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Absence of bacterial resistance to medical-grade manuka honey

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Abstract: Clinical use of honey in the topical treatment of wounds has increased in Europe and North America since licensed wound care products became available in 2004 and 2007 respectively. Honey-resistant bacteria have not been isolated from wounds, but there is a need to investigate whether honey has the potential to select for honey resistance. Two cultures of bacteria from reference collections (*Staphylococcus aureus* NCTC 10017 and *Pseudomonas aeruginosa* ATCC 27853) and four cultures isolated from wounds (*Escherichia coli*, MRSA, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*) were exposed to sub-lethal concentrations of manuka honey in continuous and stepwise training experiments to determine whether susceptibility to honey diminished. Reduced susceptibilities to manuka honey in the test organisms during long-term stepwise resistance training were found, but these changes were not permanent and honey-resistant mutants were not detected. The risk of bacteria acquiring resistance to honey will be low if high concentrations are maintained clinically.

Response to Reviewers: Dear Editor,

Thank you for the reviewers' comments on our paper entitled "Absence of bacterial resistance to medical-grade manuka honey". We were pleased that they liked the design and execution of our study and we understood the reservations that reviewer had about our conclusions. We have made the following changes in response to each specific observation, which we feel strengthen our paper:

Reviewer 1.

We have inserted line numbers into the paper.

In order to rebut the reservations about our conclusions we have recently performed further tests on the four clinical isolates that were collected at the end of the recovery period and stored at -80°C. For each of the thawed cultures, MICs and MBCs were determined in duplicate on three separate occasions. We found that three cultures (*P.aeruginosa*, *S. epidermidis* and MRSA) had returned to pre-training levels of susceptibility and that the MIC of *E. coli* was only 1.4 times higher than at time 0. That information is included as Table 2 (line 370) and described in lines 201 to 209. We respectfully maintain that honey-resistant mutants were not recovered. To support this conclusion as advised we noted (lines 241-243) that antibiotic-resistance training led to MICs that increased by factors of either 32 or 64 (citation 17-Blair et al, 2009), while honey resistance training led to an increase of 1.4. We

also used the EUCAST definition of clinical resistance to show that honey susceptibility which had increased by a factor of 1.4 is unlikely to lead to therapeutic failure when wound care products normally contain at least 80% manuka honey and normally 95%(w/v) (lines 258 to 260).

We have removed the statement "gradually increased towards pre-treatment levels".

We have made clear the proportional changes in MICs (lines 195 to 197 and 208 and 251). We have commented on the need to maintain high concentrations of manuka honey in wounds to avoid the selection of resistant strains (lines 278 to 284).

We have modified the Abstract (lines 42 to 45) to explain that reduced susceptibility was found during long-term training and we have commented on the need to maintain high concentrations of manuka honey during clinical use (lines 45 to 46). In the concluding paragraph we also note that prolonged exposure to antimicrobial agents should be avoided.

We have deleted statements about the development of antibiotic resistance from the Introduction and the Discussion.

We have inserted "preceding days' culture" into line 169, and "day" into line 194.

We have removed the reference to high sugar content, low water content and acidity.

We have changed the captions to Figure 1 and 2.

Reviewer 2.

We were encouraged that our conclusions were considered to be robust.

We have changed MIC against manuka honey to "MICs to manuka honey" throughout the paper.

We have clarified which honey was used in which experiments by changing "preliminary" to "short-term"(line 91) and explained the activity associated with each honey (lines 100 to 103).

We have inserted "preceding days' culture" in line 133.

In order to clarify the number of MIC tests performed and the experimental design we have inserted a brief explanation in lines 210 to 217.

We hope that you will now be able to accept our manuscript for publication,

Yours sincerely,
Rose Cooper

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1 Absence of bacterial resistance to medical-grade manuka honey

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6 31 **Abstract**

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8 32 Clinical use of honey in the topical treatment of wounds has increased in
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10 33 Europe and North America since licensed wound care products became
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12 34 available in 2004 and 2007 respectively. Honey-resistant bacteria have not
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14 35 been isolated from wounds, but there is a need to investigate whether honey
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16 36 has the potential to select for honey resistance. Two cultures of bacteria from
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18 37 reference collections (*Staphylococcus aureus* NCTC 10017 and
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20 38 *Pseudomonas aeruginosa* ATCC 27853) and four cultures isolated from
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22 39 wounds (*Escherichia coli*, MRSA, *Pseudomonas aeruginosa* and
23
24 40 *Staphylococcus epidermidis*) were exposed to sub-lethal concentrations of
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26 41 manuka honey in continuous and stepwise training experiments to determine
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28 42 whether susceptibility to honey diminished. Reduced susceptibilities to
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30 43 manuka honey in the test organisms during long-term stepwise resistance
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32 44 training were found, but these changes were not permanent and honey-
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34 45 resistant mutants were not detected. The risk of bacteria acquiring resistance
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36 46 to honey will be low if high concentrations are maintained clinically.

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38 47 **Introduction**

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40 48 For thousands of years wounds were treated topically with relatively poorly
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42 49 characterised preparations of antimicrobial agents derived from animal,
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44 50 vegetable and mineral sources [1]. Apart from honey, many of those
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46 51 substances were too toxic for internal use [2]. During the nineteenth century,
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48 52 the chemical industry yielded a variety of antiseptic compounds that were
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50 53 utilised for preventing and treating wound infections [3], but in recent times
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52 54 cytotoxicity has limited their acceptance [4]. A systematic strategy of

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4 55 discovering and evaluating promising chemotherapeutic antimicrobial agents
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6 56 was initiated by Paul Ehrlich at the beginning of the twentieth century; it paved
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8 57 the way for the advent of antibiotics, which revolutionised the treatment of
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10 58 infection. These antimicrobial agents with their specific mechanisms of
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12 59 inhibition exhibited selective toxicity against microbial species, rather than
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14 60 patients and were initially effective in controlling infections. Although not
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16 61 universally susceptible to all antibiotics, most microbial species were
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18 62 susceptible to at least one type of antibiotic. To date seventeen classes of
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20 63 antibiotic have been introduced, but the rate of discovery of new antibiotics
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22 64 has slowed within the past twenty years. The therapeutic value of many
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24 65 antibiotics has been curtailed by the acquisition of resistance in species that
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26 66 were once susceptible, as well as the emergence of species with resistance to
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28 67 multiple antibiotics. This is a real concern because species resistant to every
29
30 68 known antibiotic have emerged [5], antiseptic resistant strains have been
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32 69 isolated and organisms with both antibiotic and antiseptic resistance
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34 70 determinants have been discovered [6]. The speed of emergence of
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36 71 antimicrobial resistance is unpredictable [7], but whenever a new inhibitory
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38 72 agent is introduced, resistant organisms emerge in due course. With fewer
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40 73 effective antimicrobial agents to manage wound infections, impacts such as
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42 74 increased morbidity, treatment costs and mortalities have ensued [8]. Novel
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44 75 antimicrobial remedies are needed, but there is always the possibility that
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46 76 organisms with resistance to these agents will arise.
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56 78 Since medical-grade honey was first licensed for treating wounds in Australia
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58 79 in 1999, the clinical use of wound care products containing honey has
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4 80 extended to Canada, Europe, Hong Kong, New Zealand and United States of
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6 81 America. As with antibiotics, extensive use of honey may well provide a
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8 82 selective pressure for the emergence of honey resistant strains of wound
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10 83 pathogens. This can be tested *in vitro* by either repeated cultivation of
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12 84 bacteria in a sub-lethal concentration of the agent, or by incubation in
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14 85 stepwise increasing concentrations. The aim of this study, therefore, was to
15
16 86 determine whether bacteria continuously exposed to low concentrations of
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18 87 manuka honey changed their susceptibility to manuka honey and whether
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20 88 resistant strains were selected.

24 89 **Methods**

26 90 Organisms tested

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28 91 In short-term resistance training experiments *Staphylococcus aureus* NCTC
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30 92 10017 and *Pseudomonas aeruginosa* ATCC 27853 were utilised with a
31
32 93 sample of sterile manuka honey provided by the New Zealand Natural Food
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34 94 Company. For the rest of the study clinical isolates of MRSA, *Staphylococcus*
35
36 95 *epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* were tested with
37
38 96 a sample of manuka honey that was provided by Comvita UK (Manukacare
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40 97 18+). This product is available as Medihoney in Canada and the United
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42 98 States. The clinical isolates were cultures of bacteria that had been isolated
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44 99 from patients with chronic wounds attending a local out-patient clinic at
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46 100 University Hospital of Wales, Cardiff. Using a bioassay [9], the antibacterial
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48 101 activity of New Zealand Natural Food company honey sample was found to be
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50 102 equivalent to 19%(w/v) phenol and Manukacare 18+ was equivalent to
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52 103 18%(w/v) phenol.

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59 104 Determination of Minimum Inhibitory Concentrations (MICs)
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4 105 MICs were determined in 96 well, flat bottomed microtitre plates (Nunc,
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6 106 Roskilde, Denmark) using manuka honey dissolved in either nutrient broth
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8 107 (NB; Oxoid, Basingstoke, UK) or isosensitest broth (ISB; Oxoid, Basingstoke,
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10 108 UK). Honey concentrations varying by 1%(w/v) were used in successive wells
11
12 109 and all wells contained 200 µl. Wells were inoculated with 1µl undiluted
13
14 110 overnight broth cultures of test organisms (approximately 1×10^6 cfu/ml) and
15
16 111 incubated at 37°C for 24 h. Positive (broth and inoculum) and negative
17
18 112 controls (broth and honey) were included. MICs were determined visually and
19
20 113 confirmed by measuring absorbance at 400 nm in a microtitre plate reader
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22 114 (Anthos Labtec Instruments).

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27 115 Short-term resistance training by exposure to a single sub-lethal concentration
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29 116 of manuka honey.

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31 117 To one 100 ml conical flask with 20 ml NB containing 2.5%(w/v) manuka
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33 118 honey, 40 µl overnight broth culture of *S. aureus* was inoculated and to
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35 119 another 100 ml conical flask with 20 ml NB containing 8%(w/v) manuka
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37 120 honey, 40 µl *P. aeruginosa* was inoculated. Both flasks were incubated at
38
39 121 37°C in a shaking water bath (100 cycles per min) for 24 h. On 10 successive
40
41 122 days similar, freshly prepared flasks were inoculated with 40 µl from each
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43 123 preceding days' culture, respectively. MICs for each test organism were
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45 124 determined on days 0 and 10 and compared by the Mann-Whitney test using
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47 125 Minitab version 14.

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51 126 Short-term resistance training by exposure to stepwise increasing
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53 127 concentrations of manuka honey.

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56 128 Cultures were set up as above using an initial concentration of 1%(w/v)
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58 129 manuka honey in NB for *S. aureus* and 5%(w/v) manuka honey in NB for *P.*

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4 130 *aeruginosa* and incubated at 37°C with shaking for 24 h. At 24 h intervals on
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6 131 10 consecutive days, freshly prepared broths with honey concentrations
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8 132 increased by 0.5%(w/v) for *S. aureus* and 1%(w/v) for *P. aeruginosa* were
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10 133 inoculated with 40 µl from the preceding days' culture. Throughout the
11
12 134 training period the identity of each culture was confirmed daily by BBL Crystal
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14 135 kits (Becton Dickinson, Basingstoke, UK). For both test organisms MICs of
15
16 136 manuka honey were determined before and after the training period.
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18 137 Stepwise resistance training of clinical isolates by long-term exposure to sub-
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20 138 inhibitory concentrations of manuka honey.
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22 139 MICs of manuka honey for each of the clinical isolates were determined
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24 140 before continuous exposure to honey. In a 96 well, flat bottomed microtitre
25
26 141 plate for each of the 4 test organisms a range of 15 concentrations of manuka
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28 142 honey (Comvita manukacare 18+) in ISB that varied by 1%(w/v) intervals and
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30 143 included concentrations above and below the initial MIC of each test organism
31
32 144 was prepared. Each well contained 200 µL ISB and honey and was inoculated
33
34 145 on day 0 with 5 µl overnight broth culture, then incubated at 37°C for 24 h.
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36 146 MIC was determined visually as the lowest concentration of honey to prevent
37
38 147 growth. A fresh microtitre plate was prepared every 24 h with a new range of
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40 148 15 honey concentrations contained in ISB that increased by 1%(w/v)
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42 149 increments and included the last MIC in the middle of the range. With time,
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44 150 increasingly higher concentrations of honey in ISB were required, but the
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46 151 range always included honey concentrations above and below the expected
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48 152 MIC. On each successive day of the experiment, wells in the new microtitre
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50 153 plate were inoculated with 5 µl of each respective test organism taken from
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52 154 the well of the previous microtitre plate containing the highest concentration of
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4 155 honey with viable cells (i.e. one well below the actual MIC). Also, the purity of
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6 156 each inoculum was checked by dropping 20 µl onto nutrient agar plates and
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8 157 streaking out to achieve isolated colonies after incubation at 37°C for 24
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10 158 hours. The experiment was continued for 28 successive days with each test
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12 159 organism using an appropriate range of honey concentrations above and
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14 160 below the last recorded MIC value. Cultures obtained after 28 days
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16 161 continuous exposure to a sub-lethal concentration of honey were frozen and
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18 162 stored at -80°C for future reference.

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22 163 Recovery of cultures of clinical isolates following long-term exposure to
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24 164 honey.

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26 165 Each 'honey-trained' culture obtained after 28 days continuous exposure to
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28 166 manuka honey was inoculated into 20 ml isosensitest broth (ISB) contained
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30 167 within a 100 ml conical flask and incubated in a shaking water bath at 37°C for
31
32 168 24 hours. On each successive day, one of four fresh flasks of ISB were
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34 169 inoculated with 100 µl of each culture transferred from the preceding days'
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36 170 culture, respectively until 28 days had elapsed. At each daily transfer MIC
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38 171 against manuka honey was determined. Cultures obtained after the 28 day
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40 172 recovery period were frozen and stored at -80°C.

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44 173 Cultural characteristics.

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46 174 Identity of the test organisms before the start of the long-term training regime,
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48 175 collected after the 28 day training period and after the 28 day recovery period
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50 176 was determined by Crystal kits (Becton Dickinson, Basingstoke, UK) and by
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52 177 RAPD typing.

53 178 **Results**

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57 179 Short-term resistance training with reference cultures.
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4 180 When *S. aureus* NCTC 10017 was cultured in a sub-lethal concentration of
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6 181 manuka honey for 10 consecutive days, susceptibility to manuka honey did
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8 182 not decrease significantly (Table 1). Conversely the MIC for *P. aeruginosa*
9
10 183 ATCC 27853 increased significantly from 15.7 to 19.7%(w/v), indicating that
11
12 184 the culture had become less susceptible to manuka honey. However, after 6
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14 185 subcultures in honey free NB, there was no significant difference in MIC
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16 186 compared to the starting value.

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20 187 When these two test organisms were successively cultured in stepwise
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22 188 increasing concentrations of honey, viable bacteria were not recovered above
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24 189 their starting MIC values. Hence honey resistance was not observed in either
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26 190 *S. aureus* or *P.aeruginosa*.

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31 192 Resistance training of clinical isolates by long-term exposure to honey.
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33 193 The susceptibility of the 4 clinical isolates tested in this study for manuka
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35 194 honey decreased during the 28 day resistance training period (Fig. 1), with the
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37 195 greatest proportional change observed for *E. coli* (2.1) and the least in *P.*
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39 196 *aeruginosa* (1.4). MICs of MRSA and *S. epidermidis* increased by factors of
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41 197 1.6 and 1.7, respectively . However during the recovery period, MIC values
42
43 198 returned to pre-training values within 9 and 14 days for *S. epidermidis* and *P.*
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45 199 *aeruginosa* respectively, demonstrating that honey-resistant strains of these
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47 200 bacteria had not been selected (Fig. 2). Neither MRSA nor *E. coli* returned to
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49 201 the pre-training MIC values within the 28 day recovery period. Several months
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51 202 later the cultures frozen at the end of the recovery period were thawed and
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53 203 MICs were determined in duplicate on three separate occasions (Table 2).
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55 204 MBCs were also determined by streaking out samples from microtitre plate
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4 205 wells showing no growth onto nutrient agar. It was seen that the susceptibility
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6 206 of three of the clinical isolates was similar to those values obtained before
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8 207 resistance training (Fig. 1), but the MIC of manuka honey in *E.coli* remained
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10 208 1.4 times higher. MBC values were close to MICs indicating that manuka
11
12 209 honey was bactericidal for the recovered cultures.

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15 210 It is perhaps worth noting that by exposing each test organism to a range of
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17 211 honey concentrations simultaneously during the long-term training
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19 212 experiment, MIC values were obtained daily at each transfer step. This meant
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21 213 that only one determination was obtained at each time point. MICs obtained
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23 214 later on stored cultures were done in replicate (Table 2). Unlike antibiotic
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25 215 testing where serial doubling dilutions series are normally utilised, here honey
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27 216 concentrations differing by 1%(w/v) intervals were used to get a more precise
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29 217 estimate of MIC.

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33 218 Purity plates and biochemical characteristics confirmed that culture identities
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35 219 were maintained throughout the training and recovery periods; RAPD profiles
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37 220 remained constant.

38 221 **Discussion**

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41 222 The excessive use and misuse of antibiotics is known to favour the selection
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43 223 of resistant strains and to inhibit sensitive strains. In Norway, for example,
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45 224 where mupirocin was not available, mupirocin-resistant strains of *S. aureus*
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47 225 were not detected [10]. Restricting mupirocin use in an American medical
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49 226 centre caused an immediate reduction in the occurrence of mupirocin-
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51 227 resistant MRSA [11] and an association between exposure to fusidic acid and
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53 228 fusidic acid-resistance in *S.aureus* has been established [12]. Resistance to
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55 229 biocides used for skin and wound care has also been demonstrated [6].In the
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4 230 first 'acclimatization' experiments in 1887, reduced sensitivity to mercuric
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6 231 chloride and boric acid was noted [13]; increased tolerance towards many
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8 232 other antibiotics and antiseptics has since been investigated. Inability to select
9
10 233 for resistance to povidone iodine has been reported [13], yet *Pseudomonas*
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12 234 *stutzeri* was shown to develop stable resistance to each of chlorhexidine
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15 235 diacetate and cetylpyridinium chlorine when exposed to stepwise increases in
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17 236 concentration [14]. Bacterial resistance to silver, which is often used in wound
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19 237 care, is well defined and debated [15 and16]. Furthermore, short-term
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21 238 stepwise resistance training of *P. aeruginosa* ATCC 27853 to each of
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23 239 tetracycline, ciprofloxacin and honey, and of *S.aureus* ATCC 9144 to each of
24
25 240 tetracycline, oxacillin and honey demonstrated acquisition of antibiotic
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27 241 resistance but not honey resistance [17]. In that study antibiotic-resistant
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29 242 strains recovered after stepwise resistance training to antibiotics exhibited
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31 243 antibiotic susceptibilities significantly reduced by factors of 32 and 64 [17].
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38 245 Our study showed that continuous exposure to sub-lethal concentrations of
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40 246 manuka honey for up to 28 days failed to select for honey resistant mutants in
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42 247 any of two reference cultures or four clinical isolates. Reduced susceptibility to
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44 248 manuka honey was observed throughout the long-term training period in each
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46 249 of the clinical isolates. Unlike the large changes in susceptibilities seen in
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48 250 antibiotic resistance training [17], honey MICs increased in *P. aeruginosa*,
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50 251 MRSA, *S. epidermidis* and *E. coli* by factors of 1.4, 1.6, 1.7 and 2.1,
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52 252 respectively (Fig. 1). These changes, however, were not permanent because
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54 253 susceptibility to honey was found to increase during cultivation in honey-free
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56 254 media and during storage at -80°C. MICs of final cultures (Fig. 2 and Table 2)
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4 255 were close to pre-training MIC values (Fig. 1), except for *E. coli* which
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6 256 remained 1.4 times higher than at the start of the honey resistance training
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8 257 period. It is not known why susceptibility to honey increased in *E.coli* and
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10 258 MRSA during storage at -80°C. The European consensus of a clinically
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13 259 resistant organism is one in which the level of antimicrobial susceptibility has
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15 260 a high likelihood of clinical failure [18]. Since the concentrations of medical-
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17 261 grade manuka honey that are contained in contemporary licensed wound care
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19 262 products usually exceed 80% (w/v) and many are 95% (w/v), the honey
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21 263 susceptibilities found in our experiments are not likely to give rise to clinical
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23 264 failure.
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29 266 Topical application of honey to wounds will invariably result in dilution,
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31 267 depending on the extent of exudation, and concentrations *in vivo* must always
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33 268 exceed those shown to be inhibitory *in vitro* by a significant margin. Providing
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35 269 that the concentration of manuka honey in practice exceeds the highest MBC
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37 270 recorded here by a factor of three, the risk of selecting honey-resistant
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39 271 organisms should be low. Regular dressing changes will help to keep levels of
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41 272 manuka honey high, particularly in highly exuding wounds. Maintaining high
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43 273 levels of antimicrobial agents in the wound environment is always necessary
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45 274 to effectively inhibit viable bacteria before resistant strains emerge. For some
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47 275 biocides cytotoxicity may then become a limitation, but this should not be an
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49 276 issue with manuka honey.
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55 277 Our findings confirm and extend those of Blair and colleagues [17]. With
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57 278 increasing topical use of honey in wounds, the possibility of selecting for
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59 279 honey-resistant wound pathogens is raised. This study suggests that this
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4 280 outcome will be remote if high concentrations of manuka honey are
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6 281 maintained in clinical practice. Prolonged exposure to antimicrobial agents,
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8 282 including honey, should always be avoided. Hopefully manuka honey will
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10 283 continue to be used as a whole product and specific active components [19-
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12 284 21] will not be purified and used alone, because this is more likely to promote
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14 285 the development of honey-resistant bacteria.
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18 286 **Acknowledgements**

19
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21

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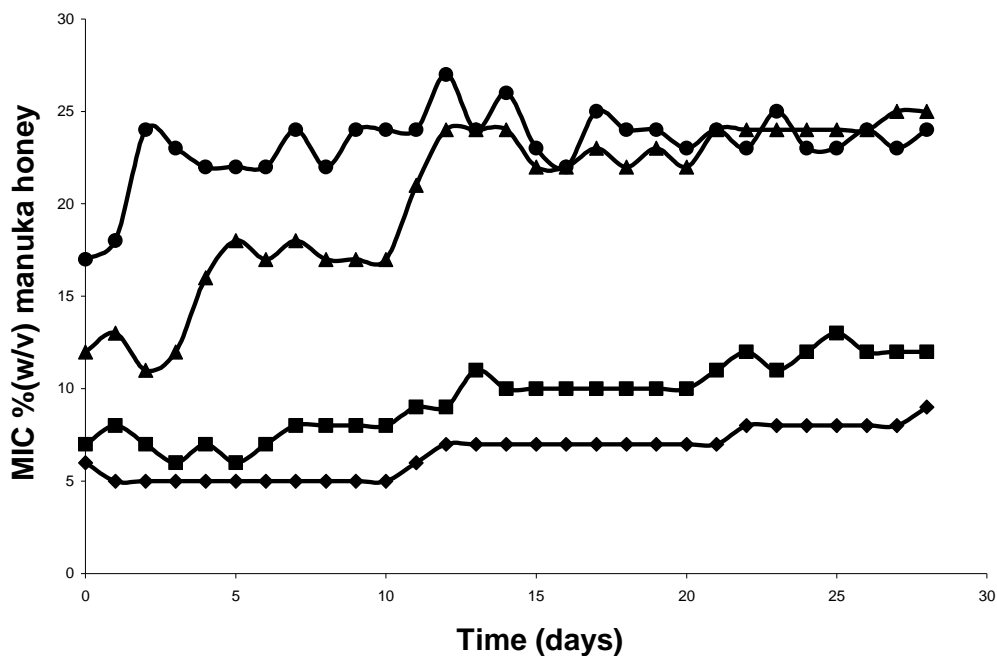
354 Table 1: Sensitivity of two reference cultures to manuka honey before and
 355 after culture in sub-lethal concentrations of honey for 10 successive days.

	Mean MIC %(w/v) ± sd (n)	
	<i>P.aeruginosa</i> ATCC 27853	<i>S.aureus</i> NCTC 10017
Before training	15.7 ± 2.0 (8)	2.8 ± 0.4 (5)
After training	19.7 ± 1.4 (7)	3.8 ± 0.08 (5)
<i>p</i>	0.03	>0.05

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357 sd = standard deviation; n = number of assays

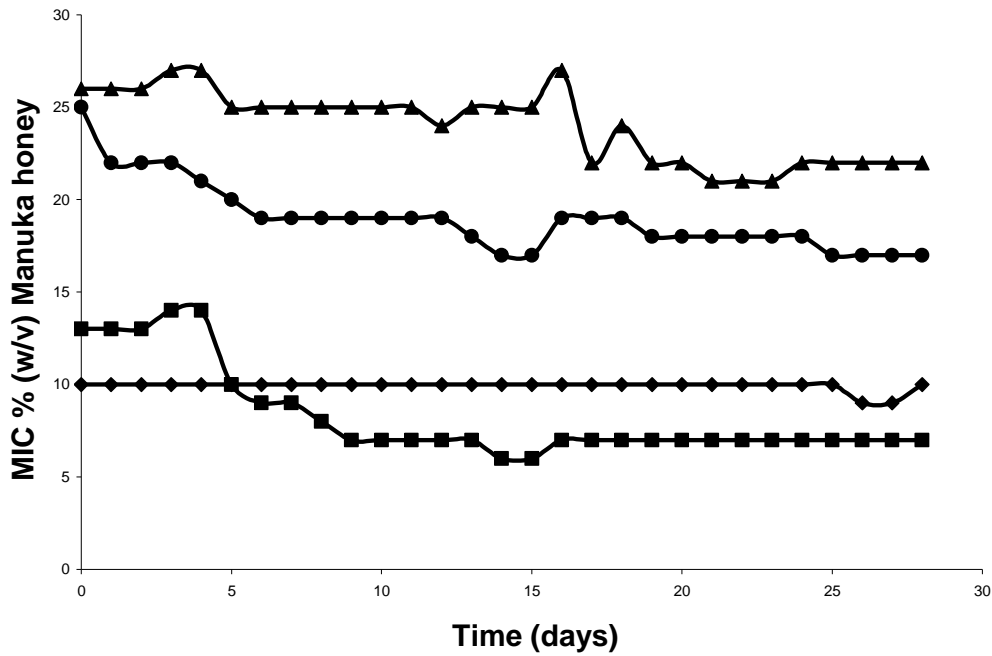
358 Fig.1: Sensitivity of four clinical isolates to manuka honey during exposure to
 359 sub-lethal concentrations of manuka honey for 28 days



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361 MICs of manuka honey in subcultures of four clinical isolates during a long-
 362 term honey resistance training experiment. Cultures were isolated from
 363 patients with chronic wounds: *E. coli* (triangles), MRSA (diamonds), *P.*
 364 *aeruginosa* (circles) and *S. epidermidis* (squares). .

365 Fig. 2: Sensitivity of four clinical isolates to manuka honey during recovery by
 366 cultivation in nutrient broth following long-term honey-training



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 368 MICs of manuka honey in subcultures of four 'honey-trained' clinical isolates
 369 during a 28 day recovery period in honey-free media: *E. coli* (triangles),
 370 MRSA (diamonds), *P. aeruginosa* (circles) and *S. epidermidis* (squares)
 371 Table 2: Sensitivity of clinical isolates to manuka honey following long-term
 372 exposure, recovery in honey free media and storage at -80°C.

Test organism	MIC*	MBC*
<i>E. coli</i>	16.17 ± 1.47	18.0 ± 1.67
MRSA	5.83 ± 1.6	8.5 ± 2.5
<i>P. aeruginosa</i>	15.33 ± 2.25	15.67 ± 3.2
<i>S. epidermidis</i>	5.67 ± 1.63	8.33 ± 2.56

373 *Mean manuka honey concentration as %(w/v) ± standard deviation (tests
 374 were done in duplicate on three separate occasions)