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Antibacterial potential of Manuka honey against three oral bacteria *in vitro*

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Running title: Antibacterial effects of Manuka honey and

Key words: Honey, Antimicrobial, Periodontitis, Caries

Abstract

Honey is an ancient natural remedy for the treatment of infected wounds. It has regained attention in the medical profession, as it has recently been reported to have a broad-spectrum inhibitory effect against bacteria. Data concerning Manuka honey of New Zealand origin, which is claimed to provide additional non-peroxide antimicrobial activity (so-called standard NPA), against oral pathogens is still scarce.

Therefore, this study aimed to screen for the antibacterial efficacy of different Manuka honey products against *S. mutans* (OMZ 918), *P. gingivalis* (OMZ 925) and *A. actinomycetemcomitans* (OMZ 299). Chlorhexidine and saline served as positive and negative controls, respectively, whereas a Swiss multifloral honey served as control honey without intrinsic non-peroxide activity.

Chlorhexidine showed the highest inhibiting potential against all specimens tested. Manuka honey below an NPA value of 15 showed the least bacterial growth-inhibiting potential, even less – although not significantly so – than multi- floral Swiss honey. Manuka honey above an NPA value of 15 showed a significantly higher antibacterial effect compared to the other honeys tested. All Manuka honey preparations were more effective in inhibiting the growth of *P. gingivalis* and *A. actinomycetemcomitans*, rather than *S. mutans*.

In conclusion, the study showed an NPA dose-dependent antibacterial efficacy of Manuka honey. Further investigations of this natural product are now open for scrutiny.

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Introduction

Gingivitis and periodontitis are bacterial infections modified by systemic and environmental factors (PAGE & KORNMAN 1997). The main treatment target remains the effective reduction of the supra- and subgingival pathogenic flora, mainly by mechanical means. A variety of antibiotics and antiseptics have been introduced as adjunct to mechanical therapy (GREENSTEIN 2005). Whereas the use of systemic antibiotics is warranted in certain cases, it is associated with risks, notably the development of antibiotic resistance in various bacterial species (BIDAULT et al. 2007). However, administration of subgingival and topical antimicrobial agents as a quick and inexpensive means of supporting mechanical periodontal debridement is worth consideration (SLOTS 2002). Apart from well-investigated and established antiseptics frequently used in gingivitis/periodontitis-prophylaxis and treatment protocols like chlorhexidine or iodine, natural products would be a desirable alternative (MOLAN 2001). Among them, natural products such as bee honey have a great potential to be used as adjuncts to mechanical periodontal treatment and infection control.

Honey is an ancient remedy for the experiential treatment of infected wounds and has recently been 'rediscovered' by the medical profession. It has been reported to have a broad-spectrum inhibitory effect against bacteria and yeasts (ALLEN et al. 1991, WILLIX et al. 1992) and contains an enzyme that produces hydrogen peroxide when diluted (WHITE et al. 1963). The importance of the additional antibacterial activity of honey is demonstrated in several studies comparing the therapeutic effects of honey and sugar (POSTMES 1997, BOSE 1982, SOMERFIELD 1991, FRENCH et al. 2005). The hydrogen peroxide concentration produced in honey activated by dilution is around 1 mmol/l (MOLAN 1992), which is about 1000 times less than in the 3% solution commonly used as an antiseptic (GUPTA 1973). Although the level of enzymatic hydrogen peroxide production in honey is very low, it is still effective as an antimicrobial agent and produces no harmful effects on tissue due to its excellent biocompatibility (MARTIN & PILEGGI 2004, OZAN et al. 2007). However, most honey loses its antibacterial activity after treatment with catalase which eliminates hydrogen peroxide activity.

But additional non-peroxide antibacterial factors have been identified. Manuka honey, in particular, demonstrates this type of activity. Manuka is the common name of *Leptospermum scoparium*, a tea tree from New Zealand (LUSBY et al. 2002). This non-peroxide antibacterial effect is caused by methylglyoxal, found in high doses in Manuka honey (ADAMS et al. 2008, MAVRIC et al. 2008). The level of non-peroxide

activity in Manuka honey is referred to by a standard NPA (Non Peroxide Activity) value, also trademarked in New Zealand as UMF ("Unique Manuka Factor"). This number refers to the equivalent percentage of phenol antiseptic in water. i.e. NPA 15 (or UMF 15) equates to an antibacterial activity greater or equal to a 15% solution (%w/v) of phenol/water.

The antimicrobial potential of honey has been evaluated and a wide range of MIC values (minimum concentration of honey necessary for complete inhibition of bacterial growth) have been reported in studies comparing different honeys against single species of bacteria (FRENCH et al. 2005). However, although there is evidence of its antimicrobial efficacy on non-oral pathogens, data concerning the specific activity of Manuka honey against oral pathogens is scarce (ENGLISH et al. 2004). The aim of the present study was therefore to evaluate the antibacterial efficacy of different Manuka honey products against three well established putative oral pathogens *in vitro* using an agar diffusion method as well as determinations of the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC). We hypothesized that Manuka honey would exhibit superior antibacterial efficacy than other commonly consumed honey types, in a dose-dependent manner.

Materials and methods

Semi-liquid honey formulations were tested on *Aggregatibacter actinomycetemcomitans* (OMZ 299), *Porphyromonas gingivalis* (OMZ 925) and *Streptococcus mutans* (OMZ 918). For the inoculum, the three strains were grown separately in growth medium containing fluid universal medium (FUM; Gmür & GUGGENHEIM 1983), supplemented with 67 mmol/l Sørensen's buffer, final pH 7.2) and 0.3% glucose. Towards the end of the exponential growth phase the optical density of the cell suspensions was measured at a wavelength of 550 nm and adjusted by dilution with FUM to an optical density of 1.0. Columbia Blood Agar (CBA) without addition of blood (Becton Dickinson Microbiology Systems, Sparks, MD, USA) was prepared according to the manufacturer's instructions. The agar was kept in a water bath at 50°C until further use. For each strain, Petri dishes were prepared by mixing 20 ml of agar with 1 ml of the corresponding inoculum. After hardening of the culture medium, wells measuring 5 mm in diameter were punched out. These cavities were then filled with the different materials. The following honey products were tested:

- a) Swiss multi-floral honey (Apiary Halbheer, Rüti, Switzerland)
- b) Manuka honey NPA 5+ (Watson & Son, Masterton, New Zealand)
- c) Manuka honey NPA 15+ (Watson & Son, Masterton, New Zealand)
- d) Manuka honey Label „MGO 400+“, equivalent to NPA 20+ (Manuka Health New Zealand Ltd, Te Awamutu, New Zealand)
- e) Manuka honey NPA 25+ (Watson & Son, Masterton, New Zealand)
- f) 'MediHoney'TM Medical Honey (Comvita® Derma Sciences Maidenhead, UK)
- g) MediHoney'TM Gel Sheet (Comvita® Derma Sciences Maidenhead, UK)

All honey preparations were maintained at 35° C for 1 hour before use to make them more flowable and then the products were filled in the vial to completely fill the well (Figure 1). Chlorhexidine (0.2%, Kantonsapotheke, Zürich, Switzerland) and sterile physiologic saline served as controls (filling the well corresponded to 40 µl). In the case of the gel sheet, a standardized punch sample 4 mm was taken and placed in the well, which was then filled with sterile saline (40 µl). After 48 hours of anaerobic incubation at 37 °C, the zones of inhibition were measured using a caliper. The experiments were performed in triplicate.

In addition, MIC and MBC of the best performing honey in the agar diffusion test were determined with liquid cultures in 96-well flat-bottom polystyrene microtitre test plates (Milian, Wohlen, Switzerland) as described in details earlier (SHAPIRO et al. 1994). In short, Manuka NPA 25+ honey solutions were prepared using FUM at concentrations of 50, 20, 10, 4, 2, 1, and 0% ^{w/v} and were tested on the same three microorganisms as described above for the agar diffusion tests. For each bacterial strain one microtitre plate was used and for each dilution 6 wells were used. Five of the latter served to determine the MIC whereas the 6th well was needed for MBC determinations. Plates with wells containing 180 µl of growth medium (100% growth controls) and 180 µl of honey solution were covered with plastic lids and pre-reduced for 45 min at 37°C in an anaerobic incubator (Microincubator MI22NK, Scholzen Microbiology Systems AG, Necker, Switzerland) then inoculated with 20 µl of a bacterial culture adjusted to an optical density at 550 nm (OD₅₅₀ = 0.5 ± 0.05). After 48 h of anaerobic incubation at 37°C (Microincubator MI22NK) each plate was covered with a plastic foil (Plate Sealers, Microtec, Embrach, Switzerland) and

vigorously shaken for 10 seconds (model AM 69 micro-shaker, Dynatech, Kloten, Switzerland) and immediately scanned (492 nm) using an Epoch microplate spectrophotometer, (BioTek, Luzern, Switzerland). The MIC was defined as the minimal honey concentration that precluded growth ($OD_{492} < 0.05$).

For MBC 25 μ l of the corresponding honey concentrations used for MIC testing were withdrawn from the microtitre plate, streaked on Columbia Blood Agar plates (CBA) (Difco, Basingstoke, Hamps, UK) and incubated anaerobically for 48 h at 37°C. Bacterial growth was visually analysed using a stereo-microscope (Stemi 2000, Carl Zeiss, Feldbach, Switzerland). The MBC was defined as the minimal concentration of the honey that reduced the colony-forming units/ml by 4 orders of magnitude relative to the 100% growth controls.

Data presentation and statistical analyses

The zones of inhibition (mm) were calculated according to the following formula $(D-5\text{mm})/2$, where D represents the greatest extent of the diameter including the well diameter. Therefore the diameter of the well was subtracted. The statistical analysis was performed using computer software (StatView® 4.02, Abacus Concepts, Berkeley, CA, USA). Results were presented in mean values and standard deviations. One-way ANOVA followed by Scheffé post-hoc testing was used to determine the significant differences between groups. P values smaller than 5% were considered to be statistically significant.

Results

The results of the agar diffusion test are summarized in Table 1.

Chlorhexidine showed the highest inhibiting potential against all specimens tested. As expected, water, which was used as a negative control, showed no antibacterial effect. Manuka honey below an NPA value of 15 showed the least bacterial growth-inhibiting potential, even less – although not significantly so – than multi-floral Swiss honey, which also showed a considerable antibacterial effect. Manuka honey above an NPA value 15+ showed a significantly higher antibacterial effect compared to the other honeys tested, with the exception of 'MediHoney' that showed a comparable antimicrobial effect as Manuka honey for *P. gingivalis*. However, there was no or little significant difference between the three

Manuka honey samples with an NPA $\geq 15+$, indicating an optimal effect at this NPA value. It is also worth noting, that all Manuka honey preparations were more effective in inhibiting the growth of *P. gingivalis* and *A. actinomycetemcomitans*, rather than *S. mutans*.

MIC and MBC of Manuka honey 25+ were determined, because this compound showed the best activity against all bacterial strains under investigation. Since – from a practical perspective - honey was dissolved in fluid, dilutions instead of concentrations are reported. The average optical densities (OD₄₉₂) of the tested bacteria after growth on Manuka honey 25+ dilutions used for MIC determinations are listed in Table 2. The MIC for *A. actinomycetemcomitans*, *P. gingivalis*, and *S. mutans* accounted for 1:25, 1:10, and 1:5, respectively. The lowest dilution required to kill these bacteria was $>1:2$ for all three bacterial strains.

Discussion

This study tested the growth inhibiting effect of different Manuka honey products against three well established oral pathogens *in vitro* using an agar diffusion method. The study showed antibacterial efficacy, which was dose-dependent, but an NPA level greater than 15+ showed comparable properties, which were more pronounced than the control honey and – surprisingly – than the 'MediHoney'.

In general, an NPA (Non-Peroxide Activity) rating of 10 is considered the minimum for therapeutic efficacy. Our study supports this, in finding that NPA 5+ showed no superior effect. Therefore, 'ordinary' Manuka honey has only hydrogen peroxide antibacterial properties, which are common to most [types of honey](#), as corroborated in this study. In contrast, Manuka honey with an NPA of 10+ and greater has both the natural hydrogen peroxide antibacterial property and its own natural non-peroxide antibacterial properties, giving it increased antibacterial potency.

In addition, when discussing the therapeutic effects of honey, a difference between 'normal' honey (meant for alimentary consumption) and so-called 'medical' honey (approved for therapeutic use) has to be made. Most of the 'normal' honey products undergo heat treatment before landing on supermarket shelves, which destroys the enzyme responsible for the hydrogen peroxide production; whereas the 'medical' honey is sterilized by gamma irradiation, which ensures persisting activity of hydrogen peroxidase (LUSBY et al. 2002). In addition, antimicrobial activity of different honeys varies considerably, even if gained from the

same floral source. However, using this agar diffusion test, the differences at high concentrations were not considerably different. The percentages of the various components depend on the plant of origin and its geographic location, the season it was collected, its treatment since harvest and its age (Lusby et al. 2002). In this study, we used the agar diffusion method as well as MIC/MBC determinations. Different screening methods to determine antibacterial activity are available: E.g. variants of the agar diffusion method (well and disc), bioautographic methods (direct and indirect) and microdilution assay. A comparative study has found that the well-variant of the diffusion method was more sensitive than the disc-variant (VALGAS et al. 2007). Bioautographic and diffusion techniques, however, were found to have similar sensitivity. But the latter technique provided even more suitable conditions for the microbial growth. Therefore, the agar diffusion method may still offer good option to screen the antibacterial effect of natural products (SCHMIDLIN et al. 2003). Since the antimicrobial effects of the Manuka honey upon single oral pathogens are confirmed by this study, the next step would be to validate their efficacy of these honey preparations, on an *in vitro* multi-species biofilm model.

Despite the positive results of this study, the therapeutic usage of Manuka honey in periodontal pockets remains questionable and still needs critical evaluation. Although Manuka honey displayed an antibacterial potential, it was still weaker than the control compound chlorhexidine. Therefore, the clinical usefulness can be questioned. However, the ongoing search and evaluation of natural products is still warranted (National policy on traditional medicine and regulation of herbal medicines: Report of a WHO global survey. World Health Organization. ISBN 92 4 159323 7) and Manuka one option to be considered as a natural antiseptic alternative, especially for patients seeking complementary agents and alternatives for synthetic chemicals.

Preparation of the dressing is sticky, and handling in clinical situations (e.g. application into the depth of the periodontal pocket) will be challenging. Saliva and sulcus fluid may lead to quick liquefaction of the honey, and cause insufficient resting time in the periodontal pocket for the development of antibacterial effects. Honey allergy, even though very rare, can lead to various clinical symptoms including anaphylaxis (KARAKAYA & FUAT KALYONCU 1999). Another factor to consider is root caries development. Especially when treating difficult-to-clean furcation areas, honey can pose a considerable caries risk that should not be underestimated. However as shown in the present study, Manuka honey even displayed an antimicrobial activity against the cariogenic bacteria *S. mutans*. Our findings are corroborated by past research demonstrating activity of Manuka honey against organisms in dental plaque that cause decay, along with inhibition of acid formation (BADET & QUERO 2011). In addition, it must be emphasized that the application of the honey would be indicated only after careful

debridement, i.e. removal of plaque, which also reduces the potential risk of caries development and that no longtime applications are possible, which also minimizes the risk of pain and hypersensitivity on exposed root surfaces, following contact with highly osmolar honey.

All these clinical aspects should be taken into consideration and critically assessed before proceeding further to clinical applications. Therefore, tests using an in vitro oral biofilm model are planned, for further validation of the antimicrobial activities of the Manuka honey and the demineralizing effect of this natural antibacterial compound. These studies will also focus on the incorporation of other natural antibacterial products (TAKARADA et al. 2004), e.g propolis.

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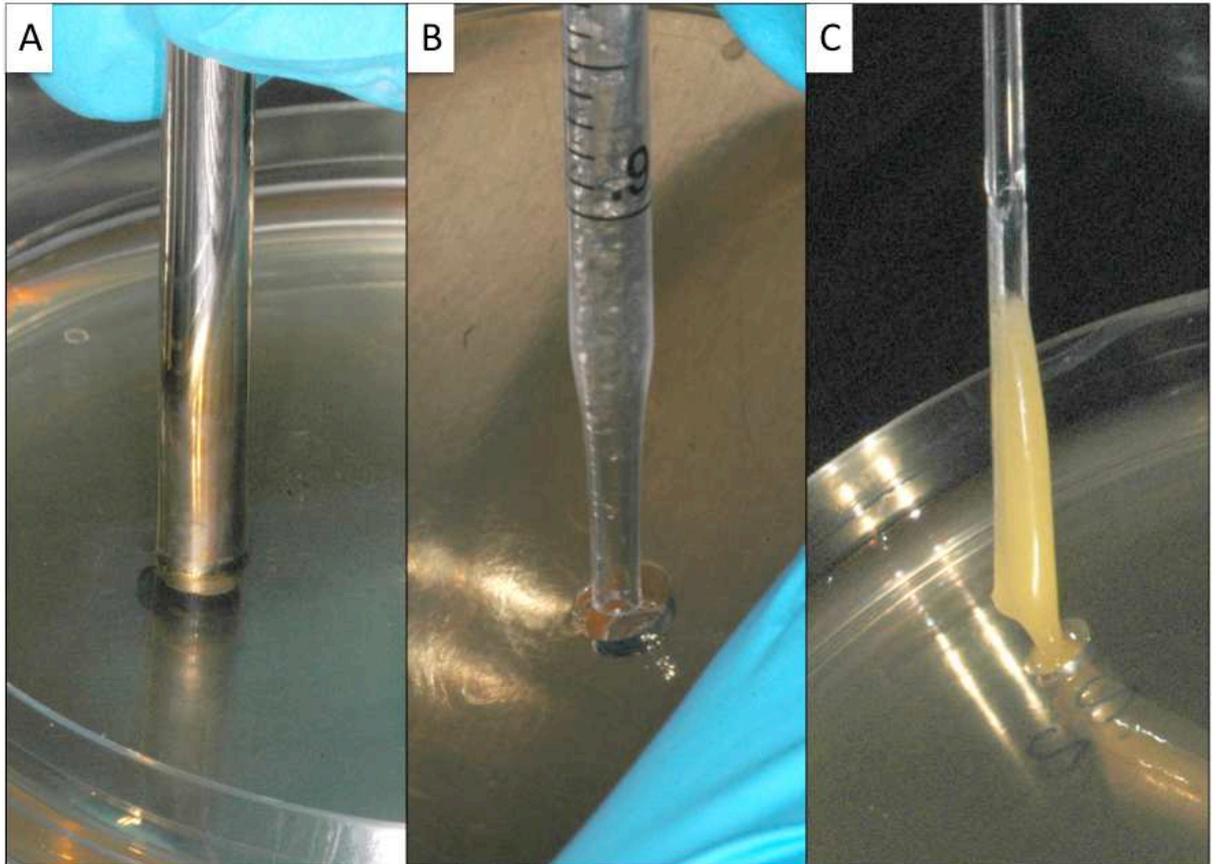
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Figure 1

Preparation of the agar plates: After hardening of the agar plate, wells were punched (A) and the agar was sucked (B). Finally the materials were carefully applied to fill the vial (C).



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Table 1

Zones of inhibition in mm (mean ± standard deviation) around the different preparations (Identical superscript capitals represent values, which do not differ statistically significantly from each other (ANOVA, Scheffé, read vertically) with regard to the bacterial strain tested).

	<i>S. mutans</i>	<i>P. gingivalis</i>	<i>A. actinomycetem-comitans</i>
Multi-floral honey (CH)	1.2 ± 0.1 ^{AB}	3.1 ± 1.1 ^A	3.5 ± 0.5 ^{AB}
Manuka honey 5+	0.8 ± 0.5 ^{AC}	2.8 ± 0.6 ^A	3.9 ± 0.3 ^{BC}
Manuka honey 15+	3.1 ± 0.3 ^D	4.3 ± 0.1 ^B	4.9 ± 0.2 ^{DE}
Manuka honey 20+	3.2 ± 0.2 ^D	5.2 ± 0.3 ^B	4.7 ± 0.7 ^{BCD}
Manuka honey 25+	3.7 ± 0.6 ^D	5.6 ± 0.6 ^B	5.8 ± 0.1 ^F
MediHoney	2.0 ± 0.1 ^B	5.0 ± 0.3 ^B	5.3 ± 0.1 ^F
MediHoney, gel sheet	0.0 ± 0.0 ^C	2.0 ± 0.6 ^A	2.3 ± 0.4 ^A
Chlorhexidine 0.2%	10.3 ± 0.1 ^E	11.8 ± 0.4 ^C	9.7 ± 0.3 ^G
Water	0.0 ± 0.0 ^C	0.0 ± 0.0 ^D	0.0 ± 0.0 ^H

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1 **Table 2**
 2 Average optical densities (OD₄₉₂) after growth on different concentrations of Manuka
 3 honey 25+ (n=5).

Bacterial strains tested				
Manu ka dilution	A. <i>actinomycetemcomitans</i>	<i>P. gingivalis</i>	<i>S. mutans</i>	
1:100	0.593	0.877	1.163	
1:50	0.541	0.839	1.077	
1:25	0.010*	0.790	0.952	
1:10	0.010	0.014	0.687	
1:5	0.005	0.019	0.009	
1:2	0	0	0.006	

5 *Bold values indicate MICs

6