

Antibiofilm and Antimicrobial Efficacy of DispersinB[®]-KSL-W Peptide-Based Wound Gel Against Chronic Wound Infection Associated Bacteria

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Abstract The medical importance of bacterial biofilms has increased with the recognition of biofilms as one of the major contributors to the slow or non-healing chronic wounds such as diabetic foot ulcers, venous leg ulcers, and pressure ulcers. Being a protected community of microorganisms, biofilms are notoriously refractory to antibiotic treatments. As the conventional treatment modalities have proven ineffective, this study provides the in vitro evidence to support the use of a novel combination of DispersinB[®] antibiofilm enzyme that inhibits biofilm formation and disperses preformed biofilm, and thus making the biofilm bacteria more susceptible to a broad-spectrum KSL-W antimicrobial peptide. The combination of DispersinB[®] and KSL-W peptide showed synergistic antibiofilm and antimicrobial activity against chronic wound infection associated biofilm-embedded bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, Coagulase-negative Staphylococci (CoNS), and *Acinetobacter baumannii*. In addition, the wound gel formulation comprising DispersinB[®], KSL-W peptide, and a gelling agent Pluronic F-127 showed a broad-spectrum and enduring antimicrobial activity against test organisms. Furthermore, as compared to commercial wound gel

Silver-Sept[™], DispersinB[®]-KSL-W peptide-based wound gel was significantly more effective in inhibiting the biofilm-embedded MRSA, *S. epidermidis*, CoNS, Vancomycin-resistant Enterococci, *A. baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* ($P < 0.05$). Thus, this study provides promising evidence for the potential application of antibiofilm-antimicrobial DispersinB[®]-KSL-W wound gel in chronic wound management.

Introduction

With an epidemic increase in obesity combined with an aging population, chronic wounds such as diabetic foot ulcers, pressure ulcers, and venous leg ulcers are of increasing clinical concern. Over 2 % of the US population suffers from such chronic, non-healing wounds and it costs the US health care system \$20 billion a year [1]. Although chronic and acute wounds progress through similar stages of healing, chronic wounds appear to stall in the inflammatory stage of wound healing, likely because of persistent colonization by bacteria [2]. The most common bacteria observed in chronic wound infections are *S. aureus* (in 93.5 % of the ulcers), *Enterococcus faecalis* (71.7 %), *P. aeruginosa* (52.2 %), Coagulase-negative Staphylococci (CoNS) (45.7 %), *A. baumannii* (13 %), and *K. pneumoniae* (6.5 %) [3, 4]. Colonization by bacteria contributes to slow- or non-healing of the wound. However, recent evidence suggests that healing of a chronic wound is dependent on infections involving biofilms [3–5]. In chronic wounds, the biofilm mode of growth is characterized by adherence to biotic or abiotic surfaces, slow development of overt symptoms, lack of resolution by the host defense, and resistance to antibiotic therapy. James et al. [4] reported the presence of biofilms in 60 % of chronic

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wounds. Also, Singh and Barbul [5] demonstrated biofilms as the potential reason why chronic wounds do not heal.

Biofilms are highly differentiated and spatially organized three-dimensional structures consisting of matrix-enclosed communities of microorganisms (mono- or mixed-species) formed on colonizable surfaces. The metabolic activity in a biofilm is highly heterogeneous and in general it is a function of depth within the structure, ranging from highly active cells at the surface close to the nutrient supply to dormant cells that lie deep within the structure [6]. Since the adaptation by biofilm bacteria has resulted in the failure of treatment using various antibiotics/antimicrobials, synergistically acting combinations of antibiofilm compounds causing the destruction of biofilms and antimicrobials that are functional independent of the metabolic state of the target cells would have the greatest likelihood of being efficacious in the clinical settings. Wolcott and Rhoads [7] observed that the chronic wound treatments that specifically target biofilms transformed non-healable wounds into healable wounds. When combined with antibiofilm compounds, the use of antibiotics declined ~25 % during the 4-year study period. They concluded that the use of suitable topical agents that inhibit biofilm formation and/or disrupt preformed biofilm should be an integral part of the management of chronic wound infections.

Antiseptic wound dressings are currently the most common clinical strategy employed to address wound infection with limited success against chronic wounds involving biofilms. Although systemic antibiotic administration has shown some efficacy against wound infections, the growing concern regarding bacteria that are resistant to antibiotic therapy shows the need for developing alternative and possibly better ways to treat chronic wound associated infections involving biofilms. Realizing the need for a wound care product with both the antibiofilm and antimicrobial activity, we formulated a novel DispersinB[®] antibiofilm enzyme-based wound gel containing a broad-spectrum cationic antimicrobial decapeptide KSL-W (KKVVFVWKFK), an analog of KSL peptide (KKVVFVKVKFK) [8, 9]. DispersinB[®] is a naturally occurring enzyme produced by an oral bacterium *Aggregatibacter actinomycetemcomitans*, which is associated with the juvenile periodontitis [10]. The enzyme inhibits biofilm formation and disperses preformed biofilm in chronic wound infection associated bacteria such as Methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, *K. pneumoniae*, and *A. baumannii* without affecting growth [11]. We sought to combine DispersinB[®] with a broad-spectrum antimicrobial peptide KSL-W that is effective as an anti-plaque agent in vitro [12].

The objectives of this study were to (i) examine the synergistic antibiofilm-antimicrobial activity of the combination of DispersinB[®] and KSL-W peptide, and (ii) compare the in vitro efficacy of DispersinB[®] and KSL-W peptide-based wound gel with that of commercial Silver-

SeptTM wound gel against chronic wound infection associated biofilm-embedded bacteria.

Materials and Methods

Reagents, Microorganisms, and Culture Conditions

DispersinB[®] enzyme was purified from a recombinant *Escherichia coli* fermentation as previously described [10]. The enzyme had a specific activity of ~10³ units/mg of protein. All the chemicals (including media ingredients) were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or BD Diagnostic Systems (Sparks, MD, USA). The commercial Silver-SeptTM wound gel was purchased from Source Medical (Edmonton, AB, Canada). The KSL-W peptide (NH₂-Lys-Lys-Val-Val-Phe-Try-Val-Lys-Phe-Lys-COOH; Molecular weight ~1.31 kDa) sample was custom synthesized by CPC Scientific (Sunnyvale, CA, USA). The wound isolates of *S. epidermidis* 1457, Vancomycin-resistant Enterococci (VRE) 143, *K. pneumoniae* P30, *P. aeruginosa* 232, and *A. baumannii* 63270 were provided by Dr. George G. Zhanel (Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada). In addition, the wound isolates of MRSA Gav 16a and CoNS 42 were obtained from Dr. Randolph D. Wolcott (Southwest Regional Wound Care Center, Lubbock, TX, USA). All the strains were maintained at -80 °C in 15 % glycerol and recovered onto tryptic soy agar (TSA). For inoculum preparation, an isolated colony was inoculated into tryptic soy broth (TSB) and incubated at 37 °C for 16–18 h. Water was used as a solvent to dissolve DispersinB[®] enzyme, KSL-W peptide, and a gelling agent Pluronic F-127.

DispersinB[®]-KSL-W Peptide-Pluronic F-127 Gel

The aqueous-based wound gel was formulated by combining antimicrobial peptide KSL-W 1 mg/ml, antibiofilm enzyme DispersinB[®] 0.2 mg/ml, and a gelling agent Pluronic F-127 200 mg/ml. Pluronic F-127 is a block copolymer of polypropylene oxide and ethylene oxide, which has a molecular weight of about 12.6 kDa and forms a semisolid gel at room temperature but is fluid at a lower temperature (4 °C). According to the FDA, Pluronic F-127 is an inactive ingredient for different types of preparations (e.g., intravenous, inhalation, oral solution, suspensions, ophthalmic, or topical formulations). An aqueous Pluronic F-127 solution was prepared by intermittent mixing at 4 °C for 18–24 h, and autoclaved for 15 min at 121 °C and 15 psi [13]. After cooling the Pluronic F-127 solution to 4 °C, it was mixed with the filter sterilized KSL-W peptide solution and DispersinB[®] enzyme solution to formulate the wound gel.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of KSL-W Peptide Solution and DispersinB®-KSL-W Wound Gel

The MICs of KSL-W peptide solution and DispersinB®-KSL-W wound gel for chronic wound infection associated bacteria were determined using a broth microdilution assay in 96-well microtiter plate as described previously with slight modifications [14]. Briefly, bacterial strains were grown overnight at 37 °C with 100 rpm shaking in TSB and diluted tenfold with fresh TSB. The cultures were further incubated to reach a mid-log growth phase (OD at 600 nm 0.3–0.6). The mid-log phase cultures were further diluted with TSB to achieve 4×10^6 cfu/ml. The twofold dilutions of the wound gel and peptide solution were performed in separate 96-well microtiter plates to achieve concentrations ranging from 250 to 0.9 µg/ml. A 100 µl diluted bacterial suspension was added to each well of 96-well microtiter plate and the plates were incubated at 37 °C for 24 h and read at 600 nm using a microtiter plate reader (Multiskan Ascent Labsystems, Helsinki, Finland). The MIC was determined as the lowest concentration of antimicrobial peptide that completely inhibited the growth. The suspension from clear wells (greater than or equal to MIC) was plated on TSA. Following the incubation at 37 °C for 18–24 h, the growth on TSA was recorded. The lowest concentration that did not permit the visible growth on TSA was recorded as MBC.

Quantitative Culture Method

Bacterial suspensions were prepared by inoculating 3 ml TSB with 10^7 – 10^8 cfus of the bacterial cultures. One gram of either placebo or DispersinB®-KSL-W gels were added to each of these tubes and incubated at 37 °C for 72 h [15]. The samples (0.1 ml) were removed at 24, 48, and 72 h, diluted 10 times to reduce the antimicrobial carryover, and plated on TSA and incubated for 48 h at 37 °C. Colonies were counted and expressed as cfu/ml. In this study, $<1 \log_{10}$ cfu/ml reduction was considered as a low bactericidal activity, between 1 and 3 $\log_{10}$ cfu/ml and $>3 \log_{10}$ cfu/ml reductions were considered as moderate and high activity, respectively.

Biofilm Kill Assay

Biofilms were grown in 1.5 ml polypropylene microcentrifuge tubes as described previously [16]. Tubes were filled with 200 µl of inoculum (diluted 1:100 in fresh broth 10^6 – 10^7 cfu/ml) and incubated at 37 °C for 24 h. The planktonic growth was aspirated; the remaining surface attached biofilm was washed once with phosphate buffered saline and treated with either 200 µl of KSL-W peptide

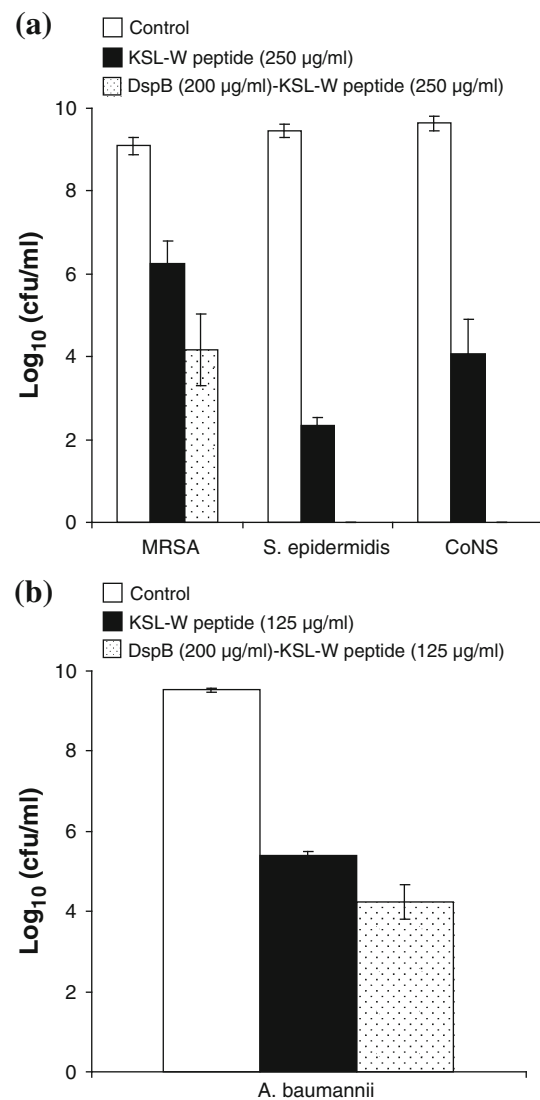


Fig. 1 Effect of DispersinB® on the antimicrobial activity of KSL-W peptide against biofilm-embedded bacteria. **a** Gram-positive MRSA, *S. epidermidis* and CoNS, **b** Gram-negative *A. baumannii*. The biofilm was grown in 1.5 ml polypropylene microcentrifuge tubes and treated with either water as control or KSL-W peptide or a combination of DispersinB® and KSL-W peptide. After 3 h treatment at 37 °C, total viable bacteria were determined by plating on TSA plates. The error bars indicate SD (not visible where the SD was less than the line thickness)

solution with or without DispersinB®. For comparing the efficacy of DispersinB®-KSL-W wound gel with that of Silver Sept™, the gels were diluted tenfold before the treatment to reduce the viscosity. After 3 h incubation at 37 °C, the cells were vortexed for 1 min, pelleted and rinsed with the sterile PBS. Cell pellets were resuspended in 200 µl of PBS containing 20 µg/ml of DispersinB® and incubated for 5 min at 37 °C, followed by vortexing for 1 min to desegregate the cells [16, 17]. The bacterial cell suspension was serially diluted in PBS and plated on TSA to determine the number of cfu/ml.

To study the effect of undiluted DispersinB®-KSL-W wound gel on preformed biofilm, biofilm was treated with the gel, 200 µl of chilled distilled water was added after 3 h treatment and tubes were incubated at 4 °C for 10 min to reduce the viscosity of gel. The viable bacterial cells were pelleted, washed, and plated as described above. The results were calculated as mean $\pm$ standard deviation (SD) from three independent experiments. Statistical analysis was performed with two-tailed Student's *t* test using Microsoft Excel. *P* values of <0.05 were considered statistically significant.

Results

Antibiofilm-Antimicrobial Activity of KSL-W Peptide Alone and in Combination with DispersinB®

The biofilm kill assay was performed to determine the effect of DispersinB® in combination with KSL-W peptide in solution on increasing the susceptibility of biofilm-embedded bacteria to antimicrobial peptide. DispersinB® significantly enhanced the antimicrobial activity of KSL-W peptide against biofilm-embedded chronic wound infection associated bacteria, including Gram-positive bacteria MRSA, *S. epidermidis*, CoNS, and Gram-negative bacteria *A. baumannii* (Fig. 1). The preformed biofilm of *S. epidermidis* and CoNS were completely eradicated when DispersinB® and KSL-W were used together at a concentration of 200 and 250 µg/ml, respectively.

Antimicrobial Activity of KSL-W Peptide Solution and DispersinB®-KSL-W Wound Gel

After having established that DispersinB® enhances the antimicrobial activity of KSL-W peptide in solution, we formulated a wound gel comprising antibiofilm enzyme DispersinB®, cationic antimicrobial decapeptide KSL-W, and a gelling agent Pluronic F-127. The MIC and MBC of peptide solution were compared with that of wound gel using a 96-well microtiter plate assay (Table 1). The MIC and MBC values for both the peptide solution and wound gel were <10 µg/ml against all the test organisms. Furthermore, the wound gel formulation showed 50 % lower MIC as well as MBC against MRSA, *S. epidermidis*, CoNS, and *A. baumannii* as compared to that of peptide solution alone, which could be due to DispersinB® potentiating the antimicrobial activity of KSL-W peptide. In addition, the durability of broad-spectrum antimicrobial activity of DispersinB®-KSL-W wound gel was tested using a quantitative culture method against chronic wound infection associated bacteria (Table 2). DispersinB®-KSL-W wound gel reduced viable counts of Gram-positive

Table 1 MIC and MBC of KSL-W peptide solution and DispersinB®-KSL-W wound gel against chronic wound infection associated bacteria

Bacteria	KSL-W Peptide solution		DispersinB®-KSL-W gel	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
MRSA	7.81	7.81	3.9	3.9
<i>S. epidermidis</i>	3.9	7.8	1.95	3.9
CoNS	1.95	3.9	<0.97	1.95
<i>K. pneumoniae</i>	3.9	3.9	3.9	3.9
<i>A. baumannii</i>	3.9	3.9	<0.97	<0.97
<i>P. aeruginosa</i>	3.9	3.9	3.9	7.81

As determined by microtiter broth dilution method. The initial inoculum was adjusted to 4×10^6 cfu/ml in the growth media

bacteria MRSA, *S. epidermidis*, CoNS, and VRE and Gram-negative bacteria *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* to undetectable level over a period of 72 h, showing the sustained broad-spectrum antimicrobial activity of the test wound gel.

In Vitro Antibiofilm Activity of DispersinB®-KSL-W Wound Gel Versus Silver-Sept™ Gel

The antibiofilm activity of tenfold diluted DispersinB®-KSL-W wound gel was compared with that of a commercial wound gel Silver-Sept™ against chronic wound infection associated bacteria using a biofilm kill assay. As compared with the control, DispersinB®-KSL-W wound gel showed significantly ($P < 0.05$) more antibiofilm activity against all the test organisms. By contrast, Silver-Sept™ was moderately effective against *S. epidermidis* and CoNS biofilm (Fig. 2). In addition, DispersinB®-KSL-W wound gel was significantly ($P < 0.05$) more effective in killing biofilm-embedded bacteria as compared with Silver-Sept™. In addition, undiluted DispersinB®-KSL-W gel was tested against preformed biofilms of chronic wound infection associated bacteria. As expected, the undiluted wound gel was more effective than tenfold diluted gel and achieved a reduction of $2.14 \log_{10}$ MRSA, $4.66 \log_{10}$ *S. epidermidis*, $6.81 \log_{10}$ CoNS, $4.26 \log_{10}$ *K. pneumoniae*, and $3.57 \log_{10}$ *A. baumannii* in biofilm-embedded bacterial cells.

Discussion

Bacterial bioburden in the form of contaminating biofilms has been demonstrated to be a major factor contributing to nonhealing chronic wounds, and therefore, biofilm targeted therapies in wound care are highly relevant. It is well

Table 2 Antimicrobial activity of DispersinB®-KSL-W wound gel against chronic wound infection associated bacteria

Bacteria	Incubation time (h)	CFU/ml	
		Placebo gel	DispersinB®-KSL-W Gel
MRSA	24	7.73×10^7 ($\pm 1.54 \times 10^7$)	NG
	48	1.12×10^8 ($\pm 1.42 \times 10^7$)	NG
	72	1.07×10^8 ($\pm 2.0 \times 10^7$)	NG
<i>S. epidermidis</i>	24	1.31×10^8 ($\pm 8.5 \times 10^6$)	NG
	48	1.27×10^8 ($\pm 1.23 \times 10^7$)	NG
	72	1.13×10^8 ($\pm 1.95 \times 10^7$)	NG
CoNS	24	1.3×10^8 ($\pm 2.1 \times 10^7$)	NG
	48	1.22×10^8 ($\pm 1.06 \times 10^7$)	NG
	72	1.43×10^8 ($\pm 1.11 \times 10^7$)	NG
VRE	24	1.04×10^8 ($\pm 1.79 \times 10^7$)	NG
	48	1.16×10^8 ($\pm 1.86 \times 10^7$)	NG
	72	1.26×10^8 ($\pm 2.78 \times 10^7$)	NG
<i>K. pneumoniae</i>	24	9.63×10^7 ($\pm 1.42 \times 10^7$)	NG
	48	1.19×10^8 ($\pm 1.91 \times 10^7$)	NG
	72	1.21×10^8 ($\pm 1.97 \times 10^7$)	NG
<i>A. baumannii</i>	24	2.38×10^8 ($\pm 4.24 \times 10^7$)	NG
	48	3.37×10^8 ($\pm 1.11 \times 10^8$)	NG
	72	5.1×10^8 ($\pm 1.2 \times 10^8$)	NG
<i>P. aeruginosa</i>	24	2.7×10^8 ($\pm 1.9 \times 10^7$)	NG
	48	5.4×10^8 ($\pm 2.19 \times 10^8$)	NG
	72	6.83×10^8 ($\pm 1.81 \times 10^8$)	NG

As determined by quantitative culture method. The wound gel was inoculated with 10^7 – 10^8 cfu/ml and viable counts were determined after every 24 h

NG no growth

recognized that infections involving biofilms are difficult to eradicate, as sessile bacteria employ mechanisms that enhance the survival and resistance to antimicrobials up to 1,000 times more than their planktonic counterparts [18]. Treatments to the biofilm-infected chronic wounds are managed primarily through mechanical manipulation, such as debridement and choice of wound dressing [7]. Rationally designing-wound dressings with effective antibiofilm control have the potential to significantly improve wound therapy and lead to successful wound healing. Thus, inhibition of biofilm formation and/or disruption of preformed biofilms may make biofilm-embedded bacteria more susceptible to antimicrobial agents. DispersinB® inhibits biofilm formation and disrupts/disperses preformed biofilms by depolymerizing a polysaccharide, poly- β -1, 6-*N*-acetyl-D-glucosamine (PNAG/PGA), which is essential for biofilm formation of *E. coli*, *A. baumannii* and *Staphylococci* [19, 20].

In this study, the combination of DispersinB® and KSL-W peptide showed better efficacy in reducing the chronic wound infection associated bacterial biofilm burden as compared to KSL-W peptide alone. The increased antimicrobial activity of KSL-W peptide in the presence of

DispersinB® could be due to DispersinB® making biofilm-embedded bacteria more susceptible to the KSL-W antimicrobial peptide. Although the confocal or electron microscopy images to show biofilm reduction could provide supporting evidence, it would be beyond the scope of this study. Furthermore, DispersinB®-KSL-W peptide gel showed lower MIC and MBC values as compared to KSL-W peptide solution alone against MRSA, *S. epidermidis*, CoNS, and *A. baumannii*. Although DispersinB® has no antimicrobial activity, it could enhance the antimicrobial activity of KSL-W peptide against planktonic bacteria by inhibiting the biofilm formation and thus making them more susceptible to antimicrobial. These findings are in agreement with the previous reports describing the synergistic inhibitory activity of DispersinB® in combination with antibiotics (cefamandole nafate and ampicillin) and antimicrobials (triclosan and SDS) [16, 21–23].

The DispersinB®-KSL-W wound gel exhibited a sustained antibacterial activity over a period of 72 h against all the chronic wound infection associated bacteria tested. In addition, DispersinB®-KSL-W wound gel was more effective against preformed biofilms of MRSA, *S. epidermidis*, CoNS, *A. baumannii*, and *K. pneumoniae* compared

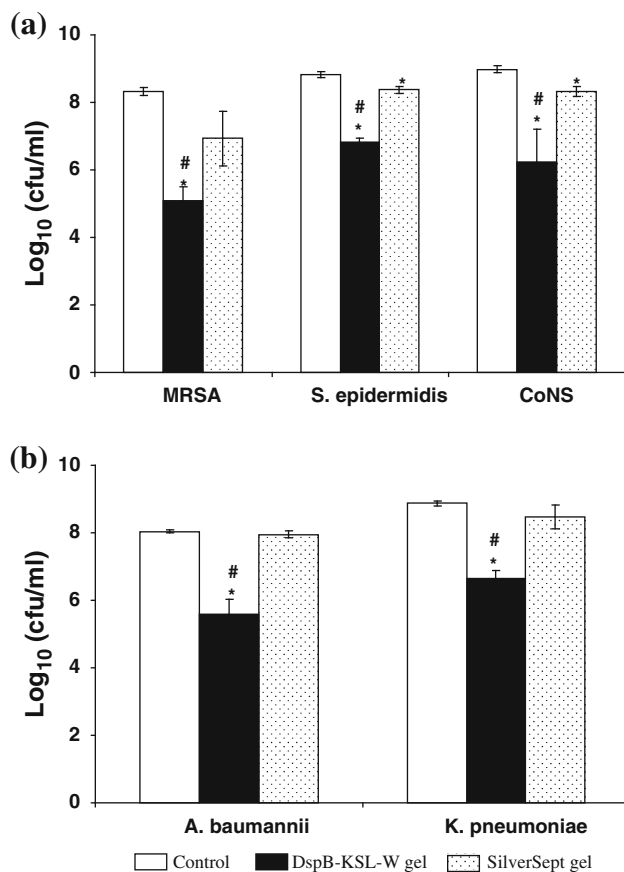


Fig. 2 Antibiofilm activity of tenfold diluted DispersinB®-KSL-W wound gel (DspB-KSL-W gel) compared to commercial Silver-Sept™ wound gel (Silver-Sept gel) against biofilm-embedded bacteria. **a** Gram-positive MRSA, *S. epidermidis*, CoNS and VRE, **b** Gram-negative *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*. The biofilm was grown in 1.5 ml polypropylene microcentrifuge tubes and treated with either water as control, or tenfold diluted DispersinB®-KSL-W peptide gel or tenfold diluted Silver-Sept™ gel. After 3 h treatment at 37 °C, total viable bacteria were determined by plating on TSA plates. The error bars indicate SD (not visible where the SD was less than the line thickness). Asterisks indicate a significant difference ($P < 0.05$) in the reduction of biofilm-embedded cells compared to control. Number sign indicates a significant difference ($P < 0.05$) in the reduction of biofilm-embedded cells by DispersinB®-KSL-W wound gel compared to Silver-Sept™ wound gel

with the commercial wound gel Silver-Sept™ containing silver. While Silver-Sept™ wound gel was moderately effective against *S. epidermidis* and CoNS, it was not effective against MRSA. The *S. aureus* that accounts for 93.5 and 40 % infections of venous leg ulcer and diabetic foot ulcer, respectively, has been implicated in slow and nonhealing of chronic wounds [3]. In addition, *S. epidermidis* and *S. aureus* are usually good biofilm formers [24], and MRSA has already developed resistance to silver [25]. The antibiofilm-antimicrobial efficacy of DispersinB®-KSL-W combination in the form of gel as well as in solution varied against different test organisms because of

the variability in the composition of extracellular polymeric substances. In addition to PNAG, extracellular matrix of bacterial biofilm also contains double-stranded extracellular DNA [26], surface-active lipopeptides [27], lipopolysaccharides, extracellular enzymes, and proteins [28].

In conclusion, this study demonstrates that DispersinB®-KSL-W wound gel provides both the antibiofilm and antimicrobial activity and is effective against bacteria embedded in preformed biofilms as compared to some of the currently available antimicrobial wound care products on the market. DispersinB® is an antibiofilm enzyme lacking antibacterial activity and thus is not prone to bacterial resistance [11]. Likewise, antimicrobial peptides in general exhibit low propensity for microbial resistance [29]. Furthermore, this kind of combination wound care product containing antibiofilm and antimicrobial agents with different modes of action not only lowers the probability of the emergence of bacterial resistance but also increases the spectrum of antimicrobial activity [30]. This DispersinB®-KSL-W wound gel formulation comprising a naturally occurring biocompatible DispersinB® enzyme and a broad-spectrum antimicrobial peptide could meet the unmet clinical need for prevention and treatment of chronic wound infections involving biofilms [22, 31]. Also, this wound gel, with both the antimicrobial and antibiofilm activity, has potential applications in wound and skin care products such as wound dressings, wound sprays, bandages, creams, lotions, and ointments for the unmet clinical as well as market need. Future work will include further development of the formulation to improve the bioavailability of the antimicrobial peptides used in infected wounds wherein host and bacteria-derived proteinases are present.

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