Inhibition of biofilms of *Pseudomonas aeruginosa* by Medihoney™ *in vitro*.

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Short title: inhibition of biofilms by Medihoney™

ABSTRACT

Objective: *Pseudomonas aeruginosa* has been linked to chronic wound infections, where its ability to form biofilms and to tolerate antimicrobial agents helps to facilitate its persistence. This study aimed to investigate the susceptibility of biofilms of *P. aeruginosa* to Medihoney™ *in vitro*.

Method: Biofilms were cultivated in microtitre plates with and without a range of concentrations of Medihoney™ and effects on biofilm were monitored by optical density (at 650 nm), biomass (by staining with crystal violet), metabolic activity (by assaying esterase) and viability (by determining total cell counts). Structural effects on established biofilms were examined by scanning electron microscopy and epifluorescence following staining by LIVE/DEAD® BacLight™, which also showed effects on vitality.

Results: The lowest concentration of Medihoney ™ found to prevent biofilm formation was 17%(w/v) whereas on average 35.5%(w/v) was required to inhibit established biofilms. Susceptibility did not vary with length of biofilm establishment between 24 and 72 h. Extensive structural changes in established biofilm were seen ≤30%(w/v) Medihoney™ using scanning electron microscopy and loss of viability was found at ≤20%(w/v) Medihoney™ using fluorescent staining, together with loss of biofilm structure.

Conclusions: Using a range of methods to evaluate biofilm integrity, this study demonstrates that Medihoney™ inhibits *P. aeruginosa* biofilms *in vitro* at concentrations that are attainable in clinical use. Whether Medihoney™ has the potential to disrupt *P. aeruginosa* biofilms in cutaneous wounds must now be tested in patients.

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Keywords: manuka honey, biofilms, wounds, biofilm viability, LIVE/DEAD® BacLight™

INTRODUCTION

There are many factors that influence the rate at which wounds heal, and the discovery that failure to heal may be associated with the presence of biofilm in the majority of chronic wounds[1, 2] has provided new insight for wound care professionals. Antibiofilm strategies may help in managing chronic wounds, but diminished susceptibility of established biofilms to conventional antimicrobial agents[3] indicates that present antibiotic and antiseptic therapies may be of limited value. This provides an impetus to search for additional antimicrobial agents. The continued emergence and increased prevalence of antibiotic-resistant strains, as well as the difficulties in eradicating wound biofilms, has increased the urgency to find effective antimicrobial interventions for wounds. Honey has been used topically in wound care for thousands of years. Its popularity waned in the 1970s as clinicians relied on antibiotics and more sophisticated dressings started to become available. Within the past ten years honey has been re-introduced into modern medicine in many developed countries. Although varying types of floral honey have been used clinically, manuka honey from New Zealand is the most frequently used medical grade honey at present. It has been shown to exhibit equivalent effectiveness in inhibiting planktonic antibiotic resistant bacteria and antibiotic sensitive strains[4, 5]. Manuka honey prevents cell division in *Staphylococcus aureus*[6] and methicillin-resistant *S. aureus*[7] by failing to cleave cell wall components due to loss of activity of autolytic enzymes. A different mode of action has been suggested for manuka honey against *Pseudomonas aeruginosa*, where changes in the cell surface led to cell lysis[8] following disorganisation of the cell wall by down-regulation of an outer membrane protein normally involved in structural stabilisation.[9] This pathogen has been implicated in large leg ulcers[10], in the failure of skin grafts[11] and in perpetuating chronic inflammatory responses in chronic wounds.[12] Some preliminary reports suggest that manuka honey is more effective than other honeys at inhibiting biofilms.[13, 14] Within the variety of licensed wound dressings now available Medihoney™ is an example that uses active *Leptospermum* honey, which is otherwise known as manuka honey. This study, therefore, aimed to investigate the effect of Medihoney™ on biofilms of *Pseudomonas aeruginosa in vitro* using a range of methods.

METHODS

Materials

Throughout this study a culture of *Pseudomonas aeruginosa* (LE08) that had been isolated from an out-patient with a chronic leg ulcer of more than two years duration who was attending a local wound care clinic was used. Tubes of Medihoney™ were provided by Comvita UK and a solution of artificial honey was used to determine the contribution of the four main constituent sugars in honey to the inhibition of biofilms.[4]

Prevention of biofilm formation

To determine the concentration of honey required to prevent a biofilm of *P. aeruginosa* forming *in vitro* a range of concentrations of honey in tryptone soya broth (TSB; Oxoid, Cambridge, UK) were freshly prepared from a stock solution of 20%(w/v) Medihoney™ and ranged from 5 to 20% with 1% intervals. 50 µl of diluted honey was dispensed into wells of 96 well microtitre plates (Nunc, Roskilde, Denmark) and inoculated with 1 µl of a 1in 5 diluted overnight culture of test organism normally at a population density of 3 x 10^8 cfu/ml. Each microtitre plate included 8 wells without inoculum (negative control), 8 inoculated wells without added honey (positive control) and at least 8 wells with 50 µl 20%(w/v) artificial honey solution prepared in TSB. Each honey concentration was tested in quadruplicate for each plate, and each experiment was conducted on at least three occasions. Microtitre plates were incubated statically at 37°C for 24 h and the extent of biofilm formed was evaluated by determining optical density, biofilm biomass, biofilm activity and biofilm viability.

Determination of biofilm density

The lowest concentration of Medihoney™ to prevent biofilm formation was determined using optical density at 650 nm using the Spectrostar nano plate reader (BMG Labtech, Buckinghamshire, UK).

Estimation of biofilm biomass

The extent of biofilm biomass was estimated by gently discarding well contents and washing the well with 100 µl phosphate buffered saline (PBS; Oxoid, Cambridge, UK) to remove planktonic cells.

Biofilm was then fixed by adding 100 µl 99% methanol to each well for 15 min. Fixative was removed and the plates allow to air dry before adding 50 µl 0.25%(w/v) crystal violet for 15 min to stain adherent biofilm. The dye was carefully removed, each well was washed three times with 100 µl PBS, dried by blotting onto paper towels and 100 µl 7% acetic acid was added to solubilise the dye contained in adherent cells. Absorbance was measured at 570 nm a Spectrostar nano plate reader. The Minimum Inhibitory Concentration (MIC_{50}) was determined as the concentration of Medihoney™ that reduced biofilm biomass by at least 50% compared to untreated controls.

Estimation of biofilm metabolic activity

The metabolic activity of biofilm was determined by assaying esterase activity in biofilms of *P. aeruginosa* by the conversion of non-fluorescent fluorescein diacetate (FDA) to yellow fluorescent fluorescein.[15] The Minimum Inhibitory Concentration (MIC_{90}) was determined as the concentration of Medihoney™ that reduced biofilm activity by at least 90% compared to untreated controls.

Determination of biofilm viability (by total cell count)

To determine the effect of honey on biofilm viability the liquid phase from wells was discarded and contents were washed with 100 µl sterile maximum recovery diluent (MRD; Oxoid, Cambridge, UK) to remove planktonic cells. Then 50 µl MRD was added to the washed biofilm and a sterile pipette tip was then used to scrape the bottom of the well to release adherent biofilm. The total viable count of the resulting suspension was determined using the surface drop count.[16] Diluted suspensions were plated onto tryptone soya agar (TSA; Oxoid, Cambridge, UK), incubated at 37°C for 24 h and colony forming units (cfu) per well calculated.

Cultivation of established biofilms

Biofilms were established in either 96 well microtitre plates or in 24 well microtitre plates (Nunc, Roskilde, Denmark) containing sterile plastic coverslips (Agar Scientific, Stansted, UK). An overnight culture in TSB was diluted 1 in 5 with TSB and 50 µl was inoculated into wells in 96 well plates, or 200 µl into wells in 24 well plates. All plates were incubated statically at 37°C for 24 h. If 48 h biofilms were required culture medium in microtitre plate wells of 24 h established biofilms was

gently removed, discarded, replaced by 50 µl of fresh TSB and incubation was continued for a further 24h. For 72 h established biofilms spent medium was similarly replaced with fresh TSB after 24 and 48 h and the plate incubated for another 24 h at 37°C.

Inhibition of established biofilms

To determine the effect of Medihoney™ on established biofilms, a range of concentrations (5 to 50%w/v with 5% intervals) were freshly prepared aseptically in TSB from a stock 50 %(w/v) solution and used to replace the liquid phase in wells of microtitre plates containing biofilm that had been cultivated for 24, 48 or 72 h. All plates were incubated statically at 37°C for 24 h and the effects on biofilm evaluated by determining optical density, biofilm biomass, biofilm activity and biofilm vitality. Each microtitre plate included 8 wells without inoculum (negative control), 8 inoculated wells without added honey (positive control) and at least 8 wells with 50 µl 50%(w/v) artificial honey solution prepared in TSB. Each honey concentration was tested in either 4 or 7 wells in each plate, and each experiment was conducted on at least three occasions.

Examination of biofilm by scanning electron microscopy

To determine the effect of Medihoney™ on biofilm structure, 24 h biofilm was established on plastic coverslips contained in 24 well microtitre plates. Then the liquid phase was replaced by 200 µl Medihoney™ concentrations ranging from 0 to 50 % (w/v) and incubated at 37°C for 24 h. Coverslips were processed for scanning electron microscopy as described previously (17) and examined in a 5200LV Jeol scanning electron microscope (Jeol Ltd, Hertfordshire, UK).

Vitality of biofilm assessed by epifluorescent microscopy

The vitality of biofilms cultivated on coverslips and exposed to varying manuka honey concentrations as described above was assessed using LIVE/DEAD® BacLight™ Bacterial Viability Kits (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Coverslips were mounted onto glass slides and visualised by a Nikon Eclipse 80i fluorescent microscope with oil immersion and x100 lens. For detection of SYTO 9 (green channel) a 488 nm excitation and 520 nm emission filter was used. For

propidium iodide detection (red channel) a 543 nm excitation and 572 nm emission filter was used. Images analysis used Volocity software (Perkin Elmer, Cambridge, UK).

RESULTS

The effect of Medihoney™ in preventing biofilm formation

The lowest concentration of Medihoney™ that prevented *P. aeruginosa* forming a biofilm *in vitro* was found to be 17%(w/v) (Table 1). Inhibitory effects are normally expressed as the Minimum Inhibitory Concentration (MIC); here this was determined by assessing optical density (Fig. 1a), biofilm biomass following staining with crystal violet (Fig. 1b) and by determining biofilm metabolic activity with an esterase assay (Fig. 1c). All methods gave similar endpoints, although slighter wider variation was seen with biomass determinations than for biofilm metabolic activity. On one occasion the total number of viable bacterial cells attached to the wall of the microtitre plate wells was estimated (Fig. 2): MIC_{90} was 13%(w/v) Medihoney™, and 20%(w/v) Medihoney™ gave a 5.14 log reduction compared to untreated cells after 24 h at 37°C. Concentrations of Medihoney™ lower than the MICs promoted increased biofilm formation compared to untreated cells (Fig. 1 and 2). Incubating *P. aeruginosa* with 20% (w/v) artificial honey elicited the formation of greater biofilm biomass and activity than untreated controls (Fig. 1), which indicated that biofilm inhibition was not caused by the sugar content of honey alone.

The effect of Medihoney™ in inhibiting established biofilm

Higher concentrations of Medihoney™ were required to inhibit established biofilms than those that prevented the formation of biofilm (Table 1), but the susceptibility of *P. aeruginosa* biofilm did not vary with the length of biofilm establishment. MICs ranged from 31 to 40%(w/v) Medihoney™ and mean of means for all methods was 35.3%(w/v). Contrary to experiments for preventing biofilm formation (Fig. 1a), measuring optical density did not give clearly defined MICs (Fig. 3a). As above concentrations of Medihoney™ below the MICs enhanced biofilm growth and assays of biofilm biomass (Fig. 3b) gave more variable results than those of biofilm metabolic activity (Fig. 3c). Treating established biofilm with 50%(w/v) artificial honey did not cause complete dispersion of the

biofilm biomass (Fig. 3b) nor loss of all biofilm activity (Fig. 3c). Changes in the total viable cell count of biofilm was related to Medihoney™ concentration (Fig.4): MIC_{90} was 30%(w/v) and a 3.62 log reduction was found in biofilm exposed to 50% Medihoney™ for 24 h compared to untreated biofilm.

Structural changes in biofilms exposed to Medihoney™

Using scanning electron microscopy (SEM) it was observed that the extent of biofilm bound to coverslips decreased with increasing honey concentration (Fig. 5). Untreated biofilm (Fig. 5a) and biofilm treated with 20%(w/v) Medihoney™ (Fig. 5b) was composed of extensive layers of rod shaped cells and extracellular material, yet cells in biofilm exposed to 30%(w/v) Medihoney™ (Fig. 5c) were noticeably shorter and more rounded. Intact biofilm was difficult to find in samples of biofilm treated with 40 (Fig. 5d) and 50 %(w/v) Medihoney™, yet it was not entirely dispersed (Fig. 5e). This is the first study in which the effects of manuka honey on biofilms has utilised electron microscopy, and marked disruption of biofilm was noticed.

Vitality of biofilms exposed to Medihoney™

The images obtained by electron microscopy did not provide any information on biofilm vitality, so biofilm was cultured on coverslips for 24 h , treated with a range of Medihoney™ concentrations for 24 h, stained with LIVE/DEAD® BacLight™ and examined by epifluorescence to search for viable cells. Untreated biofilm (Fig. 6A) contained mostly viable cells stained with green fluorescent stain with few non-viable, red cells; however, the proportion of viable cells decreased markedly with increased Medihoney™ concentration. Following exposure to 10% (w/v) manuka honey (Fig. 6B) viable cells exceeded non-viable cells, but at all of the higher concentrations of Medihoney™ tested here non-viable cells outnumbered viable cells and biofilm appeared to have been extensively disrupted (Fig. 6C – 6F).

DISCUSSION

In this laboratory study Medihoney™ was found to prevent the formation of *P. aeruginosa* biofilm, as well as inhibiting and disrupting established biofilm. As expected, a lower concentration of

Medihoney™ was required to prevent the formation of *P. aeruginosa* biofilms than to inhibit established biofilms (Table 1); interestingly the susceptibility of established biofilm to Medihoney™ did not vary with the age of the biofilm between 24 and 72 h.

Of the methods utilised to evaluate the extent of biofilm, estimates of biomass, biofilm metabolic activity and optical density gave comparable endpoints in all assays (Fig. 1 and 3). Although determining the density of the bacterial growth in each well yielded information rapidly, the entire well contents contributed to turbidity, rather than only the biofilm that was adherent on the walls of each well. Whereas this was appropriate in experiments to estimate the concentration of honey needed to prevent a biofilm forming (Fig. 1a), it was unsuitable in investigating inhibitory effects of Medihoney™ on existing biofilm (Fig. 2a) because both bacteria dispersed from the biofilm and adherent bacteria contributed to density.

Although crystal violet has long been used to determine biofilm biomass[18] and to quantify biomass [19], it was considered to be the least reliable method used here because it gave the greatest variations (Fig. 1b and Fig. 3b). The assay included several washes with PBS and occasionally biofilm was inadvertently removed during these steps. Rarely was biomass reduced by more than 50% in test wells compared to the wells with untreated biofilms, allowing only MIC_{50} to be deduced. One important limitation of crystal violet staining is that the resulting estimations of biofilm biomass did not discriminate between viable and non-viable biofilm. Hence estimating biofilm metabolic activity of biofilm is important.

MICs determined by enumerating total cell counts (Fig. 2 and Fig. 4) yielded lower MICs than other methods, perhaps indicating lower sensitivity of the method. However, estimating esterase activity by the reduction of fluorescein diacetate to fluorescein gave distinct endpoints which allowed MIC_{90} to be determined. The assay was relatively easy to perform in the laboratory and it gave consistent

results and showed that more than 90% of metabolic activity of the biofilm was lost with higher concentrations of Medihoney™.

Investigation into the effect of Medihoney™ on biofilm structure showed that extensive changes were associated with increasing concentration. The images of established biofilms obtained by scanning electron microscopy demonstrated that cells within the biofilm were shortened by 30%(w/v) Medihoney™ and that little recognisable biofilm remained at 40 %(w/v) or 50%(w/v) Medihoney™. With SEM viability of biofilm was not measurable, but fluorescence microscopy provided convincing evidence of loss of viability/vitality with increasing Medihoney™ concentration; it also confirmed the disruption in biofilm integrity with increasing Medihoney™ concentrations. Hence it is reasonable to deduce that the biomass detected in biofilms exposed to concentrations greater than 30%(w/v) Medihoney for 24 h observed in Figure 3b was likely to be comprised largely of non-viable biofilm. Similar observations have been made in testing the effect of glucose oxidase and lactoperoxidase activity on biofilms established on inert surfaces.[20, 21]

Attachment is an important step in the initiation of infection, and also in the initiation of biofilm formation. It has been proposed that fructose alone, which is the most abundant sugar in honey, impedes binding of planktonic cells of *P. aeruginosa* via receptor sites to the surface of erythrocytes.[22] Our observations here, as with studies into the inhibition of planktonic cultures of *P. aeruginosa*[4], indicated that artificial honey did not inhibit *P. aeruginosa* biofilm as effectively as equivalent concentrations of Medihoney™ (Fig. 1b and 1c; Fig. 3b and 3c). The apparent conflict between our data and previous observations[22] might be that our experimental system depended on attachment of *P. aeruginosa* to an inert surface, rather than to erythrocytes. Tests with further animal models and cell lines are needed to qualify this anomaly. Yet our data does confirm that additional components in honey contribute to antibiofilm activity. A recent review of the range of

antibacterial components currently identified in honey illustrates the complexity of this phenomenon and explains some of the variations due to floral origin.[23]

By monitoring changes in biofilm biomass, metabolic activity, viability, structural integrity and vitality, this study gave more detailed information on the effects of manuka honey on *P. aeruginosa* biofilm than previous studies.[13,14] It emphasises the need to monitor inhibitory effects with more than one methodology.

Laboratory studies can only ever provide insight into what might happen *in vivo*. Here we have shown that 17%(w/v) Medihoney™ prevented formation of *P. aeruginosa* biofilms on inert surfaces and that 35.5% (w/v) Medihoney™ inhibited established biofilms *in vitro*. Since biofilms are tolerant to many antimicrobial agents, this suggests that manuka honey may play a role in the clinical management of chronic wounds containing *P. aeruginosa*. In clinical use Medihoney™ may be applied to cutaneous wounds topically either from a tube or incorporated in a dressing and the concentration is usually at least 95%. Before definitive predictions can be made it is important to determine whether honey does inhibits *P. aeruginosa* biofilms effectively in wounds and this will only be known when wounds proven to contain such a biofilm are treated with Medihoney™ and monitored for effects on biofilm persistence. The hygroscopic nature of honey will attract water molecules, such that honey will be diluted by wound exudate. Since low concentrations of sugars will support biofilm growth, it is clear that it will be necessary to maintain sufficiently high concentrations of honey to prevent and inhibit biofilms *in vivo* by appropriate timing of dressing changes. The efficacy of Medihoney™ in inhibiting biofilms in highly exudating wounds must also be explored *in vivo*.

One limitation of this experimental study is that the effects of Medihoney™ were studied only for biofilms of *P. aeruginosa,* whereas chronic wounds often support polymicrobial biofilms [24,25,26]. The ability of manuka honey to inhibit biofilms of single cultures of Gram positive cocci *in vitro* has been demonstrated,[13,27,28] but its effect on mixed cultures of microbial species within polymicrobial

biofilms is not yet known. The development of models of polymicrobial biofilms [29,30,31] provides the technology for more detailed research to be done on the effects of Medihoney™ on polymicrobial biofilms in the future.

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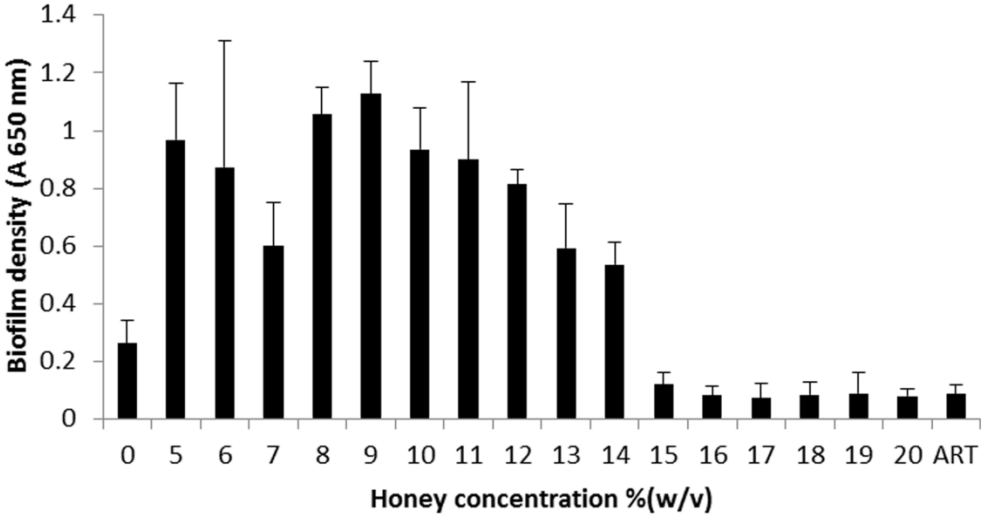
Table 1: Inhibition of *Pseudomonas aeruginosa* biofilms by Medihoney™

	*MIC_{50} %(w/v) biofilm biomass	*MIC_{90} %(w/v) biofilm activity	*MIC_{50} optical density 650 nm
Prevention of biofilm formation	16.8 ± 1.3 (5)	15.2 ± 0.4 (5)	15.7 ± 1.2(3)
24 hour established biofilm	33 ± 5.7 (5)	36 ± 4.2(5)	33.8 ±2.5 (4)
48 hour established biofilm	31.3 ± 2.5 (4)	35 ± 4.1 (4)	40 (1)
72 hour established biofilm	35 ± 3.5 (5)	36.6 ± 2.9(3)	36.7 ± 2.9 (3)

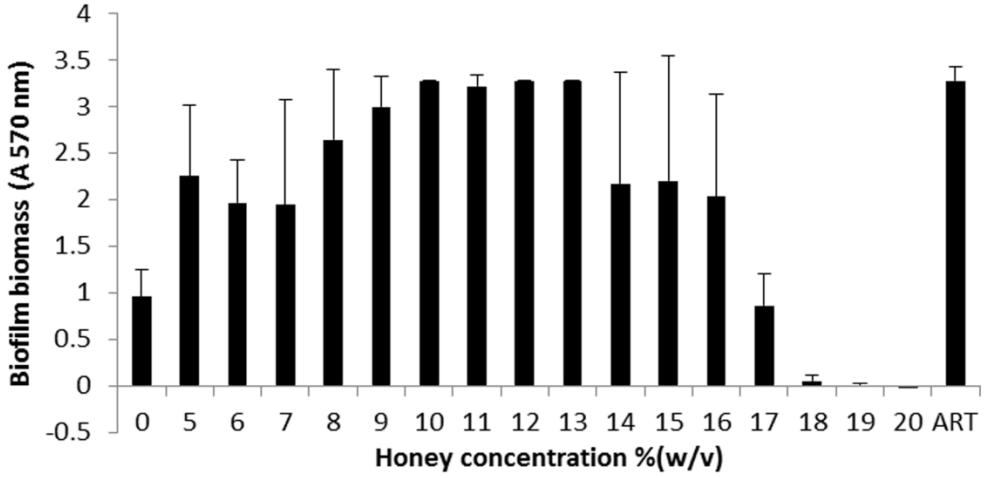
*mean MIC ± standard deviation (number of assays)

Figure 1: The effect of Medihoney ™ on biofilm formation. Varying concentrations of Medihoney™ were incubated with *P. aeruginosa* to determine the lowest concentrations to prevent biofilm formation. The extent of biofilm was assayed by optical density (a), biofilm biomass (b) and biofilm activity (c).

1a


Biofilm density (A 650 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 ART
Honey concentration %(w/v)

1b


Biofilm biomass (A 570 nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 ART
Honey concentration %(w/v)

1c

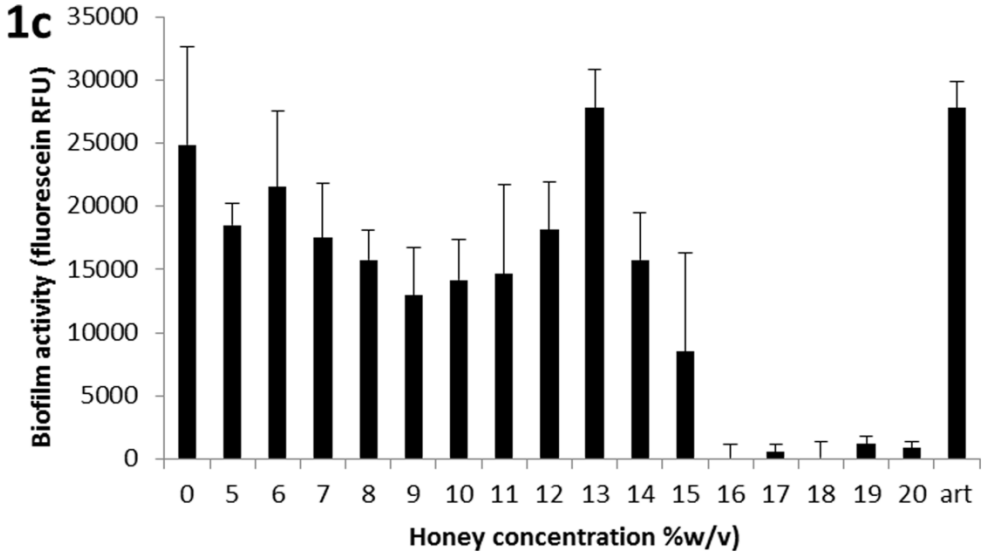

Biofilm activity (fluorescein RFU)
35000
30000
25000
20000
15000
10000
5000
0
0 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 art
Honey concentration %w/v)

Figure 2: The effect of Medihoney™ on biofilm formation determined by total cell viability.

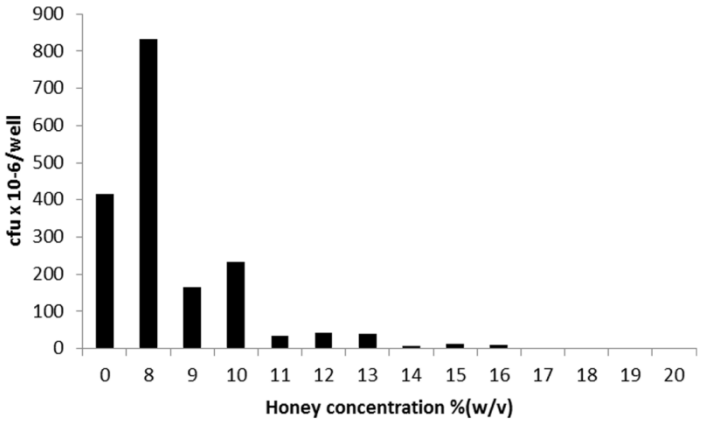
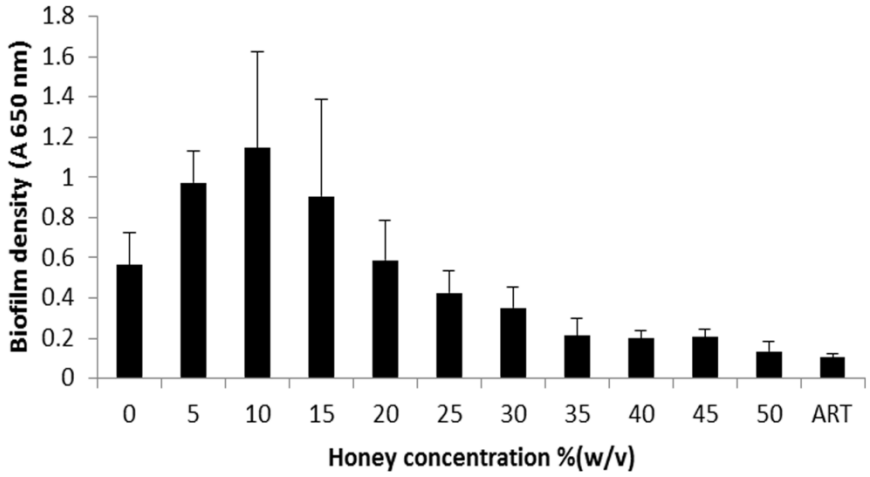
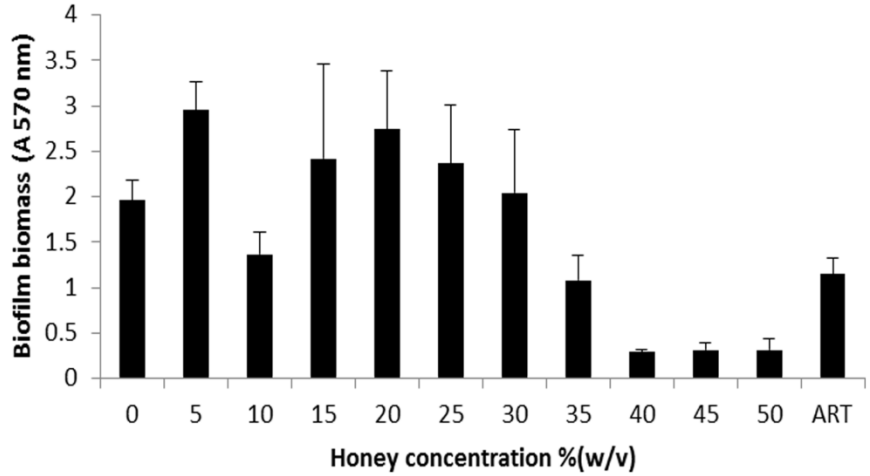


Figure 3: Inhibition of established biofilms by Medihoney™. Biofilms of *P. aeruginosa* were established in microtitre plate wells and treated with varying concentrations of Medihoney™ for 24 h. The extent of biofilm was assayed by optical density (a), biofilm biomass (b) and biofilm activity (c).

3a


Biofilm density (A 650 nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25 30 35 40 45 50 ART
Honey concentration %(w/v)

3b


Biofilm biomass (A 570 nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
0 5 10 15 20 25 30 35 40 45 50 ART
Honey concentration %(w/v)

3c

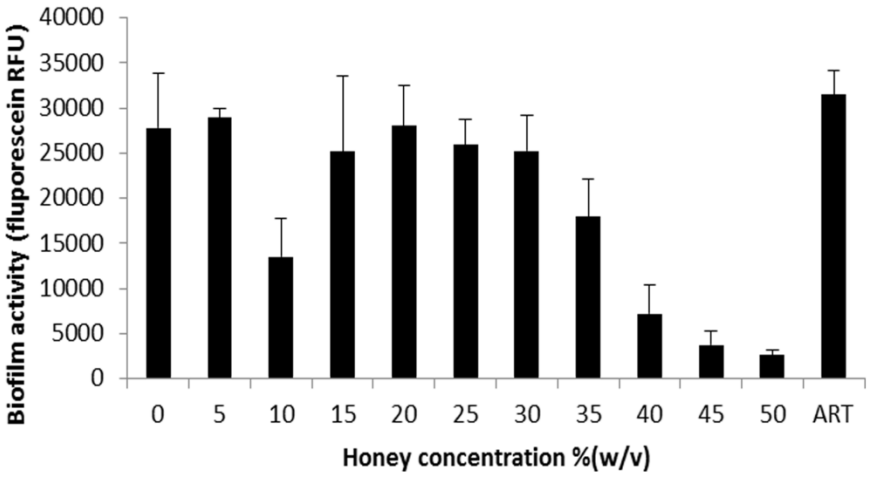

Biofilm activity (fluporescein RFU)
40000
35000
30000
25000
20000
15000
10000
5000
0
0 5 10 15 20 25 30 35 40 45 50 ART
Honey concentration %(w/v)

Figure 4: The effect of Medihoney™ on viability of 24 h established biofilm of *P. aeruginosa.* 24 h established biofilm of *P. aeruginosa* was treated for 24 h with varying concentrations of Medihoney™ and the presence of viable bacteria enumerated by total cell counts.

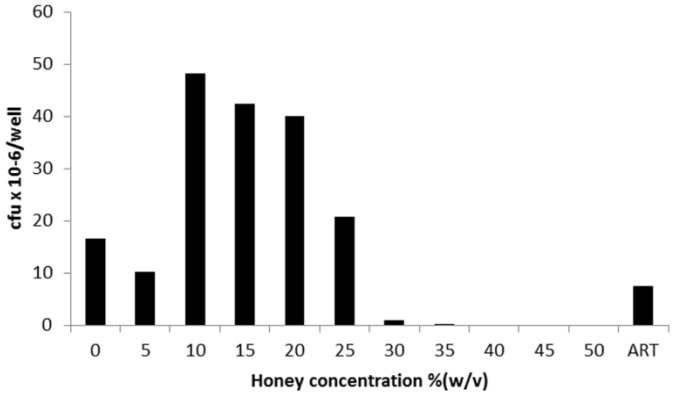


Figure 5: The effect of Medihoney™ on biofilm structure determined by scanning electron microscopy. Biofilms of *P. aeruginosa* were established on plastic coverslips for 24 h, treated for 24 h without Medihoney™ (A), with 20%(w/v) Medihoney™ (B), with 30%(w/v) Medihoney™ (C), with 40%(w/v) Medihoney™ (D) and with 50%(w/v) Medihoney™ (E) and then processed for SEM.

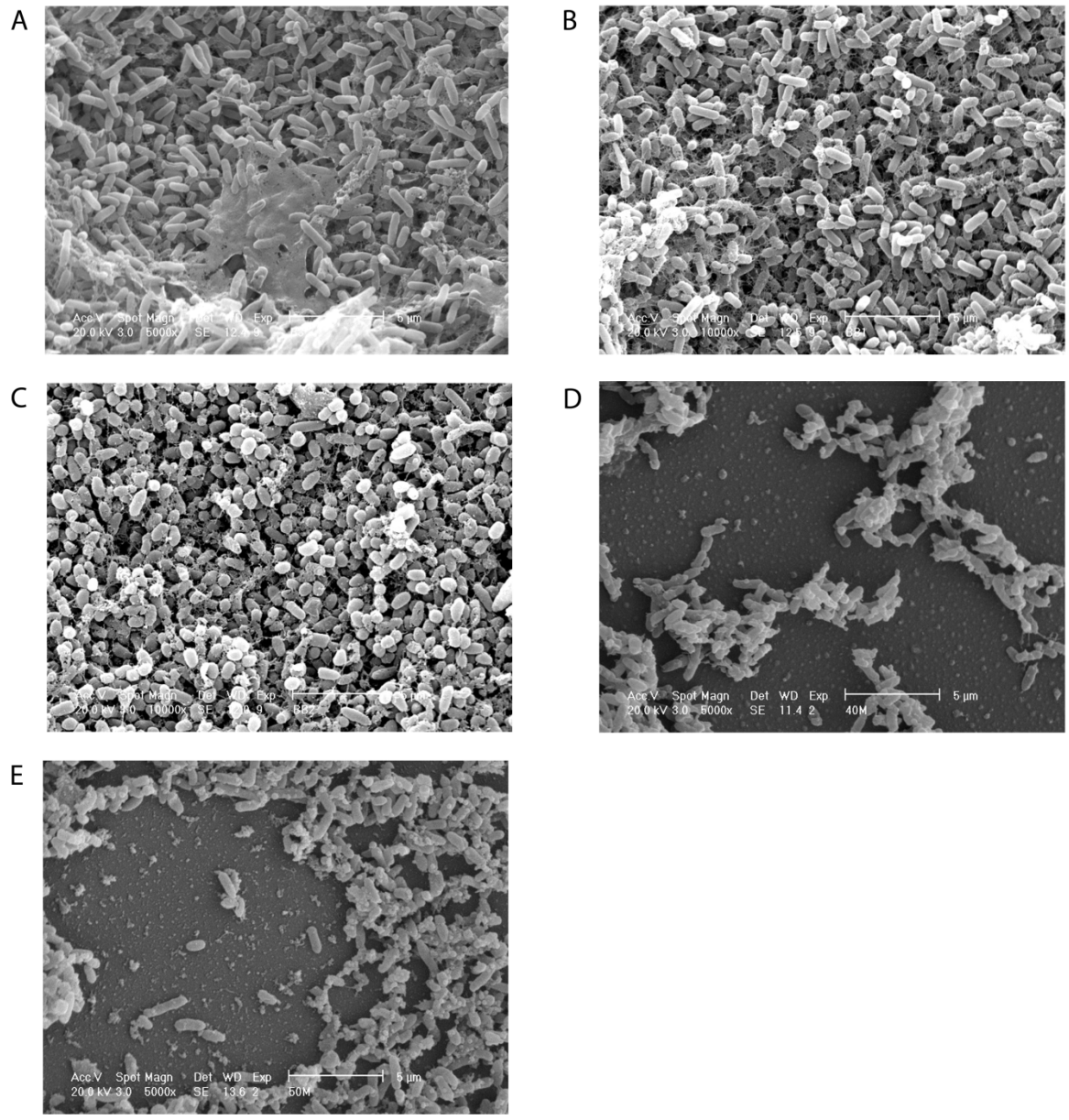
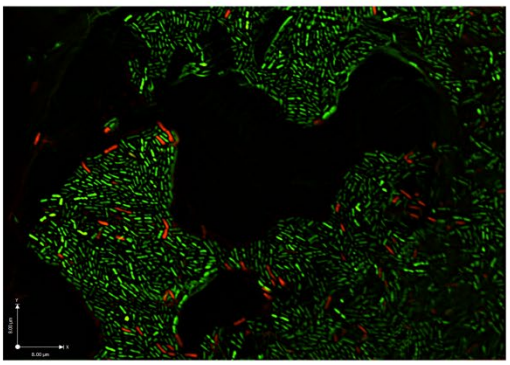


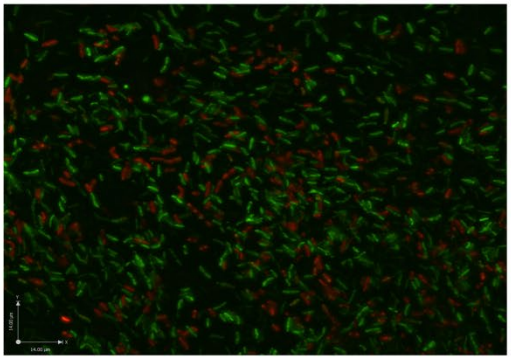
Figure 6: The effect of Medihoney™ on biofilm vitality as determined by epifluorescence.

Biofilms of *P. aeruginosa* were established on plastic coverslips for 24 h and treated for 24 h without Medihoney™ (A), with 10%(w/v) Medihoney™ (B), with 20%(w/v) Medihoney™ (C), with 30%(w/v) Medihoney™ (D), with 40%(w/v) Medihoney™ (E) and with 50%(w/v) Medihoney™ (F). After 24 h at 37 °C all coverslips were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kits ; viable cells stained green and non-viable cells stained red.

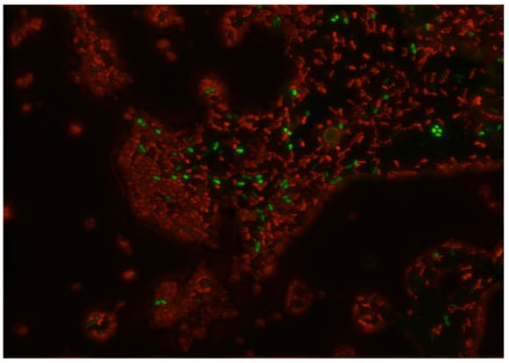
A



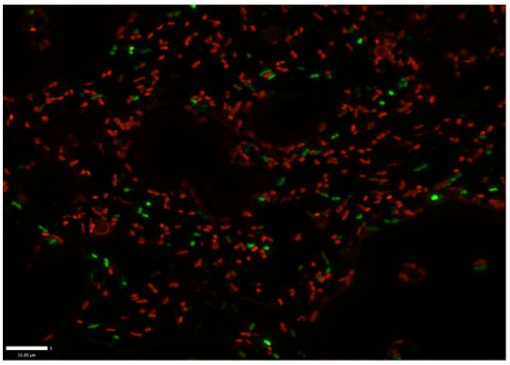
B



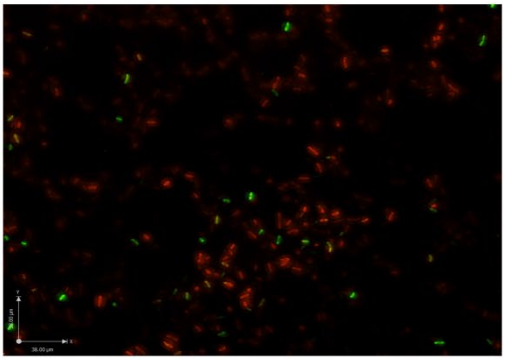
C



D



E



F