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

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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 WHO. 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 not been described. The ultimate purpose of this work is to continue to validate the use of nisin, incorporated in guar gum gel, in 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 to 24 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 importance of correct antimicrobial doses in preventing development of resistant-mutants during therapeutic regimens.

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

## Introduction

Periodontal disease is one the most prevalent and undertreated inflammatory diseases in dogs (Niemiec, 2008; Albuquerque *et al.*, 2012; 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). Moreover, in 2017 the World Health Organization (WHO) classified these bacteria as high priority pathogens for drug development (WHO, 2017). Hence, preventing the emergence of antimicrobial resistance should be the main goal regarding any antimicrobial protocol under investigation for clinical uses. A promising strategy concerns antimicrobial

peptides (AMPs), like nisin, since resistance and cross-resistance have not been described (Batoni *et al*., 2011; Kang *et al*., 2014). The ultimate purpose of this work is to continue to validate the use of nisin, incorporated in guar gum gel, in the prevention of canine periodontal disease. 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). 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 the mutant prevention concentration (MPC) (Drlica, 2003). 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). Afterwards, an antimicrobial susceptibility profiling was 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 was used to test 12 different antibiotics and results will be compared with the CLSI standard breakpoints. Lastly, MIC and MBC values of the collection of mutants were determined using the broth microdilution method (Santos *et al*., 2016).

## Materials and methods

### <u>Assessing the influence of dog's saliva in nisin antimicrobial activity</u>

#### 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, plus a human reference strain (Tavares, 2014). During this study, all isolates were kept at -20°C in a solution of buffered peptone water with 20% glycerol. When needed they were inoculated onto unspecific enrichment growth medium, namely Brain Heart Infusion (BHI) agar medium (Brain heart infusion broth, VWR Chemicals; Agar, VWR Chemicals) followed by a 24-hour incubation period at 37°C.

#### Preparation of nisin standard solution and nisin incorporated in guar gum gel

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 Tong and collaborators (2010), to obtain a solution of 40mg/mL, sterilized by filtration (Frilabo, 0.22µm). 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. 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. 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.

**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) and stored at -20°C. To optimize salivary enzymatic activity, before each assay samples were placed at 37°C for 1 hour. 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) which were then 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 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, 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.

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

**Bacterial strains**

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

**Preparation of nisin standard solutions**

Stock solutions of nisin were also prepared as previously described order to obtain a solution of 40 mg/mL.

**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. 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) plates containing different concentrations of nisin. These MH plates were supplemented with two-fold concentration increments of nisin ranging from 0,25 to 48x MIC value (0.5 mg/ml). 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 concentration was 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).

**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 and the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017) the susceptibility profile regarding the following 12 different antibiotics: Ampicillin-10μg, Amoxicillin/clavulanic acid-30μg, Vancomycin-30μg, Imipenem-10μg, Cefotaxime-30μg, Ciprofloxacin-5μg, Enrofloxacin-5μg, Tetracycline-30μg, Doxycycline-30μg, Gentamicin-10/120μg and Streptomycin-300μg.

**Determination of the mutants' minimum inhibitory concentration and minimum bactericidal concentration 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. In accordance with the protocol previously established by Pinheiro (2016). the wells of a 96-well microplate (VWR Tissue culture plates) were filled with 20 μL of nisin at different concentrations, apart from the columns designated for the positive and negative controls. Subsequently, 0.5 McFarland bacterial suspensions were prepared for each mutant, which were then diluted (1:100) in Tryptic Soy Broth (TSB, VWR Chemicals). Afterwards, 180 μL of the previously prepared bacterial suspensions were placed in each well, except for the negative control column, which was filled with 180 μL of TSB. 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 to determine MIC value, defined as the lowest nisin concentration capable of preventing bacterial multiplication in vitro (Jorgensen & Ferraro, 2009). After the 24-hour incubation period, 5 μL of the bacterial suspension from each well where no visible growth was observed, were plated onto TSA (Tryptic Soy Agar, VWR Chemicals) followed by incubation at 37 °C for 24 hours, to determine

the minimum bactericidal concentration (MBC). MBC is 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 the reproducibility.

## Results and Discussion

### Canine saliva influence in the antimicrobial activity of nisin

The first part of this work consisted in evaluating if dog's saliva exerted any influence in the antimicrobial activity of nisin. When nisin and nisin incorporated in guar gum gel were diluted in saliva at a concentration of 4.0mg/mL, 85% (n=17/20) of the isolates were inhibited. Saliva alone, used as a control, exerted no effect on bacterial multiplication and thus, these results indicate that saliva by itself does not affect enterococci growth. Nevertheless, the addition of saliva to the solutions of nisin and of nisin incorporated in guar gum gel promoted the increase of the required concentration for nisin's inhibitory activity. Nisin antimicrobial activity is 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). The canine oral cavity has a more basic pH than the human one, which might explain why nisin's antimicrobial is delayed. (Iacopetti *et al.*, 2017). In conclusion, results showed that 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., 2013; 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, the mutant selection window (MSW) of nisin was established by determining the MPC value, which ranged from 16.0 to 24.0 mg/mL for 85% (n= 17) of the isolates (Figure 1). For strains EZ36, EZ40 and EZ43 the MPC was higher than 24.0 mg/mL.

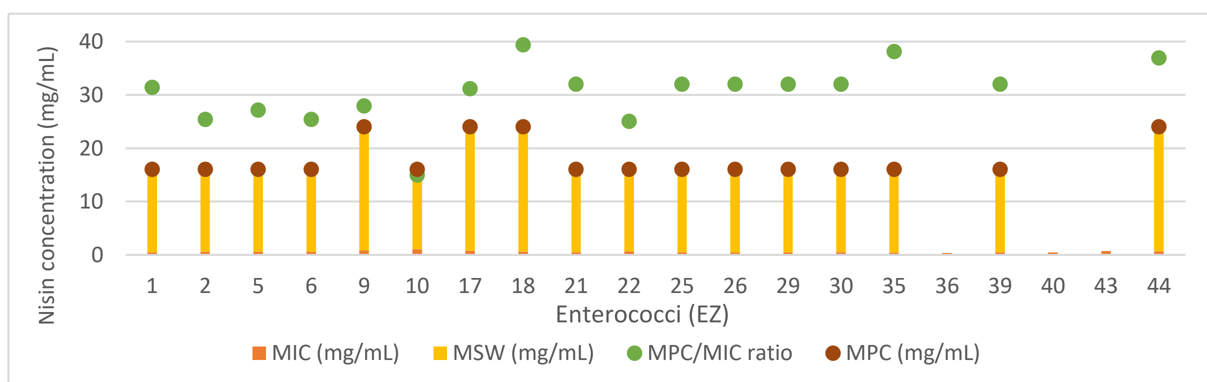


**Figure 1-** Nisin Mutant Selection Window for the collection of oral-enterococci isolates obtained from dogs with periodontal disease. MIC values were previously determined (Pinheiro, 2016).

So far, MPC protocols have been performed with antibiotics and, from those, only one was tested against *Enterococcus* spp. Consequently, it is not possible to directly compare the results obtained in this work. Nevertheless, because daptomycin and vancomycin are antibiotics with a mode of action similar to nisin, similar results were expected. Daptomycin (DAP) acts by irreversibly altering the bacterial cell membrane, resulting in pore formation and, subsequently, membrane depolarization (Sinel *et al.*,2016). Vancomycin (VA) is a tricyclic, bactericidal glycopeptide that inhibits cell-wall biosynthesis (Gupta et al., 2011; Rubinstein & Keynan, 2014). 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 results described in the literature for daptomycin and vancomycin, the results obtained in this study are close to the MSW values described in the literature. The MSW can be viewed as a dangerous range of antimicrobial concentrations, which promote the development of resistant mutants (Blondeau, 2009). Hence, the results obtained with this protocol denote the importance of determining MPC values to establish precise and effective therapeutic regimens and ultimately limit the further emergence and spread of resistances.

## Antimicrobial susceptibility testing

The emergence and dissemination of resistances has mainly been attributed to the overuse and misuse of antimicrobials (Richardson, 2017). The antimicrobial susceptibility pattern of the original enterococci isolates obtained from dogs with PD was compared against the one of the mutants derived from the MPC protocol to understand if the stress promoted by the high nisin concentrations altered the susceptibility profiles of the initial isolates.

**Table 1-** Resistance profile of the two collections of enterococci isolates: Group 1 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 | 20,0 | 75,0 |
| **G2 (n=20)** | 4 | 1 | 3 | 3 | 20 | 11 | 18 | 19 | 17 | 20 | 6 | 14 |
| **%** | 19,0 | 5,0 | 15,0 | 15,0 | 100,0 | 55,0 | 90,0 | 95,0 | 85,0 | 100,0 | 30,0 | 70,0 |

In 2012, Magiorakos and collaborators published an article with the objective of harmonizing and standardizing the international nomenclature of the acquired resistance profiles in many serious 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 (Magiorakos *et al.*, 2012). As such, the initial enterococci and the mutant collections were classified regarding their resistance profiles and results are presented below, in Table 2:

**Table 2-** Classification of the enterococci collections: Group 1 formed by the initial isolates retrieved from the oral cavity of dogs with PD (**G1**) and Group 2 with the mutants-obtained following the MPC protocol (**G2**), according to the criteria established by Magiorakos *et al.*, 2012.

| EZ | 1 | 2 | 5 | 6 | 9 | 10 | 17 | 18 | 21 | 22 | 25 | 26 | 29 | 30 | 35 | 36 | 39 | 40 | 43 | 44 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **G1** | - | - | MDR | MDR | - | - | MDR | - | - | - | - | - | - | MDR | MDR | MDR | MDR | MDR | MDR | MDR |
| **G2** | - | - | MDR | MDR | - | MDR | MDR | MDR | - | - | MDR | - | - | - | MDR | MDR | - | MDR | - | MDR |

Enterococci are intrinsically resistant to cefotaxime (CTX) and low-concentrations of aminoglycosides such as gentamicin (CN, 10 µg), thus the high resistance levels (95.2-100%) to these antibiotics were expected (Gilmore *et al.*, 2013). Furthermore, both enterococci collections exhibited higher resistance to: tetracycline (95.0%), doxycycline (85.0%), enrofloxacin (80.0-90.0%) and streptomycin (70.0-75.0%). Low resistance levels in both collections were observed for ampicillin (15.0%), amoxicillin (0-5.0%) and gentamicin-120µg (20.0-30.0%). Despite the reduced number of isolates resistant to vancomycin and imipenem, these resistances are of great concern since the antibiotics are used as last-line therapeutic agents. According to the results presented in Tables 1 and 2, the original enterococci (G1) displayed a preliminary high-level resistant profile. These isolates, in group 1, were retrieved from the oral cavity, which is an open environment, highly colonized by a variety of bacteria in close contact (Roberts & Mullany, 2010; Kolenbrander *et al.*, 2010). The close proximity between microorganisms facilitates the exchange of genetic material, that can be silent until exposure to certain stresses (Roberts & Mullany, 2010; Kolenbrander *et al.*, 2010; Huang & Agrawal, 2016). Indeed, enterococci possess an easiness to acquire and transmit resistance determinants (Oliveira *et al.*, 2016). Furthermore, considering the results obtained in this part of the work, it would 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.

**<u>Mutants' minimum inhibitory concentration and minimum bactericidal concentration for nisin</u>**

MIC determination is a standard procedure for susceptibility testing of an antimicrobial agent, reflecting the susceptibility profile of a certain microorganism (EUCAST, 2003). According to the results presented in Figure 2 The collection of mutants derived from the MPC protocol

presented higher MIC (100%) and MBC (66.7%) values than the ones determined previously for the initial enterococci collection (Pinheiro, 2016). Moreover, statistical differences (*p*-value<0.05) were found between the two collections, further differentiating the mutants from the initial enterococci isolates. 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). In this study the MBC/MIC ratio was always bellow 4, meaning that nisin exerted a bactericidal action in all the mutants, whereas for the majority of the initial isolates nisin was a bacteriostatic agent, as previously established by Pinheiro (2016).

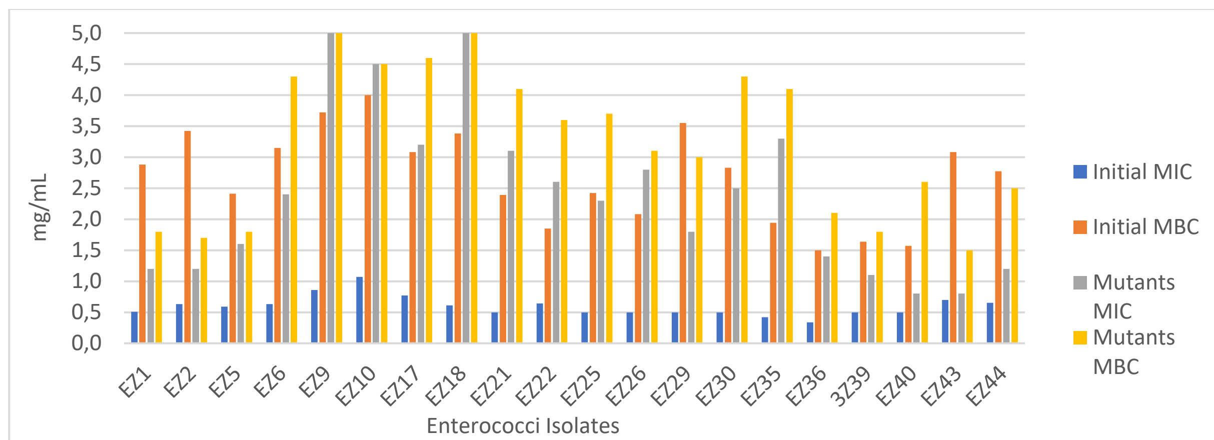


**Figure 2 –** Comparison between MIC and MBC values of nisin (mg/mL) for the original collection of oral enterococci determined by Pinheiro (2016), respectively blue and orange, with the MIC and MBC values obtained for the collection of oral-enterococci mutants derived from the MPC protocol, respectively grey and yellow.

## Conclusions

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 determination of the MPC value has only been applied to antibiotics so far. In fact, it was possible to observe that not only the mutants were more resistant to the tested antibiotics than the initial isolates but also, the minimum inhibitory and minimum bactericidal concentrations were higher. As such, at 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. The results here presented 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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