

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



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degree of Doctor of Philosophy

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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.

I also certify that the thesis has been written by me. Any help that I have received in my research work and preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Jing Lu

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Contents

| | |
|--|-------------|
| Certificate of Original Authorship..... | i |
| Acknowledgements..... | ii |
| Contents | iv |
| Tables of Figures and Tables | viii |
| Publications..... | x |
| Abbreviations | xiii |
| Abstract..... | xvi |
| Chapter 1 | 1 |
| Introduction | 1 |
| 1.1 Chronic wounds..... | 1 |
| 1.2 Revisiting the ancient remedy – honey | 3 |
| 1.3 The known antibacterial components of honey..... | 5 |
| 1.3.1 The ‘non-peroxide’ activity of honey..... | 8 |
| 1.3.2 Assays to characterize the antibacterial activity of honey | 11 |
| 1.3.3 The bacterial response to honey treatment..... | 13 |
| 1.4 Antibiofilm properties of honeys | 14 |
| 1.4.1 Bacterial biofilm development in <i>P. aeruginosa</i> | 15 |
| 1.4.2 Bacterial biofilm development in <i>S. aureus</i> | 16 |
| 1.4.3 How does honey prevent formation of biofilms and eradicate them? | 17 |
| 1.5 Antifungal properties of honeys..... | 19 |
| 1.6 Anti-inflammatory properties of honeys..... | 19 |
| Chapter 2 | 21 |
| The effect of New Zealand kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species | 21 |
| 2.1 Introduction | 23 |
| 2.2 Materials and Methods | 25 |
| 2.2.1 Honey samples..... | 25 |
| 2.2.2 Hydrogen peroxide assay | 28 |
| 2.2.3 Bacterial strains and growth media..... | 28 |

| | |
|---|-----------|
| 2.2.4 Growth of bacterial cultures | 29 |
| 2.2.5 Growth curve data analysis | 30 |
| 2.2.7 Image data analysis..... | 34 |
| 2.3 Results | 34 |
| 2.3.1 Growth responses to honey, MGO, sugar and catalase | 34 |
| 2.3.1 Growth dynamics in response to controls: MGO and sugar in the presence and absence of catalase | 40 |
| 2.3.2 Growth response in the presence of natural honeys..... | 40 |
| 2.3.2.1 Manuka honey | 41 |
| 2.3.2.2 Kanuka honey..... | 42 |
| 2.3.2.3 Manuka-kanuka honey blends | 43 |
| 2.3.2.4 Clover honey | 44 |
| 2.3.3 Other observations not fitting growth inhibition trends..... | 44 |
| 2.3.4 Cellular morphology response in the presence of natural honeys..... | 45 |
| 2.3.4.1 High-level MGO honey and cell morphology..... | 45 |
| 2.3.4.2 High-level hydrogen peroxide honey and cell morphology | 51 |
| 2.4 Discussion | 54 |
| 2.4.1 High-throughput analysis of growth dynamics reveals that MGO in honey extends the duration of lag phase..... | 54 |
| 2.4.2 Growth and morphology of different bacteria are affected by honey in markedly different ways..... | 55 |
| 2.4.3 MGO and hydrogen peroxide production cannot account for all activity present in manuka, kanuka and clover honey..... | 57 |
| 2.4.4 Clinical applications of antibacterial honey | 57 |
| Chapter 3 | 59 |
| Manuka-type honeys can eradicate biofilms produced by <i>Staphylococcus</i> <i>aureus</i> strains with different biofilm-forming abilities | 59 |
| 3.1 Introduction | 61 |
| 3.2 Materials and Methods | 63 |
| 3.2.1 Honey samples..... | 63 |
| 3.2.2 Other tested solutions..... | 65 |
| 3.2.3 Hydrogen peroxide assay | 65 |
| 3.2.4 Bacterial strains and growth conditions..... | 65 |
| 3.2.5 Susceptibility of <i>S. aureus</i> to NZ honeys: growth response assays | 66 |
| 3.2.6 Biofilm formation assays | 66 |

| | |
|--|-----------|
| 3.2.7 Biofilm elimination assays | 67 |
| 3.2.8 Determination of bacterial cell viability in biofilms | 67 |
| 3.2.9 Visualizing live/dead stained <i>S. aureus</i> biofilms using confocal laser scanning microscope (CLSM) | 69 |
| 3.2.10 Assaying honey resistance in cells recovered from biofilms | 70 |
| 3.2.11 Statistical analysis..... | 71 |
| 3.3 Results | 71 |
| 3.3.1 The effect of NZ manuka-type honeys on the planktonic growth of <i>S. aureus</i> | 71 |
| 3.3.2 The effect of NZ manuka-type honeys on <i>S. aureus</i> biofilm formation..... | 72 |
| 3.3.3 The effect of MGO on <i>S. aureus</i> biofilm prevention | 76 |
| 3.3.4 The Effect of NZ manuka-type honeys on established <i>S. aureus</i> biofilms | 77 |
| 3.3.5 The effect of NZ manuka-type honeys on cell viability within <i>S. aureus</i> biofilms..... | 80 |
| 3.3.6 The effect of MGO on established <i>S. aureus</i> biofilms | 81 |
| 3.3.7 Visualizing the effects of NZ manuka-type honeys on established <i>S. aureus</i> biofilms..... | 82 |
| 3.4 Discussion | 87 |
| 3.5 Conclusions..... | 90 |
| Chapter 4 | 91 |
| New Zealand honeys can inhibit and eliminate biofilms of <i>Pseudomonas aeruginosa</i> wound isolates..... | 91 |
| 4.1 Introduction | 91 |
| 4.2 Materials and Methods | 93 |
| 4.2.1 Honey samples | 93 |
| 4.2.2 Other tested solutions..... | 94 |
| 4.2.3 Hydrogen peroxide assay | 94 |
| 4.2.4 Bacterial strains and growth conditions..... | 94 |
| 4.2.5 Susceptibility of <i>P. aeruginosa</i> to NZ honeys: minimum inhibitory concentrations (MICs) | 95 |
| 4.2.6 Biofilm formation assays | 95 |
| 4.2.7 Biofilm eradication assays | 96 |
| 4.2.8 Determination of bacterial cell viability in biofilms | 96 |
| 4.2.9 Visualizing live/dead stained <i>P. aeruginosa</i> biofilms using confocal laser scanning microscope (CLSM)..... | 98 |
| 4.2.10 Test for the development of resistance to honey treatment..... | 99 |

| | |
|--|------------|
| 4.2.11 pH of the tested NZ honeys, sugar, and other tested solutions | 100 |
| 4.2.11 Statistical analysis..... | 100 |
| 4.3 Results | 101 |
| 4.3.1 The effects of NZ honeys and sugar solution on <i>P. aeruginosa</i> cell growth and biofilm formation | 101 |
| 4.3.2 The effects of NZ honeys and sugar solution on established <i>P. aeruginosa</i> biofilms..... | 104 |
| 4.3.3. The contribution of MGO to inhibition and eradication of <i>P. aeruginosa</i> biofilms | 108 |
| 4.3.4 Determining the pH of honeys, sugar, and other tested solutions used against <i>P.</i> <i>aeruginosa</i> biofilms | 113 |
| 4.3.5 Visualizing the effects of NZ honeys and sugar solution on established <i>P.</i> <i>aeruginosa</i> biofilms..... | 114 |
| 4.3.6 Assessing the resistance of <i>P. aeruginosa</i> to manuka-type honey treatments after exposure of biofilms to sub-inhibitory concentrations..... | 118 |
| 4.4 Discussion | 119 |
| 4.5 Conclusions..... | 124 |
| Chapter 5 | 126 |
| General Discussion..... | 126 |
| 5.1 The importance of honeys | 126 |
| 5.2 Potential mechanisms of how honey kills bacteria and affects biofilms | 127 |
| 5.3 Future directions | 130 |
| 5.4 Acceptance of honey as a wound treatment in clinical settings | 131 |
| 5.5 Concluding remarks..... | 133 |
| References..... | 134 |
| Appendices..... | 148 |
| Appendix I | 148 |
| Appendix II..... | 164 |
| Appendix III | 176 |

Tables of Figures and Tables

| | |
|---|-----|
| Figure 1.1 Manuka flower..... | 3 |
| Figure 1.2 Chronic foot ulcers treated with medical-grade manuka honey..... | 4 |
| Figure 1.3 Chemical structure of methylglyoxal (MGO)..... | 9 |
| Figure 1.4 The five-stage <i>P. aeruginosa</i> biofilm development cycle | 15 |
| Figure 2.1 Transformation of data obtained for bacterial growth with honey treatment | 32 |
| Figure 2.2 Effects of sugar, MGO and catalase on growth of bacteria..... | 37 |
| Figure 2.3 Effect of New Zealand, kanuka and manuka-kanuka blended honeys on bacterial growth..... | 39 |
| Figure 2.4 Cellular morphology of bacterial cells treated with a high-MGO honey and high-hydrogen peroxide honey | 48 |
| Figure 3.1 Correlation of level of intracellular ATP to colony forming units (CFU) in static biofilms of <i>S. aureus</i> | 69 |
| Figure 3.2 Quantification of biofilm formation by different strains of <i>S. aureus</i> | 73 |
| Figure 3.3 Effects of NZ honeys and sugar on <i>S. aureus</i> biofilm formation | 75 |
| Figure 3.4 Effects of MGO on <i>S. aureus</i> biofilm formation | 77 |
| Figure 3.5 Effects of NZ honeys on established <i>S. aureus</i> biofilms and cell viability within the biofilms..... | 79 |
| Figure 3.6 Effects of MGO on established <i>S. aureus</i> biofilms..... | 82 |
| Figure 3.7 Live/dead staining of different honey treated established biofilms | 84 |
| Figure 3.8 Quantitative analysis of live/dead stained honey treated biofilms..... | 85 |
| Figure 4.1 Relationship between levels of intracellular ATP to colony forming units (CFU) in static biofilms of <i>P. aeruginosa</i> | 98 |
| Figure 4.2 Quantification of <i>P. aeruginosa</i> biofilm adherence..... | 101 |
| Figure 4.3 Effects of NZ honeys and sugar solution on <i>P. aeruginosa</i> biofilm formation..... | 103 |
| Figure 4.4 Effects of NZ honeys and sugar solution on established <i>P. aeruginosa</i> biofilms and cell viability within the biofilms..... | 106 |
| Figure 4.5 Effects of MGO on <i>P. aeruginosa</i> biofilm formation..... | 110 |
| Figure 4.6 Effects of MGO on established <i>P. aeruginosa</i> biofilms..... | 111 |

| | |
|--|-----|
| Figure 4.7 Live/dead staining of different honey treated established biofilms..... | 116 |
| Figure 4.8 Live/dead staining of 64% and 80% of manuka and Medihoney treated <i>P. aeruginosa</i> established biofilms..... | 117 |
| Table 1.1 The antibacterial composition of different honeys | 5 |
| Table 2.1 Floral source, MGO and H ₂ O ₂ levels of honeys | 27 |
| Table 2.2 Average cell length after different honey treatment (µm)..... | 48 |
| Table 2.3 Cell morphology changes with high-MGO honey and high-hydrogen peroxide honey treatment ^a | 50 |
| Table 2.4 Summary of growth and morphological effects honey and control treatments on all organisms | 53 |
| Table 3.1 Harvesting and chemical information for the tested NZ honey samples | 64 |
| Table 3.2 Concentration of honey required to inhibit <i>S. aureus</i> growth | 72 |
| Table 3.3 Resistance of <i>S. aureus</i> cells recovered from biofilms after 8% manuka honey treatments | 87 |
| Table 4.1 Minimum inhibitory concentrations of NZ honeys and sugar solution on inhibiting <i>P. aeruginosa</i> PAO1 and PA14 cell growth and biofilm formation | 102 |
| Table 4.2 Effects of 16% and 32% NZ honeys and sugar solution on <i>P. aeruginosa</i> biofilm biomass | 107 |
| Table 4.3 pH of media and solutions used in this study | 114 |
| Table 4.4 Resistance of <i>P. aeruginosa</i> cells recovered from biofilms after 8% manuka- type honey treatments | 119 |

Publications

Journal articles

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Lu, J., Turnbull, L., Burke, C.M., Liu, M., Carter D. A., Schlothauer, R. C., Whitchurch, C. B., and Harry, E. J. (2014) Manuka-type honeys can eradicate biofilms produced by *Staphylococcus aureus* strains with different biofilm-forming abilities. *PeerJ* 2:e326.

Conference proceedings

Harry, E. J., **Lu, J.**, Turnbull, L. and Whitchurch, C. B. (May, 2010) Biofilm Prevention with Medical Honey. **SEMINAR**, 2010 Comvita Science Seminar, Auckland, New Zealand.

Harry, E. J., **Lu, J.**, Whitchurch, C. B., Turnbull, L., Blair, S., and Carter, D. (July, 2010) The Science Behind Honey, A Sweet Remedy for Antibiotic Resistant Bacteria. **SEMINAR**, 2010 Annual Scientific Meeting & Exhibition of the Australian Society of Microbiology, Sydney, Australia.

Johnson, M., **Lu, J.**, Turnbull, L., Whitchurch, C. B. (July, 2010) Super-resolution Microscopy coupled with an OptiPuter highlights a previously unseen world.

SEMINAR, *Conference of the Australian Science Teachers Association CONASTA 59, Sydney, Australia*

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Harvey, K. L., **Lu, J.**, Padula, M. P. and Harry, E. J. (November, 2011) The Effects of Manuka Honey on the *Staphylococcus aureus* Proteome. **Poster**, *The 28th Annual Scientific Research Meeting, Sydney, Australia*

Harvey, K. L., **Lu, J.**, Santos, J., Padula, M. P. and Harry, E. J. (February, 2012) The Effects of Manuka Honey on the *Staphylococcus aureus* Proteome. **Poster**, *The 17th Lorne Proteomics Symposium, Lorne, Australia*

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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 <i>Staphylococcus aureus</i> |
| 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 <i>in situ</i> hybridization |
| g | Gram(s) |
| GlxI | Glyoxalase I |
| h | Hour(s) |
| H ₂ O ₂ | Hydrogen peroxide |
| HA-MRSA | Hopital-acquired methicillin resistant <i>Staphylococcus aureus</i> |
| 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 <i>Staphylococcus aureus</i> |
| MSCRAMMs | Microbial surface componenets recognising adhesive matrix molecules |
| MSSA | Methicillin-Sensitive <i>Staphylococcus aureus</i> |
| NMR | Nuclear magnetic resonance |
| NZ | New Zealand |
| °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 violet 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.