The Effects of New Zealand Manuka-Type Honeys on Bacterial Growth and Morphology, Biofilm Formation and Biofilm Eradication

Jing Lu

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The i3 Institute

University of Technology, Sydney

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Certificate of Original Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledge within the text.

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Abbreviations

%	Percentage
AGPs	Arabinogalactan proteins
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
a_w	Water activity
C	Clover honey
CA-MRSA	Community-acquired methicillin resistant *Staphylococcus aureus*
CAMHB	Cation-adjusted Mueller-Hinton broth
CFU	Colony Formation Unit
CLSI	Clinical Laboratory Standards Institute
CLSM	Confocal Laser Scanning Microscopy
DHA	Dihydroxyacetone
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
FDA	Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
g	Gram(s)
GlxI	Glyoxalase I
h	Hour(s)
H_2O_2	Hydrogen peroxide
HA-MRSA	Hopital-acquired methicillin resistant *Staphylococcus aureus*
HPLC	High-performance liquid chromatography
HS-LBA	High-salt Luria-Bertani agar
K	Kanuka honey
kg	Kilogram(s)
LB	Luria-Bertani
M	Manuka honey
MBIC(s)	Minimum biofilm inhibitory concentration(s)
mg	Milligram(s)

MGO	Methylglyoxal
MIC(s)	Minimum Inhibitory Concentration(s)
min	Minute(s)
MK	Manuka-kanuka blends
mL	Millilitre
mm	Millimetre
mM	Millimolar
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface componenets recognising adhesive matrix molecules
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
NMR	Nuclear magnetic resonance
NZ	New Zealand
^{o}C	Degree Celsius
OD_{595}	Optical Density at 595 nm
PBS	Phosphate buffered saline
PI	Propidium iodine
PIA	Polysaccharide intercellular adhesion
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
ROW	Reverse osmosis water
rpm	Revolutions per minute
SD	Standard deviation
SEM	Standard error of the mean
TGA	Therapeutic Goods Administration
TNF- α	Tumour necrosis factor-alpha
TSB	Tryptone soya broth
TSBG	Tryptone soya broth plus 1% glucose
UMF	Unique Manuka Factor
USA	United States of America
USD	United States dollar
UV	Ultra violet

v	Volume
w	Weight
w/v	Weight per volume
µL	Microlitre
µm	Micrometer
μm^2	Square micrometer
μm^3	Cubic micrometer
µmol	Micromole

Abstract

Bacterial pathogenesis is a major threat to human health due to the increase antibiotic resistance among disease-causing bacteria. Effective and alternative therapeutics are urgently required to combat this problem. Honey is a natural product that has been used for over 2,000 years, as an effective topical chronic wound treatment. Numerous studies in the last 30 years have revealed its potent antibacterial properties (due to high sugar content, low pH and hydrogen peroxide production upon dilution). Honeys sourced from the *Leptospermum scoparium* bush in New Zealand (NZ), also referred to as manuka-type honeys, have been known to contain additional 'non-peroxide' antibacterial components (including methylglyoxal (MGO) and various phenolic compounds).

However, for honey to be considered as a mainstream wound treatment by medical professionals, the mechanism behind its antibacterial activity needs to be determined. Moreover, bacteria produce biofilms that is a matrix of extracellular polymeric substance and allow cells to adhere to a surface such as a wound. Biofilms are the preferred mode of life in wounds because it also offers protection from antibiotic treatment. It is therefore essential to evaluate honeys' effects on bacterial biofilms. Unfortunately, almost all previous studies have utilized honeys that are ill-defined chemically. Thus, the objectives of this work were to use a range of well-defined NZ manuka-type honeys and their specific antibacterial components (such as methylglyoxal and sugars) to firstly examine their antibacterial effects on bacterial cell growth and cellular morphology, across a range of different bacteria. Subsequently, the antibiofilm activities on different strains of the same organism were also investigated on preventing biofilm formation and eradicating the pre-established biofilms.

The bacterial cell growth and cellular morphology of three clinically relevant bacteria; the Gram-positive *Staphylococcus aureus*, and, the Gram-negative organisms *Escherichia coli* and *Pseudomonas aeruginosa* were examined against the selected range of NZ honeys, by cell growth assays and fluorescent microscopy.

In addition, a Gram-positive organism, *Bacillus subtilis*, was also studied because it is a model organism where the functions of many genes associated with cellular growth and morphology have been documented. Moreover, *B. subtilis* is often used as a Gram-positive representative organism, typically in drug discovery studies in the industry. Results presented in this work indicate that different bacterial species are susceptible to different components or concentrations of honey and therefore respond in different ways. It is proposed that the complexity of honey makes it hard for bacteria to become resistant to honey's antibacterial effects.

The second and third parts of this work examined the effectiveness of manuka-type honeys in preventing and eradicating preformed bacterial biofilms in *S. aureus* and *P. aeruginosa*. This was performed by using a crystal violate based static biofilm formation assay in combination with Confocal Laser Scanning Microscopy (CLSM) to visualise the integrity of the biofilms after honey treatment. It was found that very low levels of NZ manuka-honey enhanced both *S. aureus* and *P. aeruginosa* biofilm formation, which could possibly due to the evoke of a stress response similar to that seen with some conventional antibiotics. When higher concentrations of honey were used, NZ manuka-honeys were able to prevent or eliminate biofilms. This appears to be influenced by MGO levels and the presence of sugar. However, MGO and sugar content alone does not account for all of the antibiofilm properties observed. Finally, an ATP-based viability assay suggested that both *S. aureus* and *P. aeruginosa* planktonic cells, which were released after honey treatment of pre-formed biofilms were significantly reduced. The development of resistance or tolerance from these recovered planktonic cells was also determined by exposing these cells to the same previously exposed honey agents. Results indicated that the recovered *S. aureus* planktonic cells did not display any resistance to honey. However, the recovered *P. aeruginosa* planktonic cells had an increased tolerance to the same honey treatment. Altogether, these results show that at an appropriate level of manuka-type honey as a whole agent, can be used to kill *P. aeruginosa* and *S. aureus* when present in the biofilm, thereby supporting the use of this honey as an effective topical treatment for chronic

wound infections. Lastly, this work also provided guidelines and strategies for new formulation of wound treatment managements and products, respectively.

Chapter 1

Introduction

1.1 Chronic wounds

A superficial wound of the skin and mucosal layer is generally considered chronic if it has not started to heal within four weeks or has not completely healed within eight weeks [1]. Patients suffering from chronic wounds have a lower quality of life because these wounds which produce odour and exude [2]. Furthermore, patients suffering from a chronic wound are also susceptible to long-term pain, poor sleep, reduced mobility, social isolation and in the worst-case scenario, require limb amputation [2, 3]. In general, the life expectancy following amputation is shortened to only 2-5 years for 60% of the chronic cardiovascular disease patients [4, 5]. In addition, chronic wounds are a major global threat to human society due to economic loss. Chronic wounds affect 6.5 million people in the United States of America (USA) alone. They are often difficult to treat and are estimated to cost in excess of $25 billion (USD) annually, with significant increases expected in the future [6]. Increased longevity and health complications due to obesity and diabetes have made chronic wound infections particularly problematic [1, 4, 5].

Chronic wounds are caused by a variety of factors, but bacterial infections are a significant problem associated with these wounds not healing properly [7]. One factor, which contributes to the inability of chronic wounds to properly heal, is that bacterial pathogens are becoming increasingly resistant to antibiotics [7, 8]. Antibiotic resistance in bacteria can rapidly emerge and can be induced by cellular adaptation responses such as efflux pumps, receptor mutation or transduction [9, 10]. Some bacteria acquire these resistance mechanisms through the inheritance of ‘mobile gene cassettes’ [11]. Such rapid development of antibiotic resistance is partially caused by the intensive and inappropriate use of antibiotics [12]. The

second major factor, which makes it difficult to treat chronic wounds, is that most of these infections contain complex bacterial communities [8]. These bacterial communities are embedded in a matrix known as a biofilm, which are intrinsically recalcitrant to antibiotic treatment [13] [14, 15]. Therefore, there is an urgent need for new approaches to treat bacterial infections to allow chronic wounds to heal. The cost of new drug discovery and development is high and time consuming. It estimated to cost $500 million (USD) and takes 10-20 years from identification of a compound to become commercially available [16]. Thus, an alternative approach is needed to treat chronic wounds.

One alternative for chronic wound treatment is honey. Honey has been used for medicinal purposes on superficial wound sites throughout history [17-19]. However, since the discovery of antibiotics in the 1940s, the use of honey as a medicine has declined [17-19] [7]. Studies suggest that honey can potentially be used for combating bacterial-associated topical infections [18, 20, 21].

Honeys are derived from a wide range of floral sources in different geographic locations around the world. One honey in particular that is produced from native New Zealand *Leptospermum scoparium* bush (Figure 1.1) is commonly known as manuka honey. Manuka honeys have been noted for their ability to kill a wide range of bacteria [22, 23]. It is one of the most studied honeys and known for its active antibacterial properties.

Figure 1.1 Manuka flowers.

The renowned manuka honey is produced from *Leptospermum scoparium,* which is native to New Zealand and some parts of Australia. It has broad-spectrum antibacterial properties and studies have also shown that it has antifungal and anti-inflammatory properties.

1.2 Revisiting the ancient remedy – honey

Although used by an increasing number of health professionals, the use of honey as an effective wound healing agent is still under-utilized in healthcare [18]. Honey is often only used to treat chronic wounds as a last resort for wound treatment, usually after conventional antibiotic treatments have failed [19, 24]. This is concerning given the fact that research supporting the use of honey in treating chronic wounds has begun to grow rapidly in recent years; and that its use instead of antibiotics in some cases at least could reduce the rate of antibiotic-resistant bacteria in the environment. For example, there have been over 100 reports published on manuka honey and over half of them are on its medicinal use (Web of Science, 2014). In addition, patented creams and wound dressing containing

medical-grade manuka honey have obtained approval from authorities such as the Therapeutic Goods Administration (TGA) in Australia and the Food and Drug Administration (FDA) in USA. Honey can also be used as a wound dressing to treat burns and ulcers [20, 25]. Honey is non-toxic to humans and many clinical case studies have demonstrated the superior *in vivo* activity of honey for promoting wound healing [25-27].

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Figure 1.2 Chronic foot ulcers treated with medical-grade manuka honey.

The 88 year-old woman suffered from choric leg ulcers and it was lack of response to conventional antibiotic treatment. Treatment with medical-grade manuka honey was commenced and 5 weeks into treatment, the size of ulcer was decreased. Healing of the skin was almost completed after 10 weeks of honey treatment.

Despite all of the benefits of using honey to treat chronic wounds the literature lacks double-blinded clinical trials [20, 21, 28, 29]. It is believed there is a lack of

funding to perform such double-blinded clinical trials. This is because the profit margin is much less than a new wound treatment (e.g. a drug) that would be expected to earn more of a profit. Another problem with the use of honeys in treating chronic wounds is that there is insufficient information about the chemical composition of honey samples, and of how it works (section 1.3). However, this is beginning to change with the identification of several antibacterial compounds in honey [30-32] [33, 34]. Below is a discussion of the known antibacterial properties of honey including its antibiofilm, antifungal and anti-inflammatory properties.

1.3 The known antibacterial components of honey

Not all honeys have the same antibacterial properties as it varies according to floral source, geographic location and storage conditions [30, 32, 34, 35]. However, there are three basic properties of honey, which contribute to its antibacterial activity. These include low pH, low water activity and hydrogen peroxide activity upon dilution. These are summarized in Table 1.1.

Table 1.1 The antibacterial compositions of different honeys.

Concentrations of the antibacterial components	Types of honey	Literature
High sugar; low water activity	Uniform in all honeys	[36, 37] [38]
Low pH	Uniform in all honeys	[39] [19]
Hydrogen peroxide	Varies amongst different honeys	[35]; [40, 41]
MGO	*Leptospermum spp.*; some New Zealand manuka, manuka/kanuka honeys	[30-32, 42-44]
Bee defensin-1	Some honeys, e.g. Revamil	[33, 45]
Phenolic compounds	Most of the honeys	[39, 41] [35]

Honey is a complex solution, composed of more than 150 components and is mainly a saturated solution of different types of sugars, consisting of approximately 38% fructose, 30% glucose, and 1.3% sucrose [40]. It also contains a small proportion of other components, such as acids, minerals and proteins, and only about 17% water. Such high osmolarity leads to low water activity (a_w), with an average of 0.6 [36] of undiluted honey. Many bacterial species have optimum growth at a water activity of 0.94-0.99 [46, 47], and are therefore not able to grow in undiluted honey. Typically, a honey would require to be diluted down to about 2-12% for most microorganisms to survive [37]. Thus, it is likely that at least one of the antibacterial properties of honeys is due to its high sugar content resulting in the low water activity [37, 38]. However, the high osmolarity and low water activity may not be considered as a stable antibacterial component [45]. Once a honey is diluted, the water activity would increase and the osmolarity is therefore no longer an inhibitory factor [45]. Moreover, in the wound environment the sugar component of honey would also be readily diluted by wound exudate [48].

The second common antibacterial property of honey is acidity. The pH of honey generally ranges between 3.2 – 4.5 [19, 24, 39]. The acidity of honeys is primarily due to the ripening nectar, and when gluconic acid is produced as the result of an enzymatic reaction of glucose with glucose oxidase (this reaction will be discussed in detail in the following paragraph). The acidity is believed to inhibit the growth of some common microorganisms [39]. For example, *Escherichia coli, Staphylococcus aureus, Salmonella spp, Pseudomonas aeruginosa,* and *Streptococcus pyogenes* cannot grow below a pH of 4.0 – 5.05 [19, 39]. However, it is unlikely that pH would be the major component responsible for the antibacterial activity of honey. This was evidenced, as the low pH was not revealed after other major antibacterial components (Table 1.1) of honeys were neutralized [45].

The third and most commonly known antibacterial component of a variety of honeys is hydrogen peroxide. Hydrogen peroxide kills bacteria by oxidizing the cellular components [49]. It is produced enzymatically from glucose upon dilution,

in the presence of the glucose oxidase under aerobic conditions [40]. The hydrogen peroxide is produced by the following reaction:

$$\text{Glucose} + \text{Water} \xrightarrow[\text{(Lowers pH)}]{\text{Glucose oxidase}} \text{Gluconic acid} + \text{Hydrogen peroxide}$$

The catalytic enzyme glucose oxidase is believed to be deposited into the honey by the bees, as it is similar to the enzymes found in the hypo-pharyngeal glands of honey bees [50] [40]. This enzyme is inactive when honey is undiluted, suggesting there is a negligible amount of hydrogen peroxide in undiluted honey. This was demonstrated by Schepartz and Subers in 1964, in a study that revealed glucose oxidase activity peaks at pH 6.1, but decreases below pH 5.5 and is zero at pH 4.0 [51]. However, the enzymatic activity of glucose oxidase varies considerably as discussed below.

Two different measurements can be performed to describe the hydrogen peroxide content in honey. The amount of hydrogen peroxide or the rate of production can be used. The rate of hydrogen peroxide production in various honey samples differs among different floral sources and ultimately depends on the dilution of the honey sample [35, 52]. For example, the amount of hydrogen peroxide in New Zealand manuka honey is very low compared to New Zealand kanuka honey [35]. The production of hydrogen peroxide is at its highest level when it is diluted between 30-50%, but its activity is reduced dramatically at concentrations below 30% [52]. Furthermore, the rate of hydrogen peroxide production upon honey dilution varies on the storage conditions [40, 41]. Prolonged storage and exposure to heat and UV light can reduce the hydrogen peroxide activity of honey [52, 53]. In addition, the hydrogen peroxide activity of honey can also be affected by the heat and filtration process, which occurs during the production of commercially made honey products [53]. Lastly, hydrogen peroxide is also neutralized by the addition of the enzyme catalase, which can be produced by some bacteria. Generally, the majority of Gram-negative organisms produce catalase as a protective mechanism

to prevent oxidative damage by reactive oxygen species [54]. However, the amount of catalase produced by these bacteria is thought to be not enough to neutralize the hydrogen peroxide in the honey [26].

It is clear that the hydrogen peroxide activity of honey is susceptible to a number of factors such as heat and UV light [52, 53]. Accordingly, it is unclear whether the hydrogen peroxide activity in honey is the sole responsible component for killing bacteria. A study by Brudzynski and colleagues indicated that inhibition of bacterial growth was caused by honey hydrogen peroxide, however, this effect could not occur without other honey components [55]. One aspect of the work described in this thesis was to investigate the effect of the hydrogen peroxide in manuka honey on growth inhibition and cellular morphology bacteria as presented in Chapter 2.

1.3.1 The 'non-peroxide' activity of honey

Although hydrogen peroxide was thought to be the major antibacterial component of most of the honeys, some honey samples tested contain antibacterial activity even after the neutralization of hydrogen peroxide by catalase [22, 56]. Such 'non-peroxide' components were later on identified, characterized and found to be present in only certain honey samples.

Methylglyoxal (MGO) (Figure 1.3) is the aldehyde form of pyruvic acid and was identified in high concentrations in New Zealand manuka honey and demonstrated to be required for the potent non-peroxide antibacterial activity [30-32] of manuka honey. MGO in manuka honey arises from the non-enzymatic conversion of dihydroxyacetone (DHA), which is present in high concentrations in the nectar of the flowers of the manuka *L. scoparium* bush [30-32]. MGO itself is cytotoxic to both prokaryotes and eukaryotes [57, 58]. Its primary target is protein synthesis, whilst further studies identified that MGO also kills cells by irreversibly damaging cellular macromolecules such as DNA and proteins [59]. However, bacteria also produce MGO as a by-product of glycolysis and are capable of neutralizing MGO

activity [60]. MGO can be detoxified by the glyoxylase system which is composed of two enzymes, glyoxylase I and II, and is widely distributed among bacteria and eukaryotes [61].

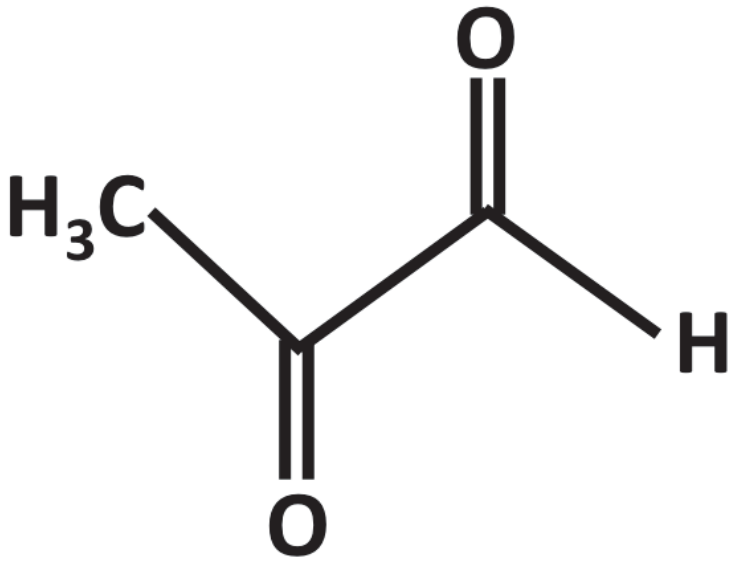


Figure 1.3 Chemical structure of methylglyoxal (MGO).

MGO is present in high concentrations in New Zealand manuka honey and is demonstrated to be the major non-peroxide antibacterial component [30, 32].

Although it is well documented that manuka honey possess MGO, not all honeys have MGO. The manuka bush is found primarily in New Zealand but also in areas of Australia. Mavric and colleagues first identified the MGO component in manuka honeys using High-Performance Liquid Chromatography (HPLC) in 2008, and showed they contained between 300-700 mg MGO/kg honey. In comparison, several other types of honeys contain, on average, 1-2 mg/kg or negligible amounts of MGO [32]. The first study to demonstrate the ability of MGO to kill bacteria was performed by Adams and colleagues. MGO was assayed for inhibition of *S. aureus* cell growth, and results suggested that MGO was the major component responsible for the non-peroxide activity of manuka honeys [30]. Later studies with other bacteria, e.g. *P. aeruginosa, E. coli,* and *S. pyogenes*, also indicated that

MGO was the major antibacterial component of manuka honey, which can effectively inhibit cell growth [42, 62]. Controversially, Molan published a study, which reported that the antibacterial activity of MGO is lower in honey compared to MGO only in a water solution [63]. This was determined by examining the antibacterial activity of manuka honey against an equivalent concentration of MGO in solution. These results suggest that other elements within honey can change MGO activity [63]. Since the antibacterial property of MGO was not revealed until 2008, the amount of MGO in different honey samples tested prior to 2008 was largely unknown. This has became a major problem as it is unclear whether MGO is always present, or if present, at what concentration in different manuka honey samples, have been used to test the antibacterial property of honey. Problem of undefined MGO content in honeys can be addressed by chemical analysis. Being able to chemically define the MGO content in honey samples is important. This is because accurate quantification of any antibacterial components allows us to fully understand how honeys kill or inhibit the growth of bacteria.

Other antibacterial compounds have also been identified in honey from different geographic regions. The British honey Revamil has been shown to display non-peroxide activity due to the presence of the antibacterial peptide bee defensin-1 [34, 64]. The same level of defensin-1 alone was capable of inhibiting growth of Gram-positive *Bacillus subtilis* and *S. aureus* bacterial cell growth, but displayed little effect on Gram-negative cell growth [33, 45]. Originally it was thought that bee defensin-1 was only found in Revamil honey and not in any other honey samples tested. However, a more recent study has shown that bee defensin-1 does indeed exist in the one manuka honey sample tested [41]. Bee defensin-1 in manuka honey appears to be modified and the antibacterial activity is masked by the presence of MGO [65]. Additionally, a second bee defensin peptide, bee defensin-2, was also identified in honeybees and it may also contribute to honeys antibacterial activity [66].

Honeys also contain other components, such as phenolic compounds, flavonoid constituents and peptides, which together may contribute to honeys unique

antibacterial activities [35, 65]. Several studies indicated that phenolic compounds originating from the plant nectar might contribute significantly to the non-peroxide activity of the honeys as some of these phenolic compounds are shown to be antibacterial [39, 41]. For example, honeys like Portobello, lavender and several Cuban honeys were all found to exhibit antibacterial properties, and believed to contain different phenolic compounds [39, 41, 67]. The phenolic compound and flavonoid content in these honeys seem to correspond to the observed antibacterial activity, where the minimum inhibitory test was performed [67]. However, the role of these phenolic compounds in contributing to the overall antibacterial activity of honeys still remains unclear. This is because other antibacterial components like MGO or bee defensin, may also presence and contribute to the antibacterial properties of these honeys [68]. Further analysis is therefore required to conclude the potential presences of antibacterial components in the above tested honeys.

Lastly, a recent study that employed Nuclear Magnetic Resonance (NMR) has identified an additional component in manuka honey, leptosin that potentially exhibit antibacterial activity [69]. The antibacterial activity of leptosin was predicted as the concentration of leptosin correlates to the UMF of the tested manuka honey. Thus, leptosin was predicted to exhibit antibacterial activity. However, *in vitro* testing is essential to conclude the antibacterial activity of leptosin. The discovery of new components in honey, which are responsible for its antibacterial activity, is in constant progress. In general, honey is a complex solution that contains multiple antibacterial components (Table 1.1). The multifactorial nature of honeys makes it not only effective against a wide range of microorganisms but also difficult to induce resistance against *in vitro* [70].

1.3.2 Assays to characterize the antibacterial activity of honey

Honey is a broad-spectrum antibacterial agent, effective against a wide range of bacteria. The antibacterial activity of honey is usually characterized by performing an agar well diffusion assay against *S. aureus*, it is also known the phenol

equivalence test. These assays have demonstrated that honey is effective against over 70 strains of *S. aureus* including methicillin-resistant (MRSA) and sensitive (MSSA) clinical strains, wound isolates and standard laboratory reference strains [71-73]. This agar diffusion assay was first introduced in 1991 [22]. It works by preparing a nutrient agar plate, which has been inoculated with a microbial culture previously, and subsequently, applying a honey solution to a small region of the agar to allow the honey to diffuse out into the agar. A growth inhibition zone, where bacterial growth is inhibited is then determined at the point where the honey concentration is sufficient to inhibit bacterial growth [22].

The agar diffusion assay is the most commonly used method to characterize antibacterial activity of honey [33, 39, 72]. However, there are several limitations to the agar diffusion assay. It has become apparent in various studies that different bacterial organisms respond differently to honey treatments [19, 34, 70]. Thus, *S. aureus* is no longer solely considered to be the model bacterium for studying the antibacterial activity of any particular honey. Another limitation of the agar diffusion assay is that some antibacterial components may not properly diffuse through the agar since a larger molecule would diffuse more slowly through the agar than a smaller molecule [45]. Therefore, a direct comparison of the antibacterial activity amongst honeys is difficult due to the complex composition of the honeys.

More recently, a modified broth micro-dilution assay is being utilized to determine the minimum inhibitory concentrations (MICs) of honeys [23]. The broth micro-dilution assay is similar to the antibiotics standard testing protocol, which was developed by the Clinical Laboratory Standards Institute (CLSI). Similar broth micro-dilution tests have been performed to determine the MIC values of honeys against other bacterial and yeast species [70, 74, 75]. However, it should be noted that one of the limitations associated with performing research with natural products is a lack of uniformity, in terms of data, and the materials and methods other studies use to perform their research. The first challenge is that the complex nature of honey makes it difficult to determine what is responsible for the

antibacterial activity of honey. Secondly, studies that utilize the agar diffusion method would be difficult to compare to other studies, which use the micro-dilution assay.

1.3.3 The bacterial response to honey treatment

Proteomics and transcriptomics, coupled with microscopic imaging has previously been carried out to determine how manuka honeys kills or inhibits the growth of bacterial cells [70, 76-79]. Different studies have examined the antibacterial activity at either MIC or sub-MIC concentrations of manuka honeys. At sub-MIC levels of manuka honey, *E. coli* respond with up-regulated gene expression in genes involved in stress responses and down-regulated gene expression in genes involved in protein synthesis. These changes were unique to the manuka honey treatment, compared to other tested non-active control honey [70]. Later on, the same team published another *S. aureus* study using proteomics rather than transcriptomics. These two studies results were consistent, where sub-MIC levels of manuka honey inducing stress responses and affected protein synthesis. Moreover, the proteomics data revealed that *S. aureus* cells treated with sub-MIC levels of manuka honey displayed a different proteomic profile compared to other antibacterial agents, suggesting a unique mode of action of manuka honey on *S. aureus* [76].

Interestingly, a separate study by Henrique and colleagues reported that cell division was interrupted in both *P. aeruginosa* and MRSA when treated with inhibitory concentrations (MIC) of manuka honey (UMF 18) [80, 81]. Subsequent follow up studies of how MIC levels of manuka honey affect *S. aureus* cells was investigated. It was shown that the universal stress protein A (UspA) was down-regulated compared to untreated samples. The up-regulation of the UspA was also reported earlier by Blair and colleagues (2009) in *E. coli* cells treated with MIC of manuka honey [70]. Together, these findings suggest one of the inhibitory effects of manuka honey on bacterial cells is the down-regulation of UspA, in both *E. coli* and *S. aureus*. In *P. aeruginosa,* atomic force microscopy, fluorescence microscopy

and qPCR were performed to understand how MIC levels manuka honey affects this Gram-negative organism [77]. Moreover, the *P. aeruginosa* cells exhibited membrane structural damage upon exposure to MIC levels of manuka honey [77]. Genomic analysis suggested these structural changes occurred due to the down-regulation of *oprF*, which is an integral membrane protein required for structural stability in Gram-negative cells [60].

Recently Jenkins and colleagues published a study on the protein levels and gene expression profiles of MRSA after exposure to MIC levels of manuka honey [79]. This study suggests that a variety of different genes and proteins were found to be either up-regulated or down-regulated after exposing to manuka honey at MIC level [79]. However, any correlations that were observed in the proteomic data when compared to the gene expression data remain to be fully understood, as these data do not seem to always agree.

A functional understanding of how bacteria respond to manuka honey treatment is currently not clear. In this thesis fluorescence microscopy techniques will be used to assess the phenotypic changes in bacterial cells after they have been treated with manuka and other honey samples (Chapter 2).

1.4 Antibiofilm properties of honeys

Bacteria do not exist freely in nature but in self-produced matrices termed biofilms [82, 83]. These biofilms are formed to protect bacterial cells from environmental stresses, such as nutrient limitation or altered pH [84]. Bacterial biofilms also exist in chronic wounds and impede wound healing. They are resistant to common treatments like conventional antibiotics or silver impregnated wound dressings [85]. In support of the idea that honey is effective in the treatment of chronic wounds, literature has emerged since 2009 that describes the effects of honeys directly on biofilms and the bacteria within. First, a brief overview of how biofilms are formed in the two organisms is reported in this thesis – *P. aeruginosa* and *S.*

aureus. Subsequently, an overview of how honey may eradicate biofilms will be discussed.

1.4.1 Bacterial biofilm development in P. aeruginosa

The biofilm structure is a complex and dynamic microbial system. The developmental cycle contains five distinct stages, which are well studied and understood in the nosocomial opportunistic pathogen *P. aeruginosa* [86]. Figure 1.4 illustrates the stages of the *P. aeruginosa* biofilm development cycle, from initial attachment, maturation, dispersal and re-colonization to a new area [87].

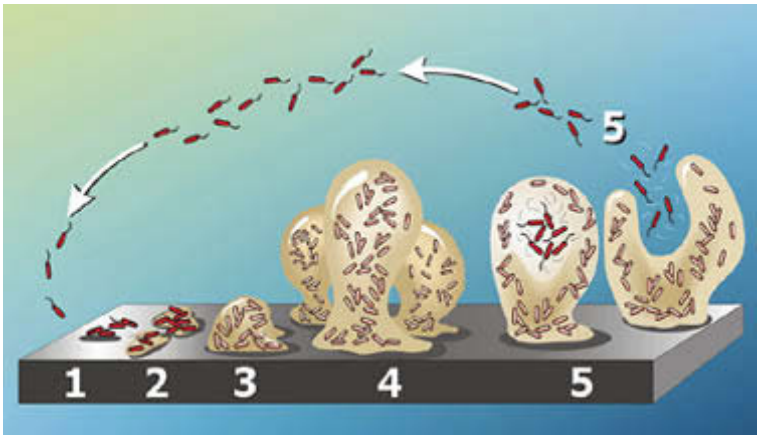


Figure 1.4 The five-stage *P. aeruginosa* biofilm development cycle.

Stage 1 is the initial, reversible attachment of cells to a surface. Stage 2 is the formation of microcolonies and extracellular polysaccharide matrix production. Stage 3 is the early development of the three-dimensional biofilm structure. Stage 4 is the biofilm maturation stage and stage 5 is the dispersal of biofilm embedded cells that disperse into planktonic mode [87]. Figure obtained from Hall-Stoodley (2004).

The first stage of biofilm development is the reversible attachment of free-living planktonic cells to a surface [83, 88]. In the second stage of biofilm development the planktonic cells irreversibly adhere to a surface to form microcolonies using type IV pili [83]. During the third stage of development, the microcolonies start to produce the extracellular polysaccharide, which is composed of different proteins,

carbohydrates, nucleic acids and extracellular DNA [89]. An early three-dimensional biofilm structure is subsequently formed. The embedded cells are able to multiply, despite the fact that they exhibit a slowed metabolic rate. Quorum sensing, which is a cell-to-cell communication system, also plays a role in the third stage of biofilm development [89, 90].

The biofilm matrix is not considered mature until it reaches the 4th stage, when the biofilm structure displays a highly differential but complex structure [7]. Water channels within this structure are also created and can transport waste products and nutrients where required [7]. Cells that are embedded in the deepest of the biofilm layers have limited or no access to oxygen, so this layer of the matrix is therefore considered to be an anaerobic environment [13, 91]. These deeply embedded cells grow more slowly than cells embedded in the upper layers of the biofilm matrix [92, 93]. Moreover, a subpopulation of cells in this deep layer of the biofilm structure does not multiply or die but remains viable and may later transform into persister cells. These persister cells can become planktonic cells after they are released from the biofilm and can re-seed in new locations [94, 95]. This is the fifth stage of the biofilm development cycle [96].

1.4.2 Bacterial biofilm development in S. aureus

The biofilm development cycle is a dynamic and complicated process as demonstrated above with *P. aeruginosa*. The other most commonly studied bacterial biofilm model, as well as being a common isolate from chronic wound sites is *S. aureus*. *S. aureus* is a non-motile bacterium and, unlike *P. aeruginosa*, does not possess any pili or flagella.

Yet, the generalized process of attachment, aggregation and dispersal are believed to be the similar for all microbes [97]. A study by Foster *et al.* in 1998 describes the initial attachment process of *S. aureus* on the surface as being mediated by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [98]. The polysaccharide intercellular adhesion (PIA) is composed of

a glucosamine polymer, which constitutes the extracellular matrix of the *S. aureus* biofilm [98]. PIA is encoded by the *ica* locus, and is present in the majority of biofilm forming *Staphylococci* strains [99]. However, *ica*-independent biofilm forming mechanisms have also been identified, suggesting that biofilm formation in Staphylococci is a multifactorial process [99]. Small colony variants of *S. aureus* have been shown to be associated with the formation of persistent cells, which leads to recurrent infections [100], similar to the last stage of *P. aeruginosa* biofilm dispersal.

1.4.3 How does honey prevent formation of biofilms and eradicate them?

Bacterial biofilm is a protected mode of life, where the embedded cells are shielded from the host immune system and other 'environmental' stresses such as nutrient limitation, altered pH, antibiotics and osmolarity [7]. The metabolic rate, gene and protein expression are generally decreased in these embedded cells compared to that of planktonic cells [7, 101]. The matrix, the physical structure of the biofilm biomass also limits access of antibiotics to the embedded cells [102]. Altogether, cells within a biofilm can be up to 1,000 times more recalcitrant to antibiotics than planktonic cells [103]. Treatment with conventional antibiotics is difficult when biofilms are involved because they often harbour a poly-microbial community [7]. Different species, genera or even kingdoms of organisms can co-exist in a single biofilm matrix and are more than likely not all sensitive to one antibiotic [7]. However, various studies have suggested that different floral types of honeys from different geographic locations are capable of eradicating bacterial biofilms. This includes *Klebsiella*, *E. coli* O157:H7, *S. pyogenes, Streptococcus mutants*, *Proteus mirabilis, Enterobacter cloacae* and the biofilms made from wound common isolates *P. aeruginosa* and *S. aureus* [43, 77, 104-108].

The mechanism of preventing biofilm formation in *P. aeruginosa* and *S. pyogenes* has been investigated and they appear to be different between these two organisms [62, 109]. *P. aeruginosa* biofilms are inhibited partly by fructose, which is able to bind the lectin PA-IIL (*Pseudomonas* lectin II, LecB) and subsequently

prevent biofilm formation [109]. A study by Wang and colleagues also showed that the sugar content in honey, is not only able to actively kill *P. aeruginosa* cells, but is also able to disrupt the quorum sensing (QS) system [110]. At sub-inhibitory concentrations of honey, it was demonstrated to affect the expression of *mvfR*, *las,* and *rhl* genes, and also the associated virulence factors among the QS network [110]. However, the two surface adhesion proteins of *S. pyrogenes*, Sfo and SfbI, which associated with the initial development of a biofilm, were found to be present at lower levels than normal by exposing to honey [62]. Moreover, the reported inhibitory concentration of honey against *S. pyogenes* biofilms was 2-fold less compared to the growth inhibitory concentrations [62]. This suggests that the sub-MIC of manuka honey prevents *S. pyogenes* biofilm formation. Therefore cells are 'forced' to remain in planktonic mode, where they are more easily accessed by treatment agents or immune defense system [82].

Honey has also been demonstrated to eradicate established and mature bacterial biofilms. Studies have found that the concentration of honey required to achieve such results is consistently higher than the MICs for planktonic bacterial cells [43, 77, 104-106, 108, 111]. However, the levels of reported antibiofilm activity are not consistent among these studies [43, 77, 104-106, 108, 111]. This is likely to be in part due to the differences in the levels of the principle antibacterial components in the honey (MGO and hydrogen peroxide), which varies with the floral and geographic source of nectar, the honey storage time and conditions, and any possible other treatments (such as excessive heating) that may have occurred [30, 32, 34, 35, 52]. All these conditions affect the antibacterial activity of honey [30, 35, 112, 113], but are often not reported. Therefore, it is imperative to use well-characterized honeys to enable both accurate comparisons among studies, and the rigorous assessment of the potential of medical-grade honey to be used in wound treatment in the clinic.

1.5 Antifungal properties of honeys

Fungi are important wound pathogens and often isolated from chronic wound sites [114]. Like antibiotic resistance, it appears that antifungal resistance is also on the rise [74]. Moreover, the real challenge is to find an antifungal agent that also is not toxic to humans as both are eukaryotes. Once again, honey is a good alternative candidate as studies have shown that honey possesses antifungal activity [74, 115, 116]. A study by Irish and colleagues, demonstrated that Australian and New Zealand honeys also have antifungal activity. They tested two of the medical-grade honeys and a natural honey - Jarrah, against the clinical isolates of *Candida albicans, Candida glabrata* and *Candida dubliniensis* and showed that all honeys were effective in inhibiting growth of these strains. MICs were determined using the modified broth microdilution methods according to the CLSI standard protocol. However, much higher MICs were detected compared to those for bacteria, with MICs ranging between 35 - 43% (w/v) [74] compared to 4%-20% for bacteria (depending on the type of honey) [72, 117]. Moreover, medical-grade manuka honey did not display improved antifungal activity over other tested honeys [74]. Similar studies have also shown that both Turkish and South African honeys have antifungal activity [115, 116]. The Turkish honey was effective against 40 different yeast strains, including both *Candida spp.* and *Trichosporon spp.*, whereas the South African honeys were shown to be effective against *C. albicans* only [115, 116]. However, none of these studies have identified components in their tested honey samples, which were responsible for the antifungal activity.

1.6 Anti-inflammatory properties of honeys

An impaired immune system also prevents a chronic wound from healing [118]. Studies have demonstrated that honey is able to stimulate the local immune response and promote wound healing [119-121]. At first, Tonks and colleagues showed that both manuka and pasture honey stimulates the release of tumour necrosis factor-alpha (TNF-α) in monocytes, which is involved in the primary response of macrophages to inflammatory stimuli [119]. Later on, they also found

that these honeys are also able to stimulate inflammatory cytokine production from monocytes, which further promotes wound healing [120]. Gannabathula and colleagues later identified the immuno-stimulatory property of New Zealand kanuka honey was due to the arabinogalactan proteins (AGPs), which are derived from the nectar of kanuka flowers [121]. Moreover, the antioxidants and free radicals in honey are believed to be responsible for the anti-inflammatory activities of honeys [122]. However, one problem is that only a handful of studies have been carried out on this area. Moreover, among the available studies, the floral species, age, and the important wound healing associated proteins profiles of honey tested were not reported, except in one study only. Again, by not knowing the details of studied honeys make direct comparison among studies difficult. In the future it would be good to characterize whether different honeys contain similar anti-inflammatory properties just like the antibacterial properties of the same honeys.

In this work, the effect of different well-characterized New Zealand manuka-type honeys (including MGO) was investigated on the growth and morphological effects across a range of different bacteria. Subsequently, these NZ honeys' ability to affect bacterial biofilm formation and the preformed biofilms were determined.

Chapter 2

The effect of New Zealand kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species

Declaration

I declare that the following publication (please refer to Appendix II for published manuscript) included in this thesis in lieu of a chapter meets the following criteria:

- The majority of the content in the following publication included in this chapter has been executed and prepared for publication by me. For detailed contribution, please refer to statement below.
- The work presented here has been peer-reviewed and accepted for publication.
- The initial draft of the work has been written by me and any subsequent changes in response to co-authors and editors reviews were performed by me.
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis.

Publication title: The Effect of New Zealand Kanuka, Manuka and Clover Honeys on Bacterial Growth Dynamics and Cellular Morphology Varies According to the Species

Authors: Jing Lu, Dee A. Carter, Lynne Turnbull, Douglas Rosendale, Duncan Hedderley, Jonathan Stephens, Swapna Gannabathula, Gregor Steinhorn, Ralf C. Schlothauer, Cynthia B. Whitchurch, Elizabeth J. Harry

Candidate's contribution: I performed all the experiments that were presented in this study; performed a majority of the data analysis, wrote the paper, prepared all the figures and tables by myself and edited numerous drafts of the paper.

Journal name: PLoS One
Volume/ page numbers: Volume 8, Issue 02, pages e55898
Status: Published 13 February 2013

I declare that the publication above meets the requirements to be included in the thesis.

Candidate's name: Jing Lu
Candidate's signature:
Date (dd/mm/yy):

2.1 Introduction

Wounds of the skin and mucosal layers can be generated by accidental trauma, surgery, maceration, inflammation and some cosmetic procedures (e.g. tattooing and piercing). For most superficial wounds, healing is prompt and requires no intervention. However, in some instances wounds can become infected, and in persons with impaired immunity or circulation, wounds can become non-healing, progressive and chronic. There is growing evidence that chronic wounds result from a complex interplay of host immunity and bacterial infection, and that infection can be due to a consortia of different species of bacteria embedded in a biofilm matrix that is highly resistant to antimicrobial therapy [123]. Planktonic bacteria are also important in chronic and acute wounds, and their release from biofilms has been proposed to maintain the inflammatory response within the wound [124, 125], as well as allowing seeding to other areas. The emergence of bacterial pathogens resistant to multiple antibiotics has exacerbated the problems associated with treating infected wounds, particularly in the hospital setting [14, 15]. There is an increasing need for new approaches to treat these infections, which are estimated to affect 6.5 million patients in America and to cost US$25 billion annually, with significant increases expected in the future [6].

Antimicrobial honey produced from the *Leptospermum scoparium* (manuka) plant from New Zealand has many features that make it a promising therapy for wound care. Manuka honey is broad in spectrum and able to inhibit a diverse range of bacterial and yeast pathogens, and is equally effective against multi-drug resistant bacteria [70, 75, 80]. This honey has been found to prevent the formation of biofilms and can disrupt pre-formed biofilms [62] [105]. Resistance to manuka honey has never been observed and could not be attained under laboratory conditions that rapidly induced resistance to conventional antibiotics [70] [80]. And finally, honey stimulates the immune system and can promote wound healing [121]. There are a number of medicinal honey products on the market in the form

of ointments, creams and impregnated gels. However their use in mainstream medicine remains limited [126].

Honey has several antibacterial features that are distinct from classical antibiotics, including high osmolarity, low pH, and the generation of hydrogen peroxide by the bee-derived enzyme glucose oxidase [22]. Some honeys also contain levels of bee defensin-1 that are sufficient to inhibit bacteria [34, 45]. Active manuka honey contains high levels of the reactive dicarbonyl methylglyoxal (MGO) [30, 32], which forms non-enzymatically from nectar-derived dihydroxyacetone (DHA) during ripening. A diverse range of phenolics, complex carbohydrates and peptides have also been reported in honey samples, and these may contribute to or modulate antibacterial activity [35] [65].

The antibacterial activity of honey is generally assessed by measuring the extent to which the indicator bacterium, *S. aureus*, is inhibited using agar diffusion or broth micro-dilution methods [23]. Similar tests have been performed to determine the inhibition of other bacterial and yeast species [70] [34, 74]. Manuka honey marketed for medicinal use generally uses a potency rating based on the "Unique Manuka Factor" (UMF), which measures antibacterial activity that is unrelated to the content of hydrogen peroxide, and is based on the *S. aureus* inhibition test. Alternatively, some medicinal honeys express potency as a direct assessment of MGO levels. While it has been established that manuka honey can inhibit the growth of bacterial cells, its effect on growth and cellular physiology among different bacterial pathogens, and how these change when the levels of the major antibacterial components, MGO and hydrogen peroxide, vary in natural honeys are unclear. These are important considerations for optimizing honey for wound care since sub-lethal levels of honey may have unanticipated effects, and there is emerging evidence that different organisms infecting a wound may respond quite differently to the active honey components [45] [34].

To address these issues this study set out to examine the growth response and cellular morphology of four different bacterial species, including three of the

clinically relevant pathogens – *E. coli*, *S. aureus* and *P. aeruginosa*, where the latter two are common wound associated organisms. The Gram-positive cell division model organisms – *B. subtilis* is also included in this study, where it has been studied extensively on cell morphology and division mechanisms. These four organisms are tested against to a suite of natural honey samples that differ in their levels of MGO and hydrogen peroxide production. This included samples of monofloral manuka honey with moderate to high MGO levels, samples of honey produced from the New Zealand kanuka *Kunzea ericoides* bush [35], where MGO levels are negligible but hydrogen peroxide is present, and manuka-kanuka blends that contain both active components at moderate levels. We included a set of controls to mimic the effects of sugar, to neutralize the effect of hydrogen peroxide, and to examine how MGO might act outside the honey milieu. We report here that while clinically relevant concentrations of honey are effective at inhibiting growth of all four bacteria, the growth and morphological responses at sub- lethal levels varied significantly between species. Furthermore, *P. aeruginosa* responded strikingly differently to the other three species (*B. subtilis*, *E. coli* and *S. aureus*). When present in sub-lethal concentrations, MGO extended the lag phase of bacterial growth in a dose-dependent manner, and the organisms eventually resumed normal growth, presumably by detoxifying the MGO. Topical wound dressings should therefore contain a high level of active honey to ensure wound pathogens are eliminated.

2.2 Materials and Methods

2.2.1 Honey samples

Table 2.1 lists the New Zealand honey samples used in this study, which included monofloral manuka (M1, M2, M3), monofloral kanuka (K1 and K2), manuka-kanuka blends (MK1, MK2, MK3, MK4) and clover (C) honey. Samples were chosen based on their levels of methylglyoxal (MGO; previously reported in Stephens *et al.* 2010), and hydrogen peroxide, determined in this study. Manuka, kanuka and

manuka-kanuka honey samples were supplied by Comvita New Zealand Ltd. (Te Puke, New Zealand) and the clover honey sample was a commercially-packaged New Zealand white clover honey [35]. Native New Zealand honey is produced by bees foraging in their local environment and cannot be guaranteed to be 100% monofloral, however the supplied samples were considered to be as representative of pure honey from a single floral origin as possible. Details of other chemical components in the manuka and kanuka honeys have been described previously [35]. All samples were stored in the dark at 4 ^{o}C and were diluted fresh for use in any assays. All honey concentrations are expressed as % w/v.

Table 2.1 Floral source, MGO and H_2O_2 levels of honeys

Code	Previous code[a]	Honey	Floral source	Antibacterials	
				MGO [b] (mg/kg)	H_2O_2 [c] (µM/h)
M1	2	Manuka [d]	*Leptospermum scoparium var incanum*	651.4	0.532
M2	13	Manuka [e]	*L. scoparium var incanum + Kunzea (?)*	1004.3	0.282
M3	7	Manuka [e]	*L. scoparium var incanum*	1541.3	0.239
K1	22	Kanuka [e]	*Kunzea ericoides*	5.6	0.36
K2	21	Kanuka [e]	*K. ericoides*	37.1	0.327
MK1	23	Manuka-Kanuka [e]	*K. ericoides + manuka (?)*	173.6	0.583
MK2	-	Manuka-Kanuka [e]	*K. ericoides + manuka (v. likely)*	229.8	0.448
MK3	18	Manuka-Kanuka [e]	*L. scoparium var 'triketone' + Kunzea*	269.9	0.345
MK4	15	Manuka-Kanuka [d]	*L. scoparium var 'triketone'*	307.8	0.38
C	24	Clover [f]	*Trifollium spp.*	trace	0.029

[a] As per 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.

2.2.2 Hydrogen peroxide assay

The level of hydrogen peroxide produced by the honey samples was determined using a hydrogen peroxide/peroxidase assay kit (Amplex Red, Molecular Probes, Invitrogen, Oregon, USA). The assay, which measures the oxidation by hydrogen peroxide of the non-fluorescent substrate Amplex Red to highly fluorescent resorufin [127], was conducted in 96-well microtitre plates according to the manufacturer's instructions. Resorufin fluorescence was measured at 530 nm excitation/590 nm emissions using a SpectraMax Gemini EM (Molecular Devices) fluorometer. Hydrogen peroxide standards from 5-20 μM were used to produce a standard curve, which was then used to assess production in duplicate samples of 2.5% and 5% w/v dilutions of the honey samples. The results were normalized to μM H_2O_2/h in 1mL of 10% w/v honey solution.

2.2.3 Bacterial strains and growth media

Four different bacterial species were examined: the Gram-positive bacteria *B. subtilis* 168 [128] and *S. aureus* ATCC 25923 (American Type Culture Collection), and the Gram-negative bacteria *E. coli* O157:H7 [129] and *P. aeruginosa* PAO1 (ATCC 15692). *B. subtilis* is a well-studied model organism for bacterial cell division and it is often used as a Gram-positive representative organisms, typically in drug discovery studies in the industry, where the other three species are clinically relevant pathogens. Growth media were selected to allow optimal growth of the different bacterial species: Luria-Bertani (LB) (Oxoid Ltd., Basingstoke, Hampshire, UK) broth and agar were used for *E. coli, P. aeruginosa* and *B. subtilis*, while Tryptone Soya Broth and agar (Oxoid Ltd., Basingstoke, Hampshire, UK) was used for *S. aureus*.

2.2.4 Growth of bacterial cultures

Planktonic bacteria in wounds, while viable, are likely to be growing very slowly, if at all. We therefore added honey to diluted stationary-phase bacterial cultures so that it would more accurately represent the addition of a honey dressing to a chronic wound. Single colonies of bacteria grown on agar were used to inoculate broth cultures. These were grown overnight at 37°C on an orbital shaker at 250 rpm (Bioline™, Australia) except *B. subtilis,* which was grown overnight at 30°C with slower shaking using a gyrotory water bath shaker (New Brunswick Scientific, N.J., USA). The slower shaking conditions for *B. subtilis* ensure that this culture does not spend too long in stationary phase, which would delay entry into exponential growth upon dilution. Cell density of the overnight cultures was assessed using serial-dilution plating and was approximately 10^9 colony-forming units (CFU)/mL. A suspension from the overnight culture was then diluted to a cell density of 10^3 CFU/mL in fresh media containing honey to give a final volume of 150 µL. For each growth assay, a freshly prepared 50% (w/v) honey stock solution was made by weighing the appropriate amount of honey and mixing this with an equivalent amount of sterilized distilled water. This stock solution was then further diluted with the appropriate growth medium to give the required honey concentration. Growth of each bacterial species was tested in six concentrations of each honey (1%, 2%, 4%, 8%, 16% and 32% w/v) in a 96-well microtitre plate format. A microtitre plate reader (Biotek PowerWave HT®) programmed to measure the optical density hourly at 595nm (OD_{595nm}) (Gen5®, BioTek) was used to assay cell growth over 24 hours at 37°C, with moderate shaking (1800 rpm, amp. 0.549 mm x-axis). Two biological replicates, each with four technical replicates were performed for the growth assays and each growth curve produced in Figure 2.2 represents the average of all data. Growth curves were presented using GraphPad Prism 5.

A comprehensive range of control treatments was included for each organism in the microtitre-plate growth assays. These included: (i) a no-treatment control; (ii) a sugar solution comprising 45% glucose, 48% fructose and 1% sucrose, (diluted

as above for honey) to identify any effects on bacterial growth due to the high sugar content in honey; (iii) honey plus catalase (1 mg/mL) to neutralize hydrogen peroxide [22]; (iv) a catalase-only control; (v) MGO diluted in water to concentrations similar to those present in honeys M1-3 (600, 1,000 & 1,500 mg/kg undiluted honey) at the various tested concentrations, to assess the effect of MGO alone on bacterial growth; vi) the same MGO dilutions plus catalase; and (vii) MGO diluted in sugar solution to the same concentrations as above and with added catalase. MGO was obtained as a ~40% solution in water (Sigma, St Louis, MO, USA).

2.2.5 Growth curve data analysis

Initial inspection of the bacterial growth data indicated that the consistent major effect of honey on growth dynamics was an extended lag phase, such that entry into exponential growth was delayed, and this increased with increasing honey concentrations. Thus, we focused on how honey altered the duration of lag phase. Lag-phase was calculated as the period from inoculation to onset of log phase, or to 10% of maximal culture absorbance. Given the large number of different growth curves (128 individual growth assays with 6 different honey concentrations per assay), we automated the calculation of these parameters by fitting the absorbance values from the bacterial growth experiments to a generalized logistic curve (equation 1), a sigmoid function used for growth modeling, using the Genstat program (Release 11.1 (PC/Windows) 28 January 2011, VSN International Ltd, UK). Due to variable T values, this generalized logistic curve fitted better than a corresponding Gompertz curve (not shown).

$$Y = A + \frac{C}{\left(1 + T e^{-B(x-M)}\right)^{1/T}} \qquad (1)$$

Here, A = the lower asymptote; C = the upper asymptote; M = time of maximum growth; B = growth rate, and T = time near which maximum (stationary phase) growth occurs.

With these parameters, we were able to compare the effect of the different honey samples on growth simply by plotting the duration of lag phase (time (h)) in the presence of varying honey concentrations (% w/v). This conversion from growth curve to lag phase duration is illustrated in Figure 2.1, where a sample curve of *E. coli* growth in response to manuka honey M3 dilution series (Figure 2.1A, onset of log phase at each honey concentration shown by "x") is converted to the corresponding lag-phase dose response to honey (Figure 2.1B).

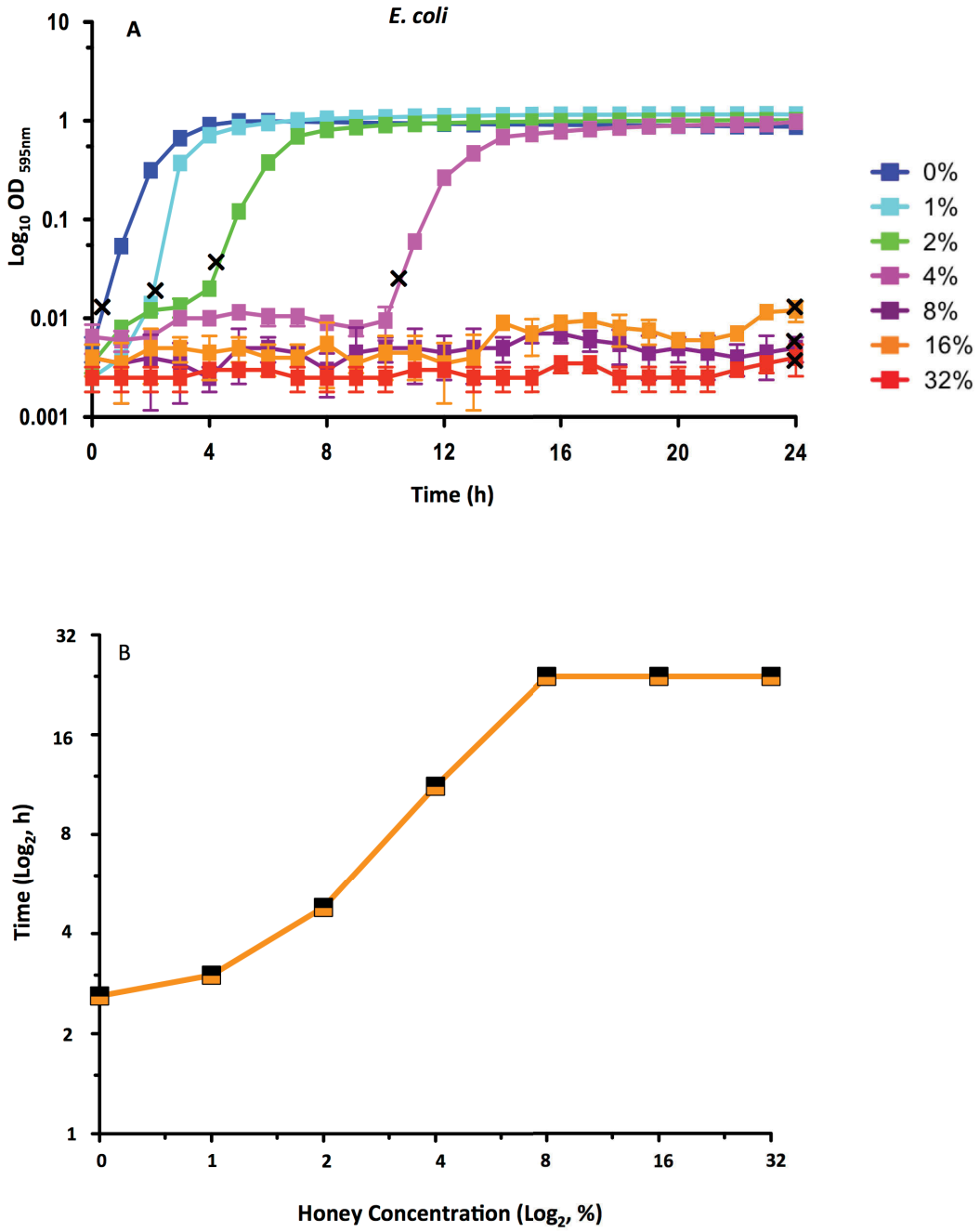


Figure 2.1 Transformation of data obtained for bacterial growth with honey treatment

Panel A illustrates the effect of various (1-32% (w/v)) concentrations of honey M3 on *E. coli* growth over 24 h as a simple log OD_{595nm} *versus* incubation time. The point at which 10% of the total OD_{595nm} is reached is shown by an 'x' on each growth curve. Panel B summarizes all the data from panel A as a simple relationship between honey concentration and the time it takes to reach 10% of the total OD_{595nm}. A value of 24 hours on the y-axis denotes 'no growth'.

In the vast majority of cases when growth of a culture was detected by absorbance measurement, the maximal culture absorbance was very similar to the no-honey

control culture. However in a few cases the maximal absorbance of the treated culture was less than 10% of the maximal culture absorbance of the no-honey control. In these cases, it was assessed as 'no growth' over the 24-hour period.

2.2.6 Cell staining and microscopy

Bacterial cultures treated with either 4% (w/v) honey M3 or honey MK1 (Table 2.1) were harvested from samples obtained from the middle of the prolonged lag phase induced by honey treatments, and at mid-log phase when cultures had resumed apparently normal growth. If a prolonged lag phase was not observed, lag phase cells were obtained from within the first half hour of incubation. Untreated cells from lag and mid-log phases of growth cells were also harvested for analysis, with the lag-phase cells collected 30 min after inoculation as described above. Harvested cells were treated for microscopy as described previously [130] but with the following modifications: 20 µL of fixed cells were diluted 1:1 with the DNA staining agent DAPI (4',6-diamidino-2-phenylindole; Molecular Probes®, Life Technologies), to give a final DAPI concentration of 0.4 µg/mL for *E. coli*, *B. subtilis* and *P. aeruginosa*, and 0.8 µg/mL for *S. aureus*. Triplicate 10 µL aliquots of the stained cells were then placed in separate wells of a multi-well microscope slide (MP, Biomedicals, LLC, Eschewege, Germany) that had been treated with 0.01% poly-L-lysine (Electron Microscopy Sciences, Hatfield, PA, USA). After 15 min at room temperature, the liquid was removed and 50% glycerol was placed on each sample. A coverslip was 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 microscope equipped with a Plan ApoChromat (100x, NA 1.4; Zeiss) objective lens, and images were captured using a Zeiss AxioCam MRm cooled CCD camera controlled by AxioVision software (version 4.5; Carl Zeiss). Fluorescence microscopy to visualize DNA stained with DAPI used a 100 W high-pressure mercury lamp passed through filter set 02 (Zeiss) as a light source. Image processing was performed using AxioVision version 4.5 (Carl Zeiss).

2.2.7 Image data analysis

Cell length, cell lysis and DAPI staining were assessed by digital analysis of the captured images. Cell length and DAPI staining were scored only for unlysed cells. A total of 152 fields of cells were imaged and analyzed. Cell length (or diamater in the case of *S. aureus*) was measured using MicrobeTracker (version 0.929) [131]. We used this MATLAB-based software to detect and outline bacterial cells in the microscopy images and measure cell lengths automatically. The optimized parameters (incorporated into the MicrobeTracker software) included a modification to algorithm 4 to enable accurate cell length measurements of rod-shaped organisms in the case of *E. coli*, *B. subtilis* and *P. aeruginosa.* For *S. aureus* algorithm 1 was optimized to enable measurement of the size of these spherical cells [131]. The individual cell length information was then extracted and statistical analysis was performed in GraphPad Prism. One-way ANOVA and Tukey's multiple comparison tests were performed with the non-honey treated cells as controls. Cells that appeared lysed due to changes in their contrast under phase-contrast microscopy were scored and cell lysis was expressed as a percentage of the whole population. Only cells that remained intact but appeared to lose their cytoplasmic contents were scored, thus underestimation of cell lysis was possible, but this was consistent across all samples. For all experiments, triplicate results were obtained and at least 100 cells were scored, except for M3-treated *B. subtilis* and *S. aureus* cells, where at least 50 cells were scored.

2.3 Results

2.3.1 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 Gram-negative bacteria, *E. coli* O157:H7 and *P. aeruginosa* PAO1 (ATCC 15692) to the 10 honeys and various control solutions were assessed. These data comprised nearly 900 growth curves in 128 graphs (Appendix I). For

the honey treatments each graph represents a particular honey at six concentrations with a single organism as well as a no-honey control, which was carried out alongside each honey sample on the multi-well plates. Comprehensive ranges of control treatments were included in the growth assays to determine the effect of various honey components on growth of the four bacteria. These data are summarized and presented in Figure 2.2 and 2.3.

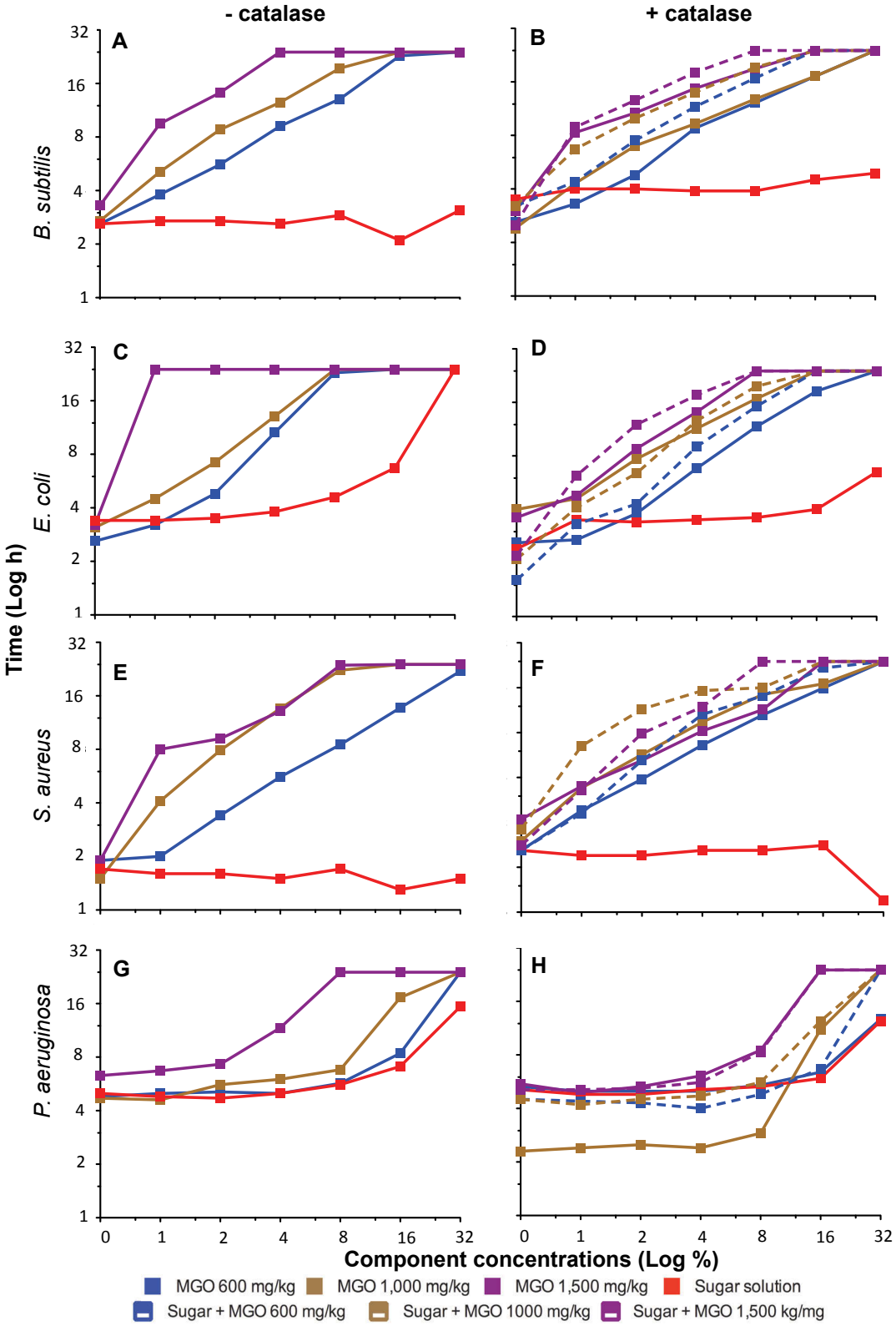

- catalase
+ catalase
A
B
C
D
E
F
G
H
B. subtilis
E. coli
S. aureus
P. aeruginosa
Time (Log h)
32
16
8
4
2
1
0
1
2
4
8
16
32
Component concentrations (Log %)
MGO 600 mg/kg
MGO 1,000 mg/kg
MGO 1,500 mg/kg
Sugar solution
Sugar + MGO 600 mg/kg
Sugar + MGO 1000 mg/kg
Sugar + MGO 1,500 kg/mg

Figure 2.2 Effects 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 components, including catalase, MGO, sugar, and a combination of MGO and sugar at various concentrations equivalent to honeys at the corresponding concentrations shown on the x-axis. The MGO/sugar experiments were performed in the absence (left-hand graphs) and presence (right-hand graphs) of catalase as indicated. The MGO levels correspond to honeys M1 (651.4 mg/kg MGO), M2 (1004.3 mg/kg MGO) and M3 (1541.3 mg/kg MGO) at 1% - 32% (w/v). Optical density was recorded at 595 nm every hour for 24 hours. For each component concentration, the time it takes for the culture to reach log phase (assessed as at least 10% of the final culture absorbance of the untreated culture) is plotted on the x-axis. The derivation of this value is described in Materials and Methods. A value of 24 hours on the y-axis denotes 'no growth'. An untreated control was performed alongside each particular treatment, and the starting OD_{595} (zero time-point on x-axis) is plotted for that particular honey experiment.

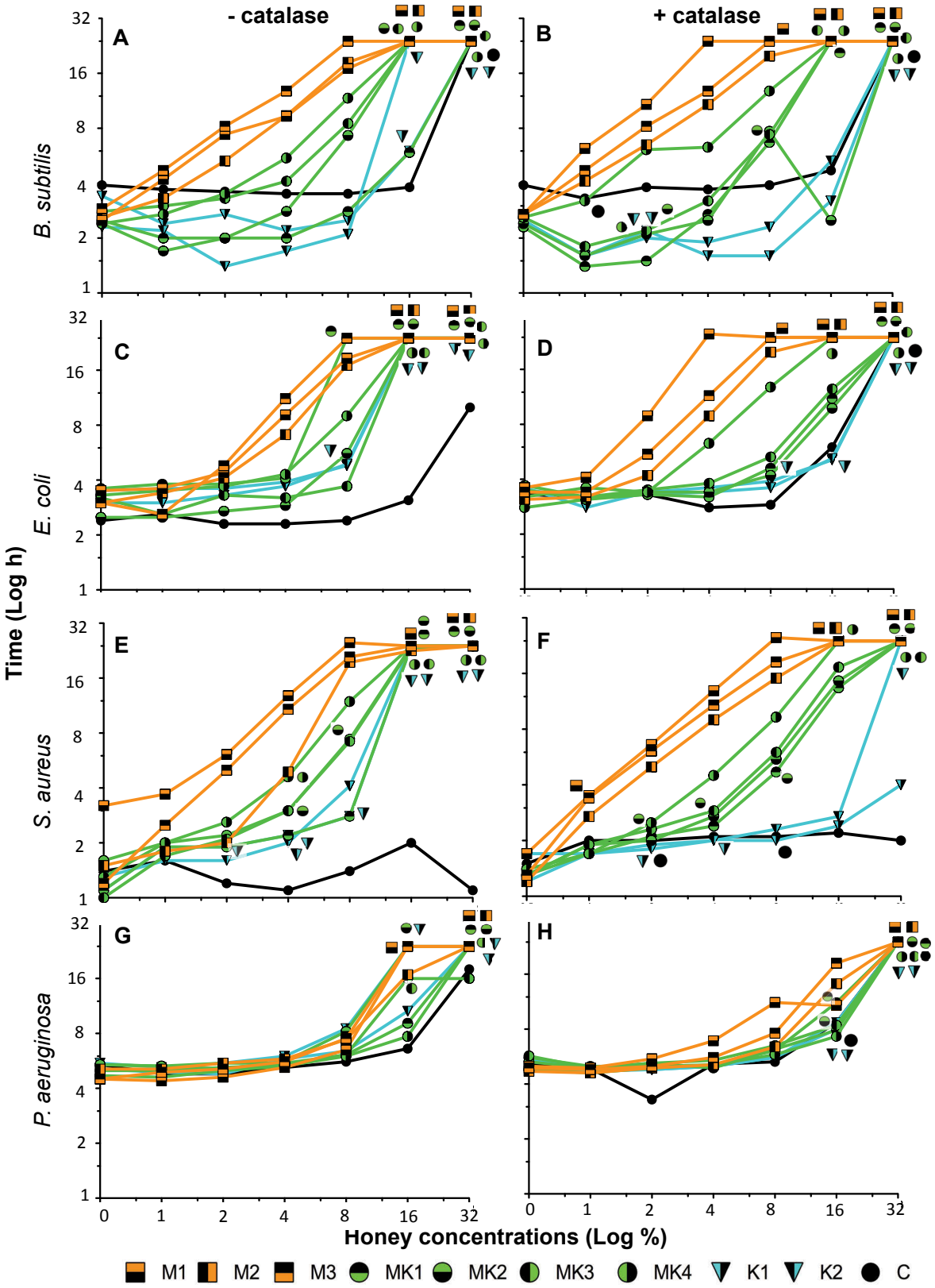

- catalase
+ catalase
A
B
C
D
E
F
G
H
B. subtilis
E. coli
S. aureus
P. aeruginosa
Time (Log h)
32
16
8
4
2
1
0
1
2
4
8
16
32
Honey concentrations (Log %)
M1
M2
M3
MK1
MK2
MK3
MK4
K1
K2
C

Figure 2.3 Effect of New Zealand, kanuka and manuka-kanuka blended honeys on bacterial growth.

Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with ten different honeys, plus or minus catalase: three manuka honeys, M1, M2 and M3; two kanuka honeys, K1 and K2; four manuka-kanuka blended honeys, MK1, MK2, MK3 and MK4; and one clover honey, C, at various concentrations (from 1% - 32% (w/v), increasing in 2-fold series). Optical density was recorded at 595 nm every hour for 24 hours. For each honey concentration, the time it takes for the culture to reach log phase (assessed as at least 10% of the final culture absorbance of the untreated culture) is plotted on the x-axis. The derivation of this value is described in Materials and Methods. A value of 24 hours on the y-axis denotes 'no growth'. Where symbols for a particular honey overlap, we have surrounded the point on the graph by all the symbols relevant to that point. An untreated control was also performed alongside each particular honey treatment, and the starting OD_{595} (zero time-point on x-axis) is plotted for that particular honey experiment.

Time spent in lag phase before entry into exponential growth emerged as the most notable difference among bacteria in their response to the different honey types. We therefore focused our analysis on growth inhibition on this parameter, expressed as the time taken (in hours) for the bacterial culture to reach 10% maximal culture absorbance. The graphs presented in Figure 2.2 and Figure 2.3 summarize the growth responses of the four different organisms to the control solutions (Figure 2.2) and the honeys (Figure 2.3). In these graphs, the time (h) taken for a culture to enter logarithmic growth (measured as at least 10% maximal culture absorbance; y-axis) is plotted against the honey (or component) concentration (x-axis) for each organism both in the absence (left panel) and presence (right panel) of catalase. Note that the faster the rise of the line, the longer the cells are arrested in lag phase at lower honey concentrations, and hence the more effective a particular honey is at inhibiting the growth of that organism. Culture growth was monitored over 24 hours, and if no growth occurred over 24 hours, it is referred to as 'no growth' or complete inhibition.

The starting absorbance values differ in each case in Figure 2.2 and Figure 2.3 because for each honey, the no-honey control was included to more accurately reflect the experimental conditions during that particular experiment.

2.3.1 Growth dynamics in response to controls: MGO and sugar in the presence and absence of catalase

The addition of catalase to an overnight culture of bacterial cells had essentially no effect on the duration of the lag phase, or any other aspect of growth of the four organisms when compared with an untreated control culture (Figure 2.2). Sugar alone had a small but variable effect on the growth of the bacteria: the Gram-positive species *B. subtilis* and *S. aureus* were unaffected even at high concentrations, while the two Gram-negatives became inhibited at 16–32% (Figure 2.2A, C, E and G).

The addition of MGO at 0–32% of the concentrations present in manuka honey samples M1, M2 and M3 (starting concentrations of 600, 1,000 and 1,500 mg/kg honey) generally showed a dose-dependent extension of lag phase (Figure 2.2A, C, E and G). This was most severe for *E. coli*, followed by *B. subtilis* and *S. aureus*, and was lowest in *P. aeruginosa*. The difference in sensitivity to MGO alone between the organisms was most obvious at the lowest MGO concentration used (blue lines in Figure 2.2A, C, E and G).

The addition of catalase to the MGO treatments shortened the lag-phase extension in most cases, so that the onset of log phase occurred earlier. With the exception of *P. aeruginosa,* the bacteria were still increasingly sensitive to increasing MGO concentrations in the presence of catalase (Figure 2.2, compare left with right panels). The addition of sugar to MGO in the presence of catalase had a small but noticeable effect on delaying the onset of log phase further for all organisms except *P. aeruginosa*, particularly at the lower sugar concentrations tested (equivalent to ≤ 8% honey; Figure 2.2).

2.3.2 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 shown in Figure 2.3. From these, four key features are

particularly apparent: first, there is a general trend of greater growth inhibition by honeys containing more MGO, with M > MK > K > clover honey; second, the addition of catalase causes a shift of the curves to the right for most honey types indicating a rescue of growth inhibition; third, *P. aeruginosa* has a completely different pattern of growth inhibition compared to *B. subtilis*, *E. coli* and *S. aureus*; and fourth, the effect of clover honey is different for the different bacteria. These points will be explored further below, where the response of the bacteria to each honey type is described.

2.3.2.1 Manuka honey

Manuka honey samples M1, M2 and M3 (Table 2.1) have the highest MGO concentrations of the honeys tested, at 651.4, 1004.3 and 1541.3 mg/kg honey, respectively. These three honeys were the most effective in inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus* and all resulted in similar levels of growth inhibition. Low honey concentrations (1–4%) caused significant lag-phase extension and growth was completely inhibited once concentrations reached 8–16%.

In the presence of catalase the monofloral manuka honeys remained the most effective of the natural honeys at inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus*, indicating that the non-peroxide components in these honeys is the over-riding component responsible for their high levels of growth inhibition.

In contrast to *B. subtilis*, *E. coli* and *S. aureus*, there was very little or no lag-phase extension when *P. aeruginosa* was treated with low concentrations of honeys M1-M3, and complete inhibition only occurred at 16% of honeys M3 and M1 and 32% of honey M2. *P. aeruginosa* was relatively sensitive to sugar (Figure 2.2G), which likely accounts for some of the inhibition. The addition of catalase increased the concentration of honeys M1 and M3 required for complete growth inhibition 2-fold (to 32%). These data and those shown in Figure 2.3 indicate that *P. aeruginosa* is relatively insensitive to both hydrogen peroxide and MGO, and that at 32% manuka honey inhibition can be attributed to non-peroxide components.

2.3.2.2 Kanuka honey

The kanuka honeys, K1 and K2, had very low levels of MGO (5.6 and 37.1 mg/kg, respectively), but moderate rates of hydrogen peroxide production (0.360 and 0.327 mM/h, respectively) compared to the other honeys tested. At low concentrations (1–8%), and particularly in the presence of catalase, K1 and K2 were amongst the least effective of the honeys at inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus*, with very little or no lag-phase extension compared to the no-honey cultures. 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. coli* and *S. aureus*, complete growth inhibition still occurred at 32%; the exception being honey K1, where lag phase was only extended by 4 hours (Figure 2.3F). The non-peroxide component causing this growth inhibition is not likely to be MGO, since the amount present in 32% K1 and K2 is 1.8 and 11.9 mg/kg of Table 2.1 respectively, which is equivalent to 0.3% and 1.8 % of 650 mg/kg MGO (M1) (Table 2.1) and is therefore too low to affect growth of these bacteria (Figure 2.2A, C and E; light blue line). The inability of honey K1 to inhibit the growth of *S. aureus* suggests that the components that contribute to complete growth inhibition of *B. subtilis* and *E. coli* are not active against *S. aureus*. Alternatively there may be a component in honey that is specifically active against *S. aureus* but requires hydrogen peroxide for its production and/or activity. Note that *S. aureus* was also the only species that was not inhibited by clover honey.

P. aeruginosa growth was completely inhibited by kanuka honeys at 16% (K1) and 32% (K2), and very little lag-phase extension was observed. Catalase addition rescued this effect to some extent, but at 32% the kanuka honeys completely inhibited growth of *P. aeruginosa*. Again, this suggests components additional to MGO or peroxide are present in these honeys that affect growth. The most striking observation for *P. aeruginosa* that was distinctly different from the other

organisms was that growth was similarly affected by kanuka honeys as by manuka honeys.

2.3.2.3 Manuka-kanuka honey blends

The responses of bacteria to the manuka-kanuka honey blends, designated MK1–MK4, are shown in green in Figure 2.3. These honeys have intermediate levels of MGO (ranging from 173.6–307.8 mg/kg) that are between those of the pure manuka and kanuka honeys, and variable but significant levels of hydrogen peroxide (Table 2.1). Treatment with these honeys gave a level of inhibition that was generally between that of the pure manuka and pure kanuka honeys, especially when the hydrogen peroxide was removed by catalase. In addition, the degree of growth inhibition related largely to the level of MGO, with MK4, which has the highest level of MGO of the blended honeys (Table 2.1), normally being the most effective at inhibiting growth.

While the overall pattern of inhibition of *B. subtilis*, *E. coli* and *S. aureus* by the MK honeys was similar, there were some notable differences in how *E. coli* responded to the different blends. In the absence of catalase MK1 inhibited *E. coli* to a similar extent as the manuka honeys, with complete growth inhibition at 8%. MK1 has a low level of MGO (173.6 mg/kg) compared to the manuka honeys but has the highest hydrogen peroxide production rate of all honeys. Catalase addition to MK1 reduced the level of growth inhibition for *E. coli* to a level well below that of all three manukas. These observations suggest that *E. coli* growth can be maximally inhibited by honeys that either have a high level of hydrogen peroxide production or have high levels of MGO.

P. aeruginosa displayed little or no lag-phase extension or growth inhibition for any of the blended honeys until concentrations reached 16% or 32%. Overall, there was no clear trend in how *P. aeruginosa* responded to the varying levels of MGO and hydrogen peroxide in the different blends, however complete growth

inhibition was achieved at 32% in the presence of catalase, indicating that the inhibition does not require hydrogen peroxide.

2.3.2.4 Clover honey

The clover honey sample had no detectable MGO and almost no hydrogen peroxide production (0.029 mM H_2O_2/h; Table 2.1). Up to 16% clover honey had little effect on the growth of the four organisms (Figure 2.3). At 32%, *S. aureus* growth remained unaffected, while the two Gram-negative species, *E. coli* and *P. aeruginosa*, showed a significant lag-phase extension. This is commensurate with the response of these two organisms to 32% sugar (Figure 2.2A, C, E and G). However, while the addition of catalase to clover honey slightly increased lag phase extension, this was not seen for the corresponding sugar control. Interestingly, 32% clover honey completely inhibited growth of *B. subtilis*, both in the presence and absence of catalase even though sugar alone at equivalent concentrations had no effect on the growth of this organism. This suggests the presence of one or more components in clover honey to which *B. subtilis* is particularly sensitive.

2.3.3 Other observations not fitting growth inhibition trends

Although there were clear trends in growth inhibition in response to treatment with honeys and control solutions discussed above, there were certain observations that did not fit these trends that are worth acknowledging. This includes: M1, which has the lowest level of MGO, was the most active manuka honey for *B. subtilis, E. coli* and *S. aureus* in the presence of catalase (Figure 2.3B, D and F); the apparent abrupt (and reproducible) decrease in growth inhibition of the MK2 honey against *B. subtilis* at 16% in the presence of catalase (Figure 2.3B); the incomplete inhibition of *P. aeruginosa* by honey MK3 only observed when catalase was not present (Figure 2.3G); and a higher level of inhibition of *E. coli* by clover honey in the presence of catalase (Figure 2.3D). Given the complexity of

honey it is likely that the growth inhibition we observe in these analyses cannot always be solely accounted for by the presence MGO and hydrogen peroxide, and other components may exert independent action or may modulate the response of bacteria to MGO- and hydrogen peroxide-based toxicity.

2.3.4 Cellular morphology response in the presence of natural honeys

To determine morphological changes that occur in response to honey containing MGO or hydrogen peroxide, *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa* were exposed to honey samples M3 (highest MGO with lowest hydrogen peroxide production of the tested honeys) and MK1 (highest rate of hydrogen peroxide and relatively low MGO; Table 2.1). Cells were treated with 4% (w/v) of each honey, which is the highest concentration that still allowed growth of all four bacteria (Figure 2.3). Cell morphology was analysed during lag- and log-phase growth and included measures of cell shape changes (length or width), cell lysis (breakage of cells or leakage of cytoplasm indicating cell envelope or growth abnormalities), and detection of chromosomal DNA abnormalities by DAPI staining.

2.3.4.1 High-level MGO honey and cell morphology

Treatment with honey M3 induced an extended lag phase for all bacterial cultures except *P. aeruginosa* (Figure 2.2; Figure 2.3). Morphological changes are shown in Figure 2.4 and charted in Table 2.3, and mean cell lengths are recorded in Table 2.2. During the extended lag phase (or the initial lag phase for *P. aeruginosa*), cells of *B. subtilis*, *E. coli* and *S. aureus* were significantly shorter ($p < 0.05$) than untreated cells, while *P. aeruginosa* cells were longer (Table 2.3; Figure 2.4). In addition, a significant percentage of the shorter cells of *B. subtilis* (29%) and *S. aureus* (57%) had a condensed chromosome (as indicated by green arrows in Figure 2.4). In the *B. subtilis* cells only one bright region of DAPI staining occurred instead of the characteristic two regions that represent replicating chromosomes (Figure 2.4; Table 2.3). Likewise the *S. aureus* cells with condensed chromosomes

showed one or two very small spots of DAPI-stained DNA, unlike the two larger lobes of DNA that represent replicating chromosomes in the no-treatment control cells. No changes to DNA appearance under these conditions were observed for *E. coli* or *P. aeruginosa*.

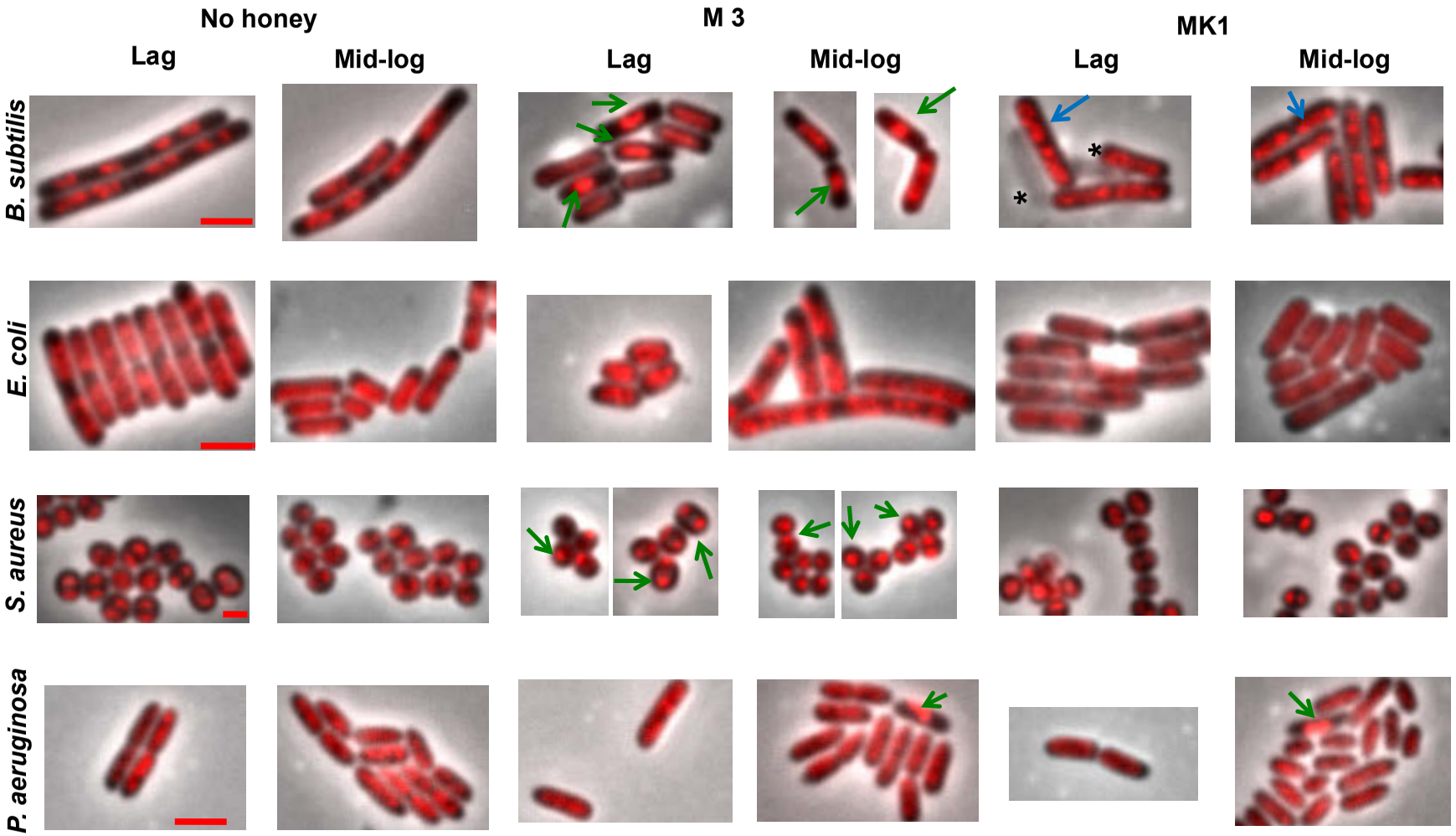

No honey
M 3
MK1
Lag
Mid-log
Lag
Mid-log
Lag
Mid-log
B. subtilis
E. coli
S. aureus
P. aeruginosa

Figure 2.4 Cellular morphology of bacterial cells treated with a high-MGO honey and high-hydrogen peroxide honey.

The effects of 4% a high-MGO honey (M3) and a high-hydrogen peroxide honey (MK1) on bacterial cellular morphology were examined. Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with these honeys, cells collected at both lag- and mid-log phases of growth as indicated in Figure 2.1, fixed with glutaraldehyde, stained with DAPI and imaged using fluorescence microscopy. All images are overlays of the phase-contrast image and the DAPI-stained (red) fluorescence image. The two left-hand panels show the no-honey treated control cells, the two middle panels M3 honey-treated cells, and the two right-hand panels show the MK1 honey-treated cells. In all images, condensed DNA is shown by green arrows; and dispersed DNA in *B. subtilis* cells is shown by blue arrows. An asterisk indicates lysed cells for *B. subtilis*. The scale bar represents 2μm, except for *S. aureus* images, where it represents 1 μm.

Table 2.2 Average cell length after different honey treatment (µm).

	B. subtilis			*E. coli*			*S. aureus*			*P. aeruginosa*		
	0%	**M3**	**MK1**	**0%**	**M3**	**MK1**	**0%**	**M3**	**MK1**	**0%**	**M3**	**MK1**
Lag	4.4±1.8	2.5±0.6	3.8±0.8	3.3±1.1	1.8±0.3	3.4±1.1^	1.2±0.2	1.0±0.2	1.1±0.2	2.5±0.6	2.7±0.5	2.4±0.6^
Mid-log	4.2±1.1	3.0±0.9	3.0±0.7	2.6±1.5	4.1±3.6	2.4±2.2^	1.1±0.1	0.9±0.2	1.1±0.2^	2.5±0.5	2.3±0.5	1.6±0.4

^ Cell lengths were not significantly affected by the honey treatments ($p > 0.05$); all other values are significantly different ($p < 0.05$); n ≥ 50.

M3 – 4% manuka M3 (high-MGO) honey treatment

MK1 – 4% manuka-kanuka blended (high-hydrogen peroxide) honey treatment

Table 2.3 Cell morphology changes with high-MGO honey and high-hydrogen peroxide honey treatment[a]

	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%)

[a] Actual mean cell lengths and statistics are shown in Table 2.2.

↓ 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

Following entry into log phase, cells treated with M3 honey were still significantly different to untreated cultures (Table 2.2 and Figure 2.4). *B. subtilis* and *S. aureus* cells remained shorter, to a similar degree observed in lag phase, and chromosomes remain condensed. *E. coli* cells became significantly longer than their untreated counterparts, while *P. aeruginosa* cells became slightly but significantly shorter (Table 2.3). In addition, 2% of the *P. aeruginosa* cell population now showed a condensed chromosome by DAPI staining (as indicated by green arrows in Figure 2.4; Table 2.3).

In summary, treatment with 4% M3 honey changed mean cells lengths of all four populations of bacteria in both lag and log phases of growth, but the direction and the extent of change varied. The greatest changes to cell length were observed with *B. subtilis* and *E. coli* (Table 2.3). Only the Gram-positive organisms had condensed DNA for both growth phases.

2.3.4.2 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 honey was particularly inhibitory to *E. coli* (Figure 2.3). The most significant changes to cell morphology induced by MK1 during the initial stages of growth were observed in *B. subtilis*, where cells were on average significantly shorter, as was seen with M3 but to a lesser extent. A significant proportion of cells (34%) appeared lysed as judged by a change in contrast visualized using phase-contrast microscopy (as indicated by asterisks in Figure 2.4; Tables S2.1 and 2.2), and the DNA in the vast majority of unlysed cells (90%) appeared dispersed compared to untreated cells (Figure 2.4; Table 2.3). No morphological changes were observed in *E. coli* or *P. aeruginosa* cultures, and the only change to *S. aureus* cells was a slight but significant decrease in cell diameter (Table 2.3).

In log-phase MK1-treated *B. subtilis* cultures, the extent of cell lysis was reduced from 34% in the lag-phase cultures to 2%. However, cells were still shorter on

average compared to control cells (Table 2.3 and Table 2.2; Figure 2.4), and the frequency of cells with a dispersed DNA appearance remained very high (99%). Log-phase *S. aureus* cells showed a normal morphology (Figure 2.4; Table 2.3), while *P. aeruginosa* cells were significantly shorter (Figure 2.4; Table 2.2 and 2.2). Two percent of the log-phase MK1-treated *P. aeruginosa* cells had the same condensed chromosome phenotype seen in the log-phase M3-treated *P. aeruginosa* cells*. E. coli* log-phase cells remained similar in appearance to the control cells (Table 2.3).

In summary, MK1 honey caused less alteration to cellular morphology than the high-MGO honey, M3. *B. subtilis* cells were the most significantly affected, with dispersed DNA, cell lysis and cell length changes. *E. coli* and *S. aureus* had little or no apparent change. *P. aeruginosa* cells were shorter in log phase only.

2.3.5 Correlation of growth inhibition and morphological changes induced by honey

A summary of combined growth and morphology data is given in Table 2.4. Overall this shows that MGO and manuka honeys are the most effective at inhibiting growth of all organisms except *P. aeruginosa*, followed by manuka-kanuka blended honeys, kanuka honeys and then clover. *P. aeruginosa* is much less sensitive to the honeys compared to the other three organisms, with little difference in growth inhibition by the different honeys, including clover. *B. subtilis* shows more morphological changes than the other bacteria for both the high-MGO and the high-hydrogen peroxide honeys, followed by *S. aureus*, *P. aeruginosa* and then *E. coli*. Thus, with the exception of *B. subtilis,* where both growth and morphology are profoundly affected by honey, the number and severity of morphological changes do not link clearly to the level of MGO or hydrogen peroxide in the honey, or to the effectiveness of the honey to inhibit growth. *E. coli* had very little apparent morphological changes even though its growth was affected in a similar way to *B. subtilis* and *S. aureus* and to a much greater extent than *P. aeruginosa*, with the latter conversely showing more profound morphological changes.

Table 2.4 Summary of growth and morphological effects honeys and control treatments on all organisms

Organism	Growth Inhibition						Cell Morphology	
	MGO	**Sugar**	**Clover**	**M**	**K**	**MK**	**High-MGO Honey (M3)**	**High-H_2O_2 Honey (MK1)**
B. subtilis	XXX[a]	-[b]	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%)

[a] The number of crosses increases the more growth is inhibited.

[b] - Means no effect

2.4 Discussion

With the dearth of development of new classes of antibiotics to treat infections caused by resistant organisms, honey is increasingly valued for its broad-spectrum antibacterial activity and effectiveness as a treatment for chronic wound infections. However, as with all natural products, there is significant chemical variation between different honey preparations [35] and this is likely to affect the level of antibacterial activity, and possibly, treatment outcomes. In this study, we have therefore assessed a series of geographically- and chemically-defined New Zealand manuka, kanuka and manuka-kanuka blended honeys with varying concentrations of MGO and hydrogen peroxide to determine their effectiveness in inhibiting the growth of different species of bacteria. We show here that, in general, the manuka honeys were the most effective at inhibiting growth, followed by the manuka-kanuka blends and then the kanuka honeys. However, the response of bacteria in the presence of sub-inhibitory concentrations of these different honeys varied with bacterial species, with each having a unique growth and morphological response. *P. aeruginosa* was very different to the other three bacteria in being both less sensitive overall and in having a similar response to the different honey types.

2.4.1 High-throughput analysis of growth dynamics reveals that MGO in honey extends the duration of lag phase

A high-throughput approach was used to assess the growth and morphological effects of a large number of natural honeys on multiple organisms. This approach is novel in honey studies and was employed here to address the challenge of assessing multiple parameters in a complex natural product. This system allowed us to explore the heterogeneous and variable composition of natural honey by analyzing large numbers of samples and control solutions, and showed the dynamic response of cell growth in response to the effects of honey toxicity. Such an approach may be useful in the study of other natural products where activity is modulated by various interacting factors.

Visual inspection of the resulting large number of growth curves revealed a distinctive dose-dependent extension of lag-phase of growth when cultures of *B. subtilis*, *E. coli* and *S. aureus* were treated with manuka honey. This growth behaviour was also observed when MGO alone was added to these bacterial cultures (Figure 2.2), and is consistent with a previous study where *E. coli* was subjected to MGO treatment [57]. Lag-phase extension was not seen for clover or pure kanuka honeys; growth was either unaffected or was completely inhibited by these two honeys, and there was no evidence for dose-dependent recovery over time (Figure 2.3). Thus the extended duration of lag-phase is presumed to be largely or completely due to MGO and is likely to be unique to honey derived from manuka and other *Leptospermum* species that contain this compound.

2.4.2 Growth and morphology of different bacteria are affected by honey in markedly different ways

The dynamics of growth in the presence of the different honey types was relatively similar for *B. subtilis, E. coli* and *S. aureus* but differed markedly in *P. aeruginosa* (Figure 2.2 and Figure 2.3). The extended duration of lag phase and the eventual resumption of logarithmic growth in the presence of MGO likely reflect induction of the glyoxylase system used to detoxify MGO [132]. All organisms produce MGO, which appears to be important in allowing them to regulate growth and maintain carbon flux as their environment changes [133] [134]. However, as MGO is toxic, cells detoxify this compound to D-lactate using two metalloenzymes, GlxI and GlxII [132]. The ability of *P. aeruginosa* to grow in the presence of higher MGO levels than the other bacteria may reflect more efficient detoxification of MGO; a suggestion supported by the discovery, through genome sequencing, that *P. aeruginosa* is unique among eubacteria in its possession of three (rather than one) fully functional GlxI homologs [135].

To date, few microscopy studies have been performed to identify morphological changes to bacterial cells treated with honey, and none have used high-throughput

phase-contrast and fluorescence microscopy that allows a large number of cells to be imaged and measured rapidly. We observed bacterial cell length changes in all organisms treated with manuka (high-MGO) honey. This is caused by an adjustment to the frequency of cell division relative to growth rate, often due to a change in nutritional state, such that division occurs at a different cell length to untreated cells [136]. Condensed DNA was also observed in a significant proportion of *B. subtilis* and *S. aureus* cells treated specifically with manuka honey. This could be a consequence of inhibition of initiation of DNA replication [137, 138]; a suggestion consistent with previous studies demonstrating that MGO alone inhibits this phase of DNA replication in bacterial cells [139]. MGO Treatment with honey that contained the highest level of hydrogen peroxide (MK1) caused significant changes to the morphology of *B. subtilis* cells, including a dispersed appearance of the DNA. This could reflect a degree of DNA degradation due to hydrogen peroxide in the honey causing oxidative DNA damage [55].

With the exception of *B. subtilis,* the number and severity of morphological changes do not link clearly to the level of MGO or hydrogen peroxide in the honey, or to the effectiveness of the honey to inhibit growth. This is not entirely unexpected since cell morphology often reflects a response to changes in the environment that allows the organism to adapt to that environment without having to change its rate of growth. Different organisms do this differently when faced with a variety of nutritional and environmental conditions, such as oxidative or nutrient stress. This might reflect, at least in part, the degree of variation of the environment that these organisms inhabit [140]. We therefore speculate that the differences in morphology that we observe in response to a particular honey reflect species-specific differences in the regulatory circuits that coordinate growth with cellular physiology.

2.4.3 MGO and hydrogen peroxide production cannot account for all activity present in manuka, kanuka and clover honey

Commensurate with previous studies [31, 34, 75, 141], we found that even when the peroxide activity was neutralized with catalase and there were negligible levels of MGO present, honey could inhibit bacterial growth. Even clover honey, with only trace levels of MGO and hydrogen peroxide, had variable effects on the four bacteria that in most cases could not be attributable to sugar. In addition, to further investigate the antibacterial effects of MGO on bacterial cell growth, MGO can be added to honeys that have little H_2O_2 and MGO, e.g. clover. More so, these observations are in line with previous studies suggesting the presence of additional antibacterial components that may be directly active or may modulate the activity of the dominant active components [31, 75, 141]. These additional components may include: (i) phenolics derived from the floral source [35]; (ii) bee-derived antimicrobial peptides (although note that bee defensin-1, an antibacterial component of Revamil honey [75], could not be identified in manuka or kanuka honeys) [75] [41]; and (iii) as yet undefined synergistic compounds identified in other studies, including transition metals [53, 55, 142].

2.4.4 Clinical applications of antibacterial honey

The range of effects induced by the different honeys in the bacterial species tested reflects a diversity of responses that could be expected by bacteria present in chronic wounds. Our findings here have important implications for the clinical application of honey in the treatment of these wounds. First, sub-inhibitory concentrations of MGO may be neutralized by bacteria, which then resume normal growth, thus any honey formulation should contain sufficient active honey to sustain inhibition. Second, honey without significant levels of MGO or hydrogen peroxide, such as clover honey, may be able to inhibit some bacteria but is not broad-spectrum and is therefore not recommended for infected wounds where

multiple species may be present. Third, 32% of MGO at 600 mg/kg honey achieves almost as much inhibition as 32% of MGO at 1,000 and 1,500 mg/kg honeys, and increasing MGO above this threshold may not result in a more effective honey.

And finally, in honey containing both MGO and hydrogen peroxide, MGO provides an over-riding activity and if this level is high enough, hydrogen peroxide does little to augment activity.

To date, more than 80 different microbial species, including bacteria and yeast pathogens known to infect wounds, have been shown to be inhibited by honey [39, 74, 143]. In the current study, the use of sub-inhibitory concentrations of honey has enabled us to examine the nature of honey inhibition, however these concentrations are well below those that would be used in a clinical situation, where whole honey is generally applied and complete and irreversible inhibition would be expected.

Emerging evidence from clinical studies suggests that honey is at least as effective as conventional treatments in healing wounds, particularly in very refractory cases such as in diabetics, the elderly, and extensively burned patients [144, 145], but more clinical data are necessary for robust statistical appraisal [107]. Here, we have demonstrated that the potency of natural honey as an antimicrobial wound dressing, and that multiple effects arise from a variety of active compounds, which not only allows active honey to be uniquely broad in spectrum, but also reduces the potential for resistant microbial populations to evolve. Use of the full honey matrix is therefore recommended for the treatment of infected wounds. Understanding the complex nature of honeys and its effects on bacterial pathogens may eventually allow the development of specific blends with an optimal combination of antibacterial components, thus ensuring a highly effective and resilient antibacterial wound treatment option.

Chapter 3

Manuka-type honeys can eradicate biofilms produced by *Staphylococcus aureus* strains with different biofilm-forming abilities

Declaration

I declare that the following publication (please refer to Appendix III for published manuscript) included in this thesis in lieu of a chapter meets the following criteria:

- The majority of the content in the following publication included in this chapter has been executed and prepared for publication by me. For detailed contribution, please refer to statement below.
- The work presented here has been peer-reviewed and accepted for publication.
- The initial draft of the work has been written by me and any subsequent changes in response to co-authors and editors reviews were performed by me.
- The publication is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in the thesis.

Publication title: Manuka-type Honeys can Eradicate Biofilms Produced by *Staphylococcus aureus* Strains with Different Biofilm-forming Abilities.

Authors: Jing Lu, Dee A. Carter, Lynne Turnbull, Douglas Rosendale, Duncan Hedderley, Jonathan Stephens, Swapna Gannabathula, Gregor Steinhorn, Ralf C. Schlothauer, Cynthia B. Whitchurch, Elizabeth J. Harry

Candidate's contribution: I performed all the experiments that were presented in this study; performed a majority of the data analysis, wrote the paper, prepared all the figures and tables by myself and edited numerous drafts of the paper.

Journal name: PeerJ
Volume/ page numbers: 2:e326
Status: Published 26/03/2014

I declare that the publication above meets the requirements to be included in the thesis.

Candidate's name: Jing Lu
Candidate's signature:
Date (dd/mm/yy):

3.1 Introduction

Chronic wounds currently affect 6.5 million people in the US. These wounds are difficult to treat and estimated to cost in excess of US $ 25 billion annually, with significant increases expected in the future [6]. A wound is generally considered chronic if it has not started to heal by four weeks or has not completely healed within eight weeks [1]. Such prolonged, non-healing wounds are caused by a variety of factors, and with bacterial infection being a significant contributor.

In chronic wounds, as with everywhere on earth, bacterial cells predominantly exist as biofilms, where cells are embedded within a matrix of polysaccharides and other components. This matrix affords resistance to environmental stresses such as altered pH, osmolarity, and nutrient limitation [101]. The matrix also limits access of antibiotics to the biofilm embedded cells [102], which are up to 1,000 times more recalcitrant to these compounds than planktonic cells [103]. Planktonic bacteria may also contribute to pathogenesis, as their release from biofilms has been proposed to maintain the inflammatory response within the wound [124, 146], as well as allowing seeding to other areas [147, 148]. Along with the difficulties of treating biofilm infections, the emergence of resistance to multiple antibiotics has exacerbated the problem of chronic wound treatment [14, 15]. Thus, there is an increasing need for new approaches to combat bacterial biofilms in chronic wounds.

Honey has been used to treat acute and chronic wound infections since 2500 BC [17-19]. Honey possesses a number of antimicrobial properties including high sugar content, low pH, and the generation of hydrogen peroxide by the bee-derived enzyme glucose oxidase [35]. However, not all honeys are the same and their antimicrobial properties vary with floral source, geographic location, weather conditions, storage (time and temperature) and various treatments, such as heat [18, 22, 113, 149]. These factors lead to differences in the levels of various antibacterial components. Manuka honey is derived from *Leptospermum scoparium* bush and is particularly potent [22, 30, 75]. This is believed to be largely

due to the high levels of the reactive dicarbonyl methylglyoxal (MGO) [30, 32], which is inhibitory to bacterial growth (Chapter 2). Other antimicrobial compounds in honeys include bee defensin-1 [33, 45], various phenolic compounds and complex carbohydrates [18, 30, 32, 41, 44]. The combination of these diverse assaults may account for the inability of bacteria to develop resistance to honey [70, 150], in contrast to the rapid induction of resistance observed with conventional single-component antibiotics [151, 152].

A few studies have examined the effect of manuka honey on biofilms, showing it to be active against a range of bacteria, including Group A *Streptococcus pyogenes*, S*treptococcus mutans, Proteus mirabilis*, *Pseudomonas aeruginosa, Enterobacter cloacae* and *Staphylococcus aureus* [62, 104, 106, 153]. However, the levels of reported antibiofilm activity are not consistent among these studies. This is highly likely to be at least in part due to differences in the levels of the principle antibacterial components in the honey, MGO and hydrogen peroxide, which varies with the floral and geographic source of nectar, the honey storage time and conditions, and any possible other treatments that may have occurred. All these conditions affect the antimicrobial activity of honey [30, 35, 112, 113], but are often not reported. Importantly, medical-grade honeys, while often composed primarily of manuka, can also contain honey derived from other flora sources, which can alter the levels of various antimicrobial components. Therefore, it is imperative to use well-characterized honeys to enable both accurate comparisons among studies, and the rigorous assessment of the potential of medical-grade honey to be used in wound treatment in the clinic.

Here we have performed biomass and viability assays, as well as confocal scanning light microscopy to examine the antibiofilm activity of four NZ manuka-type honeys, clover honey and an isotonic sugar solution on a range of *S. aureus* strains that differ widely in their biofilm-forming ability. These honeys have been well characterized in terms of their geography, floral source and the level of the two principal antibacterial components found in honey, MGO and hydrogen peroxide. We demonstrate that the manuka-type honeys are highly active in both the

prevention and elimination of methicillin-sensitive and methicillin-resistant *S. aureus* biofilms. The antibiofilm activity was highest in the honey blend that contained the highest level of manuka-derived honey; although the same level of MGO, with or without sugar, could not eradicate biofilms. This suggests that additional factors in these manuka-type honeys are required for their potent antibiofilm activity; and emphasise the importance of characterizing honey in order to understand and choose the best honey product for wound management.

3.2 Materials and Methods

3.2.1 Honey samples

The New Zealand (NZ) honey samples used in this study are listed in Table 3.1, and include monofloral manuka honey, Medihoney (a manuka-based medical grade honey; Comvita Ltd), a manuka/kanuka blend, and clover honey (a white New Zealand honey). All honey samples were supplied by Comvita New Zealand Ltd. (Te Puke, New Zealand). The harvesting and geographic information for these honeys, as well as the levels of the three major antimicrobial components: methylglyoxal (MGO), dihydroxyacetone (DHA) and hydrogen peroxide, are listed in Table 3.1. All samples were stored in the dark at 4 ^{o}C and were freshly diluted in Tryptone Soya Broth (TSB) immediately before use in assays. All honey concentrations are expressed as % w/v.

Table 3.1 Harvesting and chemical information for the tested NZ honey samples

Honey type	Harvest period	Area	Floral source	Major Composition		Antimicrobial
				DHA [a]	MGO [a]	H_2O_2 [b]
Manuka	Spring 2010	Hokianga, Northland, NZ	*Leptospermum scoparium var. incanum*	4277	958	0.34
Medihoney	Spring 2010	Northland, NZ	*Leptospermum scoparium var. incanum* + *Kunzea ericoides*	883	776	0.31
Manuka/kanuka	Summer 2010/11	Hokianga, Northland, NZ	*Leptospermum scoparium var. incanum* + *Kunzea ericoides*	652	161	0.68
Clover	N/A*	Balcutha, Otago, NZ	*Trifolium spp.*	< 20	< 10	0.11

[a] MGO (methylglyoxal) levels were analyzed against di-hydroxyacetone (DHA) and expressed as mg MGO per kg of honey.

[b] Rate of production of H_2O_2 (hydrogen peroxide) is expressed as µmol/h in 1 mL of 10% w/v honey.

* Information not available

3.2.2 Other tested solutions

A series of other solutions were included for investigation alongside the honey samples: i) a sugar solution designed to mimic the concentration and composition of honey sugars (45% glucose, 48% fructose, 1% sucrose) diluted as above for honey; ii) MGO diluted in TSB to concentrations similar to those present in the manuka-type honeys (100 mg/kg, 700 mg/kg and 900 mg/kg honey) to assess the effect of MGO alone on bacterial growth; iii) MGO diluted in sugar solution to the same concentration as (ii). MGO was obtained as a ~40% (w/w) solution in water (Sigma-Aldrich Co., MO, USA).

3.2.3 Hydrogen peroxide assay

The level of hydrogen peroxide produced by the NZ honey samples was determined using a hydrogen peroxide/peroxidase assay kit (Amplex Red, Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA) as previously reported (Chapter 2).

3.2.4 Bacterial strains and growth conditions

Four strains of *S. aureus* were examined. These include two laboratory reference strains: NCTC 8325 (National Collection of Type Cultures) [154] and ATCC 25923 (American Type Culture Collection) which are methicillin-sensitive, and two clinical isolates: MW2 (Hospital-Acquired Methicillin-resistant *Staphylococcus aureus,* HA-MRSA) [155] and USA300 (Community-Acquired Methicillin-resistant *Staphylococcus aureus*, CA-MRSA) [156]. All *S. aureus* strains were grown in TSB at 37^{o}C. For optimal biofilm formation, 1% (w/v) glucose was added to this medium (TSBG) except for strain NCTC 8325 which was found to produce optimal biofilm in the absence of added glucose.

3.2.5 Susceptibility of S. aureus to NZ honeys: growth response assays

In this study, growth response assays were carried out to assess whether the NZ honeys affected cell growth of the different strains of *S. aureus* (at concentrations of 1-32%; prepared as serial 2-fold dilutions in TSB(G)). Details of the growth assay methods are described in Chapter 2. TSB(G) media without honey was included as a control. Unless otherwise stated, all assays were performed with three biological replicates and three technical repeats of each replicate.

3.2.6 Biofilm formation assays

The effects of NZ honeys and other solutions on *S. aureus* biofilm formation were determined using crystal violet static biofilm formation assays in microtitre plates according to published studies with the following modifications [154, 157]. Crystal violet stains all biomass including live and dead cells and the biofilm matrix. *S. aureus* strains were cultured in 2 mL of TSB(G) with shaking (250 rpm) overnight at 37^{o}C . A suspension from the overnight culture was then diluted to a cell density of approximately 10^7 CFU/mL in fresh TSB containing the appropriate test solution (honey, sugar solution, MGO or MGO in combination with sugar) to give a final volume of 150 µL. The suspension was added to each well of a 96-well tissue-culture treated microtitre plate (BD Falcon, NJ, USA). Media-only and media with the appropriate test solution without *S. aureus* inoculation were included as negative controls. The microtitre plates were sealed with AeraSeal (Excel Scientific, CA, USA) and incubated in a humidified incubator for 24 h at 37^{o}C. Following this, planktonic cell growth was assessed by transferring the planktonic phase into a new 96-well microtitre plate and reading the optical density at 595 nm with a microplate reader (VersaMax, Molecular Devices, California, USA). This step was required as *S. aureus* forms biofilms on the bottom of the microtitre plate wells, which interferes with optical density readings of the planktonic culture. The microtitre plates with residual biofilm were then washed three times with sterile phosphate buffered saline (PBS) to remove unattached cells and air-dried at 65 ^{o}C for 1 h, to fix the *S. aureus* biofilm to the bottom of the well surface. The plate was

then stained with 0.2% (w/v) crystal violet at room temperature for 1 h, excess crystal violet solution was decanted and the plates were washed as above with PBS. Stain that was bound to the adherent biomass was resolubilized with 200 µL 33% acetic acid and transferred into a new 96-well microtitre plate to measure the OD_{595}.

3.2.7 Biofilm elimination assays

S. aureus biofilms were first formed in the wells of a 96-well microtitre plate for 24 h at 37^{o}C as described above. Biofilms were then washed three times with PBS. Various concentrations (0% - 32% in two-fold serial dilutions) of honey and other test solutions were then added to the established *S. aureus* biofilms. The assay plates were then incubated for a further 24 h at 37^{o}C, and planktonic cell growth and biofilm mass were quantified as described above.

3.2.8 Determination of bacterial cell viability in biofilms

Crystal violet stains all the components of the biofilm [158]. To quantify the viability of cells within the *S. aureus* biofilms following honey treatment, we used a BacTitre Glo Microbial Cell Viability Assay Kit (Promega, WI, USA), which measures ATP levels as a proxy for viability. The assay reagents lyse the bacterial cells to release intracellular ATP, the levels of which are quantified via a luminescence-based luciferase activity assay [159, 160]. The BacTitre Glo protocol involved the same steps as crystal violet staining (above), however, instead of drying and staining the biofilms, plates were incubated with BacTitre Glo reagent in TSB(G) for 10 minutes at 37 ^{o}C in the dark. The contents of each well were then transferred into white solid-bottom 96-well microtitre plates (Cellstar, Greiner Bio-one, France) for luminescence measurement. Luminescence, which is proportional to the amount of ATP produced by metabolically active cells, was recorded using a 96-well microplate reader (TeCan, Infinite 200Pro, Männedorf, Switzerland).

To ensure the validity of this assay, a standard curve was constructed to assess the correlation between bacterial cell numbers and the luminescent signal in the biofilm. This was performed on the untreated control (containing *S. aureus* in TSB(G) only). Biofilms produced as above were washed and cells within the biofilm dispersed using a small probe sonicator (Sonics and Materials VC-505) to enable quantification by direct enumeration [161]. The recovered cell suspension was serially diluted 10-fold and a 20µL aliquot was plated on Tryptone Soya Agar (TSA) for CFU determination. Luminescence of cells in the remaining suspension was assessed using the BacTitre Glo kit. From this, a correlated standard curve was constructed between calculated CFU/well and the relative luminescence readings. According to the standard curve shown in Figure 3.1, the detection limit of the BacTitre Glo is at a luminescence reading below 1,000, which is equivalent to 10^3 CFU/well (linear range from 10^3 -10^7 CFU/well). An upper limit was not detected.

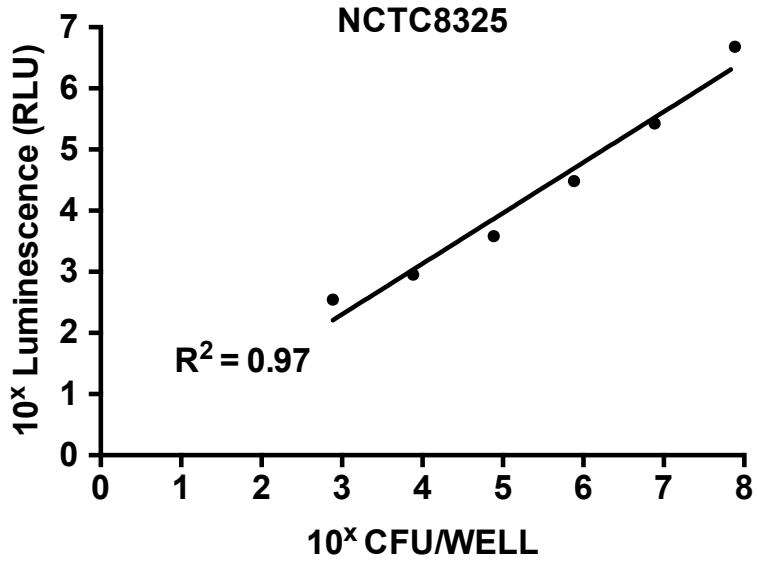


Figure 3.1 Correlation of level of intracellular ATP to colony forming units (CFU) in static biofilms of *S. aureus*.

Static biofilms of *S. aureus* were formed in the wells of a microtitre plate for 48 h (with media replenishment at 24 h). After removal of the biofilm from the wall of each well, intracellular ATP levels were measured by the BacTitre Glo Viability Kit and CFU were determined for each well. The intracellular levels of ATP are plotted as a function of CFU and validate that the BacTitre Glo Viability Kit can be used as a surrogate measure of biofilm cell viability in subsequent assays.

3.2.9 Visualizing live/dead stained S. aureus biofilms using confocal laser scanning microscope (CLSM)

S. aureus biofilms were treated with TSB containing 1%, 2%, 16%, and 32% NZ honeys or sugar solution for 24 h in black polystyrene 96-well microtitre plates with μClear bottoms (Cellstar, Greiner Bio-One, France) as described above, except the biofilm mass was not fixed by air-drying. The treated biofilm mass was washed three times with PBS and cells within the biofilm structure were fluorescently stained with 2.5 μM Syto9 (Invitrogen, CA, USA) and 4.3 μM propidium iodine (PI) (Becton Dickinson, NJ, USA), which identify live and dead cells in the biofilm structure, respectively. After 30 min of incubation in the dark at room temperature, the wells were washed thoroughly with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, MO, USA) for 15 minutes. The wells were then

rinsed and stored in PBS for imaging. Biofilms were imaged using confocal laser scanning microscopy imaging (CLSM) on a Nikon A1 confocal microscope. The Syto9 and PI fluorophores were excited at 488 nm and 561 nm, and the emissions were collected at 500-550 nm and 570-620 nm, respectively. For quantitative analysis, at least eight separate CLSM image stacks of each NZ honey treated biofilms were acquired with a resolution of 512 x 512 pixels. Biofilm biomass was calculated using COMSTAT [162] and is expressed as volume of the biofilm over the surface area ($\mu m^3/\mu m^2$). Representative presentation image stacks of each treatment were acquired at a resolution of 1024 x 1024 pixels and three dimensional biofilm images reconstructed using NIS-elements (Version10, Nikon Instruments Inc., USA). It should be noted that due to the incomplete displacement of Syto9 by propidium iodine in dead cells that there will remain some Syto9 staining of dead cells. Therefore the absolute level of live cells detected in the Syto9 channel will be somewhat overestimated using this co-staining method [163].

3.2.10 Assaying honey resistance in cells recovered from biofilms

The development of resistance is a great concern in clinical settings, where bacteria can become resistant to inhibitory compounds after exposure to sub-inhibitory concentrations [164, 165]. Planktonic cells that appeared after 24 h manuka-type honey treatment of established biofilms were assumed to have been released from the biofilm matrix. Cells recovered from biofilms treated with sub-eliminatory concentrations of manuka-type honeys were collected and tested for their ability to grow and form biofilms under the static growth conditions described above. Cell growth and biofilm formation were defined as not detected when the $OD_{(x)} - OD_{(\text{media only blank})} \leq 0.1$. Each experiment was performed with three biological replicates and three technical repeats of each biological replicate.

3.2.11 Statistical analysis

Data sets tested and passed the normality test (alpha = 0.05) for Gaussian distributions using D'Agostino-Pearson normality test in GraphPad Prism versions 5 and 6. Further statistical analysis to determine significant differences between treatments and among honey samples were performed using One-Way ANOVA with Tukey Test in GraphPad Prism versions 5 and 6. Statistical significance was set at $p < 0.05$.

3.3 Results

3.3.1 The effect of NZ manuka-type honeys on the planktonic growth of S. aureus

Planktonic growth and biofilm mass were assessed to examine the ability of four NZ honeys, three manuka-types and one clover, and a sugar solution to prevent biofilm formation by different strains of *S. aureus*. Following 24 h incubation under static conditions, *S. aureus* cells formed biofilms at the bottom of microtitre plates, with very little or no planktonic growth detected, indicating that the concentration of honey needed to prevent *S. aureus* planktonic cell growth could not be calculated under these conditions. Shaking broth cultures were instead used to assess the effect of the treatments on planktonic growth. The results are shown in Table 3.2. Planktonic growth of the four *S. aureus* strains, NCTC 8325, ATCC 25923, MW2 and USA300, was completely inhibited by 8% manuka honey and Medihoney, 16% manuka/kanuka honey and 32% clover honey. The 32% sugar solution was only effective at inhibiting growth of the MRSA strain MW2, with no inhibition of growth of the other strains at the concentrations tested (1-32%). These data are in agreement with the results of our previous study using the standard *S. aureus* reference strain, ATCC 25923, which used a similar suite of honey types and the same experimental conditions (Chapter 2).

Table 3.2 Concentration of honey required to inhibit *S. aureus* growth

Honeys	NCTC 8325	ATCC 25923	MW2	USA300
Manuka honey	8*	8	8	8
Medihoney	8	8	8	8
Manuka/kanuka honey	16	16	16	16
Clover honey	32	32	32	32
Sugar solution	>32	>32	32	>32

* All numbers in the table are honey concentrations (%, w/v).

3.3.2 The effect of NZ manuka-type honeys on S. aureus biofilm formation

All strains of *S. aureus* were assessed for their biofilm-forming ability after 24 h and 48 h. Under static conditions, biofilm-forming ability varied between strains, with NCTC 8325 forming the most robust biofilms, and generating significantly more biofilm mass than the other three tested strains (Figure 3.2A and Figure 3.2B). This was followed by ATCC 25923 and USA300, with MW2 forming the thinnest biofilms (Figure 3.2A and 2B; $p < 0.05$). The effects of the four NZ honeys and the sugar solution on biofilm formation of strain NCTC 8325 are shown in Figure 3.3A. Manuka honey was the most effective at preventing biofilm formation by *S. aureus* NCTC 8325, resulting in ~95% reduction ($p < 0.001$) in biofilm formation at 8% honey (Figure 3.3A) compared to the untreated (0%) control. At this concentration, the other honeys and the sugar solution did not significantly reduce biofilm formation. Medihoney and manuka/kanuka honey were highly effective at 16%, preventing biofilm formation by ~95% ($p < 0.001$). Clover honey displayed a less effective trend in preventing *S. aureus* biofilm formation, except *S. aureus* NCTC 8325 (Figure 3.3A), than the sugar solution at 32% (w/v) (Figure 3.3B - D).

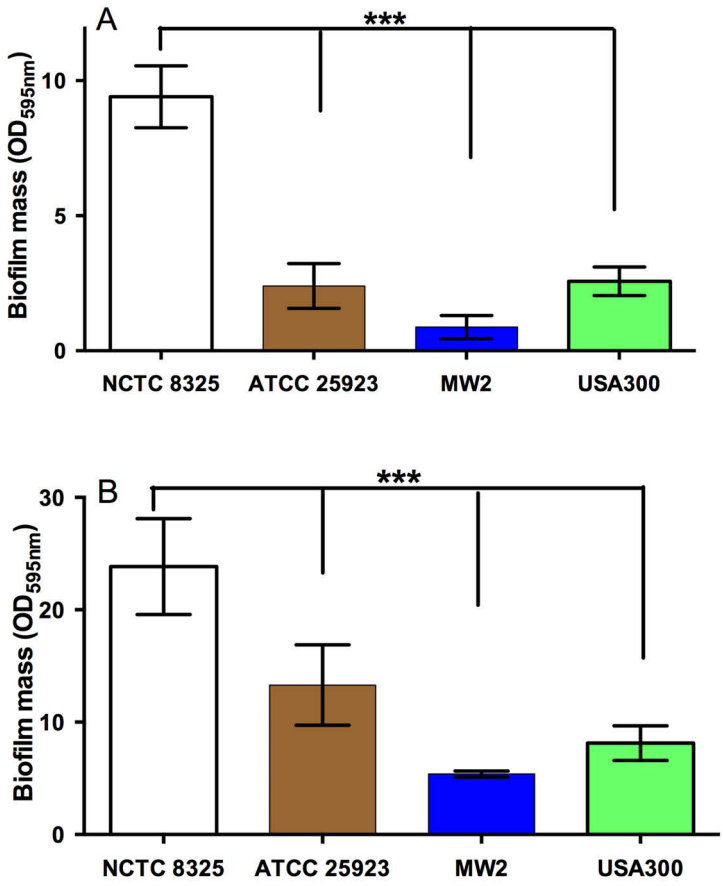


Figure 3.2 Quantification of biofilm formation by different strains of *S. aureus*

The ability of different strains of *S. aureus* to form biofilms on the plastic surface of tissue-culture treated 96-well microtitre plate was assessed in TSB(G) at 24 h and 48 h. Biofilm adherence was determined using a static biofilm formation assay over 24 h (A) and 48 h (with media replenished after 24 h incubation) (B). Biofilm formation was quantified by staining with 0.2% crystal violet solution and measured at an optical density of 595 nm. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate, *** represents $p < 0.001$, compared to NCTC 8325, as assessed by One-Way ANOVA with Tukey test described in Section 3.2.11.

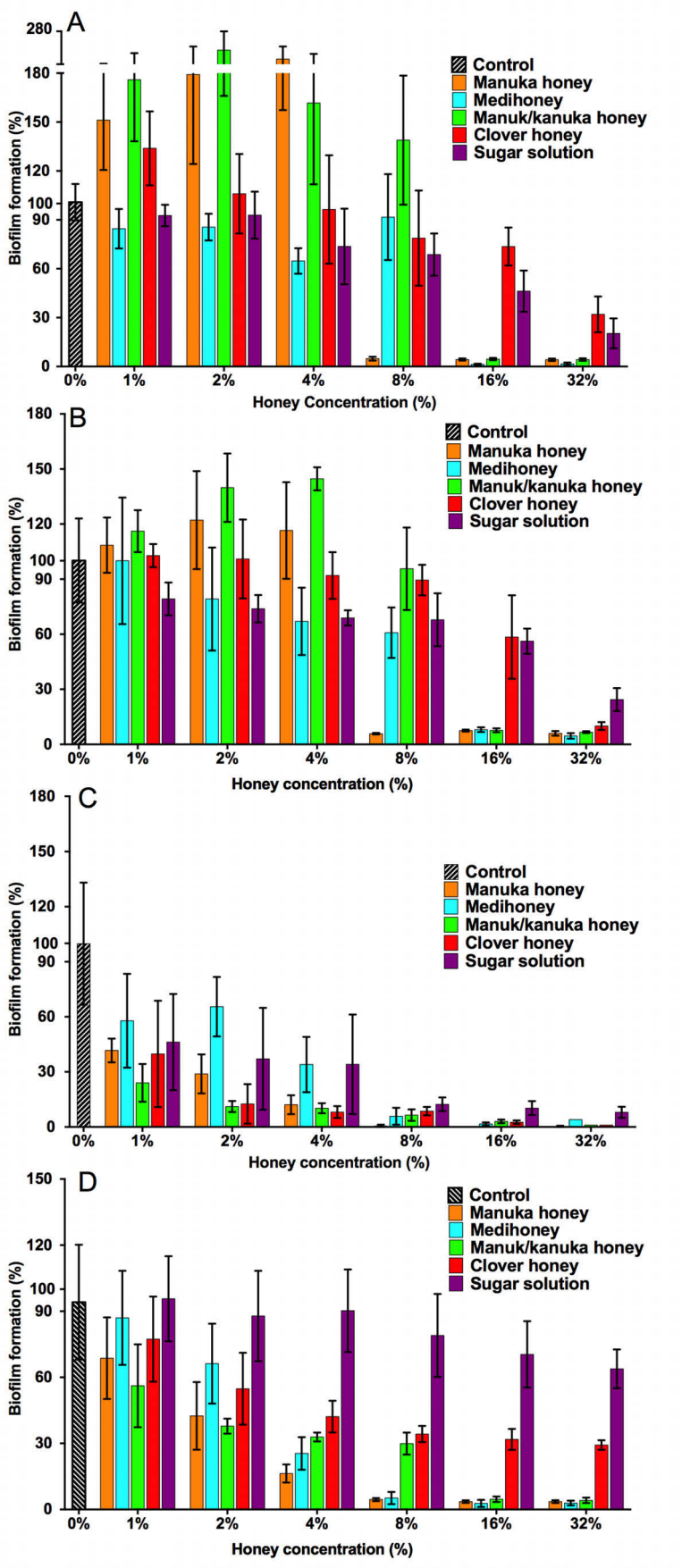

A
Biofilm formation (%)
280
180
150
120
100
90
60
30
0
Control
Manuka honey
Medihoney
Manuk/kanuka honey
Clover honey
Sugar solution
0%
1%
2%
4%
8%
16%
32%
Honey Concentration (%)
B
Biofilm formation (%)
180
150
120
100
90
60
30
0
Control
Manuka honey
Medihoney
Manuk/kanuka honey
Clover honey
Sugar solution
0%
1%
2%
4%
8%
16%
32%
Honey concentration (%)
C
Biofilm formation (%)
180
150
120
100
90
60
30
0
Control
Manuka honey
Medihoney
Manuk/kanuka honey
Clover honey
Sugar solution
0%
1%
2%
4%
8%
16%
32%
Honey concentration (%)
D
Biofilm formation (%)
150
120
100
90
60
30
0
Control
Manuka honey
Medihoney
Manuk/kanuka honey
Clover honey
Sugar solution
0%
1%
2%
4%
8%
16%
32%
Honey concentration (%)

Figure 3.3 Effects of NZ honeys and sugar on *S. aureus* biofilm formation

S. aureus biofilms were allowed to form in the presence of four different NZ honey types (manuka, Medihoney, manuka/kanuka or clover) or a sugar solution. Biofilm formation was assessed using a static biofilm formation assay with crystal violet staining to quantify biomass. *S. aureus* strains are: (A) NCTC 8325; (B) ATCC 25923; (C) MW2 (HA-MRSA) and (D) USA300 (CA-MRSA). Biofilm formation is expressed as a percentage relative to that produced by the untreated control, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate.

For NCTC 8325, the addition of sub-inhibitory concentrations of manuka and manuka/kanuka honey significantly enhanced biofilm formation, increasing it by 1.5- and 2-fold, compared to the untreated control ($p < 0.001$). In contrast, Medihoney, clover honey and the sugar solution did not enhance biofilm formation by strain NCTC 8325 at any concentration tested, which will be discussed in more details later.

S. aureus strain ATCC 25923, which is a standard clinical reference strain, produced similar results to NCTC 8325, including the enhancement of biofilm formation following sub-inhibitory honey treatment (Figure 3.3B). The hospital-acquired MRSA strain MW2, the weakest biofilm former out of all four tested strains (Figure 3.2A and 2B; $p < 0.05$), displayed a very sensitive profile to all of the NZ honeys and the sugar solution at all tested concentrations (Figure 3.3C). Even with only 1% honey or sugar solution, a ~50% reduction in biofilm formation was observed for MW2 ($p < 0.001$). At higher concentrations (≥ 8%), all four NZ honeys were significantly more effective than the sugar solution at preventing MW2 biofilm formation (Figure 3.3C). The other MRSA strain, USA 300 (Figure 3.3D), responded similarly to NCTC 8325 (Figure 3.3A), with approximately the same concentration of manuka-type honey being required to reduce biofilm formation by ~95%. However, unlike NCTC 8325 (Figure 3.3A), sub-inhibitory concentrations of manuka-type honey reduced biofilm formation of USA 300 (Figure 3.3D) rather than enhancing it (e.g. 4% manuka-type honeys exhibited ~50-80% biofilm inhibition of USA 300). Moreover, in USA 300 (Figure 3.3D),

biofilm formation was not affected by the sugar solution at any tested concentration.

The results above can be summarized as follows: i) all three manuka-type honeys are effective at inhibiting biofilm formation of a range of MSSA and MRSA strains; with (monofloral) manuka honey being generally more effective than the other maunka-type honeys; and ii) the manuka-type honeys are generally more effective than clover honey and the isotonic sugar solution, although clover honey was just as inhibitory as the manuka-type honeys for the weakest biofilm former, *S. aureus* MW2.

3.3.3 The effect of MGO on S. aureus *biofilm prevention*

MGO is a principle antibacterial component of manuka honey that has inhibitory effects on the growth of *S. aureus* and other bacterial species. This is evidenced by the correlation between the MGO level that is proportion of manuka-derived honey [43]. To determine whether MGO is solely responsible for the inhibitory effect of the three manuka-type honeys on *S. aureus* biofilm formation, biofilm assays were performed using MGO at equivalent concentrations to those present in each of the manuka-based honey samples, with and without the addition of the sugar solution (Figure 3.4). *S. aureus* NCTC8325 biofilm formation was not significantly ($p > 0.05$) affected by MGO at concentrations equivalent to that present in 1- 16% manuka-type honeys. MGO at medium (700 mg/kg) and high (900 mg/kg) levels at the equivalent concentration to 32% manuka-kanuka honey and Medihoney prevented approximately 50% and 75% biofilm formation, respectively ($p < 0.05$). The addition of the sugar solution to MGO at the same levels present in 16% of all three manuka-type honeys, led to a dramatic decrease (~95%) in biofilm formation.

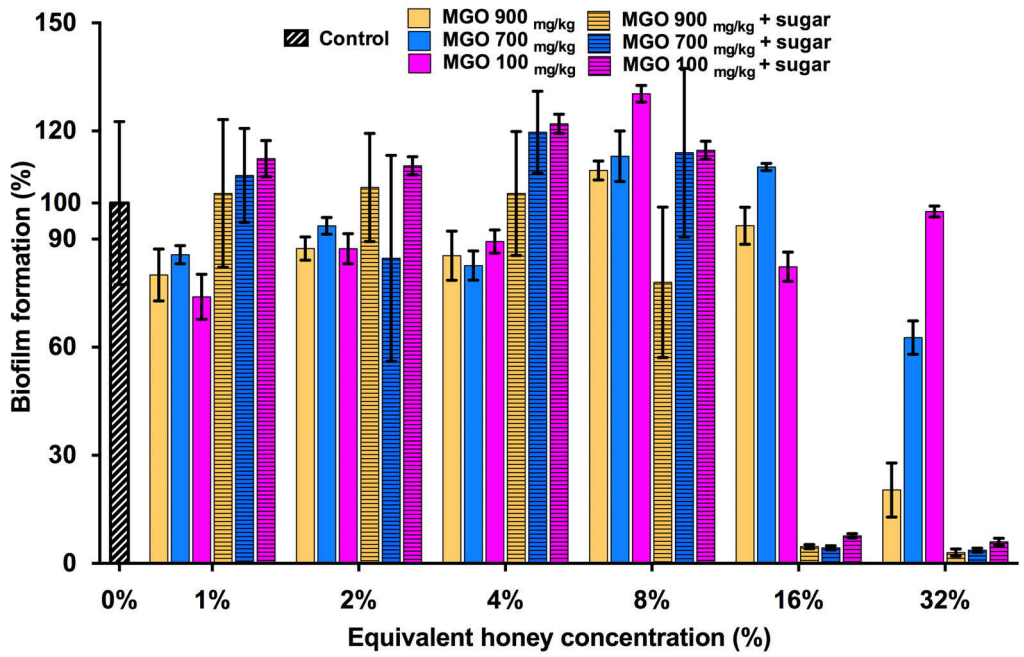


Figure 3.4 Effects of MGO on *S. aureus* biofilm formation.

Biofilm formation by *S. aureus* NCTC 8325 grown in the presence of MGO and MGO plus sugar solution. MGO stock solutions were prepared to correspond to the MGO levels in undiluted manuka-type honeys (100 mg/kg of manuka/kanuka honey, 700mg/kg of Medihoney, and 900 mg/kg of manuka- honey; Table 3.1). Biofilm formation was assessed using the described static assay with crystal violet staining to quantify biomass. Biofilm formation is expressed as a percentage relative to the untreated control, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate.

3.3.4 The Effect of NZ manuka-type honeys on established S. aureus *biofilms*

Bacterial biofilms are usually already established in open, chronic wounds prior to presentation to the clinic for medical treatment. In fact, infection is one of the reasons why these wounds become chronic. We therefore assessed the ability of the four NZ honeys to remove established biofilms produced by the four strains of *S. aureus.* These results are presented in Figure 3.5, with coloured lines showing biofilm mass present following treatment with different concentrations of the various honey types. While there was variation among the *S. aureus* strains in their response to the different honeys, there are some important general trends. First, manuka honey was consistently the most effective at removing biofilm, eliminating

almost all of the established *S. aureus* biofilms at concentrations of 16%–32%, ($p < 0.001$ compared to the untreated control sample; Figure 3.5 top panel, orange lines). Second, Medihoney and manuka/kanuka honey were also effective at these concentrations for some *S. aureus* strains, but only consistently effective across all four strains at 32% (Figure 3.5, blue and green lines). Third, both the clover honey and the sugar solution did not significantly reduce ($p > 0.5$) established biofilm mass until their concentration reached 32%. However, the sugar solution did not remove significant amount of established USA 300 biofilm biomass at 32% (Figure 3.5, purple line).

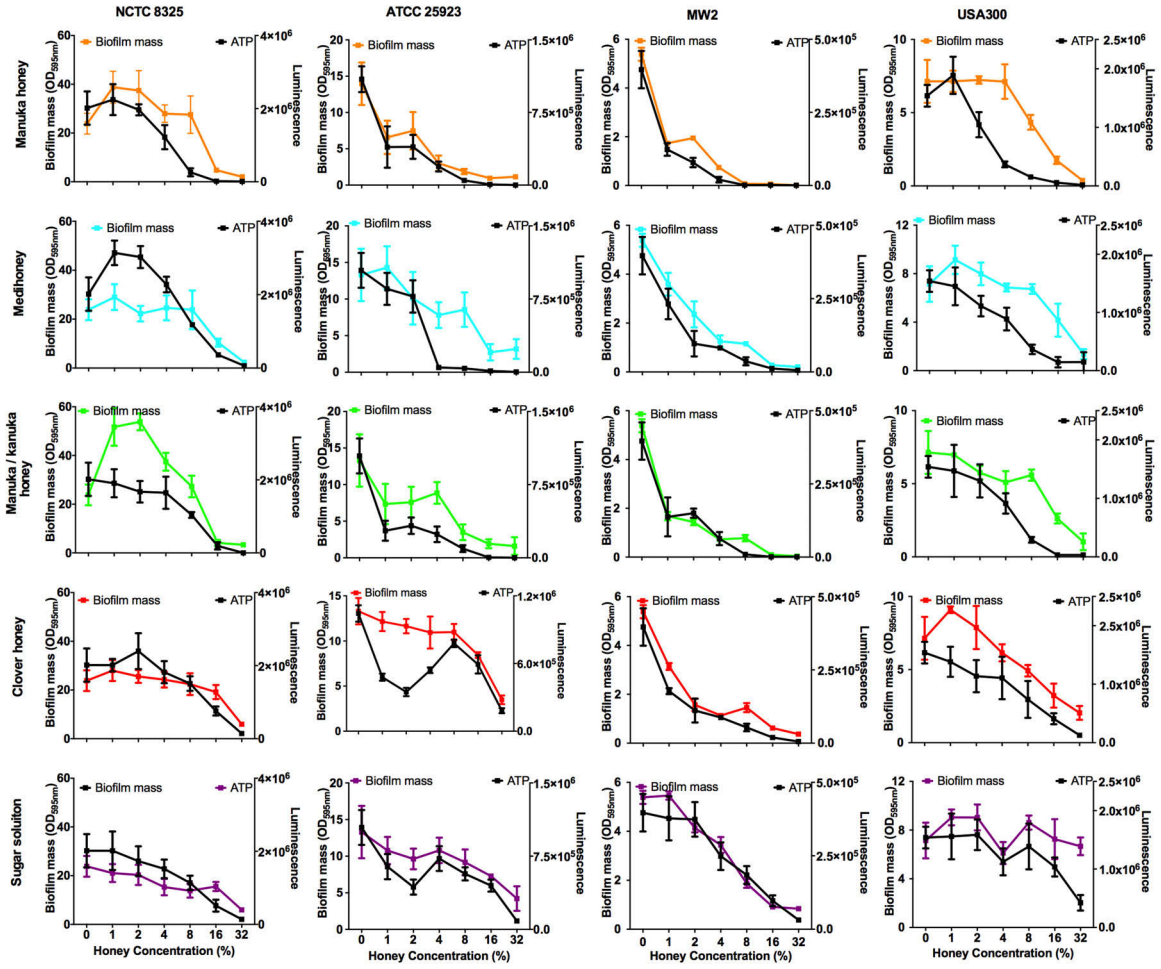


Figure 3.5 Effects of NZ honeys on established *S. aureus* biofilms and cell viability within the biofilms.

Established *S. aureus* NCTC 8325, ATCC 25923, MW2 and USA300 biofilms were treated with NZ honeys – manuka, Medihoney, manuka/kanuka, clover, and a sugar solution. The remaining biofilm masses were quantified using crystal violet staining (left y-axis) and cell viability within these remaining biofilms were assessed using the BacTitre Glo Viability Kit (right y-axis). Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate.

NCTC 8325, the most efficient biofilm former out of all tested strains, gave a slightly different response toward honey treatment compared to the other three strains. Significant biofilm enhancement occurred in this strain at sub-inhibitory concentrations of manuka honey (1-2%) and manuka/kanuka honey (1-4%) ($p < 0.001$; Figure 3.5). In addition, this strain was the least sensitive to the manuka-type honeys. For example, at 8% manuka honey treatment, the NCTC 8325 biofilm

mass remained similar to the untreated control ($p > 0.05$; Figure 3.5), while the biofilms produced by the other three strains were significantly reduced at this concentration ($p < 0.001$; Figure 3.5). Furthermore, 32% manuka honey and Medihoney were required to eradicate established NCTC 8325 biofilm mass.

3.3.5 The effect of NZ manuka-type honeys on cell viability within S. aureus *biofilms*

Elimination of biofilm mass was assessed using crystal violet, a cationic dye that stains all the components of the biofilm. However, this assay cannot assess the viability of cells remaining within the biofilm structure [158]. To determine this, we used a BacTitre Glo assay, which measures ATP levels as a proxy for viability. Side-by-side CFU measurements showed that the level of ATP detected in these assays was proportional to the count of viable cells per well, in the range 10^3-10^7 CFU/well (Figure 3.1).

The viability of cells remaining in the biofilm after the various treatments is shown in Figure 3.5. In general, cell viability decreased as the elimination of biofilm biomass increased (Figure 3.5, black lines). However, several exceptions to this general trend were observed. In some cases, biofilm biomass increased but cell viability did not, e.g. NCTC 8325 biofilms with low concentrations of manuka (2%) and manuka/kanuka honey (1-4%) (Figure 3.5). In others, biofilm biomass remained relatively constant while cell viability increased, e.g. NCTC 8325 with 1-4% Medihoney ($p < 0.05$; Figure 3.5), and ATCC 25923 with 4% and 8% clover honey. Another deviation from the general trend was a significant reduction of cell viability while biofilm biomass remained unaffected, seen for NCTC 8325 and USA 300 with 4% and 8% manuka honey treatment (Figure 3.5; $p < 0.05$). This emphasizes the importance of assessing viability alongside crystal violet assays for biofilm assessment.

Overall, at concentrations easily attainable in the clinic, the tested four NZ honeys were effective at eliminating biofilm biomass and at killing both MSSA and MRSA *S. aureus* cells in the residual biofilm. Among the honey types, manuka honey was the

most effective, where the elimination of biofilm biomass largely paralleled the reduction in viability. Following treatment with 8% manuka honey only ~10% of cells were viable in the remaining ATCC 25923 and USA 300 biofilms, compared to the untreated control (i.e. 0% honey), and no generation of ATP could be detected from MW2 (Figure 3.5). This is similar to the degree of biofilm biomass removal, where 85-98% of biofilm biomass was removed following 8% manuka honey treatment. Although NCTC 8325 biofilm biomass was seemingly unaffected at 8% manuka honey compared to the untreated control (Figure 3.5), the number of viable cells detected within this biofilm was drastically reduced by approximately 80% ($p < 0.001$; Figure 3.5).

3.3.6 The effect of MGO on established S. aureus *biofilms*

To assess the contribution of MGO alone, as well as MGO plus sugar, to biofilm removal, these components were tested on established *S. aureus* NCTC 8325 biofilms (Figure 3.6). MGO levels equivalent to the presence of 1-8% manuka/kanuka honey (Table 3.1) caused biofilm biomass to increase approximately 2-fold, relative to the untreated control ($p < 0.001$). However, the established biofilm biomass was not reduced significantly ($p > 0.05$), for any of the tested concentrations (1-32%) of MGO by itself, or in combination with the sugar solution. Thus, neither MGO nor the combination of MGO with sugar is solely responsible for the elimination of biofilms observed with these manuka-type honeys.

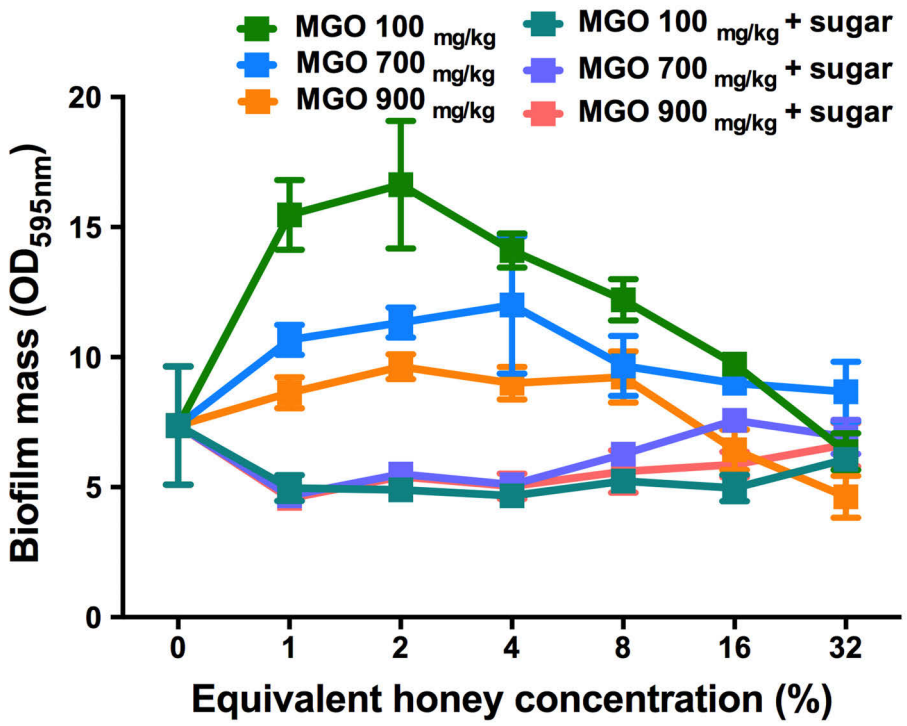


Figure 3.6 Effects of MGO on established *S. aureus* biofilms.

S. aureus NCTC 8325 biofilms were treated with MGO and a combination of MGO and the sugar solution. MGO stock solutions were prepared to correspond to the MGO levels in undiluted honey (100 mg/kg of manuka/kanuka honey, 700mg/kg of Medihoney, and 900 mg/kg of manuka honey; Table 3.1). The crystal violet stained residual biofilm mass after 24 h treatment was quantified using optical density (OD_{595nm}). Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate.

3.3.7 Visualizing the effects of NZ manuka-type honeys on established S. aureus *biofilms*

To assess the effect of the NZ honeys on *S. aureus* NCTC 8325 biofilms at the cellular level, we used confocal laser scanning microscopy (CLSM) of biofilms stained with fluorescent dyes for the detection of live and dead bacteria. This allows visualization of individual cells within the biofilm in three dimensions and allows the effect of treatments on cell viability to be determined. Treatment by sub-inhibitory (1% and 2%) and inhibitory (16% and 32%) concentrations of NZ honeys was visualized by viewing fluorescently-labelled live (Syto9; green) and

dead (propidium iodine; red) cells. Representative images of each treatment are presented in Figure 3.7 and quantification of live and dead cell biofilm biomass for several samples for each treatment is shown in Figure 3.8. In general, the established biofilm biomass decreased with increasing concentrations of manuka-type honey. More specifically, manuka honeys were effective in reducing the live cells in established *S. aureus* biofilms. Sub-inhibitory concentrations of all the manuka-type honeys (1% and 2%) and the sugar solution did not reduce the amount of biomass compared to the non-treated control cells (Figure 3.8). This is shown in Figure 3.7 where the untreated control cells displayed a green (live-cell) lawn that covered nearly the entire surface and this remained following treatment with 1% and 2% manuka-type honey. At concentrations of 16% and 32%, the manuka-type honeys substantially reduced the density and depth of the biofilm, along with the amount of live cells, compared to the untreated control (Figure 3.7 and Figure 3.8). For example, the 32% manuka honey significantly reduced the Syto9 stained (live) biofilm biomass to 10% ($p < 0.001$) compared to the non-treated live biofilm biomass (Figure 3.8) [163].

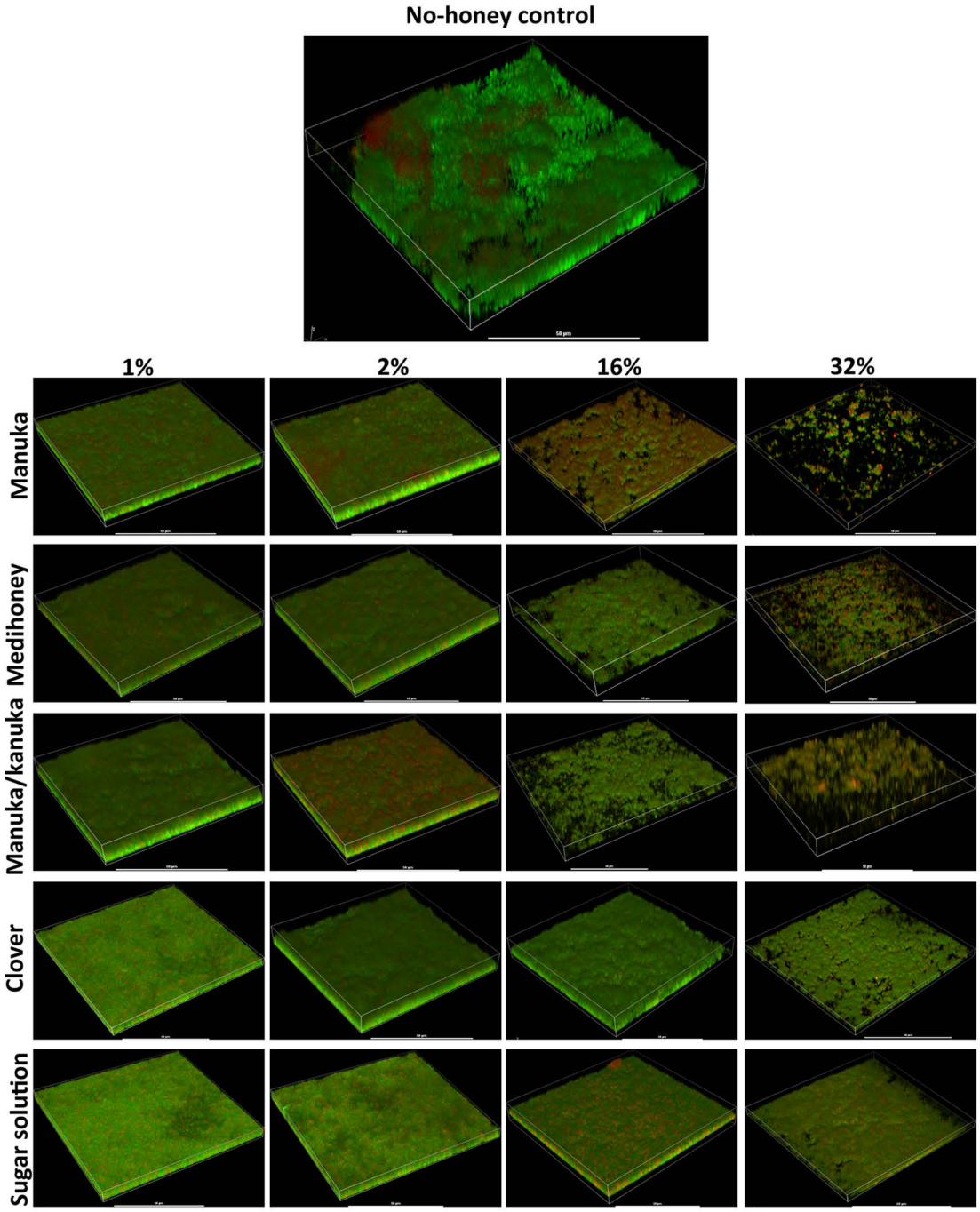


Figure 3.7 Live/dead staining of different honey treated established biofilms.

Established biofilms produced by *S. aureus* NCTC 8325 were treated with TSB containing honey (manuka, Medihoney, manuka/kanuka or clover) or sugar solution at 1%, 2%, 16%, and 32% (w/v). Syto9 (green; viable cells) and propidium iodine (red; dead cells) stained images were acquired using Nikon A1 Confocal Laser Scanning Microscope. The 3D- images were reconstructed using NIS-elements (version 10). Scale bar represents 50 µm.

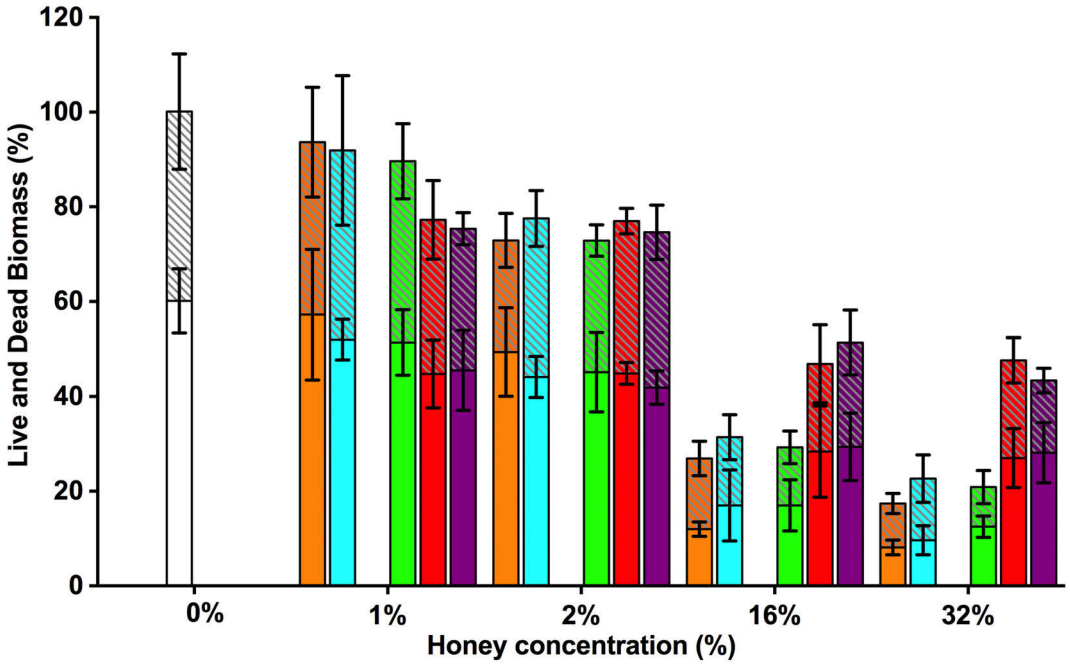


Figure 3.8 Quantitative analysis of live/dead stained honey treated biofilms.

The established *S. aureus* NCTC 8325 biofilm was treated with New Zealand honeys (manuka honey, Medihoney, manuka/kanuka honey, and clover honey) and a sugar solution at 1%, 2%, 16%, and 32% (w/v) concentrations. Biofilms were co-stained with Syto9 (S, viable cells) and propidium iodide (P, dead cells) and analyzed using COMSTAT. The estimated live (S) and dead (P) biomass (volume of the biofilm over the surface area ($\mu m^3/\mu m^2$)) are expressed as a percentage of the non-treated control live and dead biomass, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples where eight representative images were acquired.

Only small micro-colonies were present following treatment with 32% manuka honey, and the colour of the biofilms was predominantly yellow (where both the green and red dye were retained within cells), indicating mostly dead cells. In contrast, 32% clover honey and sugar solution reduced the total biomass by a maximum of 30% ($p < 0.001$) compared to the non-treated control (Figure 3.8). This result corresponds to the 3D reconstructed images, where the Syto9 stained cells remained dominant after treatment (Figure 3.7). At 32%, clover honey or sugar solution, a substantially larger Syto9 stained (live) lawn remained in comparison to the 32% manuka-type honeys, although the biomass was less confluent than in the untreated control. These results are consistent with the

results obtained with the crystal violet stained biofilm biomass and ATP viability assays.

3.3.8 Assessing the development of resistance to manuka-type honeys in S. aureus *biofilms*

Bacteria that are exposed to sub-inhibitory concentrations of antimicrobial agents generally develop resistance to these agents [166, 167]. The ability of cells released from *S. aureus* NCTC 8325 biofilms to develop resistance after exposure to sub-inhibitory concentrations of honey was investigated, and the results are summarized in Table 3.3. All cells recovered from the *S. aureus* biofilm after 24 h with 8% of all three manuka-type honeys were viable and able to form biofilms in media (TSB). However, they were unable to grow planktonically when subsequently exposed to 8% manuka, or 16% Medihoney and manuka/kanuka honey (the MIC levels for these honeys). Biofilm formation was also inhibited by 8% manuka honey, and by 16% Medihoney and manuka/kanuka honey. These growth- and biofilm-inhibitory concentrations of manuka-type honeys are the same as those observed for cells that had not previously been treated with these honeys. These results indicating that planktonic cells released from the biofilms with exposure to sub-inhibitory concentrations did not acquire resistance to the same honey treatment.

Table 3.3 Resistance of *S. aureus* cells recovered from biofilms after 8% manuka honey treatments[a]

Honey (%)	Type of assay	Manuka honey	Medihoney	Manuka/kanuka honey
0	Growth	✓	✓	✓
	Biofilm formation	✓	✓	✓
8	Growth	×	×	✓
	Biofilm formation	×	✓	✓
16	Growth	×	×	×
	Biofilm formation	×	×	×

[a] A tick means that there was normal growth or biofilm formation and a cross means that there was no growth or no biofilm formation.

3.4 Discussion

Chronic wounds are costly and difficult to treat [6, 102, 103], and bacterial biofilms are important contributors to the delay in healing. Honey is a promising alternative treatment for these wounds, and studies have indicated that it is able to prevent bacterial biofilms and eliminate established biofilms *in vitro* [62, 104, 106, 153]. However, the effective concentration of honey reported by these studies varies significantly, making it hard to establish a foundation for the efficacy of honey on chronic wound-associated bacterial biofilms in the clinic. This is probably largely due to the fact that, in most of these studies, very little information is reported on the honey itself, including the floral source, geographic location, storage conditions, and the level of the two principle antibacterial components, MGO and hydrogen peroxide. To be effective as a wound treatment, honey must be standardized as much as possible in terms of the chemical and geographical definition. Here we utilize a suite of well-defined NZ honeys,

including manuka-type honeys (manuka, Medihoney and manuka/kanuka honey) and clover honey, to investigate their antibiofilm activity on a range of *S. aureus* biofilms that differ in their ability to form biofilms. We show that manuka-type honeys can be used to kill all MSSA and MRSA cells when present as a biofilm in a chronic wound, supporting the use of this honey as an effective topical treatment for chronic wound infections.

Our study has shown that prevention of *S. aureus* biofilm formation occurred at honey concentrations that also inhibit planktonic growth (Figure 3.3A-D; Table 3.2), suggesting that biofilm prevention was a consequence of planktonic growth inhibition, as opposed to any specific effects on biofilm development. Other studies have also shown that manuka-type honeys can inhibit bacterial biofilm formation, however, the concentrations required were higher than those reported to inhibit growth [62, 104, 106, 153].

We found that higher concentrations of all honeys were necessary to eliminate established biofilms compared to those needed for prevention, as assessed by both quantification of biofilm biomass and cell viability. Elimination of biofilms was visually confirmed using CLSM of fluorescently-stained live and dead cells. The sugar content of honey clearly causes some biofilm mass eliminatory effect, as the sugar solution and clover honey were able to eliminate established biofilms at high concentrations (32%), as has been shown in other studies [36, 168]. However, manuka-type honeys consistently achieved biofilm elimination at lower concentrations, suggesting that components specifically within manuka-type honeys contribute towards biofilm elimination. The concentrations of manuka-type honeys that show significant antibiofilm activity are easily achievable in the clinic, since honey dressings typically contain > 80% honey [150].

The use of assays for total biofilm biomass and cell viability to examine the effects of the various treatments on biofilm elimination afforded some other interesting observations. We observed that in some cases, sub-inhibitory concentrations of two of the manuka-type honeys enhanced biofilm formation; however, cell viability

did not increase. The biofilm reduction effect could be due to a stress response, as has been previously observed when bacteria are exposed to sub-inhibitory concentrations of antibiotics [169-172]. In other cases, no reduction of biofilm biomass was observed but cell viability was significantly reduced. This suggests that unlike antibiotics, the manuka-type honeys (or active components therein) are able to penetrate through the biofilm matrix, killing the bacterial cells whilst leaving intact matrix.

It is believed that MGO is the primary component in manuka-type honeys responsible for its antibiofilm activity [42, 43]. The effectiveness of the different manuka-type honeys tested here did increase with MGO content. However, the same degree of biofilm prevention and elimination could not be reproduced with equivalent amounts of MGO either alone or in combination with sugar. In the case of prevention, MGO alone was generally ineffective, although a significant amount of biofilm prevention was achieved in combination with sugar. This suggests that the MGO and sugar do contribute to biofilm prevention, but their effects are not as strong as those observed with manuka honey. This might possibly due to that fact that the pH of the tested MGO solutions was different to the diluted manuka honey solutions. Moreover, it is suspecting that MGO might work synergistically with other components in the honey matrix. It is therefore ideal to add MGO into a non-MGO honey like clover, with similar pH.

Unlike the three NZ manuka-type honeys, neither MGO alone nor MGO with sugar at honey-equivalent concentrations showed significant *S. aureus* biofilm elimination. This indicates that the ability of manuka-type honeys to eliminate biofilms of this organism is due to one or more components present in the honey other than MGO and sugar, such as low pH, hydrogen peroxide, phenolics and other unknown components [42, 43, 173, 174]. Interestingly, while the kanuka/manuka honey had a relatively high rate of hydrogen peroxide production compared to the manuka and Medihoney (Table 3.1), but low MGO levels, it was not any more active against biofilms of *S. aureus*. This suggests that, at least in this

study, hydrogen peroxide within these manuka-type honeys does not provide significant antibiofilm activity.

3.5 Conclusions

This study is the first to use a suite of well-characterized manuka-type honeys against a range of strains of *S. aureus* that differ in their ability to form biofilms. We demonstrate that: 1) =at very low levels, some honeys can enhance biofilm formation, this could be attributed by evoking from stress response similar to that seen with some antibiotics; 2) the ability to prevent or eliminate biofilms is influenced by MGO levels and the presence of sugar, but these alone do not account for all of the antibiofilm effect; 3) honey is able to reduce biofilm mass and also to kill cells that remain embedded in the biofilm matrix; and 4) planktonic cells released from biofilms following honey treatment do not have elevated resistance to honey. Taken together our results show that if used at an appropriate therapeutic level, manuka-type honey can be used to kill *S. aureus* when present as a biofilm in a chronic wound, supporting the use of this honey as an effective topical treatment for chronic wound infections.

Chapter 4

New Zealand honeys can inhibit and eliminate biofilms of *Pseudomonas aeruginosa* wound isolates

4.1 Introduction

Chronic wound management has a huge impact on the global health care system [4, 6], and microbial biofilms play a significant role in chronic wound infections [7]. While in some cases conventional antibiotics may be effective [7, 175, 176], bacterial biofilms are highly tolerant to conventional antibacterial agents [103]. As such, these biofilm-related infections are often persistent and very difficult to treat.

Chronic wounds are often colonized with polymicrobial biofilms, composed of multiple bacterial species, including *Enterococcus faecalis, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus spp.* and anaerobic bacteria [177]. *P. aeruginosa* is commonly isolated from these biofilms, and is also a well-studied model biofilm organism [124, 177]. It is a motile, Gram-negative, nosocomial opportunistic human pathogen [94, 178]. Colonization with *P. aeruginosa* has been shown to result in significantly larger wound size, compared to wounds without *P. aeruginosa* colonization [177], and many studies have shown *P. aeruginosa* to be a virulent wound colonizer [179-181].

Part of the reason that *P. aeruginosa* is so prevalent in chronic wounds is that it employs a wide variety of virulence factors. This includes its biofilm forming ability and the production of other factors, such as elastase, phospholipase C and exotoxin A [182-184]. *P. aeruginosa* is also often characterized by the unique toxins visible as pigments on growth media. For example, the toxin pyocyanin is a blue/green pigment with redox activity, which is able to kill nearby competing microbes [185]. Pyocyanin also has a wide range of effects on the host, such as

inhibiting cellular respiration and accelerating neutrophil apoptosis [186]. The combination of biofilm forming ability and virulence factor production can result in persistent and devastating infections.

Another aspect of *P. aeruginosa* biology, which makes infections difficult to treat, is the emergence of resistance to multiple antibiotics [14, 15]. For example, *P. aeruginosa* contains multidrug resistance (efflux) pumps, which are used to remove antibiotics from inside the cell before they can act on specific targets [187, 188]. However, studies have shown that *P. aeruginosa*'s resistance to antibiotics in biofilms does not depend on the presence of these pumps [189, 190], but is due to the physical structure of the extracellular biofilm matrix, which makes it nearly impossible for antibiotics to penetrate through. These factors combine to make *P. aeruginosa*-associated chronic wound infections very difficult to treat with conventional antibiotics [191, 192]. It is therefore crucial to seek alternative wound management agents that are effective against *P. aeruginosa* biofilms.

Honey is known to have antibacterial and wound healing properties, and amongst all different types of honeys, the New Zealand manuka-based honeys are the most studied [25, 39, 48]. They demonstrate potent antibacterial activities against a range of bacteria [22, 30, 75], including growth and biofilm inhibition against *P. aeruginosa in vitro* [62, 77, 104-108]. The reported effective concentrations for antibiofilm activities of medical-grade manuka honey vary between studies (ranging from 12 – 50%) [104, 105]. Direct comparison among studies is difficult, partly due to the lack of reported detail with regards to the honey samples used. As reviewed in other studies, not all manuka honeys are the same, as they vary in the levels of known antibacterial components such as hydrogen peroxide, methylglyoxal and bee defensin-1 [30, 32, 34, 35]. Furthermore, different strains of bacteria have been used amongst studies, often with varying assay conditions [30, 32, 104, 105].

In this work, the antibiofilm study presented in Chapter 3, showed that NZ manuka-type honeys were effective against *S. aureus* biofilms. The study in

Chapter 2 showed that *P. aeruginosa* displayed a different susceptibility profile to manuka-type honeys compared to other tested organisms, including *S. aureus*. It is therefore important to determine whether manuka-type honeys are effective against *P. aeruginosa,* and whether this species displays a similar or different susceptibility profile to that observed with *S. aureus* in Chapter 3.

Thus, the aim of this chapter is to determine the effects of four well-characterized NZ honeys, against *P. aeruginosa* cell growth and biofilm formation, using similar experimental approaches as against *S. aureus* in Chapter 3. Three of the tested NZ honeys are manuka-type honeys, which contain various levels of the well-known antibacterial components hydrogen peroxide and methylglyoxal, while one does not (clover honey). Sugar solution was also included in this study as a control for the effect of the sugar content of the honeys. Therefore, the other aim of study in this chapter is to determine the likely responsible components of the antibacterial and antibiofilm activity of the tested NZ honeys, with respect to any activity against *P. aeruginosa*.

4.2 Materials and Methods

4.2.1 Honey samples

The New Zealand (NZ) honey samples used in this study are listed in Table 3.1, and include the three manuka-type honeys: monofloral manuka, Medihoney (a manuka-based medical-grade honey; Comvita Ltd), a manuka/kanuka blend, and clover honey (a white New Zealand honey). All honey samples were supplied by Comvita New Zealand Ltd. (Te Puke, New Zealand). The harvesting and geographic information for these honeys as well as the levels of the three major antibacterial components, methylglyoxal (MGO), di-hydroxyacetone (DHA), and hydrogen peroxide, have been described in the previous chapter (Table 3.1). All samples were stored in the dark at 4^{o}C upon arrival and were diluted in Cation-adjusted

Mueller-Hinton Broth (CAMHB) immediately before use in assays. All honey concentrations are expressed as % w/v unless otherwise indicated.

4.2.2 Other tested solutions

A series of other solutions were included for investigation along with the honey samples: i) A sugar solution designed to mimic the concentration and composition of honey sugars, to control for the effects of sugar (45% glucose, 48% fructose, 1% sucrose), diluted as above for honey. ii) MGO diluted in CAMHB to concentrations broadly corresponding to concentrations of those present in the manuka-type honeys: 100 mg/kg as in manuka/kanuka (active MGO concentration is 161 mg/kg), 700 mg/kg as in Medihoney (active MGO concentration is 776 mg/kg), and 900 mg/kg as in manuka honey (active MGO concentration is 958 mg/kg) to assess the effect of MGO alone on bacterial biofilm formation. iii) MGO diluted in sugar solution to the same concentrations as (ii), to assess the effect of MGO in the presence of a similar sugar content as in honey. MGO was obtained as a ~40% (w/v) solution in water (Sigma-Aldrich Co., MO, USA).

4.2.3 Hydrogen peroxide assay

The level of hydrogen peroxide produced by the NZ honey samples was determined using a hydrogen peroxide/peroxidase assay kit (Amplex Red, Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA) as previously reported [193].

4.2.4 Bacterial strains and growth conditions

Two commonly used laboratory reference strains of *P. aeruginosa* were examined. PAO1 and PA14, which were originally isolated from burn wounds [194, 195]. Both *P. aeruginosa* strains were grown in CAMHB at 37^{o}C.

4.2.5 Susceptibility of P. aeruginosa *to NZ honeys: minimum inhibitory concentrations (MICs)*

In this study, the minimum inhibitory concentrations (MICs) of NZ honeys on *P. aeruginosa* planktonic cell growth were determined according to CLSI standard protocol for dilution antibacterial susceptibility test (M07-A8, vol. 29 no. 2) with modifications. *P. aeruginosa* strains were cultured in 2 mL of CAMHB with shaking (250 rpm) overnight at 37°C. A suspension from the overnight culture was diluted to a cell density of approximately 10^7 CFU/mL in fresh CAMHB containing the appropriate test solution (honey or sugar) to give a final volume of 150 µL. For each of the assays, stock concentrations of the four different NZ honeys were prepared fresh prior to the assay in CAMHB. The stock concentrations were subsequently diluted down to working concentrations, in serial 2-fold dilutions, ranging from 1-32%. CAMHB media alone, i.e. without test solution was included as a control. The suspension was added to each well of a 96-well tissue culture treated microtitre plate (BD Falcon, NJ, USA), which was sealed with AeraSeal (Excel Scientific, CA, USA) and incubated in a humidified incubator for 24 h at 37°C. Following this, the cell growth was assessed by reading the optical density at 595 nm (OD_{595}) with a microplate reader (VersaMax, Molecular Devices, California, USA). The MIC was defined as no growth compared to untreated control. Unless otherwise stated, all assays were performed with three biological replicates and three technical repeats of each replicate in this study.

4.2.6 Biofilm formation assays

The effects of NZ honeys and other solutions on *P. aeruginosa* biofilm formation were determined using static biofilm formation assays in microtitre plates according to published studies with the following modifications [161]. *P. aeruginosa* overnight growth, suspension, and inoculation were the same as described above for MIC determination. The tested solutions were honey (four NZ honeys), sugar, MGO alone, or MGO in combination with sugar. After 24 h incubation at 37°C, the microtitre plates with biofilm were washed three times

with sterile phosphate buffered saline (PBS) to remove unattached cells using a programmed microtitre plate washer (Bio-Tek, ELX405, Winooski, VT, USA). The plates were then stained with 0.2% (w/v) crystal violet at room temperature for 1 h, and excess crystal violet solution was washed using the same washing program as with PBS. The stain that was bound to the adherent biofilm biomass was resolubilized with 200 µL 33% (v/v) acetic acid and transferred into a new 96-well microtitre plate to measure the OD_{595}. In this study, the MBIC (minimum biofilm inhibitory concentration) was determined as the concentration at which the measured OD_{595} was less than or equal to 5% of that of the untreated control, indicating at least 95% inhibition of biofilm formation.

4.2.7 Biofilm eradication assays

P. aeruginosa biofilms were first allowed to form in the wells of a 96-well microtitre plate for 24 h at 37°C in CAMHB media as described above. They were then washed three times with PBS. Various concentrations (from 0%, 1%, 2%, 4%, 16% and 32%) of the four honeys and other test solutions were then added to the established *P. aeruginosa* biofilms. The assay plates were incubated for a further 24 h at 37°C, and the biofilm biomass was quantified using crystal violet as described above (section 4.2.6).

4.2.8 Determination of bacterial cell viability in biofilms

Crystal violet is a cationic dye that stains all the components of the biofilm matrix and can therefore only assess the influence of a treatment on the total mass of the biofilm but not the viability of cells remaining within the biofilm structure [158]. The viability of cells remaining within established *P. aeruginosa* biofilms following honey treatment was determined using an ATP activity-based BacTitre Glo Microbial Cell Viability Assay Kit (Promega, WI, USA). The assay reagents lyse the bacterial cells to release intracellular ATP, the levels of which are quantified via a luminescence-based luciferase activity assay [159, 160]. This assay added

important additional information to the quantification of biofilm biomass using the crystal violet dye. After treatment, bacterial biofilms were washed as described above (section 4.2.7), followed by incubation with BacTitre Glo reagent in CAMHB for 10 minutes at 37^{o}C in the dark. The contents of each well were then transferred into white solid-bottom 96-well microtitre plates (Cellstar, Greiner Bio-one, France) for luminescence measurement. Luminescence, which is proportional to the amount of ATP produced by metabolically active cells, was recorded using a 96-well microplate reader (TeCan, Infinite 200Pro, Männedorf, Switzerland).

To ensure the validity of the ATP-based BacTitre Glo assay, a standard curve was constructed that assessed the correlation between bacterial cell numbers and the luminescent signal in the biofilm; this was performed on the untreated control (containing *P. aeruginosa* in CAMHB media only). Biofilms produced were washed as above and cells within the biofilm dispersed using a small-probe sonicator (Sonics and Materials VC-505) to enable quantification by direct enumeration [161]. The sonicator microtip was inserted into each wells and sonicated for 8 sec at 40% power. The recovered cell suspension was serially diluted 10-fold and 20 µL aliquots were plated on 1.6 % high-salt Luria Broth agar (HS-LBA) for measurement of Colony Formation Units (CFUs). Luminescence of cells in the remaining suspension was assessed using the BacTitre Glo kit. From this, a correlated standard curve was constructed between calculated CFU/well and the relative luminescence readings. According to the standard curve shown in Figure 4.1, the detection limit of the BacTitre Glo is at a luminescence reading below 1000, which is equivalent to 10^3 CFU/well (linear range from 10^3 -10^7 CFU/well). An upper limit was not determined.

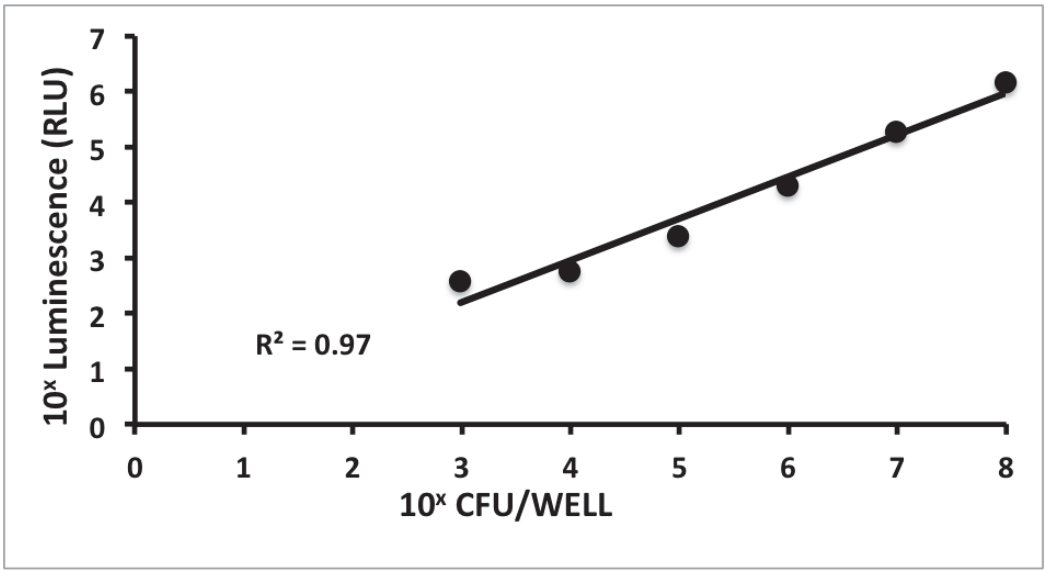


Figure 4.1 Relationship between levels of intracellular ATP to colony forming units (CFU) in static biofilms of *P. aeruginosa*.

Static biofilms of *P. aeruginosa* were allowed to form in the wells of a microtitre plate for 48 h (with media replenishment at 24 h). After removal of the biofilm from the wall of each well, the cells within were recovered using sonication, to break open the biofilm matrix. Intracellular ATP levels were measured by the BacTitre Glo Viability Kit. The viable count (CFU) of the recovered cells for each well was also enumerated using a microdilution plate method on HS-LBA plates. The intracellular levels of ATP are plotted as a function of CFU and validate that the BacTitre Glo Viability Kit can be used as a surrogate measure of biofilm cell viability in subsequent assays.

4.2.9 Visualizing live/dead stained P. aeruginosa biofilms using confocal laser scanning microscope (CLSM)

P. aeruginosa biofilms were treated with CAMHB containing 1%, 2%, 16%, and 32% NZ honeys or sugar solution for 24 h in black polystyrene 96-well microtitre plates with µClear bottoms (Cellstar, Greiner Bio-One, France) as described above in 4.2.7. However, the biofilm biomass was not quantified by staining with crystal violet solution. Instead, the honey- and sugar solution-treated biofilm biomasses were washed three times with PBS and cells within the biofilm structure were fluorescently stained with 2.5 µM Syto9 (Invitrogen, CA, USA) and 4.3 µM propidium iodine (PI) (Becton Dickinson, NJ, USA), which stains live and dead cells

in the biofilm structure, respectively. After 30 min of incubation in the dark at room temperature, the wells were washed thoroughly with PBS and fixed with 4% w/v paraformaldehyde (Sigma-Aldrich, MO, USA) for 15 minutes. The wells were then rinsed and stored in PBS for imaging.

Since higher concentrations of honey are commonly used in the commercially available dressings, high concentrations of MGO monofloral manuka honey and the commercially available Medihoney were included to determine the effect of these higher concentrations (64% and 80%) of these honeys on established *P. aeruginosa* PAO1 and PA14 biofilms.

Biofilms were imaged using confocal laser scanning microscopy imaging (CLSM) on a Nikon A1 confocal microscope. The Syto9 and PI fluorophores were excited at 488 nm and 561 nm, and the emissions were collected at 500-550 nm and 570-620 nm, respectively. Representative presentation image stacks of each treatment were acquired at a resolution of 1024 x 1024 pixels and three dimensional biofilm images reconstructed using NIS-elements (Version10, Nikon Instruments Inc., USA). It should be noted that due to the incomplete displacement of Syto9 by propidium iodine in dead cells that there will remain some Syto9 staining of dead cells. Therefore the absolute level of live cells detected in the Syto9 channel will be somewhat overestimated using these co-staining methods [163].

4.2.10 Test for the development of resistance to honey treatment

The development of resistance to antibacterial compounds in clinical settings is increasingly alarming. In particular bacteria are more likely to become resistant after exposure to such compounds at sub-inhibitory concentrations [164, 165]. The planktonic cells that appeared post-24 h exposure to sub-inhibitory concentrations of (8%) manuka-type honey treatments, from biofilm eradication assay, were assumed derived from the biofilm matrix. These cells were collected by aspirating the liquid from the top of the biofilm, and were tested for their ability to grow and form biofilms in the presence of inhibitory concentrations (16% and

32%) of honey under the static growth conditions described above (section 4.2.5 and 4.2.6). Cell growth was defined as not detected when the $OD_{(x)}$ - $OD_{(\text{media only blank})}$ ≤ 0.1 and MBIC was defined as described above (section 4.2.6). Each experiment was performed with three biological replicates and three technical repeats of each biological replicate.

4.2.11 pH of the tested NZ honeys, sugar, and other tested solutions

Low pH is one of the antibacterial characteristics of honeys. The pH of all the tested solutions was determined using a pre-calibrated pH meter (Radiometer Analytical, MeterLab®, PHM210, Lyon, France). The tested solutions include the four NZ honeys, sugar, and the three manuka-type honey equivalent MGO solution alone, or in combination with sugar. All solutions were diluted and prepared at the concentrations used in this study (ranging from 0%, 1%, 2%, 4%, 8%, 16% and 32%).

4.2.11 Statistical analysis

Data sets tested and passed the normality test (alpha = 0.05) for Gaussian distributions using D'Agostino-Pearson normality test in GraphPad Prism versions 5 and 6. Further statistical analysis to determine significant differences between treatments and among honey samples were performed using One-Way ANOVA with Tukey Test in GraphPad Prism versions 5 and 6. Statistical significance was set at $p < 0.05$.

4.3 Results

4.3.1 The effects of NZ honeys and sugar solution on P. aeruginosa *cell growth and biofilm formation*

The effects of the four NZ honeys and other honey components on *P. aeruginosa* on both planktonic cell growth and biofilm formation were examined. The two strains of *P. aeruginosa* tested in this study were isolated from wound sites, and are also commonly used as standard reference strains for *P. aeruginosa* biofilm related studies. These two strains differ in their biofilm forming ability (Figure 4.2), representing a range of *P. aeruginosa* biofilms, and how they respond to various honey treatments. All honeys were effective at inhibiting cell growth and biofilm formation of the two wound isolates of *P. aeruginosa,* PAO1 and PA14.

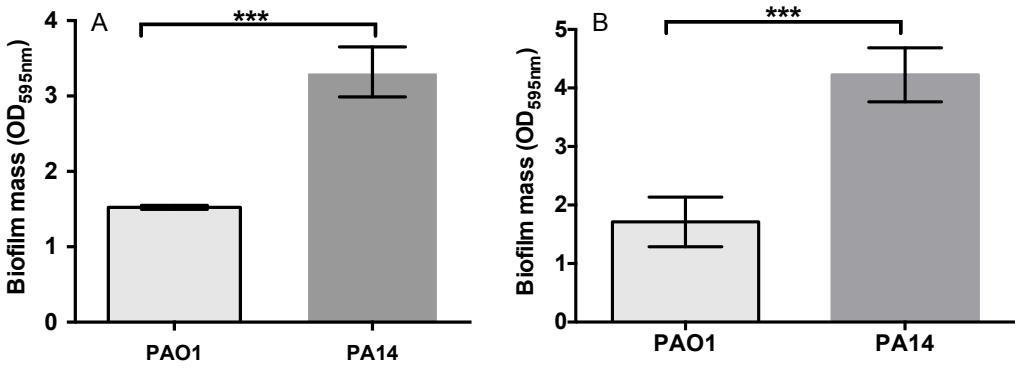


Figure 4.2 Quantification of *P. aeruginosa* biofilm adherence

Biofilm adherence was determined using a static biofilm formation assay over a 24 h period (A) and a further 24 h incubation (total of 48 h) was also performed with replenished fresh media after the first 24 h incubation (B). Biofilm adherence was quantified by staining with 0.2% crystal violet solution and measured at an optical density of 595 nm. Error bars represent ± standard deviation (SD) of three biological samples performed in triplicate, *** represents $p < 0.001$, compared between the two strains. This was assessed by One-Way ANOVA with Tukey test after confirming the normality of the data set for each, using D'Agnostino-Pearson normality test.

The planktonic growth of both *P. aeruginosa* PAO1 and PA14 was completely inhibited by 16% of all three manuka-type honeys, whereas the MIC for clover

honey was 32% (Table 4.1). Sugar solution also inhibited PAO1 planktonic cell growth at 32%. However PA14 planktonic cell growth was not completely inhibited (less than 95% of the growth was inhibited compared to control) at any of the tested sugar concentrations (1% - 32%) (Table 4.1).

Table 4.1 Minimum inhibitory concentrations of NZ honeys and sugar solution on inhibiting *P. aeruginosa* PAO1 and PA14 cell growth and biofilm formation.

Honeys	PAO1		PA14	
	MIC*	MBIC^	MIC	MBIC
Manuka	16	16	16	8
Medihoney	16	32	16	16
Manuka/kanuka	16	16	16	16
Clover	32	32	32	16
Sugar solution	32	32	>32	32

* MIC = minimum inhibitory concentration

^ MBIC = minimum biofilm inhibitory concentration, defined as ≥ 95% biofilm inhibition compared to untreated control.

Like the cell growth inhibition, biofilm formation was effectively inhibited (≥ 95%, compared to untreated control) by the four tested NZ honeys, and by the sugar solution. Generally, the minimum biofilm inhibitory concentrations (MBICs) were the same as the MICs, where ≥ 95% biofilm formation was inhibited by 16% of all three manuka-type honeys. However, MBIC for PAO1 with Medihoney and PA14 with manuka honey were 2-fold higher (32%) and 2-fold less (8%) compared to the MIC (16%), respectively (Table 4.1). The MBIC for the clover honey and sugar solution was 32% (Figure 4.3). There were a couple of exceptions to this trend. For example, the MBIC for PA14 with clover honey was 16%, 2-fold less compared to an MIC of 32%. Moreover, the MBIC of sugar solution was 32%, in comparison, the MIC was determined to be higher than 32% (Table 4.1). Nonetheless, all tested honeys and sugar solutions were effective at inhibiting planktonic cell growth and biofilm formation of *P. aeruginosa* at 32%.

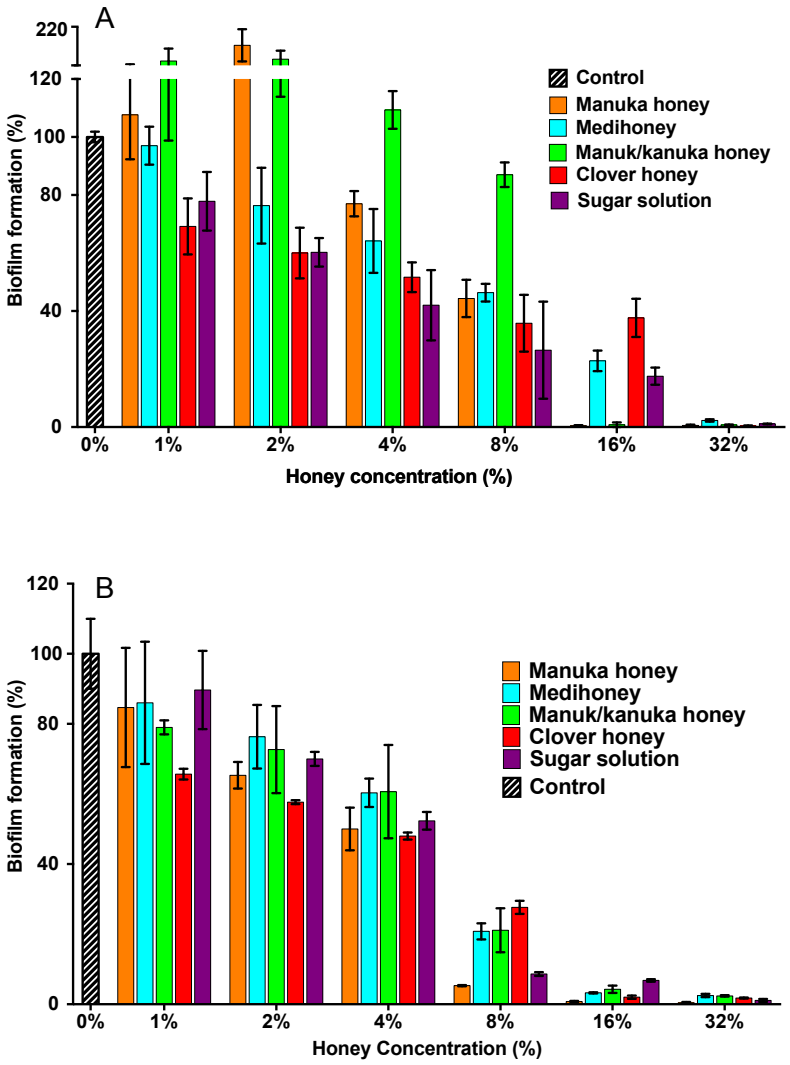


Figure 4.3 Effects of NZ honeys and sugar solution on *P. aeruginosa* biofilm formation.

P. aeruginosa biofilms were formed in the presence of four different NZ honeys (manuka, Medihoney, manuka/kanuka, or clover), or a sugar solution. Biofilm formation was assessed using a static biofilm formation assay with crystal violet staining to quantify biomass. The *P. aeruginosa* strains are (A) PAO1 and (B) PA14. Biofilm formation is expressed as a percentage relative to that produced by the untreated control, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples preformed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

In some cases, biofilm enhancement was observed at lower concentrations (1% and 2%) of manuka and manuka/kanuka honeys (Figure 4.3). PAO1 biofilm

biomass was significantly enhanced ($p < 0.05$) by the sub-MBIC concentrations of manuka (2%) and manuka/kanuka (1% and 2%) honeys (Figure 4.3). This effect was not observed with PA14.

4.3.2 The effects of NZ honeys and sugar solution on established P. aeruginosa *biofilms*

Bacterial biofilms are often already established in chronic wounds, so it is important to know if honey is effective at eradicating already established *P. aeruginosa* biofilms. We therefore tested the ability of honey and other solutions to eradicate preformed biofilms of *P. aeruginosa*. The coloured lines in Figure 4.4 show the amount of biofilm biomass remaining following incubation with different concentrations of the various treatments. In general, significant ($p < 0.05$) reductions in biofilm mass were only observed at concentrations of 32% for most treatments, and in some cases at 16% (Figure 4.4). The two strains of *P. aeruginosa* showed different profiles of susceptibility at 16% treatment. For example, with the exception of manuka honey, less than a 40% reduction in biofilm mass was achieved for the other honeys (Figure 4.4 and Table 4.2), while all four NZ honeys and sugar solution caused larger reductions of established PA14 biofilms (≥ 75% biofilm reduction, compared to untreated control; Figure 4.4 and Table 4.2).

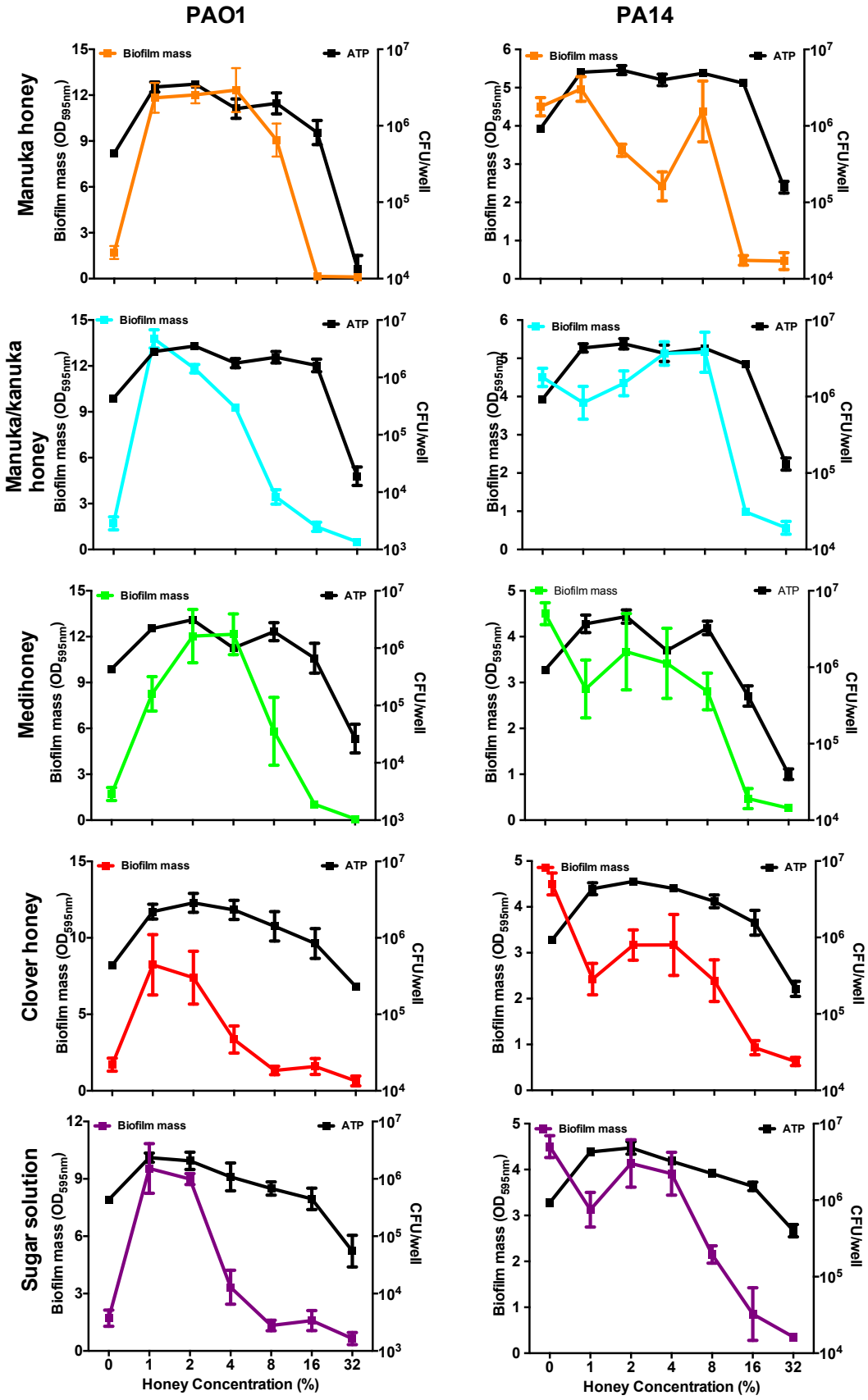

PAO1
PA14
Manuka honey
Manuka/kanuka honey
Medihoney
Clover honey
Sugar solution
Biofilm mass
ATP
Biofilm mass (OD595nm)
CFU/well
Honey Concentration (%)
0
1
2
4
8
16
32

Figure 4.4 Effects of NZ honeys and sugar solution on established *P. aeruginosa* biofilms and cell viability within the biofilms.

Established *P. aeruginosa* PAO1 (A) and PA14 (B) biofilms were treated with NZ honeys – manuka, Medihoney, manuka/kanuka, clover – or a sugar solution. The remaining biofilm masses were quantified using crystal violet staining and expressed as OD_{595} (left y-axis). The corresponding cell viability within these remaining biofilms was assessed using BacTitre Glo Viability Kit; the CFU/well values were interpreted according to the previously established standard curve (right y-axis). Error bars represent ± standard deviation (SD) of three biological samples preformed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

Table 4.2 Effects of 16% and 32% NZ honeys and sugar solution on *P. aeruginosa* biofilm biomass.

Honeys	PAO1				PA14			
	16%*		32%		16%		32%	
	Biofilm biomass	Cell viability	Biofilm biomass	Cell viability	Biofilm biomass	Cell viability	Biofilm biomass	Cell viability
Manuka	9%^	187%	6%	32%	12%	389%	12%	17%
Medihoney	87%	372%	29%	4%	25%	283%	14%	14%
Manuka/Kanuka	61%	153%	5%	6%	25%	283%	14%	14%
Clover honey	93%	194%	38%	53%	23%	168%	16%	23%
Sugar solution	93%	103%	38%	13%	21%	163%	9%	43%

* Honey concentrations in %, w/v

^ The after-treatment remaining biofilm biomass is expressed as a percentage relative to the untreated control, which is set at 100%.

It was also observed that sub-inhibitory concentrations (1-8%) of the four honeys and sugar solution significantly enhanced ($p < 0.05$) PAO1 biofilm biomass, but this effect was only seen with 4% and 8% Medihoney for PA14 (Figure 4.4).

The ATP-based viability assay was used to approximate the number of viable cells (ranging from 10^3 – 10^7 CFU/well; Figure 4.4), remaining within the biofilm after the various treatments (Figure 4.4, black lines). Table 4.2 expresses the percentage of cell viability, normalized to the untreated control, remaining after established biofilms were incubated with 16% and 32% honey solutions. In general cell viability only decreased significantly at 32% for all of the treatments. The lower detection limit of 10^3 CFU/ well of cells was not reached, meaning that more than 10^3 CFU/well remained alive in the remaining biofilm, even at a concentration of 32% honey or sugar solution (Figure 4.1, Figure 4.4 and Table 4.2). Despite the fact that 16% of manuka-type, clover honey and sugar solution reduced the established biofilm biomass of PAO1 and PA14 to some degree (Figure 4.4 and Table 4.2), cell viability either significantly increased ($p < 0.05$) or there was no change compared to the untreated control (16% sugar solution for PAO1) (Table 4.2). Additionally, sub-inhibitory concentrations (1% and 2%) of different honeys and sugar solution treatments enhanced PA14 cell viability within the established biofilms ($p < 0.05$), but not the biofilm biomass ($p > 0.05$) (Figure 4.1).

4.3.3. The contribution of MGO to inhibition and eradication of P. aeruginosa biofilms

Methylglyoxal (MGO) is thought to be the major antibacterial component of manuka-type honeys [31, 32], and has previously been demonstrated to be effective at both inhibiting biofilm formation and eliminating established *P. aeruginosa* biofilms [42]. To determine whether MGO is responsible for the antibiofilm activity of manuka-type honeys observed here, MGO was tested at broadly corresponding concentrations to those present in each of the manuka-type

honey samples (Table 3.1), both with and without the addition of the sugar solution (Figure 4.5 and Figure 4.6).

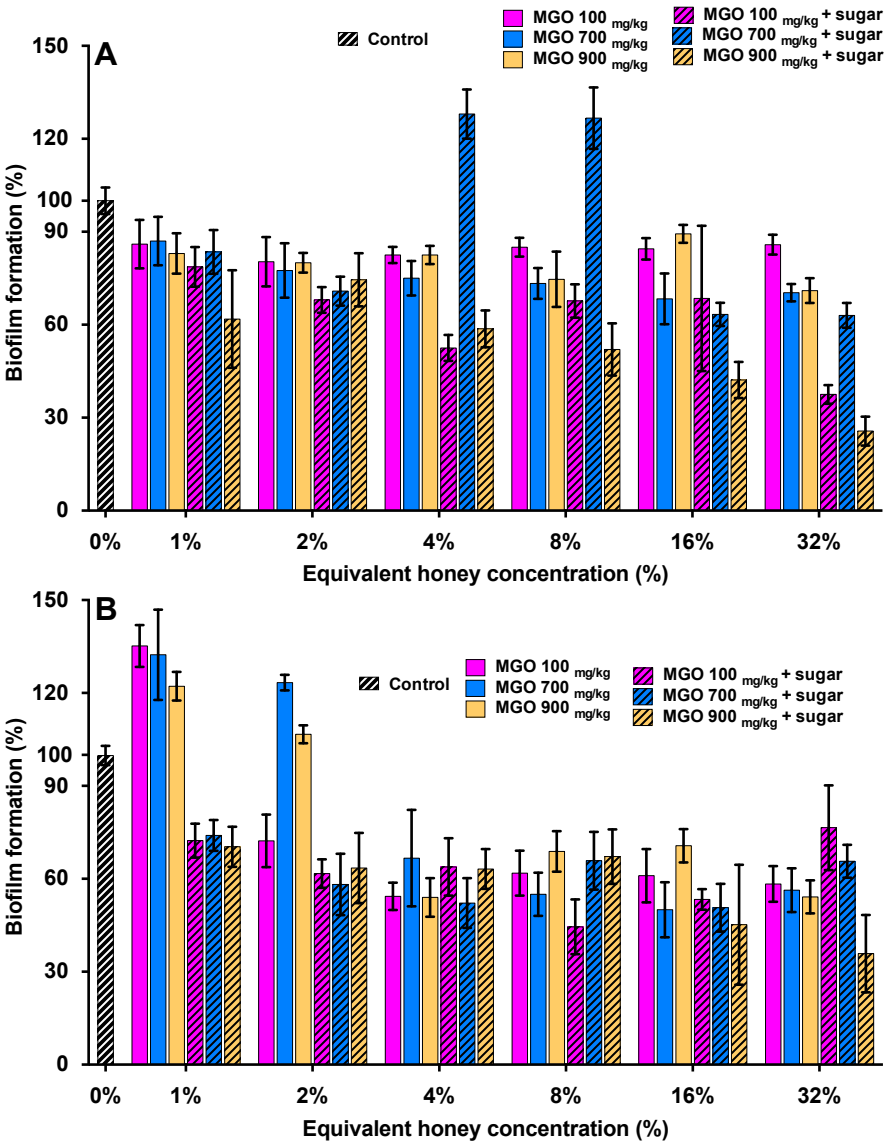


Figure 4.5 Effects of MGO on *P. aeruginosa* biofilm formation.

Biofilm formation by *P. aeruginosa* PAO1 (A) and PA14 (B) was grown in the presence of MGO and MGO plus sugar solution. MGO stock solutions were prepared to correspond to the MGO concentration in undiluted manuka-type honeys (100 mg/kg as in manuka/kanuka honey, 700mg/kg as in Medihoney, and 900 mg/kg as in manuka- honey; Table 3.1). Biofilm formation was assessed using the described static assay with crystal violet staining to quantify biomass. Biofilm formation is expressed as a percentage relative to the untreated control, set at 100%. Error bars represent deviation (SD) of three biological samples preformed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

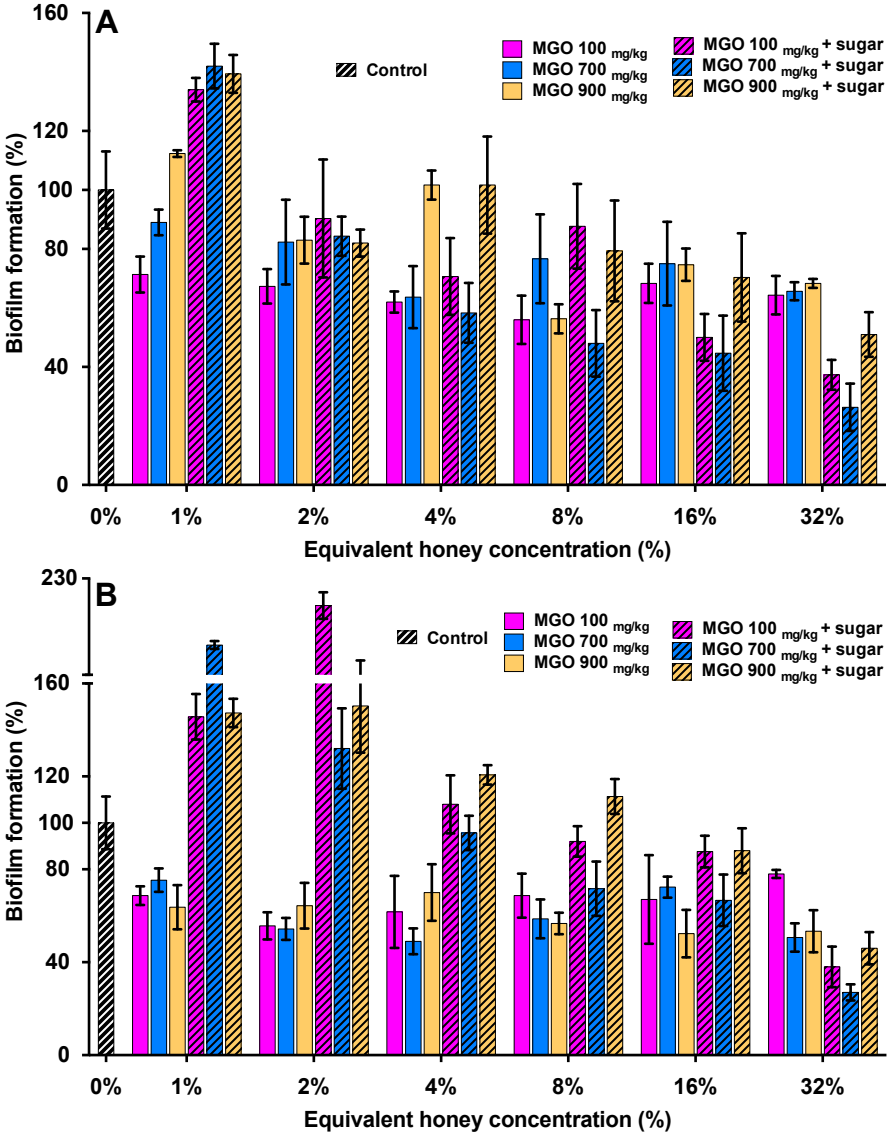


Figure 4.6 Effects of MGO on established *P. aeruginosa* biofilms.

P. aeruginosa PAO1 (A) and PA14 (B) biofilms were treated with MGO and a combination of MGO with sugar solution. MGO stock solutions were prepared to correspond to the MGO concentrations in undiluted honey (100 mg/kg as in manuka/kanuka honey, 700mg/kg as in Medihoney, and 1,000 mg/kg as in manuka honey; Table 3.1). The remaining biofilm biomass is expressed as a percentage relative to the untreated control, which is set at 100%. Error bars represent ± standard deviation (SD) of three biological samples preformed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

Whilst some reduction in biofilm formation was observed using MGO alone or in combination with sugar (Figure 4.5), the MGO solutions could not reproduce the biofilm inhibitory effects observed with manuka-type honeys (Figure 4.2 and Table

4.1). A maximum of 50% and 75% reduction in biofilm formation occurred at 16% and 32% MGO and MGO with sugar treatments, respectively, (Figure 4.5) compared to > 95% inhibition with honey and sugar solutions at the equivalent concentrations (Figure 4.3). Furthermore, the addition of MGO to the sugar solution appeared to counteract the effect of the sugar solution alone for biofilm formation inhibition.

In addition, both the *P. aeruginosa* PAO1 and PA14 biofilm biomasses were enhanced by sub-inhibitory concentrations of MGO alone or in combination with sugar solution, respectively. PAO1 biofilm mass was enhanced by 4% and 8% MGO (at the equivalent concentration to Medihoney) in combination with sugar ($p < 0.05$), where similar biofilm enhancement effect was observed at the same concentrations of Medihoney. PA14 biofilm formation was only enhanced by MGO in the absence of sugar, which was observed at 1% treatment for all MGO concentrations, and 2% at the Medihoney and manuka honey equivalent concentrations ($p < 0.05$) (Figure 4.5).

The contribution of MGO to the observed removal effects of manuka-type honeys on established *P. aeruginosa* biofilms was also assessed (Figure 4.6). Again, like the biofilm inhibitory assays, MGO at concentrations broadly corresponding to those found in the three manuka-type honeys (Table 3.1) did not result in a similar degree of biofilm biomass reduction as observed with manuka-type honeys (Figure 4.4 and Figure 4.6). Whilst some reduction in biofilm mass was observed in both strains for 32% MGO alone or in combination with sugar (Figure 4.6), the degree of reduction was less than compared to the manuka-type honeys at the equivalent concentrations (Figure 4.4 and Figure 4.6). In addition, MGO with sugar at 32% showed less removal of biofilm than 32% sugar alone (Figure 4.6; Figure 4.4 and Table 4.2).

4.3.4 Determining the pH of honeys, sugar, and other tested solutions used against P. aeruginosa *biofilms*

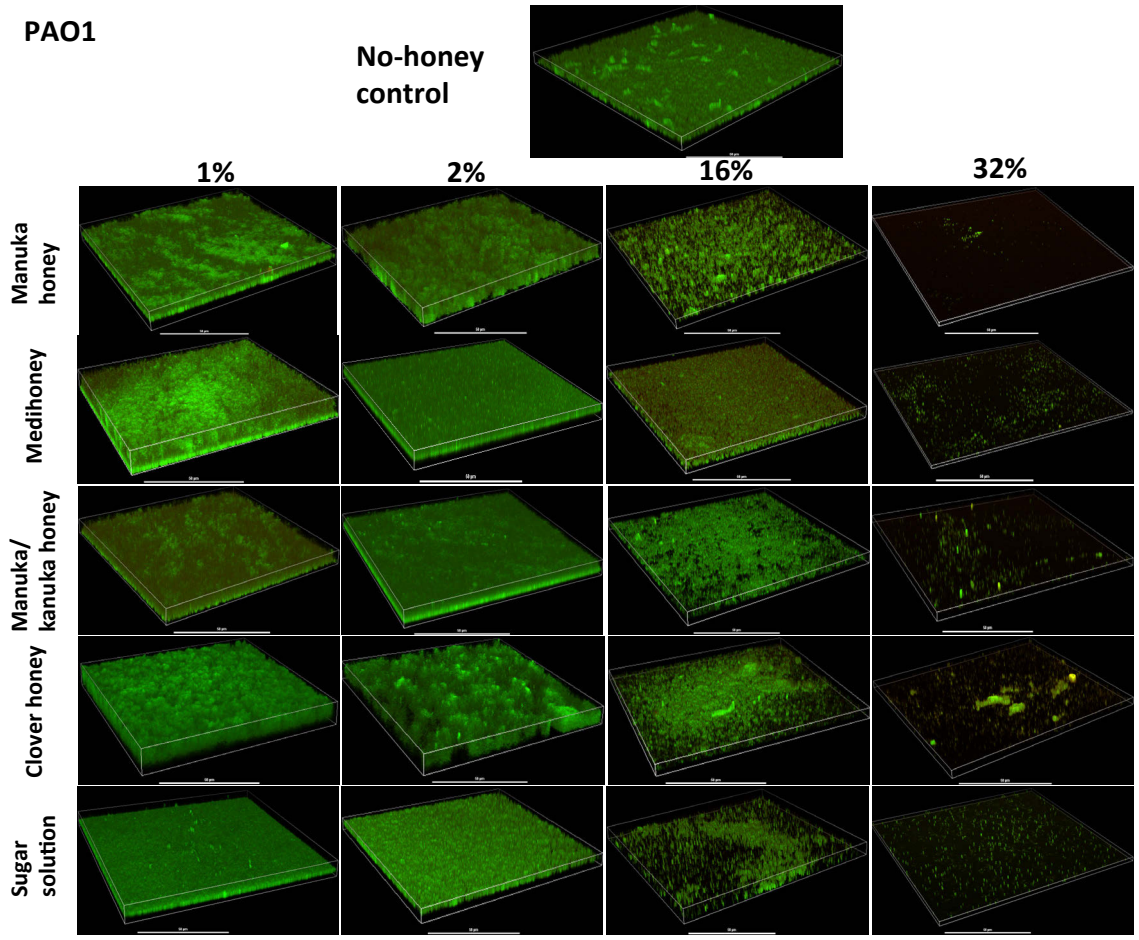
The pH of the various solutions was determined as the addition of MGO to sugar reduced the antibiofilm activity of sugar. It could possibly be due to a pH change in the sugar solution upon the addition of MGO, which might in turn decrease the antibiofilm activity compared to sugar alone. Thus, if the pH of the MGO or MGO plus sugar solutions was different to the pH of the honey solutions used, this could ultimately affect the results obtained. Therefore the pH of different honeys and test solutions used in this study were measured and results are presented in Table 4.3. The untreated control, CAMHB media, had a neutral pH of 7.2. All MGO solutions diluted in CAMHB media at concentrations equivalent to those tested for manuka-type honeys retained a neutral pH (~ 7.0 – 7.2) across the concentrations tested. All four NZ honeys and sugar solution, as well as the sugar with MGO (6.50 – 7.30) at lower concentrations, but displayed a relatively more acidic pH (ranging from 5.80 – 6.54) when present in more concentrated solutions (32%) (Table 4.3).

Table 4.3 pH of media and solutions used in this study.

Media and solutions	Concentrations					
	1%	2%	4%	8%	16%	32%
Manuka	7.13	7.15	7.13	7.04	6.88	6.54
Manuka / kanuka	7.22	7.22	7.14	7.05	6.81	6.31
Medihoney	7.14	7.14	7.09	6.97	6.76	6.26
Clover	7.15	7.16	7.13	7.07	6.92	6.59
Sugar	7.02	7.02	6.98	6.83	6.53	5.77
MGO 100	7.13	7.30	7.15	7.12	7.11	7.04
MGO 700	7.21	7.18	7.17	7.16	7.17	7.07
MGO 900	7.26	7.26	7.23	7.19	7.13	7.01
MGO 100 + sugar	7.18	7.14	7.07	6.88	6.52	5.81
MGO 700 + sugar	7.18	7.15	708	6.88	6.51	5.80
MGO 900 + sugar	7.20	7.14	7.05	6.85	6.50	5.80
CAMHB	7.20					

4.3.5 Visualizing the effects of NZ honeys and sugar solution on established P. aeruginosa *biofilms*

The effects of various NZ honeys and sugar treatments ((both sub- (1%, 2%) and inhibitory (16%, and 32%) concentrations)) were visualized using confocal laser scanning microscopy (CLSM). Higher, clinically obtainable concentrations of manuka honey and Medihoney (64% and 85%) were also tested. Cells were stained with fluorescent dyes for the detection of live (Syto9; green) and dead (propidium iodine; red) bacterial cells. The individual cells within the biofilm were visualized and representative images of each treatment are presented in three dimensions in Figure 4.7 and Figure 4.8.


PAO1
No-honey control
1%
2%
16%
32%
Manuka honey
Medihoney
Manuka/ kanuka honey
Clover honey
Sugar solution

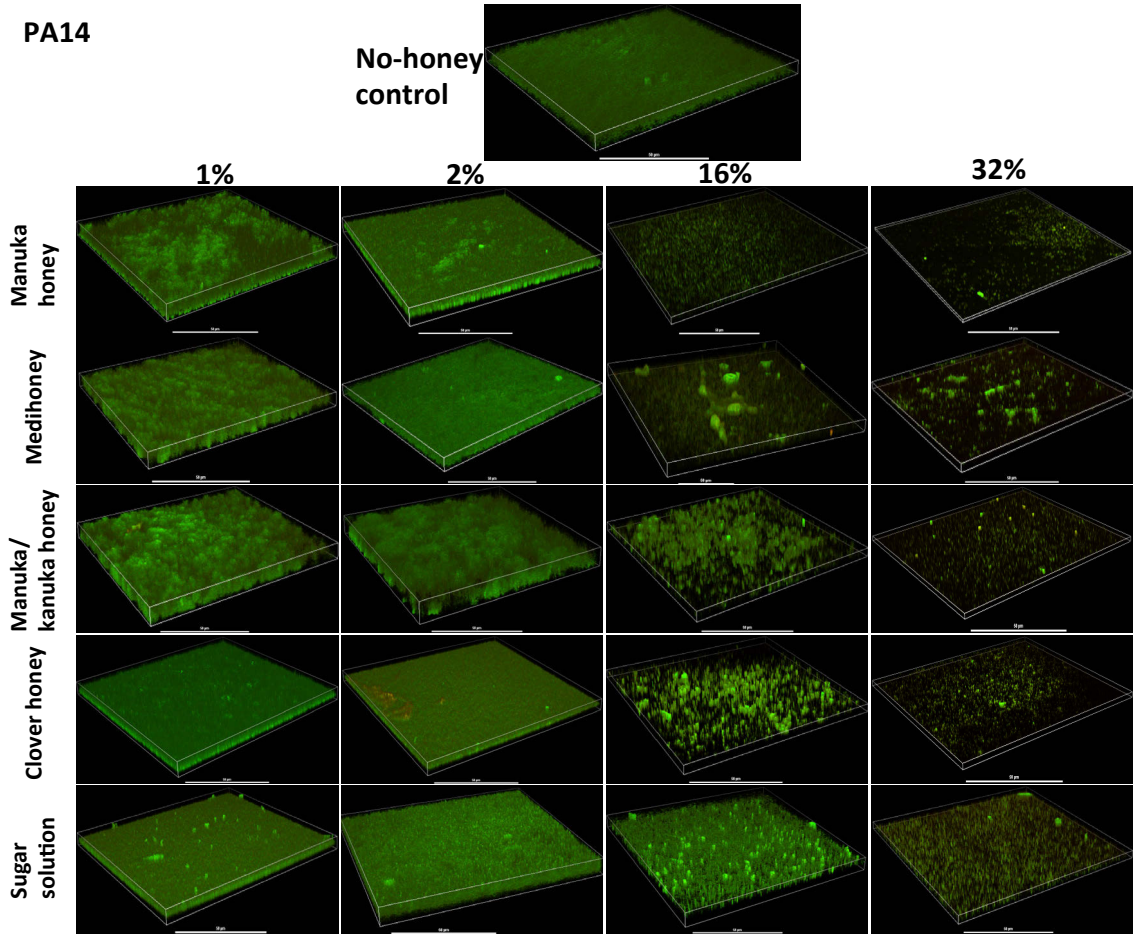


Figure 4.7 Live/dead staining of different honey treated established biofilms.

Established biofilms produced by *P. aeruginosa* PAO1 and PA14 biofilms were treated with CAMHB containing honey (manuka, Medihoney, manuka/kanuka, or clover) or sugar solution at 1%, 2%, 16%, and 32% (w/v). Syto9 (green; viable cells) and propidium iodine (red; dead cells) stained images were acquired using Nikon A1 Confocal Laser Scanning Microscope. The 3D images were reconstructed using NIS-elements (version 10). Scale bar represents 50 µm.

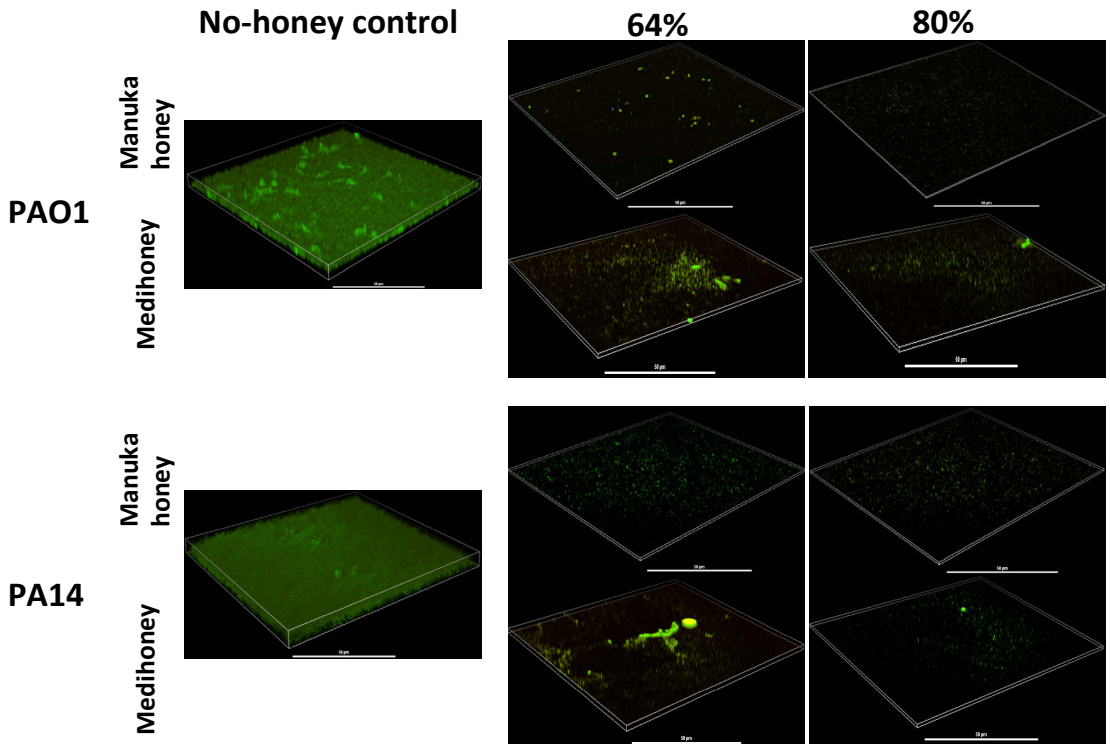


Figure 4.8 Live/dead staining of 64% and 80% of manuka and Medihoney treated *P. aeruginosa* established biofilms.

Established biofilms produced by *P. aeruginosa* PAO1 and PA14 biofilms were treated with CAMHB containing manuka and Medihoney at 64% and 80% (w/v). Syto9 (green; viable cells) and propidium iodine (red; dead cells) stained images were acquired using Nikon A1 Confocal Laser Scanning Microscope. The 3D images were reconstructed using NIS-elements (version 10). Scale bar represents 50 µm.

Generally, biofilm mass decreased with 16% and 32% of all four NZ honeys, and the sugar control. The untreated control displays cells in a green (live-cell) lawn that covers nearly the entire imaging surface. Similar biofilms were observed with 1% and 2% of all treatments, with red cells (dead-cells) becoming more visible in some cases (e.g. 1% manuka-type honey against PAO1, 1% and 2% all honeys and sugar solution against PA14). At concentrations of 16%, the total amount of cells was visibly decreased and more dead cells (red) were observed. At 32%, all treatments caused a substantial visual reduction in the amount of green lawn (live cells), with a far smaller amount of cells alive or attached to the imaged surface area (Figure 4.7). These results are consistent with the observation of biofilm eradication assay, where the tested honeys and sugar solution were able to

significantly remove, established *P. aeruginosa* biofilm biomass (Figure 4.4 and Table 4.2). Moreover, complete removal of established *P. aeruginosa* biofilm biomass was not observed at the highest concentration range (32%).

In clinical practice, however, a much higher concentration (> 32%) of medical-grade honeys are typically used. Thus, the effect of concentrations that are easily obtained in clinical settings (64% and 80%) for manuka honey and commercially available Medihoney were examined (manuka/kanuka honey was excluded as it does not meet the medical-grade honey standard). CLSM imaging indicated that the medical-grade manuka-based honeys were effective at reducing established *P. aeruginosa* biofilms, as there were very few cells remaining on the imaged surface, compared to the untreated control (Figure 4.7). Visually, there less fluorescently stained cells remaining attached on the imaged surfaces, compared to 32% of the same honey treatment (Figure 4.7 and Figure 4.8).

4.3.6 Assessing the resistance of P. aeruginosa *to manuka-type honey treatments after exposure of biofilms to sub-inhibitory concentrations*

It has been reported that sub-inhibitory concentrations of antibiotics can lead to an enhancement of *P. aeruginosa* biofilm formation, as well as the development of resistance [166, 167]. Figure 4.4 demonstrated that, like antibiotics, sub-inhibitory concentrations of the honeys and sugar solution dramatically increased biofilm biomass and the number of embedded cells of both *P. aeruginosa* strains (Figure 4.3 and Figure 4.4). Moreover, since the medical-grade manuka-type honey wound dressings are commercially available as wound management products, it is essential to determine whether bacterial cells can develop resistance to manuka-type honey treatments following exposure of these biofilms to sub-inhibitory concentrations. *P. aeruginosa* cells in the established biofilms were exposed to 8 % (sub-inhibitory concentration) manuka-type honeys, recovered, and then tested to determine whether the MIC or MBIC had changed. All recovered cells remained susceptible to the manuka-type honeys (Table 4.4), however, almost all the MICs and MBICs increased by at least 2-fold (Table 4.3).

Table 4.4 Resistance of *P. aeruginosa* cells recovered from biofilms after 8% manuka-type honey treatments

Honeys	Prior exposure to honey				After exposure to			
	PAO1		PA14		PAO1		PA14	
	MIC*	MBIC^	MIC	MBIC	MIC	MBIC	MIC	MBIC
Manuka honey	16	16	16	8	32	32	32	32
Medihoney	16	32	16	16	32	32	32	32
Manuka/kanuka honey	16	16	16	16	32	32	32	32

* MIC = minimum inhibitory concentration

^ MBIC = minimum biofilm inhibitory concentration, defined as ≥ 95% biofilm inhibition compared to untreated control.

4.4 Discussion

Due to the rise in antibiotic resistance, there has been renewed interest in the use of manuka-type honeys for the treatment of chronic wounds. They are available and licenced for use in countries like America, Australia, Japan, Hong Kong and some European countries. In this study, a set of manuka-type honeys were assessed to determine their ability to inhibit and eradicate biofilms of one of the most commonly isolated wound pathogens - *P. aeruginosa*. This opportunistic wound pathogen generally exhibits limited susceptibility towards currently available conventional antibiotics [190, 196].

Manuka-type honeys were shown to be effective at inhibiting cell growth and biofilm formation, and removing large proportions of established *P. aeruginosa* biofilms at high concentration (32 %), as tested in this study. This is in general agreement with the literature [43, 104, 105] as several studies have reported on the effectiveness of manuka-type honeys against *P. aeruginosa*. However, there are contrasting reports regarding the amount of manuka-type honey necessary to inhibit *P. aeruginosa* growth and biofilm formation; the honey concentrations range from 12 – 50% [104, 105]. In comparison, the determined growth and biofilm inhibitory concentration against *P. aeruginosa* in this study is an average of 16%. This is except the MBIC of PAO1 and PA14 were 32% Medihoney and 8%

manuka honey, respectively (Table 4.1). The observed disagreement is likely due to differences in assay conditions, as well as differences between specific manuka-type honeys tested. E.g. the age, floral source or even the storage condition of the honey samples. This highlights the importance of measuring and reporting the concentrations of the major known antibacterial components for ease of comparison between studies. Moreover, different types of growth assays, strains of testing or growth media were used. There is therefore a need for a standardized assay system, as used for antibiotic testing.

The MICs and MBICs of manuka-type honeys for *P. aeruginosa* are higher than those observed for *S. aureus* (Chapter 3). This suggests higher concentrations of honeys are required to inhibit *P. aeruginosa* planktonic cell growth and biofilm formation compared to *S. aureus* biofilms. This is in agreement with a study performed by Merckoll and colleagues, which also demonstrated that *S. aureus* biofilms were effectively inhibited by honey solutions at lower concentrations relative to *P. aeruginosa* [105]. Such a phenomenon might due to the fact that *P. aeruginosa* is often hard to treat as it is intrinsically resistant to many single compounds like antibiotics, where the efflux systems were identified might contribute to the intrinsic resistance [197-199].

MGO is believed to be one of the major antibacterial components of manuka honey, with demonstrated antibacterial effects against a range of bacteria [42]. MGO has previously been demonstrated to be the primary component responsible for the anti-*S. aureus* and *P. aeruginosa* biofilm activity of manuka –type honey [32, 43]. Under the conditions used here however, MGO treatment at concentrations broadly corresponding to those in the manuka-type honeys (both with and without sugar) did not induce similar biofilm inhibitory or eliminatory effects for both species, suggesting that MGO is not the major component responsible the antibacterial effects of manuka-type honey (as seen in Figure 4.3 and Figure 4.4).

The observations from this study suggest that MGO is not the major contributor of antibacterial activity in manuka-type honey, and in fact, had little contribution to the anti-*P. aeruginosa* biofilm activities of manuka-type honeys under the assay

conditions tested. This is in disagreement with study by Kilty and colleagues, where MGO was found to have activity against *P. aeruginosa* [42]. However, this might be because the effective concentrations were approximately 10 times higher than the highest concentration used in this study; the effective concentrations ranged between 1800 – 7300 mg/kg in the former study, whereas in this study, the highest MGO concentration tested was 288 mg/kg (equivalent to 32% of manuka honey). Thus, although MGO may be effective against *P. aeruginosa* at very high concentrations, it does not account for the antibiofilm activity of manuka-type honeys against *P. aeruginosa,* which is typically effective at concentrations that equate to much lower MGO concentrations.

The findings from this chapter show that *P. aeruginosa* has the ability to grow in the presence of higher MGO levels than other bacteria, compared to *S. aureus* for example (Chapter 3). This finding is consistent with a previous study by Kilty and colleagues [42]. This could be explained by that fact that *P. aeruginosa* has the ability to detoxify MGO via the glyoxylase system [61]. The glyoxylase system is composed of two enzymes, glyoxylase I and II, and is widely distributed within bacteria and eukaryotes [61]. However, while most bacteria contain only one glyoxylase I gene, *P. aeruginosa* has three fully functional glyoxylase I homologues [135]. This is one possible reason that *P. aeruginosa* displayed a less susceptible profile to MGO.

Moreover, low pH is one of the antibacterial properties of honey. At sugar concentration (32%) that significantly eradicated established *P. aeruginosa* biofilms (p <0.05; Figure 4.4 and Table 4.2), the pH of MGO plus sugar solution is not much different to sugar alone. According to Table 4.3, this is not likely to be due to pH differences since they are not that different, for example MGO at 32% (pH 5.80) in comparison to sugar at 32% (pH 5.77). On the other hand, the pH of the MGO vs honey may explain the different antibiofilm activities. For example, at 32% manuka honey pH is 6.54 and MGO at the same concentration with a pH of 5.8. This is 0.7 difference so could be important. Nevertheless, since the pH was not the same, further experimentation would be necessary to allow a direct comparison, for example by buffering the pH of the tested solutions. Adding MGO

to a non-MGO honey, e.g. clover honey, would overcome the pH and other environmental differences between honey and sugar, to allow for a more thorough examination of the effects of MGO specifically on inhibiting and eliminating *P. aeruginosa* biofilms.

Under the conditions tested here, sugar alone was generally almost or as effective as manuka-type honey treatments. This suggests that the sugar component of the manuka-type honeys was primarily responsible for the antibiofilm effects observed here. This may have been due to the high osmolarity created by the sugars, or a nutrient effect. Moreover, study by Wang and colleagues showed that the sugar content in honey is also able to disrupt the quorum sensing (QS) system [110]. However, the observed effective sugar solution on *P. aeruginosa* biofilms is in contrast to the results obtained for *S. aureus* biofilms. Sugar does exhibit some antibiofilm activity (32%) against some of the *S. aureus* strains, but manuka-type honeys (effective range between 8-16%) are consistently more effective (Chapter 3). *P. aeruginosa* planktonic cells have been observed to be more sensitive to the effects of sugar in comparison to *Bacillus subtilis* and *S. aureus*, where *P. aeruginosa* planktonic cell growth was inhibited by 32% sugar but not the other two organisms (Chapter 2). A study by Lerrer and colleagues also demonstrated the high concentration of fructose, present in the honey and sugar solutions, was capable of preventing *P. aeruginosa* biofilm formation [109]. Fructose has the ability to block the fructose-binding lectin PA-IIL adhesion, which is responsible for mediating *P. aeruginosa* biofilm formation and adhesion [109]. Additionally, it is also possible that *P. aeruginosa* is more sensitive to the sugar solution due to its deficit in sugar transporters [197]. *P. aeruginosa* contains only two sugar transporters in its phosphotransferase system (for fructose and *N*-acetylglucosamine). In comparison to *E. coli*, which contains more than twenty [200].

The 32% honey and sugar solutions were able to significantly removed established *P. aeruginosa* biofilm biomass (Section 3.2). However, complete biofilm biomass removal, or cellkilling effects were not observed, in contrast to the effects observed with established *S. aureus* biofilms (Chapter 3, section 3.4). However, higher

concentrations of manuka-type honeys (64% and 85%) were shown to result in the complete removal of established *P. aeruginosa* biofilms (Figure 4.8). This was assessed visually using CLSM imaging. However, further analysis should be performed in order to validate these observations. For example, it would be worthwhile performing the static biofilm formation assays and determining the cell viability as described in Section 3.2. In addition, both clover honey and sugar solution should be included and tested at similar concentrations (64% and 85%) as manuka-type honeys, since both clover honey and sugar solution displayed similar antibiofilm activities against established *P. aeruginosa* at 32% (Section 3.2).

Honey is an attractive alternative to antibiotics, in part because it has been shown that a range of free-living bacterial cells like *S. aureus, P. aeruginosa, E. coli* and *Acinetobacter calcoaceticus* do not develop resistance to medical-grade manuka honeys [70, 150], as so often happens with antibiotics [201]. The lack of development of resistance may due to the complexity of honeys, which act in a multifactorial way to target cells [70]. However, tests for resistance development have typically only been tested on planktonic cell growth, not with exposed biofilm cells. Treatment of biofilm-associated chronic wounds often requires continued disruption of biofilms, where multiple doses of antibacterial agents are needed [202]. Therefore, assessing the susceptibility of cells that were previously exposed to the same treatment agent (i.e. honey) is important. Biofilm cells recovered from sub-inhibitory manuka-type honey treatments were therefore tested for the development of resistance. These biofilm cells exhibited a 2-fold increase in MIC and MBIC. These results indicate that *P. aeruginosa* cells exposed to sub-inhibitory concentrations of the manuka-type honey can acquire tolerance or resistance. When *P. aeruginosa* cells are treated with antibiotics, some of the cells are able to survive the antibiotic exposure by slowing down their growth rate, providing a temporary tolerance. This is a reversible process, and cells once again become susceptible when the cell growth rate resumes to normal [201]. The increased tolerance of biofilm derived *P. aeruginosa* cells to manuka-type honey could be seen as a temporary tolerance – such as seen with the slow growth rate. Alternatively, this could represent a more permanent change, which renders the

cells more resistant to honey treatment. Further experiments are required to test which of these possibilities is true.

The resistance assays can be performed by exposing the biofilm-derived cells to multiple rounds of the same honey treatment, with sub-inhibitory, inhibitory and higher than inhibitory concentrations to determine their resistance to the manuka-type honeys and sugar treatments. These experiments would provide further evidence as to whether there is a limit to the concentration of honey that *P. aeruginosa* can develop resistance to, which would help provide guidance in relation to the minimum concentration of honey that should be maintained in the honey dressing. It is possible that a higher concentration, close to full-strength manuka-type honey (i.e. 64% or 85%), as opposed to the concentrations tested here (32%), is required to prevent resistance development.

In addition, clover honey and sugar solution should be included in future testing for resistance assays, since both the clover honey and sugar solution contain significant fewer bioactive components compared to manuka honey (phenolic compounds, hydrogen peroxide, MGO). Including these two solutions in the resistance assays will identify whether components of these solutions are responsible for the development of resistance in *P. aeruginosa* when biofilms are treatment with them.

4.5 Conclusions

This study showed that the tested NZ honeys were able to inhibit *P. aeruginosa* biofilm formation, and were able to significantly reduce established biofilms. While the sugar solution was demonstrated to be similarly effective to the tested NZ honeys, this does not remove the need to use manuka-type honeys over sugar in clinical practice. Wounds are commonly colonised by multiple different species, and sugar alone is not as effective against the other common wound pathogen *S. aureus*. *P. aeruginosa* biofilm cells displayed increased tolerance on exposure to sub-inhibitory concentrations of manuka-type honeys, however, whether the increased tolerance is reversible or these cells become permanently resistant is yet

to be tested. Importantly, manuka-type honeys at clinically obtainable concentrations (64% and 80%) are highly effective at preventing and eradicating established *P. aeruginosa* biofilms. Thus, in appropriate concentrations, wound dressings saturated with manuka-based honeys should be effective in inhibiting and eradicating *P. aeruginosa* biofilms from chronic wounds.

Chapter 5

General Discussion

5.1 The importance of honeys

The management of chronic wounds in hospitals and clinics is becoming an increasingly difficult and costly problem [6]. One of the major reasons for this is the development of antibiotic resistance, along with the decreased rate of new antibiotic discovery [7, 8]. Complex natural products like honey are now been revisited as alternative treatments [18, 20, 21]. Previous studies have shown that honey has broad-spectrum antibacterial activity [33, 70]. In addition, bacteria are unable to develop resistance to honey [70, 150]. However, a number of issues continue to limit the use of honey for treating chronic wounds in hospitals (Chapter 1).

Not all honeys are the same and most of the studies examining the antibacterial effects of honey did not thoroughly characterize the honeys tested. There has been a lack of focus on the importance and chemical composition, at least of the major antibacterial components like hydrogen peroxide or MGO, believed to be responsible for the antibacterial effects of honeys [30, 32, 34, 35]. Furthermore, the reported minimal inhibitory concentration (MIC) of different honey samples varies amongst studies [143]. This makes it difficult to demonstrate how effective certain honeys are as an alternative treatment agent for chronic wounds. Altogether being part of the issues for the lack of acceptance of honeys in clinical use [18].

To overcome these problems, this work was performed in collaboration with the international industry partner, Comvita Pty. Ltd. Comvita is a prominent natural product company that produces New Zealand honeys for both consumption as a functional food and medical use. The company has provided us access to honey samples and their detailed chemical and floral profiles. Consequently, the levels of

major antibacterial components like hydrogen peroxide and MGO in the honey samples used in this work are known, as well as what plant the nectar is derived from. This has allowed us to examine the antibacterial (Chapter 2) and antibiofilm (Chapter 3 and 4) properties of these honey samples, and correlate it with the specific chemistry and floral source of the honey. In addition, the geographic locations of honeys used in this study are first reported. Comvita has in fact launched a program where honey, harvesting locations are marked and tracked by GPS (Global Positioning System). The GPS marked geographic locations can then be linked with the chemical, antibacterial and antibiofilm profiles of the harvested honeys. Thus, this leads to better production strategies in the future. In brief, our findings support the use of well-defined manuka-type honeys as a topical treatment for the effective management of wound healing.

5.2 Potential mechanisms of how honey kills bacteria and affects biofilms

Chapter 2 of this work deals with an investigation of the effect of New Zealand honeys on the growth and cellular morphology of four bacterial species: *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*. In general, New Zealand manuka honeys with relatively high MGO levels (651.4 – 1541.3 mg/kg) were consistently more effective at inhibiting bacterial growth in comparison to the other tested New Zealand honeys. However, *P. aeruginosa* cells required higher concentrations of these honeys to inhibit growth. This may be explained by the fact that *P. aeruginosa* cells possess a more efficient detoxification system against MGO than other eubacteria [61]. It was also found that the morphological response of the bacteria after honey treatment was different amongst the different species examined. This likely reflects adaptation of these bacteria to their specific environmental niches.

Chapters 3 and 4 of this thesis focused specifically on the ability of New Zealand honeys to inhibit and eradicate *S. aureus* and *P. aeruginosa* biofilms, respectively. These two bacterial species were chosen because they are the most common isolates found in chronic wounds [124, 177]. This work showed that the New Zealand manuka-type honeys, at concentrations that can be applied in medical-

grade honey-impregnated wound dressings, were highly effective in preventing both *S. aureus* and *P. aeruginosa* biofilm formation. Biofilm formation of these two bacteria was inhibited at the concentrations that their planktonic growth was also inhibited (Chapter 3 and 4). This suggests biofilm inhibition was solely a consequence of planktonic growth inhibition. Furthermore, *S. aureus* biofilms were found to be more sensitive to the same manuka honey concentration than *P. aeruginosa* biofilms. This is in agreement with the results obtained in Chapter 2, where *P. aeruginosa* growth was less susceptible to manuka-type honeys than that of *S. aureus*. This could also be the result of a more efficient MGO detoxification system in *P. aeruginosa* [135].

The manuka-type honeys tested here were able to penetrate through the established *S. aureus* biofilm structure at sub-eradication concentrations without disrupting the established biofilm structure (Chapter 3, section 3.5). This finding is exciting because one of the major limitations of conventional antibiotics is their inability to penetrate through the established biofilm structure [85, 203]. At biofilm eradication concentrations of manuka-type honey, the established *S. aureus* biofilm structure was completely eradicated, killing all cells within the biofilm (Chapter 3, section 3.5). This provides further support for the use of manuka-type honeys in effective treatment for chronic wounds. Lastly, exposing the established *S. aureus* biofilms to the tested manuka-type honeys did not result in cells within the biofilms becoming resistant (Chapter 3, section 3.8).

Interestingly, at the same levels (e.g. 32%) of honey treatment, complete eradication of established *P. aeruginosa* biofilms was not observed (Chapter 4, section 3.2), unlike *S. aureus* biofilms (Chapter 3, section 3.5). The embedded cells within the pre-established *P. aeruginosa* biofilms were not completely killed, yet their numbers were significantly reduced (Chapter 4, section 3.2). These cells displayed increased tolerance to the same treatment agent (Chapter 4, section 3.6). Whether this increased tolerance is irreversible or whether these cells became resistant was not tested. Both of these concerns should be addressed in future experiments. In clinical practice, the honey concentration in many honey wound dressings and gels is close to 100%. Furthermore, the concentration of honey can

be diluted down by wound exudate itself [52]. Thus, the effects of high manuka-type honey concentrations (e.g. 64% and 80%, which were close to the concentrations used in clinical practice) were tested. Complete eradication of *P. aeruginosa* biofilms was only observed at the high concentrations (64% and 80%) of manuka-type honeys as determined by confocal laser scanning microscopy (Chapter 4, section 3.5). Presumably, the high concentration of honey (~80 – 100%) in wound dressing, would be required for a biofilm that contains *P. aeruginosa* to be eradicated in a chronic wound.

Another important observation was that the sugar-only solution was just as effective as the tested manuka-type honeys, in preventing *P. aeruginosa* biofilm formation as well as in reducing the amount of established biofilm biomass (Chapter 4, section 3.1 and Chapter 4, section 3.2). This could be due to MGO in the honey being less effective in eradicating the persister cells in the biofilm. The similar activity of sugar solution and manuka-type honeys on the *P. aeruginosa* biofilms could be due to *P. aeruginosa* containing extra functional Glyoxalase I (GlxI), which is responsible for detoxifying MGO [135]. This may explain why the established *P. aeruginosa* biofilm were susceptible to both manuka-type honey and the sugar solution.

However, the question still remains whether components of honey other than MGO are responsible for reducing the amount of established *P. aeruginosa* biofilm. Two lines of evidence suggest that the high content of sugars in manuka-type honey (which is also present in the sugar solution) may be responsible. The first is that *P. aeruginosa* is deficient in sugar transporters when compared to other bacterial species [200]. This may make *P. aeruginosa* cells equally susceptible to manuka-type honey and the sugar-only solution. Secondly, the high fructose content in both the manuka-type honeys and sugar solution, may also be responsible for preventing the *P. aeruginosa* biofilm formation. A previous study has shown that fructose (40% w/v) was responsible for blocking the fructose-binding lectin PA-IIL adhesion that is responsible for mediating *P. aeruginosa* biofilm formation and adhesion [109]. Presumably there is not enough fructose in either the manuka-type honey or the sugar solution given that *P. aeruginosa* biofilms were not

completely eradicated at concentrations ranging between 1 – 32%. Future experiments will be required to test these hypotheses. This would be performed by preparing fructose solutions that are equivalent to the tested manuka-type honeys (1 - 32% and >32%), and determine the effects of these solutions against established *P. aeruginosa* biofilms.

5.3 Future directions

The work presented in this thesis has contributed towards a greater knowledge of the antibacterial and antibiofilm properties of NZ honeys. However, there are several experiments, which could be performed to provide significant value to this field. Firstly, in this work, static biofilm screening assays were performed to identify the appropriate concentrations of the manuka-type honeys that affect both bacterial biofilm formation and the eradication of established biofilms (Chapter 3 and 4). A limitation of this assay is that biofilms cannot fully mature under these static conditions *in vitro*. However, the bacterial biofilm, which colonizes a chronic wound in a clinical setting, is typically mature [204]. To combat this problem, a flow-system biofilm assay could be performed, in order to determine in more detail, the anti-biofilm properties of honey on mature bacterial biofilms. This flow-system biofilm technology allows a biofilm to receive a continuous supply of nutrients, which is needed for maturation [87, 205]. The flow-system also allows the dispersed cells and waste products to be continually removed. This is important for mimic the actual (wound) environment, where a planktonic cell free environment can be created, as well as it allows biofilm maturation. A study by Agostinho and colleagues also shown the flow model is ideal and versatile *in vitro* model for testing wound management products [206]. Moreover, this system also enables detailed analysis of any detailed cellular (morphological) or spatial and temporal alterations to the biofilm.

It would also be worthwhile evaluating the efficacy of honey in eradicating biofilms derived from a polymicrobial community. In this work, biofilms were induced to form from a single bacterial species, which typically does not occur in a chronic wound [177], or in any natural environment. Although Chapter 2 showed that

manuka-type honey could effectively kill *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*, it remains to be determined whether or not honey is effective when used against polymicrobial biofilm. Consequently, the effective honey concentrations to combat polymicrobial biofilms are predicted to be higher than ones required for single species bacterial biofilms, which may be due to synergistic interactions between different bacterial species. These further experiments could be performed in conjunction with real time fluorescence microscopy to visualize the morphological changes and viability of the different bacteria within a polymicrobial biofilm. Different bacterial species can be distinguished using different fluorescent probes, where Fluorescence *in situ* Hybridization (FISH) technique can be used [207, 208].

Lastly, the bacteria that normally colonize skin are thought to play a role in protecting this anatomical barrier from becoming infected with a wound [209, 210]. However, the question that still remains unanswered is whether the composition of different bacterial species present on skin before, during and after chronic wound treatment has changed. Several studies have demonstrated that conventional treatments using antibiotics can alter the composition of flora, which were previously present [211, 212]. It is likely that honey would induce similar changes. Therefore, it would be worthwhile performing 16S ribosomal RNA and metagenomic sequencing to identify the skin microbiome of patients who have had a chronic wound, with and without honey treatment.

Interestingly, it has previously been shown, using *in vitro* assays, that manuka, kanuka and jellybush honeys stimulates the local immune response and promote wound healing in patients who have previously suffered from a chronic wound [119-121]. Once again, this suggests that these tested honeys are a superior treatment option for chronic wounds compared to conventional antibiotics.

5.4 Acceptance of honey as a wound treatment in clinical settings

For many years it has been known that honey cannot spoil and possesses antibacterial and antibiofilm properties [18, 72]. Research focusing on the anti-

bacterial and anti-biofilm properties of honey was strengthened with the discovery of MGO as an active component in New Zealand manuka-type honeys in 2008 [30, 32]. Accordingly, many studies have focused on understanding how MGO in honey contributes to these properties. However, numerous concerns still limit the acceptance of honeys in a clinical setting. Many studies report remarkably little information in regards to the chemical profiles of the honey being tested for its antibacterial and antibiofilm properties [43, 104-106, 153]. It is therefore rather difficult to understand how these honeys kill bacteria and eradicate biofilms. Furthermore, a study by Stephens and colleagues has shown that different honeys display different chemical profiles, which would likely impact its anti-bacterial and anti-biofilm properties [35].

Progress is being made to promote the acceptance of honeys in clinical settings by showing that the increased efficacy and stemming of resistance in combining some conventional antibiotics with honey [213]. This research is important because it shows how bacteria, which were resistant to certain antibiotics, e.g. rifampicin, can become susceptible again if honey is used synergistically. Furthermore, the drug-honey combinations can prevent development of future resistance and lower the therapeutic dose and toxicity of both honey and antibiotics used in a clinical setting.

The research reported in this thesis demonstrates for the first time that sub-inhibitory concentrations of tested different New Zealand honeys have different impacts on different bacteria (Chapter 2, section 3.4.1 and Chapter 2, section 3.4.2). It is also suggests that sub-inhibitory concentrations of the tested manuka-type honeys should be avoided in clinical practice, as it appears to promote biofilm formation (Chapter 3 section 3.2, 3.5 and Chapter 4, section 3.2). It would be worthwhile establishing whether the combination of sub-inhibitory concentrations of honeys and conventional antibiotics induce biofilm formation.

5.5 Concluding remarks

The communication strategy used in this work is believed to have led to a better acceptance of using honey by the general public and medical professions. Two of the three result chapters (Chapter 2 and 3) were published in prestigious, peer-reviewed scientific research journals. One of these publications (Chapter 2) resulted in physicians from Westmead hospital in Sydney planning to include medical-grade honey products as part of their treatment plans for diabetic patients. Furthermore, these two publications have also attracted international multimedia coverage. This coverage effectively delivered the message that honey can be a good alternative treatment option for chronic wounds and should be utilized more often if possible.

To date, almost all honey research has been conducted on the chemistry and antibacterial properties of New Zealand manuka (*Leptospermum*) rather than Australian honey species. This limited research on Australian *Leptospermum* honeys is intriguing given that Australia has more than 83 species of *Leptospermum* honeys in comparison to only 2 in New Zealand [214]. With the wide range of *Leptospermum* spread across Australia, there is enormous potential for the Australian honey industry to also contribute on such growing demand, as a new source for potential medical usage. It is likely that in the future there will be an undersupply of medicinal honey from New Zealand in the face of growing demand worldwide for antibacterial honey.

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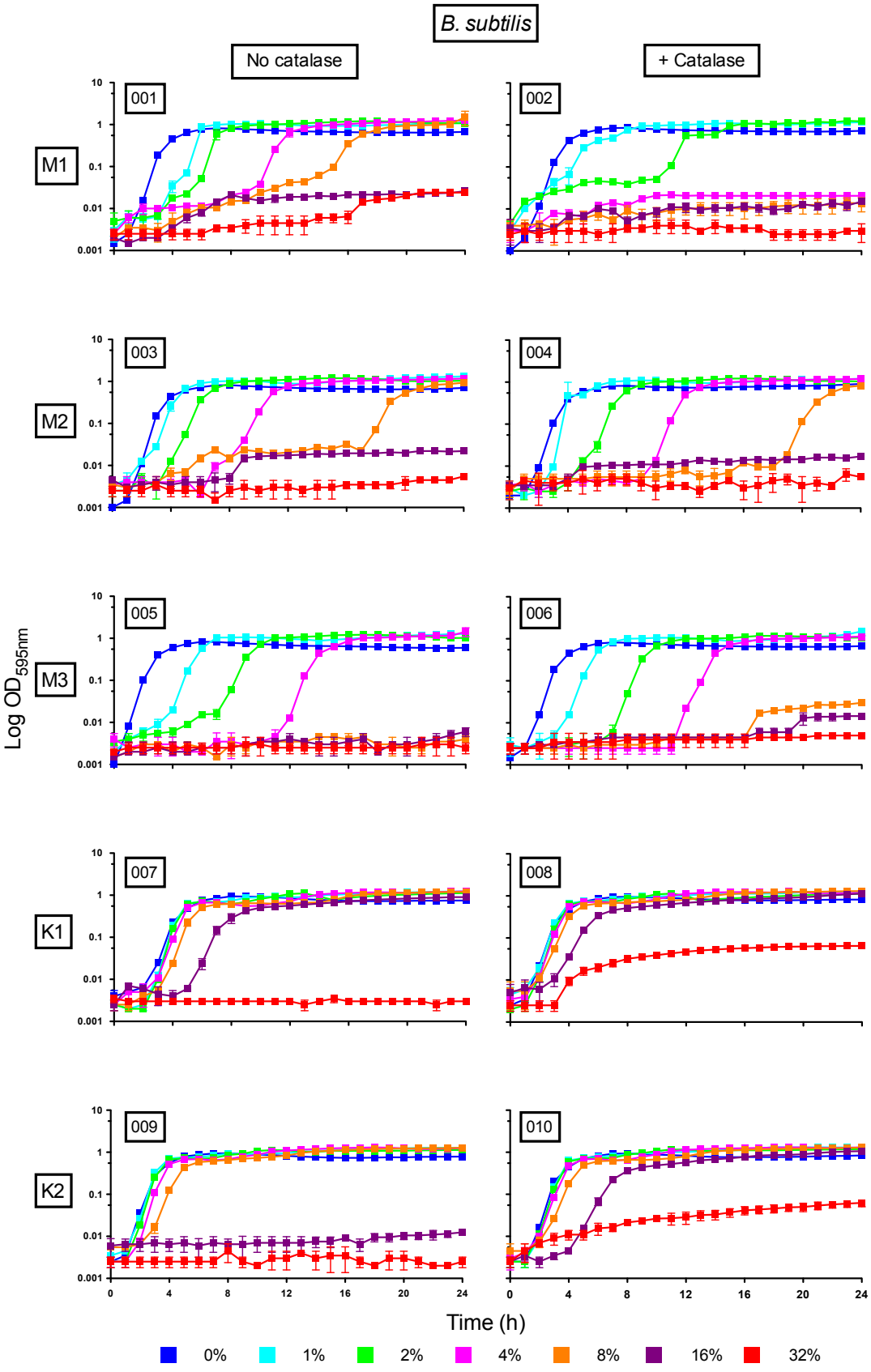
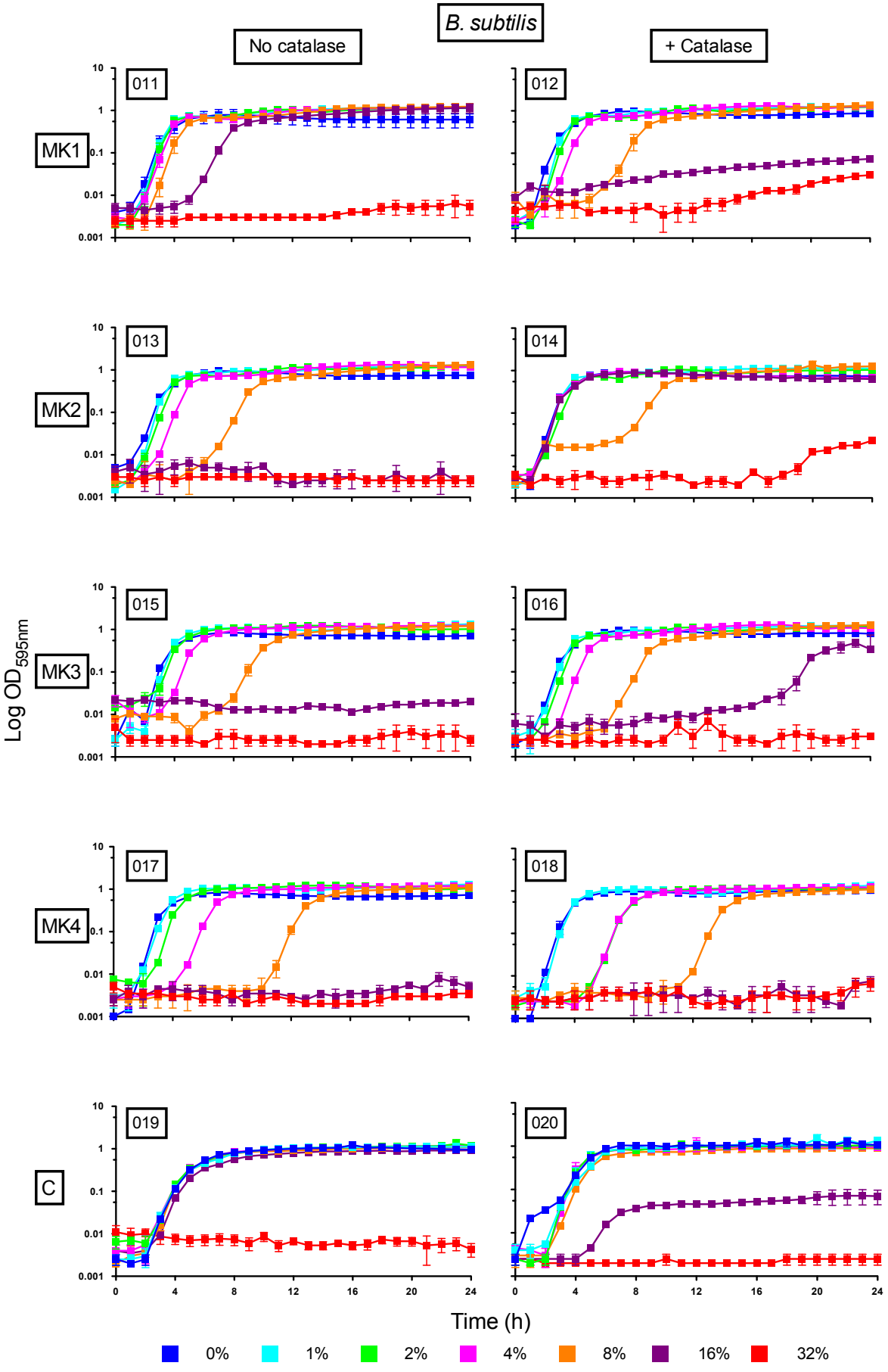
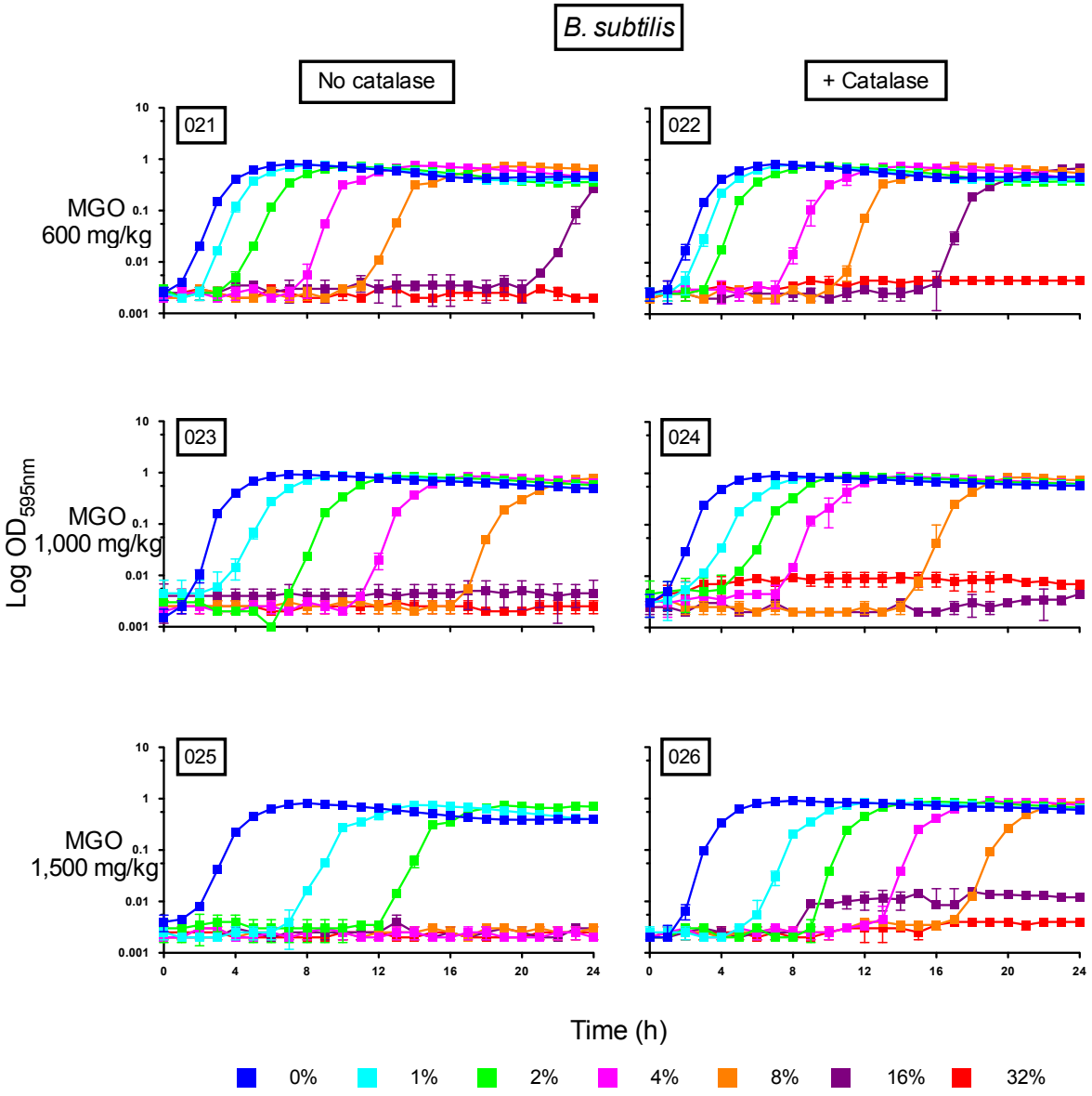
214. **Leptospermum - family Myrtaceae** [http://www.cpbr.gov.au/leptospermum/index.html]

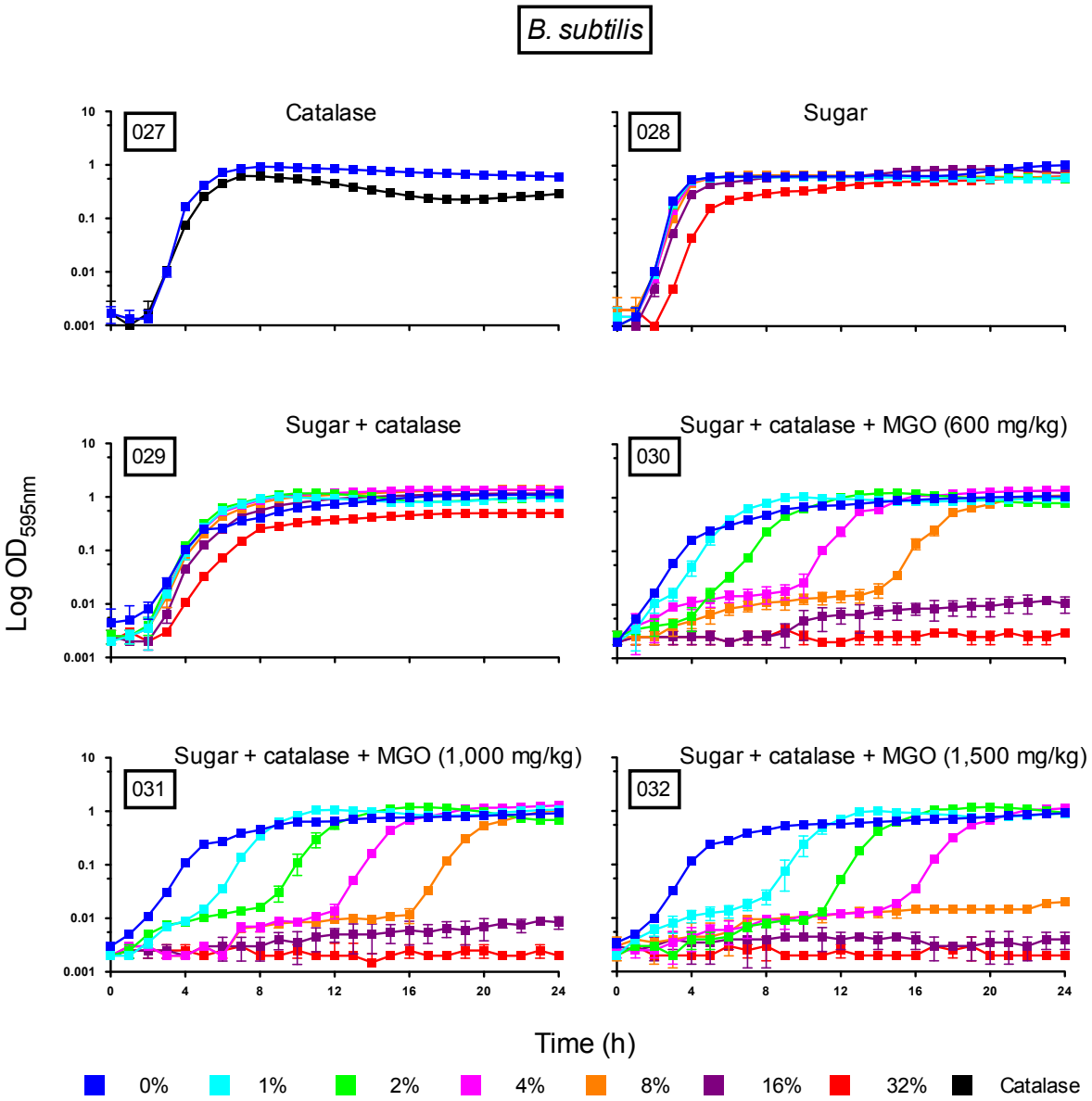
Appendices

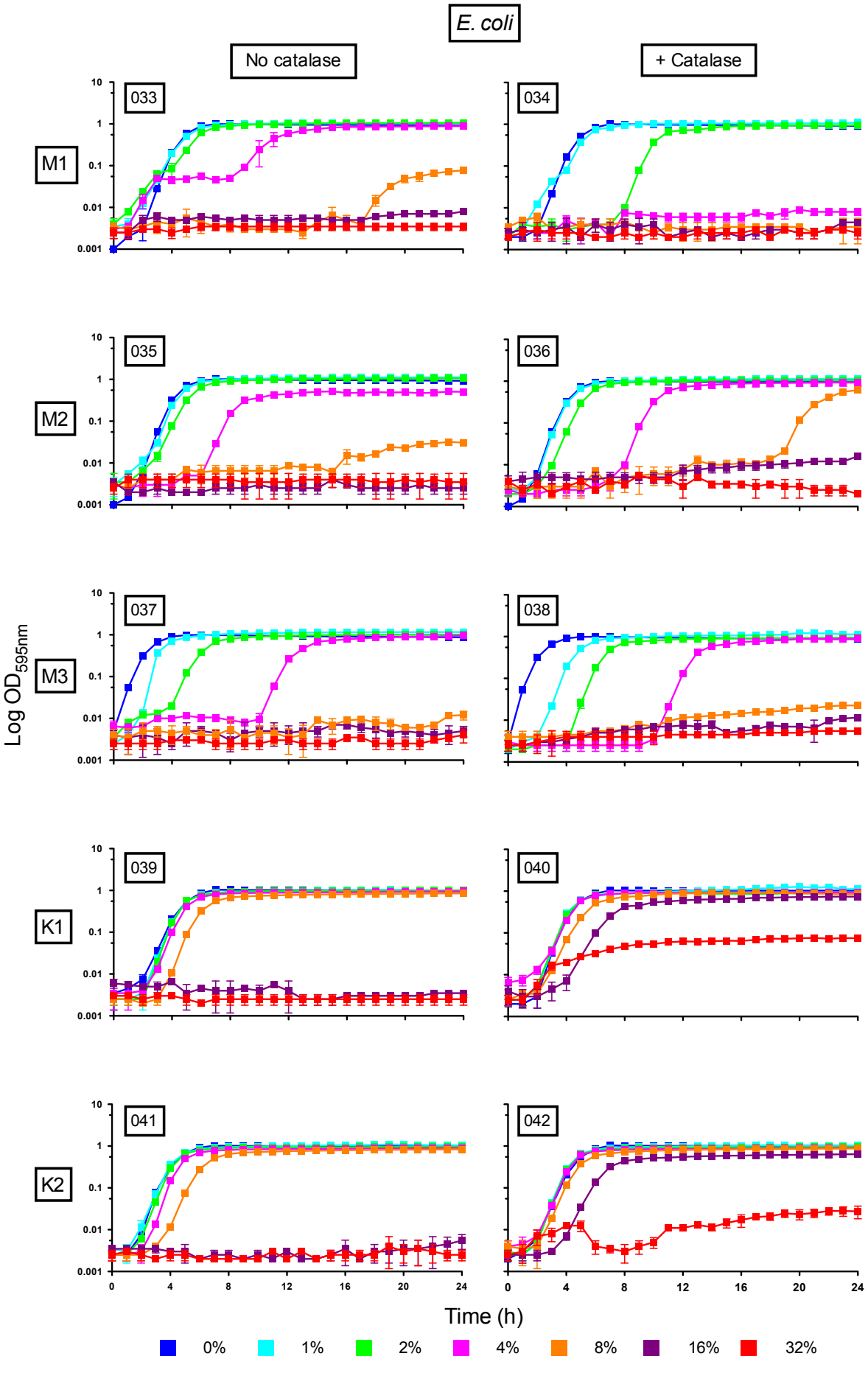
Appendix I

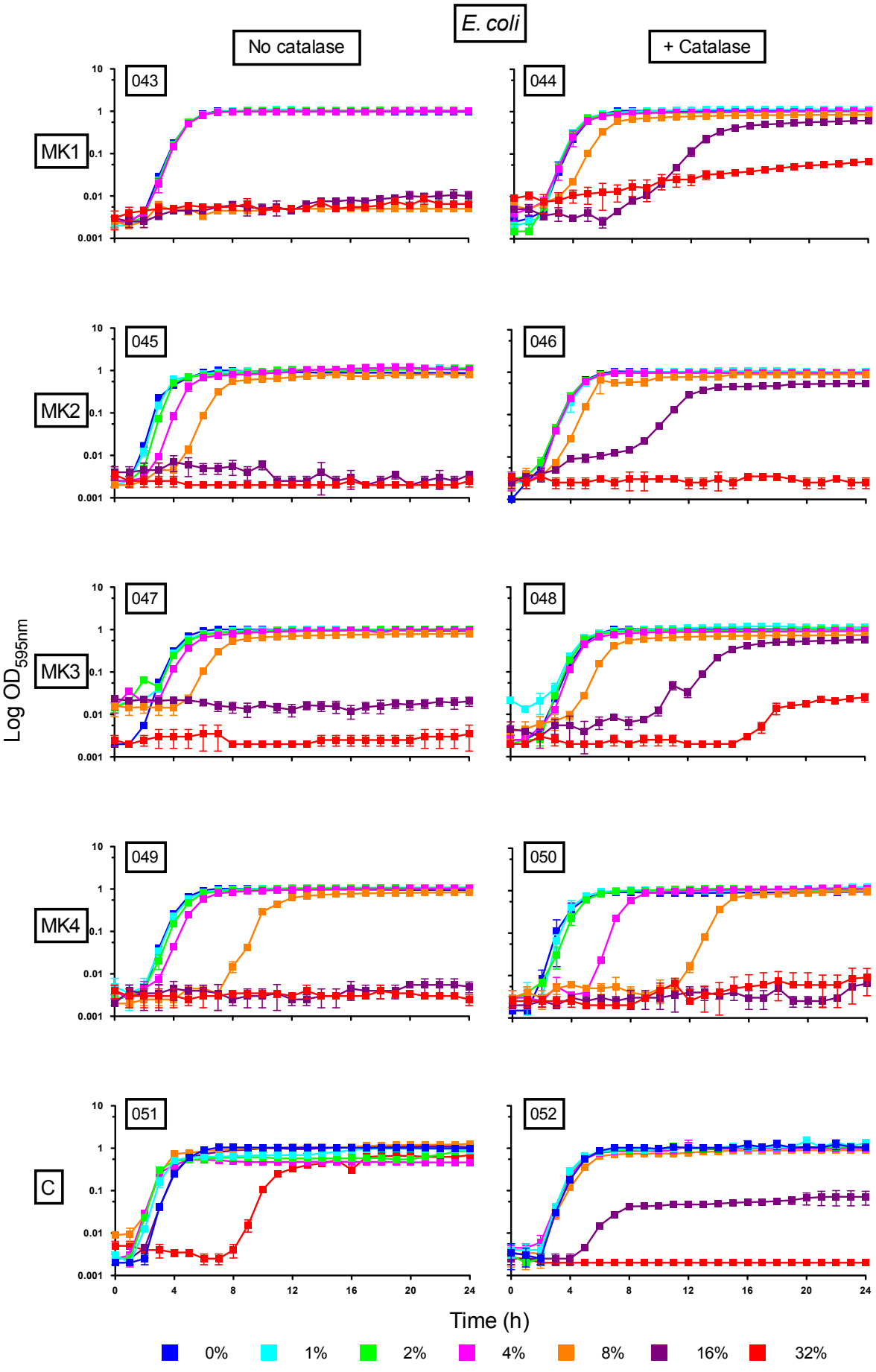


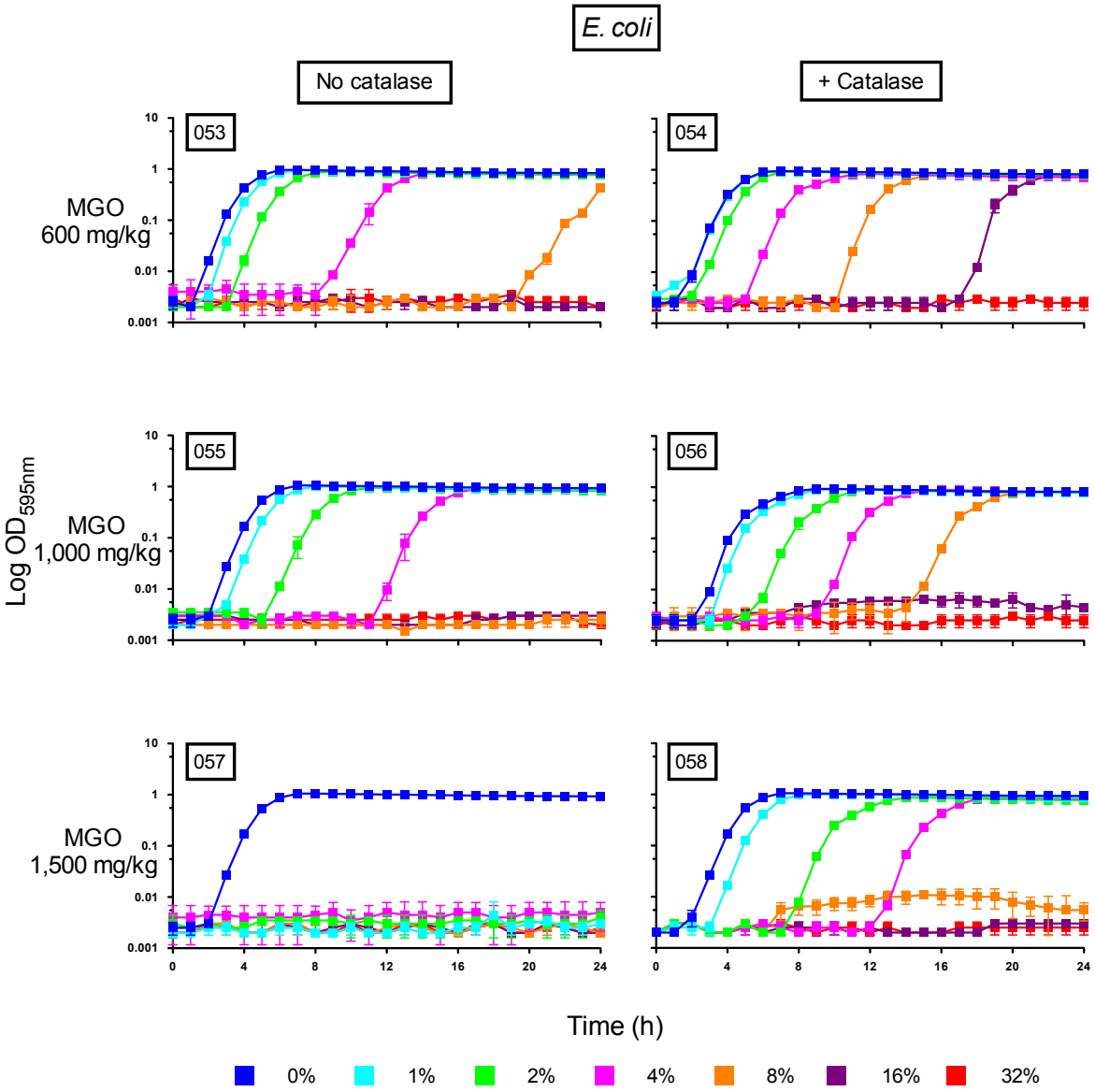

B. subtilis
No catalase
+ Catalase
011
012
013
014
015
016
017
018
019
020
MK1
MK2
MK3
MK4
C
Log OD595nm
Time (h)
0%
1%
2%
4%
8%
16%
32%

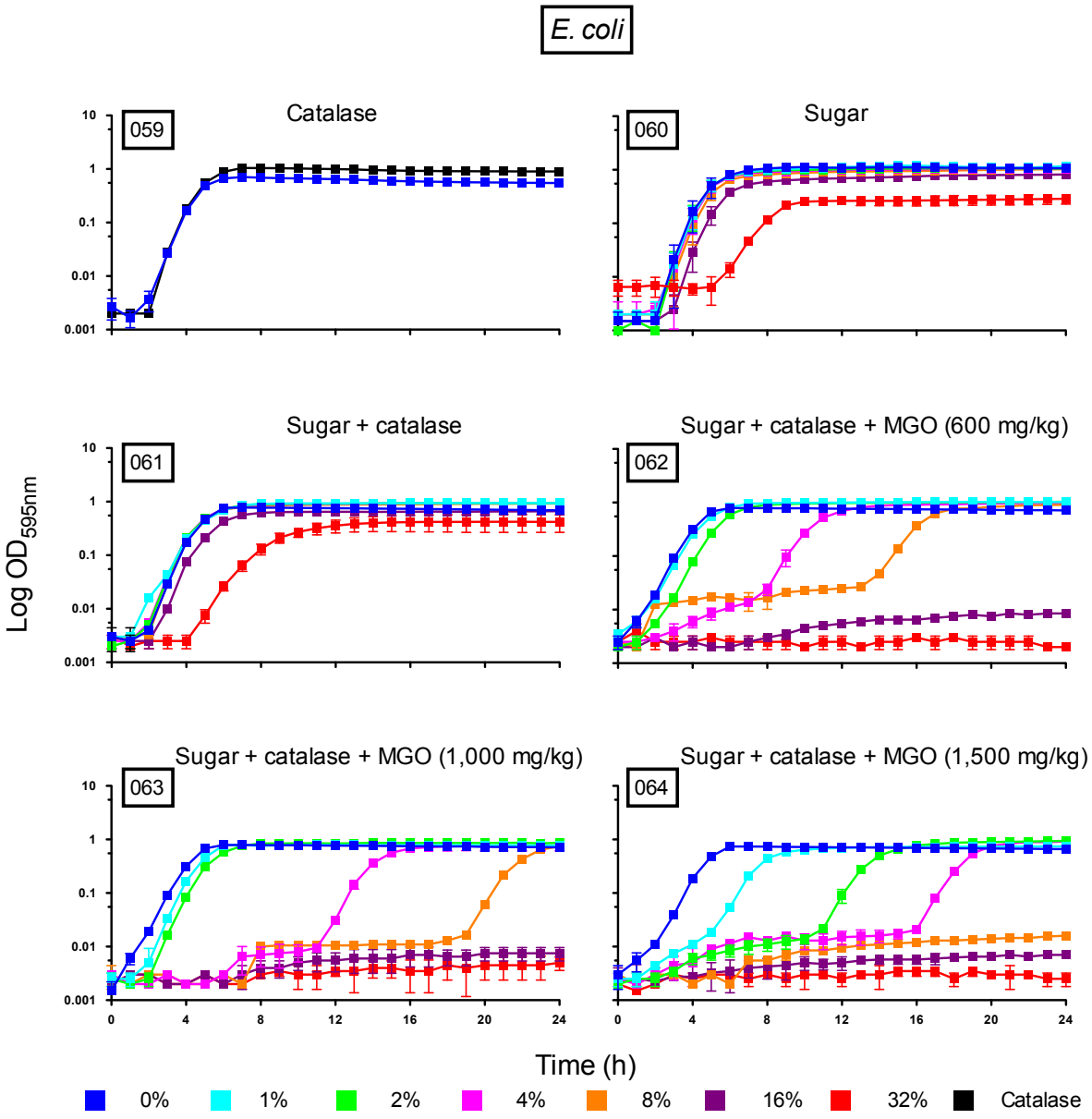

B. subtilis
No catalase
+ Catalase
021
022
023
024
025
026
MGO 600 mg/kg
MGO 1,000 mg/kg
MGO 1,500 mg/kg
Log OD595nm
10
1
0.1
0.01
0.001
0
4
8
12
16
20
24
Time (h)
0%
1%
2%
4%
8%
16%
32%

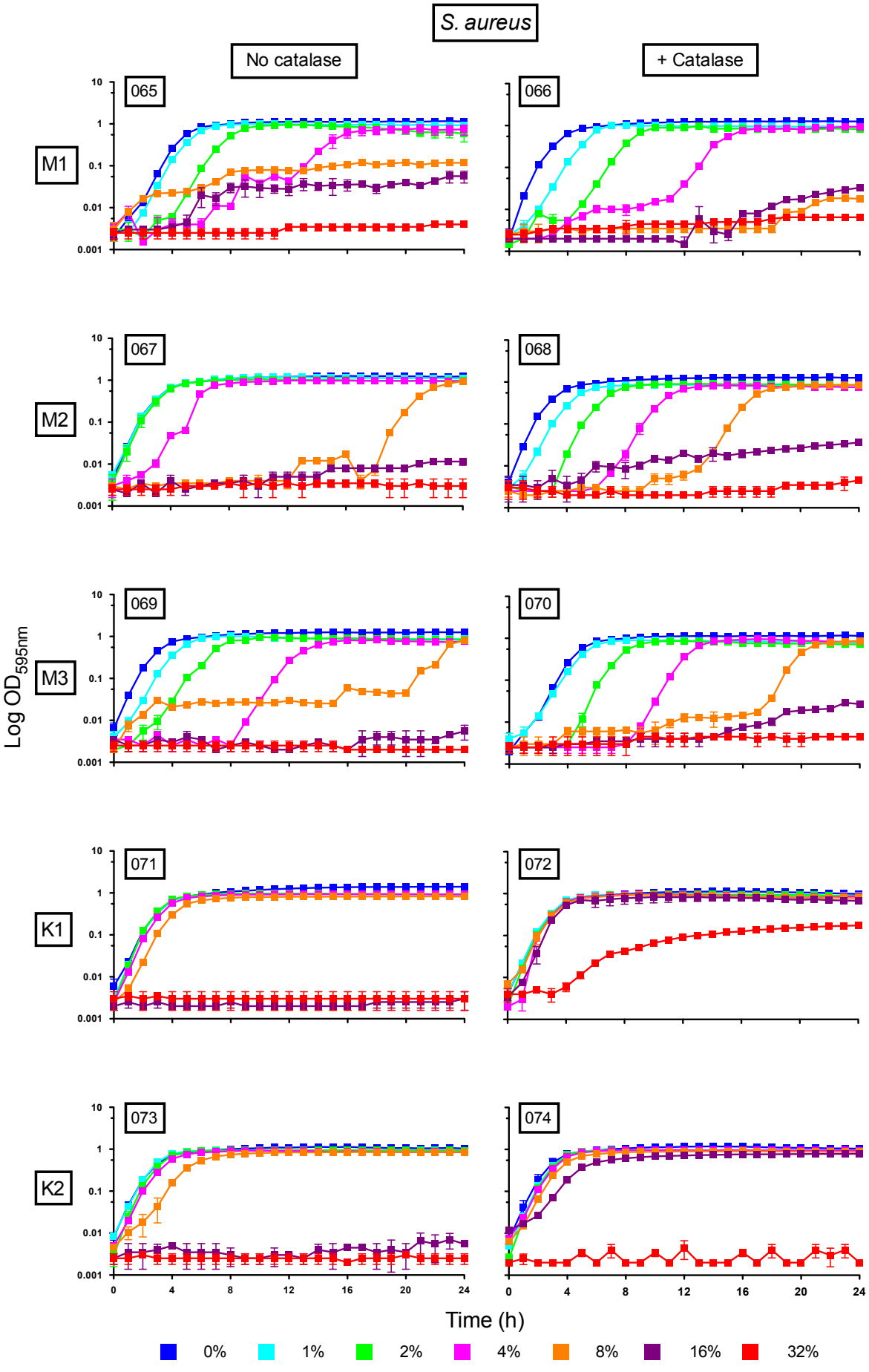

B. subtilis
Catalase
027
Sugar
028
Sugar + catalase
029
Sugar + catalase + MGO (600 mg/kg)
030
Sugar + catalase + MGO (1,000 mg/kg)
031
Sugar + catalase + MGO (1,500 mg/kg)
032
Log OD595nm
Time (h)
0%
1%
2%
4%
8%
16%
32%
Catalase

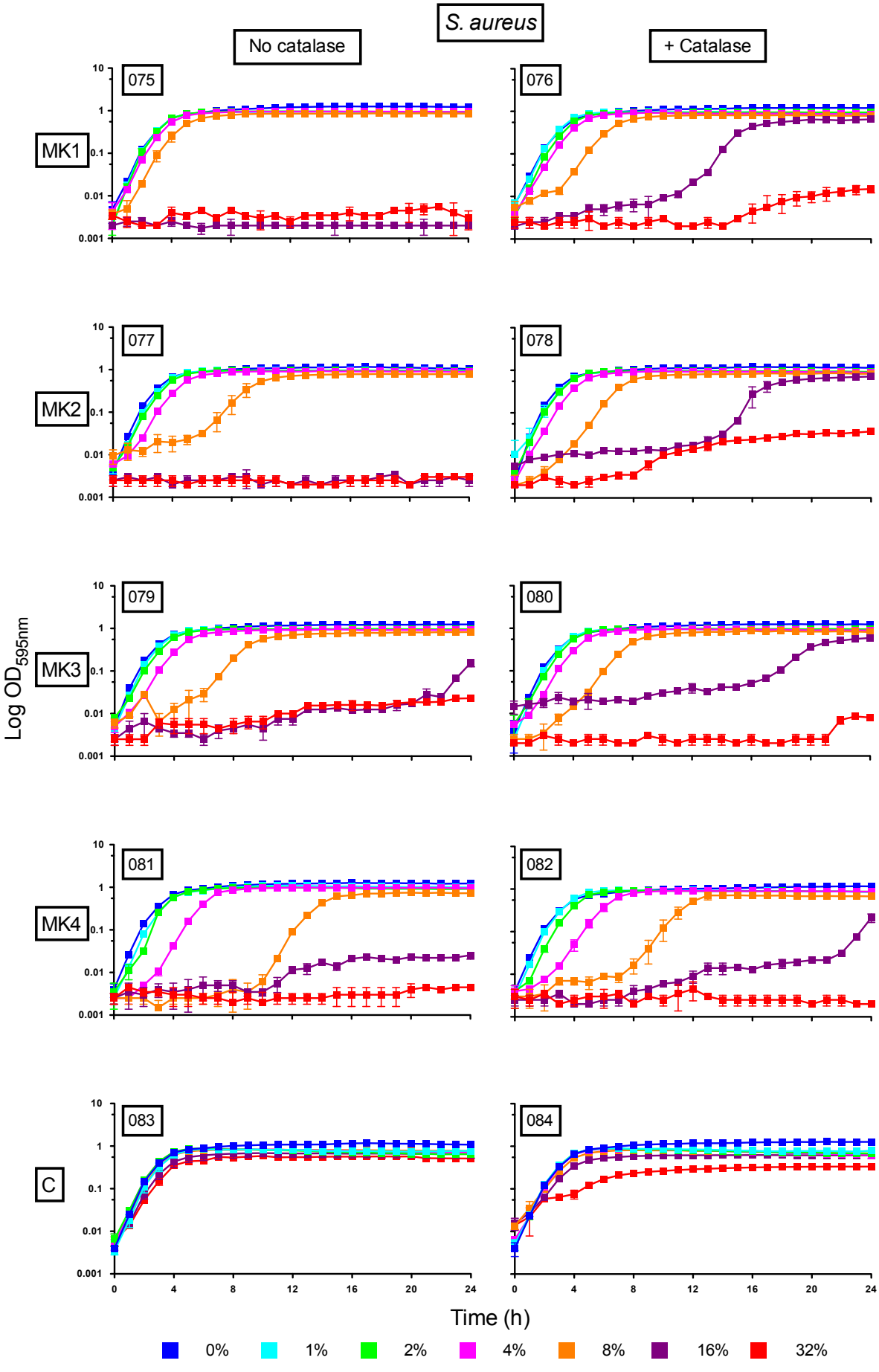

E. coli
No catalase
+ Catalase
033
034
035
036
037
038
039
040
041
042
M1
M2
M3
K1
K2
Log OD595nm
10
1
0.1
0.01
0.001
0
4
8
12
16
20
24
Time (h)
0%
1%
2%
4%
8%
16%
32%


E. coli
No catalase
+ Catalase
043
044
045
046
047
048
049
050
051
052
MK1
MK2
MK3
MK4
C
Log OD595nm
Time (h)
0%
1%
2%
4%
8%
16%
32%


E. coli
No catalase
+ Catalase
053
054
055
056
057
058
MGO
600 mg/kg
MGO
1,000 mg/kg
MGO
1,500 mg/kg
Log OD595nm
10
1
0.1
0.01
0.001
0
4
8
12
16
20
24
Time (h)
0%
1%
2%
4%
8%
16%
32%


E. coli
059
Catalase
060
Sugar
061
Sugar + catalase
062
Sugar + catalase + MGO (600 mg/kg)
063
Sugar + catalase + MGO (1,000 mg/kg)
064
Sugar + catalase + MGO (1,500 mg/kg)
Log OD595nm
10
1
0.1
0.01
0.001
0
4
8
12
16
20
24
Time (h)
0%
1%
2%
4%
8%
16%
32%
Catalase


S. aureus
No catalase
+ Catalase
065
066
067
068
069
070
071
072
073
074
M1
M2
M3
K1
K2
Log OD595nm
Time (h)
0%
1%
2%
4%
8%
16%
32%


S. aureus
No catalase
+ Catalase
075
076
077
078
079
080
081
082
083
084
MK1
MK2
MK3
MK4
C
Log OD595nm
Time (h)
0%
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8%
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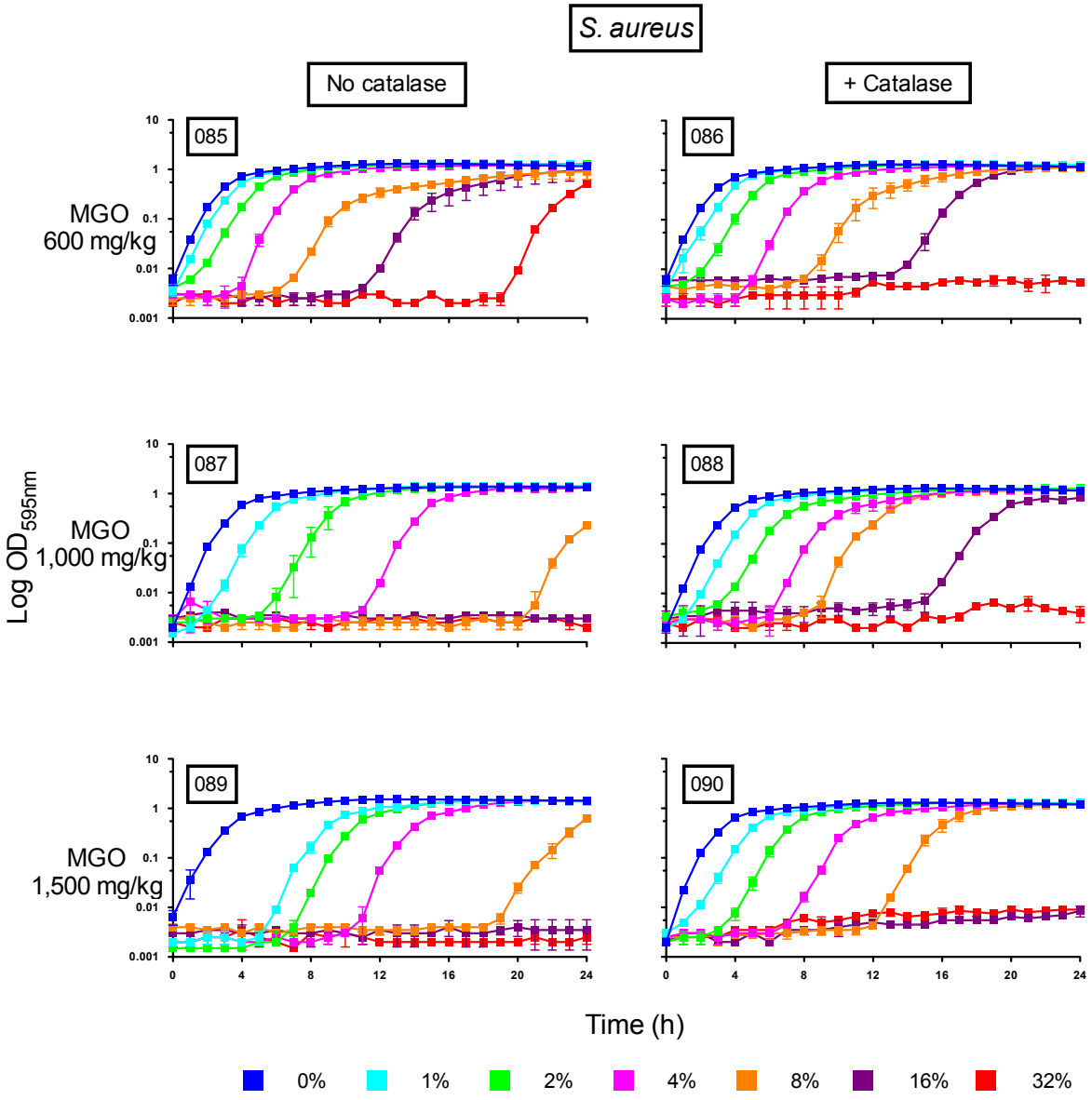
S. aureus
No catalase
+ Catalase
085
086
087
088
089
090
MGO 600 mg/kg
MGO 1,000 mg/kg
MGO 1,500 mg/kg
Log OD595nm
10
1
0.1
0.01
0.001
0
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Time (h)
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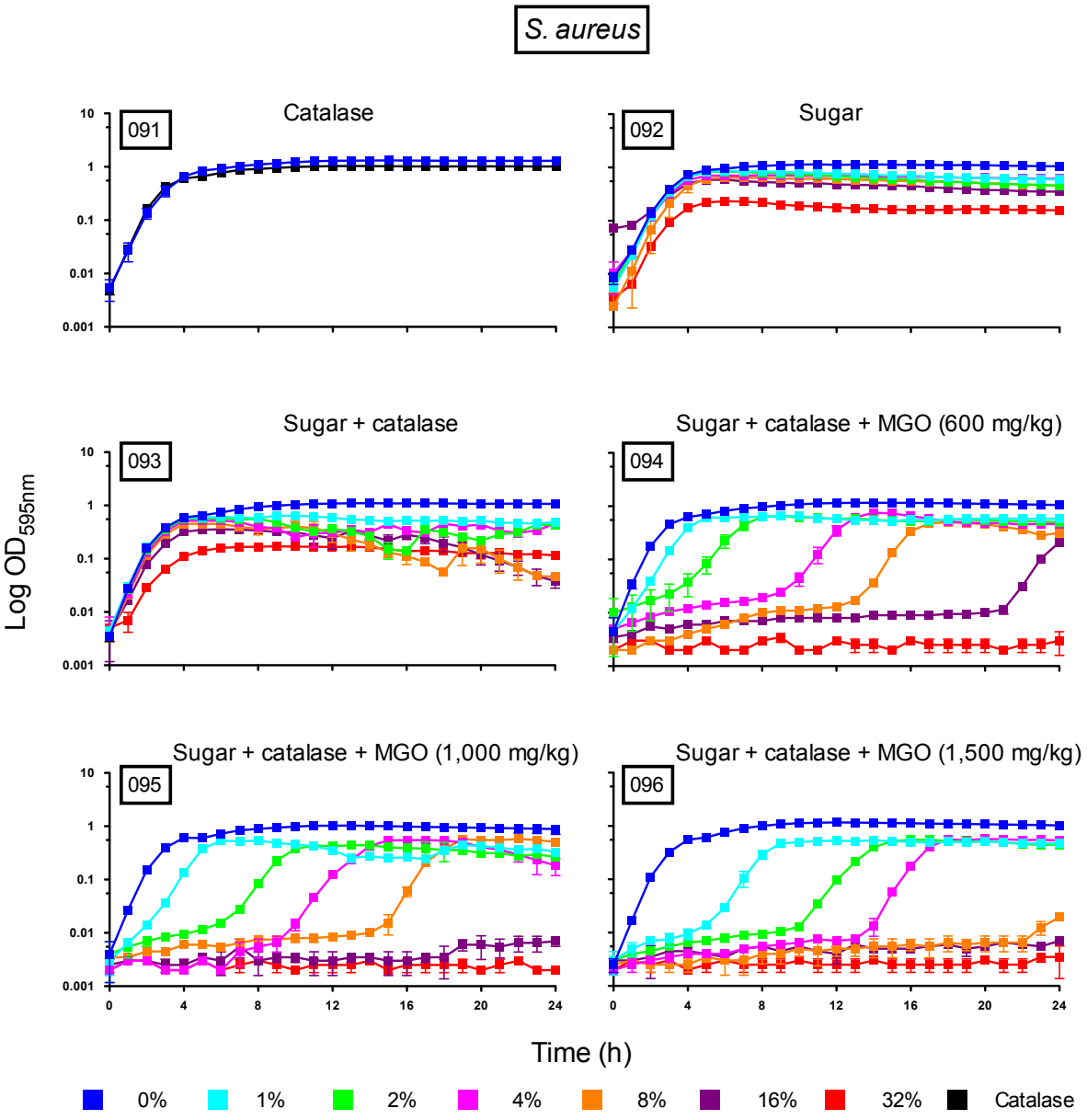
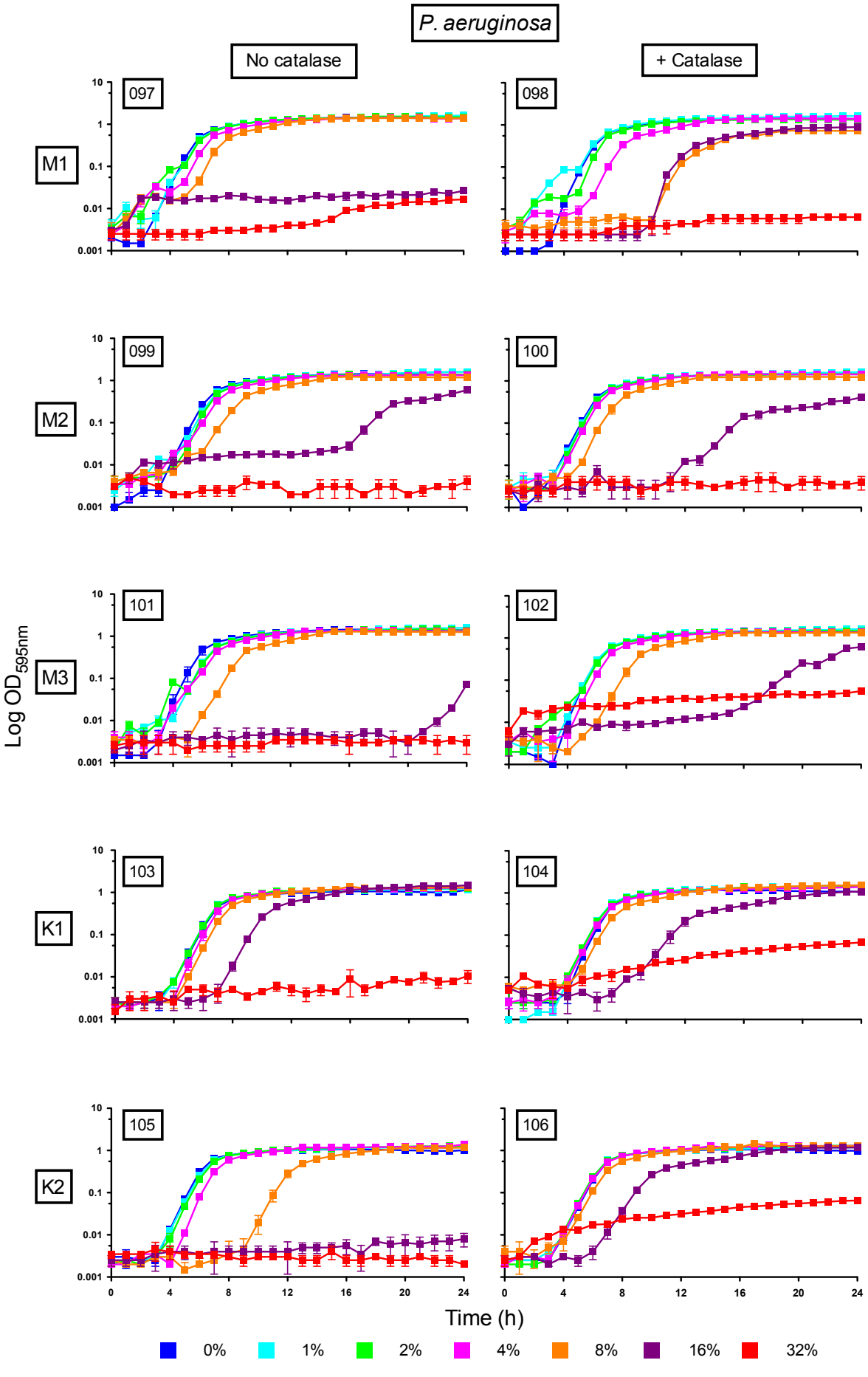

S. aureus
091
Catalase
092
Sugar
093
Sugar + catalase
094
Sugar + catalase + MGO (600 mg/kg)
095
Sugar + catalase + MGO (1,000 mg/kg)
096
Sugar + catalase + MGO (1,500 mg/kg)
Log OD595nm
Time (h)
0%
1%
2%
4%
8%
16%
32%
Catalase


P. aeruginosa
No catalase
+ Catalase
097
098
099
100
101
102
103
104
105
106
M1
M2
M3
K1
K2
Log OD595nm
Time (h)
0%
1%
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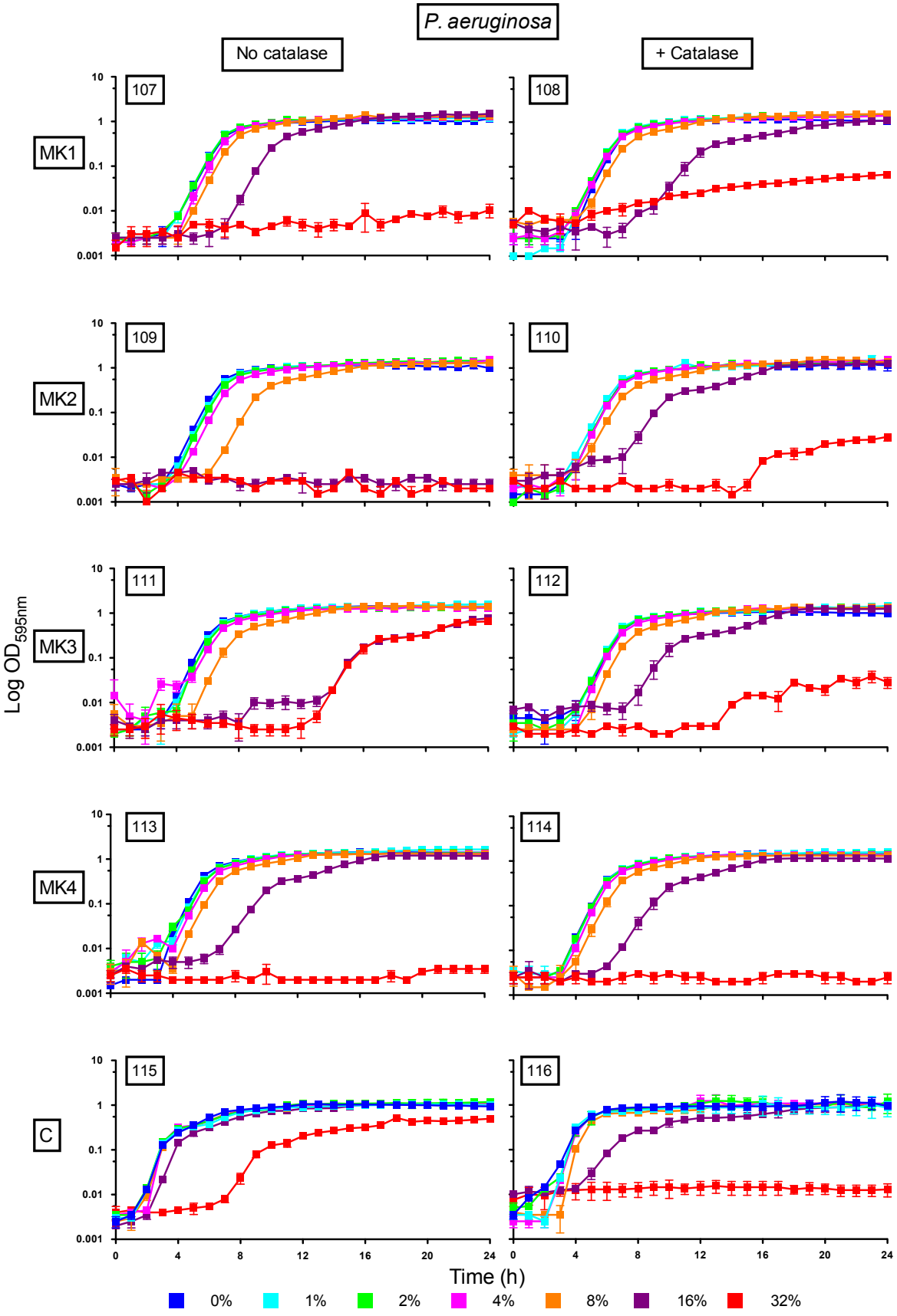
8%
16%
32%


P. aeruginosa
No catalase
+ Catalase
107
108
109
110
111
112
113
114
115
116
MK1
MK2
MK3
MK4
C
Log OD595nm
10
1
0.1
0.01
0.001
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Time (h)
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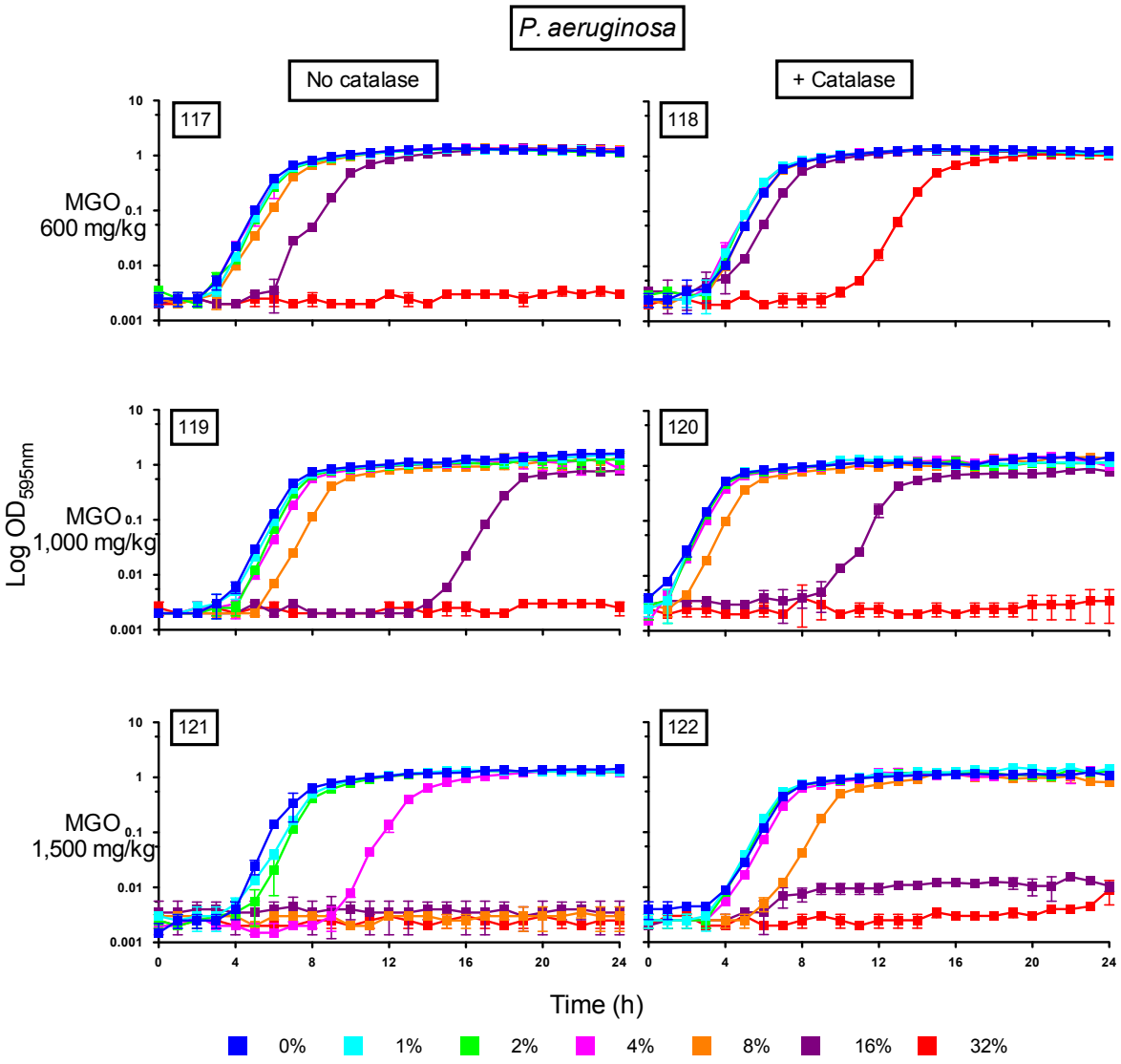
P. aeruginosa
No catalase
+ Catalase
117
118
119
120
121
122
MGO 600 mg/kg
MGO 1,000 mg/kg
MGO 1,500 mg/kg
Log OD595nm
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0.1
0.01
0.001
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Time (h)
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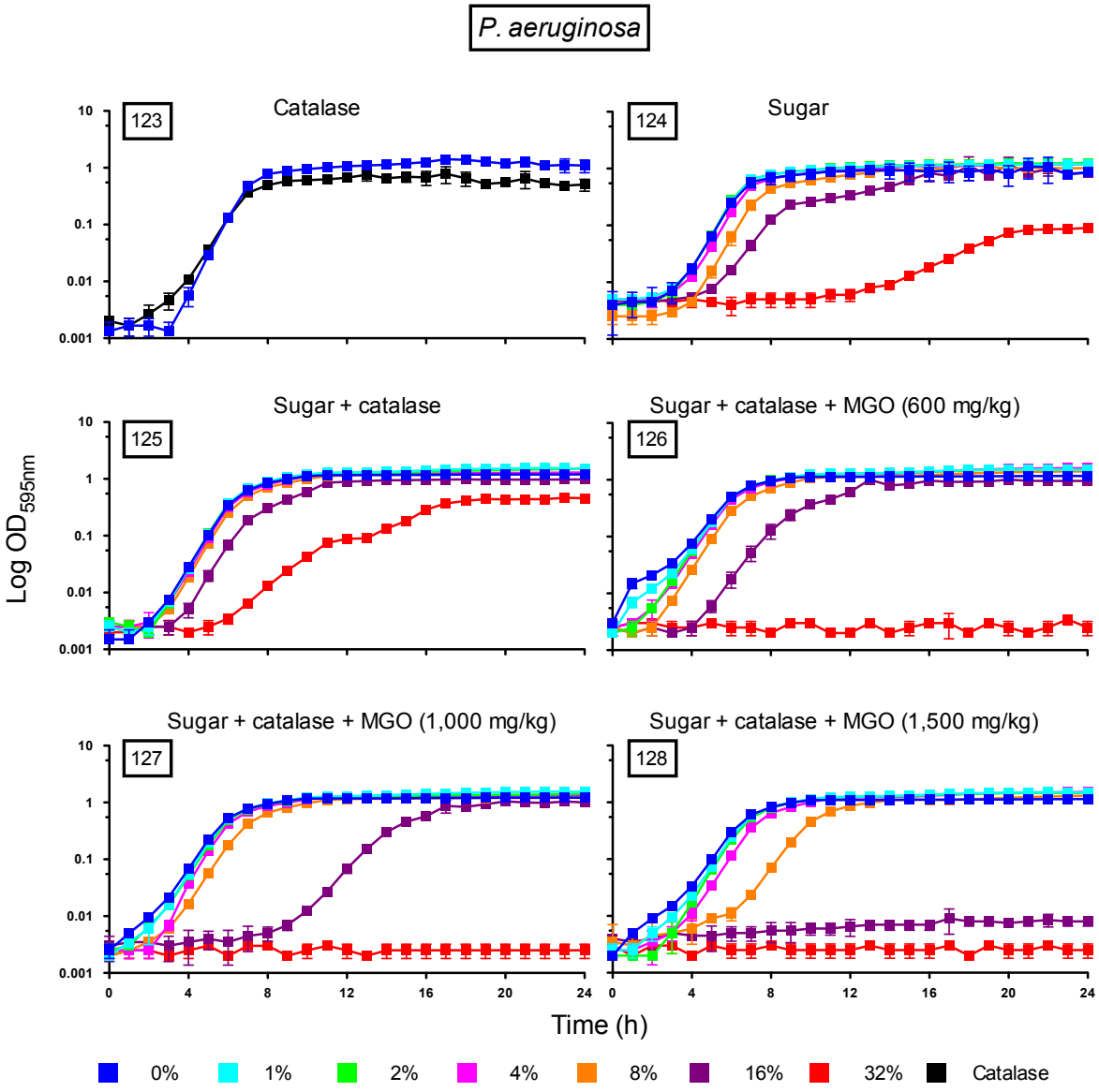
32%



Appendix I: The effect of New Zealand honey treatments on bacterial growth. Growth curves of *B. subtilis* (001-032), *E. coli* (033-064), *S. aureus* (065-096) and *P. aeruginosa* (097-128) were treated with 10 different honeys (three manuka honeys, M1, M2, M3; four manuka/kanuka blended honeys, MK1, MK2, MK3, MK4; two kanuka honeys, K1, K2; and a clover honey, C) and a comprehensive range of controls, which included (i) a sugar solution comprising 45% of glucose, 48% of fructose and 1% of sucrose; (ii) honey plus catalase (1 mg/mL); (iii) a catalase-only control; (iv) three MGO solutions at starting concentrations matching that present in 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 plus catalase; and finally (vi) different MGO concentrations in the presence of both catalase and sugar solution at various concentrations (0% - as no honey control, 1%, 2%, 4%, 8%, 16% & 32% (w/v), represent by dark blue, light blue, green, pink, orange, purple and red color respectively) . Optical density was recorded at 595 nm every h for 24 h. The optical density was then log-transformed and plotted against time using GraphPad Prism 5.0.

Appendix II

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The Effect of New Zealand Kanuka, Manuka and Clover Honeys on Bacterial Growth Dynamics and Cellular Morphology Varies According to the Species

Jing Lu[1], Dee A. Carter[2], Lynne Turnbull[1], Douglas Rosendale[3], Duncan Hedderley[3], Jonathan Stephens[4], Swapna Gannabathula[5], Gregor Steinhorn[4], Ralf C. Schlothauer[4], Cynthia B. Whitchurch[1], Elizabeth J. Harry[1]*

1 The ithree institute, University of Technology Sydney, Sydney, Australia, **2** School of Molecular Bioscience, University of Sydney, Sydney, Australia, **3** The New Zealand Institute for Plant and Food Research Ltd., Food Industry Science Centre, Palmerston North, New Zealand, **4** Comvita NZ Limited, Te Puke, New Zealand, **5** Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, Auckland, New Zealand

Abstract

Treatment of chronic wounds is becoming increasingly difficult due to antibiotic resistance. Complex natural products with antimicrobial activity, such as honey, are now under the spotlight as alternative treatments to antibiotics. Several studies have shown honey to have broad-spectrum antibacterial activity at concentrations present in honey dressings, and resistance to honey has not been attainable in the laboratory. However not all honeys are the same and few studies have used honey that is well defined both in geographic and chemical terms. Here we have used a range of concentrations of clover honey and a suite of manuka and kanuka honeys from known geographical locations, and for which the floral source and concentration of methylglyoxal and hydrogen peroxide potential were defined, to determine their effect on growth and cellular morphology of four bacteria: *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. While the general trend in effectiveness of growth inhibition was manuka>manuka-kanuka blend>kanuka>clover, the honeys had varying and diverse effects on the growth and cellular morphology of each bacterium, and each organism had a unique response profile to these honeys. *P. aeruginosa* showed a markedly different pattern of growth inhibition to the other three organisms when treated with sub-inhibitory concentrations of honey, being equally sensitive to all honeys, including clover, and the least sensitive to honey overall. While hydrogen peroxide potential contributed to the antibacterial activity of the manuka and kanuka honeys, it was never essential for complete growth inhibition. Cell morphology analysis also showed a varied and diverse set of responses to the honeys that included cell length changes, cell lysis, and alterations to DNA appearance. These changes are likely to reflect the different regulatory circuits of the organisms that are activated by the stress of honey treatment.

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Competing Interests: The authors have read the journal's policy and have the following conflicts: JS, GS and RS are employees of Comvita New Zealand (NZ) Limited which trades in medical grade manuka honey (Medihoney) Comvita NZ Ltd. have partially funded the work through a contribution to Linkage Project LP0990949 funded by the Australian Research Council. Chief Investigators on this project include EJH, CW, LT, DC and Partner Investigator RS. Some of the work reported in this manuscript performed by DR was conducted as a fee for service from Comvita Ltd., NZ. SG's current PhD scholarship is funded by Comvita Ltd., NZ. The competing interests mentioned in our submission do not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: liz.harry@uts.edu.au

Introduction

Wounds of the skin and mucosal layers can be generated by accidental trauma, surgery, maceration, inflammation and some cosmetic procedures (e.g. tattooing and piercing). For most superficial wounds, healing is prompt and requires no intervention. However, in some instances wounds can become infected, and in persons with impaired immunity or circulation, wounds can become non-healing, progressive and chronic. There is growing evidence that chronic wounds result from a complex interplay of host immunity and bacterial infection, and that infection can be due to a consortia of different species of bacteria embedded in a biofilm matrix that is highly resistant to antimicrobial therapy [1]. Planktonic bacteria are also important in chronic and acute wounds, and their release from biofilms has been proposed to maintain the inflammatory response within the wound [2,3], as well as allowing seeding to other areas. The emergence of bacterial pathogens resistant to multiple antibiotics has exacerbated the problems associated with treating infected wounds, particularly in the hospital setting [4,5]. There is an increasing need for new approaches to treat these infections, which are estimated to affect 6.5 million patients and to cost US$25 billion annually, with significant increases expected in the future [6].

Antimicrobial honey produced from the *Leptospermum scoparium* (manuka) plant from New Zealand has many features that make it

a promising therapy for wound care. Manuka honey is broad in spectrum and able to inhibit a diverse range of bacterial and yeast pathogens, and is equally effective against multi-drug resistant bacteria [7–9]. This honey has been found to prevent the formation of biofilms and can disrupt pre-formed biofilms [10–11]. Resistance to manuka honey has never been observed and could not be attained under laboratory conditions that rapidly induced resistance to conventional antibiotics [7] [9]. And finally, honey stimulates the immune system and can promote wound healing [12]. There are a number of medicinal honey products on the market in the form of ointments, creams and 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, low pH, and the generation of hydrogen peroxide by the bee-derived enzyme glucose oxidase [14]. Some honeys also contain levels of bee defensin-1 that are sufficient to inhibit growth of bacteria [15,16]. Active manuka honey contains high levels of the reactive dicarbonyl methylglyoxal (MGO) [17,18], which forms non-enzymatically from nectar-derived dihydroxyacetone (DHA) during ripening. A diverse range of phenolics, complex carbohydrates and peptides have also been reported in honey samples, and these may contribute to or modulate antibacterial activity [19–20].

The antibacterial activity of honey is generally assessed by measuring the extent to which the indicator bacterium, *S. aureus*, is inhibited using agar diffusion or broth micro-dilution methods [21]. Similar tests have been performed to determine the inhibition of other bacterial and yeast species [7] [22,23]. Manuka honey marketed for medicinal use generally uses a potency rating based on the "Unique Manuka Factor" (UMF), which measures antibacterial activity that is unrelated to the content of hydrogen peroxide, and is based on the *S. aureus* inhibition test. Alternatively, some medicinal honeys express potency as a direct assessment of MGO levels. While it has been established that manuka honey can inhibit the growth of bacterial cells, its effect on growth and cellular physiology among different bacterial pathogens, and how these change when the levels of the major antibacterial components, MGO and hydrogen peroxide, vary in natural honeys are unclear. These are important considerations for optimizing honey for wound care since sub-lethal levels of honey may have unanticipated effects, and there is emerging evidence that different 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 different bacterial species, including two of the most common wound pathogens, to a suite of natural honey samples that differ in their levels of MGO and hydrogen peroxide production. This included samples of monofloral manuka honey with moderate to high MGO levels, samples of honey produced from the New Zealand kanuka *Kunzea ericoides* bush [19], where MGO levels are negligible but hydrogen peroxide is present, and manuka-kanuka blends that contain both active components at moderate levels. We included a set of controls to mimic the effects of sugar, to neutralize the effect of hydrogen peroxide, and to examine how MGO might act outside the honey milieu. We report here that while clinically relevant concentrations of honey are effective at inhibiting growth of all four bacteria, the growth and morphological responses at sub-lethal levels varied significantly between species. Furthermore, *P. aeruginosa* responded strikingly differently to the other three species (*B. subtilis*, *E. coli* and *S. aureus*). When present in sub-lethal concentrations, MGO extended the lag phase of bacterial growth in a dose-dependent manner, and the organisms eventually resumed normal growth, presumably by detoxifying the MGO. Topical wound dressings should therefore contain a high level of active honey to ensure wound pathogens are eliminated.

Materials and Methods

Honey Samples

Table 1 lists the New Zealand honey samples used in this study, which included monofloral manuka (M1, M2, M3), monofloral kanuka (K1 and K2), manuka-kanuka blends (MK1, MK2, MK3, MK4) and clover (C) honey. Samples were chosen based on their levels of methylglyoxal (MGO; previously reported in Stephens *et al.* 2010), and hydrogen peroxide, determined in this study. Manuka, kanuka and manuka-kanuka honey samples were supplied by Comvita New Zealand Ltd. (Te Puke, New Zealand) and the clover honey sample was a commercially-packaged New Zealand white clover honey [19]. Native New Zealand honey is produced by bees foraging in their local environment and cannot be guaranteed to be 100% monofloral, however the supplied samples were considered to be as representative of pure honey from a single floral origin as possible. Details of other chemical components in the manuka and kanuka honeys have been described previously [19]. All samples were stored in the dark at 4°C and were diluted fresh for use in all assays. All honey concentrations are expressed as % w/v.

Hydrogen Peroxide Assay

The level of hydrogen peroxide produced by the honey samples was determined using a hydrogen peroxide/peroxidase assay kit (Amplex Red, Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA). The assay, which measures the oxidation by hydrogen peroxide of the non-fluorescent substrate Amplex Red to highly fluorescent resorufin [24], was conducted in 96-well microtitre plates according to the manufacturer's instructions. Resorufin fluorescence was measured at 530 nm excitation/590 nm emission using a SpectraMax Gemini EM (Molecular Devices, LLC, Sunnyvale, CA, USA) fluorometer. Hydrogen peroxide standards from 5–20 μM were used to produce a standard curve, which was then used to assess production in duplicate samples of 2.5% and 5% w/v dilutions of the honey samples. The 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. aureus* ATCC 25923 (American Type Culture Collection), and the Gram-negative bacteria *E. coli* O157:H7 [26] and *P. aeruginosa* PAO1 (ATCC 15692). *B. subtilis* is a well-studied model organism, and the other three species are clinically relevant pathogens. Growth media were selected to allow optimal growth of the different bacterial species: Luria-Bertani (LB) (Oxoid Ltd., Basingstoke, Hampshire, UK) broth and agar were used for *E. coli, P. aeruginosa* and *B. subtilis*, while Tryptone Soya Broth and agar (Oxoid Ltd., Basingstoke, Hampshire, UK) was 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 added honey to diluted stationary-phase bacterial cultures so that it would more accurately represent the addition of a honey dressing to a chronic wound. Single colonies of bacteria grown on agar were used to inoculate broth cultures. These were grown overnight at 37°C on an orbital shaker at 250 rpm (Bioline^{TM}, Australia) except *B. subtilis,* which was grown overnight at 30°C with slower shaking using a gyrotory

Table 1. Floral source, MGO and H_2O_2 Levels of Honeys.

Code	Previous code[a]	Honey	Floral source	Antibacterials	
				MGO[b] (mg/kg)	H_2O_2[c] (mM/h)
M1	2	Manuka[d]	*Leptospermum scoparium var incanum*	651.4	0.532
M2	13	Manuka [e]	*L. scoparium var incanum+Kunzea (?)*	1004.3	0.282
M3	7	Manuka[e]	*L. scoparium var incanum*	1541.3	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[e]	*Kunzea ericoides+manuka (?)*	173.6	0.583
MK2	–	Manuka-Kanuka	*Kunzea ericoides+manuka (v. likely)*	229.8	0.448
MK3	18	Manuka-Kanuka[e]	*L. scoparium var 'triketone'+Kunzea*	269.9	0.345
MK4	15	Manuka-Kanuka[d]	*L. scoparium var 'triketone'*	307.8	0.380
C	24	Clover[f]	*Trifollium spp.*	trace	0.029

[a]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.
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waterbath shaker (New Brunswick Scientific, Enfield, CT, USA). The slower shaking conditions for *B. subtilis* ensure that this culture does not spend too long in stationary phase, which would delay entry into exponential growth upon dilution. Cell density of the overnight cultures was assessed using serial-dilution plating and was approximately 10^9 colony-forming units (CFU)/mL. A suspension from the overnight culture was then diluted to a cell density of 10^3 CFU/mL in fresh media containing honey to give a final volume of 150 µL. For each growth assay, a freshly prepared 50% (w/v) honey stock solution was made by weighing the appropriate amount of honey and mixing this with an equivalent amount of sterilized distilled water. This stock solution was then further diluted with the appropriate growth medium to give the required honey concentration. Growth of each bacterial species was tested in six concentrations of each honey (1%, 2%, 4%, 8%, 16% and 32% w/v) in a 96-well microtitre plate format. A microtitre plate reader (Biotek PowerWave HT®, Bioteck Instrunents Inc, Winooski, VT, USA) programmed to measure the optical density hourly at 595 nm (OD_{595nm}) (Gen5®, BioTek) was used to assay cell growth over 24 hours at 37°C, with moderate shaking (1800 rpm, amp. 0.549 mm x-axis). Two biological replicates, each with four technical replicates were performed for the growth assays and each growth curve produced in Figure S1 represents the average of all data. Growth curves were presented using GraphPad PrismV. 5.0c (Graphpad Software, San Diego, CA, USA).

A comprehensive range of control treatments was included for each organism in the microtitre-plate growth assays. These included: (i) a no-treatment control; (ii) a sugar solution comprising 45% glucose, 48% fructose and 1% sucrose, (diluted as above for honey) to identify any effects on bacterial growth due to the high sugar content in honey; (iii) honey plus catalase (1 mg/mL) to neutralize hydrogen peroxide [14]; (iv) a catalase-only control; (v) MGO diluted in water to concentrations similar to those present in honeys M1, M2 and M3 (600, 1,000 & 1,500 mg/kg undiluted honey) at the various tested concentrations, to assess the effect of MGO alone on bacterial growth; vi) the same MGO dilutions plus catalase; and (vii) MGO diluted in sugar solution to the same concentrations as above and with added catalase. 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 dynamics was an extended lag phase, such that entry into exponential growth was delayed, and this increased with increasing honey concentrations. Thus, we focused on how honey altered the duration of lag phase. Lag phase was calculated as the period from inoculation to onset of log phase, or to 10% of maximal culture absorbance. Given the large number of different growth curves (128 individual growth assays with 6 different honey concentrations per assay), we automated the calculation of these parameters by fitting the absorbance values from the bacterial growth experiments to a generalized logistic curve (equation 1), a sigmoid function used for growth modeling, using the Genstat program (Release 11.1 (PC/Windows) 28 January 2011, VSN International Ltd, UK). Due to variable T values, this generalized logistic curve fitted better than a corresponding Gompertz curve (not shown).

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

Here, A = the lower asymptote; C = the upper asymptote; M = time of maximum growth; B = growth rate, and T = time near which maximum (stationary phase) growth occurs.

With these parameters, we were able to compare the effect of the different honey samples on growth simply by plotting the duration of lag phase (time (h)) in the presence of varying honey concentrations (% w/v). This conversion from growth curve to lag phase duration is illustrated in Figure S2, where a sample curve of *E. coli* growth in response to a series of manuka honey M3 dilutions (Fig. S2A, onset of log phase or 10% of maximal culture absorbance at each honey concentration shown 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 maximal culture absorbance was very similar to the no-honey control culture. However in a few cases the maximal absorbance of the treated culture was less than 10% of the maximal culture absorbance of the no-honey control. 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 samples obtained from the middle of the prolonged lag phase induced by honey treatments, and at log phase (which we will refer to as log phase) when cultures had resumed apparently normal growth. If a prolonged lag phase was not observed, lag phase cells were obtained from within the first half hour of incubation. Untreated cells from lag and log phases of growth were also harvested for analysis, with the lag-phase cells collected 30 min after inoculation as described above. Harvested cells were treated for microscopy as described previously [27] but with the following modifications: 20 μL of fixed cells were diluted 1:1 with the DNA staining agent DAPI (4',6-diamidino-2-phenylindole; Life Technologies), to give a final DAPI concentration of 0.4 μg/mL for *E. coli*, *B. subtilis* and *P. aeruginosa*, and 0.8 μg/mL for *S. aureus*. Triplicate 10 μL aliquots of the stained cells were then placed in separate wells of a multi-well microscope slide (MP Biomedicals, LLC, Eschewege, Germany) that had been treated with 0.01% poly-L-lysine (Electron Microscopy Sciences, Hatfield, PA, USA). After 15 min at room temperature, the liquid was removed and 50% glycerol was placed on each sample. A coverslip was 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 microscope equipped with a Plan ApoChromat (100x, NA 1.4; Carl Zeiss AG, Oberkochen, Germany) objective lens, and images were captured using a Zeiss AxioCam MRm cooled CCD camera controlled by AxioVision software (version 4.5; Carl Zeiss). Fluorescence microscopy to visualize DNA stained with DAPI used a 100 W high pressure mercury lamp passed through filter set 02 (Carl Zeiss) as a light source. Image processing was performed using AxioVision software version 4.5 (Carl Zeiss).

Image Data Analysis

Cell length, cell lysis and DAPI staining were assessed by digital analysis of the captured images. Cell length and DAPI staining were scored only for unlysed cells. A total of 152 fields of cells were imaged and analyzed. Cell length (or diameter in the case of *S. aureus*) was measured using MicrobeTracker (version 0.929) [28]. We used this MATLAB-based software to detect and outline bacterial cells in the microscopy images and measure cell lengths automatically. The optimized parameters (incorporated into the MicrobeTracker software) included a modification to algorithm 4 to enable accurate cell length measurements of rod-shaped organisms in the case of *E. coli*, *B. subtilis* and *P. aeruginosa*. For *S. aureus* algorithm 1 was optimized to enable measurement of the size of these spherical cells [28]. The individual cell length information was then extracted and statistical analysis was performed in GraphPad Prism. One-way ANOVA and Tukey's multiple comparison tests were performed with the no-honey treated cells as controls. Cells that appeared lysed due to changes in their contrast under phase-contrast microscopy were scored and cell lysis was expressed as a percentage of the whole population. Only cells that remained intact but appeared to lose their cytoplasmic contents were scored, thus underestimation of cell lysis was possible, but this was consistent across all samples. For all experiments, at least 100 cells were scored, except for M3-treated *B. 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 Gram-negative bacteria, *E. coli* O157:H7 and *P. aeruginosa* PAO1 (ATCC 15692) to the 10 honeys and various control solutions were assessed. These data comprised nearly 900 growth curves in 128 graphs (Fig. S1). For the honey treatments each graph represents a particular honey at six concentrations with a single organism as well as a no-honey control, which was carried out alongside each honey sample on the multi-well plates. A comprehensive range of control treatments were included in the growth assays to determine the effect of various honey 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 among bacteria in their response to the different honey types (see Materials and Methods). We therefore focused our analysis on growth inhibition on this parameter, expressed as the time taken (in hours) for the bacterial culture to reach 10% maximal culture absorbance. The graphs presented in Figure 1 and Figure 2 summarize the growth responses of the four different organisms to the control solutions (Fig. 1) and the honeys (Fig. 2). In these graphs, the time (h) taken for a culture to enter logarithmic growth (measured as at least 10% maximal culture absorbance; y-axis) is plotted against the honey (or component) concentration (x-axis) for each organism both in the absence (left panel) and presence (right panel) of catalase. Note that the faster the rise of the line, the longer the cells are arrested in lag phase at lower honey concentrations, and hence the more effective a particular honey is at inhibiting the growth of that organism. Culture growth was monitored over 24 hours, and if no growth occurred over 24 hours, it is referred to as 'no growth' or complete inhibition.

The starting absorbance values differ in each case in Figures 1 and 2 because for each honey, the no-honey control was included to more accurately reflect the experimental conditions during that particular experiment.

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

The addition of catalase to an overnight culture of bacterial cells had essentially no effect on the duration of the lag phase, or any other aspect of growth of the four organisms when compared with an untreated control culture (Fig. S1). Sugar alone had a small but variable effect on the growth of the bacteria: the Gram-positive species *B. subtilis* and *S. aureus* were unaffected even at high concentrations, while the two Gram-negatives became inhibited at 16–32% (Fig. 1A, 1C, 1E, 1G).

The addition of MGO at 0–32% of the concentrations present in manuka honey samples M1, M2 and M3 (starting concentrations of 600, 1,000 and 1,500 mg/kg honey) generally showed a dose-dependent extension of lag phase (Fig 1A, 1C, 1E, 1G). This was most severe for *E. coli*, followed by *B. subtilis* and *S. aureus*, and was lowest in *P. aeruginosa*. The difference in sensitivity to MGO alone between the organisms was most obvious at the lowest MGO concentration used (blue lines in Fig. 1A, 1C, 1E, 1G).

The addition of catalase to the MGO treatments shortened the lag-phase extension in most cases, so that the onset of log phase

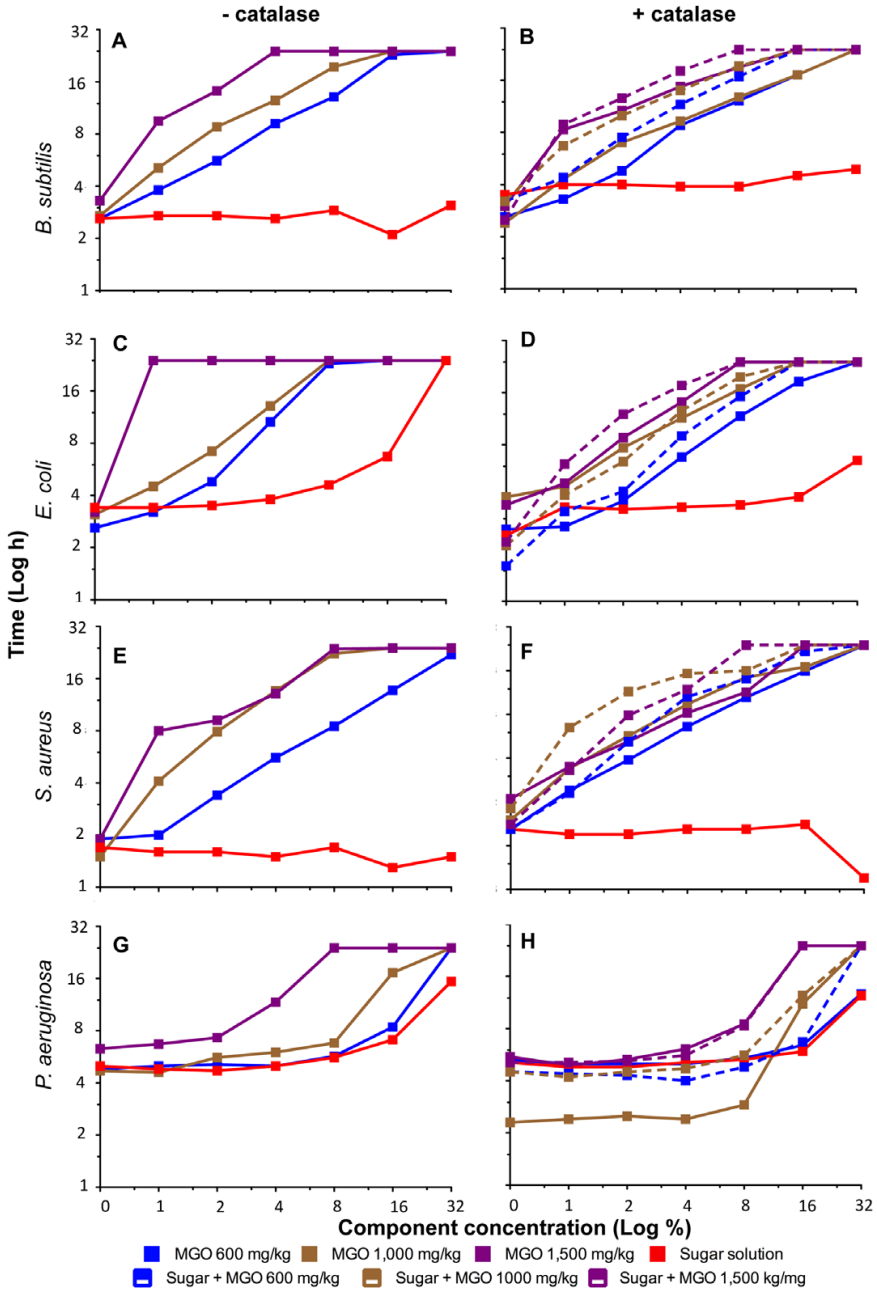

Figure 1. Effect of sugar, MGO and catalase on growth of bacteria. Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with various components, including catalase, MGO, sugar, and a combination of MGO and sugar at various concentrations equivalent to honeys at the corresponding concentrations shown on the x-axis. The MGO/sugar experiments were performed in the absence (left-hand graphs) and presence (right-hand graphs) of catalase as indicated. The MGO levels correspond to honeys M1 (651.4 mg/kg MGO), M2 (1004.3 mg/kg MGO) and M3 (1541.3 mg/kg MGO) at 1%–32% (w/v). Optical density was recorded at 595 nm every hour for 24 hours. For each component concentration, the time it takes for the culture to reach log phase (assessed as at least 10% of the final culture absorbance of the untreated culture) is plotted on the x-axis. The derivation of this value is described in Materials and Methods. A value of 24 hours on the y-axis denotes 'no growth'. An untreated control was performed alongside each particular treatment, and the starting OD_{595} (zero time-point on x-axis) is plotted for that particular honey experiment.
doi:10.1371/journal.pone.0055898.g001

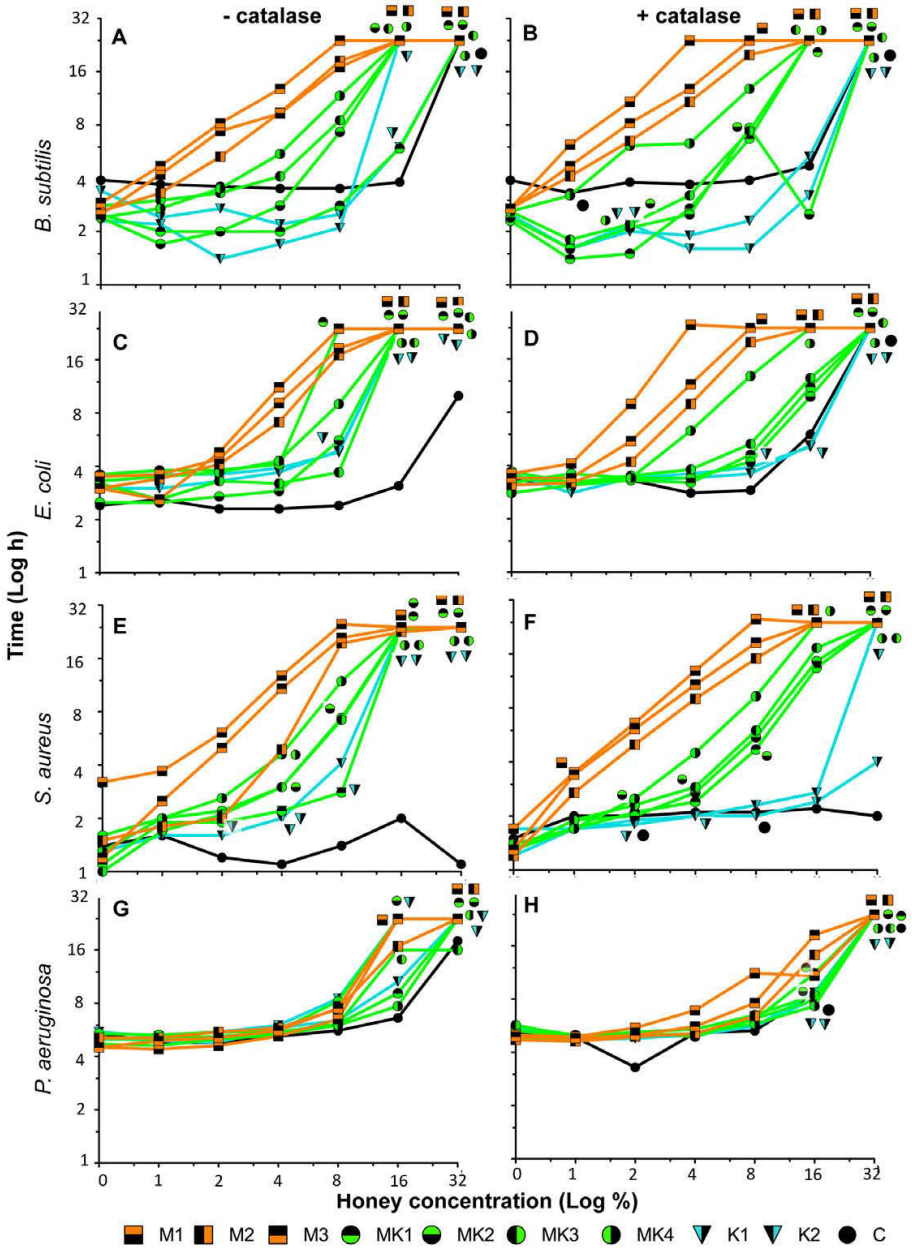

Figure 2. Effect of New Zealand manuka, kanuka and manuka-kanuka blended honeys on bacterial growth. Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with ten different honeys, plus or minus catalase: three manuka honeys, M1, M2 and M3; two kanuka honeys, K1 and K2; four manuka-kanuka blended honeys, MK1, MK2, MK3 and MK4; and one clover honey, C, at various concentrations (from 1%–32% (w/v), increasing in 2-fold series). Optical density was recorded at 595 nm every hour for 24 hours. For each honey concentration, the time it takes for the culture to reach log phase (assessed as at least 10% of the final culture absorbance of the untreated culture) is plotted on the x-axis. The derivation of this value is described in Materials and Methods. A value of 24 hours on the y-axis denotes 'no growth'. Where symbols for a particular honey overlap, we have surrounded the point on the graph by all the symbols relevant to that point. This occurs in several cases for 16% and 32% honey treatments. An untreated control was also performed alongside each particular honey treatment, and the starting OD_{595} (zero time-point on x-axis) is plotted for that particular honey experiment.
doi:10.1371/journal.pone.0055898.g002

occurred earlier. With the exception of *P. aeruginosa,* the bacteria were still increasingly sensitive to increasing MGO concentrations in the presence of catalase (Fig. 1, compare left with right panels). The addition of sugar to MGO in the presence of catalase had a small but noticeable effect on delaying the onset of log phase further for all organisms except *P. 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 shown in Figure 2. From these, four key features are particularly apparent: first, there is a general trend of greater growth inhibition by honeys containing more MGO, with M>MK>K>clover honey; second, the addition of catalase causes a shift of the curves to the right for most honey types indicating a rescue of growth inhibition; third, *P. aeruginosa* has a completely different pattern of growth inhibition compared to *B. subtilis*, *E. coli* and *S. aureus*; and fourth, the effect of clover honey is different for the different bacteria. These points will be explored further 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 tested, at 651.4, 1004.3 and 1541.3 mg/kg honey, respectively. These three honeys were the most effective in inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus* and all resulted in similar levels of growth inhibition. Low honey concentrations (1–4%) caused significant lag-phase extension and growth was completely inhibited once concentrations reached 8–16%.

In the presence of catalase the monofloral manuka honeys remained the most effective of the natural honeys at inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus*, indicating that the non-peroxide component/s in these honeys is the over-riding component responsible for their high levels of growth inhibition.

In contrast to *B. subtilis*, *E. coli* and *S. aureus*, there was very little or no lag-phase extension when *P. aeruginosa* was treated with low concentrations of honeys M1–M3, and complete inhibition only occurred at 16% of honeys M3 and M1 and 32% of honey M2. *P. aeruginosa* was relatively sensitive to sugar (Fig. 1G), which likely accounts for some of the inhibition. The addition of catalase increased the concentration of honeys M1 and M3 required for complete growth inhibition 2-fold (to 32%). These data and those shown in Figure 2 indicate that *P. aeruginosa* is relatively insensitive to both hydrogen peroxide and MGO, and that at 32% manuka honey 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 moderate rates of hydrogen peroxide production (0.360 and 0.327 mM/h, respectively) compared to the other honeys tested. At low concentrations (1–8%), and particularly in the presence of catalase, K1 and K2 were amongst the least effective of the honeys at inhibiting growth of *B. subtilis*, *E. coli* and *S. aureus*, with very little or no lag-phase extension compared to the no-honey cultures. 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. coli* and *S. aureus*, complete growth inhibition still occurred at 32%; the exception being honey K1, where lag phase was only extended by 4 hours (Fig. 2F). The non-peroxide component causing this growth inhibition is not likely to be due to MGO, or at least MGO acting alone, since the amount present in 32% K1 and K2 is 11.8 and 1.8 mg/kg respectively, which is equivalent to 1% of 660 mg/kg MGO and is therefore too low to affect growth of these bacteria (Fig. 1A, 1C, 1E; light blue line). The inability of honey K1 to inhibit the growth of *S. aureus* suggests that the component/s that contribute to complete growth inhibition of *B. subtilis* and *E. coli* are not active against *S. aureus*. Alternatively there may be a component in honey that is specifically active against *S. aureus* but requires hydrogen peroxide for its production and/or activity. Note that *S. aureus* was also the 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 lag-phase extension was observed. Catalase addition rescued this effect to some extent, but at 32% the kanuka honeys completely inhibited growth of *P. aeruginosa*. Again, this suggests component/s additional to MGO or peroxide are present in these honeys that affect growth of this organism. The most striking observation for *P. aeruginosa* that was distinctly different from the other 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 in Figure 2. These honeys have intermediate levels of MGO (ranging from 173.6–307.8 mg/kg) that are between those of the pure manuka and kanuka honeys, and variable but significant levels of hydrogen peroxide (Table 1). Treatment with these honeys gave a level of inhibition that was generally between that of the pure manuka and pure kanuka honeys, especially when the hydrogen peroxide was removed by catalase. In addition, the degree of growth inhibition related largely to the level of MGO, with MK4, which has the highest level of 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 similar, there were some notable differences in how *E. coli* responded to the different blends. In the absence of catalase MK1 inhibited *E. coli* growth to a similar extent as the manuka honeys, with complete growth inhibition at 8%. MK1 has a low level of MGO (173.6 mg/kg) compared to the manuka honeys but has the highest hydrogen peroxide production rate of all honeys. Catalase addition to MK1 reduced the level of growth inhibition for *E. coli* to a level well below that of all three manukas. These observations suggest that *E. coli* growth can be maximally inhibited by honeys that either have a high level of hydrogen peroxide production or have high levels of MGO.

P. aeruginosa displayed little or no lag-phase extension or growth inhibition for any of the blended honeys until concentrations reached 16% or 32%. Overall, there was no clear trend in how *P. aeruginosa* responded to the varying levels of MGO and hydrogen peroxide in the different blends, however complete growth inhibition was achieved at 32% in the presence of catalase, indicating that the inhibition does not require hydrogen peroxide.

Clover honey. The clover honey sample had no detectable MGO and almost no hydrogen peroxide production (0.029 mM H_2O_2/h; Table 1). Up to 16% clover honey had little effect on the growth of the four organisms (Fig. 2). At 32%, *S. aureus* growth remained unaffected, while the two Gram-negative species, *E. coli* and *P. aeruginosa,* showed a significant lag-phase extension. This is commensurate with the response of these two organisms to 32% sugar (Fig. 1A, 1C, 1E and 1G). However, while the addition of catalase to clover honey slightly increased lag phase extension, this was not seen for the corresponding sugar control. Interestingly, 32% clover honey completely inhibited growth of *B. subtilis*, both in the presence and absence of catalase even though sugar alone at equivalent concentrations had no effect on the growth of this

organism. This suggests the presence of one or more components in clover honey to which *B. subtilis* growth is particularly sensitive.

Other observations not fitting growth inhibition trends. Although there were clear trends in growth inhibition in response to treatment with honeys and control solutions discussed above, there were certain observations that did not fit these trends that are worth acknowledging. This includes: M1, which has the lowest level of MGO, was the most active manuka honey for *B. subtilis*, *E. coli* and *S. aureus* in the presence of catalase (Fig. 2B, 2D, 2F); the apparent abrupt (and reproducible) decrease in growth inhibition of the MK2 honey against *B. subtilis* at 16% in the presence of catalase (Fig. 2B); the incomplete inhibition of *P. aeruginosa* by honey MK3 only observed when catalase was not present (Fig. 2G); and a higher level of inhibition of *E. coli* by clover honey in the presence of catalase (Fig. 2D). Given the complexity of honey it is likely that the growth inhibition we observe in these analyses cannot always be solely accounted for by the presence MGO and hydrogen peroxide, and other components may exert independent action or may 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 MGO or hydrogen peroxide, *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa* were exposed to honey samples M3 (highest MGO with lowest hydrogen peroxide production of the tested honeys) and MK1 (highest rate of hydrogen peroxide and relatively low MGO; Table 1). Cells were treated with 4% (w/v) of each honey, which is the highest concentration that still allowed growth of all four bacteria (see above; Fig. 2). Cell morphology was analysed during lag- and log-phase growth and included measures of cell shape changes (length or width), cell lysis (breakage of cells or leakage of cytoplasm indicating cell envelope or growth abnormalities), and detection of chromosomal DNA abnormalities by DAPI staining.

High-level MGO honey and cell morphology. Treatment with honey M3 induced an extended lag phase for all bacterial cultures except *P. aeruginosa* (Fig. S1; Fig. 2). Morphological changes are shown in Figure 3 and charted in Table 2, and mean cell lengths are recorded in Table S1. During the extended lag phase (or the initial lag phase for *P. aeruginosa*), cells of *B. subtilis*, *E. coli* and *S. aureus* were significantly shorter ($p<0.05$) than untreated cells, while *P. aeruginosa* cells were longer (Table 2; Fig. 3). In addition, a significant percentage of the shorter cells of *B. subtilis* (29%) and *S. aureus* (57%) had a condensed chromosome (green arrows in Fig. 3). In the *B. subtilis* cells only one bright region of DAPI staining occurred instead of the characteristic two regions that represent replicating chromosomes (Fig. 3; Table 2). Likewise the *S. aureus* cells with condensed chromosomes showed one or two very small spots of DAPI-stained DNA, unlike the two larger lobes of DNA that represent replicating chromosomes in the no-honey control cells. No changes to DNA appearance under these conditions were observed for *E. coli* or *P. aeruginosa*.

Following entry into log phase, cells treated with M3 honey were still significantly different to untreated cultures (Table S1 and Fig. 3). *B. subtilis* and *S. aureus* cells remained shorter, to a similar degree observed in lag phase, and chromosomes remain condensed. *E. coli* cells became significantly longer than their untreated counterparts, while *P. aeruginosa* cells became slightly but significantly shorter (Table 2). In addition, 2% of the *P. aeruginosa* cell population now showed a condensed 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 both lag and log phases of growth, but the direction and the extent of change varied. The greatest changes to cell length were observed with *B. subtilis* and *E. coli* (Table 2). Only the Gram-positive organisms had condensed 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 honey was particularly inhibitory to *E. coli* (Fig. 2). The most significant changes to cell morphology induced by MK1 during the initial stages of growth were observed in *B. subtilis*, where cells were on average significantly shorter, as was seen with M3 but to a lesser extent. A significant proportion of cells (34%) appeared lysed as judged by a decrease in contrast visualized using phase-contrast microscopy (asterisks in Fig. 3; Tables S1 and 2), and the DNA in the vast majority of unlysed cells (90%) appeared dispersed compared to untreated cells (Fig. 3; Table 2). No morphological changes were observed in *E. coli* or *P. aeruginosa* cultures, and the only change to *S. aureus* cells was a slight but significant 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 cultures to 2%. However, cells were still shorter on average compared to control cells (Table 2 and Table S1; Fig. 3), and the frequency of cells with a dispersed DNA appearance remained very high (99%). Log-phase *S. aureus* cells showed a normal morphology (Fig. 3; Table 2), while *P. aeruginosa* cells were significantly shorter (Fig. 3; Table S1 and 2). Two percent of the log-phase MK1-treated *P. aeruginosa* cells had the same condensed chromosome phenotype seen in the log-phase M3-treated *P. aeruginosa* cells. *E. coli* log-phase 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. subtilis* cells were the most significantly affected, with dispersed DNA, cell lysis and cell length changes. *E. coli* and *S. aureus* had little or no apparent change. *P. aeruginosa* cells were shorter in log phase only.

Correlation of Growth Inhibition and Morphological Changes Induced by Honey

A summary of combined growth and morphology data is given in Table 3. Overall this shows that MGO and manuka honeys are the most effective at inhibiting growth of all organisms except *P. aeruginosa*, followed by manuka-kanuka blended honeys, kanuka honeys and then clover. *P. aeruginosa* is much less sensitive to the honeys compared to the other three organisms, with little difference in growth inhibition by the different honeys, including clover. *B. subtilis* shows more morphological changes than the other bacteria for both the high-MGO and the high-hydrogen peroxide honeys, followed by *S. aureus*, *P. aeruginosa* and then *E. coli*. Thus, with the exception of *B. subtilis*, where both growth and morphology are profoundly affected by honey, the number and severity of morphological changes do not link clearly to the level of MGO or hydrogen peroxide in the honey, or to the effectiveness of the honey to inhibit growth. *E. coli* had very little apparent morphological changes even though its growth was affected in a similar way to *B. subtilis* and *S. aureus* and to a much greater extent than *P. aeruginosa*, with the latter conversely showing more profound morphological changes.

Discussion

With the dearth of development of new classes of antibiotics to treat infections caused by resistant organisms, honey is increasingly

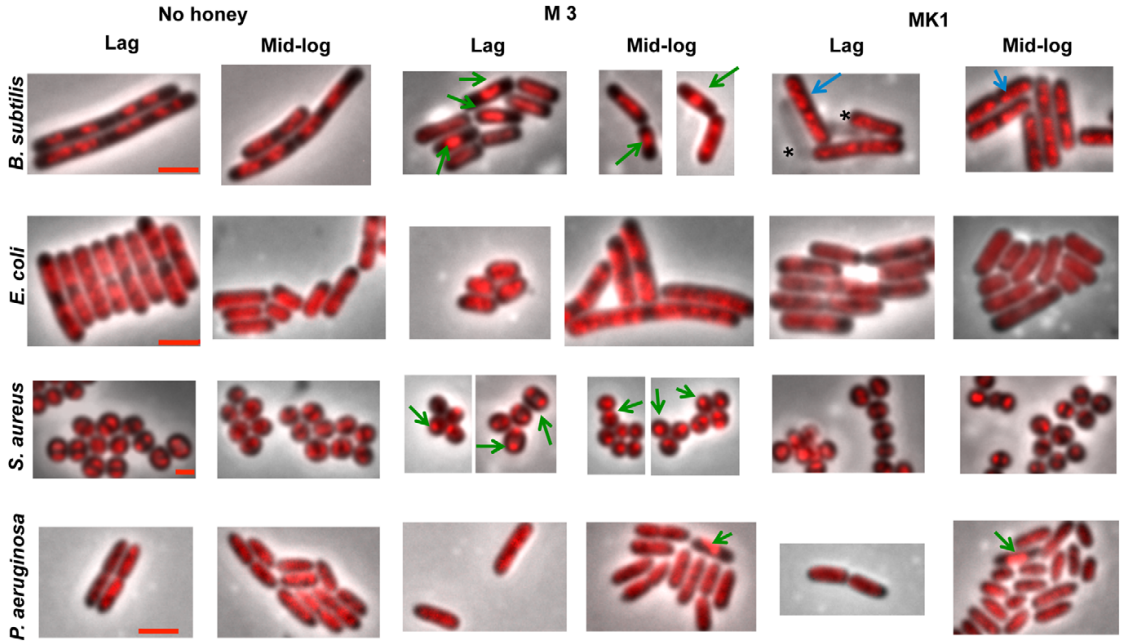

Figure 3. Cellular morphology of bacterial cells treated with a high-MGO honey and a high-hydrogen peroxide honey. The effects of 4% (w/v) of a high-MGO honey (M3) and a high-hydrogen peroxide honey (MK1) on bacterial cellular morphology were examined. Overnight cultures of *B. subtilis, E. coli, S. aureus* and *P. aeruginosa* were treated with these honeys, cells collected at both lag and log phases of growth as indicated in Figure S2, fixed with glutaraldehyde, stained with DAPI and imaged using fluorescence microscopy. All images are overlays of the phase-contrast image and the DAPI-stained (red) fluorescence image. The two left-hand panels show the no-honey treated control cells, the two middle panels M3 honey-treated cells, and the two right-hand panels show the MK1 honey-treated cells. In all images, condensed DNA is shown by green arrows; and dispersed DNA in *B. subtilis* cells is shown by blue arrows. An asterisk indicates lysed cells for *B. subitlis* (MK1, lag-phase cells). The scale bar represents 2 μm, except for *S. aureus* images, where it represents 1 μm.
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valued for its broad-spectrum antibacterial activity and effectiveness as a treatment for chronic wound infections. However, as with all natural products, there is significant chemical variation between different honey preparations [19] and this is likely to affect the level of antibacterial activity, and possibly, treatment outcomes. In this study, we have therefore assessed a series of geographically- and chemically-defined New Zealand manuka, kanuka and manuka-kanuka blended honeys with varying concentrations of MGO and hydrogen peroxide to determine their effectiveness in inhibiting the growth of different species of bacteria. We show here that, in general, the manuka honeys were the most effective at inhibiting growth, followed by the manuka-kanuka blends and then the kanuka honeys. However, the response of bacteria in the presence of sub-inhibitory concentrations of these different honeys varied with bacterial species, with each having a unique growth and morphological response. *P. aeruginosa* was very different to the other three bacteria in being both less sensitive overall and in having a similar response to the different honey types.

Table 2. Cell morphology changes with high-MGO honey and high-hydrogen peroxide honey treatment[a].

	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%)

[a]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.
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Table 3. Summary of growth and morphological effects of honeys and control treatments on all organisms.

	Growth Inhibition						Cell Morphology[a]	
Organism	**MGO**	**Sugar**	**Clover**	**M**	**K**	**MK**	**High-MGO Honey (M3)**	**High-H_2O_2 Honey (MK1)**
B. subtilis	XXX[b]	–[c]	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%)

[a]this data includes data from both the log and lag phases of growth.
[b]The number of crosses increases the more growth is inhibited.
[c]Means no effect.
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High-throughput Analysis of Growth Dynamics Reveals that MGO in Honey Extends the Duration of Lag Phase

A high-throughput approach was used to assess the growth and morphological effects of a large number of natural honeys on multiple organisms. This approach is novel in honey studies and was employed here to address the challenge of assessing multiple parameters in a complex natural product. This system allowed us to explore the heterogeneous and variable composition of natural honey by analyzing large numbers of samples and control solutions, and showed the dynamic response of cell growth in response to the effects of honey toxicity. Such an approach may be useful in the study of other natural products where activity is modulated by various interacting factors.

Visual inspection of the resulting large number of growth curves revealed a distinctive dose-dependent extension of lag phase of growth when cultures of *B. subtilis*, *E. coli* and *S. aureus* were treated with manuka honey. This growth behavior was also observed when MGO alone was added to these bacterial cultures (Fig. 1), and is consistent with a previous study where *E. coli* was subjected to MGO treatment [29]. Lag-phase extension was not seen for clover or pure kanuka honeys; in these growth was either unaffected or was completely inhibited, and there was no evidence for dose-dependent recovery over time (Fig. S1). Thus the extended duration of lag phase is presumed to be largely or completely due to MGO and is likely to be unique to honey derived from manuka and other *Leptospermum* species.

Growth and Morphology of Different Bacteria are Affected by Honey in Markedly Different Ways

The dynamics of growth in the presence of the different honey types was relatively similar for *B. subtilis*, *E. coli* and *S. aureus* but differed markedly in *P. aeruginosa* (Figs. 1 and 2). The extended duration of lag phase and the eventual resumption of logarithmic growth in the presence of MGO likely reflect induction of the glyoxylase system used to detoxify MGO [30]. All organisms produce MGO, which appears to be important in allowing them to regulate growth and maintain carbon flux as their environment changes [31–32]. However, as MGO is toxic, cells detoxify this compound to D-lactate using two metalloenzymes, GlxI and GlxII (Cooper, 1984). The ability of *P. aeruginosa* to grow in the presence of higher MGO levels than the other bacteria may reflect more efficient detoxification of MGO; a suggestion supported by the discovery, through genome sequencing, that *P. aeruginosa* is unique among eubacteria in its possession of three (rather than one) fully functional GlxI homologs [33].

To date, few microscopy studies have been performed to identify morphological changes to bacterial cells treated with honey, and none have used high-throughput phase-contrast and fluorescence microscopy that allows a large number of cells to be imaged and measured rapidly. We observed bacterial cell length changes in all organisms treated with manuka (high-MGO) honey. This is caused by an adjustment to the frequency of cell division relative to growth rate, often due to a change in nutritional state, such that division occurs at a different cell length to untreated cells [34]. Condensed DNA was also observed in a significant proportion of *B. subtilis* and *S. aureus* cells treated specifically with manuka honey. This could be a consequence of inhibition of initiation of DNA replication [35,36]; a suggestion consistent with previous studies demonstrating that MGO alone inhibits this phase of DNA replication in bacterial cells [37]. Treatment with honey that contained the highest level of hydrogen peroxide (MK1) caused significant changes to the morphology of *B. subtilis* cells, including a dispersed appearance of the DNA. This could reflect a 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 level of MGO or hydrogen peroxide in the honey, or to the effectiveness of the honey to inhibit growth. This is not entirely unexpected since cell morphology often reflects a response to changes in the environment that allows the organism to adapt to that environment without having to change its rate of growth. Different organisms do this differently when faced with a variety of nutritional and environmental conditions, such as oxidative or nutrient stress. This might reflect, at least in part, the degree of variation of the environment that these organisms inhabit [39]. We therefore speculate that the differences in morphology that we observe in response to a particular honey reflect species-specific differences in the regulatory circuits that coordinate growth with cellular physiology.

MGO and Hydrogen Peroxide Production cannot Account for All Activity Present in Manuka, Kanuka and Clover Honey

Commensurate with previous studies [8,15,40,41], we found that even when the peroxide activity was neutralized with catalase and there were negligible levels of MGO present, honey could inhibit bacterial growth. Even clover honey, with only trace levels of MGO and hydrogen peroxide, had variable effects on the four bacteria that in most cases could not be attributed to sugar alone.

These observations are in line with previous studies suggesting that the presence of additional antibacterial components that may be directly active or may modulate the activity of the dominant active components [8,40,41]. These additional components may include: (i) phenolics derived from the floral source [19]; (ii) bee-derived antimicrobial peptides (although note that bee defensin-1, an antibacterial component of Revamil honey [8], could not be identified in manuka or kanuka honeys) [23] [42]; and (iii) as yet undefined synergistic compounds identified in other studies, including 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 responses that could be expected by bacteria present in chronic wounds. Our findings here have important implications for the clinical application of honey in the treatment of these wounds. First, sub-inhibitory concentrations of MGO may be neutralized by bacteria which then resume normal growth, thus any honey formulation should contain sufficient active honey to sustain inhibition. Second, honey without significant levels of MGO or hydrogen peroxide, such as clover honey, may be able to inhibit some bacteria but is not broad-spectrum and is therefore not recommended for infected wounds where multiple species may be present. Third, MGO at 600 mg/kg honey achieves almost as much inhibition as much higher concentrations, and increasing MGO above this threshold may not result in a more effective honey. And finally, in honey containing both MGO and hydrogen peroxide, MGO provides an over-riding activity and if this level is high enough, hydrogen peroxide does little to augment activity.

To date, more than 80 different microbial species, including bacteria and yeast pathogens known to infect wounds, have been shown to be inhibited by honey [22,45,46]. In the current study, the use of sub-inhibitory concentrations of honey has enabled us to examine the nature of honey inhibition, however these concentrations are well below those that would be used in a clinical situation, where whole honey is generally applied and complete and irreversible inhibition would be expected.

Emerging evidence from clinical studies suggests that honey is at least as effective as conventional treatments in healing wounds, particularly in very refractory cases such as in diabetics, the elderly, and extensively burned patients [47,48], but more clinical data are necessary for robust statistical appraisal [49]. Here, we have demonstrated the potency of natural honey as an antimicrobial wound dressing, and that multiple effects arise from a variety of active compounds, which not only allows active honey to be uniquely broad in spectrum, but also reduces the potential for resistant microbial populations to evolve. Use of the full honey matrix is therefore recommended for the treatment of infected wounds. Understanding the complex nature of honeys and its effects on bacterial pathogens may eventually allow the development of specific blends with an optimal combination of antibacterial components, thus ensuring a highly effective and resilient antibacterial wound treatment option.

Supporting Information

Figure S1 The effect of New Zealand honey treatments on bacterial growth. Growth curves of *B. subtilis* (001–032), *E. coli* (033–064), *S. aureus* (065–096) and *P. aeruginosa* (097–128) were treated with 10 different honeys (three manuka honeys, M1, M2, M3; four manuka/kanuka blended honeys, MK1, MK2, MK3, MK4; two kanuka honeys, K1, K2; and a clover honey, C) and a comprehensive range of controls, which included (i) a sugar solution comprising 45% of glucose, 48% of fructose and 1% of sucrose; (ii) honey plus catalase (1 mg/mL); (iii) a catalase-only control; (iv) three MGO solutions at starting concentrations matching that present in 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 plus catalase; and finally (vi) different MGO concentrations in the presence of both catalase and sugar solution at various concentrations (0% - as no honey control, 1%, 2%, 4%, 8%, 16% & 32% (w/v), represented by dark blue, light blue, green, pink, orange, purple and red color respectively). Optical density was recorded at 595 nm every h for 24 h. The optical density was then log-transformed and plotted against time using GraphPad Prism 5.0.
(PDF)

Figure S2 Transformation of data obtained for bacterial growth with honey treatment. Panel A illustrates the effect of various (1–32% (w/v)) concentrations of honey M3 on *E. coli* growth over 24 h as a simple log OD_{595nm} *versus* incubation time. The point at which 10% of the final OD_{595nm} is reached is shown by an 'x' on each growth curve. Panel B summarizes all the data from panel A as a simple relationship between honey concentration and the time it takes to reach 10% of the total OD_{595nm}. A value of 24 hours on the y-axis denotes 'no growth'.
(TIF)

Table S1 Average cell length after different honey treatment (μm). Cell lengths were not significantly affected by the honey treatments ($p>0.05$); all other values are significantly different ($p<0.05$); n ≥50. M3–4% manuka M3 (high-MGO) honey treatment. MK1–4% manuka-kanuka blended (high-hydrogen peroxide) honey treatment.
(DOCX)

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Author Contributions

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

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Appendix II: Published manuscript of Chapter 2

Appendix III

PeerJ

Manuka-type honeys can eradicate biofilms produced by *Staphylococcus aureus* strains with different biofilm-forming abilities

Jing Lu[1], Lynne Turnbull[1], Catherine M. Burke[1], Michael Liu[1], Dee A. Carter[2], Ralf C. Schlothauer[3], Cynthia B. Whitchurch[1] and Elizabeth J. Harry[1]

[1] The ithree institute, University of Technology Sydney, Sydney, NSW, Australia
[2] School of Molecular Bioscience, University of Sydney, Sydney, NSW, Australia
[3] Comvita NZ Limited, Te Puke, New Zealand

ABSTRACT

Chronic wounds are a major global health problem. Their management is difficult and costly, and the development of antibiotic resistance by both planktonic and biofilm-associated bacteria necessitates the use of alternative wound treatments. Honey is now being revisited as an alternative treatment due to its broad-spectrum antibacterial activity and the inability of bacteria to develop resistance to it. Many previous antibacterial studies have used honeys that are not well characterized, even in terms of quantifying the levels of the major antibacterial components present, making it difficult to build an evidence base for the efficacy of honey as an antibiofilm agent in chronic wound treatment. Here we show that a range of well-characterized New Zealand manuka-type honeys, in which two principle antibacterial components, methylglyoxal and hydrogen peroxide, were quantified, can eradicate biofilms of a range of *Staphylococcus aureus* strains that differ widely in their biofilm-forming abilities. Using crystal violet and viability assays, along with confocal laser scanning imaging, we demonstrate that in all *S. aureus* strains, including methicillin-resistant strains, the manuka-type honeys showed significantly higher anti-biofilm activity than clover honey and an isotonic sugar solution. We observed higher anti-biofilm activity as the proportion of manuka-derived honey, and thus methylglyoxal, in a honey blend increased. However, methylglyoxal on its own, or with sugar, was not able to effectively eradicate *S. aureus* biofilms. We also demonstrate that honey was able to penetrate through the biofilm matrix and kill the embedded cells in some cases. As has been reported for antibiotics, sub-inhibitory concentrations of honey improved biofilm formation by some *S. aureus* strains, however, biofilm cell suspensions recovered after honey treatment did not develop resistance towards manuka-type honeys. New Zealand manuka-type honeys, at the concentrations they can be applied in wound dressings are highly active in both preventing *S. aureus* biofilm formation and in their eradication, and do not result in bacteria becoming resistant. Methylglyoxal requires other components in manuka-type honeys for this anti-biofilm activity. Our findings support the use of well-defined manuka-type honeys as a topical anti-biofilm treatment for the effective management of wound healing.

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Corresponding author
Elizabeth J. Harry,
liz.harry@uts.edu.au

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INTRODUCTION

Chronic wounds currently affect 6.5 million people in the US. These wounds are difficult to treat and estimated to cost in excess of US $25 billion annually, with significant increases expected in the future (*Sen et al., 2009*). A wound is generally considered chronic if it has not started to heal by four weeks or has not completely healed within eight weeks (*McCarty et al., 2012*). Such prolonged, non-healing wounds are caused by a variety of factors, with bacterial infection being a significant contributor.

In chronic wounds, as with everywhere on earth, bacterial cells predominantly exist as biofilms, where cells are embedded within a matrix of polysaccharides and other components. This matrix affords resistance to environmental stresses such as altered pH, osmolarity, and nutrient limitation (*Fux et al., 2005*). The matrix also limits access of antibiotics to the biofilm embedded cells (*Ranall et al., 2012*), which are up to 1,000 times more recalcitrant to these compounds than planktonic cells (*Hoyle & Costerton, 1991*). Planktonic bacteria may also contribute to pathogenesis, as their release from biofilms has been proposed to maintain the inflammatory response within the wound (*Ngo, Vickery & Deva, 2012*; *Wolcott, Rhoads & Dowd, 2008*), as well as allowing seeding to other areas (*Battin et al., 2007*; *Costerton et al., 2003*). Along with the difficulties of treating biofilm infections, the emergence of resistance to multiple antibiotics has exacerbated the problem of chronic wound treatment (*Engemann et al., 2003*; *Projan & Youngman, 2002*). Thus, there is an increasing need for new approaches to combat bacterial biofilms in chronic wounds.

Honey has been used to treat acute and chronic wound infections since 2500 BC (*Forrest, 1982*; *Molan, 1999*; *Simon et al., 2009*). Honey possesses a number of antimicrobial properties including high sugar content, low pH, and the generation of hydrogen peroxide by the bee-derived enzyme glucose oxidase (*Stephens et al., 2009*). However, not all honeys are the same and their antimicrobial properties vary with floral source, geographic location, weather conditions, storage (time and temperature) and various treatments, such as heat (*Al-Waili et al., 2013*; *Allen, Molan & Reid, 1991*; *Molan, 1999*; *Sherlock et al., 2010*). These factors lead to differences in the levels of various antibacterial components. Manuka honey is derived from *Leptospermum scoparium* bush and is particularly potent (*Adams et al., 2008*; *Allen, Molan & Reid, 1991*; *Kwakman et al., 2011*). This is believed to be largely due to the high levels of the reactive dicarbonyl methylglyoxal (MGO) (*Adams et al., 2008*; *Mavric et al., 2008*), which is highly inhibitory to bacterial growth (*Lu et al., 2013*). Other antimicrobial compounds in honeys include bee defensin-1 (*Kwakman et al., 2010*; *Kwakman & Zaat, 2012*), various phenolic compounds and complex carbohydrates (*Adams et al., 2008*; *Gresley et al., 2012*; *Mavric et al., 2008*; *Molan, 1999*; *Weston, Brocklebank & Lu, 2000*). The combination of these diverse assaults may account for the inability of bacteria to develop resistance to honey (*Blair et al., 2009*; *Cooper et al., 2010*), in contrast to the rapid induction of resistance observed with conventional single-component antibiotics (*Colsky, Kirsner & Kerdel, 1998*; *Cooper, 2008*).

A few studies have examined the effect of manuka honey on biofilms, showing it to be active against a range of bacteria, including Group A *Streptococcus pyogenes*, *Streptococcus mutans, Proteus mirabilis*, *Pseudomonas aeruginosa, Enterobacter cloacae* and *Staphylococcus aureus* (*Alandejani et al., 2008*; *Maddocks et al., 2013*; *Maddocks et al., 2012*; *Majtan et al., 2013*). However, the levels of reported anti-biofilm activity are not consistent among these studies. This is highly likely to be at least in part due to differences in the levels of the principle antibacterial components in the honey, MGO and hydrogen peroxide, which varies with the floral and geographic source of nectar, the honey storage time and conditions, and any possible other treatments that may have occurred. All these conditions affect the antimicrobial activity of honey (*Adams et al., 2008*; *Al-Waili, Salom & Al-Ghamdi, 2011*; *Sherlock et al., 2010*; *Stephens et al., 2009*), but are often not reported. Importantly, medical-grade honeys, while often composed primarily of manuka, can also contain honey derived from other flora sources, which can alter the levels of various antimicrobial components. Therefore, it is imperative to use well-characterized honeys to enable both accurate comparisons among studies, and the rigorous assessment of the potential of medical-grade honey to be used in wound treatment in the clinic.

Here we have performed biomass and viability assays, as well as confocal scanning light microscopy to examine the anti-biofilm activity of four NZ manuka-type honeys, clover honey and an isotonic sugar solution on a range of *S. aureus* strains that differ widely in their biofilm-forming ability. These honeys have been well characterized in terms of their geography, floral source and the level of the two principal antibacterial components found in honey, MGO and hydrogen peroxide. We demonstrate that the manuka-type honeys are highly active in both the prevention and elimination of methicillin-sensitive and methicillin-resistant *S. aureus* biofilms. The antibiofilm activity was highest in the honey blend that contained the highest level of manuka-derived honey; although the same level of MGO, with or without sugar, could not eradicate biofilms. This suggests that additional factors in these manuka-type honeys are responsible for their potent anti-biofilm activity; and emphasise the importance of characterizing honey in order to understand and choose the best honey product for wound management.

MATERIALS AND METHODS

Honey samples

The New Zealand (NZ) honey samples used in this study are listed in Table 1, and include monofloral manuka honey, Medihoney (a manuka-based medical grade honey; Comvita NZ Ltd), a manuka/kanuka blend, and clover honey (a white New Zealand honey). All honey samples were supplied by Comvita NZ Ltd. (Te Puke, New Zealand). The harvesting and geographic information for these honeys, as well as the levels of the three major antimicrobial components: methylglyoxal (MGO), di-hydroxyacetone (DHA) and hydrogen peroxide, are listed in Table 1. All samples were stored in the dark at 4 °C and were freshly diluted in Tryptone Soya Broth (TSB) immediately before use in assays. All honey concentrations are expressed as % w/v.

Table 1 Harvesting and chemical information for the tested NZ honey samples.

Honey type	Harvest period	Area	Floral source	Major antimicrobial composition		
				DHA[a]	MGO[a]	H_2O_2[b]
Manuka	Spring 2010	Hokianga, Northland, NZ	*Leptospermum scoparium var. incanum*	4277	958	0.34
Medihoney	Spring 2010	Northland, NZ	*Leptospermum scoparium var. incanum* + *Kunzea ericoides*	883	776	0.31
Manuka/kanuka	Summer 2010/11	Hokianga, Northland, NZ	*Leptospermum scoparium var. incanum* + *Kunzea ericoides*	652	161	0.68
Clover	N/A[*]	Balcutha, Otago, NZ	*Trifolium spp.*	<20	<10	0.11

Notes.

[a] MGO (methylglyoxal) levels were analyzed against di-hydroxyacetone (DHA) and expressed as mg MGO per kg of honey.

[b] Rate of production of H_2O_2 (hydrogen peroxide) is expressed as µmol/h in 1 mL of 10% w/v honey.

[*] Information not available.

Other tested solutions

A series of other solutions were included for investigation alongside the honey samples: (i) a sugar solution designed to mimic the concentration and composition of honey sugars (45% glucose, 48% fructose, 1% sucrose) diluted as above for honey; (ii) MGO diluted in TSB to concentrations similar to those present in the manuka-type honeys (100 mg/kg, 700 mg/kg and 900 mg/kg honey) to assess the effect of MGO alone on bacterial growth; (iii) MGO diluted in sugar solution to the same concentration as (ii). MGO was obtained as a ∼40% (w/w) solution in water (Sigma-Aldrich Co., MO, USA).

Hydrogen peroxide assay

The level of hydrogen peroxide produced by the NZ honey samples was determined using a hydrogen peroxide/peroxidase assay kit (Amplex Red; Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA) as previously reported (*Lu et al., 2013*).

Bacterial strains and growth conditions

Four strains of *S. aureus* were examined. These include two laboratory reference strains: NCTC 8325 (National Collection of Type Cultures) (*Stepanovic et al., 2000*) and ATCC 25923 (American Type Culture Collection) which are methicillin-sensitive, and two clinical isolates: MW2 (Hospital-Acquired Methicillin-resistant *Staphylococcus aureus*, HA-MRSA) (*Baba et al., 2002*) and USA300 (Community-Acquired Methicillin-resistant *Staphylococcus aureus*, CA-MRSA) (*Kazakova et al., 2005*). All *S. aureus* strains were grown in TSB at 37 °C. For optimal biofilm formation, 1% (w/v) glucose was added to this medium (TSBG) except for strain NCTC 8325 which was found to produce optimal biofilm in the absence of added glucose.

Susceptibility of *S. aureus* to NZ honeys: growth response assays

In this study, growth response assays were carried out to assess whether the NZ honeys affected cell growth of the different strains of *S. aureus* (at concentrations of 1–32%;

prepared as serial 2-fold dilutions in TSB(G)). Details of the growth assay methods are described in our previous publication (*Lu et al., 2013*). TSB(G) media without honey was included as a control. Unless otherwise stated, all assays were performed with three biological replicates and three technical repeats of each replicate.

Biofilm formation assays

The effects of NZ honeys and other solutions on *S. aureus* biofilm formation were determined using crystal violet static biofilm formation assays in microtitre plates according to published studies with the following modifications (*Christensen et al., 1985*; *Stepanovic et al., 2000*). Crystal violet stains all biomass including live and dead cells and the biofilm matrix. *S. aureus* strains were cultured in 2 mL of TSB(G) with shaking (250 rpm) overnight at 37 °C . A suspension from the overnight culture was then diluted to a cell density of approximately 10^7 CFU/mL in fresh TSB containing the appropriate test solution (honey, sugar solution, MGO or MGO in combination with sugar) to give a final volume of 150 μL. The suspension was added to each well of a 96-well tissue-culture treated microtitre plate (BD Falcon, NJ, USA). Media-only and media with the appropriate test solution without *S. aureus* inoculation were included as negative controls. The microtitre plates were sealed with AeraSeal (Excel Scientific, CA, USA) and incubated in a humidified incubator for 24 h at 37 °C. Following this, planktonic cell growth was assessed by transferring the planktonic phase into a new 96-well microtitre plate and reading the optical density at 595 nm with a microplate reader (VersaMax, Molecular Devices, California, USA). This step was required as *S. aureus* forms biofilms on the bottom of the microtitre plate wells, which interferes with optical density readings of the planktonic culture. The microtitre plates with residual biofilm were then washed three times with sterile phosphate buffered saline (PBS) to remove unattached cells and air-dried at 65 °C for 1 h, to fix the *S. aureus* biofilm to the bottom of the well surface. The plate was then stained with 0.2% (w/v) crystal violet at room temperature for 1 h, excess crystal violet solution was decanted and the plates were washed as above with PBS. Stain that was bound to the adherent biomass was resolubilized with 200 μL 33% acetic acid and transferred into a new 96-well microtitre plate to measure the OD_{595}.

Biofilm elimination assays

S. aureus biofilms were first formed in the wells of a 96-well microtitre plate for 24 h at 37 °C as described above. Biofilms were then washed three times with PBS. Various concentrations (0%–32% in two-fold serial dilutions) of honey and other test solutions were then added to the established *S. aureus* biofilms. The assay plates were then incubated for a further 24 h at 37 °C, and planktonic cell growth and biofilm mass were quantified as described above.

Determination of bacterial cell viability in biofilms

Crystal violet stains all the components of the biofilm (*Bauer et al., 2013*). To quantify the viability of cells within the *S. aureus* biofilms following honey treatment, we used a BacTitre Glo Microbial Cell Viability Assay Kit (Promega, WI, USA), which measures

ATP levels as a proxy for viability. The assay reagents lyse the bacterial cells to release intracellular ATP, the levels of which are quantified via a luminescence-based luciferase activity assay (*Haddix et al., 2008*; *Junker & Clardy, 2007*). The BacTitre Glo protocol involved the same steps as crystal violet staining (above), however, instead of drying and staining the biofilms, plates were incubated with BacTitre Glo reagent in TSB(G) for 10 min at 37 °C in the dark. The contents of each well were then transferred into white solid-bottom 96-well microtitre plates (Cellstar; Greiner Bio-one, France) for luminescence measurement. Luminescence, which is proportional to the amount of ATP produced by metabolically active cells, was recorded using a 96-well microplate reader (Infinite 200Pro; TeCan, Männedorf, Switzerland).

To ensure the validity of this assay, a standard curve was constructed to assess the correlation between bacterial cell numbers and the luminescent signal in the biofilm. This was performed on the untreated control (containing *S. aureus* in TSB(G) only). Biofilms produced as above were washed and cells within the biofilm dispersed using a small probe sonicator (Sonics and Materials VC-505) to enable quantification by direct enumeration (*Merritt, Kadouri & O'Toole, 2005*). The recovered cell suspension was serially diluted 10-fold and a 20 µL aliquot was plated on Tryptone Soya Agar (TSA) for CFU determination. Luminescence of cells in the remaining suspension was assessed using the BacTitre Glo kit. From this, a correlated standard curve was constructed between calculated CFU/well and the relative luminescence readings. According to the standard curve shown in Fig. 1, the detection limit of the BacTitre Glo is at a luminescence reading below 1,000, which is equivalent to 10^3 CFU/well (linear range from 10^3–10^7 CFU/well). An upper limit was not detected.

Visualizing live/dead stained *S. aureus* biofilms using confocal laser scanning microscope (CLSM)

S. aureus biofilms were treated with TSB containing 1%, 2%, 16%, and 32% NZ honeys or sugar solution for 24 h in black polystyrene 96-well microtitre plates with µClear bottoms (Cellstar; Greiner Bio-One, France) as described above, except the biofilm mass was not fixed by air-drying. The treated biofilm mass was washed three times with PBS and cells within the biofilm structure were fluorescently stained with 2.5 µM Syto9 (Invitrogen, CA, USA) and 4.3 µM propidium iodide (PI) (Becton Dickinson, NJ, USA), which identify live and dead cells in the biofilm structure, respectively. After 30 min of incubation in the dark at room temperature, the wells were washed thoroughly with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich, MO, USA) for 15 min. The wells were then rinsed and stored in PBS for imaging. Biofilms were imaged using confocal laser scanning microscopy imaging (CLSM) on a Nikon A1 confocal microscope. The Syto9 and PI fluorophores were excited at 488 nm and 561 nm, and the emissions were collected at 500–550 nm and 570–620 nm, respectively. For quantitative analysis, at least eight separate CLSM image stacks of each NZ honey treated biofilms were acquired with a resolution of 512 × 512 pixels. Biofilm biomass was calculated using COMSTAT (*Heydorn et al., 2000*) and is expressed as volume of the biofilm over the surface area ($\mu m^3/\mu m^2$). Representative

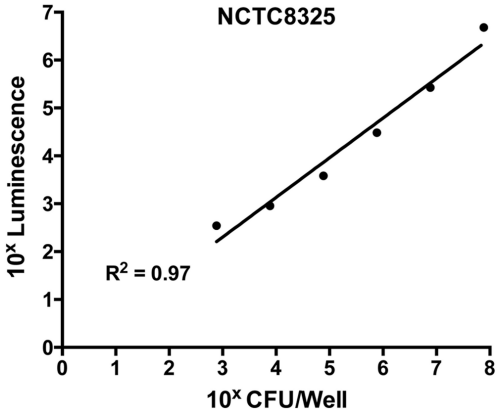

Figure 1 Correlation of levels of intracellular ATP to colony forming units (CFU) in static biofilms of *S. aureus*. Static biofilms of *S. aureus* were formed in the wells of a microtitre plate for 48 h (with media replenishment at 24 h). After removal of the biofilm from the wall of each well, intracellular ATP levels were measured by the BacTitre Glo Viability Kit and CFU were determined for each well. The intracellular levels of ATP are plotted as a function of CFU and validate that the BacTitre Glo Viability Kit can be used as a surrogate measure of biofilm cell viability in subsequent assays.

presentation image stacks of each treatment were acquired at a resolution of 1024 × 1024 pixels and three dimensional biofilm images reconstructed using NIS-elements (Version10, Nikon Instruments Inc., USA). It should be noted that due to the incomplete displacement of Syto9 by propidium iodide in dead cells that there will remain some Syto9 staining of dead cells. Therefore the absolute level of live cells detected in the Syto9 channel will be somewhat overestimated using this co-staining method (*Stocks, 2004*).

Assaying honey resistance in cells recovered from biofilms

The development of resistance is a great concern in clinical settings, where bacteria can become resistant to inhibitory compounds after exposure to sub-inhibitory concentrations (*Cars & Odenholt-Tornqvist, 1993*; *Pankuch, Jacobs & Appelbaum, 1998*). Planktonic cells that appeared after 24 h manuka-type honey treatment of established biofilms were assumed to have been released from the biofilm matrix. Cells recovered from biofilms treated with sub-eliminatory concentrations of manuka-type honeys were collected and tested for their ability to grow and form biofilms under the static growth conditions described above. Cell growth and biofilm formation were defined as not detected when the $OD_{(x)}$–$OD_{(\text{media only blank})} \leq 0.1$. Each experiment was performed with three biological replicates and three technical repeats of each biological replicate.

Statistical analysis

Statistical analysis to determine significant differences between treatments and among honey samples were performed. First, data sets were checked for normality (Gaussian Distribution) using the D'Agostino-Pearson normality test (alpha = 0.05) in GraphPad

Table 2 Concentration of honey required to inhibit *S. aureus* growth.

Honeys	NCTC 8325	ATCC 25923	MW2	USA300
Manuka honey	8*	8	8	8
Medihoney	8	8	8	8
Manuka/kanuka honey	16	16	16	16
Clover honey	32	32	32	32
Sugar solution	>32	>32	32	>32

Notes.

* All numbers in the table are honey concentrations (%, w/v).

Prism (versions 5 and 6). Once normality was confirmed, significant differences between treatments and among honey samples were performed using One-Way ANOVA with Tukey Test using the same software. Statistical significance was set at $p < 0.05$.

RESULTS

The effect of NZ manuka-type honeys on the planktonic growth of *S. aureus*

Planktonic growth and biofilm mass were assessed to examine the ability of four NZ honeys, three manuka-types and one clover, and a sugar solution to prevent biofilm formation by different strains of *S. aureus*. Following 24 h incubation under static conditions, *S. aureus* cells formed biofilms at the bottom of microtitre plates, with very little or no planktonic growth detected, indicating that the concentration of honey needed to prevent *S. aureus* planktonic cell growth could not be calculated under these conditions. Shaking broth cultures were instead used to assess the effect of the treatments on planktonic growth. The results are shown in Table 2. Planktonic growth of the four *S. aureus* strains, NCTC 8325, ATCC 25923, MW2 and USA300, was completely inhibited by 8% manuka honey and Medihoney, 16% manuka/kanuka honey and 32% clover honey. The 32%, sugar solution was only effective at inhibiting growth of the MRSA strain MW2, with no inhibition of growth of the other strains at the concentrations tested (1–32%). These data are in agreement with the results of our previous study using the standard *S. aureus* reference strain, ATCC 25923, which used a similar suite of honey types and the same experimental conditions (*Lu et al., 2013*).

The effect of NZ manuka-type honeys on *S. aureus* biofilm formation

All strains of *S. aureus* were assessed for their biofilm-forming ability after 24 h and 48 h. Under static conditions, biofilm-forming ability varied between strains, with NCTC 8325 forming the most robust biofilms, and generating significantly more biofilm mass than the other three tested strains (Figs. 2A and 2B). This was followed by ATCC 25923 and USA300, with MW2 forming the thinnest biofilms (Figs. 2A and 2B; $p < 0.05$). The effects of the four NZ honeys and the sugar solution on biofilm formation of strain NCTC 8325 are shown in Fig. 3A. Manuka honey was the most effective at preventing biofilm formation by *S. aureus* NCTC 8325, resulting in ~95% reduction ($p < 0.001$) in biofilm

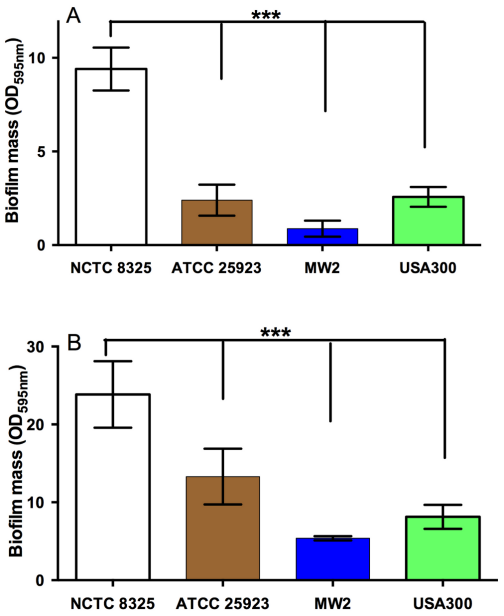

Figure 2 Quantification of biofilm formation by different strains of *S. aureus*. The ability of different strains of *S. aureus* to form biofilms on the plastic surface of tissue-culture treated 96-well microtitre plate was assessed in TSB(G) at 24 h and 48 h. Biofilm adherence was determined using a static biofilm formation assay over 24 h (A) and 48 h (with media replenished after 24 h incubation) (B). Biofilm formation was quantified by staining with 0.2% crystal violet solution and measured at an optical density of 595 nm. Error bars represent ±standard deviation (SD) of three biological samples performed in triplicate, *** represents $p < 0.001$, compared to NCTC 8325, as assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

formation at 8% (Fig. 3A) compared to the untreated (0%) control. At this concentration, the other honeys and the sugar solution did not significantly reduce biofilm formation. Medihoney and manuka/kanuka honey were highly effective at 16%, preventing biofilm formation by ∼95% ($p < 0.001$). Clover honey was much less active and was less able to prevent biofilm formation than the sugar solution, even at the highest concentration used (32%).

For NCTC 8325, the addition of sub-inhibitory concentrations of manuka and manuka/kanuka honey significantly enhanced biofilm formation, increasing it by 1.5- and 2-fold, compared to the untreated control ($p < 0.001$). In contrast, Medihoney, clover honey and the sugar solution did not enhance biofilm formation by strain NCTC 8325 at any concentration tested.

S. aureus strain ATCC 25923, which is a standard clinical reference strain, produced similar results to NCTC 8325, including the enhancement of biofilm formation following

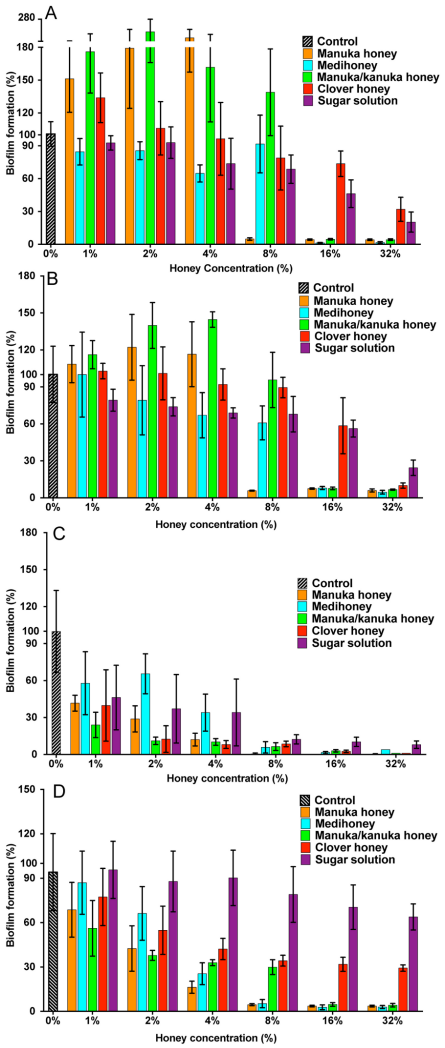

Figure 3 Effects of NZ honeys and sugar on *S. aureus* biofilm formation. *S. aureus* biofilms were allowed to form in the presence of four different NZ honey types (manuka, Medihoney, manuka/kanuka or clover) or a sugar solution. Biofilm formation was assessed using a static biofilm formation assay with crystal violet staining to quantify biomass. *S. aureus* strains are: (A) NCTC 8325; (B) ATCC 25923; (C) MW2 (HA-MRSA) and (D) USA300 (CA-MRSA). Biofilm formation is expressed as a percentage relative to that produced by the untreated control, which is set at 100%. Error bars represent ±standard deviation (SD) of three biological samples performed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

sub-inhibitory honey treatment (Fig. 3B). The hospital-acquired MRSA strain MW2—the weakest biofilm former out of all four tested strains (Figs. 2A and 2B; $p < 0.05$)—displayed a very sensitive profile to all of the NZ honeys and the sugar solution at all tested concentrations (Fig. 3C). Even with only 1% honey or sugar solution, a ∼50% reduction in biofilm formation was observed for MW2 ($p < 0.001$). At higher concentrations (≥8%), all four NZ honeys were significantly more effective than the sugar solution at preventing MW2 biofilms. The other MRSA strain, USA 300, responded similarly to NCTC 8325, with approximately the same concentrations of manuka-type honey being required to reduce biofilm formation by ∼95%. However, unlike NCTC 8325, sub-inhibitory concentrations of manuka-type honey reduced biofilm formation of USA 300 rather than enhancing it (e.g., 4% manuka-type honeys exhibited ∼50–80% biofilm inhibition of USA 300). Moreover, in USA300, biofilm formation was not affected by the sugar solution at any tested concentration.

The results above can be summarize as follows: (i) all three manuka-type honeys are effective at inhibiting biofilm formation of a range of of MSSA and MRSA strains; with (monofloral) manuka honey being generally more effective than the other maunka-type honeys; and (ii) the manuka-type honeys are generally more effective than clover honey and the isotonic sugar solution, although clover honey was just as inhibitory as the manuka-type honeys for the weakest biofilm former, *S. aureus* MW2.

The effect of MGO on *S. aureus* biofilm prevention

MGO is a principle antibacterial component of manuka honey responsible for its inhibitory effects on the growth of *S. aureus* and other bacterial species. This is evidenced by the correlation between the MGO level and the proportion of manuka-derived honey in a honey blend (*Jervis-Bardy et al., 2011*; *Lu et al., 2013*). To determine whether MGO is solely responsible for the inhibitory effect of the three manuka-type honeys on *S. aureus* biofilm formation, biofilm assays were performed using MGO at equivalent concentrations to those present in each of the manuka-based honey samples, with and without the addition of the sugar solution (Fig. 4). *S. aureus* NCTC 8325 biofilm formation was not significantly ($p > 0.05$) affected by MGO at concentrations equivalent to that present in 1–16% manuka-type honeys. MGO at medium (700 mg/kg) and high (900 mg/kg) levels at the equivalent concentration to 32% manuka-kanuka honey and Medihoney prevented approximately 50% and 75% biofilm formation, respectively ($p < 0.05$). The addition of the sugar solution to MGO at the same levels present in 16% of all three manuka-type honeys, led to a dramatic decrease (∼95%) in biofilm formation.

The effect of NZ manuka-type honeys on established *S. aureus* biofilms

Bacterial biofilms are usually already established in open, chronic wounds prior to presentation to the clinic for medical treatment. We therefore assessed the ability of the four NZ honeys to remove established biofilms produced by the four strains of *S. aureus*. These results are presented in Fig. 5, with coloured lines showing biofilm mass present following treatment with different concentrations of the various honey types. While

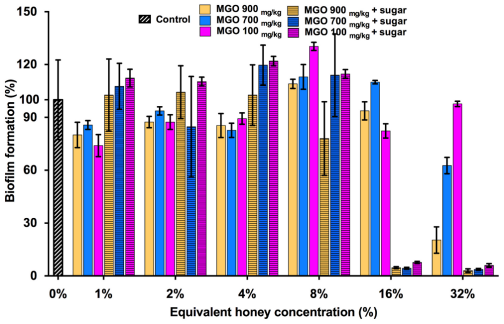

Figure 4 Effects of MGO on *S. aureus* biofilm formation. Biofilm formation by *S. aureus* NCTC 8325 grown in the presence of MGO and MGO plus sugar solution. MGO stock solutions were prepared to correspond to the MGO levels in undiluted manuka-type honeys (100 mg/kg of manuka/kanuka honey, 700 mg/kg of Medihoney, and 900 mg/kg of manuka-honey; Table 1). Biofilm formation was assessed using the described static assay with crystal violet staining to quantify biomass. Biofilm formation is expressed as a percentage relative to the untreated control, which is set at 100%. Error bars represent ±standard deviation (SD) of three biological samples performed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

there was variation among the *S. aureus* strains in their response to the different honeys, there are some important general trends. First, manuka honey was consistently the most effective at removing biofilm, eliminating almost all of the established *S. aureus* biofilms at concentrations of 16%–32%, ($p < 0.001$ compared to the untreated control sample; Fig. 5 top panel, orange lines). Second, Medihoney and manuka/kanuka honey were also effective at these concentrations for some *S. aureus* strains, but only consistently effective across all four strains at 32% (Fig. 5, blue and green lines). Third, both the clover honey and the sugar solution did not significantly reduce ($p > 0.5$) established biofilm mass until their concentration reached 32%. However, the sugar solution did not remove the USA 300 biofilm, with no significant reduction in biofilm mass at 32% (Fig. 5, purple line).

NCTC 8325, the most efficient biofilm former out of all tested strains, gave a slightly different response toward honey treatment compared to the other three strains. Significant biofilm enhancement occurred in this strain at sub-inhibitory concentrations of manuka honey (1–2%) and manuka/kanuka honey (1–4%) ($p < 0.001$; Fig. 5). In addition, this strain was the least sensitive to the manuka-type honeys. For example, at 8% manuka honey treatment, the NCTC 8325 biofilm mass remained similar to the untreated control ($p > 0.05$; Fig. 5), while the biofilms produced by the other three strains were significantly reduced at this concentration ($p < 0.001$; Fig. 5).

The effect of NZ manuka-type honeys on cell viability within *S. aureus* biofilms

Elimination of biofilm mass was assessed using crystal violet, a cationic dye that stains all the components of the biofilm. However, this assay cannot assess the viability of cells

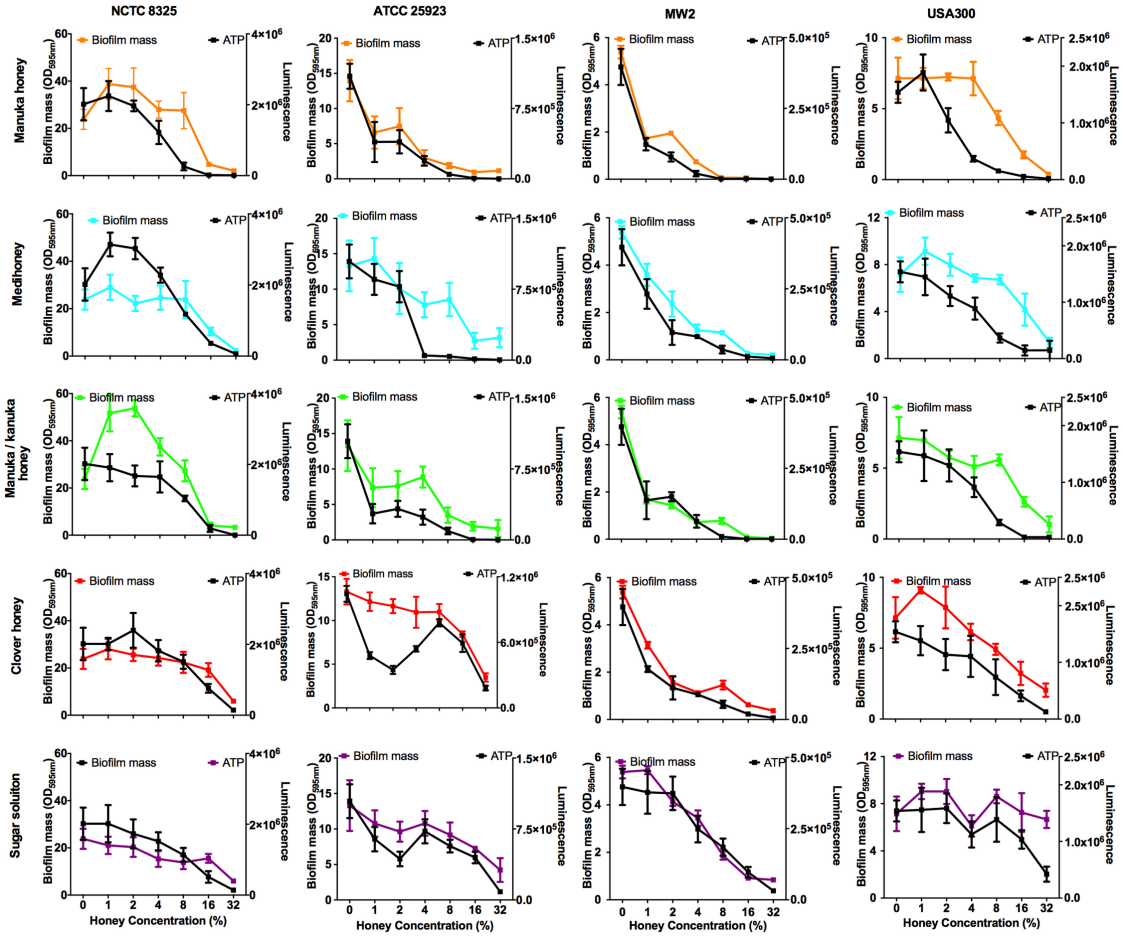

Figure 5 Effects of NZ honeys on established *S. aureus* biofilms and cell viability within the biofilms. Established *S. aureus* NCTC 8325, ATCC 25923, MW2 and USA300 biofilms were treated with NZ honeys – manuka, Medihoney, manuka/kanuka, clover, and a sugar solution. The remaining biofilm masses were quantified using crystal violet staining (left *y*-axis) and cell viability within these remaining biofilms were assessed using the BacTitre Glo Viability Kit (right *y*-axis). Error bars represent ±standard deviation (SD) of three biological samples performed in triplicate Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

remaining within the biofilm structure (*Bauer et al., 2013*). To determine this, we used a BacTitre Glo assay, which measures ATP levels as a proxy for viability. Side-by-side CFU measurements showed that the level of ATP detected in these assays was proportional to the count of viable cells per well (ranging from 10^3 to 10^7 CFU/well) (Fig. 1).

The viability of cells remaining in the biofilm after the various treatments is shown in Fig. 5. In general, cell viability decreased in proportion to the elimination of biofilm biomass (Fig. 5, black lines). However, several exceptions to this general trend were observed. In some cases, biofilm biomass increased but cell viability did not, e.g., NCTC 8325 biofilms with low concentrations of manuka (2%) and manuka/kanuka honey (1–4%) (Fig. 5). In others, biofilm biomass remained relatively constant while cell viability increased, e.g., NCTC 8325 with 1–4% Medihoney ($p < 0.05$; Fig. 5), and ATCC 25923 with 4% and 8% clover honey. Another deviation from the general trend was a significant reduction of cell viability while biofilm biomass remained unaffected, seen for NCTC 8325 and USA 300 with 4% and 8% manuka honey treatment (Fig. 5; $p < 0.05$). This emphasizes the importance of assessing viability alongside crystal violet assays for biofilm assessment.

Overall, at concentrations easily attainable in the clinic, the tested four NZ honeys were effective at eliminating biofilm biomass and at killing both MSSA and MRSA *S. aureus* cells in the residual biofilm. Among the honey types, manuka honey was the most effective, where the elimination of biofilm biomass largely paralleled the reduction in viability. Following treatment with 8% manuka honey only ∼10% of cells were viable in the remaining ATCC 25923 and USA 300 biofilms, compared to the untreated control (i.e., 0% honey), and no generation of ATP could be detected from MW2 (Fig. 5). This is similar to the degree of biofilm biomass removal, where 85–98% of biofilm biomass was removed following 8% manuka honey treatment. Although NCTC 8325 biofilm biomass was seemingly unaffected at 8% manuka honey compared to the untreated control (Fig. 5), the number of viable cells detected within this biofilm was drastically reduced by approximately 80% ($p < 0.001$; Fig. 5).

The effect of MGO on established *S. aureus* biofilms

To assess the contribution of MGO alone, as well as MGO plus sugar, to biofilm removal, these components were tested on established *S. aureus* NCTC 8325 biofilms (Fig. 6). MGO levels equivalent to the presence of 1–8% manuka/kanuka honey (Table 1) caused biofilm biomass to increase approximately 2-fold, relative to the untreated control ($p < 0.001$). However, the established biofilm biomass was not reduced significantly ($p > 0.05$), for any of the tested concentrations (1–32%) of MGO by itself, or in combination with the sugar solution. Thus, neither MGO nor the combination of MGO with sugar is solely responsible for the elimination of biofilms observed with these manuka-type honeys.

Visualizing the effects of NZ manuka-type honeys on established *S. aureus* biofilms

To assess the effect of the NZ honeys on *S. aureus* NCTC 8325 biofilms at the cellular level, we used confocal laser scanning microscopy (CLSM) of biofilms stained with fluorescent dyes for the detection of live and dead bacteria. This allows both the visualization of

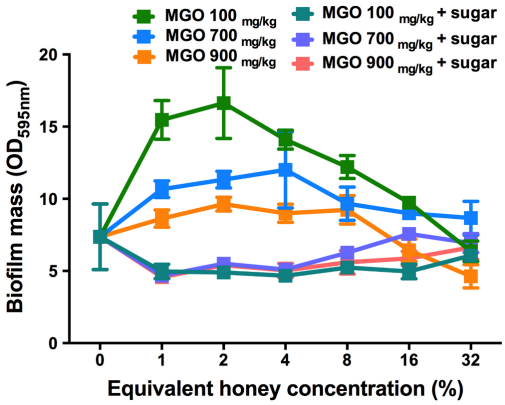

Figure 6 Effects of MGO on established *S. aureus* biofilms. *S. aureus* NCTC 8325 biofilms were treated with MGO and a combination of MGO and the sugar solution. MGO stock solutions were prepared to correspond to the MGO levels in undiluted honey (100 mg/kg of manuka/kanuka honey, 700 mg/kg of Medihoney, and 900 mg/kg of manuka honey; Table 1). The crystal violet stained residual biofilm mass after 24 h treatment was quantified using optical density ($OD_{595\ nm}$). Error bars represent ±standard deviation (SD) of three biological samples performed in triplicate. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

individual cells within the biofilm in three dimensions and the effect of treatments on cell viability to be determined. Treatment by sub-inhibitory (1% and 2%) and inhibitory (16% and 32%) concentrations of NZ honeys was visualized by viewing fluorescently-labelled live (Syto9; green) and dead (propidium iodide; red) cells. Representative images of each treatment are presented in Fig. 7 and quantification of live and dead cell biofilm biomass for several samples for each treatment is shown in Fig. 8. In general, the established biofilm biomass decreased with increasing concentrations of manuka-type honey. More specifically, manuka honeys were effective in reducing the live cells in established *S. aureus* biofilms. Sub-inhibitory concentrations of all the manuka-type honeys (1% and 2%) and the sugar solution did not reduce the amount of biomass compared to the non-treated control cells (Fig. 8). This is shown in Fig. 7 where the untreated control cells displayed a green (live-cell) lawn that covered nearly the entire surface and this remained following treatment with 1% and 2% manuka-type honeys. At concentrations of 16% and 32%, the manuka-type honeys substantially reduced the density and depth of the biofilm, along with the amount of live cells, compared to the untreated control (Figs. 7 and 8). For example, the 32% manuka honey significantly reduced the Syto9 stained (live) biofilm biomass to 10% ($p < 0.001$) compared to the non-treated live biofilm biomass (Fig. 8).

Only small micro-colonies were present following treatment with 32% manuka honey, and the colour of the biofilms was predominantly yellow (where both the green and red dye were retained within cells), indicating mostly dead cells. In contrast, 32% clover honey and sugar solution reduced the total biomass by a maximum of 30% ($p < 0.001$) compared to

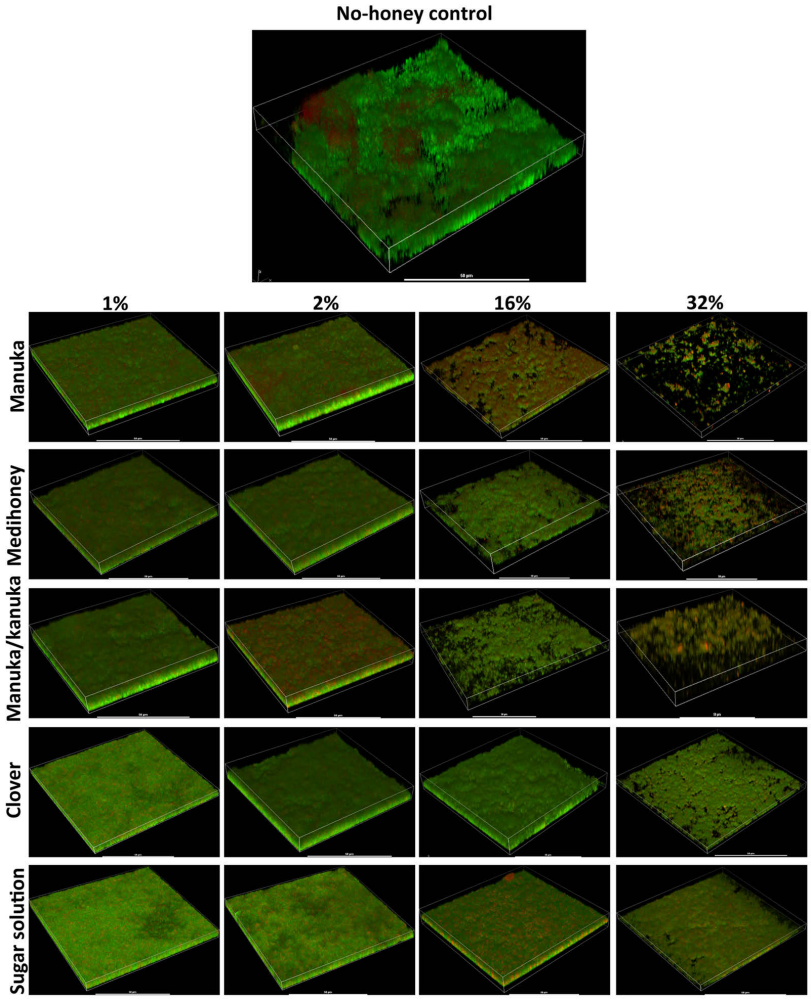

Figure 7 Live/dead staining of different honey treated established biofilms. Established biofilms produced by *S. aureus* NCTC 8325 were treated with TSB containing honey (manuka, Medihoney, manuka/kanuka or clover) or sugar solution at 1%, 2%, 16%, and 32% (w/vol). Syto9 (green; viable cells) and propidium iodine (red; dead cells) stained images were acquired using Nikon A1 Confocal Laser Scanning Microscope. The 3D- images were reconstructed using NIS-elements (version 10). Scale bar represents 50 μm.

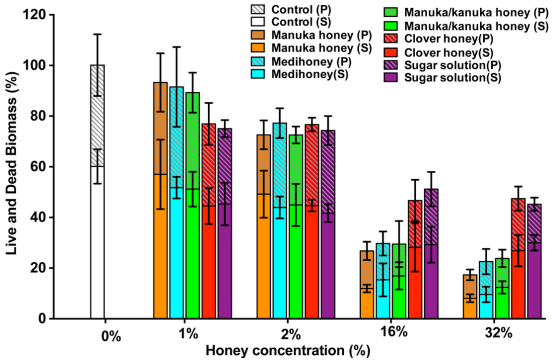

Figure 8 Quantitative analysis of live/dead stained honey treated biofilms. The established *S. aureus* NCTC 8325 biofilm was treated with New Zealand honeys (manuka honey, Medihoney, manuka/kanuka honey, and clover honey) and a sugar solution at 1%, 2%, 16%, and 32% (w/v) concentrations. Biofilms were co-stained with Syto9 (S, viable cells) and propidium iodide (P, dead cells) and analyzed using COMSTAT. The estimated live (S) and dead (P) biomass (volume of the biofilm over the surface area ($\mu m^3/\mu m^2$)) are expressed as a percentage of the non-treated control live and dead biomass, which is set at 100%. Error bars represent ±standard deviation (SD) of three biological samples where eight representative images were acquired. Statistical significance ($p < 0.05$) was assessed by One-Way ANOVA with Tukey test after confirming normality of the data set for each treatment using the D'Agnostino-Pearson normality test.

the non-treated control (Fig. 8). This result corresponds to the 3D reconstructed images, where the Syto9 stained cells remained dominant after treatment (Fig. 7). At 32%, clover honey or sugar solution, a substantially larger Syto9 stained (live) lawn remained in comparison to the 32% manuka-type honeys, although the biomass was less confluent than in the untreated control. These results are consistent with the results obtained with the crystal violet stained biofilm biomass and ATP viability assays.

Assessing the development of resistance to manuka-type honeys in *S. aureus* biofilms

Bacteria that are exposed to sub-inhibitory concentrations of antimicrobial agents generally develop resistance to these agents (*Braoudaki & Hilton, 2004*; *Davies, Spiegelman & Yim, 2006*). The ability of cells released from *S. aureus* NCTC 8325 biofilms to develop resistance after exposure to sub-inhibitory concentrations of honey was investigated, and the results are summarized in Table 3. All cells recovered from the *S. aureus* biofilm after 24 h with 8% of all three manuka-type honeys were viable and able to form biofilms in media (TSB). However, they were unable to grow planktonically when subsequently exposed to 8% manuka, or 16% Medihoney and manuka/kanuka honey (the MIC levels for these honeys). Biofilm formation was also inhibited by 8% manuka honey, and by 16% Medihoney and manuka/kanuka honey. These growth- and biofilm-inhibitory concentrations of manuka-type honeys are the same as those observed for cells that had not previously been treated with these honeys. These results indicating that planktonic cells

Table 3 Resistance of *S. aureus* cells recovered from biofilms after 8% manuka honey treatments[a].

Honey (%)	Type of assay	Manuka honey	Medihoney	Manuka/kanuka honey
0	Growth	√	√	√
	Biofilm formation	√	√	√
8	Growth	×	×	√
	Biofilm formation	×	√	√
16	Growth	×	×	×
	Biofilm formation	×	×	×

Notes.
[a] A tick means that there was normal growth or biofilm formation and a cross means that there was no growth or no biofilm formation.

released from the biofilms with exposure to sub-inhibitory concentrations did not acquire resistance to the same honey treatment.

DISCUSSION

Chronic wounds are costly and difficult to treat (*Hoyle & Costerton, 1991*; *Ranall et al., 2012*; *Sen et al., 2009*), and bacterial biofilms are important contributors to the delay in healing. Honey is a promising alternative treatment for these wounds, and studies have indicated that it is able to prevent bacterial biofilms and eliminate established biofilms *in vitro* (*Alandejani et al., 2008*; *Maddocks et al., 2013*; *Maddocks et al., 2012*; *Majtan et al., 2013*). However, the effective concentration of honey reported by these studies varies significantly, making it hard to establish a foundation for the efficacy of honey on chronic wound-associated bacterial biofilms in the clinic. This is probably largely due to the fact that, in most of these studies, very little information is reported on the honey itself, including the floral source, geographic location, storage conditions, and the level of the two principle antibacterial components, MGO and hydrogen peroxide. Here we utilize a suite of well-defined NZ honeys, including manuka-type honeys (manuka, Medihoney and manuka/kanuka honey) and clover honey, to investigate their anti-biofilm activity on a range of *S. aureus* biofilms that differ in their ability to form biofilms. We show that manuka-type honeys can be used to kill all MSSA and MRSA cells when present as a biofilm in a chronic wound, supporting the use of this honey as an effective topical treatment for chronic wound infections.

Our study has shown that prevention of *S. aureus* biofilm formation occurred at honey concentrations that also inhibit planktonic growth (Figs. 3A–3D; Table 2), suggesting that biofilm prevention was a consequence of planktonic growth inhibition, as opposed to any specific effects on biofilm development. Other studies have also shown that manuka-type honeys can inhibit bacterial biofilm formation, however, the concentrations required were higher than those reported to inhibit growth (*Alandejani et al., 2008*; *Maddocks et al., 2013*; *Maddocks et al., 2012*; *Majtan et al., 2013*).

We found that higher concentrations of all honeys were necessary to eliminate established biofilms compared to those needed for prevention, as assessed by both quantification of biofilm biomass and cell viability. Manuka honey was the most effective,

closely followed by both Medihoney and manuka/kanuka honey. Elimination of biofilms was visually confirmed using CLSM of fluorescently-stained live and dead cells. The sugar content of honey clearly mediates some effect, as sugar solution and clover honey were able to eliminate established biofilms at high concentrations (32%), as has been shown in other studies (*Chirife et al., 1983*; *Chirife, Scarmato & Herszage, 1982*). However, manuka-type honeys consistently achieved biofilm elimination at lower concentrations, suggesting that components specifically within manuka-type honeys contribute towards biofilm elimination. The concentrations of manuka-type honeys that show significant anti-biofilm activity are easily achievable in the clinic, since honey dressings typically contain >80% honey (*Cooper et al., 2010*).

The use of assays for total biofilm biomass and cell viability to examine the effects of the various treatments on biofilm elimination afforded some other interesting observations. We observed that in some cases, sub-inhibitory concentrations of two of the manuka-type honeys enhanced biofilm formation; however, cell viability did not increase. This could be due to a stress response, as has been previously observed when bacteria are exposed to sub-inhibitory concentrations of antibiotics (*Haddadin et al., 2010*; *Kaplan et al., 2012*; *Mirani & Jamil, 2011*; *Subrt, Mesak & Davies, 2011*). In other cases, no reduction of biofilm biomass was observed but cell viability was significantly reduced. This suggests that unlike antibiotics, the manuka-type honeys (or active components therein) are able to penetrate through the biofilm matrix, killing the bacterial cells whilst leaving intact matrix.

It is believed that MGO is the primary component in manuka-type honeys responsible for its anti-biofilm activity (*Jervis-Bardy et al., 2011*; *Kilty et al., 2011*). The effectiveness of the different manuka-type honeys tested here did increase with MGO content. However, the same degree of biofilm prevention and elimination could not be reproduced with equivalent amounts of MGO either alone or in combination with sugar. In the case of prevention, MGO alone was generally ineffective, although a significant amount of biofilm prevention was achieved in combination with sugar. This suggests that the MGO and sugar do contribute to biofilm prevention, but their effects are not as strong as those observed with manuka honey.

Unlike the three NZ manuka-type honeys, neither MGO alone nor MGO with sugar at honey-equivalent concentrations showed significant *S. aureus* biofilm elimination. This indicates that the ability of manuka-type honeys to eliminate biofilms of this organism is due to one or more components present in the honey other than MGO and sugar, such as low pH, hydrogen peroxide, phenolics and other unknown components (*Jagani, Chelikani & Kim, 2009*; *Jervis-Bardy et al., 2011*; *Kilty et al., 2011*; *Zmantar et al., 2010*). Interestingly, while the kanuka/manuka honey had a relatively high rate of hydrogen peroxide production compared to the manuka and Medihoney (Table 1), but low MGO levels, it was not any more active against biofilms of *S. aureus*. This suggests that, at least for this organism, hydrogen peroxide within these manuka-type honeys does not provide significant anti-biofilm activity.

CONCLUSIONS

This study is the first to use a suite of well-characterized manuka-type honeys against a range of strains of *S. aureus* that differ in their ability to form biofilms. We demonstrate that: (1) at very low levels, some honeys can enhance biofilm formation, presumably by evoking a stress response similar to that seen with some antibiotics; (2) the ability to prevent or eliminate biofilms is influenced by MGO levels and the presence of sugar, but these alone do not account for all of the anti-biofilm effect; (3) honey is able to reduce biofilm mass and also to kill cells that remain embedded in the biofilm matrix; and (4) planktonic cells released from biofilms following honey treatment do not have elevated resistance to honey. Taken together our results show that if used at an appropriate therapeutic level, manuka-type honey can be used to kill *S. aureus* when present as a biofilm in a chronic wound, supporting the use of this honey as an effective topical treatment for chronic wound infections.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Ralf C. Schlothauer is an employee of Comvita New Zealand (NZ) Limited which trades in medical grade manuka honey (Medihoney). Comvita NZ Ltd. have partially funded the work through a contribution to Linkage Project LP0990949 funded by the Australian Research Council. Chief Investigators on this project include Elizabeth J. Harry, Cynthia B. Whitchurch, Lynne Turnbull, Dee A. Carter and Partner Investigator Ralf C. Schlothauer. Our competing interests do not alter our adherence to all the PeerJ policies on sharing data and materials. We also note that co-author Associate Professor Dee A. Carter is an Academic Editor for PeerJ.

Author Contributions

- Jing Lu performed the experiments, analyzed the data, wrote the paper, prepared figures and tables, reviewed drafts of the paper.
- Lynne Turnbull and Cynthia B. Whitchurch conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper, revised the manuscript.
- Catherine M. Burke analyzed the data, wrote the paper, reviewed drafts of the paper.
- Michael Liu analyzed the data, wrote the paper, reviewed drafts of the paper.
- Dee A. Carter analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.
- Ralf C. Schlothauer conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, revised the manuscript.
- Elizabeth J. Harry conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper, revised the manuscript and coordinated the research.

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Appendix III: Published manuscript of Chapter 3