In vitro activity of an engineered honey, medical-grade honeys, and antimicrobial wound dressings against biofilm-producing clinical bacterial isolates

Objective: Honey is recognised to be a good topical wound care agent owing to a broad-spectrum of antimicrobial activity combined with healing properties. SurgihoneyRO™ (SH1) is a product based on honey that is engineered to produce enhanced reactive oxygen species (ROS) and has been reported to be highly antimicrobial.The objective was to investigate the ability of the engineered honey and its comparators to prevent biofilm formation *in vitro.*

Method: We tested the ability of three medical-grade honeys SH1, Activon manuka honey (MH) and Medihoney manuka honey (Med), alongside five antimicrobial dressings (AMDs) to prevent the formation of biofilms by 16 isolates. Honeys were serially double diluted from 1:3 down to 1:6144 and the lowest dilution achieving a statistically significant reduction in biomass of at least 50%, compared with untreated controls, was recorded.

Results: Although all the honeys were antibacterial and were able to prevent the formation of biofilms, SH1 was the most potent, with efficacy at lower dilutions than the medical honeys for five isolates, and equivalent dilutions for a further six. Additionally, SH1 was superior in antibacterial potency to three commercially available AMDs that contain honey.

Conclusion: SH1 is effective at preventing bioflms from forming and is superior to medical honeys and AMDs in *in vitro* tests.

Declaration of interest: SurgihoneyRO™ was provided free of charge for testing by Matoke Holdings, UK and the hospital pharmacy provided the other honeys and dressings.This paper presents independent research funded by the National Institute for Health Research (NIHR).The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

antibacterial; biofilms; honey; hydrogen peroxide; wounds

Honey has been recognised to be a good wound care agent, possessing both antimicrobial and healing properties.[1,2] The spectrum of antibacterial activity is broad (with 37 genera of bacteria shown to be susceptible),[3–5] and a range of medical honeys exist on the market (for example, chestnut, manuka, thyme, Revamil, manufactured by Bfactory Health Products, Multifloral and Medihoney).[6] Their mechanism of action is thought to be multifactorial; due to the honey preparation itself (in terms of physical properties including pH and hyperosmolarity), and the innate antibacterial components of honey (methylglyoxal, bee defensin-1, and hydrogen peroxide (H_2O_2).[6] To date, antibacterial resistance to honey has not been detected.[7,8]

Despite a 5000-year history as a topical antiseptic, a major limitation of natural honeys is that the honey may not be of a predictable and consistent quality, since the production depends on a large number of factors (for example, the floral source, the species of bee, geographical location, harvesting process, and subsequent storage conditions).[6] This has implications for large-scale production. There are also variation between the formulations of medical-grade honeys, although these are generally standardised in preparation.

SurgihoneyRO™ (SH1) is a licensed sterile product based on natural organic honey from a variety of sources. It has been developed for wound care and as a prophylactic dressing agent for wounds. It has been engineered (through a proprietary engineering process) so that it produces consistently high levels of antibacterial activity through reactive oxygen species (ROS).[9] ROS causes oxidative damage due to the production of hydroxyl radicals, leading to restricted bacterial growth and DNA degradation.[10,11] Further prototype formulations exist, in addition to SH1, which have enhanced production of H_2O_2. This means, theoretically, that antibacterial activity can be set at a higher potency if required.[12]

In vitro studies by Dryden et al.[13,14] have shown

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that SH1 has microbicidal action against both Gram-positive and Gram-negative bacterial wound isolates, and have indicated that it is superior to other honeys in terms of potency. In one study, Dryden et al.,[13] tested the *in vitro* efficacy of the formulations of SH against bacterial wound isolates by determining the minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBC), and compared these results with those obtained from a variety of other honeys. A total of 48 isolates were tested (comprising *Staphylococcus aureus* (MRSA and MSSA), ß-haemolytic *streptococci*, *Enterococcus spp*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, along with a range of other Gram-positive organisms and fungi) and the results demonstrated that all the SH formulations had higher antimicrobial activity than the other honeys tested, as evidenced by the larger inhibitory zones present around the test honey using an agar diffusion method. Time-kill experiments in this same study furthermore demonstrated the speed of the cidal activity of SH, with undetectable bacterial growth (for a range of Gram-positive and Gram-negative organisms) within 30 minutes for the most potent prototype SH formulation, and within 2 hours for the currently marketed and licensed version.

Clinical outcomes have also been favourable with SH1. In a pilot study looking at the prevention of surgical site infection (SSI) in patients undergoing caesarean section (CS) surgery,[12] SH1 was offered to all patients who had undergone a CS. Recipients were then monitored over 30 days for the development of infection, and rates of infection were compared with a previous cohort of patients who received no SH1. It reduced SSI by 60% compared with normal wound dressings, and was found to offer considerable cost savings over other preparations.[12] Further favourable outcomes were observed when SH1 was used to reduce bacterial colonisation in long lines in oncology patients.[14]

Although these *in vitro* and clinical studies provide good evidence to support the antimicrobial activity of SH1, no work has yet been performed to assess the activity of this engineered honey against biofilms. Biofilms are associated with wound chronicity, and are present in 60% of those with chronic wound infections.[15]

This *in vitro* study was undertaken to assess whether SH1 has any antibacterial activity against biofilms, in terms of ability to prevent their formation. To evaluate this, SH1 was tested on a range of biofilm-producing bacteria and the activity compared against two standard medical-grade honeys and commercially available honey- and silver-containing antimicrobial wound dressings.

Table 1. List of the control and clinical isolates used in this study

Study Identifier	Organism	Description
PS_PA01	*Pseudomonas aeruginosa*	ATCC_15692
PS_6749	*Pseudomonas aeruginosa*	NCTC_6749
PS_1054	*Pseudomonas aeruginosa*	QEHB clinical burn isolate
PS_1586	*Pseudomonas aeruginosa*	QEHB clinical burn isolate
ACI_AYE	*Acinetobacter baumannii*	MPR clinical Isolate (Paris)
ACI_19606	*Acinetobacter baumannii*	ATCC_19606
ACI_C59	*Acinetobacter baumannii*	NCTC_13420
ACI_C60	*Acinetobacter baumannii*	NCTC_13424
MDR_B	CRE *Klebsiella pneumoniae* (ESBL+ with additional permeability changes)	QEHB clinical isolate
MDR_C	*Escherichia coli* (ESBL+)	NCTC_13451
MDR_D	*Pseudomonas aeruginosa* (VIM+)	Royal Free Hospital clinical isolate
EC_042	*Escherichia coli* (enteroaggregative)	EAEC_042 – prototypical strain[17]
MSSA_10788	*Staphylococcus aureus*	NCTC_10788
MSSA_F77	*Staphylococcus aureus*	NCTC_8532
MRSA_ F475	*Staphylococcus aureus*	Isolate 252 of EMRSA-16
MRSA_F483	*Staphylococcus aureus*	MW2 strain

CRE–Carbapenem resistant Enterobacteriaceae; ESBL–Extended-Spectrum β-lactamase
VIM– Verona integron-encoded metallo-β-lactamase

Methods

In vitro experiments were conducted on a panel of important wound pathogens (Table 1) to determine the antibacterial effects of SurgihoneyRO™ (Matoke Holdings, UK), Medihoney (Derma Sciences, UK) and Activon tube 100% medical grade Manuka honey (Advancis Medical, UK), these will be referred to as SH1, Med and MH, respectively.

There were five antimicrobial dressings (AMDs) tested alongside SH1 on a limited panel of the isolates, so that any observed effects of SH1 could be compared with commercially available dressings containing honey, or alternative antimicrobial agents. The selection of honey-containing AMDs reflects those available in the formulary at our hospital, a major tertiary referral centre.

A total of 16 organisms (previously identified as good biofilm-producers)[16] were tested against the three honey formulations (Table 1):

- Four *Pseudomonas aeruginosa*
- Four *Acinetobacter baumannii*
- Four *Staphylococcus aureus*
- Three multidrug resistant organisms (MDR). *Klebsiella pneumoniae* (carbapenem-resistant Enterobacteriaceae), *Escherichia coli* (ESBL positive), *Pseudomonas aeruginosa* (carrying the Verona integron-encoded β-lactamase (VIM) plasmid conferring resistance to the carbapenems)
- Standard *Escherichia coli* isolate for reference.

Table 2. List of the dressings/agents used in this study, their supplier, antimicrobial agent and formulation, and reports on their activity

Dressing/agent	Supplier	Antibacterial agent and formulation	Reports/references
SurgihoneyRO™ (Honey and enhanced production of hydrogen peroxide)	Matoke Holdings, UK	Natural honey (not a manuka honey) that has been engineered to produce high levels of reactive oxygen species (ROS)	Research shows it is highly active against a range of Gram-positive and Gram-negative bacteria, including multidrug resistant ones. More potent than other honeys, including Medihoney.[13]
Activon tube 100% medical-grade manuka honey (Honey)	Advancis Medical	100% Manuka Honey (without additives) from New Zealand	Biofilms of *Staphylococcus aureus*, MRSA and VRE were prevented and inhibited *in vitro* at concentrations that could be used in clinical practice.[28]
Medihoney 'antibacterial medical honey' (Honey)	Derma Sciences	Medical-grade honey predominantly sourced from Leptospermum species (Manuka honey)	The minimum inhibitory concentration (MIC) was tested against 130 clinical strains and it was effective at concentrations ranging from 4–14% v/v. Organisms tested included *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Enterobacter* spp., and *Acinetobacter baumannii*.[29] It was also effective at preventing the formation of *Pseudomonas aeruginosa* biofilms *in vitro* when used at concentrations attainable in clinical use.[30]
Aquacel Ag (Silver)	Convatec	Ionic silver impregnated hydrofibre pad composed of sodium carboxymethylcellulose and 1.2% ionic silver	Contain ionic silver to kill a wide variety of microorganisms (including certain tested antibiotic-resistant bacteria), within 30 minutes, and provide sustained bacterial killing for up to seven days.[31,32]
Aquacel Ag+ extra (Silver)	Convatec	Ionic silver impregnated hydrofibre pad composed of a combination of silver and 'anti-biofilming agents'	The ionic silver in the dressing kills pathogenic microorganisms, both planktonic and within bacterial biofilms, including wound bacteria, yeasts and moulds.The dressing also disrupts and absorbs biofilm, prevents biofilm formation/ reformation and increases the efficiency of silver transfer to microorganisms.[31,32]
Actilite (Honey)	Advancis Medical	Light viscose net dressing coated with antibacterial Manuka honey and Manuka oil	The antibacterial effect has been enhanced by combining high grade antibacterial Manuka oil with Manuka honey. Dressing has been demonstrated *in vitro* to be effective against MRSA,VRE and *Providentia stuartii*.[33]
L-Mesitran Net (Honey)	L-Mesitran Wound Care	Non-adherent open polyester mesh coated with a thin layer of L-Mesitran Hydro gel.	L-Mesitran is a broad-spectrumantimicrobial, effective against most bacteria including MRSA and VRE.[34]
L-Mesitran Hydro (Honey)	L-Mesitran Wound Care	Hydrogel sheet (1mm thick) attached to a semi-polyurethane membrane by a thin fibrous bonding layer.The hydrogel contains 30% medical-grade honey.	As above.

The panel contained a mixture of well-character- ised control strains (PS_PA01, PS_6749, ACI_C59, ACI_C60, ACI_19606, EC_042,[17] MSSA_10788, MSSA_F77, MRSA_F475, MRSA_F483, and MDR_C) and clinical isolates from burns patients with large open wounds (PS_1054, PS_1586, ACI_AYE, MDR_B, MDR_D), and represent a diverse range of strains (data not shown).

The isolates were all varied in terms of antibio-gram (data not shown), stored at –80°C on Protect beads, and were cultured on cysteine lactose electro- lyte deficient (CLED) agar, or blood agar (as appro- priate; Biomerieux, France) before each experiment. Honeys and AMDs (Table 2) were freshly opened and within date when used. Experiments were per- formed using at least two biological replicates, and at least six technical replicates of each isolate per test dilution.

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Preparation of the honey

The honeys were prepared in the same way and tested at a range of dilutions from 1:3 down to 1:6144. The strongest concentration (1:3) was made by placing 6ml of honey into a universal tube and adding 14ml of water to make a total volume of 20ml, which was then serially double diluted down in sterile autoclaved distilled water until 1:6144 was reached. Dilution from the neat product was necessary since the viscosity of the neat honey meant that it was impossible to standardise the volume used in the experiments at this concentration. The dilution chosen (1:3) was the most concentrated solution that could be accurately pipetted into the test wells.

Impact of the honey on biofilm formation

The ability of the honey formulations to prevent biofilm formation was assessed using a crystal violet (CV) biofilm formation assay[18] with the endpoint measurement being the 'minimum biofilm inhibitory concentration' (MBIC).

Overnight cultures of the test strains (grown in 5ml of Lysogeny broth (LB; Oxoid)) were diluted in fresh antibiotic-free Muller-Hinton broth (Oxoid) to an optical density at 600nm (OD600) of 0.1, and then 100µl seeded into wells of a 96-well microtiter tray (MTT) (Fisher Scientific), alongside 100µl of either diluted honey (with water as diluent) or sterile distilled water. The honeys were tested at the following dilutions: 1:3, 1:6, 1:12, 1:24, 1:48, 1:96, 1:192, 1:384, 1:768, 1:1536, 1:3072, and 1:6144. Suitable controls were included in each assay, comprising 100µl overnight bacterial culture with 100µl water (for the positive control), or 200µl Muller-Hinton broth alone (for the negative control). This composition of the positive control was selected so that both the positive control and the test wells contained the same volume and concentration of bacteria, and the same amount of broth. Two biological and three technical replicates were performed for each strain and each honey dilution, respectively.

Plates were sealed and statically incubated at 33°C; the temperature of the surface of a wound.[19] After 72 hours, the liquid was removed from the wells and the plates rinsed in tap water to remove any unbound cells. Any existing biofilms were then visualised through staining with 200µl of 1% CV (Sigma Aldrich, Poole, UK), further rinsed (as above) to remove unbound CV, and dye solubilised by the addition of 200µl of 70% ethanol. The OD600 of the solubilised CV solution was then measured using a FLUOstar Optima (BMG Labtech) to assess the biomass of the biofilms.

If positive and negative controls for each test plate were within a normal range, the rest of the data were analysed for statistical significance by comparing values at each dilution of honey with untreated (positive) controls using the students' t-test. The MBIC was defined as the lowest dilution of honey where there was both statistical significance in the t-test (p value <0.05) and a prevention of biofilm biomass accumulation ≥50% compared with the positive control.

Preparation of antimicrobial dressings

The following AMD were prepared for testing: Aquacel Ag, Aquacel Ag+ extra (both Convatec), Actilite (Advancis Medical), L-Mesitran Net, and L-Mesitran Hydro (both from L-Mesitran Wound Care). These dressings were chosen as they either i) represent the most commonly used silver dressings in our burns unit (Aquacel Ag, and Aquacel Ag + extra), or ii) are composed of honey (Actilite, L-Mesitran Net, and L-Mesitran Hydro).

Each sterile dressing was carefully cut into a number of 1cm^2 pieces (sufficient for one piece per test well) using a sterile scalpel or a pair of flame -sterilised scissors. Details of these dressings (and references to published work) are in Table 2.

Impact of antimicrobial dressings on biofilm formation

Overnight cultures of the test strains (grown in 5ml LB) were diluted in fresh antibiotic-free Muller-Hinton broth to an optical density at 600nm of 0.1, and then 1ml was seeded into wells of a 24-well MTT (Corning, New York), with 1ml of either diluted honey (from 1:3 to 1:1536) or sterile water. For the AMD test wells, one piece of dressing was placed into the well containing the 2ml bacterial suspension and water.

The plates were processed and analysed using the above methods. No MBIC values were possible for AMD, so the change in biofilm biomass (compared with positive control) was calculated.

Results

All 16 bacterial isolates were tested against all three honeys, and a subset of four were additionally tested against the AMDs, achieving at least two but up to six technical replicates per dilution. The number of replicates can be seen in Tables 3 and 4, respectively.

The mean average optical densities of the solubilised CV were plotted per species and for all honeys and/or AMD to visually represent any prevention in the accumulation of biofilm biomass achieved by the treatment at the range of dilutions. Sample data are shown in Fig 1 (showing the difference in the abilities of the three honeys to prevent biofilm formation of PS_1586), Fig 2 showing the similarities in the abilities of the three honeys to prevent biofilm formation of EC_042 and Fig 3 showing the performance of the AMD compared with SH1 for a range of *Pseudomonas aeruginosa* isolates.

The MBIC for the honeys was determined and is reported in Table 3. All honeys demonstrated antibacterial activity against the formation of biofilms. The

Fig 1. The mean average (ave) biomass of the biofilms produced by *Pseudomonas aeruginosa* isolate PS_1586 when planktonic cells were coincubated with three types of honey (of a range of dilutions) for 72 hours. Optical density on the y axis refers to the average biofilm biomass for the *Pseudomonas aeruginosa* isolate with different dilutions of the honeys as shown on the x axis. Mean ± standard error mean

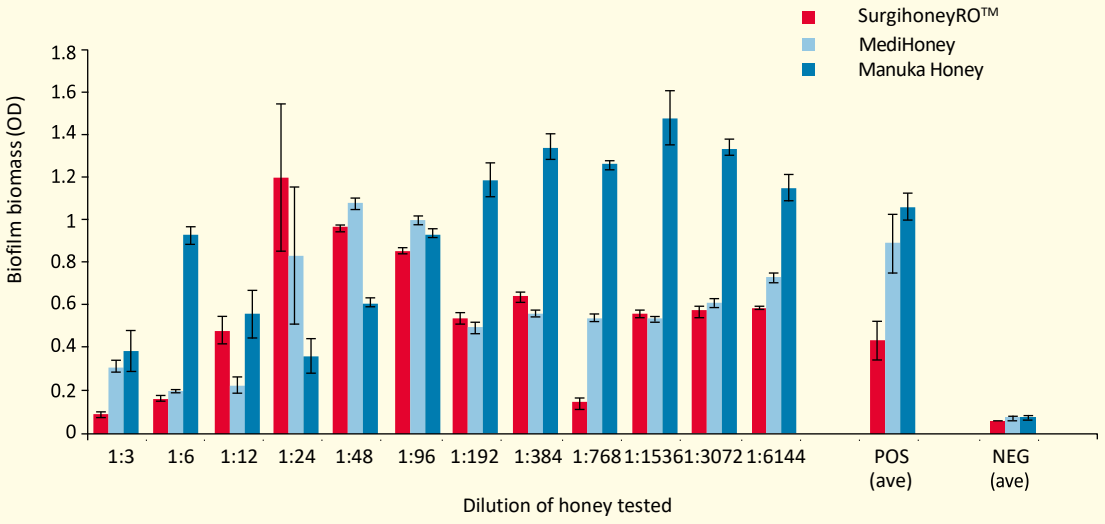
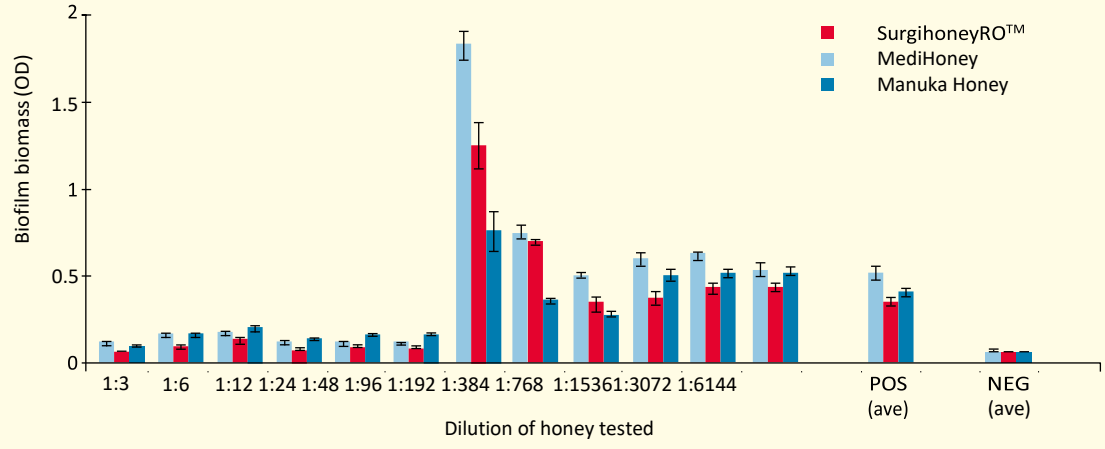


Fig 2. Graph showing the mean average (ave) biomass of the biofilms produced by *Escherichia coli* isolate EC_042 when planktonic cells were coincubated with a three types of honey (of a range of dilutions) for 72 hours. Optical density on the y axis refers to the average biofilm biomass for the *Escherichia coli* isolate with different dilutions of the honeys as shown on the x axis. Mean ± standard error mean



% change in biofilm biomass has been reported for the AMD data (Table 4).

Impact of the honeys on biofilm formation

SH1 was able to prevent biofilm formation for all 16 of the isolates, with MBIC values ranging from 1:6 (PS_PA01 and PS_1586) to 1:192 (MDR_C, and MRSA_F483) (Table 3). The dilution of 1:12 was the most commonly observed MBIC value and was present in six of the isolates (two *Pseudomonas aeruginosa* and four *Acinetobacter baumannii*). The remaining Gram-negative isolates were highly susceptible to SH1, with MBICs of 1:24 (MDR_D), 1:48 (MDR_B), and 1:96 for EC_042. Similar MBICs were seen with the Gram-positive isolates; 1:24 (MSSA_10788), 1:48 (MRSA_F475, MSSA_F77), and 1:192 (MRSA_F483).

MH was able to prevent biofilm formation of 14 of the 16 isolates, but was ineffective for two isolates (PS_PA01 and MRSA_F475). Here, there was no statistically significant prevention of biomass accumulation even when the strongest dilution of honey was used (1:3). This finding was repeatable.

Of the 14 isolates, MH was effective at preventing biofilms for eight (PS_1054, PS_6749, ACI_AYE, MDR_C, EC_042, MSSA_10788, MRSA_F483, and MSSA_F77) when used at the same dilution as SH1 (denoted by * Table 3), but required a stronger dilution than SH1 for the remaining six isolates (PS_1586,

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Fig 3. The mean average (ave) biomass of the biofilms produced by the *Pseudomonas aeruginosa* isolates when tested with the range of agents shown on the x axis. Mean ± standard error mean, significant reductions in biofilm biomass *p<0.05

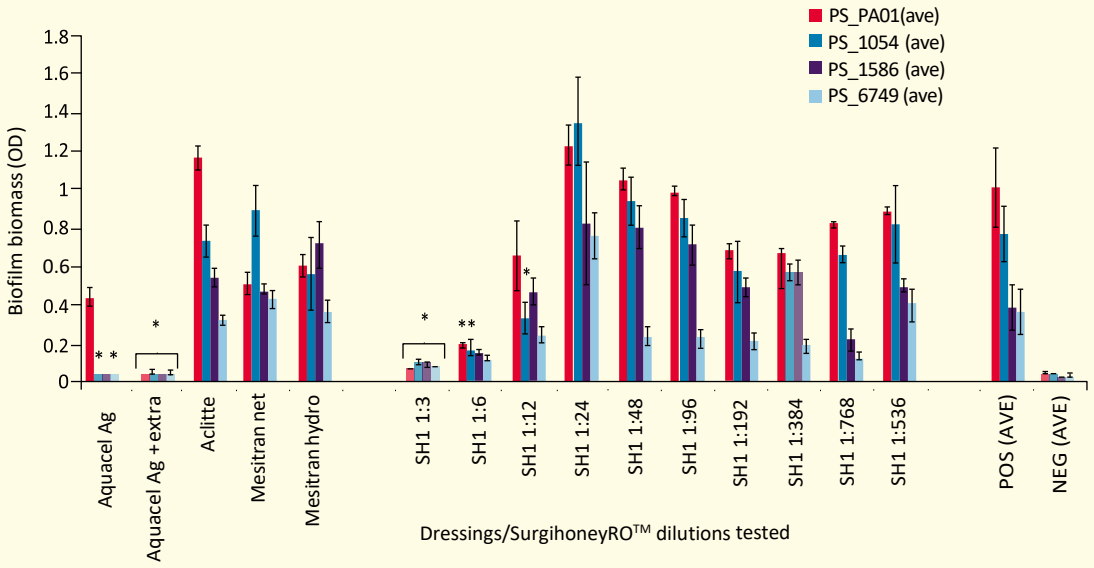
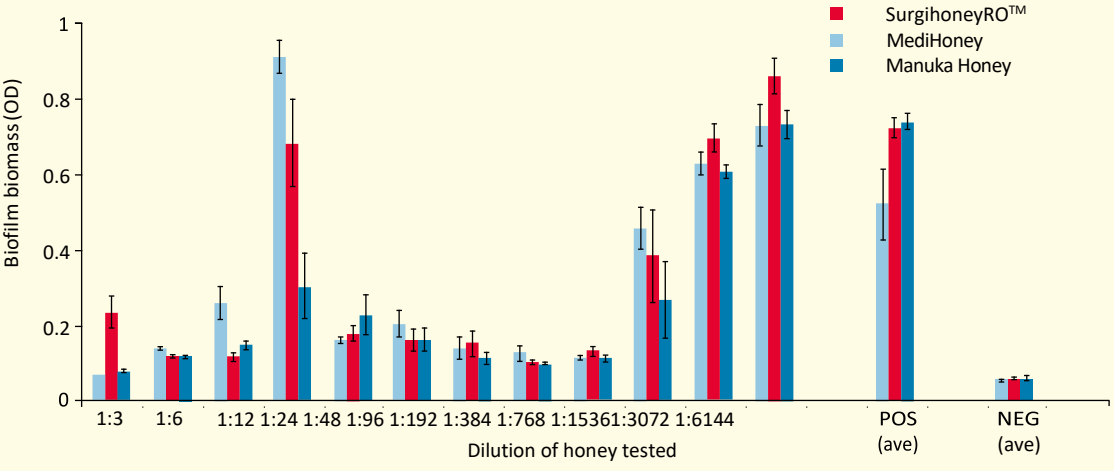


Fig 4. The mean average (ave) biomass of the biofilms produced by *Pseudomonas aeruginosa* isolate PS_6749 when planktonic cells were coincubated with three types of honey (of a range of dilutions) for 72 hours. Optical density on the y axis refers to the average biolfim biomass for the *Pseudomonas aeruginosa* isolate with the different dilutions of the honeys on the x axis. Mean ± standard error mean.



ACI_C59, ACI_C60, ACI_19606, MDR_B, and MDR_D; denoted by ⁺Table 3).

In summary, six of the isolates (PS_1586, ACI_C59, ACI_C60, ACI_19606, MDR_B and MDR_D) have MBICs of ≤1:6, three of 1:12 (PS_1054, PS_6749 and ACI_AYE), with the remainder of the MBICs ranging from 1:24 to 1:192 (Table 3). There did not appear to be a trend or pattern between susceptibility and pathogen group in the data.

Med performed similarly to MH, and was able to prevent biofilm formation for 15 of the 16 isolates, when used at equivalent (6/15 isolates), stronger (seven isolates), and also weaker (two isolates) dilutions compared with SH1 (denoted by *,⁺,‡Table 3).

Med was ineffective against biofilm production by one isolate (PS_PA01), where there was no statistically significant reduction in biofilm biomass, even when the strongest dilution of honey was used (1:3).

With Med, five of the isolates had an MBIC of ≤1:6 (all *Acinetobacter baumannii* isolates, and MDR_D), which is similar to MH where five of the isolates also have MBICS of ≤1:6. The highest MBIC observed with Med was with PS_6749, where the lowest effective concentration to inhibit biofilm formation was 1: 1536. This was statistically significant (p=0.009) and there was a greater than 50% reduction in biofilm biomass compared with the positive control (data not shown).

Table 3. Showing the MBIC for each of the honeys tested against the 16 isolates included in the study

Organism	SH1			MH			Med		
	MBIC	n	p	MBIC	n	p	MBIC	n	p
PS_PA01	1:6	6	<0.001	>1:3	n/a	n/a	>1:3	n/a	n/a
PS_1054	1:12	6	<0.001	1:12*	6	<0.001	1:12*	6	**<0.001**
PS_1586	1:6	6	<0.001	1:3†	6	0.02	1:12‡	6	0.019
PS_6749	1:12	6	<0.001	1:12*	6	<0.001	1:1536‡	6	0.009
ACI_AYE	1:12	6	<0.001	1:12*	6	<0.001	1:3†	6	<0.001
ACI_C59	1:12	6	<0.001	1:3†	6	<0.001	1:3†	6	<0.001
ACI_C60	1:12	6	<0.001	1:3†	6	<0.001	1:3†	6	<0.001
ACI_19606	1:12	6	<0.001	1:6†	6	<0.001	1:6†	6	<0.001
MDR_B	1:48	6	<0.001	1:6†	6	<0.001	1:24†	6	<0.001
MDR_C	1:192	6	<0.001	1:192*	6	<0.001	1:192*	6	<0.001
MDR_D	1:24	6	<0.001	1:3†	6	0.023	1:3†	6	0.043
EC_042	1:96	6	<0.001	1:96*	6	<0.001	1:96*	6	<0.001
MRSA_F475	1:48	6	0.04	None	n/a	n/a	1:24†	6	0.036
MSSA_10788	1:24	6	0.04	1:24*	6	0.001	1:24*	6	<0.001
MRSA_F483	1:192	6	<0.001	1:192*	6	<0.001	1:192*	6	<0.001
MSSA_F77	1:48	6	<0.001	1:48*	6	0.006	1:48*	6	0.02

MBIC–mean biomass inhibition concentration; MH–Manuka Honey; Med–Medihoney: SH1–SurgihoneyRO™ *MH or Med MBIC equivalent to the SH1 MBIC; ‡MBIC for MH and Med is a weaker dilution of honey than SH1; †MH and Med MBIC is a stronger dilution of honey than SH1; p values from Student's T–test

Comparison of the honeys

Although all the honeys were antibacterial and able to prevent the formation of biofilms for the majority of the isolates tested, SH1 was generally the most potent. SH1 was effective at lower dilutions than both the other honeys for five of the isolates (ACI_C59, ACI_C60, ACI_19606, MDR_B, and MDR_D), and was equivalently effective to the other honeys for a further six isolates (PS_1054, MDR_C, EC_042, MSSA_10788, MRSA_F483, and MSSA_F77). For the remaining five isolates, SH1 was either the only effective honey (PS_PA01), was one of two effective honeys (MRSA_F475), or gave concordant/discordant results compared with MH and Med (PS_1586, PS_6749, ACI_AYE).

Generally, MH and Med have similar MBIC for all isolates. However, for ACI_AYE, MH was superior to Med, and could prevent biofilm formation at 1:12 (the same effective dilution as SH1), compared with 1:3 for Med. There was also a difference in MBIC between MH (MBIC of 1:6) and Med (MBIC of 1:24) for MDR_B. All these results are statistically signifi- cant ($p \leq 0.05$).

On two occasions (PS_1586 and PS_6749), Med demonstrated superior potency to the other honeys. For the latter, the MBIC was 1:1536 ($p=0.009$), compared with 1:12 for both SH1 and MH (Fig 4). This may be explained by the enhanced growth at 1:24 observed with SH1 and MH for this isolate.

Comparison of the antibacterial activity of engineered honey and the antimicrobial dressings

The activity of SH1 was additionally tested and compared with a range of AMDs. This honey was chosen owing to the higher potency observed early on in the experiments, as it was not logistically possible to test all honeys in this experiment.

Pseudomonas aeruginosa isolates, PS_PA01, PS_6749, PS_1054, and PS_1586 were tested against SH1 and the range of AMD, achieving at least two, and up to six replicates per isolate (Table 4). The % change in biofilm biomass with each dressing or SH1 dilution was calculated based on the untreated positive control and are listed alongside those that were statistically significant ($p<0.05$; Table 4). Fig 3 shows this information with the statistically significant reduced accumulations of biomass denoted by an asterisk.

These data show that there is a large variation in the ability of the test agents to prevent the formation of biofilms of the *Pseudomonas* isolates tested. SH1 was effective at preventing biofilm formation of all isolates when used at a dilution of 1:3 with reductions in accumulations ranging from 79% (with PS_1586) to 94.1% (with PS_PA01). All of these reductions are statistically significant ($p<0.05$). In fact, SH1 still resulted in reduced biofilm forma- tion (that was statistically significant) when used down to 1:6 (where there was an 82% reduction for

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Table 4. Table showing the % change in biofilm biomass for each of the *Pseudomonas aeruginosa* isolates when incubated with SH1 and each of the antimicrobial dressings for 72 hours, compared with an untreated, positive control

	Pseudomonas aeruginosa PS_PA01			*Pseudomonas aeruginosa* PS_1054		
	% change	**n**	**p‡**	**% change**	**n**	**p‡**
Aquacel Ag	−38.4	3	0.33	**−94.1**	**2**	**0.001**
Aquacel Ag + Extra	**−95.3**	**4**	**0.007**	**−86.8**	**4**	**<0.001**
Actilite	+62.3	4	0.063	**+87.2**	**4**	**0.048**
L-Mesitran Net	− 27.8	4	0.132	**+129.7**	**4**	**0.047**
L-Mesitran Hydro	−15.8	4	0.488	+43.1	4	0.431
SH1 1:3	**−94.1**	**6**	**0.005**	**−86.7**	**6**	**0.01**
SH1 1:6	**−82.8**	**6**	**0.01**	**−76.6**	**6**	**0.029**
SH1 1:12	−34.9	6	0.16	**−57.08**	**6**	**0.005**
SH1 1:24	+22	6	0.25	**+7.5**	**6**	**0.02**
SH1 1:48	+4.5	6	0.81	+22	6	0.12
SH1 1:96	−1.6	6	0.93	+9.7	6	0.37
SH1 1:192	−32.1	6	0.68	−25.4	6	0.5
SH1 1:384	−34.2	6	0.184	−25.5	6	0.29
SH1 1:768	−19.6	6	0.39	−14.2	6	0.64
SH1 1:1536	−11.4	6	0.58	+6.03	6	
	Pseudomonas aeruginosa PS_1586			*Pseudomonas aeruginosa* PS_6749		
Aquacel Ag	−88.2	2	0.175	**−85.2**	**4**	**0.002**
Aquacel Ag + Extra	**−84.2**	**4**	**0.01**	**−72.3**	**4**	**0.004**
Actilite	**+155.6**	**4**	**0.001**	**+90.4**	**4**	**0.002**
L–Mesitran Net	+123.1	4	0.007	**+154.8**	**4**	**0.015**
L–Mesitran Hydro	**+233.2**	**4**	**0.02**	**+117.1**	**4**	**0.048**
SH1 1:3	**−79.8**	**6**	**0.04**	**−80.9**	**6**	**0.05**
SH1 1:6	−59.6	6	0.08	−65.6	6	0.089
SH1 1:12	+18.5	6	0.49	−32.7	6	0.293
SH1 1:24	+112.1	6	0.3	**+102.5**	**6**	**0.04**
SH1 1:48	**+106.3**	**6**	**0.002**	−35.2	6	0.425
SH1 1:96	**+82.3**	**6**	**0.035**	−39.4	6	0.38
SH1 1:192	+24.8	6	0.5	−42.7	6	0.312
SH1 1:384	+45.6	6	0.19	−48.5	6	0.22
SH1 1:768	−43.8	6	0.29	−63.9	6	0.127
SH1 1:1536	+29.7	6	0.342	+9.5	6	0.78

% Change of biofilm biomass compared with an untreated positive control; SHI–SurgihoneyRO™; ‡p values from Student's

PS_PA01) and 1:12 (with a 57% reduction for PS_1054). When SH1 was diluted beyond these values, there were no statistically significant reductions in biofilm formation (Fig 3).

Of the AMD, Aquacel Ag, and Aquacel Ag + extra (the silver-containing dressings) were most effective at preventing biofilm formation for the majority of isolates. Apart from the result for PS_PA01, Aquacel Ag was associated with reductions of 84–95%, and the reduction was statistically significant for PS_6749, and PS_1054. Similar results were obtained with Aquacel Ag + extra. The performance of the honey-containing dressings (Actilite, L-Mesitran Net, and L-Mesitran Hydro) was disappointing, with average increases in biofilm formation of 98% for all isolates with Actilite (range: 62–155.6%), and increases in biofilm biomass for three isolates (PS_1054, PS_1586 and PS_6749) with L-Mesitran Net, and L-Mesitran Hydro (Table 4).

Discussion

Although several studies have shown the antibacterial effects of a range of medical-grade honeys on biofilms,[20,21] none have tested SH1: an engineered natural honey has enhanced production of ROS. Through a series of *in vitro* experiments on a panel of 16 clinically important burn-wound pathogens, we have shown that all the honeys (SH1, MH and Med) are able to reduce biofilm formation *in vitro*, but that the MBICs differ per honey and per organism.

Generally, the Gram-negative organisms were most susceptible to the honeys, including those MDR organisms. SH1 was the most potent of all the honeys, being effective at lower concentrations (weaker dilutions) than those required for MH and Med, and compared favourably against the two commonly used silver dressings. SH1 was also more effective at preventing biofilm formation *in vitro* of four *Pseudomonas aeruginosa* isolates than the three commercially available honey-containing dressings. While we did not conduct an extensive evaluation against all available honey-containing AMDs, we have compared all those available in our hospital that contain honey of some variety.

Honeys (including those of medical grade) are diverse, and it is known that potency can vary as much as 100-fold.[22] Cooke et al.[9] showed that the antibacterial activity of SH1 was due to the generation of H_2O_2 and reactive oxygen species (ROS),[23] and that release was sustained over a period of at least 24 hours. H_2O_2 is produced on dilution of the honey[24] by the action of glucose oxidase,[25] and reaches maximum levels when the honey is diluted to between 50 and 30% w/v.[26] Sustained ROS activity in SH1 has been demonstrated *in vitro* for over 3 days through the use of peroxide testing strips.[13] Of note, MH is a non-peroxide honey and H_2O_2 has been shown to be absent.[27] This may explain the differences in potency between the three honeys.

SH1 represents a highly effective and promising topical antibacterial as evidenced by both *in vitro* experiments, and *in vivo*.[12,14]

A Cochrane review[2] into 'honey as a topical treatment for wounds' concluded that there is high-quality evidence that honey accelerates the healing of partial-thickness burn wounds com- pared with conventional dressings, and is also more effective than antiseptic for treating infected surgical wounds. The evidence for the clinical benefits of honey for wound healing, however, was equivocal, although SH1 was not available for inclusion in the referenced studies. These data pre- sented in this and other studies[9,12,13] strongly sup- port the use of SH1 as an antibacterial, and suggest further clinical evaluation is warranted. A rigorously controlled clinical trial is currently being planned at our centre to investigate the clinical use of SH1 further.

Limitations

However, there are limitations that must be considered. For example, the *in vitro* experiments all involved single species of bacteria, which have been artificially grown in the presence of the required nutrients. Biofilms in the clinical setting are likely to be vastly different in terms of their composition (for example they are likely to be composed of mul- tiple bacterial species) and physiology (in terms of metabolic rate and presence of nutrients). Our data have shown that SH1 can prevent biofilm formation *in vitro* but not the impact it has on established biofilms. Preliminary work we have performed, has shown that SH1 can reduce the seeding (the release of planktonic bacterial cells from the biofilm surface so that new sites can be colonised) of pre-formed biofilms of the same panel of 16 isolates, after 24-hour exposures. Therefore, some evidence exists that honey can disrupt biofilms *in vitro* (Halstead et al. unpublished), however comparable *in vivo* activity against biofilms remains to be tested.

The *in vitro* data from this study also highlights that for some isolates (PS_1054, PS_1586, PS_6749, EC_042 and MSSA_10788) the test honey may actually enhance growth at certain dilutions. For these six isolates, all honeys were equally likely to enhance biofilm growth with this typically occurring at values lower than the MBIC (1:24 for the *Pseudomonas* isolates, 1:96 for MSSA_10788, and 1:192 for EC_042). This may prove problematic in the clinical setting if the honey is applied at an effective concentration but is then diluted down to subinhibitory concentrations by wound exudates, or other secretions from the wound surface. An additional limitation is that the majority of clinical reports to date are anecdotal reports of patients with chronic wounds recovering after application of SH1—it would need to be demonstrated that this was due to the SH1 and not simply because the wound was already healing.

Conclusions

The work presented here supports previous *in vitro* findings and is consistent with the anecdotal clinical evidence. All the honeys tested, SH1, MH and Med, demonstrated antibacterial activity against the formation of biofilms. However, SH1, appears to be the most potent against a range of Gram- positive and Gram-negative bacteria, including MDR organisms.

Although further randomised controlled trials are required, SH1 clearly has considerable thera- peutic and infection-prevention potential for the management and treatment of chronically colonised and infected wounds, and may help to reduce the use of antibiotics and selection pressure while promoting wound healing and infection prevention. n

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