*, *ace*, *gls24*, *efaAfs*, *ebpA*, *ebpB*, *ebpC*) virulence factors present, respectively. Regarding MBEC values, the most susceptible strains are *E. faecium* EZC6d and *E. faecium* EZC15a, both with two out of twelve virulence factors (*acm* and *efaAfm*), while the most resistant are *E. faecalis* EZC21a and *E. faecalis* EZC28c with *gls24* and *efaAfs* and *gls24*, *efaAfs*, *ebpA*, *ebpB* and *ebpC* genes, respectively. All these virulence factors are mainly related to bacterial adherence and tissue degradation. The aggregation substance (Agg) on the surface of *E. faecalis*, has been shown *in vivo* to form large aggregates, contributing to pathogenesis, as well as Ace that is a collagen-binding protein that may also play a role in the pathogenesis of endocarditis; GelE role is to provide nutrients to the bacteria by degrading host tissue; and the formation of pili (EbpABC) by enterococci contributes to pathogenesis as it is necessary for biofilm formation, that is essential in causing endocarditis.[48] Thus, if it has higher capacity to form biofilms, probably it also has higher MBEC.

In 38 out of the 43 biofilm producer strains, MBEC values are equal or inferior to MBC values. These results were not expected as it is well-known that biofilms have an increased tolerance to antibiotics relative to the planktonic form; however, MBEC values equal or inferior to MBC values have already been reported in other studies.[49–51] These values may be explained by nisin's different mode of action and the differences between biofilms and planktonic cells. In a biofilm, diffusion of nutrients and oxygen is not homogeneous, resulting in chemical gradients that restrict bacterial growth, thus metabolic activity within the biofilm is heterogeneous and decreases with depth.[49] In order for some antimicrobials to inhibit bacteria, cells have to be metabolically active; however, nisin does not have this requirement. Nisin acts at the cell membrane level, forming pores that allow the efflux of small cytoplasmic compounds leading to cell death. Also, *E. faecalis* forms a biofilm with a substantial amount of eDNA but a low level of extracellular polysaccharides, leading to low resistance to penetration by antimicrobial agents, allowing them to easily enter these biofilms and access bacteria within them.[38] These characteristics of enterococci biofilms make them good targets for the action of nisin, which may be applied for the controlling of the dental plaque.

Also, antibiotic resistance is not a concern regarding the use of nisin; unlike antibiotics, nisin does not induce drug resistance or cross resistance.[23] Nisin resistant mutants may randomly appear, but they do not show any cross-resistance to therapeutic antibiotics probably due to the difference between the modes of action of nisin and conventional antibiotics. Also, in spite of the prolonged use of nisin by the food industry there are no reports of increasing resistance. Nisin resistance is not transferable between microorganisms and spontaneous nisin-resistant bacterial strains are not resistant to other antibiotics, being that some of those strains are even more susceptible to conventional antibiotics.[19]

Potential of a toothpaste to be used as an oral administration vehicle to nisin

To prevent and control PD, the plaque and calculus removal from the tooth and gingiva is crucial and it can be accomplished with tooth brushing with dentifrices, special diet and professional periodontal therapeutics, aiming at controlling the bacterial plaque.[2,52] Periodontal therapy often involves invasive methods which allow bacteria to enter the blood stream, thus increasing the chance of systemic diseases.[1,52] Therefore, it is imperative to develop new methods to treat PD and to prevent systemic diseases, such as IE. With this in mind, this study evaluated the potential of a veterinary toothpaste (C.E.T.® Enzymatic Toothpaste for Dogs and Cats, Virbac) as an oral administration vehicle of nisin as it has the advantage of acting directly at the infection site.

First, it was necessary to evaluate if nisin is capable of keeping its antimicrobial activity when dissolved in toothpaste, so an agar well diffusion method was used to test the reference strains, *E. faecalis* OG1-10 and *E. faecalis* V583 (figure 3). This method allows the diffusion of the antimicrobial agent tested through the agar medium resulting in bacterial inhibition. As expected, it was observed that the inhibitory zone diameters promoted by the toothpaste alone or mixed with nisin were different and increased along with nisin's concentration. Therefore, nisin kept its activity against *Enterococcus* spp. when incorporated in toothpaste and enhanced the toothpaste activity. It was also observed *that E. faecalis* OG1-10, PD's reference strain, was more susceptible to nisin than *E. faecalis* V583, bacteremia's reference strain, which contributes to confirming the potential of this supplemented veterinary toothpaste to treat and prevent enterococcal PD in dogs.

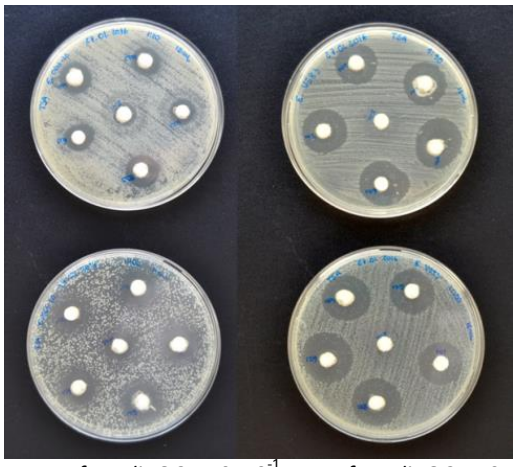

Figure 3 - Agar well diffusion assay: A - *E. faecalis* OG1-10, 10^{-1}; B - *E. faecalis* OG1-10, 10^{-2}; C - *E. faecalis* V583, 10^{-1}; D - *E. faecalis* V583, 10^{-2}; [nisin] = 0, 5, 10, 20, 30, 40 mg/mL

INHIBITORY POTENTIAL OF NISIN INCORPORATED IN TOOTHPASTE IN TOOTH BRUSHING PERIOD

To confirm its potential for *in vivo* application, it was also important to understand if nisin was able to kill bacteria in short periods of time, like the tooth brushing period. Ten random strains were tested, including 4 strains isolated from the oral cavity (EZB) and 6 isolated from the heart (EZC) of infected dogs.

Toothpaste alone was able to inhibit more than 50% of the bacterial growth on 7 out of 10 strains tested, but if supplemented with nisin the inhibitory effect was enhanced. In general, 25 mg/mL of nisin allowed the decrease of bacterial concentration of most of the isolates in more than 90% (table 1).

Table 1 - Percentual bacterial decrease after 2 to 3 minutes of contact with toothpaste (C+ to C) and with toothpaste incorporating two different concentrations of nisin (C+ to 25 mg/mL and C+ to 40 mg/mL)

Strain	C+ to C	C+ to 25 mg/mL	C+ to 40 mg/mL
OG1-10	64.7%	99.8%	100%
V583	59.7%	92.8%	88.8%
EZB3b	58.3%	75.5%	90.1%
EZC3d	65.5%	90.7%	91.1%
EZB4a	72.1%	97.5%	96.7%
EZC4c	75.8%	100%	99.9%
EZC15c	8.3%	94.2%	96.9%
EZB25a	62.2%	99.8%	99.7%
EZC26ea	34.5%	99.9%	99.6%
EZB28a	67.9%	99%	99.2%
EZC28c	48.8%	55.1%	50.2%
EZC32da	60.4%	69.2%	66%

The difference between supplementing the toothpaste with 25 mg/mL or 40 mg/mL appears to be minor; therefore it does not justify increasing nisin's concentration in the toothpaste as it seems that it would not affect the bacterial decrease significantly, but further tests and statistical analysis are necessary to confirm this.

These results once again allowed confirming the potential of the toothpaste as an oral delivery vehicle for nisin to the oral cavity of dogs.

The use of nisin as an effective compound to decrease or even eliminate oral bacteria that cause PD depends on its antimicrobial activity when dissolved in saliva. PD is majorly caused by Gram-positive microorganisms that form the dental plaque. The acidic microenvironment in these microbial communities enhances the activity of nisin since this compound has higher antibacterial activity and stability at low pH,[23] hence nisin is expected to have good activity *in vivo*, being able to contribute for the prevention of PD. Saliva has enzymes that could inhibit nisin's activity, but a study from Tong et al. (2010)[23] already showed that nisin keeps its activity when dissolved in saliva. Besides keeping its activity when dissolved in saliva, nisin has an Acceptable Daily Intake of 0.13 mg pure nisin/kg bw for a product with a potency of 40 000 IU/mg and is also quickly inactivated by digestive enzymes after entering the gastrointestinal tract, thus being safe to use.[19,23] Nisin also has low hemolytic activity and targets specifically Lipid II that is only present in bacteria cell wall, thus it is unlikely to be toxic to mammalian cells.[25] Finally, nisin has already been described as a proper antimicrobial to prevent and treat dental caries.[14,17]

The incorporation of nisin in the toothpaste to be used in the prevention of PD-driven IE also depends on its cost. Once 25g of commercial nisin costs almost 500€ and 2 mg/mL are enough to inhibit bacterial growth (data not shown), the addition of nisin, in this concentration, to a tube of toothpaste (approximately 100 mL) would increase the price of the toothpaste in only 4€, being economically viable.

CONCLUSION

A great deal of effort is being carried out to overcome the problems associated with their use as therapeutics, such as development of efficient delivery systems, poor biodistribution and fast decomposition, thus it is expected that antimicrobial peptides will become the drug of choice for emerging bacterial infections in the future.[47,53]

Nisin has the potential to be administered through toothpaste contributing to prevent and treat enterococcal periodontal disease in dogs, as well as prevent other systemic diseases, such as infective endocarditis. Further studies are necessary to determine if toothpaste can really be an efficient AMP delivery system, such as stability tests to confirm maintenance of AMP antimicrobial activity in toothpaste formulation as well as *in vitro* and *in vivo* efficacy/toxicity bioassays and oral environment tolerance tests.

Since dogs are considered models of PD in humans, these results can be extrapolated to human medicine. This study can play a key role in the establishment of therapeutic protocols, since the possibility of using nisin as a therapeutic agent in the future can help to relieve the use of antibiotics, eventually contributing for the decrease in bacterial antibiotic resistance that currently impairs the treatment of numerous bacterial infectious diseases.

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