Unveiling the role of nisin on resistance development by Diabetic Foot staphylococci: mutant selection window and horizontal gene transfer

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Abstract

The most prevalent microorganism in diabetic foot infections (DFI) is *Staphylococcus aureus*, an important pathogen due to its frequent antibiotic multi-resistant profile. As such, it is mandatory to develop alternative compounds for DFI treatment. The antimicrobial peptide nisin is considered a promising alternative because it has been showed to be effective against *S. aureus* DFI isolates and due to its use in food industry for 90 years. However, correct drug therapeutic doses must be established before instituting a new DFI therapeutic protocol based on nisin, to avoid the selection and amplification of resistant mutants.

The mutant selection window (MSW) of nisin was determined for 24 DFI *S. aureus* isolates. MSW ranged from 11.25-360 μg/mL for two isolates, from 11.25-540 μg/mL for three isolates and from 11.25-720 μg/mL for one isolate. It was not possible to determine the MSW for the remaining 18 isolates since they were able to grow at the highest nisin concentration tested (720 μg/mL).

Results are in accordance with the previously determined MSW for vancomycin regarding *S. aureus* isolates which is relevant since the action mode of these antimicrobials is similar. To understand if nisin could potentiate the transfer of resistant genes from *Enterococcus* to the clinical *S. aureus* isolates, a protocol aiming to prompt the horizontal gene transfer of *vanA* between these bacterial species was performed. In the presence of nisin sub-MIC values no transconjugates were obtained, indicating that nisin sub-MIC values do not promote *vanA* transfer, supporting nisin future application to DFI treatment.

Key-words: DFI; *S. aureus*; Nisin; MSW; Horizontal Gene Transfer.

1. Introduction

DFI are one of the major complications of diabetes (Singh, David, & Benjamin, 2005; Skrepnek, Mills, Lavery, & Armstrong, 2017). DFI are characterized by their polymicrobial feature, being *S. aureus* the most frequent isolated species (Citron, Goldstein, Merriam, Lipsky, & Abramson, 2007; Hobizal & Wukich, 2012).

One of the biggest concerns about the treatment of *S. aureus* infections is the resistance ability of this bacterial species to antibiotics action. Since Methicillin Resistance *Staphylococcus aureus* (MRSA) strains are commonly resistant to other classes of β-lactam antibiotics, one of the alternatives found for the treatment of infections caused by these strains was vancomycin (Sujatha & Praharaj, 2012; Weigel et al., 2003). It is through conjugation with enterococci, followed by *vanA* transfer, that *S. aureus* become resistant to vancomycin. Researchers believe that Vancomycin Resistant *S. aureus* (VRSA) develop due to single and independent acquisitions of *Enterococcus Tn1546* transposon (which carries *vanA*) by MRSA from the clonal complex 5 (CC5). Almost all VRSA isolated so far were obtained from patients with DFU (Gardete & Tomasz, 2014; Kos et al., 2012; Mendes et al., 2011). In 2013, Zhu et al. associated the transfer of the transposon *Tn1546*, that contains the *vanA* operon, from *Enterococcus* to *S. aureus*, with the pSK41-like plasmid, a class of conjugative staphylococci plasmids that can integrate multiple mobile genetic elements (McDougal et al., 2010; Zhu, Clark, & Patel, 2013).

The treatments currently applied to DFI are expensive and could be therapeutically more effective, rendering the development of new treatments a challenge to the scientific community (Gottrup & Apelqvist, 2012).

The antimicrobial peptides (AMPs) are oligopeptides naturally produced by prokaryotes and eukaryotes, being part of their innate immune response against several microorganisms (Bahar & Ren, 2013; Mahlapuu, Håkansson, Ringstad, & Björn, 2016; Zhang & Gallo, 2016).

Nisin is one of the better described AMPs, being produced by *Lactococcus lactis* subsp. *lactis* (Abee & Delves-Broughton, 2003; Hassan, Kjos, Nes, Diep, & Lotfipour, 2012; Mitchell, Truscott, Dickman, Ward, & Tabor, 2018). Nisin possesses in its constitution five lanthionine rings, a positive overall charge and is an amphiphilic peptide (Abee & Delves-Broughton, 2003; Hassan et al., 2012). It acts by two independent forms, producing pores on the bacterial membrane and blocking the cell wall synthesis (Field, Cotter, Hill, & Ross, 2015; Gough et al., 2017; Hassan et al., 2012). Inhibition of cell wall synthesis and pore formation are improve through bonding of nisin with lipid II (peptidoglycan subunit), although nisin may disturb the membrane independently of lipid II presence (Field et al., 2015; Hassan et al., 2012; Shin et al., 2016). Nisin has been used in food industry for 90 years, being a promising product for biomedical applications (Shin et al., 2016). It has also demonstrate to be effective against a wide range of Gram-positive bacteria and it can also present antimicrobial activity against antibiotic resistant strains, such as MRSA, VRE and VRSA (Field et al., 2015; Lagedroste, Reiners, Smits, & Schmitt, 2019; Zhou, Fang, Tian, & Lu, 2013). Bacterial biofilms are also susceptible to this AMP, pointing out for its potential use against biofilm-related infections (Cunha et al., 2018; Santos et al., 2016).

As previously referred, in the last decades antimicrobial resistance has been a growing problem as demonstrated by the increment in reported resistances. To avoid a selective mutant environment, Zhao and Drlica proposed the mutant selection window (MSW) concept (Zhao & Drlica, 2002), referring to an antibiotic concentration range that has as lower limit the minimum inhibitory concentration (MIC) and as the higher limit the mutant prevention concentration (MPC) (Cairns & Payne, 2008). The MIC is the lowest concentration of an antimicrobial that inhibits the growth of the majority of the susceptible cells, while the MPC is the concentration that inhibits the growth of the least susceptible mutant (Drlica, 2003). These are usually single-step mutants, being difficult for a cell to multiply in the presence of antibiotic concentrations above MPC values, that would require the simultaneous occurrence of two or more mutations, which is a rare event (Zhao & Drlica, 2002).

2. Materials and Methods

2.1. Bacterial Isolates

A collection of 23 *S. aureus* isolates was used in this study. These isolates were previously collected from patients with DFI (Mendes et al., 2011) and further selected and characterized (Mottola, Semedo-Lemsaddek, et al., 2016). Additionally, the reference strain *S. aureus* ATCC 29213 was also included in this study as a control. Each isolate was maintained at - 20 ºC in buffered peptone water with 20% of glycerol during this study.

2.2. Nisin

The nisin (ref N5764; Sigma-Aldrich, USA) used has a purity of 2.5% (1000 IU/mg). To obtain a stock solution of 1000 µg/mL, 1 g were dissolved in 25 mL of 0.02M HCl (Merck, Germany). After dilution, nisin was filtered with 0.22 µm filters (Frilabo, USA) and stored at 4ºC.

2.3. Determination of the Mutant Prevention Concentration

A modified version of the protocol elaborated by Sinel et al. in 2016 was used to determine the MPC of nisin regarding the 24 *S. aureus* DFI isolates under study (Sinel, Jaussaud, Auzou, Giard, & Cattoir, 2016).

Each isolate was inoculated in Brain Heart Infusion agar (BHI) (Brain heart infusion broth, VWR Chemicals, ref 84626.0500; Agar, VWR Chemicals, ref 84609.0500), and after a 24h incubation at 37ºC, a suspension of 0.5 MacFarland ($1x10^8$ CFU/mL) was performed and used to inoculate two plates. After a 24h incubation at 37ºC, the bacterial lawn was collected from the two plates and resuspended in 1mL of Brain Heart Infusion broth (BHIB) to achieve a bacterial suspension with a concentration of 10^{10} CFU/mL. In order to confirm the concentration values, serial dilutions of the suspensions 10^0 to 10^{-8} were performed, after witch, 100 µL of the dilutions 10^{-7} and 10^{-8} were inoculated in BHI agar and incubated for 24h at 37ºC, for viable cell count.

Afterwards, 50 µL from the original suspension, were inoculated in Mueller Hinton agar (MHA) (Mueller-Hinton Agar, OXOID, ref CM0337) supplemented with the following nisin concentrations: 5.63, 11.25, 22.5, 45, 90, 180, 360 and 720 µg/mL. These concentrations were selected considering a two-fold increase of the MIC value (11.25 µg/mL) that was previously determined (Santos et al., 2016). A sub MIC value was also included (5.63 µg/mL). Finally, plates were incubated for 72h at 37ºC for MPC determination.

The MPC corresponded to the minimum concentration of nisin that prevented the growth of resistant mutants after the incubation period. For each isolate, the mutants grown at the concentration below the MPC of nisin were isolated and stored at -20°C and - 80°C in a solution of buffered peptone water with 20% glycerol (Peptone water buffered, VWR Chemicals, ref 84600.0500; Glycerine 87%, VWR, ref 24385.295). The MPC values of nisin were determined in two different and independent rounds.

2.4. Horizontal Gene Transfer

2.4.1. DNA extraction

DNA extraction was performed based on the protocol described by Mottola (Mottola, Semedo-Lemsaddek, et al., 2016).

All isolates were inoculated in BHI agar for 24h at 37ºC. Four to five bacterial colonies were collected using a sterile loop and resuspended in 100 µL of TBE buffer (0.9 M Tris-Borate, 0.01 M EDTA, pH 8.3 – Omega, ref. AC10078) supplemented with 0.1% Tween 20 (Merck-Schuehardt, ref. 8.22184.0500) solution. After homogenization, the solution was incubated for seven minutes at 97ºC and centrifuged at 15000 rpm for 5 minutes (Hermle Labortechnik). The supernatant was collected for PCR screening.

2.4.2. Multiplex PCR for *vanA* detection

Before the Horizontal Gene Transfer protocol, it was necessary to confirm the absence of *vanA* gene in the 24 *S. aureus* isolates, using a multiplex PCR (Ramos-Trujillo, Pérez-Roth, Méndez-Alvarez, & Claverie-Martín, 2003).

Two pairs of primers, targeting *vanA* (5' GGG AAA ACG ACA ATT GC 3') with 732 bp and *mecA* (5' TCCAGATTACAACTTCACCAGG 3') with 162 bp were used in this PCR, synthesized by STABVIDA® (Mottola, Matias, et al., 2016; Ramos-Trujillo et al., 2003).

The PCR mixture had a final volume of 28.5 µL, 10 µL of the Supreme NZYTaq 2x Green Master Mix (Nzytech®) consisting in 1x reaction buffer (50 mM Tris – HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP), 0.29 µL (0.5 uM) of the *vanA* primer, 0.23 µL (0.4 uM)

of the MecA primer, 16.88 µL of PCR-grade water and 5 µL (170 ng/µL) DNA template.

PCR amplification was completed in a MyCycler Thermal Cycler (BioRad®) using the following conditions: initial denaturation at 94°C for 4 min; 10 cycles involving denaturation at 94°C for 30s, annealing at 64°C for 30s and elongation at 72°C for 45s; 25 cycles involving denaturation at 94°C for 30s, annealing at 50 °C for 45s and elongation at 72°C for 2min, and a final extension step at 72°C for 10min.

An electrophoresis gel was performed to perceive the amplified products, using a 1.5% agarose gel (Nzytech, ref. MB14402) and a buffer stained with GreenSafe (Nzytech®) at 90V for 45 min. A molecular weight marker, NZYDNA ladder VI (Nzytech®) was also included. Results were visualized by transillumination (ChemiDoc XRS+, Bio-rad).

Two positive control strains, *Staphylococcus aureus* 01-00694 (*mecA* positive) and *Enterococcus faecium* CCUG 36804 (*vanA* positive), were included in each PCR amplification protocol, as well as a negative control, with no DNA.

2.4.3. Horizontal Gene Transfer protocol

To test if nisin selective pressure induces horizontal gene transfer, a protocol adapted from Niederhäusern in 2011 was performed (Niederhäusern et al., 2011). Mating experiments were performed in three rounds, using the VRE rifampicin susceptible (Van^r Rif^s) *Enterococcus faecium* (*E. faecium)* CCUG 36804 strain as a donor of the *vanA* gene and as recipients all the 24 *S. aureus* isolates, obtained in the previous task, which were resistant to rifampicin (previously induced) and susceptible to vancomycin (Van^s Rif^r).

After performing a 0.5 MacFarland suspension for each isolate, 500 µL of the donor and 500 µL of one of the recipients were added to 5 mL of TSB (Tryptic Soy broth, VWR Chemicals, ref. 84675.0500) and incubated at 35ºC for 18h.

After incubation, 1 mL of the bacterial suspension was added to 5 mL of TSB and further incubated for 6h at 37ºC. Afterwards, 2 mL of each suspension were inoculated in TSA and incubated for 5h at 37ºC on a shaker, to promote mating. Then, the plates were incubated at 37ºC for 24h. The bacterial suspension that remained at the surface of the agar plates was removed and inoculated in 5 mL of TSB. After an incubation period of 12h at 37ºC, 100 µL of the solution was inoculated in MSA (Mannitol Salt agar, PanReac AppliChem, ref 413783.1210) supplemented with 64 µg/mL of rifampicin and 8 µg/mL of vancomycin (Vancomycin hydrochloride, Abcam, ref. ab141224) to select the transconjugants. If mating occurred, recombinant isolates that developed on these plates should be resistant to rifampicin and vancomycin. The transconjugants were stored at -20°C and - 80°C in a solution of buffered peptone water with 20% glycerol and a PCR analysis was performed to confirm the presence of the *vanA* gene.

The second mating round was performed in the presence of nisin, with all the media used being supplemented with nisin at sub-MIC (5.63 µg/mL) concentration. The third mating round was performed in the presence of a sub-MIC value of 0.28 µg/mL of vancomycin, based on the previous MIC determination (Mottola, Matias, et al., 2016).

2.4.4. PCR for pSK41-like plasmid detection

To evaluate the presence of the pSK41-like plasmid in the 23 clinical isolates under study, a PCR protocol was performed, using a pair of primers targeting the *traE* (5' ACA AAT GCG TAC TAC AGA CCC TAA ACG A 3') which has 317 bp; the primer was synthesized by STABVIDA® (Albrecht et al., 2014; Zhu et al., 2013).

The PCR mixture had a final volume of 50 µL, 10 µL of the Supreme NZYTaq 2x Green Master Mix (Nzytech®) consisting in 1x reaction buffer (50 mM Tris – HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP), 0.4 µL (0.4 uM) of *traE* primer and 39.2 µL of PCR-grade water and 5 µL (170 ng/µL) DNA template.

PCR amplification was completed in a MyCycler Thermal Cycler (BioRad®) using the following conditions: initial denaturation at 94°C for 2 min; 30 cycles involving denaturation at 95°C for 15s, annealing at 53°C for 90s and elongation at 72°C for 90s, and a final extension step at 72°C for 7min.

A positive control strain, *Staphylococcus aureus* RN4220 (pGO1 positive), gently provided by Dr. Alex O'Neill, from University of Leeds, was included in the PCR amplification protocol, as well as a negative control, with no DNA (Caryl & O'Neill, 2009).

An electrophoresis gel was performed to perceive the amplified products, using a 1.5% agarose gel (Nzytech, ref. MB14402) and a buffer stained with GreenSafe (Nzytech®) at 90V for 45 min. A molecular weight marker, NZYDNA ladder VII (Nzytech®) was also included. Results were visualized by transillumination (ChemiDoc XRS+, Bio-rad).

3. Results and Discussion

3.1. Mutant Selection Window

Antibiotic resistance is a worldwide concerning problem. Nowadays the antibiotic concentrations established in the therapeutic protocols for *in vivo* administration, have as reference the MIC determination. However, the clinical application of antimicrobial doses based on MIC values, could exert a selective pressure on bacteria, allowing the selection of resistant mutants (Drlica & Zhao, 2007). The fact that some cases of resistance to nisin have already been reported shows the importance of determining the MSW in order to establish proper therapeutic concentrations to be applied at the clinical settings and to avoid promoting resistance.

MPC determination was performed for all the 23 *S. aureus* isolates and for the reference strain *S. aureus* ATCC 29213. To our knowledge, the determination of the MPC of nisin regarding *S. aureus* was not performed previously.

The MPC values ranged from 360 µg/mL to more than 720 µg/mL. The distribution of the MPC values obtained in the two rounds is shown in table 5, being observed that nisin MPC average values was of 360 µg/mL for 8.33% of the isolates (n=2), of 540 µg/mL for 12.5% of the isolates (n=3) and of 720 µg/mL for 4.17% (n=1) of the isolates. MPC value could not be determined regarding 18 isolates (75%), since they were able to grow in the presence of the highest concentration of nisin tested (720 µg/mL).

Our results are in accordance with a previous study

Isolates	A1.1	A5.2	A6.3	B3.2	B3.3	B7.3	B13.1	B14.2	Z1.1	Z2.2	Z3.1
MPC average values	>720	>720	>720	>720	>720	>720	720	>720	>720	>720	>720

Isolates	Z5.2	Z12.2	Z14.1	Z16.1	Z17.2	Z21.1	Z21.3	Z23.2	Z25.2	Z27.2	Z27.3	Z32.2	ATCC 29213
MPC average values	>720	540	>720	>720	360	>720	>720	540	540	>720	>720	360	>720

Figure 1- Nisin MPC values for the 24 S. aureus isolates under study.

that determined the vancomycin MPC_{80} value for 855 *S. aureus* clinical isolates, which was 64 times higher than the MIC_{80} (Fujimura, Nakano, & Watanabe, 2014). Vancomycin and nisin have similar modes of action since they both act on lipid II, although through different mechanisms (Hasper et al., 2006). Vancomycin inhibits the cell wall synthesis by binding to the sequence of the C-terminal D-ala-D-ala of the lipid II, while on the other hand, the lanthionine rings of nisin bind to the pyrophosphate of lipid II, using it as a docking molecule to form pores on the target membranes (Breukink & Kruijff, 2006; Hasper et al., 2006).

Nisin MPC values regarding most isolates was superior to 720 µg/mL. It cannot be stated, yet, that this dose can be applied *in vivo* or that will not be toxic for diabetic patients presenting infected ulcers.

In a study performed in 2008, nisin was applied to the nipple and mammary areola of four women with clinical signs of mastitis infection by *S. aureus* (Fernández, Delgado, Herrero, Maldonado, & Rodríguez, 2008). The values of nisin applied in the previous referred study were based on the study performed on the toxicity of nisin published in 2006 by the European Food Safety Authority (EFSA), which determined the toxicity related to the oral administration of nisin. The acceptable daily intake of nisin determined by EFSA was of 0.13 mg/kg body weight. Since the nipples presented infected fissures (infected wound) and no signs of toxicity were observed after the application of nisin, the EFSA acceptable daily intake was also considered in this study for comparison purposes. EFSA recently updated the acceptable daily intake of nisin to 1 mg/kg body weight (Younes et al., 2017), which means that a person with medium weight (65 kg) can ingest a maximum of 65 mg of nisin per day. As the MPC average concentration of nisin determined in this study was of 0.72 mg/mL (720 µg/mL), if 2 ml of a biogel supplemented with nisin at this concentration were applied to DFI 3 times a day, this would correspond to the application of 4 mg of nisin to the wound, which is 16 times below the acceptable daily intake for a medium weight individual.

The emergence of mutants resistant to this AMP can be prevented if the administration doses remains above the MPC value, being the recommended dose determined in this study probably safe, since the acceptable daily intake of nisin is above the MPC value.

3.2. Horizontal Gene Transfer

The emergence of VRSA is a current problem, since vancomycin is often a last resort antibiotic applied in the treatment of several types of infections promoted by resistant bacteria, including DFI (Bader, 2008; Butler, Hansford, Blaskovich, Halai, & Cooper, 2014; Hsu et al., 2004). It is known that the rate of resistant mutants increases with prolonged antimicrobial treatments (Giraud, Matic, Radman, Fons, & Taddei, 2002). For this reason, and considering that nisin binds to the same molecule that vancomycin, it is important to understand if a new therapeutic protocol based on nisin would promote the transfer of resistant genes, in particular of *vanA* (Breukink & Kruijff, 2006; Giraud et al., 2002).

The horizontal gene transfer protocol was performed, using the 23 *S. aureus* clinical isolates as potential recipients and the *E. faecium* CCUG 36804 as the donor of the *vanA* gene. PCR analysis was performed regarding all isolates recovered from the media used to select the possible transconjugants. A band matching the *vanA* positive control was obtained from the mating between the recipient *S. aureus* Z5.2 and *E. faecium* CCUG 36804. This clinical isolate is a methicillin susceptible *S. aureus* (MSSA) and belongs to the Clonal Complex 5, as the majority of the clinical isolates under study (69.5%) (Mottola, Semedo-Lemsaddek, et al., 2016). Clones belonging to the CC5 are the predominant cause of hospital acquired MRSA (HA-MRSA) infections, being also present in community. Additionally, the majority of the VRSA strains reported so far belong to the clonal complex 5 (King, Kulhankova, Stach, Vu, & Salgado-Pabón, 2016; Rossi et al., 2014).

Since the pSK41 plasmid has been described as required for the transfer of the *vanA* gene from enterococci to staphylococci, a PCR analysis was performed regarding all the clinical *S. aureus* isolates to evaluate the presence of this plasmid. pSK41 was already detected in multiple strains, including CA-MRSA (ex. CC8) and HA-MRSA (ex. CC5) (Albrecht et al., 2014; McDougal et al., 2010). Surprisingly, in our collection all the isolates were negative for pSK41-plasmid, even *S. aureus* Z5.2.

Another interesting fact is the methicillin susceptible profile of transconjugant *S. aureus* Z5.2, since almost all the VRSA reported are also MRSA (Friães et al., 2015; Kohler, Vaishampayan, & Grohmann, 2018). The association between the emergence of VRSA with MRSA is probably due to the fact that treatment with vancomycin is only recommended when semi-synthetic penicillin fail, which indicates the presence of methicillin-resistant mutants at the site of infection when the new vancomycin-based antibiotherapy is started. Results from this study seems to indicate that the MSSA strains also have the ability to acquire other resistant determinants besides *mecA*.

In 2012, in Brazil, two different clinical *S. aureus* isolates obtained from blood samples of one patient were found to be resistant to vancomycin. Researchers believe that both of these isolates resulted from the mating of enterococci with two *S. aureus* isolates presented different characteristics: one was susceptible to methicillin and the other was a MRSA without a pSK41 plasmid; the MSSA belonged to CC5 and the MRSA to CC8 (Panesso et al., 2015; Rossi et al., 2014). The researchers found that both VRSA presented a 55,7 bp plasmid denominated pBRZ01, which is not related with the pSK41 plasmid. This plasmid is a rearranged Tn1546-like element and holds a insertion region flaking the *vanA* gene cluster, which could be responsible for providing mobility to pBRZ01 (Rossi et al., 2014). Therefore, it would be interesting to evaluate the role of PBRZ01 in gene transference on our bacterial collection.

The fact that strains that belong to CC5 are repeatedly acquiring resistance to vancomycin is probably related with some predisposition of these strains to horizontal gene transfer (Kos et al., 2012). In our study, the acquisition of *vanA* was accomplished by a MSSA that belongs to CC5 but does not present pSK41, which supports the hypotheses that other plasmids could be related with the transfer of the *vanA* gene. This phenomenon may contribute for the increasing virulence of the *S. aureus* strains found in Portugal at the hospital settings, since CC5 is the second most predominant clone in Portuguese hospitals; as such, the transfer of resistant elements can be more widespread than expected (Semedo-Lemsaddek et al., 2015). Although these strains were considered to be specific of the hospital settings, they have also been found in the community, which raises more concerns about these bacteria, since they seem to have a genetic or biological predisposition to acquire resistance factors (Friães et al., 2015; King et al., 2016). Additionally, the fact that MSSA can also be involved in horizontal gene transfer increases the range of possible events.

Despite the highlight that has been given to the role of the pSK41 plasmid in the horizontal gene transfer between enterococci and *S. aureus*, it could have a lower relevance than researchers believe, since other plasmids, like pBRZ01, appear to have the ability to acquire resistance determinants such as the *vanA* gene. It is important to understand the mechanisms of resistance genes transference, since infections, in particular the ones promoted by biofilms, present perfect conditions for the transfer of resistance determinants. It is also important to highlight that the transfer only occur in 4.16% (n=1) of the clinicals isolates under study. If a bacteria is susceptible to first line antibiotics, the use of last resort antibiotics decrease, not promoting the emergence of new resistances to the last resort antibiotics.

It was not possible to obtain a *vanA* positive transconjugate in the second mating round (with selective pressure of nisin) and in the third mating round (with selective pressure of vancomycin).

The presence of nisin at a sub-MIC value of 5.63 µg/mL appears to not promote the transfer of the *vanA* gene which is an important characteristic to

support the future clinical application of this AMP for DFI treatment.

The presence of vancomycin at a sub-MIC value (0.55 µg/mL) appears to not selectively enhance the transfer of the *vanA* gene, which is a surprising result, since antibiotics at low levels, like sub-MIC values, appears to promote the emergence of resistant bacteria (Wistrand-Yuen et al., 2018). This result may be related to the fact that the acquisition of resistance genes has a fitness cost for bacteria, with several genes being activated to acquire and maintain resistance factors (Hernando-Amado, Sanz-García, Blanco, & Martínez, 2017).

It is important to refer that the donor-recipient ratio used in this study was of 1:1, which could have influenced the low rate of transconjugants obtained.

The HGT is a concerning problem in our days, mainly between *S. aureus* and enterococci, since these microorganisms were classified by WHO as high priority pathogens due to the ability to acquire new resistance factors (Tacconelli, Carrara, Savoldi, Kattula, & Burkert, 2017). For that reason it is important that the mechanisms under these processes are understood.

To our knowledge this was first time that nisin ability to induce transferability of resistance genes was evaluated, being interesting to observe that nisin at sub-MIC values does not seem to induce *vanA* gene transfer.

The characteristics of the microenvironment of DFI may prompt the acquisition of resistant determinants, including vancomycin resistance, as the majority of VRSA isolated so far were obtained from patients with DFI (Kos et al., 2012). Diabetic patients with these types of infections are often hospitalized and under antibiotherapy, which could promote the emergence of new resistant strains, becoming important and vehicles for the dissemination of resistant isolates in and out of the hospital setting (Kos et al., 2012; Mottola, Semedo-Lemsaddek, et al., 2016).

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