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ARTICLE

# The effect of manuka honey on the structure of *Pseudomonas aeruginosa*

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**Abstract** The purpose of this study was to investigate the effects of manuka honey on the structural integrity of *Pseudomonas aeruginosa* ATCC 27853. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of manuka honey for *P. aeruginosa* were determined by a microtitre plate method, and the survival of bacteria exposed to a bactericidal concentration of manuka honey was monitored. The effect of manuka honey on the structure of the bacteria was investigated using scanning and transmission electron microscopy (SEM and TEM, respectively). The MIC and MBC values of manuka honey against *P. aeruginosa* were 9.5% (w/v) and 12% (w/v) respectively; a time–kill curve demonstrated a bactericidal rather than a bacteriostatic effect, with a 5 log reduction estimated within 257 min. Using SEM, loss of structural integrity and marked changes in cell shape and surface were observed in honey-treated cultures. With TEM, these changes were confirmed, and evidence of extensive cell disruption and lysis was found. Manuka honey does not induce the same structural changes in *P. aeruginosa* as those observed in staphylococci. Our results indicate that manuka honey has the potential to be an effective inhibitor of *P. aeruginosa*.

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## Introduction

*Pseudomonas aeruginosa* is an opportunist pathogen that is ubiquitously distributed throughout the environment, particularly in moist habitats. It has been implicated in a wide range of infections, such as endocarditis, folliculitis, keratitis, meningitis, pneumonia, urinary tract infections and wound infections. In wounds, *P. aeruginosa* has emerged as a multidrug-resistant organism that gives rise to persistent infections in burns patients [1, 2] and chronic venous leg ulcers [3]. Novel antimicrobial interventions are needed.

Honey has been used for thousands of years as a topical treatment for wounds. Although ancient remedies may have been crude preparations, modern wound care products are standardised to satisfy regulatory constraints. The use of honey in modern clinical practice is based on its broad antimicrobial properties and its ability to stimulate rapid wound healing. Antimicrobial activity extends to more than 80 species [4, 5]. The inhibition of pathogens capable of causing wound infection has been demonstrated [6–10], with both antibiotic-resistant and antibiotic-sensitive strains exhibiting susceptibility to honey [6, 7, 11].

The complex chemistry of honey has complicated attempts to understand its mode of action, but the contributions of individual antibacterial components have been identified [12]. Recently, macroarray transcriptome analysis with *Escherichia coli* has shown that a blend of *Leptospermum* honeys induces a unique response that involves multiple cellular targets [13]. Manuka honey prevents cell division in *Staphylococcus aureus* and leads to the accumulation of cells with septa [14]; its effect on the structure of Gram-negative bacteria has not been studied. This pilot study, therefore, aims to investigate the effects of

manuka honey on the structural integrity of a bacterium commonly associated with wound infection, in order to gain insight into its action as an inhibitory agent.

## Materials and methods

### General

A culture of *P. 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. The antibacterial potency of the sample was equivalent to 18% (w/v) phenol using a bioassay developed in New Zealand [15].

### Minimum inhibitory concentrations and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) was determined in 96-well, flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) using double-strength nutrient broth (Oxoid, Basingstoke, UK) and honey concentrations varying by 1% (w/v) intervals in a total volume of 200 μL. Overnight broth cultures of the test organism (1 μL) was used as an inoculum without dilution, and total viable cell counts were performed to check retrospectively that each well had received approximately $10^6$ cells. Plates were incubated at 37°C for 24 h 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 concentration without growth were recorded as the MIC. The minimum bactericidal concentration (MBC) was determined by plating 20 μL from wells without growth onto nutrient agar (Oxoid, Basingstoke, UK) and incubating at 37°C for 24 h to find the lowest concentration without viable bacteria. Experiments were performed in duplicate on each of three occasions.

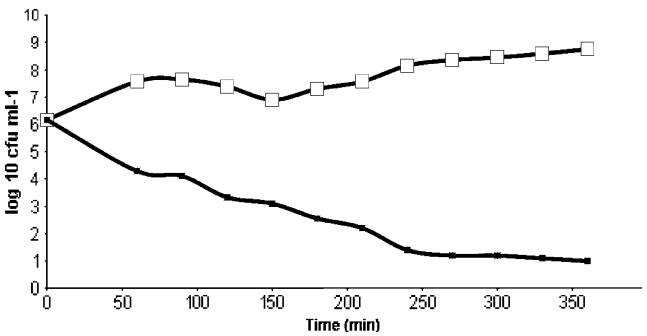


**Fig. 1** Time–kill curve. *Pseudomonas aeruginosa* in nutrient broth (*open squares*), *P. aeruginosa* in nutrient broth containing 20% (w/v) M109 manuka honey (*filled squares*)

### Time–kill curve

A time–kill study was performed by inoculating 40 μL of an overnight culture of *P. aeruginosa* ATCC 27853 into 20 mL nutrient broth with and without 20% (w/v) M109 and incubating at 37°C for 24 h in a shaking water bath (120 cycles $min^{-1}$) (the honey concentration was approximately twice the MIC value). Samples were removed at known intervals and Miles and Misra surface drop counts

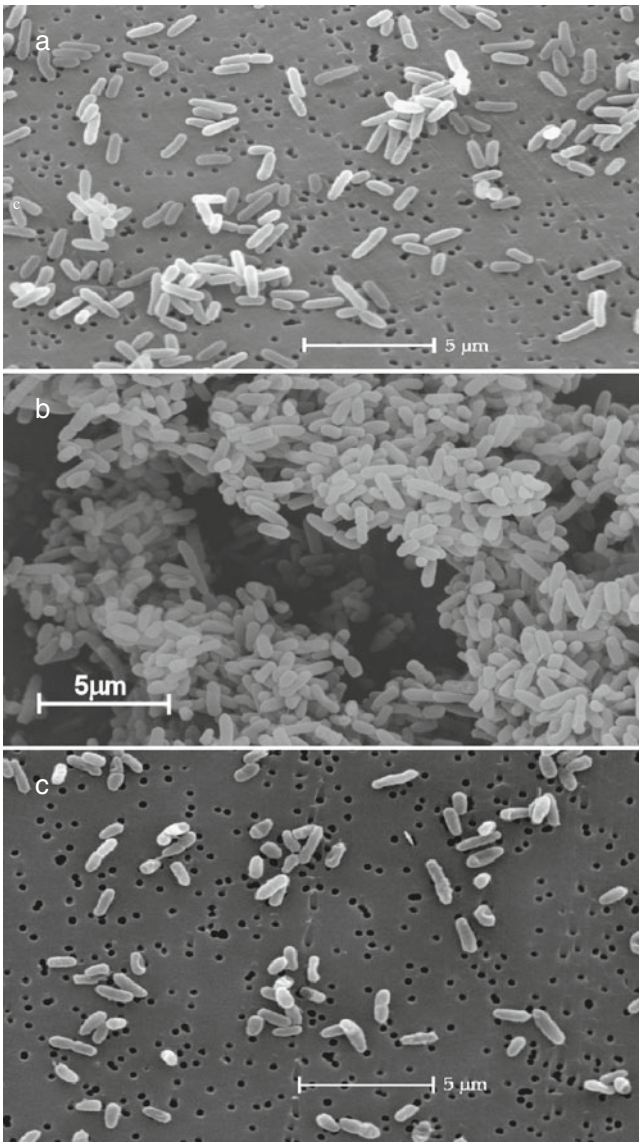


**Fig. 2a–c** The effect of manuka honey on the structure of exponential phase bacteria as seen by scanning electron microscopy (SEM). **a** Exponential phase cells of *P. aeruginosa* incubated with MOPS buffer for 8 h at 37°C as viewed by SEM at 10,000× magnification. **b** Exponential phase cells *P. aeruginosa* incubated with MOPS buffer containing 20% (w/v) artificial honey for 8 h at 37°C as viewed by SEM at 10,000× magnification. **c** Exponential phase cells of *P. aeruginosa* incubated with MOPS buffer containing 20% (w/v) manuka honey for 8 h at 37°C as viewed by SEM at 10,000× magnification

**Table 1** Structural changes observed in scanning electron micrographs of *Pseudomonas aeruginosa*

| | % of exponential phase cells with structural changes | % of stationary phase cells with structural changes |
|---|---|---|
| No honey | 2 ($n$=1,568) | 2 ($n$=2,414) |
| Artificial honey | 7 ($n$=2,566) | NT |
| Manuka honey | 80 ($n$=1,100) | 60 ($n$=283) |
| Comparison between untreated and manuka-honey-treated cells (Mann–Whitney test) | $p$=0.02 | $p$=0.001 |

$n$ = number of cells observed; NT = not tested

were performed by serial decimal dilution in quarter-strength Ringer's solution, plating onto nutrient agar and incubating at 37°C for 24 h.

### Electron microscopy

Electron microscopy was performed using the test organism in either the exponential or stationary phase of growth following cultivation in Iso-Sensitest broth (Oxoid, Basingstoke, UK) at 37°C in a shaking water bath for either 3 h or overnight, respectively. Cells were harvested by centrifugation at 3,000g for 30 min (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 h, or in MOPS buffer containing 20% (w/v) artificial honey solution to determine the effect of sugars in honey on the cell structure [6, 7]. Cells were examined using scanning (5200LV Jeol, Herts, UK) and transmission electron microscopy (SEM and TEM, respectively) (1210 Jeol, Herts, UK) by the method of Lemar et al. [16], except that harvested cell pellets for TEM were embedded in Araldite resin, not Spurr.

### Analysis of images

Electron micrographs of untreated and treated cells were examined to identify structural changes such as altered shape, modified surface layers, the presence of electron-dense material and cellular debris. Typically, at least six photographs, each with approximately 160 cells, were observed, so that more than 1,000 cells were counted in total for each sample. The data were analysed for statistically significant differences by the Mann–Whitney test using Minitab (version 15).

## Results

### Inhibition studies

The MIC and MBC were found to be 9.5% (w/v) and 12% (w/v) manuka honey, respectively. The close proximity of these two values indicates a bactericidal mode of inhibition. This was confirmed by time–kill studies (Fig. 1), where cells exposed to manuka honey were found to lose viability

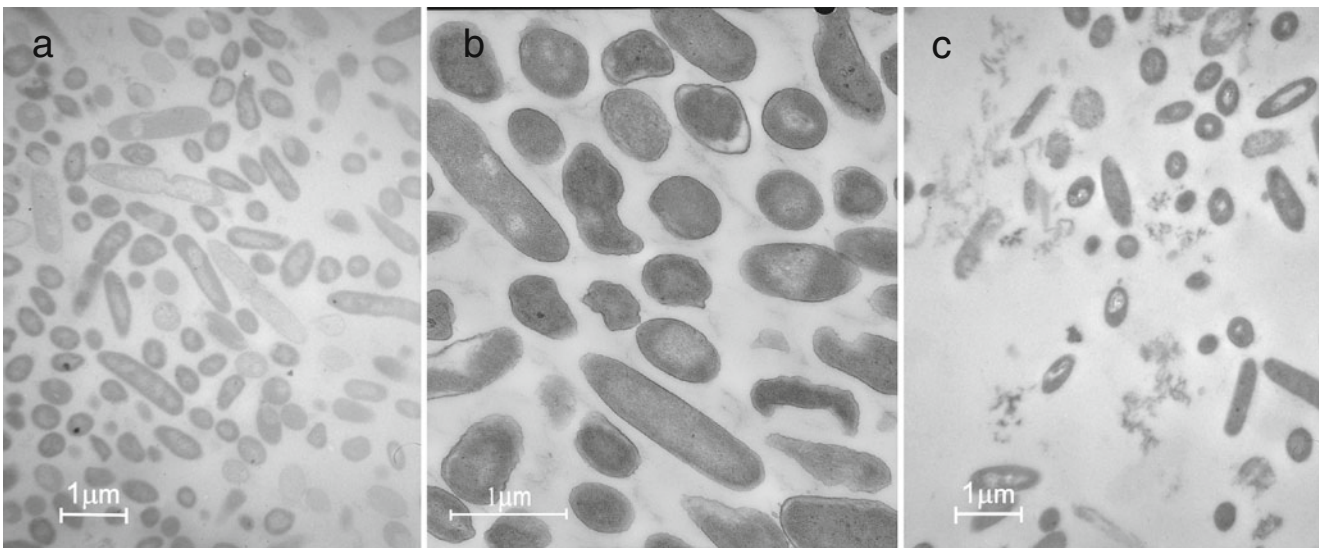


**Fig. 3a–c** The effect of manuka honey on the structure of exponential phase bacteria as seen by SEM. **a** Exponential phase cells of *P. aeruginosa* incubated with MOPS buffer for 8 h at 37°C as viewed by transmission electron microscopy (TEM) at 10,000× magnification. **b** Exponential phase cells of *P. aeruginosa* incubated with MOPS buffer containing 20% (w/v) artificial honey for 8 h at 37°C as viewed by TEM at 20,000× magnification. **c** Exponential phase cells of *P. aeruginosa* incubated with MOPS buffer containing 20% (w/v) manuka honey for 8 h at 37°C as viewed by TEM at 10,000× magnification

with time, yet the numbers of untreated cells increased. The time estimated to achieve a 5 log reduction of test organism incubated with nutrient broth containing 20% (w/v) manuka honey was 257 min.

### Structural studies

The effect of manuka honey on cell structure was investigated in both exponential and stationary phase cultures because stationary phase cells are often less susceptible to antimicrobial agents than exponential cells. However, the structural changes observed in both of these stages of growth were similar and, therefore, only electron micrographs of exponential cells are presented here.

Using SEM, the smooth surface layers of untreated cells (Fig. 2a) and cells exposed to 20% (w/v) artificial honey (Fig. 2b) contrasted with those of honey-treated *P. aeruginosa* cells, which exhibited marked cell surface changes as furrows and blebs (Fig. 2c). Honey-treated cells also appeared to be shortened and to have distorted shapes (Fig. 2c). In untreated samples, 2% of cells were found to have structural irregularities, whereas 80% and 60% cells of exponential and stationary cultures, respectively, exhibited irregular cell structure. These differences were statistically significant (Table 1). For exponential phase cells exposed to 20% (w/v) artificial honey, 7% of cells were found to exhibit structural irregularities. This suggests that the effect of manuka honey on *P. aeruginosa* is not due exclusively to the sugars contained in honey.

Using TEM, untreated cells (Fig. 3a) and cells incubated in MOPS containing 20% (w/v) artificial honey (Fig. 3b) were entire cells with relatively densely stained contents. In the TEM images of honey-treated *P. aeruginosa* (Fig. 3c), cellular debris was clearly evident and whole cells with evacuated areas were observed.

## Discussion

### Inhibition studies

The MIC obtained in this study concurred with previous studies [6, 9, 10]. The MBC and time–kill curve (Fig. 1) indicated a bactericidal rather than bacteriostatic effect of manuka honey on *P. aeruginosa*. The loss of viability of cells exposed to 20% (w/v) manuka honey in vitro provided a guide to its clinical efficacy, where in licensed wound dressings undiluted honey is usually used. Since part of the weight of the dressing is absorptive material, honey is not diluted in terms of its antibacterial activity, even though the quantity of honey in terms of the total weight of the dressing applied to the wound is decreased.

### Structural studies

The loss of structural integrity in *P. aeruginosa* treated with manuka honey was unlike that observed in *S. aureus*, where increased numbers of cells with septa were evident [14]. The presence of cellular debris in TEM images indicated that *P. aeruginosa* had lysed and whole cells in SEM images were short, distorted and displayed cell surface abnormalities.

The results of this preliminary study demonstrate that manuka honey is an effective inhibitor of *P. aeruginosa*. Further investigation into its effect on cellular and molecular targets is warranted, and its clinical efficacy must be confirmed.

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