Activity and distribution of the antimicrobial peptides nisin and pexiganan in a Diabetic Foot Infection 3D model

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Abstract

Diabetic Foot Ulcers (DFU) constitute one of the major and devastating complications of Diabetes *mellitus*. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the most important pathogenic agents isolated from infected DFU and their increased resistance to traditional antibiotic-based treatments prompts the development of new therapeutic alternatives, with antimicrobial peptides (AMP) being a promising strategy.

A three-dimensional (3D) collagen model was developed aiming at mimicking the ulcer microenvironment, to evaluate the inhibitory potential of a guar gum biogel supplemented with nisin, pexiganan and the antibiotic (AB) gentamicin, being tested individually and in several combinations. Results retrieved from the collagen 3D model allowed to observe that the guar gum gel supplemented with nisin plus pexiganan was able to eradicate one of the clinical isolates present in the model. Several combinations including AMP, AB and AMP plus AB presented an inhibitory activity against one of the isolates, but none of them allowed its eradication from the model, being required further studies in order to develop new antimicrobial alternatives.

In conclusion, the dual AMP biogel constitutes a promising alternative or complement to antibiotic-based therapy, for topical application in diabetic foot infection (DFI) treatment.

Key-words: antimicrobial peptides; biofilm; collagen model; diabetic foot ulcer; *Pseudomonas aeruginosa*; *Staphylococcus aureus*.

1. Introduction

Diabetic foot ulcers (DFUs) constitutes one of the most frequent complications of diabetes [1,28] and *Staphylococcus aureus* is considered the most prevalent bacterial species isolated from infected DFU, followed by *Pseudomonas aeruginosa*. They are frequently co-isolated [2,3] and produce biofilms structures [4,5,27], increasing their resistance to conventional treatments, namely antibiotics, impairing diabetic foot infection (DFI) treatment. Therefore, the development of new alternatives is required [6,29].

Antimicrobial peptides (AMP) are produced by all living organisms [7], with a broad action spectrum [8,9], acting through bactericidal activity and immunomodulatory effect, constituting a promising alternative [9,10].

Two examples of AMP are nisin and pexiganan. Nisin is produced by Gram-positive bacteria, namely *Lactococcus lactis* [11,12] and acts through the connection with lipid II, inhibiting the cell wall synthesis or through pore formation, killing the bacteria [13-15]. Regarding pexiganan, an analog from magainin, it presents a broad action range, namely Gram-positive and Gram-negative bacteria, acting through pore formation on the cytoplasmatic membrane [16].

Several studies have demonstrated that the efficacy of individual lantibiotics [11,17] can be enhanced by their combination with other AMP [17,18], presenting three types of possible interactions [19,20]. Also, it is known that AMP and antibiotics have been combined in a synergistic form, in order to increase their action against bacterial species [12].

In spite of the potential of AMP, their inhibitory action can be affected by their inhibition or degradation before it reaches the target zone at therapeutic concentrations. Therefore, to guarantee its successful action, it is necessary to find a proper delivery system [21,22], such as guar gum [22,23].

Several *in vitro* studies have been performed to evaluate new possibilities for DFI treatment. These studies allow an improved understanding of the product in study, being essential before *in vivo* studies. For DFI, the studies performed so far were, generally, based in microtiter plates protocols used for evaluating the effect of a potential treatment in test, including of nisin incorporated in a guar gum

gel, in a two-dimensional model (2D) [24]. Therefore, it is necessary to evaluate the characteristics of the same product in a representative three-dimensional model (3D) aiming at mimicking the *in vivo* conditions. This model will allow to mimetize the environment conditions present in DFI, allowing to evaluate bacteria dissemination and treatments efficacy in deeper tissues. For the construction of 3D models, one material that can be used is collagen [1,25].

The main aim of this work was to establish an *in vitro* collagen-based three-dimensional DFI model, to evaluate the inhibitory activity of the guar gum gel supplemented with antimicrobial compounds, alone and in combination against selected DFI isolates present in the 3D DFI model.

This model allowed to study the AMP efficacy in *in vitro* conditions that better mimetize *in vivo* conditions, representing a further step in the evaluation of the therapeutic potential of these AMP to be applied as an alternative or as a complementary therapy to antibiotherapy in DFI treatment.

2. Material and Methods

2.1. Bacterial isolates

Two DFI clinical isolates that belong to a collection previously obtained from DFU samples [2] were used. These isolates were already characterized [2,3,26].

2.2. Antimicrobial Peptides and Antibiotic preparation

Pexiganan solution

A stock solution of pexiganan (Innovagen, Sweden) was used, and provided by Castanho's Laboratory at the Institute of Molecular Medicine (IMM), in Lisbon.

Nisin solution

A stock solution of nisin (Sigma-Aldrich, USA) was prepared and stored at 4ºC until further use [24].

Antibiotic preparation

The stock solution of gentamicin (ITW Reagents; Italy) was previously prepared according to the manufacturer PanReac Appli Chem. and kept at -80ºC until use.

Guar gum gel

A guar gum gel of 1.5% (w/v) was prepared. Before the assays, dilutions of nisin, pexiganan and antibiotic were incorporated within the gel in a proportion of 1:1.

2.3. Establishment of a Collagen DFI 3D Model

For the establishment of the DFI 3D model, a collagen suspension were prepared using Collagen I High Concentration from rat tail (Corning, US), cold Simulated Wound Fluid (SWF), being composed by 50% of fetal bovine serum (FBS; biowest; France) plus 50% of peptone water (PW; Biokar Diagnostics; France), acetic acid at 0.1% (Sigma-Aldrich; USA) and sodium hydroxide at 0.1M (NaOH, Merck; Germany), with a final pH of 7.5 (Macherey-Nagel; Germany) (Price *et al*., 2016).

The system used to reproduce the collagen ulcer model was composed by a 6 well-plate (Corning; Falcon, USA), in which an insert (High Density translucent PET Membrane, 6 well 3.0 µm pore size; Corning, Falcon; USA) was placed in the wells. A volume of the collagen suspension previously prepared was placed in the insert, and afterwards a peg-lid, previously washed and sterilized was placed on the plate, followed by incubation to allow collagen polymerization (Price *et al*., 2016).

2.3.1. Evaluation of Inhibitory Potential of the AMP/Antibiotic biogel in a DFI 3D model

The evaluation of the inhibitory activity of antimicrobial solutions was performed with different incubation periods, namely one with a single antimicrobial solution and other assay with intervals of antimicrobial solution.

For both incubation periods, after polymerization of the collagen model (2.3.), bacterial suspension was added to the model after which the plate was incubated to allow bacterial diffusion.

In the first assay, after incubation, an antimicrobial solution was added to the insert, following incubation. Afterwards, bacterial quantification was performed.

In the assay performed with antimicrobial solution intervals, after incubation, an antimicrobial solution was added to the insert, following incubation. Afterwards, another antimicrobial solution was added to the insert, after removing a volume of the inoculated SWF present in the well, that was used for bacterial quantification. The 6-well plate were posteriorly incubated. This process was repeated one more time, after which bacterial quantification was performed.

Evaluation of the inhibitory activity of antimicrobial biogel using the 3D DFI model

Bacterial quantification was performed in the liquid and solid phases of the collagen model of both assays. For the bacterial quantification in the liquid phase, a volume of the inoculated SWF was removed from the well, and 10-fold serial dilutions were performed. Regarding the solid phase, it was performed after sectioning the collagen model into three areas. Afterwards, each area was placed in falcons, to which a volume of collagenase solution was added, followed by incubation [1]. Then, each suspension was centrifuged (HERMILE Z383K) to obtain the pellet [25]. Then, a volume of the resuspended pellet was 10-fold serial diluted.

For both phases, a volume of each bacterial dilution was inoculated in TSA plates in duplicate and posteriorly incubated. After incubation, bacterial colonies were quantified.

The inhibitory potential evaluation occurred for the AMP nisin and pexiganan and for the antibiotic gentamicin. The experiments were performed in duplicate.

2.3.2. Histochemical Evaluation of the supplemented biogel inhibitory activity in the DFI 3D Model

Bacteria and antimicrobial solutions diffusion were also evaluated using the collagen 3D model by histochemical analysis These protocols were performed with the collaboration of the Laboratory of Pathology of the Faculty of Veterinary Medicine of the Lisbon University.

Statistical Analysis

The average and standard deviation of the results obtained from the collagen models were determined using Microsoft Office Excel 2016.

3. Results

3.1. Evaluation of the inhibitory activity of antimicrobial solutions using a DFI 3D model

The inhibitory activity of antimicrobial solutions against the clinical isolates was determined according to the performed assays (Table 1).

Table 1. Evaluation of antimicrobial solutions inhibitory potential for the clinical isolates (individually and for a dual inoculum) using a DFI 3D model (results in average according to the performed assays).

Evaluation of antimicrobial solution biogel inhibitory potential using a DFI 3D model (CFU/mL)							
Antimicrobial biogel solution	**Bacterial Strains**		**Before Antimicrobial addition**	**Liquid Phase**	**Solid Phase**		
					Area 1	**Area 2**	**Area 3**
Nisin	Clinical isolate		4.2×10^8	3.7×10^7	8.3×10^5	1.7×10^6	5.1×10^6
			4.2×10^8	2.9×10^6	2.6×10^4	9.2×10^4	1.9×10^5
Pexiganan	Clinical isolate		1.0×10^9	1.9×10^9	2.8×10^8	1.6×10^8	3.2×10^8
	Clinical isolate		2.2×10^8	1.1×10^8	7.5×10^6	1.3×10^7	1.5×10^6
	Clinical isolate		5.0×10^8	Uncountable	1.7×10^8	9.0×10^7	3.3×10^8
Dual AMP	**Dual inoculum**	Clinical isolate	5.2×10^7	0	0	0	0
		Clinical isolate	3.0×10^8	1.3×10^9	3.6×10^7	1.1×10^8	1.1×10^8
Gentamicin	Clinical isolate		8.0×10^7	2.6×10^8	6.6×10^7	1.4×10^8	7.0×10^7
	Clinical isolate		6.5×10^9	6.0×10^9	1.4×10^8	1.7×10^8	5.6×10^8
	Clinical isolate		1.6×10^8	1.0×10^8	8.7×10^6	1.2×10^7	3.6×10^7
	Clinical isolate		7.0×10^8	4.5×10^8	8.5×10^7	8.8×10^7	7.8×10^7
	Dual inoculum	Clinical isolate	8.5×10^6	1.1×10^7	7.5×10^6	4.1×10^7	1.5×10^7
		Clinical isolate	6.2×10^8	1.1×10^9	5.2×10^7	3.0×10^8	Uncountable
Nisin plus Pexiganan plus Gentamicin	**Dual inoculum**	Clinical isolate	3.0×10^7	5.0×10^6	1.9×10^6	8.5×10^5	6.0×10^6
		Clinical isolate	1.7×10^9	1.4×10^9	2.5×10^8	1.8×10^8	1.8×10^8

Legend. **AMP**: Antimicrobial Peptides; **AB:** Antibiotic; **Areas of the model:** Area 1, Area 2, Area 3; **Uncountable:** plates without possibility of counting/number of colonies superior to 300.

Regarding the evaluation of the inhibitory potential of nisin biogel using a DFI 3D model (Table 1), the bacterial diffusion occurred across the collagen 3D model. However, in the first assay, the bacterial concentration increased from Area 1 (8.3×10^5 CFU/mL) to Area 2 (1.7×10^6 CFU/mL), stabilizing in Area 3 (5.1×10^6 CFU/mL). The same was observed when the AMP was added to the model 3 times. Nevertheless, it is important to refer that in this assay bacterial counts were always lower than in the first assay, as follows: 2.6×10^4 CFU/mL (Area 1); 9.2×10^5 CFU/mL (Area 2); and 1.9×10^5 CFU/mL. Considering the liquid phase of each assay, it was possible to observe a high bacterial concentration, which demonstrated diffusion from the insert to the well.

The evaluation of pexiganan biogel inhibitory potential demonstrated bacterial diffusion across the collagen 3D model (Table 1) for both assays. In the first assay with a clinical isolate, the bacterial concentration was similar among the three areas of the collagen model, as follows: Area 1 (2.8×10^8 CFU/mL); Area 2 (1.6×10^8 CFU/mL) and Area 3 (3.2×10^8 CFU/mL); regarding the assay with AMP addition within intervals, for the same isolate, the bacterial counts decreased from Area 1 (1.7×10^8 CFU/mL) to Area 2 (9.0×10^7 CFU/mL), increasing in Area 3 (3.3×10^8 CFU/mL), in comparison with the first assay. Nevertheless, for the other clinical isolate, the bacterial concentration decreased in the three areas in comparison with the bacterial concentration before the AMP addition, as follows: Area 1 (7.5×10^6 CFU/mL); Area 2 (1.3×10^7 CFU/mL) and Area 3 (1.5×10^6 CFU/mL). Considering the liquid phase of each assay, it allowed to observe a high bacterial concentration, demonstrating diffusion from the insert to the well.

Regarding the results of the inhibitory potential of the dual AMP biogel (Table 1), it presented a high impact on one of the clinical isolates, since it allowed its eradication in the collagen model. Nevertheless, the other isolate diffused across the three areas of the collagen model and although the AMPs presented some inhibitory activity against this strain, it was not high enough to eradicate it.

Considering the evaluation of gentamicin biogel inhibitory potential (Table 1) using a DFI 3D model, the bacterial diffusion occurred across the collagen 3D model, the bacterial concentration of one of the isolates increased from Area 1 (6.6×10^7 CFU/mL) to Area 2 (1.4×10^8 CFU/mL), decreasing in Area 3 (7.0×10^7 CFU/mL). For the other isolate, the bacterial concentration among the three areas presented some variability, as follows: 1.4×10^8 CFU/mL (Area 1); 1.7×10^8 CFU/mL (Area 2) and 5.6×10^8 CFU/mL (Area 3); in the second assay, the results were similar. Nevertheless, the gentamicin biogel presented a higher inhibitory activity against one of the isolates without promoting its complete eradication. In fact, for this isolate, the bacterial concentration presented a ten-fold decrease in Area 1; concerning the dual inoculum and comparing both isolates, it was also observed that gentamicin biogel does not present an effective inhibitory activity against these two isolates. Regarding the liquid phase of both assays, it was possible to observe a high bacterial concentration of both isolates as well as in the dual inoculum, demonstrating that occurred diffusion between the insert and the well.

The evaluation of inhibitory potential of the multiple combination biogel composed by nisin, pexiganan and gentamicin (Table 1), it was possible to observe a higher decrease of bacterial concentration of one of the isolates. Bacterial diffusion occurred across the collagen model, with bacterial concentrations decreasing ten to twenty-fold. Regarding one of the isolates, the bacterial concentration was maintained throughout the model, with a ten-fold decrease in all areas, being also in higher concentration in the liquid phase.

Therefore, these results confirmed that both bacterial species selected for this study are able to diffuse across the collagen 3D model and demonstrated that the triple combination of nisin plus pexiganan plus gentamicin biogel did not allow the complete inhibition of the dual inoculum.

3.2. Histochemical Evaluation of the inhibitory activity of antimicrobial biogel solutions in the DFI 3D Model

Regarding the results of histochemical analysis (Figure 1), it allowed to observe the clinical isolates individually and in combination in the three areas of the collagen model through the staining protocols.

In conclusion, this type of analysis allowed to confirm the results obtained in bacterial quantification.

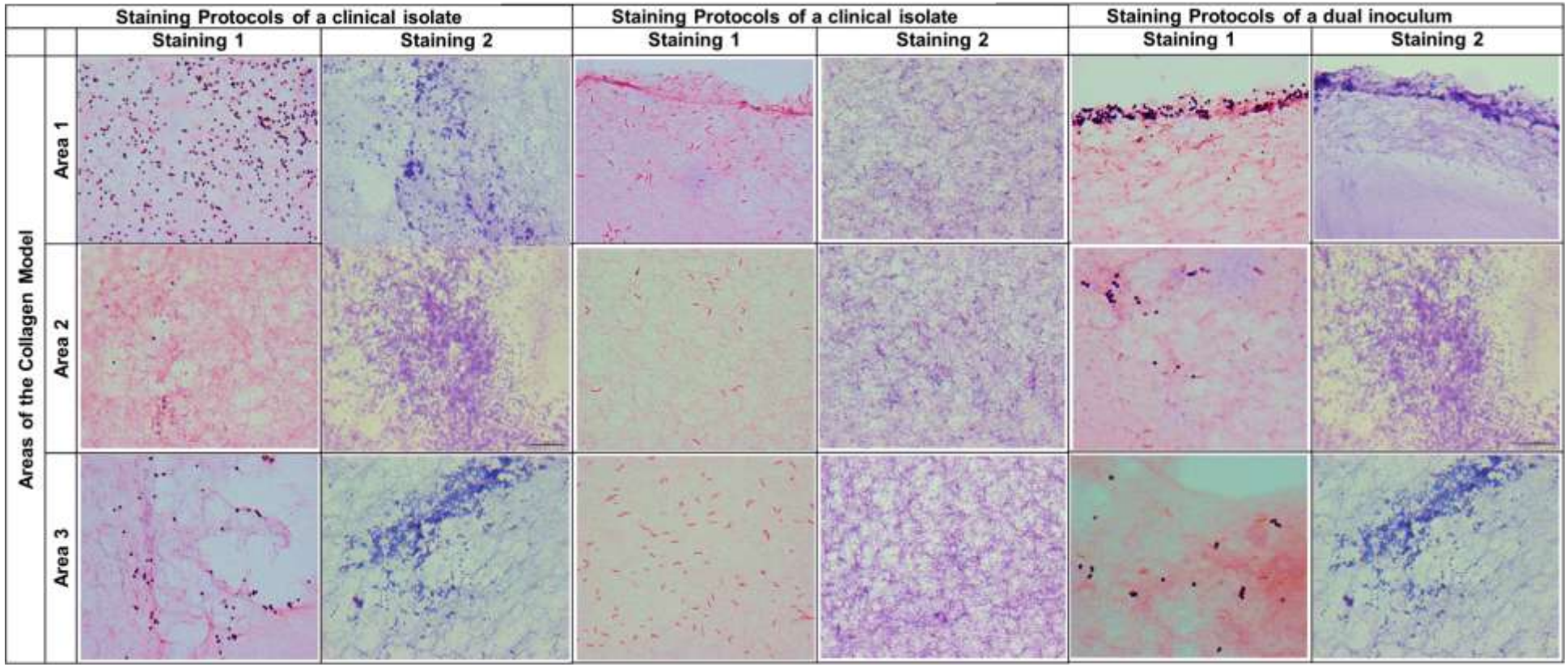

Figure 1. Evaluation of the clinical isolates individually and in a dual inoculum. through histochemical analysis. **Legend**. **Areas of the model:** Area 1, Area 2, Area 3 (Original, 1000x).

4. Discussion/Conclusion

The development of a three-dimensional (3D) representation of an ulcer [1] was considered important to evaluate several parameters that are directly related with the success of DFI treatment, such as the diffusion of bacteria, of antimicrobial peptides (AMP) and of antibiotics. Therefore, a 3D ulcer model aiming at better mimetizing the *in vivo* conditions of a diabetic foot ulcer (DFU) was developed using collagen.

The 3D model was established using collagen due to several properties [30-34], allowing to evaluate the distribution and the inhibitory activity of a guar gum gel supplemented with antimicrobial compounds, tested individually or in several combinations, through the collagen 3D model.

The inhibitory activity of nisin biogel was evaluated by mimicking its application to a DFI infected with a clinical isolate. Two protocols were tested. Some variability was found across the 3D model, which could be due to the bacteria diffusion ability bacteria, to the AMP action and to the properties of the collagen matrice [33]. In general, a lower concentration of bacteria was detected in the three areas of the collagen model in the second assay. These results were expected, since a high AMP concentration was present in the model, namely in Area 1 that was four times higher in this assay in comparison with the first assay. Also, the bacterial concentration was lower in comparison with the results from the bacteria diffusion evaluation assay (without AMP), with a 10 to 20-fold decrease of bacterial concentration, demonstrating the inhibitory effect of nisin biogel.
The final aim of the development of this gel is its topical application to infected mucosa. At the concentration used in the collagen model, the application of this supplemented guar gum gel can be considered to have a low toxic potential, considering the acceptably daily intake (ADI) of 0 to 2 mg/kg defined by WHO and FAO in 2013. This ADI value was determined for the oral administration of nisin [37], but they can be extrapolated for its topical application to DFI, considering that the absorption by digestive tract mucosa is similar [24]. Moreover, the widely application of nisin as food preservative suggests that it could be safely used not only in food industry as well as in the clinical setting [11,36]. Therefore, the application of nisin incorporated in a guar gum gel with the aim of topical application to DFI could be considered safe and effective for DFI patients [24].

Considering the pexiganan inhibitory ability regarding other clinical isolate, a single addition of pexiganan followed by incubation did not allow the eradication of this isolate from the model. Pexiganan is an AMP with inhibitory activity against Gram-positive and Gram-negative bacteria. However, as previously referred, the cell wall composition of Gram-negative bacteria is more complex in comparison with Gram-positive bacteria, which may influence pexiganan action [10,38,39], together with the presence of an increased Lipid A concentration [40] or with modifications on Lipid A [41]. The inhibitory potential of pexiganan against both clinical isolates was also evaluated. Results suggested that pexiganan biogel presented a higher inhibitory activity against one of the isolates. This may be due to not only to the fact that the interaction between bacteria and the AMP depends on the bacterial groups, since in Gram-positive bacteria it occurs through teichoic acids and in Gram-negative bacteria occurs through the LPS present in the outer membrane, but also it could be due to differences in the biofilm production mechanisms. In a bacterial species, biofilm production involves the presence of alginate, which impairs the AMP action as previously referred [41]. Additionally, pexiganan may not have been able to correctly align with the bacterial membrane surface and promoting a rearrangement between the bacterial membrane and the AMP, not allowing its intake and consequently the formation of pores in the cytoplasmic membrane [8,10].

Afterwards, a guar gum simultaneously supplemented with nisin and pexiganan was prepared, aiming at producing a biogel with increased antimicrobial potential, being tested against the two bacterial species under study. The dual AMP biogel was added to the polymicrobial DFI model three times, being observed that it presented an effective inhibitory action against one of the isolates, promoting the eradication of this strain in all areas of the model, which allowed to confirm the higher inhibitory effect of this dual AMP biogel [19,20]. However, the other clinical isolate was not eradicated from the ulcer model, as it remained in the three areas of the collagen model. Therefore, considering the dual AMP solution composition, only pexiganan acts against Gram-negative bacteria whereas against Gram-positive bacteria, both nisin and pexiganan present antimicrobial activity [24,42], constituting an explanation for the

results. Additionally, one of the bacterial species presents a higher survival ability which could be due to its ability to produce toxins that can also inhibit another bacterial species [43], as well as its cell wall properties and biofilm production ability, as previously referred [41].

Therefore, a final assay was performed, with the further incorporation of an antibiotic in the guar gum gel aiming at promoting the eradication of one of the isolates from the model. Gentamicin was the antibiotic chosen for this assay, as it constitutes a promising treatment for topical application to DFI treatment [44]. The inhibitory activity of a gentamicin biogel in the collagen DFI 3D model was evaluated, being observed that this biogel did not present a high antibacterial effect, particularly against this isolate. This antibiotic acts through the inhibition of protein synthesis [45], being effective against Gram-negative and Gram-positive bacteria [35,45]. However, this bacterial species resistance to aminoglycosides was already described and related to the presence of the outer membrane, since it presents low permeability acting as a selective barrier, impairing its action [46]. When gentamicin biogel was applied three times, the results were similar to the previously described as also observed in the assay aiming at evaluating its inhibitory effect against a dual inoculum.

Finally, a guar gum biogel supplemented with Nisin, Pexiganan and Gentamicin was prepared, aiming at evaluating its distribution in the collagen DFI 3D model, as well as its inhibitory potential against a polymicrobial ulcer. In this assay, it was possible to observe a lower concentration of one of the isolates in all areas of the model, demonstrating the inhibitory potential of this combination against Gram-positive bacteria [3,24,42,44]. However, regarding the remaining isolate, the inhibitory activity of the multiple supplementation of the biogel with the two AMP plus the Antibiotic did not present relevant improvements, since it did not allow the inhibition of this species.

Although several studies have demonstrated that the combination of AMP with antibiotics promote an enhanced action [18], the combination of nisin plus pexiganan plus gentamicin was not studied until now. Results similar to the ones obtained with the dual AMP biogel were expected; however, since the eradication of both bacterial species was not observed, the results suggested that the addition of gentamicin could have had an antagonist impact on the multiple guar gum biogel, since a lower inhibitory activity was observed as it did not eradicate one of the isolates [20]. Therefore, other antibiotics must be evaluated in further work.

The collagen 3D models from all assays were also evaluated by histochemical analysis, confirming the results obtained in the bacterial quantification process.

This work represents a novelty regarding the other studies [1,25], since it was not only based on the evaluation of bacterial diffusion across the collagen model but also focused on the study of new potential alternatives to conventional DFI treatments. In conclusion, the 3D representation of an ulcer is an important step in order to obtain a better understanding of the bacteria diffusion in the DFU environment *in vivo* [1,25], aiming at the development and testing of new alternatives to conventional treatments [2]. In this work, the study of the inhibitory potential of a biogel supplemented with several antimicrobial combinations, including AMP and the antibiotic gentamicin, was evaluated using a 3D collagen model. In spite the supplemented biogel ability to eradicate one of the bacterial species present in the 3D collagen ulcer model, further studies are required to develop new strategies for the other bacterial species and biofilm eradication.

It is important to refer that a 0.8% pexiganan acetate cream (Locilex®) has already been subjected to clinical trials aiming at its clinical application, however, its approval failed [47]. Our results suggest that the further supplementation of this cream with a complementary AMP, such as nisin, may allow to increase its inhibitory potential. Finally, the 3D representation of an ulcer allowed to understand the bacterial diffusion as well as the AMP and antibiotic diffusion *in vitro*, constituting an important tool aiming at the development of innovative DFI treatment strategies.

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References

1. Price, B. L., Lovering, A. M., Bowling, F. L & Dobson, C. B. Development of a novel collagen wound model to simulate the activity and

distribution of antimicrobials in soft tissue during diabetic foot infection. *Antimicrob Agents Chemother*. (2016); 60:6880–6889.
2. Mendes, J. J., Marques-Costa, A., Vilela, C., Neves, J., Candeias, N., Cavaco-Silva, P. & Melo-Cristino J. Clinical and bacteriological survey of diabetic foot infections in Lisbon. *Diab Res Clin Pract.* (2012); 95, 153–161.
3. Mottola, C., Matias, C. S., Mendes, J. J., Melo-Cristino, J., Tavares, L., Cavaco-Silva, P. & Oliveira, M. Susceptibility patterns of *Staphylococcus aureus* biofilms in diabetic foot infections. *BMC Microbiol.* (2016); 16:119.
4. Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. (2002); 15, 167–193.
5. Banu, A., Hassan, M., Rajkumar, J. & Srinivasa, S. Spectrum of bacteria associated with diabetic foot ulcer and biofilm formation: a prospective study. *AMJ*. (2015); 8, 280-285.
6. Kumar, P., Kizhakkedathu, J. N. & Straus, S. K. Antimicrobial Peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. *Biomolecules*. (2018); 1-24.
7. Baltzer, S. A. & Brown, M. H. Antimicrobial peptides-promising alternatives to conventional antibiotics. *J Mol Microbiol Biotechnol.* (2011); 20, 228–235.
8. Hancock, R. E. W. Cationic peptides: Effectors in innate immunity and novel antimicrobials. *Lancet Infect Dis.* (2001); 1, 156–164.
9. Moual, L. H., Thomassin, J. L. & Brannon, J. R. Antimicrobial peptides as an alternative approach to treat bacterial infections. *J Clin Cell Immunol*. (2013); S13.
10. Gottler, L. M. & Ramamoorthy, A. Structure, membrane orientation, mechanism, and function of pexiganan – a highly potent antimicrobial peptide designed from magainin. *Biochim. Biophys. Acta - Biomembr.* (2009); 1788, 1680–1686.
11. Shin, J. M., Gwak, J.W., Kamarajan, P., Fenno, J.C., Rickard, A. H. & Kapila, Y. L. Biomedical applications of nisin. *J Appl Microbiol.* (2015); 1364-5072.
12. Field, D., O' Connor, R., Cotter, P. D., Ross, R. P. & Hill, C. *In vitro* activities of nisin and nisin derivatives alone and in combination with antibiotics against *Staphylococcus* biofilms. *Front Microbiol.* (2016); 7, 508.
13. Breukink, E. & Kruijff, B. Lipid II as a target for antibiotics. *Nat Rev Drug Discov.* (2006); 1-12.
14. Okuda, K., Zendo, T., Sugimoto, S., Iwase, T., Tajima, A., Yamada, S., Sonomoto, K. & Mizunoe, Y. Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* biofilm. *Antimicrob Agents Chemother.* (2013); 57, 5572–5579.
15. Kramer, N. E., Smid, E. J., Kok, J., Kruijff, B., Kuipers O. P. & Breukink, E. Resistance of Gram-positive bacteria to nisin is not determined by Lipid II levels. *FEMS Microbiol Lett.* (2004); 239, 157–161.
16. Gopinath, D., Kumar, M. S., Selvaraj, D. & Jayakumar, R. Pexiganan-incorporated collagen matrices for infected wound-healing processes in rat. *J Biomed Mater Res*. (2005); 320-331.
17. Cavera, V. L., Arthur, T. D., Kashtanoc, D. & Chikindas, M. L. Bacteriocins and their position in the next wave of conventional antibiotics. *Int J Antimicrob Agents*. (2015); 46, 494-501.
18. Grassi, L., Maisetta, G., Esin, S. & Batoni, G. Combination strategies to enhance the efficacy of antimicrobial peptides against bacterial biofilms. *Front Microbiol*. (2017); 8:2409.
19. Worthington, R. J. & Melander, C. Combination approaches to combat multi-drug resistant bacteria. *Trends Biotechnol*. (2013); 31, 177-184.
20. Yu, G., Baeder, D. Y., Regoes, R. R. & Rolff, J. Combination effects of antimicrobial peptides. *Antimicrob Agents Chemother.* (2016); 60, 3: 1717-1724.
21. O'Driscoll, N.H., Labovitiadi, O., Cushnie, T.T., Matthews, K.H., Mercer, D.K. & Lamb, A.J. Production and evaluation of an antimicrobial peptide-containing wafer formulation for topical application. *Curr Microbiol*. (2013); 66(3), 271–278.
22. Reddy, K., Mohan, G. K., Satla, S. & Gaikwad, S. Natural polysaccharides: versatile excipients for controlled drug delivery systems. *AJPS*. (2011); 6 (6): 275-286.
23. Thombare, N., Jha, U., Mishra, S. & Siddiqui, M. Z. Guar Gum as a promising starting material for diverse applications: a review. *Int J Biol Macromol.* (2016); 88, 361–372.
24. Santos, R., Gomes, D., Macedo, M., Barros, D., Tibério, C., Veiga, A. S., Tavares, L., Castanho, M. & Oliveira, M. Guar gum as a new antimicrobial peptide delivery system against diabetic foot ulcers *Staphylococcus aureus* isolates. *J Med Microbiol*. (2016); 65, 1092–1099.
25. Werthén, M., Henriksson, L., Jensen, P, Ø., Sternberg, C., Givskov, M. & Bjarnsholt, T. An *in vitro* model of bacterial infections in wounds and other soft tissues. *APMIS*. (2010); 118: 156-164.
26. Mottola, C., Mendes, J. J., Melo Cristino, J., Cavaco-Silva, P., Tavares, L. & Oliveira, M. Polymicrobial biofilms by diabetic foot clinical isolates. *Folia Microbiol.* (2015); 61:35–43.
27. Arciola, C. R., Campoccia, D., Speziale, P., Montanaro, L. & Costerton, J. W. Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials*. (2012); 33, 5967-5982.
28. Jneid, J., Lavigne, J. P., La Scola, B. & Cassir, N. The diabetic foot microbiota: A review. *Human Microbiome Journal*. (2017); 1–6.
29. Wiegand, I., Hilpert, K. & Hancock, R. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc*. (2008); 3, 163.

30. Ruszczak, Z. & Friess, W. Collagen as a carrier for on-site delivery of antibacterial drugs. *Adv Drug Deliv Rev*. (2003); 55(12):1679-98.
31. Fleck, C. A. & Simman, R. Modern collagen wound dressings: function and purpose. *J Am Col Certif Wound Spec*. (2010); 2, 50–54.
32. Gorgieva, S. & Kokol, V. Collagen- vs. gelatine-based biomaterials and their biocompatibility: review and perspectives. *InTech*. (2011); 17-52.
33. Antoine, E. E., Vlachos, P. P. & Rylander, M. N. Review of collagen I hydrogels for bioengineered tissue microenvironments: characterization of mechanics, structure and transport. *Tissue Eng*. (2014); 20,6: 683-696.
34. Walters, B. D. & Stegemann, J. P. Strategies for directing the structure and function of 3D collagen biomaterials across length scales. *Acta Biomater*. (2014); 10(4):1488-1501.
35. Duarte, N. & Gonçalves, A. Pé diabético. *Angiologia e Cirurgia Vascular*. (2011); 7:2.
36. Fernández, L., Delgado, S., Herrero, H., Maldonado, A. & Rodríguez, J. M. The bacteriocin nisin, an effective agent for the treatment of staphylococcal mastitis during lactation. *J Hum Lact*. (2008); 24(3):311-316
37. World Health Organization (WHO) & Food and Agriculture Organization (FAO). WHO FOOD ADDITIVES SERIES: Safety evaluation of certain food additives and contaminants/ prepared by the Seventy-seventh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA); (2013); 91-96.
38. Goering, R. V., Dockrell, H. M., Zuckerman, M., Roitt, I. M., & Chiodini, P. L. Mims 'Medical Microbiology. Fifth Edition. *Elsevier Saunders*. (2013c); 2; 7-9.
39. Schmidtchen, A., Pasupuleti, M. & Malmsten, M. Effect of hydrophobic modifications in antimicrobial peptides. *Adv Colloid Interface Sci*. (2014); 1-41.
40. Erridge, C., Benett-Guerrero, E. & Poxton, I. R. Structure and function of lipopolysaccharides. *Microbes Infect*. (2002); 4, 837–851.
41. Guilhelmelli, F., Vilela, N., Albuquerque, P., Derengowski, L., Silva-Pereira, I. & Kyaw, C. Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. *Front Microbiol*. (2013); 1-12.
42. Ge, Y., MacDonald, D. L., Holroyd, K. J., Thornsberry, C., Wexler, H. & Zasloff, M. In vitro antibacterial properties of pexiganan, an analog of magainin. *Antimicrob Agents Chemother*. (1999); 43, 4: 782–788.
43. Nair, N., Biswas, R., Götz, F. & Biswas, L. Impact of *Staphylococcus aureus* on pathogenesis in polymicrobial infections. *Infect Immun*. (2014); 82 (6): 2162-2169.
44. Lipsky, B. A., Kuss, M., Edmonds, M., Reyzelman, A. & Sigal, F. Topical Application of a Gentamicin-Collagen Sponge Combined with Systemic Antibiotic Therapy for the Treatment of Diabetic Foot Infections of Moderate Severity. *J Am Podiatr Med Assoc*. (2012); 102 (3): 223-232.
45. Goering, R. V., Dockrell, H. M., Zuckerman, M., Roitt, I. M. & Chiodini, P. L. Mims 'Medical Microbiology. Fifth Edition. *Elsevier Saunders*. (2013b); 33; 449-464.
46. Breidenstein, E., Fuente-Nunez, C. & Hancock, R. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microb*. (2011); 19 (8): 419-426.
47. Gomes, A., Teixeira, C., Ferraz, R., Prudêncio, C. & Gomes, P. Wound-Healing Peptides for Treatment of Chronic Diabetic Foot Ulcers and Other Infected Skin Injuries. *Molecules*. (2017); 1-18.