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## **A cathelicidin-2-derived peptide effectively impairs *Staphylococcus epidermidis* biofilms**

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## ABSTRACT

*Staphylococcus epidermidis* is a major cause of nosocomial infections owing to its ability to form biofilms on the surface of medical devices. Biofilms are surface-adhered bacterial communities. In mature biofilms these communities are encased in an extracellular matrix composed of bacterial polysaccharides, proteins and DNA. The antibiotic resistance of bacteria present in biofilms can be up to 1000-fold higher compared with the planktonic phenotype. Host defence peptides (HDPs) are considered to be excellent candidates for the development of novel antibiotics. Recently, we demonstrated that a short variant of the HDP chicken cathelicidin-2, peptide F<sub>2,5,12</sub>W, has potent antibacterial and lipopolysaccharide-neutralising activities. This study reports on the antibiofilm activity of peptide F<sub>2,5,12</sub>W against two strains of *S. epidermidis*, including a multiresistant strain. Peptide F<sub>2,5,12</sub>W potently inhibited the formation of bacterial biofilms in vitro at a low concentration of 2.5 µM, which is below the concentration required to kill or inhibit growth (minimal inhibitory concentration = 10 µM). Moreover, peptide F<sub>2,5,12</sub>W also impaired existing *S. epidermidis* biofilms. A 4-h challenge of pre-grown biofilms with 40 µM F<sub>2,5,12</sub>W reduced the metabolic activity of the wild-type strain biofilm completely and reduced that of the multiresistant strain biofilm by >50%. It is concluded that F<sub>2,5,12</sub>W prevents biofilm formation and impairs mature *S. epidermidis* biofilms.

## 1. Introduction

*Staphylococcus epidermidis* is a major cause of infections related to indwelling medical devices such as intravascular catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, intraocular lenses, cardiac pacemakers and prosthetic joints [1]. It is an important member of the human skin and mucous membrane microflora and can easily be transmitted to the surfaces of these devices when they are implanted or manipulated. *Staphylococcus epidermidis* adheres to and grows on medical devices, which may lead to a mature biofilm consisting of cells embedded in a sticky extracellular slime composed of bacterial polysaccharides, proteins and DNA. This matrix renders the *S. epidermidis* biofilm resistant to antibiotics and host defence systems and makes it difficult or almost impossible to eradicate. Bacteria in biofilms can be up to 1000-fold more resistant to antibiotic treatment than the planktonic phenotype [2].

The rising number of infections caused by bacterial isolates resistant to conventional antibiotics has led to an intense search for novel antimicrobials and chemotherapeutics, including the use of host defence peptides (HDPs). HDPs comprise a class of antibiotic compounds, i.e. naturally occurring peptides, that exhibit broad antibacterial and/or immunomodulatory properties [3].

Recently, the chicken HDP cathelicidin-2 (CATH-2) has been identified [4]. We demonstrated that CATH-2 displays potent antibacterial activity against a variety of bacteria, including biowarfare agents, and is able to inhibit lipopolysaccharide (LPS)-induced cytokine release by peripheral blood mononuclear cells [5]. Furthermore, we

found that the peptide C1-15, comprising the first 15 N-terminal amino acids of CATH-2, is the core element of CATH-2 required for antibacterial activity. The antibacterial and LPS-neutralising activities of C1-15 were subsequently improved by tryptophan substitution of all three phenylalanine residues, resulting in peptide F<sub>2,5,12</sub>W [6,7].

Here we report the inhibitory effect of F<sub>2,5,12</sub>W on *S. epidermidis* biofilm formation as well as on existing biofilms.

## 2. Material and methods

### 2.1. Peptide

F<sub>2,5,12</sub>W (RWGRWLRKIRRWPK) was purchased from Pepscan Therapeutics (Lelystad, The Netherlands). Purity was determined using high-performance liquid chromatography (HPLC) and was >90%.

### 2.2. Micro-organisms

The strains used were the *S. epidermidis* clinical isolate BM185 and the *S. epidermidis* multiresistant strain BM492.

### 2.3. Bacterial susceptibility assay

Antimicrobial susceptibility of *S. epidermidis* was determined using the broth microdilution method. Briefly, two-fold serial dilutions of F<sub>2,5,12</sub>W were prepared in

Muller–Hinton broth (MHB) containing 0.5% glucose at a volume of 50  $\mu\text{L}$ /well in 96-well flat-bottom tissue culture plates (Costar<sup>®</sup>; Corning Inc., Corning, NY). Overnight cultures of *S. epidermidis* were diluted 1:50 in MHB containing 0.5% glucose and 50  $\mu\text{L}$  of this diluted culture was added to each well and plates were incubated for 24 h at 35 °C. The final concentration of F<sub>2,5,12</sub>W ranged between 0.6  $\mu\text{M}$  and 40  $\mu\text{M}$ . The minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration that yielded no visible growth.

#### 2.4. Biofilm formation and quantification of activity against biofilms

Biofilm formation was induced in 96-well flat-bottom tissue culture plates (Costar<sup>®</sup>). First, overnight cultures of *S. epidermidis* strains were diluted 1:100 in fresh MHB containing 5% glucose and a 100  $\mu\text{L}$  aliquot of the diluted culture was added to each well. Following incubation at 35 °C for 24 h without shaking, each well was washed twice with phosphate-buffered saline (PBS) to remove planktonic bacteria.

The biomass of the biofilm was quantified by semiquantitative crystal violet (CV) staining. After removal of planktonic bacteria, wells were stained with 0.4% CV for 15 min. Excess CV was removed by washing each well three times with PBS. Bound CV was solubilised in 100  $\mu\text{L}$  of 33% acetic acid and the optical density at 630 nm was measured.



Metabolic activity of the biofilm was determined with the alamarBlue<sup>®</sup> assay (Invitrogen, Carlsbad, CA). Following removal of planktonic bacteria, 100  $\mu$ L of MHB containing 5% glucose and 5% alamarBlue was added to each well. alamarBlue is a redox indicator that both shows fluorescence and changes colour in response to chemical reduction. The extent of reduction is a reflection of bacterial viability [8]. Following 1 h incubation at 37 °C, fluorescence was measured at excitation and emission wavelengths of 550 nm and 590 nm, respectively.

The effects of F<sub>2,5,12</sub>W on biofilm formation were determined by addition of different concentrations of peptide (0–40  $\mu$ M) during the 24-h biofilm formation period. Cell growth was determined by measuring the absorbance at 595 nm.

Similarly, the effects of F<sub>2,5,12</sub>W on mature biofilms were determined by incubating washed 24-h-old biofilms with different concentrations of peptides (0–40  $\mu$ M) for 4 h. Positive controls were untreated biofilms with only MHB plus 0.5% glucose. All experiments were performed in triplicate.

### *2.5. Statistical analysis*

Differences between mean values of groups were evaluated using one-way analysis of variance (ANOVA) and Dunnett's post hoc test and were considered significant at  $P < 0.05$ .

### 3. Results

#### 3.1. Minimal inhibitory concentration

The concentration of  $F_{2,5,12}W$  required to inhibit the growth of planktonic bacteria (MIC) was 10  $\mu$ M (data not shown). No differences in MIC values were found between the antibiotic-sensitive strain and the multiresistant strain. Both the clinical isolate and the multiresistant strain showed no visible growth at a concentration of 10  $\mu$ M.

#### 3.2. Biofilm formation of two *Staphylococcus epidermidis* strains

Both strains formed biofilms in vitro. Culturing for 24 h resulted in a significantly larger biomass and greater metabolic activity for the multiresistant strain (BM492) compared with the clinical wild-type strain (BM185). Furthermore, a strong correlation was observed between biomass quantified by CV staining (Fig. 1A) and biofilm metabolic activity quantified by alamarBlue reduction (Fig. 1B) in the 24-h-old biofilm.

#### 3.3. Peptide $F_{2,5,12}W$ impairs *Staphylococcus epidermidis* biofilm formation

An in vitro assay was performed to investigate the effects of  $F_{2,5,12}W$  on biofilm formation of *S. epidermidis*. As shown in Fig. 2A,B, all tested concentrations of the peptide inhibited biofilm formation. No differences were found between the two strains tested. The amount of biofilm biomass as well as the metabolic activity of the biofilm was significantly inhibited at 2.5  $\mu$ M peptide, a concentration four times below the MIC. This

inhibitory effect was increased to control levels at 10  $\mu\text{M}$ , a concentration equal to the MIC (Fig. 2).

#### 3.4. $F_{2,5,12}W$ impairs the metabolic activity of existing biofilms

Mature biofilms are known to be more resistant to eradication compared with inhibition of de novo biofilm formation. Therefore, the activity of peptide  $F_{2,5,12}W$  against mature biofilms was evaluated. Fig. 3 show the amount of biomass quantified by CV staining as well as metabolic activity quantified by the alamarBlue assay in treated and untreated biofilms.

Metabolic activity of *S. epidermidis* strain BM185 was significantly suppressed at 5  $\mu\text{M}$  peptide, a concentration two times below the MIC (Fig. 3C). Higher concentrations further suppressed the metabolic activity; at 40  $\mu\text{M}$  the metabolic activity was similar to the negative control levels. The effect of  $F_{2,5,12}W$  treatment on the biomass of strain BM185 was also dose-dependent (Fig. 3A). The CV staining method is less reproducible than the alamarBlue assay and therefore was only significant at a concentration of 20  $\mu\text{M}$ .

Mature biofilms of the multiresistant strain of *S. epidermidis* BM492 were less susceptible to  $F_{2,5,12}W$  treatment. However, a significant suppression of metabolic activity of the biofilm was found following treatment with 40  $\mu\text{M}$   $F_{2,5,12}W$  (Fig. 3D).

## 4. Discussion

The involvement of *S. epidermidis* in infections associated with indwelling medical devices as well as the rapid development of multiple antibiotic resistance have continued to receive significant attention [9]. These types of infections are mediated by the propensity of *S. epidermidis* to adhere to surfaces and form biofilms. In general, bacteria grown as a biofilm are protected from host defences and often exhibit reduced susceptibility to antibiotics, contributing to the persistence of the biofilm [2].

F<sub>2,5,12</sub>W, a short peptide based on chicken CATH-2, has been reported to have broad antibacterial activity [7], but so far its effects on bacterial biofilms had not been evaluated.

The primary aim of this study was to investigate the effects of F<sub>2,5,12</sub>W on *S. epidermidis* biofilms using a simple screening method to quantify biofilms in vitro. The viability of biofilm cells was considered as the most important aspect when evaluating the effect of antimicrobial agents. CV stains polysaccharides in the extracellular matrix but does not distinguish between living and dead cells. Therefore, a quantification model based on reduction of alamarBlue by metabolically active cells was also included.

First, the antibacterial activity of F<sub>2,5,12</sub>W against two *S. epidermidis* strains, including a multiresistant strain, was determined. The results demonstrated that F<sub>2,5,12</sub>W exhibits activity with a MIC value of 10  $\mu$ M for both strains.

In addition, it was demonstrated that  $F_{2,5,12}W$  suppressed biofilm formation of both strains at a concentration four times below the MIC. Inhibition by  $F_{2,5,12}W$  of biofilm formation may be the result of the inhibition of bacterial growth. However, inhibition of biofilm formation was observed at concentrations that did not affect bacterial growth. Therefore, the inhibitory effect of  $F_{2,5,12}W$  on biofilm formation likely reflects decreased initial adhesion of bacteria to the surface and subsequent development of the biofilm.

Mature biofilms are known to be more refractory to eradication relative to inhibition of de novo biofilm formation. Therefore, the effect of peptide  $F_{2,5,12}W$  on mature biofilms was evaluated. Low concentrations of  $F_{2,5,12}W$  had a better antimicrobial effect on the weak biofilm-producing *S. epidermidis* strain BM185 than on the strong biofilm-forming strain BM492. Both the architecture of a thinner biofilm and the total number of bacteria in such a biofilm may explain this finding.

Several other groups have investigated the effect of natural HDPs or synthetic analogues on different types of biofilms [10–15]. It was reported that various peptides could be used to prevent biofilm formation. However, only three groups reported that peptides were effective in killing bacteria in a 24-h-old biofilm [12,14,15].

In summary, we have demonstrated that at low concentrations, below those that kill or inhibit growth,  $F_{2,5,12}W$  strongly prevents bacterial biofilm formation in vitro. Moreover,  $F_{2,5,12}W$  also impairs existing *S. epidermidis* biofilms.

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**Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Biomass of a 24-h-old biofilm of two different *Staphylococcus epidermidis* strains, the clinical isolate BM185 and the multiresistant strain BM492. (A) Quantification of biomass by crystal violet (CV) staining and (B) metabolic activity determined by the alamarBlue<sup>®</sup> reduction assay. Values represent the mean  $\pm$  standard error of the mean (S.E.M.) of three experiments. \* Significantly different compared with BM185 ( $P < 0.05$ ). OD<sub>630nm</sub>, optical density at 630 nm.

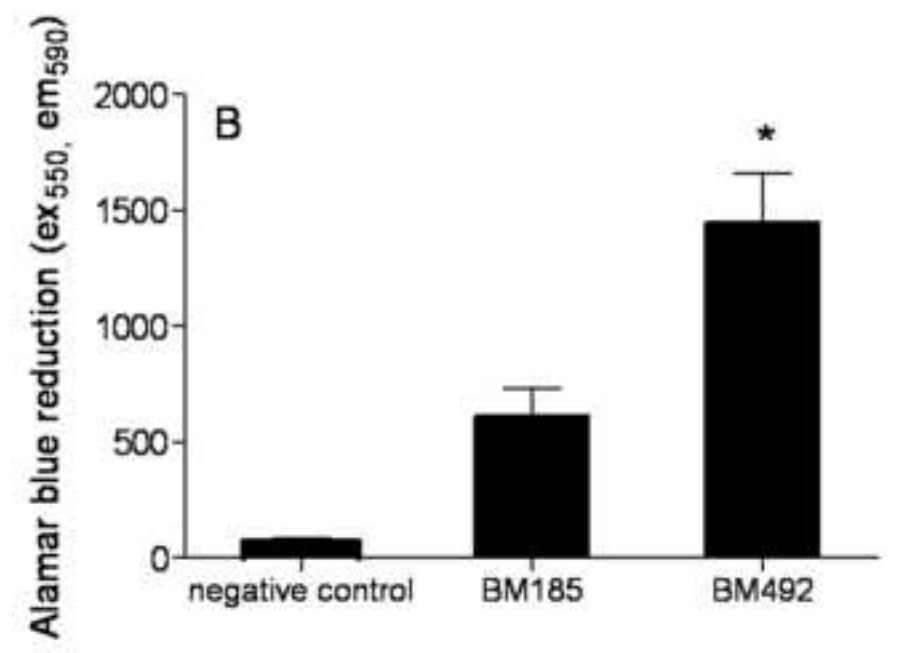
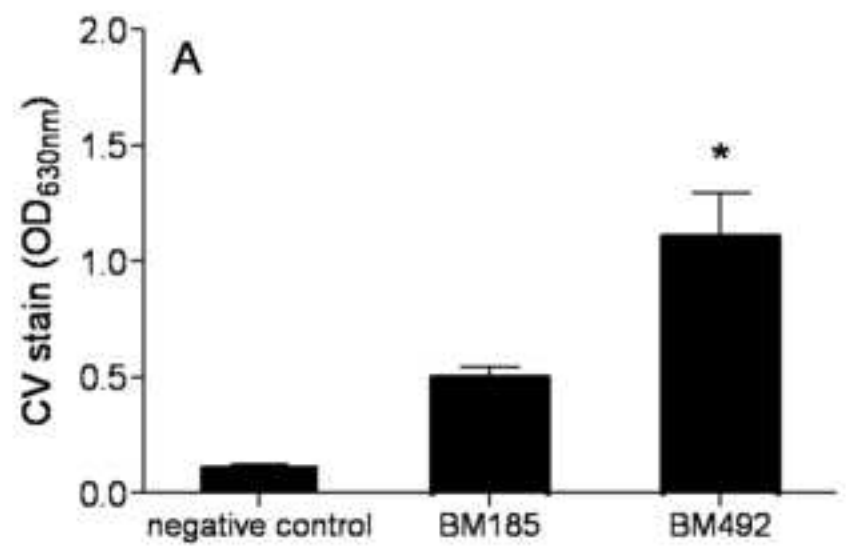
**Fig. 2.** Effect of F<sub>2,5,12</sub>W on *Staphylococcus epidermidis* biofilm formation by (A,C) strain BM185 and (B,D) strain BM492. Bacterial cells were inoculated in a 96-well plate containing different concentrations of peptides. Culture without F<sub>2,5,12</sub>W was used as a positive control, and media without culture was used as a negative control. Following incubation at 35 °C for 24 h, absorbance at 595 nm ( $A_{595}$ ) was recorded to assess cell growth (line graph); biofilm biomass was determined by crystal violet (CV) staining (white bars) (A,B). Metabolic activity of the biofilm was determined by the alamarBlue<sup>®</sup> reduction assay (grey bars) (C,D). Data represent the mean  $\pm$  standard error of the mean (S.E.M.) of at least three experiments. \* Significantly different compared with controls without peptide ( $P < 0.05$ ). OD<sub>630/595</sub>, optical density at 630/595 nm.

**Fig. 3.** Effect of F<sub>2,5,12</sub>W on *Staphylococcus epidermidis* pre-formed biofilm of (A,C) strain BM185 and (B,D) strain BM492. Bacterial cells were inoculated in a 96-well plate and were incubated for 24 h to develop a mature biofilm. Biofilms were rinsed and different concentrations of F<sub>2,5,12</sub>W were transferred onto the biofilm. Following 4 h of incubation at 35 °C, biofilm biomass was determined by crystal violet (CV) staining

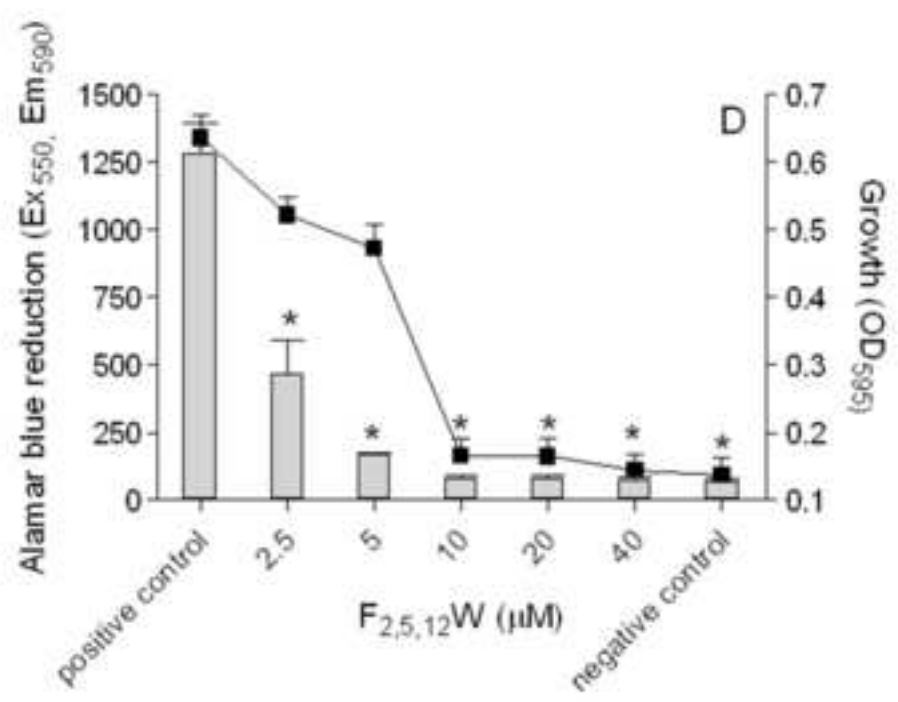
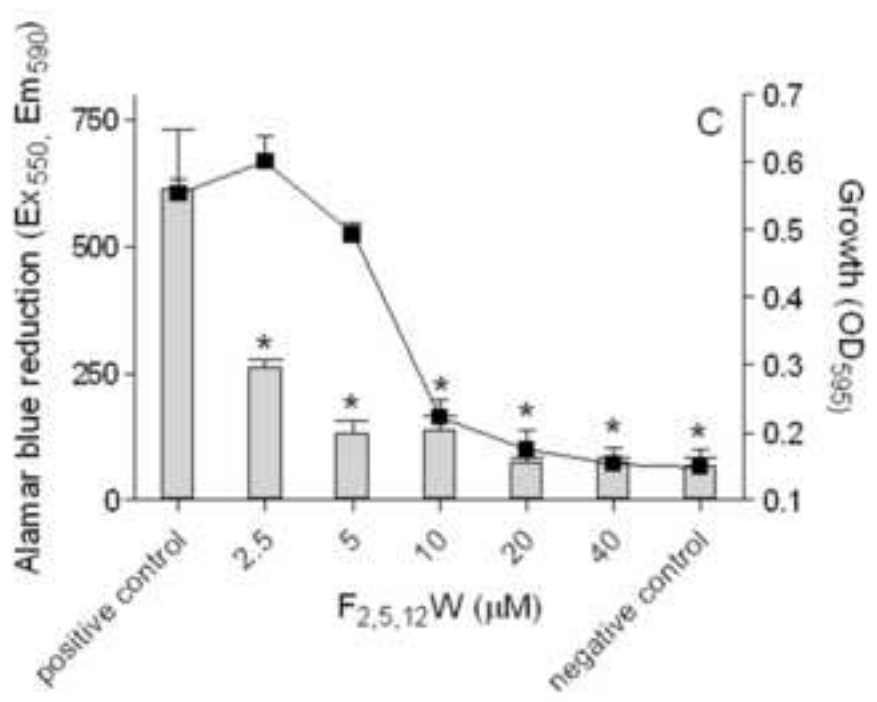
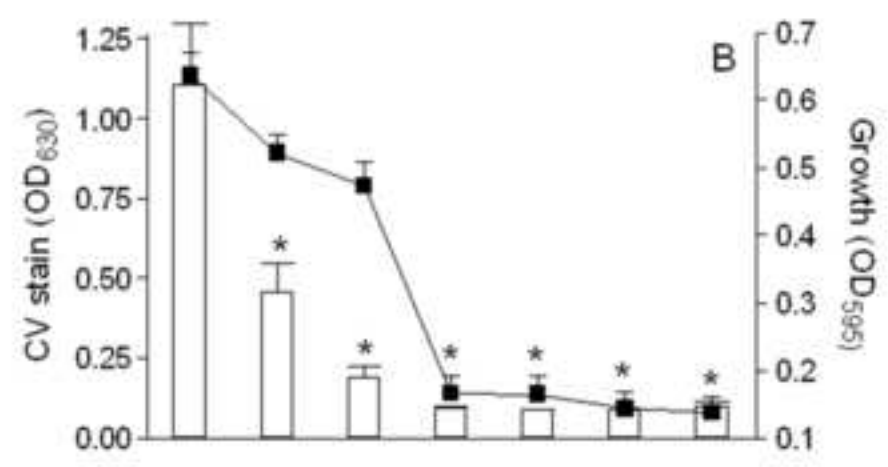
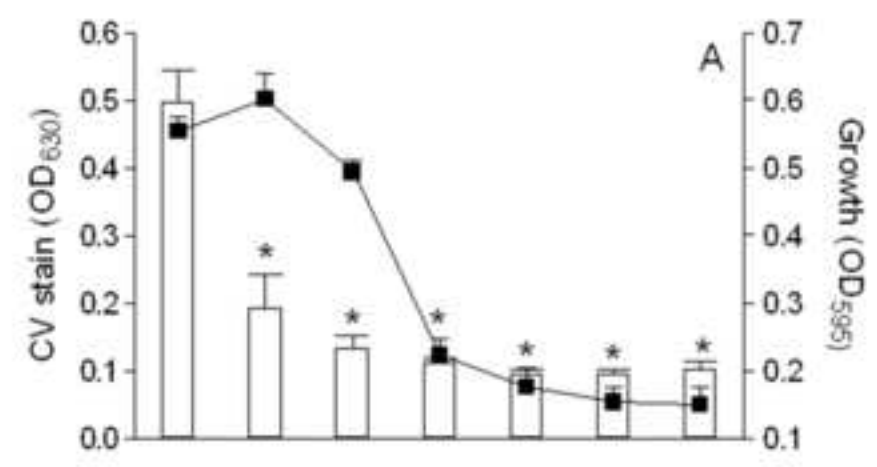
(white bars) (A,B) and metabolic activity was determined by the alamarBlue<sup>®</sup> reduction assay (grey bars) (C,D). OD<sub>630</sub>, optical density at 630 nm.

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