**Diluted Honey Inhibits Biofilm Formation: Potential Application in Urinary Catheter Management?**

Somadina Emineke, Alan J. Cooper, Sarah Fouch, Brian R. Birch, Bashir A. Lwaleed

1. School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, UK

1. Department of Urology, University Hospital Southampton NHS Foundation Trust, Southampton, UK

1. Faculty of Health Sciences, University of Southampton, Southampton, UK

Corresponding author:

Dr Bashir A. Lwaleed

Faculty of Health Sciences

University of Southampton

South Academic and Pathology Block (MP 11)

Southampton General Hospital

Tremona Road

Southampton

SO16 6YD

United Kingdom

Tel: (++ 44) 02381 206559

Fax: (++ 44) 02381 206922

E-mail: [bashir@soton.ac.uk](mailto:bashir@soton.ac.uk)

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**ABSTRACT**

**Introduction:** Biofilms are ubiquitous and when mature have a complex structure of microcolonies in an extracellular plolysaccharide and extracellular DNA matrix. Indwelling medical devices harbour biofilms which have been shown to cause infections and act as reservoirs for pathogens. Urinary catheters are often in place for considerable periods of time and are susceptible to both encrustation and biofilm formation. Strategies for minimizing biofilm occurrence underpin an active research area in biomedicine. Manuka honey has, *inter alia,* well established antibacterial properties. This study aims to assess the influence of honey on early biofilmformation in an established *in vitro* model.

**Materials and Methods:** An established model of early biofilm formation using static bacterial cultures in vinyl 96-well plates was used to grow *Escherichia coli*, strain ATC 25922 and *Proteus mirabilis*, strain 7002. Planktonic cells were removed and the residual biofilm stained with crystal violet, which is subsequently eluted and quantified spectrophotometrically. Manuka “UMF15+” was added either with the bacteria or up to 72 hours after.

**Results:** Biofilms in this model developed over three days, after which growth stalled. Mixed (1:1) cultures of *Escherichia coli* and *Proteus mirabilis* grew slower than monocultures. In mixed cultures honey gave a dose-dependent reduction in biofilm formation (between 3.3 and 16.7 %w/v). At 72 hours all concentrations inhibited maximally (p<0.001). Application of honey to cultures after 24 and 48 hours also reduced the adherent bacterial biomass (p<0.05 – p<0.01).

**Conclusion:** Manuka honey at dilutions as low as 3.3% w/v in some protocols and at 10% or above in all protocols tested, significantly inhibits bacterial attachment to a vinyl substrate and reduce further early biofilm development. No augmentation of growth over untreated controls was observed in any experiment.

**WHAT THE PAPER ADDS**

Strategies for biofilm prevention have for some time centred round modified surfaces or materials that are inimical to bacterial adhesion with limited success, or to antibiotic use, trends in which are currently towards promoting alternatives. We present evidence from an *in vitro* model that honey is a promising alternative candidate.

**INTRODUCTION**

Biofilms are ubiquitous [[1](#_ENREF_1)]. Indwelling medical devices as well as epithelial layers exposed to the environment engender and harbour biofilms and in the case of devices, they have been shown to cause infections and act as environmental reservoirs for pathogens [[2](#_ENREF_2)]. Within a mature biofilm, bacteria are enclosed in a largely self-produced extracellular matrix, accounting for about 90% of the biomass [[3](#_ENREF_3)]. The matrix is made up of extracellular polymeric substances that, along with pili, flagella, carbohydrate-binding proteins, extracellular DNA (eDNA) and other adhesive fibers, act as a stabilizing scaffold for the three-dimensional biofilm structure [[4](#_ENREF_4)]. Enzymes secreted adaptively by the bacteria customize biofilm architecture to the current environment. The result is a highly robust structure with high tensile strength that keeps bacteria in close proximity, allowing cell to cell interactions and DNA exchange, at the same time protecting the biomass from damaging agents [[3](#_ENREF_3)]. The maturation processes in biofilms bestows survival advantages [[5](#_ENREF_5)], achieved in part by quorum sensing [[6](#_ENREF_6)] through gene transfer, biofilm attachment and the production of virulence factors. The result is bacterial microcolonies exhibiting their own cyclical existence [[7](#_ENREF_7)].

Indwelling urinary catheters are commonly used in medical and nursing care. Long-term catheterisation is associated with frequent complications, many arguably linked to inflammation and/or infection, encrustation and biofilm formation [[8](#_ENREF_8)]. *Escherichia coli* is the cause of 80–85% of urinary tract infections [[9](#_ENREF_9)]. Strategies for minimizing their occurrence and impact underpin a currently active research area in biomedicine [[10](#_ENREF_10)].

Honey has been used as a remedy for centuries [[11](#_ENREF_11)], but the active ingredients including glucose oxidase catalase and a range of polyphenols are more recently described [[12](#_ENREF_12)]. Potentially therapeutic properties include antibacterial and anti-inflammatory effects [[12](#_ENREF_12)] as well as modulation of angiogenesis [[13](#_ENREF_13)] and inhibition of induced histamine release by mast cells [[14](#_ENREF_14)]. We acknowledge that despite ample evidence of potential therapeutic properties, validation of topical honey applications in medicine have not been robust, as evidenced in three Cochrane reviews [[15-17](#_ENREF_15)]. However, in these reviews honey does not fare worse than classical and well accepted compounds, such as povidone iodine and silver or peroxide based products. In fact no drug or dressing receives ringing endorsement, suggesting that the field is under-investigated or difficult to address. Resistance is an issue in antimicrobial therapies; studies generally assert the inability of bacteria to develop resistance to honey [[18](#_ENREF_18)]. This study addresses in a reproducible model system, fundamentally that described by O’Toole and colleagues [[19](#_ENREF_19)], the hypothesis that relatively dilute and therefore acceptably non-viscous dilutions of honey might have a role as a flushing agent to minimise the initial establishment and early development of biofilms on implanted devices such as urinary catheters. Maturation is another issue, not well addressed by static culture; models incorporating some sort of flow, through or across the affected surface, are required for progression to a complex structure [[19](#_ENREF_19)]. Prevention, however is determined by inhibition of attachment and early biofilm development.

**MATERIALS AND METHODS**

**Bacteria**

Two microorganisms from genera commonly associated with catheter-associated urinary tract infections were used in this study. *Escherichia coli*, strain ATC 25922 and *Proteus mirabilis*, strain 7002 were available in-house on agar slopes and grown on in Luria-Bertani (LB) broth. The inoculated broth was incubated for 24 hours without shaking. 0.1% and 1.0% concentrations of *Escherichia coli* and *Proteus mirabilis* were prepared in LB broth, vortexed and 100µl of the bacterial suspension was pipetted into each well of the 96-well plate. Initial experiments establishing the method were performed with monocultures and 1:1 v:v mixed cultures, all adjusted to a McFarlane 1% standard.

**Honey**

Manuka honey UMF (Unique Manuka Factor) 15+ from Comvita (U.K.) Ltd was purchased from a local health food shop. A 50%w/v stock dilution was prepared in distilled water.

**Bacterial growth assay**

Round bottomed 96 well polyurethane plates (Fisher Scientific) were used to assess bacterial biofilm formation from a final 150µl culture volume. At the termination of the experiment supernatant medium containing planktonic bacteria was gently aspirated to clear flat bottom 96 well plates for measurement of planktonic bacteria using absorbance at 620nm in a plate reader. Each well of the experimental plates was rinsed three times with 200µl of distilled water without disturbing the adherent biofilm. The plate was air dried for 5 minutes. Crystal violet (125µl of 0.1%, 15 minutes, ambient temperature) was used to stain bacteria. The crystal violet was removed and each well rinsed three times with 200µl of distilled water and left to air dry. 200µl of 95% ethanol per well was subsequently added and the plates incubated at room temperature for 15 minutes. The contents of each well were mixed and 125µl of the crystal violet/ethanol solution was transferred to clear flat bottom 96 well plate. The extent of biofilm was determined by measuring absorbance at 593nm.

To assess the contribution of each species to the biofilms in a temporally separate series of experiments, wells were washed free of non-adherent organisms, adherent bacteria were wiped off and plated and incubated on Mackonkey agar with neutral red as a discriminant colour indicator for lactose fermenting organisms .

**Experimental protocols**

Preliminary experiments demonstrated that bacteria, either as monocultures or mixed, adhered to the plate walls and that these biofilms developed over three days, after which absorbance from eluted stain decreased. A maximum of 3 days was therefore imposed on further experiments. All cultures were incubated aerobically at 37oC.

Treatment with honey present throughout

Five concentrations of honey (10, 20, 30, 40, 50%) were added in 50µl of medium to two columns of each plate. This gave final concentrations, as reported in the results section of 16.7, 13.3, 10.0, 6.6 and 3.3% w/v. For controls, the first column had 50µl of plain medium added and in the second row, 50µl of “half strength artificial honey” (45% glucose, 48% fructose, 1% sucrose w/v final). Plates were sealed and incubated for 24, 48 and 72 hours to assess the effect of honey on bacterial biofilm formation.

Addition of honey after initial biofilm establishment

Plates were seeded with bacteria as described above. After 24 hours of biofilm formation the medium (containing planktonic bacteria) was discarded, 100µl of fresh LB broth added to each well and treatment with honey initiated as described above. The treated plates were incubated for 4 or 24 hours with honey before being prepared for staining.

**Statistical analysis**

Data were included in a database and analyzed by GraphPad Prism™. Results are normally distributed and expressed as means ± SEM. Differences between two or more groups were assessed by One-Way ANOVA, with pairings of each honey concentration vs controls assessed by the Quickcalcs™ post-hoc calculator. Setting α=0.05, pairs are reported as significant or not.**RESULTS**

Preliminary experiments established that, under the conditions employed, early biofilm formation assessed by bacterial content was optimal for study after 3 days at 37oC. Further incubation yielded no further growth, indeed a tendency to reduced optical density readings was observed.

Figure 1 shows honey at all five concentrations used reducing the optical density readings obtained in the culture supernatants (planktonic cells) after incubation by a minimum of 35% (day 2, 3.3% honey), the greatest reduction being 77% (day 3, 16.7%honey). Days 2 and 3 results exhibited dose dependency across the range of dilutions tested.

Adherent biofilm crystal violet stain was also reduced by continuous exposure to honey (Figure 2), but in this situation dose dependency was observed at 24 and 48 hours, giving reductions in optical density between 15 and 70%. At 72 hours all the honey concentrations employed gave approximately 70% suppression of optical density.

Application of honey dilutions to 24 hour-old established cultures for both 4 and 24 hours illustrate a pronounced dose response to honey for both exposure times (Figure 3). However, the lowest (3.3%) honey concentration applied over 4 hours gave the only groups of treated wells in the study where the mean optical density was higher numerically (by 3% and 1.5% for the two lowest honey concentrations) than the untreated control. These two columns were rated “not significant” on post-hoc testing. The maximum effects, at 16.7% honey, were 38% and 46% for 4 hour and 24 hour exposures respectively.

Differential colony counting was performed as separate experiments, by different operatives, using different stock bacterial cultures and using a stored (18 month) batch of honey. The results gave responses to the *Escherichia coli* in line with the were in line with the *crystal violet*  measurements, but the *Proteus mirabilis* was resistant to honey, at least to the level of the upper colony counting limit (Table 1).

**DISCUSSION**

Indwelling urinary catheters are commonly used in medical and nursing care, for the management of bladder drainage. Approximat100 million catheters are sold annually worldwide [20] and 15-25% of patients in acute settings may be catheterized [21]. Chronic problems with urinary control affect up to 20% of the general population, rising to 25% or more in those over the age of 75 [[22](#_ENREF_20)].

It was to be expected that manuka honey would prove bacteriostatic [[23](#_ENREF_21)], as illustrated by the results on planktonic bacteria. There is a suggestion in the results that the active constituent(s) may be consumed or degraded over time, as dose responsiveness increases with length of incubation.

Adherence of bacteria, representing early biofilm formation was strongly inhibited by honey. The shorter incubations showed a strong dose response, but inhibition was maximal at the lowest concentration tested, 3.3% honey, after 72 hours at 37oC. This is not intuitively consistent with the effects noted for planktonic bacteria, but may represent a lasting effect of early damage. Further growth of biofilms established for 24 hours was inhibited by exposure to honey for both 4 and 24 hours, although this was a weaker inhibition and the dose responsiveness rather less smooth. Honey inhibited 48 hour-old biofilms with a steeper and more even dose dependency, with the caveat that 4 hour treatment with 3.3 and 6.6% honey was ineffective. This pattern of results could be taken to indicate that honey sticks more effectively with establishment of the biofilm.

It is important to note that these results relate to bacterial adhesion and early biofilm formation. Moreover this biofilm model is, as used here, self-limiting and not capable of developing a complex matrix. According to reports from O’Toole’s group [[19](#_ENREF_19)] these require an element of flow, through or over the substrate. However, the model used demonstrates a capability of honey to inhibit the formation and early development of biofilms on solid plastic surfaces at concentrations that are not unduly viscous. In clinical applications honey instillation would also confer benefit from its independent anti-inflammatory properties.

Studies in our laboratories on inhibition of histamine release from mast cells indicate that such activities can occur at relatively high dilutions of honey [24, 25]. Another outcome from these studies that requires further enquiry is that honeys from different floral sources have varying activity in assays for different bioactivities that do not align. Antibacterial activity is generally found to be highest in dark honey such as Manuka, whereas the suppression of mast cell activation was maximal with the relatively light coloured eucalyptus honey [24, 25]. Such differential activity presents a problem, but also opportunities for commercial exploitation, in terms of processing, standardization and blending.

The apparent resistance of *Proteus mirabilis* in the colony forming assay was surprising, as other studies have demonstrated antibacterial activity of Manuka honey against this species [26,27]. It arguably highlights the variability of raw honeys, even from the same floral source, with storage time and conditions a likely factor. An alternative explanation for the lack of dose responsiveness is that the counting system was too sensitive to detect any changes that may have occurred. Further studies in this area are ongoing.

In conclusion, our study demonstrates that dilute honey is potentially a useful agent for reducing biofilm formation on indwelling plastics devices such as urinary catheters, probably by using as a periodic flushing agent. This application would require the following further pre-clinical developments: further standardisation of medical grade honeys (or derivatives), storage requirements and assessments of honeys from other floral sources. Honey would also need to be subjected to *in vivo* tolerability trials, probably in rodents) at dilutions that demonstrate efficacy *in vitro*, yet are not too viscous for instillation.

**TAKE-HOME MESSAGE**

Dilute honey inhibiting early biofilm formation on a vinyl substrate lends credence to a putative use in urinary catheter management.

**COMPETING FINANCIAL INTEREST STATEMENTS**

We do not have any direct conflict of interest that we should disclose.

**FUNDING**

**The study was funded internally.**

**Table 1:** Colony formation after 24 hour biofilm formation and subsequent contact with honey for 24 hours.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Honey Concentrations | Planktonic Bacteria | | Biofilm Bacteria | |
|  | *Escherichia coli* | *Proteus mirabilis* | *Escherichia coli* | *Proteus mirabilis* |
| 3.33% honey | >1000 CFU | >1000 CFU | >1000 CFU | >1000 CFU |
| 6.67% honey | >1000 CFU | >1000 CFU | >1000 CFU | >1000 CFU |
| 10% honey | >1000 CFU | >1000 CFU | 800 CFU | >1000 CFU |
| 13.33% honey | 170 CFU | >1000 CFU | 180 CFU | >1000 CFU |
| 16.67% honey | 0 CFU | >1000 CFU | 0 CFU | >1000 CFU |

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**LEGENDS TO FIGURES**

**Figure 1: The Effect of Honey on Planktonic Growth**

Dilutions of honey (50µl) were added to columns of 100µl bacterial cultures. A bank column was left untreated and second control column was treated with artificial sugar solution. Honey dilutions (H) were expressed as final concentration. Optical density of unstained aspirated supernatants plotted at 620nm over 3 days, each time point represents one plate, 8 replicate wells. Columns represent means and standard deviations One-way ANOVA yielded p<0.001; post-hoc analysis (GaphPad Prism™) of individual results against untreated control were all recorded as significant (α=0.05).

**Figure 2: Effect of Honey on Biofilm Formation over 72 hours**

Dilutions of honey (50µl) were added directly to wells containing bacterial culture. Cultures were washed to remove planktonic cells, then dried. Plates were then stained with crystal violet, washed and the stain eluted with 90% ethanol. Plots include reagent blank (no bacteria) and a positive control with untreated bacteria. Optical densities were read at 593nm. Columns represent means and standard deviations. The three plots represent 1, 2 and 3 day incubations. Honey dilutions are expressed as final concentration. One-way ANOVA yielded p<0.001; post-hoc analysis (GaphPad Prism™) of individual results against untreated control are recorded as significant (α=0.05) under asterisk.

**Figure 3: Effect of honey dilutions applied to 24hr adherent biofilm**

Plates incubated for 24hr to establish a biofilm. Honey dilutions (50µl) per well were applied in and incubation was for 4 and 24 hours. Planktonic cells were removed and plates dried. Reagent blank and untreated positive control are included. Optical densities read after staining and elution at 593nm for crystal violet. Columns represent means and standard deviations. One-way ANOVA yielded p<0.001, post-hoc analysis (GaphPad Prism™) of individual results against untreated control are recorded as significant (α=0.05) under asterisk.