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The effect of manuka honey on *Pseudomonas aeruginosa*

Running Title: **Manuka honey and bacteria**

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25 **Abstract** Licensed wound care products containing manuka honey are available in Europe
26 and Australasia for the topical treatment of wounds. Bactericidal activity of manuka honey
27 on *Pseudomonas aeruginosa* has been reported, but structural effects have not. The aim of
28 this study was to investigate the inhibitory effects of manuka honey on the structural integrity
29 of *Psuedomonas aeruginoas* ATTC 27853. The minimum inhibitory concentration (MIC) and
30 the minimum bactericidal concentration (MBC) of manuka honey for *Ps. aeruginosa* were
31 determined by a microtitre plate method, and the survival of bacteria exposed to a
32 bactericidal concentration of manuka honey was monitored. The effects of a bactericidal
33 concentration of manuka honey on the structure of the bacteria were investigated using
34 scanning and transmission electron microscopy. MIC and MBC values of manuka honey
35 against *Ps. aeruginosa* were 9.5 % (w/v) and 12% (w/v) respectively, and incubation at 37^0C
36 in 20 % w/v manuka honey contained in nutrient broth demonstrated a bactericidal rather
37 than a bacteriostatic effect. Using scanning electron microscopy, loss of structural integrity
38 and marked changes in cell shape and surface were observed in honey-treated cultures.
39 With transmission electron microscopy these changes were confirmed, and evidence of
40 extensive cell disruption and lysis was found.

41

42 **Introduction.** Honey is an ancient remedy that has recently been re-introduced for the
43 treatment of acute and chronic wounds. In modern times it has been used in both remote
44 and conventional medical practice, with evidence in medical journals documenting the
45 clinical use of honey applied topically to the wounds of more than 1000 patients (Molan,
46 2006). Within the past two years wound care products containing honey from three
47 distributors have attained drug tariff status in the UK, with Australasian honey derived from
48 the nectar of the genus *Leptospermum* (manuka and jellybush) predominantly utilised.
49 Tubes of gamma irradiated honey, honey impregnated tulle, honey and alginate dressings

50 and honey based ointments are available as licensed medical devices. Further wound care
51 products are under development in several European and Australasian countries and clinical
52 trials are in progress in the UK, Ireland, Denmark, Australia, New Zealand and South Africa.
53 The availability of regulated products is expected to promote increased clinical use in
54 conventional medicine.
55
56 The benefits of honey for wounds are based on its antimicrobial properties and its ability to
57 stimulate rapid wound healing (Blair & Carter, 2005; Molan, 2002). Antimicrobial activity
58 extends to more than 80 species (Blair & Carter, 2005; Molan, 1992). Inhibition of pathogens
59 capable of causing wound infection has been demonstrated (Cooper, Halas & Molan, 2002;
60 Cooper, Molan & Harding, 2002; French, Cooper & Molan, 2005; Wahdan, 1998; Willix,
61 Molan & Harfoot, 1992), with both antibiotic-resistant and antibiotic-sensitive strains
62 exhibiting susceptibility to honey (Cooper, Halas & Molan, 2002; Cooper, Molan & Harding,
63 2002; Karayil, Deshpande, & Koppikar, 1998). There are many clinical reports of the
64 clearance of infection by topical application of honey, including eradication of MRSA from
65 colonised and infected wounds.
66
67 Honey is a complex and variable natural product that contains more than 600 components
68 (Bogdanov, Ruoff, & Oddo, 2004), so the search for specific inhibitors is complicated. The
69 antimicrobial activity of honey is derived from multiple factors, with contributions from high
70 sugar content, low water content, low acidity, hydrogen peroxide and phytochemicals (Molan
71 1992). Using artificial honey preparations, bactericidal effects of diluted natural honey
72 solutions that are independent of sugar content have been demonstrated (Cooper, Halas &
73 Molan, 2002; Cooper, Molan & Harding, 2002). New Zealand manuka honey is derived from
74 nectar collected by honeybees (*Apis mellifera*) foraging on Western tea tree (*Leptospermum*

75 *scoparium*). Unlike many honeys, the activity of this honey at low dilutions is not dependent
76 on the production of hydrogen peroxide by glucose oxidase (Molan, 1992). However the
77 identity of the components responsible for the inhibition of micro-organisms has not yet been
78 discovered. Hence, cellular target sites and mechanisms of action have not yet been
79 established, and structural effects of honey on bacteria have not been published. This study,
80 therefore, aims to investigate the effects of manuka honey on a bacterium commonly
81 associated with wound infection, in order to gain insight into its effectiveness as an inhibitory
82 agent.
83
84
85 **Materials and methods.** A culture of *Ps. aeruginosa* ATCC 27853 was tested with a sample
86 of manuka honey (M109) that was a gift from Prof. Molan of the University of Waikato, New
87 Zealand. A sample of sterile manuka honey (SH) obtained from New Zealand Natural Food
88 Company was used to determine MBC in the presence of protein because germination of
89 endospores contained in M109 caused turbidity that masked the end-point. Antibacterial
90 potency of the samples was related to phenol using a bioassay developed in New Zealand
91 (Allen, Molan & Reid, 1991). M109 and SH had non-peroxide activity equivalent to 18 %
92 (w/v) and 17 % (w/v) phenol, respectively.
93 MIC was determined in 96-well, flat bottomed microtitre plates using double strength nutrient
94 broth (Oxoid) and honey concentrations varying by 1 % (w/v) intervals in a total volume of
95 200 µl. Overnight broth cultures of the test organism was used as an inoculum without
96 dilution, and total viable cell counts were performed to check retrospectively that each well
97 had received approximately 10^6 cells. Plates were incubated at 37 ^{0}C for 24 hours and
98 turbidity measured at 400 nm in a plate reader (Anthos Labtec Instruments). Positive (broth
99 and inoculum) and negative (broth and honey) controls were included. Wells with the lowest

100 concentration without growth were recorded as MIC. MBC was determined by plating 20 µl
101 from wells without growth onto nutrient agar (Oxoid) and incubating at 37 ^{0}C for 24 hours to
102 find the lowest concentration without viable bacteria. Experiments were performed in
103 duplicate on each of three occasions.
104 A time-kill study was performed by inoculating 40 µl of an overnight culture of *Ps. aeruginosa*
105 ATCC 27853 into 20 ml nutrient broth with and without 20 % (w/v) M109 and incubating at
106 37 ^{0}C in a shaking water bath (120 cycles min^{-1}) hours (the honey concentration was
107 approximately twice the MIC value). Samples were removed at known intervals and Miles
108 Misra surface drop counts performed.
109 Electron microscopy was performed using test bacteria in either the exponential or stationary
110 phase of growth by cultivation in isosensitest broth (Oxoid) at 37 ^{0}C in a shaking water bath
111 for either 3 hours or overnight. Cells were harvested by centrifugation at 3000 g for 30
112 minutes (MSE harrier 15/80 centrifuge, Sanyo) at room temperature and suspended in
113 MOPS buffer (pH 7.2) with and without 20 % (w/v) manuka honey for 8 hours. Cells were
114 examined in scanning (SEM) (5200LV Jeol, Herts, UK) and transmission electron
115 microscopy (TEM) (1210 Jeol, Herts, UK) by the method of Lemar, Turner & Lloyd (2002),
116 except that for TEM, pellets were embedded in Araldite resin, not Spurr. Electron
117 micrographs of untreated and treated cells were compared to identify structural changes
118 such as altered shape, modified surface layers, the presence of electron dense material,
119 and cellular debris.
120
121 **Results.** Inhibition studies: MIC and MBC were found to be 9.5 and 12 % (w/v) manuka,
122 which agreed well with previous reports (Cooper & Molan 1999; Cooper Halas &
123 Molan.2002). The close proximity of these two values indicated a bactericidal mode of
124 inhibition, which was confirmed by time-kill studies (Fig. 1). The time estimated to achieve a

125 3 log reduction was 257 minutes, and a 5 log reduction was be expected to be achieved
126 within 24 hours.
127 Structural studies: Cells in both the exponential and stationary phases of growth were used
128 in electron microscopy experiments, because stationary phase cells are often less
129 susceptible to antimicrobial agents than exponential cells. However the structural changes
130 observed in exponential and stationary phase cells were similar and therefore only electron
131 micrographs of exponential cells are presented here.
132 Observations with SEM: Compared to the smooth surface layers of untreated cells, honey
133 treated *Ps. aeruginosa* cells exhibited marked cell surface changes as furrows and blebs
134 (Fig 1a). Cell length was shortened and extruded cellular material was present. In untreated
135 samples 2% cells were found to have cell surface irregularities, whereas 80 and 60 % cells
136 of exponential and stationary cultures, respectively were irregular (Table 1).
137 Observations with TEM: Damaged cell surfaces and debris were clearly evident in TEMs of
138 honey treated *P. aeruginosa* (Fig. 3d), and cell interiors appeared to be more densely
139 stained.
140 **Discussion.** MICs obtained in this study were similar but not identical to values previously
141 reported (Cooper, Halas & Molan, 2002; Cooper, Molan & Harding, 2002; Wahdan, 1998;
142 Willix, Molan & Harfoot, 1992), but this is not unexpected as honey is a natural product and
143 never consistent in quality. For medicinal purposes it is usually collected from identified hives
144 and only samples of proven antibacterial potency are utilised (Allen, Molan & Reid, 1991).
145 MBCs (Table 1), time-kill plots (Fig.1) and the determination of the time of commitment to
146 death confirmed a bactericidal rather than bacteriostatic mode of inhibition for both bacteria.
147 Previously published work shows that *S. aureus* was more susceptible to manuka honey
148 than *P. aeruginosa*, *S. aureus* at approximately three times MIC values lost viability at a

149 slower rate than *P. aeruginosa* exposed to twice the MIC. These observations suggest that
150 manuka affects each species differently.
151 The structural studies conclusively demonstrate that manuka honey elicited distinct
152 cytological changes *P. aeruginosa*. The changes to cell surface and shape seen in SEMs of
153 pseudomonads were extensive. The presence of shortened rods indicated a stress
154 response (Oliver, 2005).
155 In TEMs of treated cells, cell debris provided evidence of leakage of cellular material and
156 lysis of whole cells, and was obvious with *Pseudomonas*. Honey seems to have a greater
157 affect on the surface layers of *P. aeruginosa* than it does for *S. aureus*.
158
159 **Acknowledgements.** We wish to thank Drs. Hann and Turner of the Electron Microscope
160 unit at Cardiff University.
161
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195 other honey. *J Appl Bacteriol* **73**:388-394.

196

197

198

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

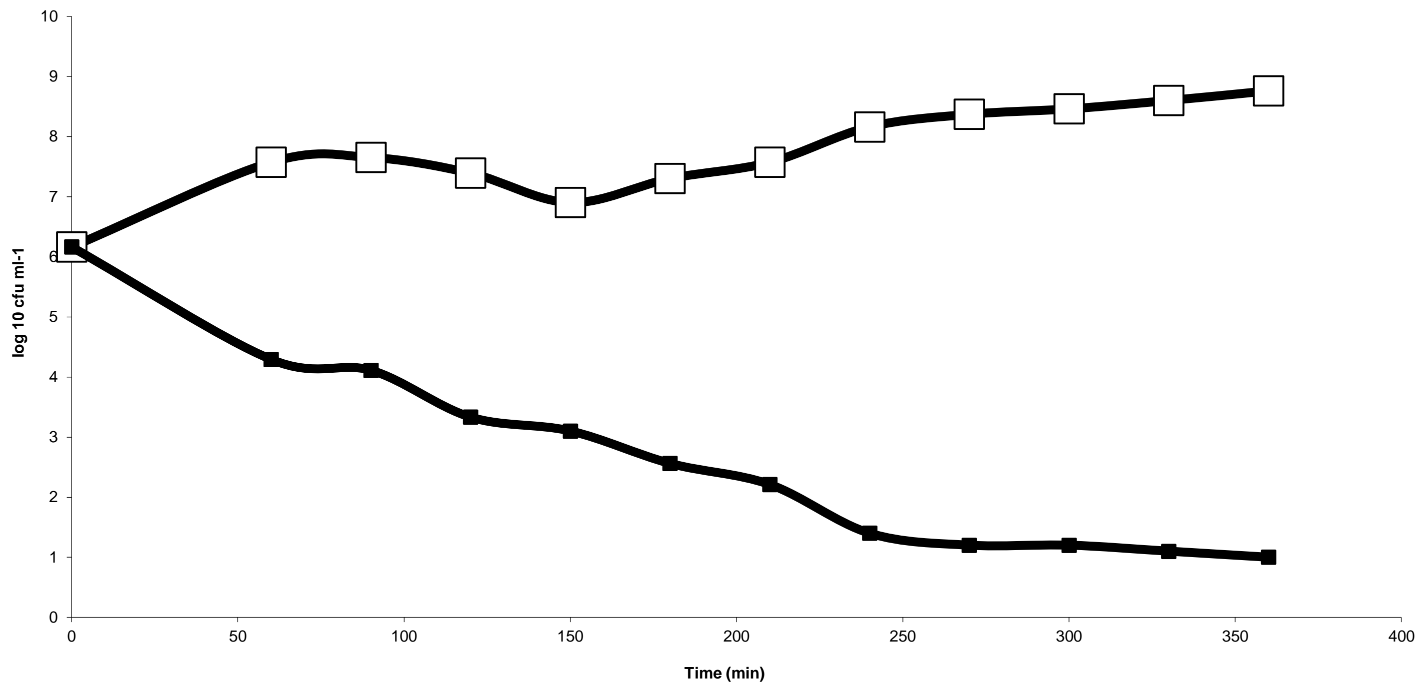
	% of exponential phase cells with structural changes	% of stationary phase cells with structural changes
No honey	2 (n=1568)	2 (n=24514)
Honey	80 (n=1100)	60 (n=283)
Mann-Whitney test	p = 0.02	p = 0001

200

201 n = number of cells observed

202

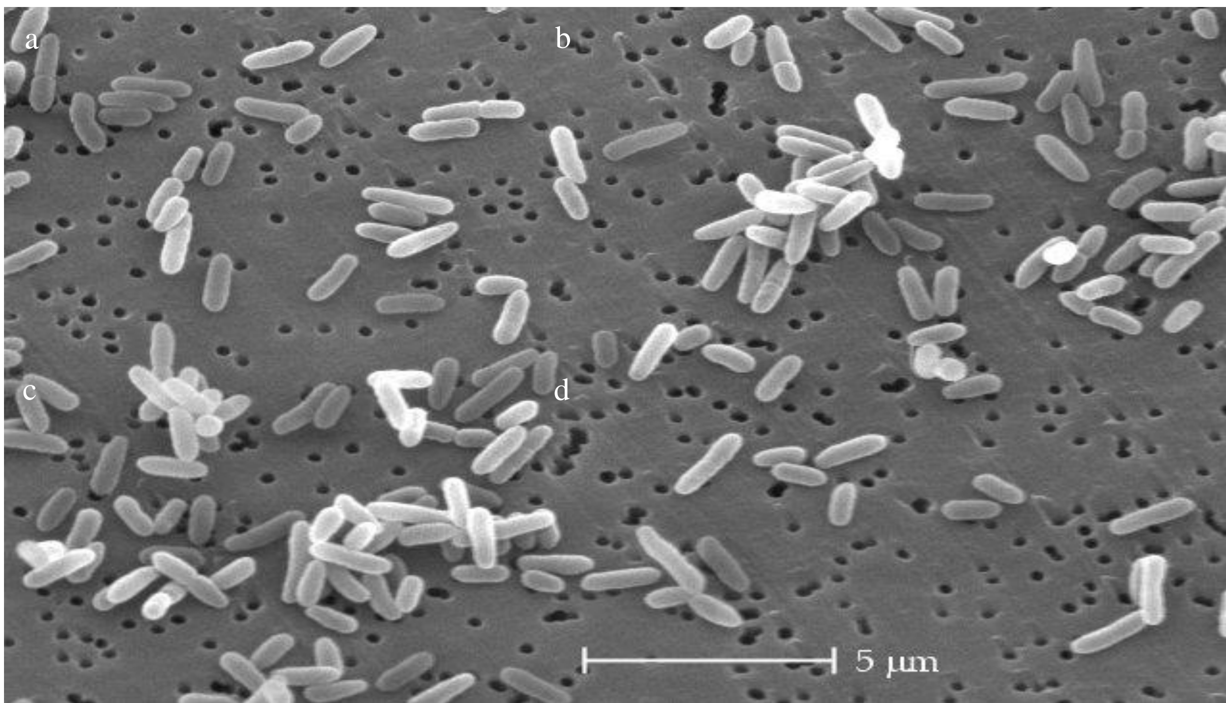
203 **Figure 1: time-kill experiments**



204

205 *Ps. aeruginosa* in nutrient broth (□), *Ps.aeruginosa* in nutrient broth containing 20 % (w/v)

206 M109 Manuka honey (□),



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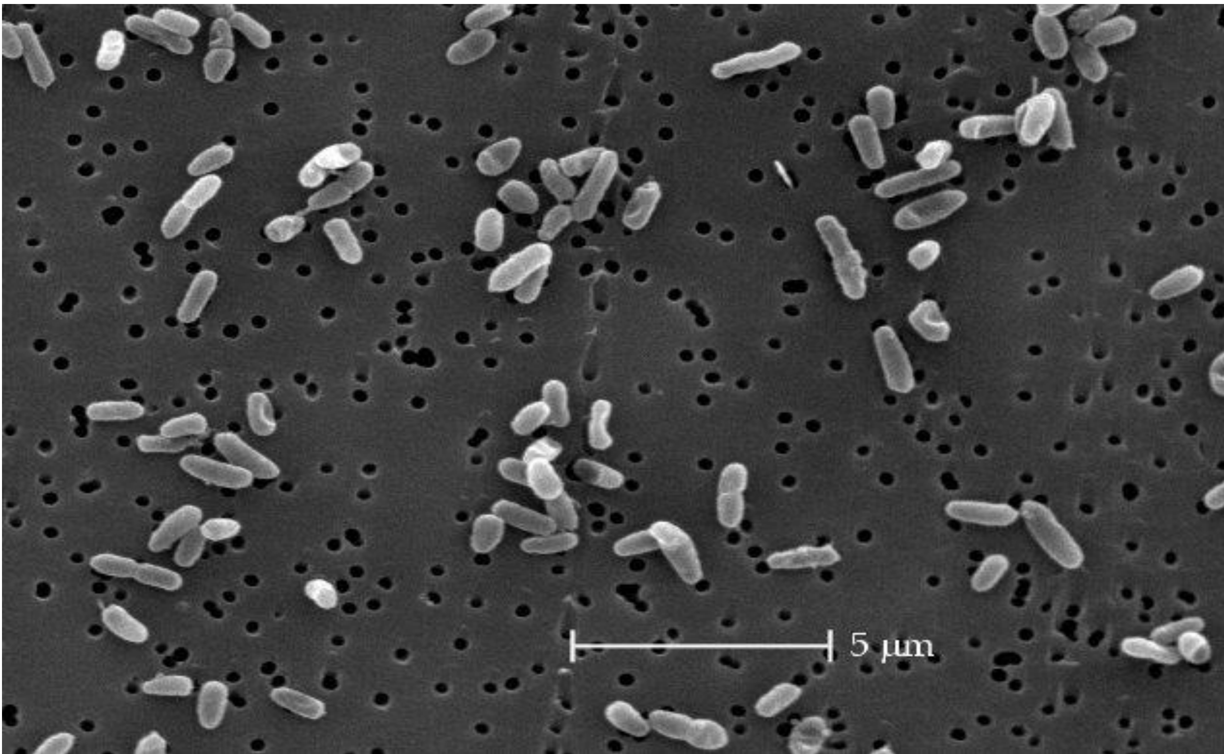

210

211 **Figure 2: The effect of manuka honey on the structure of exponential phase**

212 **bacteria as seen by SEM**

213

214 a Exponential phase cells of *Ps. .aeruginosa* incubated with buffer for 8 hours at 37°C

215 as viewed by SEM at 10,000 magnification

216 b Exponential phase cells of *Ps. aeruginosa* incubated with buffer containing 20 % (w/v)

217 manuka honey for 8 hours at 37°C as viewed by SEM at 10,000 magnification

218

219

220

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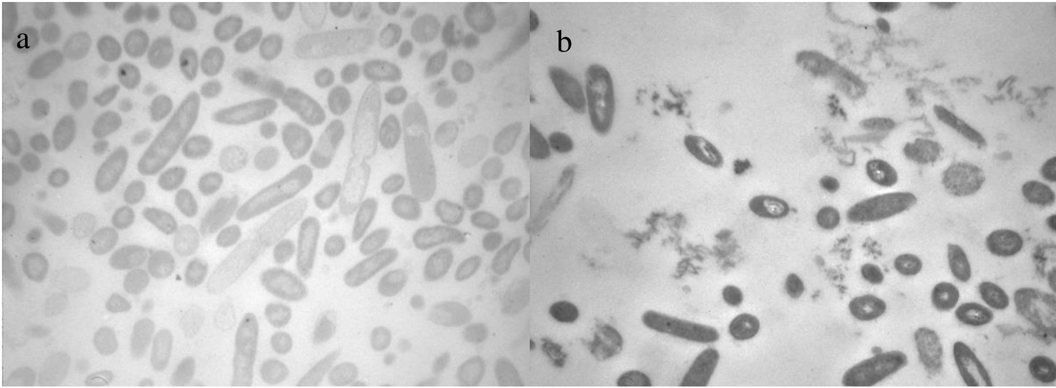
222



223 **Figure 3: The effect of manuka honey on the structure of exponential phase**
224 **bacteria as seen by TEM**

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

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

229

230

231