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ORIGINAL ARTICLE

Farnesol increases the susceptibility of *Burkholderia pseudomallei* biofilm to antimicrobials used to treat melioidosis

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Abstract

Aims: The aim of this study was to analyse the *in vitro* activity of farnesol alone and combined with the antibacterial drugs amoxicillin, doxycycline, ceftazidime and sulfamethoxazole–trimethoprim against *Burkholderia pseudomallei* biofilms.
Methods and Results: Susceptibility was assessed by the broth microdilution test and cell viability was read with the oxidation–reduction indicator dye resazurin. The biofilms were evaluated through three microscopic techniques (optical, confocal and electronic microscopy). The minimum biofilm erradication concentration (MBEC) for farnesol was 75–2400 mmol l^{-1}. In addition, farnesol significantly reduced the MBEC values for ceftazidime, amoxicillin, doxycycline and sulfamethoxazole–trimethoprim by 256, 16, 4 and 4 times respectively ($P < 0{\cdot}05$). Optical, confocal and electronic microscopic analyses of farnesol-treated *B. pseudomallei* biofilms demonstrated that this compound damages biofilm matrix, probably facilitating antimicrobial penetration in the biofilm structure.
Conclusions: This study demonstrated the effectiveness of farnesol against *B. pseudomallei* biofilms and its potentiating effect on the activity of antibacterial drugs, in particular ceftazidime, amoxicillin, doxycycline and sulfamethoxazole–trimethoprim.
Significance and Impact of the Study: The intrinsic antimicrobial resistance of *B. pseudomallei* is a serious challenge for the treatment of melioidosis. Thus, this paper reports the inhibitory potential of farnesol against *B. pseudomallei* biofilms, as well as highlights the favourable pharmacological interaction of farnesol with antibiotics tested, not only on cell viability, but also in the structural morphology of biofilms.

Introduction

Burkholderia pseudomallei is a Gram-negative bacillus that causes melioidosis, a severe and often fatal infectious disease that is endemic in Southeast Asia, northeastern Australia (Currie 2015) and northeastern Brazil (Rolim *et al.* 2011; Macedo *et al.* 2012).

Melioidosis treatment comprises two phases: first, the acute phase, when the aim is to prevent death in patients with sepsis; and, second, the elimination phase, when the goal is to avoid clinical relapse due to persisting bacteria (Dance 2014), which are commonly within biofilms (Limmathurotsakul *et al.* 2014).

Ceftazidime and imipenem are used in the acute stage, with the latter being indicated for serious infections or treatment failures; while sulfamethoxazole–trimethoprim and doxycycline are used in the eradication phase. Amoxicilin–clavulanate has been used as an alternative for

children and for pregnant women (Dance 2014). This limited number of antibacterial agents available for the treatment of melioidosis is mainly due to the inherent resistance of *B. pseudomallei* to many drugs (Foong *et al.* 2014).

Biofilm formation is an aggravating factor for the development of antimicrobial resistance among *B. pseudomallei*, since this type of bacterial growth is often associated with decreased antimicrobial susceptibility (Lazar *et al.* 2013). It is important to highlight that the relapse of human melioidosis is commonly associated with biofilm growth of *B. pseudomallei* within the host (Limmathurotsakul *et al.* 2014), which can also be responsible for the increased probability of occurring asymptomatic infections (Koh *et al.* 2013). In addition, the resilience of *B. pseudomallei* in the environment may also be associated with biofilm growth (Chen *et al.* 2014).

Therefore, it is necessary to search for new antimicrobial agents, mainly compounds with greater effectiveness against the biofilm form of *B. pseudomallei*. In this context, there is the prospect of using farnesol, a sesquiterpene alcohol known for its antimicrobial properties (Brilhante *et al.* 2012b; Horev *et al.* 2015), which has previously shown inhibitory activity against planktonic *B. pseudomallei* (Brilhante *et al.* 2012b). Additionally, some studies have demonstrated the ability of farnesol to increase the susceptibility of bacterial biofilms to antimicrobials (Bell and Chappell 2014). Therefore, this study aimed to analyse the susceptibility of *B. pseudomallei* biofilms to farnesol alone and in combination with antimicrobials conventionally used in the treatment of melioidosis.

Materials and methods

Micro-organisms

In this study, we used 13 strains of *B. pseudomallei*. Clinical strains were isolated from blood ($n = 8$), bone marrow ($n = 1$), peritoneal fluid ($n = 1$), lymph node aspirate ($n = 1$) and bronchoalveolar lavage ($n = 1$). The environmental strain ($n = 1$) was isolated from soil. All strains are deposited in the bacterial collection of the Laboratory of Emerging and Reemerging Pathogens of the Federal University of Ceará (Fortaleza, Brazil), and were chosen based on their strong biofilm-forming ability. All strains were previously identified through the VITEK® 2 (BioMérieux, Marcy-l'Étoile, France) automated method, and/or through amplification and sequencing of the 16S rDNA region, as described by Brilhante *et al.* (2012a,b).

Antimicrobial susceptibility testing

In order to evaluate the antibacterial susceptibility of *B. pseudomallei* biofilms, the susceptibility of the planktonic form was initially evaluated. Thus, the antibacterial drugs were diluted according to M100-S22 (CLSI 2012b) in the following concentration ranges: 0·5–256 $\mu g\ ml^{-1}$ for amoxicillin (AMOX, Sigma-Aldrich, São Paulo, Brazil) diluted with sterile phosphate buffer; 0·25–128 $\mu g\ ml^{-1}$ of ceftazidime (CAZ, Sigma-Aldrich) diluted with sterile water; 0·03–16 $\mu g\ ml^{-1}$ of doxycycline (DOX, Sigma-Aldrich) diluted with sterile water, 0·25–128 $\mu g\ ml^{-1}$ of imipenem (IPM, Sigma-Aldrich, São Paulo) diluted with sterile water and 0·29/0·015–152/8 $\mu g\ ml^{-1}$ of sulfamethoxazole-trimethoprim (SXT, Sigma-Aldrich), diluted with 0·1 mol l^{-1} NaOH (Labsynth, São Paulo, Brazil) and 10% Acetic acid (GQ – Grupo Química, São Paulo, Brazil) respectively. Farnesol (Sigma-Aldrich, São Paulo, Brazil) was tested at concentrations ranging from 0·58 to 600 mmol l^{-1}, diluted with sterile water with 7·5% of Dimethylsulfoxide (DMSO, LGC Biotecnologia, São Paulo, Brazil). Antimicrobial susceptibility assays were carried out as described by the document M07-A9 (CLSI 2012a). Minimum inhibitory concentrations (MIC) were defined as the lowest concentration capable of inhibiting 100% of bacterial growth for AMOX, CAZ, DOX, IPM (CLSI 2010) and farnesol (Brilhante *et al.* 2012b). For SXT, MIC was defined as the lowest concentration capable of inhibiting 80% of bacterial growth, when compared to drug-free growth control (CLSI 2010). The strains *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 29213 were used as control (CLSI 2010).

For the drug combination assays with farnesol and the antibacterial drugs (AMOX, CAZ, DOX, SXT), the initial concentration of drugs in combination was the MIC obtained for each individual isolate (Brilhante *et al.* 2012b). IPM was not tested in association with farnesol because its MIC were lower than the interpretive breakpoints established by the CLSI.

Antimicrobial susceptibility of mature biofilms

In order to obtain the biofilm form of *B. pseudomallei*, the strains were subcultured on brain heart infusion agar (BHI, HIMEDIA, India) and incubated at 37°C, for 24 h. Then, a bacterial suspension was prepared in saline solution, reaching a turbidity equivalent to 6 on McFarland scale. In parallel, 96-well flat-bottomed polystyrene microtitre plates were prepared by adding 175 μl of BHI broth supplemented with 1% glucose (BHIGlic, Vetec, Brazil) (Bandeira *et al.* 2013). Afterwards, 25 μl of the bacterial suspension were added to each well, and the plates were incubated at 37°C, for 48 h (Roveta *et al.* 2007; Peeters *et al.* 2008; Bandeira *et al.* 2013). *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis*

ATCC 12228 were used as positive and negative control for biofilm formation respectively (Bandeira *et al.* 2013).

After incubation, the wells were washed three times with 300 μl of 1× PBS in order to remove nonadhering cells. The results for the planktonic form were used to select the concentration range for each drug against mature biofilms. Thus, the following concentration ranges were tested: 8–4096, 1–4096, 0·03–16, 1–512, 0·29/0·015–152/8 μg ml^{-1} and 4·68–2400 mmol l^{-1} for AMOX, CAZ, DOX, IPM, SXT, and farnesol respectively. Each well of the microdilution plate received 300 μl of a given drug concentration and all plates were incubated in an aerobic atmosphere, at 37°C, for 24 h (Bandeira *et al.* 2013).

Afterwards, 20 μl of a sterile aqueous solution of resazurin (0·025%, Invitrogen™, MA) were added to each well, and incubated for 1 h at 37°C, in order to detect biofilm metabolic activity (Bandeira *et al.* 2013). The minimum biofilm erradication concentration (MBEC) was defined as the lowest concentration capable of completely inhibiting biofilm metabolic activity, which was detected by the absence of colour change suffered by resazurin (Bandeira *et al.* 2013). Finally, the content of the plate was transferred to another microplate for spectrophotometric reading, to confirm the results. The absorbance was measured in a microplate spectrophotometer at two wavelengths, 540 nm (reduced state) and 630 nm (oxidized state). The absorbance at 630 nm was subtracted from that at 540 nm in order to obtain the specific absorbance (OD), due to the overlap between the absorbance at these wavelengths. The absorbance obtained for the growth-free wells, which contained culture medium and antibacterial drugs, were subtracted from all the ODs (Yu *et al.* 2003).

For the drug combination assays with farnesol and the antibacterial drugs (AMOX, CAZ, DOX, SXT), the initial concentration of drugs in combination was the MBEC obtained for each individual isolate (Bandeira *et al.* 2013).

Biofilm microscopic evaluation

For the microscopic analyses of farnesol-treated *B. pseudomallei* biofilms, these structures were initially grown in a 12-well polystyrene plate containing a Thermanox® (Thermo Fisher Scientific, New York City, NY) coverslide at the bottom of the well. For such, the same methodology described for biofilm formation was used, adjusting the total volume of culture medium to 1000 μl. Then, the mature biofilms were exposed to farnesol MBEC and the total volume of culture medium and farnesol was also adjusted to 1000 μl. These biofilms were evaluated through three microscopic techniques (optical, confocal and electronic microscopy) and a drug-free biofilm growth control was also included in the analysis. Optical and scanning electron microscopy were chosen in order to evaluate the biofilm structure, including matrix and bacterial cells, while confocal scanning microscopy was used to evaluate biofilm viability and distinguish live and dead cells. It is important to emphasize that all assays were carried out according to biosafety level 3 procedures.

For the evaluation of the biofilm matrix, the methodology proposed by Allison and Sutherland (Allison and Sutherland 1984) was used. Coverslides were stained for 15 min with a 2 : 1 saturated aqueous solution of Congo Red (Sigma-Aldrich, São Paulo, Brazil), containing 10% (v/v) Tween 80 (ISOFAR, Rio de Janeiro, Brazil) (Allison and Sutherland 1984). The stained coverlides were then evaluated in an optical microscope OLYMPUS BX41 and camera OLYMPUS DP71. For the confocal microscopic analysis, the methodology described by Saini *et al.* (2014) was used. Then, the Live/Dead (Invitrogen™, MA) fluorescent dye was added to cover the surface of the Petri dish containing the Thermanox coverslide. Afterwards, the coverslides were evaluated under a Confocal Microscope Nikon C2 (NIKON, Tokyo, Japan), at 488 nm for the detection of the SYTO9 fluorescent dye, which identifies live bacterial cells, and at 561 nm for the detection of the propidium iodide, which identifies dead bacterial cells. Software COMSTAT was used for quantitative analysis of the image stacks produced by CLSM (Heydorn *et al.* 2000). Parameters evaluated were: total biomass, biomass average thickness, total average thickness, roughness coefficient and surface-volume ratio, whereas thresholding was calculated automatically by Otsu's method.

Finally, the scanning electronic microscopy was performed according to the methodology described by Tran *et al.* (2009). The slides were observed in a FEI Inspect S50 scanning electron microscope, in high vacuum mode at 20 kV. The images were processed with the PHOTOSHOP software (Adobe Systems, San José, CA, USA).

Statistical analysis

The nonparametric Wilcoxon test was used to analyse the antibacterial MIC and MBEC reductions, after the combination with farnesol. These reductions were compared pairwise, with the nonparametric Friedman test. In all cases, the maximum significance level adopted for affirmative conclusion was 5%.

Results

The antibacterial MICs obtained against planktonic *B. pseudomallei* presented geometric means of 196·09 μg ml^{-1} for AMOX, 2·75 μg ml^{-1} for CAZ, 1·11 μg ml^{-1}

for DOX, 0·45 μg ml^{-1} for IPM, 15·35/0·80 μg ml^{-1} for SXT and 185·65 mmol l^{-1} (41·28 mg ml^{-1}) for farnesol. The combination of farnesol with antibacterial drugs, when tested at decreasing concentrations starting at the MIC against each strain, reduced the MIC geometric means of the drugs, as follows: 142·40 μg ml^{-1} for AMOX (P = 0·0394), 2·24 μg ml^{-1} for CAZ (P = 0·0256), 0·90 μg ml^{-1} for DOX, and 8·09/0·42 μg ml^{-1} for SXT (P = 0·0074; Table 1).

Regarding biofilm susceptibility, the MBEC values showed geometric means of 784·36 μg ml^{-1} for AMOX, 16 μg ml^{-1} for CAZ, 1·80 μg ml^{-1} for DOX, 1·11 μg ml^{-1} for IPM, 19/1 μg ml^{-1} for SXT and 826·20 mmol l^{-1} (183·72 mg ml^{-1}) for farnesol. The combination of farnesol with antibacterial drugs, when tested at decreasing concentrations starting at the MBEC against each strain, led to a reduction in MBEC geometric means, as follows: 242·70 μg ml^{-1} for AMOX (P = 0·0021), 4·45 μg ml^{-1} for CAZ (P = 0·0113), 1·24 μg ml^{-1} for DOX (P = 0·0260) and 8·53/0·44 μg ml^{-1} for SXT (P = 0·0319).

The microscopic evaluation of the effects of farnesol on mature biofilms of *B. pseudomallei* through the Congo Red technique demonstrated the disaggregation of the biofilm matrix, when biofilms were treated with MBEC (1200 mmol l^{-1}), as evidenced by the presence of unstained areas within the biofilm structure, suggesting the absence of matrix (Fig. 1a,b). The confocal analysis demonstrated the loss of biofilm structure, as shown by the thin layer of bacterial cells, with a reduced number of viable cells (Fig. 1c,d). Mature biofilms incubated at MBEC of farnesol presented a 99·2% reduction in the total biomass, a 58·18% reduction in the biomass average thickness and a 99·4% reduction in the total average thickness, when compared to untreated mature biofilms. In addition, there was an 18·6% increase in the roughness coefficient of the farnesol-treated biofilm, when compared to drug-free biofilm, indicating larger cell motion in the former, and a 28·4% increase in the surface-volume ratio, indicating the disruption of cellular agglomeration, hence an increase in cell dispersion.

The SEM analysis also demonstrated the disaggregation of the biofilm matrix and biofilm destruction, with areas containing only the outline of a disaggregated matrix with few bacterial cells adhered. In addition, exposure to farnesol led to alterations in the format and size of bacterial cells (Fig. 1e,f).

Discussion

Biofilm formation is a very relevant inducer of antimicrobial resistance and this ability has been observed for many strains of *B. pseudomallei* (Lazar *et al.* 2013). Limmathurotsakul *et al.* (2014) analysed the biofilm-forming

Table 1 Increased antimicrobial susceptibility of *Burkholderia pseudomallei* treated with farnesol

Compounds	MIC – drug and farnesol alone		Antimicrobial drugs in combination with farnesol				MBEC – drug and farnesol alone		Antimicrobial drugs in combination with farnesol			
	MIC range	Geometric mean	FARN MIC range	MIC range	Geometric mean	Reduction range	MBEC range	Geometric mean	FARN MBEC range	MBEC range	Geometric mean	Reduction range
FARN, mmol l^{-1}	150–300	185·65	–	–	–	–	75–2400	826·20	–	–	–	–
AMOX, μg ml^{-1}	128–256	196·09	75–150	64–256	142·40*	2–4×	256–4096	784·36	9·37–1200	64–512	242·70*	2–16×
CAZ, μg ml^{-1}	1–8	2·75	75–300	0·5–4	2·24*	2×	2–4096	16	4·68–1200	2–256	4·45*	2–256×
IPM, μg ml^{-1}	0·25–1	0·45	–	–	–	–	0·5–2	1·11	–	–	–	–
DOX, μg ml^{-1}	0·5–2	1·11	75–300	0·25–2	0·90	2×	0·5–8	1·80	75–1200	0·25–2	1·24*	2–4×
SXT, μg ml^{-1}	4·75/0·25–76/4	15·35/0·80	37·5–150	2·37/0·125–19/1	8·09/0·42*	2–4×	9·5/0·5–152/8	19/1	37·5–1200	2·37/0·125 – 38/2	8·53/0·45*	2–4×

FARN, farnesol; AMOX, amoxicillin; CAZ, ceftazidime; IPM, imipenem; DOX, doxycycline; MIC, minimum inhibitory concentrations; MBEC, minimum biofilm erradication concentration; SXT, sulfamethoxazole–trimethoprim.
*Indicates statistically significant reductions in MIC or MBEC values.

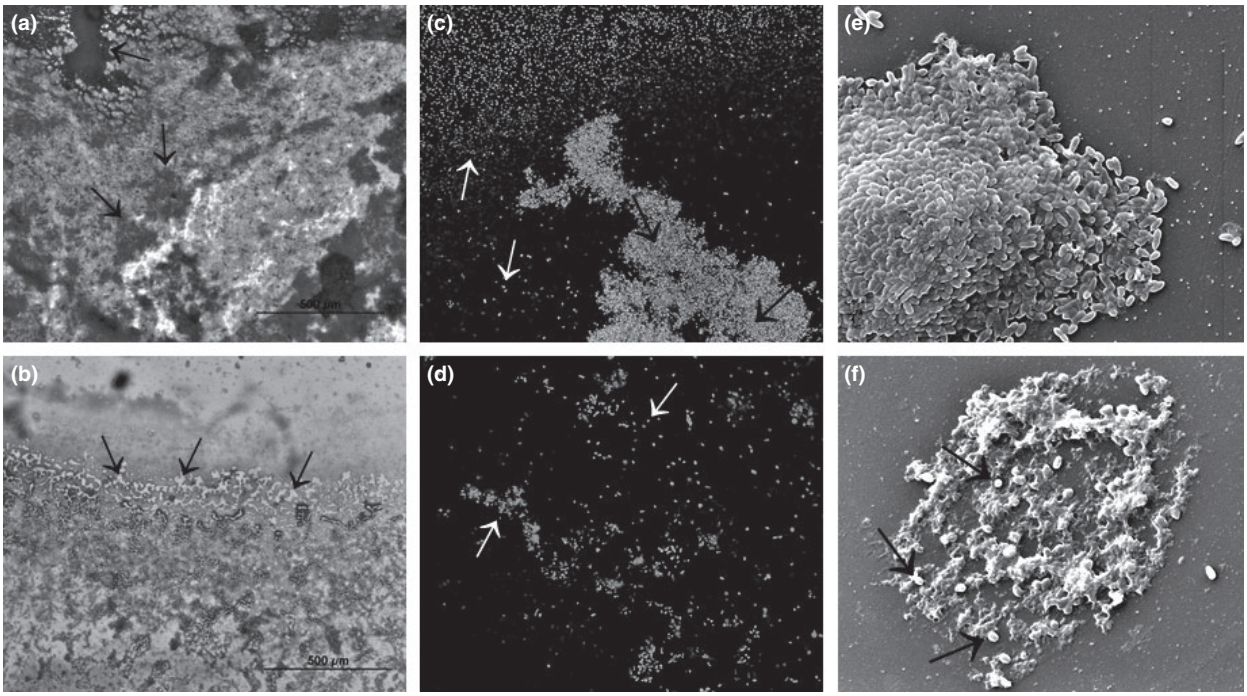

Figure 1 (a and b) Mature *Burkholderia pseudomallei* biofilms stained with the Congo Red technique. (a) Biofilm growth control, without farnesol exposure (100×): note regular homogenous, exuberant extracellular polysaccharide matrix (arrows); (b) Biofilm matrix after incubation with farnesol at minimum biofilm erradication concentration (MBEC) (100×): note the presence of unstained areas in the biofilm matrix, suggesting its perforation and destruction (arrows); (c and d) Mature *B. pseudomallei* biofilms under confocal laser scanning microscopy (600×, excitation at 488 nm for SYTO9, which reveals viable cells), (c) Biofilm growth control, without farnesol exposure: note the biofilm structure (black arrow), with an abundant number of viable cells (white arrows); (d) Biofilm after incubation with farnesol at MBEC: note the destruction of the mature biofilm and the dispersion of few viable bacterial cells (white arrows); (e and f) Mature *B. pseudomallei* biofilms under scanning electron microscopy (8000×). (e) Biofilm growth control, without farnesol exposure: note the exuberant cellular agglomeration, involved by the extracellular polysaccharide matrix; (f) Biofilm after incubation with farnesol at MBEC: note the destruction of the biofilm structure, associated with matrix erosion and alterations in the format and size of bacterial cells (arrow).

ability of strains from patients with recurrent melioidosis and observed that these strains presented greater biofilm production, when compared to those recovered from patients with acute disease. These authors provided the first evidence that the ability of *B. pseudomallei* to produce biofilm *in vitro* is associated with the occurrence of chronic recurrent melioidosis *in vivo*.

Moreover, previous studies of our group demonstrated that farnesol, a sesquiterpene alcohol, is capable of enhancing the susceptibility of *B. pseudomallei* to β-lactam antibiotics (Brilhante *et al.* 2012b). Therefore, considering that biofilm formation is a common feature among *B. pseudomallei* (Bandeira *et al.* 2013), which leads to decreased antimicrobial susceptibility and the occurrence of clinical relapses (Limmathurotsakul *et al.* 2014), it was decided to evaluate the effects of farnesol alone and in combination with antimicrobial drugs on mature biofilms of *B. pseudomallei*.

In this study, the biofilm-associated tolerance to the antibacterial drugs was mainly observed for the β-lactam antibiotics amoxicillin and ceftazidime. It is believed that this tolerance is not only associated with the biofilm structure itself and the decreased permeability to antimicrobial drugs, which prevents high drug concentrations from interacting with the bacterial cells. β-lactamase production and accumulation within the biofilm extracellular polysaccharide matrix (Hengzhuang *et al.* 2013) also seems to be an important mechanism of biofilm-mediated resistance. The accumulated β-lactamase inactivates the antibiotic as it penetrates the biofilm, thus protecting all the layers of bacterial cells against β-lactam drugs. This impairs the effectiveness of β-lactam antibiotics, which are included in the current therapeutic recommendations for melioidosis (Brilhante *et al.* 2012a,b; Sarovich *et al.* 2012; Bandeira *et al.* 2013).

Thus, the aim of this study was to analyse the *in vitro* activity of farnesol alone and combined with the antibacterial drugs amoxicillin, doxycycline, ceftazidime and sulfamethoxazole–trimethoprim against *B. pseudomallei* biofilms. It was observed that combination with farnesol reduced the MBEC values for amoxicillin from 2 to 16 times and those for ceftazidime from 2 to 256 times. It

has been shown that farnesol interferes with peptidoglycan biosynthesis and cell wall synthesis, enhancing the effect of β-lactams, and also decreases the production of β-lactamases (Kuroda *et al.* 2007). These properties may potentiate the inhibitory activity of β-lactams against mature *B. pseudomallei* biofilms.

In addition, it has been reported that farnesol is capable of disaggregating bacterial biofilm structure, reducing its biomass (Cerca *et al.* 2012), and allowing the contact of antibiotics with the cells entrapped within the biofilm structure (Katragkou *et al.* 2014). The results of the present research corroborate these findings, since the microscopic analyses of mature *B. pseudomallei* biofilms revealed that exposure to MBEC concentrations of farnesol promoted disaggregation of the matrix and, consequently, destruction of the biofilm. As reported by Katragkou *et al.* (2014), the damage to the matrix is secondary to the farnesol-mediated formation of gaps in the matrix, due to its lipophilic properties, incompatible with the exopolysaccharide matrix, which mainly composed of water. These findings suggest that the β-lactamase accumulated in the matrix may be released by the action of farnesol, thereby preventing cleavage of β-lactam antibiotics. This hypothesis seems to explain the results of this study, where β-lactam antibiotics showed the most significant MBEC reductions against *B. pseudomallei* biofilms, when combined with farnesol.

Concerning the drug doxycycline, no tolerant strains were seen when in biofilm growth, while for the combination sulfamethoxazole–trimethoprim one strain was tolerant. The association of farnesol with doxycycline and sulfamethoxazole–trimethoprim led to a decrease in the MBECs obtained for both of these drugs against biofilms of *B. pseudomallei*. Previous papers showed that farnesol inhibits fungal (Sharma and Prasad 2011) and bacterial efflux pumps (Jin *et al.* 2010), hence, suggesting that this reduction in doxycycline and sulfamethoxazole–trimethoprim MBECs is associated with farnesol-mediated inhibition of the efflux pumps responsible for the efflux of these groups of drugs, that is, tetracyclines and sulfonamides, in *B. pseudomallei* (Mima and Schweizer 2010).

This study showed the antimicrobial effects of farnesol against *B. pseudomallei* mature biofilms. This compound disrupts biofilm extracellular polymeric matrix, most likely leading to the increased susceptibility of these bacterial structures to amoxicillin, ceftazidime, doxycycline and sulfamethoxazole–trimethoprim, which are routinely used to treat melioidosis.

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Conflict of Interest

The authors have no conflict of interest to declare.

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