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

**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 *Pseudomonas 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 650nm), biomass (by staining with crystal violet), metabolic activity (using an esterase assay) 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) of Medihoney was required to inhibit established biofilms. Susceptibility did not vary with length of biofilm establishment between 24 and 72 hours. Extensive structural changes in established biofilms were seen in the sample with ≤30%(w/v) Medihoney using scanning electron microscopy and loss of viability was found in test samples with ≤20%(w/v) Medihoney concentration using fluorescent staining, together with loss of biofilm structure.

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

**Declaration of interest:** This study was sponsored by Derma Sciences Inc, NJ. An unrestricted grant was provided and the sponsors were not involved in the design of the experiments or the preparation of this manuscript.

**manuka honey; biofilms; wounds; biofilm viability; LIVE/DEAD BacLight**

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 a majority of chronic wounds¹ ² has provided new insight for wound care professionals. Anti-biofilm strategies may help in managing chronic wounds, but diminished susceptibility of established biofilms to conventional antimicrobial agents² indicates that current antibiotic and anti-septic 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 predominantly on antibiotics, and more sophisticated dressings started to become available. In the past decade, honey has been reintroduced 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 *Staphylococcus 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 downregulation 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 grafts¹¹ 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.

Method
A culture of Pseudomonas aeruginosa that had been isolated from a wound care clinic outpatient with a chronic leg ulcer of more than two years duration 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 Pseudomonas 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 5–20%, with 1% intervals. Approximately 50µl of diluted honey was dispensed into wells of 96 well microtitre plates (Nunc, Roskilde, Denmark) and inoculated with 1µl of a 1 in 5-diluted overnight culture of the test organism (population density of 3x10^8 colony forming units (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 50µl 20%(w/v) artificial honey solution prepared in TSB. Each concentration of honey was tested in quadruplicate for each plate, and each experiment was conducted at least three times. Microtitre plates were incubated statically at 37°C for 24 hours 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 by assessing optical density at 650nm using the Spectrostar nanoplate 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 100µl phosphate buffered saline (PBS; Oxoid, Cambridge, UK) to remove planktonic cells. Biofilm was then fixed by adding 100µl of 99% methanol to each well for 15 minutes. The fixative was removed and the plates were allowed to air dry before the addition of 50µl of 0.25%(w/v) crystal violet for 15 minutes to stain the adherent biofilm. The dye was carefully removed, each well was washed three times with 100µl PBS, dried by blotting onto paper towels and 100µl of 7% acetic acid was added to solubilise the dye contained in adherent cells. Absorbance was measured at 570nm on a Spectrostar nanoplate reader. The Minimum Inhibitory Concentration (MIC<sub>50</sub>) 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 Pseudomonas aeruginosa by the conversion of non-fluorescent fluorescein diacetate (FDA) to yellow fluorescent fluorescein.15 The Minimum Inhibitory Concentration (MIC<sub>50</sub>) 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 100µl sterile maximum recovery diluent (MRD; Oxoid, Cambridge, UK) to remove planktonic cells. A further 50µl of 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 hours and cfu per well was 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 50µl was inoculated into wells in 96 well plates, or 200µl into wells in 24 well plates. All plates were incubated statically at 37°C for 24 hours. For 48 hour biofilms, the culture medium in the microtitre plate wells of 24 hour established biofilms was gently removed, discarded, replaced by 50µl of fresh TSB and incubation was continued for a further 24 hours. For 72 hour established biofilms, spent medium was similarly replaced with fresh TSB after 24 and 48 hours and the plate incubated for another 24 hours 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
All plates were incubated statically at 37°C for 24 hours 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 50µl 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, a 24 hour biofilm was established on plastic coverslips contained in 24 well microtitre plates. The liquid phase was then replaced by 200µl Medihoney concentrations ranging from 0–50 % (w/v) and incubated at 37°C for 24 hours. Coverslips were processed for scanning electron microscopy as described previously 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 488nm excitation and 520nm emission filter was used. For propidium iodide detection (red channel) a 543nm excitation and 572nm emission filter was used. Images were analysed using Volocity (Perkin Elmer, Cambridge, UK).

Table 1. Inhibition of Pseudomonas aeruginosa biofilms by Medihoney

<table>
<thead>
<tr>
<th>Treatment</th>
<th>*MIC50 (w/v)</th>
<th>*MIC90 (w/v)</th>
<th>*MIC50 optical density 650 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention of biofilm formation</td>
<td>16.8 ± 1.3</td>
<td>15.2 ± 0.4</td>
<td>15.7 ± 1.2</td>
</tr>
<tr>
<td>24 hour established biofilm</td>
<td>33 ± 5.7</td>
<td>36 ± 4.2</td>
<td>33.8 ± 2.5</td>
</tr>
<tr>
<td>48 hour established biofilm</td>
<td>31.3 ± 2.5</td>
<td>35 ± 4.1</td>
<td>40 (1)</td>
</tr>
<tr>
<td>72 hour established biofilm</td>
<td>35 ± 3.5</td>
<td>36.6 ± 2.9</td>
<td>36.7 ± 2.9</td>
</tr>
</tbody>
</table>

*mean MIC ± standard deviation (number of assays)
Results

The effect of Medihoney in preventing biofilm formation

The lowest concentration of Medihoney that prevented *Pseudomonas aeruginosa* forming a biofilm *in vitro* was found to be 17%(w/v) (Table 1). Inhibitory effects are normally expressed as the 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 *Pseudomonas 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 *Pseudomonas aeruginosa* biofilm did not vary with the length of biofilm establishment. MICs ranged from 31–40%(w/v) Medihoney and the mean 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). The 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 hours compared to untreated biofilm.

Structural changes in biofilms exposed to Medihoney

Using scanning electron microscopy, 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\%(w/v) 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 hours, treated with a range of Medihoney concentrations for 24 hours, 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 Pseudomonas aeruginosa biofilm, as well as inhibiting and disrupting established biofilm. As expected, a lower concentration of Medihoney was required to prevent the formation of Pseudomonas 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 hours.

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 2 and 3). Although determining the density of the bacterial growth in each well yielded information rapidly, the entire contents of the well 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 and to quantify biomass, 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\(_{90}\) 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 the 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 esthetic 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, the viability of biofilm was not measurable, but fluorescence microscopy provided convincing evidence of loss of viability/vitality with increasing Medihoney concentration and further confirmed the disruption in biofilm integrity with increasing Medihoney concentrations. Hence it is reasonable to deduce that the biomass detected in biofilms exposed to concen-

![Fig 4. The effect of Medihoney on viability of 24 h established biofilm of Pseudomonas aeruginosa. 24 h established biofilms were treated for 24 h with varying concentrations of Medihoney and the presence of viable bacteria enumerated by total cell counts.](image-url)

References
Fig 5. The effect of Medihoney on biofilm structure determined by scanning electron microscopy. Biofilms of *Pseudomonas aeruginosa* were established on plastic coverslips for 24 hours, treated for 24 hours 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.
shown that 17% (w/v) Medihoney prevented formation of *Pseudomonas 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 *Pseudomonas 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 inhibit *Pseudomonas 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.

**Limitations and conclusion**

One limitation of this study is that the effects of Medihoney were studied only for biofilms of *Pseudomonas aeruginosa*, whereas chronic wounds often support polymicrobial biofilms. The ability of manuka honey to inhibit biofilms of single cultures of Gram positive cocci in vitro has been demonstrated, but its effect on mixed cultures of microbial species within polymicrobial biofilms is not yet known. The development of models of polymicrobial biofilms provides the technology for more detailed research to be done on the effects of Medihoney on polymicrobial biofilms in the future.

27 Maddocks, S.E., Lopez, M.S., Rowlands, R.S., Cooper, R.A. Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins. Microbiology 2012; 158: 3, 781–790.

28 Cooper, R., Jenkins, L., Rowlands, R. Inhibition of biofilms through the use of manuka honey. Wounds UK 2011; 7: 1, 24–32.

