



Swansea University  
Prifysgol Abertawe



## Cronfa - Swansea University Open Access Repository

---

This is an author produced version of a paper published in:  
*European Journal of Clinical Microbiology & Infectious Diseases*

Cronfa URL for this paper:  
<http://cronfa.swan.ac.uk/Record/cronfa37986>

---

### Paper:

Henriques, A., Jenkins, R., Burton, N. & Cooper, R. (2011). The effect of manuka honey on the structure of *Pseudomonas aeruginosa*. *European Journal of Clinical Microbiology & Infectious Diseases*, 30(2), 167-171.  
<http://dx.doi.org/10.1007/s10096-010-1065-1>

---

This item is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence. Copies of full text items may be used or reproduced in any format or medium, without prior permission for personal research or study, educational or non-commercial purposes only. The copyright for any work remains with the original author unless otherwise specified. The full-text must not be sold in any format or medium without the formal permission of the copyright holder.

Permission for multiple reproductions should be obtained from the original author.

Authors are personally responsible for adhering to copyright and publisher restrictions when uploading content to the repository.

<http://www.swansea.ac.uk/library/researchsupport/ris-support/>

1 **The effect of manuka honey on *Pseudomonas aeruginosa***

2

3 Running Title: **Manuka honey and bacteria**

4

5

6 Ana Henriques, Rowena Jenkins, Neil Burton, Rose Cooper\*,

7 University of Wales Institute, Cardiff.

8

9

10

11

12

13 Corresponding author:

14 RA Cooper,

15 Centre for Biomedical Sciences,

16 Cardiff School of Health Sciences,

17 University of Wales Institute Cardiff,

18 Western Avenue,

19 Cardiff

20 CF5 2YB,

21 UK.

22 Tel: +44 (0) 2920 416845

23 Fax:+44 (0) 2920 416982

24 Email:rcooper@uwic.ac.uk

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 *Pseudomonas aeruginosa* 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°C  
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  $\mu$ l. 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 °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  $\mu$ l  
101 from wells without growth onto nutrient agar (Oxoid) and incubating at 37  $^{\circ}$ 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  $\mu$ l 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  $^{\circ}$ C in a shaking water bath (120 cycles  $\text{min}^{-1}$ ) 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  $^{\circ}$ 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

## 162 **References.**

163 **Allen, K., Molan P., Reid G. (1991)** A survey of the antibacterial activity of some New  
164 Zealand honeys. *Journal of Pharmacology* **43** (12): 817-882

165 **Blair, S. & Carter, D. A. (2005).** The potential for honey in the management of wounds  
166 and infection. *Australian Infection Control* **10**:24-31.

167 **Bogdanov, S., Ruoff, K. & Oddo, L. P. (2004).** Physico-chemical methods for the  
168 characterisation of unifloral honeys: a review. *Apidologie* **35**:S4-S17.

169 **Cooper, R. A., Halas, E. & Molan, P. C. (2002).** The efficacy of honey in inhibiting strains  
170 of *Pseudomonas aeruginosa* from infected burns. *J Burn Care Rehabil.* **23**:366-370.

171 **Cooper, R. A., Molan, P. C. & Harding, K. G. (2002).** The sensitivity to honey of Gram-  
172 positive cocci of clinical significance isolated from wounds. *J. Appl Microbiol* **93**:857-863.



- 173 **Errington J., Daniel R.A., & Scheffers D-J. (2003).** Cytokinesis in bacteria. *Microbiol Mol*  
174 *Biol Rev* **67**: 52-65
- 175 **French, V. M., Cooper, R. A. & Molan, P. C. (2005).** The antibacterial activity of honey  
176 against coagulase-negative staphylococci. *J Antimicrob Chemother* **56**:228-231.
- 177 **Karayil, S., Deshpande, S. D. & Koppikar, G. V. (1998).** Effect of honey on multidrug  
178 resistant organisms and its synergistic action with three common antibiotics. *J*  
179 *Postgrad Med* **44**:93-96.
- 180 **Lemar, K. M., Turner, M. P. & Lloyd, D. (2002).** Garlic (*Allium sativum*) as an anti-  
181 *Candida* agent: a comparison of the efficacy of fresh garlic and freeze-dried extracts.  
182 *J Appl Microbiol* **93**:398-405.
- 183 **Molan, P. C. (1992).** The antibacterial activity of honey .1. The nature of the antibacterial  
184 activity. *Bee World* **73**:5-28.
- 185 **Molan, P. C. (2002).** Re-introducing honey in the management of wounds and ulcers -  
186 theory and practice. *Ostomy Wound Manage* **48**:28-40.
- 187 **Molan, P.C. (2006)** The evidence supporting the use of honey as a wound dressing.  
188 *International Journal of Lower Extremity Wounds* **5** (1) : 40-54
- 189 **Oliver, J. D. (2005).** The viable but nonculturable state in bacteria. *J Microbiol* **43** Spec  
190 No:93-100.
- 191 **Wahdan, H. A. L. (1998).** Causes of the antimicrobial activity of honey. *Infection* **26**:30-  
192 35.

193 **Willix, D. J., Molan, P. C. & Harfoot, C. G. (1992).** A comparison of the sensitivity of  
194 wound-infecting species of bacteria to the antibacterial activity of manuka honey and  
195 other honey. *J Appl Bacteriol* **73**:388-394.

196

197

198

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

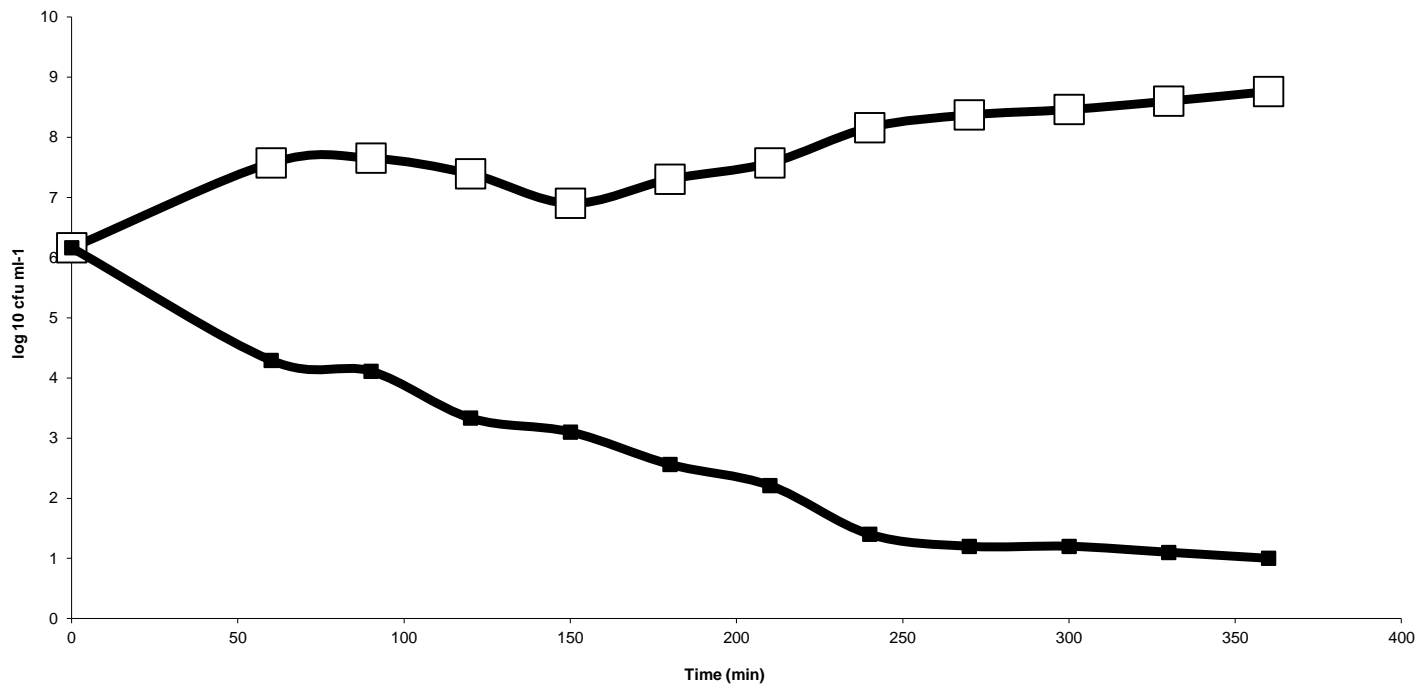
	% of exponential phase cells with structural changes	% of stationary phase cells with structural changes
No honey	2 (n=1568)	2 (n=24514)
Honey	80 (n=1100)	60 (n=283)
Mann-Whitney test	p = 0.02	p = 0001

200

201 n = number of cells observed

202

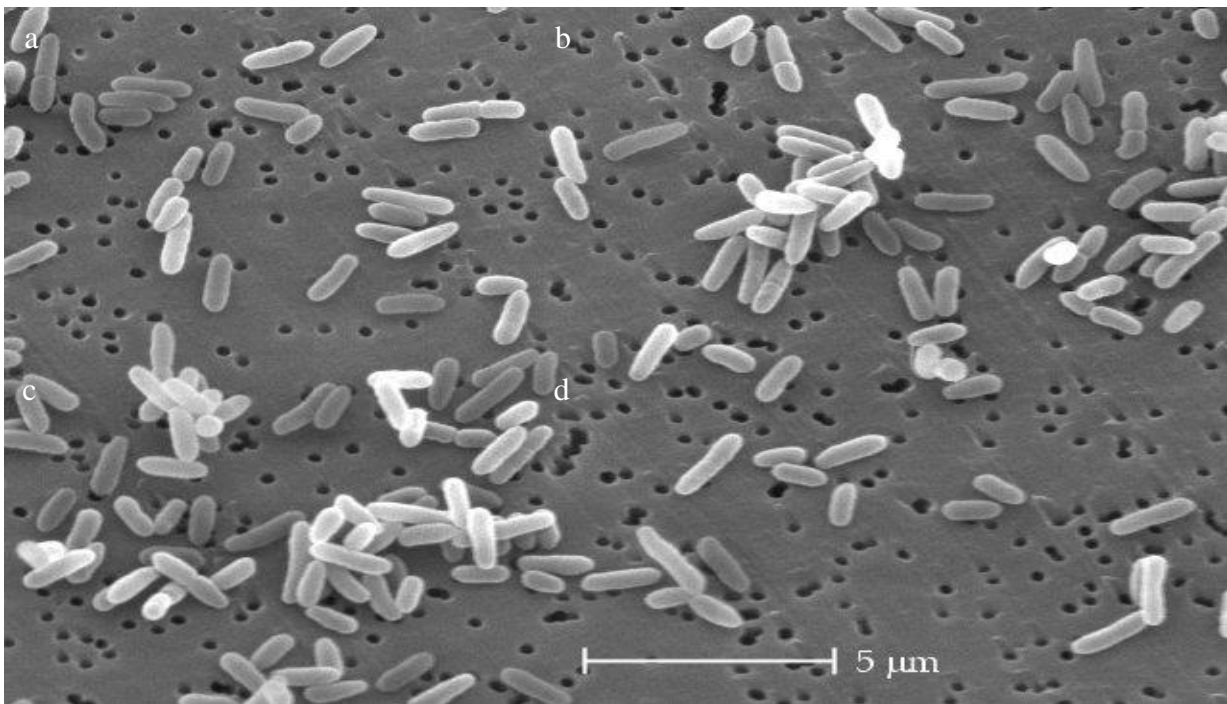
203 **Figure 1: time-kill experiments**



204

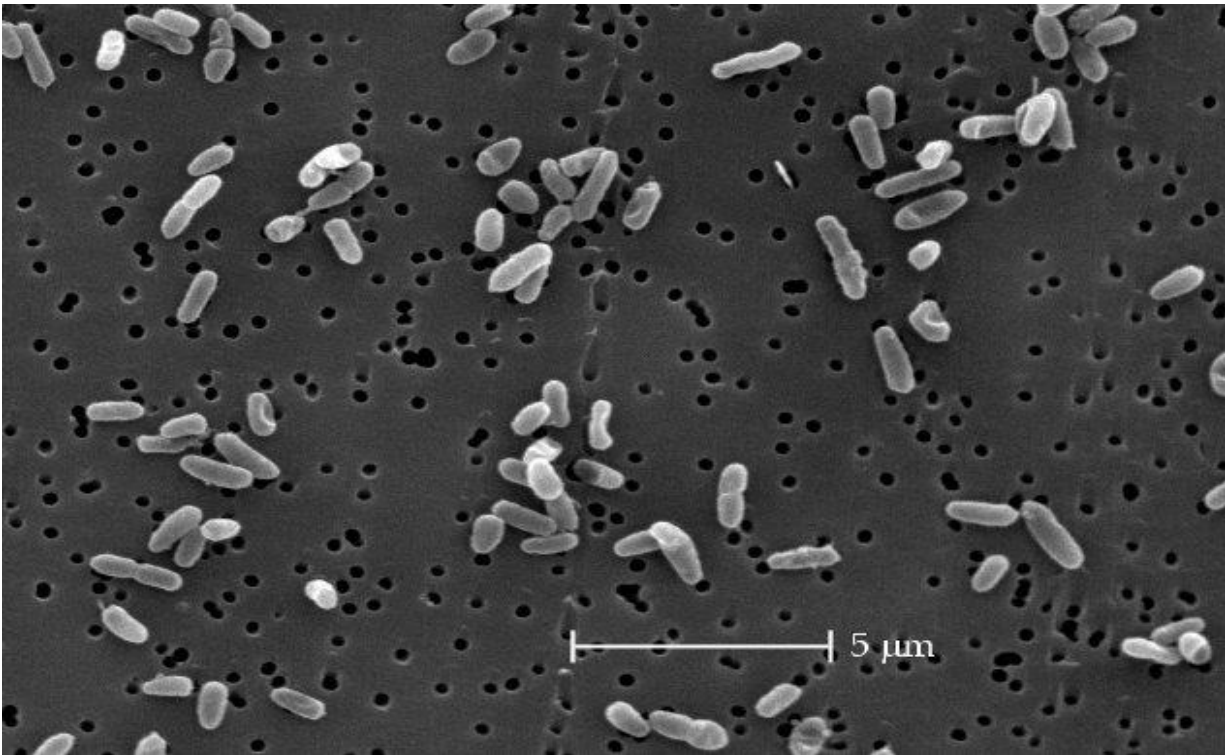
205 *Ps. aeruginosa* in nutrient broth (□), *Ps.aeruginosa* in nutrient broth containing 20 % (w/v)

206 M109 Manuka honey (■),



207

208



209

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

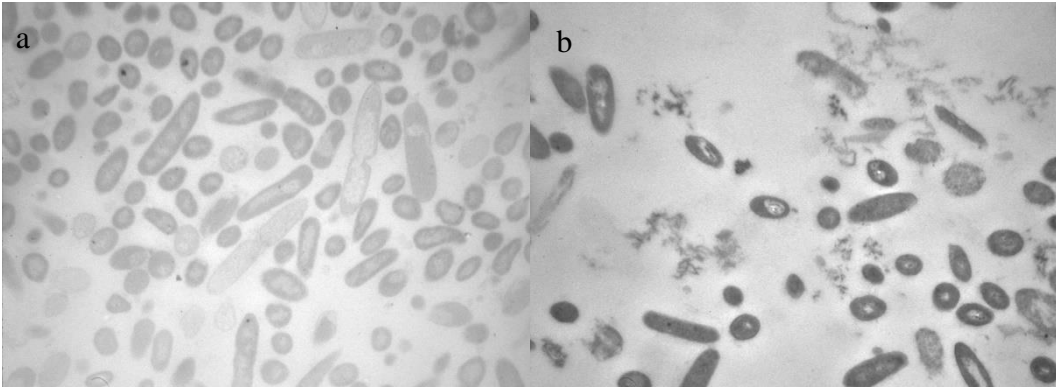
216 b Exponential phase cells of *Ps. aeruginosa* incubated with buffer containing 20 % (w/v)  
217 manuka honey for 8 hours at 37°C as viewed by SEM at 10,000 magnification

218

219

220

221



222

223

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

224

225

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

226

227

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

228

229

230

231