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ARTICLE

The controlled *in vitro* susceptibility of gastrointestinal pathogens to the antibacterial effect of manuka honey

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Abstract The susceptibility of common gastrointestinal bacteria against manuka honey with median level non-peroxide antibacterial activity (equivalent to that of 16.5% phenol) was investigated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a standardized manuka honey with the broth microdilution method. The measured sensitivity of bacteria showed that manuka honey is significantly more effective than artificial honey (a mixture of sugars as in honey), indicating that osmolarity is not the only factor that is responsible for the antibacterial activity of the honey. Most tested gastrointestinal pathogens have MIC and MBC values in the range of 5–10% of honey, other than *Enterobacter* spp. which was in the range of 10–17%. The difference in efficacy between the honey with and without hydrogen peroxide removed was also studied, and it was found that both hydrogen peroxide and the non-peroxide components contribute to the bacteriostatic and bactericidal activity of the honey. It was also found that treatment against multi-antibiotic resistant microorganisms such as *Salmonella typhimurium* DT104 and ESBL-producing organisms with manuka honey may be promising.

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Introduction

Honey has been reported to show a significant antibacterial activity against a wide range of bacteria including some antibacterial-resistant species [1], but the efficacy of different honey samples in these reports is usually not comparable with each other for several reasons. On the one hand, neither the details on the honey being tested are clearly given, nor are the honey samples standardized in the studies. For instance, Badawy et al. [2] compared the antibacterial activity of four Egyptian clover honey samples, each of which had been stored for a different period of time (7 months, 12 years, 16 years and 21 years) against *Escherichia coli* O157:H7 and *Salmonella typhimurium*, and concluded that the activity of honey declined with time. However, Badawy et al. [2] did not state whether their honey samples had been stored at room temperature or in a dark refrigerator whereas it is known that antibacterial activity of honey is sensitive to light and to heat [3]. In some reports [4, 5], the floral source of the tested honey was not given whilst the activity of honey can vary greatly among different floral types [6]. It has also been known that even the potency of honeys sharing the same floral source could differ in activity by up to 100-fold [6]. Note also that some bacterial species can be inhibited by low levels of osmolarity, so inhibition by honey that is observed may be due to the sugar content rather than to hydrogen peroxide or non-peroxide factors. Therefore, it is essential to have the antibacterial activity of honey standardized, and also to include an artificial honey as a reference, so that the antibacterial efficacy of factors other than the osmolarity can be distinguished.

Another variable that is commonly missed in the studies that are in the literature is the cell density of the bacteria

being tested in the susceptibility assay. It is generally observed that the higher the cell number, the more resistant the cells are to antimicrobial agents. Depending on the species of bacteria or the antibacterial agents being tested, the MIC could rise 4- to 16-fold with as little as 0.5 $\log_{10}$ increase in inoculum density [7]. Wiegand and Burak [8] reported that the MIC of eight tested antibiotics against *Plesiomonas shigelloides* dramatically increased from ≤0.03 mg/l with 10^5 CFU/ml up to 16 mg/l with 10^6 CFU/ml. The time-to-kill may also reach from 1 h with a 6 $\log_{10}$ CFU/ml starting inoculum to ≥24 h with an 8 to 9 $\log_{10}$ CFU/ml starting inoculum with 10× the MIC at 24 h for some microorganisms [9]. It has also been observed that the influence of inoculum size substantially increased if the inoculum exceeded 9×10^7 CFU/ml [10], if the antibacterial agents were tested under anaerobic conditions [7, 10], or if the antibacterial agents were not rapidly bactericidal [11]. The influence of inoculum density on sensitivity studies is understandable because an increase in the inoculum would reduce the effective concentration or the per-cell concentration of antibacterial agents [12]. Alternatively, a high density may lead to biofilm formation which renders a high resistance against the agents [13]. Therefore, without the information on the cell density being given, it would be difficult to determine if the reported sensitivity of the microbes to the antibacterial agents being tested is actually over-estimated or under-estimated.

The objective of this study was, therefore, to investigate the antibacterial activity of a standardized manuka honey against some type strains and clinical isolates of gastrointestinal pathogens that had the inoculum density standardized. As manuka honey is usually regarded as a non-peroxide antibacterial [6, 14], the difference in the efficacy between the honey with and without its hydrogen peroxide being removed was also studied.

Materials and methods

Honey samples

Manuka honey samples (internally labeled as M115 in the Honey Research Unit laboratory collection) from Summer-Glow Apiaries Ltd. were used for testing the susceptibility of gastrointestinal bacteria to manuka honey. The honeys were labeled "UMF16+" on their packages, and had antimicrobial activity equivalent to that of 16.5% phenol when checked with the method reported by Allen et al. [6]. An artificial honey was included in this study to simulate the sugar composition of honey [15]. The honey samples were stored in a dark refrigerator until used.

Microbiological materials

Escherichia coli ATCC 25923, *Salmonella typhimurium* Phage type 4 and *S. typhimurium* DT104 were obtained from the Institute of Environmental Science and Research (ESR), Christchurch, New Zealand. *Salmonella enteritidis*, *Salmonella mississippi* and *Yersinia enterocolitica* were obtained from Medlab, Hamilton, New Zealand. *Enterobacter aerogenes*, *Enterobacter cloacae*, *Shigella flexneri* and *Shigella sonnei* were provided by Waikato Hospital, Hamilton, New Zealand. Extended spectrum β-lactamase (ESBL)-producing organisms were obtained from Pathlab, Hamilton, New Zealand (Table 1). Mueller-Hinton broth (MHB; Difco Becto Dickinson) was used as the culture media.

Susceptibility test

Inoculum preparation

Each culture was recovered from cryopreservative beads (TSC Ltd.) by transferring one bead into 10 ml MHB then

Table 1 The extended spectrum β-lactamase (ESBL)-producing organisms and their antibiograms provided by Pathlab, Hamilton, New Zealand

Species	ID	Susceptible to	Resistant to
E. coli	ESBL-1	Nitrofurantoin, Gentamicin	Amoxycillin, Amoxy/Clavulanate, Cefaclor, Trimethoprim, Ceftriaxone, Cefuroxime, Ciprofloxacin, Cotrimoxazole
E. coli	ESBL-2	Nitrofurantoin, Meropenem	Amoxycillin, Amoxy/Clavulanate, Cefaclor, Trimethoprim, Ceftriaxone, Cefuroxime, Ceprofloxacin, Cotrimoxazole, Gentamicin
Enterobacter cloacae	ESBL-3	Trimethoprim, Ciprofloxacin, Nitrofurantoin (Intermediate susceptibility[a])	Amoxycillin, Amoxy/Clavulanate, Cefaclor
E. coli	ESBL-4	Nitrofurantoin, Ceprofloxacin	Amoxycillin, Amoxy/Clavulanate, Cefaclor, Trimethoprim, Ceftriaxone, Cefuroxime, Cotrimoxazole, Gentamicin
Enterobacter spp.	ESBL-5	Nitrofurantoin, Trimethoprim	Amoxycillin, Amoxy/Clavulanate, Cefaclor

[a] Intermediate susceptibility implies that clinical effect may be obtainable with usual doses as antibiotic concentration in urine is high

incubating at 37°C overnight. The optical density of the recovered culture was adjusted to 0.08 at 625 nm by addition of MHB and then was further diluted 300-fold (10 μl culture in 3 ml MHB). The broth cultures prepared in this way had a final culture density of approximately 3×10^5 cells/ml which was confirmed with the track dilution method as described previously [16]. This was used as the inoculum for the susceptibility testing.

Susceptibility test

Honey preparation Double-strength solutions of manuka honey and artificial honey were prepared with distilled water, and then an equal amount of double-strength MHB (prepared by adding twice the recommended amount of broth powder in distilled water) was added to make single-strength manuka honey and artificial honey solutions. These single strength honey solutions were considered as the "starting" honey solutions. For example, to make a starting honey solution of 40% v/v, 4 ml of honey (measured as 5.48 g, as honey has an average density of 1.37 g/ml) was added to 1 ml of pure water in a universal bottle and then placed in a 37°C water bath for 5 min to aid dissolving. After mixing with a wooden stick, the 5 ml of double strength honey (80%) was added with 5 ml of double strength MHB to achieve the desired concentration (40%). The starting honey solution was then filter-sterilised with 0.2 μm filters (Sartorius Co.) before serial dilution.

Starting honey solutions with several concentrations were made, and a higher concentration of starting honey solution was used if the MIC appeared to be higher than the detectable range of the MIC test. Up to 50% starting honey solution (16.6% after inoculating broth culture as stated in the following sections) was made. It is unlikely that the ingested honey would be at as high level as 20%, and therefore if an even higher concentration of honey was found to be required to inhibit the growth of the tested microorganisms, the MIC was recorded as >16.6%.

To investigate the effect of hydrogen peroxide activity in manuka honey on the microorganisms, manuka honey solution containing catalase was also included in the test. A solution of 0.2% w/v catalase solution was made by adding 0.02 g of catalase in 10 ml of distilled water and then this was filtered through a 0.22 μm filter membrane. The same amount of the 0.2% w/v catalase stock solution and double-strength honey were mixed so that there was 0.1% catalase in the final honey solution. The amount of catalase used was enough to eliminate any hydrogen peroxide activity in the honey [6]. This solution of manuka honey with catalase was treated in the same way as that without catalase, and had the same final concentrations as those without catalase in the microplate after serial dilution and inoculation.

Honey serial dilution A broth microdilution method on microplates was used as previously described [16]. Of the 12 wells in each row in a microplate, to the first was added 40 μl honey solution, and to the remaining 11 wells were added 40 μl MHB. For serial dilution, 160 μl honey solution was added into the second well in the column and then 160 μl was sequentially transferred to the following wells till the tenth well. The last two wells served a growth control (culture and broth added but no honey) and sterility check (plain MHB). After that 80 μl of inoculum was added into each well except the last well in which 80 μl plain broth was added instead.

MIC and MBC determination The growth of the microorganisms in the microplates was monitored at 37°C for 18 hours using a microplate reader (BMG FLUOStar OPTIMA) and the results were observed as the monitored growth curves. The lowest concentration of honey needed to completely inhibit the growth of a microorganism in the 18-h period of incubation was defined as the minimum inhibitory concentration (MIC) of honey for the microorganism.

After the MIC determination, from each well 10 μl was subcultured on Mueller-Hinton agar plates without honey present to see if the various concentration of honey had been bacteriostatic or bactericidal for the tested organisms. The lowest concentration of honey the bacteria had been exposed to that completely inhibited the growth of the subcultured microorganism was considered to be the minimum bactericidal concentration (MBC). If the MIC was greater than 16.6%, which was the highest final concentration available in the MIC test, then subculturing was omitted as this suggested that the microorganism is relatively resistant to the honey and also that it would be of no practical importance to further investigate the MBC with a yet higher level honey. The broth cultures in the growth control wells were also subcultured on agar plate as positive controls. The susceptibility test for each species was replicated five times.

The difference between manuka honey M115 and the artificial honey for each species in results was analysed by the Wilcoxon test in the statistical package R (http://www.r-project.org) [17]. Two-sided $p<0.05$ was considered statistically significant. Statistical analysis was omitted for any species for which the MIC or the MBC was higher than 16.6%.

Results

All the tested gastrointestinal pathogens had a lower MIC value with manuka honey M115 than with artificial honey

(Table 2). Generally, manuka honey at a concentration less than 8% could inhibit the growth of the tested gastrointestinal bacteria. Although *Enterobacter* spp. had higher MICs than other tested microorganisms, the concentrations of manuka honey required to inhibit their growth were still lower than those of sugar syrup (approx. 11% cf. >16%). The MBCs of manuka honey were generally higher than the MICs of the same honey by one or two dilution steps. The manuka honey with its peroxide activity being removed revealed higher MIC and MBC than normal manuka honey, but these are still lower than those of artificial honey. Antibiotic-resistant organisms (*S. typhimurium* DT104 and ESBL-producing organisms) were found susceptible to manuka honey as were other bacteria ($p>0.05$).

Discussion

Because of the shortcomings of modern antibiotics therapy such as side effects and the emergence of antibiotic resistant bacterial species, alternative therapies have been increasingly drawing the public's interest. However, alternative therapies have not been widely accepted by medical professions largely due to their mechanisms not being well understood and also due to the studies on their efficacy usually not being well controlled. In this study, we analysed the antibacterial activity of manuka honey with having both antibacterial potency and the cell density standardized, and this makes the findings from this study of greater value than those from other similar works where this was not done.

The controlled study revealed that most facultative anaerobes tested were inhibited by the manuka honey with antimicrobial potency near the median level (equivalent to approx. 16% phenol) even when the honey was diluted 10-fold or more. On the other hand, the artificial honey which imitates the sugar content of a normal honey failed to inhibit the growth of all tested microorganisms even at the highest concentration used in the test (16.6%). This suggests that it is not the osmolarity but other antibacterial factors in the honey that are responsible for the inhibition of the growth of the bacteria.

Note also that, although manuka honey is usually regarded as a non-peroxide antibacterial in contrast to most other types of honey, the removal of hydrogen peroxide in this study resulted in both statistical and numerical increase in the MIC and MBC on some bacterial species. This suggests that the antibacterial potency of manuka honey is contributed by both peroxide and non-peroxide activities.

In this controlled susceptibility test, *S. typhimurium* DT104 and antibiotic-resistant Enterobacteriaceae obtained from a local medical laboratory were included to make a comparison with other non-resistant strains because the

Table 2 The minimum inhibitory and bactericidal concentration (% v/v) of manuka honey M115 and artificial honey for non-fastidious gastrointestinal pathogens. The values are represented as means of replicates±standard deviation. The determinations of the MIC and MBC values for each isolate were carried out after 18 h incubation, and were replicated five times

Species	Minimum inhibitory concentration (MIC)			Minimum bactericidal concentration (MBC)		
	Manuka	Manuka+catalase	Artificial honey	Manuka	Manuka+catalase	Artificial honey
E. coli	6.87±0.97*	7.48±0.83*	>16.6	7.48±0.83*	8.5±0*	NT
Y. enterocolitica	4.79±0.54*	7.48±0.83*	>16.6	5.45±0*	8.5±0*	NT
S. typhimurium Phage type 4	6.8±0*	6.8±0*	>16.6	8.5±0*	16.6±0	NT
S. mississippi	6.8±0*	8.5±0*	>16.6	8.5±0*	16.6±0	NT
S. enteritidis	6.8±0*	6.8±0*	>16.6	8.5±0*	16.6±0	NT
E. aerogenes	11.89±2.15*	16.6±0	>16.6	16.6±0	16.6±0	NT
E. cloacae	10.65±0*	15.99±1.34*	>16.6	16.6±0	16.6±0	NT
S. flexneri	7.58±1.14*	9.36±1.05*	>16.6	8.5±0*	16.6±0	NT
S. sonnei	6.61±0.47*	8.93±0.86*	>16.6	8.5±0*	10.65±0*	NT
S. typhimurium DT104	7.48±0.83*	7.48±0.83*	>16.6	10.65±0*	16.6±0	NT
ESBL-1	4.72±0.64*	5.88±0.75*	>16.6	5.45±0*	8.53±0*	NT
ESBL-2	5.08±0.64*	6.91±1.55*	>16.6	5.88±0.75*	8.53±0*	NT
ESBL-3	5.88±0.75*	9.24±1.22*	>16.6	6.75±0*	13.3±0*	NT
ESBL-4	5.45±0*	6.91±1.55*	>16.6	5.88±0.75*	10.83±2.39*	NT
ESBL-5	5.88±0.75*	8.05±2.25*	>16.6	5.88±0.75*	11.53±1.53*	NT

NT not tested

*$p<0.05$ for comparison with artificial honey

prevalence of multi-antibiotic resistant bacteria has been increasing for the last few years [18–22]. Interestingly, it is revealed that all these strains can be inhibited by manuka honey at a concentration less that 10%, and the MIC/MBC parameters are not significantly different from the non-resistant Enterobacteriaceae being tested. Prior studies have also shown that honey is effective on MRSA and VRE [23–25].

Bacteria with antibiotic-resistance properties were reported as soon as antibiotics were commonly used among the medical professions [26–28], whereas resistance to honey has not been reported regardless of it having been used as a medicine for millennia. The fact that bacteria have failed to develop resistance to honey can be due to several reasons. First, the antibacterial efficacy of honey has not been widely known by medical professionals. Second, many of the reported antibacterial activities of honey being used had not been well standardized using a reference antiseptic. The result has been that a wide range of MIC values have been reported in the literature (in some cases these have ranged from less than 20% up to 100% for the same bacterial species [4, 29–31]), and consequently it is impossible to detect whether or not resistance to honey has developed. Third, and perhaps most importantly, microorganisms are unlikely to acquire antibacterial resistance if they are treated with compounds targeting multiple loci [32]. As reviewed by Molan [1], honey is a complex substance and the antibacterial activity is multi-factorial. A long-term study was conducted to select wound pathogens resistant to manuka honey by continuously exposing bacteria to a sub-lethal concentration of honey, but honey-resistant bacteria have not yet developed successfully [33]. A similar study conducted by other researchers also failed to develop honey-resistant strains of *S. aureus* and *P. aeruginosa* whereas these bacteria had increased their resistance to other antibacterial agents under similar conditions in the study [34].

Although the number of antibacterial resistant strains that have been tested with honey is relatively limited so far and whether or not bacteria would eventually develop resistance to honey was not evaluated in our study, the sensitivity of *S. typhimurium* DT104 and of ESBL-producing organisms to manuka honey in this study may partially, if not fully, suggest the usefulness of honey on treating multi-resistant strains of bacteria increasingly seen in medical disciplines. It would be of value to further investigate the potency of the honey with more antibiotic-resistant bacterial species in future.

In short, most gastrointestinal bacteria are susceptible to the antimicrobial activity of manuka honey but not to artificial honey. Most tested organisms can be inhibited by manuka honey even if it is diluted 10–20 fold, and can also be killed with slightly higher concentration of the honey. The efficacy of manuka honey against gastrointestinal pathogens, and possibly their antibiotic-resistant strains too, seems promising.

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