Manuka honey inhibits adhesion and invasion of medically important wound bacteria in vitro

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Aim: To characterize the effect of manuka honey on medically important wound bacteria in vitro, focusing on its antiadhesive properties. Materials & methods: Crystal violet biofilm assays, fluorescent microscopy, protein adhesion assay and gentamicin protection assay were used to determine the impact of manuka honey on biofilm formation, human protein binding and adherence to invasion into human keratinocytes. Results: Manuka honey effectively disrupted and caused extensive cell death in biofilms of Staphylococcus aureus, Pseudomonas aeruginosa and Streptococcus pyogenes. Sublethal doses of manuka honey inhibited bacterial adhesion to the fibronectin, fibrinogen and collagen. Manuka honey impaired adhesion of laboratory and clinical isolates of S. aureus, P. aeruginosa and S. pyogenes to human keratinocytes in vitro, and inhibited invasion by S. pyogenes and homogeneous vancomycin intermediate S. aureus. Conclusion: Manuka honey can directly affect bacterial cells embedded in a biofilm and exhibits antiadhesive properties against three common wound pathogens.

Surgical site infections account for approximately 25% of all hospital or healthcare-acquired infections, and nonhealing wounds (as a result of infection) comprise 2–4% of all healthcare expenses [1,2]. Wounds are host to a wide range of microorganisms and, even in the absence of infection, are colonized with bacteria. The most commonly isolated wound pathogens include staphylococci, streptococci, clostridia, pseudomonads, coliforms and anaerobes. Uncomplicated wound infections, if not treated in a timely and appropriate manner, can rapidly develop into systemic or invasive infection due to the breakdown of the skin and mucosal membranes, with potentially fatal consequences.

Chronic or persistent wound infections are often associated with the presence of a biofilm, which hinders the wound healing process [3,4]. Biofilm formation is a multistep process relying upon adhesion, proliferation and the development of a stable community; however, the capacity for microorganisms to form a biofilm can differ greatly [5]. Surface adhesins facilitate adherence of pathogens to target host cells via pili or cell wall surface proteins, and are regarded as part of the bacterial virulence repertoire. Many wound-associated pathogens are also invasive, a process that is mediated by the secretion of extracellular invasins, such as collagenase, streptokinase, phospholipase C and hyaluronidase, and results in extensive tissue damage.

In recent years, emerging antibiotic-resistant pathogens have forced healthcare providers to search for alternative forms of treatments. Multidrug-resistant bacteria are often isolated from wounds and have resulted in an unexpected rise in wound infection, sepsis and associated death worldwide. Manuka honey has had a valued place in traditional medicine for many years. It has a broad-spectrum antibacterial activity and has been reported to be effective against numerous species of bacteria, which includes methicillin-resistant Staphylococcus aureus (MRSA), Lancefield groups A, C and G streptococci, vancomycin-resistant enterococci, Pseudomonas aeruginosa and Actinomyces spp. [6,7]. The precise antimicrobial mechanisms of manuka honey are only just starting to be unravelled and it appears that cellular damage is mediated through what appear to be genus-specific processes [8–11].

Studies of Streptococcus mutans and uncharacterized isolates of Streptococcus pyogenes demonstrate that manuka honey disrupts biofilm development [12,13]. Recent evidence has shown that sublethal doses of manuka honey specifically inhibit binding of S. pyogenes M28 to human fibronectin and that this process is in part mediated by a concomitant reduction in the

Keywords

- biofilm
- collagen
- fibrinogen
- fibronectin
- Pseudomonas aeruginosa
- Staphylococcus aureus
- Streptococcus pyogenes
expression of two major surface-bound fibronectin proteins, SfbI and Sof [14]. These proteins are known to mediate biofilm development.

No current paradigm exists that coherently describes the antimicrobial mechanisms of manuka honey against bacterial biofilms. Many studies have focused on uncharacterized clinical isolates, and only a few on well-characterized bacterial strains. This work provides a direct comparison between well-characterized laboratory strains of bacteria and their uncharacterized clinical isolate counterparts, thus highlighting similarities/differences in the effect of manuka honey treatment. The aim was to ascertain whether there is a common mechanism of biofilm disruption and inhibition of protein binding for the wound pathogens *S. pyogenes*, *S. aureus* and *P. aeruginosa*. In addition, this is the first study in which manuka honey has been demonstrated to impact upon the adhesion and invasion of wound-associated pathogens into human keratinocytes, which is highly relevant to manuka honey’s application as a topical antimicrobial to treat wound infection and demonstrates, for the first time, this possible mode of action in vitro.

**Materials & methods**

**Bacterial strains & growth conditions**

MRSA (NCTC13142), methicillin-sensitive *S. aureus* (MSSA; NCTC6571), homogeneous vancomycin intermediate *S. aureus* (VISA; clinical isolate) and heterogeneous VISA (hVISA; clinical isolate) were cultured in Mueller–Hinton media. *S. pyogenes* MGAS6180 and the clinical isolate *S. pyogenes* 74721 were cultured in Tryptic soy broth; it should be noted that strain MGAS6180 is used only in the adhesion and invasion assays, as data regarding biofilm and protein binding have been previously established [14]. *P. aeruginosa* NCIMB 8626 (ATCC 9027) and the clinical isolate *P. aeruginosa* 867 were cultured in nutrient broth. All cultures were incubated at 37°C, aerobically throughout the study.

**Manuka honey**

Sterile medical-grade manuka honey was provided in 50-g portions (Medihoney™; Comvita, London, UK).

**MIC**

The MIC for manuka honey against planktonic bacteria was established using serial dilution (0–50% weight/volume [w/v]) in 2% increments in 5 ml of appropriate media (Oxoid, Cambridge, UK; according to the British Society for Antimicrobial Chemotherapy methodology for determining MIC [15]). Cultures were incubated aerobically for 16 h at 37°C.

**Static biofilm model**

Bacterial strains were initially grown for 16 h and then harvested by centrifugation and adjusted to an optical density (650 nm = 0.1). To ascertain the ability of manuka honey to prevent biofilm formation, biofilms were cultured in 96-well microtiter plates (Greiner, Gloucester, UK) in 50 µl of appropriate media, supplemented with manuka honey (0–60% w/v in 2% increments), by inoculating each well with 5 µl of harvested cells. Plates were incubated aerobically at 37°C for 48 h. Biomass was determined by aspirating and discarding nonadherent cells; the remaining adherent bacteria were washed twice with phosphate-buffered saline (PBS) and then stained for 10 min with crystal violet (0.25% w/v). Following an additional two washes with PBS, cell-bound crystal violet was resolubilized with 7% acetic acid and the absorbance measured at 595 nm (A595) [16].

**Live/dead staining**

Biofilms were grown on glass coverslips in 24-well microtiter plates, essentially as described above. Media and planktonic cells were removed by aspiration and biofilms were stained using backlight FilmTracer™ (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Images were collected for untreated biofilms, biofilms grown in the presence of manuka honey and established biofilms treated with manuka honey for 2 h to determine the effect of manuka honey on viability. The concentration of manuka honey used varied for each bacterium studied and was established using the crystal violet assay, described above. Fluorescence microscopy imaging used a Nikon Eclipse 80i fluorescent microscope (Nikon, Surrey, UK) with oil immersion (Nikon, Surrey, UK) with oil immersion and ×100 lens. SYTO 9 detection (green channel; detects live cells) used a 488-nm excitation and 520-nm emission filter. Propidium iodide detection (red channel; detects dead cells) used a 543-nm excitation and 572-nm emission filter. Volocity Software was used for image analysis (PerkinElmer Inc., Cambridge, UK).

**Protein binding assays**

The outcome of manuka honey treatment on the ability of bacteria to adhere to immobilized fibronectin, fibrinogen or collagen was established by means of crystal violet assay (as...
plates and incubated at 37°C with 5% CO₂.

were seeded at 500,000 cells/well in 24-well microplates (Nunc High-Bind; Thermo Scientific, Hemel Hempstead, UK) were coated with a solution of each protein dissolved in coating buffer (20 mM of Na₂CO₃, 20 mM of NaHCO₃; pH: 9.3) to achieve a concentration of 1 µg per well. This is the optimal concentration for bacterial adhesion (data not shown). Manuka honey was dissolved in Tris-buffered saline (pH: 7.4) to attain concentrations corresponding to the MIC for each bacterial strain used to determine whether binding was inhibited. Bacterial cells were cultured for 16 h prior to the assay, harvested as described, then re-suspended in Tris-buffered saline, with or without manuka honey. Subsequently, 50 µl of bacterial suspension was added to the protein-coated microtiter plate. A PBS control was used as a comparison for the absorbance values obtained (A₅95) throughout the assay. The assay also included a control containing only coating buffer, to which no protein ligands were added, to determine protein-coating efficiency. Triplicate sets of biological replicates were used for each experiment and each assay was repeated three-times; statistical analysis utilized Minitab (version 14; Student’s t-test; Minitab Ltd, Coventry, UK).

Cell culture
The human keratinocyte cell line HaCaT was grown in Dulbecco's modified Eagle medium (DMEM) at 37°C and 5% CO₂. Cells were subcultured once a week.

Adhesion & invasion assays
The assays followed the method of Rasigade et al. [19] with some modifications. HaCaT cells were seeded at 500,000 cells/well in 24-well plates and incubated at 37°C with 5% CO₂ for 48 h in culture medium. Bacterial strains were grown for 16 h in appropriate medium and harvested by centrifugation for 5 min at 13,000 x g. Bacterial cells were resuspended in DMEM. Honey-treated bacterial cells were resuspended in DMEM, supplemented with the appropriate concentration of manuka honey. All honey concentrations corresponded to the MIC for planktonic bacteria. HaCaT cells were washed twice with DMEM and infected with bacterial cells at a multiplicity of infection of 200:1, as confirmed by bacterial count. Infected cells were incubated for 2 h at 37°C to enable adhesion and internalization of bacteria; they were subsequently washed with PBS to remove any unbound bacterial cells. For adhesion assays, pure water was used to osmotically shock cells to release cell-associated bacteria. For invasion assays, infected cells were incubated for a further 1 h in culture medium containing 200 µg ml⁻¹ gentamicin to kill extracellular, but not internalized, bacteria. Cells were then washed twice in PBS and treated with pure water as described above to release internalized bacteria. Bacterial enumeration used the method of Miles et al. [20]. To calculate the number of adherent bacteria, the number of internalized bacteria was subtracted from the total number of cell-associated bacteria.

Results
MICs of manuka honey for planktonic & biofilm growth
The MIC varied between each bacterial species, as well as between strains of the same species, and was consistently higher for bacterial biofilms than planktonically grown cells (Table 1). Clinical isolates did not show a consistently higher or lower resistance to manuka honey compared with the laboratory strains.

Manuka honey causes biofilm disruption & bacterial killing
For all of the bacteria studied, growth in the presence of manuka honey equivalent to the concentration that caused reduced biofilm development (MIC for biofilms [MIC-B]) correlated with an observed decrease in biomass and a reduction in the number of both adherent and viable bacterial cells in established (48 h) biofilms (Figure 1). Bacterial biomass was higher in biofilms that had been cultured for 48 h and then treated with manuka honey for 2 h compared with those that had been grown in the presence of manuka honey, suggesting that cells were perhaps killed but not detached from the established biofilms. Live/dead staining supported this, as more cells were visible in the established, treated biofilms compared with those grown in the presence of manuka honey, and a higher proportion of these cells were viable (Figure 1). MRSA appeared to be more resistant to honey treatment than MSSA; however, both VISA and hVISA exhibited equal or more susceptibility to treatment than MSSA (Figure 1A-D & Table 2). The observed reduction in biomass was compared for all three conditions with two-way analysis of variance and post-hoc analysis using Tukey’s method; in each case, the reduction was found to be statistically significant (Table 2).

Biofilms of S. pyogenes were effectively inhibited and disrupted using 30% (w/v) manuka honey (Figure 1E); this supports previous data
showing that the invasive strain MGAS6180 is also inhibited using this concentration (Table 1) [14]. Again, the reduction in biomass for established biofilms was less than that of those grown with manuka honey. However, live/dead staining showed similar numbers of bacterial cells, with only a few remaining viable following both manuka honey treatments, and very little biofilm structure; more nonviable cells were present in the established biofilms that were treated for 2 h, suggesting that perhaps nonviable cells comprised much of the biomass observed by crystal violet staining.

The clinical isolate of P. aeruginosa was more susceptible to honey treatment than the laboratory strain and also exhibited a smaller reduction in biofilm biomass for established biofilms than for those grown in the presence of manuka honey (Figure 1F & G). Numerous cells were visible by live/dead staining under both treatment conditions, suggesting that there was limited bacterial detachment as a consequence of manuka honey treatment, despite attached cells being mainly comprised of nonviable organisms (Figure 1F & G & Table 2).

Manuka honey inhibits bacterial adhesion to human wound-associated proteins

In all cases, the isolates adhered to the microtiter plate better when coated with each of the proteins than without them (data not shown). A statistically significant (p < 0.05) reduction in binding of MRSA to fibrinogen, fibronectin and collagen was observed for honey-treated cells (Figure 2A). For MSSA, a similar reduction was observed, but for fibronectin and collagen only (Figure 2B). VISA exhibited similar fibrinogen and collagen binding to both MRSA and MSSA; however, honey treatment did not result in a significant reduction in binding. A reduction in fibronectin binding (p < 0.05) was evident following honey treatment, but was less than the observed effect for other S. aureus (Figure 2C). hVISA exhibited a relatively poor capacity for binding to fibrinogen and collagen, which was unchanged following honey treatment, but binding to fibronectin was inhibited (p < 0.05) and to a similar extent to that observed for MRSA and MSSA (Figure 2D).

S. pyogenes bound well to each protein ligand, and binding to each was significantly reduced (p < 0.05 in each case) with honey treatment (Figure 2E). Manuka honey has been previously shown to inhibit binding of S. pyogenes MGAS6180 to fibronectin, but this is the first time a reduction in binding to fibrinogen and collagen has been observed.

The laboratory strain of P. aeruginosa bound to each of the protein ligands better than the clinical strain and showed a statistically significant reduction in binding to each one following honey treatment (Figure 2F). For the clinical isolate, there was a slight increase in binding to fibronectin, but this was not statistically significant (p > 0.05); both collagen and fibrinogen binding were significantly reduced (p < 0.05) (Figure 2G).

Manuka honey inhibits adhesion & invasion into human keratinocytes

All of the isolates adhered well to the keratinocytes (Figure 3). Following honey treatment, there was a statistically significant reduction

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC-B-eliminating concentration (manuka honey, %)</th>
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<tbody>
<tr>
<td></td>
<td>Planktonic cells, MIC</td>
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<tr>
<td>Staphylococcus aureus EMRSA-15 (NCTC13141)</td>
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<td>Homogeneous vancomycin intermediate S. aureus</td>
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<td>Heterogeneous vancomycin intermediate S. aureus</td>
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<tr>
<td>Streptococcus pyogenes MGAS6180</td>
<td>20</td>
</tr>
<tr>
<td>S. pyogenes (74721)†</td>
<td>20</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 9027 (8626)</td>
<td>30</td>
</tr>
<tr>
<td>P. aeruginosa (867)†</td>
<td>10</td>
</tr>
</tbody>
</table>

All findings are from the present study unless otherwise indicated.

†Clinical isolates.

EMRSA: Epidemic methicillin-resistant Staphylococcus aureus; MIC-B: MIC for biofilms.
(p < 0.05) in binding for each Staphylococcus strain, but a reduction in bacterial internalization was only seen for VISA (Figure 3A-D). The S. pyogenes clinical isolate exhibited reduced binding and internalization following manuka honey treatment; in this experiment, the S. pyogenes laboratory strain MGAS6180 was included as a comparison for the clinical isolate (74721), as this is a novel set of experiments, and no other relative data set is currently available for comparison. The characterized laboratory strain also showed a statistically significant (p < 0.05) reduction in both adherence and internalization following manuka honey treatment and at similar levels to those observed for the clinical isolate (Figure 3E & F). For the two P. aeruginosa strains, there was a reduction in binding to keratinocytes following honey treatment; however, a reduction in internalization was only observed for the clinical isolate (p < 0.05) (Figure 3G & H & Table 3).

**Discussion**

Bacterial biofilms are a major barrier to healing and play a significant role in chronic, recalcitrant wound infection [21–23]. The damaged epithelia of patients with underlying medical conditions provide an ideal environment for bacteria to form a biofilm, which affords a physiological niche in which bacteria exhibit intrinsic resistance to the killing effects of antimicrobial agents [24,25]. While biofilm development is not a prerequisite for persistent infection, eradication of biofilm-based infections is particularly difficult, as treatments cannot always effectively permeate the extracellular polymeric substance (EPS); slow growing persister cells comprising up to 1% of the population are found embedded deep within the biofilm and are tolerant to high concentrations of antimicrobials [22,26].

In this study, manuka honey was able to effectively prevent biofilm formation of S. aureus, S. pyogenes and P. aeruginosa. In every instance, the MIC was higher than that required to inhibit planktonic bacterial growth; this is in keeping with current hypotheses [27,28]. The data strongly suggest that the observed reduction in biomass and decreased numbers of bacterial cells observed by fluorescence microscopy resulted from the combined effects of inhibition of growth, cell death and impaired adhesion. Jenkins et al. have previously shown that the growth rate of MRSA is reduced following honey treatment [30]; a phenomenon that was also observed for S. pyogenes [14]. In addition, a recent study using P. aeruginosa has shown that extensive cell damage occurs following treatment with subinhibitory concentrations of manuka honey, which impairs growth and abrogates microcolony development, which is an adhesion-dependent process similar to that seen in early-stage biofilm development [29].

The resistance of biofilms can be attributed in part to the preclusion of the antimicrobial from the target site because of limited diffusion through the biofilm matrix [30–32]. The EPS layer also impedes biofilm penetration; it is known that some antibiotics, such as ciprofloxacin, cannot pervade the EPS layer of the biofilm, but others, such as vancomycin and rifampicin, can [33,34]. In established biofilms of S. aureus, S. pyogenes and P. aeruginosa manuka honey (at concentrations equivalent to the MIC-B) appeared to be able to permeate the biofilm structure, mediating a reduction in biomass. For S. aureus and S. pyogenes, a quite extensive disruption of biofilm structure was observed, whereas for P. aeruginosa, the biofilm structure was mostly retained following honey treatment, even though the remaining bacterial cells were largely nonviable (Figure 1). Given that the application of manuka honey was observed to kill biofilm-embedded bacterial cells that had been growing for 48 h, it is likely than honey or some of its bactericidal components permeated the EPS and gained access to the biofilm-embedded cells.

Manuka honey treatment did not completely eradicate the established biofilms of the three test bacteria and larger numbers of bacteria remained following treatment as compared with the biofilms grown in the presence of manuka honey; this was especially evident for P. aeruginosa. Similar studies to this one have investigated the efficacy of silver-impregnated dressings on bacterial biofilms and have likewise shown that treatment did not clear all bacterial cells, but that the remaining population was susceptible to subsequent antibiotic treatment [27]. It was inferred from these studies that silver dressings could be utilized most effectively in combination with antibiotics, and this might also be the case for manuka honey [35]. Merckoll et al. have similarly demonstrated the capacity for biocidal components of Medihoney™ and Norwegian honey to permeate biofilms of wound-associated bacteria, including MRSA and P. aeruginosa [36].

Adherence of potential pathogens to host cells or components of the extracellular material is a widespread strategy in establishing bacterial colonization, often followed by invasion...
Biofilm biomass (A595 nm)

Post-treatment

2.5
1.5
2
1
0.5
0

No honey
Honey
Post-treatment

Pseudomonas aeruginosa
ATCC 9027

Streptococcus pyogenes
74721

MRSA
MSSA
VISA
hVIS
AVISA
MSSA
P. aeruginosa 867

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has been observed adhesion to host cells via fibronectin bridges. ginosa are available regarding the capacity of Gram-positive microorganisms, far fewer data into associated tissues [37,38]. S. aureus express numerous surface-bound adhesins, collectively termed microbial surface components, recognizing adhesive matrix molecules; each adhesin apparently has specificity for a single host protein [39]. S. pyogenes similarly display a plethora of surface adhesins capable of recognizing numerous adhesive matrix molecules, which show affinity for more than one kind of extracellular material protein [40]. Compared to the Gram-positive microorganisms, far fewer data are available regarding the capacity of P. aeruginosa to bind to human proteins; however, adhesion to host cells via fibronectin bridges has been observed [41].

Despite the apparent diversity in attachment to host proteins for the microorganisms used in this study, it is possible that there is a common mechanism of action by which manuka honey inhibits adhesion of these pathogens to one or more of the human proteins fibronectin, fibrinogen and collagen. The amount of observed inhibition of binding differed between strains of the same microorganism and does not appear to be an 'umbrella' phenomenon. For example, the S. aureus strains used in this study each showed a statistically significant reduction in binding to fibronectin, but not all had reduced binding to fibrinogen or collagen, and any reduction in binding that was observed appeared to be strain specific. Similarly for P. aeruginosa, no reduction in binding to fibronectin was observed for the clinical isolate, but was evident for the laboratory strain.

It is understood that different strains of both S. pyogenes and S. aureus exhibit unique affinities for host proteins that are dependent upon the surface proteins they express; different strains of the same species do not always carry the same arsenal of surface adhesins [42,43]. It is likely that in the host wound environment, these microorganisms utilize fibronectin as a binding ligand prior to the development of a biofilm and that this process is disrupted by manuka honey treatment.

S. aureus is known to bind to collagen in a specific, saturable manner, but there is reported variability between the different strains with regards to the degree of binding observed, which is also seen here [44]. Evidence has previously shown that some strains of S. aureus carrying the mec gene, which confers resistance to methicillin, were defective in fibronectin binding; however, this does not appear to be the case for the strain used in this study [45]. Fibronectin is known to mediate adherence of S. pyogenes to eukaryotic cells, and fibronectin-binding proteins have been associated with biofilm accumulation and maturation [46].

P. aeruginosa has been found to bind poorly to epithelial cells that express a large amount of fibronectin, preferentially binding where lower levels are present; it is possible that binding is mediated by other protein ligands rather than fibronectin directly [41,47]. In this study, both strains of P. aeruginosa bound well to soluble, immobilized fibronectin, supporting the accepted understanding that this organism has an affinity for soluble fibronectin. Both strains also adhered well to human keratinocytes, but it is unknown by what mechanism this was

Table 2. Effect of manuka honey treatment either pre- or post-biofilm formation, expressed as a percentage of the untreated biofilm biomass for the seven isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Biofilm biomass of untreated biofilm (%)</th>
<th>Pretreated with honey</th>
<th>Postbiofilm formation treatment</th>
<th>Significant*</th>
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<tbody>
<tr>
<td>Staphylococcus aureus EMRSA-15 (NCTC13141)</td>
<td></td>
<td>7</td>
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<td></td>
<td>7</td>
<td>53</td>
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<td>7</td>
<td>72</td>
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<td>Streptococcus pyogenes (74721)†</td>
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<td>2</td>
<td>27</td>
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<td>Pseudomonas aeruginosa ATCC 9027 (8626)</td>
<td></td>
<td>2</td>
<td>43</td>
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<tr>
<td>P. aeruginosa (867)†</td>
<td></td>
<td>2</td>
<td>33</td>
<td>Yes</td>
</tr>
</tbody>
</table>

†Clinical isolates
*Output from Minitab (Minitab Ltd, Coventry, UK) analysis stated p-values of <0.0001. EMRSA: Epidemic methicillin-resistant Staphylococcus aureus.
Adherent cells (A_595 nm)

MRSA

MSSA

VISA

hVISA

Streptococcus pyogenes 74721

Pseudomonas aeruginosa ATCC 9027 (8626)

P. aeruginosa 867

PBS

No ligand

Fibronectin

Fibrinogen

Collagen

No honey treatment

Honey treatment
facilitated. Manuka honey treatment was able to disrupt binding to immobilized fibronectin and keratinocytes for the laboratory strain, but only the latter for the clinical isolate. Studies utilizing a nonmanuka honey have previously shown that adhesion and biofilm development by *P. aeruginosa* is disrupted by competitive blocking of mannose-binding lectins, but it is not known how fibronectin binding might be disrupted [48].

Some antibiotics are known to decrease bacterial attachment to human proteins, such as collagen or fibronectin; these include bacitracin, erythromycin, chloramphenicol and polymyxin B, which inhibit the attachment of *P. aeruginosa* and *S. aureus* to collagen and fibronectin [19,37,49]. Importantly, inhibition of bacterial binding has been observed using levels of antibiotics regarded as being subinhibitory for growth. Therefore, the effect observed for manuka honey treatment in this study supports the idea that antimicrobial treatments have the capacity to prevent bacterial attachment and therefore colonization and subsequent infection. It is the first time that manuka honey has been shown to exert this effect on a variety of diverse bacteria encompassing the firmicutes and γ-proteobacteria.

It is well documented that there is some level of redundancy among bacterial adhesins [5,50]. This may go some way to explaining the clear differences in binding to the protein ligands used in this investigation for each of the microorganisms studied, but does not explain why binding should be so uniquely affected by manuka honey. It is not known how attachment is inhibited, and it is possible that the observed reduction in binding might be due to differential expression of surface adhesins, as has been previously shown for *S. pyogenes* [14]. Further studies will be necessary to determine the precise mechanism by which adherence is disrupted.

The binding of pathogenic microorganisms to human tissue proteins is often a prerequisite for internalization and invasive disease [42,51]. This study demonstrates for the first time that manuka honey decreases adhesion of *S. pyogenes*, *S. aureus* and *P. aeruginosa* to human keratinocytes *in vitro*. Reduced binding is observed for both the characterized laboratory strains and clinical isolates of each bacteria studied. Epithelial cells are known to present proteins, such as fibronectin and fibrinogen, on their surface, and so the reduced binding may in part be facilitated by inhibited adherence to these surface-bound proteins, reflecting the protein-binding data described above. By inhibiting adhesion to soluble and surface-bound ligands, manuka honey has the potential to prevent bacterial binding to the wound bed and, in doing so, prevent colonization of the wound, offering an effective, prophylactic, anti-infective treatment.

In addition to reducing adherence to human keratinocytes, manuka honey also prevented the invasion of some of the bacterial strains studied into these cells *in vitro* and it is the first time that this phenomenon has been reported. Much like the observed inhibition of bacterial adhesion, this appeared to be both a species- and strain-specific occurrence. Where invasion was not inhibited, approximately the same number of adherent bacterial cells were internalized as compared with the untreated control. Of the *S. aureus* strains studied, only hVISA exhibited lower intracellular numbers (*p < 0.05*) following manuka honey treatment; for MSSA, MRSA and VISA, the reduction was negligible. Fibronectin-binding proteins are known to promote internalization of *S. aureus* into epithelial cells that are not usually phagocytic, but are not the only proteins that trigger the internalization process [42,52].

*S. pyogenes* MGAS6180 is a well-characterized Lancefield GAS laboratory strain and is known to cause invasive disease; it was used in the final part of this study as a comparison for the clinical isolate (Figure 3). Following manuka honey treatment, invasion of both strains of *S. pyogenes* was significantly reduced (*p < 0.05*). Previous studies have shown that manuka honey reduces the expression of SfbI, which might contribute to the reduction in the internalization observed during this study [64]. Only the clinical isolate of *P. aeruginosa* appeared to display reduced keratinocyte invasion following manuka honey treatment.
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**No honey treatment**

**Honey treatment**

<table>
<thead>
<tr>
<th></th>
<th>Adherent</th>
<th>Internalized</th>
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</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>8</td>
<td>7.5</td>
</tr>
<tr>
<td>MSSA</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>VISA</td>
<td>8</td>
<td>7.5</td>
</tr>
<tr>
<td>hVISA</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>S. pyogenes MGAS 6180</td>
<td>8</td>
<td>7.5</td>
</tr>
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<td>S. pyogenes 74721</td>
<td>8</td>
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</tr>
<tr>
<td>P. aeruginosa ATCC 9027 (8626)</td>
<td>6.5</td>
<td>6</td>
</tr>
<tr>
<td>P. aeruginosa 867</td>
<td>6.5</td>
<td>6</td>
</tr>
</tbody>
</table>

Legend:

- Red: No honey treatment
- Blue: Honey treatment

**Log_{10} CFU/ml**

- Adherent
- Internalized
P. aeruginosa is well known to invade human epithelial cells in vitro and in vivo [53]. Again, it is evident that manuka honey applied at the MIC has the capacity to prevent invasion of P. aeruginosa into human keratinocytes and thus could prevent invasive wound infection; this is especially significant as the clinical isolate rather than the laboratory isolate was affected. However, to determine whether clinical isolates are more susceptible to this process of inhibition, much larger studies encompassing numerous clinical isolates are necessary.

What is abundantly evident from this study is that manuka honey is effective in vitro at both preventing biofilm development and disrupting established biofilms of each of the microorganisms studied. Within the honey-treated biofilm, there is extensive cell death, which is concurrent with a loss in biomass. However, given that honey treatment did not result in complete clearance of established biofilms, this suggests that for chronic wound infections, manuka honey applied topically might be better used in conjunction with other systemic antibiotics. Chronic wounds are often comprised of polymicrobial flora, rather than single species, as described in this study. Microbial community profiling has demonstrated the presence of Gram-negative rods, Gram-positive cocci, aerobes and anaerobes in chronic wounds, forming highly organized biofilms [3,54,55]. Further studies of such polymicrobial biofilms will demonstrate whether manuka honey might be efficacious against multispecies biofilms and is currently the subject of extensive research by the authors.

**Conclusion & future perspective**

Taken collectively, the data presented here describe, for the first time, a paradigm in which manuka honey has the capacity to prevent bacterial adhesion in vitro via a mechanism that relies on inhibition of bacterial binding to host proteins found at the wound site and on the surface of keratinocytes, in some cases preventing bacterial invasion. Past studies have shown that bacterial manuka honey targets are unique to specific microorganisms and no common mechanism of action has been previously identified. This investigation has found that despite the precise molecular targets perhaps remaining diverse in terms of bacterial colonization, the mechanisms of antibacterial or antiadhesive action for manuka honey appear to be shared. Even so, diversity of function remains apparent, as is evident from the fact that for each microorganism studied, manuka honey inhibited binding to a distinct repertoire of human tissue proteins.

**Table 3. Reduction in adhesion to and invasion into human keratinocytes following treatment with sublethal doses of manuka honey.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Reduction in bacterial adhesion to human tissue proteins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Staphylococcus aureus EMRSA-15 (NCTC13141)</td>
<td>35</td>
</tr>
<tr>
<td>Methicillin-sensitive S. aureus</td>
<td>51</td>
</tr>
<tr>
<td>Vancomycin intermediate S. aureus†</td>
<td>14</td>
</tr>
<tr>
<td>Variable vancomycin intermediate S. aureus†</td>
<td>43</td>
</tr>
<tr>
<td>Streptococcus pyogenes (74721)†</td>
<td>67</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 9027 (8626)</td>
<td>56</td>
</tr>
<tr>
<td>P. aeruginosa (867)†</td>
<td>5 (increase*)</td>
</tr>
</tbody>
</table>

†Clinical isolates.  
*The observed increase is not statistically significant (p > 0.05).  
EMRSA: Epidemic methicillin-resistant Staphylococcus aureus.
Therefore, it is possible that if the process does not rely entirely on steric hindrance, at the molecular level, the expression of adhesins might be altered in response to manuka honey treatment, ultimately resulting in the reduced binding observed in this study. This paves the way for future studies to investigate the specific molecular changes that are taking place during these processes.

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**Executive summary**

**Manuka honey causes biofilm disruption & bacterial killing**
- Manuka honey prevents the formation of biofilms of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*.
- Manuka honey can effectively permeate biofilms of *P. aeruginosa*, *S. aureus* and *S. pyogenes*.
- Manuka honey can disrupt established biofilms of the above-named micro-organisms.

**Manuka honey inhibits bacterial adhesion to human wound-associated proteins**
- Sublethal doses of manuka honey prevent the binding of the above-named micro-organisms to human tissue proteins.
- Inhibition of binding to human tissue proteins by manuka honey appears to be a species- and strain-specific process.

**Manuka honey inhibits adhesion & invasion into human keratinocytes**
- Sublethal doses of manuka honey reduces the binding of all of the above-named micro-organisms, but inhibits invasion of only some.
- Low levels of manuka honey treatment impacts on bacterial adhesion without affecting viability.
- Manuka honey could represent a novel antivirulence and bactericidal treatment.

**References**
Papers of special note have been highlighted as:
- of interest
- **of considerable interest**


**** Describes, in detail, the role of the biofilm in chronic wound infection. It is the first study to suggest a link between wound chronicity and the presence of a biofilm.
18. Jakubovics NS, Brittan JL, Durton LC, Jenkinson HF. Multiple adhesin proteins on
Documents bacterial viability within the biofilm and provides an understanding of how bacteria persist within the biofilm environment.

29. Roberts AE, Maddocks SE, Cooper RA. Manuka honey is bactericidal against Pseudomonas aeruginosa and results in differential expression of oprF and algD. Microbiology 158, 3005–3013 (2012).


Thoroughly describes the way in which diffusion occurs within the biofilm community and how antimicrobial agents might penetrate the complex biofilm structure.


Describes how doses of antibiotics that are regarded as being subinhibitory to bacterial growth can still impact upon the organism’s ability to adhere to human proteins, therefore also impacting on the host–pathogen relationship during infection.


