Sains Malaysiana 51(5)(2022): 1325-1337
*http://doi.org/10.17576/jsm-2022-5105-05*

# Comparing Manuka and Other Medical Honeys as Adjunct to Antibiotic Therapy against Facultative Anaerobes

(Membandingkan Madu Manuka dan Madu Perubatan yang Lain sebagai Adjung kepada Terapi Antibiotik terhadap Anaerob Fakultatif)

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*Received: 3 November 2020/Accepted: 21 September 2021*

ABSTRACT

The development of antibiotic resistance in pathogenic bacteria has created a push for new treatments, with honeys (especially Manuka) becoming a common focus due to their strong antimicrobial action. However, alternatives to Manuka are necessary, as its production is vulnerable. Additionally, research is lacking on how honey affect facultative anaerobic bacteria grown in anaerobic conditions and how honey and antibiotics interact in these conditions. In order to understand these interactions and find novel honey candidates, we investigated the antibacterial effects of four honeys (two Manuka, one Chilean and one 'Santa Cruz' honeydew honey) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* grown aerobically and anaerobically in broth cultures, and how the honeys affected the action of common antibiotics against these bacteria using agar diffusion assays. We found all honeys to be highly effective at 75% honey, with no significant differences between honeys, showing that other honeys were suitable alternatives to Manuka at such high concentrations. At 20%, oxygen availability and bacterial species impacted the effectiveness of honeys as Santa Cruz honey was most effective aerobically but failed anaerobically, while Manuka honeys were effective against *S. aureus* but not *P. aeruginosa* in both conditions, and Chilean honey was ineffective against all samples. The addition of honey increased bacterial sensitivity to antibiotics in some cases, varying with aerobic conditions. The antibacterial activity of the honeys, and differences in conditions whether aerobically or anaerobically, were not correlated with pH, antioxidant capacity or total phenolic count. Since in all cases honeys were either beneficial or of no effect, these results supported the use of honey as adjunct to antibiotic therapy in scenarios such as on bandages, with honeys other than Manuka also being worth consideration.

Keywords: Antibiotic resistance; honey; Manuka; *Pseudomonas aeruginosa; Staphylococcus aureus*

ABSTRAK

Perkembangan kerintangan antibiotik oleh bakteria patogen telah mendorong penekanan untuk rawatan baru dengan madu (terutama Manuka) menjadi tumpuan umum disebabkan tindakan antimikrobnya yang kuat. Walau bagaimanapun, alternatif untuk Manuka diperlukan kerana pengeluarannya yang tidak terjamin. Selain itu, penyelidikan mengenai bagaimana madu mempengaruhi bakteria anaerob fakultatif yang tumbuh dalam keadaan anaerob serta bagaimana madu dan antibiotik berinteraksi dalam keadaan ini adalah masih kurang. Untuk memahami interaksi ini dan mencari calon madu yang baharu, kami mengkaji kesan antibakteria bagi empat madu (dua Manuka, satu madu Chile dan satu madu 'Santa Cruz') terhadap *Staphylococcus aureus* dan *Pseudomonas aeruginosa* yang tumbuh secara aerobik serta anaerobik

dalam kultur kaldu dan bagaimana madu mempengaruhi tindakan antibiotik biasa terhadap bakteria ini menggunakan ujian penyerapan agar. Kami mendapati semua madu sangat berkesan pada 75% madu, tanpa perbezaan yang signifikan antara madu, menunjukkan bahawa madu lain adalah alternatif yang sesuai untuk Manuka pada kepekatan tinggi. Pada kepekatan 20%, kehadiran oksigen dan spesies bakteria mempengaruhi keberkesanan madu kerana madu Santa Cruz paling berkesan secara aerobik tetapi gagal secara anaerob, sementara madu Manuka berkesan terhadap *S. aureus* tetapi tidak berkesan ke atas *P. aeruginosa* dalam kedua-dua keadaan dan madu Chile tidak berkesan terhadap semua sampel. Penambahan madu meningkatkan kesensitifan bakteria terhadap antibiotik dalam beberapa kes, berbeza dengan keadaan aerobik. Aktiviti antibakteria madu dan perbezaan keadaan sama ada aerobik atau anaerob, tidak berkorelasi dengan pH, kapasiti antioksidan atau jumlah fenol. Oleh kerana dalam semua kes madu adalah sama ada bermanfaat atau tidak mempunyai sebarang kesan, hasil ini menyokong penggunaan madu sebagai tambahan kepada terapi antibiotik dalam senario seperti aplikasi di atas bahan pembalut, serta mempertimbangkan madu selain daripada Manuka.

Kata kunci: Kerintangan antibiotik; madu; Manuka; *Pseudomonas aeruginosa; Staphylococcus aureus*

## Introduction

Bacterial resistance to antibiotics is widely recognised as a major health concern, as infections with resistant strains of bacteria are more likely to result in fatality than non-resistant strains and the occurrence of bacterial infections which prove resistant to even last resort treatments is on the rise globally (World Health Organization 2020). This has created pressure to develop ways to combat bacterial growth that do not rely on antibiotics. One proposed alternative treatment is the use of honey, which has a long history of therapeutic use in ancient cultures (Zumla & Lulat 1989) and has more recently been shown to inhibit microbial growth, in part due to its low pH, high osmolarity and hydrogen peroxide activity (Bang et al. 2003). The use of honeys under compression bandages is now also highly recommended for burns victims, as honey helps sterilise the wound and demonstrates anti-inflammatory, anti-oxidative and pain-reducing effects (Zbuchea 2014). Over the last decade, research has especially highlighted the positive effects of honey produced in New Zealand from the *Leptospermum scoparium* brush, which is also known as Manuka honey. Manuka honey has been found to impede the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), as well as Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli* (Bulman et al. 2017), while being resistant to bacterial defensive mechanisms such as biofilm formation (Lu et al. 2014). The exact mechanisms by which honeys inhibit bacterial growth seem to vary. Methylglyoxal, a compound common in Manuka honey, has been found to suppress bacterial growth by itself, though only at a 20-fold increased concentration of that with which it is present in Manuka honey (Bulman et al. 2017). Manuka honey has also been found to remain active when methylglyoxal has been removed, suggesting that this honey's mode of action is multifactorial (Kwakman et al. 2011a). More generally, honeys tend to be highly acidic and contain hydrogen peroxide ($H_2O_2$), both of which have been found to inhibit bacterial growth (Bang et al. 2003). Honeys also tend to have a high antioxidant capacity and total phenolic count (TPC), a higher concentration of which is generally correlated with stronger antibacterial action (Stagos et al. 2018).

One little-explored aspect of research on the antibacterial effects of honey is the effect of conditions such as oxygen availability. Low oxygen and anoxic conditions are common in wounds surrounded by traumatised tissue and lead to a higher risk of infection and slower wound healing (Bowler et al. 2001). The use of honeys on these types of wounds, as well as chronic wounds such as ulcers, has been shown to increase wound healing rates with lower rates of infection than existing non-honey methods in several studies and clinical case reports (Al-Waili & Saloom 1999; Dunford & Hanano 2004; Molan 2006). These studies did not, however, examine the specific effects of oxygen conditions on the antibacterial activity of honey. As the activity of honey is attributed in large part to its antioxidant effects, oxygen conditions may have an impact on its effectiveness in lower concentrations. In particular, there is no previous research conducted on honey using facultative anaerobic bacteria, such as *S. aureus* and *P. aeruginosa*, in anaerobic conditions. There is, however, a growing body of studies interested in the use of honey as an adjuvant to antibiotic therapy.

A combination of these treatments allows broader protection, as the honey directly inhibits bacterial growth at the site where it is applied, while an antibiotic can combat any bacteria that have infiltrated into deeper tissue or the bloodstream. Hayes et al. (2018) found synergistic effects between the aforementioned methylglyoxal present in Manuka honey and the antibiotic linezolid. The researchers reported that exposure to Manuka honey was found to increase the sensitivity of *S. aureus* to linezolid, as the methylglyoxal increased intracellular concentrations of the antibiotic. This warrants further studies in order to determine synergistic, or anti-synergistic, effects between different honeys and common antibiotics. It is also notable that so much of the current literature is focused on Manuka honey, which is reliant on a small population of a single species of plant. Should this population be suddenly affected by a new epidemic disease or if the export of this honey becomes restricted for other reasons, adapted therapies using Manuka would be impossible. It is therefore advisable to attempt to identify honeys from other sources which could serve as an alternative to Manuka honey. In summary, there is a lack of research on changes in the antibacterial effects of honey against facultative anaerobic bacteria in differing oxygen conditions, and how this affects their combination with antibiotics, all of which are important to consider in the context of topical application of honeys on bandaged wounds. Previous honey research has focused on the monofloral Manuka honey, whose production is potentially vulnerable, and thus viable alternatives should be identified.

This study aimed to contribute to these areas of the literature by comparing the antibacterial activity of four honeys on *S. aureus* and *P. aeruginosa* in aerobic and anaerobic conditions in order to identify how oxygen availability might affect the activity of the honeys against facultative anaerobic bacteria, as well as the combined effects of honeys and common antibiotics. A branded 'active' floral honey from the Chilean Andes, and a honeydew honey from local pine trees from a private manufacturer in Santa Cruz (CA, USA) were compared to two Manuka honeys approved by NHS Scotland for use in clinical settings (Medihoney and Activon) in order to determine their viability as alternatives to Manuka honey. The characteristics of these honeys, including pH, sugar content, hydrogen peroxide activity, antioxidant capacity and total phenolic count, were measured in order to establish whether these measures were correlated with the antibacterial activity of these honeys.

## Materials and Methods

### Bacterial stock cultures

The bacterial strains used were *P. aeruginosa* NCTC 10782 and *S. aureus* NCTC 6571. These were supplied by the National Collection Type Culture, Porton Down, Salisbury, UK. Bacterial stock cultures were prepared by using an inoculation loop to transfer colonies from previously-prepared plates of pure bacterial colonies into a bijou container containing 5 mL of sterile tryptone soya broth (TSB, Oxoid™, Thermo Scientific, Loughborough, Leicestershire, UK). These were incubated overnight (approximately 16 h) at 37 °C in order to attain exponential growth of bacteria but prevent stagnation and plateauing (Zwietering et al. 1990). These cultures were used immediately the next day. Cultures were prepared in duplicate for each bacterial species to increase the chances of successful growth. One stock culture was then selected for each species to be used in the assays detailed below.

### Antibacterial effect of honey assay

A total of 4 honeys were used in the experiment. Two types of 'medical-grade' Manuka honey from New Zealand were used, Activon® (Advancis Medical, Kirkby-in-Ashfield, Nottinghamshire, UK) and Medihoney® (Derma Sciences Europe, Maidenhead, Berkshire, UK). Chilean 'Active Honey' was acquired from The Active Honey Company (LifePlan, Lutterworth, Leicestershire, UK). 'Santa Cruz' honeydew honey was donated by Bob Bencini (Sunnyvale, CA, USA). Following the methods evaluated in Schneider et al. (2013), 75 and 20% (w/v) honey mixtures were prepared using sterile TSB in a 5 mL bijou container and 50 µL of stock culture was added. Plain (control) samples were prepared by adding 50 µL of stock culture to 5 mL of sterile TSB in a bijou container. Aerobic samples were incubated at 37 °C for 24 h in a shaking incubator, while anaerobic samples were incubated at 37 °C for 72 h in airtight containers prepared with anaerobic sachets (Oxoid™ AnaeroGen™ Sachets, Thermo Fischer Scientific). Anaerobic samples were incubated for longer periods in order to attain similar growth to aerobic samples, as facultative anaerobic bacteria grow slower in anaerobic conditions (Bailey et al. 1984). Following the method of Okoro et al. (2015), after incubation, the samples were serially diluted by factor 10 using 0.1 M sterile phosphate buffered saline (PBS, Sigma/Merck, Gillingham, Dorset, UK). Diluted samples (100 µL) were plated in duplicate

on tryptone soya agar (TSA, Oxoid™, Thermo Fisher Scientific) plates. These plates were incubated in identical conditions as the bijou containers before. Following incubation, bacterial growth on the plates was recorded as viable counts in colony forming units per milliliter (cfu/mL) using a colony counting pen. All samples were plated in duplicate on three separate days, with a total of six readings per sample.

### ANTIBIOTIC SENSITIVITY ASSAY

In order to investigate the effects of 20% honey on the antibiotic sensitivity of the bacteria, 100 µL of undiluted test and control samples were plated on duplicate TSA plates. This honey concentration was used as previous evaluations had shown concentrations above this to eliminate antibacterial colonies to the point that results would have been unreadable (Schneider et al. 2013). Following thorough spreading, Mastring-S M5 antibiotic rings (Mast Diagnostic, Amiens, France) were placed centrally on the plates with sterile tweezers and pressed down along the edges to insure adherence to the agar. These ringlets contained sulphatriad (S, a mixture of sulfathiazole, sulfadiazine and sulfamerazine) (Grey & Hamilton-Miller 1977)), penicillin G (PG), chloramphenicol (C), ampicillin (AP), tetracycline (T) and streptomycin (ST). Aerobic plates were incubated for 24 h at 37 °C after which the diameter of the zones of inhibition were recorded in mm for each antibiotic. Anaerobic plates were incubated in airtight jars with anaerobic sachets for 72 h at 37 °C. This was repeated on three separate days for a total of six readings per sample.

### ANTIOXIDANT CAPACITY

Following the method of Benzie and Strain (1996), the working ferric ion reducing antioxidant power (FRAP) solution was prepared by mixing 100 mL of 300 mM acetate buffer (pH 3.6), 10 mL of 10 mM tripyridyl triazine (TPTZ), 10 mL of 20 mM ferric chloride and 12 mL of distilled water until the colour of the solution turned to a dark orange. The solution was briefly allowed to develop the color change. Standards were prepared according to the amount of 1 mM ferrous sulphate used, ranging from 1.0 to 10.0 mL. One gram of honey sample was diluted with 9 mL of working distilled water and vortexed for 2 min. The honey samples (10 µL) were added to a 96-well plate with 250 µL of working FRAP solution and incubated for 4 min at 37 °C. Absorbance was read at 600 nm and the FRAP values were calculated in ferrous sulphate equivalent concentration (FSE fmM/kg of honey). All samples were tested in duplicate.

### TOTAL PHENOLIC COUNT (TPC)

Total phenolic count was performed for each honey using the Folin-Ciocalteau method, described by Schneider et al. (2013). One gram of honey sample was diluted with 9 mL of distilled water and vortexed for 2 min. Each diluted honey (200 µL) was added to 10 mL of diluted (1:10) Folin and Ciocalteau reagent. After 5 min, 7 mL of sodium carbonate solution was added and left at room temperature for 2 h to let color develop. Absorption was read at 765 nm against a water blank using a spectrophotometer. The optical density was compared to a standard curve made with 50 to 500 mg/L of gallic acid standards in the range of 1.0 to 10.0 mL. The phenolic count concentration was determined as milligrams of gallic acid equivalents per kilogram of each honey (mg GAE/kg). All samples were tested in triplicate.

### PRESENCE OF $H_2O_2$

Following the method proposed by Okoro et al. (2015), 1 g of neat honey was gently mixed with 100 µL of each inoculum to detect presence of $H_2O_2$ as the catalase in bacteria created carbon dioxide in the form of visually observable bubbles. Incubation at 37 °C for 30 min was used to promote catalase activity. A blossom honey from Capstone Valley Apiaries (Dunfermline, UK) was used as a positive control for comparison, as it contains a very high amount of $H_2O_2$ (Okoro et al. 2015). This was repeated on three separate days for triplicate readings.

### pH OF HONEYS

As described in Schneider et al. (2013), neat honey was set onto pH strips (pH range 1 to 14, Fisherbrand FB33003, Fisher Scientific) for 1 h to let color develop. After removing the honey from the surface of the strip, results were read against the manufacturer's scale. This was repeated on three separate days for triplicate readings.

### pH OF BACTERIAL SAMPLES

pH readings were also taken of the 20% honey samples post-incubation in order to allow the assessment of whether a change in pH related to anaerobic conditions may be responsible for a significant difference between samples in other assays. Each sample (10 µL) was pipetted onto each square of pH strips and left for 5 min to develop. After removing any excess, the results were read against the manufacturer's scale. This was repeated for each sample on two separate days for duplicate readings.

### SUGAR CONTENT OF HONEYS

The total sugar content of each honey was determined using a pocket refractometer (Bellingham & Stanley Limited/Xylem, Tunbridge Wells, Kent, UK) for each honey. This was repeated on three separate days for triplicate readings.

### STATISTICAL ANALYSIS

Data analysis was done in SPSS Statistics (v24, IBM Corp., Armonk, NY, USA) and Prism (v8, GraphPad Software, San Diego, CA, USA). Differences between antibacterial action of honeys at 75 and 20% concentrations were analysed using three-way ANOVAs (with honey type, bacterial species and oxygen availability as independent variables), followed by post-hoc analysis with Bonferroni-corrected p-values. Correlation between honey characteristics (pH, sugar content, antioxidant capacity, and TPC) and inhibition of bacterial growth at 20% honey was analysed using Pearson's r test. For the antibiotics assay, differences between honeys were tested using one-way ANOVAs for each antibiotic – bacterial species – oxygen availability pairing (for example, chloramphenicol – *S. aureus* – aerobic), with Bonferroni-adjusted post-hoc analysis. Two-way ANOVA analysis was not possible due to the missing values from samples in which bacterial growth was not sufficient to measure zone of inhibition. Normality and homoscedasticity were tested using Shapiro-Wilk W and Brown-Forsythe tests, respectively, and tests used were adjusted accordingly. To test for differences in antibiotic and honey action due to oxygen availability and bacterial species, multiple independent t-tests were run, with a false-discovery step-up rate of 1%. A significance alpha of 5% was used in all cases.

## RESULTS

The results of the tests on neat honeys showed that the Santa Cruz honey had the highest sugar concentration, as well as antioxidant capacity and total phenolic count, while maintaining the most basic pH at 4.7 (Table 1). By comparison, the Chilean honey had the lowest sugar concentration, as well as antioxidant capacity and TPC, though the Medihoney was the most acidic at a mean pH of 3.0. All honeys tested positive for hydrogen peroxide ($H_2O_2$), though as this assay was qualitative, not quantitative, it was not possible to compare these honeys further in this regard. A visual comparison of the honeys showed that Activon was the lightest color, followed by Medihoney, then Santa Cruz and Chilean honey (not shown), indicating that honey color was not related to the concentration of antioxidants or polyphenols (Table 1).

TABLE 1. Mean sugar concentration, pH, antioxidant capacity (ferrous sulphate equivalents (FSE) in mM/kg), total phenolic count (gallic acid equivalents (GAE) in mg/g) and hydrogen peroxide test results per honey

| Honey | Mean sugar content (% ±SD) | Mean pH (±SD) | Mean FSE Concentration (mM/kg ±SD) | Mean GAE (mg/g ±SD) | Presence of $H_2O_2$ |
|---|---|---|---|---|---|
| Activon | 80.0 ± 0.0 | 3.7 ± 0.6 | 9.29 ± 0.68 | 1.30 ± 0.05 | ✓ |
| Medihoney | 78.0 ± 0.0 | 3.0 ± 0.0 | 7.92 ± 0.24 | 1.07 ± 0.01 | ✓ |
| Chilean | 77.0 ± 0.0 | 3.3 ± 0.6 | 6.92 ± 0.47 | 0.98 ± 0.04 | ✓ |
| Santa Cruz | 83.7 ± 0.6 | 4.7 ± 0.6 | 10.76 ± 0.04 | 1.46 ± 0.05 | ✓ |

SD = Standard deviation

At 75% honey, a three-way ANOVA found honey, bacterial species and aerobic condition to be significant (all $p \leq 0.01$) factors, with an interaction effect between honey and bacterial species. Post-hoc analysis showed that all honeys achieved significant ($p < 0.05$) reductions in bacterial growth compared to the control in all conditions (Figure 1), although there were no significant differences in the honeys' effectiveness in different conditions or on different bacteria.

At 20% honey, the ANOVA analysis again found all independent variables to be significant (all $p < 0.01$) factors, with interaction effects in all cases except between oxygen availability and bacterial species. Post-hoc analysis also showed more apparent differences between honeys and conditions. Aerobically, all honeys except Chilean still significantly ($p < 0.01$) reduced bacterial growth compared to the control samples, though this growth was often higher than at 75% (Figure 1).

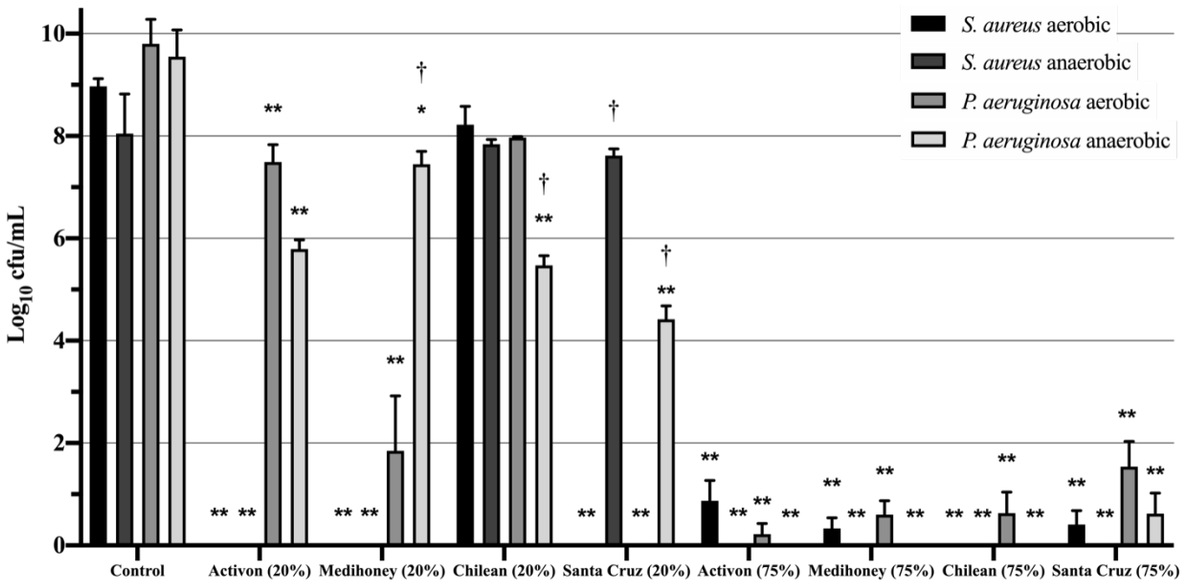


FIGURE 1. Mean viable counts (in $\log_{10}$ cfu/mL) of *S. aureus* and *P. aeruginosa* following incubation in aerobic and anaerobic conditions with varying concentrations of honey. Error bars represented Standard Error of the Mean (SEM). *$p < 0.05$ compared to control. **$p < 0.01$ compared to control. †$p < 0.05$ compared to equivalent aerobic sample

Both Manuka honeys performed significantly worse on *P. aeruginosa* than on *S. aureus*, while Santa Cruz honey performed similarly in both species aerobically, with Chilean honey being ineffective in most cases. Notably, Santa Cruz honey was significantly less effective in anaerobic conditions against both species, not reducing growth in *S. aureus* compared to the control. The only honey that performed better anaerobically than aerobically was Chilean against *P. aeruginosa*, with all others performing the same or worse anaerobically. Compared to controls, all honeys significantly reduced bacterial growth of *P. aeruginosa* anaerobically, with Medihoney performing significantly worse than Chilean and Santa Cruz honey, but not Activon.

None of the characteristics of honeys measured here (Table 1) were significantly correlated with bacterial growth in 20% honey samples, regardless of bacterial species and aerobic condition (Table 2).

There were no significant differences in pH between any of the 20% honey samples post-incubation, regardless of whether they were incubated aerobically or anaerobically (Table 3). Any differences between aerobic and anaerobic samples therefore could not be explained by a significant difference in pH during incubation.

Due to the lack of bacterial growth at 20% honey in some samples (Figure 1), the antibiotic sensitivity assay was not readable in some cases (Table 4). Nonetheless, the results showed significant effects of incubation conditions and honey use on the effectiveness of some antibiotics. Most evidently, bacterial sensitivity to sulphatriad was significantly ($p < 0.05$) reduced in anaerobic conditions in all readable samples, including controls, for both bacterial species. Only the Santa Cruz anaerobic *P. aeruginosa* sample showed some sensitivity to the antibiotic, though no aerobic sample was

available for comparison. None of the samples showed sensitivity to penicillin G, while the only sample which showed sensitivity to ampicillin was the one treated with Medihoney in aerobic conditions.

TABLE 2. Correlation analyses of honey characteristics with inhibition of bacterial growth at 20% honey

| Honey | Sugar content | Honey pH | Antioxidant capacity | Phenolic count |
|---|---|---|---|---|
| | $r^2$ (p) | $r^2$ (p) | $r^2$ (p) | $r^2$ (p) |
| *S. aureus* aerobic | 0.36 | 0.11 | 0.52 | 0.46 |
| | (0.40) | (0.66) | (0.28) | (0.32) |
| *S. aureus* anaerobic | 0.06 | 0.24 | <0.01 | <0.01 |
| | (0.76) | (0.51) | (0.94) | (0.93) |
| *P. aeruginosa* aerobic | 0.38 | 0.21 | 0.34 | 0.24 |
| | (0.38) | (0.55) | (0.42) | (0.52) |
| *P. aeruginosa* anaerobic | 0.44 | 0.74 | 0.32 | 0.37 |
| | (0.33) | (0.14) | (0.44) | (0.39) |

TABLE 3. Mean pH reading from 20% honey samples after incubations in different conditions

| Honey | Mean pH post-incubation (±SD) | | | |
|---|---|---|---|---|
| | *S. aureus* | | *P. aeruginosa* | |
| | Aerobic | Anaerobic | Aerobic | Anaerobic |
| Activon | 5.5 ± 0.5 | 6.0 ± 0.0 | 5.5 ± 0.5 | 5.0 ± 0.0 |
| Medihoney | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 5.0 ± 0.0 |
| Chilean | 4.0 ± 0.0 | 5.0 ± 0.0 | 4.5 ± 0.5 | 4.5 ± 0.5 |
| Santa Cruz | 5.0 ± 0.0 | 5.0 ± 0.0 | 5.0 ± 0.0 | 4.5 ± 0.5 |

Meanwhile, *P. aeruginosa* sensitivity to chloramphenicol was significantly increased in anaerobic conditions both in the control and the Activon sample, which also showed significantly increased sensitivity compared to control. Chilean honey significantly increased *P. aeruginosa* sensitivity to tetracycline in aerobic conditions compared to control, but not so in anaerobic conditions. Meanwhile, Medihoney significantly increased *S. aureus* sensitivity to tetracycline in aerobic conditions compared to control. Lastly, Activon significantly increased *P. aeruginosa* sensitivity to streptomycin compared to controls aerobically, but not anaerobically. Notably, the Activon sample was the only sample that showed sensitivity to streptomycin from both species, though *S. aureus* readings are not available for this honey.

TABLE 4. Mean diameter of zone of inhibition (mm ±SEM) of antibiotics for 20% honey samples and controls

| Honey | S | | PG | | C | | AP | | T | | ST | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aero | An | Aero | An | Aero | An | Aero | An | Aero | An | Aero | An |
| *P. aeruginosa* | | | | | | | | | | | | |
| Activon | 15.1 ± 0.5 | 0.0† ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 15.6 ±1.7 | 21.9*† ±0.5 | 0.0 ±0.0 | 0.0 ±0.0 | 19.7 ±0.6 | 27.0† ±0.0 | 20.8* ±1.3 | 0.0† ±0.0 |
| Medi | 16.3 ±1.4 | 0.0† ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 22.3 ±2.7 | 18.8 ±0.9 | 0.0 ±0.0 | 0.0 ±0.0 | 19.8 ±1.1 | 21.0 ±2.1 | 0.0 ±0.0 | 0.0 ±0.0 |
| Chilean | 15.0 ±0.4 | 0.0† ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 17.5* ±0.6 | 18.5 ±1.0 | 0.0 ±0.0 | 0.0 ±0.0 | 20.3* ±0.5 | 25.5† ±1.0 | 0.0 ±0.0 | 0.0 ±0.0 |
| Santa Cruz | - | 5.5 ±3.2 | - | 0.0 ±0.0 | - | 16.5 ±3.2 | - | 0.0 ±0.0 | - | 28.3 ±1.5 | - | 0.0 ±0.0 |
| Control | 15.3 ±0.6 | 0.0† ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 15.8 ±0.3 | 17.3† ±0.5 | 0.0 ±0.0 | 0.0 ±0.0 | 18.0 ±0.6 | 26.8 ±5.0 | 0.0 ±0.0 | 0.0 ±0.0 |
| *S. aureus* | | | | | | | | | | | | |
| Activon | - | - | - | - | - | - | - | - | - | - | - | - |
| Medi | 6.0 ±6.0 | - | 0.0 ±0.0 | - | 31.5* ±0.5 | - | 18.0* ±12.0 | - | 32.0* ±0.0 | - | 6.0 ±6.0 | - |
| Chilean | 10.0 ±0.4 | 0.0† ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 25.0 ±4.1 | 20.0 ±0.4 | 0.0 ±0.0 | 0.0 ±0.0 | 27.3 ±0.8 | 29.8 ±1.7 | 0.0 ±0.0 | 0.0 ±0.0 |
| Santa Cruz | - | 0.0 ±0.0 | - | 0.0 ±0.0 | - | 19.0 ±1.5 | - | 0.0 ±0.0 | - | 28.3 ±4.2 | - | 0.0 ±0.0 |
| Control | 10.1 ±0.4 | 0.0† ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 20.8 ±0.6 | 17.8† ±0.9 | 0.0 ±0.0 | 0.0 ±0.0 | 25.4 ±0.6 | 27.5 ±4.7 | 0.0 ±0.0 | 0.0 ±0.0 |

Missing fields indicated a lack of bacterial growth at 20% honey, making readings impossible (Figure 1). Aero = aerobic, An = anaerobic, Medi = Medihoney, S = sulphatriad, PG = penicillin G, C = chloramphenicol, AP = ampicillin, T= tetracycline, ST = streptomycin. *$p < 0.05$ compared to control. †$p < 0.05$ compared to aerobic equivalent sample

## DISCUSSION

The results of the bacterial growth assay showed that at a high concentration, such as 75%, all tested honeys were effective in significantly inhibiting bacterial growth compared to controls, no matter in what condition. These results suggested that Chilean and Santa Cruz honeys would be suitable alternatives to Manuka honey against *S. aureus* and *P. aeruginosa*, as long as a high concentration (such as 75%) could be maintained. This was also consistent with previously reported findings on the same or similar honeys, which had reported growth of $< 1 \log_{10}$ cfu/mL at these concentrations (Schneider et al. 2013). The only sample which did not perform at this level was the Santa Cruz honey acting on *P. aeruginosa* aerobically, though it still significantly reduced bacterial growth compared to control and was not significantly different from the anaerobic sample or the *S. aureus* samples. As there was no significant difference between the honeys in any condition, it was not possible to draw many further conclusions. In order to determine which honey was the most effective in the shortest time at this concentration, it would have been necessary to take viable counts from the samples at shorter intervals, as had been done in other studies (Kwakman et al. 2011b).

While the results from these honeys at such a high concentration were very promising, in a clinical setting

it is necessary for honeys to have rapid and strong antimicrobial action at much lower concentrations, as wound exudate will cause dilution of honey which has been applied to a bandage (Kwakman et al. 2011b). For this purpose, the 20% honey assay was likely more representative of a clinical setting, though other studies have suggested that even lower concentrations, as low as 5% to 10% honey, can occur in wounds (Kwakman et al. 2011b). At 20%, differences became much clearer between the honeys. Except for Chilean honey, all samples save one still significantly reduced growth of both species compared to controls. It was however noteworthy that the previously mentioned level of $< 1 \log_{10}$ cfu/mL was only attained by Santa Cruz honey against both species aerobically, and by the Manuka honeys in the *S. aureus* samples. Notably, Activon performed significantly worse against *P. aeruginosa* aerobically than against *S. aureus*, while Medihoney performed similarly against both, suggesting that the performance of different Manuka honey products may vary greatly. From these results, it seemed that the Santa Cruz honey would be the best honey to use in an aerobic setting, as it showed zero growth in both species, performing the same as Medihoney and better than Activon and Chilean honey. Anaerobically, only the Manuka honeys significantly reduced growth of *S. aureus*, preventing any colony from forming. While all honeys significantly reduced *P. aeruginosa* growth, none could achieve 'bactericidal' levels of $< 1 \log_{10}$ cfu/mL. Interestingly, Activon and Chilean honey both performed better against *P. aeruginosa* anaerobically than aerobically, while Medihoney and Santa Cruz honey performed better aerobically. It was also worth noting that differences between the aerobic and anaerobic samples could not be attributed to the differences in bacterial growth in these conditions, as the control samples for both species did not show significant difference in growth. These results suggested that while the Manuka honeys may be viable bactericidal agents against *S. aureus* anaerobically, none of the honeys were able to effectively kill off *P. aeruginosa* anaerobically.

The results from the 20% honey assay suggested that the Chilean honey was not a viable bactericidal agent at this concentration, as it did not reduce bacterial growth to $<1 \log_{10}$ cfu/mL in any sample. This honey was also observed to have the lowest antioxidant capacity and TPC, while Santa Cruz honey, which was the most effective in aerobic conditions, was observed to have the highest antioxidant capacity and TPC. While these results were in line with previous studies which had identified both antioxidant capacity and TPC to be positively correlated with antimicrobial action (Stagos et al. 2018), the anaerobic samples did not show this trend and our correlation analyses suggested that these factors were not solely responsible for the observed antibacterial effects. This in turn supported the observation from other studies that these factors are not the only determinants of honey's antibacterial action. For example, the Manuka honeys were more effective against *S. aureus* anaerobically than the other honeys, which might be due to the action of the aforementioned methylglyoxal (Bulman et al. 2017). These results might also indicate that the action of antioxidants and polyphenols in honey were diminished in anaerobic conditions, though further research would be needed to support this claim. Previous studies had also identified a positive relationship between darker colored honey and higher levels of antioxidants and polyphenols (Kaškonienė et al. 2009; Schneider et al. 2013). This was not the case in this study, as Chilean honey was the darkest honey despite containing the lowest TPC and antioxidant capacity (Table 1). The colours for the other honeys were also not correlated with their TPC or antioxidant capacity.

The Manuka honey was consistently more effective against *S. aureus* than *P. aeruginosa*, which might be in part due to the differences in the way that Manuka honey affected these bacterial cells. Other studies have observed that while Manuka honey prevents cell division of *S. aureus*, it deforms cells and promotes cell lysis of *P. aeruginosa* (Henriques et al. 2009; Salonen et al. 2017). Other studies also suggested that Gram-positive bacteria, like *S. aureus*, were generally more susceptible to the action of honey than Gram-negative ones, possibly due to their increased membrane permeability to exogenous substances (Fidaleo et al. 2011). Previous studies have also highlighted the significance of other antimicrobial compounds in honey, such as $H_2O_2$ and bee defensin-1 (Kwakman et al. 2011a). As all the honeys in this study tested positive for $H_2O_2$, a quantitative assay would be necessary to determine how far differences between the honeys could be attributed to its action. Additional testing for bee defensin-1 and methylglyoxal concentration would also have enabled further analyses in this regard. A common point of contention in the discussion of research surrounding the antibacterial action of honey is the effect of honey pH. Some studies have found that a higher pH leads to stronger antibacterial action (Gallardo-Chacón et al. 2008), while others have observed the opposite (Salonen et al. 2017). Some data in this study supported the former theory, as Santa Cruz honey, which

performed the best against both species aerobically at 20%, also had the highest pH at 4.7. However, the poor performance of this honey anaerobically, where it performed worse than the Manuka honeys against *S. aureus*, contradicted this observation, and honey pH was also not significantly correlated with bacterial growth. Additionally, there were no significant differences in pH between the aerobic and anaerobic samples at 20% after incubation, suggesting that pH was not primarily responsible for the better performance of Santa Cruz honey aerobically. The poor performance of the Manuka honeys, especially Activon, against *P. aeruginosa* with no associated changes in pH also suggested that some bacterial species may be more sensitive to the honey's pH than others. It should also be noted at this point that the Santa Cruz honey was not pasteurised, unlike the Chilean and Manuka honeys. If this honey were to be deployed in a clinical setting, it is likely that some pasteurisation will be necessary. Pasteurisation of honey has been linked to a reduction in antioxidant capacity in honey (Blasa et al. 2006), as well as the denaturing of enzymes which have antimicrobial effects, such as glucose oxidase (Subramanian et al. 2007). This indicated that this honey's antibacterial activity in clinical deployment may be different from the currently presented results, depending on the exact procedure and intensity of pasteurisation method used.

The antibiotics sensitivity assay showed a number of significant interactions between honeys and environmental conditions in terms of their effect on antibiotic sensitivity. Most evidently, sulphatriad was not effective in anaerobic conditions, as all samples which could be compared to their aerobic equivalent showed a significant reduction in sensitivity, to zero inhibition of growth, including in the control samples. This should, however, not be of much concern, as the use of this mixture antibiotic has drastically reduced due to the toxicity of its component sulphathiazole (Greenwood 2010). Another very clear result was that neither of the species tested in this study was at all sensitive to penicillin G. This was a matter of concern, as penicillins are still the most commonly prescribed antibiotics in countries such as England (Public Health England 2015), though not surprising as reports from 22 countries have identified an up to 51% prevalence of penicillin resistance in pathogens commonly treated with this antibiotic, including *S. aureus* and *P. aeruginosa* (World Health Organization 2018). This made the finding that Medihoney might increase sensitivity to ampicillin in aerobic conditions of particular interest. Just like penicillin G, almost all readable samples, including controls, were not sensitive to streptomycin. Again, this was not very surprising, as studies of wild bacterial populations in Europe and Nigeria have reported high prevalence of resistance to streptomycin in both species (Udo & Grubb 1995; van Overbeek et al. 2002). The one exception to this was the aerobic *P. aeruginosa* sample treated with Activon, which showed significantly higher sensitivity, indicating a possible synergistic effect.

Anaerobically, streptomycin did not inhibit growth of *P. aeruginosa*, which was in line with other studies on facultative anaerobes grown in anaerobic conditions (Kogut et al. 1965; Schlessinger 1988). Chloramphenicol and tetracycline were also found to be affected by honeys, as Chilean honey increased their effectiveness against *P. aeruginosa*, while Medihoney increased their effectiveness against *S. aureus* aerobically. Anaerobically, only Activon significantly increased the effectiveness of chloramphenicol against *P. aeruginosa*. Notably, the controls also showed chloramphenicol to be more effective against *P. aeruginosa* anaerobically than aerobically, while being significantly less effective against *S. aureus*.

One important result of this study was that there was no observed case in which the addition of a honey significantly reduced the effectiveness of an antibiotic. This observation was in line with previous research on Manuka honey and antibiotic sensitivity in MRSA and *P. aeruginosa* (Jenkins & Cooper 2012a, 2012b; Liu et al. 2015). This would suggest that in a clinical setting, the addition of one of the tested honeys to a treatment, such as in the form of a honey-impregnated bandage, would always be beneficial as an adjuvant therapy. This study can, however, only make that claim when the concentration of the honey can be maintained at 20%, as higher or lower concentrations of honeys may have different effects on the honeys.

While previous studies using honey against *E. coli* (Chaudhry & Mukherjee 2016) and *Proteus mirabilis* (Irwin et al. 2013) had shown pH to be a significant factor, the current study could not assess the effect of pH changes from honey directly as control samples were not tested. Differences in antibiotic sensitivity and inhibition by honey between aerobic and anaerobic conditions were not linked to pH changes in this study, as there were no significant differences between samples incubated in different conditions (Table 2). This study was also not able to establish whether the improvements in antibiotic effectiveness were due to direct synergistic effects between the honey and the antibiotics, or due to

changes in antibiotic sensitivity of the bacteria. Previous studies investigating MRSA have suggested that both of these mechanisms are involved. Manuka honeys alter the levels of protein synthesis components like ribosomal proteins (Blair et al. 2009; Packer et al. 2012), which may contribute to a 'like-plus-like' synergy with antibiotics like gentamicin, which also work by inhibiting these pathways (Liu et al. 2015; Schlünzen et al. 2001). Other studies have observed that exposure to low concentrations of Manuka honey reduced the expression of the *mecR1* gene in MRSA, which is responsible for resistance to the antibiotic oxacillin (Jenkins & Cooper 2012a; Meng et al. 2006). Investigating changes in gene expression of the bacteria unfortunately lay outside of the scope of this study, limiting the conclusions which can be drawn from the results presented above.

The robustness of the results presented in this study would likely have been improved with changes to the methods, such as a larger variety of honey concentrations, as well as the measurement of viable counts at more frequent time points. The results of the antibiotic sensitivity assay were also weakened by the lack of bacterial growth at 20% honey, which may have been improved by using a lower concentration of honey. The addition of a quantitative assay for the detection of $H_2O_2$ concentration, as well as testing for other antimicrobial agents highlighted by previous studies (such as bee defensin-1) would also have enabled a more detailed analysis of the mechanisms of action for the honeys tested. Other studies have also investigated these mechanisms by removing the antimicrobial agent involved from the honey and comparing its effectiveness (Bulman et al. 2017; Kwakman et al. 2011a). This has usually led to the conclusion that the effects of the honeys are multifactorial and vary highly from honey to honey (Bulman et al. 2017; Kwakman et al. 2011b; Liu et al. 2015; Salonen et al. 2017).

## Conclusions

This study found that all tested honeys were effective at a concentration of 75%. This suggested that if a high concentration could be maintained, Santa Cruz and Chilean honey are suitable alternatives to Manuka in clinical use. At lower concentrations, such as 20%, oxygen availability and bacterial species can impact the effectiveness of honey. As the addition of honey did not reduce the effectiveness of antibiotics in any sample, addition of honey as an adjuvant to antibiotic use in clinical treatments could be recommended. Care should still be taken as aerobic conditions played a role, with both sulphatriad and streptomycin being ineffective anaerobically. This study was not able to identify a mode of action of the honeys, as well as differences between their actions aerobically and anaerobically, as these were not correlated with pH, antioxidant capacity or TPC, supporting the view that honey's action is highly multifactorial.

## Acknowledgements

Anom Bowolaksono's effort was supported in part by the Research and Development Directorates of Universitas Indonesia through Publikasi Terindeks Internasional (PUTI) Grant No. NKB-1039/UN2.RST/HKP.05.00/2020 of the year 2020.

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