

Inhibition of biofilms through the use of manuka honey

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Abstract

Aims: To investigate the effectiveness of Activon® Manuka honey (Advancis Medical) in inhibiting the *in vitro* formation of clinically important Gram positive bacteria biofilms. **Methods:** Biofilms of meticillin-sensitive *Staphylococcus aureus* (MSSA), meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) were cultivated in microtitre plates with and without varying concentrations of Activon for specific intervals. The extent of biofilm biomass was estimated by staining with crystal violet. **Results:** Concentrations of Activon above 10% (w/v) interfered with the formation of biofilms in all of the test organisms. Inhibition of established biofilms by Activon Manuka honey was dependent on concentration and time. **Conclusions:** Biofilms of MSSA, MRSA and VRE can be prevented and inhibited *in vitro* with concentrations of Activon Manuka honey that could be used in clinical practice. The efficacy of Activon Manuka honey in inhibiting biofilms *in vivo* must be tested. **Conflict of interest:** This study was sponsored by Advancis Medical, Nottingham, UK.

KEY WORDS

Chronic wounds
Biofilms
Activon® Manuka honey

Although biofilms have long been associated with persistent infections (Potera, 1999), their presence in wounds was proposed more recently (Mertz, 2003). Now that the link between chronic wounds and biofilms has been established (James et al, 2008) and explanations of how biofilms impede wound healing have been suggested (Bjarnsholt et al, 2008), there is a need to find effective treatments to inhibit their development in wounds.

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Although potential biofilm inhibitors include chlorhexidine, cadexomer, garlic, honey, iodine, hydrogen peroxide, lactoferrin, octenidine, polyhexanide, povidone iodine, silver and xylitol, laboratory data is rather limited and clinical

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evidence is sparse (Cooper, 2010). This laboratory study was designed to investigate the ability of Activon® Manuka honey (Advancis Medical) to inhibit the development of those Gram positive bacteria biofilms that are of clinical significance to wounds.

Methods

Test materials

Activon® Manuka honey was used throughout this study. This irradiated, sterile honey is recommended for partial and full-thickness wounds, including sloughy wounds, pressure ulcers, leg ulcers, surgical wounds,

burns, graft sites and malodorous wounds. The test organisms used in the study were isolated from actual clinical situations — a culture of meticillin-resistant *Staphylococcus aureus* (MRSA) was taken from a chronic wound in a patient who was successfully treated with manuka honey (Natarajan et al, 2001); a culture of penicillin-resistant, meticillin-sensitive *S. aureus* (MSSA) was taken from an infected wound; and a culture of vancomycin-resistant *Enterococcus faecalis* (VRE) was taken from a contaminated hospital surface.

The MSSA and VRE were provided by the Medical Microbiology and Public Health Laboratory Service at University Hospital of Wales by the late Alan Paull. Cultures were stored at -80°C until required using the Protect system (Technical Service Consultants Ltd, Heywood, UK) by coating bacterial suspensions onto coloured beads with the cryopreservative solution provided. They were cultivated overnight in Tryptone Soya Broth (TSB) (Oxoid) and maintained on nutrient agar (Oxoid) between experiments.

Establishment of 24-hour biofilms

Methods for growing and evaluating biofilms were derived from those

described by Christensen et al (1985). Each test organism was cultured overnight in 10ml TSB and then diluted 1 in 1000 with sterile TSB; 200µl aliquots of this diluted culture were dispensed into wells in 96-well, flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) and incubated at 37°C for 24 hours without shaking to allow biofilms to establish (Figure 1). Inocula were routinely streaked out onto nutrient plates (Oxoid, Cambridge, UK) to confirm culture purity, and total viable counts (Miles and Misra, 1938) were performed to verify that a consistent inoculum size was used for each experiment (approximately 5×10^5 CFU/ml).

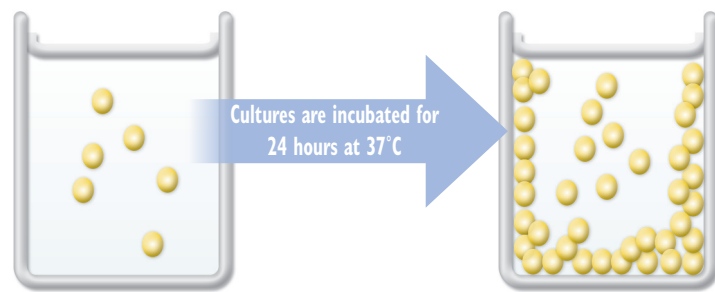
Biofilm evaluation

The extent of biofilm growth or biomass (Figure 2a) was estimated by gently removing and discarding planktonic growth (the liquid phase) from each well of the microtitre plate (Figure 2b), and fixing all remaining adherent biofilm with 2.5% glutaraldehyde (Fluka, UK) for five minutes to prevent further growth (Figure 2c). The fixative was removed and wells were washed twice with phosphate buffered saline (PBS; Oxoid, Cambridge, UK) (not shown in Figure 2). Biofilm was then stained with 0.25% crystal violet for five minutes (Figure 2d) and gently washed eight times using a multichannel pipette (Figure 2e). Stained microtitre plates were dried overnight, dye was solubilised from the stained biofilm in 200µl of solvent 1:1 acetone: absolute ethanol (Figure 2f), and 20µl of the resulting solution was added to 180µl of solvent contained in wells of a second corresponding microtitre plate. Absorbance was determined at 570nm using the Infinite M200 microtitre plate reader (Tecan, Switzerland) to indicate the extent of biofilm biomass (illustrated in Figure 3).

Biofilm inhibition

The effect of Activon Manuka honey on biofilms of each of the three test organisms was tested in three different experiments.

To determine whether Activon Manuka honey could prevent the formation of biofilm and to determine



Time 0: bacterial cells are introduced into nutritive medium (TSB)

24 hours later, attached cells represent biofilm and unattached cells are planktonic cells

Figure 1. Biofilm formation.

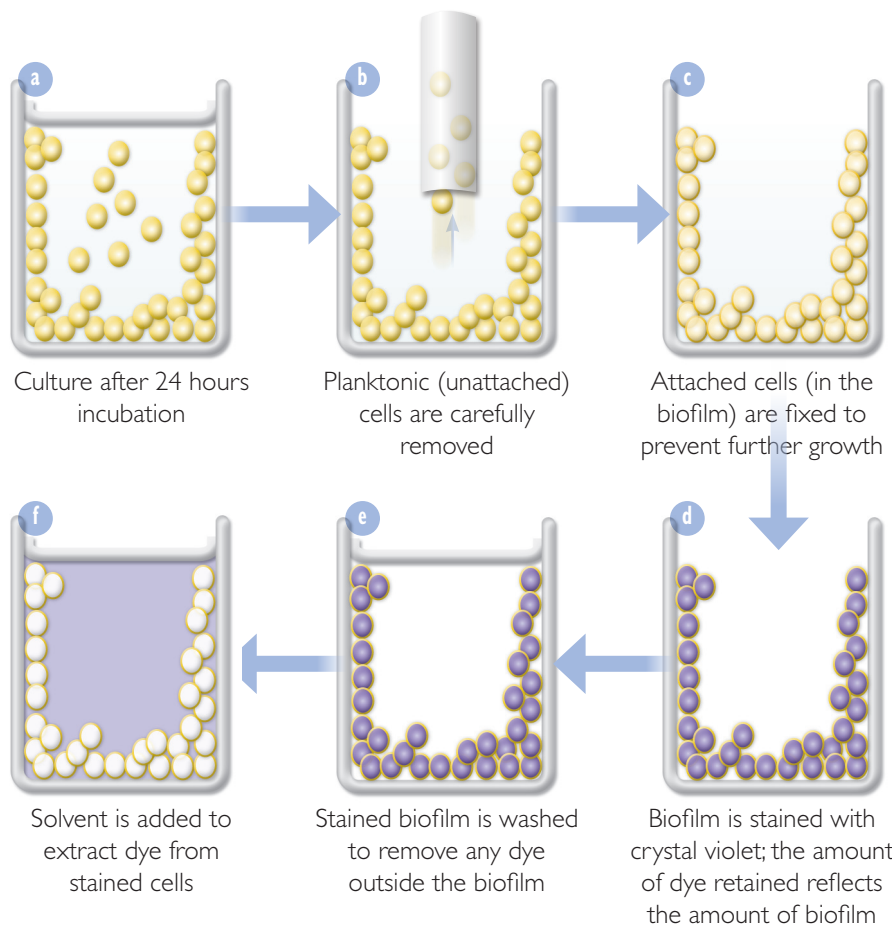


Figure 2. Biofilm evaluation.

the lowest concentration of honey capable of preventing biofilm formation, a range of concentrations of Activon Manuka honey varying by increments of 1% (w/v) were prepared in TSB, mixed with diluted inoculum and incubated in microtitre plates for 24 hours at 37°C (Figure 1). Final Activon Manuka honey concentrations between 1 and 20% (w/v) were used, and each honey concentration was tested in at least seven wells in each microtitre plate.

At the same time, seven wells in each microtitre plate contained no honey (as a positive control) and seven wells contained neither honey nor test bacteria but TSB alone (negative control). After incubation, the extent of biofilm in each well was determined (Figure 2) and mean values minus the negative control were plotted versus honey concentration (Figures 4, 5 and 6). Separate microtitre plates were used for each test organism.

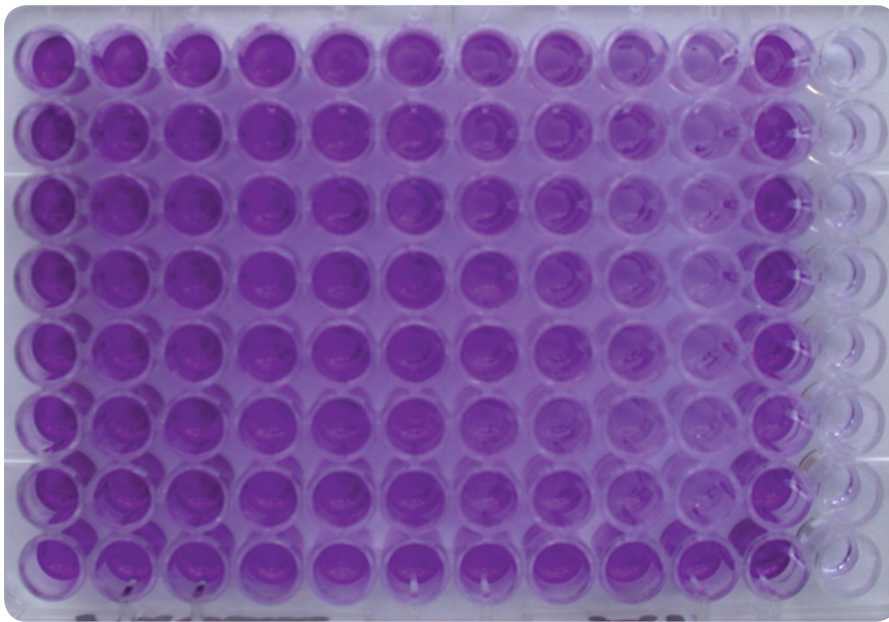


Figure 3. A microtitre plate ready for analysis in the plate reader.

To investigate the effects of Activon Manuka honey on established biofilms, 24-hour biofilms of each of the test organisms were allowed to form in wells in separate microtitre plates as described above (Figure 1). Then planktonic organisms were carefully removed from each well without disturbing adherent cells and replaced by fresh TSB containing concentrations of Activon Manuka honey ranging from 0 to 50% (w/v) and incubated for 24 hours. The extent of biofilm in each well was quantified as described above (Figure 2) and plotted versus honey concentration (Figures 7, 8 and 9).

To investigate the effect of contact time of Activon Manuka honey on established biofilms, biofilms of each of the test organisms were formed in wells in separate microplates (Figure 1). Planktonic bacteria were removed as described above. Into wells in one microtitre plate of each test organism, 200µl TSB alone was dispensed and labelled untreated. Into wells in a second microtitre plate of the same bacterium, 200µl TSB containing 40% (w/v) Activon Manuka honey was dispensed and labelled honey. The time at which the wells were refilled was noted and all plates were incubated at 37°C. At known time intervals (1, 2, 3, 4, 5, 6 and 24 hours), the amount of biofilm in eight wells of each microtitre

plate was assayed (Figure 2) and plotted versus time (Figures 10, 11 and 12).

Experiments were performed in duplicate.

Results

Prevention of biofilm formation

In this experiment, suspensions of test bacteria were inoculated into a range of concentrations of Activon Manuka honey contained in nutritive broth (TSB) and incubated at 37°C for 24 hours to determine whether biofilm formed. Concentrations above 10% (w/v) prevented biofilm formation in all of the bacteria tested (Figures 4, 5 and 6). The ability of Activon Manuka honey to inhibit the establishment of biofilm was dependent on concentration; by comparing the amount of biofilm formed in wells without honey, it was evident that concentrations of honey below 4, 5 and 10% (w/v) for MSSA, MRSA and VRE, respectively, encouraged biofilm development.

Effect of honey on established biofilms

Similarly, when 24-hour established biofilms were exposed to varying concentrations of Activon Manuka honey, the extent of biofilm biomass for each of the test bacteria was markedly reduced after 24 hours

contact with honey concentrations greater than 10% (w/v), but enhanced at concentrations less than 10% (w/v) (Figures 7, 8 and 9). It was seen that higher concentrations of Activon Manuka honey were required to disrupt established biofilms than those required to prevent biofilm formation.

Effect of contact time on established biofilms exposed to an inhibitory concentration of honey

In order to monitor the effectiveness of Activon Manuka honey with time, 24-hour established biofilms were incubated with and without 40% (w/v) Activon Manuka honey for varying time intervals and biofilm biomass was determined. This concentration was chosen because it had been found to inhibit established biofilms of each of the test organisms (Figures 7, 8 and 9). The extent of biofilm biomass was determined after exposure to 40% (w/v) Activon Manuka honey for 1, 2, 3, 4, 5, 6 and 24 hours for each organism included in the study. The results plotted in Figures 10, 11 and 12 show biofilm biomass at varying sampling times. Data derived from the plate with untreated wells is shown in blue, and the biofilm biomass for the microtitre plate treated with 40% (w/v) Activon Manuka honey is given in purple. After 24-hour exposure to honey, the amount of biofilm detected for each test organism was markedly reduced compared to untreated established biofilm and lower than at the start of the experiment. The extent of inhibition of VRE-honey treated biofilms (Figure 12) was greater than that of either MSSA honey-treated biofilm (Figure 10) or MRSA honey-treated biofilms (Figure 11). Some loss of biofilm even in untreated wells was noted within the first hour of each experiment, which was probably due to the removal of planktonic growth and the addition of fresh broth at the start of the inhibition period.

Discussion

To date, four laboratory studies have been published in which the effect of honey on biofilms has been studied (Merckoll et al, 2009; Alandejani et al, 2009; Okhiria et al, 2009; Lerrer et al, 2007).

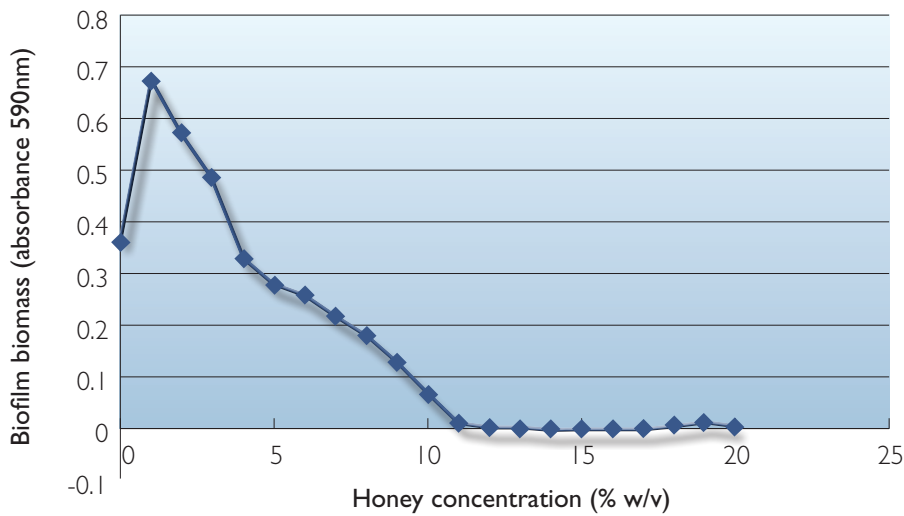


Figure 4. The effect of Activon Manuka honey in preventing biofilm formation by MSSA.

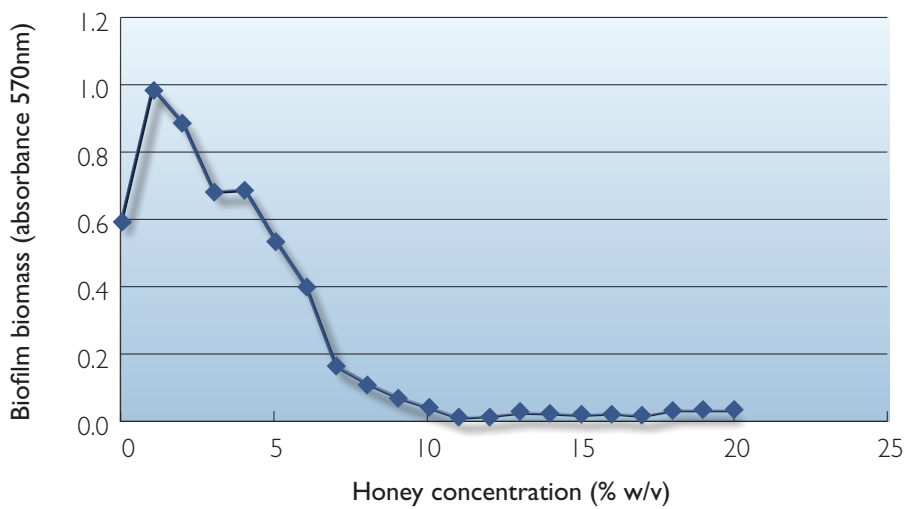


Figure 5. The effect of Activon Manuka honey in preventing biofilm formation by MRSA.

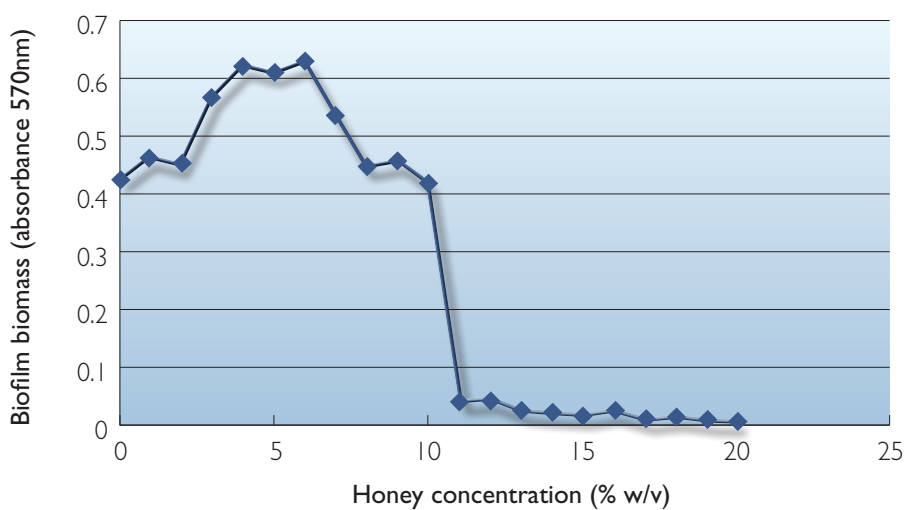


Figure 6. The effect of Activon Manuka honey in preventing biofilm formation by VRE.28

It was demonstrated that a Norwegian forest honey and Medihoney™ reduced the growth rate of planktonic cultures of each of four bacteria (MRSA, methicillin-resistant *Staphylococcus epidermidis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) in a concentration-dependent manner, with noticeable effects detected at concentrations as low as 0.8% (v/v) (Merckoll et al, 2009). The concentrations required to inhibit each of the test organisms grown as planktonic organisms were found to be half those required to inhibit established biofilms. Also, biofilm formation in MRSA was prevented by 3 and 6% (v/v) Medihoney and Norwegian honey, respectively (Merckoll et al, 2009).

Honey concentration can be expressed as % (w/v) or % (v/v). To convert honey concentration from % (v/v) to % (w/v), the density of each honey sample must be known. As an example, the density of manuka honey is 1.37, so 0.8% (v/v) is approximately equivalent to 1.1% (w/v).

The potential of honey for treating chronic rhinosinusitis was investigated by screening four honey samples in the Calgary biofilm model against four clinical isolates obtained from a childrens' hospital. One manuka honey and one Yemeni honey sample were then selected for further testing with 10 clinical isolates of each of MRSA, MSSA and *P. aeruginosa*, together with one reference strain of each. Susceptibility of established biofilms was determined with 50% honey solutions in the model, but whether % (v/v) or % (w/v) concentrations were used was not explained. The honeys were shown to be more effective in inhibiting planktonic bacteria than biofilms, and comparison with a similar study that had previously been performed in the same laboratory suggested that honey was more effective than antibiotics in disrupting biofilms (Alandejani et al, 2009).

Manuka honey has been shown to inhibit established biofilms of six clinical isolates of *P. aeruginosa* in a time- and concentration-dependent way

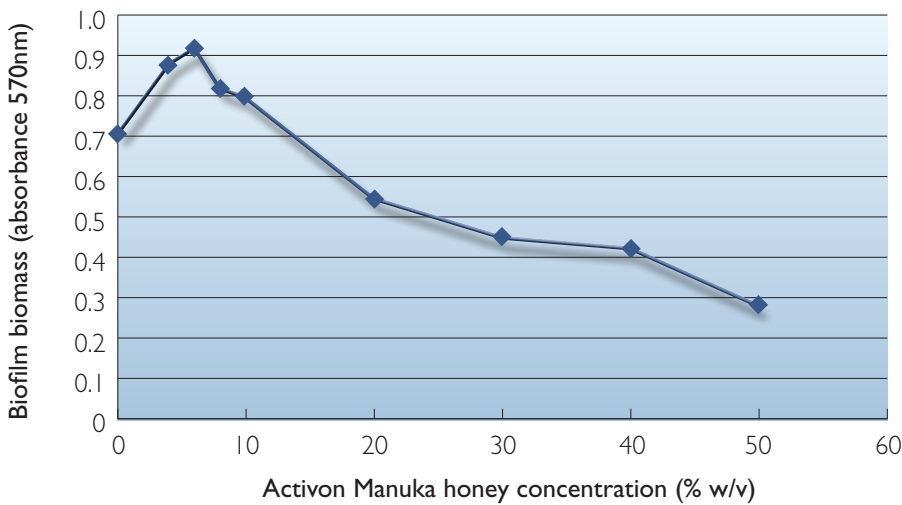


Figure 7. Effect of Activon Manuka honey on established biofilm of MSSA.

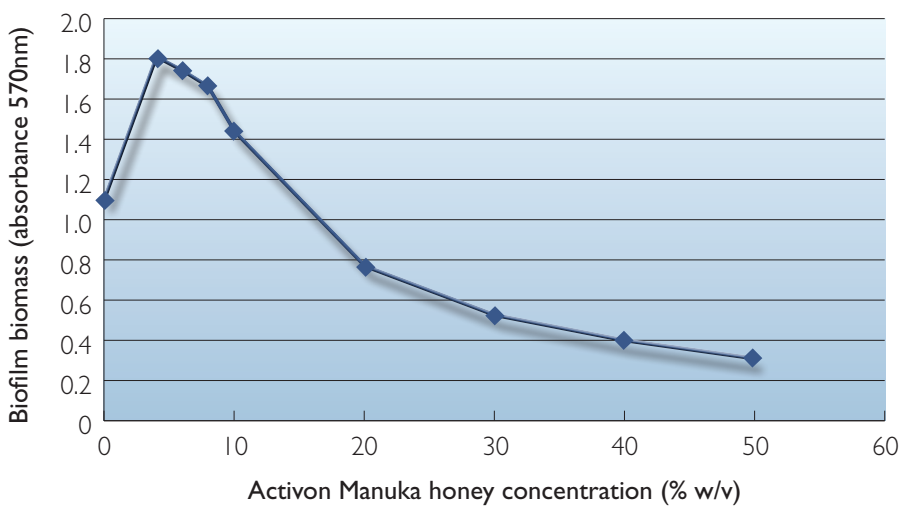


Figure 8. Effect of Activon Manuka honey on established biofilm of MRSA.

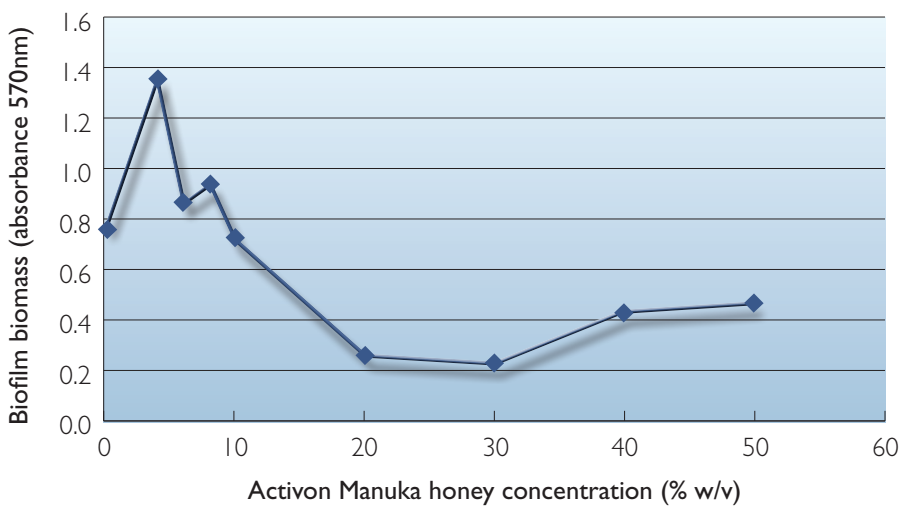


Figure 9. Effect of Activon Manuka honey on established biofilm of VRE.

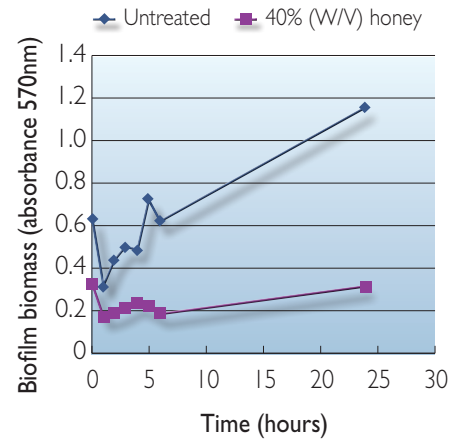


Figure 10. Survival curve of MSSA biofilm incubated with and without 40% (w/v) Activon Manuka honey.

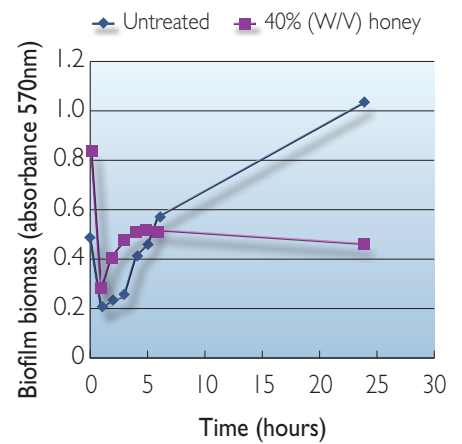


Figure 11. Survival curve of MRSA biofilm incubated with and without 40% (w/v) Activon Manuka honey.

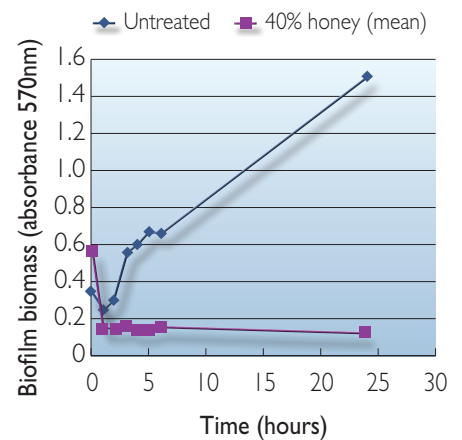


Figure 12. Survival curve of VRE biofilm incubated with and without 40% (w/v) Activon Manuka honey.

and exposure to 40% (w/v) manuka honey for 9 to 11 hours was found to be an effective inhibitory regime *in vitro* (Okhiria et al, 2009). A mechanism to explain how honey might inhibit biofilm formation in *P. aeruginosa* has been proposed (Lerrer et al, 2007). The fructose contained in four honey samples was shown to block binding mediated by PA-III lectin. Since PA-III lectin is an adhesin that is involved in binding *P. aeruginosa* to the fucosylated receptors on the surface of potential target host cells, it is important in initiating infection. It also facilitates binding of *P. aeruginosa* cells to each other, contributing to the initiation of biofilm formation.

The data reported in the authors' study supports that of previous studies. Namely, that manuka honey not only prevents biofilm formation, but also inhibits established biofilms in a time- and concentration-dependent manner.

As with *P. aeruginosa* biofilms (Okhiria et al, 2009), Activon Manuka honey concentrations below 10% promoted the growth of established biofilms of MSSA, MRSA and VRE. Furthermore, concentrations above 10% were found to prevent biofilm formation of each of the test organisms and 40% honey inhibited established biofilms over a 24-hour period. Since Activon Manuka honey is unlikely to be diluted by factors greater than ten in a wound, this is unlikely to be a problem in clinical use, but it emphasises the need to apply and maintain manuka honey at an appropriate concentration *in vivo*. However, honey should not be diluted before topical application.

In vitro tests with planktonic cultures indicate that MSSA and MRSA are more susceptible to manuka honey than VRE (Cooper et al, 1999; Cooper et al, 2002), but marked differences in the susceptibility of established biofilms of these bacteria to manuka honey have not been demonstrated in this study. Although honey has been shown to interfere with binding of *P. aeruginosa*, the mechanisms of biofilm inhibition by honey in staphylococci and enterococci is unknown and further investigation is needed.

The susceptibility of established biofilms to antibiotics has been shown to be reduced by a factor of 500 compared to planktonic forms (Stewart and Costerton, 2001), making the eradication of biofilms from patients especially difficult. In this study, three clinical isolates that exhibited antibiotic resistance have been found to be inhibited by relatively low concentrations of Activon Manuka honey, and the susceptibility of biofilms to honey was found to differ to the susceptibility of planktonic cultures by a factor of less than ten. An evaluation of the clinical efficacy of Activon Manuka honey in treating wounds with biofilms is now indicated. **WUK**

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Key points

- ▶▶ Inhibition of established biofilms of staphylococci and enterococci by Activon Manuka honey was influenced by contact time and concentration.
- ▶▶ Relatively low concentrations of Activon Manuka honey prevented biofilm formation in MSSA, MRSA and VRE.
- ▶▶ Planktonic bacteria are more susceptible to manuka honey than biofilms, but the susceptibility of biofilms of MSSA, MRSA and VRE differed to that of planktonic cultures by a factor of less than ten.
- ▶▶ Clinical studies to evaluate the potential of Activon Manuka honey to limit biofilm infections in wounds are needed.

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