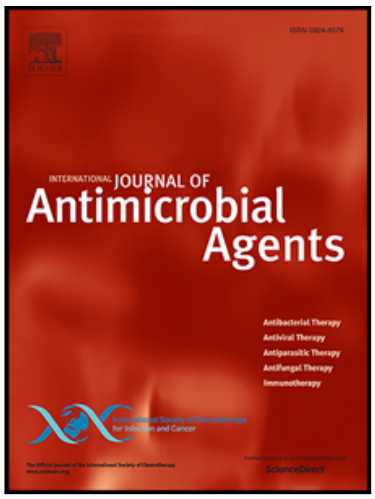

Activity of a Nitric Oxide Generating Wound Treatment System against Wound Pathogen Biofilms

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PII: S0924-8579(18)30111-0
DOI: 10.1016/j.ijantimicag.2018.04.009
Reference: ANTAGE 5424

To appear in: *International Journal of Antimicrobial Agents*

Received date: 24 November 2017
Revised date: 3 April 2018
Accepted date: 7 April 2018

Please cite this article as: Richard D. Waite , Joanne E. Stewart , Abish S. Stephen , Robert P. Allaker , Activity of a Nitric Oxide Generating Wound Treatment System against Wound Pathogen Biofilms, *International Journal of Antimicrobial Agents* (2018), doi: 10.1016/j.ijantimicag.2018.04.009

Highlights:

Antibiofilm activity of a nitric oxide generating wound dressing demonstrated.

Prevention and treatment of biofilms shown with 13 species of bacteria and yeasts.

Activity against mixed and single species biofilms including MRSA and MDR strains.

Reduction in virulence factor activity from Pseudomonas aeruginosa biofilms.

Activity of a Nitric Oxide Generating Wound Treatment System against Wound Pathogen Biofilms

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ABSTRACT

Wound bioburden plays an important role in impaired healing and the development of infection-related complications. The objective of this study was to determine the efficacy of an innovative two-layer nitric oxide generating system (NOx) to prevent and treat biofilms formed by bacterial and fungal pathogens commonly associated with wound infection, and activity against *Pseudomonas aeruginosa* virulence factors. Single and mixed species biofilms were grown for 24h on nitrocellulose filters placed on agar. Filters were covered with either NOx or placebo, before and after biofilm formation. Populations of bacteria and yeasts were determined using viable counts. Pyocyanin and elastase production from *P. aeruginosa* were determined in supernatants derived from suspended biofilms. Efficacy of NOx was demonstrated against S*taphylococcus aureus, P. aeruginosa, Acinetobacter baumannii, Escherichia coli* and *Candida* spp. Population reductions between 2 and 10 log fold were observed. Pyocyanin and elastase activities from *P. aeruginosa* were reduced 1.9 and 3.2-fold respectively. This study demonstrated activity of NOx against formation and treatment of single and mixed species biofilms, including multi-drug resistant strains. NOx represents a new generation of antimicrobial agent with potent, broad-spectrum activity, and with no evidence of resistance development.

Keywords: Biofilms; Wound infection; Nitric Oxide; Antimicrobial; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

The microbial burden of acute and chronic wounds plays an important role in impaired healing and development of infection-related complications. Prevalence of infection in chronic wounds is reported as 53 % [1]. In these patients, inadequate infection management can result in lower limb amputation and bloodstream invasion, with complications such as sepsis, multi-organ failure and death [2]. Biofilm formation is now thought to exacerbate wound infection and delay healing, not only in chronic wounds [3, 4] but also following acute injury [5]. By intensifying inflammation, biofilms are heavily implicated in the chronicity and delayed healing of venous leg ulcers, pressure ulcers and diabetic foot ulcers [6]. Biofilms form when bacterial communities deposit polymeric matrices, serving to store nutrients, enhance adherence and protect the cells within the matrix from both the host immune response and penetration by antimicrobial agents. Notoriously difficult to eradicate, the increased tolerance of biofilm microorganisms to conventional antimicrobial agents, compared with their planktonic counterparts [7] poses significant clinical problems. Exacerbating the problem is antimicrobial resistance, with the risk of developing chronic wounds and the prevalence of antimicrobial resistance rising simultaneously [8]. Of particular concern, the resistance to antibiotics of last resort, such as vancomycin and the carbapenems, is now well documented [9-11].

For any new wound treatment to be deemed effective, multiple challenges must be overcome. This would include showing efficacy against a broad range of wound-associated bacteria, including the most commonly isolated, aggressive opportunistic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [12], biofilms and multidrug-resistant (MDR) strains [13]. In certain cases, tolerance to antimicrobials is attributed to ease of biofilm formation, and the establishment of a physical barrier to antimicrobial penetration

[14]. Within a biofilm, microbial cells may exhibit altered behaviours, particularly relating to growth rate and gene transcription [15,16]. Whilst there are now a myriad of antimicrobial agents on the market, for prevention and treatment of wound bioburden and infection, guidelines currently support their use only after clinical diagnosis of infection [17]. Several wound dressings that claim to combat biofilms are currently available but there remains a distinct lack of evidence to support the effectiveness of these dressings. Rather, many commercial topical agents and wound dressings are acknowledged to be ineffective against biofilm infections [18].

NOx has been developed as an advanced wound treatment device, with the added benefit of generating nitric oxide *in situ*. Comprising a non-adherent contact layer, and an absorptive hydrogel secondary layer, nitric oxide is generated, with a total amount of approximately 3 μmoles/cm^2 of dressing produced over a 48 hour period, as an ancillary function when the two layers are placed in contact and applied to the wound. The dressing has undergone all International Standard biocompatibility tests, including skin irritation testing, demonstrating safety and enabling a phase II/III clinical study on open wounds with no adverse effects observed. The dressing provides cushioning, protection and absorption of wound exudate, and also aids healing and prevents infection via intrinsic properties of nitric oxide; including vasodilation, modulation of the host immune responses and antimicrobial activity [19].

In health, nitric oxide is produced endogenously and is critical in defence against infection [19]. Its activities are concentration dependent, where at low concentrations (< 1 μM); it promotes growth and activity of host immune cells. While at high concentrations (> 1 μM) it acts against multiple targets to inhibit or kill microbial pathogens [20]. In contrast, conventional antimicrobial agents typically have a single mode of action, for example,

targeting protein synthesis, nucleic acid metabolism or membrane function of microorganisms. Importantly, whilst high doses of nitric oxide can kill a wide variety of pathogenic bacteria, at low levels it acts as a key mediator of biofilm dispersal, capable of rendering detached bacteria sensitive to conventional antimicrobials [20-22]. As an integral and highly conserved component of host immunity, it is unsurprising that resistance to nitric oxide has yet to be demonstrated [23]. Its inherent broad-spectrum antimicrobial activity against protein, lipid and nucleic acid microbial components therefore makes it a promising target for clinical development. For planktonic bacteria, minimum inhibitory concentration (MIC) tests are the gold standard for determining susceptibility to antimicrobial agents [24]. However, for biofilms susceptibility testing is not standardised and many different models are used. For studies associated with wound infection, models that generate air/surface biofilms are the most appropriate.

We have previously demonstrated a model whereby mature *P. aeruginosa* biofilms are formed after 24 hours, and that biofilm architecture, cell density and gene expression of these populations does not alter significantly for a further 24 hours [25, 26]. Using this established model, we have tested here the effect of NOx for the prevention and treatment of biofilms formed by species of bacteria and yeasts commonly associated with wound infection.

2. Materials and methods

2.1. Strains and growth conditions

Bacterial and fungal strains used in this study are listed in Table 1. Gram negative and Gram positive bacteria were routinely grown at 37^{o}C on LB agar (Invitrogen) and tryptone soya agar (TSA; Oxoid), respectively. *Candida* strains were grown on sabouraud agar (4% glucose; Sigma). Liquid cultures used to inoculate biofilms were grown overnight aerobically

with 5% CO_2 and with agitation (200 rpm) at 37°C in LB broth (Gram –ves), tryptone soya broth (TSB) for Gram +ves and sabouraud broth (Oxoid) for *Candida*.

2.2 Filter biofilm method

Biofilms were grown on 25-mm diameter nitrocellulose filters (GSWP02500, Millipore) placed on agar (20% w/v LB agar, Gram –ves; 20% w/v Mueller Hinton agar, Gram +ves; sabouraud agar, *Candida* spp.), as previously described [25]. For biofilm prevention studies, replicate filters were inoculated with 10^5 colony-forming units (CFU), a population accepted to be in excess of the level which would initially colonise a new wound [27, 28] of the species/strain under test and incubated at 37°C for 30 or 60 minutes, as indicated. Filters were then covered with either placebo or NOx test mesh squares (5 x 5 cm) followed by the NOx hydrogel secondary layer, cut aseptically into 6 x 6 cm squares. Placebo mesh squares, NOx test mesh squares and hydrogel secondary layers were obtained from Edixomed Ltd. (Edinburgh). After 24 h incubation (37°C), dressings were removed and filters placed into 5 ml liquid medium (LB broth, Gram –ve; TSB, Gram +ves). To remove micro-organisms the resuspended filters were vortexed twice for 30 seconds, and scraped with a sterile loop. To enumerate microbial populations, viable counts were performed using solidified agar (LB agar, Gram –ve; TSA, Gram +ves; sabouraud, *Candida* spp.) incubated aerobically (37°C). For examination of effect on mature biofilms, inoculated filters were incubated for 24 h (37°C) prior to addition of test materials.

2.3 Polymicrobial biofilms

Filters were inoculated with 10^5 CFU of both *P. aeruginosa* PAO1 and MRSA NCTC 12493. For quantification of bacterial populations, viable counts were performed using Pseudomonas Isolation Agar (PIA; Difco) incubated aerobically overnight (37^oC) and blood agar base

(Oxoid) containing 5% defibrinated horse blood (TCS Biosciences Ltd., Bucks, UK) incubated anaerobically (37^{o}C) for 24 h, to select for MRSA.

2.4 Detection of P. aeruginosa virulence factor (VF) production.

VF assays were performed using culture supernatants generated from mature biofilms grown and disrupted as described above. Re-suspended biofilm cells were pelleted by centrifugation (4000 rpm, 10 minutes, 4^{o}C) and the supernatant sterilised (0.22 µm pore membranes). To assess *P. aeruginosa* elastase production, 250 µl of sterile supernatant from triplicate suspensions were screened using the quantitative elastin Congo red (ECR; Sigma) assay as previously described [29]. The supernatant pyocyanin content was quantified as previously described [30].

2.5 Statistical analyses

Differences between placebo and NOx treated conditions were analysed using the Mann Whitney U test. A significance level of $p<0.05$ was accepted.

3. Results

3.1 Prevention and treatment of S. aureus biofilms

Placebo dressings (non NOx-generating) allowed biofilm cell densities to reach between median counts of $2.1x10^7$ and $4.8x10^8$ CFU / filter for the *S. aureus* strains examined. However, the NOx device was found to prevent biofilm formation and reduce bacterial populations to below detectable levels (50 CFU/Filter) for all strains tested (Fig. 1A). Wound dressings (placebo or NOx) were also added 24 h post-inoculation. Biofilm formation was then analysed following a further 24 h incubation period. For all test strains, a median population of $\geq 6x10^7$ CFU / filter survived beneath placebo dressings. Below NOx dressings, bacterial polymer / cell debris was still observed on the filter surface. However, the surviving bacterial population was below detectable levels for MRSA (NCTC 12493) biofilms, and showed 6.1 and 2.4 log fold reductions for *S. aureus* Newman and VISA strain Mu50, respectively (Fig. 1B).

3.2 Prevention and treatment of P. aeruginosa and other enteric species biofilms

Placebo dressings allowed high cell density biofilm formation of *P. aeruginosa* and other enteric species (median populations ranged from $4x10^8$ to $6x10^9$ CFU / filter for the 7 strains). NOx not only prevented biofilm formation (Fig. 1A), but reduced bacterial populations to below detectable levels for the following bacterial strains; *P. aeruginosa* (PAO1, PA5 & PA57), *A. baumannii* (ATCC 19606 & Ab186) and *E. coli* (ATCC 25922). NOx also prevented biofilm formation (at least 7.7 log fold reduction) for *K. pneumoniae* (NCTC 13368). In treatment studies for all strains, a median bacterial population of between $1.5x10^8$ and $2.8x10^8$ CFU / filter accumulated beneath placebo dressings. Beneath the NOx dressings, bacterial polymer / cell debris was still observed on the filter surface after treatment. However, the surviving bacterial population was below detectable levels for *P. aeruginosa*

biofilms (strains PAO1, PA5 & PA57), whilst *E. coli* (ATCC 25922) and *K. pneumoniae* (NCTC 13368) showed at least 6.2 and 4.8 log fold reductions, respectively. NOx treated *A. baumannii* (ATCC 19606 and Ab186) median populations were reduced 2 to 2.9 log fold compared to placebo (Fig. 1B).

3.3 Effect of NOx on virulence factor production from P. aeruginosa

Mean PA5 strain pyocyanin activity was reduced by 1.9 fold under NOx treated biofilms, compared to placebo biofilms. Elastase activity was reduced by 3.2 fold under NOx compared to placebo.

3.4 NOx effect on mixed species biofilms

In biofilm prevention studies, populations of both *P. aeruginosa* and MRSA beneath NOx were below detectable levels (Fig. 2A). In biofilm treatment studies, placebo or NOx was added 24 h post-inoculation. NOx reduced both *P. aeruginosa* (at least 4.1 log fold reduction) and MRSA (at least 5.3 log fold reduction) populations compared to untreated biofilms (control) and placebo treated samples (Fig. 2B).

3.5 NOx effect on Candida species biofilms

In biofilm prevention studies, populations of the 3 *Candida* spp. were below detectable levels (Fig. 3A). In biofilm treatment studies, placebo or NOx was added 24 h post-inoculation. Median population reductions in the range of 2.1 to 3.9 log fold were observed for *Candida krusei*, *Candida albicans* and *Candida tropicalis* as compared to placebo treated samples (Fig. 3B).

4. Discussion

This study was designed to evaluate the antimicrobial activity of NOx to prevent and treat microbial biofilms. A well characterised anti-biofilm method was used, whereby air-surface biofilms were covered with the dressings to be evaluated and after their removal the filters could be harvested and bacteria remaining on the filter surface enumerated by quantitative bacteriology. This enables the prediction of antimicrobial efficacy at a simulated "wound" interface. Importantly, others have used a similar system to demonstrate efficacy of novel agents to treat or prevent biofilms produced by bacterial burn wound isolates [31]. Inoculation densities were chosen which would be in excess of that reported to normally colonise a wound [27].

NOx prevented biofilm formation with all species/strains of bacteria tested, including antibiotic sensitive and multidrug resistant strains. These included *S. aureus* (MSSA, MRSA and VISA), *P. aeruginosa*, *A. baumannii*, *E. coli*, *K. pneumoniae* (including antibiotic sensitive and strains resistant to many penicillin and cephalosporin antibiotics) and 3 *Candida* species. NOx was also effective in treating established biofilms formed by all strains screened. Bacterial populations were eliminated or markedly reduced for Gram positive species. Activity against carbapenem resistant bacteria and vancomycin intermediate *Staphylococcus aureus* (VISA) was observed. Similarly, efficacy was demonstrated against *P. aeruginosa*, antibiotic sensitive and MDR; *A. baumannii*, antibiotic sensitive and MDR; *E. coli*, antibiotic sensitive; *K. pneumoniae* (resistant to many penicillin and cephalosporin antibiotics). Importantly, efficacy against MRSA / *P. aeruginosa* dual species biofilms was demonstrated. This is a crucial finding as it is usual for wounds to be colonised by more than one species and subsequently develop polymicrobial infections [13].

Virulence factors from *P. aeruginosa* that aid in the resistance to host immune responses and may be associated with the prevention of wound repair were reduced as a consequence of biofilm disruption by NOx. Both pyocyanin and elastase were reduced in established *P. aeruginosa* biofilms. This may have important implications for clinical application, through the reduction of factors known to cause tissue damage [29]. In addition to the reduction in biofilm bioburden, it is suggested that NOx might facilitate healing of wounds with established *P. aeruginosa* infections, through the reduction of factors that cause persistence of infection and tissue damage.

The most resistant biofilms were formed by *A. baumanni*, where bacterial populations were not completely eradicated; high cell density biofilms were observed to re-form after removal of the dressing. This suggests that repeat exposures might be needed to eradicate biofilm populations if they are not completely cleared upon a single exposure to NOx. However, significant reductions *in vivo* may allow the host immune response to overcome the infection. Subsequently, repeat exposure tests confirmed that mature *A. baumanni* biofilms were eradicated after two exposures to NOx (Waite & Allaker, unpublished observations). This suggests that replacement of fresh NOx dressing is sufficient to eradicate mature biofilms whose bacterial population are not cleared in a single exposure.

In terms of any proposed mechanism, Barraud *et al* suggested that in addition to killing bacterial cells, nitric oxide at sub-bactericidal concentrations is able to penetrate biofilm matrices and initiate cell dispersion [21, 22]. This ability to disperse bacteria from a biofilm is promising, in that once bacterial cells have re-entered the planktonic state, they are more susceptible to standard antimicrobial agents than in their biofilm state. Hetrick *et al* [32] demonstrated nitric oxide-mediated membrane damage in *P. aeruginosa* membranes, making

them permeable to propidium iodide staining, and further suggested that rapid delivery of nitric oxide may be more effective at biofilm killing than slow/ prolonged delivery. Both of these authors suggest that greater levels of nitric oxide released over short durations are more damaging, particularly to Gram-negative bacteria, than sustained, lower-level surface fluxes. The double lipid bilayer of Gram-negative bacteria typically acts as a permeability barrier to antibiotics that function within the cell. Ironically, it is this same structural characteristic that renders these cells particularly susceptible to nitric oxide- induced membrane damage [33].

In the normal biofilm cycle, nitric oxide production coincides with cell death and dispersal, as part of a co-ordinated process of solubilisation and chemotaxis. Introducing low, non-toxic doses of nitric oxide to biofilms, in the picomolar to nanomolar range, mimics this process, triggering dispersal and the transition back to the planktonic state of growth. Additionally, nitric oxide restores the sensitivity of biofilm and dispersed bacteria towards several classes of antimicrobial agents, restoring their efficacy [22]. In their recent examination of transition in wound biofilms of the diabetic foot, Loesche *et al*. [34] concluded that instability of the wound microbiota leads to faster healing and improved repair. Any agent capable of physically and chemically disrupting a biofilm would therefore be a welcome addition to the dressing armoury.

NOx represents a new wound treatment system which has, as an ancillary aid to its wound healing properties, the rapid generation of nitric oxide. This current series of studies has demonstrated efficacy of the NOx treatment system against a wide variety of wound microbes, both antibiotic sensitive and resistant. It is the hope that this may translate to tangible clinical benefit in this field.

DECLARATIONS

Funding: This work was supported by Edixomed Ltd.

Competing Interests: None

Ethical Approval: Not required

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Figure Legends

Figure 1 Effect of NOx on prevention of single species biofilm formation (A) and on treatment of pre-formed single species biofilms (B). Bars signify the median for biofilms (n=4, except PA01 in triplicate) with error bars showing range. All strains analysed showed statistically significant ($p<0.05$) reductions with NOx.

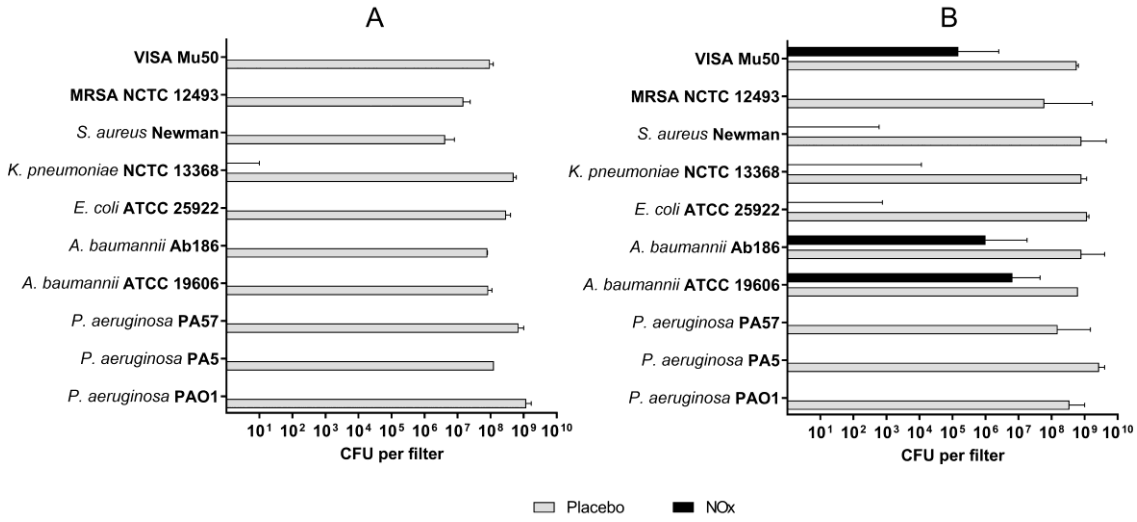

Figure 2 Effect of NOx on prevention of MRSA / *P. aeruginosa* dual species biofilm establishment (A) and treatment of pre-formed dual species biofilms (B). Bars signify the median for triplicate biofilms; error bars show range.

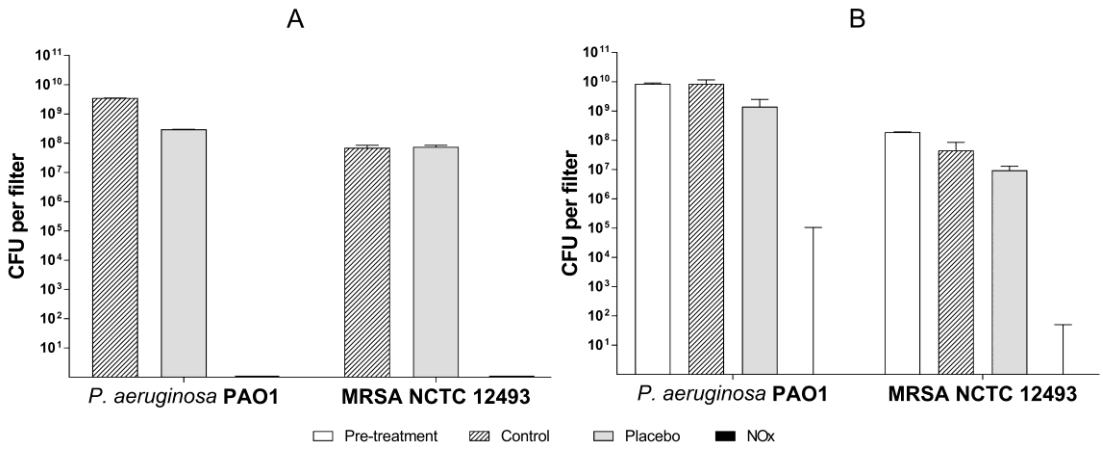

Figure 3 Effect of NOx on prevention (A) and treatment of pre-formed (B) *Candida* spp. biofilms. Bars signify the median of triplicate biofilms with error bars showing range.

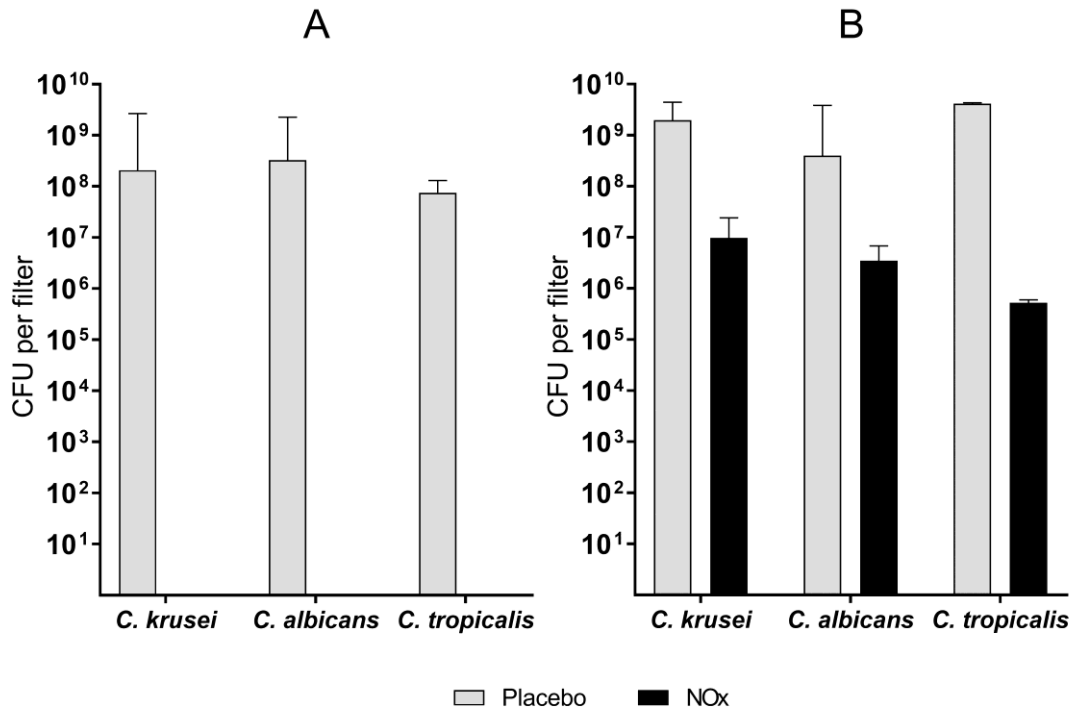

Table 1. Microbial strains

Organism	Strain name	Characteristics	Notes
P. aeruginosa	PAO1	Antibiotic sensitive wound isolate	
	PA5	Wound isolate, MDR, harbours the TEM β-lactamase.	MDR (multiple drug resistance)
	PA57	Pus isolate with VIM-2	MDR, Carbapenem resistant
A. baumannii	ATCC 19606	Antibiotic sensitive isolate	
	Ab186	MDR OXA-23 producer UK 'burn clone'	MDR, Carbapenem resistant
E. coli	ATCC 25922	Antibiotic sensitive isolate	
K. pneumoniae	NCTC 13368	Extended spectrum β-lactamase (ESBL) producer (SHV-18)	ESBL producer - resistant to many penicillin and cephalosporin antibiotics
S. aureus	Newman	Antibiotic sensitive human isolate. Methicillin-sensitive *S. aureus* (MSSA).	
Methicillin-resistant *S. aureus* (MRSA)	NCTC 12493	mecA	MDR
Vancomycin-intermediate *S. aureus* (VISA)	Mu50	vraSR	MDR
Candida albicans	ATCC 24433		
Candida krusei	ATCC 750		
Candida tropicalis	ATCC 6258		