Antibiofilm Activity of Antimicrobial Compounds from Different Classes Against Diabetic Foot Staphylococci

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Abstract

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide and its role as a major risk factor for the development of Diabetic Foot Ulcers (DFU) is well stablished. DFU are vulnerable to opportunistic infections, being that gram-positive bacteria, such as *Staphylococcus aureus*, is the most frequent microorganism isolated. Due to the emergence of drug resistant bacteria that could impair its successful treatment, it is urgent to find new therapeutics protocols that could be an alternative to the current antibiotherapy. This has aroused the interest in antimicrobial peptides (AMPs) and biocides to complement the administration of antibiotics.

It was tested the antimicrobial potential of chlorhexidine against 23 biofilm-producing strains of *S. aureus* isolated from DFU, determining minimum inhibitory (MIC) and bactericidal (MBC) concentrations. Afterwards, creating different treatments between chlorhexidine MIC, nisin incorporated in guar gum gel and antibiotics (clindamycin, gentamycin and vancomycin) biofilm inhibitory and eradication action were analyzed.

Results suggest that chlorhexidine have a good antimicrobial effect even in low concentrations, evidencing a bactericidal effect in most isolates.

The treatment with better inhibitory action against biofilm was nisin incorporated in guar gum gel combined with chlorhexidine, followed by this same treatment combined with clindamycin. Regarding eradication action of treatments, overall results were quite similar, being that vancomycin combined with chlorhexidine had the best effect. These results highlight the potential of nisin incorporated in guar gum gel and chlorhexidine as a substitute or as complementary to antibiotherapy, mainly for inhibition of biofilm in Diabetic Foot Infections (DFI).

Key-words: Diabetic Foot Ulcer, *Staphylococcus aureus*, Chlorhexidine, Nisin, Guar Gum Gel, New therapeutics

1. Introduction

Diabetes *mellitus* (DM) is a serious health problem in rapid expansion worldwide.[1] According to the International Diabetes Federation, the prevalence of diabetes is estimated to be 415 million globally, being expected to alarmingly rise to 642 million by 2040, which represents a significant rise over a small time period.[2]

The rise in the prevalence of DM is leading to an increasing problem of infections, especially foot ulcer infections, which are potentially serious. Many organisms, alone or in multispecies communities can cause diabetic foot infections (DFI).[3] *Staphylococcus aureus* is the most commonly isolated from these ulcers, either alone or as a component of mixed infections.[4,5]

S. aureus is considered the most important human pathogen among staphylococci, causing a wide range of clinical infections.[6] Although is

usually regarded as a transient microorganism in the skin, approximately 50% of the general population are either permanently or intermittently colonized in the nasal mucosa without any pathogenic event. The ability to acquire resistance to antibiotics from multiples classes makes *S. aureus* a challenging pathogen to eliminate.[7] This microorganism developed resistance to methicillin due to acquisition of the *mecA* gene which is part of a mobile genetic element found in all methicillin-resistant S. aureus (MRSA) strains.[8,9]

After adhering to tissues, *S. aureus* can grow in various ways. It can evade host defenses and the activity of antibiotics by forming biofilms on host.[10] In the health field, biofilms have been of great relevance because many pathogenic and non-pathogenic bacteria can produce it as a part of its virulence mechanism and protection against the immune system of host. Infections associated to biofilms represents 80% of nosocomial infections, being *S. aureus* the leading species in this domain.[11]

The emergence and dissemination of antibiotic resistance bacteria in DFU patients has led to a lack of response to traditional antimicrobial therapies.[12] This biological phenomenon is not recent, being the presence of MRSA and multidrug resistant (MDR) species a major problem.[13]

Biocides and antimicrobial peptides (AMP) are some of the compounds that can be applied as alternatives to classic antibiotic therapeutics or, at least, as complementary therapeutics tools, to treat infectious diseases.

Chlorhexidine is an antiseptic, which destroys or inhibits the growth of microorganisms present in or on living tissue. At low concentrations, chlorhexidine affects membrane integrity.[14] At higher concentrations, chlorhexidine exerts a bactericidal action. It enters the cytoplasm through the damaged cytoplasmic membrane, forming irreversible precipitates with intracellular adenosine triphosphate and nucleic acids, resulting in cell death.[15]

Antimicrobial peptides (AMPs), are a diverse class of molecules that function as a first line of defense against microbial threats. Nisin is produced by *Lactococcus lactis* subsp. *lactis* strains.[16] The spectrum of action includes a range of gram-positive bacteria and spore germination, but it has little or no activity against gram-negative bacteria, fungi or viruses. It exerts two mechanisms of action: interfering with cell wall synthesis and pore formation.[17]

One major impairment of the application of AMP in infectious diseases therapeutics is the lack of delivery systems. Being that now, natural polysaccharides, which are obtained from a biological origin, are recognized by their potentially influence in the rate and/or extent of absorption of a drug. Guar gum is a polysaccharide obtained from the ground endosperm of the seed of the leguminous crop *Cyamopsis tetragonolobus*.[18] Due to its thickening, emulsifying, gelling and binding properties, quick solubility in cold water, wide pH stability and film forming ability, guar gum is used in pharmaceuticals formulations, having also application as a versatile system for the delivery of bioactive agents.[19]

The present study evaluated the inhibitory potential of the biocide chlorhexidine against *S. aureus* isolates obtained from DFU. Furthermore, it was used chlorhexidine, antibiotics (clindamycin, gentamycin and vancomycin) and an antimicrobial peptide incorporated in a delivery system against the *S. aureus* isolates. This experiment allowed us to

assess the inhibitory effect that each compound or combination of compounds has against the biofilm-based isolates.

2. Materials and Methods

2.1. Bacterial Strains

Isolates under study were obtained in a previous epidemiological survey regarding DFU infections.[1] A total of 53 *Staphylococcus* spp were collected and isolated from samples obtained from 49 DFU patients. From this collection, 23 representative biofilm-producing *S. aureus* isolates were then selected, based on Pulse Field Gel Electrophoresis (PFGE) and Multilocus Sequence Type (MLST) analysis.[5] In addition to these 23 isolates, a reference strain, *S. aureus* ATCC 29313, a known biofilm producer was also included in this study.

Strains were growth in a nonselective Brain Heart Infusion (BHI) agar medium (VWR Chemicals, Belgium) at 37ºC for 24h.

2.2. Minimum Inhibitory Concentration (MIC) for Chlorhexidine

Bacterial suspensions were performed for each isolate in 5 mL of sterile normal saline (NaCl) (Merck, Germany) and their concentration were standardized visually using a 0.5 McFarland standard (BioMérieux, France). Afterwards bacterial suspensions were diluted in 9 mL of BHI broth (VWR Chemicals, Belgium). MIC were determined using the broth microdilution method, using 96-well flat-bottomed polystyrene microtiter plates (VWR, Belgium).[20,21] The set of chlorhexidine (AGA, Portugal) concentrations tested was as follows: 1, 5, 10, 50, 100 and 500 µg/mL. In all wells were distributed 25 µL of chlorhexidine solution, except for the negative control, that only contained broth medium. Afterwards, 150 µL of bacterial suspensions was also placed in each well.

2.2.1. Minimum Bactericidal Concentration (MBC) of Chlorhexidine

MBC assessment was carried out after MIC determination. It was inoculated a 3 µL of the suspensions from the wells where there was no visible growth on BHI agar plates that were incubated at 37ºC for 24h. MBC was determined as the lowest chlorhexidine concentration at which no colonies were observed.

2.3. Preparation of Inhibitory Compounds Tested

Chlorhexidine

Concentration used in this assay was the mean value obtained in the MIC assay.

Nisin incorporated in Guar Gum Gel

A stock solution of nisin was obtained by dissolving 1 g of nisin powder (2.5% purity Sigma-Aldrich, USA) in 25 mL of HCl (0.02 M) (Merck, Germany). The stock solution was then diluted with sterile water to a concentration of 45 µg/mL.

Guar gum 1,5% was prepared by dissolving 0.6 g of guar gum (Sigma-Aldrich, USA) in 40 mL of sterile distilled water and heat sterilized by autoclave.

The solution of nisin was incorporated within the gel. For this, 1.8 mL of the stock solution of nisin was diluted in 38.2 mL of sterile distilled water, which was added to the 40 mL of guar gum gel. Thus, there was obtained a final gel of 0,75% (w/v) at 22.5 µg/Ml.[20]

Antibiotics

The antibiotics used in this assay where Clindamycin, Gentamycin and Vancomycin.

MIC concentrations used in this study were previously described by Mottola et al. 2016.[5]

2.4. Combined Protocol

A modified version of the Calgary Biofilm Pin Lid Device was used.[20] For this assay, bacterial suspensions were prepared as described for MIC protocol. Afterwards bacterial suspensions were diluted in Tryptic Soy Broth (TSB) (VWR Chemicals, Belgium) medium supplemented with 0.25% (w/v) glucose (Merck, USA). Then, 200 µL of the bacterial suspensions were distributed in a 96-well flat-bottomed polystyrene microtiter plate (Nunc, Thermo Fisher Scientific, Denmark), covered with 96-peg polystyrene lids (Nunc, Thermo Fisher Scientific, Denmark) and statically incubated for 24h at 37ºC.

During the period of 24h biofilm formed in the peg lids, was rinsed periodically, at intervals of 8h, in different combinations of antiseptic, antibiotics and antimicrobial peptide solutions. This step was performed in 96-well flat-bottomed polystyrene microtiter plates (Nunc, Thermo Fisher Scientific, Denmark). The assays were done by placing the lids three times in 0.9% NaCl (Merck, Germany) for 30 seconds; one time in chlorhexidine for 15 seconds; one time in nisin incorporated in guar gum gel for 3 minutes; and a final drying step, in an empty microplate during 30 minutes.

After this drying step, peg lids were placed on microplates containing 10µL of antibiotic (clindamycin, gentamycin or vancomycin) plus 190µL of TSB+0.25% glucose broth medium. Then, the microplates were incubated at 37ºC during 8h, until the next rinsing step. A total of three rinsing steps were performed.

The inhibitory effect of compounds was determined by removing the peg lids and determining the absorbance values of the suspensions in the 96 well-plate using a microplate reader (BGM LABTECH, Germany).

Pegs lids that were removed for inhibitory action determination were rinsed three more times in 0.9% NaCl, placed in new microplates containing 200 µL of TSB medium supplemented with 0.25% (w/v) glucose (Merck, USA) and incubated in an ultrasound bath (Grant MXB14, England), at 50Hz for 15 minutes, in order to disperse the biofilm-based bacteria from the peg surface. Afterwards, pegs lids were discarded and microplates were covered with normal lids and incubated for 24h at 37 ºC. The eradication effect was determined using the same protocol applied for the inhibitory action.

2.5. Statistical Analysis

Statistical analysis was performed in IBM SPSS Statistics™ V20 Software for Windows. Minimum and maximum, mean and standard deviation values were determined for all quantitative variables. Significant differences between the variables MIC and MBC were determined using the T-test. Correlation between MIC, MBC and antibiotic resistance was evaluated through Pearson's correlation coefficient. Analysis of variance (ANOVA) for Randomized Complete Block Design (RCBD) was used for biofilm inhibition and eradication absorbance results. A two-tailed *p*-value ≤ 0.05 was considered to be statistically significant in all applied tests.

3. Results and discussion

3.1. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Chlorhexidine

The mean values of absorbance obtained for each strain regarding MIC and MBC are present in Table 1 (supplementary data). For MIC, mean values were 5.7±1.5 µg/mL, with a minimum value of 1.4 µg/mL and a maximum of 7.0. Regarding MBC values, they were higher, with a mean value of 15.5±14.9 µg/mL, with a minimum of 9.8 µg/mL and a maximum of 68.8 µg/mL.

An antimicrobial agent can be classified as bactericidal if the MBC is no more than four times de MIC value.[20] In this case, MBC values where 2.72-fold higher than MIC, therefore chlorhexidine can be considered bactericidal for 20 strains (including *S. aureus* ATCC 23213) and bacteriostatic for strains A6.3, B7.3, Z12.2.

Regarding MBC, diverse values were obtained. Vali et al 2016 described values between 0.94-60 µg/ml, Acton 2011 between 16-32 µg/ml and Liu et al 2016, 32 µg/ml for MRSA. Values obtained in this assay are within these ranges, with exception for isolate B7.3 strain.[22-24] The fact that B7.3 has high values for MIC and MBC, can be related to the fact of being a MRSA and MDR strain. Furthermore, harbor the antibiotic resistance gene *norA* which presence is related to increased tolerance to disinfectants agents, such as chlorhexidine.[24]

3.2. Biofilm Inhibition

In Table 2 (supplementary data) are represented the averages of the mean absorbance values obtained regarding the inhibition effect of antimicrobials in descending order.

Regarding antimicrobials applied alone, were observed that for antibiotics, clindamycin was the compound with higher value of absorbance, being close to the value of positive control and having no significative differences (p-value>0.05), followed by gentamycin and then vancomycin. The three antibiotics had close absorbance values, meaning that their inhibitory effect against the biofilm of the strains under study were similar, although significative differences between them (p-value<0.05) were found.

When chlorhexidine is applied alone, its inhibitory effect against biofilm producing strains is very similar to the antibiotics, as no significative differences were observed between these antimicrobials (p-value > 0.05). Taking this in account, chlorhexidine can be a good alternative to antibiotics applications.

Nisin incorporated in guar gum gel showed inhibitory results higher than chlorhexidine and antibiotics, being observed significative differences between these antimicrobials (p-value<0.05). These results demonstrated that nisin incorporated in guar gum gel had a good inhibitory effect against bacterial biofilm, being able to be an alternative to classic therapeutic for DFI. Besides that, Okuda et al 2013 studies indicated that pore formation leading to ATP efflux is important for the activity against biofilm cells. Suggesting that bacteriocins that form stable pores on biofilm cells are highly potent for the treatment of MRSA biofilm infections.[25] Concerning dual application of antimicrobials with chlorhexidine, were observed that a sharp decrease in absorbance values is not observed for antibiotics combined with chlorhexidine, though significative differences were found at a statistical level (p-value<0.05). The fact that absorbance values were similar can be related with the low chlorhexidine concentration used and the short incubation period.

The lowest absorbance values were obtained for the dual application of nisin incorporated in guar gum gel and chlorhexidine, and there were significative differences regarding other antimicrobials that were combined with chlorhexidine (p-value<0.05). Through the analysis of absorbance values is possible to observed differences. It should be noted that the inhibitory effect of chlorhexidine increased when combined with nisin incorporated in guar gum gel. The synergetic effect can be related with both compounds acting in the bacteria membrane.[25] Since this combination of antimicrobials had the best inhibitory effect against bacterial biofilm, is a hypothesis to be studied in order to substitute the use of antibiotics in DFI.

3.3. Biofilm Eradication

In Table 3 (supplementary data) are represented the average of the mean absorbance values obtained in the biofilm eradication assay.

Regarding the eradication effect of antimicrobials without combinations, it was observed that chlorhexidine had de highest absorbance value and vancomycin the lowest. However, there were not significative differences (p-value>0.05) between vancomycin, clindamycin, gentamycin and nisin incorporate in guar gum gel, meaning that these antimicrobials had a similar effect of eradication over the bacterial biofilm.

Concerning antibiotics, the low eradication effect of gentamycin can be related with the fact that aminoglycoside effectiveness relies heavily on *S. aureus* growth phase and extra bacterial factors, including the availability of oxygen and the pH in the surrounding environment.[26] Regarding clindamycin, the low eradication effect against the bacterial biofilm can be due to the presence of *erm* genes, which mediate target site modifications that leads to a reduced susceptibility to this class of antibiotics. In turn, low absorbance values of eradication for vancomycin can be related to the presence of the accessory gene regulator (*agr*) of *S. aureus.* In fact, the presence of *agr* types I and II is associated with evolution towards reduced vancomycin susceptibility; *agr* type II polymorphism is associated with vancomycin therapeutic failures and reduced bacterial killing due to diminished autolysis; and decreased *agr* function promotes organism survival especially in the hospital environment.[27]

Biofilm protection against chlorhexidine may be due to reduced penetration in the biofilm matrix.[28] For a higher eradication effect, a much longer time of contact between chlorhexidine and biofilm-formed bacteria than that for planktonic cells may be required.164 Another factor that can interfere with chlorhexidine action is the existence of bacterial biofilms at different developmental stages. Okuda et al 2013 observed that nisin has eradication activity against MRSA organized in biofilm and Santos et al 2016 suggested that nisin incorporated in guar gum is able to inhibit established biofilms of *S. aureus*.[20,25] In our study nisin incorporated in guar gum gel demonstrated some inhibitory action against the strains tested but not eradication, even when combined with other antimicrobials. Regarding combinations of antimicrobials were observed that combinations involving vancomycin and chlorhexidine, and clindamycin and chlorhexidine, had the highest eradication effect against biofilm, having no significative differences between them (p-value>0.05). These results, demonstrated that

combining these antimicrobials increased the eradication effect.

4. Conclusions

Diabetes *mellitus* is a major worldwide health problem, being observed that one of its most severe complications is the development of DFU which can subsequently infected.[12] DFI are usually polymicrobial, being promoted by several bacterial genera, principally gram-positive bacteria, being *Staphylococcus aureus* the most common specie isolated from these ulcers.[20]

Antimicrobials tested in this study aiming at inhibiting biofilm formation showed promising results. Antimicrobials combinations that include nisin incorporated in guar gum gel and chlorhexidine showed the higher inhibitory effects. Like chlorhexidine, nisin concentrations required to inhibit biofilm cells were below nisin acceptable daily intake even when incorporated in guar gum gel.[20] These antimicrobials could be applied as a complement to antibiotics, allowing to reduce their dose. Bacteria embedded within a biofilm are difficult to eradicate due to a wide variation of nutrient gradients that slow or arrest bacterial growth, protein synthesis and other physiologic activities. Although nisin incorporated in guar gum gel and chlorhexidine presented an inhibitory effect against bacterial biofilms, the same was not observed in the eradication assays. In order to achieve a better eradication effect, a good option would be to use higher antimicrobial concentrations.

Overall, results suggest that nisin incorporated in guar gum and chlorhexidine have a good inhibitory effect against *S. aureus* isolates from DFU. This can be a new therapeutic alternative, or a complement to antibiotherapy, with the advantage that there are currently no resistances described to these compounds.

5. References

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Supplementary Data

Table 1: MIC and MBC values of chlorhexidine

Strains	MIC (µg/mL)	MBC (µg/mL)	
A 1.1	5.6	9.8	Bactericidal
A 5.2	4.2	9.8	Bactericidal
A 6.3	4.2	39.2	Bacteriostatic
B 3.2	5.6	9.8	Bactericidal
B 3.3	5.6	9.8	Bactericidal
B 7.3	7.0	68.6	Bacteriostatic
B 13.1	7.0	9.8	Bactericidal
B 14.2	5.6	9.8	Bactericidal
Z 1.1	7.0	19.6	Bactericidal
Z 2.2	7.0	9.8	Bactericidal
Z 3.1	7.0	9.8	Bactericidal
Z 5.2	4.2	9.8	Bactericidal
Z 12.2	1.4	9.8	Bacteriostatic
Z 14.1	4.2	9.8	Bactericidal
Z 16.1	4.2	9.8	Bactericidal
Z 17.2	4.2	9.8	Bactericidal
Z 21.1	7.0	9.8	Bactericidal
Z 21.3	7.0	9.8	Bactericidal
Z 23.2	4.2	9.8	Bactericidal
Z 25.2	7.0	9.8	Bactericidal
Z 27.2	7.0	9.8	Bactericidal
Z 27.3	7.0	49.0	Bacteriostatic
Z 32.2	7.0	9.8	Bactericidal
ATCC 23213	7.0	9.8	Bactericidal
Mean	5.7	15.5	
Minimum	1.4	9.8	
Maximum	7.0	68.6	
Std. Deviation	1.5	14.9	

Table 2: Means of absorbance values for each biofilm inhibition effect of antimicrobials and respective standard deviation. Chx: Chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel; Abs: Absorbance; SD: Standard deviation.

	Abs	**±SD**
Positive Control	0,654	0,057
Clindamycin	0,626	0,076
Chx	0,599	0,058
Gentamycin	0,580	0,063
Vancomycin	0,563	0,060
Gentamycin, Chx	0,553	0,080
Clindamycin, Chx	0,546	0,175
Vancomycin, Chx	0,480	0,166
Vancomycin, Nisin in GGG, Chx	0,298	0,060
Vancomycin, Nisin in GGG	0,287	0,087
Gentamycin, Nisin in GGG	0,282	0,068
Clindamycin, Nisin in GGG	0,270	0,072
Nisin in GGG	0,264	0,056
Gentamycin, Nisin in GGG, Chx	0,255	0,061
Clindamycin, Nisin in GGG, Chx	0,252	0,066
Nisin in GGG, Chx	0,242	0,054
Negative Control	0,101	

Table 3: Means of absorbance values for biofilm eradication effect and respective standard deviation. Chx: Chlorhexidine; Nisin in GGG: Nisin incorporated in guar gum gel; Abs: Absorbance; SD: Standard deviation.

	Abs	**±SD**
Positive Control	0,688	0,045
Gentamycin, Nisin in GGG, Chx	0,652	0,052
Chx	0,633	0,048
Vancomycin, Nisin in GGG, Chx	0,620	0,063
Clindamycin	0,614	0,063
Gentamycin, Nisin in GGG	0,613	0,045
Gentamycin	0,604	0,050
Vancomycin, Nisin in GGG	0,603	0,041
Nisin in GGG	0,595	0,052
Nisin in GGG, Chx	0,591	0,052
Vancomycin	0,587	0,050
Gentamycin, Chx	0,586	0,053
Clindamycin, Nisin in GGG	0,565	0,064
Clindamycin, Nisin in GGG, Chx	0,552	0,061
Clindamycin, Chx	0,543	0,134
Vancomycin, Chx	0,534	0,136
Negative Control	0,101	