



TURNING NOVEL DISCOVERIES INTO  
INNOVATIVE HEALTHCARE SOLUTIONS

**DispersinB<sup>®</sup>: A Novel Antibiofilm Technology**

**5-1250 Waverley Street, Winnipeg, Manitoba, Canada R3T 6C6**

**Contact: Gord Froehlich, President & CEO**

**Tel: (204) 477-7592; Fax: (204) 453-1314; [gfroehlich@kanebiotech.com](mailto:gfroehlich@kanebiotech.com)**

## 1. DispersinB<sup>®</sup>

DispersinB<sup>®</sup> is an antibiofilm enzyme, which inhibits and disperses biofilms of major bacterial pathogens. It was discovered by Dr. Jeffrey B. Kaplan, Department of Oral Microbiology, New Jersey Dental School, NJ, USA. The discovery of this enzyme with the support of the NIH (USA) grant was listed in the “NIH Annual Performance Report of 2004” as one of the thirteen achievements of the year. The unique ability of this enzyme to specifically inhibit biofilm formation without affecting bacterial growth and to disperse preformed biofilms makes it a first of its kind antibiofilm enzyme. Kane Biotech Inc. has an exclusive worldwide license agreement with the University of Medicine and Dentistry of New Jersey, NJ, USA to commercialize DispersinB<sup>®</sup> technology for human, animal, agricultural and industrial applications. The US patent on this technology has already been issued (U.S. Pat. No.7,294,497).

## 2. Structural Chemistry

DispersinB<sup>®</sup> is a naturally occurring N-acetylglucosaminidase enzyme produced by a periodontal disease-associated oral bacterium, *Aggregatibacter actinomycetemcomitans*. This 41 kDa enzyme consists of a single chain containing 361 amino acid residues, is a highly active and stable glycoside hydrolase that functions in a narrow pH range. It is readily soluble in water. Structurally, the bowl-shaped DispersinB<sup>®</sup> molecule has a large central cavity, which exhibits a negative electrostatic potential. It consists of a single domain and can further be subdivided into substrates. The major structure (residues 1-60 and residues 91-358) folds into a  $\beta/\alpha$  structure with a typical TIM-barrel, which contains minor substrates as an insertion.

### 2.1 DispersinB<sup>®</sup> Amino Acid (AA) Sequence:

```
NCCVKGNSIYPQKTSTKQTGLMLDIARHFYSPEVIKSFIDTISLSGGNFLHLHFSDHEN  
YAIESHLLNQRAENAVQKDGIIYINPYTGKPFLSYRQLDDIKAYAKAKGIELIPELDSP  
NHMTAIFKLVQKDRGVKYLQGLKSRQVDDEIDITNADSIITFMQSLMSEVIDIFGDTSQH  
FHIGGDEFGYSVESNHEFITYANKLSYFLEKKGLKTRMWNDGLIKNTFEQINPNIEITY  
WSYDGD TQDKNEAAERRDMRVS LPELLAKGFTVLNYSYLYIVPKASPTFSQDAAF AA  
KDV IKNWDLGVWDGRNTKNRVQNTHEIAGAALS IWGEDAKALKDETIQKNTKSLLEAVI  
HKTNGDE
```

## 3. Mode of Action

DispersinB<sup>®</sup> specifically hydrolyses the glycosidic linkages of poly- $\beta$ -1, 6-N-acetylglucosamine in polysaccharide adhesins of bacteria needed for biofilm formation, and also in preformed

biofilm (a polysaccharide matrix) without affecting the growth (Itoh, *et al.*, 2005; Izano, *et al.*, 2007b). Thus, it inhibits as well as disperses bacterial biofilms. Furthermore, DispersinB<sup>®</sup>'s antibiofilm activity is specific to bacteria that produce poly- $\beta$ -1, 6-N-acetylglucosamine polysaccharide in biofilms.

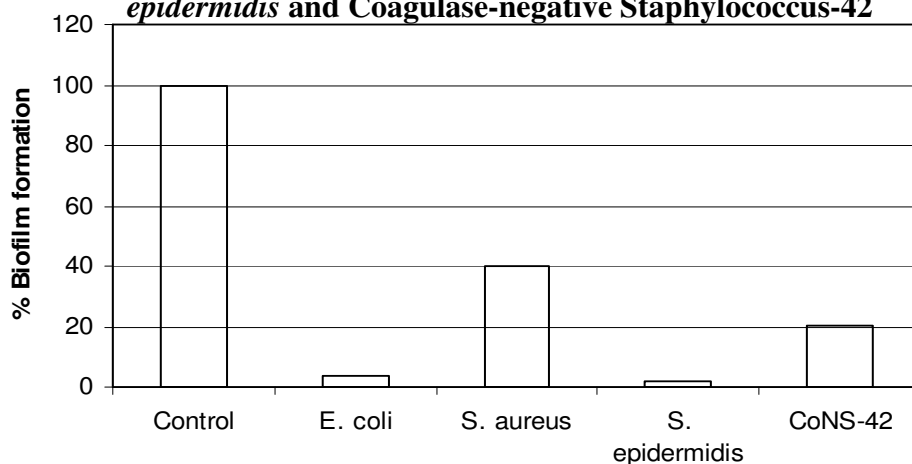
#### 4. Antibiofilm Activity

##### 4.1 Biofilm inhibition

##### 4.1.1 *Effect of DispersinB<sup>®</sup> on biofilm formation*

Biofilms were assayed by crystal violet staining, as described previously (Jackson, *et al.*, 2002). The overnight grown gram-negative *E. coli* and gram-positive bacteria such as *S. epidermidis*, *S. aureus*, and Coagulase-negative staphylococci (CoNS-42) were diluted to 5% in colony forming antigen medium (CFA) and tryptic soy broth (TSB), respectively and biofilm was grown in 96-well microtiter plates (Corning Inc., New York). Biofilm growth was determined by measuring the absorbance at 630 nm. DispersinB<sup>®</sup> at 12.5  $\mu$ g/ml was effective in significantly reducing ( $P < 0.05$ ) biofilm formation by about 96% of *E. coli*, 63% of *Staphylococcus aureus*, 94% of *S. epidermidis*, and 80% of Coagulase-negative Staphylococcus-42 (CoNS-42) (**Fig. 1**).

**Fig. 1: Effect of DispersinB<sup>®</sup> (12.5  $\mu$ g/ml) on biofilm formation in *E. coli*, *S. aureus*, *S. epidermidis* and Coagulase-negative Staphylococcus-42**

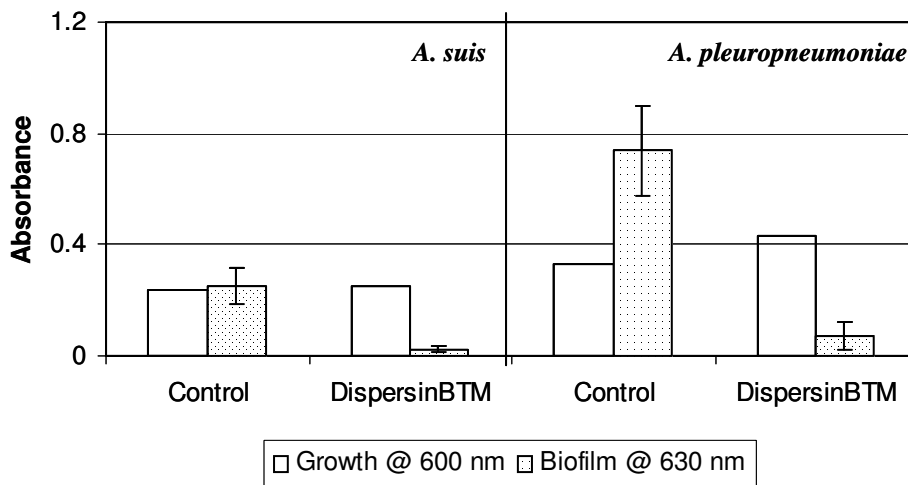


##### 4.1.2 *Effect of DispersinB<sup>®</sup> on biofilm formation in animal pathogens Actinobacillus pleuropneumoniae and A. suis*

An *in vitro* microtiter assay was performed to determine the effect of DispersinB<sup>®</sup> on the growth and biofilm formation of *Actinobacillus pleuropneumoniae* and *A. suis*. *A. pleuropneumoniae* and

*A. suis* are swine pathogens causing pleuropneumonia. *A. pleuropneumoniae* and *A. suis* biofilm was grown in brain heart infusion broth (BHI) supplemented with 10 mg/l NAD and BHI broth respectively. Biofilm was grown in 96-well microtiter plate in the absence and presence of 50 µg/ml DispersinB<sup>®</sup>. The plate was incubated at 37°C for 24 hours. Growth of planktonic cells based on the absorbance at 600 nm was determined using Labsystems Multiskan Ascent microplate reader. Biofilm was measured by discarding the medium, rinsing the wells with water (three times), and staining bound cells with crystal violet. The dye was solubilized with 33% acetic acid, and absorbance at 630 nm was determined. For each experiment, background staining was corrected by subtracting the crystal violet bound to uninoculated control (Fig. 2). The DispersinB<sup>®</sup> inhibited biofilm formation of *A. pleuropneumoniae* and *A. suis*.

**Fig. 2: Effect of DispersinB<sup>®</sup> on biofilm formation in *Actinobacillus suis* and *A. pleuropneumoniae***

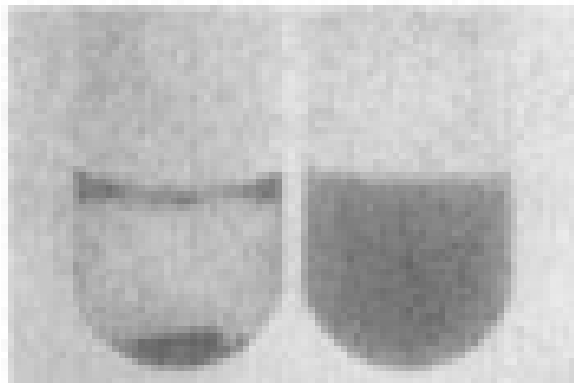


## 4.2 Biofilm dispersal

### 4.2.1 Effect of DispersinB<sup>®</sup> on *S. epidermidis* biofilm dispersal

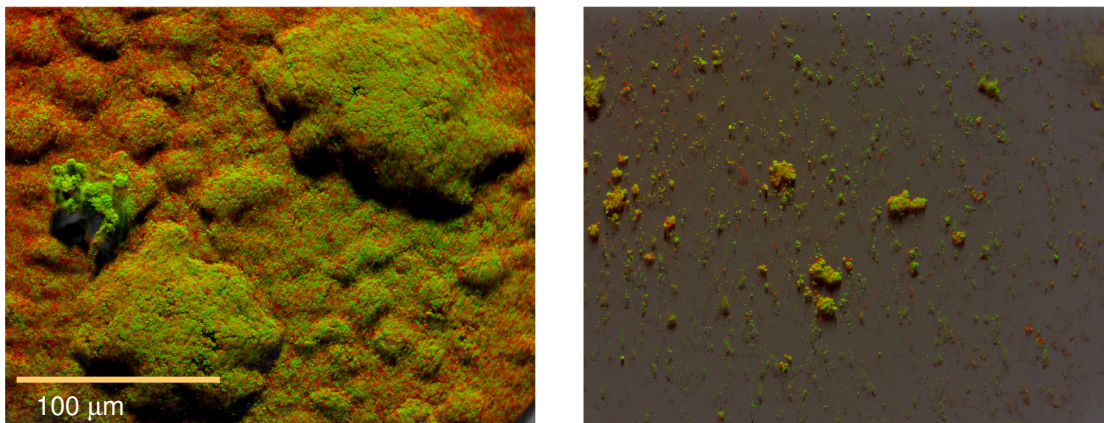
To demonstrate that DispersinB<sup>®</sup> degrades the *S. epidermidis* intracellular slime matrix, the biofilm growth from the surface of a culture vessel was scraped and transferred to a tube containing physiological saline. Under these conditions, the cells formed a sticky aggregate that rapidly settles to the bottom of the tube. Addition of DispersinB<sup>®</sup> in the tube resulted in uniformly turbid cell suspensions indicating that treatment with DispersinB<sup>®</sup> disperses biofilms (Figs. 3 & 4).

**Fig. 3: Dispersal of *S. epidermidis* biofilm by DispersinB<sup>®</sup>**



Control      DispersinB<sup>®</sup>-treated

**Fig. 4: Confocal images of *S. epidermidis* biofilm dispersal by DispersinB<sup>®</sup>**



Control

DispersinB<sup>®</sup>-treated

#### 4.3 Broad-spectrum activity

DispersinB<sup>®</sup> is effective against both gram-negative and gram-positive bacteria. It inhibits biofilm formation and/or disperses preformed biofilm in human, animal and plant bacterial pathogens (**Table 1**).

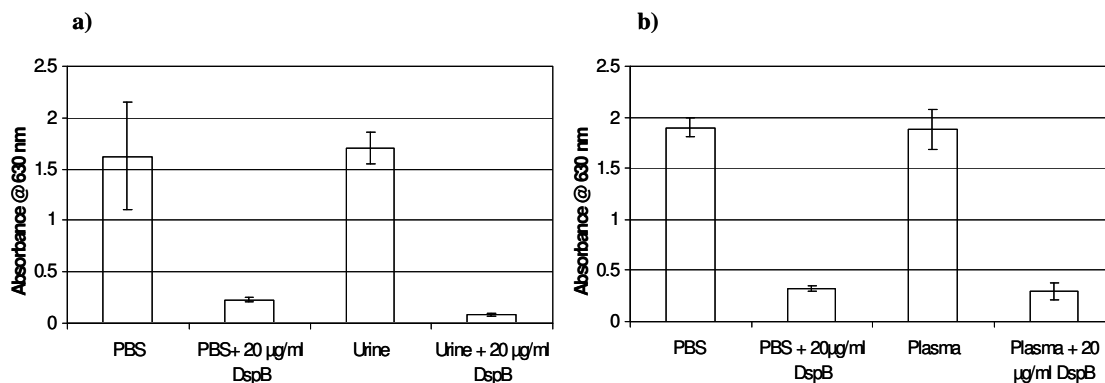
**Table 1: Antibiofilm activity of DispersinB<sup>®</sup> against human and animal pathogens**

<b>Pathogen</b>	<b>Antibiofilm activity</b>	<b>Reference</b>
Acinetobacter spp.	Inhibition, dispersal	Dr. Kaplan, J.B. (Personal Communication)
<i>Actinobacillus pleuropneumoniae</i>	Inhibition, dispersal	Izano, <i>et al.</i> , 2007b
<i>Aggregatibacter (Actinobacillus) actinomycetemcomitans</i>	Dispersal	Izano, <i>et al.</i> , 2007a
<i>Aggregatibacter (Haemophilus) aphrophilus</i>	Dispersal	Dr. Kaplan, J.B.(Personal Communication)
<i>Escherichia coli</i>	Inhibition, dispersal	Itoh, <i>et al.</i> , 2005
<i>Yersinia pestis</i>	Inhibition	Itoh, <i>et al.</i> , 2005
<i>Pseudomonas fluorescens</i>	Inhibition, dispersal	Itoh, <i>et al.</i> , 2005
<i>Bordetella spp.</i>	Inhibition	Parise, <i>et al.</i> , 2007
<i>Xanthomonas axonopodis</i>	Dispersal	Dr. Kaplan, J.B. (Personal Communication)
<i>Staphylococcus epidermidis</i>	Inhibition, dispersal	Izano, <i>et al.</i> , 2008b; Rohde, <i>et al.</i> , 2007
<i>S. aureus</i>	Inhibition	Izano, <i>et al.</i> , 2008b; Rohde, <i>et al.</i> , 2007

### 5. Compatibility of DispersinB<sup>®</sup> with biological fluids

The compatibility of DispersinB<sup>®</sup> with the biological fluids such as human urine and blood plasma (BioChemmed Services, Winchester, VA, USA) was determined using *S. epidermidis* biofilm dispersal assay. *S. epidermidis* biofilm was grown in 96-well microtiter plate containing tryptic soy broth (TSB). After 24 h incubation at 37°C, the broth containing planktonic cells was discarded. DispersinB<sup>®</sup> at 20 µg/ml was mixed with phosphate buffered saline (PBS), urine and plasma separately and added to the wells containing *S. epidermidis* biofilm. The plate was further incubated at 37°C for 4 h and the biofilm was estimated by crystal violet method. Although, urine and plasma did not disperse *S. epidermidis* biofilm, DispersinB<sup>®</sup> significantly dispersed biofilm in combination with urine and plasma. This indicates that these two biological fluids do not affect the activity of DispersinB<sup>®</sup> (Fig. 5 a and b).

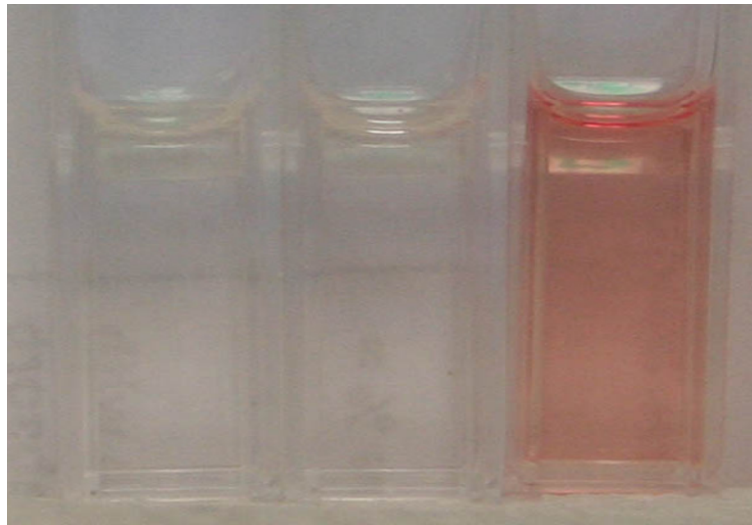
**Fig 5: Effect of human urine and human blood plasma on biofilm dispersal activity of DispersinB<sup>®</sup>**



## 6. Haemolytic activity of DispersinB<sup>®</sup>

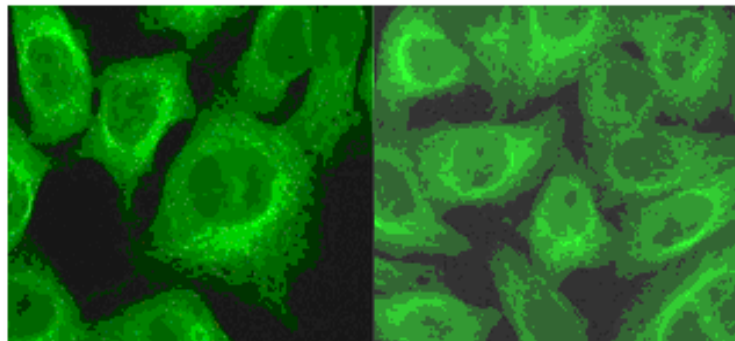
The haemolytic activity of DispersinB<sup>®</sup> was determined as previously described by Stark *et al.* (2002) and Klajnert *et al.* (2006). Briefly, erythrocytes were separated from human blood plasma and leukocytes by centrifugation at 4°C, and washed 3 times with PBS. Washed erythrocytes were centrifuged for 5 min and used immediately by suspending in PBS to obtain 4% suspension. DispersinB<sup>®</sup> was diluted to appropriate concentrations in PBS and 100 µl of it was transferred to a polypropylene tube containing 100 µl of erythrocyte suspension. Similarly, 100 µl each of PBS and 0.1% TritonX-100 were added separately to polyethylene tubes containing 100 µl of erythrocyte suspension. PBS and 0.1% TritonX-100 served as negative and positive controls, respectively. The tubes were incubated for 1 hr at 37°C and centrifuged at 4°C for 5 min, and transferred 100 µl of supernatant to cuvettes. After adding 900 µl of PBS to each cuvette, the reading was taken at 540 nm using a UV-Vis spectrophotometer.

While PBS and DispersinB<sup>®</sup> (400 µg/ml) did not show any haemolytic activity, TritonX-100 caused 100% lysis of erythrocytes (**Fig. 6**). Furthermore, DispersinB<sup>®</sup> did not seem to have any noticeable haemolytic activity when its concentration was increased up to 2 mg/ml (data not shown). Thus, the results of this haemolytic activity test indicate the haemocompatibility of DispersinB<sup>®</sup>.

**Fig. 6: Haemolytic activity of DispersinB<sup>®</sup>****PBS****400 µg/ml  
DispersinB™****0.1%  
Triton-X**

### 7. Toxicity

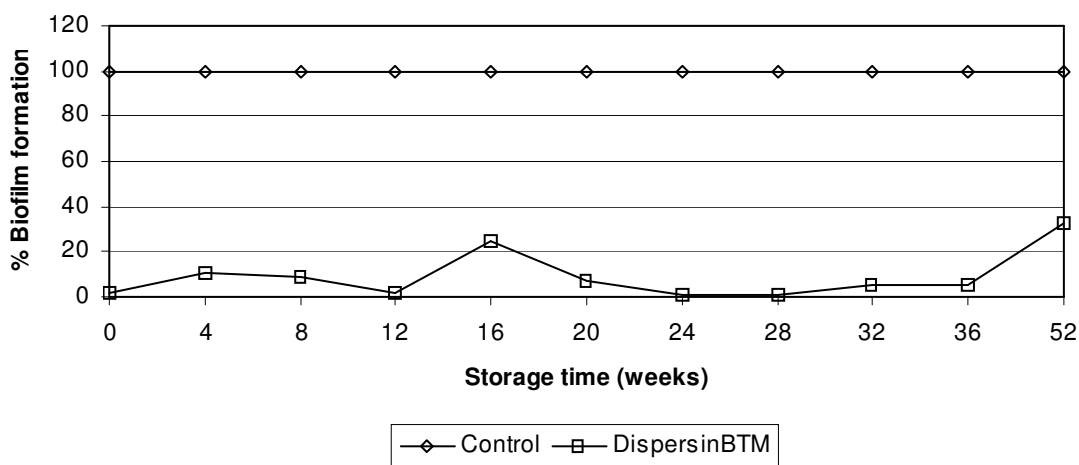
The *in vitro* cytotoxic effect of DispersinB<sup>®</sup> on eukaryotic cells was evaluated using Hep-2 cell culture (Donelli, *et al.*, 2007). Hep-2 cells were seeded on a glass coverslip in a 24-well plate, and treated with 300µg/ml DispersinB<sup>®</sup> for 24 h (**Fig. 7**). In order to assess the possible action on the cytoskeleton by DispersinB<sup>®</sup>, Hep-2 cells were stained with fluorescein isothiocyanate dye. DispersinB<sup>®</sup> did not cause any alteration in the cell cytoskeleton.

**Fig. 7: Cytotoxic effect of DispersinB<sup>®</sup> on Hep-2 cells****Control****DispersinB<sup>®</sup>-treated**

## 8. Stability

The silicone urinary catheter (14 fr) segments (1-cm) were coated with 60 µg/ml DispersinB<sup>®</sup> and stored at room temperature for 52 weeks. Every 4 weeks, the antibiofilm activity was tested by adhesion assay performed against *S. epidermidis* (Cormio, *et al.*, 2001). For adhesion assay, coated and uncoated segments were placed in 15-ml sterile culture tube, each containing 10 ml tryptic soy broth (TSB). The tubes were inoculated with 100 µl of inoculum ( $10^7$ - $10^8$  CFUs) and incubated in a water bath for 16-18 h at 37°C with shaking at 100 rpm. After incubation, the catheter segments were washed three times in saline and transferred into 2-ml tube containing 1 ml saline. Adherent cells were removed by sonication for 30 s, followed by vortexing for 1 min. The cells were serially diluted in saline and then plated on to tryptic soy agar plates. The plates were incubated at 37°C for 24 h, and the colonies were counted (**Fig. 8**). The DispersinB<sup>®</sup> coating on urinary catheter was effective in preventing catheter colonization by *S. epidermidis* for more than 52 weeks (1 year) indicating the stability of DispersinB<sup>®</sup> at room temperature.

**Fig. 8: Storage stability of silicone catheter coated with DispersinB<sup>®</sup> at room temperature**



## 9. DispersinB<sup>®</sup> in Combination with Antimicrobials

As DispersinB<sup>®</sup> can only inhibit or disperse bacterial biofilms without affecting their growth, it would be necessary to combine it with an antimicrobial (antiseptic or antibiotic or detergent or bacteriophage) in order to inhibit the growth and proliferation of biofilm-embedded bacteria (Madhyastha, *et al.* WO 2008/043195 A1; Darouiche, *et al.*, 2009). Furthermore, a composition

comprising DispersinB<sup>®</sup> and an antimicrobial with both antibiofilm and antimicrobial activity is highly effective against biofilm-embedded bacteria as DispersinB<sup>®</sup> makes them more susceptible to antimicrobial. In other words, the use of DispersinB<sup>®</sup> together with an antimicrobial is a two pronged approach wherein DispersinB<sup>®</sup> inhibits or disperses biofilms and an antimicrobial inhibits bacterial growth and proliferation. Alternatively, DispersinB<sup>®</sup> and an antimicrobial can be used sequentially for inhibiting or dispersing biofilms prior to inhibiting the growth and proliferation of bacteria with an antimicrobial.

### 9.1 DispersinB<sup>®</sup>/Antiseptics

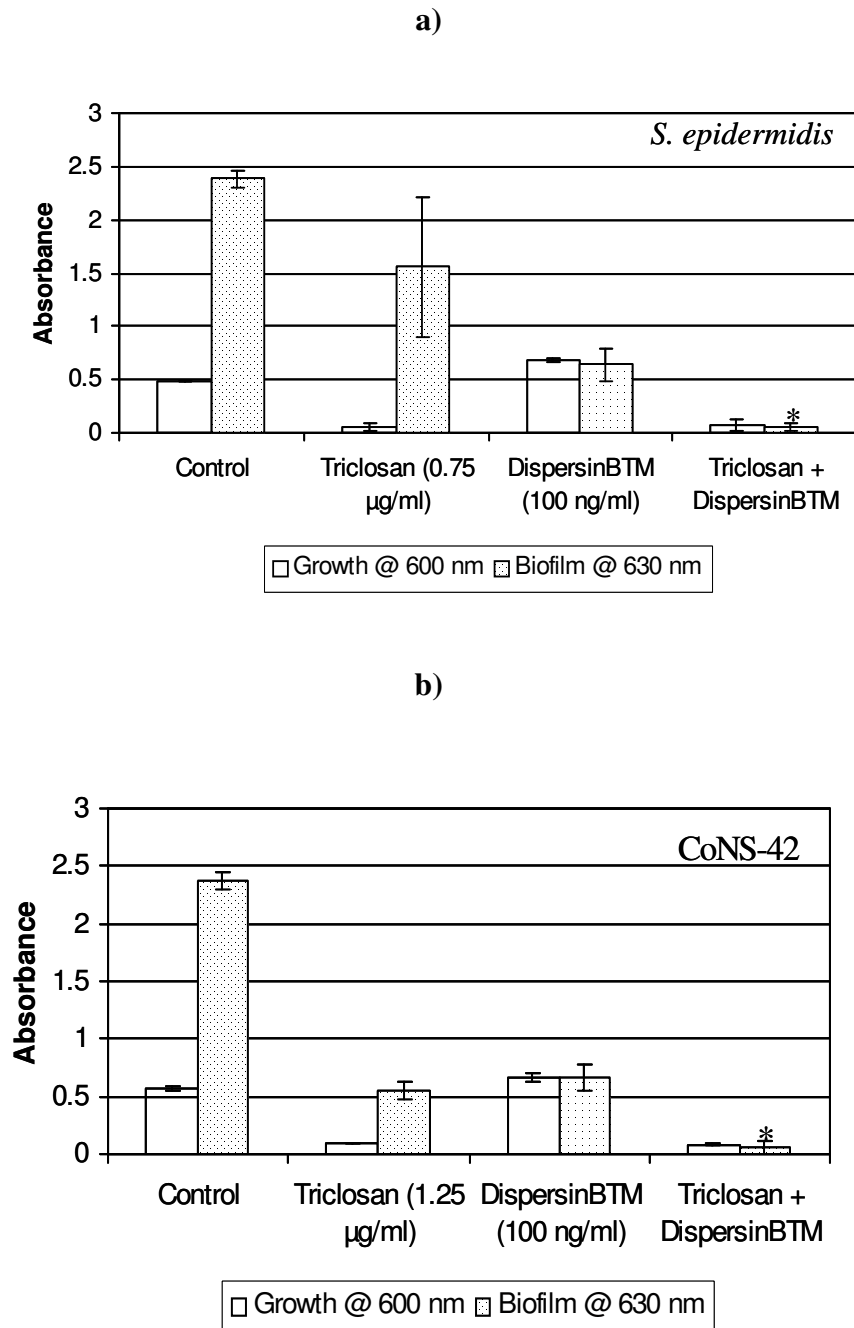
#### *9.1.1 DispersinB<sup>®</sup>/Triclosan*

Although the combination of DispersinB<sup>®</sup> and Triclosan has wide applications, we are currently focusing on its application in antibiofilm-antimicrobial coating of catheters and in wound care. The following experimental data show synergy, broad-spectrum activity, *in vitro* and *in vivo* efficacy of DispersinB<sup>®</sup> and Triclosan combination:

#### *9.1.2 Synergistic inhibitory effect of DispersinB<sup>®</sup>/Triclosan on biofilm formation*

Biofilms were assayed by crystal violet staining, as described previously (Jackson, *et al.*, 2002). The overnight grown cultures were diluted to 5% in TSB and grown in 96-well microtiter plates (Corning Inc., New York). The central venous catheter (CVC)-associated pathogens *S. epidermidis* and Coagulase-negative Staphylococci-42 (CoNS-42) biofilms were grown in the presence and absence of DispersinB<sup>®</sup> and Triclosan alone and in combination. Biofilm growth was determined by measuring the absorbance at 630 nm. Triclosan in combination with DispersinB<sup>®</sup> significantly inhibited biofilm formation by *S. epidermidis* and CoNS-42 as compared to either control or either agents alone ( $P < 0.05$ ), thereby indicating the synergy between Triclosan and DispersinB<sup>®</sup> (**Fig. 9a,b**). The *S. epidermidis* and CoNS-42 biofilms were reduced to undetectable levels when Triclosan was combined with DispersinB<sup>®</sup>.

**Fig. 9: Effect of DispersinB<sup>®</sup> and Triclosan alone and in combination on staphylococcal biofilm formation**

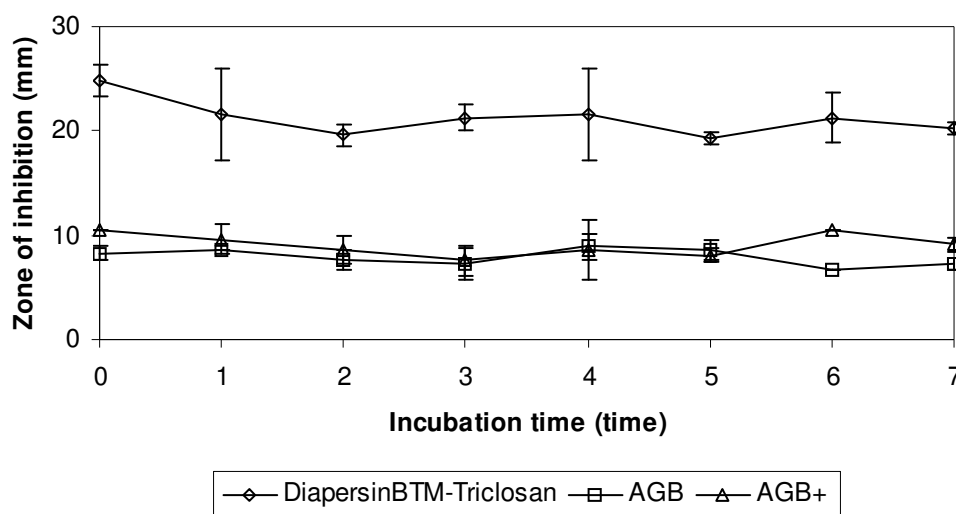


9.1.3 *In vitro* durability of DispersinB<sup>®</sup>/Triclosan coated, ARROWg+ard Blue (AGB), and ARROWg+ard Blue Plus (AGB+) catheters by serial plate transfer method against *S. aureus*

The overnight cultures were spread on to the surface of tryptic soy agar (TSA) plates and 1 cm segments of polyurethane catheter coated with DispersinB<sup>®</sup>/Triclosan; AGB and AGB+ were

placed on to the surface of agar plates separately. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured as a clear zone perpendicular to the long axis of the catheter segment. The catheter segments were transferred daily to fresh lawns on TSA. The DispersinB<sup>®</sup>/Triclosan-coated, AGB and AGB+ catheter segments were effective in inhibiting the growth of *S. aureus* for more than 7 days (Fig. 10). However, the zone sizes of AGB and AGB+ catheters were smaller than DispersinB<sup>®</sup>/Triclosan-coated catheters during 7 days serial plate transfer.

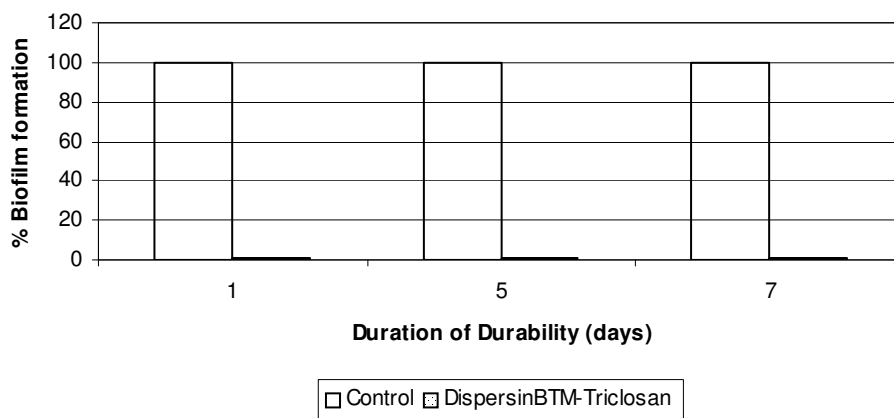
**Fig. 10: *In vitro* durability of DispersinB<sup>®</sup>/Triclosan-coated, AGB, AGB+ polyurethane catheters as determined by the zone of inhibition against *Staphylococcus aureus***



#### 9.1.4 *In vitro* durability of DispersinB<sup>®</sup>/Triclosan- coated CVC in rabbit plasma

One-cm long segments of uncoated, and DispersinB<sup>®</sup>/Triclosan coated CVCs were placed in separate bottles containing 10 ml rabbit plasma. The bottles were incubated at 37°C and 100 rpm shaking. Catheter segments were removed from the flask after 1, 5, and 7 days to perform adhesion assay against *S. epidermidis*. DispersinB<sup>®</sup>/Triclosan coated catheter segments were significantly less colonized by *S. epidermidis* as compared to control catheter segments during the 7 day experiment ( $P < 0.05$ ) (Fig. 11).

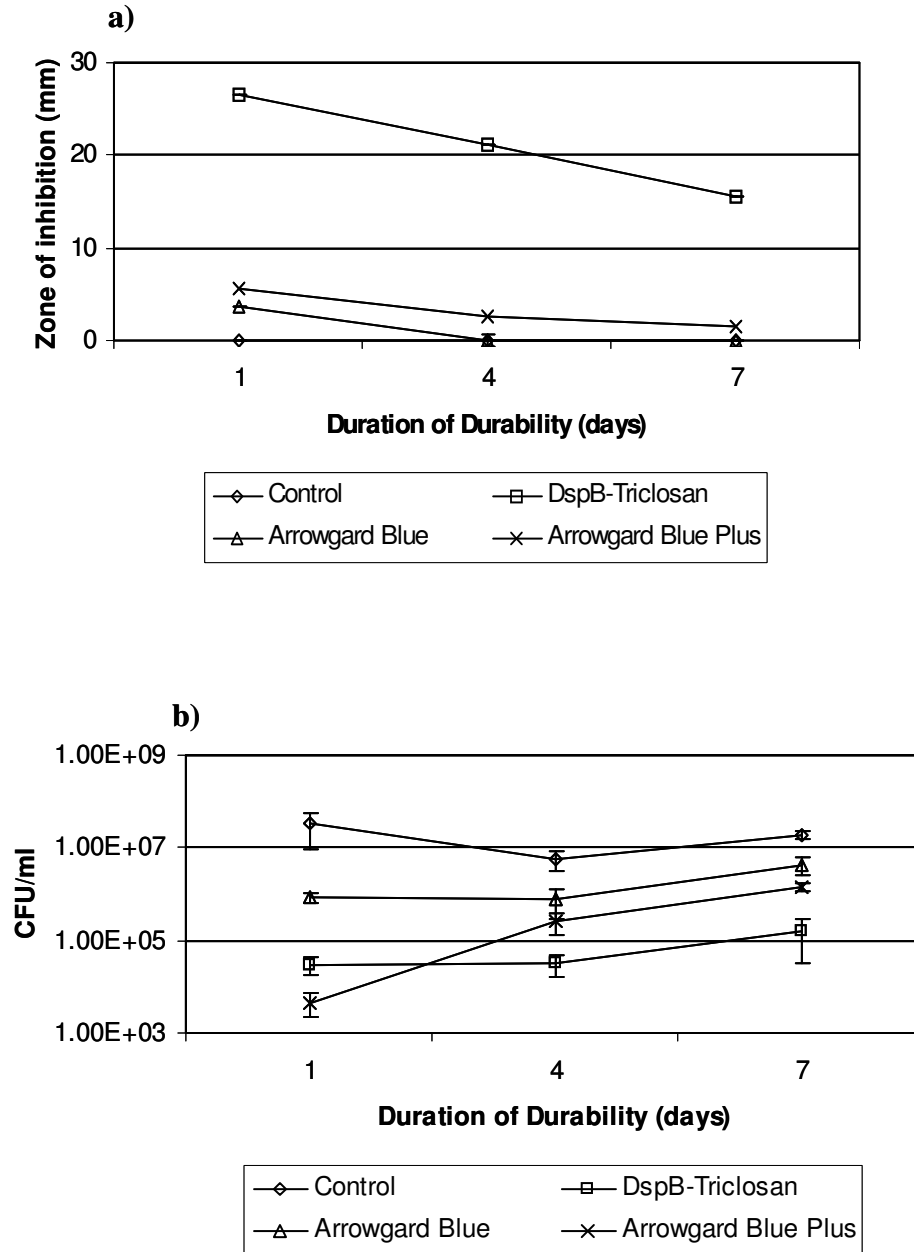
**Fig. 11: *In vitro* durability of DispersinB<sup>®</sup>/Triclosan-coated polyurethane CVC in rabbit plasma**



#### 9.1.5 *In vitro* durability of DispersinB<sup>®</sup>/Triclosan-coated CVC in bovine serum

One-cm long segments of uncoated, DispersinB<sup>®</sup>/Triclosan coated, Arrowgard Blue, and Arrowgard Blue Plus CVCs were placed in separate 500 ml flasks and soaked in 100 ml TSB containing 20% bovine serum (Kim, *et al.*, 2002). The flasks were incubated at 37°C with 100 rpm shaking. Catheter segments were removed from the flask after 1, 4, and 7 days to perform zone of inhibition and adhesion assay against *S. aureus*. On day 1, the mean zones of inhibition for DispersinB<sup>®</sup>/Triclosan, Arrowgard Blue and Arrowgard Blue Plus were 26.6, 3.6, and 5.6 mm respectively (**Fig. 12a**). After being soaked for 7 days, the mean zones of inhibition produced by DispersinB<sup>®</sup>/Triclosan-coated CVC, Arrowgard Blue, and Arrowgard Blue Plus were 15.6, 0, and 1.6 mm. Furthermore, DispersinB<sup>®</sup>/Triclosan coated, Arrowgard Blue, and Arrowgard Blue Plus catheter segments were significantly less likely to be colonized by *S. aureus* as compared to control catheter segments during the 7 days of the experiment ( $P < 0.05$ ). However, after 7 days of soaking DispersinB<sup>®</sup>/Triclosan-coated catheters were colonized approx. 1 log lower than, Arrowgard Blue, and Arrowgard Blue Plus catheter segments but the difference was not significant ( $P \geq 0.05$ ) (**Fig. 12b**).

**Fig. 12: *In vitro* durability of DispersinB<sup>®</sup> (DspB)/Triclosan-coated CVC in comparison with Arrowgard CVC as determined by (a) the zone of inhibition and (b) the adhesion assay against *S. aureus***



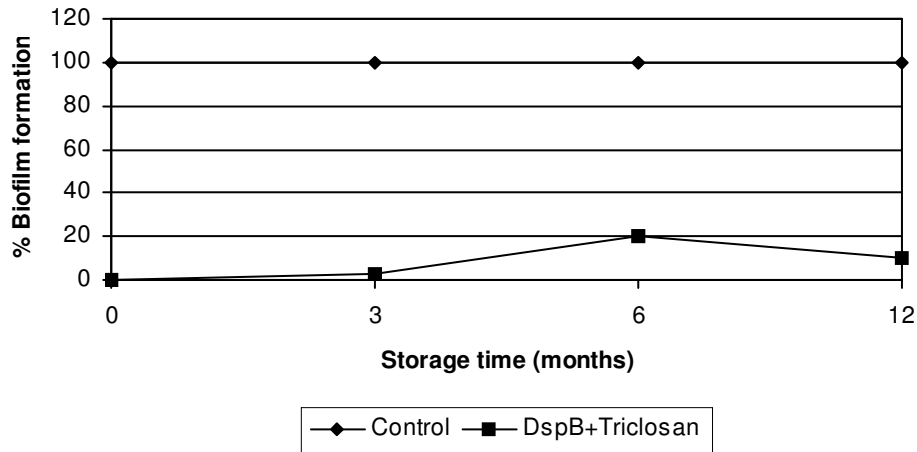
*9.1.6 In vitro storage stability of DispersinB<sup>®</sup>/Triclosan-coated polyurethane CVC and silicone urinary catheters at room temperature*

The DispersinB<sup>®</sup>/Triclosan coated and uncoated polyurethane and silicone catheter segments were stored at room temperature for one year. The stability was studied by performing adhesion

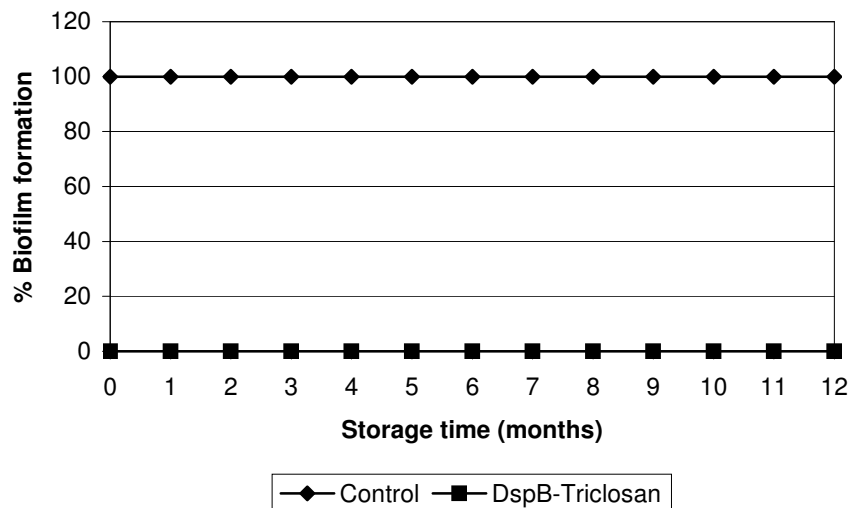
assay against *S. epidermidis*. The DispersinB<sup>®</sup>/Triclosan coating on polyurethane and silicone catheters was stable for a year at room temperature preventing 90% catheter adhesion by *S. epidermidis* (Fig. 13a,b).

**Fig. 13: Storage stability of DispersinB<sup>®</sup>/Triclosan coated catheters:**

**a) Polyurethane catheters**



**b) Silicone catheters**

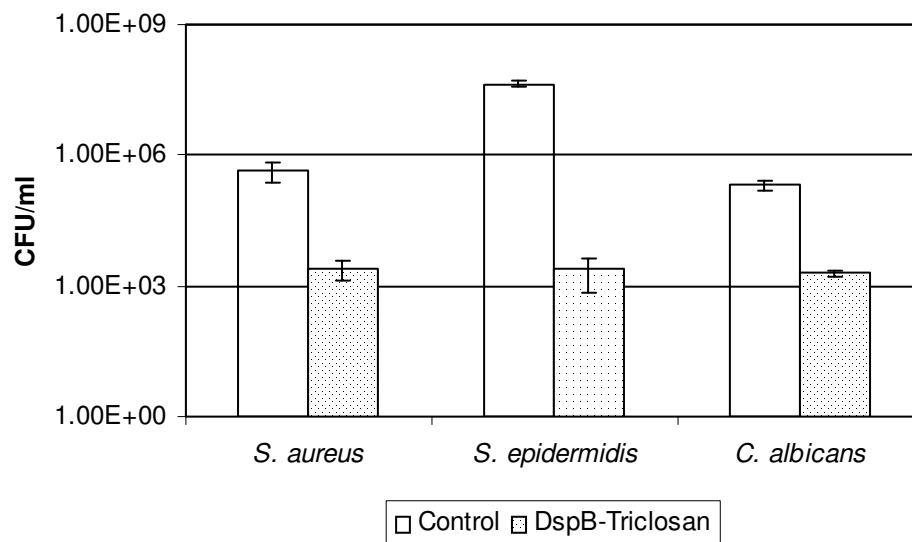


**9.1.7 In vitro efficacy of DispersinB<sup>®</sup>/Triclosan-coated CVC**

In order to develop an effective broad-spectrum antibiofilm-antimicrobial catheter coating capable of impeding bacterial colonization *in vivo*, it was first necessary to document *in vitro*

activity. The CVC segments (1-cm) were coated with DispersinB<sup>®</sup>/Triclosan combination by dipping in a solution that contained Triclosan (10 mg/ml) and DispersinB<sup>®</sup> (100 µg/ml) and then gas sterilized with ethylene oxide. We used a slightly modified method of Cormio, *et al.* (2001) to determine the adherence of Staphylococci and *Candida albicans* to the surface of CVCs. DispersinB<sup>®</sup>/Triclosan-coated catheter segments demonstrated an antiadherence ability against all tested organisms, namely, *S. aureus*, *S. epidermidis*, and *C. albicans*. The growth of tested organisms on DispersinB<sup>®</sup>/Triclosan-coated catheter segments was significantly lower than control uncoated catheter segments ( $P < 0.05$ ) (Fig. 14). The DispersinB<sup>®</sup>/Triclosan coating on catheter segments reduced catheter adhesion by 2 logs as compared to uncoated control catheter segments. In fact, Staphylococci and *Candida albicans* account for 70%-80% and 10%-15%, respectively of central venous catheter (CVC) associated infections (Darouiche, 2001).

**Fig. 14: Effect of DispersinB<sup>®</sup>/Triclosan coated CVC on adhesion of CVC-associated pathogens**



#### 9.1.8 *In vivo* efficacy of DispersinB/Triclosan-coated CVC in a rabbit model of *S. aureus* infection

The catheter segments uncoated, DispersinB<sup>®</sup>/Triclosan-coated, ARROWg+ard Blue (AGB), and ARROWg+ard Blue plus (AGB+) were subcutaneously inserted in 20 rabbits and the insertion

sites were inoculated with clinical isolates of *S. aureus* (Darouiche, *et al.*, 2002). After one week rabbits were sacrificed and catheter segments were obtained for bacteriological analysis. All 20 rabbits tolerated surgery well and exhibited no evidence of sepsis or failure to thrive, indicating the safety of antimicrobial compounds. As the table 2 shows, 1/30 (3.3%) DispersinB<sup>®</sup>/Triclosan coated catheters, 4/30 (13.3%) AGB, 1/30 (3.3%) AGB+ catheters, and 29/30 (96.7%) uncoated control catheters became colonized with *S. aureus*. DispersinB<sup>®</sup>/Triclosan-coated, AGB, and AGB+ catheters were significantly less likely to be colonized by *S. aureus* compared to uncoated control catheters ( $P < 0.001$ ). However, no significant difference was observed when catheter colonization was compared among DispersinB<sup>®</sup>/Triclosan-coated, AGB, and AGB+ catheters groups ( $P \geq 0.05$ ).

**Table 2:** *In vivo* efficacy of DispersinB<sup>®</sup>/Triclosan-coated, ARROWg+ard Blue (AGB), ARROWg+ard Blue PLUS (AGB+), and uncoated CVCs against *S. aureus*<sup>a</sup>

Outcome	No. of catheter segment with outcome/total (%)				P value
	DispersinB <sup>®</sup> /Triclosan	AGB	AGB+	Uncoated	
Catheter	1/30 (3.3%)	4/30	1/30	29/30	0.353 <sup>1</sup>
colonization		(13.3%)	(3.3%)	(96.7%)	1.0 <sup>2</sup>
					<0.001 <sup>3</sup>
					<0.001 <sup>4</sup>
					<0.001 <sup>5</sup>

<sup>a</sup> Catheter segments (1 cm long) were subcutaneously inserted in rabbits and *S. aureus* suspension ( $10^4$  CFU) was inoculated at the insertion site. After seven days, catheter segments were analyzed for catheter colonization. <sup>1</sup> $P$  value for DispersinB<sup>®</sup>/Triclosan coated vs. AGB catheter; <sup>2</sup> $P$  value for DispersinB<sup>®</sup>/Triclosan coated vs. AGB+ catheter; <sup>3</sup> $P$  value for DispersinB<sup>®</sup>/Triclosan coated vs. uncoated catheter; <sup>4</sup> $P$  value for AGB vs. uncoated catheter; <sup>5</sup> $P$  value for AGB+ vs. uncoated catheter

9.1.9 *In vivo* efficacy of catheter-lock solution containing DispersinB<sup>®</sup>/Teicoplanin antibiotic  
*Staphylococcus aureus* causes port-related bloodstream infections (PRBI) in catheterized patients. An *in vivo* experiment was performed to evaluate the activity of DispersinB<sup>®</sup> in sheep model of PRBI by administering DispersinB<sup>®</sup> as a lock solution inside the port with teicoplanin

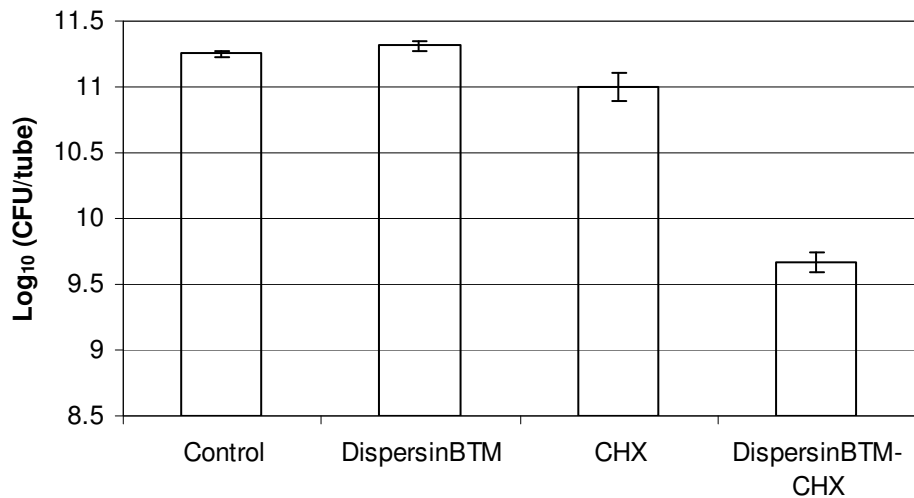
(Serrera, *et al.*, 2007). Experimental PRBI was established in 24 female sheep. Three days after the infection, animals were randomly divided into three equal groups and antimicrobial therapy was initiated as follows: (i) no treatment; (ii) Teicoplanin intravenous injection (loading dose of 6 mg/kg/12 h x 3 doses and then 4 mg/kg/24 h) plus teicoplanin locks (10 mg/ml); and (iii) Teicoplanin; I.V. and DispersinB<sup>®</sup> (40 µg/ml)/teicoplanin (10 mg/ml) locks. This treatment was administered every day for ten days. Blood samples were collected daily from venipuncture and port for microbiological analysis. Three days, after completion of therapy, sheep were sacrificed and ports were aseptically removed and cultured (swabbing of the internal lumen, septum sonication and catheter tip sonication).

All the animals in no treatment group died due to septic complications and all blood and port cultures were positive for *S. aureus*. In group II, 75% of the animals got sterile blood cultures during treatment (mean: 7, range: 3-10 days), but all the port cultures were positive after removal. In group, III 100% of the animals got sterile blood cultures during treatment (mean: 3, range: 3-8 days), and catheter cultures were completely negative in 50% of the animals. The results indicate that a combination lock of DispersinB<sup>®</sup> with teicoplanin besides systemic antibiotic therapy is active in a sheep model of PRBI.

#### 9.1.10 DispersinB<sup>®</sup>/Chlorhexidine sequential application

*In vitro* biofilm killing assay was performed to determine the effect of DispersinB<sup>®</sup> pre-treatment on susceptibility of *Staphylococcus epidermidis* to CHX. *S. epidermidis* biofilm was grown in tubes and pre-treated with PBS or DispersinB<sup>®</sup> (20 µg/ml), for 30 min and then treated with CHX (0.2 µg/ml) for 5 min at 37°C (Izano, *et al.*, 2007a). The untreated, DispersinB<sup>®</sup> or CHX alone did not significantly kill *S. epidermidis* biofilm (**Fig. 15**). However, CHX caused a 1.6-log unit decrease in the number of CFUs in tubes pre-treated with DispersinB<sup>®</sup>. DispersinB<sup>®</sup> pre-treatment makes biofilm embedded cells more susceptible to CHX treatment.

**Fig. 15: Effect of DispersinB<sup>®</sup> and chlorhexidine (CHX) on *Staphylococcus epidermidis* biofilm**

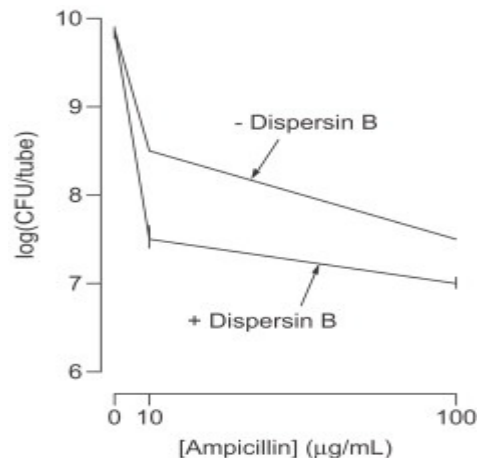


## 9.2 DispersinB<sup>®</sup>/Antibiotics

### 9.2.1 DispersinB<sup>®</sup>/Ampicillin

*Actinobacillus pleuropneumoniae* biofilm was treated with 0, 10 or 100 µg/ml ampicillin and 0 or 20 µg/ml DispersinB<sup>®</sup>. Biofilm treated with combination of DispersinB<sup>®</sup> and ampicillin exhibited a significant decrease in the number of CFU/tube when compared with biofilm treated with ampicillin alone (**Fig. 16**,  $P < 0.02$ ). Therefore, treatment of *A. pleuropneumoniae* biofilm with DispersinB<sup>®</sup> rendered them more sensitive to killing by ampicillin (Izano, *et al.*, 2007b).

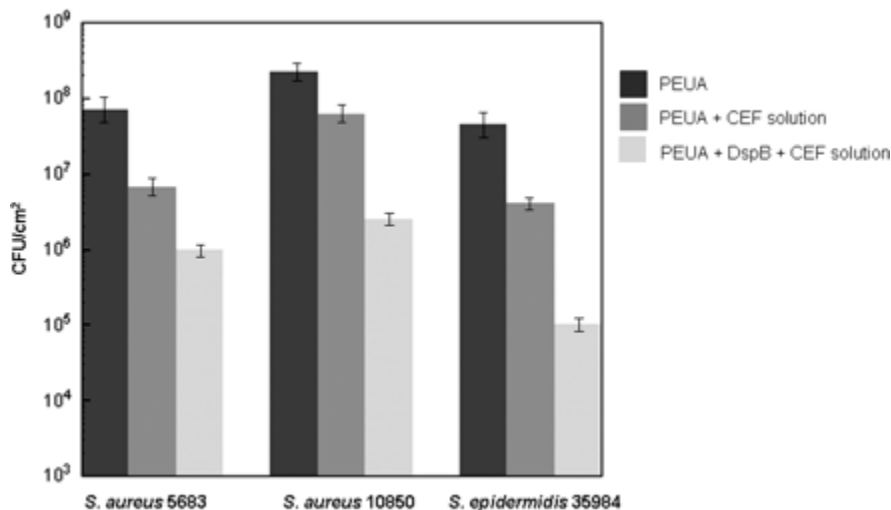
**Fig. 16: Effect of DispersinB<sup>®</sup> and ampicillin alone and in combination on *A. pleuropneumoniae* biofilm**



### 9.2.2 DispersinB<sup>®</sup>/Cefamandole nafate

Staphylococcal biofilm was developed onto polyurethane disk loaded with and without DispersinB<sup>®</sup> for 24 h. The planktonic growth was removed by washing the disks twice with PBS and treated overnight with 0.25 µg/ml cefamandole nafate. The methicillin-susceptible (strain 5683), methicillin-resistant (strain 10850) strain of *S. aureus* and *S. epidermidis* ATCC 35984 biofilm developed on DispersinB<sup>®</sup> loaded disks were more sensitive to cefamandole nafate treatment compared to unloaded disks (Donelli, *et al.*, 2007) (**Fig. 17**).

**Fig. 17: Effect of DispersinB<sup>®</sup> loaded polyurethane disk on the antimicrobial activity of cefamandole nafate against biofilm-embedded *S. aureus* and *S. epidermidis***

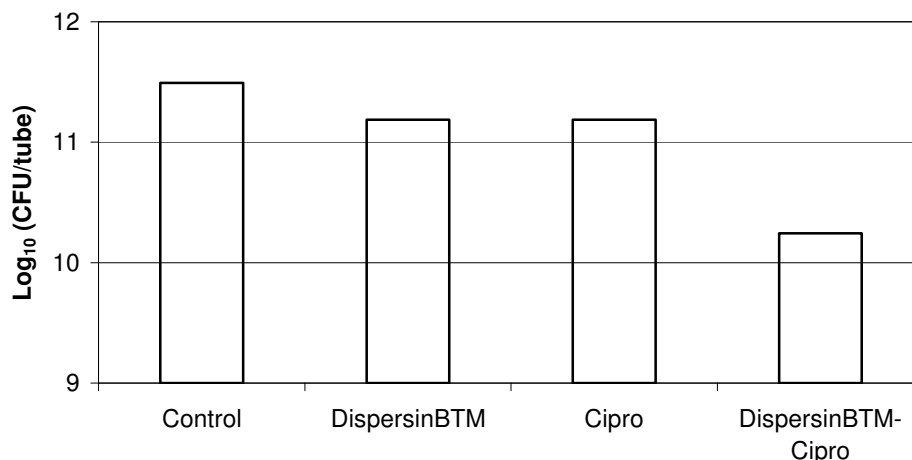


### 9.2.3 DispersinB<sup>®</sup>/Ciprofloxacin

*In vitro* biofilm dispersal assay was performed to determine the effect of DispersinB<sup>®</sup> on enhancing the sensitivity of *S. epidermidis* biofilm to ciprofloxacin. *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes were rinsed with 200 µl of fresh medium and then treated with 200 µl medium containing 200 µg/ml of ciprofloxacin and/or 20 µg/ml of DispersinB<sup>®</sup> (Izano, *et al.*, 2007b). After incubating for 3 hours at 37°C, 10 µl of 200µg/ml DispersinB<sup>®</sup> was added to each tube and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of cells were plated on TSA. When DispersinB<sup>®</sup> was used in combination with ciprofloxacin; it increased the sensitivity of *S. epidermidis* biofilm to

ciprofloxacin (**Fig. 18**). Thus, DispersinB<sup>®</sup> and ciprofloxacin combination have an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

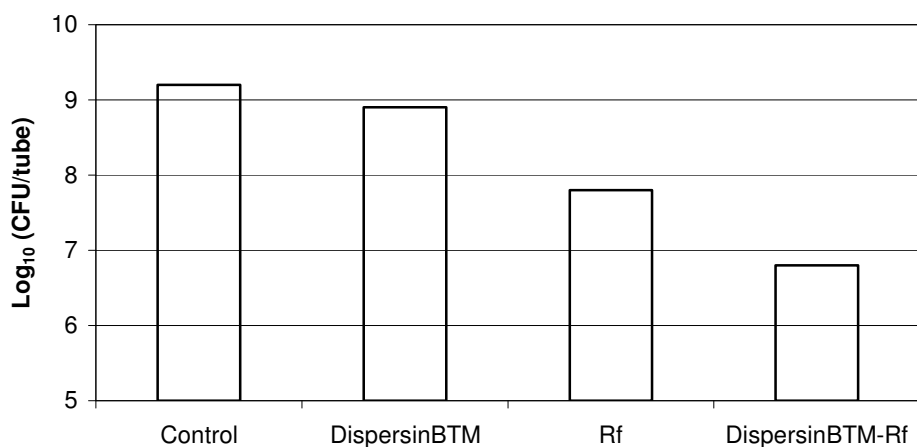
**Fig. 18: Effect of DispersinB<sup>®</sup> and ciprofloxacin (cipro) alone and in combination on *S. epidermidis* biofilm**



#### 9.2.4 DispersinB<sup>®</sup>/Rifampicin

*In vitro* biofilm dispersal assay was performed to determine the effect of DispersinB<sup>®</sup> on enhancing the sensitivity of *S. epidermidis* biofilm to rifampicin. *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes were rinsed with 200  $\mu$ l of fresh medium and then treated with 200  $\mu$ l medium containing 100  $\mu$ g/ml of rifampicin and/or 20  $\mu$ g/ml of DispersinB<sup>®</sup> (Izano, *et al.*, 2007b). After incubating for 3 hours at 37°C, 10  $\mu$ l of 200 $\mu$ g/ml DispersinB<sup>®</sup> was added to each tube and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of cells were plated on TSA. When DispersinB<sup>®</sup> was used in combination with rifampicin; it increased the sensitivity of *S. epidermidis* biofilm to rifampicin (**Fig. 19**). Thus, DispersinB<sup>®</sup> and rifampicin combination have an enhanced inhibitory effect on biofilm-embedded *S. epidermidis*.

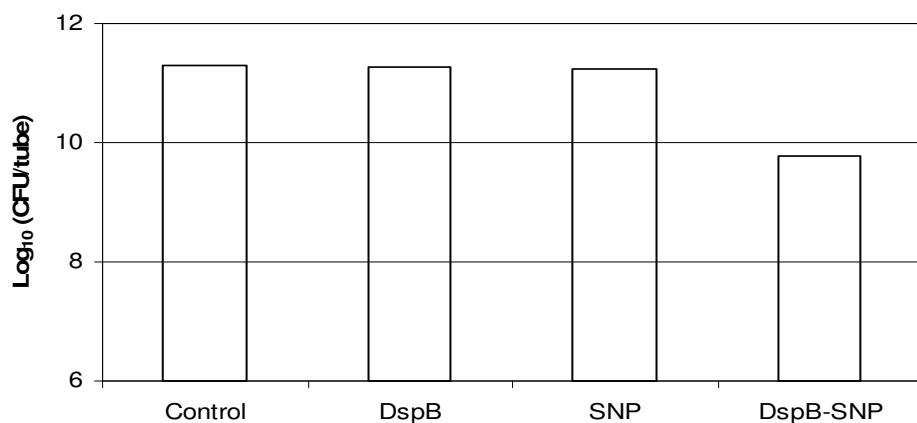
**Fig. 19: Effect of DispersinB<sup>®</sup> and rifampicin (Rf) alone and in combination on *S. epidermidis* biofilm**



### 9.3 DispersinB<sup>®</sup>/Silver nanoparticles sequential application

An *in vitro* biofilm assay was performed to determine the effect of DispersinB<sup>®</sup> pre-treatment on the susceptibility of *S. epidermidis* to silver nanoparticles (SNP). *S. epidermidis* biofilm grown in tubes was pre-treated with PBS or DispersinB<sup>®</sup> (20 µg/ml) for 30 min, and then treated with SNP (0.03125 µg/ml) for 60 min at 37°C. The untreated, DispersinB<sup>®</sup> or SNP alone did not significantly kill biofilm-embedded *S. epidermidis* (Fig. 20). However, SNP caused a 1.5 log decrease in CFU in tubes pre-treated with DispersinB<sup>®</sup>. Thus, DispersinB<sup>®</sup> pre-treatment made biofilm embedded cells more susceptible to SNP treatment.

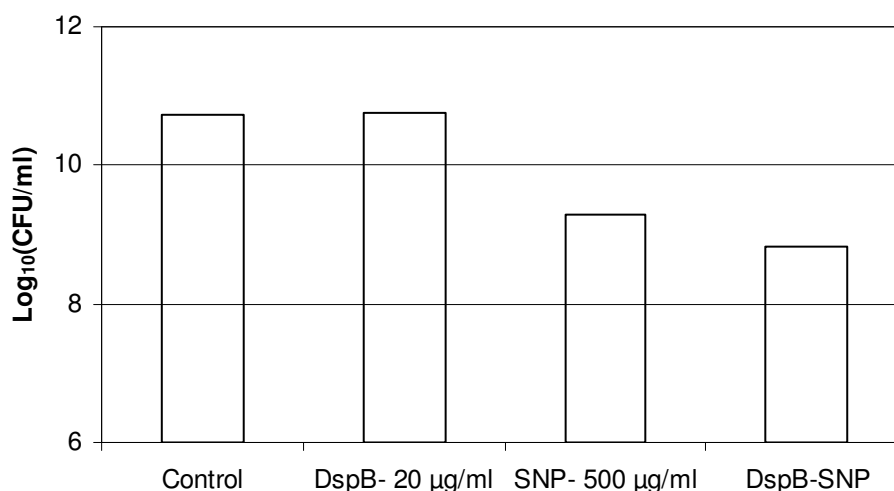
**Fig. 20: Effect sequential application of DispersinB<sup>®</sup> and silver nanoparticles (SNP) on *Staphylococcus epidermidis* biofilm**



#### 9.4 DispersinB<sup>®</sup>/ Silver nanoparticles combination

*In vitro* biofilm dispersal assay was performed to determine the enhancing effect of DispersinB<sup>®</sup> on the sensitivity of *S. epidermidis* biofilm to silver nanoparticles (SNP). *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes was rinsed with 200  $\mu$ l of fresh medium and then treated with 200  $\mu$ l medium containing SNP (500  $\mu$ g/ml) and DispersinB<sup>®</sup> (20  $\mu$ g/ml) alone and in combination (Izano, *et al.*, 2007b). After incubating for 3 hours at 37°C, 10  $\mu$ l of 200 $\mu$ g/ml DispersinB<sup>®</sup> was added to each tube and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of cells were plated on TSA. When DispersinB<sup>®</sup> was used in combination with SNP; it increased the sensitivity of *S. epidermidis* biofilm to SNP (**Fig. 21**). Thus, DispersinB<sup>®</sup> and SNP combination showed a synergistic inhibitory effect on biofilm-embedded *S. epidermidis*.

**Fig. 21: Effect of DispersinB<sup>®</sup> and silver nanoparticles (SNP) combination on *Staphylococcus epidermidis* biofilm**

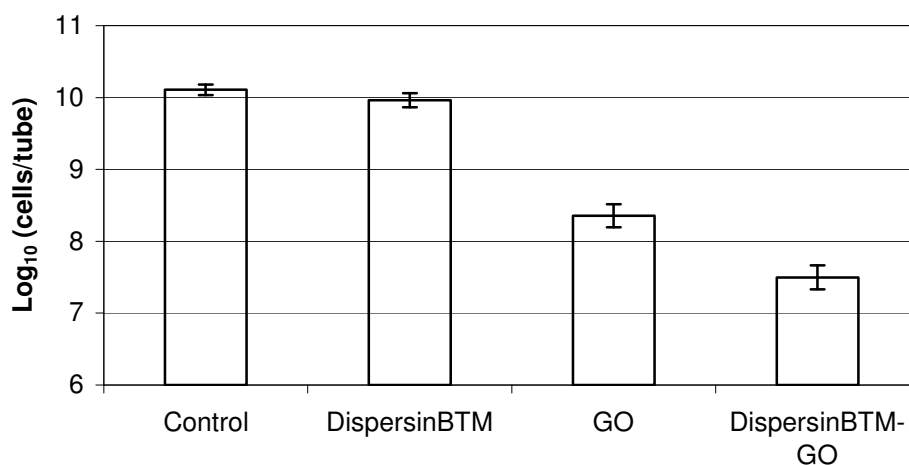


#### 9.5 DispersinB<sup>®</sup>/ Glucose Oxidase enzyme

*In vitro* biofilm dispersal assay was performed to determine the effect of DispersinB<sup>®</sup> on enhancing the sensitivity of *S. epidermidis* biofilm to antimicrobial enzyme glucose oxidase. Glucose oxidase converts glucose into hydrogen peroxide, which is an antimicrobial agent. *S. epidermidis* biofilm grown in 1.5 ml polypropylene microcentrifuge tubes were rinsed with 200

$\mu\text{l}$  of fresh medium and then treated with 200  $\mu\text{l}$  medium containing 10 U/ml of glucose oxidase and/or 20  $\mu\text{g/ml}$  of DispersinB<sup>®</sup> (Izano, *et al.*, 2007b). After incubating for 3 hours at 37°C, 10  $\mu\text{l}$  of 200 $\mu\text{g/ml}$  DispersinB<sup>®</sup> was added to each tube and tubes were incubated for additional 5 min to detach biofilm. Serial dilutions of cells were plated on TSA. When DispersinB<sup>®</sup> was used in combination with glucose oxidase; it increased the sensitivity of *S. epidermidis* biofilm to glucose oxidase (**Fig. 22**). Thus, DispersinB<sup>®</sup> and glucose oxidase combination has a synergistic inhibitory effect on biofilm-embedded *S. epidermidis*.

**Fig. 22: Effect of DispersinB<sup>®</sup> and glucose oxidase (GO) alone and in combination on *S. epidermidis* biofilm**

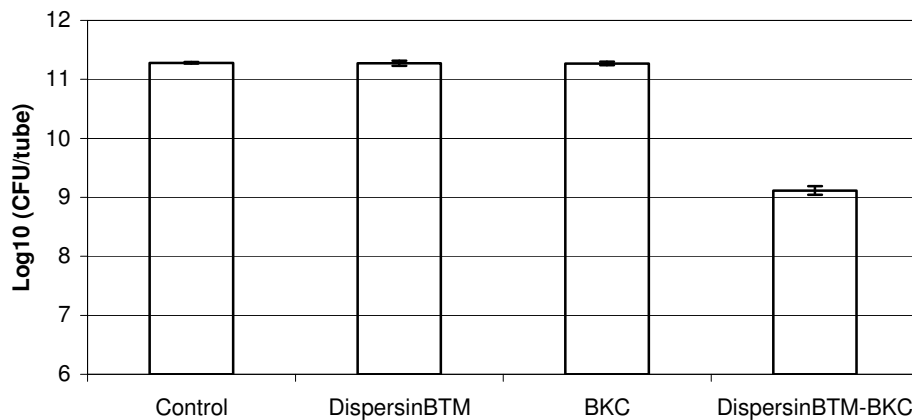


## 9.6 DispersinB<sup>®</sup>/Detergents

### 9.6.1 DispersinB<sup>®</sup>/Benzalkonium chloride sequential application

*In vitro* biofilm killing assay was performed to determine the effect of DispersinB<sup>®</sup> pre-treatment on susceptibility of *S. epidermidis* to benzalkonium chloride (BKC). *S. epidermidis* biofilm was grown in tubes were pre-treated with PBS or DispersinB<sup>®</sup> (20  $\mu\text{g/ml}$ ) for 30 min, and then treated with BKC (0.4  $\mu\text{g/ml}$ ) for 60 min at 37°C (Izano, *et al.*, 2007a). The untreated, DispersinB<sup>®</sup> or BKC alone did not significantly kill *S. epidermidis* biofilm (**Fig. 23**). However, BKC caused a 2.2 log unit decrease in the number of CFUs in tubes pre-treated with DispersinB<sup>®</sup>. DispersinB<sup>®</sup> pre-treatment makes biofilm embedded cells more susceptible to BKC treatment. Detergents are not compatible with enzymes as they precipitate proteins. Thus, DispersinB<sup>®</sup> can be used sequentially with the antimicrobial agents that are not compatible.

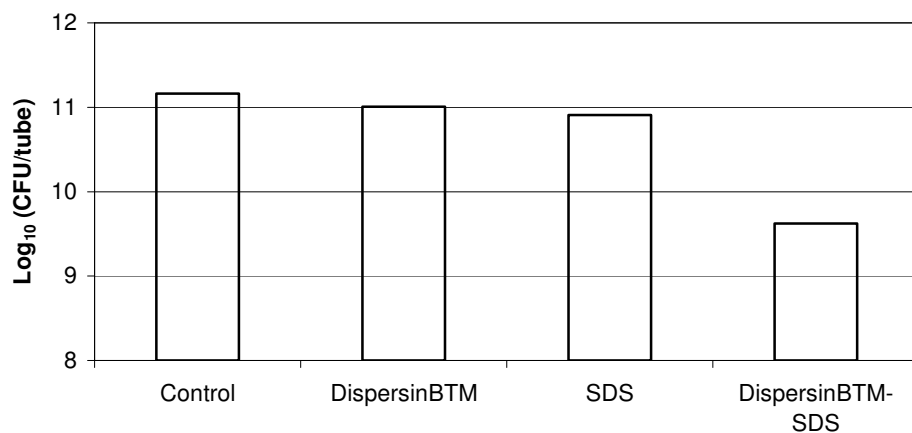
**Fig. 23: Effect of DispersinB<sup>®</sup> and benzalkonium chloride (BKC) alone and in combination on *S. epidermidis* biofilm**



#### 9.6.2 DispersinB<sup>®</sup>/SDS sequential application

*In vitro* biofilm killing assay was performed to determine the effect of DispersinB<sup>®</sup> pre-treatment on the susceptibility of *Staphylococcus epidermidis* to SDS. *S. epidermidis* biofilm was grown in tubes and pre-treated with PBS or DispersinB<sup>®</sup> (20 µg/ml), for 30 min and then treated with SDS (0.2 mg/ml) for 5 min at 37°C (Izano, *et al.*, 2007a). The untreated, DispersinB<sup>®</sup> or SDS alone did not significantly kill *S. epidermidis* biofilm (**Fig. 24**). However, SDS caused a 1.5-log unit decrease in the number of CFUs in tubes pre-treated with DispersinB<sup>®</sup>. Thus, DispersinB<sup>®</sup> pre-treatment makes biofilm embedded cells more susceptible to SDS treatment.

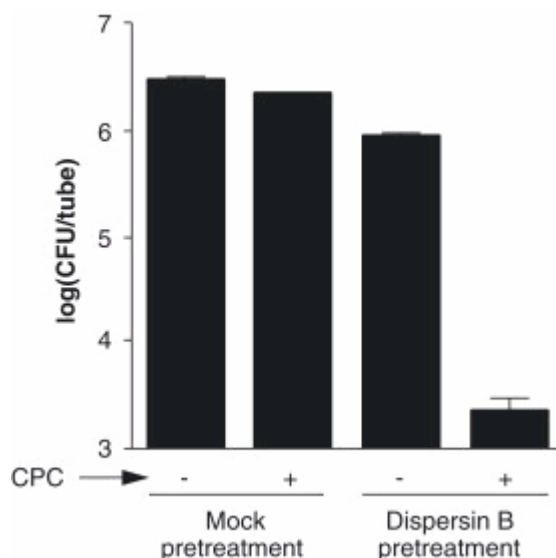
**Fig. 24: Effect of DispersinB<sup>®</sup> /SDS on *Staphylococcus epidermidis* biofilm**



### 9.6.3 DispersinB<sup>®</sup>/Cetylpyridinium chloride

*In vitro* biofilm killing assay was performed to determine the effect of DispersinB<sup>®</sup> pre-treatment on susceptibility of *Aggregatibacter actinomycetemcomitans* to cetylpyridinium chloride (CPC). *A. actinomycetemcomitans* colonizes the human oral cavity and causes periodontitis and endocarditis. *A. actinomycetemcomitans* biofilm grown in tubes were pre-treated with PBS or DispersinB<sup>®</sup> (20 µg/ml), and then were treated with CPC (0.002%) for 5 min at 37°C (Izano, *et al.*, 2008). The untreated, DispersinB<sup>®</sup> or CPC alone did not significantly kill *A. actinomycetemcomitans* biofilm. However, CPC caused a 3-log unit decrease in the number of CFUs in tubes pre-treated with DispersinB<sup>®</sup> (Fig. 25). Thus, DispersinB<sup>®</sup> pre-treatment makes biofilm embedded cells more susceptible to CPC treatment.

**Fig. 25: Effect of DispersinB<sup>®</sup> and cetylpyridinium chloride (CPC) on *Aggregatibacter actinomycetemcomitans* biofilm**

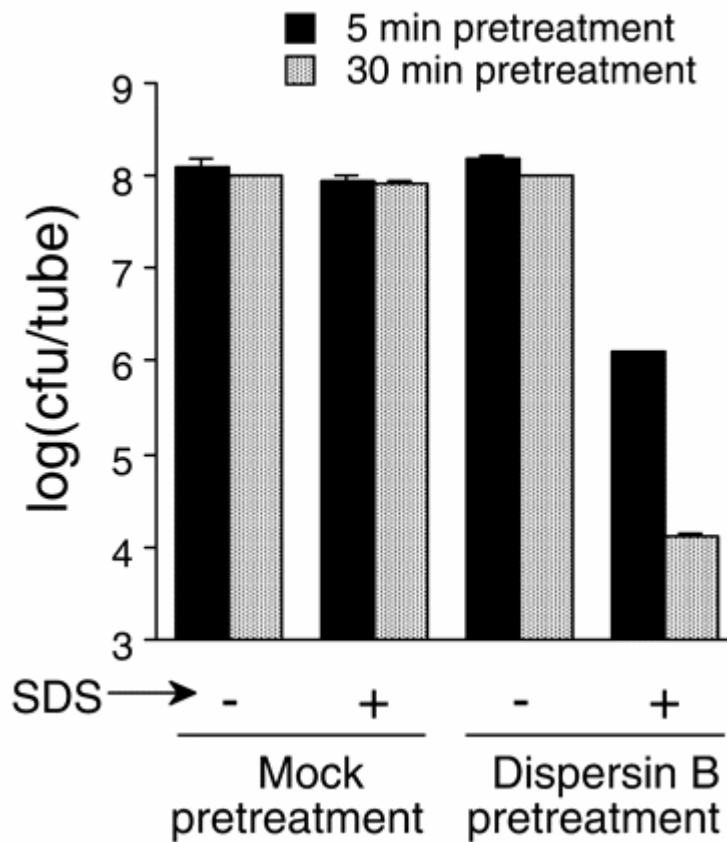


### 9.6.4 DispersinB<sup>®</sup>/SDS sequential application

*In vitro* biofilm killing assay was performed to determine the effect of DispersinB<sup>®</sup> pre-treatment on susceptibility of *Aggregatibacter actinomycetemcomitans* to SDS. *A. actinomycetemcomitans* colonizes the human oral cavity and causes periodontitis and endocarditis. *A. actinomycetemcomitans* biofilm was grown in tubes and were pre-treated with PBS or DispersinB<sup>®</sup> (20 µg/ml), for 5 and 30 min and then treated with SDS (0.01 %) for 5 min at 37°C (Izano, *et al.*, 2007a). The untreated, DispersinB<sup>®</sup> or SDS alone did not significantly kill *A.*

*actinomycetemomitans* biofilm (Fig. 26). However, SDS caused a 2-log unit decrease in the number of CFUs in tubes pre-treated with DispersinB<sup>®</sup> for 5 min, and 4-log-unit decrease in tubes pre-treated for 30 min. DispersinB<sup>®</sup> pre-treatment makes biofilm embedded cells more susceptible to SDS treatment.

**Fig. 26: Effect of DispersinB<sup>®</sup> and SDS on *Aggregatibacter actinomycetemcomitans* biofilm**

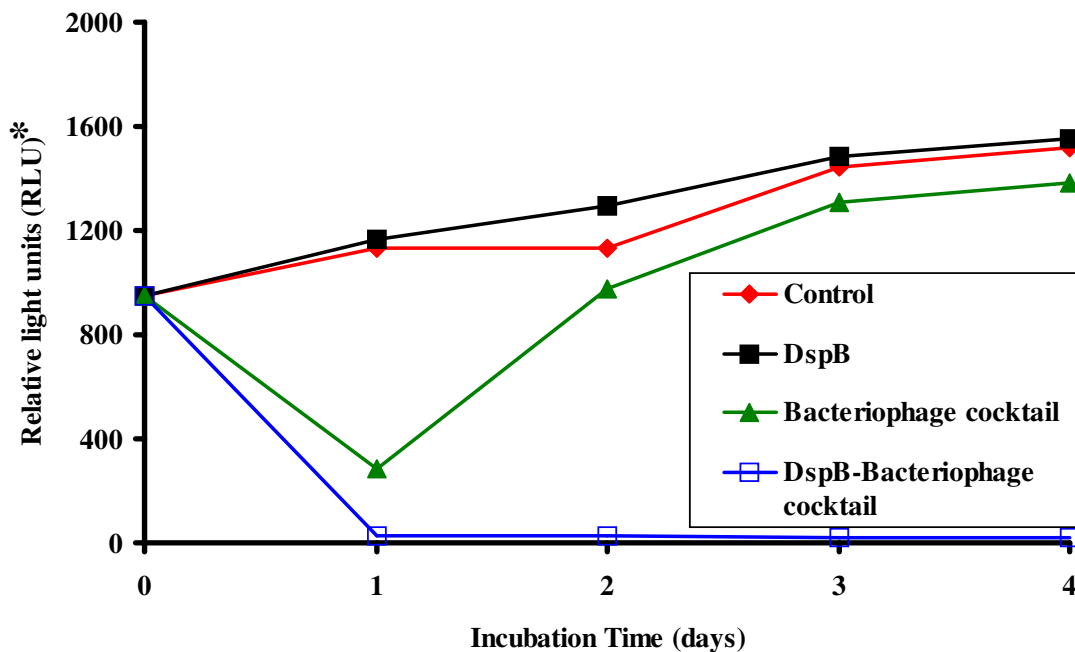


### 9.7 DispersinB<sup>®</sup>/Bacteriophage

Bacteriophages are viruses that infect bacteria. Many phages have the ability to lyse bacteria (lytic bacteriophages); usually occurring after viral assembly is complete so that fully assembled virus can exit the host cells and infect new bacterial cells. Using our DispersinB<sup>®</sup> technology, Dr. Randy Wolcott's team at the Southwest Regional Wound Care Center in Lubbock, Texas formulated a composition comprising DispersinB<sup>®</sup> enzyme and a lytic phage mixture, which disperses and kills biofilm embedded *E. coli* cells. The combination of DispersinB<sup>®</sup> and phage

mixture showed almost 99% inhibition of *E. coli* biofilm as compared to only 9% inhibition by the phage mixture alone over the four day period of treatment (Fig. 27). This combination therapy could provide a new and highly effective method of treating chronic wounds such as diabetic foot ulcers.

**Fig. 27: Effect of DispersinB<sup>®</sup> (DspB) and bacteriophage mixture on biofilm-embedded *E. coli*\***

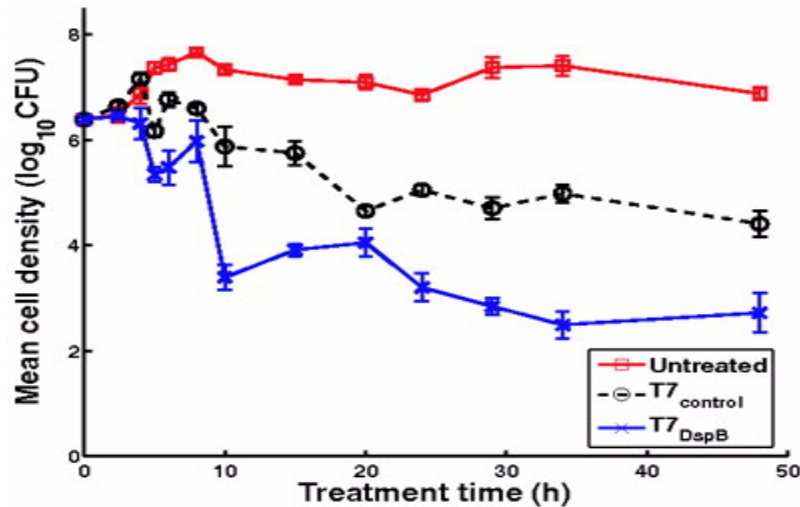


\*Biofilm embedded cells were measured in terms of bioluminescence (Relative light units, RLU)

Recently, Lu and Collins (2007) from Harvard-MIT, MA, USA engineered *E. coli* T7 bacteriophage to express DispersinB<sup>®</sup>, in order to simultaneously attack the bacterial cells in the biofilm and the biofilm extracellular polymeric substances. This two-pronged DispersinB<sup>®</sup>-expressing bacteriophage strategy removed 99.99% of bacterial biofilm, which were two orders of magnitude better than that of bacteriophage alone (Fig. 28).

Thus, this study on DispersinB<sup>®</sup>-expressing bacteriophage and Dr. Wolcott's research on DispersinB<sup>®</sup> and bacteriophage mixture combinations provide a novel strategy for treating chronic wounds more effectively than the current antibiotic/antimicrobial therapies.

**Fig. 28: Effect of engineered DispersinB<sup>®</sup> (DspB)–expressing phage treatment on *E. coli* biofilm.**

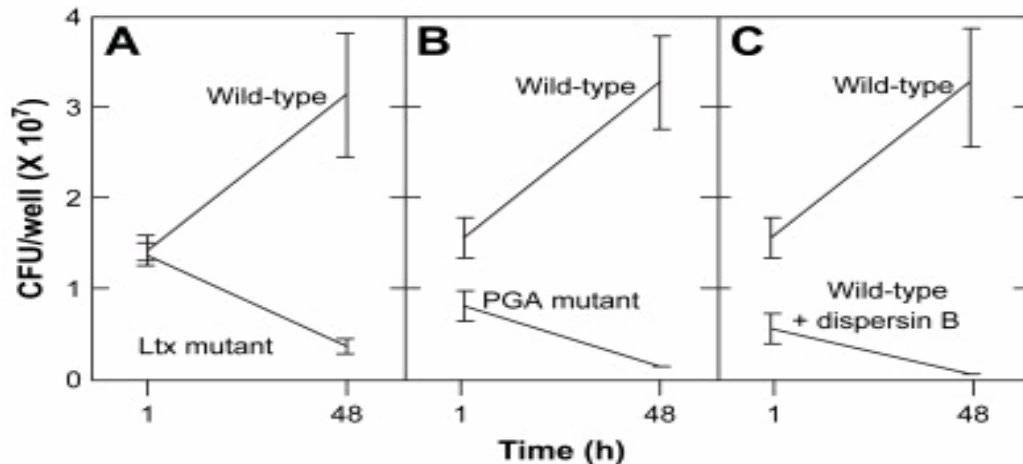


### 9.8 DispersinB<sup>®</sup>/Macrophage

Macrophages are phagocytes, acting in both non-specific defence as well as specific defence of vertebrate animals. Their role is to phagocytose (engulf and then digest) cellular debris and pathogens either as stationary or mobile cells and to stimulate lymphocytes and other immune cells to respond to the pathogen. Previously, it has been shown that bacteria in a biofilm are 100 to 1000 times more resistant to antimicrobials and host's immune responses than their planktonic counterparts. Therefore, *in vitro* biofilm killing assay was performed to demonstrate the effect of DispersinB<sup>®</sup> pre-treatment on the susceptibility of *Aggregatibacter actinomycetemcomitans* to macrophage (Venketaraman, *et al.*, 2008). The monolayers of human THP-1 macrophages were infected with single cell suspensions of wild-type, leukotoxin mutant (Ltx), and poly-N-acetylglucosamine mutant (PGA) *A. actinomycetemcomitans* strains. All infections were carried out at a ratio of 10-30 bacterial cells per macrophage. The total number of viable bacteria in each well was enumerated by lysing macrophages with water and determining the colony forming units (CFUs) by dilution plating. After 48 h, the number of *A. actinomycetemcomitans* CFUs increased approximately 100% in the wells infected with the wild-type strain, but decreased approximately 70-90% in the wells infected with leukotoxin and PGA mutant strains (**Fig. 29 A and B**). Wild type cells pre-treated with DispersinB<sup>®</sup> exhibited the same phenotype as PGA mutant cells, resulting in a significant decrease in CFUs (**Fig. 29 C**). This study showed that the

pre-treatment of biofilm-embedded *A. actinomycetemcomitans* cells with DispersinB<sup>®</sup> removes the biofilm and renders the cells sensitive to killing by the body's macrophages.

**Fig 29: Effect of DispersinB<sup>®</sup> and macrophage on *Aggregatibacter actinomycetemcomitans* biofilm**



### 9.9 DispersinB<sup>®</sup>/DNase I/FU for Cystic Fibrosis

As reported in the United States cystic fibrosis patient registry annual data report for 2001, the prevalence of *S. aureus* in respiratory secretions in cystic fibrosis patients is approximately 40% during the first year of life, rises to 58% during adolescence, and decreases throughout adulthood. *Pseudomonas aeruginosa* may be the first pathogen recovered from as many as 30% of infants. By 18 years of age, 80% of patients are infected with *P. aeruginosa*. Approximately 3% of cystic fibrosis patients of all ages now harbor *Burkholderia cepacia* complex, and 8% of adults are infected with these organisms (Saiman, *et al.*, 2003). DispersinB<sup>®</sup> inhibits as well as disperses *S. aureus* biofilm. In order to formulate a broad-spectrum antibiofilm/antimicrobial combination DispersinB<sup>®</sup> was combined with DNase I and 5-fluorouracil (FU) (Madhaystha, *et al.*, US Provisional Patent Appl. Serial No. 61/041,941). Extracellular DNA, proteins and polysaccharides act as adhesives in biofilm formation. DNase I inhibit and disperse *P. aeruginosa* and *S. aureus* biofilms (Saiman, *et al.*, 2003; Whitchurch, *et al.*, 2002). A chemotherapeutic agent FU has been in use against cancer for about 40 years also shows antimicrobial activity against *S. aureus*, *P. aeruginosa*, and *B. cepacia* (Table 3).

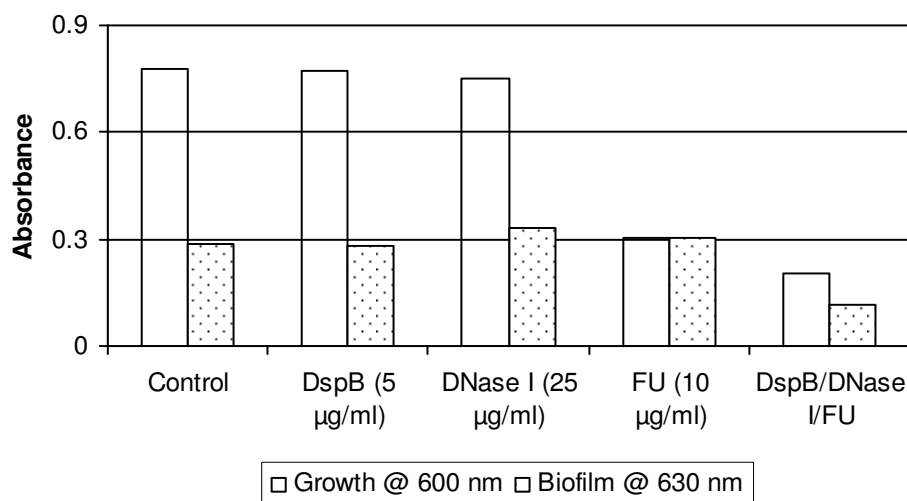
**Table 3: MIC of 5-fluorouracil against cystic fibrosis infection associated pathogens**

Pathogen	MIC ( $\mu\text{g/ml}$ )
<i>Staphylococcus aureus</i>	25
<i>Pseudomonas aeruginosa</i>	31.25
<i>Burkholderia cepacia</i>	62.5

### 9.9.1 DispersinB<sup>®</sup>/DNase I/FU

An *in vitro* microplate assay was performed to determine the effect of DispersinB<sup>®</sup>, DNase I, and FU on the growth and biofilm formation of *S. aureus*. An overnight culture of *S. aureus* in Tryptic Soy Broth (TSB) was used as inoculum. *S. aureus* biofilm was grown in TSB medium on a 96-well microtiterplate in the absence and presence of DispersinB<sup>®</sup> (5  $\mu\text{g/ml}$ ), DNase I (25  $\mu\text{g/ml}$ ), or FU (10  $\mu\text{g/ml}$ ) separately and in combination (DNase I + DispersinB + FU). The plate was incubated at 37°C for 24 hours. The planktonic growth and biofilm was estimated as described in Section 4.1.2. The DispersinB<sup>®</sup>/ DNase I/FU combinations showed synergistic inhibitory effect on *S. aureus* biofilm formation (**Fig. 30**).

**Fig. 30: Effect of DispersinB<sup>®</sup>, DNase I, 5-fluorouracil (FU) alone and in combination on *S. aureus* growth and biofilm formation**



## **10. DispersinB<sup>®</sup> Production**

Kane Biotech Inc. has retained BioVectra Inc., Charlottetown, PE, Canada to manufacture clinical grade DispersinB<sup>®</sup> to be used in the Company's wound care product. BioVectra is FDA approved cGMP facility having 35 years of experience serving a global customer base. A trial *E. coli* fermentation run performed by BioVectra in a 30 L fermentor without optimizing the conditions, showed about seven-fold increase in the production of purified DispersinB<sup>®</sup> as compared to that in a 2 L shake flask (544 vs. 79 mg/ml) indicating a great potential to increase the yield by optimizing the fermentation parameters (Yakandawala, *et al.*, 2008). Thus, the fermentation optimization as well as production scale up will increase the yield thereby decreasing the cost of DispersinB<sup>®</sup> production substantially.

## **11. Wound Gel Formulation**

As shown in Table 4 two types of DispersinB<sup>®</sup>-based wound gel were formulated. One gel contains the active ingredient DispersinB<sup>®</sup> alone (DispersinB<sup>®</sup> Antimicrobial Wound Gel) and the other contains both DispersinB<sup>®</sup> and Triclosan as active ingredients (DispersinB<sup>®</sup>/Triclosan Antimicrobial Wound Gel). DispersinB<sup>®</sup> is an antibiofilm enzyme, which inhibits biofilm formation and disperses preformed biofilms. Triclosan is a broad-spectrum antiseptic, which kills both gram-negative and gram-positive bacteria. DispersinB<sup>®</sup> Antimicrobial Wound Gel contains 0.02% DispersinB<sup>®</sup>, 1.5% sodium alginate and 98.48% water. DispersinB<sup>®</sup>/Triclosan Antimicrobial Wound Gel contains 1% Triclosan, 10% polyethylene glycol (PEG-400), 10% alcohol, 0.02% DispersinB<sup>®</sup>, 1.5% sodium alginate and 77.48% water. A table showing the compositions of wound gels is given below:

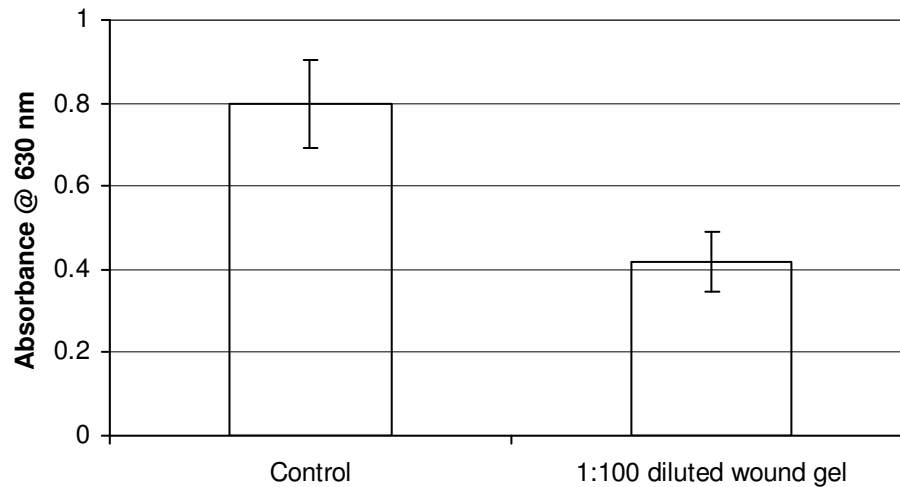
Table 4: Composition of Wound Gel

Wound Gel Type	Ingredient	% Composition
DispersinB <sup>®</sup>	DispersinB <sup>®</sup>	0.02%
Antimicrobial Wound Gel	Sodium Alginate (autoclaved)	3%
	Water	96.98%
DispersinB <sup>®</sup> /Triclosan Antimicrobial Wound Gel	DispersinB <sup>®</sup>	0.02%
	Triclosan	1.0%
	Sodium Alginate	1.5%
	Polyethylene Glycol (PEG-400)	10.0%
	Alcohol (Absolute)	10.0%
	Water	77.48%

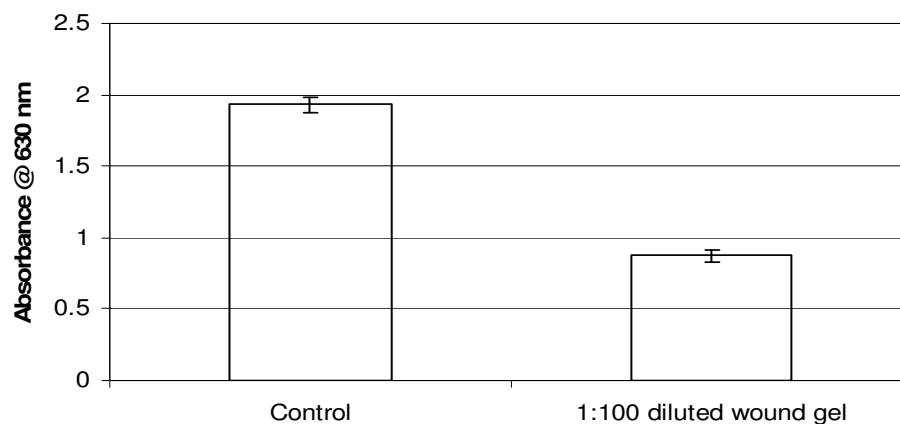
## 12. Wound Gel Activity

### 12.1 Antibiofilm activity of DispersinB<sup>®</sup> gel

*In vitro* antibiofilm activity of DispersinB<sup>®</sup> wound gel was determined by biofilm dispersal assay. The overnight grown *S. epidermidis* culture was diluted 1:100 times in TSB and 200 µl of the culture was dispensed in each well of a 96-well microtiter plate. The plate was incubated at 37°C for 24 h. After incubation, the planktonic growth was discarded and the biofilm was treated with 1:100 dilution of wound gel prepared in sterile distilled water at 37°C for 5 h. Biofilm was measured by discarding the wound gel, rinsing the wells with water (three times), and staining the bound cells with crystal violet (0.4 % w/v) for 15 minutes, followed by washing with water to remove unbound stain. The dye was solubilized in 200 µl of 33% (v/v) acetic acid and absorbance at 630 nm was determined using a microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). Six replicates were used for each sample, and each experiment was performed three times. DispersinB<sup>®</sup> wound gel dispersed 50% of *S. epidermidis* biofilm at 1:100 dilution (**Fig. 31**).

**Fig. 31: *S. epidermidis* biofilm dispersal activity of DispersinB<sup>®</sup> wound gel**

A similar experiment was performed on Coagulase-negative Staphylococci-42 (CoNS-42) a wound isolate obtained from Dr. Randall Wolcott, Southwest Regional Wound Care Centre, Lubbock, TX, USA. DispersinB<sup>®</sup> wound gel was also active in dispersing CoNS-42 biofilm (**Fig. 32**).

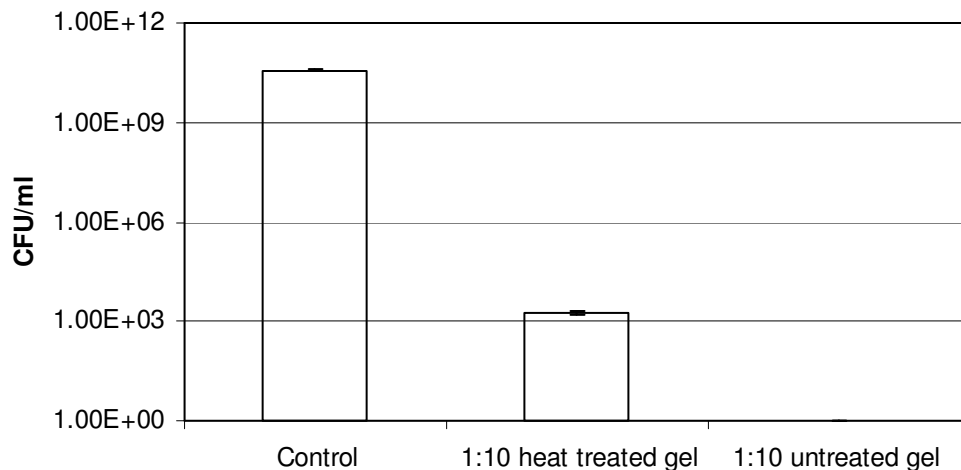
**Fig. 32: Coagulase-negative Staphylococci (CoNS-42) biofilm dispersal activity of DispersinB<sup>®</sup> wound gel**

### 12.2 Antibiofilm-antimicrobial activity of DispersinB<sup>®</sup>/Triclosan wound gel

*In vitro* biofilm dispersal assay was performed to determine the antibiofilm effect of DispersinB<sup>®</sup>/Triclosan wound gel on *S. epidermidis* biofilm. *S. epidermidis* biofilms grown in 1.5 ml polypropylene microcentrifuge tubes were rinsed with 200  $\mu$ l of distilled water and then

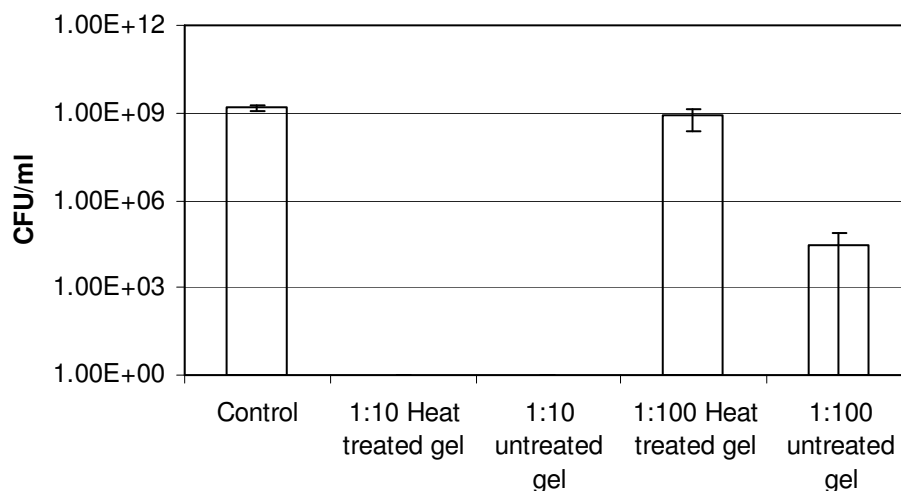
treated with 200  $\mu$ l distilled water (control), 1:10 diluted heat-treated (60°C for 45 min) wound gel, and 1:10 diluted untreated wound gel (Izano, *et al.*, 2007b). After incubating for 3 hours at 37°C, the tubes were centrifuged and the pellet was washed with distilled water. The pellet was resuspended in 200  $\mu$ l of distilled water containing 20 $\mu$ g/ml DispersinB<sup>®</sup> and tubes were incubated for an additional 5 min to disaggregate cells. Serial dilutions of cells were plated on TSA. The untreated gel was more active in killing biofilm embedded *S. epidermidis* cells than heat-treated gel, which had lost considerable activity of DispersinB<sup>®</sup>. The untreated gel dispersed biofilm embedded cells and made it more susceptible to antimicrobial Triclosan (**Fig. 33**).

**Fig. 33: Antibiofilm-antimicrobial activity of DispersinB<sup>®</sup>/Triclosan wound gel against *S. epidermidis* biofilm**



A similar experiment was performed against CoNS-42, a clinical chronic wound isolate. DispersinB<sup>®</sup>/Triclosan wound gel showed antibiofilm-antimicrobial activity against CoNS-42 (**Fig. 34**).

**Fig. 34: Antibiofilm-antimicrobial activity of DispersinB<sup>®</sup>/Triclosan wound gel against Coagulase-negative Staphylococci (CoNS-42) biofilm**



### 12.3 Antimicrobial activity of DispersinB<sup>®</sup>/Triclosan gel

The overnight grown cultures were diluted 1:10 times and 100 µl culture was spread onto TSA plate (Vandenbulcke, *et al.*, 2006). The 200 µl wound gel was overlaid onto the inoculum and the plates were incubated at 37°C for 24 h. The DispersinB<sup>®</sup>/Triclosan wound gel completely inhibited the growth of wound associated Gram-positive, Gram-negative bacteria and yeast (Table 5). In addition, the wound gel also inhibited the growth of wound isolates completely (Table 6).

**Table 5: Antimicrobial activity of DispersinB<sup>®</sup>/Triclosan wound gel against wound associated pathogens**

Pathogen	Growth (CFU/ml)	
	Control	DispersinB <sup>®</sup> /Triclosan wound gel treated
<b>Gram-positive</b>		
<i>Staphylococcus aureus</i>	1.2 x 10 <sup>8</sup>	0
<i>S. epidermidis</i>	1 x 10 <sup>8</sup>	0
<i>Enterococcus faecalis</i>	2.3 x 10 <sup>8</sup>	0
<b>Gram-negative</b>		
<i>Escherichia coli</i>	2.2 x 10 <sup>8</sup>	0
<i>Enterobacter cloacae</i>	1.5 x 10 <sup>8</sup>	0
<b>Yeast</b>		
<i>Candida albicans</i>	4.5 x 10 <sup>6</sup>	0

**Table 6: Antimicrobial activity of DispersinB<sup>®</sup>/Triclosan wound gel against clinical isolates**

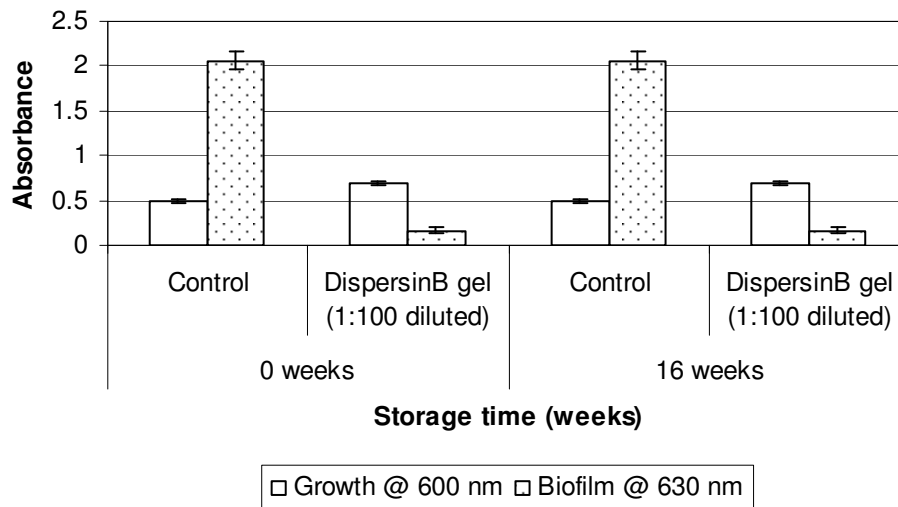
Pathogen	Characteristics	Source	Growth (CFU/ml)	
			Control	DispersinB <sup>®</sup> / Triclosan gel
<i>E. coli</i> 100	Thigh wound, Sensitive to most antibiotics	Southwest Regional Wound Care Centre, Lubbock, TX, USA	2 X 10 <sup>8</sup>	0
<i>E. coli</i> 101	Lower leg wound, Resistant to ampicillin, augmentin, levofloxacin, and doxycycline	Southwest Regional Wound Care Centre, Lubbock, TX, USA	3.5 X 10 <sup>8</sup>	0
Group D Enterococcus (GDE) Vancomycin sensitive Enterococci (VSE) # 93	Lower leg wound, Sensitive to ampicillin, Intermediate to doxycycline, vancomycin, and zyvox	Southwest Regional Wound Care Centre, Lubbock, TX, USA	2.35 X 10 <sup>8</sup>	0
Group D Enterococcus (GDE) Vancomycin resistant Enterococci (VRE) # 143	Heel wound, Sensitive to zyvox, Intermediate to doxycycline, Resistant to ampicillin and vancomycin	Southwest Regional Wound Care Centre, Lubbock, TX, USA	3.54 X 10 <sup>8</sup>	0
<i>S. aureus</i> Gav 16a	Not tested for antibiotic resistance, epidermolysis bullosa isolate	Southwest Regional Wound Care Centre, Lubbock, TX, USA	2.18 X 10 <sup>8</sup>	0
MRSA 64791	Wound isolate, Methicillin resistant	Health Science Centre, University of Manitoba, Winnipeg, MB, Canada	3.7 X 10 <sup>8</sup>	0
Coagulase Negative Staphylococci (CoNS 42)	Methicillin resistant	Southwest Regional Wound Care Centre, Lubbock, TX, USA	1.71 X 10 <sup>8</sup>	0

### 13. Wound Gel Stability

#### 13.1 DispersinB<sup>®</sup> wound gel

The stability of DispersinB<sup>®</sup>/Triclosan wound gel at room temperature 23°C with a relative humidity (RH) between 10 and 15% (unadjusted) was studied in order to determine its shelf life at 4°C using the FDA recommended accelerated aging protocol (ASTM F1980-07). The antibiofilm activity of DispersinB<sup>®</sup> wound gel was determined using a standard biofilm assay against *S. epidermidis* as described in the Section 4.1. DispersinB<sup>®</sup> wound gel inhibited about 95% of *S. epidermidis* biofilm formation after 16 weeks of storage at room temperature, which is more than equivalent to a shelf life of 1 year at 4°C (**Fig. 35**).

**Fig. 35: Accelerated aging of DispersinB<sup>®</sup> wound gel at room temperature (14 weeks of accelerated aging at 23°C = 1 year shelf life at 4°C)**

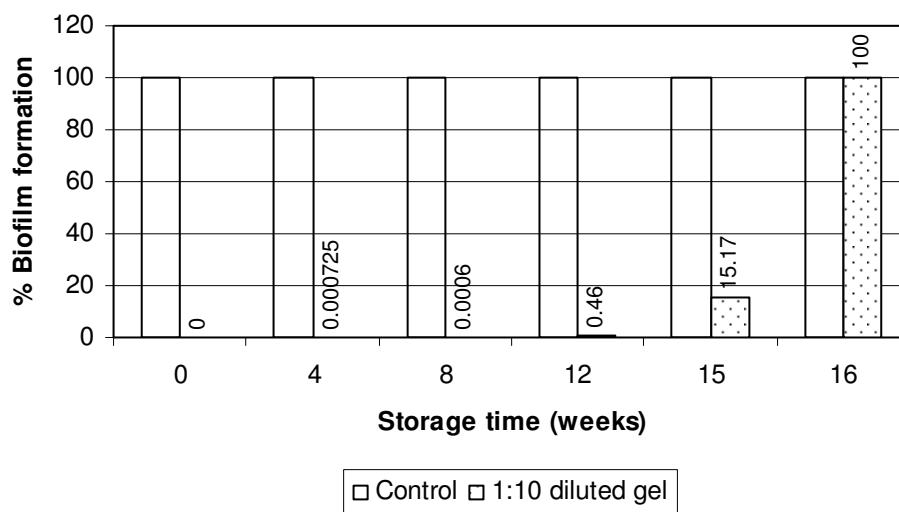


#### 13.2 DispersinB<sup>®</sup>/Triclosan gel

The stability of DispersinB<sup>®</sup>/Triclosan wound gel at room temperature 23°C with a relative humidity (RH) between 10 and 15% (unadjusted) was studied in order to determine its shelf life at 4°C using the FDA recommended accelerated aging protocol (ASTM F1980-07). The antibiofilm-antimicrobial activity of DispersinB<sup>®</sup> wound gel was determined using a standard biofilm dispersal assay against *S. epidermidis* as described in the Section 11.2. DispersinB<sup>®</sup>/Triclosan wound gel killed more than 80% of *S. epidermidis* biofilm embedded cells after 15 weeks of storage at room temperature indicating the shelf life at 4°C for at least a year

(Fig. 36). Further formulation development work is in progress to increase the shelf life of DispersinB<sup>®</sup>/Triclosan gel.

**Fig. 36: Accelerated aging of DispersinB<sup>®</sup>/Triclosan wound gel at room temperature (14 weeks of storage at 23°C = 1 year shelf life at 4°C)**



#### 14. Desirable Features

- Activity:** (i) specific to biofilms, inhibits as well as disperses biofilms, (ii) inhibition and/or dispersal of biofilms using only micro-quantities, (iii) effective against both gram-negative and gram-positive bacteria, (iv) enhances the performance of antibiotics, antiseptics, non-antibiotics, detergents and bacteriophage
- Compatibility with biological fluids:** active in presence of human urine and human blood plasma.
- Stability:** (i) stable at room temperature when coated on silicone catheters after 52 weeks, (ii) stable at mammalian body temperature as demonstrated by *in vivo* efficacy studies in rabbits and sheep, and (iii) stable in rabbit plasma and bovine serum as demonstrated by *in vitro* durability studies
- Haemolysis:** (i) no haemolytic activity was observed at a concentration as high as 2 mg/ml in rabbit blood, and (ii) no haemolytic activity was observed at a concentration as high as 1 mg/ml in human blood

- e) Toxicity: (i) a naturally occurring enzyme produced by one of the oral bacteria present in the human mouth, (ii) non-cytotoxic as demonstrated by an *in vitro* cytotoxicity study, and (iii) no toxic or other adverse effects were observed in animal studies
- f) Resistance: A naturally occurring enzyme and therefore is unlikely to pose any safety or bacterial resistance concerns

## 15. Applications

DispersinB<sup>®</sup> could be used alone or in combination with antimicrobial agents for:

- a) Medical devices, including: urinary catheters, central venous catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, vascular grafts, endotracheal tubes, intraocular lenses, prosthetic cardiac valves, cardiac pace makers, and prosthetic joints.
- b) Wound care products
- c) Oral care products
- d) Animal health care products
- e) Cystic fibrosis

## 16. References

### 16.1 Patents

**Kaplan, J.B.** “Compositions and methods for enzymatic detachment of bacterial and fungal biofilms”. U.S. Patent No. 7,294,497

**Madhyastha, et al.** “Compositions for inhibition of proliferation of biofilm embedded microorganisms and uses thereof”. PCT Publication No. WO 2008/043195 A1

**Madhyastha, et al.** “DispersinB<sup>®</sup>, 5-Fluorouracil, Deoxyribonuclease I (DNase I) and Proteinase K- based antibiofilm compositions and uses thereof”. U.S. Provisional Patent Application Serial No. 61/041,941.

### 16.2 Publications

**Cormio, L., P. L. Forgia, D. L. Forgia, A. Siitonen, and M. Ruutu.** 2001. Bacterial adhesion to urethral catheters: role of coating materials and immersion in antibiotic solution. *Eur. Urol.* **40**:354-359.

- Darouiche, R. O.** 2001. Device-associated infections: a macroproblem that starts with microadhesion. *Clin. Infect. Dis.* **33**:1567-1572.
- Darouiche, R. O., M. D. Mansouri, and R. Meade.** 2002. *In vitro* and *in vivo* activity of antimicrobial coated prosthetic heart valve sewing cuffs. *J. Heart Valve Dis.* **11**:99-104.
- Darouiche, R. O., M. D. Mansouri, P. V. Gawande, and S. Madhyastha** 2008. Antimicrobial and antibiofilm efficacy of Triclosan and DispersinB combination. *Antimicrob. Agents Chemother.* (In Press).
- Donelli, G., I. Francolini, D. Romoli, E. Guaglianone, A. Piozzi, C. Rangunath, and J. B. Kaplan.** 2007. Synergistic activity of dispersinB and cefamandole nafate in inhibition of Staphylococcal biofilm growth on polyurethanes. *Antimicrob. Agents Chemother.* **51**:2733-2740.
- Itoh, Y., X. Wang, B. J. Hinnebusch, J. F. Preston III, and T. Romeo.** 2005. Depolymerization of  $\beta$ -1, 6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *J. Bacteriol.* **187**:382-387.
- Izano, E. A., M. A. Amarante, W. B. Kher, and J. B. Kaplan.** 2008a. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl. Environ. Microbiol.* **74**: 470-476.
- Izano, E.A., I. Sadovskaya, H. Wang, E. Vinogradov, C. Rangunath, N. Ramasubbu, S. Jabouri, M.B. Perry, and J.B. Kaplan.** 2008b. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in *Aggregatibacter actinomycetemcomitans*. *Microb. Pathog.* **44**: 52-60.
- Izano, E.A., H. Wang, C. Rangunath, N. Ramasubbu, and J. B. Kaplan.** 2007a. Detachment and killing of *Aggregatibacter actinomycetemcomitans* biofilms by DispersinB and SDS. *J. Dent. Res.* **86**:618-622.
- Izano, E.A., I. Sadovskaya, E. Vinogradov, M.H. Mulks, K. Velliyagounder, C. Rangunath, W.B. Kher, N. Ramasubbu, S. Jabbouri, M.B. Perry, and J.B. Kaplan.** 2007b. Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. *Microb. Pathog.* **43**:1-9.

- Jackson, D. W., K. Suzuki, L. Oakford, J. W. Simecka, M. E. Hart, and T. Romeo.** 2002. Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *J. Bacteriol.* **184**:290-301.
- Kaplan, J. B., C. Rangunath, K. Vellyagounder, D. H. Fine, and N. Ramasubbu.** 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrob. Agents Chemother.* **48**:2633-2636.
- Kim, C. Y., A. Kumar, L. Sampath, K. Sokol, and S. Modak.** 2002. Evaluation of an antimicrobial-impregnated continuous ambulatory peritoneal dialysis catheter for infection control in rats. *Am. J. Kidney Dis.* **39**:165-173.
- Klajnert, B., J. Janiszewska, Z. Urbanczyk-Lipkowska, M. Bryszewska, D. Shcharbin and M. Labieniec.** 2006. Biological properties of low molecular mass peptide dendrimers. *Int. J. Pharm.* **309**:208-217.
- Lu, T.K., and J.J. Collins.** 2007. Dispersing biofilms with engineered enzymatic bacteriophage. *PNAS* **104**:11197-11202.
- Parise, G., M. Mishra, Y. Itoh, T. Romeo, and R. Deora.** 2007. Role of a putative polysaccharide locus in *Bordetella* biofilm development. *J. Bacteriol.* **189**: 750-760.
- Rohde, H., E.C. Burandt, N. Siemssen, L. Frommelt, C. Burdlski, S. Wurster, S. Scherpe, A.P. Davies, L.G. Harris, M.A. Horstkotte, J.K.M. Knobloch, C. Rangunath, J.B. Kaplan, and D. Mack.** 2007. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials.* **28**:1711-1720.
- Saiman, L., J. Siegel, and the cystic fibrosis foundation consensus conference on infection control participants.** 2003. Infection control recommendations for patients with cystic fibrosis: Microbiology, important pathogens, and infection control practices to prevent patient-to-patient transmission. *AJIC Supplement* **31**: S1-S62.
- Serrera, A., J.L. del Pozo, M. Alonso, R. Gonzalez, J. Leiva, M. Vergara, and I. Lasa.** 2007. DispersinB<sup>®</sup> therapy of *Staphylococcus aureus* experimental port-related bloodstream infection, abstr. R-2261, p. S654-S655. Abstr. 17<sup>th</sup> ESCMID. European Society of Clinical Microbiology and Infectious Diseases, Munich, Germany.
- Stark, M., L. Liu, and C. Deber.** 2002. Cationic Hydrophobic peptides with antimicrobial activity. *Antimicrob. Agents Chemother.* **46**:3585-3590.

- Vandenbulcke, K., L. L. Horvat, M. D. Mil, G. Slegers, and H. Beele.** 2006. Evaluation of the antibacterial activity and toxicity of 2 new hydrogels: a pilot study. *Int. J. Low Extrem. Wounds* **5**: 109-114.
- Venketaraman, V., A. K. Lin, A. Le, S. C. Kachlany, N. D. Connell, and J. B. Kaplan.** 2008. Both leukotoxin and poly-N-acetylglucosamine surface polysaccharide protect *Aggregatibacter actinomycetemcomitans* cells from macrophage killing. *Microb. Pathog.* **45**: 173-180.
- Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick.** 2002. Extracellular DNA required for bacterial biofilm formation. *Science* **295**: 1487.
- Yakandawala, N., P. V. Gawande, K. LoVetri, T. Romeo, J. B. Kaplan, and M. S. Madhyastha.** 2009. Enhanced expression of engineered ACA-less  $\beta$ -1,6-N-acetylglucosaminidase (Dispersin B) in *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* Submitted.