

# Guar gum as a new antimicrobial peptide delivery system against diabetic foot ulcers *Staphylococcus aureus* isolates

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Diabetic patients frequently develop diabetic foot ulcers (DFUs), particularly those patients vulnerable to *Staphylococcus aureus* opportunistic infections. It is urgent to find new treatments for bacterial infections. The antimicrobial peptide (AMP) nisin is a potential candidate, mainly due to its broad spectrum of action against pathogens. Considering that AMP can be degraded or inactivated before reaching its target at therapeutic concentrations, it is mandatory to establish effective AMP delivery systems, with the natural polysaccharide guar gum being one of the most promising. We analysed the antimicrobial potential of nisin against 23 *S. aureus* DFU biofilm-producing isolates. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were determined for nisin diluted in HCl and incorporated in guar gum gel. Statistical analysis was performed using the Wilcoxon matched-pair test. Nisin was effective against all isolates, including some multidrug-resistant clinical isolates, independent of whether it is incorporated in guar gum. While differences among MIC, MBC and MBIC values were observed for HCl- and guar gum- nisin, no significant differences were found between MBEC values. Inhibitory activity of both systems seems to differ only twofold, which does not compromise guar gum gel efficiency as a delivery system. Our results highlight the potential of nisin as a substitute for or complementary therapy to current antibiotics used for treating DFU infections, which is extremely relevant considering the increase in multidrug-resistant bacteria dissemination. The guar gum gel represents an alternative, practical and safe delivery system for AMPs, allowing the development of novel topical therapies as treatments for bacterial skin infections.

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## INTRODUCTION

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide. Recently, the WHO global report on diabetes demonstrated that the number of adults with this disease has almost quadrupled since 1980 to 422 million people. This dramatic increase is largely due to

the rise in type 2 diabetes, and its driving factors include overweight and obesity (Roglic, 2016). Diabetic foot ulcers (DFU) are one of the most frequent complications of diabetes, resulting from a complex interaction of several pathophysiological factors. Although ischaemic and neuropathic lesions have the initial role in DFU onset (Armstrong *et al.*, 2011; Jeffcoate & Harding, 2003; Vuorisalo *et al.*, 2009), it is the infection by pathogenic micro-organisms along with local microenvironmental conditions unfavourable to antibiotic action that ultimately cause infection chronicity and lower limb amputation (Lipsky *et al.*, 2004; Richard *et al.*, 2011).

**Abbreviations:** AMP, antimicrobial peptide; DFU, diabetic foot ulcer; MBC, minimum bactericidal concentration; MBIC, minimum biofilm inhibitory concentration; MBEC, minimum biofilm eradication concentration; MRSA, methicillin-resistant *Staphylococcus aureus*.

Diabetes-associated foot ulcer infections are usually polymicrobial, and several bacterial genera can be part of their microbiota, mainly Gram-positive bacteria, with *Staphylococcus aureus* as the most predominant species (Mendes *et al.*, 2014; Mottola *et al.*, 2016a). *S. aureus* is a commensal bacterium known to colonize the human skin and mucosal surfaces. Colonized individuals are at increased risk for developing *S. aureus* infections which range from minor skin and soft tissue infections to severe diseases, such as endocarditis, septicaemia and osteomyelitis (Jenkins *et al.*, 2015).

These bacteria have the ability to produce several virulence factors, with biofilm formation as one of the most important. Biofilms are ubiquitous and complex structures consisting of an interactive community of polymicrobial cells embedded in a self-produced extracellular matrix of hydrated polymeric substances, such as proteins, polysaccharides, nucleic acids and others, which are irreversibly attached to biological surfaces (Dickschat, 2010). Because of inefficient diffusion or sequestering of the agent within the biofilm matrix, biofilm-based bacteria are recalcitrant to the action of most antibiotics and more resistant to the innate immune system (An & Ryan, 2016; Stewart & Costerton, 2001). Moreover, in the past few decades, a major problem in treating DFU infections is the presence of antibiotic-resistant pathogens, particularly methicillin-resistant *S. aureus* (MRSA) (Akhi *et al.*, 2016; Dang *et al.*, 2003; Mottola *et al.*, 2016b; Stanaway *et al.*, 2007). The rates of isolation of multidrug-resistant pathogens vary widely between geographical area and treatment centre (Kandemir *et al.*, 2007; Richard *et al.*, 2008). However, the increasing incidence of multidrug-resistant micro-organisms, together with the incapacity of antibiotics to act on resistant and biofilm-producing bacteria at therapeutic concentrations, emphasizes the importance of developing new treatment strategies to effectively eradicate these infections.

Antimicrobial peptides (AMP) are molecules produced by the vast majority of living organisms as part of their innate immune response against a broad range of pathogens (Hancock & Sahl, 2006; Lewis, 2013; Zasloff, 2002), and unlike conventional antibiotics, AMPs can also act as modulators of the immune system (Batoni *et al.*, 2016; Kirikae *et al.*, 1998; Rosenfeld *et al.*, 2006). Additionally, some authors suggest that AMP are able to prevent biofilm formation and act on pre-formed biofilms (Overhage *et al.*, 2008; Stempel *et al.*, 2015), supporting their potential as alternatives to currently available DFU therapeutic agents (Mohammad *et al.*, 2015). One of the best studied and characterized AMP is nisin (Abts *et al.*, 2011). It belongs to the class I bacteriocins, also known as lantibiotics. These are small peptides containing unusual amino acids such as lanthionine and L-methylanthionine and a number of dehydrated amino acid residues (McAuliffe *et al.*, 2001). Nisin is produced by *Lactococcus lactis*, acts principally against Gram-positive bacteria and has been used as a food preservative for over 60 years (Cleveland *et al.*, 2001; Gharsallaoui *et al.*, 2016).

Despite all their advantages, AMP successful delivery represents a challenge, since they can be degraded or inactivated before reaching their target at therapeutic concentrations (O'Driscoll *et al.*, 2013). Natural polysaccharides have been considered as promising drug delivery systems by the pharmaceutical industries, mainly because of their non-toxicity, biodegradability, biocompatibility, abundant availability in nature and economical cost (Reddy *et al.*, 2011). Guar gum is a natural polysaccharide obtained from the endosperm of the leguminous crop *Cyamopsis tetragonolobus* and consists of a linear polymer of D-galactose and D-mannose, called galactomannan (Thombare *et al.*, 2016). This hydroxyl-group-rich polymer when added to water forms hydrogen bonds that confer a significant viscosity to the solution. Because of its thickening, emulsifying, gelling and binding properties; quick solubility in cold water; and wide pH stability and film-forming ability, it finds application as a safe and versatile system for delivery of bioactive agents (Reddy *et al.*, 2011; Thombare *et al.*, 2016).

The present study was designed not only to determine the antimicrobial activity of nisin against both planktonic and biofilm-based *S. aureus* diabetic foot clinical isolates collected in Lisbon medical centres but also to evaluate the efficiency of the peptide incorporated in a guar gum gel to be used as a delivery system for this AMP.

## METHODS

**Bacterial isolates.** In a previous epidemiological survey regarding DFU infectious microbiota conducted from January to July 2010, a total of 54 *Staphylococcus* spp. clinical isolates were collected from 49 DFU patients (Mendes *et al.*, 2012). All isolates were characterized regarding clonality, antimicrobial resistance and virulence profiles. Based on macrorestriction analysis by PFGE and multilocus sequence typing, 23 representative *S. aureus* strains were selected (Mottola *et al.*, 2016b). All the 23 strains were the subject of the current study. Additionally, a reference strain, *S. aureus* ATCC 29213, a known biofilm producer, was also included as a control strain. As a result, the number of strains analysed in this work is 24.

**AMP preparation and guar gum incorporation.** A nisin stock solution (1000 µg ml<sup>-1</sup>, corresponding to 40 000 IU ml<sup>-1</sup>) was obtained by dissolving 1 g of nisin powder (2.5% purity, 1000 IU mg<sup>-1</sup>; Sigma-Aldrich) in 25 ml of HCl (0.02 M) (Merck). The nisin stock solution was filtered using a 0.22 µm Millipore filter (Frilabo) and stored at 4 °C. A set of dilutions of nisin were prepared, corresponding to the following concentrations: 900, 800, 700, 600, 500, 400, 300, 200, 100, 40, 20, 10 and 5 µg ml<sup>-1</sup>.

A guar gum gel of 1.5% (w/v) was prepared by dissolving 0.75 g of guar gum (Sigma-Aldrich) in 50 ml of sterile distilled water and heat sterilized by autoclave. The set of dilutions of nisin were incorporated within the gel in a proportion of 1:1, obtaining a final gel of 0.75% (w/v).

**MIC and MBC determination.** The MIC value of nisin was determined by microtitre broth dilution method (Wiegand *et al.*, 2008).

Strains were grown in a non-selective brain-heart infusion (BHI) agar medium (VWR Chemicals) at 37 °C for 24 h. Bacterial suspensions of approximately 10<sup>8</sup> c.f.u. ml<sup>-1</sup> were prepared directly from plate cultures using a 0.5 McFarland standard (bioMérieux) in sterile normal

saline (Scharlau). For MIC and MBC assays, bacterial suspensions were diluted in fresh BHI broth (VWR Chemicals) to a concentration of  $\sim 10^7$  c.f.u. ml $^{-1}$ .

The set of concentrations of nisin, diluted in HCl or incorporated in the guar gum gel, ranging from 5  $\mu\text{g ml}^{-1}$  (5 IU per well) to 1000  $\mu\text{g ml}^{-1}$  (1000 IU per well), were distributed in 96-well flat-bottomed polystyrene microtitre plates (Nunc; Thermo Fisher Scientific). All the wells, except for the negative control (with only broth medium), were inoculated with 150  $\mu\text{l}$  of the  $10^7$  c.f.u. ml $^{-1}$  bacterial suspensions. Microplates were statically incubated for 24 h at 37 °C, and MIC was determined as the lowest concentration of nisin that visually inhibited the microbial growth.

MBC value was determined by inoculating a 3  $\mu\text{l}$  dot of the suspension from the wells where no bacterial growth was observed on BHI agar plates that were incubated at 37 °C for 24 h. MBC was determined as the lowest nisin concentration at which no colonies were observed. Experiments were conducted in triplicate, and independent replicates were performed at least three times on different days. For each strain, nine results were obtained and analysed.

**Minimum biofilm inhibitory concentration and minimum biofilm eradication concentration determination.** A modified version of the Calgary Biofilm Pin Lid Device (Ceri *et al.*, 1999) was used to determine the antimicrobial susceptibility of bacteria embedded in a 24 h biofilm.

For minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) assays, bacterial suspensions prepared as described before were diluted in fresh tryptic soy broth (TSB) (VWR Chemicals) plus 0.25% (w/v) glucose (Merck) medium to a concentration of  $\sim 10^6$  c.f.u. ml $^{-1}$ .

Briefly, 200  $\mu\text{l}$  of the  $\sim 10^6$  c.f.u. ml $^{-1}$  bacterial suspensions were distributed in 96-well flat-bottomed polystyrene microtitre plates, covered with 96-peg polystyrene lids (Nunc-TSP; Thermo Fisher Scientific) and statically incubated for 24 h at 37 °C, to allow biofilm formation on pegs. Peg lids were then rinsed three times in sterile normal saline to remove planktonic bacteria and placed on new microplates containing the set of nisin concentrations, diluted in HCl or incorporated in the guar gum gel, with concentrations ranging from 5  $\mu\text{g ml}^{-1}$  (5 IU per well) to 1000  $\mu\text{g ml}^{-1}$  (1000 IU per well) and 200  $\mu\text{l}$  of fresh TSB +0.25% glucose medium. Microplates were incubated for 24 h at 37 °C, without shaking. After incubation, peg lids were removed, and the MBIC value was determined as the lowest nisin concentration that visually inhibited the microbial growth.

Subsequently, in order to determine the MBEC value, peg lids were rinsed three times in sterile normal saline, placed in new microplates containing only 200  $\mu\text{l}$  fresh TSB+0.25% (w/v) glucose medium and incubated in an ultrasound bath (Grant MXB14), at 50 Hz for 15 min in order to disperse the biofilm-based bacteria from the peg surface. Afterwards, peg lids were discarded, and microplates were covered with normal lids and incubated for 24 h at 37 °C.

Next, MBEC was determined through direct observation of experimental wells, and MBEC value was defined as the lowest nisin concentration that visually eliminates the microbial growth. Additionally, MBEC quantification was also conducted according to a previously described protocol using Alamar Blue, a redox indicator that yields a colourimetric change in response to metabolic activity (Pettit *et al.*, 2005). Briefly, 5  $\mu\text{l}$  of resazurin (Alamar Blue; Thermo Fisher Scientific) were added in each well, and microplates were incubated for 1 h at 37 °C. Absorbance values at 570 nm and 600 nm were then recorded using a microplate reader (BMG LABTECH).

Percentage of Alamar Blue reduction was calculated using the following formula (Pettit *et al.*, 2005):

$$\frac{(\varepsilon_{\text{ox}})\lambda_2 A\lambda_1 - (\varepsilon_{\text{ox}})\lambda_1 A\lambda_2}{(\varepsilon_{\text{red}})\lambda_1 A'\lambda_2 - (\varepsilon_{\text{red}})\lambda_2 A'\lambda_1} \times 100$$

where  $\varepsilon_{\text{ox}}$ =molar extinction coefficient of Alamar Blue oxidized form ( $\varepsilon_{\text{ox}\lambda_1}$  = 80.586 and  $\varepsilon_{\text{ox}\lambda_2}$  = 117.216);  $\varepsilon_{\text{red}}$ =molar extinction coefficient of Alamar Blue reduced form ( $\varepsilon_{\text{red}\lambda_1}$  = 155.677 and  $\varepsilon_{\text{red}\lambda_2}$  = 14.652);  $A$ =absorbance of test wells;  $A'$ =absorbance of negative control well;  $\lambda_1$ =570 nm and  $\lambda_2$ =600 nm.

MBEC value was defined as the lowest nisin concentration resulting in  $\leq 50\%$  of Alamar Blue reduction. Experiments were conducted in triplicate, and independent replicates were performed at least three times on different days. For each strain, nine results were obtained and analysed.

**Guar gum gel viability assay.** The nisin-incorporated guar gum gel was stored at different temperatures ( $-18$  °C, 4 °C, 20 °C, 37 °C and 44 °C) for 6 months. Its efficacy as a delivery system was tested at three different time points (1, 3 and 6 months) by placing a 3  $\mu\text{l}$  drop of the nisin-incorporated guar gum gel on BHI agar plates with a lawn culture executed using  $10^7$  c.f.u. ml $^{-1}$  bacterial suspensions. Plates were incubated at 37 °C for 24 h, and inhibition halo diameters were measured.

**Statistical analysis.** Qualitative variables (presence/absence of growth) are expressed as percentages, and quantitative variables (concentrations) are expressed as means $\pm$ SD. Data analysis was performed using STATISTICA Data Miner software, version 13. Significance of the study variables was tested using Wilcoxon matched-pair tests. A two-tailed  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### MIC and MBC

MIC and MBC values are presented in Table 1 and summarized in Fig. 1.

All isolates, including the reference strain *S. aureus* ATCC 29213, were considered susceptible to nisin. MIC values for nisin diluted in HCl ranged from 40 to 100  $\mu\text{g ml}^{-1}$ , with a mean value of  $90 \pm 22.8$   $\mu\text{g ml}^{-1}$ . When incorporated in guar gum gel, nisin MIC concentrations were significantly different ( $P < 0.05$ ) and ranged from 40 to 300  $\mu\text{g ml}^{-1}$ . The mean value was  $180.8 \pm 53.9$   $\mu\text{g ml}^{-1}$  (Table 1, Fig. 1a, b).

MBC values were approximately fivefold higher than the MIC ones. For nisin diluted in HCl, the mean MBC value was  $495.2 \pm 149.9$   $\mu\text{g ml}^{-1}$ , and only three isolates presented an MBC  $> 800$   $\mu\text{g ml}^{-1}$ . For nisin incorporated in guar gum gel, MBC was also significantly different ( $P < 0.05$ ) with the mean MBC being  $766.7 \pm 272.6$   $\mu\text{g ml}^{-1}$  and only three isolates presenting an MBC  $> 1000$   $\mu\text{g ml}^{-1}$  (Table 1, Fig. 1a, b).

### MBIC and MBEC

MBIC and MBEC values are presented in Table 1 and summarized in Fig. 1.

Considering nisin diluted in HCl, MBIC values ranged from 20 to 300  $\mu\text{g ml}^{-1}$ , and the mean value was  $150.8 \pm 85.5$   $\mu\text{g ml}^{-1}$ . When delivered through guar gum gel, nisin MBIC concentrations were significantly different ( $P < 0.05$ ) and ranged from 100 to 600  $\mu\text{g ml}^{-1}$ . The mean value was  $366.7 \pm 140.4$   $\mu\text{g ml}^{-1}$  (Table 1, Fig. 1c, d).

**Table 1.** MIC, MBC, MBIC and MBEC determinations for nisin diluted in HCl and incorporated in guar gum against *S. aureus* DFU isolates

Strain ID	Strain characterization	Nisin-HCl						Nisin-guar gum					
		MIC ( $\mu\text{g ml}^{-1}$ )	MBC ( $\mu\text{g ml}^{-1}$ )	MBIC ( $\mu\text{g ml}^{-1}$ )	MBEC visual ( $\mu\text{g ml}^{-1}$ )	MBEC AB ( $\mu\text{g ml}^{-1}$ )	MIC ( $\mu\text{g ml}^{-1}$ )	MBC ( $\mu\text{g ml}^{-1}$ )	MBIC ( $\mu\text{g ml}^{-1}$ )	MBEC visual ( $\mu\text{g ml}^{-1}$ )	MBEC AB ( $\mu\text{g ml}^{-1}$ )		
A1.1	MRSA	100	600	200	>1000	>1000	200	1000	600	>1000	>1000		
A5.2		100	300	100	>1000	>1000	200	>1000	400	>1000	>1000		
A6.3		100	>800	300	>1000	>1000	100	500	400	>1000	>1000		
B3.2		100	700	100	>1000	>1000	200	1000	400	>1000	>1000		
B3.3		100	800	100	>1000	>1000	200	>1000	500	>1000	>1000		
B7.3	MDR	100	>800	100	>1000	>1000	200	900	500	>1000	>1000		
B13.1	MDR	40	400	200	>1000	>1000	100	400	300	>1000	>1000		
B14.2	MDR	100	500	100	>1000	>1000	200	1000	300	>1000	>1000		
B23.2		40	300	20	400	600	100	400	100	700	1000		
Z1.1	MRSA	100	700	100	>1000	>1000	200	900	400	>1000	>1000		
Z2.2		40	300	200	>1000	>1000	200	500	400	>1000	>1000		
Z3.1		100	400	200	1000	1000	200	1000	300	700	>1000		
Z5.2		100	600	100	700	1000	200	900	300	>1000	>1000		
Z14.1		100	300	300	>1000	>1000	300	600	600	>1000	>1000		
Z16.1	MDR	100	700	200	>1000	>1000	200	900	500	>1000	>1000		
Z16.2		100	400	20	200	200	200	1000	100	600	1000		
Z17.2		40	400	40	200	200	200	400	200	800	500		
Z21.1	MDR	100	500	200	>1000	>1000	200	1000	300	>1000	>1000		
Z21.3	MDR	100	400	200	700	1000	200	1000	500	>1000	>1000		
Z25.2		100	600	40	>1000	>1000	100	400	200	>1000	>1000		
Z27.2		100	500	200	>1000	>1000	200	1000	400	>1000	>1000		
Z27.3		100	>800	200	>1000	>1000	200	>1000	400	>1000	>1000		
Z32.2		100	500	100	>1000	>1000	100	300	200	>1000	>1000		
ATCC29213		100	500	300	>1000	>1000	200	1000	500	>1000	>1000		

AB, Alamar Blue; MDR, multidrug resistant.

MBEC values were higher than the respective MIC. No significant differences ( $P \geq 0.05$ ) were observed between the nisin diluted in HCl and the nisin impregnated in the guar gum gel. The majority of isolates presented MBEC values  $>1000 \mu\text{g ml}^{-1}$ , namely 65 % ( $n=15$ ) for nisin diluted in HCl and 87 % ( $n=20$ ) for nisin impregnated in guar gum gel (Table 1, Fig. 1c, d).

In the MBEC assay, before adding the Alamar Blue to the wells, cell growth was visually evaluated, and MBEC values were registered, for nisin diluted in HCl and for nisin incorporated in the guar gum gel. When compared to the MBEC values obtained after quantification using the Alamar Blue reduction formula (Pettit *et al.*, 2005), no significant differences were observed between results from both MBEC determination methods, neither for nisin diluted in HCl nor for nisin incorporated in guar gum gel ( $P \geq 0.05$ ).

### Guar gum gel viability assay

The effect of temperature and storage period on the antimicrobial activity of nisin incorporated in the guar gum gel was investigated using the agar diffusion method. Results

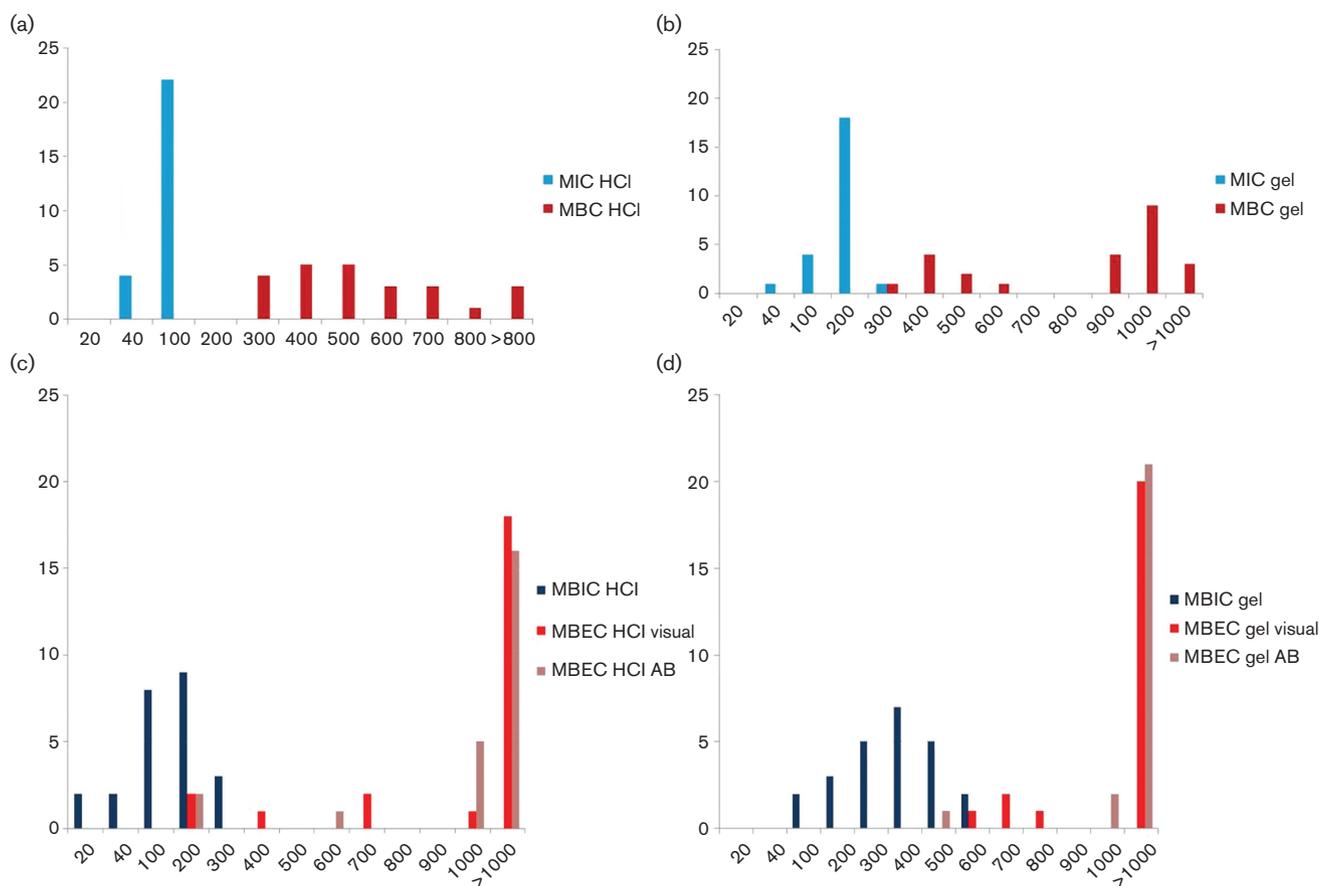
revealed that nisin kept its activity at all temperatures tested, from  $-18^\circ\text{C}$  to  $44^\circ\text{C}$ , during 6 months (Table 2).

## DISCUSSION

Multiple factors are involved in DFUs, namely neuropathy, abnormal foot biomechanics and peripheral arterial disease (Jeffcoate & Harding, 2003; Vuorisalo, 2009). Infection occurs following traumatic injury with introduction of pathogenic bacteria, mainly *S. aureus* (Mendes *et al.*, 2014; Mottola *et al.*, 2016a). Failure to recognize and control the infectious process may have devastating consequences, such as limb amputation, sepsis and even death (Lipsky *et al.*, 2004).

According to the European Centre for Disease Prevention and Control, MRSA has been the most important cause of antimicrobial-resistant, healthcare-associated infections worldwide, and Portugal is one of the European countries presenting higher rates of MRSA incidence (ECDC, 2015).

All *S. aureus* DFU isolates under analysis were previously characterized regarding their antimicrobial resistance profile (Mottola *et al.*, 2016b), observing that 35 % ( $n=8$ ) were



**Fig. 1.** MIC, MBC, MBIC and MBEC determinations ( $\mu\text{g ml}^{-1}$ ) for nisin diluted in HCl (a, c) and incorporated in guar gum (b, d) against *S. aureus* DFU isolates. The  $y$ -axis represents the number of isolates; and the  $x$ -axis, the nisin concentrations tested. AB, Alamar Blue.

**Table 2.** Guar gum gel viability assay: diameters of inhibition halos (mm) promoted by nisin incorporated in guar gum gel on BHI agar plates with  $10^7$  c.f.u. ml<sup>-1</sup> bacterial lawn cultures

T (°C)	Stored time (months)		
	1	3	6
-18	10.6±2.7	9.9±1.8	12.7±3.8
4	10.3±4.0	11.6±2.4	12.8±2.1
20	9.6±1.4	9.5±1.3	9.2±2.8
37	14.2±2.1	8.9±2.4	13.0±3.5
44	11.3±4.6	10.1±4.1	10.2±2.9

resistant to cefoxitin and carriers of the *mecA* gene and thus classified as MRSA (CLSI, 2013). Moreover, 22% ( $n=5$ ) were considered to be multidrug resistant, since they were resistant to three or more antimicrobials belonging to different antibiotic classes (Magiorakos & Srinivasan, 2012).

The biofilm mode of growth of the infecting organisms is another major contributor to the healing impediment of DFUs, since biofilm-based bacteria can resist antibiotic concentrations 10 to 10 000 times higher than those needed to kill planktonic cells (Kaplan, 2011). Besides their antimicrobial-resistant nature, all *S. aureus* strains evaluated in this study were able to create, under adequate conditions, a stable biofilm matrix in less than 24 h (Mottola *et al.*, 2016a).

Considering the overall clinical and economical burden caused by such virulent strains, it is of utmost importance to identify, develop or redesign effective alternative treatment regimens for DFUs. In recent years, AMP have attracted great interest in their potential use as new antibacterial agents mainly due to their high antibacterial activity and low AMP resistance development (Hancock & Sahl, 2006; Kirikae *et al.*, 1998; Rosenfeld *et al.*, 2006; Zasloff, 2002).

Nisin is one of these peptides, which is produced by *L. lactis* and possesses antimicrobial activity against a broad range of Gram-positive bacteria, including *S. aureus* strains. For that reason, it is regularly used for the control of pathogens in food products (Cleveland *et al.*, 2001). In fact, nisin (E234) is authorized for food preservation in the European Union by Directive 95/2/EC on food additives, and its acceptable daily intake is 0.13 mg (kg body weight)<sup>-1</sup> (EFSA, 2006).

Here, we set out to evaluate for the first time to our knowledge the ability of nisin to control a range of *S. aureus* DFU isolates when incorporated in guar gum, a natural galactomannan polymer, with the ultimate aim of identifying its efficacy as a topical delivery system for AMPs.

As results have shown, susceptibility to nisin was a characteristic of all *S. aureus* DFU clinical isolates studied. This group of bacteria includes, among others, eight MRSA isolates, five of which are also resistant to three or more antibiotic classes (Mottola *et al.*, 2016b).

Nisin presented high levels of antimicrobial activity toward planktonic bacteria, with MIC  $\leq 100$   $\mu\text{g ml}^{-1}$  and MBC 5.5 times higher. Since antimicrobial agents are usually classified as bactericidal if the MBC is no more than four times the MIC (French, 2006), our results showed that nisin is a bacteriostatic agent against *S. aureus* strains. However, since the MBC value is similar to the limit value used to classify an antimicrobial agent as bacteriostatic, its bactericidal potential cannot be disregarded, and nisin should be considered a valued AMP to kill free-floating bacteria.

When applied to biofilm cells, nisin MBIC values were  $\leq 300$   $\mu\text{g ml}^{-1}$ . Established biofilms were more difficult to eradicate, and only 35% of isolates presented MBEC values  $\leq 1000$   $\mu\text{g ml}^{-1}$ . These results are in agreement with some previous studies that have already analysed the *in vitro* activity of this AMP against biofilm-producing *S. aureus* strains (Okuda *et al.*, 2013). MBEC values were determined using two approaches, namely MBEC quantification according to the percentage of Alamar Blue reduction, which depends on bacterial cell metabolic viability (Pettit *et al.*, 2005), and the visual direct observation of microbial growth. No statistically significant differences were observed between these two approaches, suggesting that the visual direct observation of biofilm inhibition provides accurate MBEC determinations, avoiding the need for the application of a very expensive methodology. However, visual determinations should not be applied to rigorous cell metabolic activity determination.

Also, the natural polysaccharide guar gum displayed a very good efficacy as a delivery system for this peptide. In fact, nisin kept its antimicrobial activity toward *S. aureus* DFU strains when incorporated in the guar gum gel, with all strains presenting susceptibility to this AMP-delivery system combination. As observed in the MIC and MBIC determinations, the inhibitory activity of this AMP incorporated in guar gum was only twofold higher than the one from nisin diluted in HCl, proving that this delivery system acts not only in free-living cells but also in established biofilms. Similarly, MBC values of nisin incorporated in guar gum were less than twofold higher than those from nisin alone. As predicted, sessile bacteria were consistently more difficult to eliminate, and only 13% of pre-formed biofilms were eradicated by the concentrations used in this study.

Furthermore, nisin incorporated in guar gum maintained its antimicrobial activity when stored at a broad range of temperatures for a minimum of 6 months, which is probably due to the physical and chemical characteristics of the guar gum gel formulation (Reddy *et al.*, 2011; Thombare *et al.*, 2016). Besides its storage characteristics, the 0.75% (w/v) guar gum gel keeps its viscosity when applied to the human surface skin (data not shown), which shows its potential for topical therapeutic administration. Also, its eventual clinical application is strengthened by the fact that nisin minimum concentrations required to inhibit and eradicate planktonic cells and to inhibit biofilm cells are below nisin's acceptable daily intake, when the peptide is

either diluted in HCl or incorporated in the guar gum gel. Moreover, it is important to point out that Directive 95/2/EC on nisin (EFSA, 2006) was established for oral consumption. Considering that we are developing a jellified delivery system for topical application, we assume that the nisin-incorporated guar gum gel can be safely and effectively applied to clinical patients with DFUs.

In conclusion, results suggest that nisin has the ability to rapidly diffuse in the guar gum polymer and to inhibit and eradicate staphylococcal planktonic cells and established biofilms. This innovative therapeutic strategy may in the future substitute or complement antibiotic therapy, ultimately contributing to the decrease in multidrug-resistant bacteria dissemination. The use of guar gum gel as a delivery system for antimicrobial compounds can lead to the development of novel topical therapies for the treatment of generalized bacterial skin infections, particularly those promoted by pathogenic bacteria with reduced susceptibility to current antibiotic agents.

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