Use of an engineered honey to eradicate pre-formed biofilms of important wound pathogens: an *in vitro* study

**Objective:** There is a close link between chronicity of wounds, and the presence of biofilms. Honey is a well-recognised wound care agent, with demonstrable *in vitro* antibacterial activity against a broad-range of Gram-positive and Gram-negative bacteria. We previously reported on the ability of SurgihoneyRO™ (SHRO), an engineered honey, to prevent biofilm formation *in vitro*, but data was lacking regarding the activity against pre-formed biofilms. This study was undertaken to assess whether SHRO has any antibacterial activity against mature, pre-formed biofilms, and whether there is any evidence to support the observed clinical effectiveness when SHRO has been used anecdotally on acute and chronic wounds where biofilm is most likely present.

**Method:** Laboratory experiments tested the *in vitro* antibacterial activity of SHRO against mature biofilms of 16 clinically relevant wound pathogens, in terms of impacts on biofilm seeding, and biofilm biomass. The honey was 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:** All 16 bacterial isolates were susceptible to SHRO, with reduced biofilm seeding observed for all, and percentage reductions ranging from 58% (ACI_C59) to 94.3% (MDR_B) for the strongest concentration of honey (1:3). Furthermore at this concentration, biofilm seeding of the test biofilm was reduced by 80–94.3% (when compared with the positive control) for 12/16 isolates. We additionally demonstrated that SHRO has antibiofilm impacts, with the 24 hour exposure resulting in disruption of the biofilm, reduced seeding, and reduced biomass.

**Conclusion:** SHRO is effective at reducing seeding of pre-formed biofilms of clinically important wound pathogens *in vitro*, and also has antibiofilm activity. This supports the anecdotally clinical data for antibiofilm efficacy, and furthermore supports the use of SHRO as a promising topical wound care agent.

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antimicrobial • biofilms • honey • hydrogen peroxide • wounds • MBEC

There is a close link between chronicity of wounds, and the presence of biofilms, with biofilms found in >60% of those with chronic wound infections, compared with just 6% of those with acute wounds.1,2 Concomitantly, biofilm-based wound management is an avenue that needs exploring if the burden of chronic non-healing wounds is to be reduced. Given the persistence of chronic wounds, alternative wound care agents need to be explored.

Honey is a well-recognised antimicrobial wound care agent which possesses multifactorial antimicrobial properties. This is due to the honey preparation itself (e.g. physical properties including pH and osmolarity,3,4 and antibacterial components of the honey (e.g. methylglyoxal, bee defensin-1 and -2, phytochemicals and hydrogen peroxide (H2O2)).3-5 Honey also contributes significantly to wound healing processes, owing to anti-inflammatory and antioxidant properties, and a boosting effect on the immune system.3 Hydrogen peroxide in particular is a key antimicrobial component of honey, which is slowly released when wound exudate interacts with glucose oxidase (which converts glucose to H2O2).5,6 and causes oxidative damage of macromolecules of pathogens due to the production of hydroxyl radicals (resulting in restricted bacterial growth and DNA degradation).6,7 Interest in honey, as well as other ‘novel’ antimicrobial agents, has seen resurgence in recent years owing to the widespread misuse of antibiotics, together with increasing threats posed by antimicrobial resistance.

A range of medical honeys exist on the market (for example chestnut, manuka, thyme, revamil, multifloral, and Medihoney,4 which differ from natural honeys in that they are more likely to be of predictable and consistent quality (the composition of natural honey is influenced by a range of factors including e.g floral source, species of bee and geographical location).3,4 A narrative review on the therapeutic activities of honey on wound healing by Oryan et al.,3 identified 25 clinical studies where honey (of varying types) was used on patients with acute wounds, chronic wounds or a...
combination of wound types. They concluded that honey accelerates the wound healing process, and reduces the incidence of scar formation.

There have been a number of in vitro studies investigating the effect of honey (mostly Manuka) on wound bacterial biofilms. There are four studies8–11 that report that treatment with Manuka honey is effective in reducing levels of *Pseudomonas aeruginosa* and meticillin-resistant *Staphylococcus aureus* (MRSA) bacterial biofilms *in vitro*, with Majtan et al.12 investigating the antibiofilm effects of honey against *Proteus mirabilis* and *Enterobacter cloacae*. Using microtitre plate assays to quantify biofilm formation, and a variety of honeys (hawthorn, honeydew, acacia and commercially available Manuka), they found that all honeys significantly reduced the biofilm development of both isolates, and significantly decreased biofilm seeding and viability of existing biofilms.

SurgihoneyROTM (SHRO) (Matoke Holdings, UK) is a licensed CE marked sterile topical engineered honey, which differs from other medical honeys as it does not rely on a single floral pollen source, and has been designed to produce elevated levels of reactive oxygen species (ROS) (including H₂O₂). It has a broad spectrum of activity, and has been shown *in vitro* to be active against Gram-positive and Gram-negative bacteria, including multidrug resistant strains (such as carbapenem resistant enterobacteriaceae (CPE), and extended-spectrum β-lactamase producing *Escherichia coli*),13,14 as well as fungi.14 It is furthermore considered non-toxic to human tissue, and is capable of delivering ROS over a prolonged period.13 Given the importance of ROS for bacterial killing, SHRO is reported to be a more potent antimicrobial than other medical honeys. In a recent study15 we compared the *in vitro* activity of SHRO (referred to as SH1) to two other medical-grade honeys (Activon manuka honey (MH), and Medihoney Manuka honey (Med)), and five antimicrobial dressings (AMDs), in terms of the ability of the honey(s) to prevent biofilm formation. Honeys were serially doubly diluted (in sterile autoclaved distilled water) from 1:3 down to 1:6144 and their impact on supressing biofilm formation by 16 clinically relevant bacterial isolates (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*) was assessed using a crystal violet (CV) biofilm formation assay. The outcome measure was the ‘minimum biofilm inhibition concentration’ (MBIC), which 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 of ≥50% compared with the positive control. Although all the honeys were antibacterial, and could prevent the formation of biofilms, SHRO was the most potent, with MBICs at lower dilutions than the medical honeys for five isolates, and at equivalent dilutions for a further six. SHRO is indicated for use in infected wounds, chronic Wounds (pressure ulcers, venous leg ulcers, diabetic foot ulcers), and acute wounds (surgical wounds, burns, donor and recipient sites, cuts and abrasions), and reported clinical outcomes have been favourable to date. A multi-centre clinical evaluation of SHRO was performed for 114 topical wounds (from 104 patients) by Dryden et al.16 Wounds comprised a range of types (including ulcers, surgical wounds, site infections and traumatic wounds) from both the developed and developing world, and varied in their chronicity, with mean wound durations ranging from 1 month to 8 months. SHRO was applied to the wound beds and covered with a suitable sterile secondary dressing; with reapplication recommended for every 2–3 days (this however was at the discretion of the clinician). Endpoints of interest were changes in microbial loads, and wound healing. Although durations of treatment varied (from 9 days for central catheter site infections to 374.3 days for other topical infections), 24 wounds (21% healed, and the remaining 90 (79%) improved following application of SHRO. These authors have also shown that SHRO can reduce bacterial colonisation in long lines in oncology patients,17 and reduce rates of surgical site infections in patients undergoing caesarean section surgery.18

However, the clinical findings published to date on SHRO represent a series of case reports, which although demonstrate favourable outcomes, must be interpreted with caution. The clinical evaluation of SHRO on 114 wounds for example included subjective reporting, sampling bias, and there was neither randomisation nor control of the treatment. Furthermore, it was not possible to assess biofilm presence for each of the wounds. These are all acknowledged by Dryden et al.,16 as limitations to the *in vivo* study.

Continuing on from the previous study,15 this in vitro study was undertaken to assess whether SHRO has any antibacterial activity against mature, pre-formed biofilms. Biofilm seeding was used as the endpoint measure of biofilm viability, and SHRO was tested on a range of biofilm-producing bacteria, with the activity compared to a non-treated positive control. The aim was to ascertain if there is any evidence to support the observed clinical effectiveness when SHRO has been used anecdotally on acute and chronic wounds where biofilm is most likely present.

**Methods**

Minimum biofilm eradication concentration (MBEC) *in vitro* experiments were conducted on a panel of important wound pathogens, to determine the antibacterial effects of SHRO against mature biofilms. The panel contained 16 isolates, which had previously been identified as good biofilm producers,19 and had already been used to assess the in *in vitro* activity of SH for preventing biofilm formation.15 In particular, the panel comprised a mixture of well-characterised control strains and clinical isolates, including four *Pseudomonas aeruginosa*, four *Acinetobacter baumannii*, four
Staphylococcus aureus, three multi-drug resistant organisms (MDR) (Klebsiella pneumoniae (carbapenem-resistant Enterobacteriaceae), Escherichia coli (ESBL positive), and Pseudomonas aeruginosa (carrying the Verona integron-encoded β-lactamase (VIM) plasmid conferring resistance to the carbapenems)), and a standard E. coli isolate for reference (Table 1).

The isolates were stored at -80°C on Protect™ beads, and were routinely cultured on cysteine lactose electrolyte deficient (CLED) agar, or blood agar (as appropriate) (Biomerieux, France) prior to each experiment.

The SHRO sachets (10g) were freshly opened and were within date when used. Experiments were performed using at least two biological replicates, and at least six technical replicates of each isolate per test dilution.

Preparation of the honey
SHRO was tested at a range of dilutions. This 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 strongest concentration that could be accurately pipetted was 1:3 (v/v), and was made by placing 6ml of honey into a universal and adding 14ml of sterile autoclaved distilled water to make a total volume of 20mls, which was then serially double diluted down until 1:6144 was reached.

Impact of SHRO on biofilm eradication
The antibacterial activity of SHRO against mature biofilms in vitro was assessed by conducting MBEC experiments per isolate, as described by Ceri et al.21 followed by a crystal violet biofilm accumulation assay (to quantify the amount of biofilm that had formed at the time of exposure), as described by Baugh et al.22

Overnight cultures of the test strains (grown in 5ml of Lysogeny (LB) broth [Oxoid, Reading]) were diluted in fresh antibiotic-free Mueller-Hinton (MH) broth [Oxoid] to an optical density at 600nm (OD600) of 0.1, and then 200µl seeded into wells of a 96-well microtiter tray (MTT) (Fisher Scientific). A 96-well polystyrene plate [Starlabs, UK] was placed into the MTT, so that each well contained a ‘peg’ on which biofilms could form. Suitable controls were included, comprising 200µl overnight bacterial culture (for the positive control), or 200µl MH broth alone (for the negative control). The assembly was sealed with cling film, and statically incubated at 33°C for 72 hours. This temperature was chosen for biofilm formation as it represents the average temperature of the surface of the skin,23 and therefore has most relevance for wound infections.

After 72 hours, the pegs (containing the biofilm) were washed in an MTT containing 200µl sterile distilled water (to remove any unbound cells), and the peg plate then placed in a further MTT containing 200µl of diluted SHRO at the following dilutions from neat: 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. The positive and negative control pegs were placed into 200µl of water, so that the only differing variable was the presence or absence of honey. This assembly was statically incubated at 33°C for 24 hours. Two biological and three technical replicates were performed for each strain and each honey dilution, respectively. All assays were repeated, providing data for 12 technical replicates per isolate.

To assess the viability of the exposed peg biofilm, the peg plate was removed from the diluted honey, washed in sterile water as before, and placed into an MTT containing 200µl sterile MH ‘reporter’ broth for static overnight incubation at 33°C. The OD of the reporter broth was measured after 18 hours using a FLUOstar Optima [BMG Labtech], to assess the viability (seeding) of the biofilms following exposure to SHRO, and to determine the minimum concentration which significantly reduced biofilm seeding (and by inference had killed some of the cells in the biofilm).

Demonstration of biofilm presence
Crystal violet (CV) assays were performed on the exposed peg plates (after the OD of the reporter broth had been measured), in order to demonstrate biofilm presence. The peg plate was placed into an MTT containing 200µl of 1% CV [Sigma Aldrich, Poole, UK], incubated for 20 minutes at room temperature, rinsed with sterile water in a further MTT (to remove unbound CV), and finally placed into a further MTT containing 200µl of 70% ethanol. Once the dye was solubilised (20 minutes incubation at room temperature), the OD600 of the solubilised CV solution was then measured (as before) to assess whether biofilms were present on the test pegs.

Data analysis
The positive and negative controls for each test plate were examined and if within a normal range the rest of the data were analysed for statistical significance by comparing values at each dilution of honey to untreated (positive) controls using the students’ ‘t’ test. The MBEC was defined as the lowest dilution of honey where there was both statistical significance in the t-test (p value <0.05), and a reduction in biofilm seeding of greater than or equal to 50% compared to the positive control. This value has been arbitrarily chosen since to the best of our knowledge, no standard definition exists.

Results
SHRO was tested against mature biofilms of all 16 isolates, with at least two biological and 12 technical replicates tested per honey dilution. The mean average optical densities of the reporter broth were calculated across the 12 replicates for each isolate and honey dilution, and were plotted on the y axis relative to the dilution of honey (x axis) to visually represent any reduction in the seeding of the mature biofilms achieved by the treatment at the range of dilutions.
This data is shown in Figure 1. To determine the MBEC per isolate, the percentage difference in seeding of the reporter broth between the positive (unexposed) control and the exposed biofilms was furthermore calculated (Table 2), as well as the p-value (according to the Student’s t-test) (Table 3).

Impact of SHRO on biofilm eradication
All 16 bacterial isolates were susceptible to SHRO, with reductions in seeding (following 24 hour exposure to the agent) observed. MBECs (representing statistically significant reductions in seeding of at least 50%) ranged from 1:6 dilutions of honey from neat (PS_6749, ACI_C60, MDR_B, MDR_C, EC_042, MSSA_10788, and MSSA_F77), to 1:384 (ACI_C59), with 1:6 being the modal MBEC (occurring for 7/16 isolates). There does not appear to be any relationship between the MBEC values and the bacterial species, with varied MBECs present across the P. aeruginosa, A. baumannii and S. aureus isolates. Three of the four comparator Gram-negative isolates (MDR_B, MDR_C and EC_042) have MBECs of 1:6, and a plot of the biofilm seeding OD is suggestive of greater uniformity in this group, although the numbers of isolates tested is too small to make any firm conclusions.

In terms of the impact of SHRO exposure on biofilm seeding (and viability), all isolates were susceptible, with percentage reductions ranging from 58% (ACI_C59) to 94.3% (MDR_B) for the strongest concentration of honey (1:3). Furthermore (at this concentration), biofilm seeding was reduced by 80-90% for four isolates (PS_PA01, PS_6749, ACI_19606, MDR_B, MDR_C, MDR_D, EC_042, MSSA_10788, MSSA_F77, MRSA_F483). From the data there is a dose-dependent effect, with reductions in seeding of biofilms, diminishing as the concentration of SHRO gets weaker. This is apparent at the weakest concentration (1:6144) of SHRO tested, where statistically significant reductions ranging from 12.1-58.2% occurred with 9/16 isolates only, with the remainder of the isolates demonstrating (non-statistically significant) increases (PS_PA01, PS_1054, MDR_C, EC_042) or modest decreases (PS_1586, ACI_19606, MSSA_F77) in biofilm seeding.

For some of the isolates (PS_PA01, PS_6749, ACI_AYE, ACI_19606, MDR_C, and MDR_D) the data reveals reduced and inconsistent reductions in biofilm seeding, especially at concentrations 1:12 and 1:96. The isolate for which this was most prominent was PS_PA01 (Figure 2), where there was a reduction of 51% at 1:12, and 33.2% at 1:96, compared to reductions of 88.4, 86.1, 88.7 and 86% for the neighbouring concentrations (1:6, 1:12, 1:96, and 1:192, respectively). All results were statistically significant.

Demonstration of biofilm presence
Biofilm presence was confirmed for all 16 isolates using the CV assay. A plot of the biofilm biomass (y axis) against the SHRO dilutions (x axis) is shown (Supplementary Figure S1), and demonstrates that biofilm was present for all isolates at all SHRO dilutions, and was absent in the negative control. Interestingly there is a dose-dependent effect, with the biofilm biomass reducing as the SHRO concentration increases. Since the CV assay was performed following the exposure, it is possible that the SHRO has additionally reduced the biomass of the biofilm. The CV optical density values for two of the Gram positive isolates (MRSA_F475, and MRSA_F483) are a lot lower than for the other isolates, suggesting poor biofilm production in this assay. The biofilm eradication data and MBEC values for these isolates are ‘normal’ however (data not shown), so this may furthermore demonstrate eradication and reduced biomass of the biofilms following SHRO exposure.

Discussion
Using a simple and effective in vitro assay we have provided further evidence that SHRO is antimicrobial, and has significant activity against preformed, mature biofilms of a range of clinically important wound pathogens. All isolates were susceptible to SHRO, with significant reductions in biofilm seeding observed following 24 hours of exposure. This complements our earlier findings, where SHRO was tested against the same panel of 16 isolates to assess whether treatment could prevent biofilm formation. Unlike the previous study, no comparator honeys were included in the in vitro assessment.

The MBECs ranged from 1:6 (seen for 7/16 isolates) to 1:384, based on our MBEC definition of a statistically significant (p<0.05) reduction in seeding of ≥50% compared to the positive control. Comparing these results to the MBIC (biofilm formation) findings, the MBEC represents a stronger concentration of SHRO for 8/16 isolates. It is widely reported in the literature that pre-existing mature biofilms are harder to treat than planktonic bacteria, and therefore this result makes sense. For the others, the MBIC values are higher (stronger concentrations) by one to five dilutions, possibly revealing limitations in the experiment (e.g. in terms of accuracy of the SHRO dilutions that were used, or interpretation of the MBIC).

Decreased reductions in biofilm seeding occurred for six isolates, most commonly only at the 1:12 dilution (PS_PA01, PS_6749, ACI_AYE, ACI_19606, MDR_C, MDR_D), but for others (PS_PA01, PS_6749, ACI_19606), but additionally at the 1:96 dilution. This needs to be considered further since dilution of SHRO could occur in vivo (with exudate), and increased biofilm seeding and dispersal would not be a favourable outcome. There are a number of possible explanations for the decreased seeding reductions observed at these dilutions. The experimental plate layout was such that two of the six 1:12 wells were next to the positive control, and therefore it is possible that contamination might have occurred. This is unlikely however, as
Disruption of the biofilm, reduced seeding, and reduced biofilm formation and persistence in the in vivo setting. Growth conditions, and hence are unlikely to mimic of abiotic surfaces (e.g. plastic), using standardised assays were conducted on a range acknowledged. The assays were conducted on a range activity, with the 24 hour exposure resulting in increased biofilm formation and consequently increased seeding. Indeed, it is reported that in some bacterial biofilms (e.g. A. baumannii), dispersal and seeding is activated in the presence of abundant nutrition.

Dose-dependent reductions in biofilm biomass occurred as the SHRO concentration increased (Figure S1). This demonstrates that SHRO has antibiofilm activity, with the 24 hour exposure resulting in disruption of the biofilm, reduced seeding, and reduced biomass. This is an important outcome as it shows antibacterial effects against both the planktonic and sessile bacterial cells.

The limitations of the in vitro assays must be acknowledged. The assays were conducted on a range of abiotic surfaces (e.g. plastic), using standardised growth conditions, and hence are unlikely to mimic biofilm formation and persistence in the in vivo setting. Malone et al.25 address this in an elegant review, highlighting the complexities of biofilms, and the large variation, and range of factors that influence biofilm architecture from in vitro to in vivo settings (e.g. the host immunological response). For simplicity, the assays were also conducted using monomicrobial biofilms, although it is well recognised that the majority of clinically relevant biofilms are polymicrobial in nature, involving a range of bacterial species (chronic ulcers are estimated to contain an average of 6.3 bacterial species,26 as well as fungi. This work was performed as an initial evaluation of SHRO, which would have been significantly more complex with polymicrobial biofilms. Future in vitro studies should therefore investigate the antibacterial activity of SHRO against a range of polymicrobial biofilms both in terms of prevention of biofilm formation, and eradication of preformed biofilms.

To extend this work into the clinical setting, it would be prudent to perform further in vitro studies, to investigate the antibacterial activity of SHRO on polymicrobial biofilms, and to investigate the possibility of the development of bacterial resistance to SHRO. This information could then be used to support a robust randomised clinical trial, which would provide an assessment of clinical efficacy of this very promising topical antimicrobial agent.

Conclusions

There is a close link between chronicity of wounds, and the presence of biofilms. Honey is a well-recognised wound care agent, with demonstrable in vitro antibacterial activity against a broad-range of Gram-positive and Gram-negative bacteria. We previously reported on the ability of SHRO (an engineered honey) to prevent biofilm formation in vitro, but data was lacking regarding the activity against pre-formed biofilms.

The in vitro findings reported herein show that SHRO is antibacterial against biofilms, significantly reducing biofilm seeding of a range of different species of bacteria, including common wound pathogens and multi-drug resistant isolates. As well as reductions in biofilm seeding, we also observed reduced biofilm biomass (as measured through the CV assay), which together would suggest that SHRO has anti-biofilm properties, and consequently that the treatment does not just kill the outermost planktonic bacteria in the biofilm.

The findings therefore support the anecdotal clinical data for anti-biofilm efficacy, and the topical use of SHRO for the treatment of chronic wounds, where biofilms are likely to be present.

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