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Enhanced Antibacterial Activity of MGO™ Manuka Honey complexed with α- cyclodextrin (Manuka Honey with CycloPower™)

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Running Title: Manuka honey complexed with α-cyclodextrin

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Submission date: March 03, 2014; Acceptance date: May 13, 2014; Publication date: May 16, 2014

ABSTRACT

Background: Manuka honey is recognized for it's health-promoting properties and it's use in medicine is well documented. However, the actions of Manuka honey are limited by rapid digestion and the inactivation of bioactive components such as methylglyoxal. Cyclodextrins are naturally occurring glucose rings that improve stability and bioactivity of products. This study investigates the tolerability and bioactivity of α-cyclodextrin-complexed Manuka honey called Manuka Honey with CycloPower ™.

Methods: The antibacterial properties of Manuka honey complexed with α-cyclodextrin (Manuka honey with Cyclopower™) were compared to uncomplexed Manuka honey against a range of common organisms using standard measurements of minimum inhibitory (MIC) and bactericidal (MBC) concentrations. Time course growth measurements were determined using a sublethal concentration of 2% w (honey solids)/v and measuring the area under the growth curve.

Results: In tube MIC assays, Manuka honey completely inhibited *Staphylococcus aureus* (MSSA and MRSA), *Streptococcus pyogenes*, *Helicobacter pylori* and *Moraxella catarrhalis* at concentrations of 10% w/v or less, with MIC values decreasing as the methylglyoxal content of the honeys increased from 100 to 550 mg/kg. MIC values at a given methlyglyoxal level were also decreased for *S. pyogenes*, *M. catarrhalis* and *H. pylori* by complexing the Manuka honey with α-cyclodextrin. *Pseudomonas aeruginosa* was not inhibited by any of the Manuka honey or Cyclopower treatments at the concentrations tested (2-10% w/v). Manuka honey with Cyclopower™ had an increased bacteriostatic action against *S. aureus*, MRSA and *P. aeruginosa* compared with Manuka honey.

Conclusions: This study concludes that Manuka honey is an effective antibacterial agent that can be enhanced by complexing with α-cyclodextrin.

Keywords: Manuka honey, methylglyoxal, α-cyclodextrin, antimicrobial

INTRODUCTION:

The use of honey in medicine and to promote health and wellness is well documented [1-3]. The first investigations to elucidate the bioactive components of honey identified hydrogen peroxide (produced by glucose oxidase) as being the primary antibacterial agent [4], although minor roles were assigned to osmolarity and acidity [5]. Antibacterial efficacy has been demonstrated *in vitro* with high hydrogen peroxide producing honeys [6, 7]. Although their usefulness in medicine appears to be limited as glucose oxidase is rapidly broken down by heat and light [8, 9] and the hydrogen peroxide generated is rapidly degraded by catalase, an enzyme present in all body fluids [10].

Manuka (*Leptospermum scoparium*) honey from New Zealand offers potential in health and wellness as it contains a stable antimicrobial compound, originally termed "non-peroxide activity (NPA)", now identified as methylglyoxal [11]. Manuka honeys with high methylglyoxal levels have potent antibacterial action against a range of bacterial species, including those resistant to frontline antibiotics [12-14]. Further, the synergistic action of methylglyoxal with conventional antibiotics has also been demonstrated [15]. The recognition of the medical capabilities of Manuka honey has led to a number of medical grade products being produced, these including sterilised honey, skin care products, wound dressings and cough lozenges [1, 16, 18].

Unfortunately, the oral application of Manuka honey is limited because the sugars in honey are rapidly absorbed (approximately 70% of honey by weight is glucose and fructose) and methylglyoxal is rapidly degraded via reaction with other food components [19, 20]. To circumvent this, researchers have been investigating new formulations that may offer improved stability and efficacy. One such product is Manuka honey complexed with α–cyclodextrin, a naturally occurring cyclical oligosaccharide (Manuka Honey with CycloPower™). This product promises to be easier to handle due to its powdered form, and may promote enhanced delivery and slow release of active components to the gastrointestinal tract. Cyclodextrins are primarily employed as excipients for the delivery of hydrophobic drugs, improving water solubility and stability [21, 22]. The mode of action involves complexation with the drug, which may be via the hydrophobic central cavity of cyclodextrin or through the formation of non-inclusion complexes [22]. Cyclodextrins are "generally recognized as safe" and used in drug products for oral, rectal, topical and parenteral administration [22]. It is hypothesised that this formulation will retain antibacterial activity for a longer period of time, thereby allowing for enhanced action against infections. α–Cyclodextrin may also offer bioactive advantages of its own: 1) as a soluble dietary fibre because it is not hydrolyzed by salivary or pancreatic amylase [23, 24]; 2) to yield short-chain fatty acids due to fermentation by the intestinal microbiota [25]; and 3) to reduce the glycemic index of foods that it is incorporated with [26]. Thus, the ingestion of α-cyclodextrin-complexed Manuka honey could lead to some of the beneficial physiological effects that are typically associated with the intake of fermentable dietary fibres and/or make it a more suitable product for diabetic individuals.

In this study the *in vitro* antimicrobial efficacy of high methylglyoxal Manuka honeys complexed with α–cyclodextrin (Manuka Honey with CycloPower^{TM}) was tested in comparison to matched (uncomplexed) Manuka honeys against a selection of common Gram-negative and Gram-positive pathogens.

MATERIALS AND METHODS:

Honey preparations

Manuka honey (MGO™ Manuka honey), powdered Manuka honey with CycloPower™ (containing honey with methylglyoxal at minimum levels of 100, 250, 400 and 550 mg/kg), and α-cyclodextrin were supplied by Manuka Health New Zealand Ltd (Auckland, New Zealand). MGO™ Manuka honey is 83% honey solids and 17% water; Manuka Honey with CycloPower™ is 55% α–cyclodextrin plus 45% honey solids. Matched samples (uncomplexed honey and cyclodextrin-complexed honey with the same methylglyoxal level) were re-suspended by weight percent of the honey solids in either sterile water or sterile culture broth, containing 0.1% w/v catalase (Sigma Aldrich, Australia). Catalase was added to remove the effect of hydrogen peroxide produced as a result of glucose oxidase activity occurring as honey is diluted, thus ensuring that only non-peroxide antibacterial activity was being measured [27]. Preparations were sterile filtered prior to dilution, although preliminary experiments with *Staphylococus aureus* showed that aseptically prepared honey solutions were sterile prior to filtration and that filtration did not affect antibacterial activity.

Bacterial strains

Antibacterial activity was tested against *Enterobacter aerogenes* ATCC 13048, *Helicobacter pylori* NCTC 11637, *Moraxella catarrhalis* ATCC 25238, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 33593 (an MRSA strain) and *Streptococcus pyogenes* ATCC 8668.

Bacterial growth conditions

Overnight broth cultures and antibacterial assays were performed in Brain Heart Infusion (BHI) broth (Difco) for *E. aerogenes*, *M. catarrhalis*, *P. aeruginosa*, *S. aureus* and *S. pyogenes*. *H. pylori* was cultured in Brucella broth (Difco) containing 2.5% v/v foetal calf serum (FCS; Life Technologies), antibacterial assays were performed in Brucella broth containing a final concentration of 1.25% v/v FCS. Agar plate culture and MBC plate counts used horse blood agar for all species except *H. pylori* (Brucella agar containing 2.5% FCS).

All cultures were grown at 37^{o}C. *H. pylori* was cultured in 90mm Petri dishes containing 10ml broth or 35mm Petri dishes containing 2-3ml broth in a microaerophilic atmosphere generated by a CampyGen gas pack (Oxoid) in a 2.5l gas jar (Oxoid). MacConkey and Eosin Methylene Blue (EMB) agar plates were obtained from Fort Richard, Auckland.

Minimum inhibitory concentration (MIC)

For assay of activity against *E. aerogenes*, *M. catarrhalis*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* a 20% w (honey solids)/v preparation of MGO™ Manuka honey or Manuka Honey with CycloPower^{TM} powder in sterile water plus 0.1% w/v catalase was prepared by incubation with shaking at 37^{o}C for 30 to 60 minutes. The homogenous solution obtained was diluted to give a series of concentrations, which when mixed 1:1 with a suspension of bacteria at approximately 10^{6} CFU/ml in BHI, gave 10, 8, 6, 4, 2, 1 and 0 % w (honey solids)/v. Tube assays were performed in 2 ml, microplate assays in 200 μλ. Assays were incubated overnight at 37^{o}C with shaking at 100 rpm for microplates and 200 rpm for tubes. Growth was estimated by the measurement of any increase in absorbance at 600 nm using either cuvettes with a 1 cm path length (Novaspec II spectrophotometer), or microplates (μQuant, Biotek Instruments). In this study we employed tube assays to determine the MIC

rather than agar diffusion methods to avoid potential artefacts arising from the slow release of methylglyoxal from the Cyclopower™ complex.

Minimum bactericidal concentration (MBC)

MIC broths that did not show growth were used to inoculate agar plates (20 µl inocula) and grown at 37°C. The viable count of the final inoculum was compared with the count of bacteria remaining at 18-48 h to calculate the MBC, defined as the lowest concentration of the honey product giving 99.9% kill of the organism under test.

Testing anti-Helicobacter pylori *activity*

As *H. pylori* is a fastidious organism requiring culture for a number of days in a microaerophilic environment a modified methodology was employed. A 48-72 h culture of *H. pylori* in Brucella broth plus 2.5% FCS at an absorbance between 0.5 and 1.0 at 570 nm was diluted 1 in 1000 and grown overnight to produce an inoculum at an A_{570nm} around 0.1 and containing about 10^5 CFU/ml. The viable count from the inoculum was determined by plating a dilution series to Brucella agar + 2.5% FCS. Solutions of MGO™ Manuka honey and Manuka Honey with CycloPower™ were prepared in Brucella broth at 4 and 8% w (honey solids)/v. Brucella broth alone was used as a control. Inocula and honey preparations were mixed in 35mm Petri dishes in a 1:1 ratio to a final culture volume of 2 ml and incubated for 24 h at 37°C with gentle shaking (80 rpm) in a microaerophilic environment. Growth was measured as an increase in A_{570nm} and the remaining viable count was determined by plating a dilution series to Brucella agar + 2.5% FCS.

Time course experiments

For assay of antibacterial activity over time Manuka Honey with CycloPower™ was compared to matched uncomplexed MGO™ Manuka honey at 2% w (honey solids)/v and tested for growth inhibition over time against *S. aureus*, MRSA and *P. aeruginosa*. The concentration chosen was expected to give some growth, at least for the Manuka honey, and to be in the concentration window where if the form of honey used affected antimicrobial activity this would be apparent. Growth was measured as increasing absorbance at 600 nm at regular time intervals to capture any impact upon the exponential growth of bacteria in the presence of Manuka honey and Manuka Honey with CycloPower™. To illustrate differences across the many samples tested "area under the curve" measurements (GraphPad Prism 5.02, GraphPad Software, San Diego, USA) were made for each growth curve using the exponential region of the no-additive control as a reference. Data was transformed to represent a percentage of the area under the curve of the no-additive control, which was set at 100%.

Statistical Analysis

All assays were carried out at least in triplicate. Statistical analyses were performed using GraphPad Prism 5.02. Normality of data was tested by use of the D'Agostino & Pearson omnibus normality test. Differences in AUC were assessed by use of the non-parametric one-tailed Mann-Whitney test to compare the antibacterial activity of Manuka honey and Manuka Honey with CycloPower™ treatments. A p value < 0.05 was taken as significant in all cases.

RESULTS

MIC and MBC Results

The MIC and MBC values of Manuka Honey and Manuka honey with Cyclopower™ are presented in Table 1. The results demonstrated that all four methylglyoxal honeys (100, 250, 400 and 550) were capable of total inhibition of growth with ≤10% solutions for the majority of organisms tested. Complexation of Manuka honey with α-cyclodextrin did not compromise the antibacterial activity, and in some cases showed an improved *in vitro* bacteriostatic and/or bactericidal activity (refer to Table 1). Control experiments with α-cyclodextrin alone showed no inhibitory activity at concentrations up to the equivalent of that present in a Manuka honey with CycloPower™ solution with 30% honey solids by weight (36.7% w/v α-cyclodextrin by weight). Growth of *S. aureus* was measured as total viable count at 4h and 24h indicating no short term bacteriostatic or longer term inhibitory activities were apparent.

Table 1. The MIC and MBC for manuka honey and Manuka Honey with CycloPower™ against a range of bacterial species

	100H[1]	100CP	250H	250CP	400H	400CP	550H	550CP
[2]MIC								
Staphylococcus aureus	10	8	10	8	8	8	8	8
Streptococcus pyogenes	8	6	8	2	6	2	4	2
Pseudomonas aeruginosa	>10[3]	>10	>10	>10	>10	>10	>10	>10
Moraxella catarrhalis	4	4	10	3	6	2	5	2
Enterobacter aerogenes	>10	>10	>10	>10	>10	>10	>10	>10
Helicobacter pylori	nd[4]	nd	>4[5]	<2	4	<2	4	<2
[5]MBC								
Staphylococcus aureus	10	8	>10	10	8	8	8	8
Streptococcus pyogenes	8	6	8	4	8	4	6	4
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10
Moraxella catarrhalis	10	8	10	8	8	8	8	4
Enterobacter aerogenes	>10	>10	>10	>10	>10	>10	>10	>10
Helicobacter pylori	nd	nd	>4	4	>4	4	4	<2

Note [1]Number denotes MGO level, H denotes manuka honey, CP denotes Manuka Honey with CycloPower™ manuka honey. [2]MICs tested between 2 and 10% honey solids per volume, except see[5]. Values obtained were consistent across at least three assays. [3]10% honey solids per volume was the highest concentration. [4]*H. pylori* was only tested at 2 and 4% honey solids per volume. [4]nd = Precipitation in CycloPower™ manuka honey tests, probably due to serum in the media, did not allow measurement of MIC[5] MBCs tested between 2 and 10% honey solids per volume, except see[5]. Values obtained were consistent across all wells.

Neither preparation was able to inhibit the growth of *E. aerogenes* at concentrations up to 10% honey solids by weight. The experiment was repeated on three separate occasions, in both microplate and 2 ml culture formats with the same result, and streak plates on horse blood agar from cultures grown in the MIC test isolated a single colony type indistinguishable from *E. aerogenes* ATCC 13048 on MacConkey and EMB agars. The resistance of species of *Enterobacter* to Manuka honey has been demonstrated previously [14].

For *H. pylori* Manuka Honey with CycloPower™ resulted in 100% bacterial inhibition at 2 and 4% w (honey solids)/v, whereas the Manuka honey demonstrated full inhibition only for MGO400 and MGO550 honeys at 4% w (honey solids)/v. Plating of the hour incubation samples to Brucella agar + FCS showed that all three Manuka honey treatments (MGO250, 400 and 550) at 2% w (honey solids)/v, and two treatments at 4% w/v (MGO250, and 400) were bacteriostatic only. In contrast, the Manuka Honey with CycloPower™ preparations were all bactericidal at 4% w/v, as was the MGO550 Manuka honey sample.

Time Course Experiments

As MIC and MBC measure an endpoint, the effect of Cyclopower™ on the bacteriostatic duration at sub-lethal concentrations was also assessed over time (refer Table 2).

Table 2. Enhancement of the bacteriostatic duration with Manuka honey with Cyclopower™

Methylglyoxal Content (mg/kg)[a]	Growth in presence of Honey (mean ± S.D).[b]	Growth in Presence of honey with Cyclopower™ (mean ± S.D). [b]
Staphylococcus aureus		
250	79.5 (1.9)	47.2 (3.2)[c]
400	36.7 (15.3)	22.7 (2.5) [c]
550	18.9 (9.8)	11.7 (2.0)
MRSA		
250	78.5 (4.4)	55.3 (4.0) [c]
400	50.5 (8.8)	28.5 (3.4) [c]
550	32.3 (4.8)	22.2 (1.4) [c]
Pseudomonas aeruginosa		
250	96.6 (1.7)	64.1 (6.0) [c]
400	90.3 (6.7)	69.5 (9.0) [c]
550	91.9 (2.7)	61.2 (7.7) [c]

[a.]Matched (uncomplexed) honey or honey with Cyclopower™ was added to BHI at 2% honey solids by weight.

[b.]Growth, as area under the curve, is represented as a percentage of the no-additive control over the same time period. N=5.

[c.]A significant reduction in growth with honey with Cyclopower™ compared to uncomplexed honey (determined using a one tailed Mann-Whitney test; $p<0.05$).

Cyclopower™ treatments gave a significantly greater reduction in growth compared with the matched uncomplexed MGO™ honey, suggesting that it increases the bacteriostatic duration at sub-lethal concentrations when compared to honey matched for methylglyoxal content. Only the *S. aureus* treatment with 2% MGO550 honey failed to demonstrate a significant reduction in growth, where the reduction due to honey itself was sufficiently large as to preclude further significant reduction due to the Cyclopower™.

DISCUSSION

The results of this study have demonstrated that Manuka honey exhibits a potent antimicrobial effect at methylglyoxal concentrations of 100–550 mg/kg, and these data align with results observed with Manuka honey in other studies, despite the fact that a range of methodologies have been employed [28-32]. Further, these results can likely be contributed directly to the methylglyoxal content of the honeys as any peroxide factors had been eliminated by the addition of catalase to the assay, and it has been reported elsewhere that methylglyoxal is the primary antimicrobial agent in Manuka honey [28].

Importantly, the complexation of Manuka honey with α-cyclodextrin does not appear to compromise the antibacterial efficacy when matched for percent honey solids, and in some cases it resulted in an enhanced action. Currently there is little data available describing the biochemical action of Manuka honey with CycloPower; however, we suggest that the improved antibacterial action may be a result of a slow release of antimicrobial components to maintain a bacteriostatic concentration over an extended time period. This is a common property associated with the binding of molecules into a α-cyclodextrin complex [22], although it is also possible that the α-cyclodextrin may be able to deliver antimicrobial components to the bacterium allowing more efficient killing. Data from the present study indicated that □-cyclodextrin at concentrations of up to 36.7% w/v did not show any antibacterial inhibition, and this agrees with well diffusion assays carried out with the same samples (J. Ketel, personal communication). Thus, a direct inhibitory role of α-cyclodextrin is unlikely, though a synergistic action between this and other compounds in the Manuka honey cannot be ruled out. Clearly, the chemistry of Manuka honey and methylglyoxal complexation with α-cyclodextrin, and the specific mechanism of the enhancement of antimicrobial activity demonstrated for Manuka Honey with CycloPower™ are areas for further research.

Both the Manuka honey and the Manuka honey with CycloPower™ preparations have shown differing results on different organisms. *P. aeruginosa* and *E. aerogenes*, in particular, appeared to be particularly resistant to both treatments at concentrations of up to 10%, irrespective of methylgloxal content, though this does agree with previous studies [14, 15]. In contrast, *S. aureus*, *S. pyogenes* and *H. pylori* seem to exhibit relative sensitivity to Manuka honey. Jenkins et al. (2011) [30] indicated that Manuka honey can inhibit cell division via interaction of methylgloxal with *atl*, a gene coding for murein hydrolase, a peptidoglycan-degrading enzyme implicated in cell separation. It is possible that the different degrees of inhibition seen by different organisms interacting with Manuka honey treatments could be due to differences in this process, however, this needs to be investigated further.

The treatment of gastrointestinal infections (including *H. pylori* gastritis and diarrhoeal diseases) is an attractive target for Manuka honey given by the oral route [31, 32], and it has been described as part of an effective treatment for *H. pylori* [33]. In addition, the effectiveness against gastrointestinal conditions is supported by anecdotal evidence, however,

methylglyoxal is not stable in digestive fluids [20, 34] and so delivery of the active components of honey to the intestines in a protected slow release form is desirable. This study has shown that the antibacterial activity of Manuka honey is retained (and often enhanced) when complexed with α-cyclodextrin and this offers potential for improved delivery options of Manuka honey to the GI tract. It is known that a-cyclodextrin is poorly digested and can transit through to the lower gut intact [21]; thus by complexing Manuka honey with the cyclodextrin it is proposed that the bioactive components of Manuka honey can be delivered to the necessary sites of actions. Further research is clearly required to investigate the delivery of methylglyoxal by α-cyclodextrin in the human GI tract, as well as the antibacterial performance of Manuka Honey with CycloPower™ in models of gastrointestinal infection (including testing against common intestinal pathogens such as Salmonella spp., Shigella spp., enteropathogenic *E. coli* and other true pathogens of the human gut).

In conclusion, we show that Manuka Honey with CycloPower™, a Manuka honey in complex with α–cyclodextrin retains antibacterial activity, and exhibits enhanced bacteriostatic and possibly bactericidal activity against Gram-negative and Gram-positive pathogens. The combination of Manuka honey with α-cyclodextrin produces an easier to handle material that may facilitate a wider range of Manuka honey based products. Moreover, the combination of the antibacterial properties of Manuka honey and the complementary bioactive properties of α-cyclodextrin may afford controlled release and delivery that could be suitable for intestinal infections.

Competing Interests: SS and LMC have undertaken paid consultancy for Manuka Health Ltd. No other competing financial interests exist.

Authors' contributions: All authors contributed to this study.

Acknowledgements and Funding: The authors gratefully acknowledge Prof Keiji Terao, Medical Faculty, Kobe University, Japan, for the development of the manufacturing process for the manuka cyclodextrin complex used is this study. Manuka Health New Zealand Ltd has provided all study materials and funding through Uniservices Ltd., Project numbers 30086 and 31295.

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