Unlocking the Secrets of an Ancient Antimicrobial, Honey, Using Modern Transcriptomic Techniques

A Thesis

by

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DOCTOR OF PHILOSOPHY

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Certificate of Original Authorship

I, Daniel Bouzo declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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"Success, after all, loves a witness, but failure can't exist without one."
— Junot Díaz, *The Brief Wonderous Life of Oscar Wao*

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Abstract

Antibiotic resistance has been described as an 'apocalyptic' threat to human health. As resistance to antibiotics is common soon after they are introduced to clinical use, there is little investment in their development, thus prompting interest in complex natural products as antimicrobials. Honey has been used as a topical wound treatment throughout history, predominantly due to its antimicrobial activity. Manuka honey (from *Leptospermum scoparium* trees) has broad-spectrum antimicrobial activity effective against antibiotic resistant pathogens, such as ciprofloxacin-resistant *Pseudomonas aeruginosa,* and is currently approved as a wound treatment. Unlike traditional antibiotics, bacterial resistance to honey has not been reported, however honey remains underutilised in the clinic partly due to a lack of understanding of its mechanism of action.

Through passaging experiments, it was found that honey resistance cannot be induced under conditions that rapidly induced resistance to antibiotics. This is due to the complex composition of honey, which is likely to have multiple modes of action, unlike traditional single target antibiotics. The mechanism of action of honey and its key components, methylglyoxal and sugar, was investigated using a transcriptomic approach in a model organism, *P. aeruginosa*. Results indicate that no single component of honey accounts for its total antimicrobial action and that honey causes DNA and oxidative damage, and affects pathways involved in cell motility, central carbon metabolism, and quorum sensing – explained only partially by its key components.

Manuka honey uniquely upregulates genes involved in the explosive cell lysis process, an autolysis mechanism in *P. aeruginosa.* Honey also downregulates the expression of genes involved in maintaining the electron transport chain and causes protons to leak across biological membranes, ultimately collapsing the proton motive force. Flow cytometry data showed that treatment with manuka honey induces membrane depolarisation and permeabilisation in *P. aeruginosa* cells. This was confirmed by modelling membrane potential in liposomes and studying permeabilisation using electrical impedance spectroscopy of tethered lipid bilayer membranes. To investigate whether the membrane damaging action of honey could enhance the action of certain antibiotics, chequerboard assays were used to show that manuka honey acted synergistically with tetracyclines. Taken together, these data argue that manuka honey has multiple mechanisms of action, including the newly described membrane depolarising and permeabilising activity. This thesis contributes to the existing literature demonstrating the potent and unique antimicrobial activity of manuka honey which does not engender bacterial resistance, and supports its inclusion as part of the current array of wound treatments.

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List of Abbreviations

°C	Degree Celsius
AH	Artificial honey
AHMGO	Artificial honey with methylglyoxal
AKRs	Aldo-keto reductases
ATCC	American Type Culture Collection
a_W	Water activity
bp	Base pairs
CAMHA	Cation adjusted Mueller Hinton agar
CAMHB	Cation-adjusted Mueller Hinton broth
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
cDNA	Complementary DNA
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DHA	Dihydroxyacetone
$DiBAC_4(3)$	Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECL	Explosive cell lysis
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIS	Electrical Impedance Spectroscopy
FC	Fold change
FSC	Forward Scatter
FICI	Fractional Inhibitory Concentration Index
FDA	Food and Drug Administration
g	Grams
G	G-force
g/L	Grams per litre
g/mL	Grams per millilitre
GFP	Green fluorescent protein
h	Hours
H_2O_2	Hydrogen peroxide

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
kb	Kilobase
kg	Kilogram
LB	Luria Bertani (Miller)
M	Molar
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
mg	Milligram
mg/kg	Milligram per kilogram
mg/mL	Milligram per millilitre
MGO	Methylglyoxal
MH	Manuka honey
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre
NPA	Non-peroxide activity
O_2^-	Superoxides
OD	Optical Density
P	*P*-value
p.adj	Adjusted *p*-value
PCA	Principal component analysis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PMF	Proton Motive Force
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine

POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
ppm	Parts per million
PQS	Pseudomonas quinolone signalling molecule
QS	Quorum sensing
RCT	Randomised controlled trial
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNA-Seq	RNA Sequencing
RNAase	Ribonuclease A
RND	Resistance-nodulation-division
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCV	Small colony variant
SD	Standard deviation
sec(s)	Second/s
SOS	'Save-our-souls'
SSC	Side scatter
tBLM	Tethered Lipid Bilayer Membranes
TGA	Therapeutic Goods Administration
TMGG	Twitching motility gellan gum
Tris	Tris(hydroxymethyl)methylamine
UMF®	Unique Manuka Factor®
v/v	Volume per volume
WHO	World Health Organisation
w/v	Weight per volume
ΔpH	Proton gradient
Δψ	Electrical potential
µg	Micrograms
µg/mL	Micrograms per millilitre
µL	Microlitre
µm	Micrometre
µS	Microsiemens

Publications and Awards

Peer-reviewed publications

Bouzo D, Cokcetin N, Li L, Ballerin G, Bottomley A, Lazenby J, Whitchurch CB, Paulsen I, Hassan K, Harry E, 2020 (Accepted), Characterizing the mechanism of action of an ancient antimicrobial, manuka honey, against *Pseudomonas aeruginosa* using modern transcriptomics, *mSystems,* 5:e00106-20. https://doi.org/10.1128/mSystems.00106-20.

Carter D, Blair S, Cokcetin N, Bouzo D, Brooks P, Schothauer R, Harry E (2016), Therapeutic manuka honey: no longer so alternative, *Frontiers in Microbiology*, 7, 569

Publications in preparation

Williams S, Cokcetin N, Farlow R, Bouzo D, Blair S, Carter D, Brooks P, Harry E (in preparation), Active Australian *Leptospermum* honey: new sources and their bioactivity

Awards

Sydney Bioinformatics Research Symposium Runner-up in the 'Fast-forward' presentation category, 2018

SPARK Global Biomedical Innovation and Entrepreneurship Programme International Runner-up Award (University of Tokyo, Japan), 2017

Loraine Holley Essay Prize Winner, 2016

Best Oral Presentation Award at New Horizons Conference, Sydney, 2015

UTS Doctoral Scholarship Recipient, 2015

Conference and seminar proceedings

Unlocking the secrets of an ancient antibiotic, honey, *using modern transcriptomic techniques,* oral presentation at the Amateur Beekeeper Association of NSW Annual Conference, Western Sydney University, 2019

Unlocking the secrets of an ancient antibiotic, honey, *using modern transcriptomic techniques,* invited department seminar, ithree institute, University of Technology Sydney, 2019

Unlocking the secrets of an ancient antibiotic: honey, oral presentation at the Sydney Bee Meeting, Sydney, 2019

Investigating the activity of an ancient antimicrobial, honey, using modern transcriptomics, oral presentation at the Gordon Research Conference for Microbial Stress Responses, South Hadley Massachusetts USA, 2018.

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Investigating the activity of an ancient antimicrobial, honey, using modern transcriptomics, oral presentation at the Sydney Bioinformatics Research Symposium, Sydney, 2018.

Investigating the activity of an ancient antimicrobial, honey, using modern transcriptomics, poster presentation at the Sydney Bioinformatics Research Symposium, Sydney, 2018.

Unlocking the secrets of an ancient antibiotic: honey, invited department seminar, Hawkesbury Institute for the Environment, Western Sydney University, 2018.

Characterising the antibacterial mechanism of action of manuka honey on P. aeruginosa, oral presentation at the International Conference on Antimicrobial Research, Malaga, Spain, 2016.

Investigation the effects of manuka honey on bacterial growth using the model organism Pseudomonas aeruginosa, oral presentation at the New Horizons Combined Health Science Meeting, Sydney, 2015.