Paper:
http://dx.doi.org/10.1007/s10096-010-1065-1
The effect of manuka honey on *Pseudomonas aeruginosa*

Running Title: **Manuka honey and bacteria**

Ana Henriques, Rowena Jenkins, Neil Burton, Rose Cooper*,
University of Wales Institute, Cardiff.

Corresponding author:
\[ \text{RA Cooper,} \]
\[ \text{Centre for Biomedical Sciences,} \]
\[ \text{Cardiff School of Health Sciences,} \]
\[ \text{University of Wales Institute Cardiff,} \]
\[ \text{Western Avenue,} \]
\[ \text{Cardiff} \]
\[ \text{CF5 2YB,} \]
\[ \text{UK.} \]
\[ \text{Tel: +44 (0) 2920 416845} \]
\[ \text{Fax: +44 (0) 2920 416982} \]
\[ \text{Email: rcooper@uwic.ac.uk} \]
**Abstract** Licensed wound care products containing manuka honey are available in Europe and Australasia for the topical treatment of wounds. Bactericidal activity of manuka honey on *Pseudomonas aeruginosa* has been reported, but structural effects have not. The aim of this study was to investigate the inhibitory effects of manuka honey on the structural integrity of *Psuedomonas aeruginoas* ATTC 27853. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of manuka honey for *Ps. aeruginosa* were determined by a microtitre plate method, and the survival of bacteria exposed to a bactericidal concentration of manuka honey was monitored. The effects of a bactericidal concentration of manuka honey on the structure of the bacteria were investigated using scanning and transmission electron microscopy. MIC and MBC values of manuka honey against *Ps. aeruginosa* were 9.5 % (w/v) and 12% (w/v) respectively, and incubation at 37°C in 20 % w/v manuka honey contained in nutrient broth demonstrated a bactericidal rather than a bacteriostatic effect. Using scanning electron microscopy, loss of structural integrity and marked changes in cell shape and surface were observed in honey-treated cultures. With transmission electron microscopy these changes were confirmed, and evidence of extensive cell disruption and lysis was found.

**Introduction.** Honey is an ancient remedy that has recently been re-introduced for the treatment of acute and chronic wounds. In modern times it has been used in both remote and conventional medical practice, with evidence in medical journals documenting the clinical use of honey applied topically to the wounds of more than 1000 patients (Molan, 2006). Within the past two years wound care products containing honey from three distributors have attained drug tariff status in the UK, with Australasian honey derived from the nectar of the genus *Leptospermum* (manuka and jellybush) predominantly utilised. Tubes of gamma irradiated honey, honey impregnated tulle, honey and alginate dressings
and honey based ointments are available as licensed medical devices. Further wound care products are under development in several European and Australasian countries and clinical trials are in progress in the UK, Ireland, Denmark, Australia, New Zealand and South Africa. The availability of regulated products is expected to promote increased clinical use in conventional medicine.

The benefits of honey for wounds are based on its antimicrobial properties and its ability to stimulate rapid wound healing (Blair & Carter, 2005; Molan, 2002). Antimicrobial activity extends to more than 80 species (Blair & Carter, 2005; Molan, 1992). Inhibition of pathogens capable of causing wound infection has been demonstrated (Cooper, Halas & Molan, 2002; Cooper, Molan & Harding, 2002; French, Cooper & Molan, 2005; Wahdan, 1998; Willix, Molan & Harfoot, 1992), with both antibiotic-resistant and antibiotic-sensitive strains exhibiting susceptibility to honey (Cooper, Halas & Molan, 2002; Cooper, Molan & Harding, 2002; Karayil, Deshpande, & Koppikar, 1998). There are many clinical reports of the clearance of infection by topical application of honey, including eradication of MRSA from colonised and infected wounds.

Honey is a complex and variable natural product that contains more than 600 components (Bogdanov, Ruoff, & Oddo, 2004), so the search for specific inhibitors is complicated. The antimicrobial activity of honey is derived from multiple factors, with contributions from high sugar content, low water content, low acidity, hydrogen peroxide and phytochemicals (Molan 1992). Using artificial honey preparations, bactericidal effects of diluted natural honey solutions that are independent of sugar content have been demonstrated (Cooper, Halas & Molan, 2002; Cooper, Molan & Harding, 2002). New Zealand manuka honey is derived from nectar collected by honeybees (Apis mellifera) foraging on Western tea tree (Leptospermum
scoparium). Unlike many honeys, the activity of this honey at low dilutions is not dependent on the production of hydrogen peroxide by glucose oxidase (Molan, 1992). However the identity of the components responsible for the inhibition of micro-organisms has not yet been discovered. Hence, cellular target sites and mechanisms of action have not yet been established, and structural effects of honey on bacteria have not been published. This study, therefore, aims to investigate the effects of manuka honey on a bacterium commonly associated with wound infection, in order to gain insight into its effectiveness as an inhibitory agent.

Materials and methods. A culture of Ps. aeruginosa ATCC 27853 was tested with a sample of manuka honey (M109) that was a gift from Prof. Molan of the University of Waikato, New Zealand. A sample of sterile manuka honey (SH) obtained from New Zealand Natural Food Company was used to determine MBC in the presence of protein because germination of endospores contained in M109 caused turbidity that masked the end-point. Antibacterial potency of the samples was related to phenol using a bioassay developed in New Zealand (Allen, Molan & Reid, 1991). M109 and SH had non-peroxide activity equivalent to 18 % (w/v) and 17 % (w/v) phenol, respectively.

MIC was determined in 96-well, flat bottomed microtitre plates using double strength nutrient broth (Oxoid) and honey concentrations varying by 1 % (w/v) intervals in a total volume of 200 μl. Overnight broth cultures of the test organism was used as an inoculum without dilution, and total viable cell counts were performed to check retrospectively that each well had received approximately 10⁶ cells. Plates were incubated at 37 °C for 24 hours and turbidity measured at 400 nm in a plate reader (Anthos Labtec Instruments). Positive (broth and inoculum) and negative (broth and honey) controls were included. Wells with the lowest 4
concentration without growth were recorded as MIC. MBC was determined by plating 20 μl from wells without growth onto nutrient agar (Oxoid) and incubating at 37 °C for 24 hours to find the lowest concentration without viable bacteria. Experiments were performed in duplicate on each of three occasions.

A time-kill study was performed by inoculating 40 μl of an overnight culture of *Ps. aeruginosa* ATCC 27853 into 20 ml nutrient broth with and without 20 % (w/v) M109 and incubating at 37 °C in a shaking water bath (120 cycles min⁻¹) hours (the honey concentration was approximately twice the MIC value). Samples were removed at known intervals and Miles Misra surface drop counts performed.

Electron microscopy was performed using test bacteria in either the exponential or stationary phase of growth by cultivation in isosensitest broth (Oxoid) at 37 °C in a shaking water bath for either 3 hours or overnight. Cells were harvested by centrifugation at 3000 g for 30 minutes (MSE harrier 15/80 centrifuge, Sanyo) at room temperature and suspended in MOPS buffer (pH 7.2) with and without 20 % (w/v) manuka honey for 8 hours. Cells were examined in scanning (SEM) (5200LV Jeol, Herts, UK) and transmission electron microscopy (TEM) (1210 Jeol, Herts, UK) by the method of Lemar, Turner & Lloyd (2002), except that for TEM, pellets were embedded in Araldite resin, not Spurr. Electron micrographs of untreated and treated cells were compared to identify structural changes such as altered shape, modified surface layers, the presence of electron dense material, and cellular debris.

**Results.** Inhibition studies: MIC and MBC were found to be 9.5 and 12 % (w/v) manuka, which agreed well with previous reports (Cooper & Molan 1999; Cooper Halas & Molan.2002). The close proximity of these two values indicated a bactericidal mode of inhibition, which was confirmed by time-kill studies (Fig. 1). The time estimated to achieve a
3 log reduction was 257 minutes, and a 5 log reduction was be expected to be achieved within 24 hours.

Structural studies: Cells in both the exponential and stationary phases of growth were used in electron microscopy experiments, because stationary phase cells are often less susceptible to antimicrobial agents than exponential cells. However the structural changes observed in exponential and stationary phase cells were similar and therefore only electron micrographs of exponential cells are presented here.

Observations with SEM: Compared to the smooth surface layers of untreated cells, honey treated *Ps. aeruginosa* cells exhibited marked cell surface changes as furrows and blebs (Fig 1a). Cell length was shortened and extruded cellular material was present. In untreated samples 2% cells were found to have cell surface irregularities, whereas 80 and 60 % cells of exponential and stationary cultures, respectively were irregular (Table 1).

Observations with TEM: Damaged cell surfaces and debris were clearly evident in TEMs of honey treated *P. aeruginosa* (Fig. 3d), and cell interiors appeared to be more densely stained.

**Discussion.** MICs obtained in this study were similar but not identical to values previously reported (Cooper, Halas & Molan, 2002; Cooper, Molan & Harding, 2002; Wahdan, 1998; Willix, Molan & Harfoot, 1992), but this is not unexpected as honey is a natural product and never consistent in quality. For medicinal purposes it is usually collected from identified hives and only samples of proven antibacterial potency are utilised (Allen, Molan & Reid, 1991). MBCs (Table 1), time-kill plots (Fig.1) and the determination of the time of commitment to death confirmed a bactericidal rather than bacteriostatic mode of inhibition for both bacteria. Previously published work shows that *S. aureus* was more susceptible to manuka honey than *P. aeruginosa*, *S. aureus* at approximately three times MIC values lost viability at a
slower rate than *P. aeruginosa* exposed to twice the MIC. These observations suggest that manuka affects each species differently.

The structural studies conclusively demonstrate that manuka honey elicited distinct cytological changes *P. aeruginosa*. The changes to cell surface and shape seen in SEMs of pseudomonads were extensive. The presence of shortened rods indicated a stress response (Oliver, 2005).

In TEMs of treated cells, cell debris provided evidence of leakage of cellular material and lysis of whole cells, and was obvious with *Pseudomonas*. Honey seems to have a greater affect on the surface layers of *P. aeruginosa* than it does for *S. aureus*.

**Acknowledgements.** We wish to thank Drs. Hann and Turner of the Electron Microscope unit at Cardiff University.

**References.**


**Table 1: Structural changes observed in SEMs of *Ps. aeuginosa***

<table>
<thead>
<tr>
<th></th>
<th>% of exponential phase cells with structural changes</th>
<th>% of stationary phase cells with structural changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No honey</td>
<td>2 (n=1568)</td>
<td>2 (n=24514)</td>
</tr>
<tr>
<td>Honey</td>
<td>80 (n=1100)</td>
<td>60 (n=283)</td>
</tr>
<tr>
<td>Mann-Whitney test</td>
<td>p = 0.02</td>
<td>p = 0.001</td>
</tr>
</tbody>
</table>

n = number of cells observed
**Figure 1: time-kill experiments**

- *Ps. aeruginosa* in nutrient broth (□), *Ps. aeruginosa* in nutrient broth containing 20% (w/v) M109 Manuka honey (□),

---

---
Figure 2: The effect of manuka honey on the structure of exponential phase bacteria as seen by SEM

a Exponential phase cells of *Ps. aeruginosa* incubated with buffer for 8 hours at 37°C as viewed by SEM at 10,000 magnification

b Exponential phase cells of *Ps. aeruginosa* incubated with buffer containing 20% (w/v) manuka honey for 8 hours at 37°C as viewed by SEM at 10,000 magnification
Figure 3: The effect of manuka honey on the structure of exponential phase bacteria as seen by TEM

a Exponential phase cells of *Ps. aeruginosa* incubated with buffer for 8 hours at 37°C as viewed by TEM at 10,000 magnification

b Exponential phase cells of *Ps. aeruginosa* incubated with buffer containing 20 % (w/v) manuka honey for 8 hours at 37°C as viewed by TEM at 10,000 magnification