Diluted honey inhibits biofilm formation: potential application in urinary catheter management?

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Abstract

Aims

Biofilms are ubiquitous and when mature have a complex structure of microcolonies in an extracellular polysaccharide 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 minimising 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 biofilm formation in an established in vitro model.

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 was stained with crystal violet, which were subsequently eluted and quantified spectrophotometrically. Manuka honey (Unique Manuka Factor 15+) was added either with the bacteria or up to 72 hours after.

Results

Biofilms in this model was developed over 3 days, after which growth stalled. Mixed (1:1) cultures of *E. coli* and *P. 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 (<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 reduces further early biofilm development. No augmentation of growth over untreated controls was observed in any experiment.

Introduction

Biofilms are ubiquitous.[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] Within a mature biofilm, bacteria are enclosed in a largely self-produced extracellular matrix, accounting for about 90% of the biomass.[3] The matrix is made up of extracellular polymeric substances that, along with pili, flagella, carbohydrate-binding proteins, extracellular DNA and other adhesive fibres, act as a stabilising scaffold for the three-dimensional biofilm structure.[4] Enzymes secreted adaptively by the bacteria customise 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 The maturation processes in biofilms bestows survival advantages, [5] achieved in part by quorum sensing[6] through gene transfer, biofilm attachment and the production of virulence factors. The result is bacterial microcolonies exhibiting their own cyclical existence.[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 *Escherichia coli* is the cause of 80%–85% of urinary tract infections.[9] Strategies for minimising their occurrence and impact underpin a currently active research area in biomedicine.[10]

Honey has been used as a remedy for centuries[11] but the active ingredients including glucose oxidase catalase and a range of polyphenols are more recently described.[12] Potentially therapeutic properties include antibacterial and anti-inflammatory effects[12] as well as modulation of angiogenesis[13] and inhibition of induced histamine release by mast cells.[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] 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] This study addresses in a reproducible model system, fundamentally described by Merritt et al,[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] Prevention, however, is determined by inhibition of attachment and early biofilm development.

Materials and Methods

Bacteria

Two micro-organisms from genera commonly associated with catheter-associated urinary tract infections were used in this study. E. 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 *E. coli* and *P. mirabilis* were prepared in LB broth vortexed and 100 mL 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 Unique Manuka Factor 15+ from Comvita (UK) 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, UK) were used to assess bacterial biofilm formation from a final 150 mL culture volume. At the termination of the experiment, supernatant medium containing planktonic bacteria was gently aspirated to clear flat-bottomed 96-well plates for measurement of planktonic bacteria using absorbance at 620 nm in a plate reader. Each well of the experimental plates was rinsed three times with 200 mL of distilled water without disturbing the adherent biofilm. The plate was air-dried for 5 min. Crystal violet (125 mL of 0.1%, 15 min, ambient temperature) was used to stain bacteria. The crystal violet was removed and each well was rinsed three times with 200 mL of distilled water and left to air dry. About 200 mL of 95% ethanol per well was subsequently added and the plates were incubated at room temperature for 15 min. The contents of each well were mixed and 125 mL of the crystal violet/ethanol solution was transferred to clear flat-bottomed 96-well plate. The extent of biofilm was determined by measuring absorbance at 593 nm.

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 and adherent bacteria were wiped off and plated and incubated on MacConkey agar with neutral red as a discriminant colour indicator for lactosefermenting organisms.

Experimental protocols

Preliminary experiments demonstrated that bacteria, either as monocultures or mixed, adhered to the plate walls and that these biofilms developed over 3 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 37°C.

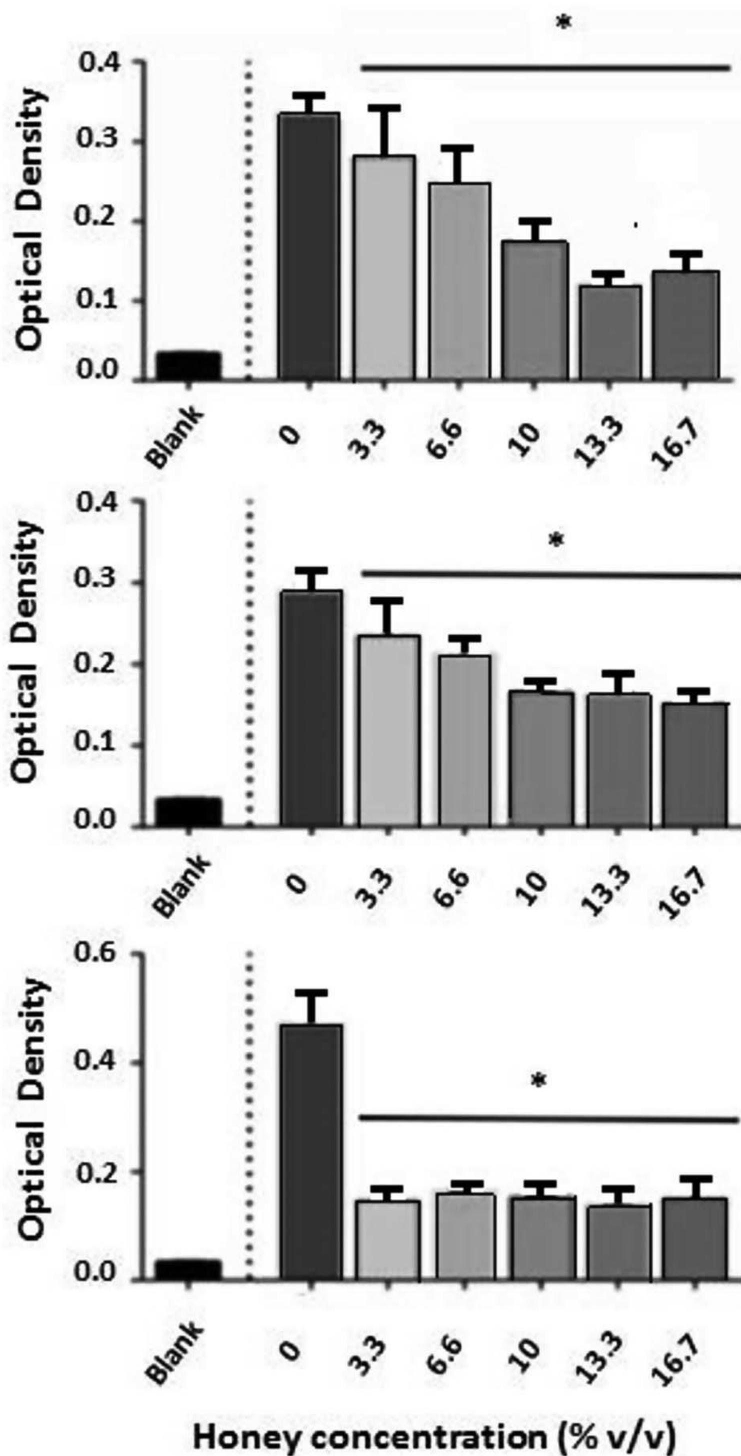

Figure 2 Bar graph showing the effect of honey on biofilm formation over 72 hours. Dilutions of honey (50 mL) were added directly to wells containing bacterial culture. Cultures were washed to remove planktonic cells and then dried. Plates were then stained with crystal violet, washed and the stain eluted with 90% ethanol. Plots include reagent blank (no bacteria) and a positive control with untreated bacteria. Optical densities were read at 593 nm. Columns represent means and SDs. The three plots represent 1, 2 and 3 days incubation. Honey dilutions are expressed as final concentration. One-way analysis of variance yielded $p<0.001$; post-hoc analysis (GraphPad Prism) of individual results against untreated control are recorded as significant ($\alpha=0.05$) under asterisk.

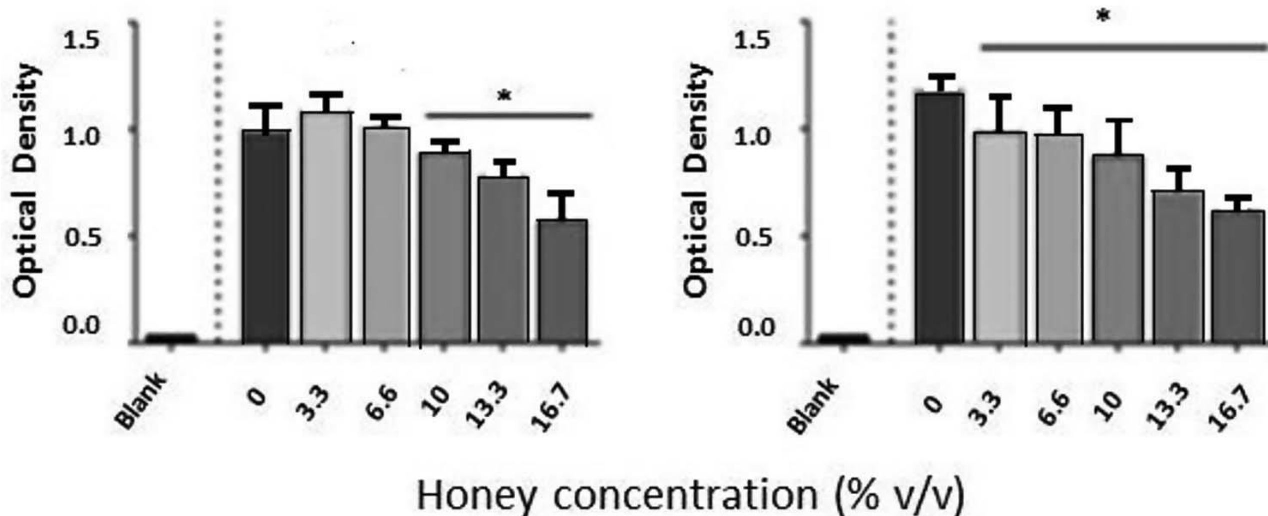

Figure 3 Bar graph showing the effect of honey dilutions applied to 24-hour adherent biofilm. Plates were incubated for 24 hours to establish a biofilm. Honey dilutions (50 mL) per well were applied in and incubation was for 4 and 24 hours. Planktonic cells were removed and plates were dried. Reagent blank and untreated positive control are included. Optical densities read after staining and elution at 593 nm for crystal violet. Columns represent means and SDs. One-way analysis of variance yielded p<0.001; post-hoc analysis (GraphPad Prism) of individual results against untreated control are recorded as significant (α=0.05) under asterisk.

Treatment with honey present throughout

Five concentrations of honey (10%, 20%, 30%, 40%, 50%) were added in 50 mL 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 50 mL of plain medium added and in the second row, 50 mL of 'half strength artificial honey' (45% glucose, 48% fructose, 1% sucrose w/v final) was added. 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, 100 mL of fresh LB broth was added to each well and treatment with honey was 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 analysed by GraphPad Prism (GraphPad Software, Inc., USA). Results are normally distributed and expressed as means±SEM. Differences between two or more groups were assessed by one-way analysis of variance, with pairings of each honey concentration versus 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 37°C. Further incubation yielded no further growth; indeed, a tendency to reduce 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 illustrates 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 *E. coli* in line with the crystal violet measurements, but the *P. 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. Approximately, 100 million catheters are sold annually worldwide[20] and 15%–25% of patients in acute settings may be catheterised. [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

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* (CFU)	*Proteus mirabilis* (CFU)	*Escherichia coli* (CFU)	*Proteus mirabilis* (CFU)
3.33	>1000	>1000	>1000	>1000
6.67	>1000	>1000	>1000	>1000
10	>1000	>1000	800	>1000
13.33	170	>1000	180	>1000
16.67	0	>1000	0	>1000

CFU, colony-forming unit

It was to be expected that Manuka honey would prove bacteriostatic, [23] 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 37°C. 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 was 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 biofilmmodel is, as used here, self-limiting and not capable of developing a complex matrix. According to reports from Merritt et al,[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 honey from different floral sources has 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 and also opportunities for commercial exploitation, in terms of processing, standardisation and blending.

The apparent resistance of *P. 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 honey, even from the same floral source, with storage time and conditions as likely factors. 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 diluted honey is potentially a useful agent for reducing biofilm formation on indwelling plastic devices such as urinary catheters, probably by using as a periodic flushing agent. This application would require the following further preclinical developments: further standardisation of medical-grade honey (or derivatives), storage requirements and assessments of honey 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.

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