The Effect of New Zealand Kanuka, Manuka and C Bacterial Growth Dynamics and Cellular Morpholc the Species

Jing Lu, Dee A. Carter, Lynne Turnbull, Douglas Rosendale, Duncan Hedderley, Jonathan Stephens, Sv Ralf C. Schlothauer, Cynthia B. Whitchurch, Elizabeth J. Harry

Abstract

Treatment of chronic wounds is becoming increasingly difficult due to antibiotic resistance. Complex natural under the spotlight as alternative treatments to antibiotics. Several studies have shown honey to have broac honey dressings, and resistance to honey has not been attainable in the laboratory. However not all honeys defined both in geographic and chemical terms. Here we have used a range of concentrations of clover hon geographical locations, and for which the floral source and concentration of methylglyoxal and hydrogen pe growth and cellular morphology of four bacteria: *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* a effectiveness of growth inhibition was manuka>manuka-kanuka blend>kanuka>clover, the honeys had varyi morphology of each bacterium, and each organism had a unique response profile to these honeys. *P. aerug* inhibition to the other three organisms when treated with sub-inhibitory concentrations of honey, being equa sensitive to honey overall. While hydrogen peroxide potential contributed to the antibacterial activity of the n complete growth inhibition. Cell morphology analysis also showed a varied and diverse set of responses to alterations to DNA appearance. These changes are likely to reflect the different regulatory circuits of the org

Citation: Lu J, Carter DA, Turnbull L, Rosendale D, Hedderley D, et al. (2013) The Effect of New Zealand K Dynamics and Cellular Morphology Varies According to the Species. PLoS ONE 8(2): e55898. doi:10.1371/j

Editor: Riccardo Manganelli, University of Padova, Medical School, Italy

Received: June 14, 2012; **Accepted:** January 4, 2013; **Published:** February 13, 2013

Funding: This work was supported by the following sources: 1) Australian Research Council http://www.arc Australian Research Council had no role in study design, data collection and analysis, decision to publish, o partial funding for the work described in the manuscript. Employees of Comvita played a role in study desigr preparation of the manuscript.

Competing interests: The authors have read the journal's policy and have the following conflicts: JS, GS a Limited which trades in medical grade manuka honey (Medihoney) Comvita NZ Ltd. have partially funded th LP0990949 funded by the Australian Research Council. Chief Investigators on this project include EJH, CW reported in this manuscript performed by DR was conducted as a fee for service from Comvita Ltd., NZ. SG

The competing interests mentioned in our submission do not alter the authors' adherence to all the PLOS O

Introduction

Wounds of the skin and mucosal layers can be generated by accidental trauma, surgery, maceration, inflam piercing). For most superficial wounds, healing is prompt and requires no intervention. However, in some ins impaired immunity or circulation, wounds can become non-healing, progressive and chronic. There is growi interplay of host immunity and bacterial infection, and that infection can be due to a consortia of different sp resistant to antimicrobial therapy [1]. Planktonic bacteria are also important in chronic and acute wounds, ar maintain the inflammatory response within the wound [2], [3], as well as allowing seeding to other areas. Th antibiotics has exacerbated the problems associated with treating infected wounds, particularly in the hospit approaches to treat these infections, which are estimated to affect 6.5 million patients and to cost US$25 bil future [6].

Antimicrobial honey produced from the *Leptospermum scoparium* (manuka) plant from New Zealand has m care. Manuka honey is broad in spectrum and able to inhibit a diverse range of bacterial and yeast pathoge bacteria [7]–[9]. This honey has been found to prevent the formation of biofilms and can disrupt pre-formed been observed and could not be attained under laboratory conditions that rapidly induced resistance to con immune system and can promote wound healing [12]. There are a number of medicinal honey products on t impregnated gels. However their use in mainstream medicine remains limited [13].

Honey has several antibacterial features that are distinct from classical antibiotics, including high osmolarity bee-derived enzyme glucose oxidase [14]. Some honeys also contain levels of bee defensin-1 that are suffi honey contains high levels of the reactive dicarbonyl methylglyoxal (MGO) [17], [18], which forms non-enzy during ripening. A diverse range of phenolics, complex carbohydrates and peptides have also been reporte modulate antibacterial activity [19]–[20].

The antibacterial activity of honey is generally assessed by measuring the extent to which the indicator bact micro-dilution methods [21]. Similar tests have been performed to determine the inhibition of other bacterial for medicinal use generally uses a potency rating based on the “Unique Manuka Factor” (UMF), which meas hydrogen peroxide, and is based on the *S. aureus* inhibition test. Alternatively, some medicinal honeys expr has been established that manuka honey can inhibit the growth of bacterial cells, its effect on growth and ce how these change when the levels of the major antibacterial components, MGO and hydrogen peroxide, va considerations for optimizing honey for wound care since sub-lethal levels of honey may have unanticipate organisms infecting a wound may respond quite differently to the active honey components [16] [23].

To address these issues this study set out to examine the growth response and cellular morphology of four common wound pathogens, to a suite of natural honey samples that differ in their levels of MGO and hydrog monofloral manuka honey with moderate to high MGO levels, samples of honey produced from the New Ze levels are negligible but hydrogen peroxide is present, and manuka-kanuka blends that contain both active controls to mimic the effects of sugar, to neutralize the effect of hydrogen peroxide, and to examine how MG while clinically relevant concentrations of honey are effective at inhibiting growth of all four bacteria, the gro significantly between species. Furthermore, *P. aeruginosa* responded strikingly differently to the other three sub-lethal concentrations, MGO extended the lag phase of bacterial growth in a dose-dependent manner, a presumably by detoxifying the MGO. Topical wound dressings should therefore contain a high level of active

Materials and Methods

Honey Samples

Table 1 lists the New Zealand honey samples used in this study, which included monofloral manuka (M1, M2 blends (MK1, MK2, MK3, MK4) and clover (C) honey. Samples were chosen based on their levels of methyl and hydrogen peroxide, determined in this study. Manuka, kanuka and manuka-kanuka honey samples wer Zealand) and the clover honey sample was a commercially-packaged New Zealand white clover honey [19] in their local environment and cannot be guaranteed to be 100% monofloral, however the supplied samples from a single floral origin as possible. Details of other chemical components in the manuka and kanuka hon stored in the dark at 4°C and were diluted fresh for use in all assays. All honey concentrations are expresse

Code	Previous code*	Honey	Floral source	Antibacterials	
				MGO[b] (mg/kg)	H_2O_2[c] (mM/h)
M1	2	Manuka[d]	*Leptospermum scoparium var incanum*	651.4	0.332
M2	13	Manuka [d]	*L. scoparium var incanum*×*Kunzea* (?)	1004.3	0.282
M3	7	Manuka[d]	*L. scoparium var incanum*	1141.3	0.239
K1	22	Kanuka[e]	*Kunzea ericoides*	5.6	0.360
K2	21	Kanuka[e]	*Kunzea ericoides*	37.1	0.327
MK1	23	Manuka-Kanuka[d]	*Kunzea ericoides*×*manuka* (?)	173.6	0.583
MK2	–	Manuka-Kanuka	*Kunzea ericoides*×*manuka* (v. likely)	229.8	0.448
MK3	18	Manuka-Kanuka[d]	*L. scoparium var* 'trikettone'×*Kunzea*	269.9	0.345
MK4	15	Manuka-Kanuka[d]	*L. scoparium var* 'trikettone'	307.8	0.380
C	24	Clover[f]	*Trifolium* spp.	trace	0.029

*As reported in Stephens *et al.* (2010).
[b]MGO (methylglyoxal) levels, reported in Stephens *et al.* (2010).
[c]H_2O_2 (hydrogen peroxide) levels are expressed as mean H_2O_2 production rate in 1 mL of 10% w/v honey.
[d]Samples collected from hive sites.
[e]Aged samples from drums supplied by apiarists and purchased as designated type.
[f]Obtained commercially.
doi:10.1371/journal.pone.0055898.t001

Table 1. Floral source, MGO and H_2O_2 Levels of Honeys.
doi:10.1371/journal.pone.0055898.t001

Hydrogen Peroxide Assay

The level of hydrogen peroxide produced by the honey samples was determined using a hydrogen peroxide Technologies Corp., Carlsbad, CA, USA). The assay, which measures the oxidation by hydrogen peroxide o fluorescent resorufin [24], was conducted in 96-well microtitre plates according to the manufacturer's instruc excitation/590 nm emission using a SpectraMax Gemini EM (Molecular Devices, LLC, Sunnyvale, CA, USA were used to produce a standard curve, which was then used to assess production in duplicate samples of results were normalized to mM H_2O_2/h in 1 mL of 10% w/v honey solution.

Bacterial Strains and Growth Media

Four different bacterial species were examined: the Gram-positive bacteria *B. subtilis* 168 [25] and *S. aureu* Gram-negative bacteria *E. coli* O157:H7 [26] and *P. aeruginosa* PAO1 (ATCC 15692). *B. subtilis* is a well-st clinically relevant pathogens. Growth media were selected to allow optimal growth of the different bacterial s Hampshire, UK) broth and agar were used for *E. coli, P. aeruginosa* and *B. subtilis*, while Tryptone Soya Bro used for *S. aureus*.

Growth of Bacterial Cultures

Planktonic bacteria in wounds, while viable, are likely to be growing very slowly, if at all. We therefore addec it would more accurately represent the addition of a honey dressing to a chronic wound. Single colonies of b cultures. These were grown overnight at 37°C on an orbital shaker at 250 rpm (Bioline™, Australia) except shaking using a gyrotory waterbath shaker (New Brunswick Scientific, Enfield, CT, USA). The slower shakin spend too long in stationary phase, which would delay entry into exponential growth upon dilution. Cell dens dilution plating and was approximately 10^9 colony-forming units (CFU)/mL. A suspension from the overnight fresh media containing honey to give a final volume of 150 µL. For each growth assay, a freshly prepared 5(appropriate amount of honey and mixing this with an equivalent amount of sterilized distilled water. This sto growth medium to give the required honey concentration. Growth of each bacterial species was tested in si 32% w/v) in a 96-well microtitre plate format. A microtitre plate reader (Biotek PowerWave HT®, Bioteck Ins

the optical density hourly at 595 nm (OD_{595nm}) (Gen5®, BioTek) was used to assay cell growth over 24 hou mm x-axis). Two biological replicates, each with four technical replicates were performed for the growth ass represents the average of all data. Growth curves were presented using GraphPad PrismV. 5.0c (Graphpad

A comprehensive range of control treatments was included for each organism in the microtitre-plate growth sugar solution comprising 45% glucose, 48% fructose and 1% sucrose, (diluted as above for honey) to iden content in honey; (iii) honey plus catalase (1 mg/mL) to neutralize hydrogen peroxide [14]; (iv) a catalase-or similar to those present in honeys M1, M2 and M3 (600, 1,000 & 1,500 mg/kg undiluted honey) at the variou on bacterial growth; vi) the same MGO dilutions plus catalase; and (vii) MGO diluted in sugar solution to the MGO was obtained as a 40% solution in water (Sigma-Aldrich Co., St Louis, MO, USA).

Growth Curve Data Analysis

Initial inspection of the bacterial growth data indicated that the consistent major effect of honey on growth dy exponential growth was delayed, and this increased with increasing honey concentrations. Thus, we focuse phase was calculated as the period from inoculation to onset of log phase, or to 10% of maximal culture abs (128 individual growth assays with 6 different honey concentrations per assay), we automated the calculatio from the bacterial growth experiments to a generalized logistic curve (equation 1), a sigmoid function used f 11.1 (PC/Windows) 28 January 2011, VSN International Ltd, UK). Due to variable T values, this generalized curve (not shown).

$$Y = A + C/[(1 + Te^{-B(x-M)})^{1/T}]$$

Here, A = the lower asymptote; C = the upper asymptote; M = time of maximum growth; B = growth rate, an occurs.

With these parameters, we were able to compare the effect of the different honey samples on growth simply presence of varying honey concentrations (% w/v). This conversion from growth curve to lag phase duration growth in response to a series of manuka honey M3 dilutions (Fig. S2A, onset of log phase or 10% of maxin by “x”) is converted to the corresponding lag-phase honey dose response (Fig. S2B).

In the vast majority of cases when growth of a culture was detected by absorbance measurement, the maxi control culture. However in a few cases the maximal absorbance of the treated culture was less than 10% o In these cases, it was assessed as ‘no growth’ over the 24-hour period.

Cell Staining and Microscopy

Bacterial cultures treated with either 4% (w/v) honey M3 or honey MK1 (Table 1) were harvested from samp induced by honey treatments, and at log phase (which we will refer to as log phase) when cultures had resu was not observed, lag phase cells were obtained from within the first half hour of incubation. Untreated cells analysis, with the lag-phase cells collected 30 min after inoculation as described above. Harvested cells we with the following modifications: 20 µL of fixed cells were diluted 1:1 with the DNA staining agent DAPI (4',6- final DAPI concentration of 0.4 µg/mL for *E. coli*, *B. subtilis* and *P. aeruginosa*, and 0.8 µg/mL for *S. aureus*. placed in separate wells of a multi-well microscope slide (MP Biomedicals, LLC, Eschewege, Germany) tha Microscopy Sciences, Hatfield, PA, USA). After 15 min at room temperature, the liquid was removed and 50 then placed on all samples and the edges of the coverslip were sealed with nail polish.

Cells were imaged using phase-contrast and fluorescence microscopy with a Zeiss Axioplan 2 fluorescence 1.4; Carl Zeiss AG, Oberkochen, Germany) objective lens, and images were captured using a Zeiss AxioCa software (version 4.5; Carl Zeiss). Fluorescence microscopy to visualize DNA stained with DAPI used a 100 (Carl Zeiss) as a light source. Image processing was performed using AxioVision software version 4.5 (Carl

Image Data Analysis

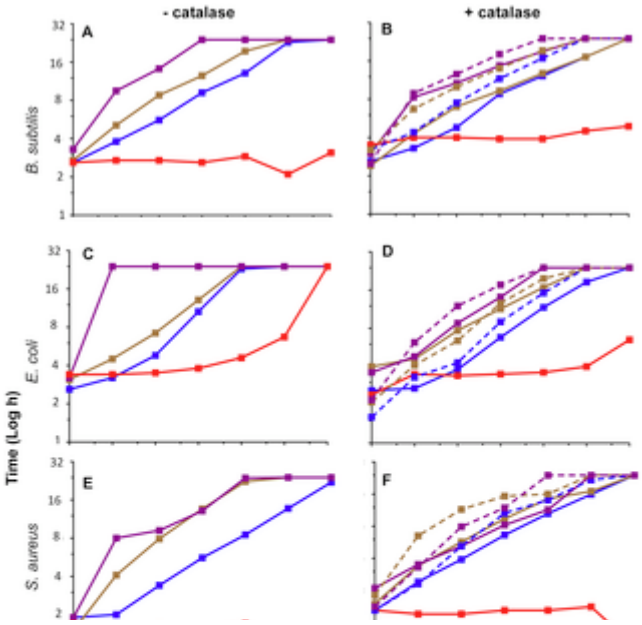
Cell length, cell lysis and DAPI staining were assessed by digital analysis of the captured images. Cell lengt total of 152 fields of cells were imaged and analyzed. Cell length (or diameter in the case of *S. aureus*) was used this MATLAB-based software to detect and outline bacterial cells in the microscopy images and measu (incorporated into the MicrobeTracker software) included a modification to algorithm 4 to enable accurate ce case of *E. coli*, *B. subtilis* and *P. aeruginosa*. For *S. aureus* algorithm 1 was optimized to enable measureme cell length information was then extracted and statistical analysis was performed in GraphPad Prism. One-v performed with the no-honey treated cells as controls. Cells that appeared lysed due to changes in their cor cell lysis was expressed as a percentage of the whole population. Only cells that remained intact but appea underestimation of cell lysis was possible, but this was consistent across all samples. For all experiments, a *subtilis* and *S. aureus* cells, where at least 50 cells were scored.

Results

Growth Responses to Honey, MGO, Sugar and Catalase

The growth response of two Gram-positive bacteria, *B. subtilis* 168 and *S. aureus* ATCC 25923, and two Gr PAO1 (ATCC 15692) to the 10 honeys and various control solutions were assessed. These data comprised honey treatments each graph represents a particular honey at six concentrations with a single organism as each honey sample on the multi-well plates. A comprehensive range of control treatments were included in components on growth of the four bacteria (see Materials and Methods).

Time spent in lag phase before entry into exponential growth emerged as the most notable difference amon (see Materials and Methods). We therefore focused our analysis on growth inhibition on this parameter, exp to reach 10% maximal culture absorbance. The graphs presented in Figure 1 and Figure 2 summarize the g control solutions (Fig. 1) and the honeys (Fig. 2). In these graphs, the time (h) taken for a culture to enter lo culture absorbance; y-axis) is plotted against the honey (or component) concentration (x-axis) for each orga panel) of catalase. Note that the faster the rise of the line, the longer the cells are arrested in lag phase at lo particular honey is at inhibiting the growth of that organism. Culture growth was monitored over 24 hours, ar 'no growth' or complete inhibition.



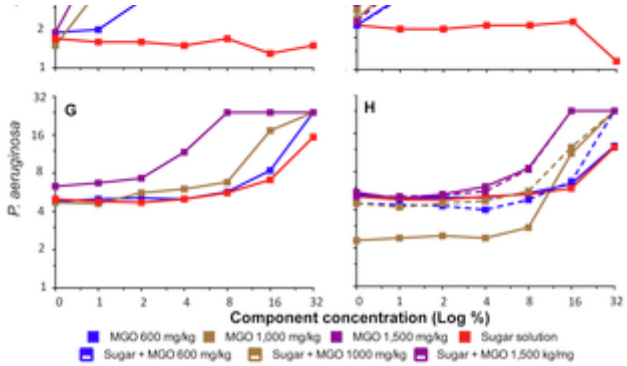


Figure 1. Effect of sugar, MGO and catalase on growth of bacteria.
Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with various compone of MGO and sugar at various concentrations equivalent to honeys at the corresponding concentrations s performed in the absence (left-hand graphs) and presence (right-hand graphs) of catalase as indicated. mg/kg MGO), M2 (1004.3 mg/kg MGO) and M3 (1541.3 mg/kg MGO) at 1%–32% (w/v). Optical density each component concentration, the time it takes for the culture to reach log phase (assessed as at least culture) is plotted on the x-axis. The derivation of this value is described in Materials and Methods. A val untreated control was performed alongside each particular treatment, and the starting OD_{595} (zero time- experiment.
doi:10.1371/journal.pone.0055898.g001

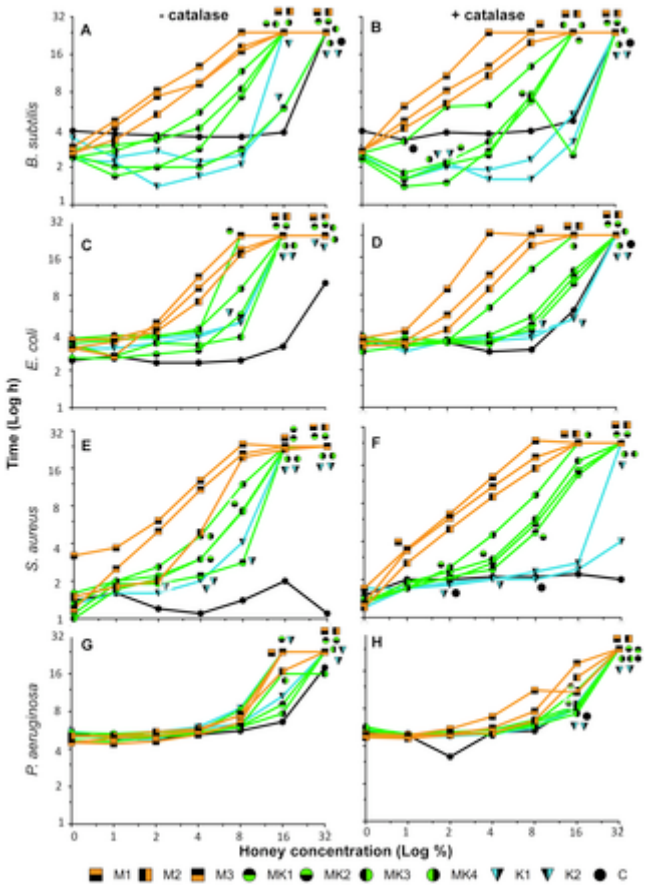


Figure 2. Effect of New Zealand manuka, kanuka and manuka-kanuka blended honeys on bacteri
Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with ten different hone

M2 and M3; two kanuka honeys, K1 and K2; four manuka-kanuka blended honeys, MK1, MK2, MK3 and
concentrations (from 1%–32% (w/v), increasing in 2-fold series). Optical density was recorded at 595 nm
concentration, the time it takes for the culture to reach log phase (assessed as at least 10% of the final
the x-axis. The derivation of this value is described in Materials and Methods. A value of 24 hours on the
particular honey overlap, we have surrounded the point on the graph by all the symbols relevant to that
honey treatments. An untreated control was also performed alongside each particular honey treatment,
plotted for that particular honey experiment.
doi:10.1371/journal.pone.0055898.g002

The starting absorbance values differ in each case in Figures 1 and 2 because for each honey, the no-hone
experimental conditions during that particular experiment.

Growth Dynamics in Response to Controls: MGO and Sugar in the Presence

The addition of catalase to an overnight culture of bacterial cells had essentially no effect on the duration of
organisms when compared with an untreated control culture (Fig. S1). Sugar alone had a small but variable
species *B. subtilis* and *S. aureus* were unaffected even at high concentrations, while the two Gram-negative

The addition of MGO at 0–32% of the concentrations present in manuka honey samples M1, M2 and M3 (st
honey) generally showed a dose-dependent extension of lag phase (Fig 1A, 1C, 1E, 1G). This was most se
was lowest in *P. aeruginosa*. The difference in sensitivity to MGO alone between the organisms was most ol
Fig. 1A, 1C, 1E, 1G).

The addition of catalase to the MGO treatments shortened the lag-phase extension in most cases, so that th
of *P. aeruginosa,* the bacteria were still increasingly sensitive to increasing MGO concentrations in the prese
addition of sugar to MGO in the presence of catalase had a small but noticeable effect on delaying the onse
aeruginosa, particularly at the lower sugar concentrations tested (equivalent to ≤8% honey; Fig. 1).

Growth Response in the Presence of Natural Honeys

Graphs summarizing the effect of the different honey types on growth of the four bacterial species are show
apparent: first, there is a general trend of greater growth inhibition by honeys containing more MGO, with M
causes a shift of the curves to the right for most honey types indicating a rescue of growth inhibition; third, *P*
inhibition compared to *B. subtilis*, *E. coli* and *S. aureus*; and fourth, the effect of clover honey is different for
below, where the response of the bacteria to each honey type is described.

Manuka honey.

Manuka honey samples M1, M2 and M3 (Table 1) have the highest MGO concentrations of the honeys teste
These three honeys were the most effective in inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus* and all re
concentrations (1–4%) caused significant lag-phase extension and growth was completely inhibited once co

In the presence of catalase the monofloral manuka honeys remained the most effective of the natural honey
indicating that the non-peroxide component/s in these honeys is the over-riding component responsible for t

In contrast to *B. subtilis*, *E. coli* and *S. aureus*, there was very little or no lag-phase extension when *P. aerug*
M1–M3, and complete inhibition only occurred at 16% of honeys M3 and M1 and 32% of honey M2. *P. aeru*
likely accounts for some of the inhibition. The addition of catalase increased the concentration of honeys M1
32%). These data and those shown in Figure 2 indicate that *P. aeruginosa* is relatively insensitive to both hy
inhibition can be attributed to non-peroxide component/s.

Kanuka honey.

The kanuka honeys, K1 and K2, had very low levels of MGO (5.6 and 37.1 mg/kg, respectively), but modera mM/h, respectively) compared to the other honeys tested. At low concentrations (1–8%), and particularly in least effective of the honeys at inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus*, with very little or no lag- Complete growth inhibition with K1 and K2 occurred at 16% or 32%.

Although the addition of catalase to K1 and K2 made them less effective at inhibiting growth of *B. subtilis*, *E* occurred at 32%; the exception being honey K1, where lag phase was only extended by 4 hours (Fig. 2F). T is not likely to be due to MGO, or at least MGO acting alone, since the amount present in 32% K1 and K2 is of 660 mg/kg MGO and is therefore too low to affect growth of these bacteria (Fig. 1A, 1C, 1E; light blue line *aureus* suggests that the component/s that contribute to complete growth inhibition of *B. subtilis* and *E. coli* a component in honey that is specifically active against *S. aureus* but requires hydrogen peroxide for its pro only species that was not inhibited by clover honey (see below).

P. aeruginosa growth was completely inhibited by kanuka honeys at 16% (K1) and 32% (K2), and very little rescued this effect to some extent, but at 32% the kanuka honeys completely inhibited growth of *P. aerugino* peroxide are present in these honeys that affect growth of this organism. The most striking observation for *P* organisms was that growth was similarly affected by kanuka honeys as by manuka honeys.

Manuka-kanuka honey blends.

The responses of bacteria to the manuka-kanuka honey blends, designated MK1–MK4, are shown in green MGO (ranging from 173.6–307.8 mg/kg) that are between those of the pure manuka and kanuka honeys, ar (Table 1). Treatment with these honeys gave a level of inhibition that was generally between that of the pure hydrogen peroxide was removed by catalase. In addition, the degree of growth inhibition related largely to th MGO of the blended honeys (Table 1), normally being the most effective at inhibiting growth.

While the overall pattern of growth inhibition of *B. subtilis*, *E. coli* and *S. aureus* by the MK honeys was simil responded to the different blends. In the absence of catalase MK1 inhibited *E. coli* growth to a similar extent 8%. MK1 has a low level of MGO (173.6 mg/kg) compared to the manuka honeys but has the highest hydro addition to MK1 reduced the level of growth inhibition for *E. coli* to a level well below that of all three manuka maximally inhibited by honeys that either have a high level of hydrogen peroxide production or have high lev

P. aeruginosa displayed little or no lag-phase extension or growth inhibition for any of the blended honeys u no clear trend in how *P. aeruginosa* responded to the varying levels of MGO and hydrogen peroxide in the d achieved at 32% in the presence of catalase, indicating that the inhibition does not require hydrogen peroxid

Clover honey.

The clover honey sample had no detectable MGO and almost no hydrogen peroxide production (0.029 mM on the growth of the four organisms (Fig. 2). At 32%, *S. aureus* growth remained unaffected, while the two G significant lag-phase extension. This is commensurate with the response of these two organisms to 32% su of catalase to clover honey slightly increased lag phase extension, this was not seen for the corresponding s inhibited growth of *B. subtilis*, both in the presence and absence of catalase even though sugar alone at equ organism. This suggests the presence of one or more components in clover honey to which *B. subtilis* grow

Other observations not fitting growth inhibition trends.

Although there were clear trends in growth inhibition in response to treatment with honeys and control soluti did not fit these trends that are worth acknowledging. This includes: M1, which has the lowest level of MGO and *S. aureus* in the presence of catalase (Fig. 2B, 2D, 2F); the apparent abrupt (and reproducible) decreas at 16% in the presence of catalase (Fig. 2B); the incomplete inhibition of *P. aeruginosa* by honey MK3 only a higher level of inhibition of *E. coli* by clover honey in the presence of catalase (Fig. 2D). Given the complexit in these analyses cannot always be solely accounted for by the presence MGO and hydrogen peroxide, and modulate the response of bacteria to MGO- and hydrogen peroxide-based toxicity.

Cellular Morphology Response in the Presence of Natural Honeys

To determine morphological changes that occur in response to honey containing relatively high levels of MG *P. aeruginosa* were exposed to honey samples M3 (highest MGO with lowest hydrogen peroxide production peroxide and relatively low MGO; Table 1). Cells were treated with 4% (w/v) of each honey, which is the higl bacteria (see above; Fig. 2). Cell morphology was analysed during lag- and log-phase growth and included (breakage of cells or leakage of cytoplasm indicating cell envelope or growth abnormalities), and detection c

High-level MGO honey and cell morphology.

Treatment with honey M3 induced an extended lag phase for all bacterial cultures except *P. aeruginosa* (Fig and charted in Table 2, and mean cell lengths are recorded in Table S1. During the extended lag phase (or t *coli* and *S. aureus* were significantly shorter ($p<0.05$) than untreated cells, while *P. aeruginosa* cells were lor of the shorter cells of *B. subtilis* (29%) and *S. aureus* (57%) had a condensed chromosome (green arrows ir DAPI staining occurred instead of the characteristic two regions that represent replicating chromosomes (Fi chromosomes showed one or two very small spots of DAPI-stained DNA, unlike the two larger lobes of DNA control cells. No changes to DNA appearance under these conditions were observed for *E. coli* or *P. aerugir*

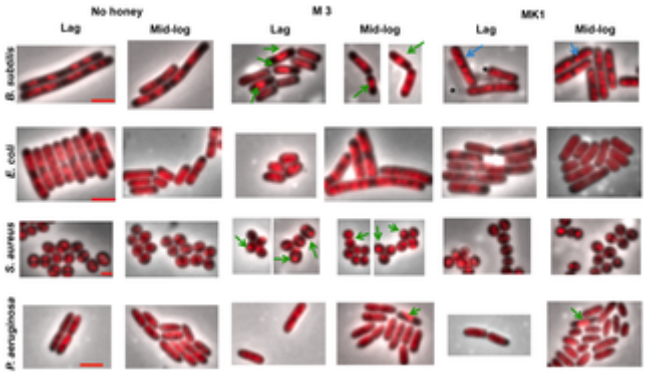
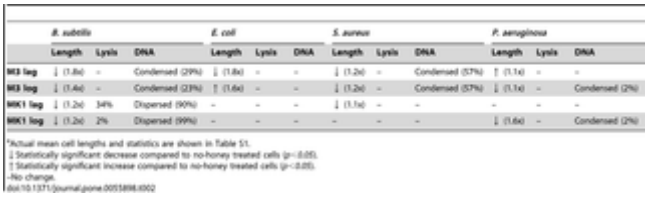


Figure 3. Cellular morphology of bacterial cells treated with a high-MGO honey and a high-hydrog
The effects of 4% (w/v) of a high-MGO honey (M3) and a high-hydrogen peroxide honey (MK1) on bacte cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with these honeys, cells collecte Figure S2, fixed with glutaraldehyde, stained with DAPI and imaged using fluorescence microscopy. All i the DAPI-stained (red) fluorescence image. The two left-hand panels show the no-honey treated control and the two right-hand panels show the MK1 honey-treated cells. In all images, condensed DNA is show cells is shown by blue arrows. An asterisk indicates lysed cells for *B. subitlis* (MK1, lag-phase cells). The images, where it represents 1 μm.
doi:10.1371/journal.pone.0055898.g003



	B. subtilis			*E. coli*			*S. aureus*			*P. aeruginosa*		
	Length	Lysis	DNA	Length	Lysis	DNA	Length	Lysis	DNA	Length	Lysis	DNA
M3 lag	↓ (1.8x)	-	Condensed (29%)	↓ (1.8x)	-	-	↓ (1.2x)	-	Condensed (57%)	↑ (1.1x)	-	-
M3 log	↓ (1.4x)	-	Condensed (23%)	↑ (1.6x)	-	-	↓ (1.2x)	-	Condensed (57%)	↓ (1.1x)	-	Condensed (2%)
MK1 lag	↓ (1.2x)	34%	Dispersed (90%)	-	-	-	↓ (1.1x)	-	-	-	-	-
MK1 log	↓ (1.2x)	2%	Dispersed (99%)	-	-	-	-	-	-	↓ (1.6x)	-	Condensed (2%)

*Actual mean cell lengths and statistics are shown in Table S1.
↓ Statistically significant decrease compared to no-honey treated cells ($p<0.05$).
↑ Statistically significant increase compared to no-honey treated cells ($p<0.05$).
-No change.
doi:10.1371/journal.pone.0055898.t002

Table 2. Cell morphology changes with high-MGO honey and high-hydrogen peroxide honey trea

doi:10.1371/journal.pone.0055898.t002

Following entry into log phase, cells treated with M3 honey were still significantly different to untreated cultu remained shorter, to a similar degree observed in lag phase, and chromosomes remain condensed. *E. coli* c counterparts, while *P. aeruginosa* cells became slightly but significantly shorter (Table 2). In addition, 2% of chromosome by DAPI staining (green arrows in Fig. 3; Table 2).

In summary, treatment with 4% M3 honey changed mean cells lengths of all four populations of bacteria in b extent of change varied. The greatest changes to cell length were observed with *B. subtilis* and *E. coli* (Tabl DNA for both growth phases.

High-level hydrogen peroxide honey and cell morphology.

Treatment with 4% MK1 honey did not result in an extended lag phase for any organism, however this hone significant changes to cell morphology induced by MK1 during the initial stages of growth were observed in shorter, as was seen with M3 but to a lesser extent. A significant proportion of cells (34%) appeared lysed a contrast microscopy (asterisks in Fig. 3; Tables S1 and 2), and the DNA in the vast majority of unlysed cells (Fig. 3; Table 2). No morphological changes were observed in *E. coli* or *P. aeruginosa* cultures, and the only decrease in cell diameter (Table 2).

In log-phase MK1-treated *B. subtilis* cultures, the extent of cell lysis was reduced from 34% in the lag-phase average compared to control cells (Table 2 and Table S1; Fig. 3), and the frequency of cells with a dispersed *S. aureus* cells showed a normal morphology (Fig. 3; Table 2), while *P. aeruginosa* cells were significantly sl log-phase MK1-treated *P. aeruginosa* cells had the same condensed chromosome phenotype seen in the lo cells remained similar in appearance to the control cells (Table 2).

In summary, MK1 honey caused less alteration to cellular morphology than the high-MGO honey, M3. *B. sul* dispersed DNA, cell lysis and cell length changes. *E. coli* and *S. aureus* had little or no apparent change. *P.*

Correlation of Growth Inhibition and Morphological Changes Induced by Hone

A summary of combined growth and morphology data is given in Table 3. Overall this shows that MGO and of all organisms except *P. aeruginosa*, followed by manuka-kanuka blended honeys, kanuka honeys and the honeys compared to the other three organisms, with little difference in growth inhibition by the different hone changes than the other bacteria for both the high-MGO and the high-hydrogen peroxide honeys, followed by exception of *B. subtilis,* where both growth and morphology are profoundly affected by honey, the number a the level of MGO or hydrogen peroxide in the honey, or to the effectiveness of the honey to inhibit growth. *E* though its growth was affected in a similar way to *B. subtilis* and *S. aureus* and to a much greater extent tha profound morphological changes.

	Growth Inhibition						Cell Morphology*	
Organism	MGO	Sugar	Clover	M	K	MK	High-MGO Honey (M3)	High-H_2O_2 Honey (MK1)
B. subtilis	XXX*	-*	X	XXXX	XX	XXX	Shorter cells; condensed DNA (25%)	Shorter cells; lysis; dispersed DNA (95%)
E. coli	XXXX	XX	X	XXXX	XX	XX	Shorter and longer cells	-
S. aureus	XXX	-	-	XXXX	XX	XX	Shorter cells; condensed DNA (57%)	Shorter cells
P. aeruginosa	XX	X	X	XX	XX	XX	Shorter and longer cells; condensed DNA (2%)	Shorter cells; condensed DNA (2%)

*this data includes data from both the log and lag phases of growth.
†The number of crosses increases the more growth is inhibited.
‡Means no effect.
doi:10.1371/journal.pone.0055898.t003

Table 3. Summary of growth and morphological effects of honeys and control treatments on all o
doi:10.1371/journal.pone.0055898.t003

Discussion

With the dearth of development of new classes of antibiotics to treat infections caused by resistant organism antibacterial activity and effectiveness as a treatment for chronic wound infections. However, as with all natu between different honey preparations [19] and this is likely to affect the level of antibacterial activity, and pos assessed a series of geographically- and chemically-defined New Zealand manuka, kanuka and manuka-ka and hydrogen peroxide to determine their effectiveness in inhibiting the growth of different species of bacter the most effective at inhibiting growth, followed by the manuka-kanuka blends and then the kanuka honeys. sub-inhibitory concentrations of these different honeys varied with bacterial species, with each having a unic very different to the other three bacteria in being both less sensitive overall and in having a similar response

High-throughput Analysis of Growth Dynamics Reveals that MGO in Honey E

A high-throughput approach was used to assess the growth and morphological effects of a large number of novel in honey studies and was employed here to address the challenge of assessing multiple parameters i explore the heterogeneous and variable composition of natural honey by analyzing large numbers of sample of cell growth in response to the effects of honey toxicity. Such an approach may be useful in the study of ot interacting factors.

Visual inspection of the resulting large number of growth curves revealed a distinctive dose-dependent exte *coli* and *S. aureus* were treated with manuka honey. This growth behavior was also observed when MGO al consistent with a previous study where *E. coli* was subjected to MGO treatment [29]. Lag-phase extension v growth was either unaffected or was completely inhibited, and there was no evidence for dose-dependent re lag phase is presumed to be largely or completely due to MGO and is likely to be unique to honey derived fr

Growth and Morphology of Different Bacteria are Affected by Honey in Marke

The dynamics of growth in the presence of the different honey types was relatively similar for *B. subtilis, E.* (Figs. 1 and 2). The extended duration of lag phase and the eventual resumption of logarithmic growth in th system used to detoxify MGO [30]. All organisms produce MGO, which appears to be important in allowing environment changes [31]–[32]. However, as MGO is toxic, cells detoxify this compound to D-lactate using t ability of *P. aeruginosa* to grow in the presence of higher MGO levels than the other bacteria may reflect mo by the discovery, through genome sequencing, that *P. aeruginosa* is unique among eubacteria in its posses [33].

To date, few microscopy studies have been performed to identify morphological changes to bacterial cells tr phase-contrast and fluorescence microscopy that allows a large number of cells to be imaged and measure organisms treated with manuka (high-MGO) honey. This is caused by an adjustment to the frequency of cell nutritional state, such that division occurs at a different cell length to untreated cells [34]. Condensed DNA w and *S. aureus* cells treated specifically with manuka honey. This could be a consequence of inhibition of init with previous studies demonstrating that MGO alone inhibits this phase of DNA replication in bacterial cells of hydrogen peroxide (MK1) caused significant changes to the morphology of *B. subtilis* cells, including a dis degree of DNA degradation due to hydrogen peroxide in the honey causing oxidative DNA damage [38].

With the exception of *B. subtilis,* the number and severity of morphological changes do not link clearly to the effectiveness of the honey to inhibit growth. This is not entirely unexpected since cell morphology often refle the organism to adapt to that environment without having to change its rate of growth. Different organisms d environmental conditions, such as oxidative or nutrient stress. This might reflect, at least in part, the degree inhabit [39]. We therefore speculate that the differences in morphology that we observe in response to a par regulatory circuits that coordinate growth with cellular physiology.

MGO and Hydrogen Peroxide Production cannot Account for All Activity Prese

Commensurate with previous studies [8], [15], [40], [41], we found that even when the peroxide activity was of MGO present, honey could inhibit bacterial growth. Even clover honey, with only trace levels of MGO and bacteria that in most cases could not be attributed to sugar alone. These observations are in line with previc antibacterial components that may be directly active or may modulate the activity of the dominant active cor include: (i) phenolics derived from the floral source [19]; (ii) bee-derived antimicrobial peptides (although not Revamil honey [8], could not be identified in manuka or kanuka honeys) [23] [42]; and (iii) as yet undefined s transition metals [38], [43], [44].

Clinical Applications of Antibacterial Honey

The range of effects induced by the different honeys in the bacterial species tested reflects a diversity of res chronic wounds. Our findings here have important implications for the clinical application of honey in the trea of MGO may be neutralized by bacteria which then resume normal growth, thus any honey formulation shou Second, honey without significant levels of MGO or hydrogen peroxide, such as clover honey, may be able t therefore not recommended for infected wounds where multiple species may be present. Third, MGO at 60(higher concentrations, and increasing MGO above this threshold may not result in a more effective honey. A peroxide, MGO provides an over-riding activity and if this level is high enough, hydrogen peroxide does little

To date, more than 80 different microbial species, including bacteria and yeast pathogens known to infect w [46]. In the current study, the use of sub-inhibitory concentrations of honey has enabled us to examine the n well below those that would be used in a clinical situation, where whole honey is generally applied and comp

Emerging evidence from clinical studies suggests that honey is at least as effective as conventional treatme such as in diabetics, the elderly, and extensively burned patients [47], [48], but more clinical data are neces demonstrated the potency of natural honey as an antimicrobial wound dressing, and that multiple effects ari allows active honey to be uniquely broad in spectrum, but also reduces the potential for resistant microbial p therefore recommended for the treatment of infected wounds. Understanding the complex nature of honeys the development of specific blends with an optimal combination of antibacterial components, thus ensuring a option.

Supporting Information

Figure_S1.pdf

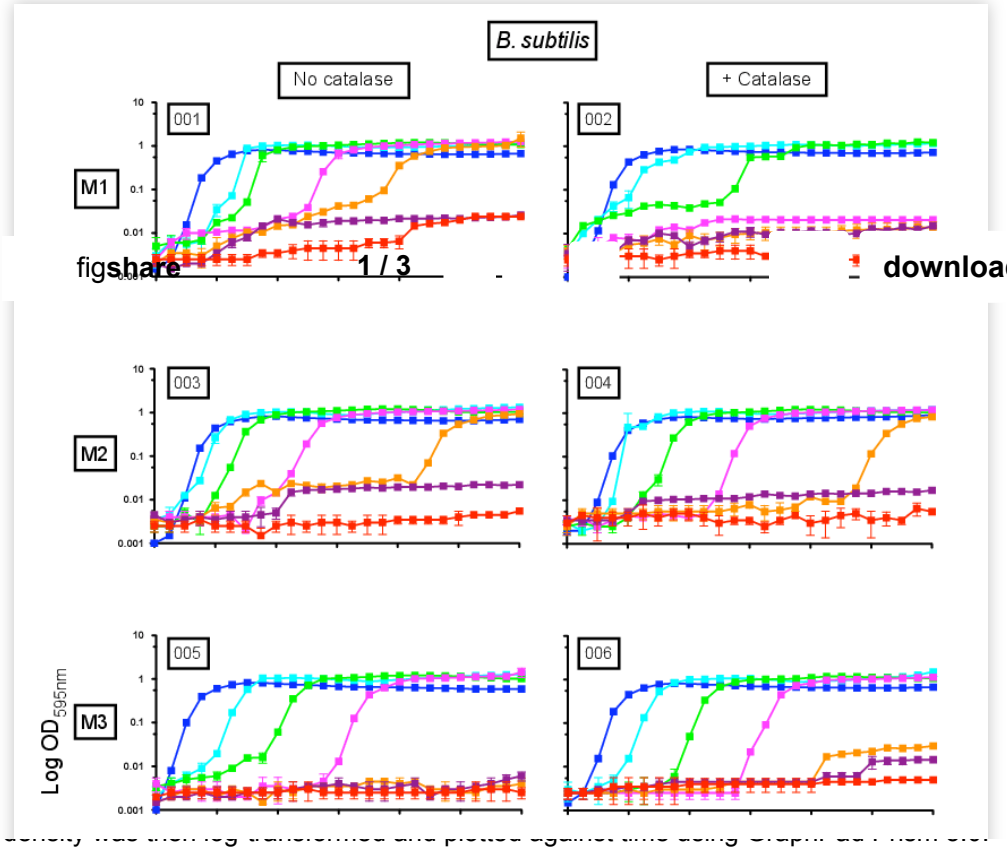


Figure S1.
The effect of New Zealand honey treatments on bacterial growth. Growth curves of *B. subtilis* (001–032 *aeruginosa* (097–128) were treated with 10 different honeys (three manuka honeys, M1, M2, M3; four manu kanuka honeys, K1, K2; and a clover honey, C) and a comprehensive range of controls, which included (i) a and 1% of sucrose; (ii) honey plus catalase (1 mg/mL); (iii) a catalase-only control; (iv) three MGO solutions undiluted honeys M1, M2 and M3 (600, 1,000 & 1,500 mg/kg) and diluted the same as honey; v) a range of MGO concentrations in the presence of both catalase and sugar solution at various concentrations (0% - as represented by dark blue, light blue, green, pink, orange, purple and red color respectively). Optical density density was then log-transformed and plotted against time using GraphPad Prism 5.0.
doi:10.1371/journal.pone.0055898.s001
(PDF)

Figure S2.
Transformation of data obtained for bacterial growth with honey treatment. Panel A illustrates the effe *E. coli* growth over 24 h as a simple log OD_{595nm} *versus* incubation time. The point at which 10% of the fina curve. Panel B summarizes all the data from panel A as a simple relationship between honey concentration value of 24 hours on the y-axis denotes 'no growth'.
doi:10.1371/journal.pone.0055898.s002
(TIF)

Table S1.
Average cell length after different honey treatment (µm). Cell lengths were not significantly affected by t significantly different ($p<0.05$); n ≥50. M3–4% manuka M3 (high-MGO) honey treatment. MK1–4% manuka-
doi:10.1371/journal.pone.0055898.s003
(DOCX)

Acknowledgments

E. coli O157:H7 was a gift from David Gally (University of Edinburgh, UK).

Author Contributions

Conceived and designed the experiments: EJH JL SG DR RS. Performed the experiments: JL SG JS DR. A
Contributed reagents/materials/analysis tools: JS DH EJH DR. Wrote the paper: EJH JL DC GS JS DR.

References

1. Dowd SE, Wolcott RD, Sun Y, McKeehan T, Smith E, et al. (2008) Polymicrobial nature of chronic di bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). PLoS One 3: e3326. doi: 10.1371/j
CrossRef • PubMed/NCBI • Google Scholar
2. Wolcott RD, Rhoads DD, Dowd SE (2008) Biofilms and chronic wound inflammation. J Wound Care
CrossRef • PubMed/NCBI • Google Scholar
3. Ngo DK, Vickery K, Deva AK (2007) Effects of Combined Topical Negative Pressure (TNP) and Anti A66. doi: 10.1111/j.1445-2197.2007.04127_23.x.
CrossRef • PubMed/NCBI • Google Scholar
4. Engemann JJ, Carmeli Y, Cosgrove SE, Fowler VG, Bronstein MZ, et al. (2003) Adverse clinical and among patients with Staphylococcus aureus surgical site infection. Clin Infect Dis 36: 592–598. doi:
CrossRef • PubMed/NCBI • Google Scholar
5. Projan SJ, Youngman PJ (2002) Antimicrobials: new solutions badly needed. Current opinion in mic 10.1016/S1369-5274(02)00364-8.
CrossRef • PubMed/NCBI • Google Scholar
6. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, et al. (2009) Human skin wounds: a major and s Repair Regen 17: 763–771. doi: 10.1111/j.1524-475X.2009.00543.x.
CrossRef • PubMed/NCBI • Google Scholar
7. Blair SE, Cokcetin NN, Harry EJ, Carter DA (2009) The unusual antibacterial activity of medical-grad and transcriptome analysis. European journal of clinical microbiology & infectious diseases : official 28: 1199–1208. doi: 10.1007/s10096-009-0763-z.
CrossRef • PubMed/NCBI • Google Scholar
8. Kwakman PH, de Boer L, Ruyter-Spira CP, Creemers-Molenaar T, Helsper JP, et al. (2011) Medical- enhanced activity against antibiotic-resistant pathogens. European journal of clinical microbiology & Society of Clinical Microbiology 30: 251–257. doi: 10.1007/s10096-010-1077-x.
CrossRef • PubMed/NCBI • Google Scholar
9. Henriques AF, Jenkins RE, Burton NF, Cooper RA (2010) The intracellular effects of manuka honey microbiology & infectious diseases : official publication of the European Society of Clinical Microbiol
CrossRef • PubMed/NCBI • Google Scholar
10. Maddocks SE, Lopez MS, Rowlands RS, Cooper RA (2012) Manuka honey inhibits the developmen expression of two fibronectin binding proteins. Microbiology 158: 781–790. doi: 10.1099/mic.0.0539
CrossRef • PubMed/NCBI • Google Scholar
11. Merckoll P, Jonassen TO, Vad ME, Jeansson SL, Melby KK (2009) Bacteria, biofilm and honey: a st embedded chronic wound bacteria. Scandinavian journal of infectious diseases 41: 341–347. doi: 1
CrossRef • PubMed/NCBI • Google Scholar
12. Gannabathula S, Skinner MA, Rosendale D, Greenwood JM, Mutukumira AN, et al. (2012) Arabinog

properties of New Zealand honeys. Immunopharmacology and immunotoxicology 34: 598–607. doi:
CrossRef • PubMed/NCBI • Google Scholar

13. Cooper R, Jenkins R (2012) Are there feasible prospects for manuka honey as an alternative to con
623–625. doi: 10.1586/eri.12.46.
CrossRef • PubMed/NCBI • Google Scholar
14. Allen KL, Molan PC, Reid GM (1991) A survey of the antibacterial activity of some New Zealand hor
10.1111/j.2042-7158.1991.tb03186.x.
CrossRef • PubMed/NCBI • Google Scholar
15. Kwakman PH, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CM, et al. (2010) How hon
10.1096/fj.09-150789.
CrossRef • PubMed/NCBI • Google Scholar
16. Kwakman PH, Zaat SA (2012) Antibacterial components of honey. IUBMB Life 64: 48–55. doi: 10.1(
CrossRef • PubMed/NCBI • Google Scholar
17. Mavric E, Wittmann S, Barth G, Henle T (2008) Identification and quantification of methylglyoxal as
(Leptospermum scoparium) honeys from New Zealand. Mol Nutr Food Res 52: 483–489. doi: 10.10
CrossRef • PubMed/NCBI • Google Scholar
18. Adams CJ, Boult CH, Deadman BJ, Farr JM, Grainger MNC, et al. (2008) Isolation by HPLC and ch
manuka (Leptospermum scoparium) honey. Carbohydrate Research 343: 651–659. doi: 10.1016/j.c
CrossRef • PubMed/NCBI • Google Scholar
19. Stephens JM, Schlothauer RC, Morris BD, Yang D, Fearnley L, et al. (2010) Phenolic compounds a
Kanuka honeys. Food Chemistry 120: 78–86. doi: 10.1016/j.foodchem.2009.09.074.
CrossRef • PubMed/NCBI • Google Scholar
20. Majtan J, Klaudiny J, Bohova J, Kohutova L, Dzurova M, et al. (2012) Methylglyoxal-induced modific
manuka honey: Possible therapeutic implications. Fitoterapia 83: 671–677. doi: 10.1016/j.fitote.2012
CrossRef • PubMed/NCBI • Google Scholar
21. Irish J, Blair S, Carter DA (2011) The antibacterial activity of honey derived from Australian flora. PL
CrossRef • PubMed/NCBI • Google Scholar
22. Irish J, Carter DA, Shokohi T, Blair SE (2006) Honey has an antifungal effect against Candida speci
International Society for Human and Animal Mycology 44: 289–291. doi: 10.1080/13693780500417(
CrossRef • PubMed/NCBI • Google Scholar
23. Kwakman PH, Te Velde AA, de Boer L, Vandenbroucke-Grauls CM, Zaat SA (2011) Two major med
activity. PLoS One 6: e17709. doi: 10.1371/journal.pone.0017709.
CrossRef • PubMed/NCBI • Google Scholar
24. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP (1997) A stable nonfluorescent derivative of r
hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxi
10.1006/abio.1997.2391.
CrossRef • PubMed/NCBI • Google Scholar
25. Monahan LG, Robinson A, Harry EJ (2009) Lateral FtsZ association and the assembly of the cytokir
1004–1017. doi: 10.1111/j.1365-2958.2009.06914.x.
CrossRef • PubMed/NCBI • Google Scholar
26. Perna NT, Plunkett G III, Burland V, Mau B, Glasner JD, et al. (2001) Genome sequence of enteroha
529–533. doi: 10.1038/35054089.
CrossRef • PubMed/NCBI • Google Scholar
27. Setlow B, Magill N, Febbroriello P, Nakhimovsky L, Koppel DE, et al. (1991) Condensation of the for
Journal of bacteriology 173: 6270–6278.
CrossRef • PubMed/NCBI • Google Scholar

28. Sliusarenko O, Heinritz J, Emonet T, Jacobs-Wagner C (2011) High-throughput, subpixel precision a temporal dynamics. Molecular Microbiology 80: 612–627. doi: 10.1111/j.1365-2958.2011.07579.x.
CrossRef • PubMed/NCBI • Google Scholar

29. Egyud LG, Szent-Gyorgyi A (1966) On the regulation of cell division. Proc Natl Acad Sci U S A 56: 2
CrossRef • PubMed/NCBI • Google Scholar

30. Cooper RA (1984) Metabolism of methylglyoxal in microorganisms. Annu Rev Microbiol 38: 49–68.
CrossRef • PubMed/NCBI • Google Scholar

31. Ferguson GP, Totemeyer S, MacLean MJ, Booth IR (1998) Methylglyoxal production in bacteria: sui 10.1007/s002030050635.
CrossRef • PubMed/NCBI • Google Scholar

32. Totemeyer S, Booth NA, Nichols WW, Dunbar B, Booth IR (1998) From famine to feast: the role of n 27: 553–562. doi: 10.1046/j.1365-2958.1998.00700.x.
CrossRef • PubMed/NCBI • Google Scholar

33. Sukdeo N, Honek JF (2007) Pseudomonas aeruginosa contains multiple glyoxalase I-encoding gen Acta 1774: 756–763. doi: 10.1016/j.bbapap.2007.04.005.
CrossRef • PubMed/NCBI • Google Scholar

34. Chien AC, Hill NS, Levin PA (2012) Cell size control in bacteria. Curr Biol 22: R340–349. doi: 10.10
CrossRef • PubMed/NCBI • Google Scholar

35. Britton RA, Lin DC, Grossman AD (1998) Characterization of a prokaryotic SMC protein involved in 10.1101/gad.12.9.1254.
CrossRef • PubMed/NCBI • Google Scholar

36. Moriya S, Rashid RA, Rodrigues CD, Harry EJ (2010) Influence of the nucleoid and the early stages Bacillus subtilis. Molecular Microbiology 76: 634–647. doi: 10.1111/j.1365-2958.2010.07102.x.
CrossRef • PubMed/NCBI • Google Scholar

37. Fraval HN, McBrien DC (1980) The effect of methyl glyoxal on cell division and the synthesis of prot Escherichia coli B/r. J Gen Microbiol 117: 127–134. doi: 10.1099/00221287-117-1-127.
CrossRef • PubMed/NCBI • Google Scholar

38. Brudzynski K, Abubaker K, Laurent M, Castle A (2011) Re-examining the role of hydrogen peroxide Microbiol 2: 213. doi: 10.3389/fmicb.2011.00213.
CrossRef • PubMed/NCBI • Google Scholar

39. Silva-Rocha R, de Lorenzo V (2010) Noise and robustness in prokaryotic regulatory networks. Annu 10.1146/annurev.micro.091208.073229.
CrossRef • PubMed/NCBI • Google Scholar

40. Adams CJ, Manley-Harris M, Molan PC (2009) The origin of methylglyoxal in New Zealand manuka 1050–1053. doi: 10.1016/j.carres.2009.03.020.
CrossRef • PubMed/NCBI • Google Scholar

41. Lin SM, Molan PC, Cursons RT (2011) The controlled in vitro susceptibility of gastrointestinal pathog journal of clinical microbiology & infectious diseases : official publication of the European Society of 10.1007/s10096-010-1121-x.
CrossRef • PubMed/NCBI • Google Scholar

42. Weston RJ (2000) The contribution of catalase and other natural products to the antibacterial activity 10.1016/S0308-8146(00)00162-X.
CrossRef • PubMed/NCBI • Google Scholar

43. Molan P (2008) An explanation of why the MGO level in manuka honey does not show the antibacte
CrossRef • PubMed/NCBI • Google Scholar

44. Chen C, Campbell LT, Blair SE, Carter DA (2012) The effect of standard heat and filtration processir

peroxide levels in honey. Front Microbiol 3: 265. doi: 10.3389/fmicb.2012.00265.
CrossRef • PubMed/NCBI • Google Scholar

45. Molan PC (1992) The antibacterial activity of honey: 2. Variation in the potency of the antibacterial a
CrossRef • PubMed/NCBI • Google Scholar
46. Blair SE, Carter DA (2005) The potential for honey in the management of wounds and infection. Hea
CrossRef • PubMed/NCBI • Google Scholar
47. Kegels F (2011) Clinical Evaluation of Honey-based Products for Lower Extremity Wounds in A Hom
CrossRef • PubMed/NCBI • Google Scholar
48. Smaropoulos E, Romeos S, Dimitriadou C (2011) Honey-based therapy for paediatric burns and der
Wounds UK 7: 33–40.
CrossRef • PubMed/NCBI • Google Scholar
49. Lee DS, Sinno S, Khachemoune A (2011) Honey and wound healing: an overview. American journa
10.2165/11538930-000000000-00000.
CrossRef • PubMed/NCBI • Google Scholar