

ORIGINAL RESEARCH ARTICLE



Honey modulates biofilms of *Pseudomonas aeruginosa* in a time and dose dependent manner.

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Summary

Biofilms are complex microbial communities associated with persistent infections that demonstrate increased resistance to immunological and antimicrobial challenges. *Pseudomonas aeruginosa* has been associated with biofilms in chronic wounds. When this opportunist pathogen is grown in suspension culture in the laboratory it is susceptible to manuka honey at concentrations below 10 % (v/v), but its susceptibility as a biofilm has not previously been reported. The effect of two concentrations of manuka honey dissolved in Luria broth (LB) on biofilms of six cultures of *Pseudomonas aeruginosa* were tested over 24 hours. One type culture (ATCC 27853) and five clinical isolates derived from five different patients with infected wounds were each incubated at 37 °C for 24 hours in LB in microtitre plates to allow biofilms to establish. Medium was then replaced with LB, or LB containing 20 % (w/v) manuka honey or LB containing 40 % (w/v) manuka honey and plates were incubated at 37 °C for 24 hours. Biofilm biomass was monitored at known intervals by fixing adherent cells with 2.5 % (w/v) glutaraldehyde and staining with 0.25 % (w/v) crystal violet. Exposure of *Pseudomonas aeruginosa* biofilms to 40 % (w/v) manuka honey in LB resulted in significantly reduced biofilm biomass for all cultures ($p > 0.05$) compared to LB alone and 20 % (w/v) manuka honey in LB. Differences in biofilm biomass were most noticeable after 9 to 11 hour exposure times. This preliminary investigation suggests that manuka honey has potential in the control of biofilms in chronic wounds and justifies further study of this subject.

Keywords: manuka honey, biofilms, *Pseudomonas aeruginosa*, wounds

Introduction

Biofilms are complex consortia of microbial species encased in slime that have been associated with persistent infections, such as cystic fibrosis, periodontal disease and osteomyelitis (Costerton, Stewart and Greenberg, 1999). Although the presence of biofilms in wounds has been discussed for some time (Serralta *et al.*, 2001), it is only relatively recently that objective evidence of their presence in wounds has been published. A clinical study utilising scanning electron microscopy and confocal laser microscopy of tissue removed from chronic wounds revealed the presence of biofilm within the necrotic layers close to the surface in 7 of 12 non-healing wounds (Ngo, Vickery and Deva, 2007). Using similar methods, biofilms were observed in 30 of 50 chronic wounds, yet they were found in only 1 of 16 acute wounds and biofilm presence in wounds was statistically associated with failure to heal (James *et al.*, 2008). There are many complex reasons why wounds fail to heal (Vowden,

Apelqvist and Moffat, 2008) but now that the chronic inflammatory status of non-healing wounds has been linked to the presence of biofilms (James *et al.*, 2008; Wolcott and Rhoads, 2008), explanations for this effect are beginning to be formulated. It has been postulated, for example, that metabolic products of bacteria such as *Pseudomonas aeruginosa* contained within biofilms contribute to wound chronicity (Bjarnsholt *et al.*, 2008) and so the need to remove biofilms has become important. However, bacteria residing in biofilms are estimated to be 500 times less susceptible to antibiotics than free living forms and they are markedly refractive to host immune responses (Costerton *et al.*, 1995). Their control in wounds presents a serious difficulty and new treatment methods are urgently required.

Honey is a wound remedy that was used by ancient civilisations, but within the past five years it has been used in the development of modern wound care products such as ointments and wound dressings, as well as in tubes containing sterile honey. Now licensed products are available on prescription throughout Europe,

America, Australia, Canada, Hong Kong, Australia and New Zealand and use is monitored by respective regulatory authorities. Honey has long been known to possess a broad spectrum of activity against a wide range of bacteria (Molan, 1992; Blair, 2009). The inhibition of suspensions of *Pseudomonas aeruginosa* in the laboratory using manuka honey has been demonstrated (Cooper and Molan, 1999; Cooper, Halas and Molan 2002), but the inhibition of these bacteria as biofilms has not been reported. The aim of this preliminary study was to determine whether established biofilms of *P. aeruginosa* were affected by manuka honey.

Materials and methods

Materials

Six cultures of *P. aeruginosa* were used in this study, one was a reference strain (ATCC 27853) and five were cultures that were isolated from infected wounds of five different patients (kindly provided by Alan Paull of University Hospital of Wales, Cardiff). The cultures were stored at -70 °C until tested. A sample of manuka honey (M109) with non-peroxide activity equivalent to 18 %w/v phenol was provided as a gift from Professor Peter Molan of the University of Waikato, New Zealand. It was stored at 4°C in the dark until used.

Establishment of 24 hour biofilms

Each organism was cultivated overnight at 37°C in universal bottles containing 10 ml Luria broth (LB) (Oxoid, Cambridge, UK) and an inoculum of these free-living (planktonic) cells was prepared by diluting the culture 1/10 with LB. 200 µl of diluted inoculum of each organism (approximately 3.9×10^9 cfu/ml) was used to inoculate discrete wells in six 96 well microtitre plates (Nunc, Roskilde, Denmark) using aseptic technique and plates were then incubated aerobically at 37°C for 24 hours without shaking to allow biofilm to develop.

Exposure of biofilms to honey

A 40 % (w/v) stock solution of manuka honey was prepared in sterile LB immediately before use and further diluted with LB to prepare a 20 % (w/v) solution of manuka honey in LB. Microtitre plates in which 24 hour biofilms of *P. aeruginosa* had been cultivated were utilised to test the effect of manuka honey. The liquid phase which contained unattached (suspended or planktonic) bacterial cells was removed from every microtitre plate well and replaced with either 200 µl sterile LB or sterile LB containing manuka honey (either 20 or 40 % w/v) and the plates were incubated at 37°C. At known intervals up to 24 hours, a plate was removed for the estimation of biofilm biomass. The experiment was performed on two occasions for each culture and time point; 4 replicates were prepared at every point. Some wells contained only LB to act as sterility controls.

Determination of biofilm biomass.

Medium was removed from each microtitre plate leaving adherent cells (biofilm) attached to the walls of the well; each well was washed 3 times with 200 µl of deionised water in order to remove all unattached cells. To fix the adherent cells, 200 µl of 2.5% (w/v) glutaraldehyde (Sigma, Poole, UK) was added and left at room temperature for 5 minutes. This fixative was then removed and all wells were washed twice with 200 µl deionised water. The biofilm left adhered to the wells was stained by adding 200 µl of 0.25% (w/v) crystal violet and incubated for 5 minutes at room temperature. The liquid phase was then removed and wells were washed 3 times with 200 µl of deionised water. Any stain that was retained by the attached cells (biofilm) was then solubilised by adding 200 µl of ethanol/acetone (20:80) to each well and incubated for 2 minutes at room temperature. The dye solution was carefully removed to semi-micro cuvettes and the last step was repeated. Finally, 600 µl of deionised water were added to each semi-micro cuvette, to make a total volume of 1ml and the absorbance was measured at 570 nm using a spectrophotometer (Cecil, CE 1021, 1000 Series). Essentially this protocol was based on that described by Joshua *et al* (2003).

Statistical analysis

Interactions between honey concentrations and contact times were determined for each culture by two way analysis of variance (ANOVA) using Minitab version 15.

Results

All of the organisms included in this study were found to form a biofilm within 24 hours (Fig. 1-6), but their capacity to develop biofilm was not uniform. Culture 4, for example, had a greater propensity to produce biofilm than the other cultures (Fig. 4). During the 24 hour monitoring period of the test, the extent of biofilm did not change significantly when established *P. aeruginosa* biofilms were incubated with nutritive LB medium alone (Fig. 1-6). When biofilms were incubated with LB containing 40 % (w/v) manuka honey, the most pronounced effects were observed in all cases after exposure to honey for at least 9 hours and maximally at 11 hours. Interestingly, and again in all cultures exposed to honey, there appeared to be an increase in biofilm biomass after 24 hours compared to 11 hours and this may be indicative of the regrowth of biofilm. Two way ANOVA of all data indicated that the mean absorbance of biofilm biomass for each culture was significantly lower with exposure to 40 % (w/v) manuka honey contained in LB compared to 20 %w/v manuka honey in LB and LB alone ($p > 0.05$). Visual inspection of the data shows that the extent of biofilm reduction was not uniform indicating that all of the six test organisms demonstrated differing susceptibilities to manuka honey. In cultures 1 and 4 the presence of 20 % (w/v) manuka honey markedly stimulated biofilm formation at 24 hours (Fig. 1 and 4, respectively).

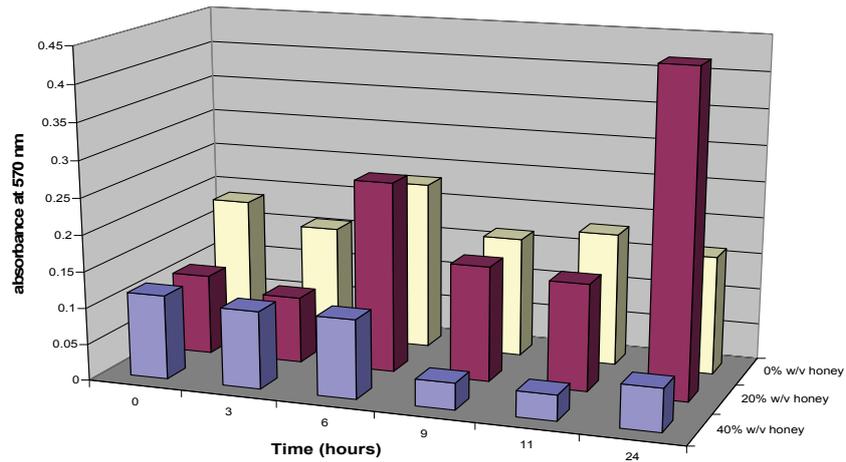


Fig. 1. Biofilm biomass (measured by absorbance at 570nm) following exposure of 24 hour biofilm of *Pseudomonas aeruginosa* ATCC 27853 to manuka honey at 20% w/v and 40% w/v in LB for 24 hours against a control of LB. Mean absorbance is shown (n=4) versus time (hours) and honey concentration

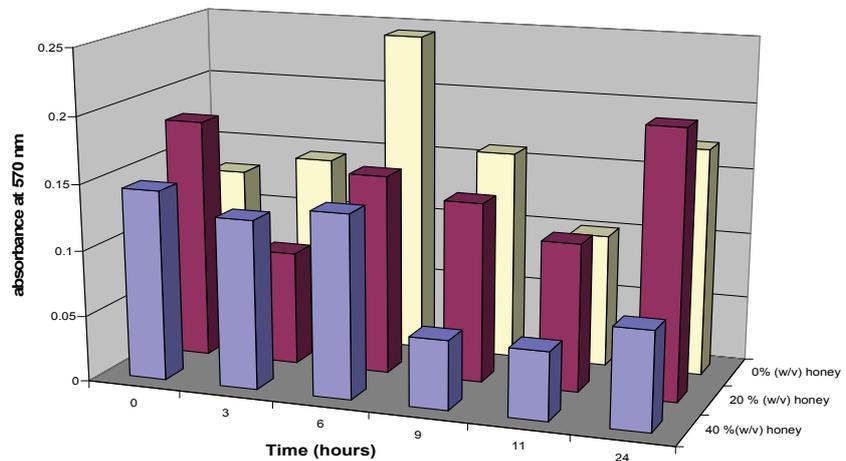


Fig. 2. Biofilm biomass (measured by absorbance at 570nm) following exposure of 24 hour biofilm of *Pseudomonas aeruginosa* (culture 2- isolated from an infected wound) to manuka honey at 20% w/v and 40% w/v in LB for 24 hours against a control of LB. Mean absorbance is shown (n=4) versus time (hours) and honey concentration.

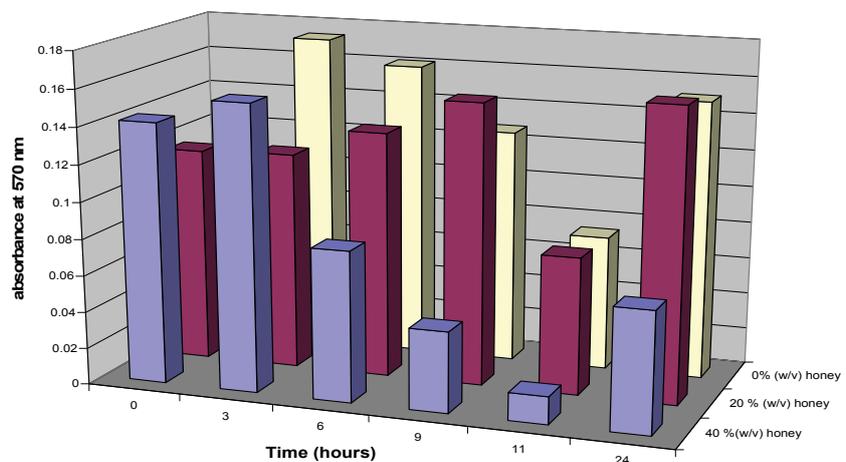


Fig. 3. Biofilm biomass (measured by absorbance at 570nm) following exposure of 24 hour biofilm of *Pseudomonas aeruginosa* (culture 3- isolated from an infected wound) to manuka honey at 20% w/v and 40% w/v in LB for 24 hours against a control of LB. Mean absorbance is shown (n=4) versus time (hours) and honey concentration

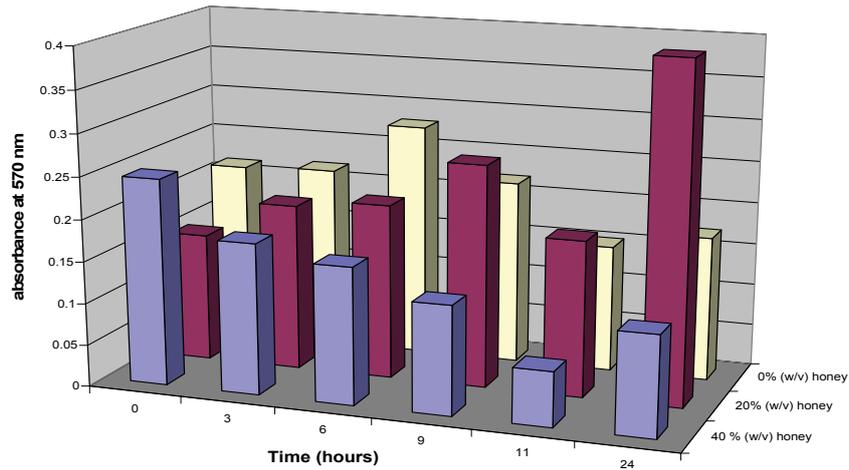


Fig. 4. Biofilm biomass (measured by absorbance at 570nm) following exposure of 24 hour biofilm of *Pseudomonas aeruginosa* (culture 4- isolated from an infected wound) to manuka honey at 20% w/v and 40% w/v in LB for 24 hours against a control of LB. Mean absorbance is shown (n=4) versus time (hours) and honey concentration.

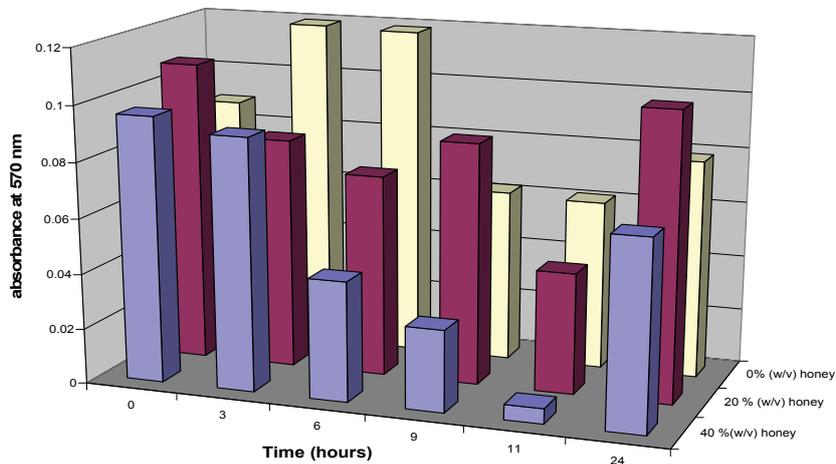


Fig. 5. Biofilm biomass (measured by absorbance at 570nm) following exposure of 24 hour biofilm of *Pseudomonas aeruginosa* (culture 5- isolated from an infected wound) to manuka honey at 20% w/v and 40% w/v in LB for 24 hours against a control of LB. Mean absorbance is shown (n=4) versus time (hours) and honey concentration.

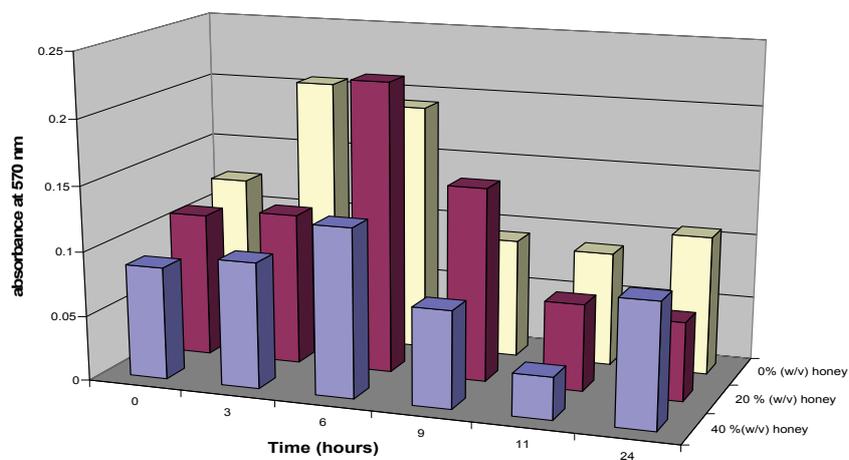


Fig. 6. Biofilm biomass (measured by absorbance at 570nm) following exposure of 24 hour biofilm of *Pseudomonas aeruginosa* (culture 6- isolated from an infected wound) to manuka honey at 20% w/v and 40% w/v in LB for 24 hours against a control of LB. Mean absorbance is shown (n=4) versus time (hours) and honey concentration.

Discussion

Cultures of planktonic cells of *P. aeruginosa* in suspension tests have been shown to be susceptible to manuka honey at concentrations less than 10 % (v/v) (Cooper and Molan, 1999; Cooper, Halas and Molan, 2002), yet in this preliminary study 24 hour *P. aeruginosa* biofilms were not significantly inhibited by 20% (w/v) solutions of manuka honey. Since biofilms are known to be less susceptible to antimicrobial agents, this observation was not unexpected, but it provides important evidence that the level of active honey within a dressing is critical if biofilm is to be inhibited rather than stimulated in a wound. Furthermore, the length of exposure to manuka honey was found to be crucial. Maximal inhibition by 40 % (w/v) honey was seen at 11 hours and increased biofilm biomass at 24 hours suggested that the inhibitory effect was not maintained.

Although inhibition of *P. aeruginosa* biofilms was found at 40 % (w/v) manuka honey, further experiments are needed to determine the minimum inhibitory concentration (MIC). Whether manuka honey would inhibit *Pseudomonas* biofilms in wounds must also be tested, but because the concentration of manuka honey that is incorporated into wounds gels, calcium alginate impregnated dressings and tubes of sterile honey ranges between 80 and 100 % it is probable that the topical application of wound care products containing manuka honey would initially reduce biofilms. When honey is applied to a wound there can be increased exudation, so it will be important to investigate the rate of dilution *in vivo* and to determine the concentration at which honey ceases to inhibit biofilms and begins to promote biofilm growth. This will influence recommendations for the frequency of dressing changes.

It will also be necessary to determine the way in which manuka honey interferes with biofilm integrity. One possible suggestion is that honey prevents attachment of the planktonic cells which is an essential step in the initiation of biofilm formation because the main sugar in honey (fructose) binds to the fucose receptor of *P. aeruginosa* (Lerrer *et al.*, 2007). Manuka honey has been demonstrated to reduce dental plaque (which is a biofilm) in a clinical study where subjects that chewed "honey leather" had lower plaque scores than those that did not (English *et al.*, 2004). It is, therefore, reasonable to continue to investigate the inhibition of *Pseudomonas* biofilms by manuka honey with a view to controlling biofilms in chronic wounds.

Conclusion

This pilot study showed that 40% (w/v) manuka honey inhibited biofilms of *P. aeruginosa* *in vitro*. Further research is indicated to elucidate the mechanisms of inhibition and *in vivo* studies are needed to evaluate the clinical potential of this effect.

References

- BJARNSHOLT, T; KIRKETERP-MØLLER, K; JENSEN, P Ø; MADSEN, K G; PHIPPS, R; KROGFELT, K; HØIBY, N; GIVSKOV, M (2008) Why chronic wounds fail to heal: a new hypothesis. *Wound Repair and Regeneration* 16(1): 2-10
- BLAIR S. (2009) Chapter 3: Antibacterial activity of honey. In: *Honey in Modern Wound Management* pp 21-46. Eds. Cooper, R; Molan, P; White, R. Wounds UK: Aberdeen. ISBN-13 978-0-9555758-5-3.
- COOPER, R; MOLAN, P (1999) The use of honey as an antiseptic in managing *Pseudomonas* infection. *Journal of Wound Care* 8 (4): 161-164
- COOPER, R A; HALAS, E; MOLAN, P C (2002) The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected burns. *Journal of Burn Care & Rehabilitation* 23 (6): 366-370
- COSTERTON, J W; LEWANDOWSKI, Z; CALDWELL, D E; KORBER, D R; LAPPIN-SCOTT, H M (1995) Microbial biofilms. *Annual Reviews in Microbiology* 49: 711-745
- COSTERTON, J W; STEWART, P S; GREENBERG, E P (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318-1322
- ENGLISH, H K P; PACK, A R C; MOLAN, P C (2004) The effects of manuka honey on plaque and gingivitis: a pilot study. *Journal of the International Academy of Periodontology* 6: 63-67
- JAMES G A; SWOGER, E; WOLCOTT, R; PULCINI, E; SECOR, P; SESTRICH, J; COSTERTON, J W; STEWART, P S (2008) Biofilms in chronic wounds. *Wound Repair and Regeneration* 16 (1): 37-44
- JOSHUA G W P; KARLYSHEV, A V; SMITH, M P; ISHERWOOD, K E; TITBALL, R W; WREN B W (2003) A *Caenorhabditis elegans* model of *Yersinia* infection: biofilm formation on a biotic surface. *Microbiology* 149: 3221-3229
- LERRER, B; ZINGER-YOSOVICH, K D; AVRAHAMI, B; GILBOA-GARBER, N (2007) Honey and royal jelly, like human milk, abrogate lectin-dependent infection preceding *Pseudomonas aeruginosa* adhesion. *ISME Journal* 1: 149-155
- MOLAN P C (1992) The antibacterial nature of honey: 1. The nature of the antibacterial activity. *Bee World* 73 (1): 5-28
- NGO, Q; VICKERY, K; DEVA, A K (2007) Role of biofilms in chronic wounds. *ANZ Journal of Surgery* 77 (suppl 1): A66
- SERRALTA, V W; HARRISON-BELESTRA, C; CAZZANIGA, A L; DAVIS, S C; MERTZ, P M (2001) Lifestyles of bacteria in wounds: presence of biofilms? *Wounds* 13(1): 29-34
- VOWDEN, P; APELQVIST, J; MOFFAT, C (2008) Wound complexity and healing. In *European Wound Management Association (EWMA) Position Document: Hard-to-heal wounds: a holistic approach*. pp 2-9. London. MEP Ltd.
- WOLCOTT, R D; RHOADS, D D; DOWD, S E (2008) Biofilms and chronic wound inflammation. *Journal of Wound Care* 17 (8) 333-341