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Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins

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Streptococcus pyogenes (group A Streptococcus; GAS) is always of clinical significance in wounds where it can initiate infection, destroy skin grafts and persist as a biofilm. Manuka honey has broad spectrum antimicrobial activity and its use in the clinical setting is beginning to gain acceptance with the continuing emergence of antibiotic resistance and the inadequacy of established systemic therapies; novel inhibitors may affect clinical practice. In this study, the effect of manuka honey on *S. pyogenes* (M28) was investigated *in vitro* with planktonic and biofilm cultures using MIC, MBC, microscopy and aggregation efficiency. Bactericidal effects were found in both planktonic cultures and biofilms, although higher concentrations of manuka honey were needed to inhibit biofilms. Abrogation of adherence and intercellular aggregation was observed. Manuka honey permeated 24 h established biofilms of *S. pyogenes,* resulting in significant cell death and dissociation of cells from the biofilm. Sublethal concentrations of manuka honey effectively prevented the binding of *S. pyogenes* to the human tissue protein fibronectin, but did not inhibit binding to fibrinogen. The observed inhibition of fibronectin binding was confirmed by a reduction in the expression of genes encoding two major fibronectin-binding streptococcal surface proteins, Sof and SfbI. These findings indicate that manuka honey has potential in the topical treatment of wounds containing *S. pyogenes*.

INTRODUCTION

Streptococcus pyogenes (group A Streptococcus) colonizes the nasopharynx and skin of healthy individuals, forming part of the commensal microbiota. Under appropriate conditions, *S. pyogenes* can be transmitted to wounds and is especially problematic after surgery, following skin grafting and for military personal with traumatic or puncture wounds. Wounds provide a route of entry to the host and damaged tissues display a matrix of proteins including collagen, albumin, fibronectin and fibrinogen, which collectively provide a plethora of ligands to which opportunistic pathogens, including streptococci, adhere (Kubo *et al.*, 2001). Surgical site infection accounts for approximately 25 % of all hospital-acquired infections and may result in the development of a non-healing or chronic wound (Werdin *et al.*, 2009). Non-healing wounds are defined as wounds that have failed to proceed through the normal, orderly and timely reparative process that results in restoration of anatomical and functional integrity, within 3 months (Cooper, 2005; Guo & Dipietro, 2010). In the developed world, approximately 1–1.5 % of populations have non-healing wounds, and these account for 2–4 % of all health care expenses (Gottrup, 2008).

Biofilms have been associated with persistent or chronic wound infections and are a major obstacle to healing (James *et al.*, 2008; Rhoads *et al.*, 2008). *Streptococcus* species readily form biofilms, by a process in which numerous cell-wall-anchored adhesins specifically attach to human tissue protein ligands and promote bacterial aggregation (Nobbs *et al.*, 2009; Maddocks *et al.*, 2011; Thenmozhi *et al.*, 2011). Examples of such surface adhesins include the pilus and F1/SfbI proteins which are encoded on the FCT (fibrinogen-collagen-T antigen) region of the genome as well as Sof, AgI/II and M protein, whose genes are located outside of the FCT region (Köller *et al.*, 2010). Streptococci differ in their capacity to form biofilms but in every instance, the process relies initially upon adhesion to a given substratum, followed by a period of proliferation prior to the establishment of a stable microbial community (Kreikemeyer *et al.*, 2011).

Honey has had a valued place in traditional medicine for many centuries and was reintroduced into modern medicine during the 21st century. Honey exhibits broad spectrum antibacterial activities and has been reported to inhibit more than 80 species of bacteria, including meticillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci,

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Lancefield groups A, C and G streptococci, *Pseudomonas aeruginosa* and *Actinomyces* species (Molan, 1992; Cooper *et al.*, 2002a, b; Blair *et al.*, 2009). The antibacterial properties of many honeys are associated with hydrogen peroxide but methylglyoxal has been shown to be important in manuka honey (Mavric *et al.*, 2008). The precise intracellular effects of manuka honey are beginning to be understood and bacterial cell damage is mediated via diverse processes that so far appear to be species-specific (Henriques *et al.*, 2010, 2011; Jenkins *et al.*, 2011a, b). Preliminary data derived from studies of *Streptococcus mutans* and clinical isolates of *S. pyogenes* have shown that biofilm development is disrupted by manuka honey, but the processes involved remain largely unknown (Badet & Quero, 2011; Cooper *et al.*, 2011). Therefore, this study was undertaken to determine the *in vitro* effect of manuka honey against biofilms of *S. pyogenes* (MGAS6180; M28), a well characterized strain associated with invasive disease and which has been the subject of numerous biofilm studies, and to establish whether manuka honey disrupted binding of streptococci to human wound proteins fibrinogen and fibronectin.

METHODS

Bacterial strains. *S. pyogenes* MGAS6180 (M28, invasive disease; Green *et al.*, 2005) was grown in Todd–Hewitt (TH) broth (Oxoid) containing 0.5 % yeast extract; Bacto agar (Difco) was added to achieve a 1.4 % (w/v) final concentration in agar plates. Cultures were incubated at 37 °C in air supplemented with 5 % CO_2. For analysis of aggregation properties, *S. pyogenes* was grown in C medium containing 0.2 % glucose (Lyon *et al.*, 1998).

Medical grade manuka honey. Sterile medical grade manuka honey (Medihoney) was kindly donated by Comvita. Medihoney was provided in sterile 50 g portions.

Minimum inhibitory and bactericidal concentrations. The MIC for manuka honey against planktonically grown *S. pyogenes* MGAS6180 was determined by serial dilution (0–95 %; w/v) in a total volume of 5 ml iso-sensitest broth (Oxoid) (according to the British Society for Antimicrobial Chemotherapy methodology for determining MIC; Andrews, 2011). Cultures were incubated for 16 h at 37 °C in aerobic conditions with 5 % CO_2. To establish the MBC, samples corresponding to the MIC and including three samples of a higher concentration were plated onto Todd–Hewitt agar and incubated for 16 h at 37 °C in aerobic conditions with 5 % CO_2. Assays were done in triplicate on each of three separate occasions.

Inhibition of bacterial growth. To determine the extent of growth inhibition by manuka honey, triplicate cultures of *S. pyogenes* MGAS6180 were grown for 8 h at 37 °C in 10 ml TH broth, in aerobic conditions with 5 % CO_2, supplemented with manuka honey (0, 20 and 40 % w/v). Bacterial growth was monitored at OD_{650} at hourly intervals. Growth experiments also utilized triplicate biological samples.

Aggregation assays. *S. pyogenes* MGAS6180 was initially grown in C medium for 16 h (Lyon *et al.*, 1998) and harvested by centrifugation at 5000 ***g*** for 10 min. Bacterial cell pellets were resuspended in either 1 ml C medium or an appropriate concentration of manuka honey (5 and 10 %; w/v) dissolved in C medium and the OD_{600} of the culture was adjusted to 1.0 ($\pm$0.05) if necessary. In both cases, manuka honey at twice the desired concentration was dissolved in double strength TH and diluted to the required concentration using TH; cell pellets were resuspended in either 5 or 10 % (w/v) honey solutions. In an untreated control, manuka honey was replaced with PBS added to maintain the appropriate volume and concentration of TH media. Aggregation assays were carried out in triplicate as described previously (Jakubovics *et al.*, 2005). Briefly, cell suspensions were incubated statically at 37 °C in 1.5 ml semi-microcuvettes and OD_{600} readings were taken at 30 min intervals over a period of 6 h to monitor the extent of bacterial aggregation. Bacterial aggregation was monitored by observing a reduction on the OD_{600} over 6 h. Controls containing no manuka honey were analysed simultaneously. Aggregation was expressed as a percentage fall in OD_{600} relative to the control; triplicate biological samples were assayed and statistical analysis (Students *t*-test to compare 5 and 10 % honey treatment, individually, to the no honey control) was undertaken using Minitab (version 14).

Static biofilm model. *S. pyogenes* MGAS6180 was initially grown in C medium for 16 h and these stationary phase cultures were harvested by centrifugation and adjusted to OD_{650} 0.1. To determine whether manuka honey prevented biofilm formation, biofilms were established in 96-well microtitre plates (Greiner) in 50 µl TH, supplemented with manuka honey (0, 10, 20 and 40 % w/v), by inoculating each well with 5 µl harvested cells. Plates were incubated at 37 °C for 24 h, aerobically with 5 % CO_2. To estimate biomass, unattached cells were gently aspirated and discarded, and adherent cells were washed twice with PBS and stained with crystal violet (0.25 %; w/v) for 10 min; following a further two washes with PBS, cell-bound crystal violet was resolubilized with 7 % acetic acid, and absorbance was measured at 595 nm (A_{595}) (Jakubovics *et al.*, 2005). To determine whether manuka honey disrupted established biofilms of *S. pyogenes* MGAS6180, biofilms were cultivated in 96-well microtitre plates for 24 h at 37 °C, as described above in TH medium in the absence of manuka honey. Biofilms were washed twice with PBS, and 50 µl manuka honey dissolved in TH medium (0, 10, 20 and 40 % w/v) was added to each well; the plates were incubated at 37 °C for a further 2 h after which time they were stained with crystal violet, as described above. To establish the effect of a sublethal dose of manuka honey on biofilm structure, biofilms were grown as described above for 24 h, but on 15 mm glass coverslips in 24-well microtitre plates. Coverslips were washed with PBS and were stained using crystal violet (0.25 %; w/v) before being visualized by light microscopy ($\times$1000 magnification). All experiments were carried out in triplicate, using three different biological samples; Minitab (version 14) was employed for statistical analysis (Students *t*-test comparing the reduction in biomass using each honey treatment, individually, to the untreated control).

Live–Dead staining. Images of bacterial cells were collected for control cells (untreated) and for cells treated with 40 % (w/v) manuka honey for 2 h, to determine the effect of manuka honey on viability. Biofilms were grown in Petri dishes in 5 ml TH media, or 5 ml 40 % (w/v) honey dissolved in TH media, as previously described; liquid was aspirated from the plates and biofilms were washed with 1 ml PBS. Biofilms were scraped from the coverslips using a cell scraper, resuspended in 1 ml PBS and stained with Live–Dead BacLight bacterial viability kit (Invitrogen), following the manufacturer's instructions. Fluorescence microscopy images were obtained using a Nikon Eclipse 80i fluorescent microscope with oil immersion and $\times$100 lens. For detection of SYTO 9 (green channel) a 488 nm excitation and 520 nm emission filter was used. For propidium iodide detection (red channel) a 543 nm excitation and 572 nm emission filter was used. Image analysis used Volocity Software (PerkinElmer).

Biofilm disruption. To determine whether manuka honey affected biofilm biomass by facilitating the dissociation of adherent cells from

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the biofilm, assays were conducted to monitor the numbers of viable planktonic cells that were released into the liquid phase, from established biofilms following treatment with manuka honey. Biofilms were grown for 24 h as described above. The liquid was aspirated from each well, biofilms were washed twice with PBS to remove any planktonic or loosely adherent cells, and manuka honey over a range of concentrations [0, 10, 20 and 40 % (w/v), respectively] was added to the 24 h established biofilms. Following the application of honey, biofilms were incubated for a further 2 h at 37 °C as above and samples of the liquid above the biofilm were collected at 30 min intervals. Bacterial cells were enumerated using the total viable cell (TVC) counting method described by Miles *et al.* (1938) with TH agar as a non-selective medium. The number of recovered cells was calculated as c.f.u. ml^{-1}.

Fibronectin- and fibrinogen-binding assays. To determine the effect of manuka honey on adherence of *S. pyogenes* cells to immobilized fibronectin and fibrinogen, a crystal violet assay was conducted as described previously, using 1 % BSA to block wells prior to assaying cell binding (Jakubovics *et al.*, 2005, 2009). Nunc high-bind 96-well microtitre plates were coated with 1 µg each protein dissolved in coating buffer (20 mM Na_2CO_3, 20 mM $NaHCO_3$; pH 9.3). This amount of fibronectin and fibrinogen was found to be optimal for bacterial binding (data not shown). Manuka honey at 20 % (w/v) was used to determine whether binding was inhibited, and the manuka honey was dissolved in Tris-buffered saline (TBS; pH 7.4). Cells were grown for 16 h in TH prior to the assay, harvested as above, resuspended in TBS with or without manuka honey (20 % w/v), and 50 µl was inoculated into a microtitre plate. Absorbance values (A_{595}) were compared against a PBS control throughout the entire procedure; the assay also included a control set of wells containing only coating buffer without protein ligands to determine whether the wells of the plate were sufficiently coated. The experiments used triplicate biological replicates and each assay was performed in triplicate; statistical analysis used Minitab (version 14; Students *t* test).

RNA extraction from *S. pyogenes* biofilms. Large scale, static biofilms of *S. pyogenes* were grown in duplicate in 5 ml C medium (with 20 % honey for the 'treated' biofilms) in sterile Petri dishes for 24 h at 37 °C, as for the small scale biofilms. The liquid was aspirated and the biofilm was scraped from the surface of the Petri dish using a sterile cell scraper. Biofilms were resuspended in 500 µl PBS and vortexed for 1 min to break up cell aggregates. Honey-treated and untreated cell suspensions were equilibrated (to approximately 2.5×10^9 c.f.u.) prior to treatment with mutanolysin (100 µg) and lysozyme (100 µg) for 20 min at 37 °C. RNA extraction was carried out using the SV Wizard total RNA extraction kit (Promega) according the manufacturer's instructions. RNA quantification was performed by spectrophotometric measurement using a NanoDrop ND-1000 (NanoDrop Technologies) and each RNA sample was adjusted to give a final concentration of 10 ng μl^{-1}.

End point RT-PCR to determine the relative expression of *sof* and *sfbI*. PCR primers were designed to amplify a 1100 bp fragment of the *sof* gene (*sof*-fwd: 5′-ACTTAGAAAGTTATCTGTAGGG; *sof*-rev: 5′-TCTCTCGAGCTTTATGGATAG) and 1200 bp fragment of the *sfbI* gene (*sbfI*-fwd: 5′-AACTGCTTTAGGAACAGCTTC; *sbfI*-rev: 5′-CCACCATAGCCACAATGCT). The complete genome sequence for *S. pyogenes* MGAS6180 was obtained from the NCBI database (www.ncbi.nlm.nih.gov) and used as a basis to design the primers used in this study. Internal control primers were designed to amplify a 900 bp internal fragment of the *glr* (*murI*; glutamate racemase) gene (*glr*-fwd: 5′-ATGGATACAAGACCAATTGGG; *glr*-rev: 5′-TCATAA-GGTGACATGCTCCAC), a known housekeeping gene in *S. pyogenes* that is commonly used for MLST (http://spyogenes.mlst.net/misc/info.asp) (Kalia *et al.*, 2001; Enright *et al.*, 2001). RNA was pre-treated with DNase I (Promega) prior to end point RT-PCR according to the manufacturer's instructions. RNA was reverse transcribed using the Access RT-PCR system (Promega). PCRs were performed in 25 µl reactions containing 50 ng template, 10 mM dNTPs, 1 µM oligonucleotides, PCR buffer, 4.5 mM $MgCl_2$ and 5 U polymerase. PCRs were performed under the following conditions: denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 53 °C for 30 s, 68 °C for 2 min, and a final elongation at 68 °C for 7 min. The positive control for the reaction was provided by the manufacturer (Promega), and nano-pure water was used to exclude the possibility of contamination. Band intensities were compared under UV light after samples had been separated by electrophoresis through a 0.8 % agarose gel. Densitometry was performed using the Bio-Rad GelDoc 2000 (Bio-Rad) and Quantity One software (Bio-Rad). This part of the study used three biological replicates and densitometric results were analysed by Students *t*-test using Minitab (version 14).

RESULTS

Inhibition of planktonic *S. pyogenes* by manuka honey

The MIC of manuka honey against *S. pyogenes* MGAS6180 was found to be 20 % (w/v) and the MBC was found to be 45 % (w/v). Growth curves with 20 % (w/v) manuka honey resulted in a reduced growth rate and reduction in overall cell number (Fig. 1) over a period of 8 h, compared with cells grown without honey. Growth curves conducted with 40 % (w/v) manuka honey resulted in no growth of *S. pyogenes* MGAS6180. Additionally, time-kill assays (Ogunmwonyi *et al.* 2010; Jenkins *et al.* 2011b) over 24 h indicated that *S. pyogenes* could not be recovered after 8 h incubation with 45 % (w/v) manuka honey (data not shown), supporting the data obtained for the MBC.

S. pyogenes aggregation and biofilm development are inhibited by sublethal concentrations of manuka honey

To test the capacity for manuka honey to inhibit intercellular aggregation, suspensions of *S. pyogenes* MGAS6180 were

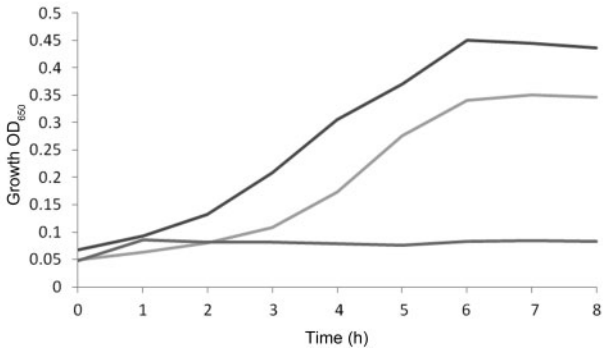

Fig. 1. Growth analysis of *S. pyogenes* MGAS6180 treated with manuka honey. Dark, light and mid grey indicate 0, 20 and 40 % (w/v) manuka honey, respectively.

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mixed with 10 % (w/v) manuka honey. In the absence of manuka honey, cells strongly aggregated but in the presence of 10 % (w/v) honey, aggregation was completely inhibited ($P<0.05$) (Fig. 2.). To determine whether this response was dose-dependent, the honey was diluted 5 % (w/v), which is four times lower than the MIC (Fig. 2). At this concentration, aggregation was inhibited as effectively as with 10 % (w/v) manuka honey ($P<0.05$).

When biofilms of *S. pyogenes* were initiated in the presence of 10, 20 and 40 % (w/v) manuka honey, a statistically significant reduction in biomass was observed in each case. A reduction of 75 % ($P=0.03$) was observed with 10 % (w/v) manuka honey; 20 and 40 % (w/v) manuka honey resulted in between 90 % ($P=0.02$) and 96 % ($P=0.01$) reduction in biomass, respectively (Fig. 3). Images of *S. pyogenes* biofilms obtained by light microscopy confirmed these observations, because cells exposed to 20 % (w/v) manuka honey showed no evidence of microcolony formation and contained only long chains of cells, some of which were enlarged (Fig. 4).

Manuka honey facilitates cell death and dissociation of bacterial cells from established *S. pyogenes* biofilms

To determine the effect of manuka honey against established biofilms of *S. pyogenes* MGAS6180, biofilms were grown for 24 h prior to honey treatment. Following 2 h treatment with 10 and 20 % manuka honey (w/v), a 72 % ($P=0.32$) and 77 % ($P=0.08$) reduction in biomass was observed, respectively. Following treatment for 2 h with 40 % (w/v) manuka honey, the observed reduction in biofilm biomass was even greater, and statistically significant at 85 % ($P=0.01$) (Fig. 5a). Biofilms were subsequently scraped from the plate and analysed using the Miles and Misra technique (Miles *et al.*, 1938). From biofilms established in the absence of manuka honey,

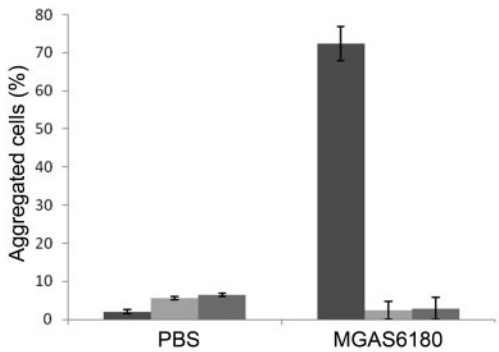

Fig. 2. Aggregation assay. Cells were assayed for aggregation after 6 h and the level of aggregation was compared with a PBS control. Dark, light and mid grey indicate 0, 5 and 10 % (w/v) manuka honey, respectively. Error bars show SEM.

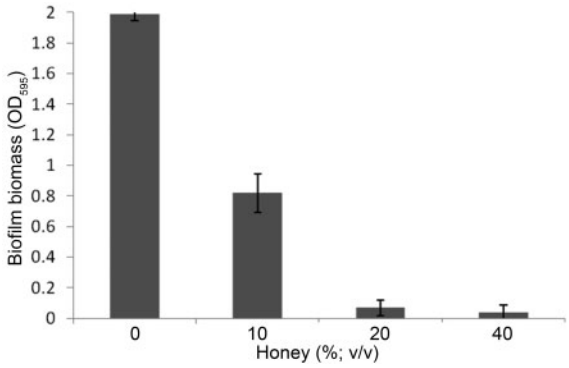

Fig. 3. The disruption of biofilm development using manuka honey. Biofilms of *S. pyogenes* MGAS6180 were grown for 24 h in media supplemented with manuka honey at 10, 20 and 40 % (w/v). Error bars show SEM.

4×10^6 c.f.u. was recoverable from biofilms. There was an approximately twofold reduction following treatment with 10 and 20 % (w/v) manuka honey, corresponding to 1.93×10^6 c.f.u. and 1.22×10^6 c.f.u., respectively (Fig. 5a). The reduction in viable cells was even more marked using 40 % (w/v) manuka honey, resulting in a viable count of 2.4×10^5 c.f.u.

Live–Dead viability staining (Invitrogen) of untreated biofilms grown for 24 h at 37 °C, honey-treated (40 % w/v) biofilms grown for 24 h at 37 °C, and biofilms that were grown for 24 h at 37 °C, then treated for 2 h with 40 % (w/v) manuka honey, showed that in both cases, the honey treatment resulted in cell death (Fig. 5b). For both honey treatment conditions, more non-viable cells were present than viable cells; in the untreated control, no non-viable cells were observed. These data suggested that the honey treatment resulted in cell death and supported the TVC data described above.

With time, bacterial cells may dissociate from a biofilm. To determine whether manuka honey facilitated the dissociation of *S. pyogenes* from biofilms, the levels of planktonic cells in the liquid phase were monitored for 2 h following the application of manuka honey (the biofilm data described above showed that the biomass was reduced following 2 h treatment, so this time period was deemed appropriate for these experiments). Immediately after application of the manuka honey (0 min) the c.f.u. was approximately the same for each condition. After 30 min and throughout the duration of the experiment (120 min), higher c.f.u. values were recovered from biofilms treated with 10 and 20 % (w/v) manuka honey (subinhibitory concentrations) compared with untreated biofilms, with a maximum recovery of 1.2×10^6 c.f.u. (Fig. 5c). However, fewer cells were recovered from biofilms exposed to 40 % (w/v) manuka honey, despite this concentration being lower than the observed MBC.

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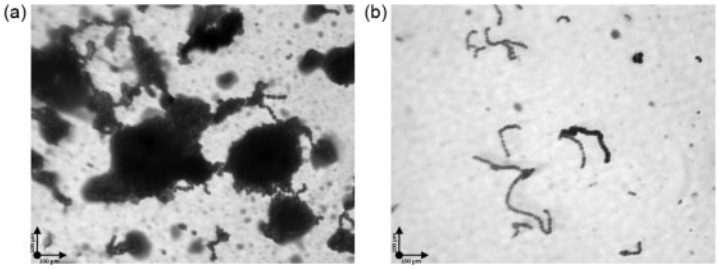

Fig. 4. Disruption of microcolony development using a sublethal dose of manuka honey (20 % w/v). Biofilms of *S. pyogenes* were grown on glass coverslips for 24 h and stained with 0.25 % (w/v) crystal violet; images were viewed by light microscopy using oil immersion at ×1000 magnification. Biofilms were grown with 0 (a) or 20 % (w/v) (b) manuka honey.

Sublethal concentrations of manuka honey inhibit binding of *S. pyogenes* to a wound-associated ligand

To establish whether manuka honey affected binding of *S. pyogenes* MGAS6180 to the wound-associated proteins fibronectin and fibrinogen, 1 µg of each of these two protein ligands was immobilized to the surface of a microtitre plate. The extent of binding inhibition using a sublethal dose (20 % w/v) of manuka honey was compared with adherence observed in the absence of manuka honey. Binding of *S. pyogenes* to fibronectin in the presence of 20 % (w/v) manuka honey was reduced by 74 %, which was found to be statistically significant (P=0.01); conversely no such reduction in binding was observed for fibrinogen (P=0.38) (Fig. 6). This is the first time that evidence to suggest that manuka honey has the potential to inhibit binding to human protein ligands has been reported.

Genes encoding the surface adhesins Sof and SbfI are differentially expressed in response to exposure to a sublethal concentration of manuka honey

To determine whether the decreased binding to fibronectin was the result of differential expression of two of the major fibronectin-binding proteins (Sof and SfbI) of *S. pyogenes* MGAS6180 in response to sublethal concentrations of honey, end point RT-PCR was employed. The PCR products were analysed by densitometry and quantified relative to the New England Biolabs 1 kb quantitative molecular mass

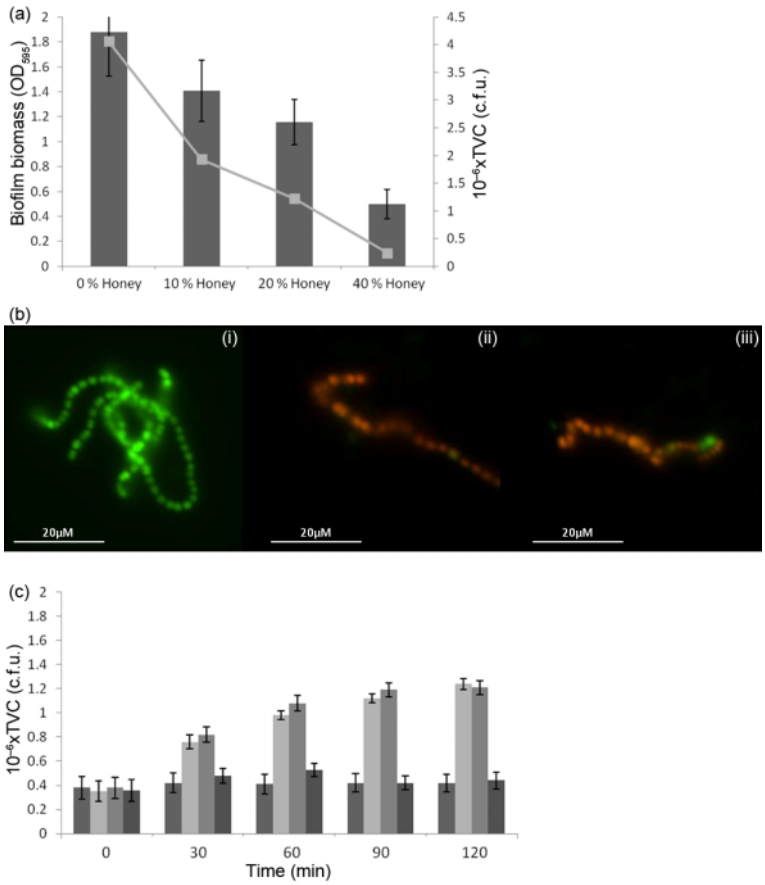

Fig. 5. (a) The disruption of established biofilms of *S. pyogenes* MGAS6180 using manuka honey and the corresponding reduction in viable cells. Dark grey bars show biofilm biomass (±SEM); the pale grey line corresponds to TVC. Biofilms were grown for 24 h and treated with manuka honey at 10, 20 and 40 % (w/v) for 2 h. TVC was determined using the method described by Miles *et al.* (1938). (b) Live–Dead viability staining of honey-treated and untreated biofilms of *S. pyogenes.* Biofilms were resuspended in PBS prior to staining. Green SYTO 9-stained cells are viable, red propidium iodide-stained cells are non-viable. (i) Untreated control cells, (ii) biofilm grown for 24 h with 40 % (w/v) manuka honey, (iii) biofilm grown for 24 h, then subsequently treated with 40 % (w/v) manuka honey for 2 h. (c) Recovery of detached bacterial cells from biofilms of *S. pyogenes* MGAS6180 that were grown for 24 h and then treated with manuka honey for 2 h. Viable cells were sampled immediately after application of honey and recovered on non-selective agar (TH). From left to right (grey, pale, mid and dark grey) indicate 0, 10, 20 and 40 % (w/v) manuka honey. Error bars show SEM.

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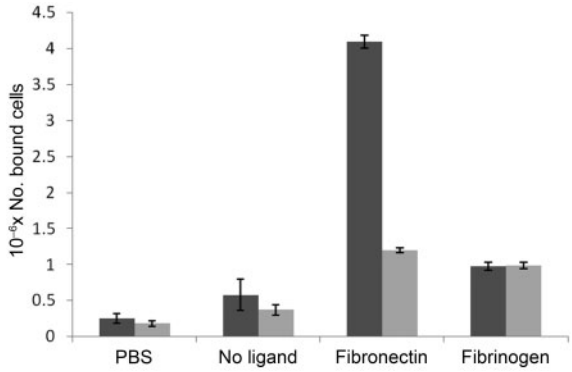

Fig. 6. Determining the effect of a sublethal concentration of manuka honey on the ability of *S. pyogenes* MGAS6180 to bind fibronectin and fibrinogen, compared with PBS as a control, and with coating buffer only (no ligand). Dark and light grey bars indicate 0 and 20 % (w/v) manuka honey, respectively. Error bars show SEM.

marker (thresholds for detection ⩾0.15 nmol). The results confirmed that both *sfbI* and *sof* were expressed to a lesser extent in the presence of 20 % (w/v) manuka honey (Fig. 7). Specifically, for *sbfI* there were 1.49 nmol PCR product in the 20 % (w/v) manuka honey conditions, compared with 2.16 nmol with 0 % manuka honey, which equates to a 31 % reduction in expression. For *sof* in the presence of 20 % (w/v) manuka honey, the amount of PCR product was below the threshold of detection; without manuka honey, 1.73 nmol was produced, suggesting that the expression of *sof* was also reduced following treatment with a sublethal concentration of honey. The equal amounts of *glr* product [2.03 nmol with no manuka honey and 2.02 nmol with 20 % (w/v) manuka honey] confirmed that equal amounts of RNA were used in these reactions and that the expression of this gene was not affected by honey treatment. Statistical analysis of the three biological replicates showed that there was no differential expression for *glr* under both conditions; for *sfbI*, the difference was statistically significant ($P<0.05$). In all instances *sof* could not be detected by densitometry following honey treatment.

DISCUSSION

Wounds that are infected with *S. pyogenes* often fail to respond to treatment. This is not always the consequence of factors that confer antibiotic resistance and a growing body of evidence strongly suggests that this phenomenon is attributed to the tendency of *S. pyogenes* to grow as a biofilm during infection. Over 90 % of invasive and non-invasive strains readily form biofilms, and despite information regarding biofilm formation in *S. pyogenes* being limited, it is becoming apparent that this characteristic is both strain- and serotype-dependent (Baldassarri *et al.*, 2006; Maddocks *et al.*, 2011). *S. pyogenes* strains routinely isolated from patients who have experienced treatment failure are all prolific biofilm formers (Lembke *et al.*, 2006) that do not necessarily exhibit resistance to commonly used systemic antibiotics; it is the biofilm community itself that appears to confer resistance (Conley *et al.*, 2003).

Currently most studies on the efficacy of honey against biofilms of *S. pyogenes* have used uncharacterized clinical isolates and in some cases local honeys (Al-Waili *et al.*, 2005; Voidarou *et al.*, 2011). This study describes the first systematic analysis of the effect of medical grade manuka honey on developing and established biofilms of a well-characterized strain of invasive *S. pyogenes* (MGAS6180). In addition to this, this study provides the first evidence, to our knowledge, that manuka honey can specifically inhibit adherence of *S. pyogenes* MGAS6180 to fibronectin. This is especially significant with regard to wound infection because high levels of fibronectin are exposed in wounds as a consequence of tissue damage, thus providing a ligand to which *S. pyogenes* can bind.

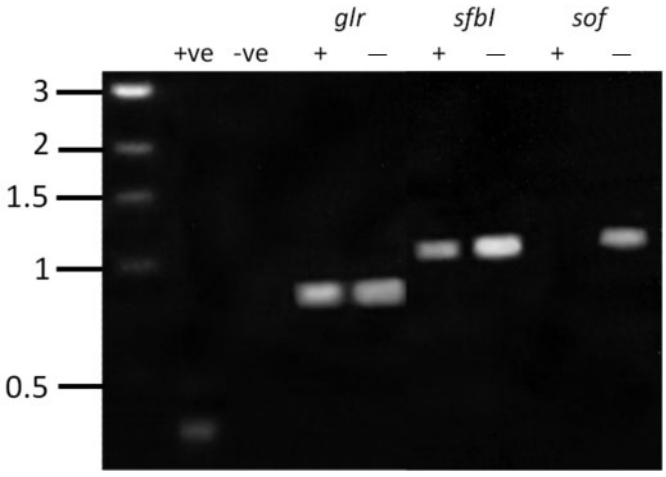

Fig. 7. End point RT-PCR to determine the amount of *sfbI* and *sof* expressed by biofilms of *S. pyogenes* MGAS6180 when treated with a sublethal (20 % w/v) concentration of manuka honey. +ve indicates the positive control (Promega); -ve indicates the negative control (dH_2O); glutamate racemase (*glr*) is an internal control. + indicates honey treatment; – indicates no honey treatment. Values for the molecular mass marker are given in kb.

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Concentrations of manuka honey equivalent to the MIC for planktonically grown cells were able to prevent biofilm development; manuka honey applied at just 5 % less than the MBC for planktonically grown cells resulted in an almost complete abrogation of biofilm development. In both cases, there was undoubtedly some degree of growth inhibition as observed from the growth studies. However, the large reduction in biofilm biomass using the MIC did not support the small reduction in growth using the same concentration of manuka honey, suggesting that the loss of biofilm biomass was the consequence of more than growth inhibition alone. Treatment of established biofilms of *S. pyogenes* with manuka honey also facilitated a reduction in biofilm biomass. Since these biofilms had been grown for 24 h prior to treatment, the observed reduction in biomass was likely the result of cell death, which was verified by Live–Dead viability staining which showed that non-viable cells were present in both developing and established biofilms that were treated with manuka honey. It was possible that the reduced TVCs could have been the result of a proportion of the population being in a viable but non-culturable state, which often occurs in response to cellular stress. This would have meant that the reduction in TVC observed might not have been attributed solely to cell death. However, the viability staining showing the presence of non-viable cells suggested that this was unlikely to be the case.

Lectins derived from *Talisia esculenta* and proteins derived from the seeds of *Labramia bojeri* are similarly known to effectively inhibit the capacity of strains of *S. sobrinus* and *S. mutans* to adhere to a polystyrene substratum and develop into a confluent biofilm (Oliveira *et al.*, 2007). These are naturally derived products that lack the antimicrobial activity observed for manuka honey. More recently, honey has also been shown to reduce biofilm formation of *S. mutans* at 12.5 and 25 % (w/v) (Nassar *et al.*, 2012). In addition to this, manuka honey is also documented to be effective against biofilms of *Staphylococcus aureus* and *P. aeruginosa*, both of which are wound-associated pathogens, making it a versatile topical treatment (Alandejani *et al.*, 2009; Merckoll *et al.*, 2009; Okhiria *et al.*, 2009).

Many streptococcal surface adhesins perform a dual role mediating intracellular aggregation and surface adhesion; this is certainly true for *S. pyogenes* in which major biofilm-associated adhesins such as AspA and pili also facilitate autoaggregation (Edwards *et al.*, 2008; Okahashi *et al.*, 2010; Maddocks *et al.*, 2011). Manuka honey was found to prevent autoaggregation of *S. pyogenes* at sublethal concentrations, of 5 and 10 % (w/v), demonstrating the significant impact that honey treatment might have on development of the early biofilm. Therefore, since biofilm development was not completely abrogated using 10 % (w/v) manuka honey, it is a possibility that arrested autoaggregation might have contributed to the prevention of biofilm development but that the process also relied on growth inhibition.

Wounds provide an environment that is rich in cell- and tissue-associated proteins such as fibronectin. Fibronectin forms part of the extracellular matrix and is associated with the epithelial cell surface. Damage caused to both cells and tissues in a wound exposes high levels of fibronectin that are available, not solely cell-bound, to which bacteria, including *S. pyogenes*, can adhere prior to colonization. Both fibronectin and high levels of fibrinogen are found in the plasma, which, as a consequence of vascular damage, is prevalent in wounds (Goodfellow *et al.*, 2000). Fibronectin-binding proteins are expressed by all streptococci (Lannergård *et al.*, 2005; Nobbs *et al.*, 2009) but they differ in affinity for their substrate, and a number of them are able to bind to more than one ligand. Eleven fibronectin-binding proteins and five fibrinogen-binding proteins have been identified in *S. pyogenes* (Kreikemeyer *et al.*, 2004; Nobbs *et al.*, 2009) and are largely conserved throughout other streptococci. Such proteins are known to enhance biofilm formation (Bonifait *et al.*, 2008) and their expression is highly regulated in response to numerous environmental stimuli (Kreikemeyer *et al.*, 2003). As well as acting as binding ligands, human tissue proteins such as fibrinogen can also serve as a bridging molecule; in the case of *S. aureus* this has been found to be especially important during attachment to epithelial cells (Bonifait *et al.*, 2008). Very little work has been undertaken to determine whether topical antimicrobial treatments can interrupt bacterial binding to human tissue proteins. To date, no studies on the effect of manuka or other honeys on bacterial biofilms have focussed on disruption of binding to a specific human protein ligand. This study has established that a sublethal concentration of manuka honey significantly reduced binding to human fibronectin ($P=0.01$), but that binding to fibrinogen was not interrupted. This is the first time that a topical antimicrobial of any type, including manuka honey has been documented to inhibit bacterial binding to a host protein.

The fibronectin-binding protein SfbI is expressed by approximately 50 % of invasive isolates of *S. pyogenes* and is regarded as one of the major adhesins and invasins (Medina *et al.*, 2000). Sof is regarded as a major virulence factor that is known to contribute to pathogenesis of streptococcal infection in animal models (Courtney & Pownall, 2010). The *sof* gene was first sequenced over 15 years ago (Rakonjac *et al.*, 1995) and its product was found to be a surface bound protein of over 100 kDa, with a C-terminal domain comprised of numerous repeating peptides that bound to both fibronectin and fibrinogen (Courtney *et al.*, 2003; Courtney & Pownall, 2010). End point RT-PCR found that the genes encoding both of these proteins were differentially expressed in response to treatment with a sublethal dose of manuka honey. In both instances, reduced expression was noticeable, with levels of *sof* below the threshold of detection. The primary end point for determining the RNA extraction efficiency prior to RT-PCR was total bacterial DNA, quantified using a NanoDrop. The half-life of bacterial mRNA is estimated to be between 0.5 and 20 min, but different RNA transcripts degrade at different rates. Studies with *S. pyogenes* have shown that

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degradation of transcripts can be broadly grouped into two classes (Bugrysheva & Scott, 2010). Information concerning the half-life of the transcripts used in this study was not available; however, all RNA samples were handled and maintained in a manner designed to minimize degradation, as recommended (Promega). With this in mind, we feel confident that while the effect of the half-life of the RNA transcripts on the observed levels of RT-PCR product cannot be ignored, it is unlikely to be the sole reason for the differential levels of PCR product observed here.

Consequently it is possible that the reduction in expression of *sof* and *sfbI* goes some way to explaining the significant reduction in fibronectin binding observed for *S. pyogenes* cells treated with 20 % (w/v) manuka honey. However, despite the reduced expression of *sof*, bacterial binding to fibrinogen was not similarly reduced. Sof is regarded as being one of the major fibrinogen binding proteins but it is not the only one, and others include M protein and Mrb (Hryniewicz *et al.*, 1972; Kehoe, 1994). It is therefore possible that there is some level of redundancy amongst these fibrinogen binding proteins that means that despite reduced expression of Sof, fibrinogen binding remained unaffected. SfbI and Sof are also not the only fibronectin-binding proteins associated with *S. pyogenes*; others include FbaA, FbaB and SfbX (Jaffe *et al.*, 1996; Terao *et al.*, 2001, 2002; Jeng *et al.*, 2003; Ramachandran *et al.*, 2004), so it remains unclear as to why fibronectin binding was so drastically altered following honey treatment. It is a possibility that the reduction in fibronectin binding was a combination of reduced expression and specific physical disruption of binding or stearic hindrance by components of the manuka honey. This is an avenue that needs further investigation, but was outside the scope of this study.

Alternatively, it is a possibility that manuka honey could be affecting many of the major transcriptional regulatory circuits that govern expression of the numerous surface adhesins, such as the Mga regulon that controls expression of both aggregation and biofilm formation (Luo *et al.*, 2008). If this is the case, it is likely that potentially many more genes than those studied here were being differentially expressed and affecting fibronectin binding. However, it was not possible to determine this during the study described here and further investigations will be necessary to confirm or dispel either of these hypotheses.

Honey is a complex substance estimated to be comprised of between 200 and 600 components, including fructose (~38.2 %), glucose (~31.3 %), sucrose (1 %) and 'other sugars' (9 %) (Bogdanov *et al.*, 2008). Additional minor constituents include acids (0.57 %), proteins (0.266 %), amino acids (0.1 %), nitrogen (0.41 %), minerals (0.17 %) and in the case of manuka honey, methylglyoxal (MGO). The antibacterial action of manuka honey is attributed to its high osmolarity, low water activity and the presence of MGO; it is the combination of these factors that is thought to provide an unsuitable environment for bacterial growth (Cooper, 2008; Adams *et al.*, 2009). The dilution of honey does not seem to affect the antimicrobial properties; MGO has been shown to remain active upon dilution but additional undefined factors are believed to maintain cidality when MGO is diluted below 0.53 mg ml^{-1} (Jervis-Bardy *et al.*, 2011). Unlike systemic antibiotics and other topical antimicrobials, the risk of bacterial resistance to manuka honey was low, even when high usage was maintained and to date, no 'honey-resistant' isolates have been found (Blair *et al.*, 2009; Cooper *et al.*, 2010). Therefore, manuka honey represents an efficacious and safe alternative to failing systemic and topical treatments that are currently used to treat wounds that are infected with *S. pyogenes*.

Taken collectively, the data described in this study indicate that manuka honey is effective at inhibiting the development of biofilms and disrupting established biofilms of *S. pyogenes*, and that this process is likely to be mediated by the specific interruption of binding to host tissue ligands, which in turn could be mediated in part by a differential expression of bacterial adhesins in response to honey treatment. Therefore, using the *in vitro* models described in this study, manuka honey was shown to have potential as a preventative measure against and treatment for wounds infected with *S. pyogenes*.

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