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Inhibition and Changes in Antibiotic Sensitivity of Bacteria Cultured Aerobically and Anaerobically in Four Different Medicinal Honeys

Research Article

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Abstract

The growing prevalence of bacterial antibiotic resistance has led to a rediscovery of the antimicrobial properties of honey. This study investigated the antibacterial activity in aerobic and anaerobic conditions, the effect on bacterial antibiotic sensitivity, and the composition of four medical-grade honeys Medihoney®, Comvita® Antibacterial Wound Gel™, Revamil® gel, and Surgihoney™RO®.

A broth assay was used to assess the antibacterial activity of the honeys against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in aerobic and anaerobic conditions. A disk diffusion test was used to investigate the effect of exposure to a subinhibitory concentration of the honeys to the sensitivity of bacteria to a range of antibiotics. The composition of each honey was characterised by measuring: sugar content, pH, hydrogen peroxide activity, total polyphenolic content and antioxidant capacity.

The honeys differed widely in antibacterial activity. Medihoney® was the most effective reducing the growth of both bacteria to < 1 compared to 9 $\log_{10}$ cfu/mL in the growth controls at all tested concentrations. Revamil® gel was the least active of the honeys only having a negligible effect on bacterial growth at the 25% honey concentration. All honeys were equally or more active in anaerobic conditions than in aerobic conditions. The polyphenolic content may influence the activity of honey. Various honey-antibiotic combinations were identified that enhanced antibiotic sensitivity in bacteria. More research is needed to clarify the role of polyphenols in honey activity and further explore the potential synergies between the honeys and antibiotics.

Keywords

Honey, Antibacterial, Aerobic, Anaerobic, Antibiotic sensitivity, Compositional analysis.

Abbreviations

CFU - colony forming units

EPS - Extracellular polymeric substances

FRAP - Ferric ion reducing antioxidant power

GAE - Gallic acid equivalents

H_2O_2 - Hydrogen peroxide

MRSA - Methicillin-resistant *Staphylococcus aureus*

P. aeruginosa - *Pseudomonas aeruginosa*

S. aureus - *Staphylococcus aureus*

SEM - Standard error of the mean

TPTZ - Ferric-2, 4, 6-tri-2-pyridyl-s-triazine

TSA - Tryptone soya agar

TSB - Tryptone soya broth

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Introduction

Honey has been used as a medicine in many parts of the world for thousands of years. Records show, for example, that ancient Egyptians, Assyrians, Chinese, Greeks and Romans already prescribed honey to treat wounds as well as certain gastrointestinal disorders [1]. A little more than a century ago, the first report of the antibacterial activity of honey shed some light on the mechanism underlying the curative effect of honey. This antibacterial activity has been investigated sporadically in the subsequent decades [2,3]. Despite this knowledge and the place of honey in traditional medicine in various cultures, modern medicine turned its back on honey as a therapeutic agent following the introduction of antibiotics in the 1930's [4,5]. More recently, however, the worsening issue of bacterial resistance to antibiotics and the urgent need to find alternative therapies for the treatment of drug-resistant bacterial infections have renewed the interest of the scientific community in the antimicrobial properties of natural substances such as honey [6]. Indeed, investigating the potential of honey as a novel antibacterial agent is a worthy initiative considering that antibiotic resistance is currently seen as one of the biggest threats to global health and food security by the World Health Organization [7].

Honey is a supersaturated sugar solution composed primarily of fructose (38.2%), glucose (31.3%) and sucrose (0.7%), as well as various other disaccharides and oligosaccharides. Overall, carbohydrates and water respectively account for 79.7% and 17.2% of the weight of an average honey [8]. In addition, honey contains small amounts of various other components including proteins, amino acids, enzymes, organic acids, vitamins, minerals, pigments, aroma compounds and polyphenols [8-11]. In fact, more than 600 individual components, some of which exhibit antibacterial properties, have been identified in honey. It is the variations in the number and concentrations of these minor constituents that differentiate one honey type from another[4]. Such compositional variations also greatly affect the nature and potency of the bactericidal activities of different honeys [12].

The antibacterial activity of honey is complex and multifactorial, involving various mechanisms and compounds. For many honeys, this activity is attributed to the combined effects of a high osmolarity, low pH (honeys are acidic with an average pH of ≈ 4), and the enzymatic production of hydrogen peroxide [13]. Other compounds have also been identified as important contributors to the high activity exhibited by certain honeys. These include methylglyoxal, a phytochemical found in high concentrations in Manuka honey, and bee defensin-1, an antimicrobial peptide present in the medical-grade honey Revamil® [12, 14-16]. In addition, novel fatty diacid glycoside derivatives have recently been detected that could potentially be responsible, at least in part, for the particularly high antimicrobial effectiveness of some Scottish honeys [6]. Evidence also suggests that polyphenols may influence the antibacterial activity of honey, however the extent and nature of their contribution remain poorly understood [6,17]. It is possible that some of these compounds work together in a synergistic manner adding an extra layer of complexity to the activity of honey. Because the antimicrobial effect of honey results from the simultaneous action of many active compounds, bacteria are deemed unlikely to develop resistance to this substance [18-20].

The antibacterial action of honey has been demonstrated *in vitro* against over 80 different bacterial species, including Gram-positive (e.g. *Staphyloccocus aureus*), Gram-negative (e.g. *Pseudomonas aeruginosa*) and antibiotic-resistant strains of bacteria [21-23]. For instance, several varieties of honey have been found to be effective against methicillin-resistant *Staphylococcus aureus* (MRSA), a common cause of healthcare-acquired infections associated with prolonged healing time, increased patient morbidity, and increased costs to public health care systems [24,26]. Moreover, interesting synergistic interactions have also been reported when combining certain honeys and antibiotics [27,28]. In one study, for example, subinhibitory concentrations of Manuka honey were shown to reverse the resistance of MRSA to oxacillin [29]. This suggests that honey could potentially be used as an adjunct treatment to prolong the useful life of existing antibiotics. Few studies on this topic have been published so far, however, and more honey/antibiotic combinations need to be evaluated. Clinically, *in vivo* evidence suggests that honey could be a valuable tool for the prevention/control of infections in wounds such as pressure ulcers or post-caesarean section wounds [30,31]. While such findings are promising, it should be noted that different honeys will vary widely in their antibacterial effectiveness [32]. Characterising the compositions, active compounds, and activities of as wide a range of honeys

as possible is therefore necessary to identify the best candidates for further research and clinical use.

Honey research has led to the appearance of several medical-grade honeys/honey products on the market over the last two decades. In the UK, for example, products currently available include Medihoney®, Comvita® Antibacterial Wound Gel™, Revamil®gel, and Surgihoney™RO® [12,21,31]. While both the composition and the antimicrobial effectiveness of Medihoney® have been well documented, relatively few studies have investigated the other medical-grade honeys. Also, no direct comparison of the compositions or activities of these four medicinal honeys has been published so far. Furthermore, honey research has often been conducted in aerobic conditions. However, many common wound infecting bacteria including *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are facultative anaerobes and thus able to infect both surface wounds as well as deep hypoxic wounds such as abscesses or necrotic tissues [33,34]. It is unclear whether the antibacterial activity of honey against such pathogens remains unchanged in an anaerobic environment. Answering this question could help to guide the use of medicinal honeys by clinicians.

The aim of this study was therefore to investigate the aerobic and anaerobic antibacterial activity, the influence on bacterial antibiotic sensitivity, and the composition of the four medical-grade honeys Medihoney®, Comvita® Antibacterial Wound Gel™, Revamil®gel, and Surgihoney™RO®.The objectives were: i) to examine the antibacterial effectiveness of the honeys against *S. aureus* and *P. aeruginosa* by a broth culture method in both aerobic and anaerobic conditions; ii) to investigate the effect of honey exposure on bacterial antibiotic sensitivity using a disk diffusion assay; and iii) to analyse the composition of each honey by measuring its sugar content, pH, hydrogen peroxide content, colour, total polyphenolic content, and antioxidant capacity.

Materials and methods

Bacterial strains

Staphylococcus aureus NCTC 10655 and *Pseudomonas aeruginosa* NCTC 10782 were supplied by the National Collection of Type Cultures, Porton Down, Salisbury, UK. These were selected to allow for comparisons with previous publications.

Honey samples

Four medical-grade honeys/honey products were used in this study: Comvita® Manuka Medihoney® distributed by Derma Sciences Europe Ltd, Maidendhead, Berkshire, UK; Comvita® Medihoney® Antibacterial Wound Gel™ distributed by Comvita UK Ltd, Maidenhead, Berkshire, UK; Surgihoney™RO® sterile medical honey which was obtained from the manufacturer Matoke Holdings Ltd, Southmoor, Abingdon, UK; and Revamil® 100% gel manufactured by Bfactory Health Products B.V., Rhenen, the Netherlands.

Bacterial maintenance and experimental inoculums

Working bacterial stocks were created by streaking 100 µL of overnight broth cultures of the bacteria onto tryptone soya agar (TSA) (Oxoid Ltd, UK) plates which were incubated at 37°C for 24 hours and then stored at 4°C for further use. The experimental inoculums were obtained by transferring colonies from the stock plates into 10 mL aliquots of sterile tryptone soy broth (TSB) (Oxoid Ltd, UK) which were then incubated overnight at 37°C under aerobic conditions.

Assessing the antibacterial activity of honeys using a broth culture assay

The antibacterial effectiveness of the honeys was assessed using a broth culture assay as described by Fyfe et al. [6] with some minor modifications. Previous publications reported that 50% and 75% honey dilutions markedly reduced the growth of bacteria in TSB[6, 17]. The present study thus used solutions of 25%, 50%, and 75% (w/v) honey in TSB; the 25% concentration giving greater sensitivity and allowing to measure differences in activity between the tested honeys. Each of the honey solutions was inoculated with 50 µL of overnight inoculum from one of the tested bacterium and left to incubate for 24 hours at 37°C in an orbital shaking incubator. Samples from these cultures were then serially diluted in phosphate buffered saline (PBS) and spread onto TSA plates which were further incubated for 24 hours at 37°C. Appropriate growth controls were also prepared. Plates containing between 30-300 colony forming units (cfu) were selected for counting. The same method was followed for the anaerobic assay except that the first incubation period in the orbital shaking incubator lasted 72 hours and was conducted using anaerobic jars (Anaerojar™ 2.5L, Oxoid Ltd, UK) and sachets (AnaeroGen™ 3.5L, Oxoid Ltd, UK) to deprive the bacteria of oxygen [35].

Antibiotic sensitivity following exposure to honey

A modified disk diffusion test was conducted to investigate the antibiotic sensitivity of bacteria following aerobic incubation either in the presence or absence of a subinhibitory concentration of honey (10% w/v) [36]. This honey concentration was used since the potent killing of bacteria in 25%, 50% and 75% honey solutions made it impossible to harvest enough cells to conduct the experiment. Furthermore, previous unpublished work in our laboratory has shown that 10% honey solutions were adequate for the purpose of this assay. Honey solutions/ TSB broth controls inoculated with 50 µL of overnight inoculum of a tested bacterium were incubated for 24 hours at 37°C in an orbital shaking incubator. Samples from these cultures (100 µL) were spread onto TSA plates and antibiotic discs (Mastring-S, Mast Group Ltd, UK) were added to the plates. The zones of inhibition (diameter in cm) were measured following 24 hrs incubation at 37°C.

Estimation of the sugar content

The sugar content of the honeys was measured using a pocket refractometer as indicated by the manufacturer (Bellingham and Stanley Ltd, UK).

Estimation of the pH

The pH of the honeys was measured using pH testing strips (FisherBrand™ pH-Fix 0-14 test strips; FB33003) as described by Schneider et al., [17] The strips were covered with the tested honeys, left to develop for 10 minutes, and compared to a colour chart supplied by the manufacturer.

Detection of hydrogen peroxide

The honeys were tested for the presence of hydrogen peroxide using a method derived from the blue peroxide slide catalase test. A drop of an overnight culture of *S. aureus* (a catalase-positive bacterium) was mixed into a small sample of pure honey spread onto a glass slide and a cover slip placed on top. After approximately 30 minutes the slide was examined visually for bubble production [17]. Catalase is an enzyme that catalyses the conversion of hydrogen peroxide into water and gaseous oxygen. The detection of oxygen bubbles following the addition of *S. aureus* to a honey sample thus indicates that it contains hydrogen peroxide.

Honey color

The honeys were photographed and their colour assessed visually for classification using the colour categories of the United States Standards for Grades of Extracted Honey (US Agricultural Marketing Service Fruit and Vegetable Division Processed Products Branch 1985). Manuka honey (amber colour) was used as the reference honey.

Total phenolic content

The total polyphenolic content of the honeys was measured using the Folin and Ciocalteau method as described by Schneider et al., [17] and using gallic acid as the standard [37]. Solutions of 10% of the tested honeys were first prepared using distilled water. Then, 100 µL aliquots of these honeys solutions (or of the gallic acid standards) were pipetted into duplicate universal tubes and 0.9 mL of distilled water as well as 5mL of Folin and Ciocalteau reagent was added to each tube. After a five minute period, 3.2 mL of a sodium carbonate solution (115 g/L) was added to each tube and the mixtures left for 2 hours for colour development. The honey samples/ standards were then transferred into cuvettes and their absorbance read at 765 nm using a spectrophotometer (Helios alpha, Thermo Electron Corporation). Using a gallic acid standard curve, the total phenolic content of the honey samples was calculated and expressed in milligrams of gallic acid equivalents per litre (mg GAE/L) of honey. Distilled water was used to provide a zero value.

Antioxidant capacity

The antioxidant capacity of the honeys was measured using the ferric ion reducing antioxidant power (FRAP) assay as described by Singleton and Rossi [38]. Aqueous solutions of ferrous sulphate (0.1 to 1.0 mM) were used as standards. Solutions of 10% of the tested honeys were first prepared using distilled water. A working FRAP reagent solution was then prepared by mixing 100 mL of 300 mM acetate buffer solution (pH 3.6), 10 mL of 10 mM TPTZ solution (0.031 g TPTZ added to 10 mL of 40 mM HCl), 10 mL of 20 mM ferric chloride solution, and 12 mL of distilled water. This FRAP solution was kept at 37°C prior to use. Aliquots of 10 µL of the honey samples/ferrous sulphate standards were pipetted into triplicate into a 96 well plate and 250 µL of FRAP solution was added to each well. Distilled water was used as a control. The plate was incubated at 37°C for 4 minutes for colour development and absorbance was read at 595 nm using a micro plate reader (MRX Revelation, Dynex). Using the ferrous sulphate standard curve, the antioxidant capacity of the samples was calculated and expressed as the concentration of Fe^{2+} produced per litre (mM Fe^{2+}/L).

Statistical analysis

All readings were taken in duplicate and the experiments were conducted in two separate occasions. The data was recorded as mean ± standard error of the mean (SEM) and was analysed in Microsoft Excel 2007. The data was compared to the relevant controls using a two-tailed independent student t-test as used in previous similar studies. A p value of ≤ 0.05 was considered statistically significant. Ethical approval for this project was granted by the Division of Health Sciences Ethics Committee, Queen Margaret University.

Results

Antimicrobial activity of honeys

The antibacterial activity of the honeys was assessed against *S. aureus* and *P. aeruginosa* in aerobic (Figures 1 and 2) and anaerobic (Figures 3 and 4) conditions using a broth culture assay. Aerobically, the most active honey against both bacteria was Medihoney®, which reduced bacterial growth at all tested concentrations from approximately 9.2 $\log_{10}$ cfu/mL in the TSB growth controls to less than 1.0 $\log_{10}$ cfu/mL ($p \leq 0.05$). The Comvita® wound gel™was

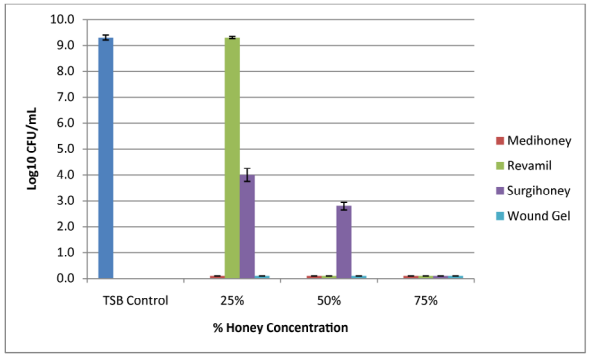

Figure 1: Inhibition of growth of *S. aureus* in honey solutions of various concentrations in aerobic conditions. Results are expressed as the mean of duplicates ± SEM of $\log_{10}$ cfu/mL after incubation at 37°C for 24 hours.

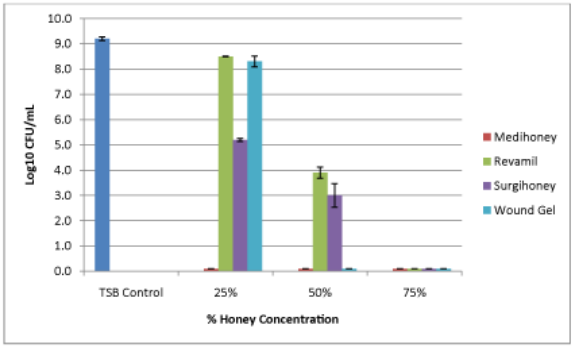

Figure 2: Inhibition of growth of *P. aeruginosa* in honey solutions of various concentrations in aerobic conditions.Results are expressed as the mean of duplicates ± SEM of $\log_{10}$ cfu/mL after incubation at 37°C for 24 hours.

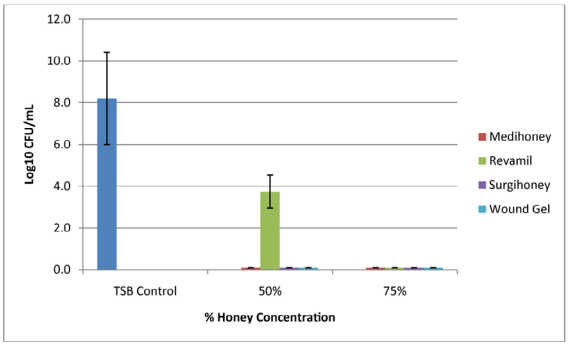

Figure 3: Inhibition of growth of *S. aureus* in honey solutions of various concentrations in anaerobic conditions. Results are expressed as the mean of duplicates ± SEM of $\log_{10}$ cfu/mL after incubation at 37°C for 72 hours.

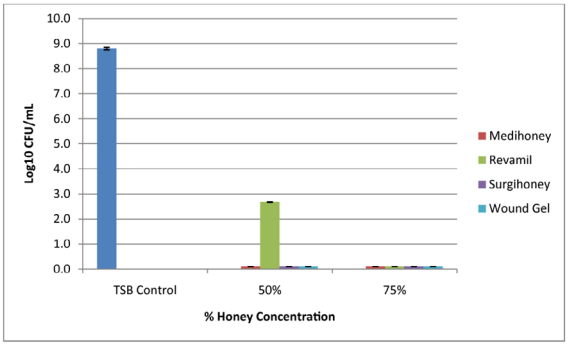

Figure 4: Inhibition of growth of *P. aeruginosa* in honey solutions of various concentrations in anaerobic conditions.Results are expressed as the mean of duplicates ± SEM of $\log_{10}$ cfu/mL after incubation at 37°C for 72 hours.

equally as effective as Medihoney® against *S. aureus* ($p \leq 0.05$) but relatively ineffective against *P. aeruginosa*, the growth of which was not significantly reduced relative to its control by exposure to a 25% concentration of the gel.

Surgihoney™RO® showed an intermediate level of activity with reductions in the growth of both *S. aureus* and *P. aeruginosa* to 4 and 5 $\log_{10}$ cfu/mL at 25% honey concentration and 2.8 and 3 $\log_{10}$ cfu/mL at 50% honey concentration. Revamil® gel was the least active honey; at the 25% concentration it had no effect on *S. aureus* growth and only reduced *P. aeruginosa* growth to 8.5 $\log_{10}$ cfu/mL. *P. aeruginosa* resisted a greater variety of honeys than *S. aureus* when exposed to the 25 and 50% honey concentrations in aerobic conditions.

Anaerobically, with the exception of Revamil® gel, all the honeys reduced the growth of both *S. aureus* and *P. aeruginosa* from more than 8 $\log_{10}$ cfu/mL in the TSB growth controls to less than 1.0 $\log_{10}$ cfu/mL ($p \leq 0.05$) at all the tested concentrations (Figures 3 and 4). Revamil® gel was again the least active honey. Overall, the data

demonstrates that the honeys were equally or more effective against the facultative anaerobes in anaerobic conditions than in aerobic conditions. Exposure to 50% Surgihoney™RO®, for example, reduced the growth of both bacteria to approximately 3.0 $\log_{10}$ cfu/mL aerobically and to less than 1.0 $\log_{10}$ cfu/mL anaerobically; a significant difference ($p \leq 0.05$). Revamil® was also slightly more active against *P. aeruginosa* in anaerobic conditions ($p \leq 0.05$). All the tested honeys were equally as active against both *S. aureus* and *P. aeruginosa* in the absence of oxygen.

Antibiotic sensitivity following exposure to honey

A disk diffusion test was conducted to investigate whether aerobic incubation of *S. aureus* and *P. aeruginosa* in subinhibitory honey concentrations (10% w/v) affected their sensitivity to antibiotics (Tables 1 and 2).

The honeys differed widely in their activity on the antibiotic sensitivity of *S. aureus*. Interestingly, Medihoney®, which was the most potent honey in terms of growth inhibition, did not significantly increase the sensitivity of the bacterium to any of the antibiotics. In fact, Medihoney® exposure even significantly ($p \leq 0.05$) decreased the sensitivity of *S. aureus* to penicillin G. Surgihoney™RO®, on the other hand, was not as efficient a growth inhibitor as Medihoney® yet significantly ($p \leq 0.05$) increased the sensitivity of *S. aureus* to four of the six antibiotics investigated relative to their respective controls (Table 1). Comvita® wound gel™ also stands out having enhanced the activity of three out of six antibiotics against the bacterium. None of the tested honeys increased the action of the antibiotics penicillin G or sulphatriad on *S. aureus*.

In contrast to the results produced by *S. Aureus* (Table 1), no single honey was particularly effective at increasing the sensitivity of *P. aeruginosa* to multiple antibiotics (Table 2). Revamil® is the only honey that did not significantly enhance the activity of any of the antibiotics against the bacterium. It is worth noting that the activity of tetracycline was significantly increased ($p \leq 0.05$) following exposure of *P. aeruginosa* to three of the four tested honeys. This antibiotic thus appears more likely to interact beneficially with honey in general against *P. aeruginosa*. Overall, with or without honey, *S. aureus* was susceptible to a greater variety of antibiotics than *P. aeruginosa*.

Compositional analysis of the honeys

As can be seen in Table 3, all the honeys were acidic (pH = 4) and had a high sugar content (≥ 79%). All four honeys

Table 1: Mean zones of inhibition (cm) of *S. aureus* when exposed to antibiotics following culture in subinhibitory concentrations of honey.
Results reported as means ± SEM (n = 2).
a Bacterium was cultured in TSB for 24 hours and then exposed to antibiotic disks on a TSA plate.
b Bacterium was cultured in 10% (w/v) honey for 24 hours and then exposed to antibiotic disks on a TSA plate.
* Result significantly different ($p \leq 0.05$) from control.

	Antibiotics					
	Tetracycline	Sulphatriad	Streptomycin	Penicillin G	Chloramphenicol	Ampicillin
Control [a]	2.25 ± 0.05	0	1.40 ± 0.00	1.38 ± 0.02	1.63 ± 0.08	2.40 ± 0.05
Medihoney [b]	2.20 ± 0.10	0	1.45 ± 0.00	1.18 ± 0.03*	1.55 ± 0.05	2.40 ± 0.00
Comvita Wound Gel [b]	3.10 ± 0.05*	0	1.33 ± 0.08	1.10 ± 0.10	2.20 ± 0.10*	2.68 ± 0.02*
Revamil [b]	2.48 ± 0.07	0	1.53 ± 0.03*	1.45 ± 0.00	1.65 ± 0.15	2.63 ± 0.13
Surgihoney [b]	3.33 ± 0.03*	0	2.05 ± 0.15*	1.45 ± 0.05	2.55 ± 0.05*	2.85 ± 0.00*

Table 2: Mean zones of inhibition (cm) of *P. aeruginosa* when exposed to antibiotics following culture in subinhibitory concentrations of honey.
Results reported as means ± SEM (n = 2).
a Bacterium was cultured in TSB for 24 hours and then exposed to antibiotic disks on a TSA plate.
b Bacterium was cultured in 10% (w/v) honey for 24 hours and then exposed to antibiotic disks on a TSA plate.
* Result significantly different ($p \leq 0.05$) from control.

	Antibiotics					
	Tetracycline	Sulphatriad	Streptomycin	Penicillin G	Chloramphenicol	Ampicillin
Control [a]	1.50 ± 0.05	0	1.58 ± 0.08	0	1.55 ± 0.05	0
Medihoney [b]	2.13 ± 0.08*	0	1.78 ± 0.03	0	1.88 ± 0.02*	0
Comvita Wound Gel [b]	2.25 ± 0.10*	0	2.08 ± 0.03*	0	1.75 ± 0.25	0
Revamil [b]	1.55 ± 0.45	0	1.88 ± 0.88	0	1.75 ± 0.15	0
Surgihoney [b]	2.13 ± 0.08*	0	1.93 ± 0.03*	0	1.63 ± 0.02	0

Table 3: Compositional data of honey samples.
The total polyphenolic content and antioxidant capacity are reported as means ± SEM (n = 2). †Because of its turbidity, the Comvita Wound Gel solution could not be assayed for its total polyphenolic content or antioxidant capacity.
*Result significantly different ($p \leq 0.05$) from Medihoney which was used as a control.

Honey	Total polyphenolic content (mg/L GAE)	Antioxidant capacity (mM Fe^{2+}/L)	Production of H_2O_2	pH	Sugar content (%)	Colour
Medihoney	1081.60 ± 73.45	5.96 ± 0.02	Yes	4	79%	Amber
Comvita Wound Gel	N/A†	N/A†	Yes	4	80%	Amber
Revamil	328.75 ± 11.02*	1.68 ± 0.27*	Yes	4	83%	Extra light amber
Surgihoney	1088.95 ± 95.48	7.17 ± 0.65	Yes	4	80%	Dark amber

tested positive for the presence of hydrogen peroxide (H_2O_2) with no obvious differences observed between the samples in the amount of gas produced following contact with the enzyme catalase. The colour of the honey samples varied from dark amber for the Surgihoney™RO® to amber for the Medihoney® and theComvita® wound gel™, and extra light amber for the Revamil® gel (Figure 5).

The honeys varied widely in their total polyphenolic content (TPC) (Table 3). While the TPC of both Medihoney® and Surgihoney™RO® was greater than 1000 mg/L GAE, the TPC of Revamil®was relatively low at 328 mg/L GAE. A similar pattern was observed for the antioxidant capacity of the honey samples: Medihoney® and Surgihoney™RO® had a relatively high capacity (≥ 5.96 mM Fe^{2+}/L) whereas the capacity of Revamil® was as little as 1.68 mM Fe^{2+}/L.

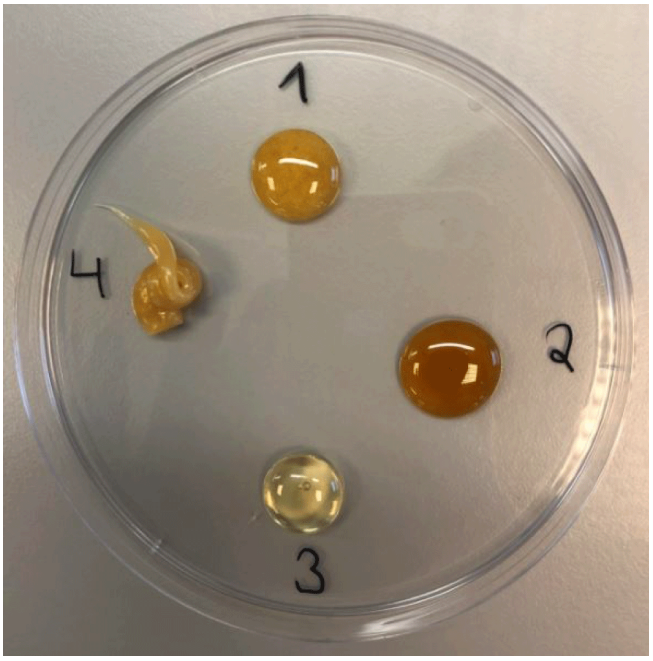

Figure 5: Colour of honeys. 1, Medihoney; 2, Surgihoney; 3, Revamil; 4, Wound Gel.

It was not possible to dissolve the Comvita® wound gel™ in the assay reagents used for the assessment of TPC or antioxidant capacity. As previously reported, the honey samples with higher TPC also had a higher antioxidant capacity and were darker in colour.

Discussion

Four medical-grade honeys were used in this study. Medihoney® and Comvita® wound gel™ are both Manuka honey based products; the former is 100% pure whereas the latter contains 80% Manuka honey in combination with some natural waxes and oils [39]. Revamil® gel contains a honey from an unknown floral source(s) produced under standardised conditions in closed greenhouses; despite its name, the product is advertised as being made up exclusively of 100% pure honey [40]. Surgihoney™RO® contains a mixture of honeys from various sites/floral sources that have been engineered to produce hydrogen peroxide and reactive oxygen species when diluted with water [41,42]. All these honeys are gamma-irradiated to eliminate microbial contamination such as bacterial spores allowing for their use in the clinical setting.

Perhaps surprisingly, this study found considerable variation in antibacterial activity among the tested medical-grade honeys. The Manuka based Medihoney® was the most effective honey, preventing the growth of *S. aureus* and *P. aeruginosa* at all tested concentrations. This result agrees with previous reports of the high antimicrobial activity of Medihoney® *in vitro* against both Gram-positive and Gram-negative bacteria (6, 21, 32, 43). While the Comvita® wound gel™ was as effective as Medihoney® against *S. aureus*, it did not fare as well against *P. aeruginosa*. For example, a 25% concentration of the gel did not significantly reduce the growth of the bacterium relatively to its control in aerobic conditions. Revamil® gel was the least effective of the honeys; aerobically the

25% honey solution had no effect on *S. aureus* growth and only exhibited negligible activity against *P. aeruginosa*. This disagree with a previous study which reported that solutions of unprocessed Revamil® source honey of 10 and 20% concentrations prevented the growth of *S. aureus* and *P. aeruginosa*. A possible explanation for these inconsistent results is that the Revamil® source honey used by Kwakman et al. (2010) [15] is some how altered during processing/storage lowering the antibacterial activity of the final product (Revamil® gel) which was tested in the present study. Surgihoney™RO® exhibited an intermediate level of activity; at the 25% concentration aerobically it was significantly more effective than Revamil® gel and significantly less effective than Medihoney®. Overall, *P. aeruginosa* exhibited a greater resistance than *S. aureus* to the activity of honey in aerobic conditions. This is probably due to the thick glycocalyx capsule produced by the bacterium as a protection against environmental threats preventing the antibacterial compounds in the honeys from reaching/affecting the cells [44]. The findings of the present study could help to inform the choice and use of medicinal honeys by clinicians. Honeys such as Revamil® or Surgihoney™RO®, which exhibited relatively low antibacterial activity, may need to be administered at higher doses and more frequently than Medihoney® for the proper treatment of a wound.

This experiment suggests that the antibacterial activity of honey remains unchanged or is enhanced in anaerobic conditions supporting the use of honey for the treatment of hypoxic wounds. Indeed, *S. aureus* and *P. aeruginosa* were more sensitive to the activity of the honeys in anaerobic conditions than in aerobic conditions. This is probably due to the fact that, although they are able to survive without oxygen, the two facultative an anaerobes stressed in such conditions and more susceptible to the multiple antibacterial compounds found in honey. Research has shown, for example, that oxygen deprivation drastically reduces the growth rate of both *S. aureus* and *P. aeruginosa* [45,46]. This indicates that the bacteria produce less energy anaerobically than aerobically, possibly decreasing their ability to survive threats in their environment. Less energy may mean that the bacteria are unable to actively excrete toxic compounds or repair damage to their membrane, for instance. Further research could confirm whether honey is also effective against obligate anaerobes in the absence of oxygen.

The broth culture assay used in this study allowed for a comparison of the antibacterial activity of the medicinal honeys against planktonic (free-living) cells. Many bacterial infections are caused by biofilms, however. When growing in biofilms, bacteria such as *S. aureus* and *P. aeruginosa* attach themselves to a surface and form aggregates encased in a matrix of extracellular polymeric substances (EPS) [47]. This EPS layer acts as a protective barrier that increases the resistance of the bacteria to a variety of factors including ultra-violet light, acidity, heavy metals, and antibiotics [48]. Bacterial biofilms are the main cause of chronic wound infections that resist antibiotic treatment, for example [27]. Repeating this experiment using bacteria grown as biofilms could help produce a more accurate picture of the antibacterial activity of the honeys when these are used to treat actual wounds.

A compositional analysis was conducted to help identify some of the factors underlying the antibacterial activity of the four medicinal honeys. The variation in antibacterial activity observed between the honeys was not due to their pH or sugar contents since these were similar in all the tested samples. The presence of hydrogen peroxide was also detected in all the honeys. The qualitative assay that was used, however, did not allow determine the level of this chemical in the different honeys making meaningful comparisons between them difficult. Furthermore, the assay was only performed on undiluted honey samples. Research has shown, however, that addition of water to certain honeys activates the hydrogen peroxide-producing enzyme glucose oxidase they contain greatly increasing the hydrogen peroxide concentration (and thus antibacterial activity) in these samples over time [12, 50]. This is clinically relevant since, when used in a dressing, the effectiveness of a honey whose activity is hydrogen peroxide dependent may be maintained even after heavy dilution by wound exudate whereas the activity of other types of honey may decrease in such situations. A recent study, for instance, has shown that hydrogen peroxide production is not an important contributor to the activity of Manuka honey[12]. Future experiments could use a quantitative assay such as described by Kwakman et al. [12] to fully characterise the hydrogen peroxide activity of both undiluted and diluted samples of the medicinal honeys.

In this study, the most active medicinal honeys had the highest total polyphenolic contents (TPC), antioxidant capacity, and were also darker in colour. For instance, the TPC was more than three times higher in Medihoney® (1081 mg/L GAE), the most active honey, than in Revamil®

(328 mg/L GAE), the least active honey. Previous studies have also reported a greater antimicrobial activity in honeys that are richer in polyphenols (17, 51). Together, these results suggest that polyphenols probably play a role in the antibacterial activity of the honeys. The TPC alone cannot completely explain the action of the honeys, however, since Medihoney® and Surgihoney™RO® greatly differed in their antibacterial effectiveness despite containing similar concentrations of polyphenols. A possible explanation is that the presence of specific polyphenols in a honey could be a more important determinant of antimicrobial effectiveness than the TPC (6). The polyphenolic profile of a honey is influenced by its floral source, among other things [51]. Coming from different floral sources, Medihoney® and Surgihoney™RO® probably contain different types of polyphenols. Further studies aimed at characterising the polyphenolic profiles of the honeys could help understanding the role of polyphenols in their antibacterial activity.

Other potential contributors to the activity of honey that have not been analyzed in the present study are methylglyoxal and bee defensin-1. Methylglyoxal is derived from dihydroxyacetone, a phytochemical present in the nectar of the flowers of the Manuka tree (*Leptospermum scoparium*) as well as other *Leptospermum* species found in New Zealand and Australia[14, 16]. This compound is found in high concentration in Manuka honey and is largely responsible for its potent antibacterial activity[12, 16]. Bee defensin-1, an antimicrobial peptide secreted into the honey by the producing bee, is one of the anti-bacterial factors in Revamil® source honey[15]. The presence of methylglyoxal probably contributed to the relatively high antibacterial effectiveness exhibited by Medihoney® and Comvita® wound gel™ in this study.

A disk diffusion assay was used to investigate the interactions between honeys and antibiotics against *S. aureus* and *P. aeruginosa*. Surgihoney™RO® and Comvita® wound gel™ were particularly effective against *S. aureus* increasing its sensitivity to multiple antibiotics including tetracycline, which target bacterial ribosomes and prevent protein synthesis, and ampicillin, an inhibitor of cell wall synthesis (Table 1) [52,53]. These multiple interactions with antibiotics from different antibiotic classes could reflect the fact that several active compounds are present in the honeys and these affect different target sites in the bacteria [54]. The absence of positive interactions between Medihoney® and antibiotics against *S. aureus* was unexpected since both Medihoney® and Comvita® wound gel™ contain Manuka honey and should have behaved in a similar manner. This result also contradicts the findings of a recent study which reported an increased sensitivity of *S. aureus* to several antibiotics, including penicillin G and tetracycline, upon exposure to subinhibitory concentrations of Medihoney®[54]. Against *P. aeruginosa*, the activity of the antibiotic tetracycline was enhanced upon exposure to three of the four tested honeys (Table 2). This suggests that tetracycline may be prone to interact positively with honey in general and should be more systematically studied in combination with a wider range of honeys. The mechanisms by which honey enhances the sensitivity of bacteria to honey remain poorly understood. Liu et al., [49] proposed that since honey-antibiotic synergies are strain- and antibiotic-specific, they are probably due to a honey affecting specific targets within a bacterium rather than causing a general weakening of the cell. Overall, the results of the present study support this hypothesis.

Various combinations of honeys and antibiotics that interact positively to inhibit the growth of *S. aureus* and/or *P. aeruginosa* were identified in this study. The disk diffusion assay used in this experiment did not allow to determine whether these interactions were synergistic or simply additive, however. Further studies using methods such as checkerboard microdilution assays would be required to establish which combinations are synergistic and, therefore, have the greatest potential as combinational therapies for the treatment of bacterial infections[49]. Clinically, synergistic honey and antibiotic combinations would have the obvious advantage of improving the efficacy of antibiotics against bacteria. Such combinations could also decrease the concentrations of antibiotics required to treat infections thus lowering the costs and side-effects of the treatments[54]. Furthermore, *in vitro* evidence suggests that synergies between honey and antibiotics may help to prevent the emergence of antibiotic resistance in bacteria and may even restore the susceptibility of a resistant bacterium to an antibiotic. Muller et al., [28] for example, showed that subinhibitory concentrations of Medihoney® acted synergistically with rifampicin against various strains of *S. aureus* including clinical isolates and MRSA. The authors also observed that while the bacterium readily developed resistance to rifampicin when exposed to the antibiotic alone, it could not do so in the presence of the honey-antibiotic combination. In another study,

Jenkins and Cooper [29] found that Manuka honey and oxacillin interacted synergistically to reverse the resistance of MRSA to the antibiotic and to inhibit the growth of the bacterium. Such findings suggest that the development of combined honey-antibiotic therapies could help to address the antibiotic resistance crisis the world is currently facing.

Most of the research on the synergistic activity of honey-antibiotic combinations has focused on the effects of Medihoney®/Manuka honey against *S. aureus*. The results of the present study, however, indicate that other honeys (e.g. Surgihoney™RO®) may be even more effective than Manuka honey in enhancing the antibacterial activity of antibiotics and should be further investigated. Ideally, such experiments will assess a variety of medicinal and non-medicinal honeys in combination with various classes of antibiotics against a range of Gram-positive and Gram-negative bacteria including clinical isolates and antibiotic resistant strains. In addition, clinical trials are required to determine whether the promising findings of *in vitro* synergy studies can be reproduced *in vivo*. More research is also required to better understand the mechanisms underlying the synergies between honey and antibiotics.

Conclusion

This study investigated the aerobic and anaerobic inhibitory activity of four medical-grade honeys against *S. aureus* and *P. aeruginosa*. The honeys varied greatly in their antibacterial effectiveness with Medihoney® exhibiting the most potent activity. The antibacterial activity of the honeys was equal or increased in anaerobic conditions and appeared to be influenced by their polyphenol content. Fully characterising the polyphenolic profile of the honeys would help to clarify the role of polyphenol in honey activity. Several honey-antibiotic combinations interacted positively to enhance the antibiotic sensitivity of the bacteria. Future research is needed to fully explore the potential synergistic interactions between these honeys and antibiotics.

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