Assessing inhibitory activity of probiotic culture supernatants against *Pseudomonas aeruginosa*: A comparative methodology between agar diffusion, broth culture and microcalorimetry

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Abstract

Cell free supernatants (CFS) obtained from probiotic species are routinely used to preliminary investigate the antimicrobial activity of potential probiotic isolates by the agar well diffusion and broth culture. Both methods have documented limitations. In this work, the potential of isothermal microcalorimetry (IMC), a technique based on the measurement of heat produced by growing bacteria was used to investigate the antimicrobial effects of two commercial probiotic species (*Lactobacillus acidophilus* LA-5® and *Bifidobacterium lactis* BB-12®) and one reference strain (*Bifidobacterium bifidum* ATCC 11863) against *Pseudomonas aeruginosa* NCIMB 8628 using unmodified, neutralised and heat-treated CFS. *P. aeruginosa* was inhibited in growth by the unmodified CFS of all the species. No inhibitory activity was recorded for neutralised CFS of all the species using the agar well diffusion assay. Plate count during co-incubation of *P. aeruginosa* with the neutralised CFS of all the species showed no inhibition. However, IMC data showed significant inhibition with neutralised CFS obtained from the two *Bifidobacterium* species suggesting presence of other non-acidic inhibitory compounds in the CFS. The results in this work demonstrated that IMC has potential in probiotic bioassay as it has the capability to record in real-time and capture even subtle effects, which could be unnoticed with traditional assays.

Keywords: agar well diffusion, broth culture, cell free supernatant, inhibition, isothermal microcalorimetry, probiotics

1. Introduction

Interest in probiotics continues to gain momentum. In 2016, the probiotic market was evaluated as USD 42 billion and is anticipated to rise to USD 64 billion by 2024

(Marketsandmarkets.com 2017). The huge market of probiotic is largely driven by increasingly understanding of the role of the normal human microbiota in human and animal health and diseases. Microorganisms are incessantly isolated from humans, animals, plants, food, and environment and assessed for probiotic properties and applications (Damaceno et al. 2017; do Vale Pereira et al. 2017; Makete et al. 2017; Marius et al. 2018). The main properties assessed in a potential probiotic organism includes antimicrobial and antagonistic activity against potential pathogens, gastrointestinal tolerance, adherence and immunomodulatory properties (Vinderola et al. 2017).

The antimicrobial and antagonistic properties of probiotics against pathogenic microorganisms are traditionally assessed by two main methods. With the culture method, the probiotic strain or its inhibitory metabolite (culture supernatant) is co-cultured with pathogens of interest and changes in the microbial growth analyzed, by selective growth and colony counting (Annuk et al. 2003; Hutt et al. 2006), turbidity assay (Lee et al. 2003), biochemical methods or by molecular biological methods (Folkers et al. 2010). Alternatively, the diffusion method is used where probiotic is seeded on an agar plate together with a test microorganism (Annuk et al. 2003) or agar plates spotted with probiotic organisms are overlaid with agar inoculated with indicator microorganism (Barbosa et al. 2005; Chapman et al. 2012; Siroli et al. 2017; Tejero-Sarinena et al. 2012). Another variant of the diffusion method is the application of probiotic culture supernatant in punched wells or impregnation into disks, which are placed on agar plates seeded with test microorganism (Jara et al. 2011; Olivares et al. 2006; Rao et al. 2015). Diffusion of antimicrobial metabolite (or compound) produced by the probiotic or from the wells/disks into the medium leads to inhibition of bacterial growth in the vicinity of the probiotic or the well/disk and to

the formation of clear zones without any bacterial lawn. The zone of clearing around well or disk or probiotic is used as an indicator of antagonism.

Both methods are well established and have several advantages, however their drawbacks are also well documented. The diffusion method is hinged on the diffusibility of the inhibitor (Pongtharangkul and Demirci 2004; Rogers and Montville 1991) hence inhibitors that are large or interact with the agar may not readily diffuse. Plate count associated with broth culture is laborious, slow and analyses retrospectively. Furthermore, some cells may exist in dormant, cryptobiotic, moribund or latent states after a particular treatment hence may not form colonies on plates even though they may have other measurable activity (Davey 2011). The use of turbidity assay for cell enumeration although relatively rapid, non-labour intensive and amenable to automation, also suffers from drawbacks such as inability to analyse heterogenous or complex samples and to distinguish viable cells from dead cells. Other rapid and automated methods and molecular techniques have been applied in the detection and enumeration of cells numbers in similar assays however, only few are able to distinguish viable cells from dead cells. In this study, the potential of isothermal microcalorimetry (IMC) was explored to determine the inhibitory activity of probiotic culture supernatant obtained from two commercial probiotic strains (*Lactobacillus acidophilus* LA-5® and *Bifidobacterium lactis* BB-12®) and one reference strain (*Bifidobacterium bifidum* ATCC 11863) against *P. aeruginosa* NCIMB 8628. Isothermal microcalorimetry (IMC) is a technique based on the principle of measurement of heat, which can measure the growth of microorganisms in real time, non-destructively. In an appropriate medium, bacteria utilize the energy sources provided for respiration and fermentation to form new cellular materials. These metabolic processes are heat evolving or consuming resulting in the exchange

of heat energy to or from the environment and this can easily be detected by the microcalorimeter. By monitoring net metabolic heat output by growing bacteria, IMC can give characteristic signatures for individual bacteria, which is proportional to their growth. It was believed that IMC could overcome some of the limitations of the diffusion and traditional broth methods in probiotic antimicrobial activity characterizations.

2. Materials and Methods

Microorganisms

Pure cultures of *Lactobacillus acidophilus* LA-5® and *Bifidobacterium lactis* BB-12® were obtained from Chr. Hansen's Culture Collection (Hørsholm, Denmark). *Bifidobacterium bifidum* ATCC 11863 was purchased from American Type Culture Collection, USA. *Pseudomonas aeruginosa* NCIMB 8628 was obtained from ConvaTec Ltd., UK.

Stock culture maintenance

B. bifidum ATCC 11863 and the commercial probiotic strains, *L. acidophilus* LA-5® and *B. lactis* BB-12® were grown respectively in Reinforced Clostridial Medium (RCM; Oxoid, Basingstoke, UK) and de Man Rogosa Sharpe broth (MRS, Oxoid) supplemented with 0.05% (w/v) L-cysteine hydrochloride (Fisher Scientific, UK) "MRSc" under anaerobic conditions (anaerobic jar with AnaeroGen, Oxoid to generate the anaerobic environment) at 37^{o}C for 48 h . *P. aeruginosa* was grown in Nutrient broth (NB; Oxoid) aerobically at 37^{o}C. The cells were harvested when they reached stationary phase of growth. The cells were washed in phosphate buffered saline (PBS), and resuspended in 15% (v/v) glycerol at an organism density of 10^{8}

CFU/mL and frozen in 1.8 mL aliquots over liquid nitrogen (Beezer et al. 1976). Aliquots were stored under liquid nitrogen until required.

Preparation of cell-free culture supernatants from probiotic cultures

The culture supernatants of *B. bifidum* and the commercial probiotic species were prepared by cultivating the respective species in Cooked meat medium (CMM; Oxoid) supplemented with 1% w/v glucose (Sigma-Adrich, UK), "CMMg" over 48 h anaerobically. The cells and debris were removed by centrifuging at 3500 *g* for 10 min at 4^{o}C. The supernatant was collected and filter-sterilized to remove cells using a 0.22 µm membrane syringe filter (Sartorius AG, Germany). The pHs of the supernatants were examined (pHenomenal®, UK) and equal aliquots modified by: adjusting the pH to neutral (pH 7.0) with 1M NaOH and heating at 100^{o}C for 60 min.

Co-incubation of *P. aeruginosa* with probiotic culture supernatants: microcalorimeter and broth culture method

1.5 mL of unmodified CFS, (repeated for the modified CFS) obtained from *B. bifidum*, *L. acidophilus* LA-5® and *B. lactis* BB-12® was added to 1.5 mL of 2-fold concentrated CMMg "dsCMMg" in 3 mL sterile calorimetric glass ampoule. The mixture was vortexed for 10 s. *P. aeruginosa* was inoculated into each CFS-broth mixture to a population density of 10^6 CFU/mL and placed in the thermal equilibration position of a Thermometric Thermal Activity Monitor 2277 (TAM 2277, TA Instruments Ltd., UK) set at 37^{o}C (±0.1^{o}C). The filled ampoules were allowed 30 min to equilibrate to temperature at this intermediate position before being lowered into the measurement position. Data were captured with Digitam 4.1 every 10 s with an amplifier range of 1000 µW for 24 h. Experiments were performed in triplicate. Data were analysed with Origin Pro 8.6 (Microcal Software Inc.). The reference

ampoule was loaded with 3 mL of sterile media. A control experiment was done by replacing the CFS with sterile distilled water. Plate counts of the species were done after the calorimetric experiments. Equivalent CFS-broth cultures inoculated with 10^6 CFU/mL *P. aeruginosa* was incubated at 37ºC for 24 h and cell counts determined at the end of incubation.

Agar well diffusion assay

Cell-free culture supernatants of *B. bifidum*, *L. acidophilus* LA-5® and *B. lactis* BB-12® were assessed for their antimicrobial activity against *P. aeruginosa* by the agar well diffusion assay as described by Jara et al. (2011) with some modifications. 10^6 CFU/mL of *P. aeruginosa* was spread onto Nutrient broth with a sterile cotton swab to give a lawn of confluent growth of cells. Wells of 9 mm diameter were made with a sterile borer and filled with 100 µL of unmodified and modified CFS. The plates were kept on the bench for 2 h for diffusion of the CFS from the wells into the agar and incubated after at 37ºC. The zones of inhibition were measured after 24 h of incubation.

3 Results

IMC data are typically shown as plots of power (µW) against time (t). Alternatively, data can be plotted as cumulative heat (J) against time (t), which is comparable to bacteria growth measured by plate count or optical density, Fig. 1. In an isothermal microcalorimeter, microorganisms growth is typically exponential (which could represent the heat produced by the growing microorganism comparable to biomass produced by plate count assay or optical density). As energy becomes limited and there is build up of toxic metabolites, power signal approaches baseline, which may represent stationary phase or cell death (Braissant et al. 2013). Bacteria utilise given

nutrients through different metabolic pathway, hence each bacteria typically produces a signature curve in the microcalorimeter which can be used for identification (Boling et al. 1973) . An inverse relationship exists between inoculum density and signal detection time. As inoculum density decreases, there is a time lag to detection of signal (Fig. 2).

Co-incubation of probiotic supernatant with *P. aeruginosa*

The supernatants produced from the probiotic species were acidic. From a pH of 7.2 ± 0.2 pre-inoculation, the pH of CMMg after the growth of the probiotic species were 4.0 ± 0.13 for *L. acidophilus* LA-5®, 4.55 ± 0.01 for *B. lactis* BB-12® and 4.79 ± 0.01 for *B. bifidum*.

IMC data for the co-incubation of *P. aeruginosa* with the CFS of the probiotics are shown in Fig. 3, 4, and 5. The total heat output (Q_t), which is the total area under the growth curve (AUC), the amplitude of the maximum signature peak of *P. aeruginosa* (P_{smax}) and the time of registration of the maximum signature peak (t_{smax}) calculated from the power-time curves are shown in Table 1. Fig. 3 compares the power-time curves of *P. aeruginosa* inoculated into CMMg (control) and dsCMMg diluted with CFS (unmodified, neutralised and heat-treated) obtained from *L. acidophilus* LA-5®. The data show increasing signal for *P. aeruginosa* (control) peaking at approximately 4.8 h before the return to baseline. When inoculated into broth with unmodified CFS and heat-treated CFS, the microorganism does not show characteristic growth but shows slight metabolic activity in the first hour that principally contributed to the Q_t value (Table 1). In neutralised CFS, the characteristic profile of *P. aeruginosa* is recognized however with a slightly delayed peak (the t_{smax} recorded is 5.55 ± 0.20 h relative to 4.69 ± 0.14 h in the control). Fig. 4 and 5 compare the power-time curves of *P. aeruginosa* inoculated into CMMg and dsCMMg diluted with unmodified and

modified CFS obtained from *B. lactis* BB-12® and *B. bifidum* respectively. Unlike the CFS obtained from *L. acidophilus* LA-5®, *P. aeruginosa* inoculated into broth with neutralised CFS obtained from *B. lactis* BB-12® and *B. bifidum* showed lower maximum power (52.21 ± 8.83 μW and 62.42 ± 7.15 μW respectively) and significant time-lag (delayed peak, >5 h) of *P. aeruginosa* which was more profound in *B. bifidum* (Table 1). The plate count data after incubation of *P. aeruginosa* in the broth-CFS mixture of the probiotic species are shown in Table 2. The data showed that only neutralised CFS of all three probiotic species supported growth of *P. aeruginosa*. Heat-treated and unmodified CFS of the probiotic species inhibited growth of *P. aeruginosa.*

Agar well diffusion assay

The diameters of growth inhibition zones of *P. aeruginosa* using the CFS obtained from the probiotic species are also shown in Table 2. Images of zones of inhibition of *P. aeruginosa* with the unmodified, neutralised and heat-treated CFS of *L. acidophilus* LA-5® are shown in Fig. 6. CFS obtained from *L. acidophilus* LA-5® was most inhibitory towards the species according to the diffusion assay. Zones of inhibition of heat-treated CFS were superior to that of unmodified CFS. The agar well diffusion assay corroborated the plate count data. Thus, neutralised CFS of all the probiotic species did not have any inhibitory potential. This was however not in agreement with the IMC data, which showed partial inhibition of *P. aeruginosa* by the neutralised CFS obtained from *B. lactis* BB-12® and *B. bifidum.*

4 Discussion

The goal of this study was to compare IMC with the agar diffusion and broth culture assays, which are routinely used in assessing the inhibitory activity of probiotic

culture supernatants. Microorganisms produce heat, about 2 pW per active cell (Braissant et al. 2010). Although this amount of heat is small, when actively metabolizing and growing, the amount of heat generated cumulatively by growing cells with initial density below 10^6 CFU/mL can be detected by microcalorimeters. Usually cell numbers below 10^6 CFU/mL eventually get to this detectable density in an appropriate medium and this is evidenced as time-lags in the IMC data (Fig. 1).

In this work, it was demonstrated that both unmodified and heat-treated CFS produced by the three probiotic species were inhibitory towards *P. aeruginosa*. The degree of effectiveness of the unmodified and heat-treated CFS in inhibiting *P. aeruginosa* could not be determined with the present data. The agar diffusion assay showed higher inhibition zones for the heat-treated samples (Table 2, Fig. 6), which could be as a result of the concentration of the CFS during heating or inhibition of an exudate in the CFS, which interacted negatively with the CFS. Neutralised CFS of the probiotic species were demonstrated by IMC to exhibit inhibitory activities towards *P. aeruginosa* although this was not registered with both the plate count and agar well diffusion data. While this inhibition was not significant in the case of CFS produced by *L. acidophilus* LA-5®, it is likely the CFS of the other probiotic species contained non-acidic inhibitory metabolites which were present in small quantities and or were not diffusible in the agar. It can also be argued that the activity of the non-acidic metabolites depended on the acidity of the supernatant and were therefore hindered when the CFS were neutralised (Messens and De 2002; Oliveira et al. 2008).

From the IMC data, it is possible these non-acidic metabolites caused cell death and reduced cell numbers of initial inocula until more than 5 h before detection, where survived cells had multiplied to 10^6 CFU/mL. This inhibition noted in the IMC could not be seen with the broth assay as once the survived cells went through a typical

growth cycle, it was difficult to determine the initial inhibition which had occurred. The final CFU/mL of *P. aeruginosa* in all three CFS therefore did not show any difference which emphases the fact that the broth culture method is limited by multiple sampling and concomitant plating to determine these effects unlike the IMC which records real-time metabolism of the microorganisms

Agar diffusion assay is the most commonly used method to determine antimicrobial activity of bacteria and bacteriocin activity (Masoumikia and Ganbarov 2015; Prabhurajeshwar and Chandrakanth 2017; Sukhina et al. 2012). However, this method is dependent on the inhibitory compound diffusing through the agar which could be affected by size, medium components and other molecules secreted by the bacteria which could possibly react (Arena et al. 2016). Different sensitivities has also been demonstrated to exist between different agar medium (Azevedo et al. 2018). Using IMC, inhibitory activity of neutralised CFS was detected which could not be verified with broth culture and the agar well diffusion methods due to lower sensitivities of the latter methods in recognizing subtle inhibitory effects and possibility of poor diffusion and interaction within the agar medium. IMC records activity in real-time in contrast to broth culture with requires sampling alongside turbidity or plate count to determine the effect of a metabolic modifier after treatment or the agar diffusion assay which requires an incubation period to determine effect and also relies on investigator's interpretation of unclear and non-perfectly circular zones of inhibition. This work shows that IMC is a valuable alternative in the determination of antimicrobial activity of bacteria or bacteriocin activity, overcoming some of the limitations of the agar diffusion and broth culture methods.

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Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Statement

This article does not contain any studies with animals or human participants.

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Table 1. Total heat output (Q_t), maximum signature peak (P_{smax}), time of registration of maximum signature peak (t_{smax}) of *P. aeruginosa* in the unmodified and modified CFS of *L. acidophilus* LA-5®, *B. lactis* BB-12® and *B. bifidum*.

Supernatant	**Q_t (J)**	**P_{smax} (µW)**	**t_{smax} (h)**
Control	2.26 ± 0.17	203.65 ± 34.08	4.69 ± 0.14
L. acidophilus LA-5® CFS	0.68 ± 0.06	-	-
L. acidophilus LA-5® CFS neutralised	2.75 ± 0.24	204.97 ± 10.65	5.55 ± 0.20
L. acidophilus LA-5® CFS heat treated	0.74 ± 0.08	-	-
B. lactis BB-12® CFS	0.66 ± 0.06	-	-
B. lactis BB-12® CFS neutralised	1.23 ± 0.04	52.21 ± 8.83	9.88 ±0.22
B. lactis BB-12® CFS heat treated	0.82 ± 0.13	-	-
B. bifidum CFS	0.90 ± 0.14	-	-
B. bifidum CFS neutralised	1.50 ± 0.04	62.42 ± 7.15	12.65 ± 0.14
B. bifidum CFS heat treated	0.85 ± 0.01	-	-

- no signature peak recorded

Table 2. Plate count and zone of inhibition of *P. aeruginosa* tested with unmodified and modified CFS of *L. acidophilus* LA-5®, *B. lactis* BB-12® and *B. bifidum*. Growth of *P. aeruginosa* was observed only in neutralised CFS of all the probiotic species. The zones of inhibition values are means (n=4) ± SD (mm)

Supernatant	**CFU of *P. aeruginosa* (CFU/mL)**	**Zone of inhibition of *P. aeruginosa* (mm)**
Control	2.8×10^7	-
L. acidophilus LA-5® CFS	0	9.0 ± 0.8
L. acidophilus LA-5® CFS neutralised	3.0×10^7	0.0 ± 0.0
L. acidophilus LA-5® CFS heat treated	0	12.0 ± 0.8
B. lactis BB-12® CFS	0	5.8 ± 1.5
B. lactis BB-12® CFS neutralised	2.3×10^7	0.0 ± 0.0
B. lactis BB-12® CFS heat treated	0	7.5 ± 1.0
B. bifidum CFS	0	6.3 ± 1.0
B. bifidum CFS neutralised	4.2×10^7	0.0 ± 0.0
B. bifidum CFS heat treated	0	9.3 ± 0.5

Fig. 1. IMC data represented as power-time curves (—) or cumulative heat–time (---) curves for the growth of *P. aeruginosa*

Fig. 2. Varying inoculum density of *P. aeruginosa* (10^6 CFU/mL - 10^3CFU/mL) in NB in the microcalorimeter. Lower concentrations of inocula is monitored as time-lags.

Fig. 3. Comparison of the power-time curves of *P. aeruginosa* in CMMg (control) and *P. aeruginosa* in dsCMMg diluted with CFS of *L. acidophilus* LA-5® unmodified, neutralised and heat-treated

Fig. 4. Comparison of the power-time curves of *P. aeruginosa* in CMMg (control) and *P. aeruginosa* in dsCMMg diluted with CFS of *B. lactis* BB-12® unmodified, neutralised and heat treated

Fig. 5. Comparison of the power-time curves of *P. aeruginosa* in CMMg (control) and *P. aeruginosa* in dsCMMg diluted with CFS of *B. bifidum* unmodified, neutralised and heat treated

Fig. 6. Inhibition of *P. aeruginosa* by unmodified [A], neutralised [B] heat-treated [C] CFS of *L. acidophilus* LA-5®

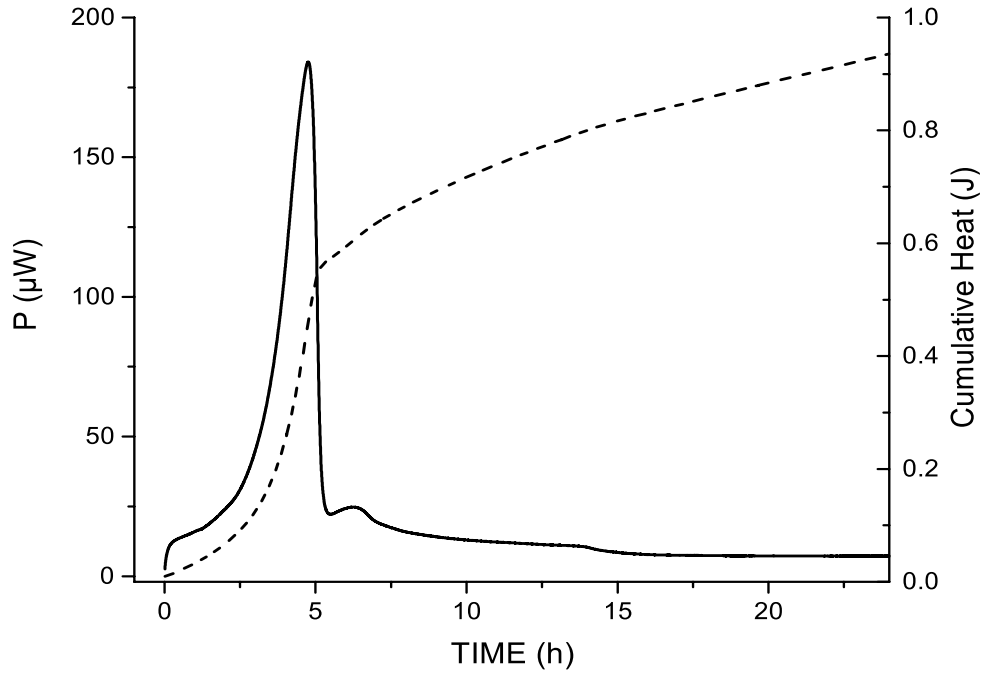
200
150
100
50
0
P (μW)
1.0
0.8
0.6
0.4
0.2
0.0
Cumulative Heat (J)
0
5
10
15
20
TIME (h)

Fig. 1

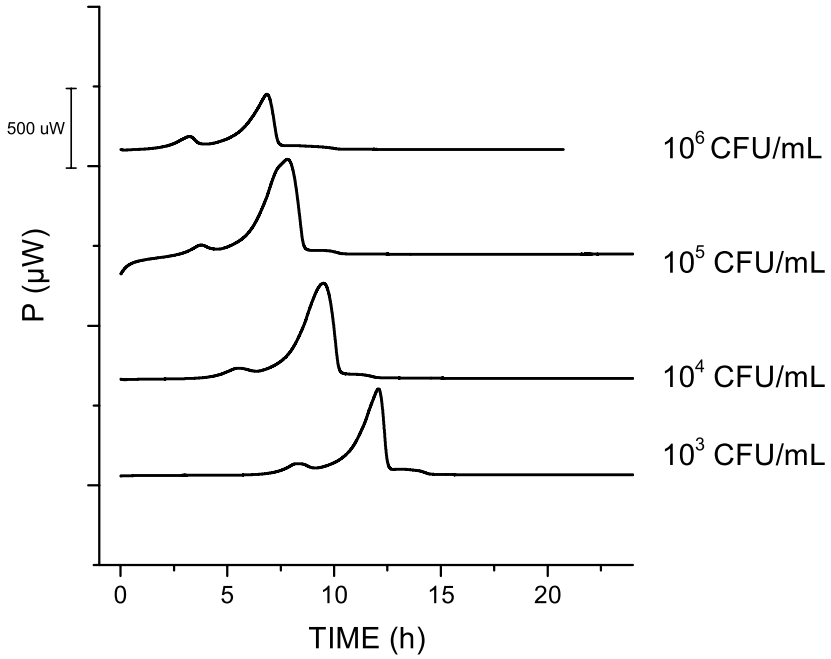
500 uW
P (μW)
10^6 CFU/mL
10^5 CFU/mL
10^4 CFU/mL
10^3 CFU/mL
0
5
10
15
20
TIME (h)

Fig. 2

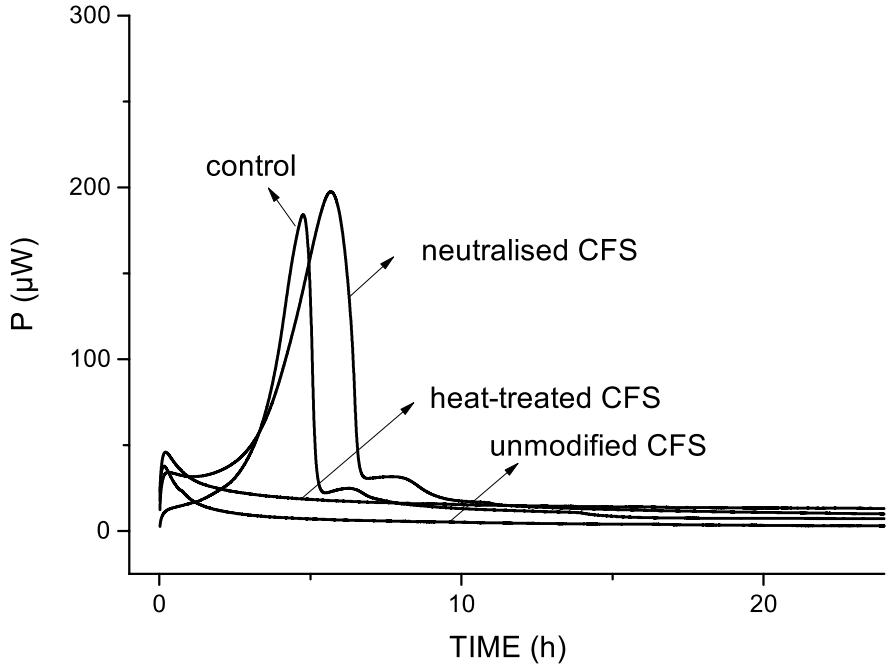
300
200
100
0
P (µW)
control
neutralised CFS
heat-treated CFS
unmodified CFS
0
10
20
TIME (h)

Fig. 3

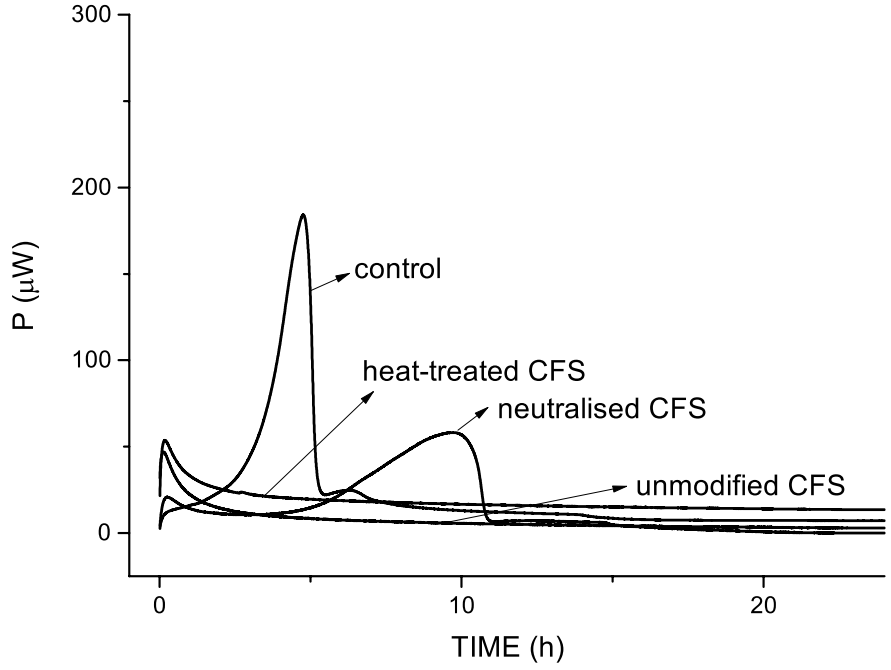
300
200
100
0
P (µW)
control
heat-treated CFS
neutralised CFS
unmodified CFS
0
10
20
TIME (h)

Fig. 4

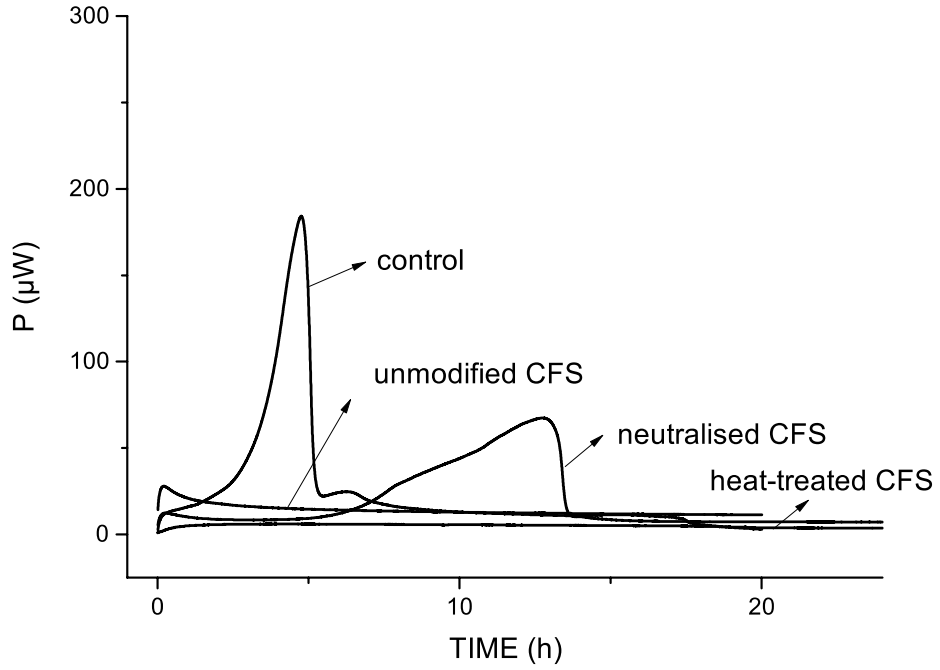
300
200
100
0
P (µW)
control
unmodified CFS
neutralised CFS
heat-treated CFS
0
10
20
TIME (h)

Fig. 5

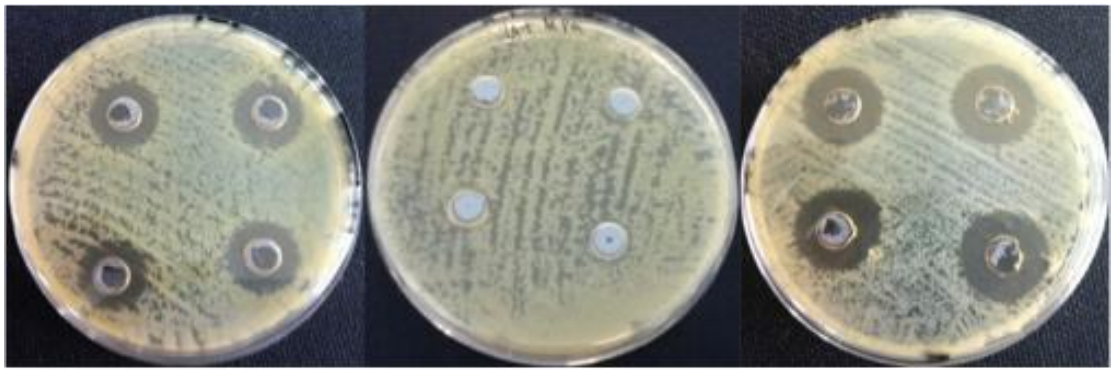
[A]
[B]
[C]

Fig. 6