THE CHARACTERISATION OF THE ANTIMICROBIAL ACTIVITY OF HONEY ON CLINICAL ISOLATES OF MULTI- DRUG RESISTANT BACTERIA IMPLICATED IN HEALTHCARE ASSOCIATED INFECTIONS

JACQUELINE M. KENNY

MAY 2013

A thesis submitted in partial fulfilment of the requirements of

Kingston University

for the degree of Doctor of Philosophy

DECLARATION

I declare that the work reputed in this thesis is entirely my own and has been carried out The Royal Marsden Foundation Trust UK in conjunction with Kingston University UK.

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Jacqueline Mary Kenny

Abstract

Bacterial resistance to antibiotics has presented increasing challenges in healthcare and the management of infection. This has resulted in alternative and traditional products that are used in other cultures being considered as an alternative to topical antibiotics. Honey, particularly Manuka honey is a product which has gained credibility as an antibacterial agent in a healthcare environment. The aim of this study was to investigate the antimicrobial capacity of syrups and honeys from different floral sources on antibiotic sensitive and resistant bacteria isolated from a clinical environment.

The antimicrobial activity of seven Manuka honeys, seven honeys from other floral sources and two syrups were assayed against antibiotic sensitive and resistant isolates of *Staphylococcus aureus, Enterococcus species, Escherichia coli* and *Pseudomonas aeruginosa* using agar diffusion and microbroth methods to determine minimum inhibitory concentrations. These assays demonstrated both the superior antimicrobial activity of the Manuka products and highlighted differences in susceptibility between sensitive and resistant strains within organism groups.

Clinical grade Manuka honeys were used to study the effect of bioload on antimicrobial efficacy on isolates from clinical polymicrobial wound populations. This demonstrated that it was the direct physical contact with the organism and not the microbial bioload which influences antimicrobial efficacy.

Bacteria may form biofilms when they come into contact with an adherent surface. Organisms in biofilms have greater resistance to antimicrobials and are recognised clinically as a feature of chronically

infected wounds. The ability of medical grade Manuka honey to remove established biofilms from a variety of surfaces was investigated. The results indicated potential activity but were inconsistent due to the fragility of biofilm adherence to artificial surfaces. To better emulate a clinical environment a wound model was designed using cooked meat and the polymicrobial bacterial populations from clinical wounds. The results of these experiments showed the Manuka honeys to have a bacteriostatic effect on the biofilms with no contamination of the surrounding honey medium.

Chemical analysis of the honey products was performed using thin layer chromatography (TLC) and diffusion ordered spectroscopy nuclear magnetic resonance (DOSY NMR). TLC demonstrated the presence of antimicrobial fractions but insufficient material was yielded for further analysis and identification using NMR. Using DOSY NMR directly on the untreated honey products enabled characterisation of the products, identifying aromatic compounds in the Manuka products which are reputed to have antimicrobial activity. There did not appear to be any single constituent proportional to the antimicrobial UMF rating (Unique Manuka Factor) of the Manuka products where a high rating indicates a high level of antibacterial activity. The results suggest that it is a combination of compounds which confer the antimicrobial properties of the Manuka products.

In conclusion this study demonstrated the superior antimicrobial activity of Manuka honey compared to syrups and honey from other floral sources and that this activity is likely due to a number of aromatic compounds present only in the Manuka products. Clinical grade Manuka

honey appears to have bactericidal activity upon planktonic organisms with mainly bacteriostatic activity on biofilms grown on a wound model.

Acknowledgments

My first thanks go to Prof. Mark Fielder, my Director of Studies for his support, advice and faith in me throughout the project. Also my thanks to the team of supervisors at Kingston University Dr. Alison Kelly and Prof. Declan Naughton for their contributions. My special thanks to Dr Claire Cassar whose support and advice came with such warmth and encouragement. I would also like to express my gratitude to Dr Adam Le Gresley for his time, training and patience with the NMR section. I would also like to thank Kingston University for contributing to the course funding and The William Harvey Hospital for providing clinical wound populations.

I have an enormous debt of gratitude to my sponsors The Royal Marsden Foundation Trust who have granted so many years of financial sponsorship, study leave and laboratory resource to enable me to do this project. My Consultant and workplace supervisor Dr Unell Riley and my mentor and manager Sue Alexander for their continued patience and encouragement and who have been hugely instrumental in me reaching this destination.

Finally thank you to all my friends and family. At KU, Tamsyn for being such a superstar when I first started and always being so willing to offer help and advice and Teri whom has, laughed, cried, drank wine and made cake as needed although not entirely in equal measure. To Julie and the Usual Suspects, my sister Jo and my children Jamie and Holly who have encouraged me, had total and relentless faith in me, put up with me and helped me hold it together, without you all I would never have got to the finishing line.

DEDICATION

I dedicate this to my father Bryn Trick who believed I could achieve anything if I wanted it badly enough

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

CHAPTER 1

Introduction

1.1 Introduction

The increasing resistance of bacteria to antimicrobial agents has become a worldwide problem (Levy, 2002). The wealthiest and most technologically advanced countries are facing the greatest challenge in the fight against infections caused by multi-drug resistant organisms (Cars *et al.*, 2008). The capacity of bacteria to develop resistance is rapidly exceeding the development of new agents with which to fight the infections they cause (Projan, 2003, Charles and Grayson, 2004).

In the 1930s at the point Penicillin was discovered, antimicrobials were considered to be the wonder drugs of modern medicine. The UK Department of Health (DoH) publication 'The Path of Least Resistance' (Standing Medical Advisory Committee, 1998) recounts how the incidence of death by post-partum sepsis and post-appendectomy dropped to almost zero within the space of ten years and the necessity for sanatoria for the treatment of tuberculosis decreased. In turn, surgical techniques developed in response to the decreasing risks of infection. Within ten years antibiotic resistance began to occur, but as new drugs were continually being developed, the threat of emerging resistance was not fully appreciated (Standing Medical Advisory Committee, 1998).The DoH publication further illustrates the emergence of resistance to almost every class of antimicrobial product that has been developed. The over use of antibiotics by the medical profession is not the sole cause for the increase in drug resistant bacteria (Stokes *et al.*, 2008). Lieberman and Wootan, (2007) proposed that the problem has been exacerbated by the widespread use of antibiotics in agriculture for crops and in the

production of meat for consumption (Gallois *et al.,* 2009). Since 2000, the DoH has recommended judicious use of antibiotics (Department of Health, 2000) and indeed the commensurate rise of resistance has encouraged the medical profession to seek alternatives to conventional therapies, thus reducing the dependence of both clinicians and their patients on antibiotic prescription (Colgan, 2001). Consequently, the scientific community has now started to consider and explore alternative methods used in other cultures and traditional lore, many of which advocate the use of natural products as a means to combat infection (Baker *et al.,* 2007). One such product that has had increasing acceptance in recent years is honey (Molan, 2006).

1.2 Historical Overview

The first reported use of honey for the treatment of wounds dates back to the Middle East and Mesopotamia between 3000 and 2000 BC (Forrest, 1982). As these ancient cultures evolved, so did their medicinal use of the natural products available at the time. Forrest (1982) describes how in turn, the ancient Egyptians favoured honey, lard and resins and the introduction of minerals such as copper in the treatment of wound infections. The shaman of Indian, African and South American tribes used herbs, barks and various animal products. Gold, frankincense and myrrh were used in South Western Arabia (Hillson, 1988). It would therefore appear that very few natural products remained unexploited in the treatment of wounds at some point by the healers of the ancient civilisations (Forrest, 1982). Animal products such as milk, blood, bile and faeces, vegetable products such as tree sap, turpentine, vinegar and wine and many minerals including alum and arsenic were

amongst the vast number of agents used on wounds in an attempt to prevent infection and aid healing (Majno, 1975, Forrest, 1982). One may speculate that some of these products would have produced disappointing results therefore their continued use does not appear to have been pursued. However, the use of honey has endured. Aristotle (*circa* 385-320 BC) is reputed to have commented that honey was 'good as a salve for sore eyes and wounds' and by Dioscorides in 50 BC as 'good for all hollow and rotten ulcers' (Molan, 1999).

To place the use and applications of honey into the context of modern wound management it is necessary to consider what is known about the healing process and how the microbiological environment of the different types of wounds, for example surgical or trauma, may influence the healing process.

1.3 Wound healing

To address the problems associated with slow healing, chronic wounds, it is first necessary to understand the normal process of wound healing in a healthy individual. At the moment of injury, the body is immediately galvanised into the inflammatory phase of healing (Enoch and Leaper, 2008). Coagulation and plasminogen cascades are activated and polymorphonuclear neutrophils (PMNs) are drawn to the wound site by kinins and growth factors triggered by platelets (Enoch and Leaper, 2008). The large numbers of PMNs help to cleanse the wound of bacteria by phagocytosis. These PMNs naturally die by apoptosis after a day or two and along with any other wound debris are cleared by macrophages which are responsible for the down regulation of the inflammatory response. This inflammatory, acute phase lasts around 4-6

days (Enoch and Harding, 2003, Schultz *et al.,* 2004). The wound bed is now prepared for the proliferative phase. Cytokines and growth factors direct fibroblasts, epithelial and endothelial cells to the wound site, which in turn produce growth factors, whereby a new extracellular matrix is synthesised and the area vascularised (Enoch and Leaper, 2008). The wound contracts and after around 14-21 days the remodelling phase takes place where collagen fibres are reorganised and scar tissue is formed (Enoch and Leaper, 2008). It can take up to 2 years for these fibres to fully mature and regain the strength of the original tissue (Rhoads *et al.,* 2007). This is the normal progression of repair of an acute wound. Wounds become chronic when there is a failure to progress from the inflammatory phase (Nwomeh *et al.,* 1998). The health of the patient, and the presence of underlying factors, such as heavy smoking, diabetes, vascular disease and ischaemia, all effect the body's ability to heal (Hollisaz *et al.*, 2004). These factors along with the nature of the wound, whether it is clean, dirty, surgical or traumatic, as well as the size and depth, influence microbial populations and healing time (Bowler *et al.*, 2001). Understanding how microbial populations differ with wound type and within acute and chronic wounds is key to effective wound management.

1.4 Bacterial contamination, colonisation and infection

Whenever and however a wound occurs there is a high probability that contamination will occur (Sibbald *et al.*, 2003). Contamination is described as the presence of non-replicating organisms within a wound (Dow *et al.,* 1999) and usually occurs with the host's normal regional flora. In itself, this has little impact on the normal healing of acute

wounds in healthy individuals, but prolonged exposure in wounds with devitalised tissue may result in colonisation. Colonisation occurs when bacteria replicate in the wound and 'outcompete' normal host defence mechanisms (Dow *et al.,* 1999, Bowler *et al.,* 2001). Colonisation levels of around 1 x 10^5 organisms per gm tissue do not generally delay healing but virulent organisms such as *Staphylococcus aureus, Pseudomonas aeruginosa, β* haemolytic streptococci and anaerobic bacteria even in low numbers, may tip the balance from localised to invasive infection (Edwards and Harding, 2004). Lack of epithelialisation, perfusion of the wound site, the presence of pathogens and total bacterial load inclusive of non pathogenic bacteria may all contribute to the features of chronic wounds. It is only relatively recently that the significance and challenges presented by bacterial biofilms have been identified as an obstacle to effective treatment and healing (Edwards and Harding, 2004, Rhoads *et al.,* 2008).

1.5 Bacterial biofilms in wounds

When organisms first contaminate a wound site they are unattached and in their planktonic form thus facilitating translocation from one site to another (Watnick and Kolter, 2000). However, in the environment, bacteria are often associated with surfaces, where profound changes occur and complex polymicrobial ecosystems are formed, increasing their resistance to external forces detrimental to their survival (Costerton *et al.,* 1999, Costerton and Stewart, 2001, James *et al.,* 2008). Should a biofilm form the organisms develop into what are described as robust, hardy bacterial communities. These biolfilms can play a significant role in their persistence in biological environments such as the human body

(Costerton *et al.,* 1999). The significance of their presence in dental decay, endocarditis and mucosal surfaces such as the gastrointestinal tract and respiratory mucosa have long been recognised (Rickard *et al.,* 2003, Hannan, *et al.,* 2010) and the significance of their role in chronic wounds and the subsequent identification of effective therapeutic regimes has only more recently been acknowledged (Costerton *et al.,* 1999, Rhoads *et al.,* 2007, James *et al.*, 2008, Cooper, 2010).

1.5.1 Biofilm formation.

The first step in biofilm formation is the adherence of an organism to a surface. The surface can be inorganic or organic, the latter providing a readily available nutritional source. Physio-chemical factors affect bacterial adherence to a surface (Cerca *et al.,* 2005). Adhesion may be mediated by non-specific forces such as Van der Waal and electrostatic forces and factors influencing the attraction of the bacteria to the solid surface (Cerca *et al.,* 2005, Todar, 2012). Hydrophobic surfaces such as plastic and materials used for catheters and indwelling devices attract the charged polymers within the bacterial cell wall (Gross *et al.,* 2001). Gross and colleagues (2001) identified teichoic acids as playing a key role in the adhesion of *S. aureus,* as the ability to adhere was compromised when the charge was altered by a lack of D-alanine esters in the teichioc acids. The second phase of adhesion is the adhesion of the bacteria using structures such as pili and fimbriae as used by *E.coli* and cell bound proteins molecules used by *S. aureus* and *Streptococcus pneumoniae* (Katsikogianni and Missirlis 2004, Todar 2012).

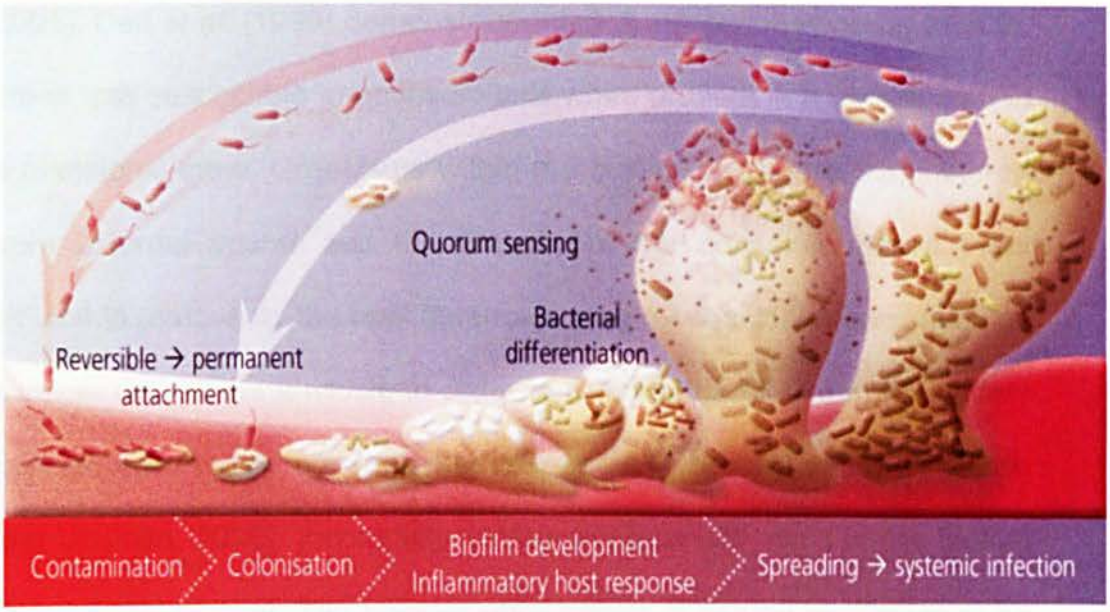
Once the bacteria adhere, phenotypic changes take place (Costerton *et al.,* 1999). In polymicrobial environments coaggregation may occur, a

symbiotic, synergistic, specific intra- species binding of surface polymers (Rickard *et al.,* 2003). This phenomenon was first observed with oral bacteria in the 1970's (McIntire *et al.,* 1978) but has since been observed in the urogenital tract (Reid *et al.,* 1988) and in many environmental habitats (Buswell *et al.,* 1997, Min *et al.,* 2010). The biofilm develops as cells start to produce an extracellular polymeric substance or matrix (EPS) facilitating 'irreversible' adherence and protection from forces hostile to the bacteria. In Staphylococci, Poly –*N*-acetyl-glucosamine (PNAG), formally known as polysaccharide intercellular adhesin (PIA) is responsible for cell to cell adhesion and it has been shown that a lack of PNAG prevents biofilm formation (Gross *et al.,* 2001, Cerca *et al., 2005*) Inter and intra- species communication between cells known as 'quorum sensing' mediates transcription of genes and proliferation of the bacterial community (Nadell *et al.,* 2008, Cooper, 2010). Quorum sensing in Gram negative bacteria utilises acyl-homoserine lactone (acyl-HSL) signals which are generally synthesised by the Lux-1 family of synthases. Acyl-HSLs travel freely through cell membranes and the levels increase with cell density (Greenberg, 2000, Parsek and Greenberg, 2005). The expression of the signal is regulated by the LuxR homologues. The Lux enzymes are named after the *Vibrio fischeri* lux system where they were first identified (Cerca *et al.,* 2005). Gram positive bacteria use peptide signalling. These are not detected intracellularly but by membrane bound proteins (Parsek and Greenberg, 2005). It has also been noted that bacterial adhesins faciliatate the coaggregation of bacteria in the developing biofilm (Rickard *et al.,* 2003).

The mature biofilm appears as a biolayer made up of mushroom-like projections encased in EPS separated by channels in which nutrients can dissolve and diffuse (Cooper and Okhiria, 2006). In some cases cells of the immune system such as Neutrophils and macrophages may be bound within the biofilm effectively creating one of the anti-phagocytic properties associated with biofilms (Mertz, 2003). Coaggregation enhances communication between organisms, facilitating the transference of genetic material such as virulence factors and antibiotic resistance (Hannan *et al.*, 2010). Cell to cell communication finally triggers the bacteria in the outer layer of the biofilm to revert back to a planktonic state allowing fragments to break off and reseed elsewhere (Phillips *et al.*, 2010).

Figure 1 is a schematic taken from an article in Wounds International by Phillips *et al.* (2010) showing the formation and subsequent dispersal of a biofilm.

Figure 1.1 A schematic diagram showing the process of biofilm formation, development and subsequent dispersion of planktonic bacteria and biofilm fragments from a mature biofilm



The biofilm is formed by contaminating organisms attaching and adhering to a surface. Bacterial differentiation occurs and formation of EPS mediates cell to cell adhesion. Quorum sensing molecules mediate transcription of genes, proliferation and ultimately the detachment of bacteria from the biofilm allowing the disseminated organisms to cause systemic infection and to reseed and form new biofilms and loci of infection. (Image taken from Wounds International, Phillips *et al.* 2010)

1.5.2 Significance of wound biofilms

In the presence of a good blood supply, healthy host defences combat bacterial contamination and colonisation (Bowler and Davis 1999). Where the bacterial load exceeds the body's capacity to hold infection in abeyance, systemic treatments can be an effective adjutant to fighting off infection. However, the wound bed, with incomplete epithelialisation, devitalised tissue and compromised vascular access, provides an ideal nutrient rich location for biofilm formation (Cooper and Okhiria, 2006).

This permits infecting organisms to proliferate in an environment that can resist assault from the immune system, systemic pharmaceuticals and topical antimicrobial therapy (Xu *et al.*, 2000, Costerton and Stewart, 2001). Ceri *et al.* (1999) demonstrated that *S. aureus* may be up to 1000 times less susceptible to antimicrobials when present in a biofilm than in a planktonic state. Organisms within the biofilm matrix are sequestered from external agents and the tenacity of the adhesion makes them difficult to remove by the host defences or by mechanical means such as surgical debridement which, in order to remove the biofilm in its entirety has the undesirable consequence of removing healthy tissue (Vowden and Vowden, 1999). Incomplete biofilm removal will result in the biofilm re-establishing itself. The polymicrobial nature of wound biofilms may be complicated and include both aerobic and anaerobic organisms living symbiotically together presenting further challenge to identifying optimal therapies (Bradshaw *et al.*, 1998). The majority of *in vitro* study and diagnostics on bacterial susceptibility is performed on planktonic bacteria as the bacterial cells may revert to their planktonic phenotypes during laboratory isolation and investigations. Hence, bacterial biofilms in chronically infected wounds pose significant challenges to both host defences and effective antimicrobial therapy (Cooper 2010)

1.6 Wound care

Whether the wound is a 'clean' surgical wound or a traumatic 'dirty' wound requiring initial cleansing, it is necessary to keep the wound germ free to expedite healing and secondary complications. Infection prevention measures may result in more radical intervention such as surgical debridment of necrotic and anoxic tissue (Werdin *et al.*, 2009).

Even in the absence of pathogenic bacteria, the bacterial load may still prolong healing (Bowler *et al.,* 2001), therefore maintaining wounds free from bacterial contamination, colonisation and biofilm formation is of primary importance. Microbiological investigations of wound swabs or aspirated fluid, pus or tissue can be used to monitor the effectiveness of the care regime and indicate when antimicrobial therapy may be required, although the integrity of these results may be compromised by bacteria reverting back to a planktonic state. The pathogens primarily responsible for wound infection are *Staphylococcus aureus, β* haemolytic streptococci, *Pseudomonas aeruginosa*, *Clostridium perfringens* and *Clostridium tetanii.* Even wounds with no clinical signs of infection, may be colonised with a substantial population of *Enterobacteriaceae* and commensal skin flora which may prolong healing (Bowler *et al.,* 2001).

Due to the difficulty in collection, sample transport and isolation of anaerobic organisms, wound culture reports tend not to present a true aerobe : anaerobe population. However, some reviews of the literature (Bowler, 1998, Bowler and Davis, 1999), explain that when the necessary isolation techniques are used, anaerobes do form a large part of the microbial population in wounds. Due to the symbiotic co-existence of aerobic and anaerobic bacteria in wounds, a decrease in the aerobic bacterial population, reduces the reduction potential in the underlying tissue and therefore assists in the reduction of the resident anaerobic microbial population (Bowler, 1998).

Successful wound repair requires that the bacterial load is minimised with no primary pathogens and that the healthy tissue has an adequate

nutritional supply (Sibbald *et al.*, 2003). Therefore criteria for wound dressings include that they prevent external contamination of the wound, reduce the bio-load, destroy pathogenic bacteria and prevent the escape of microorganisms into the surrounding area and creating an environment where the tissue can repair and regenerate (Bowler *et al.*, 2001). In this context, there are a variety of dressings and topical agents available in addition to the use of systemic antibiotics. The most popular dressings and topical agents include silver, iodine, topical antimicrobials and honey (Molan, 1999, Burrell, 2003, White *et al.*, 2006). Polyhexamethylene biguanide (PHMB) is a synthetically produced antimicrobial peptide (AMP) similar to the AMPs produced by the immune system which has recently found favour and been has promoted as a valuable addition to wound care treatment (Gray *et al.*, 2010).

1.6.1 Topical antibiotics and agents

Historically, the widespread use of systemic and topical antibiotics have been the generally accepted as treatments for infected wounds (White *et al.*, 2006), otitis media (Ho *et al.*, 2007) and along with benzoyl peroxide, acne (Del Rosso., 2007). White *et al.* (2006) underlined the value of using antiseptic agents instead of antibiotics to reduce the biological burden in wounds. Diehr *et al.* (2007) recommended antibiotic prophylaxis for surgical wounds and observed that additional topical antibiotics appear to aid healing. However, Diehr and colleagues also state that antimicrobial applications, such as silver sulfadiazine and benzoyl peroxide, hasten healing of chronic wounds and that honey may also be an acceptable dressing (Diehr *et al.*, 2007). Antibiotics generally too toxic to be of use systemically, such as Neomycin and Polymyxin B

have been put to effective use topically on wounds (Frank *et al.*, 2005). The value of topical antimicrobials is increased when ischaemia inhibits the bioavailability of systemic antimicrobials (White *et al.*, 2006). For the treatment of otitis media topical antibiotics still seem to be the treatment of choice by both general practitioners and ear, nose and throat surgeons, although opinions differ in the presence of a perforated timpanic membrane due to the ototoxicity of many topical antibiotics (Ho *et al.*, 2007). Acuin (2002) describes in his review of the evidence for treating chronic otitis media, how most clinicians would consider the use of non-ototoxic antibiotics in these cases. However, during this review no mention was made of the potential use of alternative products supporting conventional otitis media treatments. There are many acne medications currently available on prescription, including topical and systemic retinoids and antibiotics and hormonal therapies, although adverse effects are reported in some individuals (Haider *et al.*, 2004). Both light and laser treatments have been used for acne with comparable success to topical antibiotics but with faster results and fewer side effects (Elman and Lebzelter, 2004). These examples serve to show there are many products available for consideration when looking for topical agents and many of these are incorporated into wound dressings.

1.6.2 Wound dressings

1.6.2.1 Silver

The antimicrobial activity property of silver (Burrell, 2003) has been utilised for centuries in the preservation of water. The medicinal application of silver was first reported in the 19th Century as cited by Maillard and Denyer (2006). Silver sulfadiazine (SSD) and silver with

sulphonamide, seen as an improvement on silver salts, were first described by Fox in 1968 as an anti-pseudomonal agent on burn wounds. This was supported by further work by Carr *et al.*, (1973) who looked at the susceptibility of a wide range of Gram positive and Gram negative wound-associated bacteria to SSD. It was shown that SSD had a broad spectrum of activity against these organisms even at low concentrations (Carr *et al.*,1973).

There are several mechanisms which have been shown to contribute to the antimicrobial efficacy of silver. In 1974, Bragg and Rainee described the inhibition of the respiratory chain between *b*- cytochromes and cytochrome a_2 and by interferance with flavoprotein (Bragg and Rainee, 1974). The affinity of silver with thiols allows silver to interact with enzymatic proteins in the cell membrane causing leakage of potassium ions across the cell membrane (Jung *et al.*, 2008). Silver has also been shown to bind with purine and pyrimidine bases in DNA, stabilising the double helix and reducing proliferation (Krall, 1999).

The antibacterial action of silver can be inhibited by halide ions at low concentration although increasing the halide concentration has been shown to reverse the inhibitory effect by forming water soluble complexes increasing the bioavailability and bacterial toxicity of the silver ions (Silver., 2003). Plasmid borne genetic mechanisms of bacterial resistance have been identified (Silver, 2003). Silver describes nine genes conferring three resistance mechanisms, ATPase and chemiosomotic efflux pumps and a peri-plasmic metal binding protein. These are plasmid borne genes which therefore may be transferred to

other bacteria potentially increasing the incidence of organisms with resistance to silver (Silver, 2003).

Currently silver is used in a cream for topical application and until recent times, silver, has been unchallenged as the best treatment for minor or partial thickness burns (Chung, 2001). Work has been undertaken to impregnate fibres with silver (MacKeen *et al.,* 1987) and dressings made of silver sodium carboxymethyl cellulose are now widely used (Frank *et al.,* 2005). Recent studies on the effects of nanocrystalline silver in dressings show enhanced silver activity resulting in faster epithelialisation and healing (Sibbald *et al.*, 2003).

A limitation of silver treatment is the lack of debridement activity and inability to penetrate deep into the wound bed (Molan, 2006). Silver does not have any wound healing properties other than the antibacterial activity and some microbial resistance has been observed (Loh *et al.*, 2009).

1.6.2.2 Copper

Copper has a long history of use as an antibacterial agent. There is evidence dating from 400BC that it was used to purify water (Barkow and Gabbay, 2005) and amongst the heavy metals, it is second only to silver as a water purifier (Albright and Wilson 1974). Copper has been incorporated into paints (Cooney, 1999), food storage containers in food processing (Faundez *et al.,* 2004, Noyce *et al.*,2006) and into textiles such as bedding and clothing in hospital settings to aid the prevention of nosocomial infections (Borkow and Gabbay, 2004).

In addition to antimicrobial properties, copper has been shown to promote the expression of vascular endothelial growth factor (VEGF)

which stimulates angiogenesis (Sen *et al.*, 2002). Barkow and Gabbay (2010) have since demonstrated using a mouse model, that copper impregnated dressings can enhance wound healing,

The antimicrobial activity, like silver, is attributable to a greater affinity with thiol groups and structural changes to proteins and nucleic acids (Barkow and Gabbay, 2005). Likewise, mechanisms of resistance include genetically encoded efflux pumps (Dupont *et al.*, 2011).

1.6.2.3 Iodine

Iodine has been officially recognised as an antiseptic since the 19th century as cited by Forrest (1982) and is still widely used as an iodophor. Iodophors are antiseptic agents comprising of Iodine plus a stabilising agent such as povidine which releases free iodine when in solution. Iodine preparations are contra-indicated in patients with thyroid conditions, children and pregnant or lactating women (Frank *et al.*, 2005) and a case of thyrotoxicosis in an intensive care patient has been reported (Patil *et al.*, 2003).

Iodosorb, a cadexomer iodine paste, has broad-spectrum antibacterial activity and some debriding effect but is thought to increase inflammation (Frank *et al.*, 2005).

Studies looking at the efficacy of the new bioxygenerating hydrogel dressings, which deliver iodine and oxygen to the wound, have been shown to be rapidly effective, with broad-spectrum activity including yeasts and multi-drug resistant bacteria such as *Pseudomonas aeruginosa* and both meticillin sensitive and resistant *Staphylococcus aureus* (Thorn *et al.*, 2006). However, sensitivity to iodine dressings and dressings containing other products such as zinc oxide and the

antimicrobials neomycin and soframycin is known to occur with some patients (Thomas *et al.*, 1998).

1.6.2.4 Polyhexamethylene biguanide (PHMB)

PHMB is a synthetic product that resembles naturally occurring antimicrobial peptides (AMPs). Various AMPs are produced by neutrophils and other cells and their antimicrobial activity is due to them binding to and compromising bacterial cell membranes (Sorenson *et al.*, 2003). It has long been used in cleaning agents for contact lenses (Burger *et al.*, 1994) and has been found to be active against both planktonic and sessile organisms (Gilbert *et al.*, 2001). PHMB does not have the toxicity issues associated with iodine and silver which makes it increasingly attractive alternative component of dressings.

1.6.2.5 Honey

Due to the cytotoxic and bacterial resistance issues associated with the more conventional topical treatments, honey, like PHMB, has gained popularity as a viable alternative treatment (Ndayisaba *et al.*, 1993, Al-Waili and Saloom, 1999, Subrahmanyan *et al.*, 2001, Schumacher, 2004, Bangroo *et al.*, 2005, Gethin and Cowman, 2005; Simon *et al.*, 2006). Honey dressings and medical grade honey are already available commercially. Interestingly, studies comparing efficacy of honey dressings to silver report conflicting results. Baghel *et al.*, (2009) and Gupta *et al.*, (2011) found honey a superior dressing to silver when used on burns patients although their studies did not specify the bacteria present. Bradshaw (2011) compared Iodine, silver and honey dressings *in vitro* against *S. aureus, P. aeruginosa* and *E. coli* and found little difference but reported reduced antimicrobial efficacy with the honey

dressing against *P. aeruginosa.* Lund-Nielson and colleagues (2011) conducted a randomised clinical study comparing honey and silver dressings on the bacterial populations found in chronic malignant wounds and observed no difference in results between the two dressings. It is interesting to note that a pubmed search of the published literature does not show any studies using honey as an adjunct to conventional wound dressings.

Honey as an antimicrobial agent has received a lot of attention over the last ten years, with many claims made in relation to the antimicrobial action and potential healing properties (Ndayisaba *et al.*, 1993, Molan, 1999, 2001, 2006, Al-Waili and Saloom, 1999; Subrahmanyan *et al.*, 2001, Schumacher, 2004, Bangroo *et al.*, 2005, Gethin and Cowman, 2005, Simon *et al.*, 2006, Cooper 2008).

It is with these claims in mind that honey has become the focus of the current study.

1.7 Honey in healthcare

As honey has gained popularity as a healthcare product, there have been a number of studies on honeys from different floral sources to determine which may confer medicinal benefit (Al-Jabri *et al.*, 2003, Küçük *et al.*, 2007, Roland *et al.*, 2007).

1.7.1 Active honey

The historical use of honey as a wound healing agent is well documented but as science and technology progressed ancient natural remedies were displaced by manufactured antiseptics and antibiotics. Advances in defining the constituents of honeys derived from different sources has demonstrated that not all honey is the same and that

antimicrobial and healing properties might vary with floral source (Anklam, 1998, Huidobro and Sanchez, 2005, Nagai *et al.,* 2006, Truchado *et al.,* 2009, Biesaga and Pryrznska, 2009).

The content of honey varies according to the nectar source (Molan, 2001). In the beehive, enzymes are added to the nectar by the bees and water evaporates. The honey is sealed in the comb with propolis; a substance comprising antimicrobial resinous bee glue, collected from a variety of botanical sources. Grange and Davey (1990) demonstrated that propolis itself has broad-spectrum antimicrobial activity and suggest that the activity in both propolis and honey may be attributable to the flavonoid content. After being sealed in the comb the honey is left to ripen.

Typically, honey contains around 76% sugars, predominantly glucose and fructose with small amounts of other sugars such as sucrose, raffinose and trehalose depending on the floral source (Bogdanov *et al.,* 2004). Approximately 18% of honey is water and there are also small amounts of ash (0.18%) and formic acid (0.08%). Honey is also known to contain a number of preservatives such as ascorbic acid, flavonoids, other phenolics and enzymes, namely glucose oxidase, catalase and peroxidase (Nagai *et al.,* 2006). Nagai *et al.* (2006) highlight that honeys from a variety of sources such as buckwheat, acacia and Chinese milk vetch, have antimicrobial and anti-oxidant activity whilst some other reports imply that only certain types of Manuka Honey have antimicrobial activity above that attributable to its sugar content (Wahdan, 1998). A review by Bogdanov in 2004 discussed some of the difficulties in

characterising honeys from different sources as it is not always possible to find specific markers for different floral sources.

The Honey Research Unit at the University of Waikato in New Zealand set up in 1995 by Professor Peter Molan has played a major role in researching and documenting the antimicrobial and healing properties of Manuka honey and raising its profile in a worldwide market.

Thomas *et al.,* (1998) described using honey to treat the non-healing lesions of a meningococcal patient. The honey not only cleared the wounds of pathogenic bacteria but had a debriding and anti-inflammatory action. Thomas and colleagues asserted that the honey stimulated growth of new capillaries, fibroblasts and epithelial cells. The healing properties attributed to Manuka honey are threefold: antimicrobial activity above that of osmotic pressure and peroxide cleansing; natural debridement and anti-inflammatory properties due to host immune response.

1.7.1.1 Honey as an antimicrobial agent

A study by Bose (1982), comparing the effectiveness of honey treatment to sugar treatment, indicated that there may be more to honey than osmotic influence.

Analysis of the chemical composition of honey (Oka *et al.*, 1987) showed the honey under examination possessed seven tetracyclines, fatty acids, amylases, ascorbic acid, fructose and hydrogen peroxide, all of which may contribute to its activity. Its low pH and the presence of a phenolic compound may also confer activity (Al-Jabri *et al.,* 2003). Al-Jabri and colleagues compared the activity of honey from Africa and Oman which came from different floral sources and showed that honey from different

geographical areas have different composition and spectrum of activity (Al-Jabri *et al.*, 2003).

Tetracycline residues have been found in honey by researchers in Greece (Saridaki *et al.*, 2006) and Germany (Heering *et al.*, 1998). Heering and colleagues tested a variety of honeys from different geographical areas available for purchase in Germany and found residues of tetracycline, sulfathiazole and streptomycin in many of the products (Heering *et al.*, 1998). The study revealed that a higher incidence of these substances was found in honey from unstated, other European countries and North America. Only Australian and New Zealand honeys contained no traces of the other contaminating materials. Australian honey is carefully monitored for residues of 42 different substances including sulphonamides and other antibiotics, synthetic pyrethroides, organochlorines, organophosphates and chemical elements (McKee, 2003). Residues of tetracycline, particularly oxytetracycline are found as a result of its prophylactic use in bee husbandry to help prevent European and American foulbrood caused by the organisms *Streptococcus pluton* and *Bacillus larvae* respectively (Sanford, 1987). The United States Department of Agriculture (USDA) Quality Assurance International, (QAI) which certifies organic produce describes the legitimate use of oxytetracycline in bee husbandry when honey production has ended until 30 days before the production of new organic honey (Quality Assurance International, 2013). In the UK the criteria required for honey to qualify as organic, as described by the Soil Association means that effectively organic honey cannot be produced here due to proximity of apiaries to possible sources of contamination

such as motorways, industry, towns and cities (The Soil Association, 2013). The European Commission regulate the maximum residue limits (MRLs) of pharmacological products which may be found in food products, but no such levels have been made applicable to honey. This means that the use of veterinary medicine is not authorised in the treatment of honey bees. However imported honeys may be sold providing they do not contain antibiotic levels in excess of the Reference Point for Action (RPA) which is based on the amount detectable by food laboratories (European Commission Regulation (EC) No 470/2009). These irregularities give some indication of the conflicting criteria for antimicrobial usage internationally, the definitions of organic products and the possible presence of pharmacological contaminants. It is unclear if these trace antimicrobials confer any antimicrobial activity to the honey.

Manuka honey is obtained from bees feeding on the nectar of the Manuka tree (*Leptospermum scoparium*) found in New Zealand and its Australian counterpart, the jellybush (*Melaleuca alternifolia*). Manuka is the Maori name for the Tea Tree. The Honey Research Unit at the University of Waikato, New Zealand, has developed a potency rating which they apply to the different Manuka Honeys. The potency rating has been named the Unique Manuka Factor rating (UMF). This rating is based upon the activity of hydrogen peroxide generated by the enzyme glucose oxidase found in the honey, produced by the bees, along with the activity of the phytochemical compound found in the nectar (Molan, 1999). The UMF factor is equivalent to the percentage of phenol required to inhibit *Staphylococcus aureus* (Molan, 1999).

In recent years, much research has been carried out to characterise the active components of honey. The osmotic effect, due to the high sugar content does not entirely explain its action, as wound exudate dilutes the honey beyond the point at which an osmotic effect is exerted (Cooper *et al.,* 1999a). The explanation for this was given in a historical study by White *et al.,* (1963), which showed that upon dilution, honey yields small amounts of hydrogen peroxide. The reason for hydrogen peroxide production is the presence of glucose oxidase, which comes from the bee and catalyses the following reaction (Molan, 2001).

Glucose + $H_2O + O_2$ → gluconic acid + H_2O_2

Providing the enzyme is present, the dilution of the honey by wound exudates will cause peroxide to be released. The antimicrobial effect of this peroxide will depend upon the amount of catalase available to break down the peroxide to water and oxygen.

Studies carried out by Cooper and Molan (1999b) suggested that the addition of catalase to Manuka and pasture honey prior to testing anti-*Staphylococcus aureus* activity, would demonstrate the non-peroxide activity of the honey. The honey was tested by incorporating honey into agar, which involved diluting the honey in water. Cooper and Molan went on to demonstrate that the Manuka honey maintained its antibacterial activity in the presence of catalase whilst the pasture honey's activity was reduced. The damaging effects of hydrogen peroxide are limited by the ability of the honey to isolate and inactivate the free iron, which promotes the release of free radicals by hydrogen peroxide. The anti – oxidant content of honey mops up any free radicals, which are produced by the peroxide, reducing the inflammatory effect (Molan, 1999).

In vitro studies have shown the susceptibility of *S.aureus* to a number of honeys: Maeda and colleagues (2008) found undiluted honey from bee keepers in Northern Ireland to be active against Community Acquired MRSA (CA-MRSA); George and Cutting (2007) assayed Medihoney, a commercial medical grade Manuka honey, against control and clinical strains of *S. aureus, P. aeruginosa, E. faecalis* (vancomycin sensitive and resistant) and Extended Spectrum β-lactamase (ESBL) producing strains of *E. coli* and *K. pneumoniae*. All strains were susceptible at honey concentrations of 4-8% with the exception of *P. aeruginosa* which was inhibited at 12-14%. Lusby and colleagues (2005) looked at the activity of Manuka and other floral honeys against a large number of organisms including *Candida albicans, Serratia marscesans* and a large number of *Enterobactericae.* Their study included single isolates of each organism species and found all to be susceptible except *Candida albicans* and *Serratia marscesans*. However the majority of published material has been produced from Prof Rose Cooper's group at Cardiff Metropolitan University. Cooper, Molan and Harding (1999b) described the antibacterial activity of honey against strains of *S. aureus* isolated from infected wounds. A total of 58 clinical isolates were shown to have an average Minimum Inhibitory Concentration (MIC) of 2.88% with Manuka honey and 3.79% with pasture honey. In 2002 a similar study was performed by Cooper, Hallas and Molan which showed Manuka and pasture honeys were active against 17 isolates of *P. aeruginosa*; at a concentration of <10%. Cooper, Molan and Harding (2002) compared the activity of Manuka honey to an artificial honey against 18 isolates of *S. aureus,* 7 vancomycin sensitive Enterococci (VSE) and 20

vancomycin resistant strains of Enterococci (VRE). The Manuka inhibited VSE at concentrations of 4.5-5%, VRE at 3.8-5% and *S. aureus* at 3%. Pasture honey had equal activity against *S. aureus* and inhibited VSE at 9.66% and VRE at 5.6-9%. The artificial honey which comprised of glucose, fructose, maltose and sucrose dissolved in sterile distilled water, inhibited all organisms at 27 - >30% demonstrating honey has non-sugar antimicrobial properties.

A more recent study by Jenkins, Burton and Cooper (2011) looked at mechanisms of organism killing and identified that Manuka honey interrupts cell division of MRSA. This was visualised using electron microscopy on Manuka treated MRSA revealing enlarged septate cells that had failed to divide with few lysed cells or debris. Assays of murein hydrolase, an autolytic enzyme encoded by the *atl* gene were shown to be inhibited by Manuka honey and implicated in the cells failure to divide (Jenkins *et al.*, 2011).

1.7.1.2 The search for active fractions

As a health food product honey has under gone extensive chemical analysis (Pyrzynska and Biesaga, 2009). The phenolic and flavenoid compounds are natural anti-oxidants and vary between honeys from different floral sources. This has made the floral source of honey of greater interest as it influences both taste and health benefits (Anklam, 1998, Bogdanov *et al.*, 2004, Tsiapara *et al.*, 2009). However, it is the anti-bacterial properties of honeys that have given rise to many studies in order to determine which compounds confer non-peroxide activity and which floral sources the antimicrobial honeys are derived from (Conti *et al.*, 2007, Biesaga and Pyrzynska, 2009, Küçük *et al.*, 2007). Antibiotic

residues have long been known to exist in some honeys (Oka *et al.*, 1987) and may influence the antimicrobial properties in some products (Saridaki-Papakonstadinou, *et al.*, 2006). Concerns over pesticide residue and the need to be able to identify these compounds have driven the need for effective analysis of all honey products (Rezić *et al.*, 2005). Various chromatographical techniques including thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been used to identify compounds such as syringic acid, methylglyoxal (MGO) and phenylactic acid among others (Weston *et al.*, 2000, Gomez –Caravacca *et al.*, 2006). Mavric *et al.* (2008) identified MGO as the dominant antimicrobial component and stated that MGO is directly responsible for the antimicrobial activity of Manuka honey. However, the results of the 2011 study by Jenkins and colleagues compared artificial honey + MGO with Manuka and suggested that non-sugar, non -peroxide antimicrobial activity is not entirely down to MGO. Another study by this group demonstrated that Manuka down regulates the expression of the *uspA* gene which is produced in stress conditions and increases cell endurance (Jenkins *et al.*, 2010). Whilst other studies concur that MGO has a significant effect, other active compounds have also been identified (Stephens *et al.*, 2010, Fearnley *et al.*, 2011). More recently Nuclear Magnetic Resonance has been used with success to quantify MGO in honey (Donarski *et al.*, 2010). Beretta *et al.* (2009) used both quantitative NMR and Diffusion Ordered Spectroscopy NMR (DOSY NMR) to further identify the chemical composition of different floral honeys. This study aims to employ DOSY NMR to identify and

quantify the antimicrobial fraction in a number of Syrup and honey products.

1.7.1.3 Stimulation of the immune system

In vitro studies by Tonks and Cooper (2001) showed that both Manuka and New Zealand Pasture honey stimulated the production of tumour necrosis factor alpha (TNF-α) in resting human monocytic cell lines (Mono Mac6, MM6). A reduction in the production of reactive oxygen intermediates was induced by negative feedback from the hydrogen peroxide produced by diluting the honey in water. TNF-α is produced by activated macrophages which have an essential role in the healing process (Sibbald *et al.*, 2003, Tonks *et al.*, 2003). Tonks and colleagues (2003) observed the accumulation of unstimulated macrophages at the margins of chronically infected wounds and went on to look at cytokine stimulation by Manuka, New Zealand Pasture and Jellybush honey compared to an artificial honey using MM6 cells and human peripheral blood monocytes. The floral honeys, particularly the Jellybush honey, stimulated TNF-α production, which in turn was observed to have a positive influence on the levels of interleukin 6 (IL-6), IL-1, IL-1β and transforming growth factor β (TGF-β) (Tonks *et al.* 2003). Tonks and colleagues (2007) have since identified that monocyte activity required for, however literature searches have not offered any suggestions as to what this component might be. Honey activation of these cytokines and cells suggests a possible reason for how some honeys may accelerate wound healing.

1.7.2 Medicinal uses of honey

The increasing popularity of honey, particularly Manuka honey has made it a potential candidate as a treatment for numerous conditions.

1.7.2.1 Stomach ulcers

An *in vitro* study showed that clinical strains of *Helicobacter pylori* are susceptible to Manuka honey (Al Somal *et al.*, 1994). However, McGovern and colleagues (1999) had concluded that although Manuka honey might be effective against dyspepsia there was no evidence to suggest it could eradicate *H. pylori* from the gastrointestinal tract of affected patients.

1.7.2.2 Tear deficiency

In 2006, Albietz and Lenton used antibacterial honey as a topical treatment for tear deficiency conditions and reported after 3 months usage there was no statistically different numbers of organisms than in non tear deficient groups. They concluded that their study warranted further work. Similar findings were reported by Cernak and colleagues (2012) when comparing prophylactic use of honeydew honey and fluoroquinolones during the peri-operative period of eye surgery. They concluded that honey eye drops were equally as effective as fluoroquinolones and may be of value for prophylaxis but that further work was required to characterise ocular penetration. However, Ranjan *et al.* (2002) reported honey ineffective for rhinoconjuctivitis.

1.7.2.3 Skin conditions

Skin conditions such as psoriasis, seborrheic dermatitis, dandruff and fungal infections have been reported to improve with the use of honey, (Al-Waili, 2001, 2003, 2004) but these were non-randomised trials. The

lack of clinical trials has not prevented a proliferation of honey skin products appearing on the market.

The above conditions demonstrate the current interest in exploring the medicinal properties of honey but the main body of evidence for its role in healthcare is as an antimicrobial agent with biological healing properties in wound care (Ndayisaba *et al.*, 1993, Molan 1999, 2001, 2006, Al-Waili and Saloom, 1999, Subrahmanyan *et al.*, 2001, Schumacher, 2004, Bangroo *et al.*, 2005, Gethin and Cowman, 2005; Simon *et al,.* 2006, Cooper, 2008).

1.7.3 Current evidence for the use of honey in wound treatment

1.7.3.1 Burns

Throughout the 1990s Subrahmanyam documented and reported his use of honey in a series of random controlled trials, on burns victims in India (Subrahmanyan 1991, 1993, 1994, 1996a, 1996b, 1998, 1999, Subrahmanyan *et al.*, 2001) In 1991, the authors data showed that the healing time of 104 patients with superficial burns using honey dressings was superior to that of silver sulphadiazine dressings. The honey dressing was found to promote a faster wound healing time and a reduction in the incidence of infection and with patients reporting a reduction in pain (Subrahmanyam, 1991). Adesunkanmi and Oyelami (1994) and Baghel and colleagues (2009), also found honey to be equally as effective as silver sulphadiazine supporting the previous observations of Subrahmanyam (1998).

Later studies by other groups also observed success of honey dressings on partial thickness burns (Malik *et al.*, 2010; Gupta *et al.*, 2011). Further work by Subrahmanyam in 1994 and 1996 on a total of 164 patients

showed honey dressings superior to dressings made of other natural products (Subrahmanyam 1994, 1996a). He also reported in 1996 that adding antioxidants and vitamins C and E produced even better results (Subrahmanyam, 1996b). This work continued through to 2001 (Subrahmanyam, 1998, 1999, Subrahmanyam *et al.*, 2001) with only excision and skin grafting producing superior results. More recent studies confirmed the apparent superiority of honey when compared to silver dressings on superficial burns, with Malik *et al.* (2010) reporting a 25% reduction in healing time with honey (Bangroo *et al.*, 2005).

1.7.3.2 Post surgical wounds

The use of honey in Thailand in the treatment of post caesarean wound dehiscence where the wounds splits along the suture line, was reported as successful when compared to the use of saline or silver (Phupradit and Saropala, 1992). In a 50 patient study, Al-Waili and Saloom in United Arab Emirates (1999) found that honey accelerated healing and reduced scarring. Vardi and colleagues (1998) used honey on neonates in Israel who had developed post surgical wounds. Whilst success has been found using honey post operatively there is no evidence to suggest from literature searches that it has gained popularity, or been adopted for post surgery wounds in Western culture

1.7.3.3 Chronic non-healing wounds

One area where honey usage has gained momentum is in its use on chronic wounds that fail to respond to more conventional therapies. As far back as 1988, the successful use of honey was reported on 58/59 patients with non-healing ulcers (Efem, 1988). Within one week there

was a reduction in wound discharge and smell, the debridement properties of the honey were noted along with an increase in epithelialisation (Efem, 1988). A Nigerian group, (Oluwatosin *et al.*, 2000) compared the use of honey against phenytoin on ulcers and found that although the phenytoin proved better long term, honey produced better results in the short term.

Throughout the 1990s and into the 21st Century, honey has been successfully employed around the world for complicated non-healing wounds. High mortality is often associated with Fournier's gangrene, a complicated polymicrobial infection, usually of the male genitalia which includes both aerobic and anaerobic bacteria, often with underlying health issues such as diabetes or alcoholism (Thwaini *et al.*, 2006). Success has been reported where honey has been used alongside antibiotics as an alternative to surgical debridement with improvement to painful swelling, malodorous discharge and improved prognosis with reduced fatalities (Hejase *et al.*, 1996, Efem *et al.*, 1993, Tahmaz *et al.*, 2006).

Internationally honey has in many cases throughout 1990s, been found to be more effective than conventional wound treatments in chronic complicated wounds, all reporting improved healing times and in many cases reduced hospital stays (Ndayisaba *et al.*, 1993, Farouk *et al.*, 1998) and more recently its use has started to spread to the West.

In the UK, Schumacher compared honey with split skin grafting on 6 patients with chronic ulcers and found honey to be a comparable option (Schumacher, 2004).

Dunford and Hanano successfully used honey on 40 patients whose ulcers had failed to respond to compression therapy and found that patients experienced less pain, a reduction in odour and accelerated healing (Dunford and Hanno, 2004). Similar reports were made regarding wounds of varied aetiology in other European studies (Vandeputte and Van Waeyenberge, 2007, Gethin and Cowman, 2005).

1.7.3.4 Cancer care

Cancer patients present unique challenges to wound therapy regimes. The use of cytotoxic drugs in chemotherapy undermines the immune system and compromises the ability of the body to heal (Bardy *et al.*, 2008). Radiation induced mucositis is almost an inevitable side effect in patients with head and neck cancers causing painful oral mouth ulcers. Biswal and colleagues (2003) and Motallebnejad and colleagues (2008) both reported the benefits using undiluted honey to coat the oro-pharyngeal mucosa before and after radiotherapy.

The specific problems associated with cancerous fungating wounds are described by Wayne Naylor in a world wide wounds article (2002) when he was a wound management research nurse at The Royal Marsden Hospital (Naylor, 2002). The article explains the difficulties in wound management in cases where malodour, pain, exudate and bleeding can have a devastating effect on the patient's psychological welfare which is exacerbated by the fact that total resolution is unlikely unless the malignancy is eradicated. The Royal Marsden Hospital Handbook of Wound Management in Cancer Care (Naylor *et al.*, 2008) includes the use of honey dressings for malodorous offensive wounds. Simon and

colleagues (2006) in Germany have also reported the value of honey for wound healing in immune- compromised paediatric patients.

There is a significant amount of work already performed which has looked at *in vivo* applications of honey products but few high quality clinical trials (Bardy *et al.*, 2008). Clinical trials falls outside of the scope of the current study which aims to further characterise the antimicrobial properties of honey against clinical bacterial isolates directly isolated from patients.

1.8 The aims of this study

a. Compare the antimicrobial activity by agar diffusion and micro-broth MIC and MBC assay of 2 Syrups, 7 Manuka honeys of varying UMF ratings and 7 non-Manuka honeys from different floral sources against 10 clinical isolates each of: MSSA; MRSA; VSE; VRE; β lactamase negative strains of *E. coli,* β lactamase positive strains of *E. coli;* meropenem, ciprofloxacin sensitive strains of *P. aeruginosa;* and 10 strains of *Pseudomonas aeruginosa* with reduced sensitivity.
b. Determine the effect of bioload on the antimicrobial efficacy of antimicrobial honey.
c. Study the effect of medical grade honey on biofilms on inorganic material.
d. Characterise the activity of medical grade Manuka honey using *in vitro* wound modelling containing mixed populations of bacteria isolated from clinical wounds.
e. Identify the compounds conferring antimicrobial activity in the active honey products using chromatography and DOSY NMR.

CHAPTER 2

Methods and Materials

2.1 Syrup and honey products selected for use in this study.

The products chosen for this study comprised a selection of Manuka products marketed with different UMF ratings. Manuka honey is produced by bees that have fed on the nectar of *Leptospermum scoparium.* Manuka honey is marketed with an antimicrobial potency rating known as the Unique Manuka Factor or UMF. The UMF is based on the percentage of phenol required to inhibit *Staphylococcus aureus.* Other honeys tested were lavender, eucalyptus and acacia honeys which are reputed to have antimicrobial potential (Lusby *et al.*, 2005; Blasa *et al.*, 2006). Blossom honey and a generic basic supermarket own brand honey were included as honeys of non-specific or unstated floral origin. Two Syrups were included representing non-honey, high sugar controls.

Table 2.1 Syrup and honey products selected for use in this study.

Product Designation	Product
A	Tate and Lyle Golden Syrup From Sainsbury's
B	Tate and Lyle Black Treacle From Sainsbury's
C	Meloderm Active Manuka Honey 16+ ∂ irradiated. From Natures Nectar Ltd
D	Comvita Manuka Honey 20+ From 'The Honey Doctor'
E	Comvita Manuka Care 18+ sterilised From 'The Honey Doctor'
F	Haddrells Manuka honey 16+ From 'The Honey Doctor'
G	Haddrells Manuka Honey 13+ From 'The Honey Doctor'
H	The Honey Collection West Coast Organic Active Manuka Honey 12+. From 'The Honey Doctor'
I	Haddrells Manuka Honey 5+. From 'The Honey Doctor'
J	Spanish Lavender Honey From Sainsbury's *Taste the Difference* Range
K	Rowse Australian Eucalyptus Organic Honey From Sainsbury's
L	Rowse Pure Acacia Honey From Sainsbury's
M	Rowse Clear Blossom Honey From Sainsbury's
N	Sainsbury's Clear Basics Honey From Sainsbury's

2.2 The isolates used in this study: Selection and maintenance.

The Microbiology Department at The Royal Marsden Hospital has a retention and frozen archive policy for isolates of clinical significance. The isolates routinely saved include Meticillin Resistant *Staphylococcus aureus,* vancomycin resistant Enterococci, multi-drug resistant *Enterobacteriacae* and all organisms isolated from blood cultures.

2.2.1 Selection of clinical isolates.

The organisms selected for this study were selected either retrospectively from the frozen store or were isolated from samples being processed at the time this study commenced. Frozen isolates were archived in nutrient broth with 10% (*v:v*) glycerol and stored at -80° C.

Isolates originated from different patients across several wards on both Royal Marsden Hospital (RMH) locations. All samples were originally collected for diagnostic purposes and none were taken for the sole benefit of this study, thereby ensuring the study had no impact upon the patients. The organisms were selected for inclusion in this work on the basis that they were diagnostically confirmed as causative agents of nosocomial infections in patients or alternatively, they were prevalent in the contamination and increase of the bioburden in patient wounds (personal communication Dr Unell Riley / Infection Control Team).

The clinical isolates and correspondingly representative selection of control strains used in this study are listed in Tables 2.2 and 2.3 below.

Table 2.2 Clinical isolates selected for use in this study.

Organism	Number of Isolates	Comment
Meticillin Sensitive *Staphylococcus aureus* (MSSA)	25	
Meticillin Resistant *Staphylococcus aureus* (MRSA)	25	
Multi-drug resistant *Escherichia coli*	25	Initially the 25 *E. coli* isolates selected were all multi-drug resistant which was defined as organisms resistant to at least two different classes of antimicrobials
Fully sensitive *Escherichia coli*	25	Further into the study, the fully sensitive *E. coli* isolates were selected for study defined as sensitive to all first and second line antibiotics used by The Royal Marsden in 2006.
Pseudomonas aeruginosa	25	In the earlier studies the *P. aeruginosa* isolates were not selected on the basis of their antimicrobial sensitivities
Multi-drug resistant *Pseudomonas aeruginosa*	10	Further into the study isolates were separated into those sensitive to the Royal Marsden *Pseudomonas* panel of antimicrobials and those considered multi-drug resistant if there was a reduced susceptibility to Meropenem and/or Ciprofloxacin.
Vancomycin Sensitive *Enterococcus* species (VSE)	25	
Vancomycin Resistant *Enterococcus* species (VRE)	25	

Table 2.3 Control strains selected for use in this study.

NCTC Identification	Organism
NCTC 6571	Meticillin Sensitive *Staphylococcus aureus* (MSSA)
NCTC 12493	MEC A positive Meticillin Resistant *Staphylococcus aureus* (MRSA)
NCTC 12241	Standard *Escherichia coli*
NCTC 11560	TEM-1 β lactamase producing *Escherichia coli*
NCTC 121903	*Pseudomonas aeruginosa*
NCTC 12679	Vancomycin Sensitive *Enterococcus species* (VSE)
NCTC 13379	Low level Van B mediated Vancomycin Resistant *Enterococcus species* (VRE)

2.2.2 Antimicrobial profiles of isolates.

All sensitivity testing was performed and interpreted according to the British Society for Antimicrobial Chemotherapy (BSAC) Methods for Antimicrobial Testing (Version 6.1 February 2007). Briefly, each organism was tested against the antimicrobial panels used by the Royal Marsden in 2007 using the disc diffusion method recommended by BSAC. The zones of inhibition were measured using a ruler and interpreted according to the BSAC guidelines (the latest version and all archived versions are available on the BSAC website, www.BSAC.org.uk).

2.3 Determination of antimicrobial activity by agar diffusion.

Agar diffusion is a method to quantify the activity of an antimicrobial agent against a given organism. When a laboratory uses this method to determine bacterial sensitivity against an antibiotic, manufactured antibiotic discs impregnated with a known concentration of antibiotic are used. Zones of inhibition are measured and compared to BSAC standard zone sizes (BSAC Methods for Antimicrobial Susceptibility Testing Version 6.1, February 2007). Prior to agar diffusion testing a sterility and activity screen were performed described in methods 2.3.1 and 2.3.2 below.

2.3.1 Sterility Screen of Products.

To determine the presence of bacteria in the products, a sterility test was performed. All Syrup/honey products were cultured onto two blood agar plates (COH, Biomerieux, Basingstoke, UK) using a sterile Pasteur pipette giving a drop of approximately 75µl. One plate was incubated at 37°C aerobically and the other incubated anaerobically at 37°C. Cultures were examined for growth at 24 and 48 hours.

2.3.2 Activity screen of Products.

To determine the potential for antimicrobial activity a non-quantitative activity screen was performed.

To soften the product for pipetting, each Syrup/honey product was stood in the original sealed container or sealed sterile universal in a 35 ° C water bath for 15 minutes. Each isolate was seeded onto a Blood Agar plate (COH blood agar, Biomerieux, Basingstoke, England) using a sterile swab (Universal Hospital Supplies, London, England). A 3 ml

sterile Pasteur pipette (Griffith and Neilson Ltd., Sussex, England) was used to dispense 1 drop of product into the middle of each seeded plate. After overnight aerobic incubation at 37°C the cultures were examined for any visible inhibition of growth by product.

2.3.3 Comparison of horse serum and distilled water as product diluent.

As a variety of product concentrations were to be investigated a suitable diluent was required to ensure that activity was not affected by the diluent. Two different diluents were compared, sterile distilled water, selected due to its minimal cost and convenience and horse serum as this was the available product most resembling human serum, the diluent *in vivo*.

A Manuka honey sample with a UMF antimicrobial rating was diluted 50% and 25% (*w:v*) with sterile distilled water and horse serum (E&O Laboratories, Bonnybridge, Scotland) into sterile universals. Suspensions of *S. aureus* (NCTC 6561), *E. coli* (NCTC12241) and *P. aeruginosa* (NCTC 121903) were prepared using 'Preparation of inoculum Method 1' (Method 2.3.6 below) using ISO Sensitest broth (E&O Laboratories, Bonnybridge, Scotland) and seeded onto welled plates. 1 drop per dilution per diluent was inoculated into 1x3 wells and incubated aerobically overnight at 37°C. The mean of the 3 zone sizes was used to compare results obtained from the different diluents. This assay gave the first indication of what zone sizes may be expected and therefore how many wells might be bored into each plate.

2.3.4 Preparation of product dilutions for antimicrobial assay.

To investigate the antimicrobial activity of the Syrups and honeys at different concentrations, the products required diluting. All products were diluted weight to volume and made fresh on day of use.

Each product was decanted into a sterile universal container and weighed. The net weight was recorded to 2 decimal points. An equal amount weight: volume of sterile diluent was added. The mixture was stirred and vortexed until homogenous and kept in refrigerator until used. All were brought to room temperature and vortexed immediately prior to use.

2.3.5 Preparation of Iso Sensitivity plates for agar diffusion tests.

In this study, it was decided that products would not be impregnated into discs as the means to ensure standardisation were unavailable. As an alternative, 6mm wells were bored into agar plates into which a fixed volume of product could be introduced.

Using sterile disposable pipette tips 6 x 6mm holes were bored into the required amount of iso sensitivity test agar plates (ISTA, Biomerieux, Basingstoke, England). A hypodermic needle was used to remove the agar plugs. Plates were refrigerated until required.

2.3.6 Preparation of inocula method 1.

The inoculum for sensitivity testing should provide a semi confluent growth after overnight incubation.

This method was used for the activity screen and agar diffusion method 1.

An overnight culture of each selected isolate was taken and emulsified into sterile distilled water to a McFarland density of 0.5 ($1.5x10^8$ cfu/ml,

Prolab, Cheshire, England) and vortexed for 10 seconds. Using an Eppendorf pipette (Fisher Scientific, Leicestershire, England) 300 µl of each *S. aureus* suspension were inoculated into 3 ml peptone water broths (E&O Laboratories, Bonnybridge Scotland). This was repeated, using 30 µl for the Enterococci, *E. coli* and *P. aeruginosa* suspensions. All were vortexed for 10 seconds immediately prior to use.

2.3.7 Agar diffusion method 1.

The primary aim of this method was to investigate the activity of the 14 products against the 25 wild strains each of MRSA, MSSA, VSE, VRE, *P.aeruginosa* and *E.coli*. The secondary aim was to compare the variation of susceptibility between each organism type and to observe if there were any notable differences between each of the isolates within each species.

The 25 isolates each of MSSA, MRSA, VSE, VRE, multi-drug resistant *Escherichia coli* and *Pseudomonas aeruginosa* were taken and prepared as per method 2.3.6. The 14 Syrup/honey products were prepared and diluted in sterile distilled water as described previously in method 2.3.4, 'Preparation of Product Dilutions'. For each product against each isolate experiment, a 1 ½ welled ISTA plate was inoculated. Before the acquisition of a positive displacement pipette, undiluted product was dispensed using a sterile 3 ml Pasteur pipette. One drop of a non viscous solution such as water from the pasteur pipette was found to contain a 50 µl volume, but the cohesion properties of honey resulted in a larger volume being contained within each drop. A visual comparison between the well volume occupied by one drop of honey using as Pasteur pipette and 50 µl, 75 µl and 100 µl volumes of a 50% solution

dispensed with an eppendorf pipette, indicated that the Pasteur drop contained approximately 75 µl of diluted honey. This was repeated with the 50% and 25% dilutions using a 75µl drop from an Eppendorf pipette. All plates were incubated for 18-24 hours aerobically at 37°C. The zones were measured and the mean of the 3 zones used to compare each product to each isolate using Analysis of variance (ANOVA) and Tukeys's honest significance test (HSD) to identify homogenous subsets of antimicrobial activity. SPSS version 16 Software was used to analyse the resulting data. See section 2.9, 'Statistical analyses'.

2.3.8 Preparation of Inocula Method 2.

An overnight culture of each selected isolate was taken and emulsified into sterile distilled water to a McFarland density of 0.5 (Biomerieux, Basingstoke, England) and vortexed for 10 seconds. Using an Eppendorf pipette (Fisher Scientific, Leicestershire, England) 100 µl of the *S. aureus,* Enterococci, *E. coli* and *P. aeruginosa* suspensions was inoculated into the 10 ml of ISO sensitest broth (E&O Laboratories, Bonnybridge Scotland). The volume was changed to allow suspensions to be used for both agar diffusion and micro-broth MIC testing. All were vortexed for 10 seconds prior to use.

2.3.9 Agar diffusion method 2.

This method was performed as per agar diffusion method 1 with the modification of inoculating a 75 µl volume of each product into each well using a positive density pipette.

For the purposes of statistical analysis, it was decided that further testing of each product by agar diffusion and the MIC tests would be performed on 10 isolates of each species and NCTC control strains in triplicate.

The 10 isolates each of MSSA, MRSA, VSE, VRE, multi-drug resistant *E. coli* and *P. aeruginosa* were selected, along with NCTC control strains described in Section 2.2.1 and prepared as per Preparation of Inoculum Method 2 (Section 2.3.8). The 14 Syrup/honey products were prepared and diluted as described in 'Preparation of Product Dilutions' (Section 2.3.4) using ISO sensitest broth. Welled ISTA plates inoculated with each of the isolates were tested in triplicate against 100µl of each product undiluted and diluted at 50% and 25% plates. All plates were incubated for 18-24 hours aerobically at 37°C. The zones were measured and the mean of the 3 zones used to compare each product to each isolate using Analysis of Variance (ANOVA) and Tukeys's honest significance test (HSD) to identify homogenous subsets of antimicrobial activity in SPSS version 16.

2.4 Determination of Minimum Inhibitory Concentration.

Agar diffusion methods are useful as an indicator of comparable antimicrobial activity. However the results may be affected by factors such as viscosity which may influence the ability and speed of the product to diffuse into agar and the growth of organisms on a solid surface (Lo-Ten-Fo *et al.*, 2007). Tests to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) provide a measurement of antimicrobial agents against planktonic bacterial cells. The method selected was a modification of a microdilution method described by Andrews (2001).

Gamma irradiated round bottomed 96 well microtitre plates (Bibby Sterilin, Staffordshire, England) were used in the method to measure the MIC of each isolate for each Syrup/honey product.

One microtitre plate was sufficient for the testing of 10 isolates, the NCTC control, with three microtitre plates required to test in triplicate. Each product was double diluted across the plate in 100 µl volumes using a Multichannel Pipette (Scorex, obtained from Camlab, Cambridgeshire).

To each well 100 µl of isolate suspension with an organism concentration of 1.5 x 10^7 was added, ensuring thorough mixing. The final range was 50% - 1.56% product. The final column was the growth control and the final row was the honey control.

Each isolate suspension was inoculated at time of use onto a blood agar purity plate using a 1 microlitre loop. This provided an approximate organism count for each isolate inoculated. For example, 1-10 colonies = 10^4 cfu/ml; 10-100 colonies = 10^5 cfu/ml; 100-1000 colonies = 10^6 cfu/ml.

All plates were incubated overnight aerobically for 18-24 hours at 37°C. Organism growth was observed as a button of cells in the bottom the well, absence of a button indicated inhibition of growth.

Results were analysed using Analysis of variance (ANOVA) and Tukeys's honest significance test (HSD) to identify homogenous subsets of antimicrobial activity in SPSS version 16.

2.5 Determination of Minimum Bactericidal Concentration.

To determine minimum bactericidal concentration (MBC), after the MIC had been recorded, each well in each microtitre plate was sub-cultured onto blood agar using a microlitre loop and incubated overnight aerobically at 37°C. The well containing the lowest concentration of

product giving no growth of sample isolate was recorded as the MBC. This was carried out in triplicate.

2.6 Determination of effect of bioload on honey activity.

When performing efficacy assays *in vitro* it is custom and practice to use a standardised inoculum such as that described by Andrews (2001), the current versions of which can be viewed on the BSAC website. Whilst this is effective for antimicrobial assays where breakpoints have been defined or for testing products like for like against bacterial strains, it's limitations of reliability are exceeded when the bioload is higher than 10^6 or 10^7 cfu/ml. The methodologies presented here aimed to determine the maximum bioload that the medical grade Manuka honeys exhibited an antimicrobial effect.

2.6.1 Bioload assay 1.

Assay 1 describes the effect of bioload of NCTC strains on Manuka activity. The isolates used were the control strains described in Section 2.2.1.

Each isolate was defrosted and sub-cultured onto blood agar. The cultures were further sub-cultured on two consecutive days prior to use. Initially, bacterial dilutions of each strain were prepared in Iso sensitest broth (E&O) using the available McFarland's standards 0.5, 1, 2, 3, 4 and 5 giving organism counts in the region of approximately $1.5x10^8$ to $1.5x\ 10^9$ cfu/ml. A further suspension was prepared of a much heavier suspension of total opacity, viable counts of these suspensions showed the organism count to be approximately $1x\ 10^{12}$ cfu /ml.

The two commercially available medical grade Manuka honey products used in this study were Meloderm Manuka Honey UMF16+ (gamma

irradiated, Natures Nectar Ltd, Ash, Surrey) and Manuka Care 18+ (sterilised, The Honey Doctor, Devon).

Using 100 µl aliquots of each McFarland 1-5 suspension was prepared in Iso sensitest broth (E&O) and inoculated into 2 wells of a flat welled microtitre plate to which 100 µl of a honey dilution was added. To test the effect of the increase of the bioload/honey ratio, two further wells were inoculated with 150 µl and 175 µl of McFarland 5 suspension with honey added to make up to a total volume of 200 µl. Growth control wells containing organism without honey for each bacterial suspension and honey control wells product plus equal volume of sterile Iso sensitest broth were also included. The microtitre trays were incubated aerobically overnight at 37°C. Wells showing turbidity were recorded as positive for organism growth. No turbidity was recorded as growth inhibition. Each well was subcultured onto blood agar to determine inhibitory or bactericidal activity.

2.6.2 Assay to determine effect of honey volume on antimicrobial efficacy.

A suspension of fresh culture was inoculated into Iso sensitest broth until total opacity was achieved. Viable cell counts showed this suspension to contain viable organisms in the order of 10^{14} cfu /ml.

Each 100 µl organism suspension was inoculated into a row on a microtitre plate. To each row 10 fold decreasing volumes of honey from 100µl down to 10µl were added to each well. Plates were incubated and growth inhibition and cell death were measured as above.

All assays were performed in triplicate on successive days.

2.6.3 Bioload Assay 2.

Assay 2 was designed to determine the effect of bioload when working with isolates from clinical wound populations and to ascertain the bioload limits upon which medical grade Manuka honey could exert an anti-bacterial effect.

The selection of clinical wound populations is described in section 2.7.5.1.

Assays were performed in sterile U welled, 96 well microtitre plates.

Fresh overnight cultures of all isolates from all of the 10 wounds and the NCTC controls were prepared and inoculated into a Brain Heart Infusion Agar (BHIA) broth and a blood agar plate and incubated overnight aerobically at 37°C.

Medical grade Meloderm Active Manuka UMF* 16+ and Comvita Manuka Care UMF 18+ were diluted to 75% (w: v) in BHIA broth (Biomerieux, Basingstoke England). This medium was selected in preference to Iso sensitest broth to provide nutrients more representative of a biological setting.

A 100µl aliquot of undiluted honey was inoculated into the first 4 wells of the microtitre plate. 100µl of the 75% honey was inoculated into the following 8 wells.

Into the first well a sweep of 1 cm of organism growth equating with approximately 4-8 colonies were picked off with a sterile bacterial loop to achieve an organism concentration of >4 x 10^8 cfu/ml within the well and inoculated into the neat honey. The loop was rotated back and forth repeatedly to disperse the culture throughout the honey. Into the next

wells, 3 colonies, then 2, then 1 colony were inoculated using the same technique.

The next 4 wells containing 75% honey were inoculated as per the first 4 wells.

The next 4 wells were inoculated using 100µl of the BHIA broth undiluted, then at dilutions of 1:10, 1:100 and 1:1000.

This was performed for each isolate in triplicate with each of the medical grade Manuka products (Table 2.4).

Table 2.4 A summary of organism bioload and honey within each well of a microtitre plate row for bioload assay 2 (method 2.6.2). Figures are based on a 24 hour colony containing 10^8 organisms and an overnight broth containing 10^8 organisms per ml

	1	2	3	4	5		7	8	9	10	11	12
org load	>4.0E +08	3.00E +08	2.00E +08	1.00E +08	>4.00E +08	3.00E +08	2.00E +08	1.00E +08	5.00E +06	5.00E +05	5.00E +04	5.00E +03
% Honey	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5

The plates were incubated for 18-24 hours aerobically at 37°C. All wells were cultured onto blood agar and incubated overnight aerobically at 37°C to determine organism survival.

Where anaerobic organisms were isolated from clinical wounds it was not possible to perform bioload assays or MICs as there was no anaerobic workstation facility.

2.7 Biofilm growth.

There are several reported methodologies for growing biofilms *in vitro* (Alandejani *et al.*, 2009).

The aim of these methods was to ascertain the efficacy of honey products against organism within a biofilm. In order to do this it was necessary to develop a method which would enable the presence of a biofilm to be identified and a level of quantification to determine whether the honey products were able to eliminate biofilms or capable of significantly reducing the population of organisms within the biofilm structure.

2.7.1 Growing biofilms in microtitre plates

In order to conserve expensive medical grade Manuka honey various methods using 96 well microtitre plates were investigated.

2.7.1.1 Growing biofilms of NCTC strains in sterile flat well microtitre plates.

A sterile 96 flat well microtitre plate was taken and a 0.5 McFarland suspension of the NCTC controls described in 2.2.1 were prepared in BHIA broth (Biomerieux Basingstoke UK). 200 µl was of each suspension was added to one column of the plate and left to incubate at

37°C aerobically for 24 hours. A second and third microtitre plate was similarly prepared and incubated for 48 and 72 hour incubations.

After incubation, the plates were inverted to remove the medium and planktonic cells. The plate was placed on a hot plate to heat fix for 30 minutes to prevent any of the biofilm being washed off during the staining procedure. Using an eppendorf pipette, 200 µl of crystal violet was added to each well and left to stand for 5 minutes after which the plate was inverted to tip out the stain and gently rinsed in water to remove any excess. The colour density of the stain removed from the biofilm organisms was observed and optical density (OD) recorded using a plate reader at 630nm (ELX800 Biokit, Kent England).

2.7.1.2 Growing biofilms of NCTC strains in sterile fibrinogen coated flat well microtitre plates.

A sterile 96 flat well microtitre plate was prepared in the following way: Into the first 4 rows, 220 µl of porcine fibrinogen (10µg ml^{-1}) dissolved in phosphate buffer solution (PBS pH 7.0 Sigma Aldrich) was added to coat the well, 25 µl was added to the next 4 rows to coat the bottom of the well. The plate was then incubated aerobically at 37°C for 1 hour. The plate was inverted to remove the excess, rinsed in sterile saline and dried in the incubator.

Each plate was then inoculated with NCTC controls strains and incubated as described in 2.9.2.2. After incubation, the plates were inverted to remove the broth. Rows A, B, E and F were rinsed in PBS. Rows C, D, G and H were rinsed in sterile physiological saline to determine if rinsing medium affected the final results.

The plates were stained, rinsed and results observed as previously described.

2.7.1.3 Determination of optimum method for growing and quantification of biofilms of clinical strains in sterile flat well fibrinogen coated microtitre plates.

Organism suspensions of the clinical isolates and controls, as described in Section 2.2.1 were prepared to a 0.5 Mcfarland density in BHIA.

Aliquots of 200 µl of the 10 isolates of each organism cohort were inoculated across one row of a microtitre plate. The final well of each well was a negative broth control. The plates were aerobically incubated at 37°C for 48 and 72 hours. This method was carried out in triplicate.

After the allocated incubation time, the first of the three plates was inverted to remove organism broth and the planktonic cells. The second plate was inverted and gently refilled with 200 µl of sterile saline then inverted again in order to wash of excess medium and cells not bound in the biofilm. The third plate was filled with 200 µl of saline and allowed to stand for 5 minutes before inverting to empty. The plates were placed on the hotplate for 30 minutes to dry and fix. Each well in each plate was then stained with 200µl of crystal violet allowed to stand for 3 minutes then gently rinsed out with water, any stain remaining in the wells was that taken up by the organisms heat fixed in the wells. When dry, 200 µl of elutant (80:20 Ethanol: Acetone) was added and then left at room temperature for 60 minutes. The plates were then read in a plate reader at a wavelength of 630nm and OD recorded.

2.7.2 Stripping biofilms from microtitre wells with medical grade honey.

Seven fibrinogen-coated sterile flat welled microtitre plates were taken. Five were inoculated as described above in 2.7.1.3 with clinical and control isolates, and incubated aerobically at 37°C for 48-72 hours. One plate was inoculated with sterile BHIA broth and incubated and one was not inoculated with anything. After incubation, each plate was inverted to remove the broth. The plates were then inoculated with either honey to test biofilm stripping efficacy or broth which served as a negative control (Table 2.5). All plates were incubated overnight at 37°C aerobically. The plates were removed from the incubator and inverted to remove as much honey as possible. They were then filled with sterile saline and allowed to soak for 5 minutes to dissolve and remove the honey.

Table 2.5 Table showing agent added to remove biofilms grown in fibrinogen coated microtitre wells.

Plate No.	Description	Agent added	Volume
1	Biofilm control	Sterile BHIA broth	200 µl
2	Biofilm honey test	Undiluted medical grade Manuka honey with a UMF 16+.	200 µl
3	Biofilm honey test	50% dilution UMF16+ honey *w:v* in BHIA broth	200 µl
4	Biofilm honey test	Undiluted medical grade Manuka honey UMF rating 18+.	200 µl
5	Biofilm honey test	50% dilution UMF18+ honey *w:v* in BHIA broth	200 µl
6	Uninoculated broth control plate	Undiluted and 50% honeys inoculated into 2 rows each	200 µl
7	Uninoculated honey control plate	Undiluted and 50% honeys inoculated into 2 rows each	200 µl

The agents included the medical grade honeys at different concentrations and a BHIA broth control.

The plates were dried and fixed on the hotplate for 30 minutes prior to being stained, eluted and read at 630nm.

2.7.3 Growing biofilms on glass coverslips.

2.7.3.1 Preparation and visualisation of biofilms grown on coverslips in Dulbecco's Modified Eagle's Medium (DMEM).

DMEM was selected as the growth medium as it was thought to provide a medium representative of human tissue fluid.

20mm x20mm coverslips were sterilised by dipping in alcohol. Once the alcohol had evaporated the coverslip was placed inside a sterile 30 ml universal. A 5 ml aliquot of DMEM was added which was sufficient to submerge half the coverslip. A suspension of each test organism described in Section 2.2.1 was prepared to a McFarland density of 0.5 in peptone water. 50 µl of suspension was added to one of the prepared universals. A control was set up using 50 µl of sterile peptone water. The universals were incubated at 37°C for 48 hours. After incubation the colour of the growth medium in each universal was observed and 10µl was cultured onto blood agar which was incubated overnight at 37°C aerobically after which any growth was recorded. Coverslips were removed from the universals, gently rinsed in saline and allowed to air dry. When completely dry they were stained with crystal violet, the excess was rinsed off with gentle pipetting. Staining and rinsing were performed with great care to cause minimal disruption to the any biofilms growing on the slide. The stained biofilms which had successfully grown and adhered to the half submerged coverslip were visible after rinsing on with clear delineation from the un-submerged half.

2.7.3.2 Preparation and visualisation of biofilms grown on coverslips in Brain Heart Infusion Agar broth.

A 30 ml sterile universal container was filled with 10ml of Brain Heart Infusion Agar broth (BHIA) (Biomerieux Basingstoke England). A 20mm x20mm coverslip was placed in the universal. The universal was recapped and incubated aerobically for 48 hours at 37°C, after which, broth showing any turbidity were discarded.

Broths were inoculated with one colony of a fresh culture of the organism to be assayed or, with 100µl of a Robertson's cooked meat broth containing a polymicrobial community (see biofilm assay of wound flora section 2.7.5.1). The broths were incubated for 48 hours at 37°C and were agitated by swirling a total of 6 times throughout the incubation.

After incubation the coverslips were air dried and then heat fixed on a hot plate. Each coverslip was then placed in a 60ml sterile universal for quantification.

2.7.4 Quantification of biofilms grown on coverslips in BHIA broth.

Quantification of the biofilm was achieved by measuring the optical density of the stain eluted from the organisms within the biofilm on the surface of the coverslip. Establishing a method of quantification would provide a means of comparing biofilms before and after treatment with honey.

Each dry coverslip was heat fixed on a hotplate and then submerged in a beaker of crystal violet to stain any organisms fixed to the glass. After 30 seconds the excess stain was rinsed off by submerging gently several times into a large beaker of water. The coverslip was then air dried and placed in a sterile 60ml universal. To elute the stain, 2 ml of 80:20

ethanol:acetone was added which was sufficient to submerge the coverslip. This was left for at least 24 hour to allow for complete elution of stain. An elutant volume of 200 µl was pipetted into a microtitre and read in a plate reader at 630 nm to obtain the optical density

This whole process was later repeated using 1.5 ml of elutant to determine if denser colour gave better differentiation of OD between isolates.

2.7.5 Culture and quantification of biofilms from clinical wound specimens.

2.7.5.1 Selection of clinical wound isolates.

Genuine wound swabs received and processed in a large District General Hospital (the William Harvey hospital, Ashford, Kent) were taken after all clinical and diagnostic investigations were complete and inoculated into Robertson's Cooked Meat (RCM) with BHIA broth overlay (Biomerieux Basingstoke, England). Only details of the swab site and clinical details provided on the request form were recorded. Each swab was given an identity for the purpose of the study (Table 2.6).

Table 2.6 The clinical wound samples obtained from a large district general hospital showing wound site and clinical details.

Sample Identity	Site	Clinical details
Wound 1	Abdominal wound	No details
Wound 2	Axilla wound	Mother axilla - has baby with infected umbilicus
Wound 3	Leg wound	Superficial break Left calf
Wound 4	Knee wound	Scar post knee surgery
Wound 5	Thumb wound	Paronychia
Wound 6	Toe wound	Toe ulcer -bone exposed
Wound 7	Wound swab	unspecified site no details
Wound 8	Sacral wound	No details
Wound 9	Foot wound	No details
Wound 10	Ear swab	Ear infection

These samples provided the actual polymicrobial populations used for biofilm and wound modelling studies.

Each RCM was incubated overnight and subcultured onto Blood agar, Columbia +5% sheep blood agar / chromogenic agar biplate (CNA/CPS) and *S.aureus* Identification Agar (SAID), incubated aerobically overnight at 37°C, neomycin agar incubated 48hrs anaerobically at 37°C and Sabarouds dextrose plus chloramphenicol agar incubated at 37°C for 48

hours. All agars were purchased as pre poured media (Biomerieux Basingstoke, England).

The plates were read after incubation and growth recorded. All isolated organisms were identified using standard methods of identification including a VITEK 2 Compact for the Gram negative organisms. All isolates were frozen at -30°C in BHIA broth + 10% glycerol until required for further study. All RCMs were subcultured every 48hrs to ensure integrity of wound population throughout the duration of the study.

Analysis of the data produced from the above methods showed that microtitre plates were not ideal for growing biofilms across all the organisms in the study. Microtitre plates were inadequate for the honey versus biofilm analysis as the honey was too viscous and sticky and required vigorous washing to remove, which could damage the underlying biofilm.

The methods described below were adopted are the final methodological approach to generate experimental data.

2.7.5.2 Preparation of biofilm medium.

Sterile coverslips were put into sterile 30 ml universals with 10ml of BHIA broth. These were then incubated overnight at 37°C. They were then checked for turbidity which would allow any broths contaminated during preparation to be identified and discarded. They were then refrigerated at 4^0C until use. All broths were used within one month of preparation.

2.7.5.3 Testing wound sample flora for biofilm-forming ability

All wound RCMs and each individual wound isolates were inoculated into the prepared biofilm medium with coverslip described above. Each broth was incubated for 72 hours at 37°C and agitated at least 3 times daily to

prevent settling out of organisms. After incubation, the coverslips were removed and allowed to air dry.

2.7.5.4 Testing the efficacy of honey to remove biofilms formed by clinical wound populations.

Cultures of wound populations grown in Robertson's cooked meats subcultured the previous day and incubated at 37°C overnight were taken and inoculated into 7 BHIA broths containing a 22 x 22mm coverslip. All coverslips broths had been previously incubated to ensure sterility. Seven uninoculated negative control broths were incubated with the test broths. The broths were incubated for 72 hours at 37°C and agitated at least 3 times daily. Broth 1 for each culture was the biofilm control broth. After incubation, the coverslip was removed and air dried. The coverslips from three of the other broths were removed and placed in a universal containing 10ml of 75% medical grade Manuka honey UMF 16+ diluted *w: v* in BHIA broth. The remaining 3 broths with coverslips were placed in the same volume and dilution of medical grade Manuka with a UMF of 18+. The 7 negative controls were all processed in the same way.

The honey broths were incubated for 24 hours aerobically at 37°C.

After incubation the coverslips were slowly and gently submerged in a beaker of sterile saline for 10 seconds three times to remove as much honey as possible. The coverslips were then air dried.

All of the coverslips controls and tests were then heat fixed and submerged in crystal violet to stain. The coverslips were gently rinsed in water and air dried.

Each coverslip was placed in a 60ml sterile universal and 1.5ml of 80:20 ethanol:acetone was added to elute the stain. The lids were replaced and these were left overnight to completely remove all stain from the coverslip.

Using a pipette, 200µl of elutant from each of the coverslips was added to the well of a microtitre tray and put through a plate reader and read at 630nm. All ODs were recorded.

2.8 Wound Modelling.

The aim of this study was to determine the anti- microbial activity of medical grade Manuka honey on biofilms of polymicrobial wound populations growing on an organic nutrient source to emulate an *in vivo* environment.

The selection of wound populations for study is described in Section 2.7.5.1. Using methods 2.4 and 2.5 described above, MICs and MBCs were performed on all genera of organisms not previously tested in this study for Manuka honey susceptibility that is organisms other than *Staphylococcus aureus, Enterococcus* sp., *Escherichia coli or Pseudomonas aeruginosa.* This was done to determine if there were any isolates with sufficiently high or low susceptibility which may influence the concentrations of honey chosen for the wound modelling assay.

2.8.1 Wound model assay.

Robertson cooked meat broths containing clinical wound populations were taken after 48 hours incubation at 37°C. Approximately 0.5g of meat was aliquoted into each of 14 sterile bijoux, 12 of which contained a plug of blood agar covering the base of the container. The presence of

blood agar was to emulate the wound bed as a nutrient source. All bijoux were weighed to determine meat weight in each.

In order to ascertain a baseline of the average count per unit weight of meat not exposed to honey, 1ml of peptone was added to each of the 2 bijoux containing meat only. This was vortexed for 30 seconds. A volume of 10 µl of peptone was inoculated into 1ml sterile peptone and 10- fold serial dilutions were performed. Organism counts were performed by dropping three x 10µl drops onto a blood agar plate which was then aerobically incubated overnight at 37°C. The count was determined by taking an average count of the 3 drops using the dilution with the highest number of discreet colonies.

The honey products were taken and diluted 50% and 75% in BHIA. The products were diluted to emulate the dilution by wound exudates *in vivo*.

The 12 bijoux containing meat and blood agar were taken and 1ml of each honey dilution was added to 3 of the 12 bijoux.

This exercise generated triplicates of each wound for each honey at each dilution.

After 18-24 hours incubation at 37°C, the honey was removed by pipette, cultured on blood agar to determine the presence of viable organisms and the meat transferred to a fresh bijoux. 1ml of peptone was added and then vortexed for 30 seconds. Organism counts were performed as before.

Baseline Miles Misra counts were compared to counts from the honey treated meat by ANOVA using SPSS to determine if the honey had significantly had reduced, inhibited or cultured the biofilm growing on the uneven meat surface.

2.9 Statistical Analysis

Excel was used to record data during the laboratory investigations. The graphs shown throughout this study were produced using this programme.

All statistical analysis was preformed using Microsoft excel or SPSS versions 16 student version. SPSS is a comprehensive statistical analysis programme which can be used with other operating systems such as Microsoft, Mac and Linux (SPSS Inc. 2008)

2.9.1. The Independent samples t-test

The Independent samples t test is used to compare one variable across two different data sets. This was used to compare the mean MICs of MRSA and MSSA, VRE and VSE, β lactamase positive and negative *E.coli* and the sensitive and resistant *P.aeruginosa* isolates. This t-test was also used to compare the mean MICs of the clinical isolates with their corresponding NCTC controls.

2.9.2. The One way Analysis of Variance Procedure

The One way Analysis of Variance is used where there are more than two groups to be compared. ANOVA was used to compare the mean antimicrobial activity of the products to compare MICs of antibiotic sensitive and resistant isolates and MICs of clinical isolates and NCTC control strains.

2.9.3 Tukeys's Honest Significance Test

Tukeys's honest significance test (HSD) is an ad hoc test to identify homogenous subsets within groups. This grouped products together when there were no statistical differences in antimicrobial activity.

Products within different homogenous subsets demonstrated significant differences in bactericidal activity.

2.10 Chemical methods for the analysis of honey

Two different methods were used to try and isolate. and identify the antimicrobial fractions within the honey products.

2.10.1 Thin layer chromatography (TLC)

The materials used in this section of study are detailed in Table 2.7.

2.10.1.1 Sample preparation

The first honey solution to be test run in each of the developing solutions was medical grade Comvita Manuka honey UMF 18+ diluted 20% and 10% (*w:v*) in methanol.

Table 2.7 Chemicals and Materials used for TLC studies

Chemical/ Material	Grade	Catalogue number	Supplier
1D 5x20mm silica coated TLC plate			
Macherey Nagel TLC plates 10x10 cm		TLC-400-128W	Fisher Scientific
Acetic Acid			
Acetone			
Chloroform			
Chromasolv® Ethyl Acetate	HPLC grade ≥99.9%	439169	Sigma Aldrich
Chromasolv® Methanol	HPLC grade ≥99.9%	34860	Sigma Aldrich
Chromasolv® Toluene	HPLC grade ≥99.9%	34866	Sigma Aldrich
De-ionised Water			Elgar Process Water
Vanillin		94750	Sigma Aldrich
Nitro blue tetrazolium	98%	N6876	Sigma Aldrich UK

The same product was then diluted 50% (*w:v*) in 50% methanol:de-ionised water (chosen as the diluent for the study).

The highest UMF rated Manuka, Comvita UMF 20+ was diluted 25% and 50% in the chosen diluent.

2.10.1.2 Developing solutions used for mobile phase TLC.

A number of developing solutions of increasing polarity were tested. The constituents of each are shown in Table 2.8.

Table 2.8 Constituents of TLC developing solutions.

Solution designation	**Contents**		
Solution 1 TEM	Toluene 88%	Ethyl Acetate 6.6%	Methanol 5.5%
Solution 2 TEA	Toluene 70%	Ethyl Acetate 20%	Acetic Acid 10%
Solution 3 HCA	Hexane 60%	Chloroform 25%	Acetone 15%
Solution 4 EMW1	Ethyl Acetate 80%	Methanol 11%	Water 9%
Solution 5 EMW2	Ethyl Acetate 68%	Methanol 24%	Water 8%

2.10.1.3 One dimensional TLC.

A silica coated TLC plate (5x20mm) was taken and a pencil line was drawn 1cm parallel to the 5cm end of the plate. Pencil dots (n= 2 -4) were marked on this line. Aliquots of 10µl - 40µl of the selected diluted

honey were pipetted onto the pencil dot. The plate was dried with an electric fan until the honey dried. In a fume cupboard, a tank was filled to a depth of 1cm with the developing solution, covered and left for 40 – 60 minutes to equilibrate. The silica plate was stood on end with the honey at the bottom for approx 90 minutes, until the solution had migrated around 75% up the plate. The plate was left to dry. The plate was viewed under short wave and long wave UV light and any areas of fluorescence marked with pencil, and the retardation factor (RF) calculated. Plates were then sprayed with Vallinin, Ferric Chloride or Ninhydrin to further visualise any separated fractions.

This was repeated with the different developing solutions to determine the most suitable. Once the developing solution was selected, all the products were diluted 50% in methanol/water 1:1. and separated as above. The results using long wave UV were recorded.

2.10.1.4 Two dimensional TLC

A silica coated TLC 20x20 plate was taken and a pencil line was drawn 2cm in from the vertical edges parallel to each edge. A pencil dot was marked where the pencil lines crossed at the bottom right corner.10µl of the selected diluted honey was dropped onto the pencil dot.

In a fume cupboard, a tank was 1cm filled with the developing solution, covered and left for 40 – 60 minutes to equilibrate. The silica plates were stood in the tank with the honey at the bottom for 90 minutes, removed and left to dry. This was repeated after rotating the plate 90 degrees keeping the honey spot at the bottom. The plate was viewed under short wave and long wave UV light and any areas of fluorescence marked with pencil, and the RF calculated.

2.10.1.5 Agar overlay method 1

The first agar overlay method was used to visualize areas on the silica plates of antimicrobial components separated out of the honey sample.

From an overnight culture of the MSSA NCTC strain 6571 in Iso sensitest broth, 2 ml was added to 20 ml of molten nutrient agar (Oxoid). This was poured over the TLC plates after the UV visible fractions had been marked on the plate and left to set and incubated overnight at 37°C. To visualise any areas of organism killing, 40 µl of Nitro Blue tetrazolium (Sigma Aldrich) was spread over the plate using a microbiological spreader. A selection of plates had a further 100 µl added. They were kept at room temperature for 72 hours and observed at 48 and 72 hours and results recorded.

2.10.1.6 Agar overlay method 2.

The above method 2.10.1.5 was modified to produce clearer areas of microbial inhibition.

To test this modification, the diluted Manuka honey and Golden Syrup solutions used in method 2.10.1.5 were taken and 10 µl were dropped onto each of two silica coated TLC plates and left to dry. To 18 ml of Iso sens agar (Oxoid), 2 ml of an overnight culture of the NCTC MSSA control in iso sensitest broth, was added and poured over the TLC plates. They were left to set and incubated for 6 hours at 37°C.

To visualise any areas of non-viable organisms, 10 ml of Iso sensitest agar containing 1ml of NBT was poured over one of the TLC plates; on the second plate 10 ml of Iso sensitest agar was overlaid and allowed to set, 1 ml of NBT was then flooded over the plate. These were left at room temperature overnight and results observed and recorded.

This method was used on all 1D plates on all products described in Section 2.1. 2D TLC was performed plates on all products except the Syrups, acacia honey and Sainsbury's basic honey as these had shown previously to have the least anti-microbial activity.

2.10.2 Nuclear magnetic resonance spectroscopy using Diffusion Ordered Spectroscopy (DOSY) software

All the Manuka products described in Section 2.1 were selected for NMR including DOSY analysis with the exception of West Coast Organic Manuka which was no longer available at the time of analysis. The Eucalyptus and Acacia honeys were included as non-Manuka controls.

Approximately 50 mg of each product was dissolved in 2 ml of 10% Deuterium oxide (D_2O) in distilled water. The internal standard TrimethylSilylPropionate sodium salt (TSP) was added to a concentration of 1.52 mM and each sample was shaken for a minimum of 8 minutes then transferred to an NMR tube. Each sample was allowed to equilibrate within the NMR spectrometer for 5 minutes. All NMR experiments were carried out at 25°C.

Qualitative NMR analysis was performed using a Bruker Avance III 600 Hz NMR spectrometer with 5mm TXI Probe and temperature control unit with Bruker 5mm NMR tubes and Bruker Topspin 3 software to acquire spectra.

Parameters were set at 64K complex data points, 10.3112 ppm sweep width using a stimulated echo bipolar pulsed field gradient with 1 spoil gradient and 3-9-19 WATERGATE sequence to obtain diffusion series δ = 2.4 ms and Δ = 100 ms.

Relaxation delay was 3 seconds and WATERGATE pulse duration was 1000 μs with 64 linear gradient steps of 2-95% intensity consisting of 8 scans each. A sine bell shaped window function phase was applied over all data points prior to Fourier transformation (16384 points) to translate time data into the frequency domain to improve resolution.

Data was processed using Diffusion Ordered Spectroscopy (DOSY) software. Fourier transformation whilst being sufficient for normal 2D NMR, the DOSY programme require further preparation and TSP was used for Lorentzian reference deconvolution to minimize overlap of signals. TSP and acetate were used to validate the calculated diffusion coefficients of the individual components.

Where confirmation of the identity of compounds was required, the compounds were obtained from Sigma-Aldrich Ltd and used to spike matrix matched samples for comparison.

Selective 1D ^{1}H-^{1}H-TOCSY experiments were carried out using the Bruker SELMGP pulse program with a refocusing pulse (p12) duration determined for each signal of interest based on width, which ranged from 18 to 35Hz. This enabled expansion of the signals normally indistinguishable from carbohydrate components.

CHAPTER 3

Characterisation of the Antimicrobial Effects of Honey and Syrup on Clinical Isolates of Clinically Relevant Bacteria: A Comparison of Different Strain Groups

3.1 Introduction

As early as 1924, agar diffusion was described as a method for ascertaining the susceptibility of bacteria to antiseptics (Wheat, 2001). Fleming cut 'ditches' into agar which were modified to wells by Reddish several years later (Wheat, 2001). Various methods using filter paper discs were introduced in the 1940s with the 6 mm discs used today being described by Bondi *et al.* in 1947. During the 1950s, the disparity of results due to variables such as agar content, temperature, thickness of agar and strength of antimicrobial agent led the World Health Organisation to suggest the need for standardisation in antimicrobial sensitivity testing (World Health Organisation, 1961). Kirby and Bauer designed a method to reduce the variables and standardise the method which became the method of choice when the National Committee for Clinical Laboratory Standards were formed in 1975 (Bauer and Kirby *et al.*, 1966), this organisation later became the Clinical and Laboratory Standards Institute (CLSI). Meanwhile in the UK, Stokes was working on a similar method which utilised a rotary figurer or plater to seed a control organism around the outside of the plate with the test organism inside which served to provide a control for each antibiotic disc on each plate (Stokes, 1968, Pearson and Whitehead, 1974). In 1971 The British Society for Antimicrobial Chemotherapy (BSAC) was founded. As various modifications to the Stokes methods were being used around the country, over time this made comparison of data and surveillance of emerging resistances difficult (Gould, 2000). In response to this situation, the first guidelines were published in 2001 by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2001). The efforts to

standardise with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI continue.

Broth dilution methods of susceptibility testing have developed alongside the agar diffusion methods (Wheat, 2001) and have the advantage of ascertaining the Minimum Inhibition Concentrations (MICs) of individual organisms to specific antimicrobials. BSAC guidelines include methodologies for both macro and micro–dilution methods.

This study used both agar diffusion and micro-broth dilution methods, with agar diffusion providing an initial basis for comparing the activity of the products. Micro-broth dilution allowed further comparison of products using the MIC and Minimum Bactericidal Concentrations (MBC).

The choice of organism genera to include in the study was based on the multi-drug resistant screening protocols adopted by the Microbiology department at The Royal Marsden Foundation Trust, a specialist cancer hospital where patients are routinely screened for carriage of multi-drug resistant isolates. The mandatory MRSA screening plan states that patients requiring a MRSA screen include:

- All new patients to the Trust
- All electively admitted at least four weeks before admission with additional pre-surgical swabs from appropriate sites, for example, axilla swabs for breast patients or groin swabs for pelvic or lower abdominal surgery.
- All emergency admissions.
- All patients with nosocomial wound infections even if discharged.

There is additional screening on the critical care ward and out-patients are screened prior to each round of chemotherapy. The relevant section of this policy can be seen in Appendix 1.

The haemato-oncology patients are a particularly vulnerable group suffering from leukaemias and lymphomas which compromise their immune systems. The Royal Marsden is the largest haemato-oncology transplant centre in the UK. Transplantation may be autologous where the patients own stem cells are harvested and returned to them, or allogeneic where either stem cells or bone marrow comes from a donor (Passweg *et al.,* 2012). The procedures require chemotherapy to totally suppress the patient's immune system prior to them receiving the cells or marrow (Kessinger *et al.,* 1986). Transplants can take up to 42 days to engraft during which time the patients remain highly vulnerable to infection. These patients are frequently on numerous antibiotics which creates an optimal environment for the patients indigenous gut flora to develop resistances which are further selected out by ongoing antimicrobial therapy. Stool samples from this group of patients are screened weekly for VRE and multi-drug resistant *Enterobacteriacae* which provides the infection prevention team with information regarding the multi-drug resistant carriage of each individual patient. Patients carrying these organisms are barrier nursed and any patient developing diarrhoea is isolated within 2 hours. In recent years hydrogen peroxide fogging equipment has been procured by the Royal Marsden Trust which is used to decontaminate rooms between patients which have had organisms presenting particular risks of cross infection.

The antibiotic panels selected were those routinely used at The Royal Marsden for the specific organism at the time of testing detailed in Appendix 2.

The aim of this section of study was to establish and compare the antimicrobial activity between Syrup products and honeys from different floral sources, on different genera of bacteria, with different antibiotic susceptibilities, isolated from a clinical environment, alongside the corresponding genera of NCTC control strains. Statistical analysis was used to compare antimicrobial activity between products and to compare susceptibility between organism groups.

3.2 Methods

3.2.1 Product selection

The two Syrups were chosen to serve as sugar controls, the seven Manuka honey products covered a UMF rating range of 5+ to 20+ with 5+ being the lowest rated with regard to antimicrobial activity and 20+ being the highest. The other five honeys were non-Manuka products.

The products selected are described in Section 2.1.

3.2.2 Organism selection

The organisms selected for this study were the multi-drug resistant organisms routinely screened for at The Royal Marsden Hospital to aid infection prevention and control. Initially, the organisms studied were 25 MRSA and 25 VRE with their more sensitive counterparts MSSA (25 isolates) and VSE (25 isolates) for comparison, all obtained from inpatients at the Royal Marsden hospital. A Total of 25 *Escherichia coli* isolates were initially selected, each of which is resistant to 3 of the following classes of antibiotics: β-lactams; Aminoglyosides;, fluoroquinolones or Trimethoprim, a dihydrofolate reductase inhibitor]. *Pseudomonas aeruginosa* isolates were not selected on the basis of their antimicrobial sensitivities as *P. aeruginosa* is resistant to most first line oral antibiotics, therefore these 25 isolates were not chosen on the basis of their indicated resistance profiles.

Subsequently, for the second agar diffusion assay and MIC/MBC assays 40 Gram negative organisms were selected on the basis on antimicrobial susceptibility. These organisms included 10 β-lactamase positive and 10 β-lactamase negative isolates of *E. coli*. The *P. aeruginosa* isolates were selected on the basis antimicrobial resistance to meropenem and/or

ciprofloxacin, with 10 isolates showing reduced sensitivity to these antibiotics being studied alongside another 10 isolates which showed no reduced susceptibility.

3.2.3 Investigations performed prior to antimicrobial assay

Prior to the first agar diffusion assays, the method described in Section 2.3.3. were used to assess the suitability of horse serum and sterile distilled water as a product diluent. Horse serum was evaluated as it was the available product most resembling the serous fluid which might dilute honey applied to a wound. Iso sensitest broth was also tested as a product diluent for the micro-broth MIC assays and as a growth medium for the organisms in the microtitre wells that was also consistent with the recommended organism suspension medium (Andrews, 2001).

The honey products were cultured to determine if they contained any bacterial flora using the method described in Section 2.3.1 an initial antimicrobial activity screen was performed as described in 2.3.2.

3.2.4 Agar diffusion assays

The results from the first agar diffusion testing using the method described in 2.3.7 provided insight into the relative activity of the products with the different organism groups, however it also highlighted some areas in which the method could be improved. These improvements included use of a positive displacement pipette to facilitate the pipetting of accurate volumes of product being dispensed and a 25% increase in the volume of product being dispensed to provide larger zones of inhibition in order to provide greater differentiation for comparison. The 35°C water bath facilitated pipetting. This temperature was selected as representative of the temperature at which the products

would be used in a clinical setting. The first agar diffusion assays tested undiluted honey and honey diluted to 50% and 25% concentrations. In the second method only undiluted honey was used. The methods are fully described in Section 2.3.9. Once the methodology and organism panels had been optimised, the antimicrobial profile of each organism was performed using BSAC guidelines. A third round of agar diffusion testing was performed on the expanded panel of Gram negative isolates. Statistical analysis using ANOVA (Analysis of Variance) and Tukey's HSD Test (Honest Significant Difference Test) using SPSS was performed to identify if any of the products significantly differed from another or if they could be grouped into homogeneous subsets based on antimicrobial activity.

3.2.5 Minimum inhibitory and bactericidal concentrations of honey products

MIC and MBC tests were performed on the same panels of organisms that were tested by agar diffusion using the methods described in 2.4 and 2.5.

This method used doubling dilutions of the honey products which gave a large 25% difference in concentration between wells 1 and 2 and a 12.5% difference between wells 2 and 3 with similar reductions across the microtitre plate. As differences were expected to be small, more precise results were needed to compare the differences in honey susceptibility between antibiotic sensitive and resistant isolates. Intermediate MICs were performed on a reduced panel of Manuka products with 2% incremental dilutions of the honeys ranging from 2-26%.

The Manuka products selected were the two medical grade honeys as these would be the appropriate products to use in a clinical environment along with the Manukas with the highest and lowest rated UMF.

3.2.6 Statistical analysis

An Independent samples t test was used to compare the MIC of sensitive and resistant isolates within each organism group and the mean MICs of clinical isolates to the mean MICs of the corresponding NCTC control strain for each of the different honeys.

The difference was considered to be significant where $p \leq 0.05$. ANOVA analysis was performed using the combined results of all of the products to determine if the number of MICs of clinical isolates and NCTC control strains were significantly higher, lower or equal to the NCTC control strains.

3.3 Results

The antibiotic profiles of the organisms can be seen in Appendix 2.

3.3.1 Sterility of the honey products

The results of the Syrup and honey culture showed that seven of the products grew a scanty growth of *Bacillus* sp. None of the products grew *Clostridium botulinum* which has been reported as a honey contaminant in other studies (Midura *et al.*, 1979; Cooper and Molan 1999a; Nevas *et al.*, 2002). Only two of the products were described as having been sterilised and none of the products were described as being pasteurised. The aerobic and anaerobic culture results of the products used in this study are shown in the table below (Table 3.1).

Table 3.1 Results of aerobic and anaerobic cultures of products

Product Code	Product	Aerobic Growth	Anaerobic Growth
1 (A)	Golden Syrup	No growth	No growth
2 (B)	Black Treacle	<5 cfu* *Bacillus* sp.	No growth
3 (C)	Meloderm Active Manuka Honey 16+ ∂irradiated	No growth	No growth
4 (D)	Comvita Manuka Honey 20+	<5 cfu* *Bacillus* sp.	No growth
5 (E)	Comvita Manuka Care 18+ sterilised	No growth	No growth
6 (F)	Haddrells Manuka honey 16+	No growth	No growth
7 (G)	Haddrells Manuka Honey 13+	<5 cfu* *Bacillus* sp.	No growth
8 (H)	The Honey Collection West Coast Organic Active Manuka Honey 12+	No growth	No growth
9 (I)	Haddrells Manuka Honey 5+	No growth	No growth
10 (J)	Spanish Lavender Honey	<5 cfu* *Bacillus* sp.	No growth
11 (K)	Rowse Australian Eucalyptus Organic Honey	<5 cfu* *Bacillus* sp.	No growth
12 (L)	Rowse Pure Acacia Honey	No growth	No growth
13 (M)	Rowse Clear Blossom Honey	<5 cfu* *Bacillus* sp.	No growth
14 (N)	Sainsbury's Clear Basics Honey	<5 cfu* *Bacillus* sp.	No growth

*** cfu - colony forming units grown from a 75 µl volume of product plated onto blood**

3.3.2 Activity screen

Golden Syrup showed no antimicrobial activity at all using the method described in Section 2.3.2.

The black Treacle showed no activity against Gram positive organisms and negligible activity against the Gram negative organisms. All of the Manuka honeys and the Australian Eucalyptus honey demonstrated

some inhibition of growth of all organism genera. The other non-Manuka products did not inhibit organism growth in these investigations

3.3.3 Diluents

There were no discernable differences to zone sizes obtained with horse serum or water using the method described in section 2.3.3. When Iso sensitest broth was tested prior to micro-broth MIC assays there with no differences in zone size noted when compared to the horse serum and water.

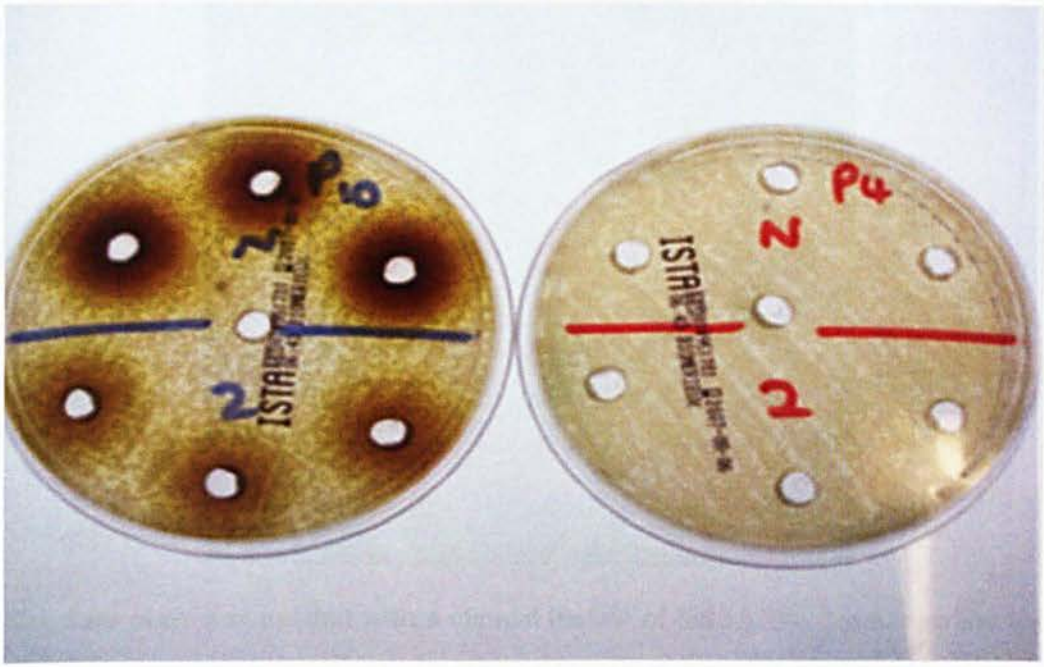
3.3.4 Determination of antimicrobial activity by agar diffusion method 1

The agar diffusion assays showed no zones of organism growth inhibition with either the Golden Syrup or Treacle Syrup against any of the organisms. All of the undiluted Manuka products produced zones of inhibition with all of the organisms when used.

When diluted to 50% concentration, only the Manuka products showed any activity against *S. aureus*. When the products were further diluted to 25% no inhibitory activity was observed with any product against any bacterial strain or isolate. The non-Manuka honey products showed either greatly diminished or indeed no activity against the isolates in the panel, when compared to the mauka honey products.

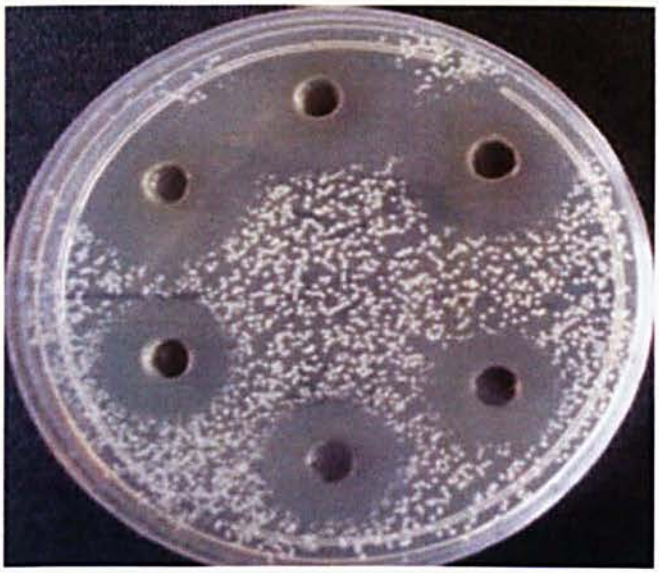
Figure 3.1 shows agar plates seeded with *Pseudomonas aeruginosa* with Treacle and Golden Syrup inoculated into the wells. Figure 3.2 shows an example of the zones obtained with a Manuka Honey and MSSA. This method assayed undiluted products and products diluted to 50% and 25% concentration.

Figure 3.1 Illustration of the activity of Treacle Syrup and Golden Syrup against clinical strains of *P. aeruginosa*



Using agar diffusion method 1, each agar plate was seeded with a clinical isolate of *P. aeruginosa*. The top three wells of each plate contained undiluted Syrup. The bottom three wells contained Syrup diluted to 50% concentration. The plate on the left shows the diffusion of the Treacle Syrup into the agar with no inhibition of organism growth. The wells in the plate on the right of the figure show the diffusion of the Golden Syrup into the agar with no inhibition of organism growth.

Figure 3.2: Zones of MSSA growth inhibition with Haddrells UMF 16+ Manuka Honey



The agar plate was seeded with a clinical isolate of MSSA.The 3 wells on the top half of the plate were inoculated with undiluted honey, the 3 wells on the bottom half of the plate contained a 50% dilution of the honey which produced smaller zones of growth inhibition.

The results of this first round of agar diffusion testing gave an indication of the relative activities of the products and the susceptibility of the different organism groups but the lack of a positive displacement pipette meant that the volumes of honey dispensed into the wells was inconsistent and the results had to be interpreted with caution. Further testing was required before conclusions could be drawn.

Table 3.2 Ranking of products using agar diffusion assay 1

Product	Efficacy Ranking (Agar Diffusion 1)*
Comvita Manuka Honey UMF 20+	5
Haddrells Manuka Honey UMF 16+	5
Comvita Mauka Honey 18+(sterilised)	4
Meloderm Manuka Honey UMF 16+(I irr)	4
Haddrells Manuka Honey UMF 13+	4
West Coast Organic Manuka Honey UMF 12+	4
Haddrells Manuka Honey UMF 5+	3
Eucalyptus Honey	1
Basic Honey	1
Blossom Honey	1
Acacia Honey	1
Lavender Honey	1
Treacle Syrup	1
Golden Syrup	1

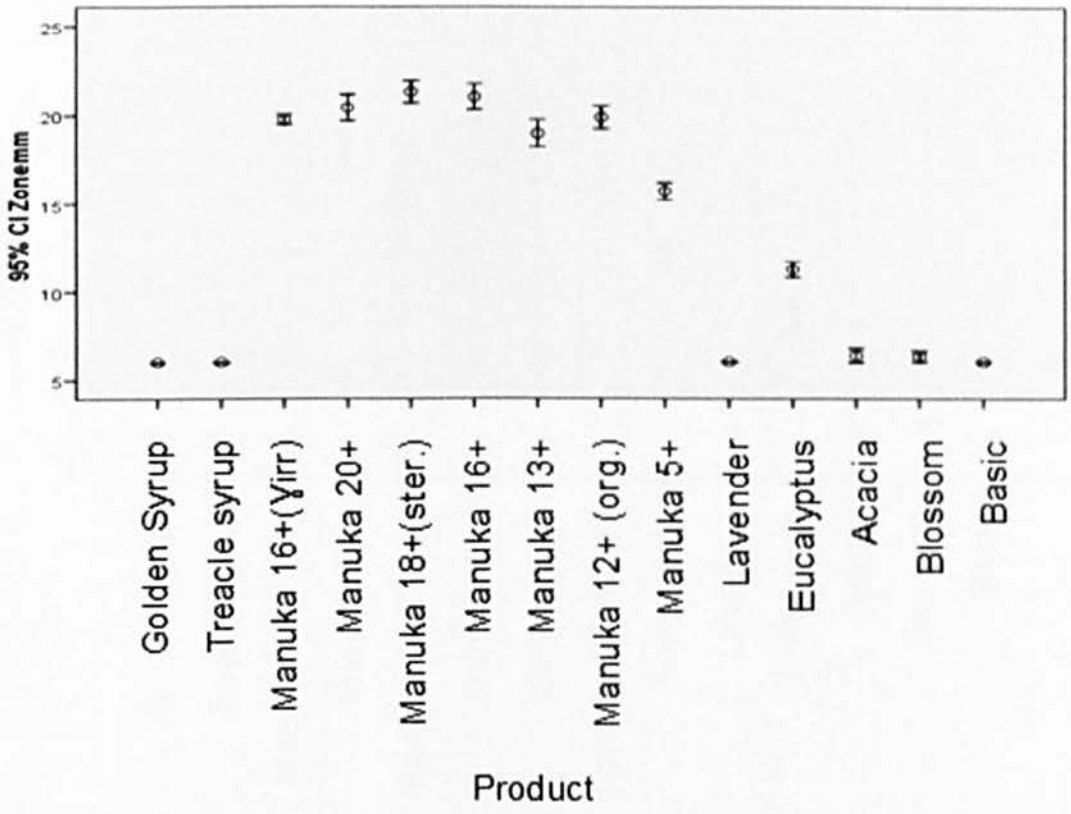
*** The scoring is based on homogenous subsets determined by Tukeys HSD analysis. The products in the subset with the best performing products were given a score of 5 and the lowest, a score of 1.**

The results of the first agar diffusion assays can be seen in the plots below (Figures 3.3 – 3.8) which show the mean zone sizes obtained with each of the products against the isolates within each organism group using each of the above methods. The diameter of the well was 6 mm so zones of inhibition are those with zones of >6mm.

This agar diffusion assay showed *S. aureus* to be the most susceptible organism with the least variation between isolates as shown by the error bars (Figures 3.3 and 3.4). The results obtained for *Enterococcus* sp.

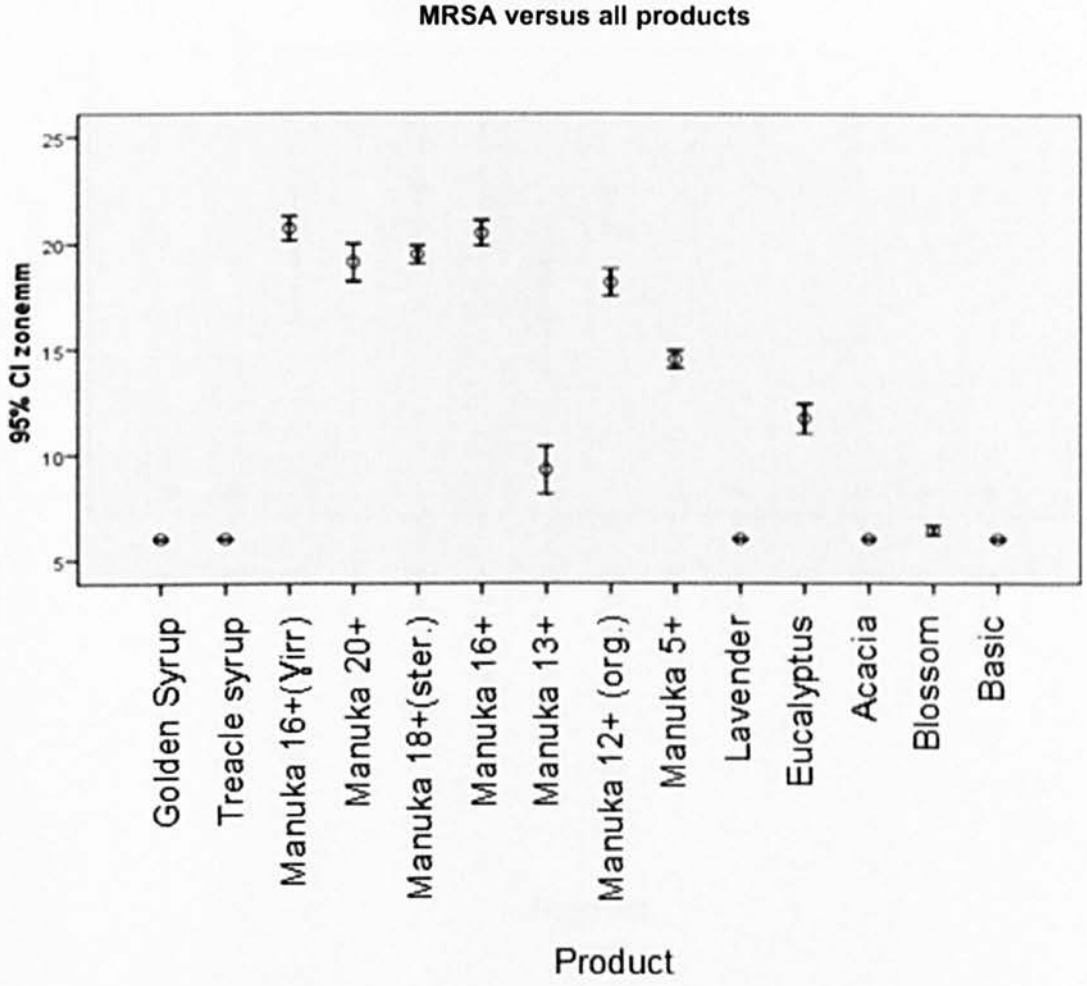
showed greater resistance to the products and greater variation between isolates as can be seen in the plots below (Figures 3.5 and 3.6)

Figure 3.3 Boxplot showing MSSA mean zone sizes of 25 clinical isolates of MSSA using agar diffusion method 1



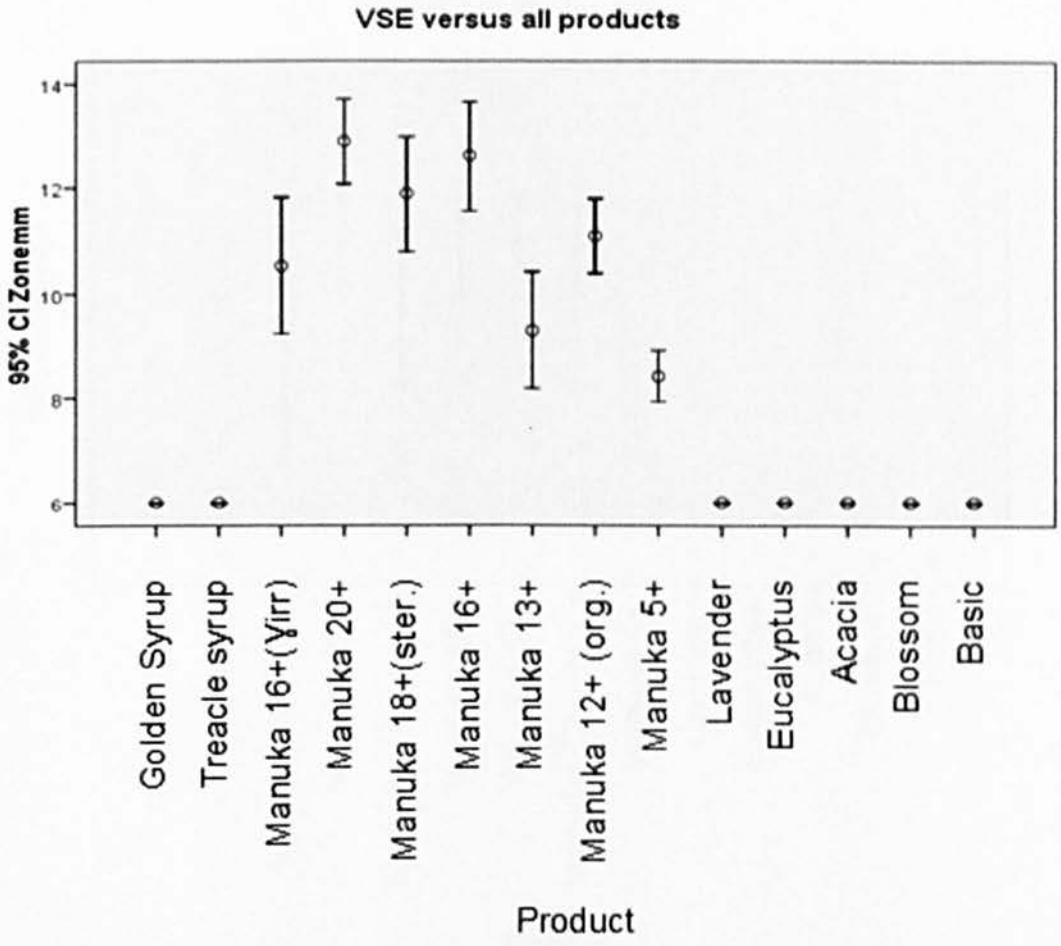
All of the Manuka honeys and the Australian eucalyptus honey produced zones of growth inhibition in excess of 10mm against MSSA. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

Figure 3.4 Boxplot showing MRSA mean zone sizes of 25 clinical isolates of MRSA using agar diffusion method 1



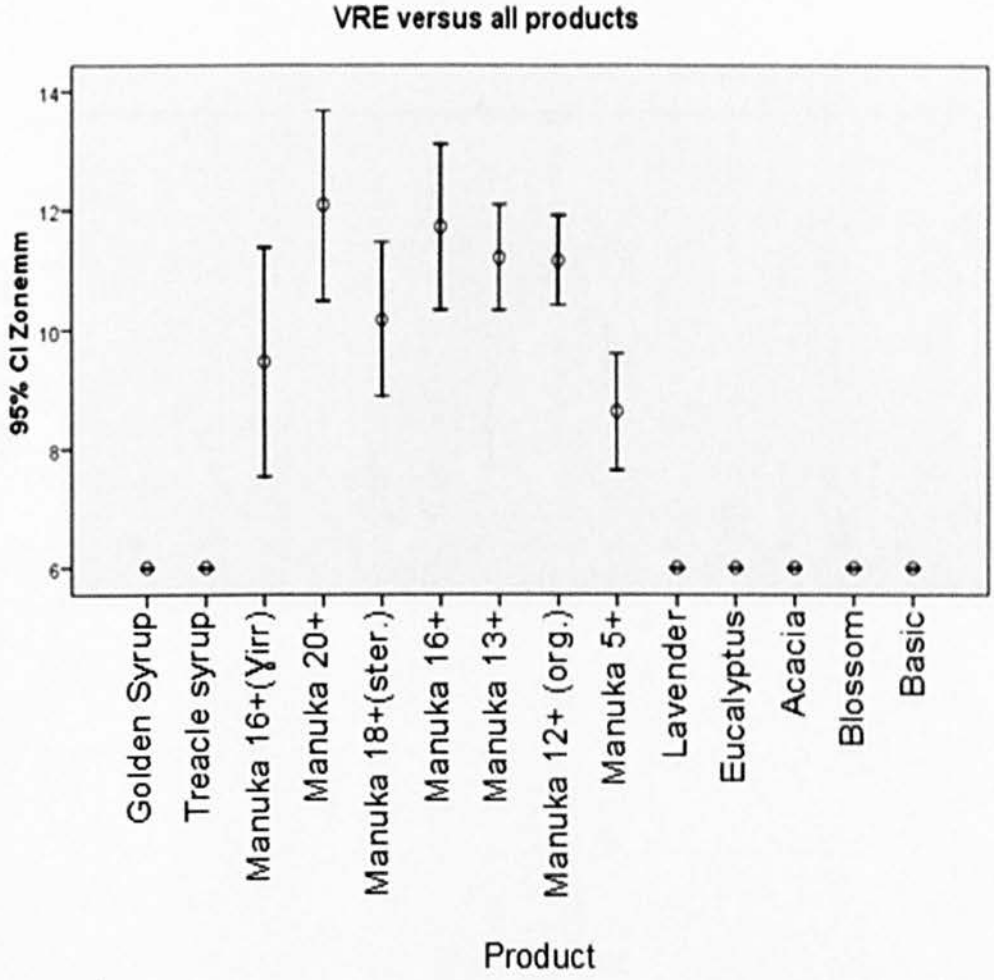
All of the Manuka honeys and the Australian eucalyptus honey produced zones of growth inhibition against MRSA. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

Figure 3.5 Boxplot showing VSE mean zone sizes of 25 clinical isolates of VSE using agar diffusion method 1



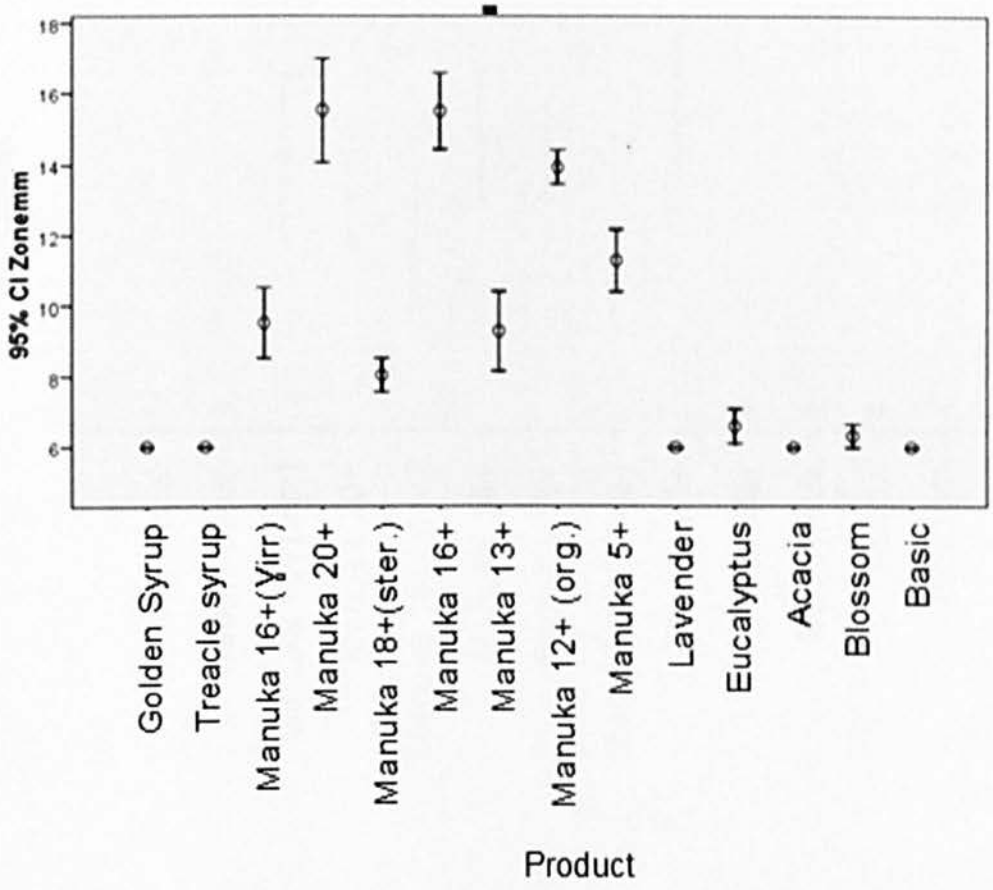
Only the Manuka honeys produced zones of growth inhibition against VSE. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and non-Manuka honeys produced no zones of growth inhibition. The error bars show the large range of zone sizes measured. The error bars show the large range of zone sizes measured.

Figure 3.6 Boxplot showing VRE mean zone sizes of 25 clinical isolates of VRE using agar diffusion method 1



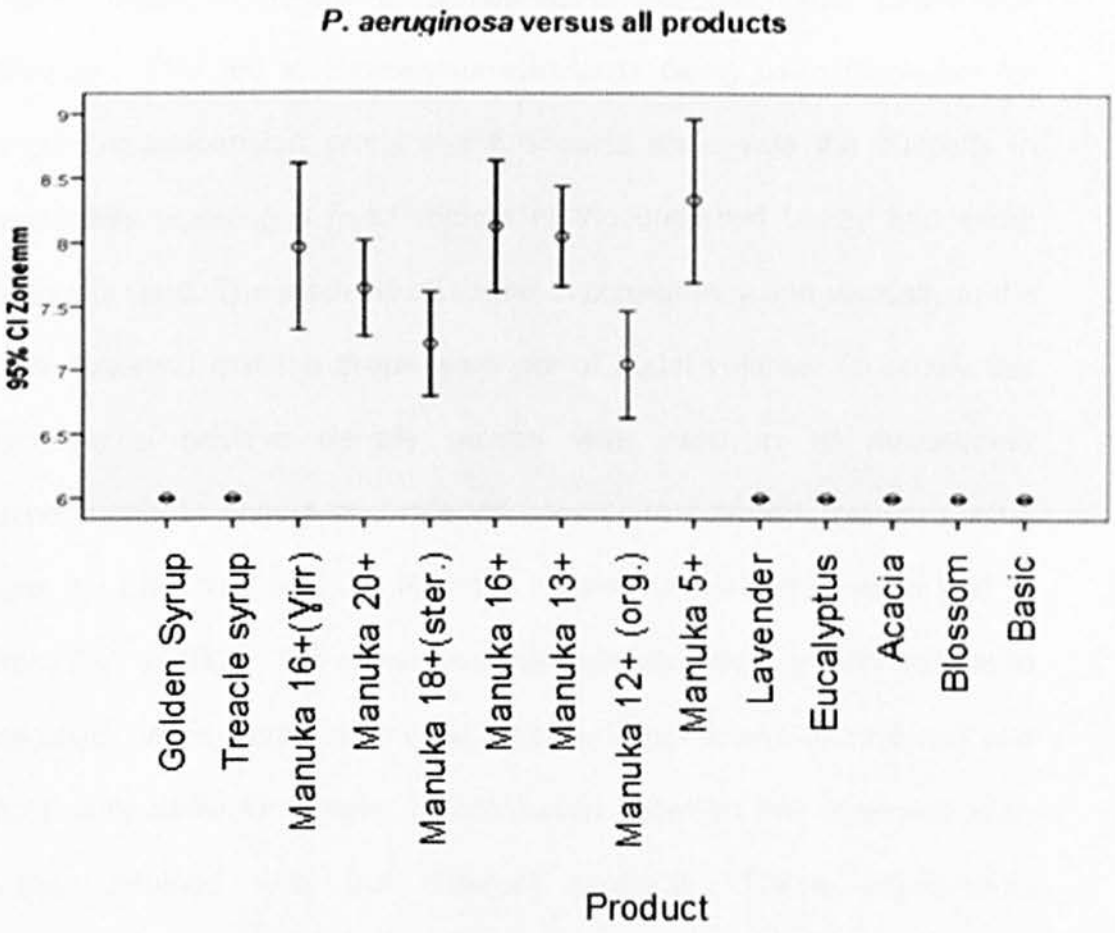
Only the Manuka honeys produced zones of growth inhibition against VRE. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and non-Manuka honeys produced no zones of growth inhibition. The error bars show the large range of zone sizes measured.

Figure 3.7 Boxplot showing *E.coli* mean zone sizes of 25 clinical isolates of *E.coli* using agar diffusion method 1



The Manuka honeys produced zones of growth inhibition against *E. coli*. The eucalyptus and blossom honeys produced zones of inhibition to some of the isolates. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the large range of zone sizes measured.

Figure 3.8 Boxplot showing *P. aeruginosa* mean zone sizes of 25 clinical isolates of *P. aeruginosa* using agar diffusion method 1



The Manuka honeys all produced zones of growth inhibition against *P. aeruginosa*. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and the non-Manuka honeys produced no zones of growth inhibition. The error bars show the large range of zone sizes measured.

The results from the first agar diffusions assays (detailed in section 3.3.4) indicated that Gram negative organisms were less susceptible to the Manuka products than the Staphylococci but more susceptible than the Enterococci used in the panel. Several methodology challenges were encountered as a consequence of the first undertaking of the agar diffusion tests. One issue was the heavy inoculum achieved with many of

the organism suspensions using Pro-lab McFarland standards. These standards were white in appearance which made degree of opacity more difficult to determine compared to the colourless Biomerieux product. This led to Biomerieux standards being used thereafter for organism suspension preparationA second issue was the difficulty in accurately pipetting a fixed volume of the undiluted honey and syrup products used. The products all varied in consistency and viscosity and it was observed that the drops were not of equal volume. To rectify this problem a positive density pipette was used in all subsequent experiments to ensure accurate volumes were pipetted The third issue was the observed need to alter the volume of product in each well to from 75µl to 100µl. The capacity of the wells allowed a greater volume to be used, which would potentially produce larger zones of inhibition and potentially allow for greater differentiation between the observed zone sizes obtained with the different products. These adjustments successfully gave a greater range of zone diameters which allowed for small variations in antimicrobial activity to be measured as demonstrated in the results below.

3.3.5 Determination of antimicrobial activity by agar diffusion method 2

The use of the positive displacement pipette yielded differences in the results with regards to ranking of products in relation to each other (Table 3.3).

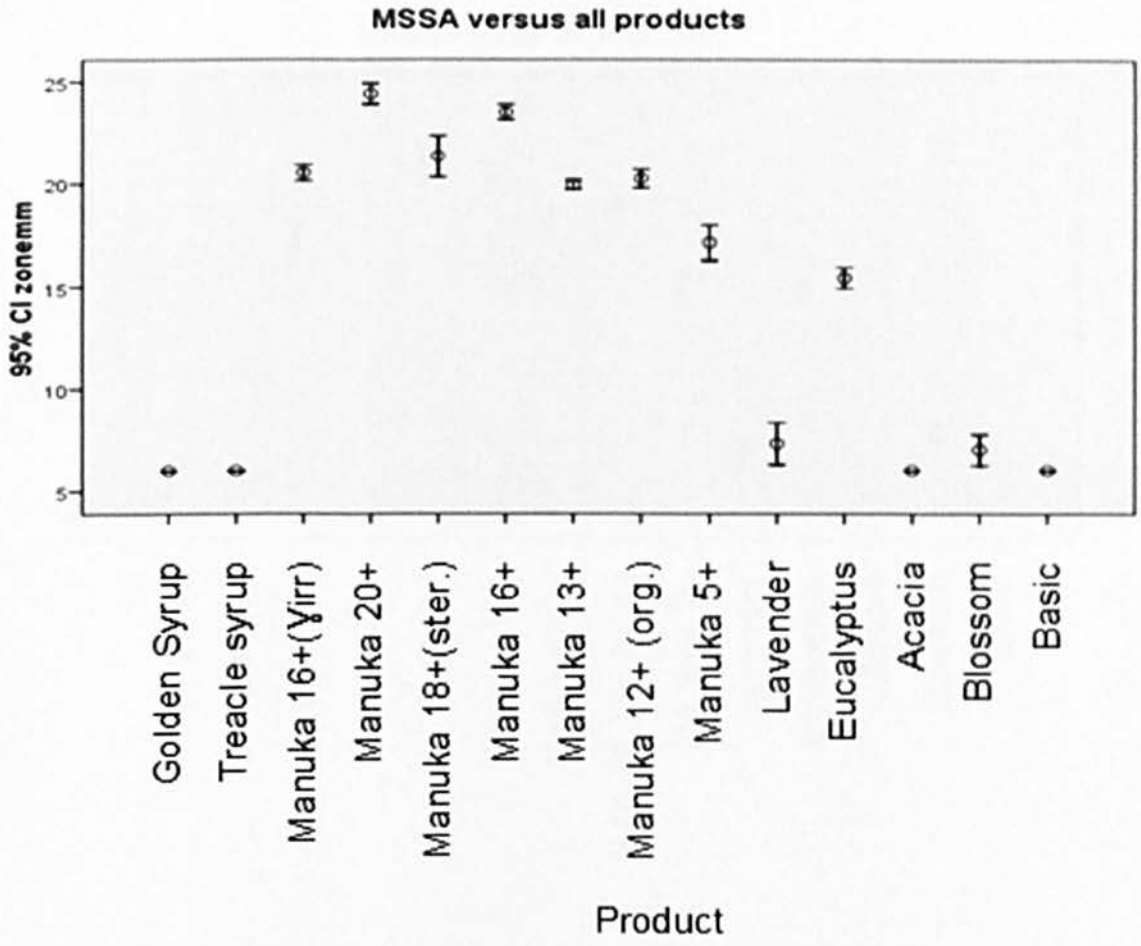
Table 3.3 Ranking of products using agar diffusion assay 2

Product	Efficacy Ranking (Agar Diffusion 2)*
Comvita Manuka Honey UMF 20+	5
Comvita Manuka Honey 18+(sterilised)	4
Meloderm Manuka Honey UMF 16+(I irr)	4
Haddrells Manuka Honey UMF 13+	4
West Coast Organic Manuka Honey UMF 12+	4
Haddrells Manuka Honey UMF 5+	4
Haddrells Manuka Honey UMF 16+	3
Eucalyptus Honey	3
Basic Honey	1
Blossom Honey	1
Acacia Honey	1
Lavender Honey	1
Treacle Syrup	1
Golden Syrup	1

*** The scoring is based on homogenous subsets determined by Tukeys HSD analysis. The products in the subset with the best performing products were given a score of 5 and the lowest, a score of 1.**

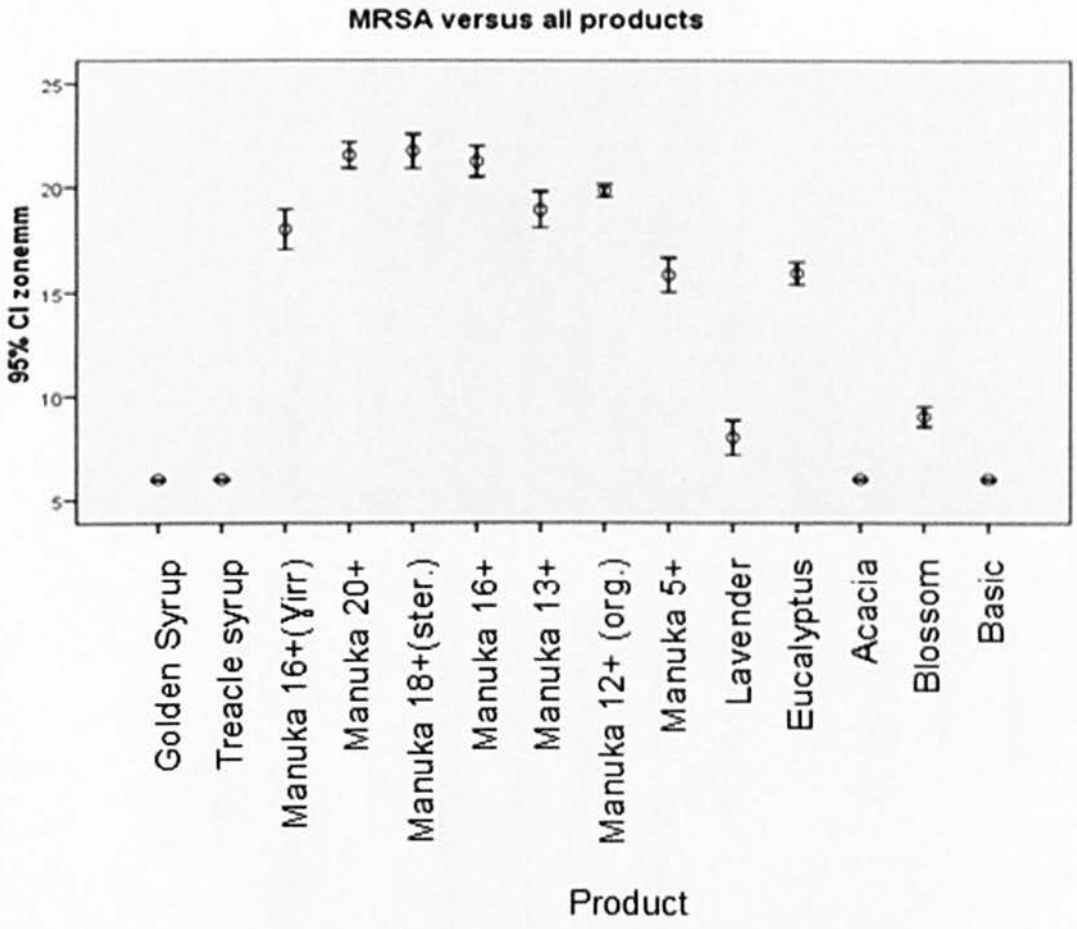
The volumes of honey were accurate and 25% larger than used in the previous method. However, the variations seen in the previous experiment with regard to susceptibility between organism groups and within organism groups remain, as shown in Figures 3.9 - 3.12.

Figure 3.9 Boxplot showing MSSA mean zone sizes of 10 clinical isolates of MSSA using agar diffusion method 2



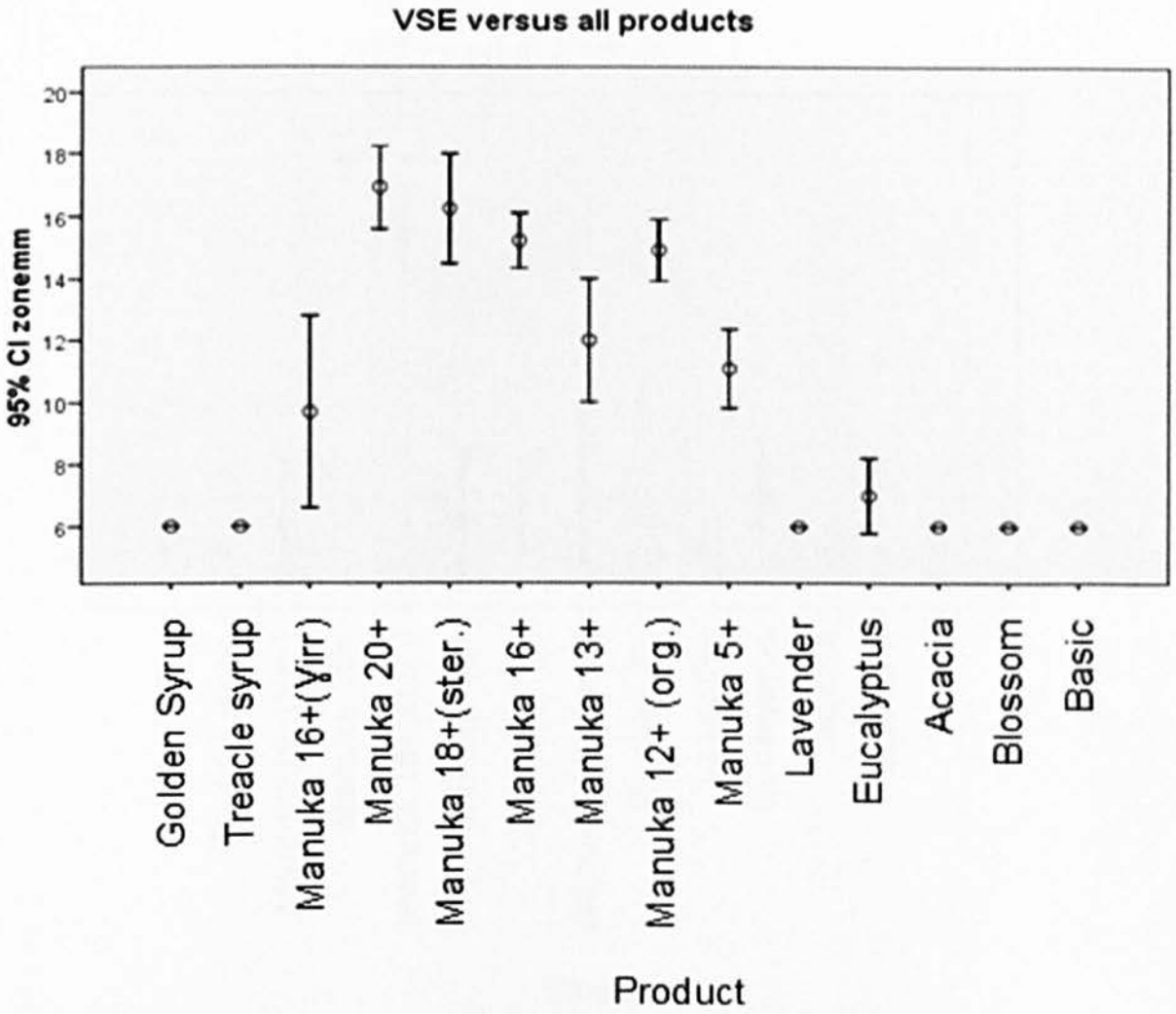
All of the Manuka honeys and the Australian eucalyptus honey produced zones of growth inhibition in excess of 10mm against MSSA, with the lavender and blossom honeys zones of less that 10mm. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

Figure 3.10 Boxplot showing MRSA mean zone sizes of 10 clinical isolates of MRSA using agar diffusion method 2



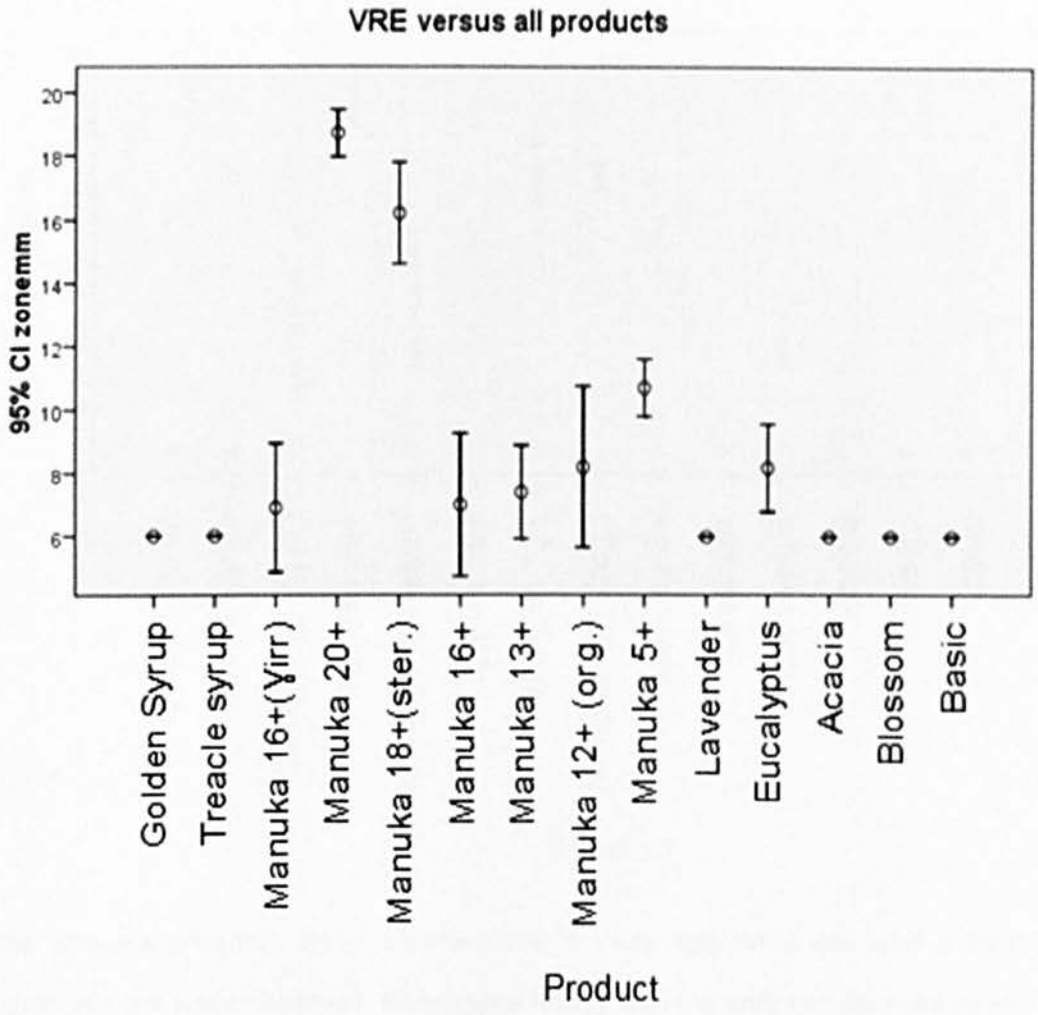
All of the Manuka honeys and the Australian eucalyptus honey produced zones of growth inhibition in excess of 10mm against MRSA, with the lavender and blossom honeys zones of less that 10mm. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

Figure 3.11 Boxplot showing VSE mean zone sizes of 10 clinical isolates of VSE using agar diffusion method 2



The Manuka products show antimicrobial activity against VSE with a large range of zone sizes observed. Of the non-Manuka honeys, only eucalyptus honey produced any zone of inhibition. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

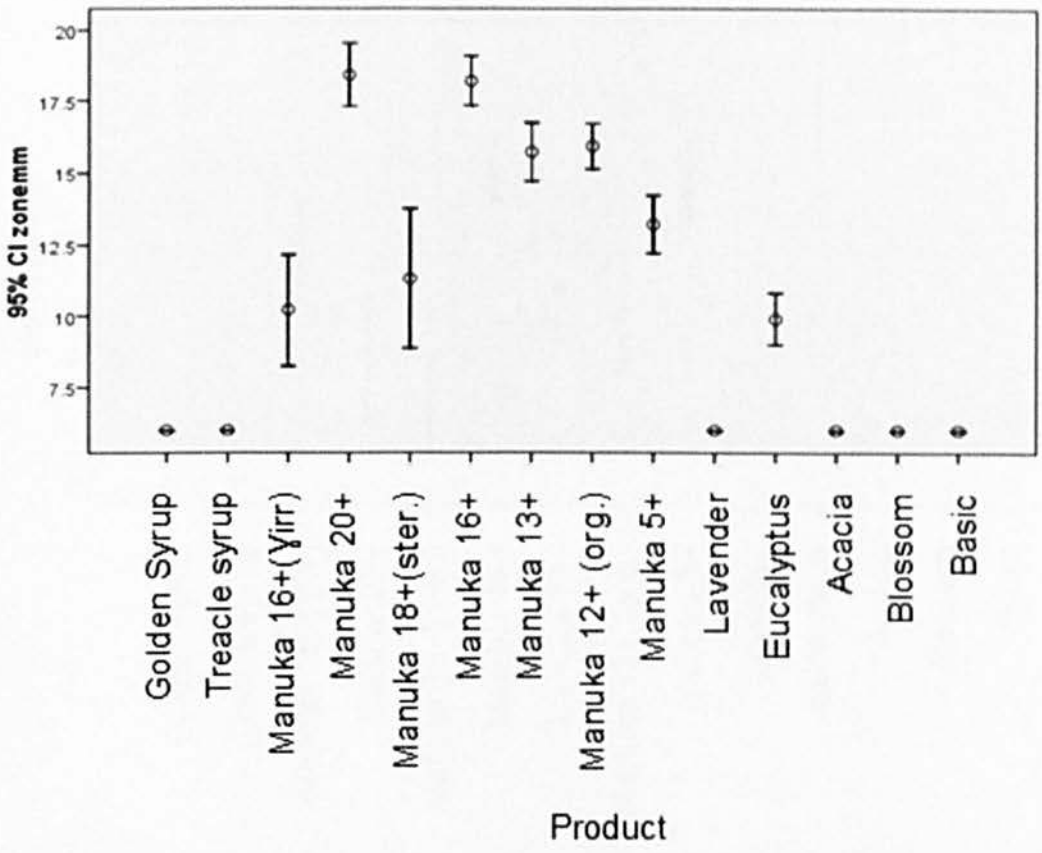
Figure 3.12 Boxplot showing VRE mean zone sizes of 10 clinical isolates of VRE using agar diffusion method 2



The Manuka products show antimicrobial activity against VRE with a large range of zone sizes observed. The two Manuka honeys with the highest UMF rating gave the largest zones. Among the non-Manuka honeys, only eucalyptus honey produced any zone of inhibition. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

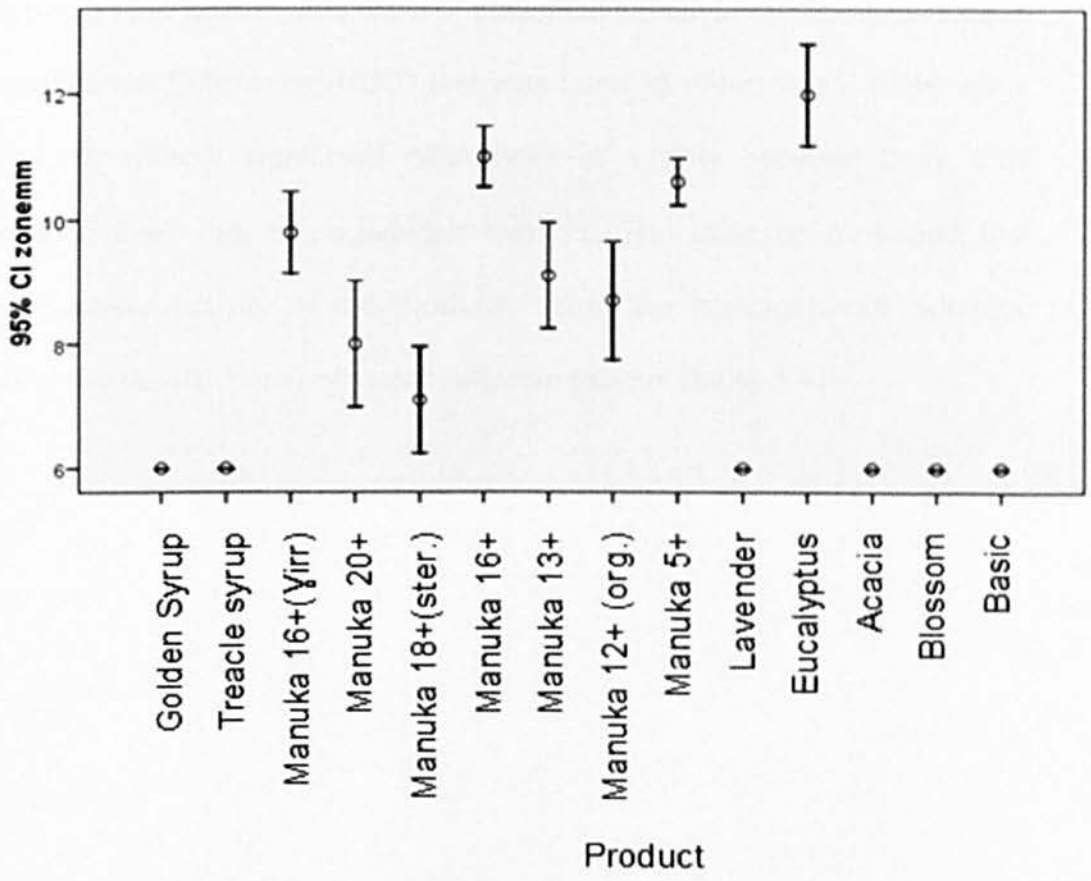
The plots below (Figures 3.13 and 3.14) show that the Gram negative isolates were similarly more susceptible to Manuka products. They also showed greater variation between products than the *S. aureus* isolates.

Figure 3.13 Boxplot showing *E. coli* mean zone sizes of 10 clinical isolates of *E. coli* using agar diffusion method 2.



The Manuka products show antimicrobial activity against *E.coli* with a large range of zone sizes observed. Eucalyptus honey was the only non-Manuka honey to produce any zone of inhibition. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

Figure 3.14 Boxplot showing *P. aeruginosa* mean zone sizes of 10 clinical isolates of *P. aeruginosa* using agar diffusion method 2



The Manuka products show antimicrobial activity against *P. aeruginosa* with a large range of zone sizes observed. Eucalyptus honey was the only non-Manuka honey to produce any zone of inhibition and demonstrated greater antimicrobial activity than all of the other products. The wells had a 6 mm diameter which shows the Golden Syrup, Treacle Syrup and other non-Manuka honeys produced no zones of growth inhibition. The error bars show the range of zone sizes measured.

3.3.6 Statistical analysis of agar diffusion assays

The zone sizes of the two agar diffusion assays were analysed using ANOVA to confirm that the differences between antibacterial activity shown on the above plots were of statistical significance. Tukey's Honest Significance Difference (HSD) test was used to determine if there were products without significant differences in activity between them and grouped them into homogeneous subsets. The table below shows the comparative activity of the products using the homogeneous subsets using the results from both agar diffusion assays (table 3.4)

Table 3.4 Ranking of comparative antimicrobial efficacy between products using the combined data from agar diffusion assays 1 and 2

Product	Efficacy Ranking (Agar Diffusion)
Comvita Manuka Honey UMF 20+	5
Haddrells Manuka Honey UMF 16+	5
Comvita Mauka Honey 18+(sterilised)	4
Meloderm Manuka Honey UMF 16+(I irr)	4
Haddrells Manuka Honey UMF 13+	4
West Coast Organic Manuka Honey UMF 12+	4
Haddrells Manuka Honey UMF 5+	3
Eucalyptus Honey	2
Basic Honey	1
Blossom Honey	1
Acacia Honey	1
Lavender Honey	1
Treacle Syrup	1
Golden Syrup	1

The scoring is based on homogenous subsets determined by Tukeys HSD analysis. The products in the subset with the best performing products were given a score of 5 and the lowest, a score of 1. The Comvita Manuka honey UMF 20+ and the Hadrells Manuka honey UMF 16+ were grouped in the homogenous subset showing the greatest antimicrobial activity. The two medical grade Manuka honeys, Comvita Manuka UMF 18+ and Meloderm Manuka UMF 16+ were grouped in the second most antimicrobial subset with Haddrells Manuka UMF 13+ and West Coast Organic Manuka UMF 12+. Hadrells Manuka UMF 5+ was in

the third most effective subset and Eucalyptus honey in the fourth. The other nin-Manuka honeys and Syrups were grouped together in the least antimicrobial subset.

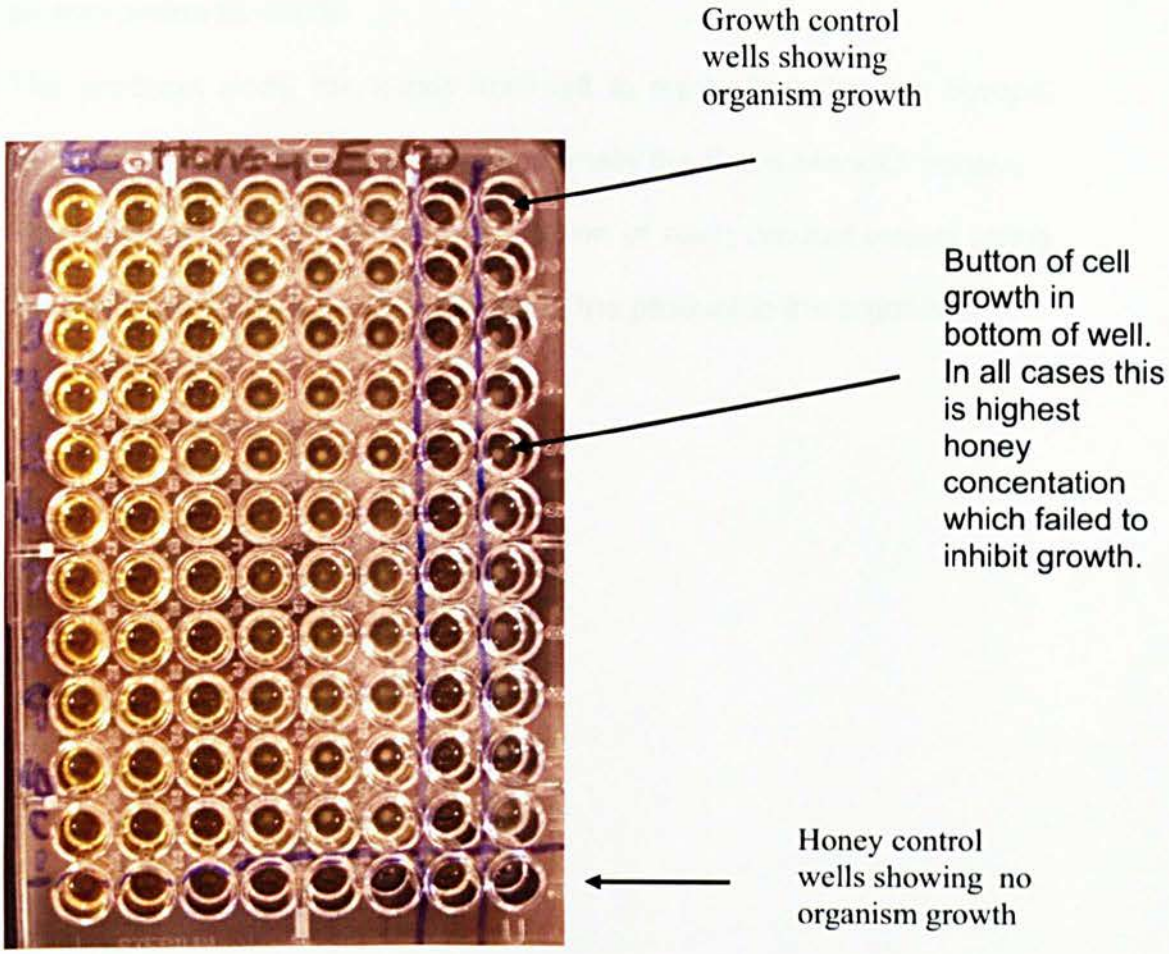
This analysis showed that the Syrups, lavender honey, acacia honey, blossom honey and the supermarket basic honey were consistently grouped in the lowest subsets with either zero activity or a small measure of variation where minute zones had been observed. The Australian eucalyptus honey consistently performed better than the other non-Manukas and performed particularly well against *Pseudomonas* using the greater volume in the second assay method. The groupings of the Manuka honeys varied and indicated that UMF was not an accurate predictor of antimicrobial efficacy or relative activity between products. The data showing the homogeneous subsets is shown in Appendix 3.

3.3.7 Minimum inhibitory and bactericidal concentrations of products

Using the same organism panels as the previous agar diffusion assays, MICs and MBCs were performed using the methods described in Sections 2.4 and 2.5.

In wells where the growth was not inhibited by the honey, a button of cells is visible in the bottom of well. Where growth was inhibited there was no button of cells (Figure 3.15)

Figure 3.15 Micro-broth MIC assay in microtitre plate.



This plate shows the activity of the □ irradiated Comvita Manuka Honey UMF 18+ against 10 isolates of *E. coli*. Buttons of cells indicating cell growth can be seen in the wells to the right of the plate where the honey is most dilute. The column to the far right is the organism growth control which contained no honey,

The results of the MIC and MBC assays are shown in the graphs below (figures 3.16 – 3.21). The MIC tests confirm the findings of the agar diffusion tests with regard to the superior efficacy of the Manuka products.

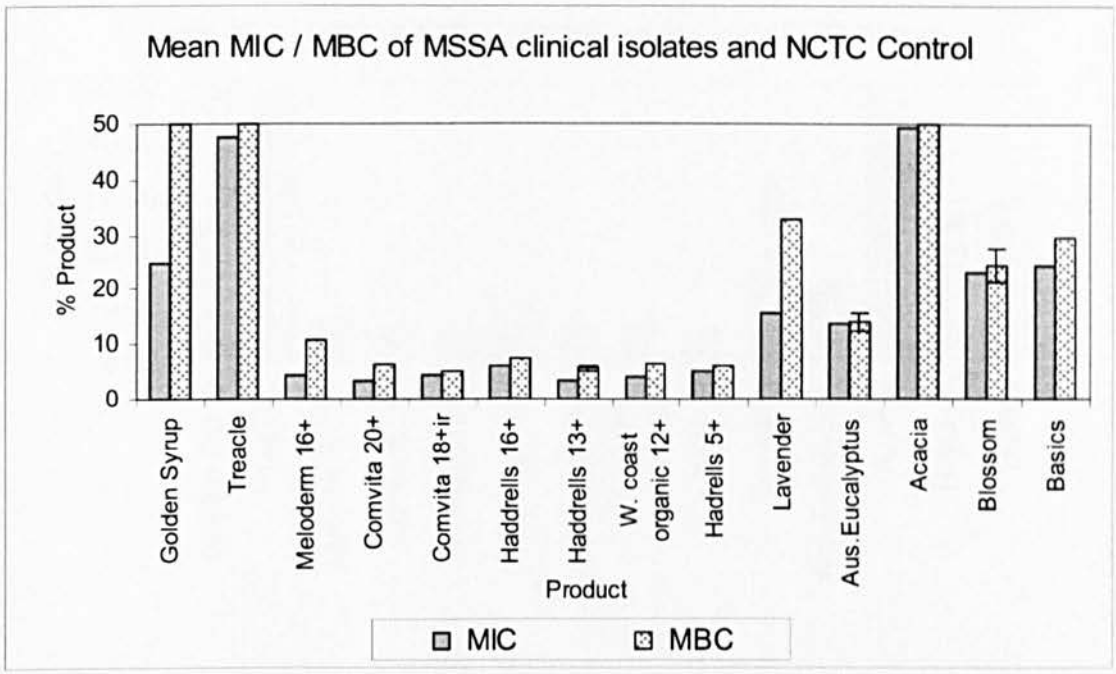
The highest concentration of product after the addition of the organism suspension was 50%. Therefore MICs and MBCs greater than the 50%

could not be measured and results of 50% on the graphs below should be interpreted as ≥50%.

The products along the x-axis from left to right show the two Syrups, followed by the 7 Manuka honeys and finally the 5 non-Manuka honeys.

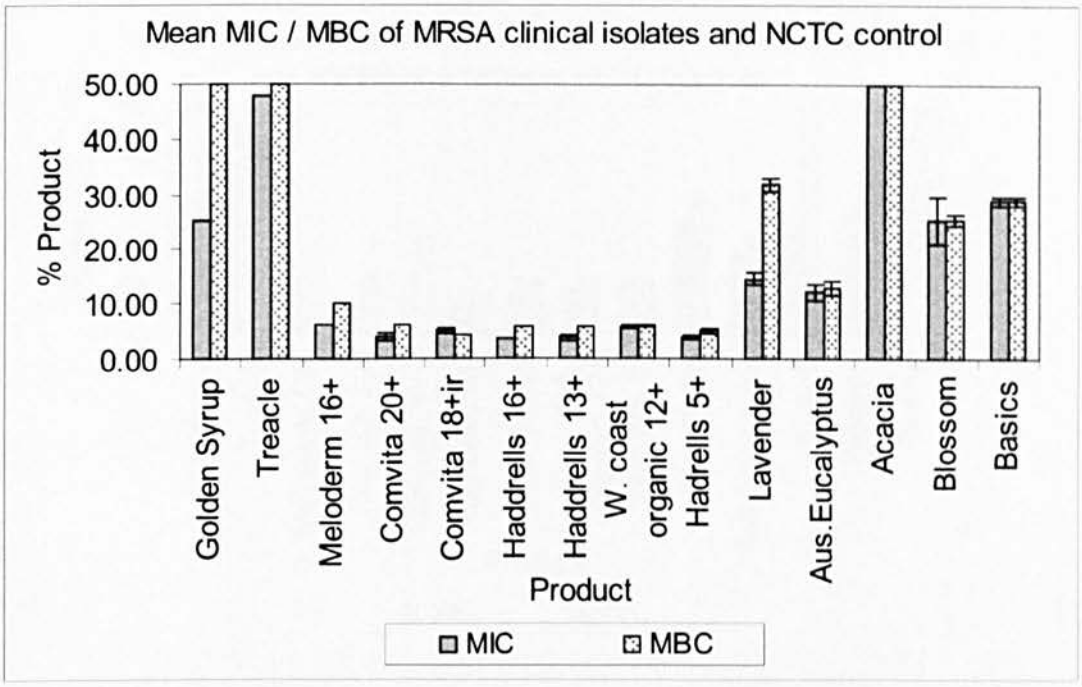
The y-axis shows the percentage dilution of each product tested giving the minimum inhibitory concentration of the product to the organism.

Figure 3.16 Mean MICs and MBCs obtained with syrup and honey products against MSSA.



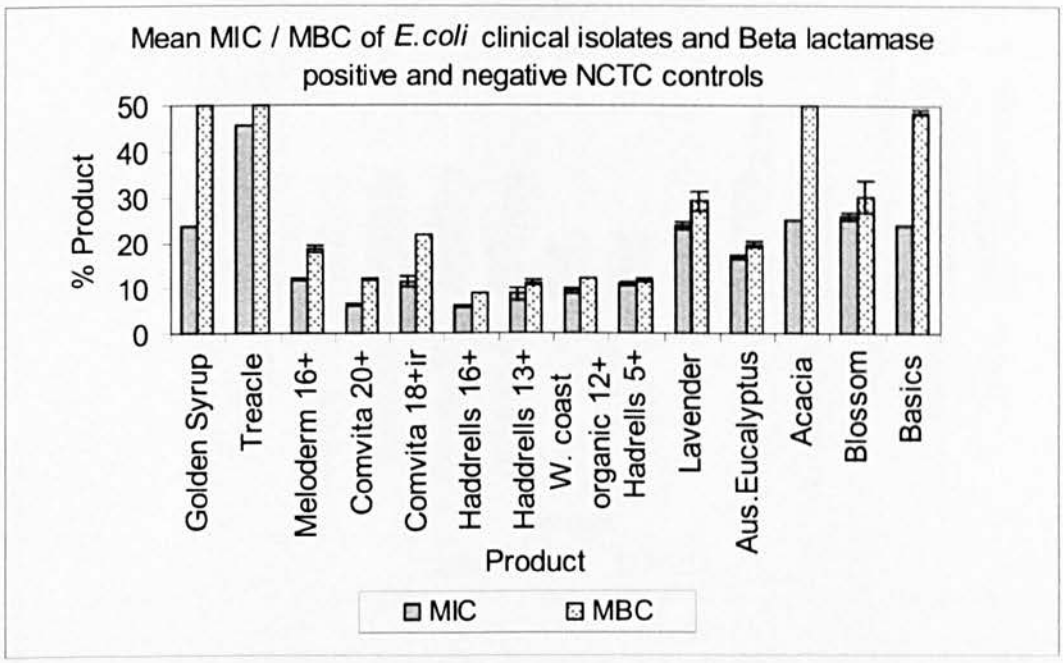
The Syrups and non-Manuka products show less antimicrobial efficacy against MSSA than the Manuka honeys with the Syrups and acacia honey failing to demonstrate bactericidal activity. All products except acacia honey showed inhibitory action. Error bars show the standard error of the mean,

Figure 3.17 Mean MICs obtained with syrup and honey products against MRSA.



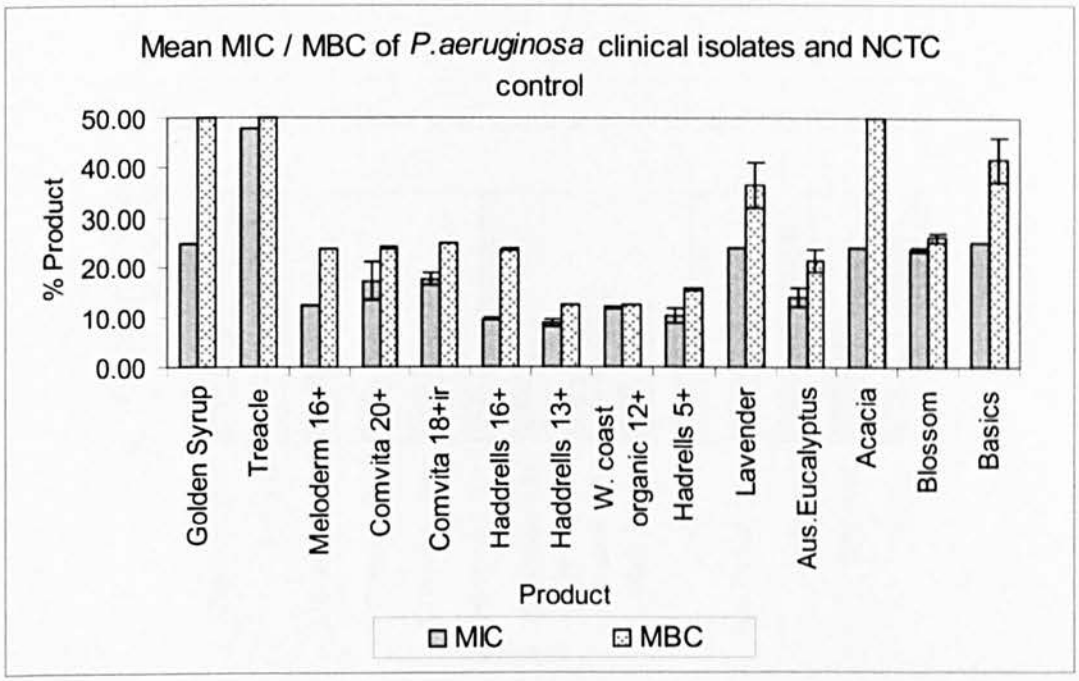
The Syrups and non-Manuka products show less antimicrobial efficacy against MRSA than the Manuka honeys, with the Syrups and acacia honey failing to demonstrate bactericidal activity. All products except acacia honey showed inhibitory action. Error bars show the standard error of the mean.

Figure 3.18 Mean MICs and MBCs obtained with syrup and honey products against *E.coli*



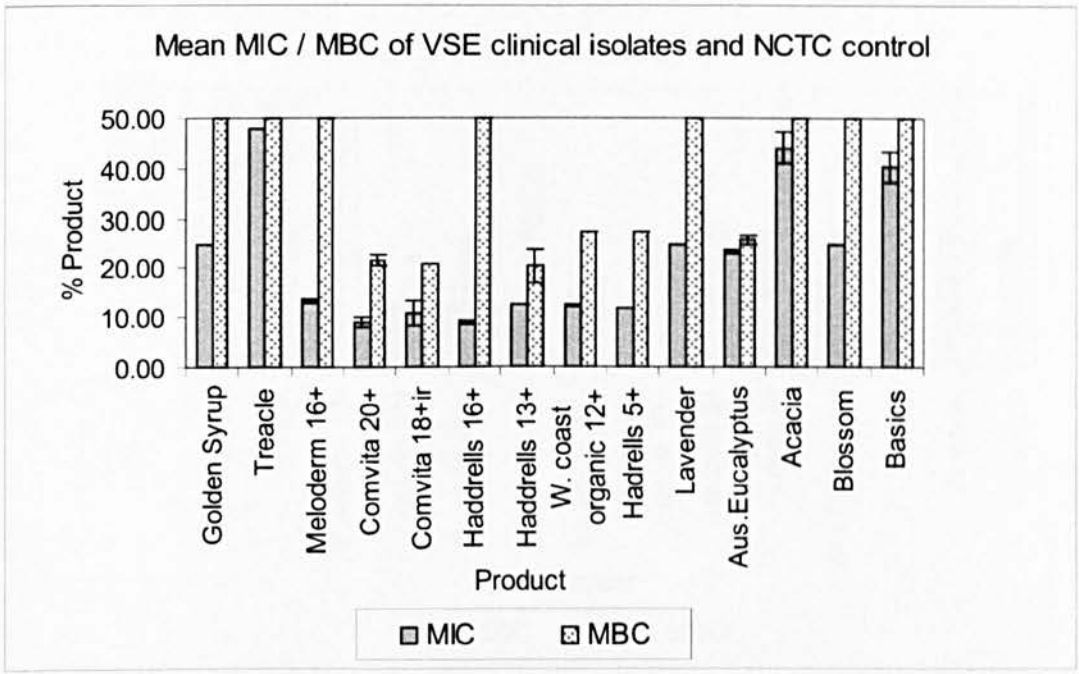
The Syrups and non-Manuka products show less antimicrobial efficacy against *E. coli* than the Manuka honeys with the Syrups and acacia honey failing to demonstrate bactericidal activity. All products showed inhibitory activity. All products demonstrated inhibitory action. Error bars show the standard error of the mean.

Figure 3.19 Mean MICs and MBCs obtained with syrup and honey products against *P.aeruginosa*



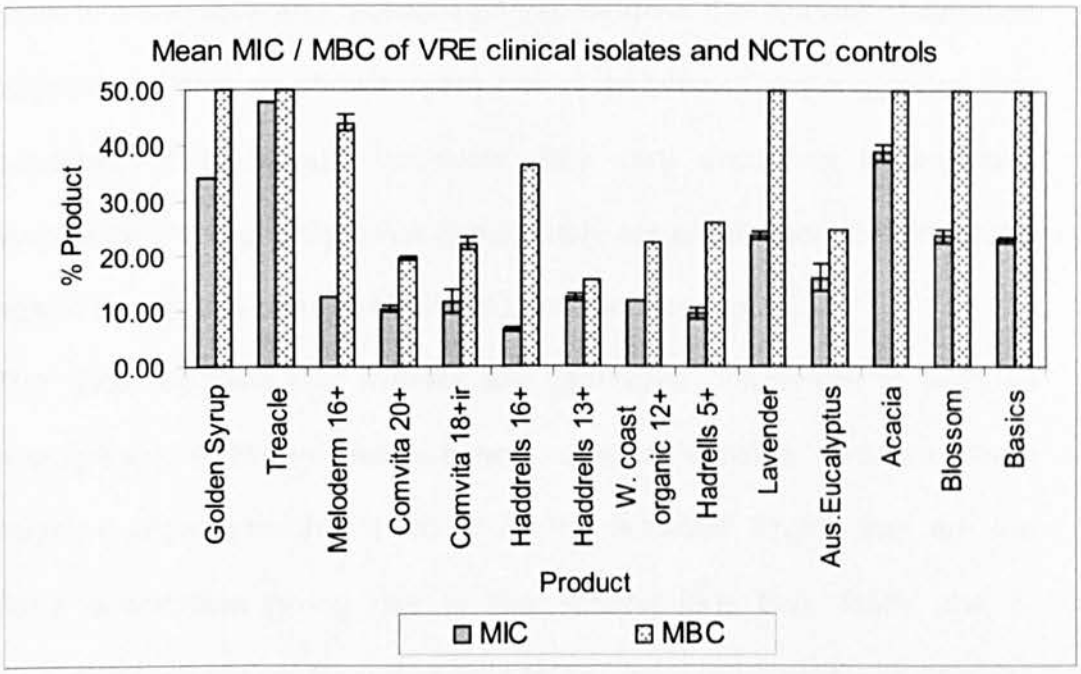
The Syrups and non-Manuka products show less antimicrobial efficacy against P. aeruginosa than the Manuka honeys. Of the non-Manuka products the Syrups and acacia honey failed to show any bactericidal activity. All products showed inhibitory action. Error bars show the standard error of the mean.

Figure 3.20 Mean MICs and MBCs obtained with syrup and honey products against VSE.



The only Manuka honey which failed to demonstrate bactericidal activity against VSE was the the Hadrells Manuka UMF 16+. Among the non-Manuka products only the eucalyptus showed bactericidal activity. All products showed inhibitory activity. Error bars show the standard error of the mean.

Figure 3.21 Mean MICs and MBCs obtained with syrup and honey products against VRE.



The Syrups and non-Manuka products show less antimicrobial efficacy against VRE than the Manuka honeys. Of the non-Manuka products only eucalyptus honey showed any bactericidal activity. All products showed inhibitory action. Error bars show the standard error of the mean.

The above results show the antimicrobial activity of the Manuka products was superior to that of the syrups and other honeys giving rise to the following observations. MRSA is less susceptible to Manuka honey than MSSA and VRE isolates appear less susceptible to Manuka honey than the VSE isolates in the panel.

In order to ascertain if there might be similar differences in inhibitory activity against the Gram negative organisms a further round of agar diffusion and MIC testing was performed on an expanded panel of Gram negative isolates described earlier in 3.2.2.

3.3.8 Agar diffusion and MIC /MBC testing of antibiotic sensitive and resistant isolates of *E. coli* and *P. aeruginosa*

Expanding the panel of *E. coli* and *P. aeruginosa* isolates and separating them into sensitive and resistant panels reduced the amount of variation between isolates as shown in the box plots below. This suggested that susceptibility to Manuka products may vary according to antibiotic resistance although this is not immediately apparent when looking at the agar diffusion box plots or MIC/MBC graphs (Appendix 6).

The data suggests that if there are significant differences in Manuka susceptibility between sensitive and resistant isolates of these Gram negative organisms that it would be the resistant strains that are the more susceptible giving rise to the observations that, firstly, the β-lactamase producing *E. coli* appear to be more susceptible to Manuka honey than β-lactamase negative isolates. Secondly, *P. aeruginosa* isolates with increased antimicrobial resistance are more susceptible to Manuka honey than antibiotic sensitive isolates.

3.3.9 MICs to compare differences in susceptibility between sensitive and resistant isolates to Manuka products.

MICs were performed using four Manuka honeys to answer the following question; is MRSA is less susceptible to Manuka honey than MSSA? Are VRE isolates are less susceptible to Manuka honey inhibition than VSE isolates? Do β-lactamase producing *E. coli* showin increased susceptibility to Manuka honey than β-lactamase negative isolates? The final question is whether *P. aeruginosa* isolates with increased antimicrobial resistance markers are more susceptible to Manuka honey inhibition than isolates with lower levels of antibiotic resistance markers?

It was difficult to ascertain if the observations were significant by looking at the results graphically (see Appendix 4 for graphical data) so an independent sample t test were performed on the data using SPSS.

3.3.9.1 Statistical analysis of MICs to compare differences in susceptibility between sensitive and resistant isolates to Manuka products.

The results of the independent samples t tests proved in some cases the observations that there are differences in susceptibility to Manuka products between antibiotic sensitive and resistant isolates within each organism group. The results are shown in Tables 3.5 – 3.8 below.

Observation 1

The independent samples t test show that MRSA are significantly less susceptible to Manuka Honey than MSSA for 3 of the 4 Manuka products using agar diffusion and for 1 of 4 Manuka products using MIC figures. Bactericidal figures did not show significantly less susceptibility for MRSA over MSSA, however it is interesting to note that the trend in all cases was for MRSA to be less susceptible to Manuka than MSSA.

Table 3.5 Susceptibility of MSSA and MRSA isolates to four Manuka products.

Product	Agar Diffusion p value	MIC p value	MBC p value
Meloderm Manuka UMF 16+	0.000	0.281	0.457
Comvita Manuka UMF 20+	0.001	0.006	0.866
Comvita Manuka UMF 18+	0.366	0.341	0.268
Haddrells Manuka UMF 5+	0.046	0.341	0.660

*** Using t tests to compare the agar diffusion zones, MICs and MBCs of MSSA and MRSA isolates a p value of <0.05 indicates that the differences were significant. Where the p value is > 0.05 the differences were not statistically significant. Where confirmation of trend is noted the data showed that the mean zones, MICs or MBCs showed MRSA to be less susceptible but the difference was too small to be considered of statistical significance.**

Observation 2

'VRE are significantly less susceptible to Manuka Honey than VSE'

The results of the independent samples t test results below do not confirm the observation using agar diffusion or MIC but in 3 out of 4 cases the honeys proved more bactericidal for the VSEs than VREs which can be seen clearly in the graphs in Appendix 4.

Table 3.6 Susceptibility of VSE and VRE isolates to four Manuka products.

Product	Agar Diffusion p value	MIC p value	MBC p value
Meloderm Manuka UMF 16+	0.165	0.000	0.000
Comvita Manuka UMF 20+	0.011	0.443	0.720
Comvita Manuka UMF 18+	0.925	0.378	0.000
Haddrells Manuka UMF 5+	0.577	0.000	0.001

Using t tests to compare the agar diffusion zones, MICs and MBCs of VSE and VRE isolates a p value of <0.05 indicates that the differences were significant. Where the p value is > 0.05 the differences were not statistically significant. Where confirmation of trend is noted the data showed that the mean zones, MICs or MBCs showed VRE to be less susceptible but the difference was too small to be considered of statistical significance.

Observation 3

'β-lactamase positive *E. coli* are more susceptible to Manuka Honey than β-lactamase negative strains.'

The independent samples t test results show that whilst there is a trend confirming the observation using agar diffusion and MIC although it is not of statistical significance' suggesting a lack of bactericidal activity.

Table 3.7 Results of t test analysis comparing susceptibility of β-lactamase positive and negative isolates with 4 Manuka products.

Observation 4

Product	Agar Diffusion p value	MIC p value	MBC p value
Meloderm Manuka UMF 16+	0.000	1.000	0.353
Comvita Manuka UMF 20+	0.118	0.009	0.720
Comvita Manuka UMF 18+	0.000	0.002	0.268
Haddrells Manuka UMF 5+	0.341	0.519	0.464

Using t tests to compare the agar diffusion zones, MICs and MBCs of β-lactamase positive and negative isolates a p value of <0.05 indicates that the differences were significant. Where the p value is > 0.05 the differences were not statistically significant. Where confirmation of trend is noted the data showed that the mean zones, MICs or MBCs showed β-lactamase positive *E.coli* to be more susceptible but the difference was too small to be considered of statistical significance.

Observation 4

'Resistant strains (i.e. reduced sensitivity to Meropenem and/or Ciprofloxacin) of *P. aeruginosa* are more susceptible to Manuka Honey than sensitive strains'

independent samples t test results confirm the observation using agar diffusion and MIC assays in each case with bactericidal activity being less conclusive.

Graphs showing all MIC and MBC results can be seen in appendix 4.

Table 3.8 Results of t test analysis comparing susceptibility of sensitive and resistant isolates of *P. aeruginosa* with 4 Manuka products

Product	Agar Diffusion p value	MIC p value	MBC p value
Meloderm Manuka UMF 16+	0.009	0.016	0.087
Comvita Manuka UMF 20+	0.000	0.000	0.810
Comvita Manuka UMF 18+	0.00	0.000	0.000
Haddrells Manuka UMF 5+	0.119	0.021	0.561

Using t tests to compare the agar diffusion zones, MICs and MBCs of sensitive and resistant isolates of *P. aeruginosa* isolates a p value of <0.05 indicates that the differences were significant. Where the p value is > 0.05 the differences were not statistically significant. Where confirmation of trend is noted the data showed that the mean zones, MICs or MBCs showed resistant *P. aeruginosa* to be more susceptible but the difference was too small to be considered of statistical significance

3.3.10 Analysis to compare differences in susceptibility between NCTC control strains and clinical isolates to Manuka products.

As with the four observed differences between sensitive and resistant isolates, four more observations were made with regard to differing susceptibility between NCTC control strains and the clinical isolates.

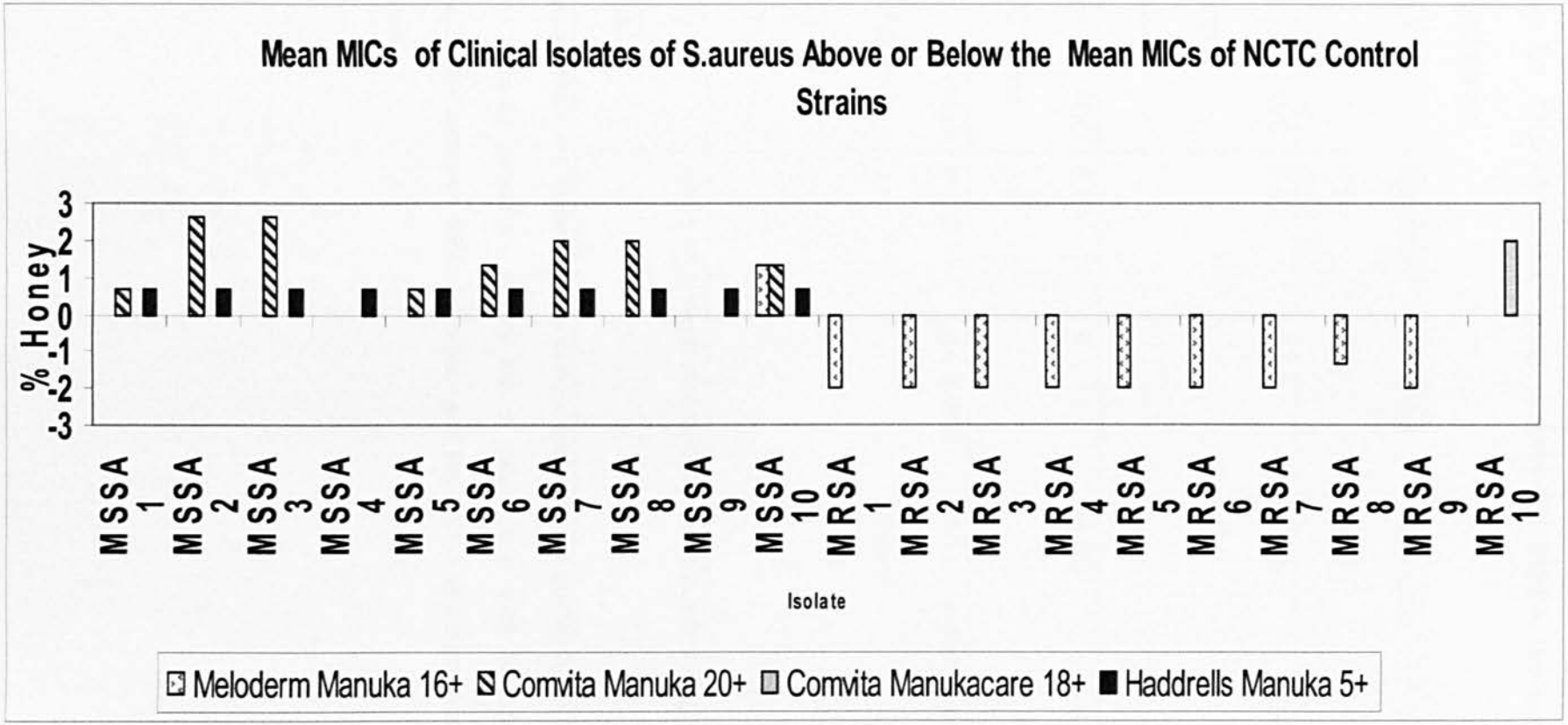
Observation 5

'MSSA isolates are less susceptible to Manuka honey that the NCTC MSSA control strain and MRSA more susceptible that the NCTC MRSA control strain'.

The graphs below (Figures 3.34 – 3.37) show the % deviation of Mean MICs of Clinical Isolates from the NCTC control mean MICs. Positive value shows the MIC is greater than that of the mean and a negative value shows the MIC is less than the NCTC control.

Tables 3.9 and 3.9 show that overall the observation was not confirmed and that MSSAs and MRSAs do not differ significantly in their susceptibility to control strains than the NCTC controls. However, the observation was confirmed for MIC of Comvita Manuka 20+ for MSSA and for the MIC of Meloderm Manuka 16+ and MRSA .

Figure 3.22 Graph showing MSSA and MRSA MICs of clinical isolates as a percentage deviation from the corresponding NCTC *Staphylococcus aureus* controls.



The zero on the y-axis is the minimum percentage of honey required to inhibit growth of the NCTC control strain of MSSA for the MSSA clinical isolates and MRSA for the MRSA isolates. Isolates requiring a higher percentage of honey to inhibit growth appear above the zero line, those below required less honey and were therefore more sucseptible

Table 3.9 ANOVA and t - test results for MSSA isolates and the control strain

MSSA	Manuka 16+	Manuka 20+	Manuka 18+	Manuka 5+
Mean MIC Clinical isolates	6.13	5.20	4.00	3.93
Mean MIC NCTC strain	6.00	4	4.00	4.0
P value Significant difference p= ≤0.05	*p*=0.651	*p*= 0.048	All values equal	*p*= 0.859
ANOVA analysis	MICs of clinical isolates *equal* to control MICs *p*=0.000			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different.

Table 3.10 ANOVA and t-test results for MRSA isolates and control strain.

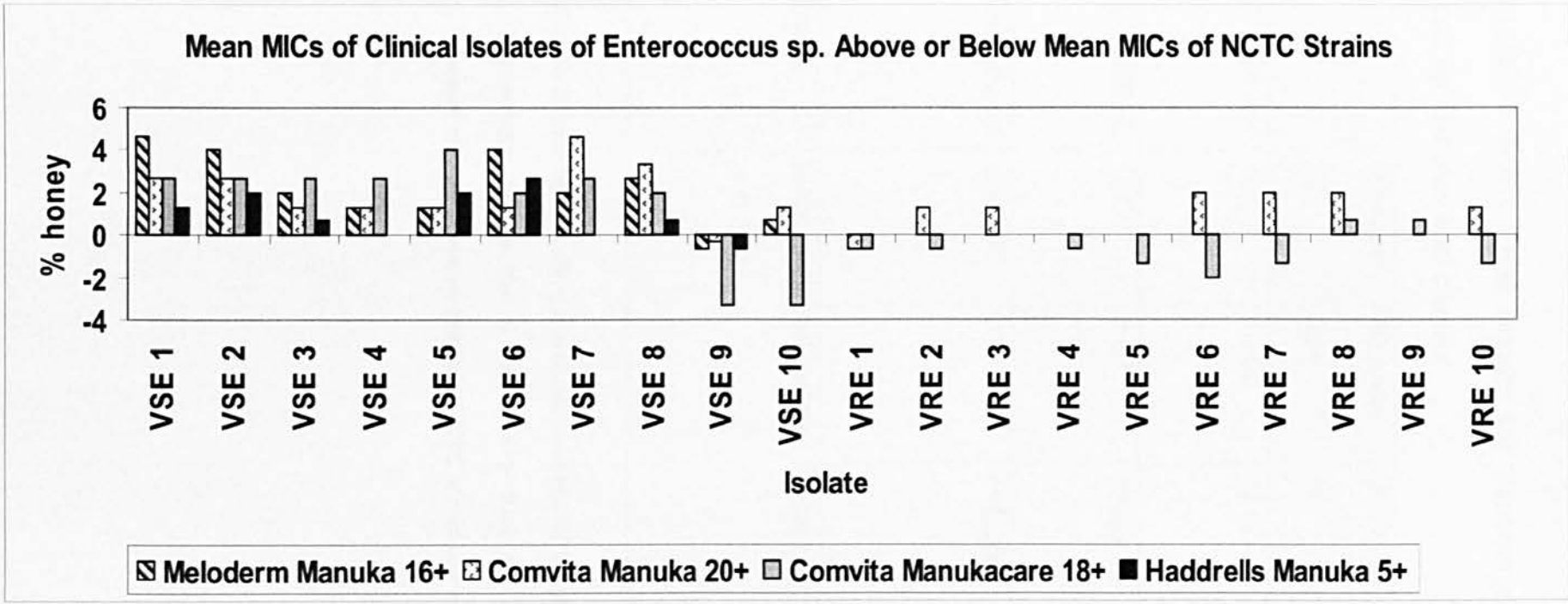
MRSA	**Manuka 16+**	**Manuka 20+**	**Manuka 18+**	**Manuka 5+**
Mean MIC Clinical isolates	6.27	6.00	4.20	4.00
Mean MIC NCTC strain	8.00	6.00	4.00	4.00
P value Significant difference $p = \leq 0.05$	$p = 0.000$	All values equal	$p = 0.580$	All values equal
ANOVA analysis	MICs of clinical isolates *equal* to control MICs $p=0.000$			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different.

Observation 6

VSE is less susceptible to the Manuka honey than the NCTC VSE control and VRE more susceptible than the NCTC VRE control.

Figure 3.23 Graph showing MICs of clinical isolates of VSE and VRE as a percentage deviation from the corresponding NCTC *Enterococcus* controls.



The zero on the y-axis is the minimum percentage of honey required to inhibit growth of the NCTC control strain of VSE for the VSE clinical isolates and VRE for the VRE isolates. Isolates requiring a higher percentage of honey to inhibit growth appear above the zero line, those below required less honey and were therefore more sucseptible

Table 3.11 ANOVA and t - test results for Vancomycin sensitive *Enterococcus* sp. isolates and control

VSE	**Manuka 16+**	**Manuka 20+**	**Manuka 18+**	**Manuka 5+**
Mean MIC Clinical isolates	16.00	10.60	12.80	12.13
Mean MIC NCTC strain	14.00	8.67	11.33	11.33
P value Significant difference p= ≤0.05	p=0.001	p=0.039	p=0.356	p=0.355
ANOVA analysis	MICs of clinical isolates *higher* than control MICs *p*=0.000			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different.

Table 3.12 ANOVA and t - test results for Vancomycin resistant *Enterococcus* sp. isolates and control.

VRE	Manuka 16+	Manuka 20+	Manuka 18+	Manuka 5+
Mean MIC Clinical isolates	12.00	10.93	13.33	10.00
Mean MIC NCTC strain	12.00	10.00	14.00	10.00
P value Significant difference p= ≤0.05	All values equal	p=0.000	p=0.430	All values equal
ANOVA analysis	MICs of clinical isolates *equal to or lower* than control MICs *p*=0.000			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different.

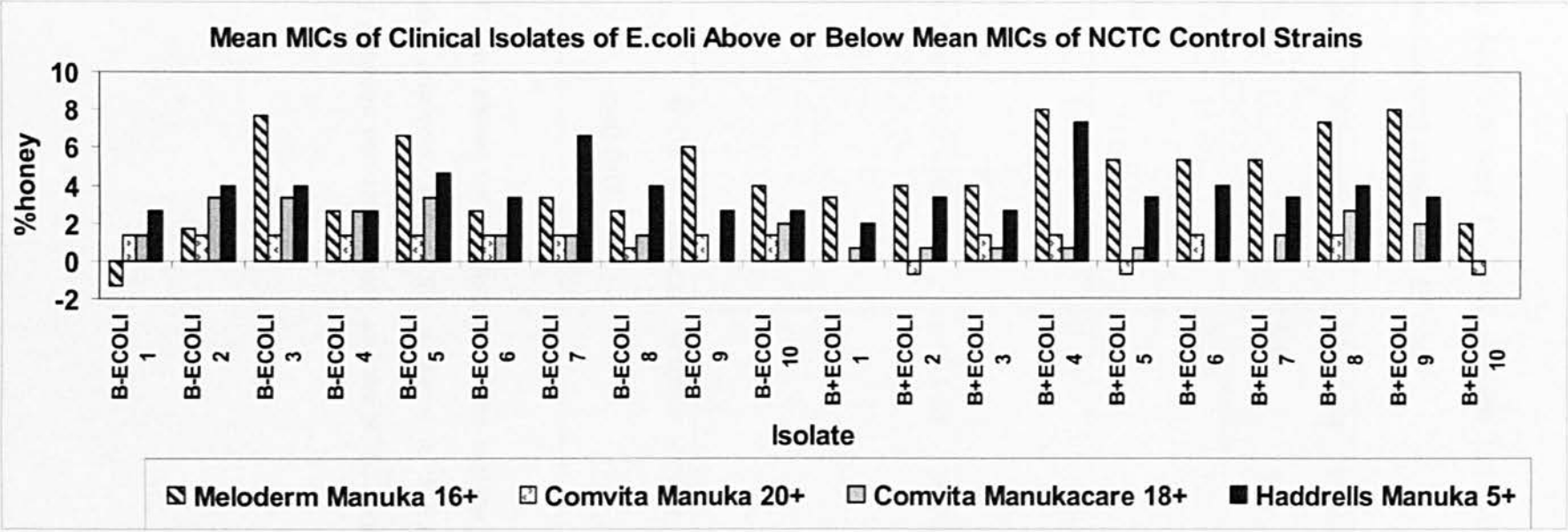
Observation 7

Clinical isolates of *E. coli* irrespective of β-lactamase producing ability were less susceptible to Manuka honey than NCTC control strains.

The graph below showing the differences of Manuka susceptibility `between clinical isolates and control isolates appear to confirm the above observation (Figure 3.36).

Tables 3.11 and 3.12 show clinical isolates of both β-lactamase negative and positive isolates of *Escherichia coli* show less susceptibility to Manuka honeys than NCTC strains. The *p* value of Meloderm Manuka 16+ was affected by one outlier in the β–lactamase sensitive isolates and the MICs of the β-lactamase positive isolates to Comvita 20+ were not significantly different from the control but did confirm the trend. ANOVA analysis of the combined values show that the clinical isolates of *E.coli* exhibited increased resistance to the Manuka products than the NCTC controls (Tables 3.11 and 3.12) confirming the hypothesis that clinical isolates are less susceptible to Manuka honeys than NCTC control strains.

Figure 3.24 Graph showing β-lactamase negative and β lactamase positive clinical isolates of *Escherichia coli* MICs as a percentage deviation from the corresponding NCTC *Escherichia coli* controls



The zero on the y-axis is the minimum percentage of honey required to inhibit growth of the corresponding NCTC control strain of β lactamase positive or negative *E. coli.* Isolates requiring a higher percentage of honey to inhibit growth appear above the zero line, those below required less honey and were therefore more susceptible

Table 3.13 ANOVA and *t* - test results for β-lactamase negative isolates and control strains of *E. coli*

β–lactamase negative *E.coli*	**Manuka 16+**	**Manuka 20+**	**Manuka 18+**	**Manuka 5+**
Mean MIC Clinical isolates	16.86	7.93	12.67	13.93
Mean MIC NCTC strain	13.33	6.66	10.67	10.00
P value Significant difference p= ≤0.05	p=0.052	p=0.005	p=0.017	p=0.007
ANOVA analysis	MICs of clinical isolates *higher* than control MICs *p*=0.007			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different.

Table 3.14 ANOVA and t - test results for β-lactamase positive isolates and control

β–lactamase positive *E. coli*	**Manuka 16+**	**Manuka 20+**	**Manuka 18+**	**Manuka 5+**
Mean MIC Clinical isolates	17.07	6.93	10.93	13.33
Mean MIC NCTC strain	12.00	6.67	10.0	10.00
P value Significant difference p= ≤0.05	p=0.000	p=0.703	p=0.215	p=0.017
Observationconfirmed Y/N	Y	N confirms trend	N confirms trend	Y
ANOVA analysis	MICs of clinical isolates *higher* than control MICs *p*=0.000			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is >0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different

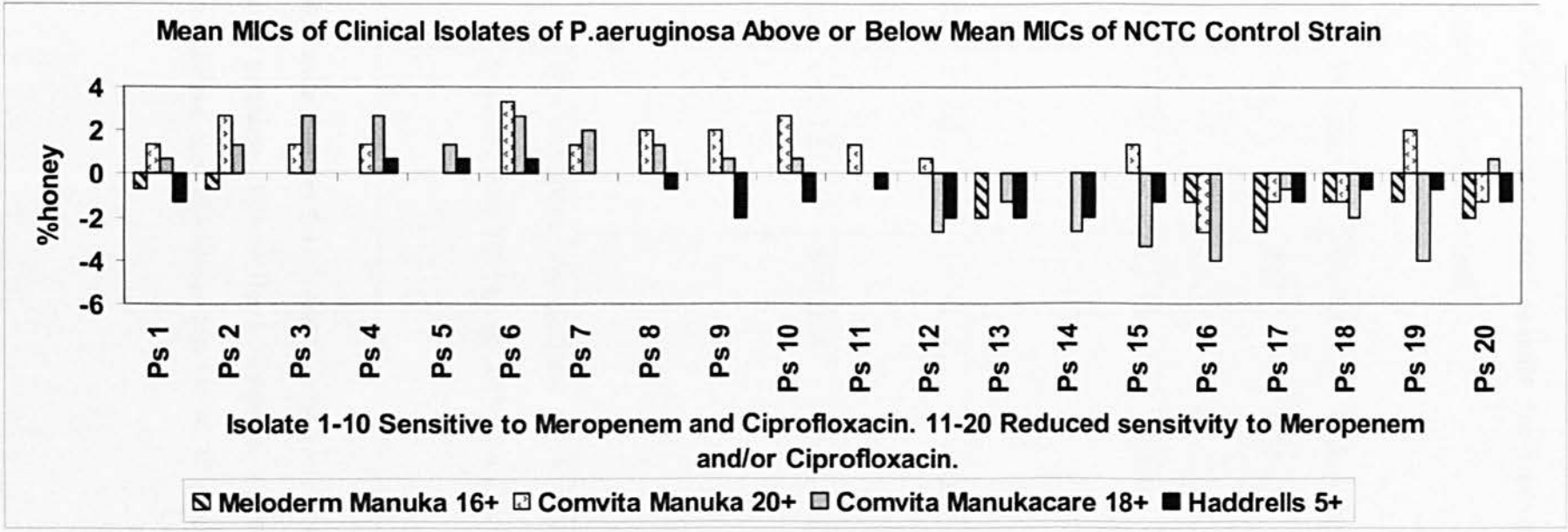
Sensitive isolates of *P. aeruginosa* are less susceptible to Manuka honey than the NCTC control strain and those strains with increased antibiotic resistance are more susceptible to Manuka honey than the NCTC control strain

The graph (Figure 3.37) shows that the MICs of the clinical isolates are not equal to the control MIC but nor can they be said to be consistently higher or lower than the control.

Statistical analysis using ANOVA showed that amongst the sensitive isolates of *P. aeruginosa* there was no statistical significance in the number of clinical isolates with MICs greater, less than or equal to the Control MICs (Table 3.13)

The ANOVA performed on the MICs of the resistant isolates showed that overall the MICs of clinical isolates were lower than the MICs of the controls even though the differences shown with the individual products were only significant with two of the products (Table 3.14).

Figure 3.25 Graph showing MICs of clinical isolates of *Pseudomonas aeruginosa* as a percentage deviation from the NCTC *Pseudomonas aeruginosa* control.



The zero on the y-axis is the minimum percentage of honey required to inhibit growth of the NCTC control strain of *P. aeruginosa.* Isolates requiring a higher percentage of honey to inhibit growth appear above the zero line, those below required less honey and were therefore more susceptible

Table 3.15 ANOVA and t - test results for sensitive *Pseudomonas aeruginosa* isolates and control

P. aeruginosa	**Manuka 16+**	**Manuka 20+**	**Manuka 18+**	**Manuka 5+**
Mean MIC Clinical isolates	11.87	13.13	15.60	7.67
Mean MIC NCTC strain	12.00	11.33	14.00	8.00
P value Significant difference p= ≤0.05	p=0.757	p=0.074	p=0.082	p=0.775
ANOVA analysis	No statistical significance in number of Clinical MICs greater, less than or equal to the Control MICs			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different

Table 3.16 ANOVA and t - test results for resistant *Pseudomonas aeruginosa* isolates and control.

***P.aeruginosa* resistant isolates**	**Manuka 16+**	**Manuka 20+**	**Manuka 18+**	**Manuka 5+**
Mean MIC Clinical isolates	10.93	10.60	11.8	6.8
Mean MIC NCTC strain	12.00	11.33	14.00	8.00
P value Significant difference p= ≤0.05	p=0.250	p=0.505	p=0.050	p=0.48
ANOVA analysis	MICs of clinical isolates significantly lower than MICs of control strains p=0.00			

The mean MICs are shown for the clinical isolates and the NCTC strain with each of the Manuka products. Where the p value is > 0.05 the differences in susceptibility between clinical isolates and the NCTC strain are not statistically different.

3.4 Discussion

The aim of this first area of study was to ascertain the antimicrobial activity of Manuka honey compared to other sugar and honey products against a variety of micro- organisms isolated from a clinical environment which have the potential to infect or delay the healing of wounds. The methods used were conventional and modified only so far as to accommodate the novel nature of the products being studied.

The initial sterility screen was performed to confirm that honey and Syrup are not necessarily naturally sterile (Midura *et al.*, 1979; Cooper and Molan 1999a; Nevas *et al.*, 2002).and to assess the nature of the organisms which can survive in such an environment. The results show that *Bacillus* species, a spore producing organism, is capable of survival in these osmotically challenging products and that they can go on to germinate when the environment becomes favourable. Other studies have reported the isolation of *Clostridium* species (Midura *et al.*, 1979; Cooper and Molan 1999a; Nevas *et al.*, 2002) from honey products. This was not a finding for the products used in this study.

The growth of *Bacillus* sp. in some of the products was a potential challenge using agar diffusion and MIC/MBC assays. On the agar diffusion Figures and the MIC microtitre wells, the bacillus growth was infrequent and sporadic, suggesting small numbers of spores distributed randomly throughout the product. When *Bacillus* contamination occurred it was clearly discernable and was discounted, when the growth obscured the target organism, that zone or well was excluded.

Confirmation that sporulating organisms can survive and be recovered from these natural products does confirm that irradiation or sterilisation of products are of value for clinical use particularly in the immunocompromised patient where even organisms of low pathogenicity can threaten the health of the patient.

The activity screen was performed to ascertain what level of microbial killing the products may exhibit. The information yielded was useful to ascertain the degree of dilution required for comparative agar diffusion study. The fact that several products had a negligible impact upon organism growth was an unexpected finding as it suggests that the activity of honey, particularly the Manuka products, is due to more than just the osmotic effect of sugar and that sugar alone is of limited value.

The agar diffusion studies clearly showed that the Manuka products had greater antimicrobial activity against all the groups of micro-organisms. The non-Manuka products showed little or no antimicrobial activity in the first round of testing but antimicrobial activity increased when the volume was increased from 75 µl to 100 µl for the second round of testing, where the Australian Eucalyptus performed particularly well against *P. aeruginosa*.

The grouping into homogenous subsets based on antimicrobial activity (Appendix 3) show that there were no statistical differences between the antimicrobial efficacy of Golden Syrup, Treacle, Acacia Honey and Sainsbury's Basics; all were equally ineffective. The Spanish Lavender and Eucalyptus Honeys performed slightly better than the other non-

Manuka products against *S. aureus* isolates, with the Eucalyptus having some measurable activity against the Gram negative organisms. Manuka products were statistically significantly better against all the organisms than the non-Manuka products, with Enterococci demonstrating the most resistance. The Comvita Manuka UMF 20+ was most regularly in the subset with greatest efficacy but none of the products consistently out performed the others. There are several reasons which could account for this. The first round of agar diffusion tests were performed without a positive displacement pipette which prevented accurate volumes being inoculated into the wells. These initial experiments provided a baseline dataset. The introduction of the positive displacement pipette addressed this accuracy issue in the subsequent activity testing experiments. The differences in honey volume and number of samples used in the first method and second methods prevent direct comparison of zone sizes between methods. An interesting technical challenge which became apparent was the difference in product consistency between the products. The Acacia honey for example was visibly the most liquid and the West Coast Organic Manuka UMF 12+ notably granular giving it the appearance of containing undissolved granulated sugar. The variation in viscosity between products created possible variations in the diffusion into the agar. These factors were particular issues with agar diffusion, but the third and possibly most significant factor which constituted a variable in all the methods used, is that honey is a natural product which means it will vary from batch to batch. Homogeneity not only cannot be

guaranteed, but can alter with age and storage conditions (Stephens *et al.*, 2010), the significance of which is discussed further in Chapter 7. The work in this chapter has sought to address many practical aspects of working productively with honeys and to seek to refine and standardise approaches to generating novel, meaningful and repeatable findings.

The results obtained using MICs and MBCs were used to analyse differences between the susceptibility to the products of antibiotic sensitive and resistant isolates and between the clinical 'wild type' isolates and the NCTC 'laboratory' control isolates. Although some of the issues with variables mentioned above would still apply, viscosity and ability to permeate the medium was reduced by the use of a liquid medium. The results of the MIC testing confirmed that Staphylococci were demonstrably the most susceptible of the organism groups with lower MICs and MBCs than the other groups. Enterococci had MICs more akin to the Gram negative organisms but with generally the highest MBCs of all the groups.

Comparative analysis of susceptibility between sensitive and resistant isolates within each organism group showed interesting trends with the resistant Gram positive organisms being more resistant to the four Manuka honeys and the resistant Gram negatives being more sensitive. β-lactamase is an enzyme which hydrolyses the β-lactam ring, neutralising the antimicrobial activity (Bush and Fisher, 2011). Meticillin is a β-lactam antibiotic which has high affinity for, and thus blocks Penicillin Binding Proteins (PBP), which are transpeptidase enzymes

required for peptidoglycan synthesis in bacterial cell walls (Llarrull, *et al.*, 2009). Meticillin resistance is conferred by the *mec-A* gene which codes for PBP 2a; this does not bind Meticillin allowing cell wall synthesis to continue (Guignard *et al.*,2005). Vancomycin is a glycopeptide and it acts by forming complexes with precursors of peptidoglycan. Resistance occurs when there is alteration of the substrate specificity of peptidoglycan precursors (Courvallin, 2006). There are many different genes that code for β-lactamase, TEM-1, SHV-1 and OXA-1 to name but a few (Livermore, 1998). The *E. coli* isolates used in these investigations were divided into those which were producers of β-lactamase and therefore showed resistance to β-lactam antibiotics and those which were not, with some of the β-lactamase producers also showing aminoglycoside resistance. The *Pseudomonas* isolates showed resistance to carbapenems, another group of β-lactam antibiotics and some had intermediate susceptibility to fluroquinolones. Aminoglycosides inhibit protein synthesis by binding to ribosomes and resistance may occur by reduced uptake or by modification enzymes which alter the structure of the drug (Mingeot-LeClerq *et al.*, 1999). Fluoroquinolones inhibit DNA gyrase and mechanisms of resistance include both modifications of the drug target and alterations in permeability (Hooper, 2001, Lister *et al.*, 2009). The organisms in this study were selected phenotypically and their genetic mechanisms of resistance were not characterised, but the observed difference in susceptibility to

antimicrobials having a bearing on their susceptibility to honey is an area which may well warrant future research.

The comparison of clinical and control organisms illustrate the necessity for including clinical isolates alongside controls when researching anti-microbial activity.

Whilst the clinical isolates of both Meticillin sensitive and resistant Staphylococci and vancomycin resistant Enterococci appear to behave comparably to the NCTC control strains, the other organism groups do not. The β –lactamase negative and positive *E.coli* were shown to be less susceptible than the controls and the clinical isolates of *Pseudomonas* MICs fluctuated evenly either side of the control values.

The reasons for this are not clear but along with the differences found between antibiotic sensitive and resistant isolates suggest that there is the possibility that in a clinical setting, micro-organisms may have the potential to develop mechanisms of resistance to honey products.

The findings in this section have provided an understanding of how micro-organisms behave in the presence of honey and sugar products.

The work has shown that Manuka products exhibit a greater antimicrobial effect than non-Manuka products which is not necessarily consistent with their marketed UMF activity rating. The findings suggest that when investigating organism susceptibility to honey products, the inclusion of wild strains with the control strains is of value. Additionally, the work presented here would indicate that there is a requirement for sterilisation of honey products destined for use in a clinical setting.

3.5 Conclusions

The Manuka honeys used in this study appearer to show greater antimicrobial activity than non-Manuka honeys and Syrups confirming that antimicrobial activity is attributable to more than just sugar content. The work also indicated that a higher UMF rating of Manuka honey does not necessarily ensure greater efficacy than a lower rated UMF Manuka honey.

Data from this chapter suggested that MRSA and VRE may be slightly less susceptible to the inhibitory effects of Manuka honey than MSSA and VSE. In contrast Gram negative such as β-lactamase positive strains of *E.coli* and isolates of *P. aeruginosa* which have reduced susceptibility to meropenem and/or ciprofloxacin may be slightly more susceptible to the inhibitory effects of Manuka honey than β-lactamase negative *E.coli* or *P. aeruginosa* isolates which are sensitive to meropenem and ciprofloxacin.

When examining the control strains it was found that there was little or no statistical significance difference between clinical isolates of VRE and NCTC strains of VRE, between MRSA and MSSA NCTC control strains and clinical isolates of *S. aureus,* in terms of susceptibility to Manuka honey. This observation was also recorded with clinical isolates of *P. aeruginosa* regardless of antimicrobial susceptibility in terms of susceptibility to Manuka honey when compared to the NCTC control strain.

However, in contrast, clinical isolates of VSE appeared less susceptible to Manuka honey than the NCTC strain of VSE Similar observations were made with both the β-lactamase positive and β-lactamase negative *E. coli* are less susceptible to Manuka honey than the NCTC control strains.

CHAPTER 4

Effect of Bioload on Antimicrobial Activity of Medical Grade Manuka Honey

4.1 Introduction

Virulent organisms can cause infection in relatively low numbers (Edwards and Harding, 2004), but a high bioload of less virulent organisms can colonise wounds challenging the ability of the body to heal (Dow *et al.*, 1999, Bowler *et al.*, 2001). Studies have shown Manuka honey to be an effective remedy for non-healing wounds (Hejase *et al.*, 1996, Efem *et al.*, 1988, Tahmaz *et al.*, 2006) but these have not investigated the maximum bioload at which topical honey maintains antimicrobial efficacy.

Methods adopted to investigate the antimicrobial efficacy of products against micro-organisms *in vitro*, involve a predetermined number of organisms per unit volume as described in the previous chapter. In order to challenge the limits of antimicrobial efficacy of medical grade Manuka honey against high concentrations of bacteria, assays were performed not only against the individual isolates previously studied but against polymicrobial populations obtained from clinical wound swabs.

To study actual polymicrobial populations one might expect to find in non-healing wounds (Bowler, 1998, Bowler and Davis, 1999, Bowler *et al.*, 2001), wound swabs were obtained from the Microbiology Department of a large District General Hospital (Section 2.7.5.1). The wound flora present was determined and their susceptibility to the medical grade honeys was determined to establish whether or not they exhibited greater susceptibility or resistance than seen with the previous bacterial MICs.

The aim of the investigations in this section of the study is to ascertain the bioload limit at which Manuka products maintain their antimicrobial efficacy against single species and polymicrobial populations and the volume of product that would be required to achieve the desired outcome.

4.2 Methods

4.2.1 Bioload assay 1 using NCTC strains

The method 2.6.1 described in Section 2 was performed to ascertain the antimicrobial efficacy of medical grade honey against suspensions of organisms of increasing McFarland densities. The experiment was performed using NCTC controls.

4.2.2 Bioload assay 2 using isolates from polymicrobial wound infections

The selected wound swabs were cultured to determine the bacteria within each wound population (See section 2.7.5.1). MICs were performed on organism species not previously included in Chapter 3. MICs were also performed on the polymicrobial populations to determine if organism susceptibly to Manuka altered in the presence of other bacterial species.

The bioload assay described in section 2.6.3 was to determine the antimicrobial capacity of Manuka honey against high concentrations of wound flora.

4.3 Results

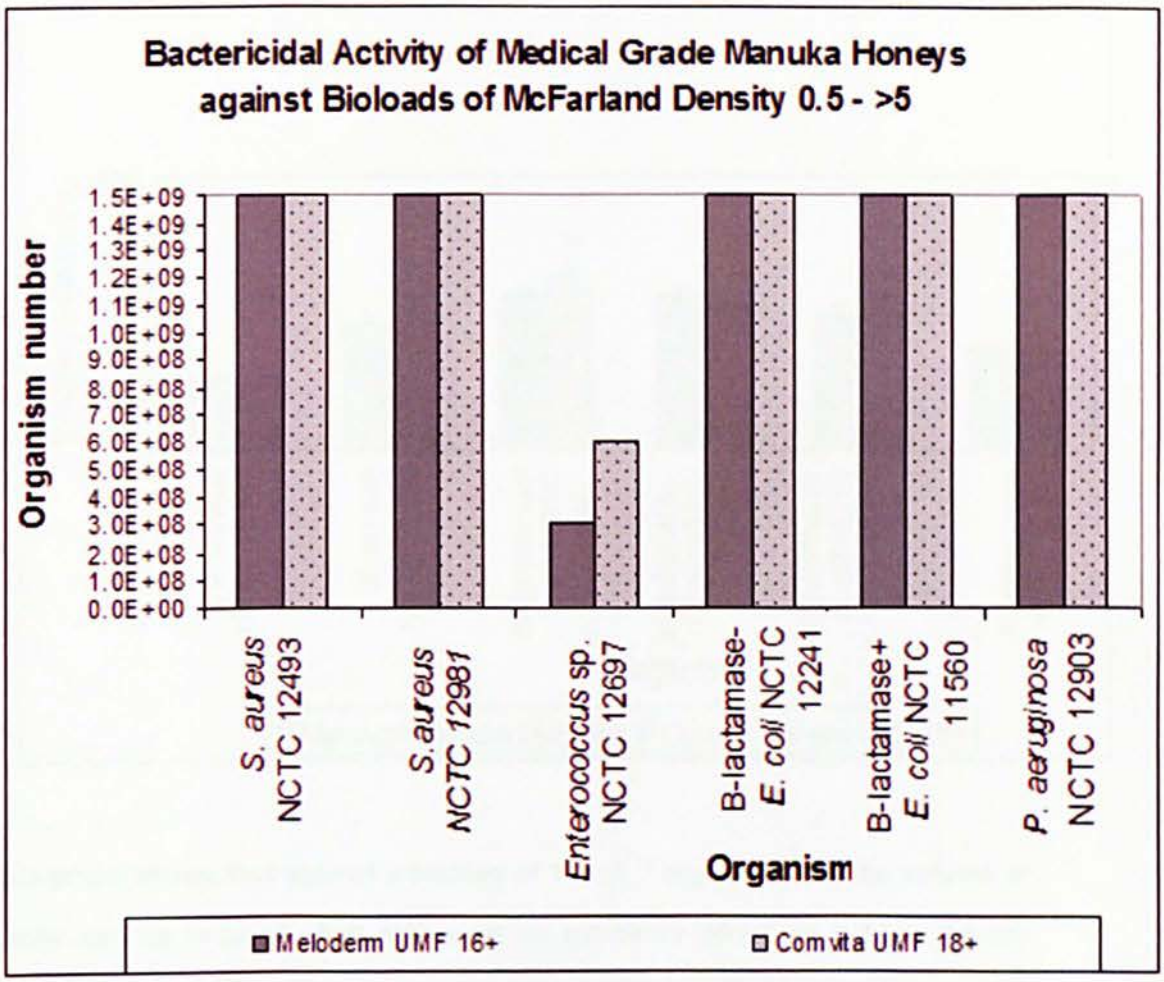
4.3.1 Bioload Assay 1

The honeys were able to exert a bactericidal effect up to a McFarland density of 5 for all NCTC isolates with the exception of *Enterococcus* sp. for which the honey of UMF18+ was bactericidal up to a McFarland 3 and the Manuka 16+ up to a McFarland 2.

Figure 4.1 below shows the organism density at which 99.9% bacterial killing was achieved. Both products were also able to kill *S. aureus, E. coli* and *P. aeruginosa* at a McFarland 5 density (approx 1.5 x 10^9 orgs/ml) whilst Enterococci were shown to be more resilient, as bactericidal activity was not achieved above 3-6 x 10^8 orgs/ml.

Using equal volumes of product and suspension was bactericidal with regard to Enterococci although a volume of 65 µl in 100µl was sufficient to inhibit the growth. Other organisms were shown to be more susceptible as can be seen in Figures 4.2 and 4.3 below.

Figure 4.1 Bactericidal activity of medical grade Manuka honey on NCTC organisms at different Mcfarland densities.

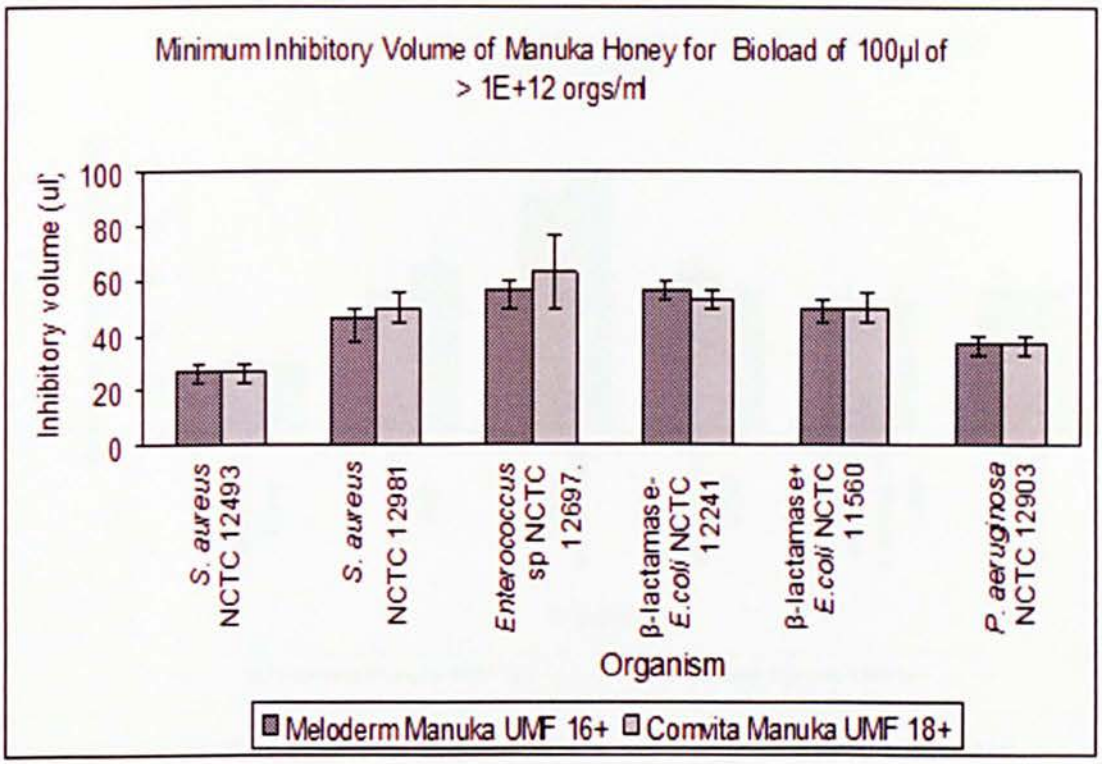


This graph shows that a 50% concentration of honey exerts an bactericidal effect against an organism concentrations greater than that used in BSAC sensitivity and MIC, MBC protocols (Andrews 2001). It can be seen that both medical grade products had bactericidal activity against a McFarland 5 density of NCTC strains of *S. aureus, E.coli* and *P. aeruginosa.* Neither product demonstrated bactericidal activity against NCTC strains of *Enterococcus* sp. Above a McFarland 2 density. The Macfarland densities equate to organisms per ml as follows.

McFarland Key: 0.5 = 1.5×10^8 1 = 3×10^8 2 = 6×10^8

3 = 9×10^8 4 = 1.2×10^8 5 = 1.5×10^9

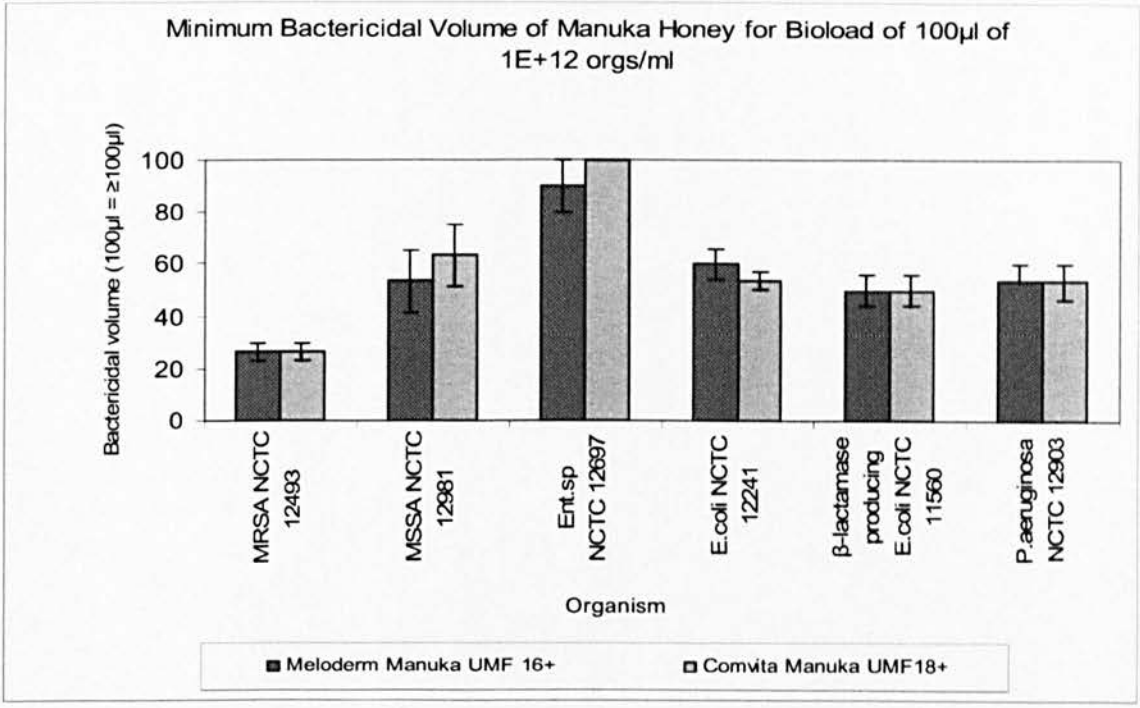
Figure 4.2 Volume of Manuka honey required to inhibit growth of 100 µl of 1 x 10^{12} organisms/ml using NCTC strains.



This graph shows that against a bioload of 1 x 10 ^{12} organisms/ml, the volume of honey can be reduced. And still exert an inhibitory effect on a high density organism population. In order to inhibit the growth 100 µl of 1 x 10^{12} organisms/ml of Enterococci a minimum volume of 65 µl was required. Of all the organism groups Staphylococci required the smallest volume of honey to inhibit growth, that being 27 µl.

Error bars show the standard error of the mean.

Figure 4.3 Volume of Manuka honey required for bactericidal activity against 100 µl of 1 x 10^{12} organisms/ml using NCTC strains..



This graph shows that against a bioload of 1 x 10 ^{12} organisms/ml, a reduced volume of honey exerted a bactericidal effect on a high density organism population An equal volume of Comvita Manuka UMF 18+ was required for bactericidal effect on Enterococci. Error bars show the standard error of the mean.

4.3.2 Characterisation of organism population

The identification of organisms within each population were determined, the results can be seen in Table 4.1 below.

4.3.2.1 Identification of wound isolates

The organisms identified in each wound are shown in Table 4.1 below

Table 4.1 Organisms isolated from selected clinical wound swabs

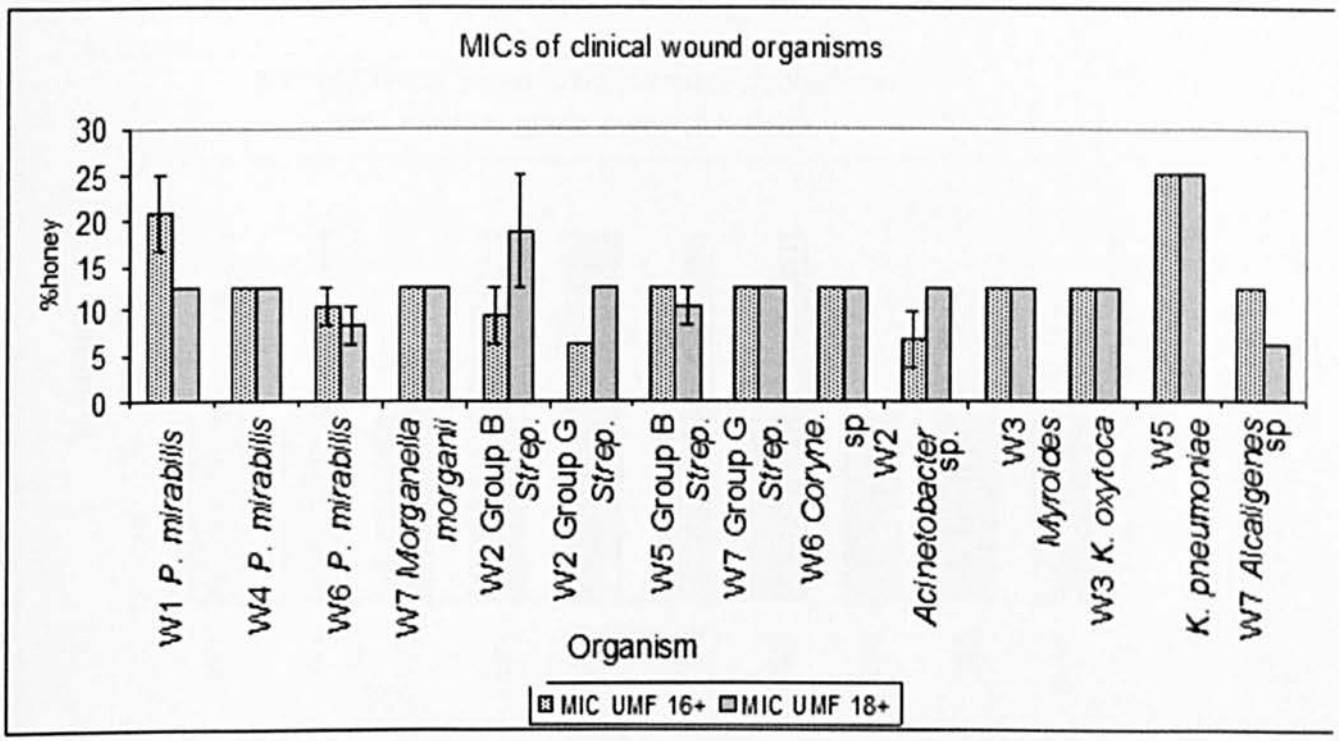
Swab number	Site	Isolates				
Wound 1	Abdominal wound	*Enterococcus* sp. *1*	*Enterococcus* sp. *2*	*Proteus mirabilis*		
Wound 2	Axilla wound	*Acinetobacter* sp.	β Haemolytic *Streptococcus* Lancefield group B	β Haemolytic *Streptococcus* Lancefield group G	*E. coli*	
Wound 3	Leg wound	*Myroides* sp.	*Klebsiella oxytoca*	*Mixed anaerobes*	*Clostridium* sp.	
Wound 4	Knee wound	*Enterococcus* sp.	*Proteus mirabilis*	*E. coli*		
Wound 5	Thumb wound	β Haemolytic *Streptococcus* Lancefield group B	*Klebsiella pneumoniae*	*S. aureus*		
Wound 6	Toe wound	*P. aeruginosa*	*Proteus mirabilis*	*Corynebacterium* spp	*Enterococcus* sp.	*Mixed anaerobes*
Wound 7	Wound swab	*Enterococcus* sp.	β Haemolytic *Streptococcus* Lancefield group G	*Alcaligenes faecalis*		
Wound 8	Sacral wound	*S. aureus*	*Enterococcus* sp.	*E. coli*		
Wound 9	Foot wound	*S. aureus*	*Enterococcus* sp.	*Morganella morganii*	*E. coli*	
Wound 10	Ear swab	*P. aeruginosa 1*	*P. aeruginosa 2*	*S. aureus*		

4.3.2.2 MICs of wound isolates with medical grade Manuka honey

The isolates collected from the polymicrobial clinical wounds included organism genera which had not been previously included in this study. MICs were performed to establish if any of these isolates showed an increased resistance or susceptibility to the Manuka products when compared to the organisms studied in Section 3 of this study. It can be seen in the figures below that the MICs of these individual wound isolates of genera included in Section 3 were all within the range of MICs demonstrated by the previously tested organism groups.

The results can be seen in figure 4.4 below.

Figure 4.4 MIC of individual wound isolates of previously unstudied genus and species.

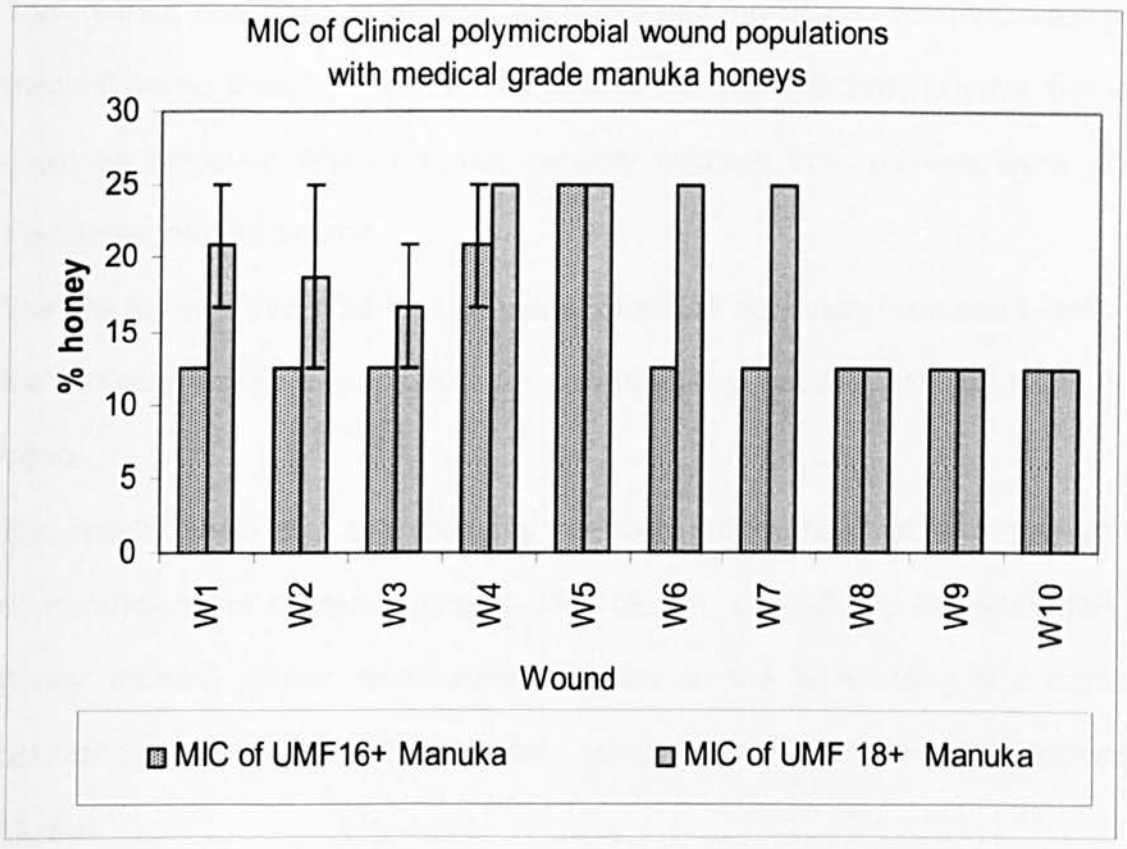


These isolates belonged to genera which had not been previously assayed. The graph shows the MICs of each organism isolated and the wound population from which it was isolated. It can be seen that all organisms were inhibited by both Manuka honeys at a honey concentration of ≤ 25% which are within the ranges of MICs of *S. aureus, Enterococcus* sp., *E. coli* and *P. aeruginosa.*

Error bars represent standard error of the mean, where no bars are shown the SEM was too small be seen.

The MICs of each polymicrobial wound population with each of the medical honeys are shown in Figure 4.5 below.

Figure 4.5 MIC of wound polymicrobial organism populations.



The MICs of each polymicrobial wound population shows that all organism populations were inhibited by both Manuka honeys at a honey concentration of ≤ 25%. These results suggest that the susceptibility of organisms to Manuka honey does not alter when they exist in a polymicrobial environment **Error bars represent standard error of the mean, where no bars are shown the SEM was too small be seen.**

4.3.2.3 Bioload assay to determine antimicrobial efficacy of medical grade Manuka honey on individual aerobic isolates from polymicrobial wound populations.

The method described in Section 2.6.3 involved inoculating colonies directly into medical grade Manuka honeys. The results showed with both honeys that killing could be achieved when colonies recently cultured from patients were directly inoculated into the product.

The results in tables 4.2a to 4.2k below recorded how many microtitre wells from the triplicate testing were positive for growth after subculture using a sterile cotton swab.

The results show that the products maintain antimicrobial activity against high concentrations of bacteria (*circa* 4 $x10^8$ cfu/ml). Overall the Manuka UMF 16+ honey showed similar antimicrobial efficacy to the UMF 18+. The increased resistance of some of the *Enterococcus* sp. isolates is consistant with the previous studies.

Interestingly the efficacy of the honey products appear to be greater at 75% concentration than when used undiluted. It also appears the organisms showing the greater resilience were the Gram positive organisms including the Staphylococci which were previously shown in chapter 3 to be highly susceptible to Manuka products.

Table 4.2 a. Bioload challenge using medical grade Manuka products against Wound 1 isolates

Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	>4.00E +08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E +08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 1												
Enterococcus sp. (1)	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
Enterococcus sp. (2)	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
P. mirabilis	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –

Each of the microtitre wells contained the stated isolate inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. The organisms isolated from Wound 1 failed to survive sub culture from all wells in each triplicate.

Table 4.2 b. Bioload challenge using medical grade Manuka products against Wound 2 isolates

Recovery of viable organisms upon subculture using a sterile cotton swab

org load	>4.00E +08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E +08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 2												
Acinetobacter sp	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
Group B *Streptococcus*	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
Group G *Streptococcus*	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
E. coli	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –

Each of the microtitre wells contained the stated isolate inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. The organisms isolated from Wound 2 failed to survive sub culture from all wells in each triplicate.

Table 4. 2 c. Bioload challenge using medical grade Manuka products against Wound 3 and 4 isolates

Recovery of viable organisms upon subculture from Wound 3 and 4 using a sterile cotton swab.

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 3												
Myroides sp	+ / +	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –
K. oxytoca	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –
Wound 4												
Enterococcus sp.	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –
E. coli	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –
P. mirabilis	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / +++	– / –	– / –	– / –

Each of the microtitre wells contained the stated isolate from Wounds 3 and 4 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. The *Myroides* sp. Isolated from Wound 3 was recovered upon subculture in one of the triplicates containing >4x 108 organisms/ml in both products at 100% concentration. The *K. oxytoca* from Wound 3 failed to survive sub culture from all wells in each triplicate. The *P. mirabilis* isolated from Wound 4 was recovered from all triplicates of the well containing an 5 x 10^6 organism /ml in Manuka honey UMF 18+ at a concentration of 37.5%. All other subcultured wells failed to yield viable organisms.

Table 4.2 d. Bioload challenge using medical grade Manuka products against Wound 5.

Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	**100**	**100**	**100**	**100**	**75**	**75**	**75**	**75**	**37.5**	**37.5**	**37.5**	**37.5**
Wound 5												
Group B *Streptococcus*	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
K. pnemoniae	– ++	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
S. aureus	– +++	– –	– –	– –	– –	– –	– –	– –	+ + –	– –	– –	– –

Each of the microtitre wells contained the stated isolate from Wound 5 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. The Lancefield Group B *Streptococcus* failed to survive subculture from all the wells in each triplicate. The *K. pneumoniae* and was recovered from two of the triplicate wells containing >4 x 10^8 organisms/ml *S.aureus* was recovered from each triplicate of the >4 x 10^8 organisms/ml in 100% Manuka UMF 18+. and in two of the triplicates containing 5 x 10^6 organisms/ml in 37.5% Manuka UMF 16+.

Table 4.2 e. Bioload challenge using m edical grade Manuka products against Wound 6 isolates.

Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 6												
P.aeruginosa	– / +	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –
Corynebacterium . Sp.	– / ++	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –
Enterococcus sp.	+ + +	+ +	-	-	+	-	-	-	+	-	-	-
P. mirabilis	– / +	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –	– / –

Each of the microtitre wells contained the stated isolate from Wound 6 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. From the wells containing the Manuka UMF 18+, the *P. aeruginosa* and *P. mirabilis* grew upon subculture from one triplicate well containing >4 x 10^8 orgs per/ml in 100% honey, the *Corynebacterium* sp. as recovered from two triplicate wells containing >4 x 108 orgs per/ml in 100% honey, the *Enterococcus* sp was recovered from all triplicates containing >4 x 10^8 and 3 x 10^8orgs. per/ml in 100% honey and in one triplicate each of the wells containing >4 x 10^8 org/ml in 75% honey and 5 x 10^6 orgs/ml in 37.5% honey. No viable organism from Wound 6 were recovered upon subculture from any wells containing the Maunuka UMF 16+.

Table 4.2 f. Bioload challenge using medical grade Manuka products against Wound 6 isolates. Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 7												
Enterococcus sp.	–	–	–	–	–	–	–	–	+ + +	+ + +	+ + +	–
	–	–	–	–	–	–	–	–	+ + +	+ + +	+ + +	+ + +
Group G *Streptococcus*	–	–	–	–	–	–	–	–	+ + +	–	–	–
	–	–	–	–	–	–	–	–	+ + +	–	–	–
Alcaligenes faecalis	–	–	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–	–	–

Each of the microtitre wells contained the stated isolate from Wound 7 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. The *Enterococcus* sp was isolated from wells in all triplicates containing 37.% Manuka UMF 18+ and from the wells containing 37.5% Manuka UMF 16+ containing > 5 x 10^3 orgs./ml. The Lancefield Group G *Streptococcus* was isolated upon subculture from all triplicates containing both honeys at 37.5% inoculated with >5 x 10^6 orgs/ml. The *A. faecalis* failed to survive subculture from any of the wells in any triplicate.

Table 4.2 g. Bioload challenge using medical grade Manuka products against Wound 8 isolates. Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 8												
E. coli	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
S. aureus	+ –	– –	– –	– –	+ –	– –	– –	– –	– –	– –	– –	– –
Enterococcus sp.	+ + + + +	+ + + + + +	+ + +	+ + + + +	– + + +	– + + +	– + + +	– +	– +	– –	– –	– –

Each of the microtitre wells contained the stated isolate from Wound 8 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+' The *E.coli* failed to survive subculture from all wells. The *S. aureus* was recovered upon subculture from one triplicate containing >4x 10^8 orgs/ml in 16+. In wells containing 100% Manuka UMF 16+, the *Enterococcus* sp. was recovered from two triplicates containing >4 x 10^8orgs/ml, three triplicates containing 3x 10^8 orgs/ml, one triplicate containing 2 x 10^8 orgs/ml and two triplicates containing 1x 10^8 orgs/ml. In wells containing Maunka UMF 18+ the *Enterococcus* sp. was recovered from all triplicates containing 100% honey and all triplicates containing ≥2 x 10^8 orgs/ml in 75% honey. The *Enterococcus* sp. was also recovered upon subculture from one triplicate each containing 1 x 10^8 orgs/ml in 75% honey and 5 x 10^6 orgs/ml in 37.5 % honey.

Table 4.2 h. Bioload challenge using medical grade Manuka products against Wound 9 isolates.

Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	**>4.00 E+08**	**3.00E+ 08**	2.00E+ 08	1.00E+ 08	**>4.00E+ 08**	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	**100**	**100**	100	100	**75**	75	75	75	37.5	37.5	37.5	37.5
Wound 9												
S. aureus	+ + + + + +	+ +	– +	– –	+ –	– –	– –	– –	– –	– –	– –	– –
Enterococcus sp.	+ + + + + +	+ + + + + +	+ –	– –	+ + +	– –	– –	– –	– + + +	– –	– –	– –
M. morganii	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
E. coli	+ + +	– +	– –	– –	– –	– –	– –	– –	+ +	– –	– –	– –

Each of the microtitre wells contained the stated isolate from Wound 9 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. In the triplicates containing Manuka UMF 16+, the *S.aureus* was recovered upon subculture from two triplicates containing >4 x 10^8 orgs/ml,1 triplicate containing 3 x 10^8 orgs/ml in 100% honey and one triplicate containing >4 x 10^8 orgs/ml in 75%honey. Enterococci was recovered from 2 triplicates of >4 x 10^8 orgs/ml, and one triplicate each of 2 and 3 x 10^8orgs/ml in 100% honey. One triplicate of >4 x 10^8 orgs/ml in 75% honey yielded Entrococcus sp. The *M. morganii* grew from one triplicate containing >4 x 10^8 orgs/ml in 100% honey and and the *E.coli* grew from one triplicate of >4x10^8 in 100% honey and one triplicate 5x10^6 orgs/ml in 37.5% honey. The Microtitre plates containing Manuka UMF 18+ honey yielded *S. aureus* from three triplicates containing >4 x 10^8 orgs/ml in 100% honey and one triplicate each of 3 and 2 x 10^8 orgs/ml in 100% honey. *Enterococcus* sp. was recovered upon subculture from all triplicates containing ≥3 x 10^8 in 100% honey, two triplicates of >4 x 10^8 in 75% honey and all triplicates containing 5 x 10^6 orgs/ml in 37.5% honey. The *M. morganii* was recovered from one triplicate containing >4 x 10^8 ors/ml in 100% honey and 5 x 10^6 orgs/ml in 37.5% honey. *E. coli* grew upon subculture from 2 triplicates containing >4 x 10^8 orgs/ml and 1 triplicate of 3x 10^8 orgs/ml in 100% honey and from one triplicate of 5 x10^6 orgs/ml in 37.5 % honey.

Table 4.2 i. Bioload challenge using medical grade Manuka products against Wound 10 isolates.

Recovery of viable organisms upon subculture from Wound 1 using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
Wound 10												
P. aeruginosa (1)	+ +	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
P. aeruginosa (2)	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
S. aureus	– + + +	+ –	– –	– –	– + +	+ + –	+ –	– –	– –	– –	– –	– –

Each of the microtitre wells contained the stated isolate from Wound 10 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'. *P. aeruginosa* (1) grew upon subculture from one triplicate with each honey containing >4 x 10^8 orgs/ml with 100%. No viable cells were recovered from any wells containing *P. aeruginosa* (2). *S. aureus* grew upon subculture from wells of one triplicate containing 3 x 10^8 cells/ml in 100% Mnauka UMF 16+, two triplicates containing 3 x 10^8 orgs/ml in 75% and one triplicate containing 2 x 10^8 orgs/ml in 75% of the Manuka UMF 16+honey. In wells containing the Manuka 18+, *S. aureus* was recovered from all triplicates containing >4 x 10^8 orgs/ml with 100% honey and 2 triplicates with the same organism load and 75% Manuka UMF 18+.

Table 4.2 j. Bioload challenge using medical grade Manuka products against Gram positive NCTC strains. Recovery of viable organisms upon subculture using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
NCTC MSSA	+ + + + + +	+ + +	– –	– –	+ + + +	+ –	– –	– –	– –	– –	– –	– –
NCTC MRSA	+ + + +	– –	– –	– –	– + + +	– –	– –	– –	– –	– –	– –	– –
NCTC VSE	+ + + + + +	+ + + +	– + + +	– + + +	+ + + + + +	+ + + +	– + + +	– + + +	+ + + + + +	+ + + + + +	+ + + + + +	+ + + + + +
NCTC VRE	+ + + + + +	+ + + –	+ + + –	+ –	+ + + +	+ –	– –	– –	+ + + –	+ –	– –	– –

Each of the microtitre wells contained the stated isolate from Wound 10 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'.

Table 4.2 k. Bioload challenge using medical grade Manuka products against Gram negative NCTC strains.

Recovery of viable organisms upon subculture using a sterile cotton swab

org load	>4.00 E+08	3.00E+ 08	2.00E+ 08	1.00E+ 08	>4.00E+ 08	3.00E+ 08	2.00E+ 08	1.00E+ 08	5.00E+ 06	5.00E+ 05	5.00E+ 04	5.00E+ 03
Honey %	100	100	100	100	75	75	75	75	37.5	37.5	37.5	37.5
NCTC β-lactamse negative	+ + +	+	–	–	+ + +	–	–	–	–	–	–	–
E. coli	+ + +	–	–	–	+ +	–	–	–	–	–	–	–
NCTC β-lactamse positive *E.*	+ +	+	–	–	–	–	–	–	–	–	–	–
coli	+ +	–	–	–	+	–	–	–	+	–	–	–
NCTC	+ +	–	–	–	–	–	–	–	–	–	–	–
P. aeruginosa	+ +	–	–	–	–	–	–	–	–	–	–	–

Each of the microtitre wells contained the stated isolate from Wound 10 inoculated into medical grade Manukas of UMF 16+ (result shown in red font) and 18+ (black font) at the concentrations and bioload indicated. The assay was completed in triplicate and where organisms grew upon subculture each triplicates yielding organism growth is recorded as a '+'.

4.4 Discussion

This section of study aimed to design an experimental model to challenge the Manuka products with bacterial numbers of a higher magnitude than is normally supported by a nutritional source. The results from Chapter 3 showed that Enterococci as a group were most able to resist the antimicrobial properties of Manuka honey which was mostly supported by the results in this section of study. The other organisms introduced in this chapter, *Proteus* sp., *Morganella* sp., β-Haemolytic Streptococci, *Acinetobacter* sp., *Alcaligenes* sp., *Klebsiella* sp. and *Myroides* sp., fell within the ranges of susceptibility of the organism panels previously adopted and did not demonstrate levels of susceptibility previously unseen and therefore could be tested with the same methodologies as the previously tested organism groups.

The first bioload assay using only control strains suggested that it is not the number of organisms that effect the bactericidal efficacy of these medical grade products so much as the concentration and volume of honey present.

The second bioload assay on the isolates cultured directly from clinical wound swabs showed that some organisms survived in 100% undiluted honey but not in 75% honey. These inconsistencies could be suggestive of organisms clumping where colonies of organisms were inoculated directly into the honey and were physically protected from coming into direct contact with the honey by adjacent organisms. It appears from the results that the Gram positive organisms were overall more resilient to the honey treatment than the Gram negative organisms. Whilst this was predictable in the case of Enterococci, it was unexpected with regard to the Staphylocci, which would be consistant with the possibility that

organism clumping contributes to organism survival. [sentence here on clumping and why you think it has happened] However this is difficult to prove without visualising organism dispersal throughout the medium. Further study using electron or confocal microscopy may be useful methods by which to determine organism dispersal.

There are many studies which look at products such as silver, iodine and polyhexamethylene biguanide in the reduction in organism numbers from wound samples (Thorn *et al.,* 2009, Miller *et al.,* 2011, Sibbald *et al.,* 2011, Hiro *et al.,*2012) [state why you have mentioned these here]. In addition, there are some studies published regarding the effective use of Manuka honey in wound care (Cooper and Molan, 1999, Dunford *et al.,* 2000, Chambers, 2006, Simon *et al.,* 2006, Blaser *et al.,* 2007, George and Cutting, 2007, Gethin *et al.,*2008) however, a review of the literature revealed no studies comparing the *in vitro* activity of these wound care products against varying bacterial bioloads. This study might well help to provide baseline data with which to initially assess appropriate selection of a topical agent. Manuka honey is often described as a useful topical therapy for sloughy or malodorous wounds where honey's debridement action is of benefit (Kingsley, 2001, Gethin and Cowman, 2005, Acton and Dunwoody, 2008, Lay-flurrie, 2008, Naylor *et al.,* 2008) and for infected post-surgical wounds (Vardi *et al.,* 1998, Al-Waili *et al.,* 1999, Blaser *et al.,* 2007), but whether this also implies that honey has a greater bactericidal activity against high bioloads than other wound dressings is not clear.

The results of this section infer that it is contact with honey that inhibits organism growth and that if the volume of honey and organisms are homogenously

dispersed and come into contact with the honey, organism death results although lesser volumes can still inhibit growth. There is no suggestion that any chemical constituents within the honey diffuse through the population to exert an antimicrobial effect. This is further explored in Chapters 5 and 6 which looks at the effect of honey on organism biofilms in *in vitro* wound models.

4.5 Conclusions

The antimicrobial activity of Manuka honey is apparently unaffected by organism bioload in this study. Where the concentration of honey is high and undiluted by a liquid medium there must be an adequate volume to permit physical contact as there is no evidence to suggest that antimicrobial constituents are able to disperse throughout bacterial colonies. Data from the work presented here suggests efficacy of the antibacterial activity of Manuka honey is at its greatest at a 75% concentration. This might be explained by the viscosity of the honey matrix being at an optimum with regards to its antimicrobial activity.

CHAPTER 5

The Activity of Medical Grade Manuka Honey on Bacterial Biofilms and Wound Modelling

5.1 Introduction

In a planktonic state, bacteria are metabolically active and in the absence of resistance genes, susceptible to antimicrobial therapeutic agents (Costerton *et al.*, 1999, Mertz 2003, Rhoads *et al.*, 2008). When these planktonic bacteria come into contact with solid surfaces, they can potentially adhere and biofilms may be formed (Costerton *et al.*, 1999). The bacterial populations of biofilms form complicated ecosystems (Costerton *et al.*, 1995) and in a clinical setting can be linked to a simple condition such as dental plaque (Socransky and Haffajee, 2002). However, resistance of organisms in biofilms to antimicrobial agents can lead to more complicated problems such as colonisation of indwelling medical devices (Donlan, 2001) and chronic infections such as gingivitis, sinusitis, necrotising fasciitis and chronic non-healing wounds (Costerton *et al.*, 1999). When biofilms form, pheromones known as quorum sensing molecules, for example acyl homoserine lactones (AHL) in Gram negative bacteria and modified oligopeptides in Gram positive bacteria (De Kievit and Iglewski, 2000, Waters and Bassler, 2005), are secreted by the bacteria. These quorum sensing molecules initiate the production of auto-inducer molecules, which activate the expression of genes releasing virulence factors and forcing bacterial metabolism to slow (Mertz, 2003). As the biofilm develops, the extracellular polymeric matrix produced increases adherence and the water channels within the biofilm layer allow the passage of plasma and wound exudate to nourish the organisms of the biofilm layer (Rhoads *et al.*, 2008). The formation of biofilms by bacteria colonising wounds challenge the healing processes, giving rise to chronic wound infections which prove difficult to treat with both topical and systemic antimicrobials (Costerton *et al.*, 1999; Cooper, 2010).

Product research shows that there are numerous honey dressings and products for use in wound care such as Medihoney and Advancis products, indicating that the use of honey on wounds has gained sufficient acceptance as a medicinal product to warrant potential commercial interest.

Biofilms act as a reservoir of infectious organisms which survive the action of antimicrobial agents effective against bacteria in a planktonic state (Costerton *et al.*, 1999). The escape of organisms from the surviving biofilm has been shown to facilitate wound reinfection and to potentially seed new infection sites (Costerton *et al.*, 1999). The findings presented in preceding chapters have focused on determining the activity of honey on bacteria from clinical sites and the influence of bioload on the antibacterial efficacy of medical grade Manuka products. This initial aim of this section of the study was to assess the ability of medical grade Manuka honeys to remove established biofilms *in vitro* by growing polymicrobial biofilms from clinical polymicrobial wounds. In order to achieve this, an effective method for growing and visualising biofilms needed to be established followed by a method which could quantify and compare the action of the two products on the cultivated wound bacterial biofilms. The final aim of this section was to replicate *in vitro* some features of a clinical wound and study the antimicrobial activity of the medical grade Manuka honeys on biofilms grown on a wound model.

5.2 Materials and Methods

In order to study the effect of Manuka honey on bacterial biofilms it was necessary to establish a suitable method for growing, measuring and quantifying biofilms. Several methods were designed and evaluated which are outlined briefly below and fully described in sections 2.7 to 2.8.

5.2.1 Growing biofilms in microtitre plates

Growing biofilms on the flat surface of wells of microtitre plates was initially investigated as the small volumes of honey required to fill a microtitre plate well had economical advantages.

5.2.1.1 Growing biofilms of NCTC strains in sterile flat well microtitre plates

Microtitre plates were inoculated with 200 µl of BHIA broth inoculated with the control strains MSSA (NCTC 6571), MRSA (NCTC 12493), β- lactamase producing *E. coli* (NCTC 11560), *E. coli* (NCTC 12241), *P. aeruginosa* (NCTC121903) and vancomycin sensitive and resistant *E. faecalis* (NCTC 12679 and 13379). The strains were incubated for 24, 48 and 72 hours. After incubation the plates were inverted to remove the broth. The plates were placed on a hotplate for 30 minutes to fix any biofilms which may have grown. Crystal violet was used to stain any organisms adhered to the well surface. See Section 2.7.1.1 for full methodology.

5.2.1.2 Growing biofilms of NCTC strains in sterile fibrinogen coated flat well microtitre plates

The second methodology utilised porcine fibrinogen to coat the microtitre wells to provide a surface which would facilitate bacterial adherence. Three methods were used to remove the broth and any remaining planktonic cells: inversion; inversion and gentle rinse with physiological saline; inversion, saline soak and rinse. Plates

were heat fixed and stained as above. See section 2.7.1.2 - 2.7.1.3 for full methodology.

5.2.1.3 Stripping biofilms from microtitre plates with medical grade honey

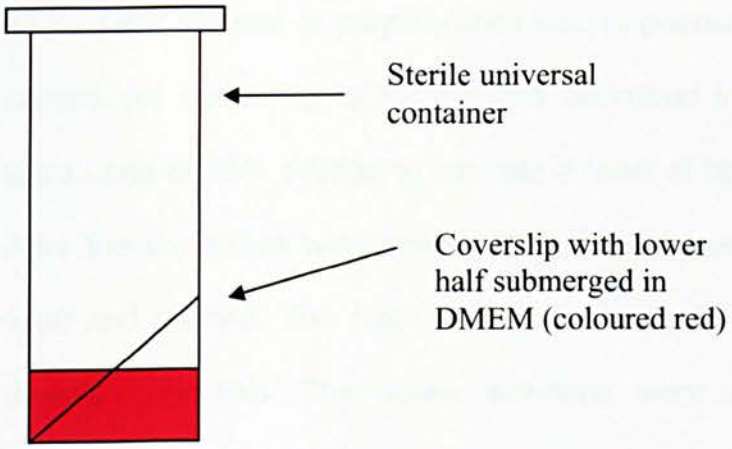
The experimentation with methodologies for growing biofilms in microtitre plates included the application of honey with the aim of optimising a method to measure the biofilm removal capacity of honey. The method is fully described in Section 2.7.2.

5.2.2 Growing biofilms on glass coverslips

Cannula lines were briefly considered as alternatives to the microtitre plates. Glass coverslips were the material selected on the basis of uniformity and simplicity of handling.

In the first instance coverslips were half immersed in Dulbecco's Modified Eagle's Medium (DMEM) (See section 2.7.3.1). DMEM is a medium used in tissue culture and was selected to represent tissue fluid, coverslips were half submerged to try to demonstrate biofilm growth and absence of growth on each coverslip (Figure 5.1).

Figure 5.1 Diagram showing coverslip in universal container half submerged in DMEM



This method was modified to use coverslips fully immersed in Brain Heart Infusion Broth (See method 2.7.3.2)

5.2.3 Growth of biofilms on glass coverslips using Brain Heart Infusion Broth

Further method modifications were attempted to ascertain optimal incubation time for biofilm formation and to determine if a rinsing step was required prior to heat fixing. For reasons of economy this test method was performed using only Meloderm Manuka Honey 16+. The volume of elutant used was 2 ml. Full methodologies are described in sections 2.7.3.2 and 2.7.4.

5.2.4 Experimentation with volumes of elutant for quantification of biofilm.

Prior to treating with honey, biofilms of each individual organism isolated from the wounds and biofilms of each polymicrobial wound flora were grown, stained and eluted in duplicate. The biofilms grown with the first set were eluted with 2 ml of elutant the second set with 1.5 ml to determine which gave a better range of optical densities. See Section 2.7.4 for full methodology.

5.2.5 The effect of Manuka honey on biofilms from polymicrobial wound infections grown on glass coverslips

A comparison of the capability of the medical grade Manuka honeys to remove the 48-72 hour biofilms of polymicrobial wound populations from glass coverslips was carried out according to the method described in section 2.7.5.4. The products were used at 75% dilution to emulate a level of honey dilution by wound exudate. After the coverslips were removed from the honey they were rinsed, dried, heat fixed and stained. The stain was eluted using 80:20 ethanol:acetone and optical density quantified. The honey solutions were cultured onto blood agar and incubated for 48 hours at 37° C in air to determine the presence of viable bacteria.

5.2.6 Wound modelling

The microbiological medium selected to represent a wound bed was Robertson's Cooked Meat (RCM). The meat pieces are small and roughly round in shape providing a large surface for biofilms to form. The microbial populations were those provided from wound swabs received in The William Harvey Hospital described in Section 2.7.5.1.

Once the organisms were grown in RCM with BHIA overlay, the meat was removed and decanted to a sterile Bijoux containing blood agar on the base. The blood agar prevented the meat pieces resting on the plastic base ensuring they were completely surrounded by a nutrient source.

The aims of this assay were to assess if honey can exert bacteriostatic or bactericidal effect on biofilms grown in a wound model and to investigate the efficacy and limitations of its use in a clinical setting. For full details of methodology see section 2.8.

5.2.7 Statistical analysis

Using SPSS a paired sample t-test was used to analyse the significance of the reduction of viable organisms resulting from the honey treatment.

5.3 Results

5.3.1 Growing biofilms of NCTC strains in sterile flat well microtitre plates

Upon inversion to remove the medium, the *Pseudomonas aeruginosa* (NCTC121903) control had produced a heavy extracellular matrix which appeared to assist in the dislodging of the biofilm from the surface of the well.

After fixing and staining, the optical densities were inconsistent and non-reproducible as a result of the weight of the slime removing the biofilm from the well.

5.3.2 Growing biofilms of NCTC strains in sterile fibrinogen coated flat well microtitre plates

The addition of porcine fibrinogen and experiments with different methods to remove the broth without damaging the biofilms failed to provide repeatable results.

5.3.3 Stripping biofilms from microtitre plates with medical grade honey

The method described in Section 2.7.2 yielded inconclusive results as it was not possible to determine if biofilms were destroyed by honey action or by the process of removing the honey.

These microtitre methods were deemed ineffective as a means for determining the ability of honey to remove established biofilms.

5.3.4 Growing biofilms on glass coverslips

The difficulties experienced using microtitre plates necessitated selecting a different surface upon which to grow biofilms. Cannula lines were considered but required cutting into sections of exactly the same size to ensure exactly the same surface area. This was in addition to the challenge of maintaining sterility during

processing. Glass coverslips were selected based on uniformity in size and ease of handling without contamination.

5.3.5 Growth of biofilms on glass coverslips in Dulbecco's Modified Eagle's Medium (DMEM)

It was observed that the DMEM medium in which the biofilms had been grown changed during incubation from its original orange colour to yellow where the *E. coli* had fermented the glucose producing acid and to pink with the non-fermenting *P.* aeruginosa (Figure 5.3). The colour of the broths after incubation varied from isolate to isolate with Gram positive bacteria. Upon subculture 10 of the 22 broths grew very small numbers of bacteria and 12 failed to yield any organisms at all indicating that this particular DMEM recipe, which contained glutamine but not pyruvate was not a suitable medium. The addition of serum may have provided additional nutrients and may have improved the outcome of the method.

Figure 5.2 Colour change of DMEM as a result of organism growth

The results of the biofilm formation on the bottom half of the coverslips in DMEM are shown in Figure 5.4 and 5.5.

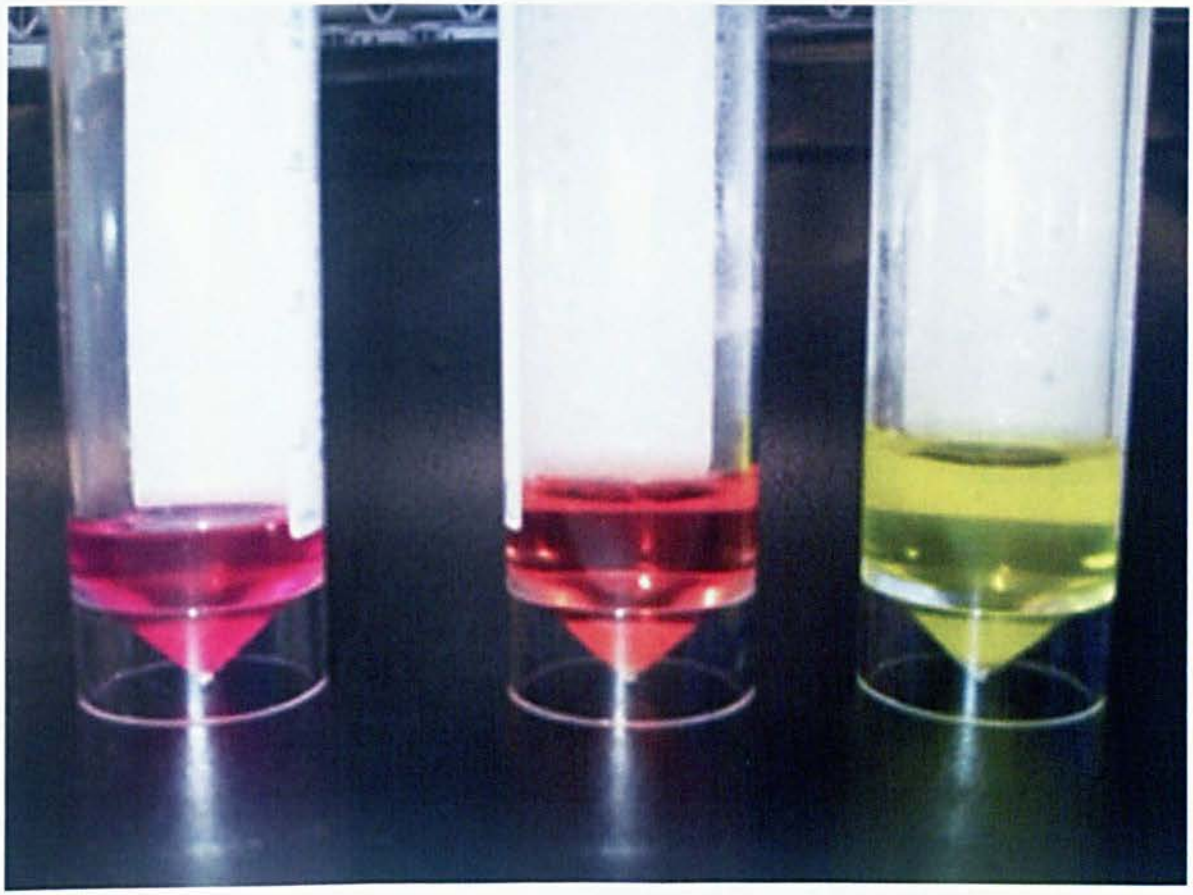
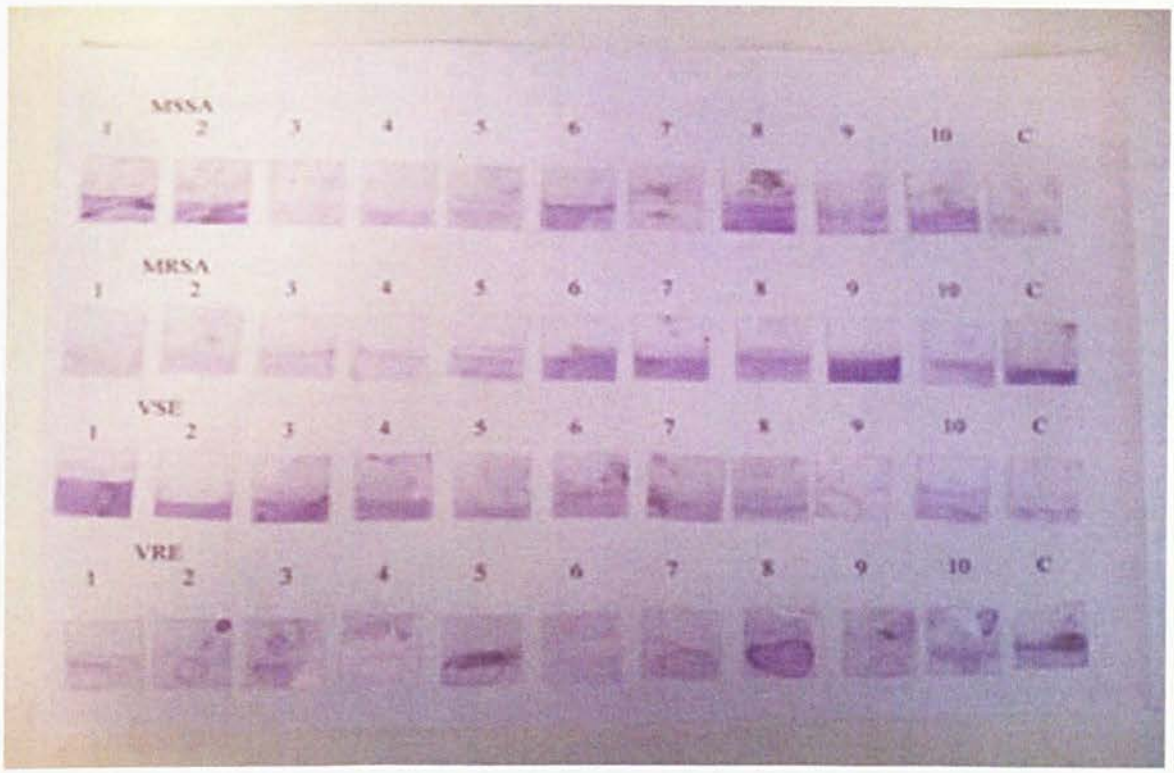
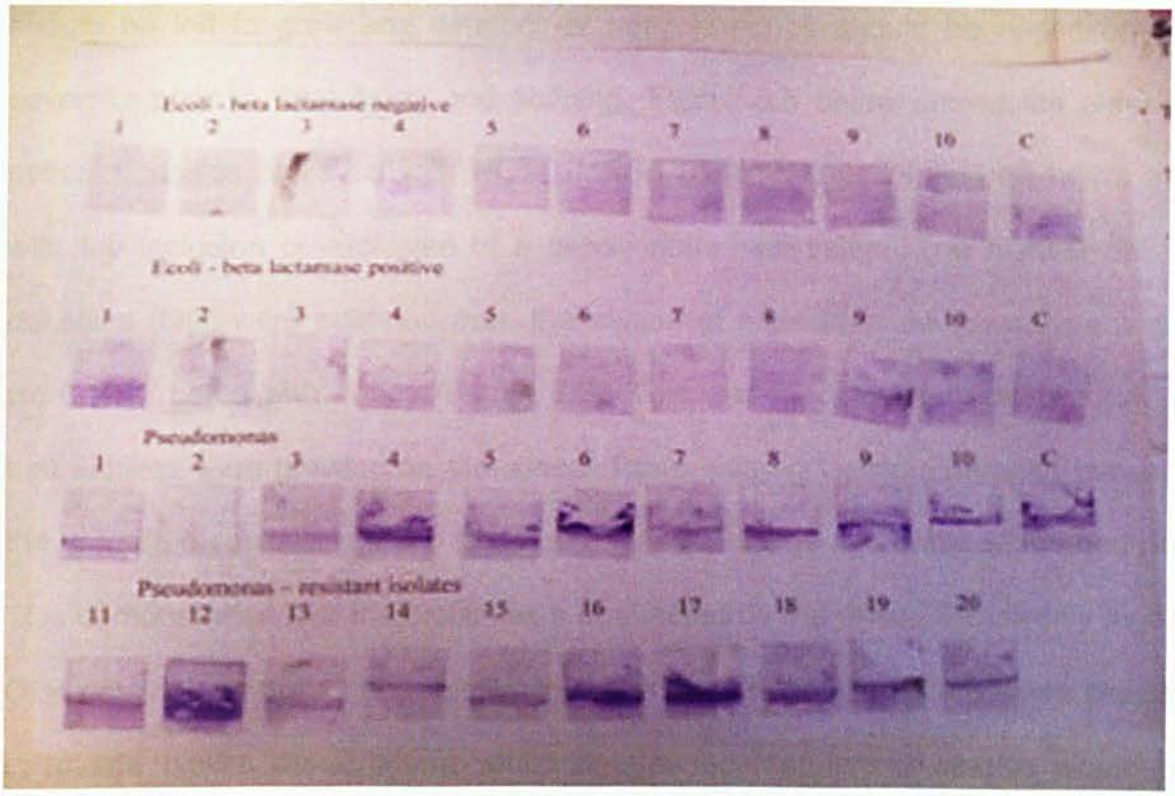


Figure 5.3 Biofilms of MSSA, MRSA, VSE and VRE grown on half coverslips in DMEM and stained with crystal violet.



The area on the bottom half of the coverslip where organisms have adhered have been visualised by staining with crystal violet. The rows of coverslips are labelled according to organism and isolate number. The final coverslip in each row is the corresponding NCTC control.

Figure 5.4 Biofilms of *E. coli* d *P. aeruginosa* grown on half coverslips in DMEM and stained with crystal violet



The area on the bottom half of the coverslip where organisms have adhered have been visualised by staining with crystal violet. The rows of coverslips are labelled according to organism and isolate number. The final coverslip in each row is the corresponding NCTC control

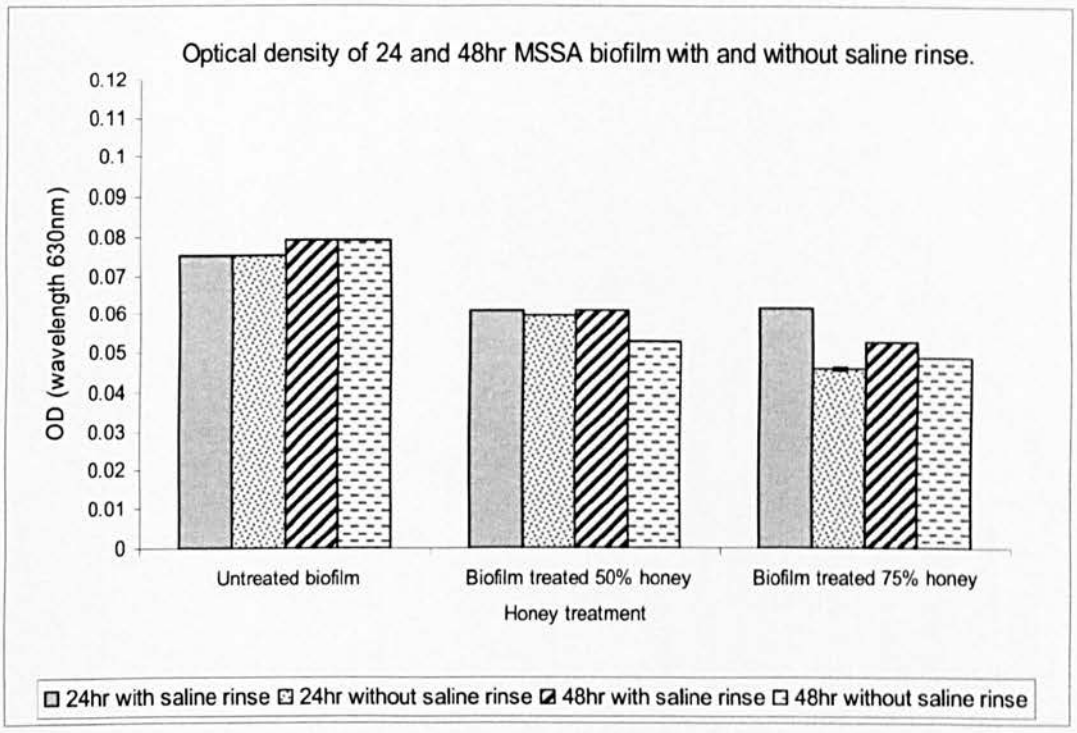
Biofilms were observed but difficulties arose with standardising the surface area when trying to grow the biofilms on half coverslips. The *Pseudomonas* isolates 16 and 17 in Figure 5.4 show the difficulties with biofilms being partially removed during stain rinsing. This did serve to show that biofilms can be effectively grown on glass coverslips. Consequently this method was modified for further study by the use of whole coverslips immersed in brain heart infusion broth cultures

5.3.6 Growth of biofilms on glass coverslips using Brain Heart infusion Broth

Further optimization of the method was required to ascertain how long the biofilm should be left to grow and whether or not the honey should be rinsed off the coverslip prior to heat fixing and staining. Figure 5.5 below shows the different optical densities achieved with NCTC MSSA biofilms after 24 and 48 hours and with the inclusion or exclusion of a gentle rinse with saline. The highest optical densities (OD) were obtained from the elution of stain from the coverslips which were not treated with honey (Figure 5.5). This was the negative control showing that biofilms were growing on the slides. There was no visible difference between the untreated coverslips which had been rinsed in saline and those which had not. This demonstrated that the biofilm was unaffected by the rinse. The slightly higher ODs obtained by the 48 hour biofilm indicated that incubating for 48 hours allowed increased biofilm development which determined that further studies would be performed on biofilms incubated for >24 hours.

The comparisons shown in figure 5.5 showed that rinsing honey treated slides with saline prior to staining raised the OD of the eluted stain. This introduced the possibility that incomplete removal of the honey prior to staining may result in incomplete staining of the biofilm. This introduced the possibility of ascribing an artificially high value to the effect of the honey on the biofilm. To avoid this, the saline rinse remained as part of the methodology when studying the biofilms of clinical wound bacterial populations.

Figure 5.5 Optical densities of 24 and 48 hour biofilms +/- honey treatment and +/- saline rinse



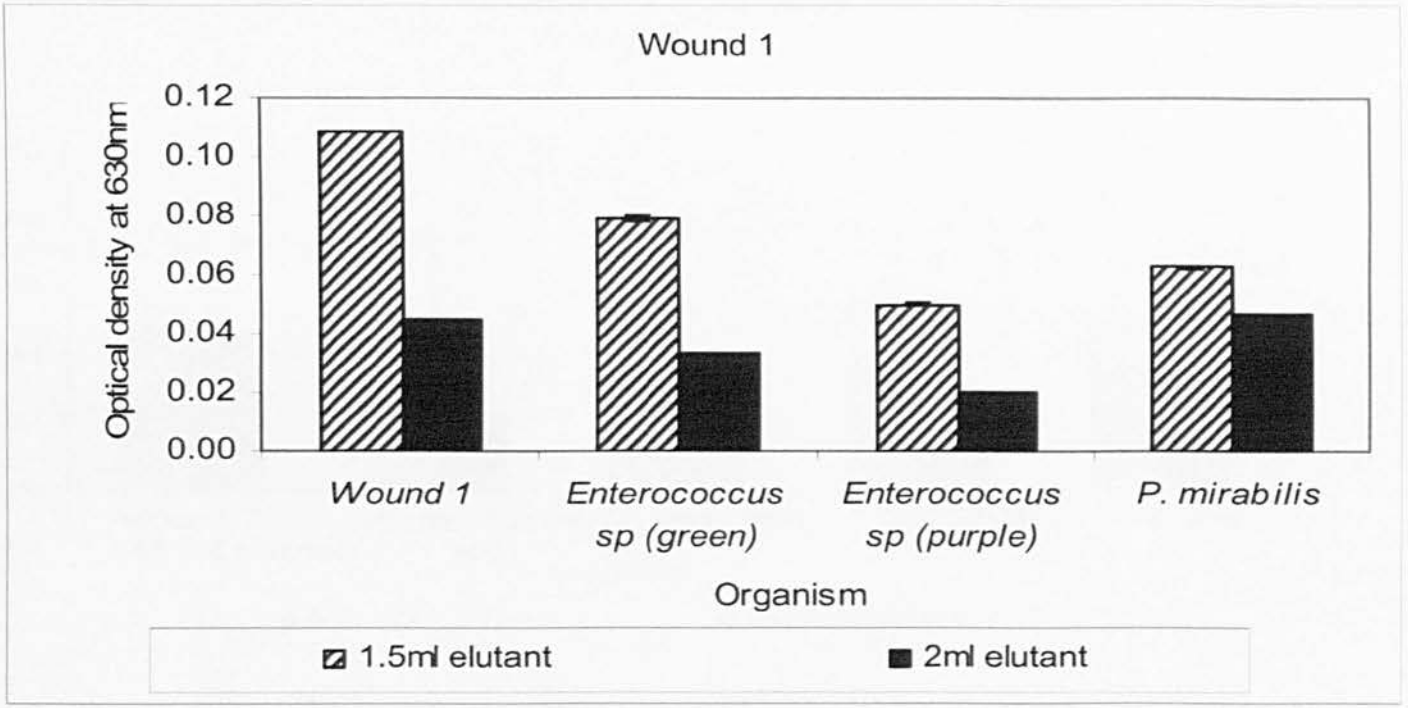
The untreated biofilms were unaffected by the saline rinse. Rinsing honey from the treated biofilms resulted in a raised OD indicating possible reduced efficiency of staining caused by residual honey. Optical density is recorded in absorbance units at λ 630nm. In all cases error bars cannot be seen due to the standard error of the mean being too small to visualise.

5.3.7 Experimentation with volumes of elutant for quantification of biofilm

Prior to treating with honey, biofilms of each individual organism isolated from the wounds and biofilms of each polymicrobial wound flora were grown, stained and eluted in duplicate. The biofilms grown with the first set were eluted with 2 ml of elutant as used above, the second set with 1.5 ml to determine if this gave a better range of optical densities. The graphs below show that the use of 1.5ml elutant in most instances provided a stronger OD than 2 ml so this method using 1.5 ml of

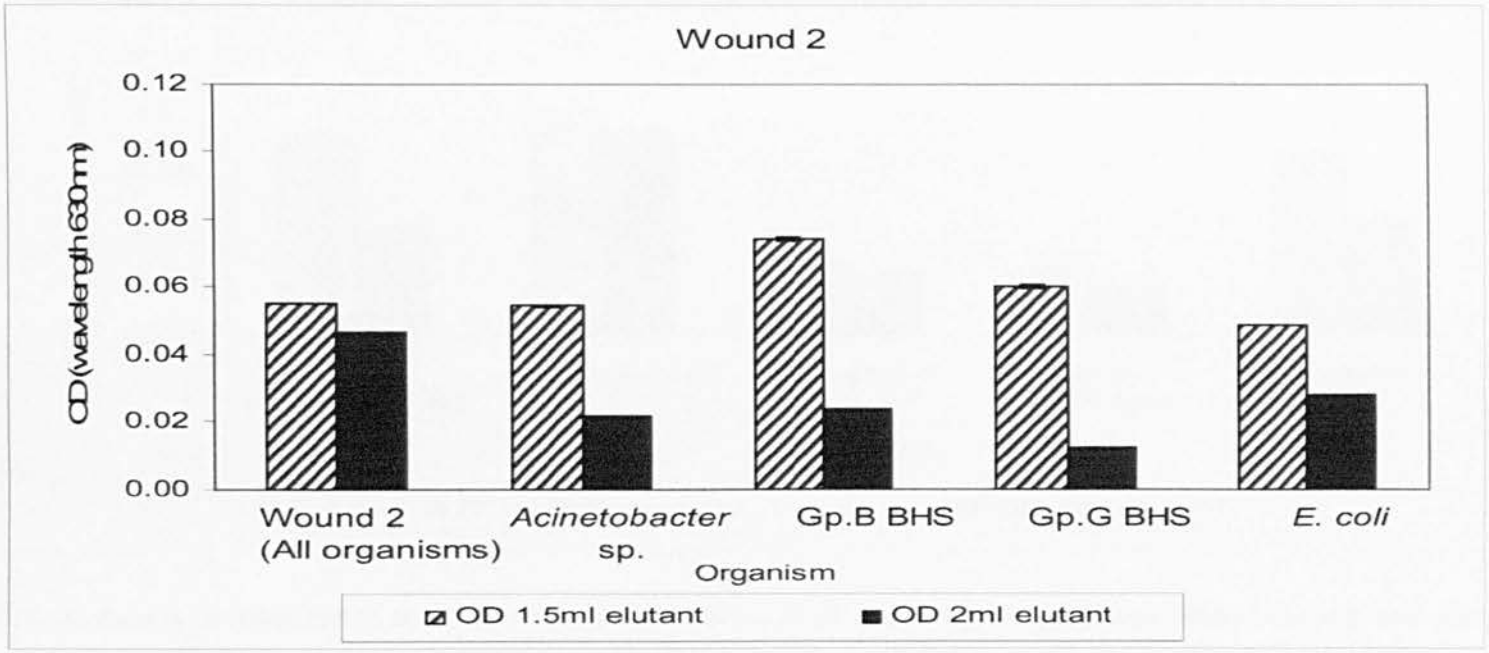
elutant was finally selected for visualising the ability of the products to remove established biofilms grown on glass.

Figure 5.6 Optical densities shown in absorbance units at 630nm obtained using 1.5ml and 2ml of elutant on Wound 1 biofilms



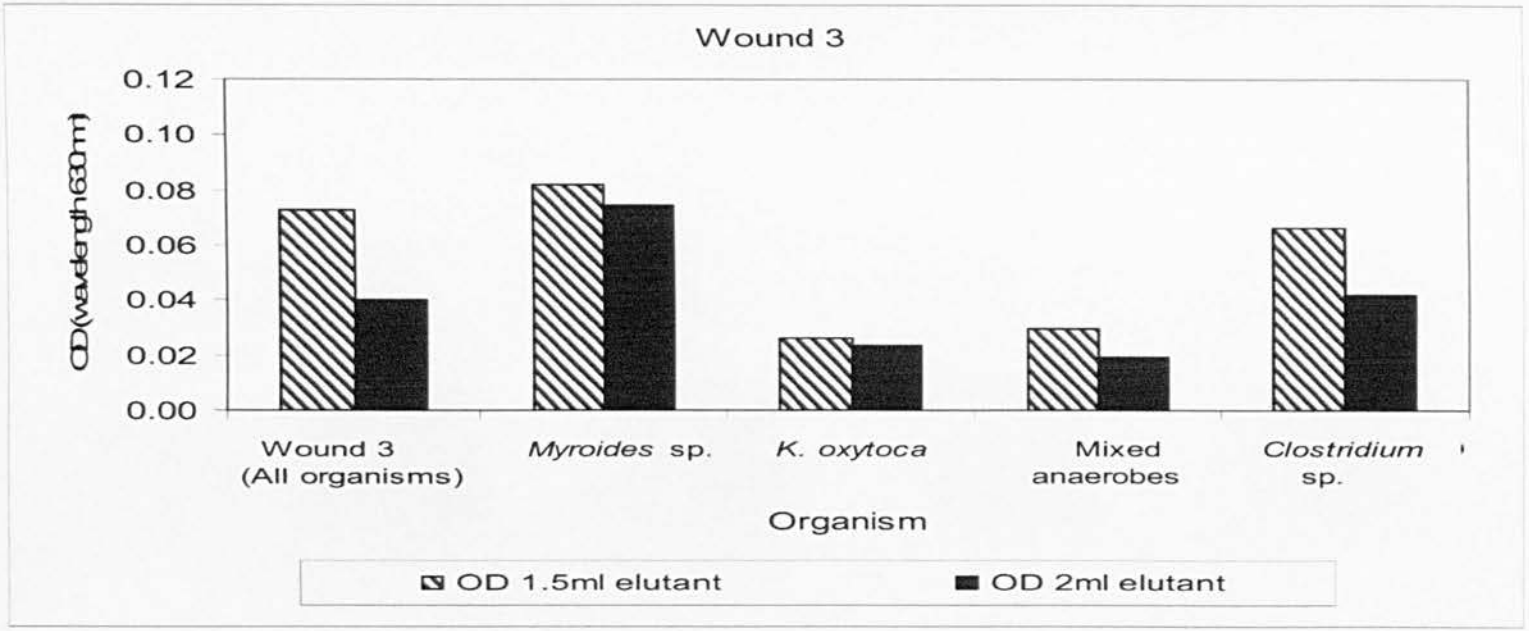
Optical density is recorded in absorbance units at λ 630nm. Green and purple denote colour of *Enterococcus* on chromogenic media. In all cases a greater OD was obtained using less elutant. The polymicrobial Wound 1 had a higher organism population than the monomicrobial biofilms using less elutant. This inconsistency between the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small.

Figure 5.7 Optical densities obtained using 1.5 ml and 2ml of elutant on Wound 2 biofilms



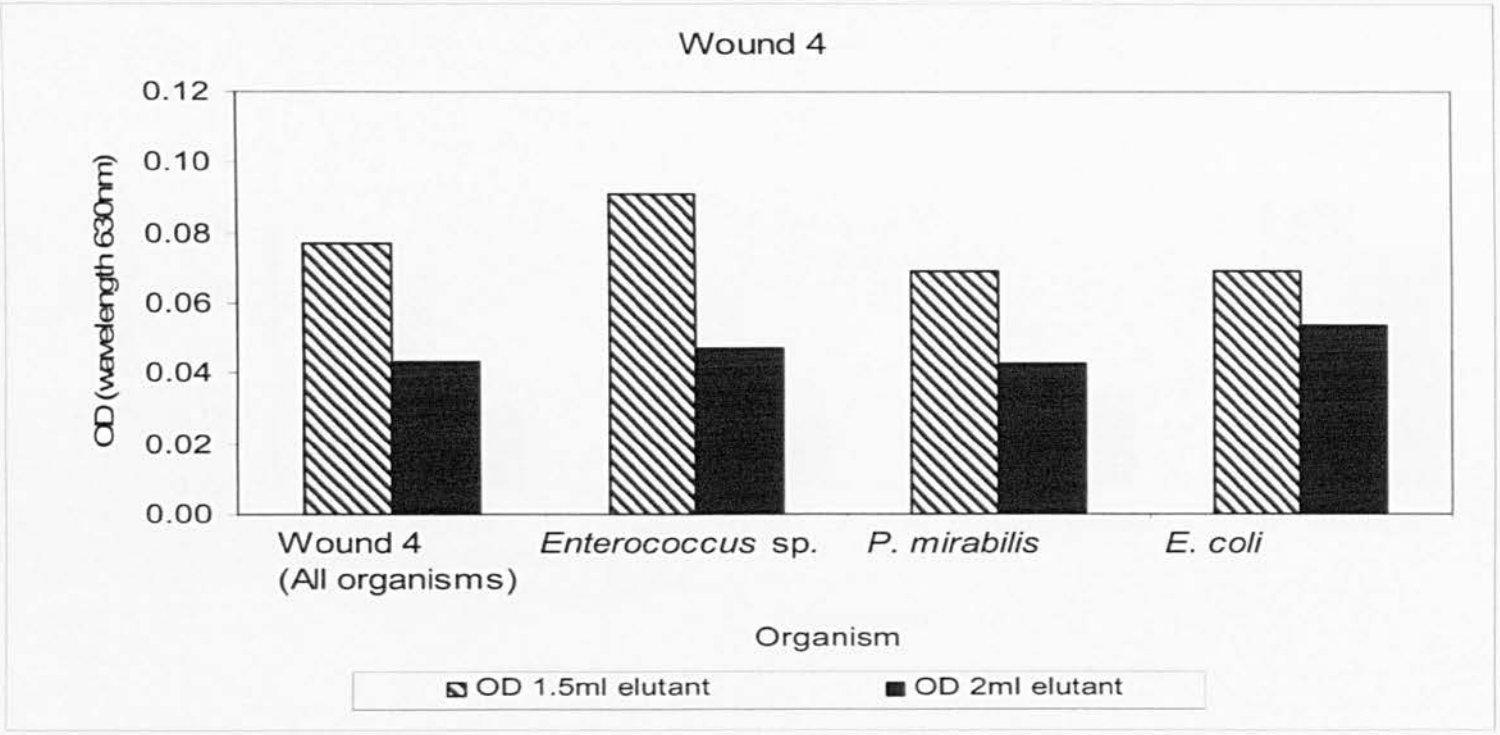
Optical density is recorded in absorbance units at λ 630nm In all cases a greater OD was obtained using less elutant. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small. GpB BHS = Lancefield Group B β haemolytic *Streptococcus*. GpG BHS = Lancefield Group G β haemolytic *Streptococcus*

Figure 5.8 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 3 biofilms



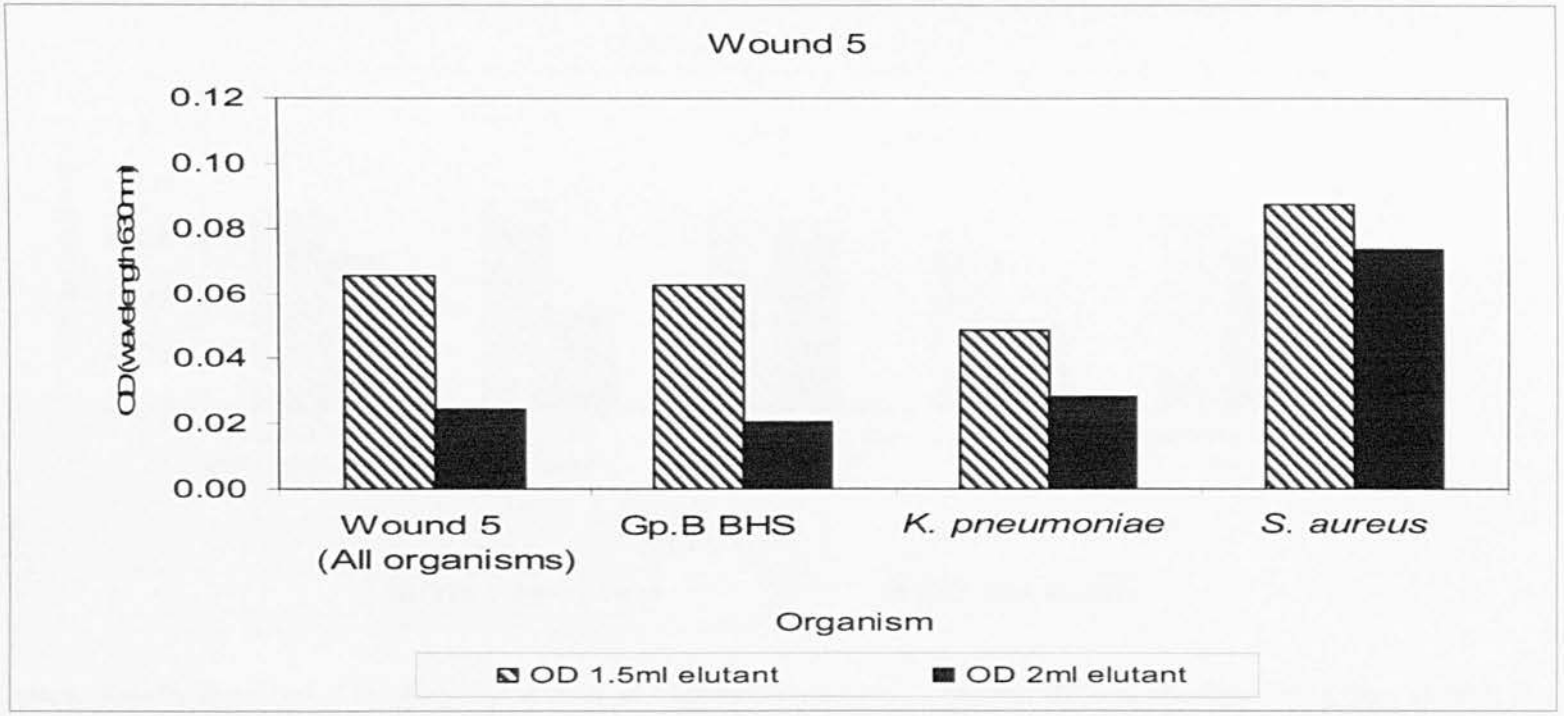
Optical density is recorded in absorbance units at λ 630nm. In all cases a greater OD was obtained using less elutant. The polymicrobial. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. However, the findings shown here is consistant with regard to *Myroides* sp., which is known to readily form biofilms. Error bars indicate standard error of the mean.Where error bars cannot be seen, SEM too small.

Figure 5.9 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 4 biofilms



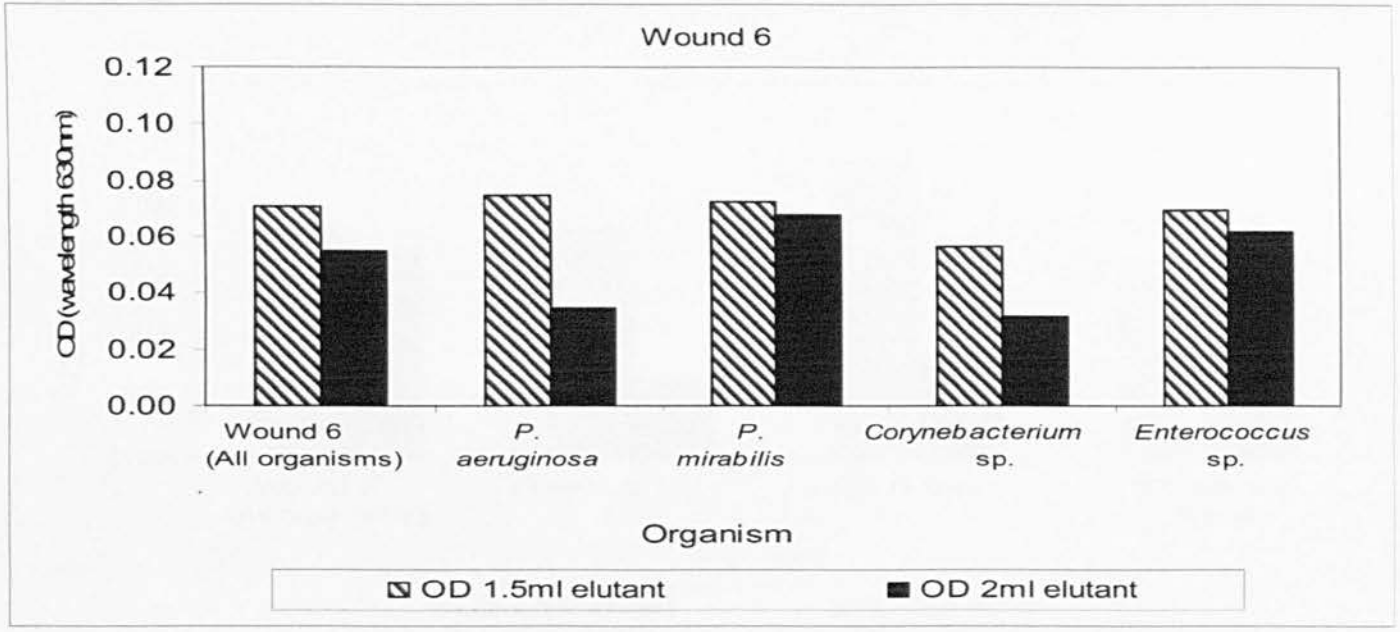
Optical density is recorded in absorbance units at λ 630n In all cases a greater OD was obtained using less elutant. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. The polymicrobial biofilm is not greater than those of the individual isolates. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small.

Figure 5.10 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 5 biofilms



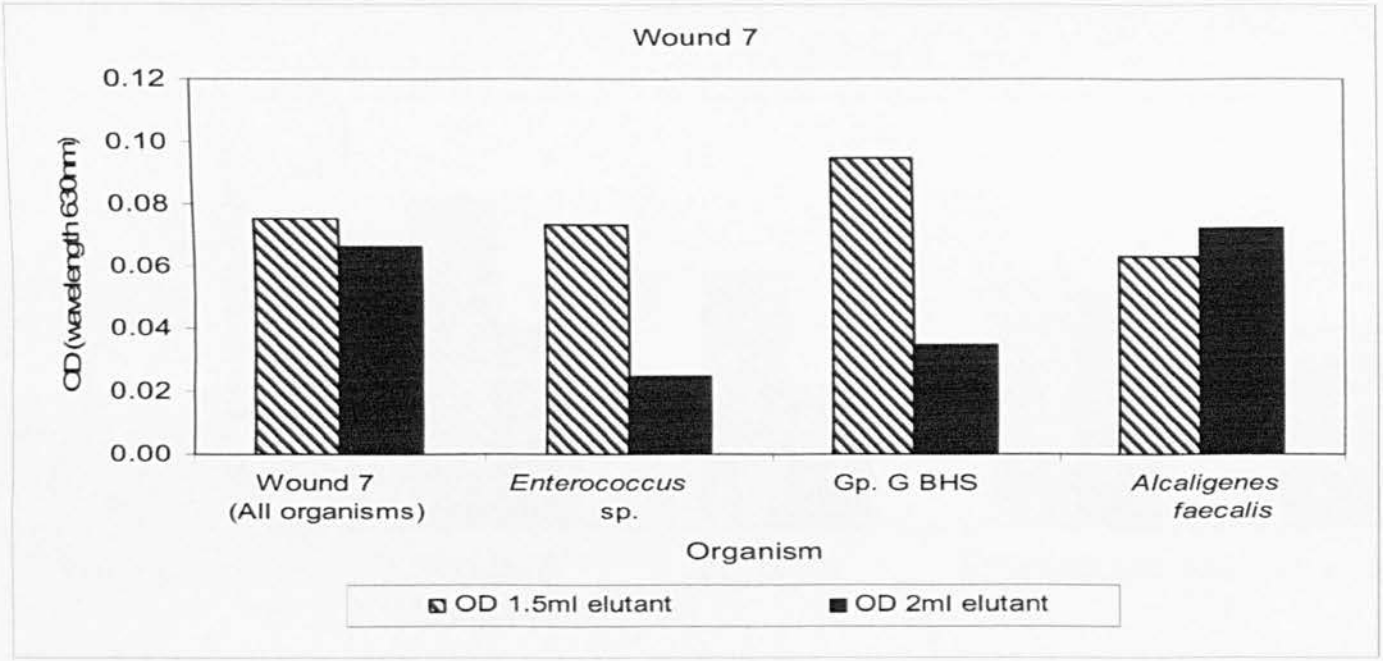
Optical density is recorded in absorbance units at λ 630nm GpB BHS = Lancefield Group B β haemolytic *Streptococcus* In all cases a greater OD was obtained using less elutant. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. However, *S. aureus* is shown to form a biofilm more effectively in the absence of the other organisms. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small.

Figure 5.11 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 6 biofilms



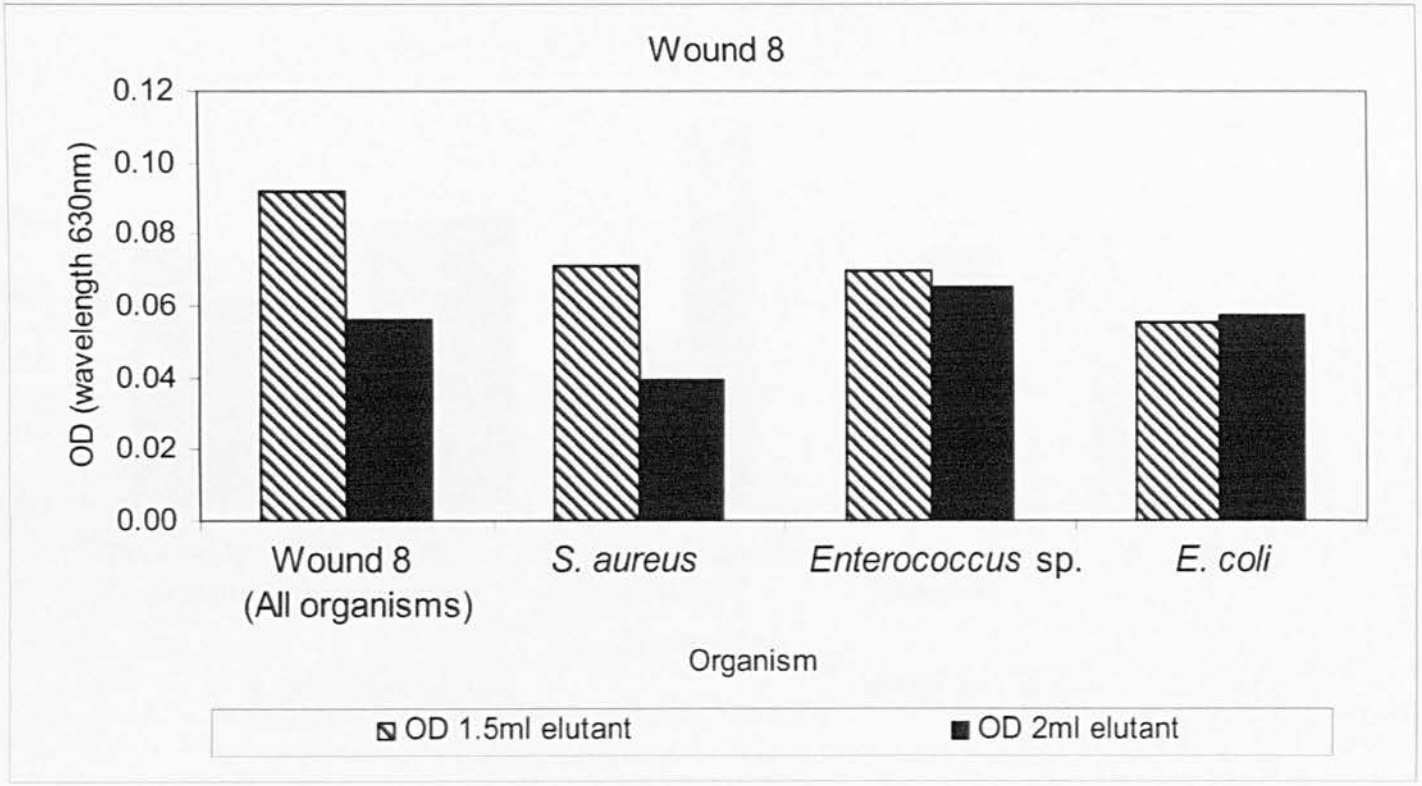
Optical density is recorded in absorbance units at λ 630nm. In all cases a greater OD was obtained using less elutant. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. The polymicrobial biofilms does not appear to have a greater organism population than the monomicrobial biofilms. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small.

Figure 5.12 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 7 biofilms



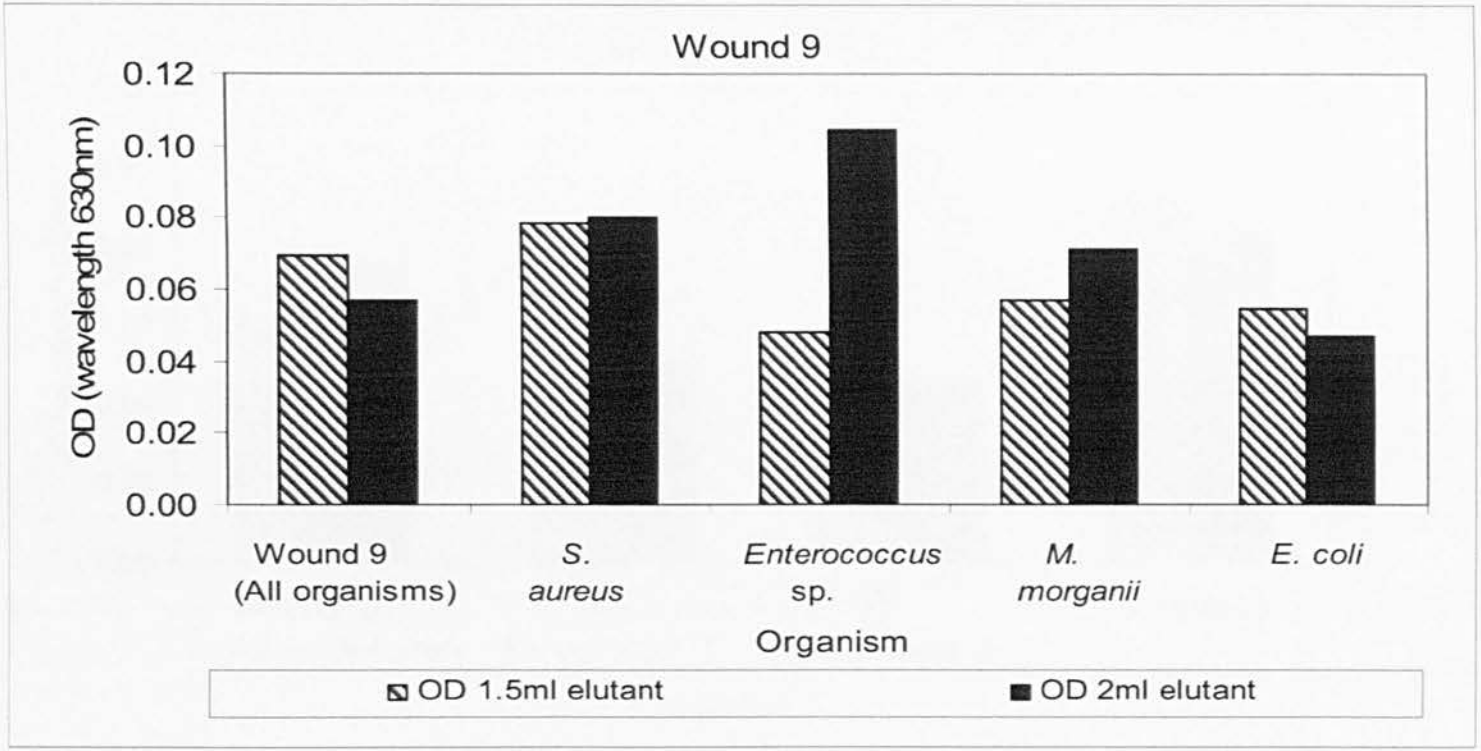
Optical density is recorded in absorbance units at λ 630nm. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. The polymicrobial biofilms does not appear to have a greater organism population than the monomicrobial biofilms. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small. GpG BHS = Lancefield Group G β haemolytic *Streptococcus*

Figure 5.13 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 8 biofilms



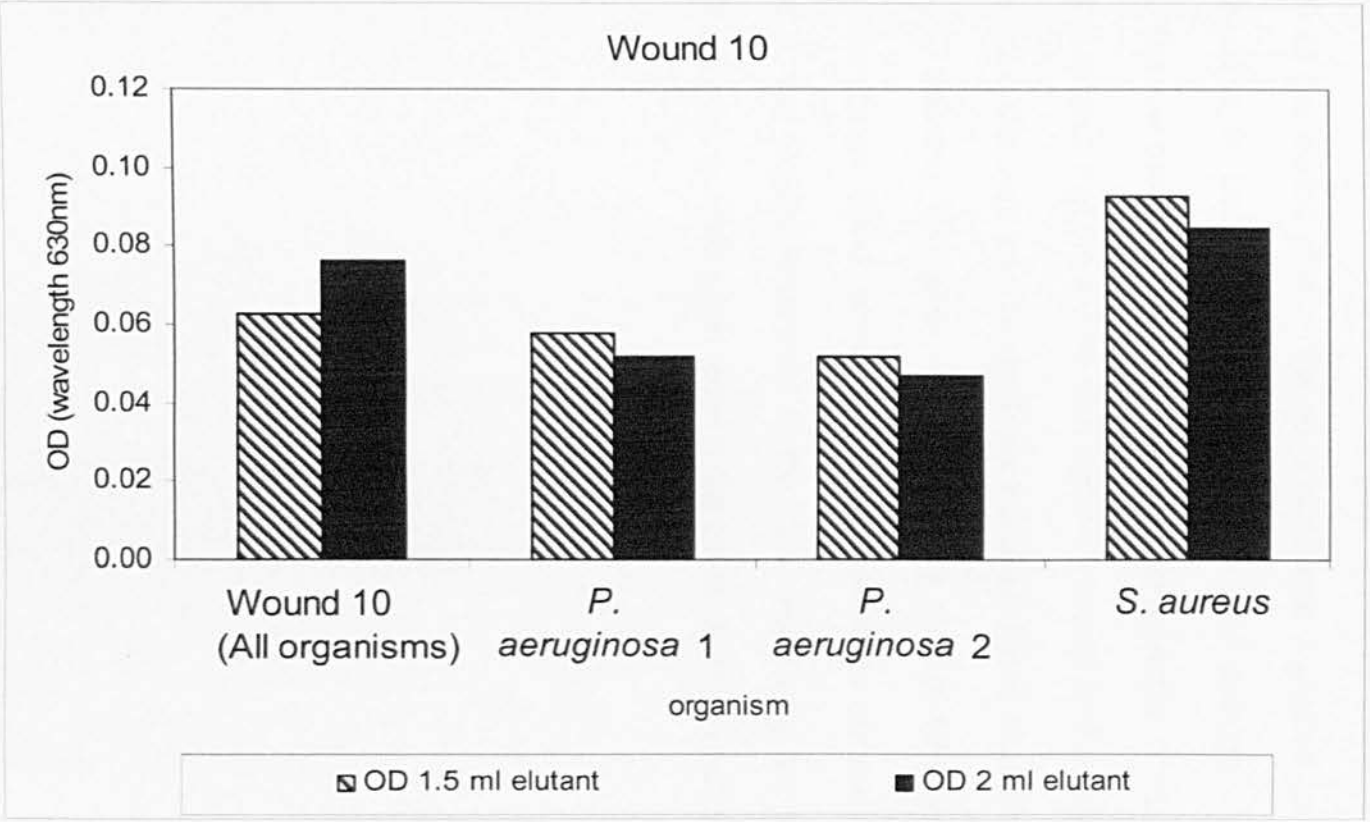
Optical density is recorded in absorbance units at λ 630nm The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small.

Figure 5.14 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 9 biofilms



Optical density is recorded in absorbance units at λ 630nm. The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. Error bars indicate standard error of the mean. Where error bars cannot be seen, SEM too small.

Figure 5.15 Optical densities obtained using 1.5ml and 2ml of elutant on Wound 10 biofilm

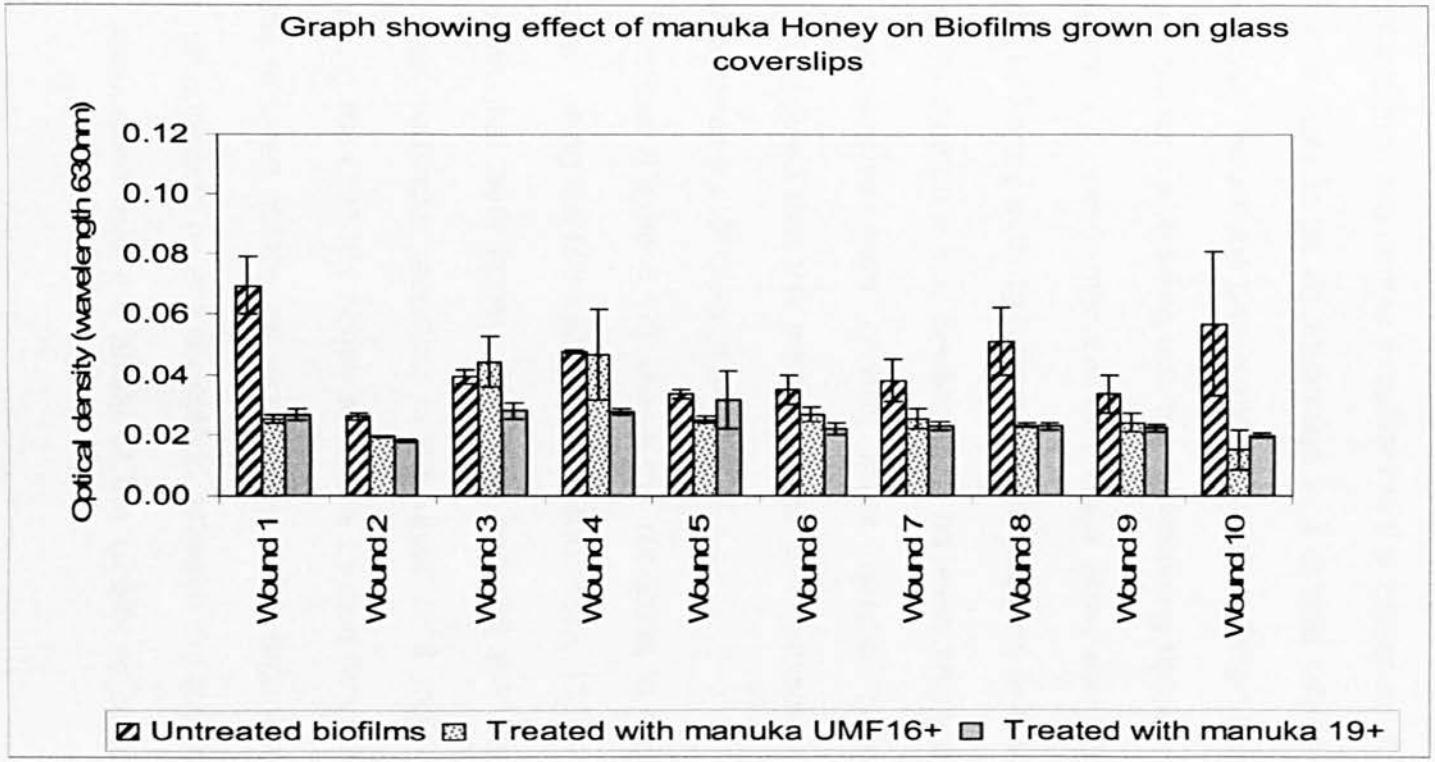


Optical density is recorded in absorbance units at λ 630nm The inconsistency of ODs between microbial populations and the two elutants is a possible indicatation that biofilm adherence to the glass was inconsistent. However, *S. aureus* is shown to form a biofilm more effectively in the absence of the other organisms.

5.3.8 The effect of Manuka honey on biofilms from polymicrobial wound infections grown on glass coverslips.

Figure 5.16 below shows the effect of submerging 48 hr biofilms on coverslips into 75% medical grade Manuka honeys after overnight incubation. Wounds 1, 8 and 10 show a dramatic decrease in optical density after honey treatment. Wound 3 indicates a lesser biofilm on the untreated coverslip as evidence by the optical density of the eluted stain.. Wound 3 contained *Myroides* sp. which is known to form biofilms in both environmental and clinical settings (Jacobs and Chenia, 2009). The low optical density of the untreated coverslip compared to wounds 1, 8 and 10 indicates that the biofilm was removed in part by the physical forces exerted as the coverslip was transferred from the brain heart infusion agar broth prior to fixing and staining. The other wounds showing a small differential in OD indicating one of two things. Either honey has a small ability to remove biofilms, or, as with Wound 3, the biofilm adherence to the glass slide was not sufficiently robust to resist the forces exerted when moving the coverslip from one medium to another.

Figure 5.16 Graph showing activity of Manuka honey on biofilms grown on glass. Optical density is recorded in absorbance units at λ 630nm.



In all but wound 3, the untreated biofilm gave a higher OD. The Manuka UMF 16+ gave a lower OD for wounds 1,5 and 10 indicating greater ability to remove biofilms. For wounds 2, 3, 4, and 6 the UMF 18+sh6wed a greater effect. For wounds 7, 8 and 9 there is no discernable difference making the overall comparison inconclusive. Error bars show +/- standard error of mean

The discrepancies make results inconclusive with regard to ascertaining the comparable efficacies of the two products used but there is evidence to show that these medical grade Manuka products may have some ability to remove biofilms.

The results of the subsequent culturing of the honey solutions after the coverslips were removed all failed to recover viable organisms showing that any organisms sloughing off a biofilm are unable to survive in the presence of 75% medical grade Manuka. This is likely to be an advantage in a clinical setting as it suggests that using honey could reduce the proliferation of an established biofilm and reduce the risk of wound bacteria spreading into the surrounding tissues or environment. To further explore this aspect further a wound model assay was designed.

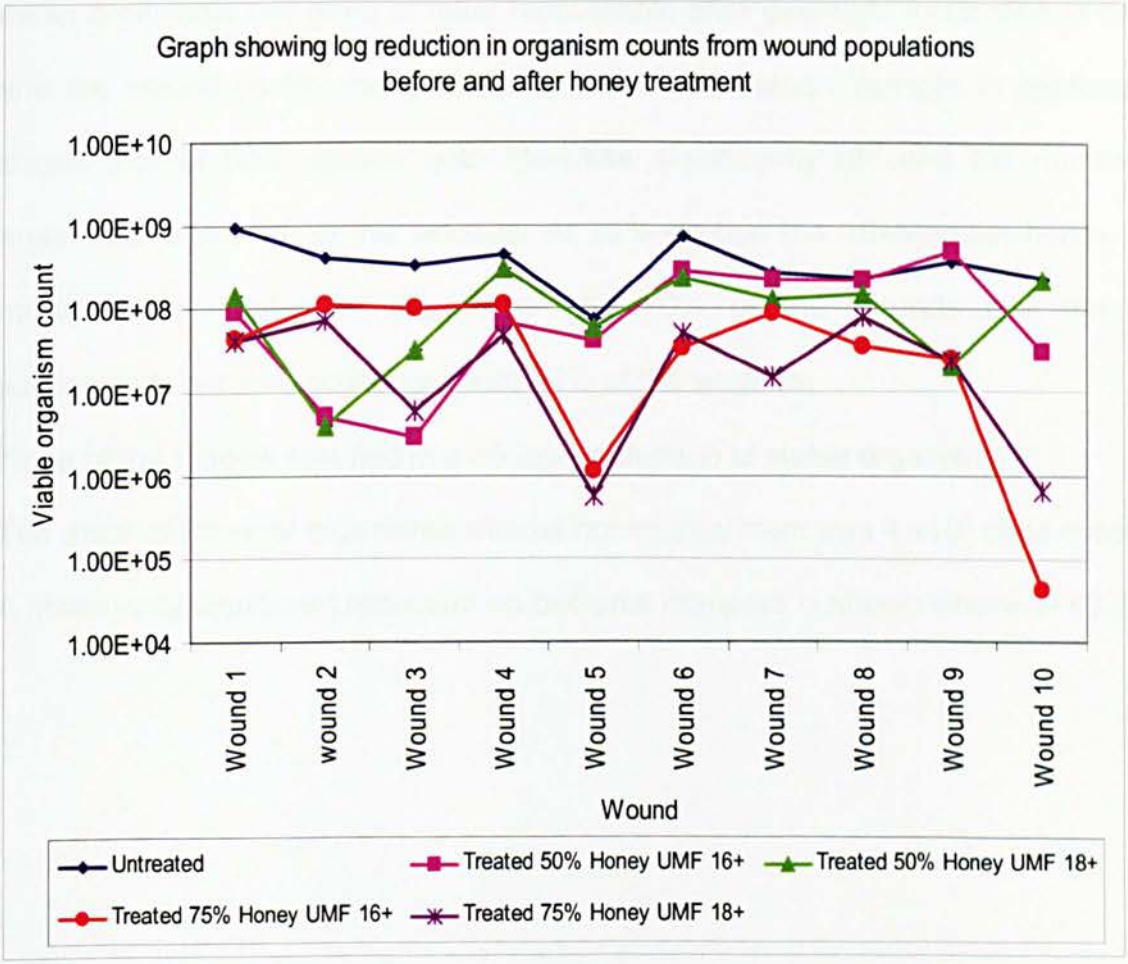
5.3.9 Results of honey activity on wound populations in a wound model

As described in method 2.8.1, Section 2, a baseline figure of number of viable organisms recoverable from cooked meat without honey treatment was determined. It showed that the mean number of organisms recoverable without honey treatment was 4 $x10^8$ cfu/g of meat.

The graph overleaf (Figure 5.17) shows the reduction in organism counts after honey treatment using the Miles Misra (Miles and Misra, 1938) drop count method. It can be seen that both honeys, at 75%, reduced the number of organisms recovered. The maximum reduction in the order of 4 log_{10} was obtained with Wound 10 using the UMF16+ honey at a 75% dilution (*w:v*). At a dilution of 50% both products reduced number of recoverable viable organisms by up to 2 log_{10}. There does not appear to be any correlation between the species of bacteria within the wound populations and the ability of the biofilm to resist honey treatment.

Figure 5.17 represents the number of viable wound bacteria recovered from wound model before and after honey treatment.

.Figure 5.17 Effect of Manuka Honey on numbers of recoverable organisms grown on cooked meat wound model using the miles misra method to count the viable organisms



The graph shows the reduction in the recovery of viable organisms from honey treated meat.which the greatest reduction being seen in organism populations from Wounds 5 and 10 after treatment with 75% honey.

5.4 Statistical analysis of reduction of biofilm organism population after honey treatment

A paired sample t-test was used to analyse the significance of the reduction of viable organisms resulting from the honey treatment.

Table 5.1a and 5.1b shows the results of a paired sample t test comparing the mean organisms per gram of meat recoverable after overnight incubation in honey and the wound control meat which had been incubated overnight in peptone. It shows that at 50% dilution both Manukas significantly reduced the number of organisms with 70% of the wounds. At 75% dilution the 16+Manuka honey also reduced the number of organisms with 70% of the wounds and the 18+ successfully reduced organisms with 90% of the wounds.

None of the models resulted in a ≥5 $\log_{10}$ reduction of viable organisms.

The mean number of organisms without honey treatment was 4 $x10^8$ cfu/g meat

A statistically significant reduction on bacterial numbers is shown where p=<0.05

Table 5.1a Wound samples 1-5. Paired sample t test using organism counts with and without honey treatment

Wound	Wound site	Organisms	50% Meloderm Manuka 16+	75% Meloderm Manuka 16+	50% Comvita Manuka 18+	75% Comvita Manuka 18+
1	Abdominal wound	2 isolates of *Enterococcus* sp. *P. mirabilis*	0.003	0.000	0.002	0.000
2	Axilla wound	Acinetobacter sp. Group B β BHS Group G β BHS *E. coli*	0.000	0.098	0.000	0.040
3	Leg wound	*Myroides* sp. *K.oxytoca* *Clostridium* sp. Mixed anaerobes	0.001	0.153	0.000	0.000
4	Knee wound	*Enterococcus* sp. *P. mirabilis* *E. coli*	0.018	0.001	0.122	0.002
5	Thumb wound	*S. alagalactiae* *K. pneumoniae* *S. aureus*	0.000	0.000	0.001	0.000

P values of >0.05 , shown in red indicate that the reduction in organism numbers was not statistically significant

Table 5.1b Wound samples 6-10. Paired sample t test using organism counts with and without honey treatment

Wound	Wound site	Organisms	50% Meloderm Manuka 16+	75% Meloderm Manuka 16+	50% Comvita Manuka 18+	75% Comvita Manuka 18+
6	Toe wound	*P. aeruginosa, P. mirabilis* *Corynebacterium* sp. *Enterococcus* sp. Mixed anaerobes	0.001	0.000	0.040	0.001
7	Wound swab	*Enterococcus* sp. Group G β haemolytic *Streptococcus* *Alcaligenes faecalis*	0.574	0.163	0.003	0.001
8	Sacral wound	*S. aureus* *Enterococcus* sp. *E. coli*	0.869	0.002	0.114	0.133
9	Foot wound	*S. aureus* *Enterococcus* sp. *M. morganii* *E. coli*	0.702	0.001	0.001	0.001
10	Ear swab	2 isolates of *P. aeruginosa* *S. aureus*	0.000	0.000	0.924	0.000

P values of >0.05 , shown in red indicate that the reduction in organism numbers was not statistically significant

5.5 Discussion

The aim of this section of study was to examine the effect of honey on established biofilms. The results have highlighted some of the difficulties of working with biofilms *in vitro*. Problems were experienced with microtitre plate methodologies. Where organisms had produced a large amount of extracellular matrix, the weight and cohesion of the biofilm caused by the matrix resulted in the biofilm detaching from the surface of the well. *S. aureus* has specific 'microbial surface components recognising adhesive matrix molecules' (MSCRAMMS) (Foster and H□□k, 1998). Fibronectin binding protein A binds to both fibrinogen and fibronectin (J□nsson *et al.*, 1991) and the experiments using porcine fibrinogen were conducted to create a more adhesive surface to overcome the problems of the biofilm weight dislodging the biofilm from the surface upon inversion. Whilst this may have improved the adhesion of the *S. aureus* biofilms, the problems were compounded by the addition of honey.Honey itself is a viscous substance and using undiluted honey in microtitre plates or on coverslips was not viable as the forces required to remove the honey also appeared to remove the biofilms. Diluted honey was trialled but did not resolve the problem. Consequently another medium on which to grow the biofilms was required.

The use of glass coverslips appeared tp be a more useful model system for robust biofilm formation, however, the problem of heavy biofilm detachment was not completely overcome. The limitation of these particular experimental approaches is in the difficulty in obtaining reproducible results. The adherence of the biofilms to glass surfaces was better but still not fully robust which meant that steps in the

methods such as removal of the coverslips from growth medium still, on occasions tore the biofilm from the smooth glass surface. Consequently the alternative approach comprising a wound model was considered to better emulate biofilms growing on an organic wound surface and therefore to be more representative of a clinical wound.

In retrospect, further modifications to this method may have addressed some of these problems. Bacteria respond to hydrodynamic forces, an ability which facilitates biofilm formation in environments such as water pipes and in aquatic environments where water is moving (Hall-Stoodley and Stoodley, 2005). In order to survive the shear forces of the moving liquid, bacteria in biofilms can alter their internal architecture to resist the environmental mechanical forces and to improve nutrient transport within the biofilm structure (Beyenal and Lewandowski, 2001). Laminar shear forces, such as those created by growing the biofilms *in vitro* in a static incubator result in weaker EPS which is readily dislodged when there is an increase in shear forces (Hall-Stoodley and Stoodley, 2005). Biofilms growing in a higher flow environs alter their viscoelastic response which reduces the EPS and increases biofilm density (Shaw *et al.*, 2004). Ceri and colleagues (1999) designed the Calgary Biofilm Device to overcome some of these problems. The device, similar in design to a 96 well microtitre plate, grows the biofilms on protruding pegs instead of if the microtitre well inner surface. The pegs are immersed into a base containing the bacterial suspensions and a growth medium. It is a sealed unit which is incubated on a rocker. This creates shear forces and uniformity of biofilm population on each of the pegs. This was

designed to perform antimicrobial MIC testing of organisms within biofilms and would perhaps have been the ideal piece of apparatus for this study. It is possible that regular refreshing of the medium would have improved biofilm formation by creating hydrodynamic challenge and replenishing bacterial nutrients.

The results of the wound modelling show that Manuka products at 50% and 75% dilutions can statistically significantly lower the number of viable organisms recoverable from polymicrobial wound populations growing on meat although significant reduction cannot be guaranteed even at 75% concentration. It can also be seen from Figure 5.17 that the maximum reduction was achieved by the Comvita Manuka UMF 18+ at 75% which achieved a 3 $\log_{10}$ reduction in organism numbers. According to Koburger *et al.* (2010) the reduction required for the product to be considered an antiseptic is 5 $\log_{10}$

The variability of efficacy visible on the Figure 5.17 can be attributed in part, to a number of causes. The organisms in each population fluctuate and have variable susceptibilities to Manuka products as observed previously (Chapter 3). *Enterococcus* sp. were shown to be generally less susceptible than other organisms to Manuka products, therefore its presence in a polymicrobial wound environment may contribute to the capacity of the biofilm to resist the effect of the honey. However, the variability of these results suggest that this is not necessarily the case. The variability may indicate that ecosystems comprising differing bacterial populations within individual biofilms may increase or decrease susceptibility to the honeys but the evidence is not conclusive.

Using coverslips showed that biofilms of organisms such as *Myroides* sp. and *P. aeruginosa* can be more easily physically disrupted than those of other organisms

and it is possibly this that accounts for some of the inconsistencies shown between honey and concentration within the models of individual wounds.

Variations in the wound model results can be attributed in part to the meat surfaces upon which the organisms were grown being crenulated and irregular. Although steps were taken to minimize the weight variation of the meat, it was not possible to compare the definitive surfaces area upon which the bacteria were growing. It is likely that there were crevices in the meat surface upon which organisms were growing which were inaccessible to the honey lending weight to the possibility discussed in Chapter 4 that honey must physically come into contact with bacteria in order to illicit an antimicrobial effect. However it is clear that a bacteriostatic effect was observed and honey use in the clinical setting could be considered at least as a supporting treatment.

The results would indicate that these products, even when diluted by 50% suppressed the propagation of polymicrobial biofilms growing on meat. The negative cultures obtained from the honey solutions after removal from the wound models suggest that any sloughed off biofilm would be unable to reseed in the presence of honey which would serve to negate the bacterial reservoir from causing persistent, chronic infection.

The products were diluted in order to formulate the minimum antimicrobial effect likely to be seen in a clinical setting where wound exudate may dilute the honey after application. There are several significant considerations with regard to the impact of wound exudate. Inflamed wounds produce exudate as a result of inflammation which causes vasodilatation and increases the permeability of vessels (Vowden and Vowden, 2003). Therefore the application of honey to

inflamed wounds will result in some level of dilution by wound exudate and the fact that honey exerts antimicrobial effects at dilutions of 50% and 75% is pertinent. The practicality of wound exudate does need to be addressed in a clinical setting as the honey needs to maintain contact with the wound and not be washed away in wound liquor. In practice this has been achieved by soaking dressings in medical grade honey (Cooper *et al.,* 2001), however the anti-inflammatory action of honey has been reported to reduce the production of wound exudate (Molan and Betts, 2004) which implies the problem decreases with continued treatment. Another influence of wound exudate effects in honey products, although not recreated as part of this wound model, is the release of hydrogen peroxide. Honeys from many different floral sources including Manuka honey have peroxide activity (Bang *et al.,* 2003) boosting antimicrobial activity..

It is reasonable to surmise that the application of honey on acute wounds could have a beneficial role in the suppression of biofilm formation and development, thus reducing the incidence of chronic wound infection. Simon *et al.* (2006) describes the use of medi-honey in paediatric haemato-oncology patients at 48 hours post surgery. Immediately post surgery, wounds are kept open and Octenidine soaked alginate dressings are applied as Octenidine is reported to kill bacteria within 5 minutes. Once cleaned of bacteria, the honey dressing is applied to keep the wound bed clean and to facilitate the healing process. However, it is unclear how often the Octenidine soaked alginate is replaced or why honey is not applied immediately post cleaning with an Octenidine solution, given that honey is reputed to reduce scarring (Phupradit and Saropala, 1992, Al-Waili and Saloom, 1999). The results of the wound modelling conducted in this study support the

reasons for using other products to initially sterilise a wound bed. These reasons are of particular relevance to immuno compromised patients whose own immune systems may be unable to clear even bacteriostatic wound populations. The value of honey in maintaining the sterile environment is of particular benefit when considered in conjunction with its anti inflammatory healing properties (Cooper and Molan, 1999a, Cooper *et al.*, 2001, Cooper, 2008, Simon *et al.*, 2006). This study demonstrated that honey will inhibit the growth of bacteria and prevent the escape of organisms from a wound bed model into the surrounding environment which further serves the control and prevention of infection supporting its use and value in a clinical setting.

5.6 Conclusions

The removal of biofilms from smoth surfaces did not reflect the efficacy of honey in removing biofilms from an uneven organics surface. The study demonstrated that honey could be useful addition to help prevent biofilm formation in a clinical wound. The study also showed that the honey was unlikely to remove the biofilm, however it was likely to arrest further development. The use of honey on infected wounds may potentially be used to prevent viable organisms escaping into the surrounding environment.

CHAPTER 6

Chemical Characterisation of Products

6.1 Introduction

Studies have shown that Manuka honey products have greater antibacterial activity than non-Manuka honeys and Syrups (Cooper *et al.*, 1999b, Lusby *et al.*, 2005) which suggests that the antimicrobial activity of Manuka is greater than that which can be attributed to the high sugar content (Wahdan, 1998). These findings were confirmed by the antimicrobial assays conducted in Chapter 3 of this thesis.

Much of the interest in identifying honey components has been generated by the food industry in order to comply with quality standards and food safety regulations (Anklam, 1998). As a food product, identification of pesticides used in honey production (Rezić *et al.*, 2005) may be necessary to determine if the honey is fit for human consumption. Honeys containing flavonoids and polyphenols are of particular interest in the health food industry as they are antioxidants which are reputed to have anti-inflammatory, anti-carcinogenic, anti-arthritic properties and antibacterial properties providing numerous benefits to health (Gómez- Caravaca *et al.*, 2006). Identification of flavonoid content can help to determine the floral origin and purity of different honey products (Truchado *et al.*, 2009).

The non-peroxide activity of Manuka honey has been attributed to methylglyoxal (MGO) (Mavric *et al.*, 2008). Adams and colleagues (2008, 2009) showed the MGO levels to have a linear relationship with the non-peroxide/UMF activity with a coefficient of determination (r^2) of 0.92, where an r^2 value of 1 is equal to perfect linearity (Adams *et al.*, 2008, 2009). The presence of phenolic compounds with antimicrobial activity such as methyl syringate, benzoic acid, cinnamic acid and phenyllactic acid have also been identified in New Zealand honeys but levels were

not considered to be antimicrobially significant (Weston *et al.*, 2000; Adams *et al.*,2009).

The methods previously used to separate and identify these compounds have included, gas chromatography (Aljadi and Karmarrudin, 2003), high performance liquid chromatography (Pyrzynska and Biesaga, 2009) and thin layer chromatography (Rezić *et al.*, 2005).

6.1.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is a basic method for separating chemicals based on the affinity between the molecules within a substance and selected stationary or mobile phases (Rezić *et al.*, 2004). TLC plates are made of glass or plastic and have an adsorbant coating such as silica or cellulose which forms the 'stationary phase'. The mobile phase is the solution which is drawn up through the stationary phase by capillary action (Fenimore and Davis, 1981). Separation occurs when the test sample is spotted onto the TLC plate which is placed in a trough containing the mobile phase solvent. The mobile phase passes through the sample as capillary action draws the solvent up the plate. Charged 'polar' molecules binding with the mobile phase are separated from the less polar components and drawn up the plate. In this study TLC was tested as a technique with the aim of separating the molecular components of the Syrup and honey products in sufficient quantity to identify them using Nuclear Magnetic Resonance (NMR) and to identify the antimicrobial fractions by agar / Nitro Blue Tetrazolium (NBT) overlay (see Section 2.10).

6.1.2 Nuclear Magnetic Resonance based Diffusion Ordered Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is a technique which capitalizes

on the ability of protons located in atomic nuclei to spin, effectively conferring them with magnetic properties (James, 1998), In the presence of a large magnetic field, it is possible to separate these spins based on whether they are aligned with (low energy) the magnetic field or against (high energy) the magnetic field. It is this energy difference between two proton spin-states, which is generated and measured in NMR spectroscopy (Jacobson, 2007). Different nuclei resonate at different frequencies proportional to the strength of the magnetic field. In a 600 MHz instrument TrimethylSilylProprionate (TSP) protons resonate at 600 MHz and the chemical shift is measured. The chemical shift is the resonance frequency of other protons compared to a reference standard, which in this study was TrimethylSilylProprionate (TSP). Shifts in frequency are minute (Hz) compared to the frequency of the magnetic field (MHz) so are reported as parts per million (ppm). Isolation and identification of individual components of complex mixtures such as honey using NMR has previously required solid or liquid phase extraction to remove impurities (Gomez-Caravaca *et al.,* 2006), preventing analysis of the product *in situ.* In addition to the information of the components afforded by different chemical shift for individual components within the honey, it is possible to perform diffusion ordered experiments, which allow the calculation of the diffusion coefficients for different components. The diffusion coefficient has a correlation to hydrodynamic radius and hence the mass of an individual molecule within the mixture (Li *et al.,* 2009). This allows differentiation between signals based on size as well as chemical shift difference of their constituent protons, making possible the direct analysis of complex mixtures such as honey.

The diffusion coefficient measured relates to the formula weight of the molecules and the density and viscosity of the mixture in the selected solvent (Li *et al.*, 2009). The size and shape of the molecules in a mixture affect how they diffuse into solvents of different viscosities. To eliminate the inconsistencies caused by variation in viscosity and concentration, this study utilized the calibration technique using three internal standards described by Li and colleagues (2009).

The aim of this section was to identify the antimicrobial components of the honey products used in this study using Thin Layer Chromatography (TLC) and to evaluate Diffusion Ordered Nuclear Magnetic Resonance Spectroscopy (DOSY NMR) as a suitable technique for the direct qualitative and quantitative analysis of honey.

6.2 Methods

6.2.1 Product selection

The honeys selected for use during the first TLC experiments were the Comvita UMF 18+ and the Comvita UMF 20+ Manuka honeys. The selection was based on the assumption that a product with a high UMF rating would contain the antimicrobial constituents the study was aiming to identify. The products were prepared as described in Chapter 2.10.1.1.

6.2.2 Selection of developing solution

The developing solutions described in Chapter 2.10.1.2 were tested in order to find the mobile phase which would provide the greatest separation of the products. The solutions were tested in order of increasing polarity. The developing solution selected for use in the study was Ethyl acetate 68%, methanol 24% and 8% deionised water which was the most polar of the developing solutions. A highly polar solution was required as the silica in the solid phase is also polar.

6.2.3 One and Two dimensional TLC

All products were tested using the 1D TLC method described in Section 2.10.1.3. The products demonstrating separation were then tested using 2 D TLC to further separate the fractions the method for which is described in Section 2.10.1.4.

6.2.4 Visualisation agents

Viewing the TLC plates under long wave and short wave UV were the first method used to visualise product fractions as this reveals UV active compounds such as aromatic compounds. Ninhydrin was used to identify amines and amino acids and ferric chloride to reveal any phenols. Vanillin was used to detect amines and amino acids (Section 2.10.1.3).

6.2.5 Agar overlay using NBT

The addition of NBT to the iso sens broth (method 2.10.1.6) resulted in a more even distribution of colour than pouring the NBT over the TLC plates after the layering of agar as described in method 2.10.1.5. The 1D and 2D TLC plates were therefore overlaid with agar containing an NCTC 6751 strain of *S. aureus* and NBT (method 2.10.1.6). *S. aureus* was selected as the organism most susceptible to the antimicrobial effects of the honey products as demonstrated in Chapter 3. NBT will only stain living cells so the absence of colour indicates organism killing which enables the determination of antimicrobial activity of the separated fractions. To ascertain this as an appropriate method to demonstrate antimicrobial activity, two 1D plates were spotted with a 10µl drop of product known to have antimicrobial activity diluted 50% in the methanol/water diluent.

6.2.6 Diffusion Ordered Spectroscopy Nuclear Magnetic Resonance

The methods and materials used were those described in Chapter 2.10.2.

Where there were overlapping signals within the carbohydrate spectra using Diffusion Ordered Spectroscopy (DOSY), Correlation Spectroscopy (COSY) and Total Correlation Spectroscopy (TOCSY) were employed which are useful methods for identifying protons on carbohydrate rings, as they can correlate the protons on an individual ring isolating it from other rings. Whilst COSY will show couplings of atoms bonded to each other, TOCSY spectra show the relationships between all nuclei within a molecule.

6.3 Results

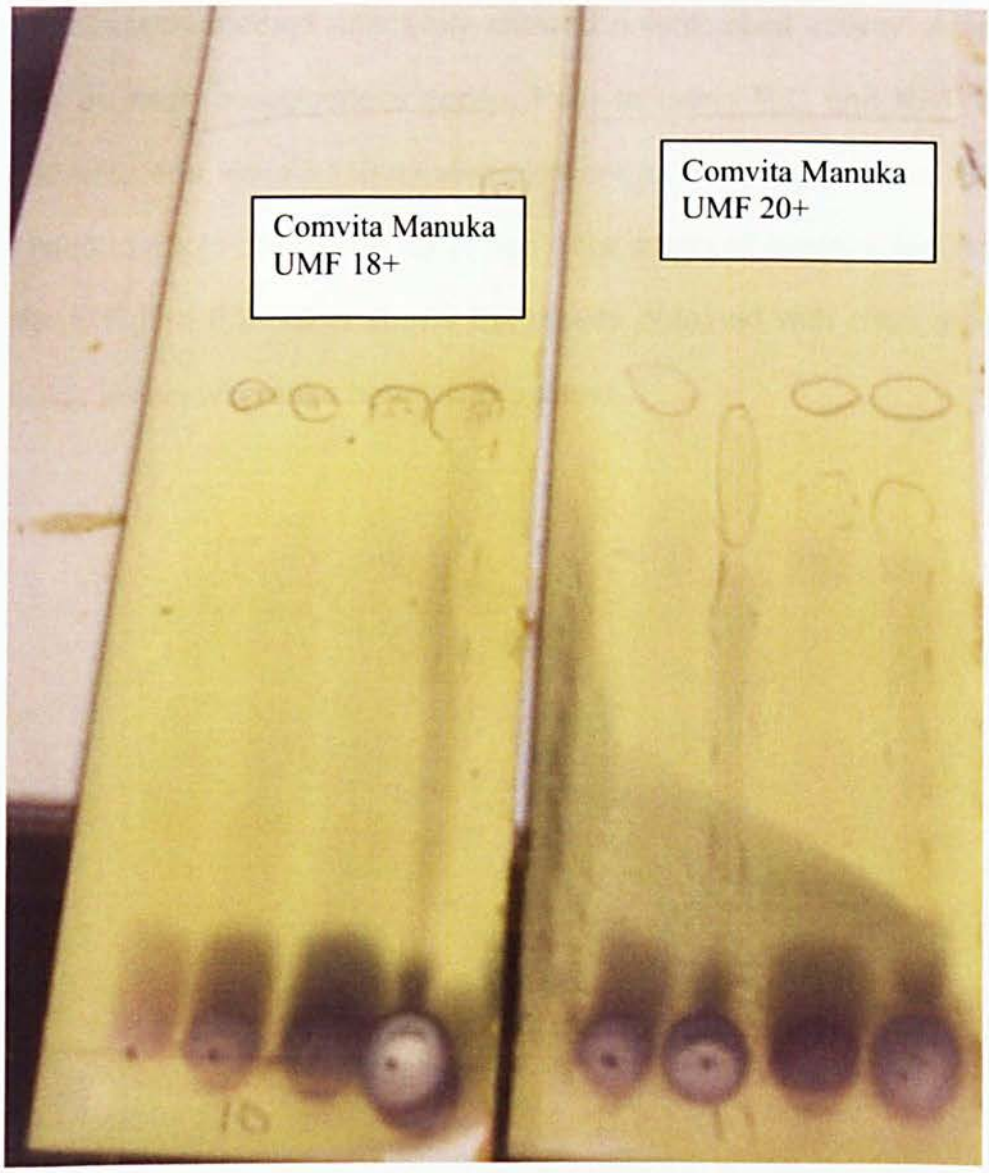
The first set of results detailed below show the separation achieved using TLC and the visualisation of antimicrobial fractions using nitro blue tetrazolium in an agar overlay. The limitations of this technique with regard to identifying the fractions can be seen and how that was overcome using NMR and DOSY software enabling the identification of the compounds within the honey products.

6.3.1 TLC Results

There were no fractions visualised using short wave UV, ninhydrin or ferric chloride. Viewing the TLC plates under long wave UV light revealed that fractions of the products had migrated up the plate. These areas turned brown after the application of vanillin and heat. The reaction with vanillin can indicate the presence of tannins. These brown areas can be seen close to the origin in Figure 6.1 below. Separation of fractions is reported as the Rf or retention factor which is the value obtained by measuring the migration from the point of origin by solvent divided by the migration of the separated fraction.

All of the products described in Chapter 2.1 were tested. Products A and B were the Syrups and showed minimal separation. Products C-I were Manuka honeys and J-N non-Manuka honeys.

Figure 6.1 One dimensional TLC plates after application of vanillin and heat.

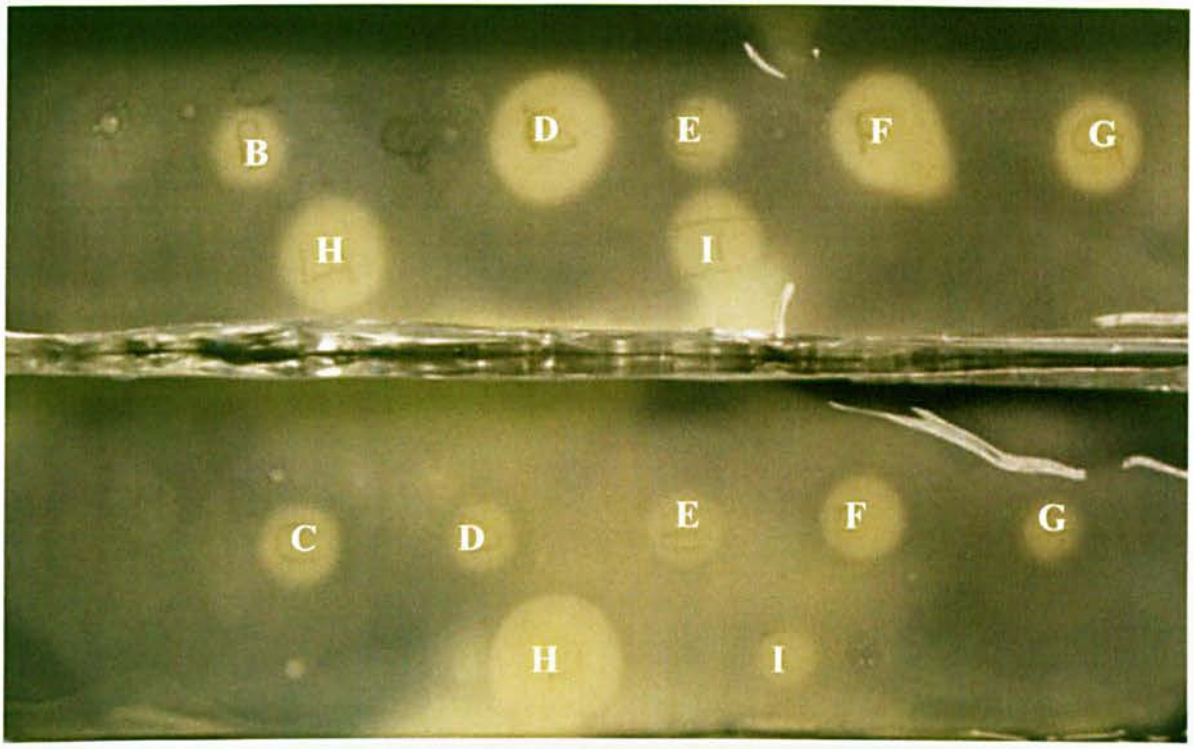


The four spots at the origin indicate the placement of 10 – 40 µl volume drops of honey. The brown areas depict possible presence of tannins in the two Manuka Honeys. It can be seen that the area and density of brown spots decrease with honey volume.The pencilled areas show regions visible under long wave UV light.

The pencilled areas seen using UV light were unique to the Manuka products. Tables showing the rf value of the separated fractions for each product can be seen in Appendix 7

The plates below show the results of the product spot test to determine whether the agar overlay method effectively showed antimicrobial activity. Areas of organism can be seen as colourless zones. Prior to using TLC and NBT agar overlay to separate and visualise separated fractions of the products, the NBT overlay was tested to spots of pure honey to see if the areas of antimicrobial activity would be clear. Figure 6.2 below shows the results obtained with clear areas of microbial killing visible where the honey was spotted.

Figure 6.2 Product spot tests with areas of organism killing appearing unstained by NBT



The labels identify the products. Manuka Honeys are labelled as MH with their UMF rating. The agar is stained blue with the NBT where the organisms are viable. The unstained areas where the honey has been dropped show the area of organism killing.

Figure 6.2 Key

B Treacle

C Manuka Honey UMF 16+ (irradiated)

D Manuka Honey UMF 20+

E Manuka Honey UMF 18+

F Manuka Honey UMF 16+ (non-irradiated)

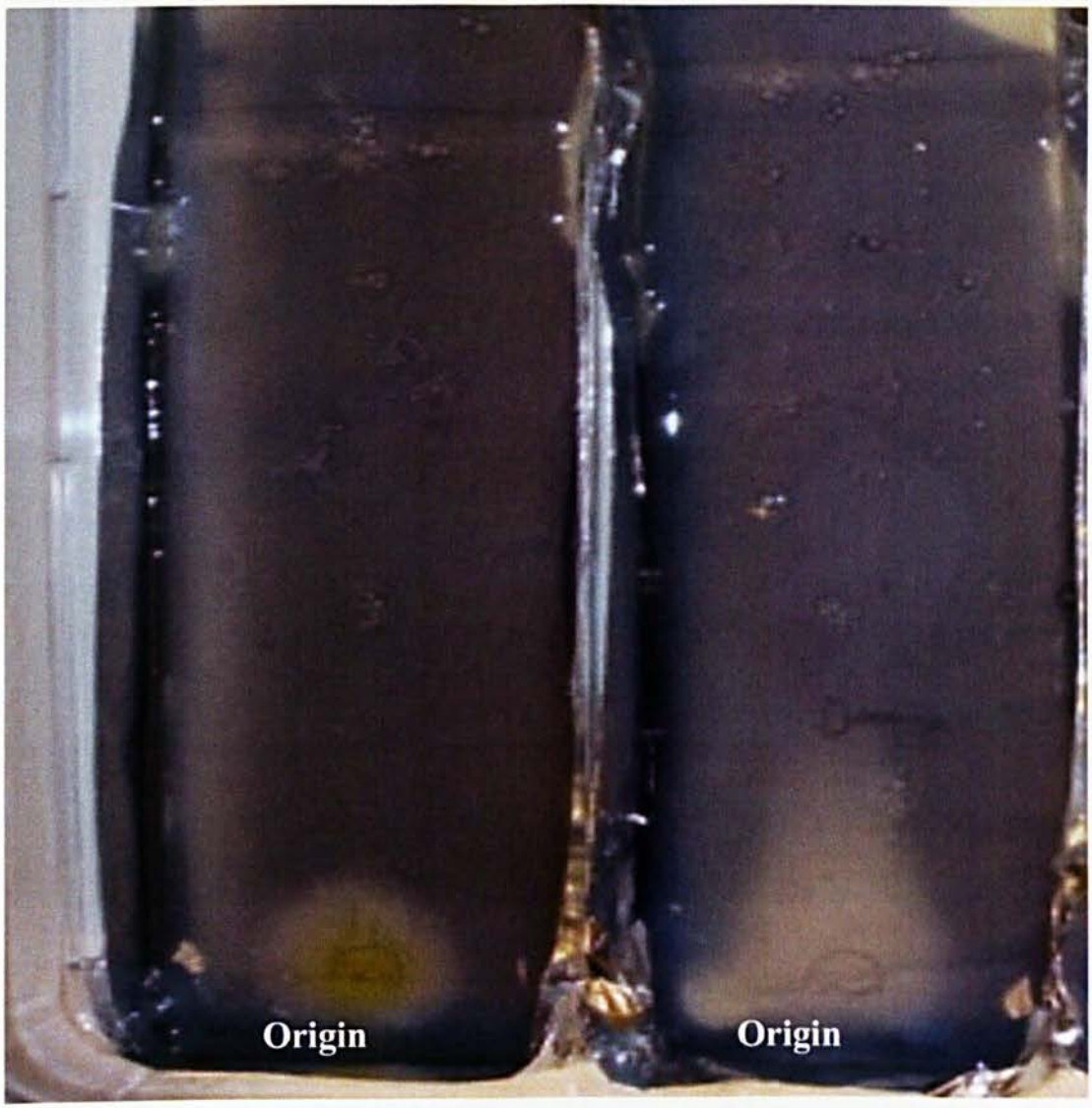
G Manuka Honey 13+

H Manuka Honey UMF 12+

I Manuka Honey UMF 5+

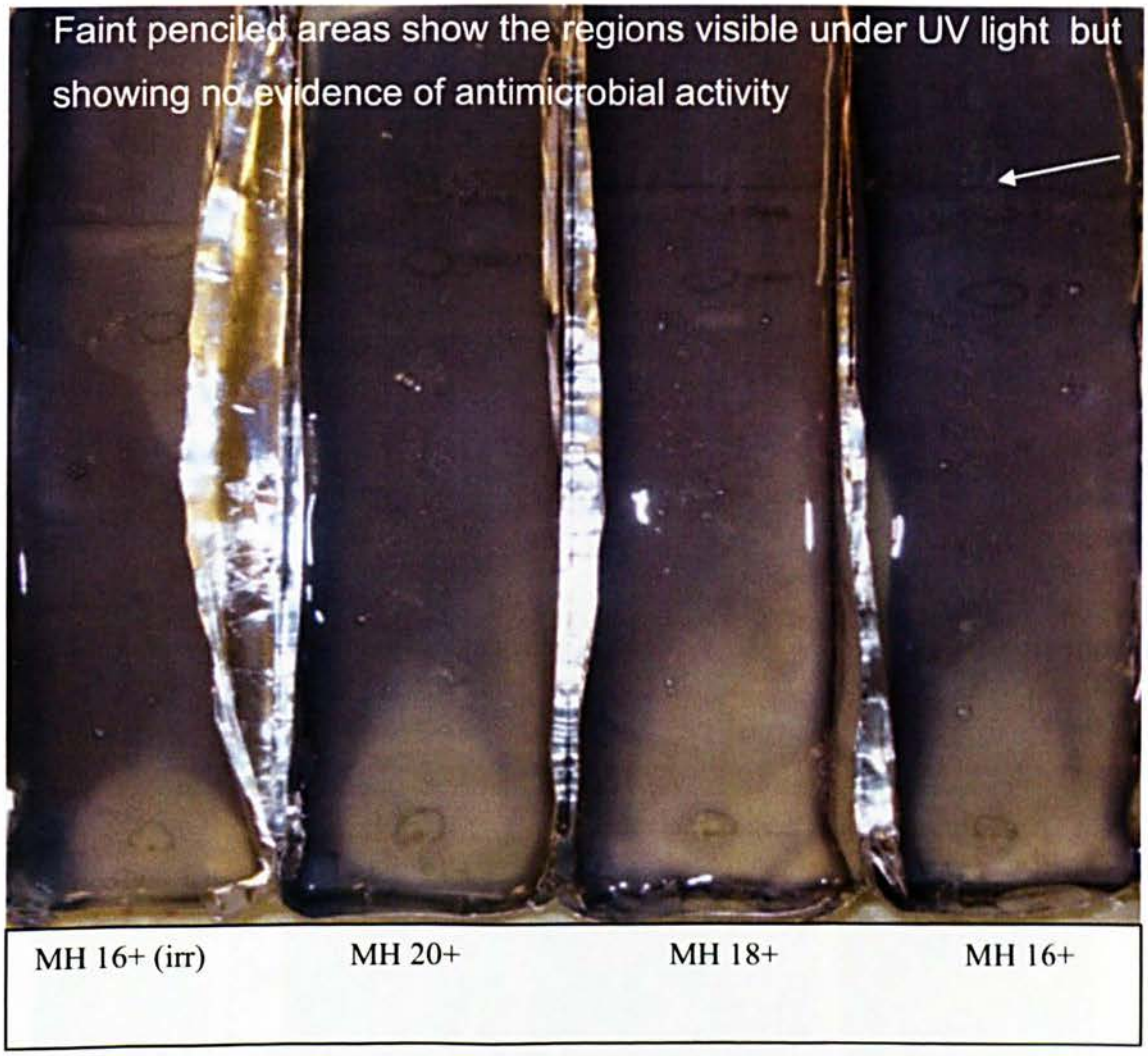
Figures 6.3 – 6.5 below show NBT agar overlay results obtained using the 1D TLC.

Figure 6.3 Treacle (left) and Golden Syrup (right) after TLC and agar overlay showing a small amount of organism inhibition around the origin



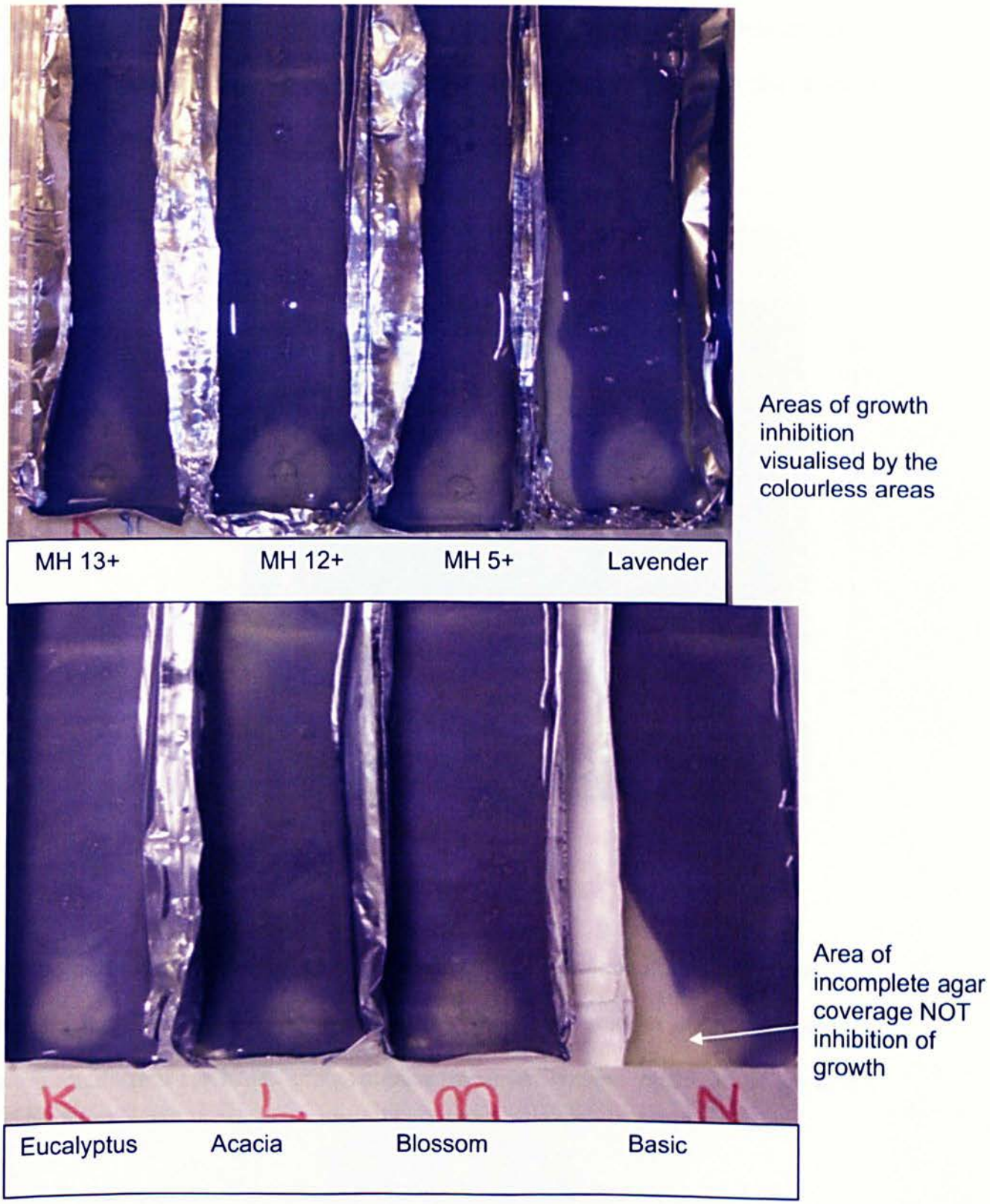
There is a clear region of antimicrobial activity around the origin, the areas where fractions migrated further up the plate which were observed using UV light, demonstrate little or no antibacterial activity using this method. These pencil marked areas are faintly visible through the blue agar.

Figure 6.4 Manuka honeys UMF 16+(irr), 20+, 18+ and 16+ after ID TLC and agar overlay showing growth inhibition around the origin



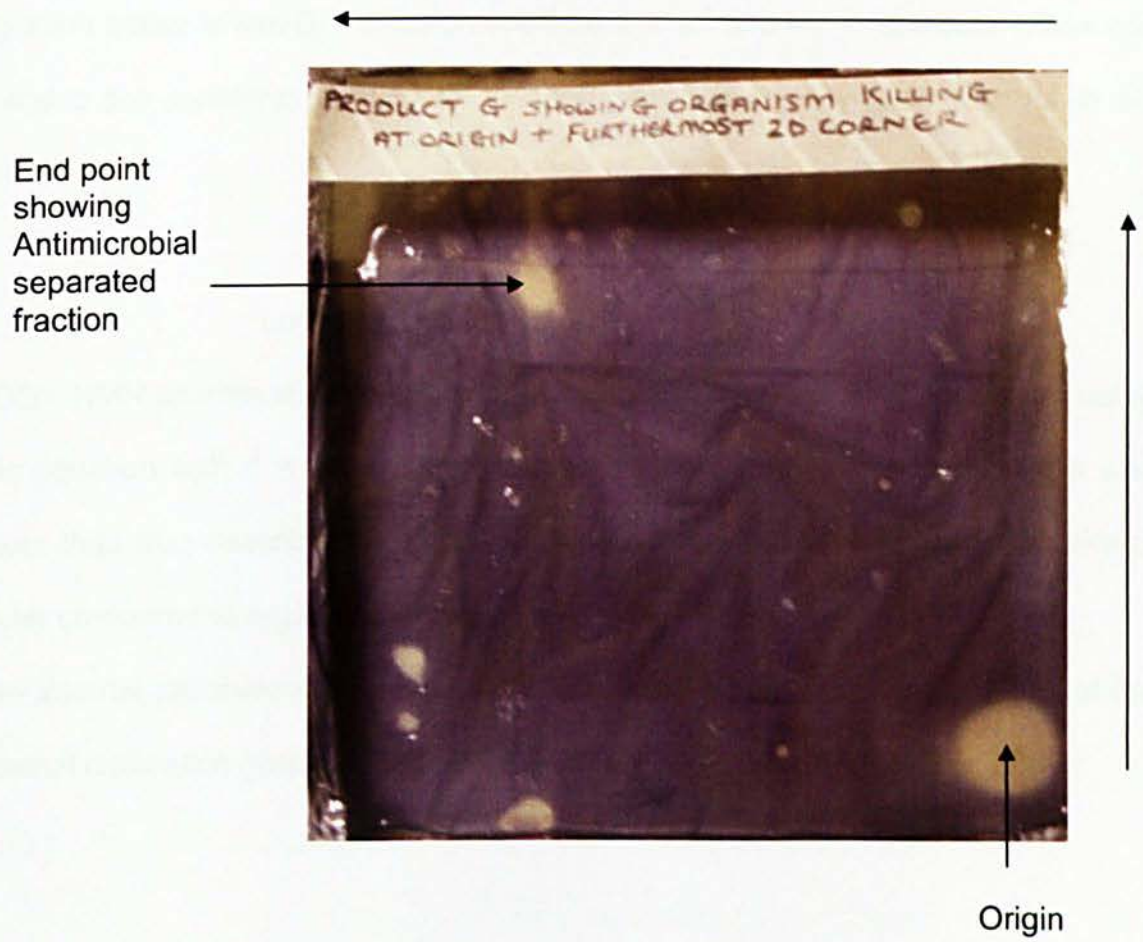
The pencilled areas indicated further up the plate which were visible with UV light and unique to the Manuka honeys show no evidence of growth inhibition.

Figure 6.5 Manuka honeys UMF 13+, 12+ and 5+ with lavender, eucalyptus, acacia, blossom and basic honeys after 1D TLC and agar overlay. The only areas of growth inhibition are those around the origin



The 2 dimensional TLC shown in figure 6.5 below using Honey G, shows an antimicrobial fraction which has migrated through the 2 dimensions. The origin can be seen bottom right of the plate and the fraction in the top left.

Figure 6.6 2D TLC of Manuka Honey 13+ with agar overlay. Arrows show direction travelled by separated fraction as it migrates through the silicon with the solvent



Having demonstrated the presence of antimicrobial fractions it was not possible to perform further analysis on these fractions due to the minute quantities involved being insufficient for NMR analysis as a minimum volume of 25 µg of extracted

substrate would have been required. However, the acquisition of DOSY software enabled analysis of unfrationated honey which allowed further identification of honey components described below.

6.3.2 DOSY NMR Results

6.3.2.1 Internal calibration

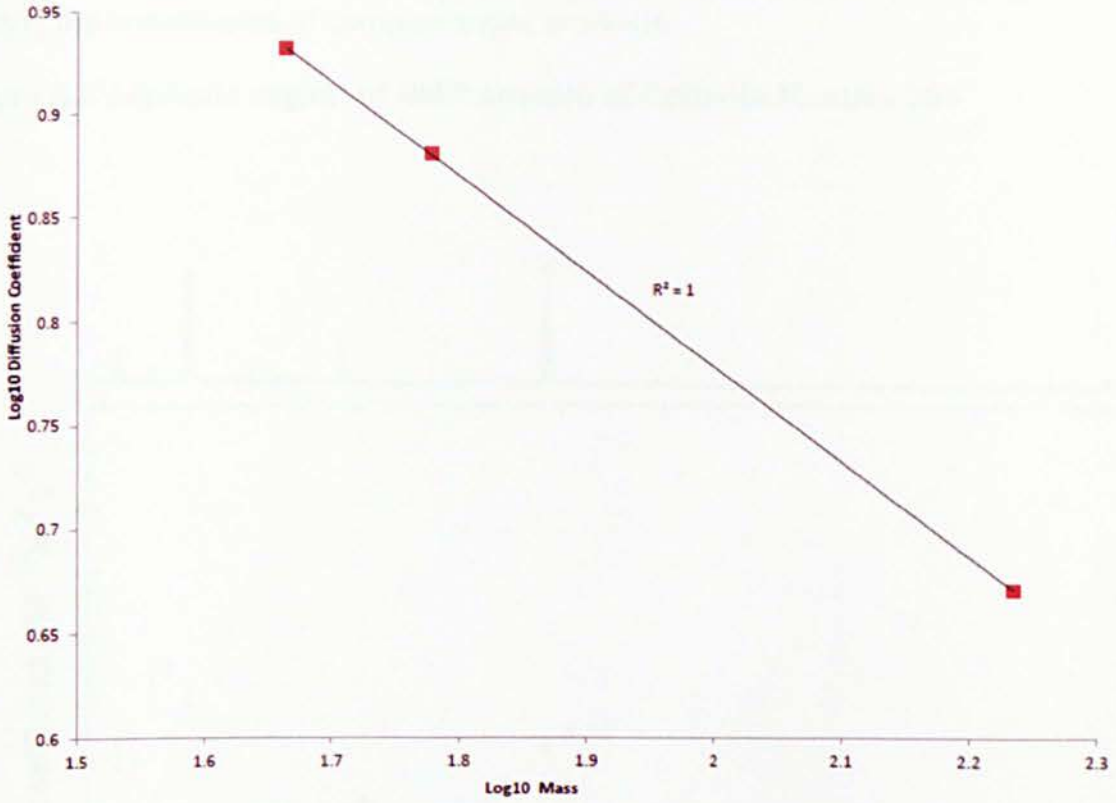
A calibration graph was produced using the three internal reference samples ethanol (48g mol^{-1}), acetic acid (60g mol^{-1}) and TSP (172g mol^{-1}) using the equation below where D = diffusion coefficient m^2s^{-1} and M_r = molecular mass and a and b are constants relating to concentration and density (viscosity) (Li *et al.*, 2009).

$$\text{Log } D = a \text{ Log } M_r + b$$

DOSY NMR spectra showed Comvita Manuka 20+)gave the best correlation using this equation with $r^2 = 1$ providing values a= - 0.46 and b = +1.69. Value b was lower than that described by Li and colleagues due to the increased viscosity of water compared to organic solvents (Figure 6.7)

The internal calibration of Manuka UMF 20+ shown below is representative of the internal calibration graphs obtained for each product using the equation above.

Figure 6.7 Internal calibration of Comvita Manuka UMF 20+



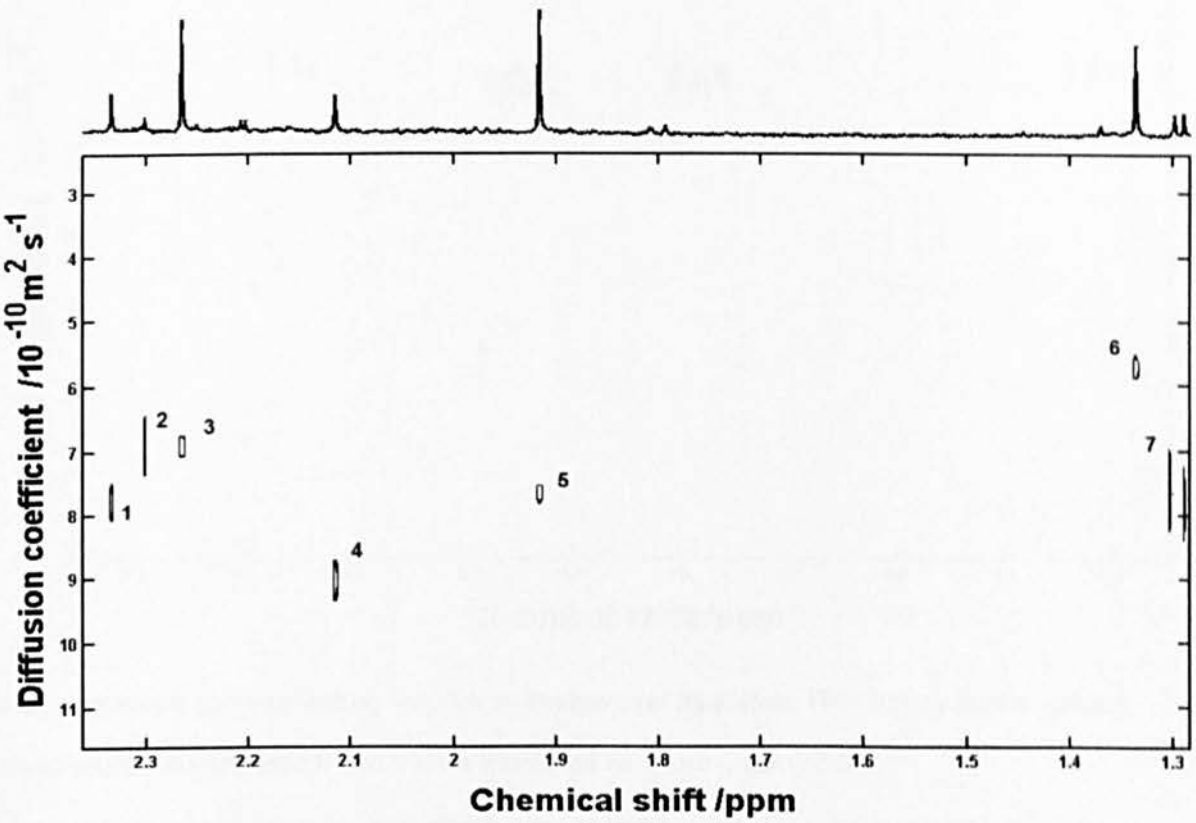
The calibration used 3 internal standards ethanol, acetic acid and TSP. The coefficient of determination value of 1 indicates excellent linearity. Components of the product can be identified by comparing their diffusion coefficients to those of the standards using the equation Log D= a Log M_r + b

6.3.2.2 Aliphatic components

As expected, all of the Manuka honeys gave signals between 1.3 and 2.3ppm which were not seen in the non Manuka products, which correlated to the CH_3 groups in methylglyoxal dihydrate and monohydrate. These were identifiable by their diffusion coefficients of 5.6 x 10^{-10} m^2s^{-1} and 6.9 x 10^{-10} m^2s^{-1} using the above

equation. These can be seen as areas 6 and 3 in Figure 6.8. This is the first clear demonstration that DOSY NMR can be used to separate and identify the constituents of complex sugar products.

Figure 6.8 Aliphatic region of NMR spectra of Comvita Manuka 20+

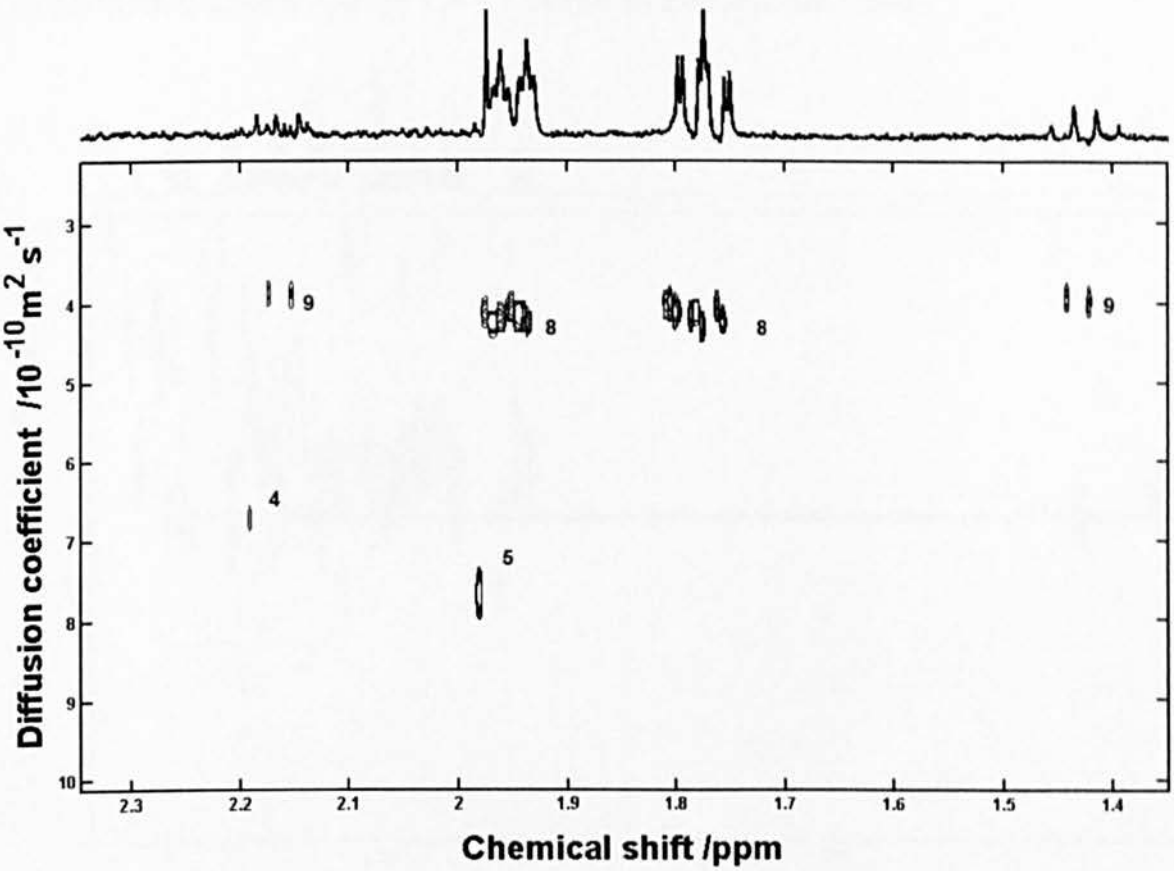


The identifiable components are:

1 – pyruvaldehyde; 2- pyruvic acid; 3- methylglyoxal monohydrate; 4- acetaldehyde; 6-methylglyoxal dehydrate; 7- lactate

The two non Manuka honeys did not give signals correlating to methylglyoxal hydrates as can be seen in Figure 6.9 below.

Figure 6.9 Aliphatic region of NMR spectra of Australian eucalyptus honey



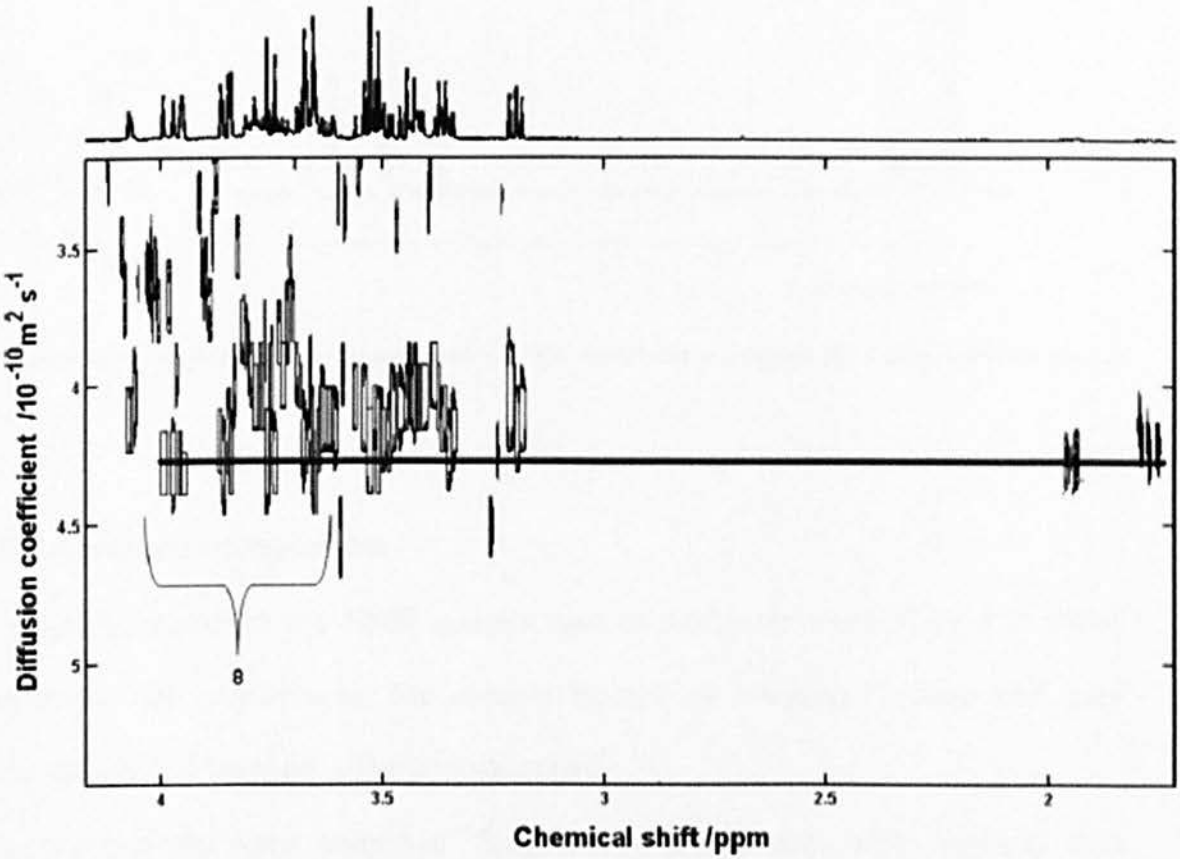
There are no areas corresponding the the methylglyoxal hydrates. This honey had a unique set of two multiplets labelled 8 and 9 later identified as *proto* – quercitol

The Australian eucalyptus honey gave two sets of multiplets (groupings) not seen in any of the other products tested (components 8 and 9 in Figure 6.9 above). Expansion of the region showed in Figure 6.10 showed 4 signals within the region of component 8 which would normally be inseparable from carbohydrate signals.

The TOCSY spectra resulted in five additional signals (Figure 6.11) however these did not correlate with any NMR data previously published on eucalyptus honey. However, a similar component was reported in oak honeydew honey by Simova and colleagues (2012) who identified the component as *proto* – quercitol. *Proto-*

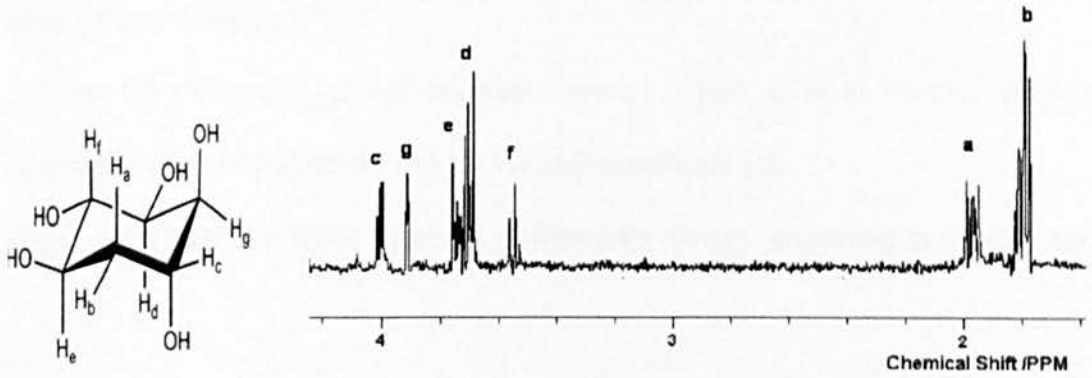
quercitol has not previously been reported in eucalyptus honey but has been isolated from the leaves of *Eucalyptus populnea* (McCassland *et al.*, 1968).

6.10 Expanded aliphatic region 1.5 – 4.3ppm of Eucalyptus honey



The 4 distinct signals of component 8 shown are normally inseparable from the other carbohydrates.

Figure 6.11 Structure of component 8 present in Eucalyptus honey and the corresponding 1D TOCSY spectra



This shows the 5 signals within component 8. This spectrum identified the component as *proto*-quercitol.

6.3.2.3 Aromatic components

The phenolic region of the NMR spectra was of particular interest as it is these compounds that characterise the unique flavour of Manuka honeys and may contribute to the enhanced antimicrobial activity.

Three compounds were identified, 3,4,5 trimethoxybenzoic acid, syringic acid phenyllactic acid, seen on Figure 6.12 and labelled 10,11,and 12 respectively and 3 unidentified molecules labelled 13-15 on the NMR spectra below (Figure 6.12).

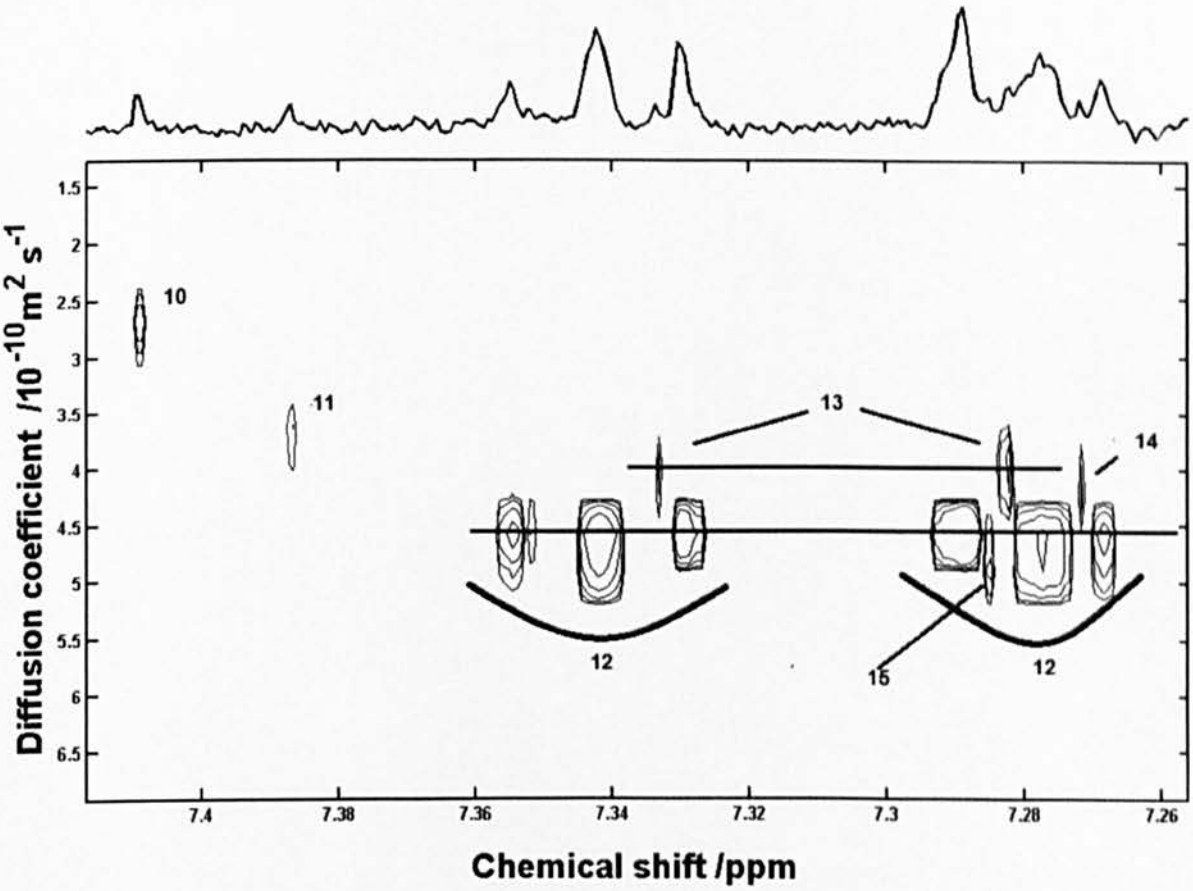
The chemical shift at 10 gave a predicted molecular mass of 330 g mol^{-1}. The high molecular mass of this compound compared to the internal standards resulted in a loss of linearity but it correlated well to the value obtained from the 3,4,5 trimethoxybenzoic acid spiked sample.

Component 11 with a predicted mass of 211g mol^{-1} correlated well to methyl syringate which has an actual mass of 212g mol^{-1}.

The signals at 12 gave a predicted mass of 163g mol^{-1} which together with the multiplets of the same diffusion coefficient identified the sample as phenyllactic acid (mass 166g mol^{-1}).

Signal 13-15 would appear to have similar mass to both methyl syringate and phenyllactic acid but could not be identified individually.

Figure 6.12 DOSY NMR spectra of Manuka honey showing aromatic regions

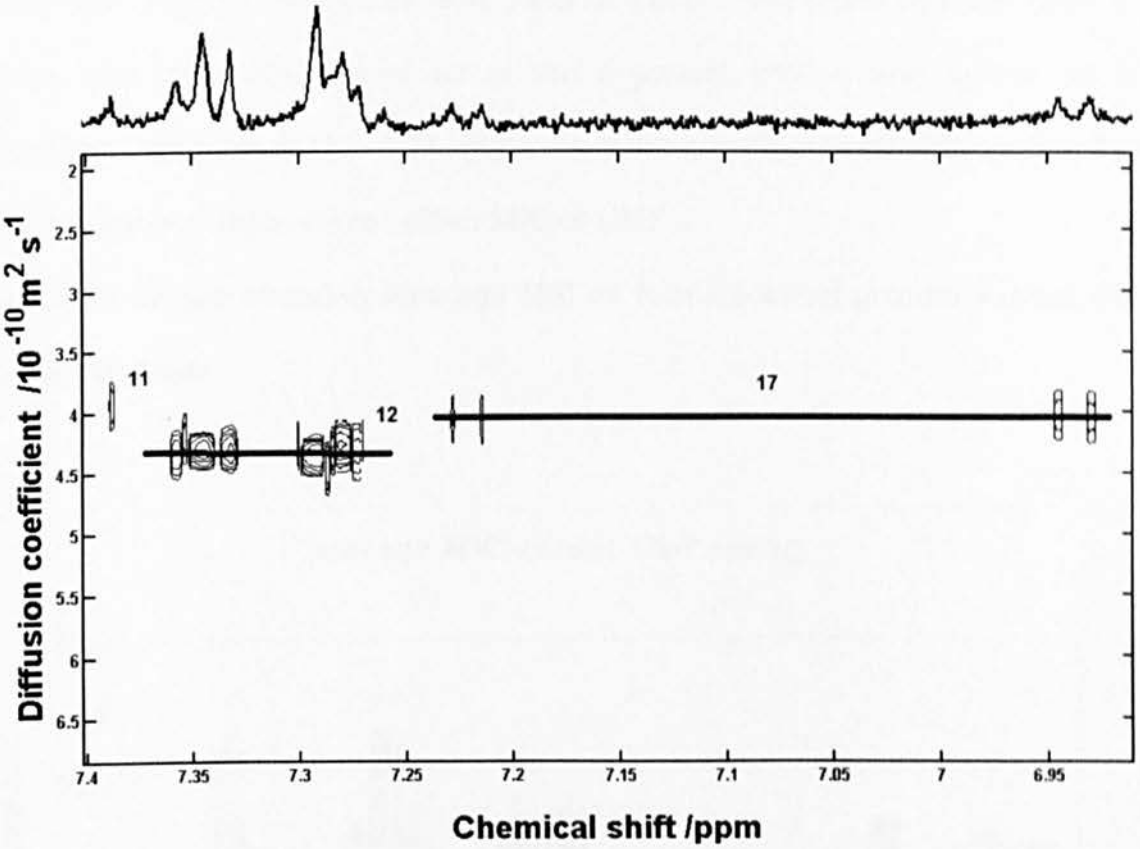


The aromatic regions shown are 3,4,5 trimethoxybenzoic acid (10) , syringic acid (11), phenyllactic acid (12) and unidentified compounds (13-15). None of these signals were seen in the non- Manuka products.

The Manuka honey with the lowest UMF rating of 5+ contained an aromatic component which was not seen in any of the other honeys. Figure 6.13 below shows the NMR spectra for the honey with the spike labelled 17.

The difference in chemical shift between the doublets, the diffusion coefficient and the predicted molecular mass of 193g mol^{-1} determined this as 4-methoxyphenyllactic acid (molecular mass 197g mol^{-1}).

Figure 6.13 DOSY NMR spectra showing aromatic region for Hadrells Manuka Honey UMF 5+



This shows the region of unique .component 4-methoxyphenyllactic acid (17), molecular mass 197 g mol^{-1}.

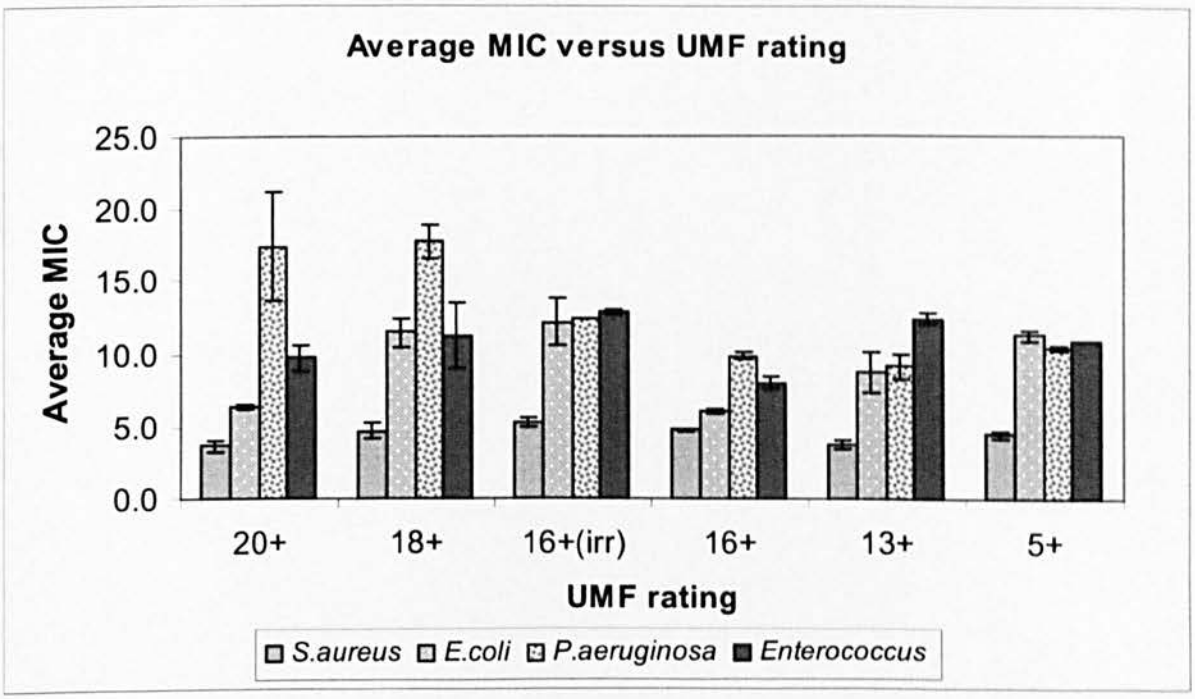
6.3.2.4 Quantification of honey components and antimicrobial activity

The study has shown that Manuka honey contains both aliphatic and aromatic components not found in non-Manuka eucalyptus and acacia honeys which have been attributed to the greater antimicrobial properties of Manuka products (Mavric *et al.*, 2008, Stephens *et al.*, 2010).

The UMF rating of Manuka products is determined by the anti staphylococcal activity of a Manuka product, however, this relationship was not confirmed by the

findings of this study described in chapter 3 which showed little difference between the anti- staphyloccal activities of the Manuka honeys of differing UMF rating. The graph below (Figure 6.14) shows clearly the lack of linearity found between UMF of Manuka and the MICs across all of the organism groups and serves as a comparison for figures 6.15 – 6.24 which show the compounds identified within the Manuka honeys plotted against either MIC or UMF.

Figure 6.14 Graph showing Average MIC of four bacterial groups versus six Manuka products



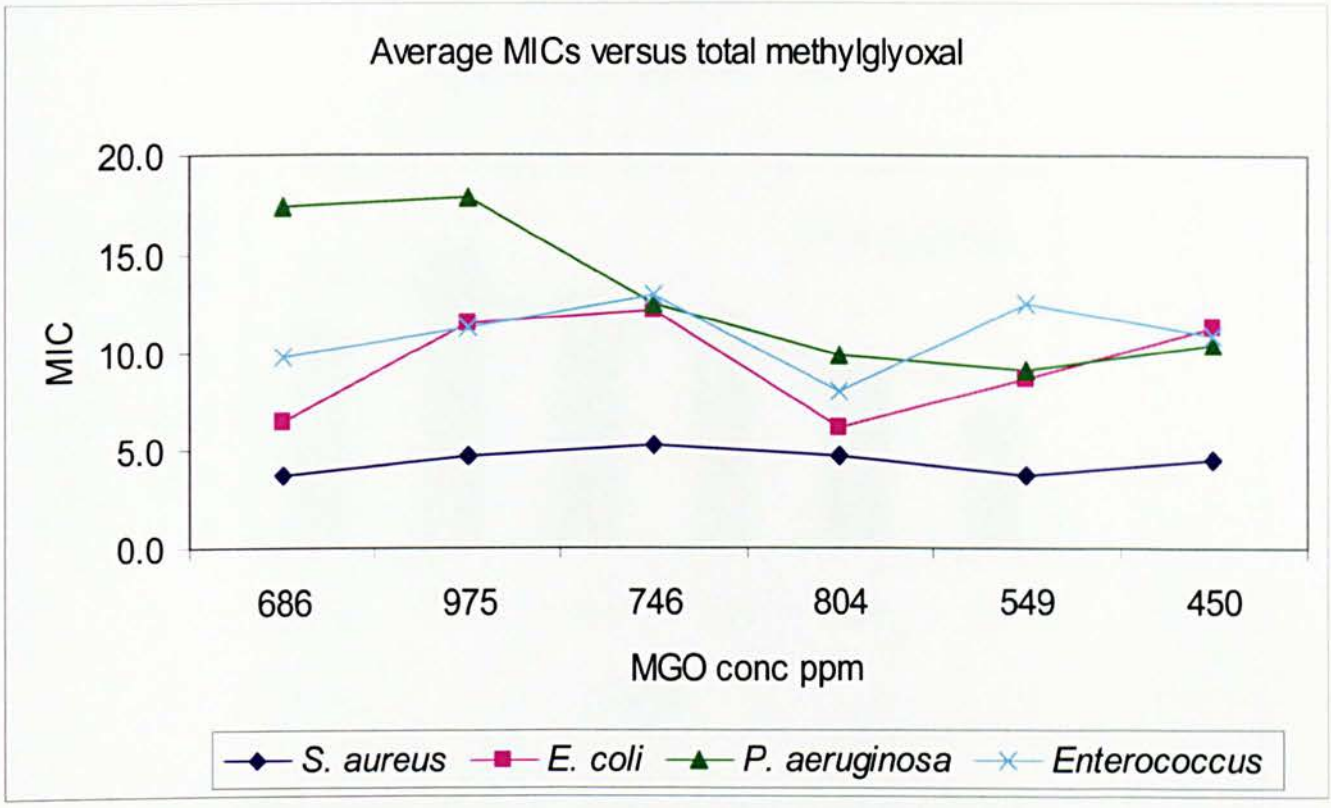
The data was obtained from the he reduced panel of Gram micro-broth MIC assays in Chapter 3 using the smaller gram negative panel of isolates. The error bars show the standard error of the mean, where error bars are not shown the SEM was too small to show

The graphs in Figures 6.15 -6.19 below look at the average MICs of *S. aureus, E. coli, P. aeruginosa* and *Enterococcus* sp. determined in Chapter 3 compared to the

quantity of the individual components to which antimicrobial activity is attributed. These products are then plotted against UMF rating.

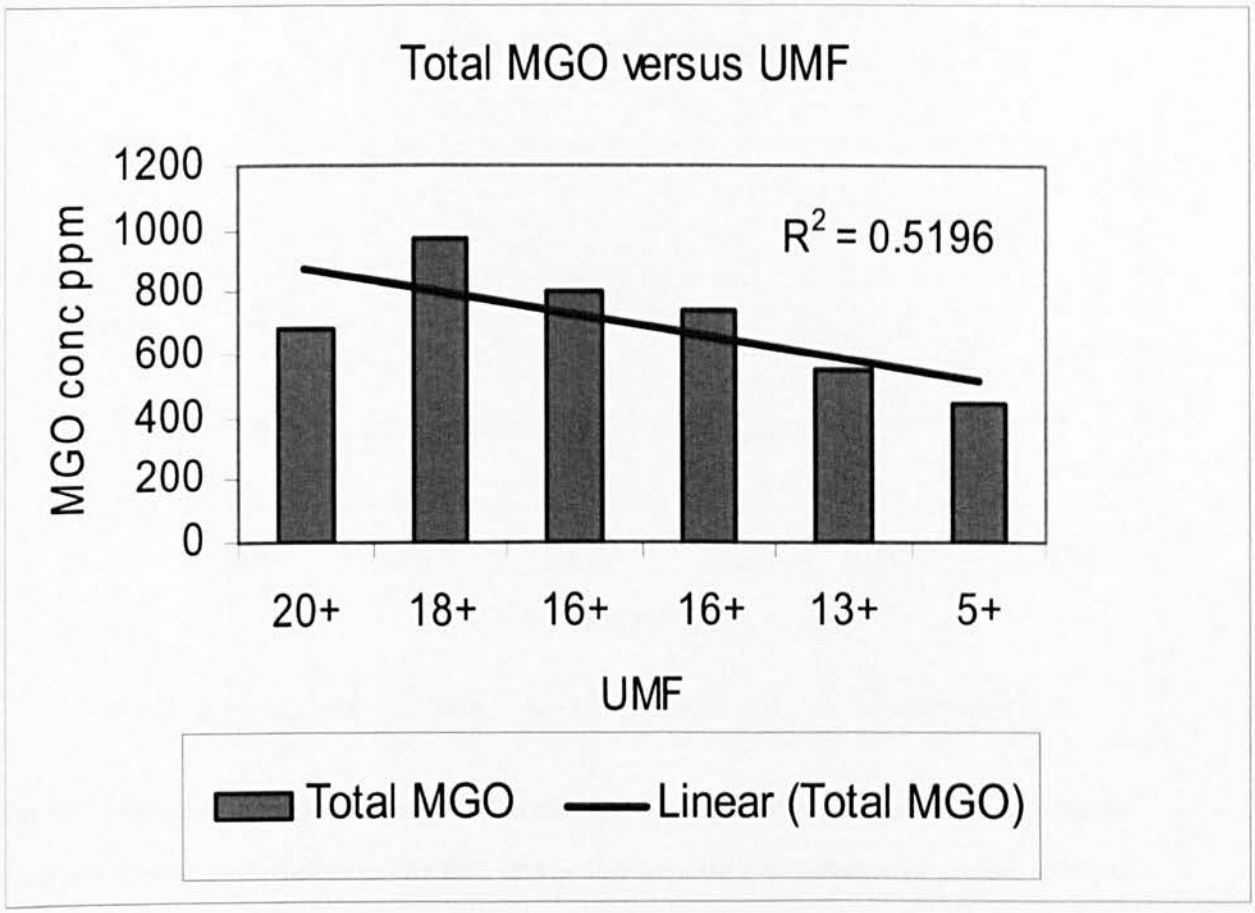
In Fig 6.16 there appears to be some level of correlation between UMF and MGO content (R^2=0.52) although this does not appear to be a directly proportional relationship with the average MICs of the different bacterial groups. Of the aromatic compounds, components 12-15, methyl syringate shows the best correlation with UMF, although still tenuous at R^2=0.39. The other aromatic components show lesser correlation with either UMF or MIC (Figures 6.17 -6.24).

Figure 6.15 Graph showing correlation between total MGO concentration and Average MIC



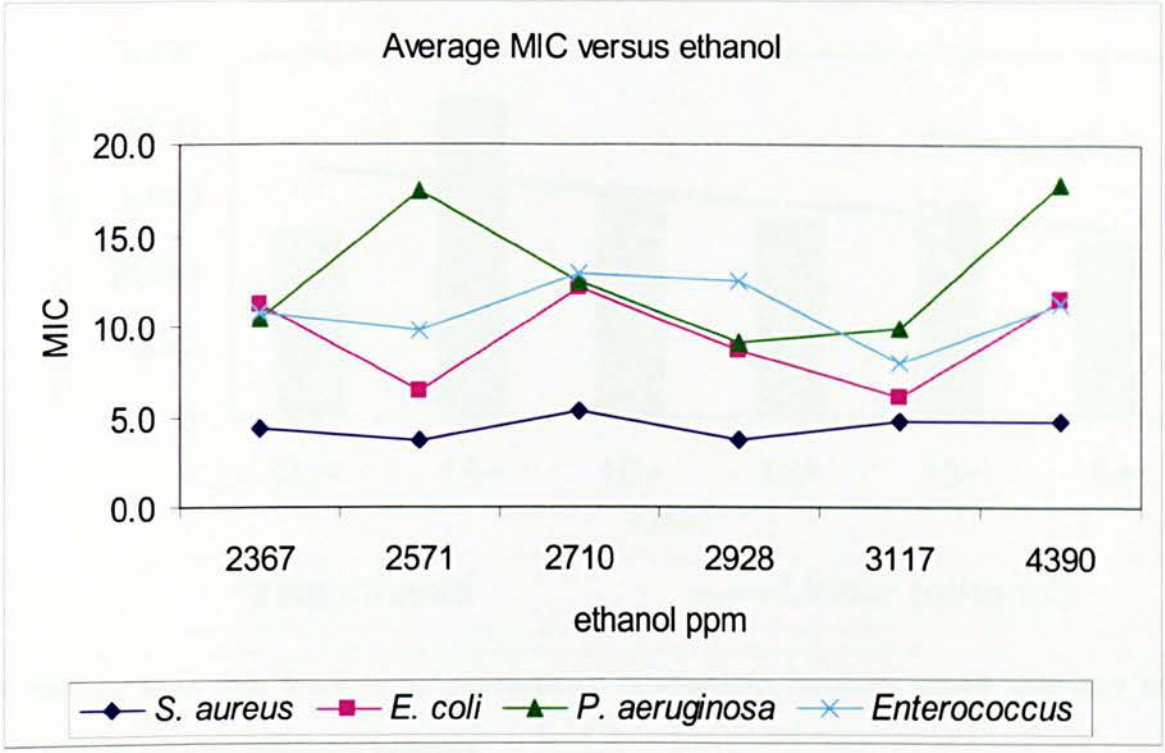
The MIC is the percentage of honey required to inhibit the organisms which is plotted against the MGO content found in the corresponding product. The plots do not show any direct correlation between MIC and the quanity of MGO.

Figure 6.16 Graph showing correlation between total MGO concentration and UMF



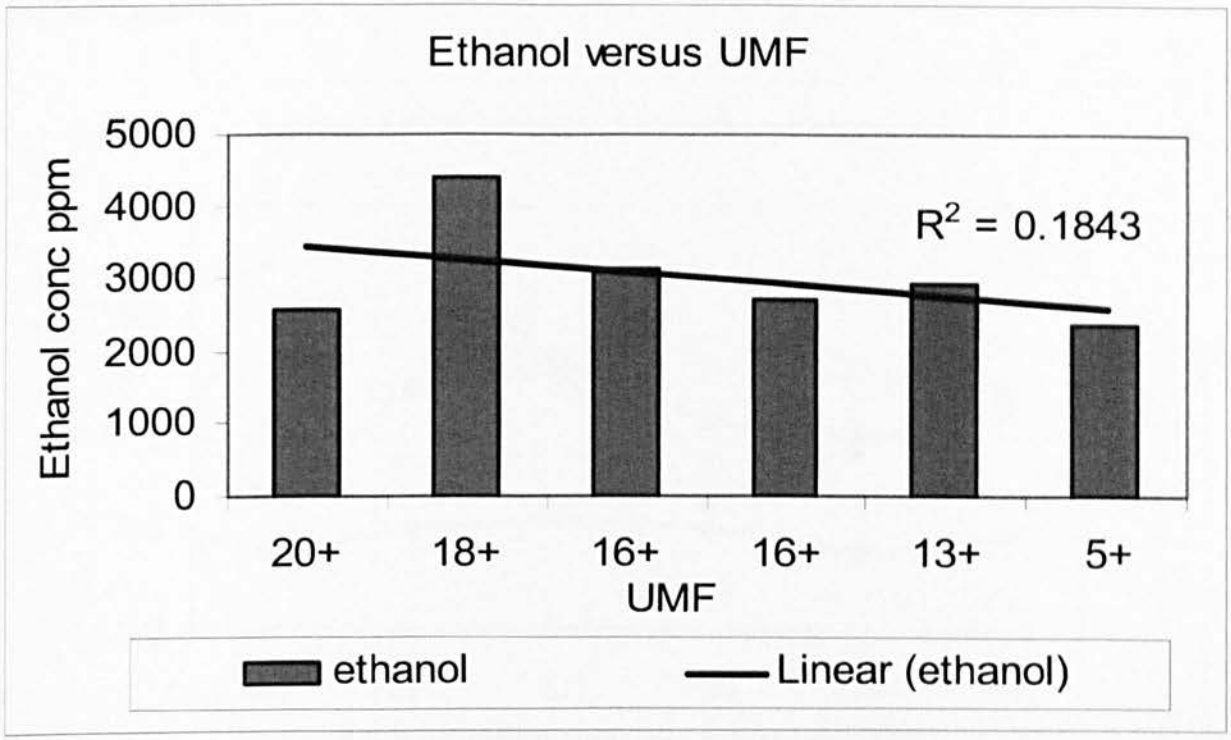
There is some level of correlation with all but the highest rated honey but quantity of MGO is not directly proportional to stated UMF.

Figure 6.17 Graph showing correlation between ethanol and average MIC of each Manuka product



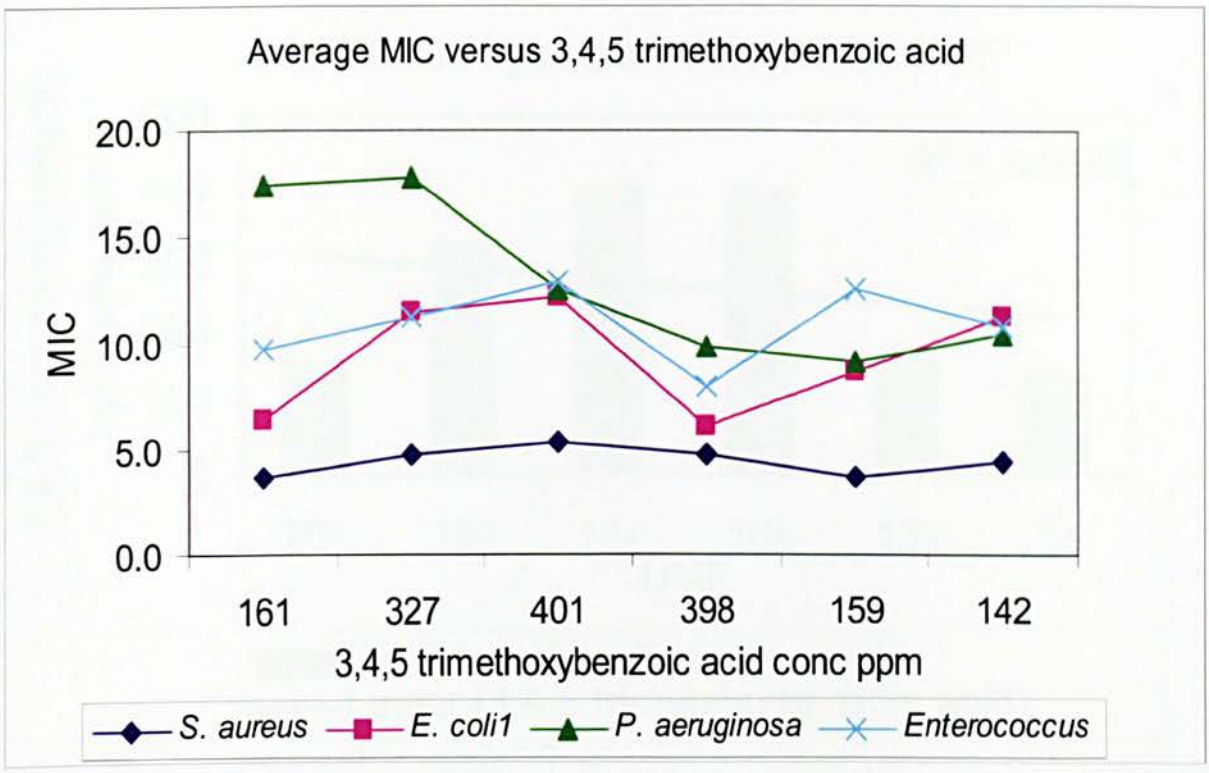
The MIC is the percentage of honey which inhibited the organism. The amount of ethanol does not appear to correlate to the MIC of the honeys with the different organism groups. This lack of correlation can be seen clearly in Figure 6.18

Figure 6.18 Graph showing correlation between ethanol and UMF



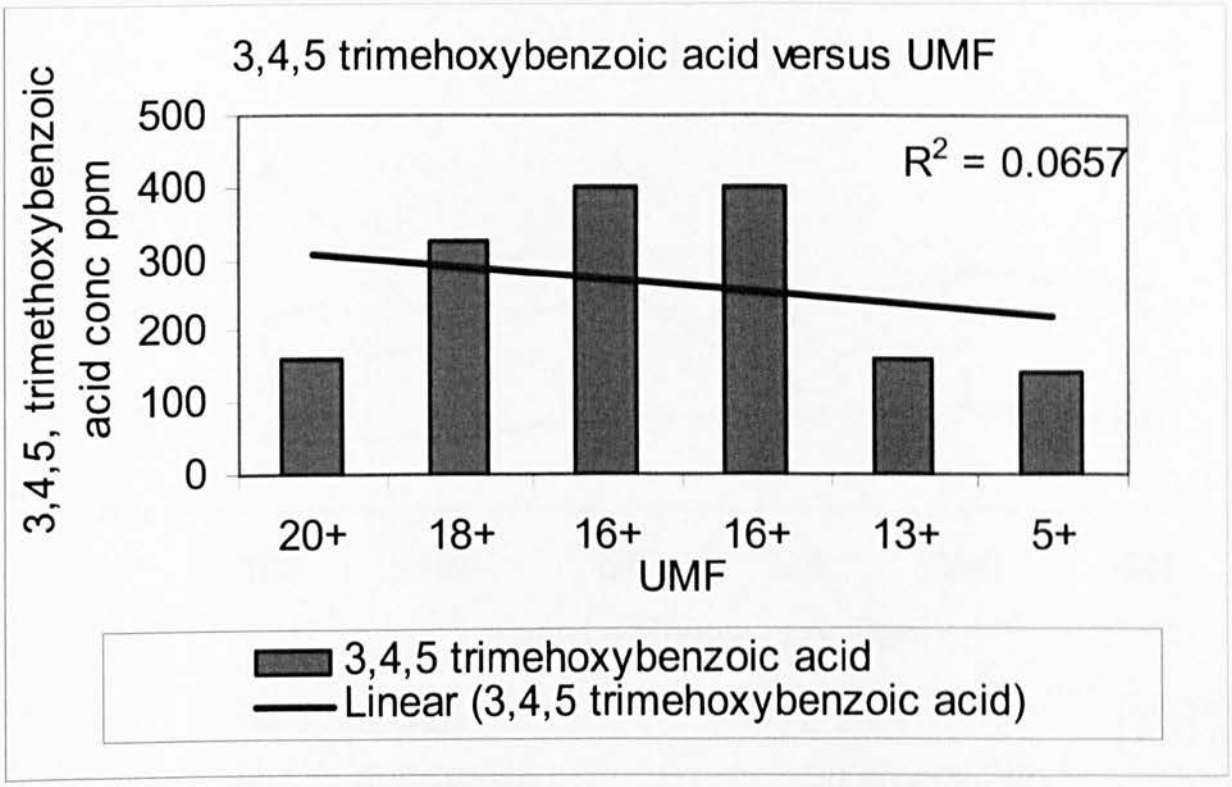
It can be seen that there is no proportional relationship between stated UMF and the quantity of ethanol within the product.

Figure 6.19 Graph showing correlation between 3,4,5 trimethoxybenzoic acid and average MIC



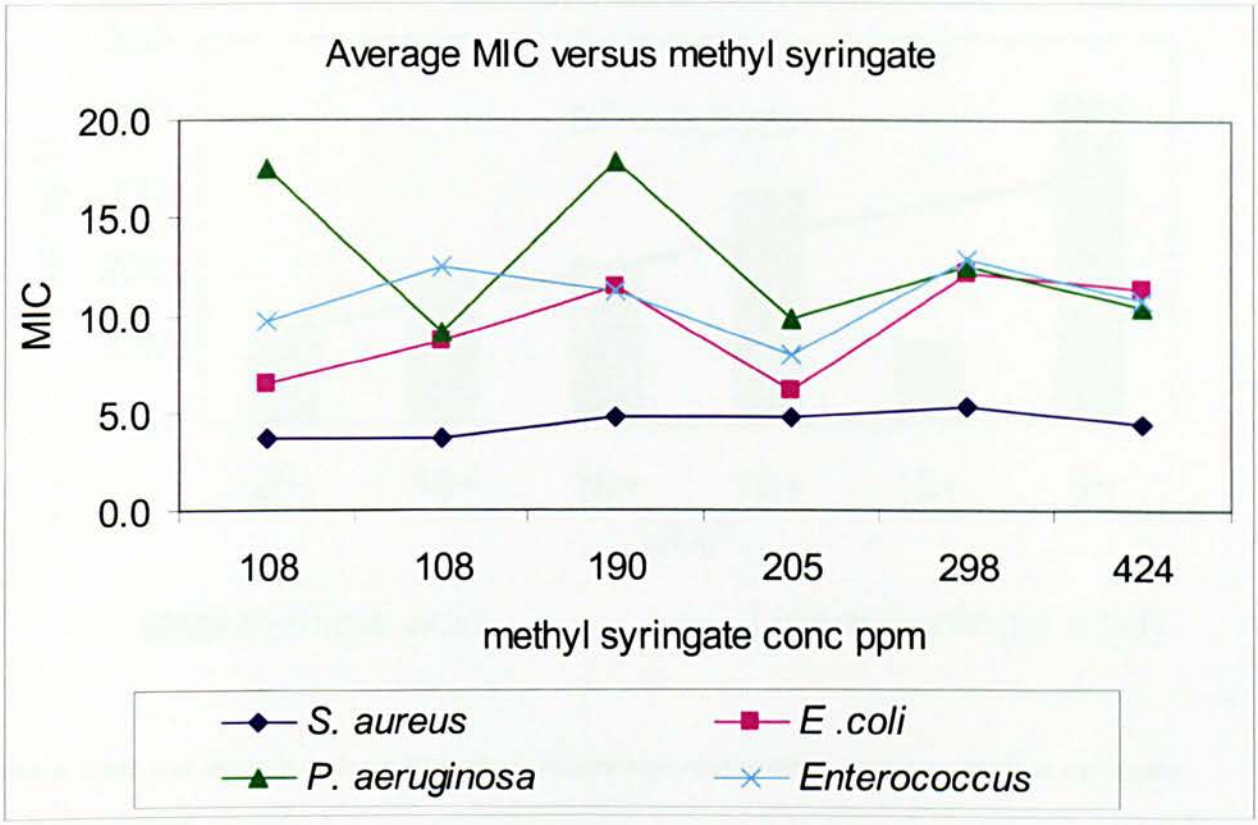
The MIC was the percentage of honey required to inhibit organism growth. This graph does not indicate any direct relationship between amount of 3,4,5 trimethoxybenzoic acid and antimicrobial activity of the Manuka honey.

Figure 6.20 Graph showing correlation between 3,4,5 trimethoxybenzoic acid and UMF



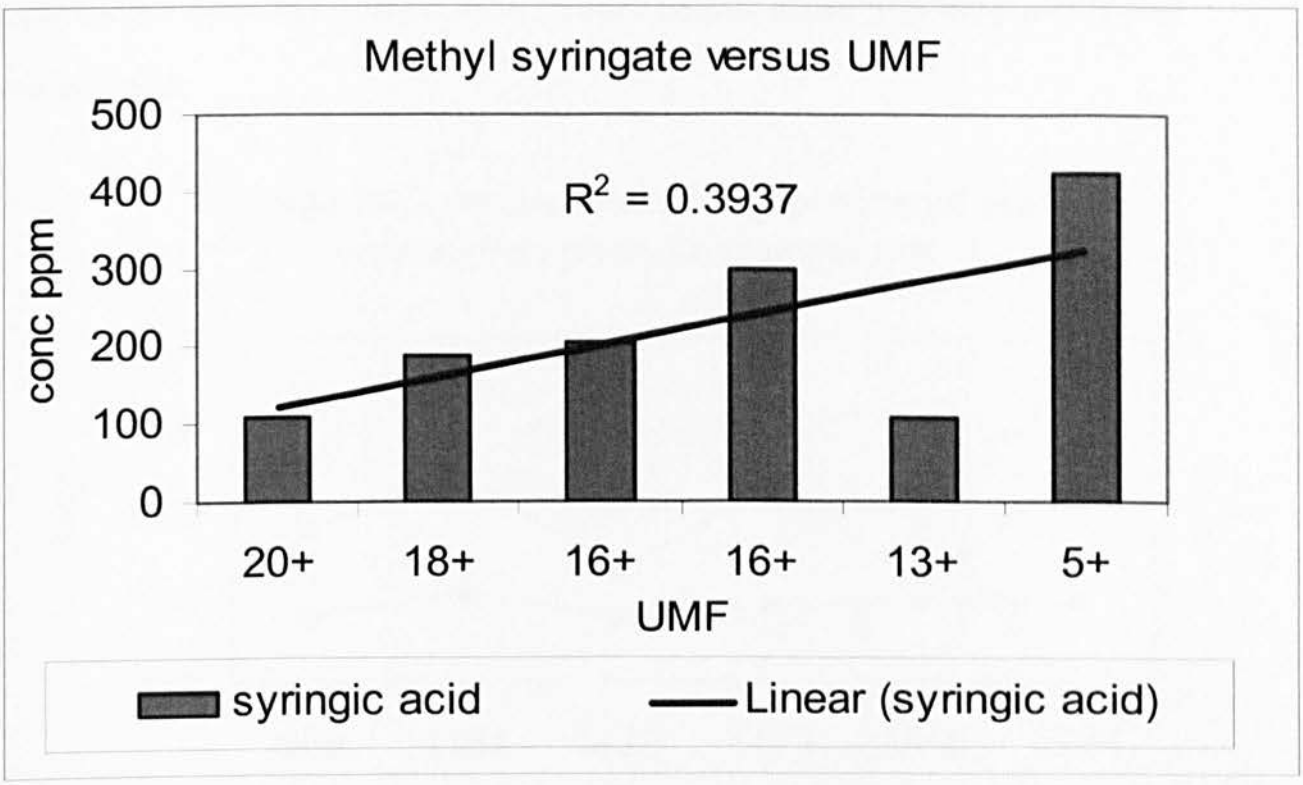
. This graph shows there is no proportional realationship between 3,4,5 trimethoxybenzoic acid and stated UMF of the product

Figure 6.21 Graph showing correlation between Syringic Acid and average MIC



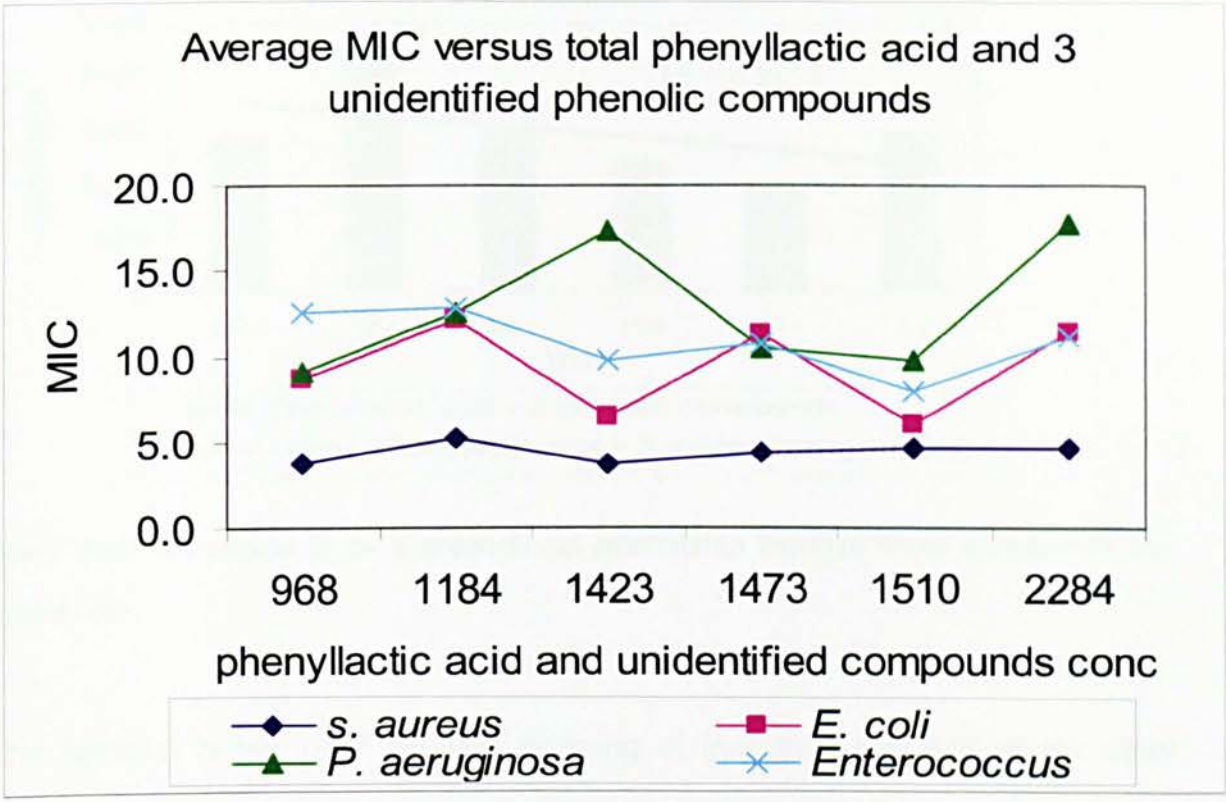
The MIC was the percentage of honey required to inhibit organism growth. There does not appear to be a relationship between antimicrobial activity and methyl syringate.

Figure 6.22 Graph showing correlation between methyl syringate and UMF



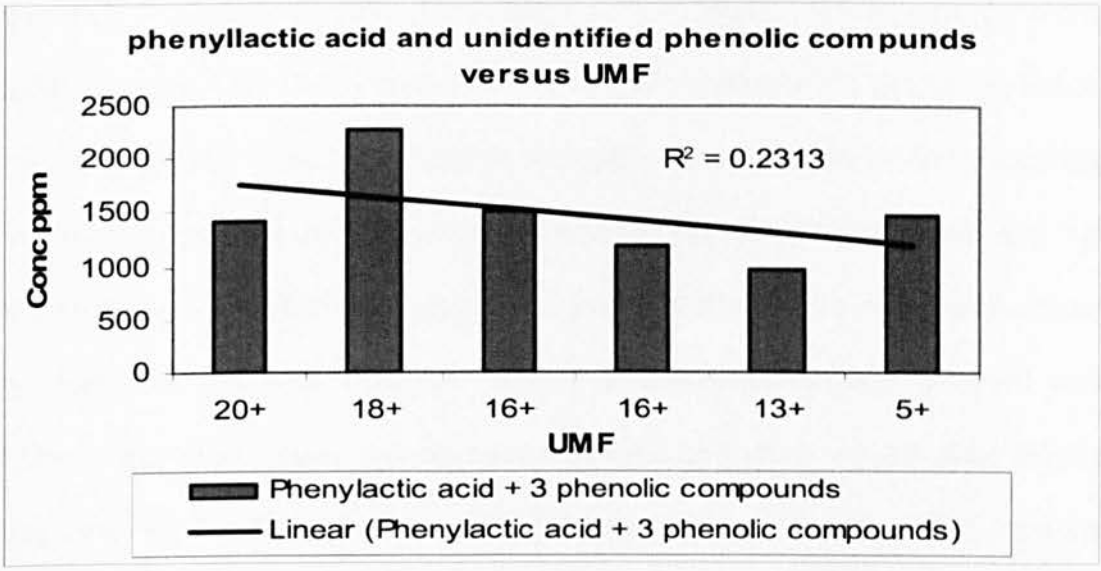
There does not appear to be a directly proportional relationship between methyl syringate and stated UMF.

Figure 6.23 Graph showing correlation between Phenyllactic acid and 3 unidentified aromatic compounds (predicted mol.mass 160-190g mol^{-1}) and average MIC



The MIC was the percentage of honey required to inhibit organism growth. There graph does not show any direct relationship between these products and antimicrobial activity.

Figure 6.24 Graph showing correlation between Phenyllactic acid 3 aromatic compounds (predicted mol. mass 160-190g mol^{-1}) and UMF



There does not appear to be a proportional relationship between these compounds and stated UMF.

The Manuka honey UMF 5+, with a rating of less than half that of the other products showed greater parity in antibacterial activity with the other Manukas than UMF implied. This study identified this product as having the highest levels of methyl syringate and the only product shown to contain 4, methoxyphenyllactic acid.

6.4 Discussion

The above results showed that there are components unique to the Manuka products. However, it was concluded that thin layer chromatography was not a suitable method for the separation and further identification of the constituents of the honey and Syrup products. Although a useful tool for assessing the antimicrobial activity of a separated fraction, the number of separated fractions observed was less than the number of sugar and phenolic compounds reputed to be present in Manuka products. Further experimentation with different solid and liquid phases may have yielded more fractions and other visualization compounds may have helped to identify the nature of the fractions. However, the main obstacle was that very small volumes of product could be processed using this method meaning that the volume of the separated fraction which could be subsequently removed from the silica was too small to process by NMR techniques so further experimentation using TLC was rejected.

In Chapter 3, Eucalyptus honey was shown to be the most antimicrobially active of the non-Manuka products. Inositol derivatives have been shown to have antimicrobial activity (Fortuna *et al.*, 2001) and one may speculate that the presence of *proto*-quercitol (1L-1,3,4/2,5-Cyclohexanepentol) an inositol derivative (Worawalai *et al.*, 2012) may account for the superior antibacterial activity of eucalyptus honey compared to non-Manuka honeys from other floral sources. Further investigation of eucalyptus honeys with regard to content and antimicrobial activity would be required to validate this tentative hypothesis.

This study shows that the antimicrobial activity and the apparent UMF rating of Manuka honey cannot be attributed to any single component and indicates that the

activity may result from a combination of compounds unique to Manuka honeys. The presence of 4-methooxyphenyllactic acid and / or higher levels of methyl syringate, phenyllactic acid and the other unidentified aromatic compounds may explain the antimicrobial efficacy of the UMF 5+ Manuka despite the low level of MGO. This study was unable to identify if any of these compounds act synergistically but this raises interesting questions which would be a worthy area of further research.

This is the first reported study using DOSY NMR to separate the components of honey without any preliminary extraction which could potentially disturb the integrity of the original product (Le Gresley *et al.*, 2012). This technique is rapid when compared to techniques such as SPE- HPLC, non-destructive and affords economical analysis of complex mixtures making it a suitable method for direct analysis of complex mixtures.

The antimicrobial action of Manuka products is clearly complex and the apparent ripening and biological changes resulting from temperature fluctuations and storage time indicate that UMF ratings should be regarded with caution. Antimicrobial efficacy appears to be a result of a number of constituents but the significance of any interdependencies between the different components would require further study.

6.5 Conclusions

The data from this study suggests that UMF is not a reliable quantitative indicator of the antimicrobial efficacy, or of the antimicrobial components within Manuka honey. DOSY NMR appears to be an effective method to identify the chemical components of complex mixtures containing high propotions of sugars without conducting pre analytical extraction. Manuka honeys contain methylglyoxal, 3,4,5 trimethoxybenzoic acid, phenylactic acid, methyl syringate, ethanol and in some cases additional aromatic compounds which contribute to antimicrobial activity. The data would indicate that no single component in Manuka honey can be attributed to antimicrobial efficacy

CHAPTER 7

General Discussion and Conclusions

7.1 Discussion

The work presented in this thesis aimed to characterise the potential therapeutic efficacy of honey on clinical multi-drug resistant isolates capable of causing healthcare associated wound infections.

Honey has a long history of being used in medicine dating back to ancient times when Aristotle allegedly favoured its use as a topical application for wounds (Majno, 1975, Forrest, 1982). The advent of antibiotics in the 20th Century displaced the use of many natural products and remedies until the spectre of antibiotic resistance was raised and pharmaceutical companies started losing the race between the development of new antibiotics and the rapid evolution of bacterial resistance to every class of antimicrobial being produced (Projan, 2003, Charles and Grayson, 2004).

It is well recognised that honeys from different floral sources and geographical regions varied in antimicrobial activity (Anklam, 1998, Al-Jabri *et al.*, 2003, Brady *et al.*, 2004, Lusby *et al.*, 2005, Huidobro and Sanchez, 2005, Nagai *et al.*, 2006, Küçük *et al.*, 2007). Manuka honey in particular had been the focus of a number of studies with the research group from the Waikato University in New Zealand establishing a rating for antimicrobial activity known as the Unique Manuka Factor (UMF) which correlates with the susceptibility of *S. aureus* to phenol (Cooper and Molan, 1999a, Cooper *et al.*, 1999b).

Previous studies had shown that Manuka honey has antimicrobial activity which cannot be explained by the sugar content alone (Cooper *et al.*, 1999b, Molan, 2001, Jenkins *et al.*, 2011) and that whilst honey from various floral sources have

antibacterial activity, few demonstrate the non-peroxide activity seen in Manuka products (Cooper and Molan, 1999, Brady *et al.*, 2004). The first part of the work presented in this thesis (Chapter 3) aimed to determine the antimicrobial efficacy of honeys from different sources on organisms in the context of a clinical environment. This group of organisms were chosen as they were expected to have high exposure to antibiotic therapy and so represent a realistic microbial challenge to the effects of the honeys under test.

The antimicrobial efficacy assays in this study were consistent with other studies demonstrating that Manuka honeys exhibit greater antimicrobial activity than non-Manuka honeys and common Syrups and that isolates of *S. aureus* show greater susceptibility compared to *Enterococcus* sp. and Gram negative organisms (Brady *et al.*, 2004, Lusby *et al.*, 2005). To understand why this may be, one must look at the different properties between the cell walls of the different organisms. Gram positive bacteria have a thick layer of peptidoglycan, a sugar/amino acid polymer which cross links to form a mesh-like layer. Gram negative bacteria have a thinner layer of peptidoglycan and are surrounded with a lipopolysaccharide layer (LPS), a highly antigenic substance which confers increased resistance to attack by external agents (Schleifer and Kandler, 1972, International Scientific Forum, 2000). *S. aureus* and Enterococci are both Gram positive organisms but Enterococci have some interesting properties which might account for their reduced susceptibility to Manuka honey (Signoretto *et al.*, 2000). When conditions become unfavorable, Enterococci are able to enter an altered phenotypic state described as a Viable but Non-Culturable (VBNC) state (Signoretto *et al.*, 2000). The cell wall of VBNC Enterococci have been shown to have increased lipotechoic acid and increased

cross linking of muropeptides within the peptidoglycan making the cell more resilient to external influences (Signoretto *et al.*, 2000). The fact that VBNCs are recoverable when conditions become favourable may explain the greater disparity demonstrated in Section 3 of this study, between the MIC and the MBC of honey against Enterococci compared to other organisms. Vancomycin acts by inhibiting cell wall synthesis and resistance in Enterococci is due to a modification decreasing the affinity to vancomycin of the cytoplasmic precursors required for building the peptidoglycan chain (Leclerq and Courvalin, 1997). The results of the investigations between antibiotic sensitive and resistant isolates discussed in Chapter 3, showed VREs had increased resistance to Manuka compared to Vancomycin sensitive isolates which raises the question that this modification may also decrease Enterococcal permeability to Manuka honey.

The lipopolysaccharide in the Gram negative cell wall is less permeable to external agents including phenol, than Gram positive cell walls (Liaqat and Sabri, 2008) which is consistent with the findings that the Gram negative isolates were less susceptible than the Staphylococci. The porin channels in the LPS of Gram negative cell walls vary with genus, with *P. aeruginosa* porins being less permeable than those of *E. coli* (Sugawara *et al.*, 2012). The isolates of *P. aeruginosa* which were considered 'resistant' for the purpose of this study were all resistant to ciprofloxacin and/or meropenem, to which mechanisms of resistance include efflux pump expression (Cabot *et al.*, 2011). Efflux pumps have also been recognised in *E. coli* resistant to β-lactams and ciprofloxacin (Borges- Walmsley *et al.*, 2003). In this study, it was the drug resistant isolates of *P. aeruginosa* that had increased susceptibility to Manuka honeys with a trend towards increased susceptibility also

shown with β-lactamase positive isolates of *E. coli.* This suggests the possibility that porin modifications and efflux pump expression mechanisms responsible for carbapenem and fluoroquinolone resistance in *P. aeruginosa* might render the organism more permeable to Manuka honey. At the time the isolates were selected for this study in 2006, it was difficult to find pseudomonad isolates which were resistant to more than one of the antimicrobials used at The Royal Marsden to treat *Pseudomonas* sp. The organisms selected for the purpose of this study were the most resistant available from the Royal Marsden Microbiology Department frozen store of clinical isolates, with all being resistant or with reduced sensitivity to meropenem and ciprofloxacin, with gentamicin resistance included where available. In recent years, there has been global spread of carbapenemase producing *Enterobacteriacae* (Nordman *et al.*, 2011). In 2011, the HPA published guidance on the management of patients carrying Carbapenem resistant organisms. In 2008, the HPA released a Resistance Alert (HPA Resistance Alert, 2008) after a rise in carbapenem resistant *Enterobacteriaceae* (CRE) asking for Microbiology laboratories to be aware of *Klebsiella pneumoniae* isolates showing decreasing MICs to carbapenems but none of these organisms were available at the time of organism selection.

It is likely that at the time of organism selection for this study that the mechanism of resistance of *Pseudomonas* isolates to meropenem was porin / efflux pump mediated and not due to carbapenemase. Russell and colleagues reported in 2002 that the use of Triclosan, a phenolic disinfectant, can cause over expression of the efflux pump inducing resistance to ciprofloxacin in *P. aeruginosa* and that *E. coli* expressing *mar*A, *soxS or acrAB* have reduced susceptibility to Triclosan (Russell,

2002). This suggests a potential for antibiotic resistant strains to be less sensitive to phenol containing products. However, this observation is not supported by the results of this study which suggest that mechanisms of antibiotic resistance will not necessarily confer additional resistance to the phenolic components in Manuka honeys. Although, Russell's findings indicate a possibility that honey usage could select out resistant strains. A review of the literature has not shown any studies investigating any link between antibiotic and Manuka honey susceptibility although this study indicates that these relationships are worth future investigations. Additionally, it would be constructive to pursue studies to look at the notably resistant isolates such as carbapenem resistant *Enterobacteriacae* (CRE) which are presenting such a threat to infection prevention and control in hospital environments today.

Microbiology studies are often performed using only NCTC control strains (Brady *et al.*, 2004, Estrada *et al.*, 2005, Rojas *et al.*, 2005, Patton *et al.*, 2006). The work presented here provides a unique opportunity to compare susceptibility of clinical and control isolates in order to determine if the studies only using controls give results representative of clinical isolates. The results of the study showed no statistical differences between controls and clinical isolates of MSSA, MRSA or sensitive isolates of *P. aeruginosa.* Clinical isolates of VSE and β-lactamase positive and negative *E. coli* were shown to be less susceptible to Manuka honey whilst VRE and resistant *P. aeruginosa* are more susceptible. However the differences in MIC were all small being ≤5% honey concentration. In a clinical setting these differences would be insignificant as honey products are used undiluted (Naylor *et al.*, 2008) and MIC values for all organisms did not exceed

25% with any of the Manuka honeys (Appendix 4). However, the fact that there is a notable difference suggests that in order to keep abreast of any increased resistance the inclusion of clinical isolates is preferable, particularly if honey has been used in the clinical environment. The presence of bacteria in a wound can cause clinical infection especially with pathogenic organisms such as *S. aureus* but a high bacterial bioload including lesser pathogens may still delay healing (Bowler *et al.*, 2001).

The antimicrobial efficacy of honey methods discussed so far were all performed on an organism population of 10^6 to 10^7 cfu/ml which may not be representative of the organism population in a clinical wound. The results of the first bioload assays using NCTC strains showed medical grade honey to be bactericidal against all but Enterococci at a bacterial concentration of 1.5 x 10^9 cfu/ml (McFarland 5). Enterococci were not eliminated above 5 x 10^8 cfu/ml; further assays used different volumes of honey against suspensions containing organism counts of 10^{12} cfu/ml. Results showed that a honey volume to organism suspension volume ratio of 6½:10 achieved inhibition of all organisms including Enterococci. The bactericidal activity ranged from a volume ratio of <3:10 with Staphylococci, 6:10 cfu/ml with Gram negatives and with Enterococci still being viable at a 1:1 ratio. These results indicated significant antimicrobial activity if sufficient product was present. Further investigations were performed on polymicrobial populations from clinical wound swabs obtained from the William Harvey hospital. MICs were performed on these polymicrobial populations and on individual isolates from those populations, the isolates were of species not previously assayed in this study. The results showed both medical grade honeys inhibited growth at ≤ 25% honey. Inoculation of the

entire wound isolates individually and as polymicrobial populations indicated that Manuka honey will kill bacteria providing there is sufficient volume for the honey to come into contact with the cells. In the context of a clinical environment, it suggests that the application of honey on wounds may inhibit or even clear the wound of large numbers of surface or planktonic bacteria but that there would be no penetrative action of honey constituents into microbial biofilms.

In addition to micro-organisms, proteinaceous wound exudate, effete leucocytes and devitalised tissue all contribute to the bioburden (Rhoads *et al.,* 2008). Clinical studies have shown honey to have a natural debriding effect on wounds (Molan, 1999, 2001, 2006, Al-Waili and Saloom, 1999, Subrahmanyan *et al.,* 2001, Schumacher, 2004, Bangroo *et al.*, 2005, Gethin and Cowman, 2005, Simon *et al.,* 2006, Cooper, 2008) which, in addition to antimicrobial activity, provides opportunity for honey to attain a credible role in the context of wound care. However, the major focus of the work presented within this part of the study is an evaluation of honey's limitations undertaken by studying the activity of medical grade Manuka honey on polymicrobial wound biofilms.

The practical difficulties experienced with the physical removal of honey could in itself explain the debriding effect of honey, as in several instances, removal of honey from the slide was accompanied by the biofilm layers. In all cases, no organisms were recoverable from the honey after the coverslips were removed indicating that the honey could prevent the spread of wound flora from the wound into the environment thereby preventing further contamination of the patients immediate environment and reducing risk of cross infection. To explore this further it was necessary to create a model more representative of a wound bed. The

inoculation of polymicrobial wound populations into cooked meat, gave the organisms a surface to grow on mimicking, in some part, the uneven fissured surface of a fleshy wound. The organisms were incubated to allow the meat to become colonised and for polymicrobial biofilms to establish and grow. The earlier results for the bioload experiments implied that there may not be a great reduction in viable organisms in a sessile state. The results of the biofilms grown on coverslips showed both a capacity to remove and kill organisms in a biofilm on a smooth artificial surface. Considered together both of those observations made the outcome of the wound modelling assay hard to predict prior to assay. The outcome of the assays showed that both honeys at both concentrations achieved a significant reduction in organism numbers in 3 of the 10 wounds. However all of the wounds showed significant reduction to at least one honey at either 50% or 75% concentration although none of the results showed the 5 $\log_{10}$ reduction in viable organisms, the reduction required to define a product as an antiseptic according to Koburger *et al.* (2010). Despite this limited reduction in organism numbers, the cultures performed on the honey solutions removed from the wound models all proved to be sterile. This suggests that the application of Manuka could prevent further propagation of the biofilm and prevent organisms escaping into the surrounding medium. The inconsistent activity of the individual honeys at either concentration can be explained by the fact that although organism counts were calculated per unit weight, the surface area of the meat in each model and in each triplicate would have presented a variable topography which was not directly comparable but which is representative of actual wound environments. The one exception was a small increase in one wound experiment using a 50%

concentration of the lower UMF rated honey. This aberrant result could be due to the differences in morphology of the meat pieces. It also raises the question that the specific combinations of species within the biofilm population may have synergistic resistance to the Manuka products. Recent studies have shown that Manuka honey at sub-inhibitory concentrations acted synergistically with oxacillin on MRSA by down regulating the *mecR1* gene (Jenkins and Cooper, 2012). The variety of organism populations studied for this thesis did not allow direct comparisons to establish any patterns of synergistic behaviour but this is an area worthy of further exploration.

These results of this study suggest that:

- Honey is only likely to inhibit or destroy organisms upon contact and unlikely to penetrate down through a biofilm matrix.
- Organisms escaping the biofilm will not survive in the presence of honey.
- Honey inhibits further development of wound biofilms but does not demonstrate a capacity to remove biofilm from a meat / wound surface.

These findings increase in significance when the differences between an *in vitro* and *in vivo* system are considered. In a live wound, the tissues are served by a blood supply which delivers the body's own defences to the wound site. If this is not the case, debridement is required. Honey has been demonstrated to induce an immune response and activate mechanisms of wound repair (Tonks *et al.*, 2003, 2007) which mean that there is an antimicrobial attack from beneath the wound bed. The debridement of wound material by honey which has been observed in clinical usage, the antimicrobial efficiency of Manuka honey on flora not encased within the biofilm and the implications for the prevention of cross contamination

justify Manuka honey as a valuable addition to wound care. However, it is important to acknowledge that although it is unlikely that honey will completely sterilise a wound bed especially in the likely presence of Enterococci, it is likely that it will help to keep the bacterial load below infection levels.

The first chemical analysis of the honey products using thin layer chromatography was performed early in the study. At that time the compounds peculiar to Manuka products which may confer antimicrobial activity were unknown and referred to in literature as an unknown phytochemical products, or the unique Manuka factor (Cooper *et al.,* 1999a,1999b; Brady *et al.,* 2004; Lusby *et al.,* 2005). In 2000, Weston and colleagues reported the presence of methyl syringate and phenyllactic acid from Manuka honeys. That these compounds were also found in non-active honeys led them to conclude that although these compounds may provide some level of antimicrobial activity, they could not be responsible for the bactericidal activity seen in active Manuka (Weston *et al.,* 2000).

From 2008, studies were published identifying methylglyoxal (Mavric *et al.,* 2008, Adams *et al.,* 2009) and numerous aromatic compounds (Pyrzynska and Biesaga, 2009; Stephens *et al.*, 2010) as active components in Manuka honey. The DOSY NMR work confirmed the presence of these compounds, although direct correlation of chemical constituents, UMF rating and antimicrobial activity was inconsistent. This was unexpected as the UMF is based on the honey's activity against *S. aureus.* Lusby and colleagues (2005) described similar inconsistencies and attributed the difference in method as a possible cause. Lusby's method incorporated honey into the agar (Lusby *et al.,* 2005) whereas the original Molan method used well diffusion (Allen *et al.,* 1991).This study utilised well diffusion

techniques but also had results which did not correlate with UMF. The results of the DOSY NMR analysis provide some explanation. The work presented here shows that none of the products contained any compounds in volumes proportional to UMF or MIC which raises questions regarding the validity of UMF as a measure of activity. Atrott and Henle (2009) demonstrated close correlation between antimicrobial activity and MGO and suggested that MGO levels could be used to quantify the antibacterial activity of Manuka honey. Mavric and colleagues (2008) also suggested that MGO alone was the compound responsible for the UMF of Manuka honey. The results of the DOSY NMR contradicted these findings but there are logical explanations for why this may be. The methylglyoxal content of honey increases over time and with increases in temperature (Stephens *et al.*, 2010). Stephens and colleagues found that Manuka of less than one year old did show linearity of MGO levels and antimicrobial activity which was lost when older honeys were tested. In a series of articles in New Zealand Beekeeper in 2008, Peter Molan expressed his opinion that replacing the UMF with the active component is misleading as it is the synergy between MGO and other compounds that produce the UMF although he did not speculate as to the identity of the synergistic partner component (Molan, 2008 a,b,c). The findings of the analysis of Manuka in this study show that the antimicrobial activity cannot be attributed to a single compound and supports the possibility that MGO levels may have altered during the time between being attributed with a UMF rating and being used in the assays and chemical analysis in this study. This might suggest that UMF can be used to qualitatively identify active honey but not quantify the level of activity. None of the products indicated the need for refrigeration and none of the products were

labelled with a date of manufacture supporting the conclusion that UMF may be a quantitative indication of antimicrobial activity at the time of production or packing but this cannot be guaranteed at the time of purchase or throughout the usage life of the product. The presence of 4 methoxyphenyllactic acid in the lowest rated Manuka honey in this study, coupled with its unexpectedly high activity suggests that the components contributing to antimicrobial activity remain incompletely defined.

7.2 Conclusions

The results of this study show Manuka honeys to have superior antimicrobial activity than the syrups and honeys from other floral sources which were included in this study. This suggests that Manuka activity is attributable to more than just sugar content. The presence of spores in unsterilised products indicate the necessity for products to be sterilised for use in a clinical environment particularly if the patient is immunocompromised.

Manuka honey was shown to be active against clinical isolates of Meticillin sensitive and resistant *S. aureus,* both sensitive and multi-drug resistant isolates of *E. coli and P. aeruginosa* and against Vancomycin sensitive and resistant *Enterococcus* sp, with *S. aureus* being the most susceptible and *Enterococcus* sp. the least. The differences in susceptibility between antibiotic sensitive and resistant isolates within each genera suggests the potential for resistance to develop if honey is to be used in a clinical setting, indicating there would be value in regular monitoring of organism susceptibility to the honey in use.

The inclusion of clinical isolates in honey studies is desirable when studying honey, particularly if honey is being used in the clinical environment as the antimicrobial susceptibility of control strains may not always be representative of that of clinical strains. Manuka honey was also shown to be active against polymicrobial wound populations, including *Acinetobacter* sp., β haemolytic Streptococci Lancefield groups B and G, *Staphylococcus aureus*, *Enterococcus* sp., *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Alcaligenes faecalis, Myroides* sp., *Morganella morganii* and *Pseudomonas aeruginosa.*

The results of this study suggested that the ability of honey to remove biofilms from an inorganic smooth surface is not representative of the action upon biofilms growing on an uneven organic surface, implying that the use of honey on a clean surgical wound could prevent the formation of a biofilm and negate the need for further antimicrobials. However, in the presence of a biofilm colonised wound the antimicrobial activity may be limited but may confer some benefit if used in conjunction with antimicrobial therapy. The application of active honey to a wound may prevent the increase in bioload and prevent the escape of pathogenic organisms into from the immediate wound environment aiding infection prevention and control. The results of this study demonstrated the volume of Manuka honey must be sufficient to come into contact with the organisms cell wall in order to inhibit of destroy it and no constituents of honey appeared able to pervade or diffuse through a biofilm or culture and destroy organism without direct contact.

The use of DOSY NMR was found to be an efficient method to analyse honey products and as it was effectively used to identify the chemical constituents of honey without the necessity for pre-analytical extraction.

This methodology showed Manuka honeys to contain Methylgloxal, 3,4,5 trimethoxybenzoic acid, phenyllactic acid, methyl syringate and ethanol and in some cases 4, methoxyphenyllactic acid all of which may contribute to its antibacterial activity with no component being the sole active agent indicating that UMF alone cannot be relied upon as an exact measure of antimicrobial activity

These conclusions support the use of Manuka honey in a clinical environment. The limitations of Manuka honey with regard to antiseptic efficacy and lack of penetrative ability do not support its use as a complete wound therapy but the

observed ability of the medical grade products to maintain a healthy environment for wound healing indicates a value in its use as a complementary therapeutic agent for use in wound care.

7.3 Further work

The importance of finding antimicrobial products suitable for use in a clinical environment cannot be underestimated as the problems associated with multi-drug resistant organisms increase. This study identified potentially significant trends linking antibiotic susceptibility and susceptibility to Manuka honey. Further work would include studying organisms with identified resistance mechanisms and comparing their susceptibility to Manuka honey to identify if particular resistance mechanisms for example, those associated with cell wall permeability, efflux pumps or the production of inhibitory enzymes may be associated with changes in Manuka susceptibility.

It was outside the scope of this study to investigate changes in morphology or organisms exposed to Manuka honey. Further work would involve electron microscopy to identify and compare changes between different organism genera to honey products from different floral sources. This could show interesting results when performed on organisms with known genetic mechanisms of resistance. Would pseudomonads with porin and efflux pump resistance to carbapenems show different changes to those with carbapenemase mediated resistance? Confocal microscopy could be used to identify penetration of honey through biofilm layers and further analysis using the techniques described by Signoretto (2000) to establish if the increased resistance of Enterococci is due to them entering a viable but non culturable state. Studying the effect of resistance enzymes such as different β lactamases on the individual constituents of Manuka honeys may provide further information regarding differences in honey susceptibility between sensitive and

resistant isolates. This study was confined to using organisms based on phenotype. Investigation of organisms of not only different genotypes but also specific ribotypes may reveal the nature of variations in susceptibility. There is an increasing use of MALDI-TOF mass spectrophotometers in diagnostic microbiology laboratories which identify organisms by their riboproteins. MALDI-TOF and the Polymerase Chain Reaction (PCR) for molecular identification open up the possibility of more detailed analysis of the mechanisms of the antimicrobial action of Manuka products and other active honeys.

Identification of the active components in Manuka honey, the interaction of components and their contribution to antimicrobial activity and the effect on honey chemistry over time and in different environmental conditions such as heat is still to be defined. DOSY NMR has been shown to be an effective method of analysis on honey as a complex mixture and using this technology to identify changes in individual products over time and to identify differences between batches of the same product would provide a valuable contribution to our current knowledge.

The wound modelling produced showed Manuka honey exhibited less biofilm removal ability than expected. Further *in vitro* work including a variety of wound models of different tissue types such as skin, muscle and mucosa with more clearly defined surface areas would contribute to understanding the scope and limitations of honey as a topical application for wounds and wound dressings.

The need for well designed random controlled clinical trials is possibly where there is greatest need for further work. The work of Subrahmanyan (1991,1993, 1994, 1996a, 1996b, 1998, 1999, 2000) with burns patients and Al-Waili and

Saloom's work (1999) on post operative healing has shown the use of honey to be beneficial. The healing properties of honey described various groups (Efem, 1988, Cooper *et al.*, 2001, Dunford and Hanano, 2004), along with the findings of this study suggest that honey might be a valuable agent to maintain wound cleanliness and warrant further investigation of its potential value as a first line dressing post surgery. Whilst there are a number of honey impregnated wound dressings commercially available, there does not appear to be any honey coated cannula products or honey impregnated sutures available, the potential of which would be worthy of investigation. The use of an elastomeric pump to deliver fresh honey underneath a hydrocolloid dressing to absorb wound exudate might facilitate the healing of chronic wounds and ulcers with the potential benefit of reducing the number of dressing changes and decreasing the risk of bacterial contamination of the patient's environment. These are all measures which positively impact upon NHS resources' without a compromise to patient care. Accelerated healing and the absence of post operative infection has economic implications with regards to reducing hospital bed days, antimicrobial usage and a faster return to work all of which have a positive impact on industry offering some economic justification in the investment into further research.

The findings of this thesis have made contributions to the current pool of knowledge with regard to the scope and limits of the antimicrobial efficacy of Syrups, Manuka and non Manuka honeys and demonstrated that diffusion ordered spectroscopy nuclear magnetic resonance is an effective method to analyse complex sugar products. It is hoped these findings may inspire further

work which will in turn contribute alternative antimicrobial therapeutic support to the current antibiotic regimens which are so challenged by increasing microbial resistance.

REFERENCES

Acuin J. (2002) 'Extracts from 'Concise clinical evidence chronic supporative otitis media', *British Medical Journal,* 325 (7373), pp. 1159-60.

Adams C. J., Boult, C. H., Deadman B. J., Farr, J. M., Grainger M. N., Manley-Harris M. and Snow M. J. (2008) 'Isolation by HPLC and characterisation of the bioactive fraction of New Zealand Manuka (*Leptospermum scoparium*) honey'. *Carbohydrate Research*, 343 (4), pp. 651-659.

Adams C.J., Manley-Harris M and Molan P.C. (2009) 'The origin of methylglyoxal in New Zealand Manuka (*Leptospermum scoparium)* honey', *Carbohydrate Research,* 344, pp. 1050-1053.

Adesunkanmi K. and Oyelami O.A. (1994) 'The pattern and outome of burn injuries at Wesley Guild Hospital, Ilesha, Nigeria. A review of 156 cases', *Journal of Tropical Medicine and Hygiene,* 97, pp. 108-112.

Alandejani, T., Marsan, J., Ferris, W., Slinger, R., & Chan, F. (2009) 'Effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms'. *Otolaryngology--Head and Neck Surgery*, 141 (1), pp. 114-118.

Albietz J.M. and Lenton L.M. (2006) 'Effect of antibacterial honey ocular flora in tear deficiency and meibomian gland disease', *Cornea,* 25, pp. 1012-1019.

Albright, L. J., & Wilson, E. M. (1974). Sub-lethal effects of several metallic salts—organic compounds combinations upon the heterotrophic microflora of a natural water. *Water Research*, *8*(2), 101-105.

Allen K. L., Molan P. C. and Reid G. M. (1991) 'A Survey of the Antibacterial Activity of Some New Zealand Honeys'. *Journal of Pharmacy and Pharmacology*, 43 (12), pp. 817–822.

Al-Jabri A.A., Nzeako Z., Al- Mahrooqi Z., Al-Nagdy A. and Nsanze, H. (2003) '*In vitro* antibacterial activity of Omani and African Honey', *Journal of Biomedical Science,* 60 (1), pp. 1-4.

Aljadi A. M and Yusoff, K. M. (2003) 'Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties', *Turkish Journal of Medical Sciences, 33* (4), pp. 229-236.

Al Somal N., Coley K.E., Molan P.C. and Hancock B.M. (1994) 'Susceptibility of *Helicobacter pylori* to the Antibacterial Activity of Manuka Honey', *Journal Royal Society of Medicine*, 87 (1), pp. 9-12

Al-Waili N.S. and Saloom K.Y. (1999) 'Effects of topical honey on post-operative wound infections due to Gram positive and Gram-negative bacteria following caesarean sections and hysterectomies', *European Journal of Medical Research*, 4 (3) pp. 126-30.

Al-Waili N.S. (2001) 'Therapeutic and prophylactic use of crude honey on seborrheic dermatitis and dandruff', *European Journal of Medical Research,* 6 (7), pp. 306-308.

Al-Waili N.S (2003) 'Topical application of natural honey beeswax and olive oil mixture for atopic dermatitis or psoriasis: partially controlled single blinded study', *Complementary Therapies in Medicine,* 11 (4), pp. 226-234.

Al-Waili N.S (2004) 'An alternative treatment for pityriasis versicolor, tinea cruris, tinea corporis and tinea faciei with topical application of honey, olive oil, and beeswax mixture: an open pilot study', *Complementary Therapies in Medicine* , 12 (1), pp. 45-47.

Andrews J.M. (2001) 'Determination of minimum inhibitory concentrations', '*Journal of .Antimicrobial Chemotherapy 48* (supplement 1), pp. 5-16.

Anklam E. (1998) 'A review of the analytical methods to determine the geographical and botanical origin of honey', *Food Chemistry.* 63 (4), pp. 549-562.

Atrott J. and Henle T. (2009) 'Methylglyoxal in Manuka Honey – Correlation with Antibacterial Properties' *Czech Journal of Food Science and Technology,* 27, S163-S165.

Baghel P.S., Shukla S., Mathur K. and Randa R. (2009) 'A comparative study to evaluate the effect of honey dressing and silver sulfadiazine dressing on wound healing in burn patients', *Indian Journal of Plastic Surgery,* 42 (2), pp. 176-181.

Baker D.D., Chu M., Oza U. and Rajgarhia V. (2007) 'The value of natural products to future pharmaceutical discovery', *Natural Products Reports*, 24, pp. 1225–1244.

Bang, L. M., Buntting, C., and Molan, P. (2003) 'The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing'. *The Journal of Alternative & Complementary Medicine*, *9* (2), pp. 267-273.

Bangroo A.K., Khatri R. and Chauhan S. (2005) 'Honey dressing in pediatric burns', *Journal of Indian Association of Pediatric Surgeons,* 10, pp. 172-175.

Bardy J., Nicholas J.S., Mais K.L., and Molassiotis A. (2008) 'A systematic review of honey uses and its potential value in oncology care', *Journal of Clinical Nursing,* 17, pp. 2604-2623.

Bauer, A. W.,. Kirby W. M. M,. Sherris J. C., Turck M. (1966) 'Antibiotic susceptibility testing by a standardised single disk method'. *American Journal of Clinical Pathology* 45 (4), pp. 493-496.

Beretta G., Artali R., Caneva E., Orlandini S., Centini M. and Facino R.M. (2009) 'Quinolone alkaloids in honey: Further analytical (HPLC-DAD-ESI-MS,

multidimensional diffusion ordered NMR spectroscopy), theoretical and chemometric studies', *Journal of Pharmaceutical and Biomedical Analysis,* 50, pp. 432-439.

Beumer R., Bloomfield S. F., Exner M., Fara G. M., Nath K. J., and Scott, E. (2000) Microbial resistance and biocides.

Beyenal, H., & Lewandowski, Z. (2001). Internal and external mass transfer in biofilms grown at various flow velocities. *Biotechnology progress*, *18*(1), 55-61.

Biesaga M. and Pyrzynska K. (2009) 'Liquid chromatography/tandem mass spectrometry studies of the phenolic compounds in honey', *Journal of Chromatography,* 1216, pp. 6620-6626.

Biswal B.M., Zakaria A. and Ahmad N.M. (2003) 'Topical application of honey in the mangement of radiation mucositis: a preliminary study', *Support Care Cancer,* 11, pp. 242-248.

Blasa M., Candiracci M., Accorsi A., Piancentini M.P., Albertini M.C., Piatti E. (2006). 'Raw *Millefiori* honey is packed full of antioxidants' *Food Chemistry* 97 (2), pp. 217-222.

Blaser G, Santos K, Bode U, et al (2007) Effect of medical honey on wounds colonised or infected with MRSA. J Wound Care 16(8): 325-8

Bogdanov S., Ruoff K. and Oddo L.P. (2004) 'Physico-chemical methods for the characterisation of unifloral honeys: a review', *Apidologie,* 35 pp. S4-S17.

Bondi A., Saplding E.H., Smith D.E., Dietz C.C. (1947) 'A routine method for the rapid determination of susceptibility to Penicillin and other antibiotics', *American Journal of the Medical Sciences,* 213, pp. 221-225.

Borges-Walmsley M.I., Mckeegan K.S. and Walmsley A.R. (2003) 'Structure and function of efflux pumps that confer resistance to drugs' *Biochemical Journal*, 376 (Pt 2), pp. 313–338.

Borkow, G., and Gabbay, J. (2004). Putting copper into action: copper-impregnated products with potent biocidal activities. *The FASEB journal*, *18*(14), 1728-1730.

Borkow, G.and Gabbay, J. (2005). Copper as a biocidal tool. *Current medicinal chemistry*, *12*(18), 2163-2175.

Borkow, G., Gabbay, J., & Zatcoff, R. C. (2008). Could chronic wounds not heal due to too low local copper levels?. *Medical hypotheses*, *70*(3), 610-613.

Borkow, G., Gabbay, J., Dardik, R., Eidelman, A. I., Lavie, Y., Grunfeld, Y. and Marikovsky, M. (2010). Molecular mechanisms of enhanced wound healing by copper oxide-impregnated dressings. *Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society*, *18*(2), 266.

Bose B. (1982) 'Honey or sugar in the treatment of infected wounds?' *Lancet*, 1 (8278) pp. 963.

Bowler P.G. (1998) 'The anaerobic and aerobic microbiology of wounds: a review', *Wounds*, 10, pp. 170-178.

Bowler P.G. and Davis B.J. (1999) 'The microbiology of acute and chronic wounds', *Wound*, 11, pp. 72-79.

Bowler P.G., Duerden B.I. and Armstrong D.G. (2001) 'Wound microbiology and associated approaches to wound management', *Clinical Microbiology. Reviews*, 14, pp. 244-269.

Bradshaw D.J., Marsh P.D., Watson G.K., and Allison C. (1998) 'Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration', *Infection and Immunity,* 66 (10), pp. 4729-4732.

Bradshaw C.E. (2011) 'An in vitro comparison of the antimicrobial activity of honey, iodine and silver wound dressings', *Bioscience Horizons* 4 (1), pp. 61-70.

Brady N., Molan P.C., Bang L. (2004) 'A survey of non-Manuka New Zealand honeys for antibacterial and antifungal activities.' *Journal of .Apicultural Research,* 43(2), pp. 47-52.

Bragg, P. D., and D. J. Rainnie. (1974) The effect of silver ions on the respiratory chain of *Escherichia coli. Can. J. Microbiol.* 20:883–889.

British Society of Antimicrobial Chemotherapy
Available at: http://bsac.org.uk/

Burger R. M., Franco R. J. and Drlica K. (1994) 'Killing acanthamoebae with polyaminopropyl biguanide: quantitation and kinetics', *Antimicrobial Agents and Chemotherapy, 38*(4), pp. 886-888.

Burrell R. (2003) 'A scientific perspective on the use of topical silver preparations', *Ostomy Wound Management,* 49 (5A Supplement), pp. 19-24.

Bush, K., & Fisher, J. F. (2011). Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. *Annual review of microbiology, 65,* 455-478.

Buswell C.M., Herlihy Y.M. Marsh P.D., Keevil C.W. and Leach S.A. (1997) 'Coaggregation amongst aquatic biofilm bacteria', *Journal of Applied Microbiology,* 87 (4), pp. 477-84.

Cabot G., Ocampo-Sosa A.A., Tubau F., Macia M.D., Rodríguez C., Moya B., Zamorano L., Suárez C., Peña C. and Martínez-Martínez L. (2011) 'Overexpression of AmpC and Efflux Pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: Prevalence and impact on resistance in a Spanish multicenter study' *Antimicrobial Agents and Chemotherapy,* 55 (5), pp. 1906-1911.

Carr H.S., Wlodkowski T.J. and Rosenkranz H.S. (1973) 'Silver sulfadiazine: *In vitro* antibacterial activity', *Antimicrobial Agents and Chemotherapy,* B4 (6), pp. 585-587.

Cars O., Högberg, L.D., Murray M., Nordberg O., Sivarman S., Stalsby Lundborg C., So A.D. and Tomson G. (2008) 'Meeting the challenge of antibiotic resistance', *British Medical Journal,* 337: a1438

Cerca, N., Pier, G. B., Vilanova, M., Oliveira, R., & Azeredo, J. (2005). Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of< i> Staphylococcus epidermidis</i>. *Research in microbiology*, *156*(4), 506-514.

Ceri H., Olson M.E., Stremick C., Read R.., Morck D. and Buret A. (1999) 'The Calgary biofilm device: New technology for rapid determination of antibiotic susceptibilities of bacterial biolfims', *Journal of Clinical Microbiology,* 37 (6), pp. 1771-1776.

Cernak, M., Majtanova, N., Cernak, A. and Majtan, J. (2012) 'Honey prophylaxis reduces the risk of endophthalmitis during perioperative period of eye surgery', *Phytotherapy Research,* 26 (4), pp. 613–616.

Chambers J (2006) Topical Manuka honey for MRSA-contaminated skin ulcers. Palliat Med 20(5):557

Charles P.G.P. and Grayson M.L. (2004) 'The dearth of new antibiotic development: why we should be worried and what we can do about it?' *Medical Journal of Australia*, 181 (10), pp. 549-553.

Chung J.Y. (2001) 'Myth: silver sulfadiazine is the best treatment for minor burns', *Western Journal of Medicine,* 175, pp. 205-206.

Colgan R. and Powers J.H. (2001) 'Appropriate antimicrobial prescribing: approaches that limit antibiotic resistance', *American Family Physician,* 64 (6), pp. 999-1004.

Conti M.E., Stripeikis J., Campanella L., Cocaine D. and Tudino M.B. (2007) 'Characterization of Italian honeys (Marche Region) on the basis of their mineral content and some typical quality parameters', *Chemistry Central Journal,* 1 (14), pp. 14.

Cooney, J. J., and Tang, R. J. (1999). [47] Quantifying effects of antifouling paints on microbial biofilm formation. *Methods in enzymology*, *310*, 637-644.

Cooper RA. and Molan P. (1999a). 'The use of honey as an antiseptic in managing *Pseudomonas aeruginosa* infection', *Journal of Wound Care*, 8(4), pp. 155.

Cooper RA, Molan P. and Harding KG (1999b) 'Antibacterial activity of honey against strains of *Staphylococcus aureus* from infected wounds', *Journal of the Royal Society of Medicine*, 92 (6), pp. 283-5.

Cooper RA, Molan PC., Krishnamoorthy I., and Harding KG (2001) 'Manuka honey used to heal a recalcitrant surgical wound' *European Journal of Clinical Microbiology and Infectious Diseases*, 20 (10), pp. 758–759.

Cooper, R. A., Halas, E., and Molan, P. C. (2002) 'The efficacy of honey in inhibiting strains of *Pseudomonas aeruginosa* from infected burns'. *Journal of Burn Care & Research*, *23* (6), pp. 366-370.

Cooper R.A., Molan P.C. and Harding K.G. (2002) 'The sensitivity to honey of Gram positive cocci of clinical significance isolated from wounds', *Journal of Applied Microbiology,* 93 (5), pp. 857-863.

Cooper R.A. and Okhiria O. (2006) 'Biofilms, wound infection and the issue of control', *Wounds UK,* 2 (3) 48-57.

Cooper RA., (2008) 'Using honey to inhibit wound pathogens', *Nursing Times,* 104 (3), pp. 46-49.

Cooper R.A. and Jenkins L. (2009) 'A comparison between medical grade honey and table honeys in relation to antimicrobial efficacy', *Wounds,* 21 (2), pp. 29-36.

Cooper R. (2010) 'Biofilms and wounds: much ado about nothing?' *Wounds UK,* 6, (4), pp. 84-90.

Costerton J.W., Lewandowski z., Caldwell D.E., Korber D.R. and Lappin-Scott H.M. (1995) 'Microbial biofilms'. *Annual .Reviews in Microbiology,* 49(1), pp. 711-745.

Costerton J.W., Stewart P.S. and Greenberg E.P. (1999) 'Bacterial biofilms: a common cause of persistent infections'. *Science*, *284*(5418), pp. 1318-1322.

Costerton J.W. and Stewart P.S. (2001) 'Battling Biofilms', *Scientific American,* 285 (1), pp. 74-81.

Courvalin, P. (2006) 'Vancomycin resistance in gram-positive cocci', *Clinical Infectious Diseases, 42*(Supplement 1), pp. S25-S34.

De Kievit T.R. and Iglewski B.H. (2000) 'Bacterial Quorum Sensing in Pathogenic Relationships' *Infection and Immunity,* 68(9), pp. 4839–4849.

Del Rosso, J. Q. (2006) Combination topical therapy in the treatment of acne. *Cutis; Cutaneous Medicine for the Practitioner, 78* (2 Suppl 1), pp. 5.

Department of Health (2000) UK Antimicrobial Resistance Strategy and Action Plan'
www.publications.doh.gov.uk/pdfs/arbstract.pdf accessed 21/09/07

Diehr S., Hamp A. and Jamieson B. (2007) 'Do Topical Antibiotics Improve Wound healing?', *Journal of Family Practitioners,* 56 (2), pp. 140-144.

Donarski J.A., Roberts D.P.T. and Charlton A.J. (2010) 'Quantitative NMR spectroscopy for the rapid measurement of methylglyoxal in Manuka honey', *Analytical Methods,* 2 (10), pp. 1479-1483.

Donlan R.M. (2001). 'Biofilms and device – associated Infections'. *Emerging Infectious Diseases.* 7 (2), pp. 277-281.

Dow G., Browne A. and Sibbald R.G. (1999) 'Infection in chronic wounds; controversies in diagnosis and treatment', *Ostomy/Wound Management, 45* (8), pp. 23-27.

Dunford C.E. and Hanano R. (2004) 'Acceptability to patients of a honey dressing for non-healing venous leg ulcers', *Journal of Wound Care,* 13 (5), pp. 193-197.

Dunford C, Cooper R, Molan P, White R (2000) The use of honey in wound management. Nurs Stand 15(11):63-68

Dupont, C. L., Grass, G., & Rensing, C. (2011). Copper toxicity and the origin of bacterial resistance—new insights and applications. *Metallomics*, *3*(11), 1109-1118.

Edwards R. and Harding K.G. (2004) 'Bacteria and wound healing'. *Current Opinion in Infectious Diseases,* 17 (2), pp. 91-96.

Efem S.E. (1988) 'Clinical observations on the wound healing properties of honey'. *British Journal of Surgery, 75* (7), pp. 679-681.

Efem S.E. (1993) 'Recent advances in the management of Fournier's gangrene: preliminary observations', *Surgery,* 113 (2), pp. 200-204.

Elman M. and Lebzelter J. (2004) 'Light therapy in the treatment of Acne Vulgaris', *Dermatologic Surgery,* 30 (2), pp. 139-146.

Enoch S. and Harding K. (2003) 'Wound bed preparation. The science behind the removal of barriers to healing', *Wounds,* 15 (7), pp. 4-11.

Enoch S. and Leaper D.J. (2008) 'Basic science of wound healing', *Surgery (Oxford),* 26 (2), pp. 31-37.

Estevinho M.L., Esteves Afonso S. and Feás X., (2011) 'Antifungal effect of lavender honey against *Candida albicans, Candida krusei* and *Cryptococcus neoformans', Journal of Food Science and Technology,* 48 (5), pp. 640-643.

Estrada H., Gamboa Mdel M., Arias M.L. and Cgaves C. (2005)'Evaluation of the antimicrobial action of honey against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, Salmonella enteritidis, Listeria monocytogenes and Aspergillus niger.* Evaluation of its microbiological charge'. *Archivos latinoamericanos de nutricion.* 55(2), pp. 167-71.

European Commission Beekeeping and Honey Production – Residues in honey. available at http://ec.europa.eu/food/animal/liveanimals/bees/residues_honey_en.htm accessed 22.07.13

Farouk A., Hassan T., Kassif H., Khalid S.A., Mutawali I. and Wadi M. (1998) 'Studies on Sudanese bee honey: laboratory and clinical evaluation', *Pharmaceutical Biology,* 26 (3), 161-168.

Faúndez, G., Troncoso, M., Navarrete, P., & Figueroa, G. (2004). Antimicrobial activity of copper surfaces against suspensions of Salmonella enterica and Campylobacter jejuni. *BMC microbiology*, *4*(1), 19.

Fearnley L., Greenwood D.R., Schmitz M., Stephens J.M., Schlothauer R.C. and Loomes K.M. (2011) 'Compositional analysis of Manuka honeys by high-resolution mass spectrometry: Identification of a Manuka-enriched archetypal molecule', *Food Chemistry,* 132 (2), pp. 948-953.

Fenimore, D. C. and Davis C. M. (1981). 'High performance thin-layer chromatography', *Analytical Chemistry*, *53*(2), pp. 252-266.

Forrest R.D. (1982) 'Early history of wound treatment', *Journal of the. Royal Society of Medicine,* 75 (3), 198-205.

Fortuna A. M., Juárez Z. N., Bach H., Nematallah A., Av-Gay Y., Sánchez-Arreola E. and Hernández L. R. (2011) 'Antimicrobial activities of sesquiterpene lactones and inositol derivatives from Hymenoxys robusta' *Phytochemistry*, 72 (18), pp. 2413-2418.

Foster, T. J., & Höök, M. (1998). Surface protein adhesins of *Staphylococcus aureus, Trends in microbiology*, *6*(12), 484-488.

Fox C.L (1968) 'Silver Sulfadiazine—A New Topical Therapy for *Pseudomonas* in Burns', *Archives of Surgery,* 96 (2), pp. 184-188.

Frank C., Bayoumi I. and Westendorp C. (2005) 'Approach to infected skin ulcers', *Canadian Family Physician,* 51 (10), pp. 1352-1359.

Gallois M. H., Rothko H.J., Bailey M., Stokes C.R. and Oswald I.P. (2009) 'Natural alternatives to in-feed antibiotics in pig production: can immunomodulators play a role? *Animal,* 3 (12), pp. 1644–1661.

George N.M. and Cutting K.F. (2007) 'Antibacterial honey (Medihoney™): *in vitro* activity against clinical isolates of MRSA, VRE and other multiresistant Gram-negative organisms including *Pseudomonas aeruginosa', Wounds,* 19 (9), pp. 231-236.

Gethin G. and Cowman S. (2005). 'Case series of Manuka honey in leg ulceration', *International Wound Journal,* 2 (1), pp. 10-15.

Gethin GT, Cowman S, Conroy RM (2008) The impact of Manuka honey dressings on the surface of chronic wounds. Int Wound J 5:185-94

Gilbert P, Das J.R., Jones M.V. and Allison D.G. (2001) 'Assessment of resistance towards biocides following the attachment of micro organisms to, and growth on surfaces', *Journal of Applied Microbiology,* 91 (2), pp. 258-254.

Gomez-Caravaca A.M., Gomez-Romero M. Arraez-Roman D., Segura-Carretero A. and Fernandez- Gutierrez A. (2006) 'Advances in analysis of phenolic compounds in products derived from bees', *Journal of Pharmaceutical and Biomedical. Analysis,* 41 (4), pp. 1220-1234.

Gould I.M. 2000. 'Towards a common susceptibility testing method'. *Journal of Antimicrobial Chemotherapy,* 45 (6), pp. 757-762.

Grange J.M. and Davey R.W. (1990) 'Antibacterial properties of Propolis (Bee Glue)', *Journal of the. Royal Society of Medicine,* 83 (3), 159-160.

Gray D., Barrett S., Battacharyya M., Butcher M., Enoch S., Fumerola S., Stephen Haynes J., Edwards-Jones V., Leaper D., Strohal R., White R., Wicks G and Young, T. (2010). PHMB and its potential contribution to wound management. *Wounds UK*, *6*(2), 40-46.

Greenberg, E. P. (2000). Acyl-homoserine lactone quorum sensing in bacteria. *Journal Of Microbiology-Seoul 38*(3), 117-121.

Guignard B., Entenza J.M. and Moreillon P. (2005) 'β-lactams against methicillin-resistant *Staphylococcus aureus'. Current Opinion in Pharmacology,* 5 (5), pp. 479-489.

Gulfraz M., Ifftikhar F., Asif S., Raja G.K., Asad J.A., Imran M., Abbasi K. and Zeenat A. (2010) 'Quality assessment and antimicrobial activity of various honey types of Pakistan.' *African Journal of Biotechnology* 9, pp. 6906-6909.

Gupta S.S., Singh O., Bhagel P.S., Moses S., Shukla S. and Mathur R.K. (2011) 'Honey dressing versus silver sulfadiazine dressing for wound healing in burn patients: A retrospective study', *Journal of Cutaneous and Aesthetic Surgery,* 4 (3), pp. 183-187.

Haider A., Shaw J.C. (2004) 'Treatment of Acne Vulgaris', *Journal of the American Medical Association,* 292 (6), pp. 726-35

Hall-Stoodley, L., & Stoodley, P. (2005). Biofilm formation and dispersal and the transmission of human pathogens. *TRENDS in Microbiology*, *13*(1).

Hannan S., Ready D., Jasni A.S., Rogers M., Pratten J. and Roberts A.P. (2010) 'Transfer of antibiotic resistance by transformation with eDNA within oral biofilms', *FEMS Immunology and Medical Microbiology,* 59 (3), pp. 345-349.

Heering W. Usleber E., Dietrich R. and Martbauer E. (1998) 'Immunochemical screening for drug residues in commercial honey', *Analyst*, 123 (12), pp. 2759-2762.

Hejase M.J., Simonin J.E., Bihrle R., Coogan C.L. (1996) 'Genital Fournier's gangrene: experience with 38 patients', *Urology,* 47 (5), pp. 734-739.

Hillson R.M. (1988) 'Gold, frankincense and myrrh', *Journal of the Royal Society of Medicine,* 81 (9), pp. 542-543.

Hiro, M. E., Pierpont, Y. N., Ko, F., Wright, T. E., Robson, M. C., & Payne, W. G. (2012). Comparative Evaluation of Silver-Containing Antimicrobial Dressings on In Vitro and In Vivo Processes of Wound Healing. *Eplasty*, *12*.

Ho E.C., Alanni A, and Irving R. (2007) 'Topical antibiotic ototoxicity: Does it Influence our practice?' *Journal of Laryngology and Otology,* 121 (4), pp. 333-7.

Hollisaz M.T., Khedmat H. and Yari F. (2004) 'A randomised clinical trial comparing hydrocolloid, phenytoin and simple dressings for the treatment of pressure ulcers', *BMC Dermatology,* 4 (1), pp. 18

Hooper, D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging infectious diseases*, *7*(2), 337.

Huidoboro J.F. and Pilar Sanchez M. (2005) 'Precise method for the measurement of catalase activity in honey', *Journal of AOAC International,* 88 (3), pp. 800-804.

Irish J, Blair S, Carter DA (2011) 'The antibacterial activity of honey derived from Australian flora'. *PLoS one,* 6(3), e18229.

Jacobs A. and Chenia H.Y. (2009). 'Biofilm-forming capacity, surface hydrophobicity and aggregation characteristics of *Myroides odoratus* isolated from South African *Oreochromis mossambicus* fish', *Journal of Applied Microbiology,* 107 (6), pp. 1957–1966.

Jacobsen, N. E. (2007) *NMR spectroscopy explained: simplified theory, applications and examples for organic chemistry and structural biology',* Wiley-Interscience.

James, T. L. (1998). Fundamentals of NMR. *Department of Pharmaceutical Chemistry University of California San Francisco, CA 94143-0446 USA.*

James G.A., Swogger E. and Wolcott R. (2008) 'Biofilms in chronic wounds', *Wound Repair and Regeneration.,* 16 (1), pp. 37-44.

Jenkins R., Burton N. and Cooper R. (2010) 'Effect of Manuka honey on the expression of universal stress protein A in meticillin resistant *Staphylococcus aureus', International Journal of Antimicrobial Agents,* 37(4), pp. 373-376.

Jenkins R., Burton N. and Cooper R. (2011) 'Manuka honey inhibits cell division in meticillin-resistant *Staphylococcus aureus', Journal Antimicrobial Chemotherapy,* 66 (11), pp. 2536-2542.

Jenkins, R. and Cooper, R. (2012) 'Synergy between oxacillin and Manuka honey sensitizes methicillin-resistant Staphylococcus aureus to oxacillin'. *Journal of antimicrobial chemotherapy, 67*(6), 1405-1407.

Jönsson K, Signäs C, Müller HP and Lindberg M (1991) 'Two different genes encode fibronectin binding proteins in Staphylococcus aureus. The complete nucleotide sequence and characterization of the second gene' *European journal of biochemistry / FEBS* 202 (3): pp. 1041–8.

Jung, W. K., Koo, H. C., Kim, K. W., Shin, S., Kim, S. H., & Park, Y. H. (2008). Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli. *Applied and environmental microbiology*, *74*(7), 2171-2178.

Katsikogianni, M., & Missirlis, Y. F. (2004). Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mater*, *8*, 37-57.

Kessinger, A., Armitage, J. O., Landmark, J. D., & Weisenburger, D. D. (1986). Reconstitution of human hematopoietic function with autologous cryopreserved circulating stem cells. *Experimental hematology*, *14*(3), 192.

Kingsley, A. (2001). The use of honey in the treatment of infected wounds: case studies. *British Journal of Nursing*, *10*(22 Suppl), S13-S20.

Krall, D. I. T. (1999). A new technology of microdispersed silver in polyurethane induces antimicrobial activity in central venous catheters. *Infection*, *27*(1), S16-S23.

Küçük M., Kolayli S., Karaoğlu Ş., Ulusoy E.M., Baltaci C. and Candan F. (2007) 'Biological activities and chemical composition of three honeys of different types from Anatolia', *Food Chemistry,* 100 (2), pp. 526-534.

Lay-flurrie, K. (2008). Honey in wound care: effects, clinical application and patient benefit. *British Journal of Nursing*, *17*(11 Suppl), S30-S36.

Leclercq R. and Courvalin P. (1997) 'Resistance to glycopeptides in enterococci'. *Clinical infectious diseases*, 24 (4), pp.545-554.

Le Gresley, A. L., Kenny, J., Cassar, C., Kelly, A., Sinclair, A., and Fielder, M. D. (2012) 'The application of high resolution diffusion NMR to the analysis of Manuka honey'. *Food Chemistry*.135 (4), pp. 2879-2876.

Levy S.B. (2002) 'Factors impacting on the problem of antibiotic resistance', *Journal of Antimicrobial Chemotherapy,* 49 (1), pp. 25-30.

Llarrull, L. I., Fisher, J. F., & Mobashery, S. (2009). Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new β-lactams that meet the challenge. *Antimicrobial agents and chemotherapy*, *53*(10), 4051-4063.

Li, D., Kagan, G., Hopson, R., and Williard P. G. (2009). 'Formula weight prediction by internal reference diffusion-ordered NMR spectroscopy (DOSY)'. *Journal of the American Chemical Society*, 131(15), pp. 5627-5634.

Liaqat I. and Sabri A.N. (2008) 'Analysis of cell wall constituents of biocide-resistant isolates from dental-unit water line biofilms.' *Current Microbiology*, 57(4), pp. 340-347.

Lieberman P. B. and Wootan M. G. (1998) *Protecting the Crown Jewels of Medicine: A strategic plan to preserve the effectiveness of antibiotics*. Center for Science in the Public Interest.
Available at www.cspinet.org/reports/abiotic.htm (accessed 14/02/07).

Lister P.D., Wolter D. J. and Hanson D.N. (2009) 'Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of

Chromosomally Encoded Resistance Mechanisms'. *Clinical Microbiology Reviews,* 22 (4), pp. 582-610.

Livermore D. (1998) 'β-lactamase-mediated resistance and opportunities for its control'. *Journal of Antimicrobial Chemotherapy,* 41 (4), pp. 25-41.

Lo-Ten-Foe J.R., de Smet A.M.J.A., Bram M. W. Diederen J.A.J., Kluytmans W. and van Keulan P.H.J. (2007) 'Comparative evaluation of the VITEK 2, disk diffusion, E test, both microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, Including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains', *Antimicrobial Agents Chemotherapy,* 51 (10), pp. 3726-3730 .

Loh J.V., Percival S.L. and Woods E.G. (2009) 'Silver resistance in MRSA from wound and nasal sources in humans and animals'. *International Wound Journal,* 6 (1), pp. 32-38.

Lund-Nielsen B., Adamsen L., Gottrup F., Rorth M., Tolver A. and Kolmos H.J. (2011) 'Qualitative bacteriology in malignant wounds - A prospective , randomised, clinical study to compare the effect of honey and silver dressings', *Ostomy Wound Management,* 57 (7), pp. 28-36.

Lusby P.E., Coombes A. and Wilkinson J.M. (2005) 'Bactericidal activity of different honeys against pathogenic bacteria', *Archives of Medical Research,* 36 (5), pp. 464-467.

MacKeen P.C., Person S., Warner S.C., Snipes W. and Stevens S.E. (1987) 'Silver coated nylon fibre as an antibacterial agent', *Antimicrobial Agents and Chemotherapy,* 31 (1), pp. 93-99.

Maeda Y., Loughrey A., Phillip J.A., Earle B., Millar C., Rao J.R. Kearns A., McConville O., Goldsmith E., Rooney P.J., Dooley J.S.G., Lowery C.J., Snelling

W.J., McMahon A., McDowell D. and Moore J.E. (2008) 'Antibacterial activity of honey against community associated meticillin resistant *Staphylococcus aureus* (CA-MRSA)', *Complementary Therapies in Clinical Practice,* 14 (2), pp. 77-82.

Maillard J-Y. and Denyer S.P. (2006) 'Focus on Silver', *World Wide Wounds.com* (Accessed 20/12/2006)

Majno G (1975) 'The Healing Hand: Man and Wound in the Ancient World', Harvard University Press. (Accessed 20/12/2006 Cambridge, Mass).

Malik K.I., Malik N. and Aslam A. (2010) 'Honey compared with silver sulphadiazine in the treatment of superficial partial thickness burns', *International Wound Journal,* 7 (5), pp. 413-417.

Mavric E., Wittman S., Barth G. and Henle T. (2008) 'Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand', *Molecular Nutrition and. Food Research,* 52 (4), pp.483-489.

McCasland, G. E., Naumann, M. O. and Durham, L. J. (1968). 'Alicyclic carbohydrates. XXXV. Synthesis of proto-quercitol. 220-MHz proton spectrum with the superconducting solenoid'. *The Journal of Organic Chemistry*, *33*(11), 4220-4227.

McIntire F.C., Vatter A.E., Baros J. and Arnold J. (1978) 'Mechanisms of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34', *Infection and immunity,* 21 (3), pp. 978-88.

McKee B. (2003) 'Prevention if residues in honey: a future perspective', *Apiacta.* 38, pp. 173-177.

McGovern D., Abbas S., Vivian G. and Dalton H. (1999) 'Manuka Honey against *Helicobacter pylori*' *Journal of the Royal Society of Medicine*, 92 (8), pp. 439.

Mertz P.M. (2003) 'Cutaneous biofilms: friends or foe?' *Wounds*, 15 (5), pp. 129-32.

Midura T. F., Snowden S., Wood R. M. and Arnon S. S. (1979) 'Isolation of *Clostridium botulinum* from Honey'. *Journal of Clinical Microbiology*, *9* (2), pp. 282-283.

Miles, A. A., Misra, S. S., & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene*, *38*(06), 732-749.

Miller, C. N., Carville, K., Newall, N., Kapp, S., Lewin, G., Karimi, L., & Santamaria, N. (2011). Assessing bacterial burden in wounds: comparing clinical observation and wound swabs. *International Wound Journal*, *8*(1), 45-55.

Min K.R., Zimmer M.N. and Rickard A.H. (2010) 'Physiochemical parameters influencing coaggregation between freshwater bacteria *Sphinomonas natatoria* 2.1 and *Micrococcus luteus* 2.13' *Biofouling*, 26 (8), pp. 931-40.

Mingeot-Leclercq M.P., Glupczynski Y., and Tulkens P.M. (1999) 'Aminoglycosides: activity and resistance'. *Antimicrobial Agents and Chemotherapy*, 43 (4), pp. 727-37.

Molan P.C. and Russell K.M. (1988) 'Non-peroxide antibacterial activity in some New Zealand honeys'. *Journal of Apicultural Research*, 27 (1), pp. 62-67.

Molan P.C. (1999) 'Selection of honey for use on wounds. A handout prepared for the Infection control Conference in New Zealand' Available at www.Manukahoney.co.uk/article4.html accessed 25/02/07.

Molan, P. C. (2001) 'Honey as a topical antibacterial agent for treatment of infected wounds'. *World Wide Wounds*, pp. 1-13..

Molan P.C. (2006) 'The evidence supporting the use of honey as a wound dressing', *The International Journal of Lower extremity wounds,* 5(1), pp. 40.

Molan P.C. (2008a). 'Improvements to the UMF assay'. *New Zealand Beekeeper.* 16 (7), pp. 24-25.

Molan, P. (2008b). 'An explanation of why the MGO level in Manuka honey does not show the antibacterial activity'. *New Zealand Beekeeper,* 16(4), pp. 11-13.

Molan, P. (2008c). 'Why using the level of the active component in Manuka honey to replace the UMF rating is misleading'. *New Zealand Beekeeper,* pp. 6-7.

Molan P.C. (2012) 'Claims for Manuka honey activity. *New Zealand Beekeeper.* October 2012 pp. 23-27

Molan, P. and Betts J. A. (2004). 'Clinical usage of honey as a wound dressing: an update'. *Journal of wound care*, 13 (9), pp. 353-356.

Molan, P. C., & Stephens, J. (2008) 'The explanation of why the level of UMF varies in Manuka honey'. *New Zealand Beekeeper,* South City Print 17-21

Motallebnejad M., Akram S., Moghadamnia A., Moulana Z. and Omidi S. (2008) 'The effect of topical application of pure honey on radiation induced mucositis: a randomised clinical trial', *Journal of Contemporary Dental Practice, 9* (3) pp. 40-47.

Nadell C.D., Xavier J.B., Levin S.A. and Foster K.R. (2008) 'The evolution of quorum sensing in bacterial biofilms', *PLoS Biology,* 6 (1), e14.

Nagai T., Inoue R., Kanamori N., Suzuki N. And Nagashima T. (2006) 'Characterisation of honey from different floral sources. Its functional properties and effects of honey species on storage of meat', *Food Chemistry*, 97 (2), pp. 256-262.

Naylor W. (2002) 'Part 1: Symptom control in the management of fungating wounds', World Wide Wounds http://www.worldwidewounds.com/2002/march/Naylor/Symptom-Control-Fungating-Wounds.html (accessed 14.10.2012)

Naylor W., Laverty D. and Mallett J. (2008) *The Royal Marsden Hospital Handbook of Wound Management in Cancer Care,* Wiley

Ndayisaba G., Bazira L., Habonimana E. and Muteganya D. (1993) 'Clinical and bacteriological outcome of wounds treated with honey', Revue de chirurgie orthopédique et réparatrice de l'appareil moteur, 79 (2), pp. 111.

Ndip R. N., Takang A. E. M., Echakachi C. M., Malongue A., Akoachere J. F. T., Ndip L. M. and Luma H. N. (2007)' In-vitro antimicrobial activity of selected honeys on clinical isolates of Helicobacter pylori', *African Health Sciences*, 7(4), pp. 228-231.

Nevas M., Hielm S., Lindstrom M., Horn H., Koivulehto K. and Korkeala H. (2002). 'High prevalence of *Clostridium botulinum* types A and B in honey samples detected by polymerase chain reaction,' *International Journal of Food Microbiology* 72 (1), pp. 45-52.

Nikaido H., Rosenburg E.Y. and Foulds J. (1983) 'Porin channels in *Eschericia coli*: studies with β-lactams in intact cells', *Journal of Bacteriology,* 153 (1), pp. 232-240.

Nordmann P, Naas T. and Poirel L. (2011) 'Global spread of carbapenemase-producing *Enterobacteriaceae', Emerging Infectious Diseases,* 17(10), pp. 1791.

Noyce, J. O., Michels, H., & Keevil, C. W. (2006). Use of copper cast alloys to control Escherichia coli O157 cross-contamination during food processing. *Applied and environmental microbiology*, *72*(6), 4239-4244.

Nwomeh B.C., Yager D.R. and Cohen I.K. (1998) 'Physiology of the chronic wound', *Clinics in Plastic Surgery,* 25 (3), pp. 341-56.

Oka H., Kawamura N., Uno K., Yamada M., Harada K. and Suzuki M. (1987) 'Improvement of chemical analysis of antibiotics: XII. Simultaneous analysis of seven tetracyclines in honey', *Journal of Chromatography A*, *400*, pp. 253-261.

Oluwatosin, O. M., Olabanji, J. K., Oluwatosin, O. A., Tijani, L. A., and Onyechi, H. U. (2000) 'A comparison of topical honey and phenytoin in the treatment of chronic leg ulcers', *African Journal of Medicine and Medical Sciences*, 29 (1), pp. 31.

Parsek, M. R., & Greenberg, E. P. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in microbiology*, *13*(1), 27-33.

Passweg, J. R., Halter, J., Bucher, C., Gerull, S., Heim, D., Rovó, A. & Tichelli, A. (2012). Hematopoietic stem cell transplantation: a review and recommendations for follow-up care for the general practitioner. *Bone Marrow Transplant*, *47*(7), 906-23.

Patil V.P., Kulkarni A.P. and Jacques T. (2003) 'Iodine induced thyrotoxicosis following povidine-iodine dressings: a case report', *Critical Care and Resuscitation,* 5 (3), pp. 186-188.

Patton, T., Barrett, J., Brennan, J. and Moran, N. (2006). 'Use of a spectrophotometric bioassay for determination of microbial sensitivity to Manuka honey'. *Journal of Microbiological Methods*, 64 (1), pp. 84-95.

Pearson C.H., Whitehead J.E. 1974. 'Antibiotic sensitivity testing: a modification of the Stokes method using a rotary plater'. *Journal of Clinical Pathology*, 27 (5), pp. 430-431.

Phillips P.L., Wolcott R.D., Fletcher J. and Schultz G. (2010) 'Biofilms made easy' *Wound International,* 1 (3), pp. 1-6.

Phuapradit W. and Saropala N. (1992) 'Topical application of honey in treatment of abdominal wound disruption', *Australian and New Zealand Journal of Obstetrics and Gynaecology*, 32 (4), pp. 381-384.

Pournaras S, Maniati M, Spanakis N, Ikonomidis A, Tassios P.T. Tsakris A. , Legakis N.J. and Maniatis A.N. (2005) 'Spread of efflux pump-overexpressing, non-metallo-β-lactamase-producing, meropenem-resistant but ceftazidime-susceptible Pseudomonas aeruginosa in a region with bla_{VIM} endemicity ' *Journal of Antimicrobial Chemotherapy*, 56, (4), pp. 761-764.

Projan, S.J. (2003) 'Why is big Pharma getting out of antibacterial drug discovery?' *Current Opinion in Microbiology,* 6 (5), pp. 427–430.

Pyrzynska K. and Biesaga M. (2009) 'Analysis of phenolic acids and flavonoids in honey', *Trends in Analytical Chemistry*, 28 (7), pp. 893-902.

Quality Assurance International (2013) 'Organic Honey Standards An example from Quality Assurance International'. *Honey Traveller.* Available at http://www.honeytraveler.com/types-of-honey/organic-honey/example-honey-quality-standard/

Ranjan T.V., Tennen H., Lindquist R.L., Cohen L. and Clive J (2002) 'Effect of ingestion of honey on symptoms of rhinoconjuctivitis', *Annals of Asthma, Allergy and Immunology,* 88 (2), pp 198-203.

Reddish G.F. (1929) 'Methods of testing antiseptics'. *Journal of Laboratory and Clinical Medicine*. 14, pp. 649-58.

Reid G., McGroarty J.A., Angotti R. and Cook R.L. (1988) '*Lactobacillus* inhibitor production against *Eschericia coli* and coaggregation ability with uropathogens', *Canadian Journal of Microbiology,* 34 (3), pp. 344-51.

Rezić I., Horvat A.J. M., Babić S. and Kaštelan-Macan M. (2005) 'Determination of pesticides in honey by ultrasonic solvent extraction and thin layer chromatography', *Ultrasonics Sonochemistry,* 12 (6), pp. 477-481.

Rojas, J., Ochoa, V., Ocampo, S., & Muñoz, J. (2006). 'Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections'. *BMC Complementary and Alternative Medicine*, *6* (1), pp. 2.

Rhoads, D. D., Wolcott, R. W., Cutting, K. F. and Percival, S. L. (2007) 'Evidence of biofilms in wounds and potential ramifications', *Biofilms: coming of age*, *8*, pp. 131-143.

Rhoads, D. D., Wolcott, R. D., & Percival, S. L. (2008). 'Biofilms in wounds: management strategies', *Journal of Wound Care*, 17(11), pp. 502-508.

Rickard A.H., Gilbert P., High N.J., Kolenbrander P.E. and Handley P.S. (2003) 'Bacterial coaggregation: an integral process in the development of multi-species biofilms', *Trends in Microbiology,* 11 (2), pp. 94-100.

Russell, A.D. (2002a). 'Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria'. *Journal of Applied Microbiology*, 92(S1), pp. 121S–135S.

Russell, A. D. (2010). 'Do Biocides Select for Antibiotic Resistance?' *Journal of Pharmacy and Pharmacology*, *52*(2), pp. 227-233.

Sanford, M. T. (1987) '*Diseases and pests of the honey bee'*. University of Florida Cooperative Extension Service, Institute of Food and Agriculture Sciences, EDIS.

Saradaki- Papakonstadinou M., Andredakis S. Burriel A. and Tsachev I. (2006) 'Determination of tetracycline residues in Greek honey', *Trakia Journal of Sciences*, 4 (1), pp. 33-36.

Schleifer K.H. and Kandler O. (1972) 'Peptidoglycan types of bacterial cell walls and their taxonomic implications' *Bacteriological Reviews,* 36(4), pp. 407–477.

Schultz Gs., Barillo D.J., Mozingo D.W. and Chin G.A. (2004) 'Wound bed preparation and a brief history of time', *International Wound Journal,* 1 (1), pp. 19-32.

Schumacher, H. H. A. (2004) Use of medical honey in patients with chronic venous leg ulcers after split-skin grafting. *Journal of Wound Care*, *13*(10), pp. 451-452.

Sen, C. K., Khanna, S., Venojarvi, M., Trikha, P., Ellison, E. C., Hunt, T. K., & Roy, S. (2002). Copper-induced vascular endothelial growth factor expression and wound healing. *American Journal of Physiology-Heart and Circulatory Physiology*, *282*(5), H1821-H1827.

Shaw, T., Winston, M., Rupp, C. J., Klapper, I., and Stoodley, P. (2004) 'Commonality of Elastic Relaxation Times in Biofilms' Physical Review Letters *93*(9), 098102.

Sibbald R. G., Orsted H., Schultz, G. S., Coutts, P. and Keast, D. (2003). Preparing the wound bed 2003: focus on infection and inflammation. *Ostomy Wound Management*, *49* (11), pp. 24-51.

Sibbald, R. G., Coutts, P., & Woo, K. Y. (2011). Reduction of bacterial burden and pain in chronic wounds using a new polyhexamethylene biguanide antimicrobial foam dressing-clinical trial results. *Advances in Skin & Wound Care*, *24*(2), 78-84.

Signoretto C., Del Mar Lleo` M., Tafi M.C. and Canepari P. (2000) 'Cell Wall Chemical Composition of *Enterococcus faecalis* in the Viable but Nonculturable State' *American Society for Microbiology.* 66 (5), pp. 1953–1959.

Silver, S. (2003). Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS microbiology reviews*, *27*(2-3), 341-353.

Simon A., Sofka K., Wiszniewsky G., Blaser G., Bode U. and Fleischhack G. (2006) 'Wound care with antibacterial honey (Medihoney) in pediatric haematology-oncology', *Support Care Cancer,* 14, pp. 91-97.

Simova, S., Atanassov, A., Shishiniova, M., & Bankova, V. (2012) 'A rapid differentiation between oak honeydew honey and nectar and other honeydew honeys by NMR spectroscopy'. *Food Chemistry*, 134 (3), pp. 1706-1710.

Socransky S.S. and Haffajee A.D. (2002) 'Dental Biofilms: difficult therapeutic targets'. *Periodontology*, 28 (1), pp. 12-55.

The Soil Association (2013) 'Is there such a thing as organic honey?' available at: http://www.soilassociation.org/frequentlyaskedquestions/yourquestion/articleid/2408/is-there-such-a-thing-as-organic-honey Accessed 21.07.2013

Sorenson O.E. Cowland J.B., Theilgaard-monch K., Liu L. and Borregaard N. (2003) 'Wound healing and expression of antimicrobial peptides/ polypeptides in human keratinocytes, a consequence of common growth factors', *Journal of Immunology,* 170 (11), pp. 5583-5589.

SPSS Inc. (2007). SPSS 16.0 Brief Guide.
ISBN-13: 978-0-13-603601-2
ISBN-1-: 0-13-603601-5

Standing Medical Advisory Committee (1998) The path of least resistance. Main report. *London: Department of Health.*
http://www.publications.doh.gov.uk/pub/docs/doh/smacrep.pdf accessed 21/09/07

Stephens J.M., Schlothauer R.C., Morris B.D., Yang D., Fearnley L., Greenwood D.R. and Loomes K.M. (2010) 'Phenolic compounds and methylglyoxal in some New Zealand Manuka and kanuka honeys', *Food Chemistry*, 120 (1), pp. 78-86.

Stokes E.J. (1968) 'Quality control in diagnostic bacteriology'. *Journal of the Royal Society of Medicine,* 61 (5), pp. 457—463.

Stokes, D. J., Kelly, A. F., Gould, S. W.J., Cassar, C. A. and Fielder, M. D. (2008) 'The withdrawal of antimicrobial treatment as a mechanism for defeating resistant microorganisms', *FEMS Immunology & Medical Microbiology*, 53 (3), pp. 300–305.

Subrahmanyan M (1991) 'Topical application of honey in treatment of burns', *British Journal of Surgery,* 78 (4), pp. 497-498.

Subrahmanyan M (1993) 'Honey impregnated gauze versus polyurethane film (OpSite) in the treatment of burns – a prospective randomised study'. *British Journal of British Surgery*, 46 (4), pp. 322-323.
Subrahmanyan M (1994) 'Honey impregnated gauze versus amniotic membrane in the treatment of burns', *Burns,* 20 (4), pp. 331-333.

Subrahmanyan M (1996 a) 'Honey dressing versus boiled potato peel in the treatment of burns', *Burns,* 22 (6), 491-493.

Subrahmanyam, M. (1996) 'Addition of antioxidants and polyethylene glycol 4000 enhances the healing property of honey in burns', *Annals of Burns and Fire Disasters*, *9* (2), pp.93-5.

Subrahmanyan M (1998) 'A prospective and randomised clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine', *Burns: Journal of the International Society for Burn Injuries*, 24 (2), pp. 157-161.

Subrahmanyan M (1999) 'Early tangential excision and skin grafting of moderate burns is superior to honey dressing: a prospective randomised trial', *Burns*, 25 (8), pp. 729-731.

Subrahmanyan M., Sahapure A.G., Nagane N.S., Bhagwat V.R. and Ganu J.V. (2001) 'Effects of topical application of honey on burn wound healing', *Annals of Burns and Fire Disasters,* 26 (1), pp. 25.2-2

Sugawara, E., Nagano, K. and Nikaido, H. (2012), 'Alternative folding pathways of the major porin OprF of *Pseudomonas aeruginosa'*. FEBS, Journal, 279 (6), pp. 910–918.

Tahmaz, L., Erdemir, F., Kibar, Y., Cosar, A. and YalcÝN, O. (2006) 'Fournier's gangrene: Report of thirty-three cases and a review of the literature', *International Journal of Urology*, *13* (7), pp. 960-967.

Thomas S., Humphries J. and Fear-Price M (1998) 'The role of moist wound healing in the management of meningococcal skin lesions', *Journal of Wound Care,* 7 (10), pp. 503-507.

Thorn RM., Greenman J. and Austin A. (2006) 'An *in vitro* study of antimicrobial activity and efficacy of iodine generating hydrogel dressings', *Journal of Wound Care*, 15 (7), pp. 305-310.

Thorn, R. M. S., Austin, A. J., Greenman, J., Wilkins, J. P. G., & Davis, P. J. (2009). In vitro comparison of antimicrobial activity of iodine and silver dressings against biofilms. *Journal Of Wound Care*, *18*(8), 343.

Thwaini A., Khan A., Malik A., Cherian J., Barua J., Shergill I. and Mammen K. (2006) 'Fournier's gangrene and its emergency management', *Postgraduate Medical Journal,* 82 (970), pp. 516-519.

Todar K. (2012) Mechanisms of bacterial pathogenicity. *Online Textbook of Bacteriology.* pp.1-8

Available at: www.textbookofbacteriology.net. Accessed 02.08.2013.

Tonks A., Cooper R.A., Price, A. J., Molan P. C. and Jones, K. P. (2001) 'Stimulation of TNF-α release in monocytes by honey', *Cytokine*, 14 (4), pp. 240-242.

Tonks A.J., Cooper, R.A., Jones K.P., Blair, S. Parton, J. and Tonks, A. (2003) 'Honey stimulates inflammatory cytokine production from monocytes', *Cytokine*, 21 (5), pp. 242-247.

Tonks, A. J., Dudley, E., Porter, N. G., Parton, J., Brazier, J., Smith, E. L., & Tonks, A. (2007). 'A 5.8-kDa component of Manuka honey stimulates immune cells via TLR4'. *Journal of Leukocyte Biology*, 82 (5), pp. 1147-1155.

Truchado P., Ferreres F. and Thomas-Baberan F.A. (2009) 'Liquid chromatography-tandem mass spectrometry reveals the widespread occurrence of flavonoid glycosides in honey and their potential as floral origin markers', *Journal of Chromatography A,* 1216 (43), pp. 7241-7248.

Tsiapara A.V., Jaakkola M., Chinou I., Graikou K., Tolonen T., Virtanen V., Moutsatsou P. (2009) 'Bioactivity of Greek honey extracts on breast cancer (MCF-7), prostate cancer (PC-3) and endometrial cancer (Ishikawa) cells: Profile analysis of extracts', *Food Chemistry,* 116 93), pp. 702-708.

Vandeputte, J., and Van Waeyenberge, P. H. (2007) 'Clinical evaluation of L-Mesitran®-a honey-based wound ointment', Professional *Nursing Today, 11*(2), pp. 26-31.

Vardi A., Barzilay Z., Linder N., Cohen H.,A., Paret G. and Barzilai A. (1998) 'Local application of honey for treatment of neonatal post-operative wound infection', *Acta Paediatrica.,* 87 (4), pp. 429-432.

Vowden K.R. and Vowden P. (1999) 'Wound debridement, Part 2: sharp techniques' *Journal of Wound Care*, 8 (6), pp. 291 – 294.

Vowden, K. and Vowden P. (2003) 'Understanding exudate management and the role of exudate in the healing process'. *British Journal of Community Nursing, 8*(11 (Exudate Supplement 2)), pp. 4-13.

Wahdan H.A.L. (1998) 'Causes of the antimicrobial activity of honey', *Infection,* 26 (1) 26-31

Waters C.M. and Bassler B.L. (2005) 'Quorum sensing: Cell to cell communication in bacteria.' *Annual Reviews. Cell Development and Biol*ogy, 21, pp. 319–46.

Watnick P. and Kolter R. (2000) 'Biofilm, city of microbes', .*Journal of Bacteriology,* 182 (10), pp. 2675-2679.

Werdin, F., Tennenhaus, M., Schaller H. E. and Rennekampff H. O. (2009) 'Evidence-based management strategies for treatment of chronic wounds', *Eplasty,* 9, e19.

Weston R.J., Brocklebank L.K. and Lu Y. (2000) 'Identification and quantitive levels of antibacterial components of some New Zealand honeys', *Food Chemistry,* 70 (4), pp. 427-435.

Wheat, P. F. (2001) 'History and development of antimicrobial susceptibility testing methodology'. *Journal of Antimicrobial Chemotherapy*, 48 (supplement1), pp. 1-4.

White Jr, J. W., Subers, M. H. and Schepartz, A. I. (1963). 'The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system', *Biochimica et Biophysica Acta (BBA)-Specialized Section on Enzymological Subjects*, *73*(1), pp. 57-70.

White RJ, Cutting K. and Kingsley A. (2006) 'Topical antibiotics in the control of wound bioburden', *Ostomy/ Wound Management,* 52 (8), pp. 26-58.

Worawalai W., Rattanangkool E., Vanitcha A., Phuwapraisirisan P. and Wacharasindhu, S. (2012) 'Concise synthesis of (+)-conduritol F and inositol analogues from naturally available (+)- proto-quercitol and their glucosidase inhibitory activity'. *Bioorganic & Medicinal Chemistry Letters*, *22* (4), pp. 1538-1540.

World Health Organization. Expert Committee on Antibiotics. (1961). *Standardization of methods for conducting microbic sensitivity tests. WHO Technical Report Series,* No.10

Xu K.D., McFeters G.A. and Stewart P.S. (2000) 'Biofilm resistance to antimicrobial agents', *Microbiology,* 146 (3), pp. 547-549.

APPENDICES

APPENDIX 1

SECTION TAKEN FROM THE ROYAL MARSDEN FOUNDATION TRUST MRSA SCREENING POLICY*

3.1 Initial Screen

The mandatory MRSA screening care plan is to be completed for all in-patients and out-patients.

'Patients requiring a MRSA screen include:

1. All new patients to the Trust – in-patient or out-patient. This includes all patients who have received out-patient care at another hospital.
2. All patients who are electively admitted to the Trust – medical, surgical or day surgery.
   a. When possible, patients awaiting elective admission should be screened before admission, by their general practitioners or in pre-admission clinics.
   b. If they have been screened but this was more than four weeks before admission, they need to be screened again.
   c. Additional, appropriate swabs should be taken from patients about to undergo surgery, e.g. axillae swabs for those undergoing breast surgery, groin swabs for those undergoing pelvic or lower abdominal surgery.
3. All patients who are an emergency admission, particularly those who have not been screened by the Trust in the last month.
5. All patients who have developed a nosocomial wound infection, even if this is post-discharge.

MRSA screening in the Community

Within the Community setting there is no requirement for routine MRSA screening unless indicated i.e.:

- If infection is suspected
- If requested by an acute hospital prior to a planned admission
- To establish clearance following an MRSA eradication regimen

When patients are discharged from hospital back into the community or care home they may still be undergoing treatment for MRSA and this must be continued as prescribed. Patient's infection status should be clearly documented in their discharge summary.

A routine screen consists of

- **Nasal swab**
- **Swabs of any breaks in skin, including wounds, vascular access device (VAD) and drain exit sites**

- **If catheterised, catheter urine and urethral swab**

3.2 Full Screen

Patients who should have full screen for MRSA include:

1. All patients who have undergone MRSA decontamination. Three consecutive full sets of negative screening specimens are required before a patient is regarded as "cleared" of MRSA colonisation. These should be a minimum of 48 hours apart and preferably separated by a week or more. These patients are still at increased risk of carriage – see points 3, 5 and 6, below.
2. Patients transferred from overseas, other hospitals, nursing and residential homes for admission, acute or elective.
3. Newly admitted patients who are known to have had MRSA in the past either at the Royal Marsden or elsewhere.
4. All patients in the Intensive Care Unit. Specimens should be taken weekly and on discharge.
5. All patients in the paediatric unit who have previously had a positive MRSA result at any time, even if they have subsequently had three or more negative sets of screening specimens. Specimens should be taken weekly and on discharge.
6. Any patient who has previously had a positive MRSA result at any time, even if they have subsequently had three or more negative sets of screening specimens, who is regarded as being at significant risk of infection.

A full screen consists of:

- **Nasal, throat, axilla and groin swabs**
- **Swabs from any breaks in skin, including wounds, VADs and drain exit sites**
- **If catheterised, catheter urine and urethral swab'**

**Excerpt used with kind permission of Rebecca Martirani, Lead Nurse, Infection Prevention and Control, The Royal Marsden Foundation Trust.*

APPENDIX 2
ANTIMICROBIAL PROFILES OF ORGANISMS

Table A2.1 Antimicrobial profile of *E. coli* isolate panel selected for Agar Diffusion 2 (Methods 2.3.9,) and MIC and MBC testing (Methods 2.4 and 2.5)

Isolate	Ampicillin 10µg	Ceftazidime 30µg	Ciprofloxacin 1µg	Tazocin 85µg	Gentamicin 10µg	Cefuroxime 30µg
Ecoli 1	R	S	R	S	R	S
Ecoli 2	R	R	R	R	R	R
Ecoli 3	R	I	R	R	R	R
Ecoli 4	R	I	S	S	R	R
Ecoli 5	R	R	R	S	S	R
Ecoli 6	R	S	R	S	R	S
Ecoli 7	R	I	R	S	R	R
Ecoli 8	R	I	R	S	R	R
Ecoli 9	R	I	S	S	S	R
Ecoli 10	R	I	R	R	S	R
Ecoli NCTC12241	S	S	S	S	S	S
β lactamase producing E.coli NCTC11560	R	I	R		S	R

S= susceptible R= resistant

Table A2.2 Antimicrobial profile of *Pseudomonas* isolate selected for Agar Diffusion 2 (Methods 2.3.9,) and MIC and MBC testing (Methods 2.4 and 2.5)

Isolate	Meropenem 10µg	Amikacin 30µg	Ceftazidime 30µg	Gentamicin 10µg	Ciprofloxacin 1µg	Tazocin 85µg	Colistin 25µg
1	S	S	S	S	S	S	S
2	S	S	S	S	S	S	S
3	S	S	S	S	S	S	S
4	S	S	S	S	S	S	S
5	S	S	S	S	S	S	S
6	S	S	S	S	I	S	S
7	R	S	S	S	S	S	S
8	S	S	S	S	S	S	S
9	R	S	S	R	S	S	S
10	S	S	S	S	I	S	S
P.aeruginosa NCTC 121903	S	S	S	S	S	S	S

S= susceptible R= resistant

Table A2.3 Antimicrobial profiles of clinical *S.aureus* isolates used in Agar Diffusion methods 1 and 2 and MIC /MBC methods (Methods 2.37, 2.39, 2.5 and 2.5)

	Cefoxitin 5µg	Erythromycin 5µg	Penicillin 1µg	Gentamicin 10µg	Vancomycin 10µg	Rifampcicn 2µg	Mupirocin 5µg
Isolate							
MSSA 1	S	S	R	S	S	S	S
MSSA 2	S	S	R	S	S	S	S
MSSA 3	S	S	R	S	S	S	S
MSSA 4	S	S	R	S	S	S	S
MSSA 5	S	R	R	S	S	S	S
MSSA 6	S	S	R	S	S	S	S
MSSA 7	S	S	R	S	S	S	S
MSSA 8	S	S	R	S	S	S	S
MSSA 9	S	S	R	S	S	S	S
MSSA 10	S	S	R	S	S	S	S
MSSA NCTC 6571	S	S	S	S	S	S	S
MRSA 1	R	R	R	S	S	S	S
MRSA 2	R	R	R	S	S	S	S
MRSA 3	R	R	R	R	S	S	S
MRSA 4	R	R	R	S	S	S	S
MRSA 5	R	R	R	S	S	S	S
MRSA 6	R	R	R	R	S	S	R
MRSA 7	R	R	R	S	S	S	S
MRSA 8	R	R	R	S	S	S	S
MRSA 9	R	R	R	S	S	S	S
MRSA 10	R	R	R	S	S	S	S
MRSA NCTC 12493	R	R	R	S	S	S	S

S= susceptible R= resistant

. **A2.4a Antimicrobial profiles expanded panel of *E.coli* isolates used to compare sensitive and resistant isolated and clinical and control strains used in Agar diffusion method 2 (Method 2.39)**

	Ampicillin 25µg	Cefalexin 30µg	Nitrofurantion 200µg	Co-amoxyclav 30µg	Cefpodoxime 10µg	Trimethoprim 2.5µg	Ampicillin 10µg	Tazocin 85µg
Isolate	S	S	S	S	S	S	S	S
Ecoli 1	S	S	S	S	S	S	S	S
Ecoli 2	S	S	S	S	S	S	S	S
Ecoli 3	S	S	S	S	S	S	S	S
Ecoli 4	S	S	S	S	S	S	S	S
Ecoli 5	S	S	S	S	S	S	S	S
Ecoli 6	S	S	S	S	S	S	S	S
Ecoli 7	S	S	S	S	S	S	S	S
Ecoli 8	S	S	S	S	S	S	S	S
Ecoli 9	S	S	S	S	S	S	S	S
Ecoli 10	S	S	S	S	S	S	S	S
Ecoli NCTC12241	S	S	S	S	S	S	S	S
Ecoli 11	R	R	S	S	S	R	R	S
Ecoli 12	R	R	S	S	R	R	R	R
Ecoli 13	R	R	R	R	R	R	R	R
Ecoli 14	R	R	S	S	R	S	R	S
Ecoli 15	R	R	S	S	R	R	R	S
Ecoli 16	R	R	S	S	S	R	R	S
Ecoli 17	R	R	S	R	R	R	R	S
Ecoli 18	R	R	S	S	R	R	R	S
Ecoli 19	R	R	S	S	R	S	R	S
Ecoli 20	R	R	S	R	R	R	R	R

S= susceptible R= resistant

A2.4b Antimicrobial profiles expanded panel of *E.coli* isolates used to compare sensitive and resistant isolated and clinical and control strains used in Agar diffusion method 2 (Method 2.39)

Isolate	Cefuroxime 30µg	Gentamicin 10µg	Ceftazidime 30µg	Ciprofloxacin 1µg	Amikacin 30µg	Tigecillin 15µg	Ertapenem 10µg	Colistin 25µg	Meropenem 10µg	C 3[illegible]µg
Ecoli 1	S	S	S	S	S	S	S	S	S	S
Ecoli 2	S	S	S	S	S	S	S	S	S	S
Ecoli 3	S	S	S	S	S	S	S	S	S	S
Ecoli 4	S	S	S	S	S	S	S	S	S	S
Ecoli 5	S	S	S	S	S	S	S	S	S	S
Ecoli 6	S	S	S	S	S	S	S	S	S	S
Ecoli 7	S	S	S	S	S	S	S	S	S	S
Ecoli 8	S	S	S	S	S	S	S	S	S	S
Ecoli 9	S	S	S	S	S	S	S	S	S	S
Ecoli 10	S	S	S	S	S	S	S	S	S	S
Ecoli NCTC12241	S	S	S	S	S	S	S	S	S	S
Ecoli 11	S	R	S	R	S	S	S	S	S	S
Ecoli 12	S	R	S	R	S	S	S	S	S	S
Ecoli 13	R	R	R	R	S	S	S	S	S	R
Ecoli 14	R	R	R	R	S	S	S	S	S	R
Ecoli 15	R	R	I	S	S	S	S	S	S	R
Ecoli 16	R	S	R	R	S	S	S	S	S	R
Ecoli 17	R	R	S	R	S	S	S	S	S	S
Ecoli 18	R	R	I	R	S	S	S	S	S	R
Ecoli 19	R	R	I	R	S	S	S	S	S	R
Ecoli 20	R	S	S	S	S	S	S	S	S	R
β lactamase positive E.coli NCTC11560	R	S	I	R	S	S	S	S	S	R

S= susceptible R= resistant

A2.5 Antimicrobial profiles expanded panel of *P. aeruginosa* isolates used to compare sensitive and resistant isolated and clinical and control strains used in Agar diffusion method 2 (Method 2.39)

Isolate	Amikacin 30µg	Meropenem 10µg	Tazocin 85µg	Ciprofloxacin 1µg	Gentamicin 10µg	Ceftazidime 30µg	Colistin 25µg
1	S	S	S	S	S	S	S
2	S	S	S	S	S	S	S
3	S	S	S	S	S	S	S
4	S	S	S	S	S	S	S
5	S	S	S	S	S	S	S
6	S	S	S	S	S	S	S
7	S	S	S	S	S	S	S
8	S	S	S	S	S	S	S
9	S	S	S	S	S	S	S
10	S	S	S	S	S	S	S
11	S	R	S	I	S	S	S
12	R	R	S	R	R	R	S
13	S	R	R	R	R	S	S
14	S	R	S	R	R	S	S
15	I	I	S	R	R	R	S
16	S	R	S	I	S	S	S
17	R	R	S	R	R	R	S
18	R	R	R	R	R	R	S
19	S	R	S	I	R	S	S
20	S	R	S	S	R	S	S
P.aeruginosa NCTC 121903	S	S	S	S	S	S	S

S= susceptible R= resistant

A2.6 Antimicrobial profile of Enterococci isolates used in Agar Diffusion methods 1 and 2 and MIC /MBC methods (methods 2.37, 2.39, 2.5 and 2.5)

Isolate	Ampicillin 10µg	Gentamicin 200µg	Teicoplanin 30µg	Tetracycline 10µg	Vancomycin 5µg	Tigecillin 15µg	Linezolid 10µg	Synercid 15µg
VSE 1	S	R	S	R	S			
VSE 2	S	R	S	R	S	S	S	R
VSE 3	S	S	S	R	S	S	S	R
VSE 4	S	S	S	R	S	S	S	R
VSE 5	S	S	S	R	S	S	S	R
VSE 6	S	R	S	R	S	S	S	R
VSE 7	S	R	S	R	S	S	S	R
VSE 8	S	R	S	R	S	S	S	R
VSE 9	R	S	S	R	S	S	S	S
VSE 10	S	S	S	R	S	S	S	R
VSE NCTC 12679	S	S	S	R	S	S	S	R
VRE 1	S	S	R	S		S	S	S
VRE 2	R	S	S	S	R	S	S	S
VRE 3	R	R	R	R	R	S	S	S
VRE 4	R	S	S	S	R	S	S	S
VRE 5	R	S	R	S	R	S	S	S
VRE 6	R	S	S	S	R	S	S	S
VRE 7	R	S	S	S	R	S	S	S
VRE 8	S	S	S	R	R	S	S	R
VRE 9	R	S	S	R	R	S	S	S
VRE 10	R	S	S	S	R	S	S	S
VRE NCTC 13379	R	R	S	S	R	S	S	R

S= susceptible R= resistant

APPENDIX 3

HOMOGENEOUS SUBSETS DETERMINED BY ANOVA AND TUKEYS HSD FOR COMPARISON OF PRODUCT EFFICACY AGAINST MICRO-ORGANISMS USING AGAR DIFFUSION METHODS 1 AND 2.

MSSA versus all products

MSSA Agar Diffusion 1.

Means for groups in homogeneous subsets are displayed

Table A3.1 Homogeneous subsets of antimicrobial activity of products against MSSA Agar diffusion 1

			Subset for alpha = 0.05					
			Zone mm					
	Product	N	1	2	3	4	5	6
Tukey HSD[a]	1	25	6.00					
	2	25	6.00					
	10	25	6.00					
	14	25	6.00					
	13	23	6.30					
	12	25	6.36					
	11	25		11.24				
	9	25			15.68			
	7	25				18.96		
	3	25				19.76	19.76	
	8	25				19.84	19.84	
	4	25					20.40	20.40
	6	25						21.00
	5	25						21.28
	Sig.		.998	1.000	1.000	.287	.791	.287

Products are grouped together where there is no significant differences in antimicrobial efficacy

.

MSSA Agar Diffusion 2

Means for groups in homogeneous subsets are displayed

Table A3.2 Homogeneous subsets of antimicrobial activity of products against MSSA Agar diffusion 2 .

			Subset for alpha = 0.05						
			Zone mm						
	Product	N	1	2	3	4	5	6	7
Tukey HSD[a]	1	10	6.00						
	2	10	6.00						
	12	10	6.00						
	14	10	6.00						
	13	10	7.00	7.00					
	10	10		7.30					
	11	10			15.40				
	9	10				17.10			
	7	10					19.90		
	8	10					20.20	20.20	
	3	10					20.50	20.50	
	5	10						21.30	
	6	10							23.50
	4	10							24.40
	Sig.		.213	1.000	1.000	1.000	.908	.108	.372

Products are grouped together where there is no significant differences in antimicrobial efficacy

MRSA versus all products

MRSA Agar Diffusion 1

Means for groups in homogeneous subsets are displayed.

Table A3.3 Homogeneous subsets of antimicrobial activity of products against MRSA Agar diffusion 1.

			Subset for alpha = 0.05						
			Zone mm						
	product	N	1	2	3	4	5	6	7
Tukey HSD[a]	1	25	6.00						
	2	25	6.00						
	10	25	6.00						
	12	25	6.00						
	14	25	6.00						
	13	25	6.44						
	7	25		9.28					
	11	24			11.71				
	9	25				14.52			
	8	25					18.16		
	4	25					19.12	19.12	
	5	25						19.48	19.48
	6	25							20.48
	3	25							20.72
	Sig.		.996	1.000	1.000	1.000	.337	.999	.051

Products are grouped together where there is no significant differences in antimicrobial efficacy

MRSA Agar Diffusion 2

Means for groups in homogeneous subsets are displayed

Table A3.4 Homogeneous subsets of antimicrobial activity of products against MRSA Agar diffusion 2.

			Subset for alpha = 0.05					
			Zone mm					
	Product	N	1	2	3	4	5	6
Tukey HSD[a]	1	10	6.00					
	2	10	6.00					
	12	10	6.00					
	14	10	6.00					
	10	10		8.00				
	13	10		9.00				
	9	10			15.80			
	11	10			15.90			
	3	10				18.00		
	7	10				18.90	18.90	
	8	10					19.80	
	6	10						21.20
	4	10						21.50
	5	10						21.70
	Sig.		1.000	.334	1.000	.511	.511	.989

Products are grouped together where there is no significant differences in antimicrobial efficacy

***E. coli* versus all products**

***E. coli* Agar diffusion 1**

Means for groups in homogeneous subsets are displayed.

Table A3.5 Homogeneous subsets of antimicrobial activity of products against *E. coli* Agar diffusion 1.

			Subset for alpha = 0.05				
			Zone mm				
	Product	N	1	2	3	4	5
Tukey HSD[a]	1	25	6.00				
	2	25	6.00				
	10	25	6.00				
	12	25	6.00				
	14	25	6.00				
	13	25	6.32				
	11	25	6.60	6.60			
	5	25		8.04	8.04		
	7	25			9.28		
	3	25			9.52		
	9	25				11.28	
	8	25					13.92
	6	25					15.48
	4	25					15.52
	Sig.		.994	.162	.132	1.000	.068

Products are grouped together where there is no significant differences in antimicrobial efficacy

***E. coli* Agar diffusion 2**

Means for groups in homogeneous subsets are displayed.

Table A3.6 Homogeneous subsets of antimicrobial activity of products against *E. coli* Agar diffusion 2 .

			Subset for alpha = 0.05				
			Zone mm				
	product	N	1	2	3	4	5
Tukey HSD[a]	1	10	6.00				
	2	10	6.00				
	10	10	6.00				
	12	10	6.00				
	13	10	6.00				
	14	10	6.00				
	11	10		9.90			
	3	10		10.20			
	5	10		11.30	11.30		
	9	10			13.20		
	7	10				15.70	
	8	10				15.90	
	6	10					18.20
	4	10					18.40
	Sig.		1.000	.677	.194	1.000	1.000

Products are grouped together where there is no significant differences in antimicrobial efficacy

***Pseudomonas aeruginosa* versus all products**

***Pseudomonas aeruginosa* Agar diffusion 1**

Means for groups in homogeneous subsets are displayed

Table A3.7 Homogeneous subsets of antimicrobial activity of products against *P. aeruginosa* Agar diffusion 1.

		Subset for alpha = 0.05			
		Zone mm			
Product	N	1	2	3	4
Tukey HSD[a] 1	25	6.00			
2	25	6.00			
10	25	6.00			
11	25	6.00			
12	25	6.00			
13	25	6.00			
14	25	6.00			
8	25		7.04		
5	25		7.20	7.20	
4	25		7.64	7.64	7.64
3	25			7.96	7.96
7	25				8.04
6	25				8.12
9	25				8.32
Sig.		1.000	.414	.094	.215

Products are grouped together where there is no significant differences in antimicrobial efficacy

***Pseudomonas aeruginosa* Agar diffusion 2**

Means for groups in homogeneous subsets are displayed

Table A3.8 Homogeneous subsets of antimicrobial activity of products against *P. aeruginosa* Agar diffusion 2

		Subset for alpha = 0.05					
		Zone mm					
product	N	1	2	3	4	5	6
1	10	6.00					
2	10	6.00					
10	10	6.00					
12	10	6.00					
13	10	6.00					
14	10	6.00					
5	10	7.10	7.10				
4	10		8.00	8.00			
8	10			8.70	8.70		
7	10			9.10	9.10		
3	10				9.80	9.80	
9	10					10.60	
6	10					11.00	11.00
11	10						12.00
Sig.		.160	.460	.160	.160	.081	.287

Products are grouped together where there is no significant differences in antimicrobial efficacy

VSE versus All Products

VSE Agar diffusion 1

Means for groups in homogeneous subsets are displayed.

Table A3.9 Homogeneous subsets of antimicrobial activity of products against VSE Agar diffusion 1

			Subset for alpha = 0.05					
			Zones mm					
	Product	N	1	2	3	4	5	6
Tukey HSD[a]	1	25	6.00					
	2	25	6.00					
	10	25	6.00					
	11	25	6.00					
	12	25	6.00					
	13	25	6.00					
	14	25	6.00					
	9	25		8.40				
	7	25		9.28	9.28			
	3	25			10.52	10.52		
	8	25				11.08	11.08	
	5	25				11.88	11.88	11.88
	6	25					12.60	12.60
	4	25						12.88
	Sig.		1.000	.842	.324	.189	.078	.685

Products are grouped together where there is no significant differences in antimicrobial efficacy

VSE Agar Diffusion 2

Means for groups in homogeneous subsets are displayed

Table A3.10 Homogeneous subsets of antimicrobial activity of products against VSE Agar diffusion 2.

			Subset for alpha = 0.05			
			Zone mm			
	product	N	1	2	3	4
Tukey HSD[a]	1	10	6.00			
	2	10	6.00			
	10	10	6.00			
	12	10	6.00			
	13	10	6.00			
	14	10	6.00			
	11	10	7.00	7.00		
	3	10		9.70	9.70	
	9	10			11.10	
	7	10			12.00	
	8	10				14.90
	6	10				15.20
	5	9				16.22
	4	10				16.90
	Sig.		.993	.058	.205	.419

Products are grouped together where there is no significant differences in antimicrobial efficacy

VRE versus all products

VRE Agar Diffusion 1

Means for groups in homogeneous subsets are displayed.

Table A3.11 Homogeneous subsets of antimicrobial activity of products against VRE Agar diffusion 1.

	Product	N	Subset for alpha = 0.05			
			Zone mm			
			1	2	3	4
Tukey HSD[a]	1	25	6.00			
	2	25	6.00			
	10	25	6.00			
	11	25	6.00			
	12	25	6.00			
	13	25	6.00			
	14	25	6.00			
	9	24		8.62		
	3	24		9.46	9.46	
	5	23		10.17	10.17	10.17
	8	25			11.16	11.16
	7	24			11.21	11.21
	6	25				11.72
	4	25				12.08
	Sig.		1.000	.426	.229	.126

Products are grouped together where there is no significant differences in antimicrobial efficacy

VRE Agar Diffusion 2

Means for groups in homogeneous subsets are displayed

Table A3.12 Homogeneous subsets of antimicrobial activity of products against VRE Agar diffusion 2.

			Subset for alpha = 0.05		
			Zone mm		
	product	N	1	2	3
Tukey HSD[a]	1	10	6.00		
	2	10	6.00		
	10	10	6.00		
	12	11	6.00		
	13	11	6.00		
	14	11	6.00		
	3	10	6.90		
	6	10	7.00		
	7	10	7.40		
	8	10	8.20	8.20	
	11	10	8.20	8.20	
	9	10		10.70	
	5	10			16.20
	4	10			18.70
	Sig.		.256	.107	.107

Products are grouped together where there is no significant differences in antimicrobial efficacy

APPENDIX 4

GRAPHS COMPARING MEAN MIC OF MANUKA HONEYS TO ANTIBIOTIC SENSITIVE AND RESISTANT CLINICAL ISOLATES

In all of the graphs below the NCTC control strain is isolate number 11.

Staphylococcus aureus

Figure A4.1 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF20+ for sensitive and resistant isolates of *S. aureus*

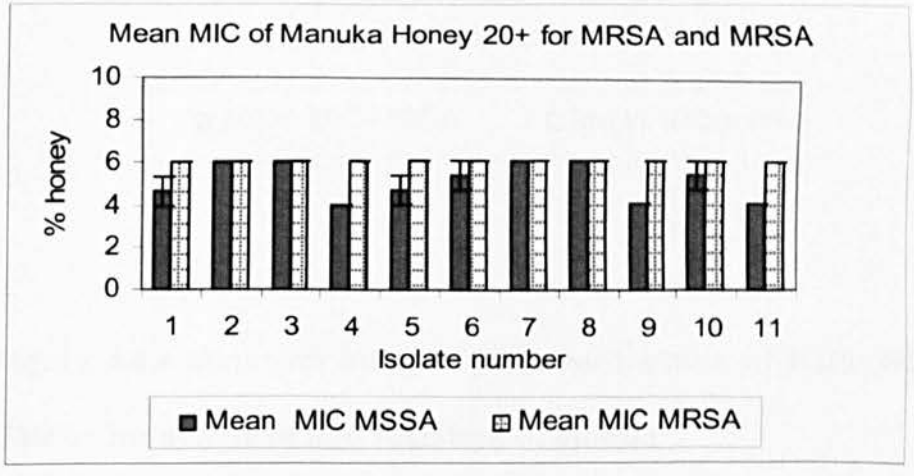


Figure A4.2 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF18+ for sensitive and resistant *S. aureus*

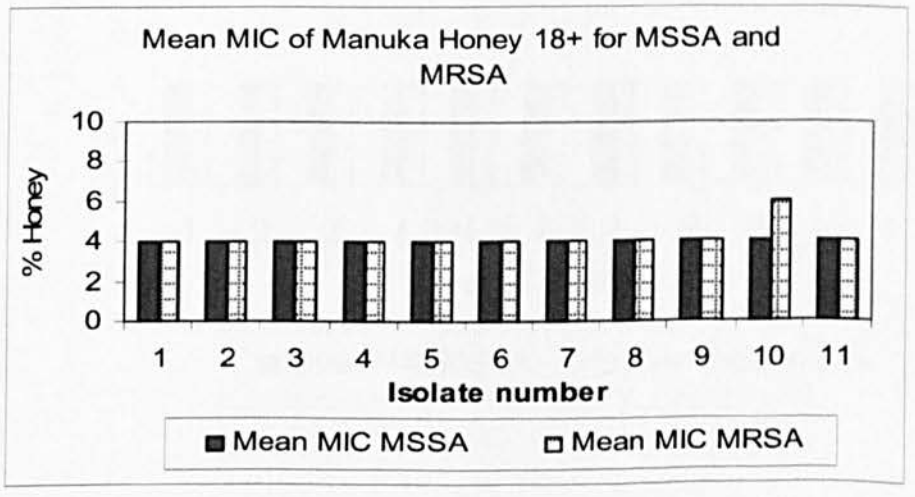


Figure A4.3 Minimum Inhibitory concentrations of Meloderm Manuka Honey UMF16+ for sensitive and resistant *S. aureus*

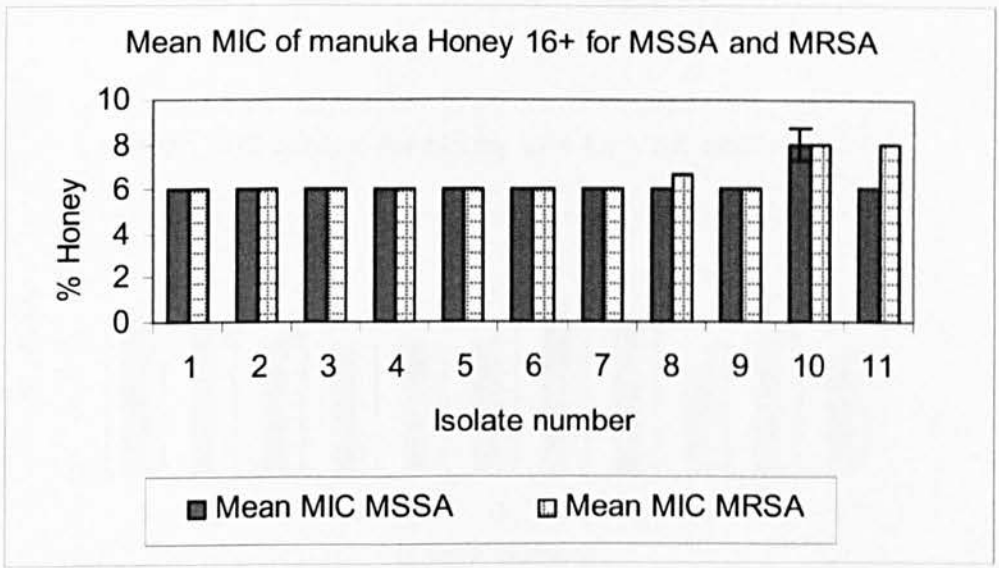
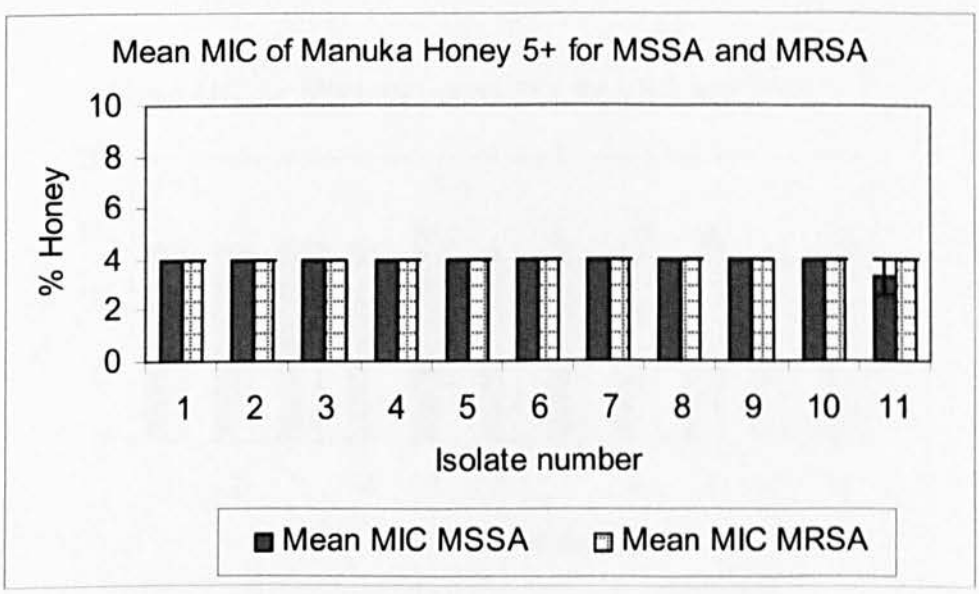


Figure A4.4 Minimum Inhibitory concentrations of Haddrells Manuka Honey UMF5+ for sensitive and resistant *S. aureus*



Enterococcus sp.

Figure A4.5 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF20+ for sensitive and resistant isolates of *Enterococcus* sp.

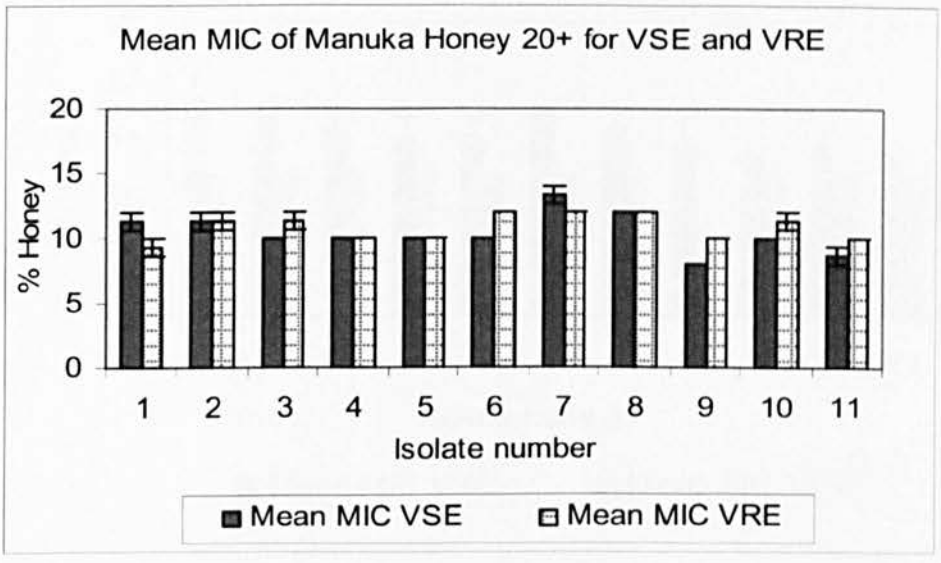


Figure A4.6 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF18+ for sensitive and resistant *Enterococcus* sp.

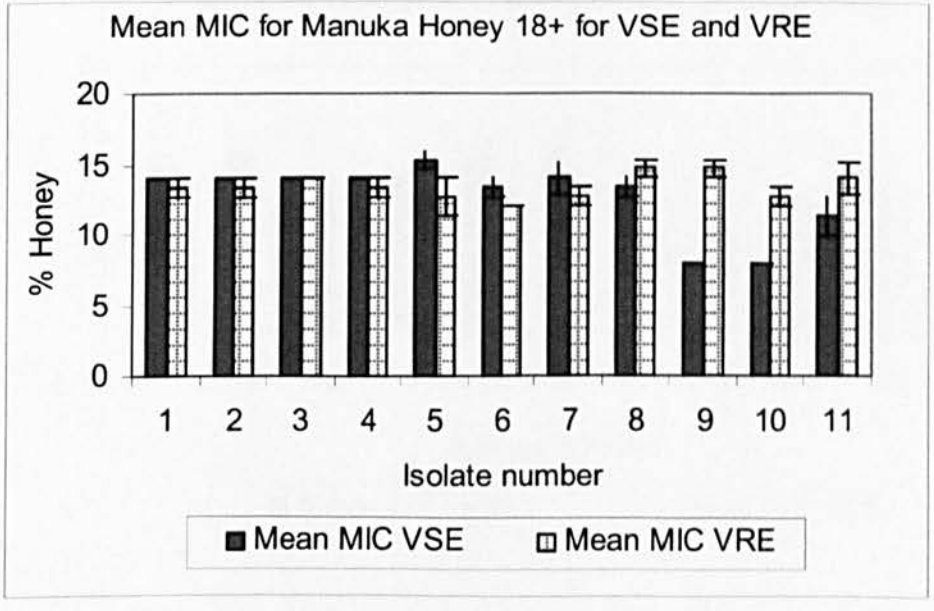


Figure A4.7 Minimum Inhibitory concentrations of Meloderm Manuka Honey UMF16+ for sensitive and resistant *Enterococcus* sp.

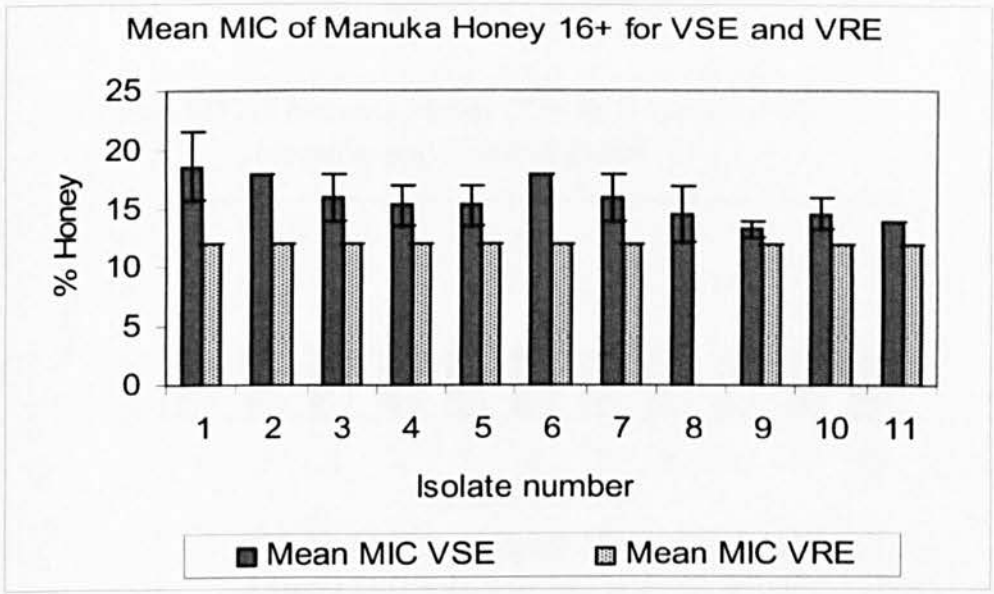
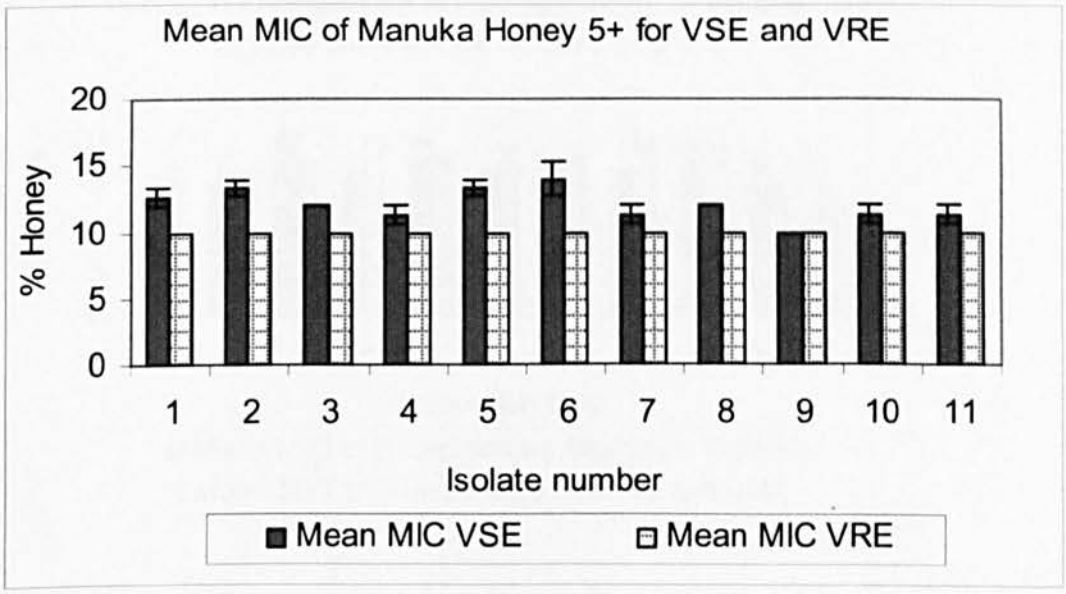


Figure A4.8 Minimum Inhibitory concentrations of Haddrells Manuka Honey UMF5+ for sensitive and resistant *Enterococcus* sp.



Escherichia coli

Figure A4.9 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF20+ for sensitive and resistant isolates of *Escherichia coli*

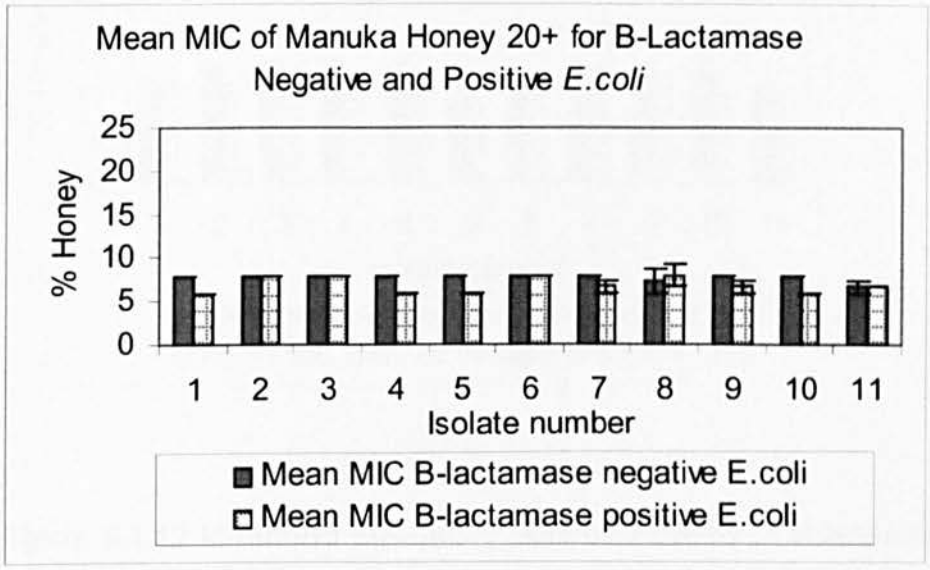


Figure A4.10 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF18+ for sensitive and resistant *Escherichia coli*

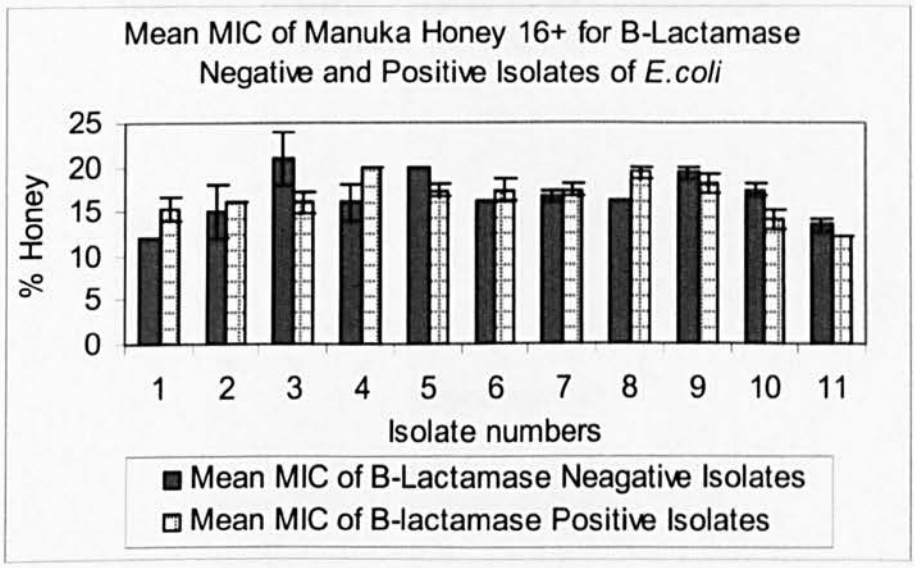


Figure A4.11 Minimum Inhibitory concentrations of Meloderm Manuka Honey UMF16+ for sensitive and resistant *Escherichia coli*

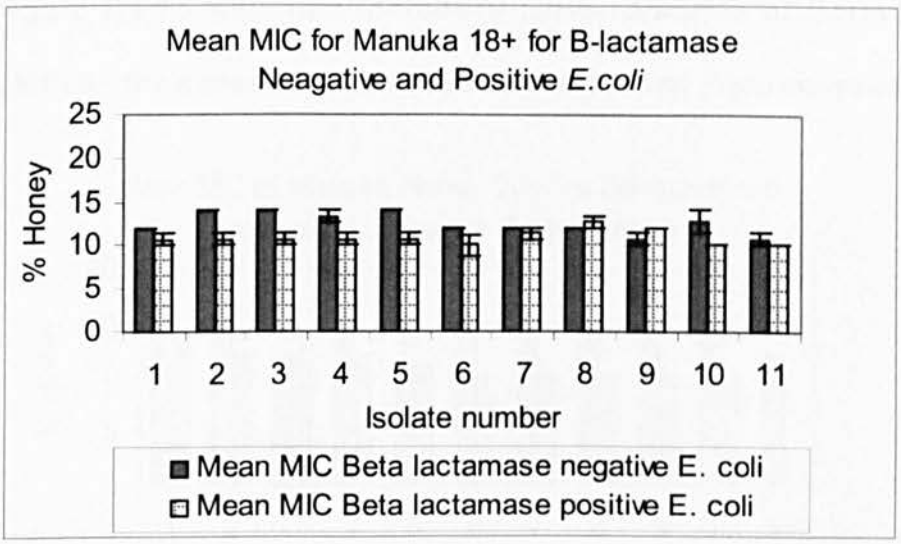
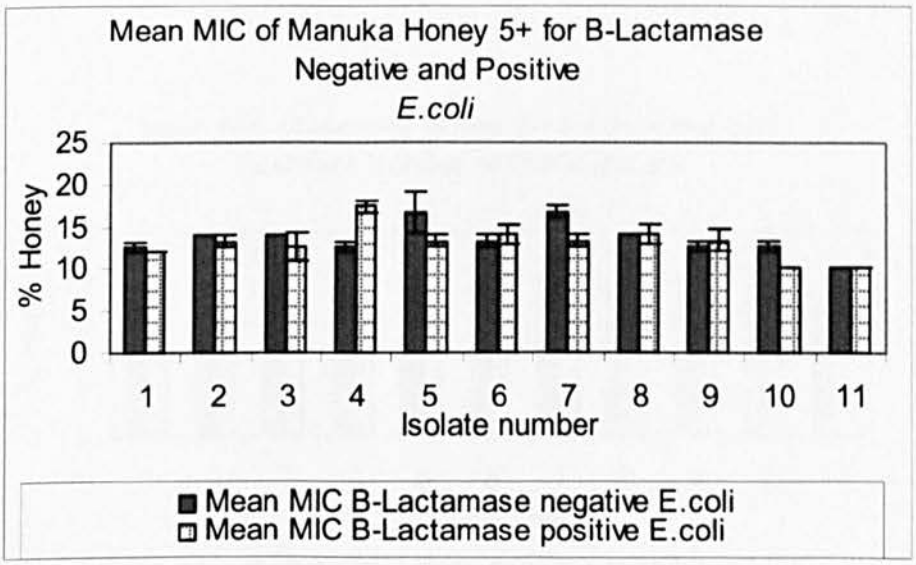


Figure A4.12 Minimum Inhibitory concentrations of Haddrells Manuka Honey UMF5+ for sensitive and resistant *Escherichia coli*



Pseudomonas aeruginosa

Figure A4.13 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF20+ for sensitive and resistant isolates of *Pseudomonas aeruginosa*

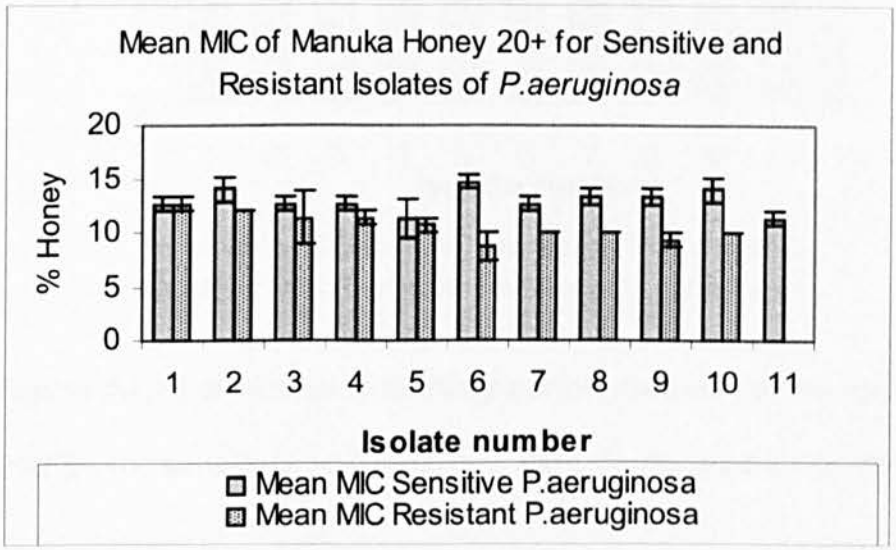


Figure A4.14 Minimum Inhibitory concentrations of Comvita Manuka Honey UMF18+ for sensitive and resistant *Pseudomonas aeruginosa*

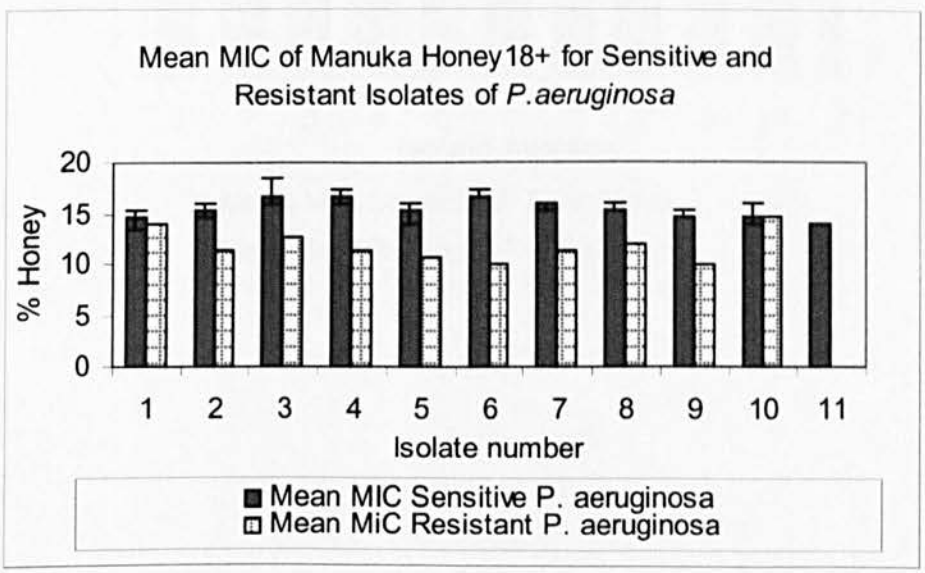


Figure A4.15 Minimum Inhibitory concentrations of Meloderm Manuka Honey UMF16+ for sensitive and resistant *Pseudomonas aeruginosa*

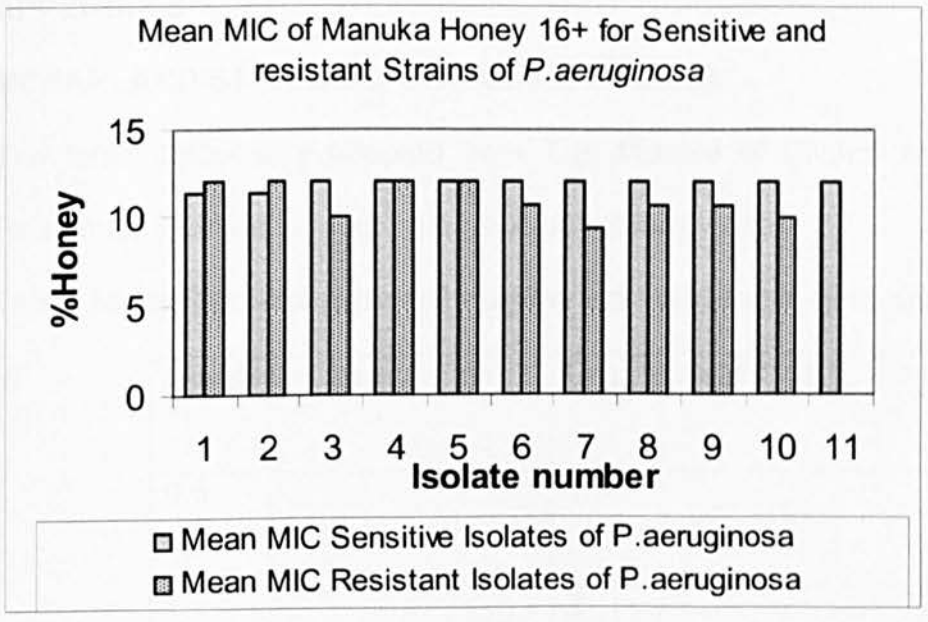
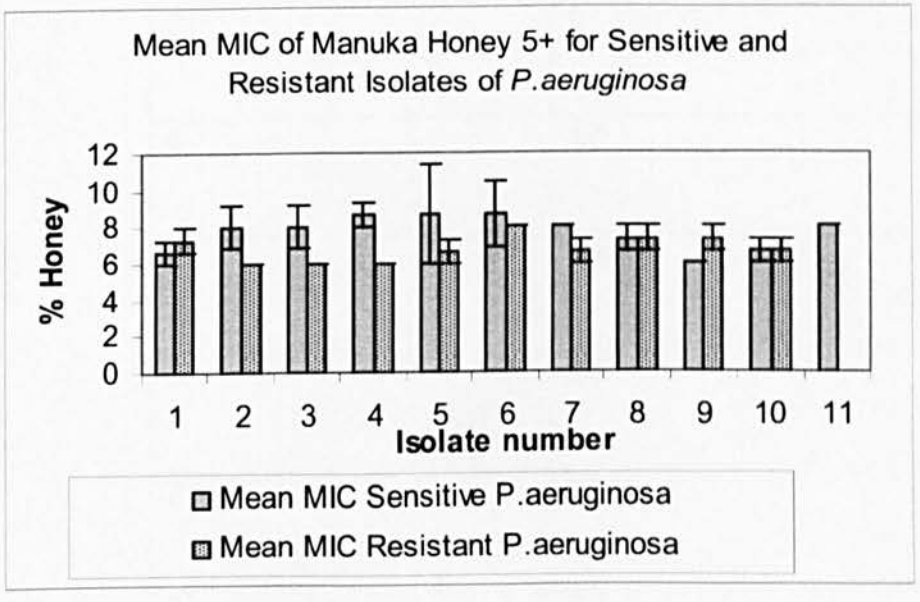


Figure A4.16 Minimum Inhibitory concentrations of Haddrells Manuka Honey UMF5+ for sensitive and resistant *Pseudomonas aeruginosa*



APPENDIX 5

MCFARLAND STANDARD ORGANISM COUNTS

The table below was adapted from The Manual of Clinical Microbiology 2003. Reagents, Stains and Media: Bacteriology (Murray *et al.*)

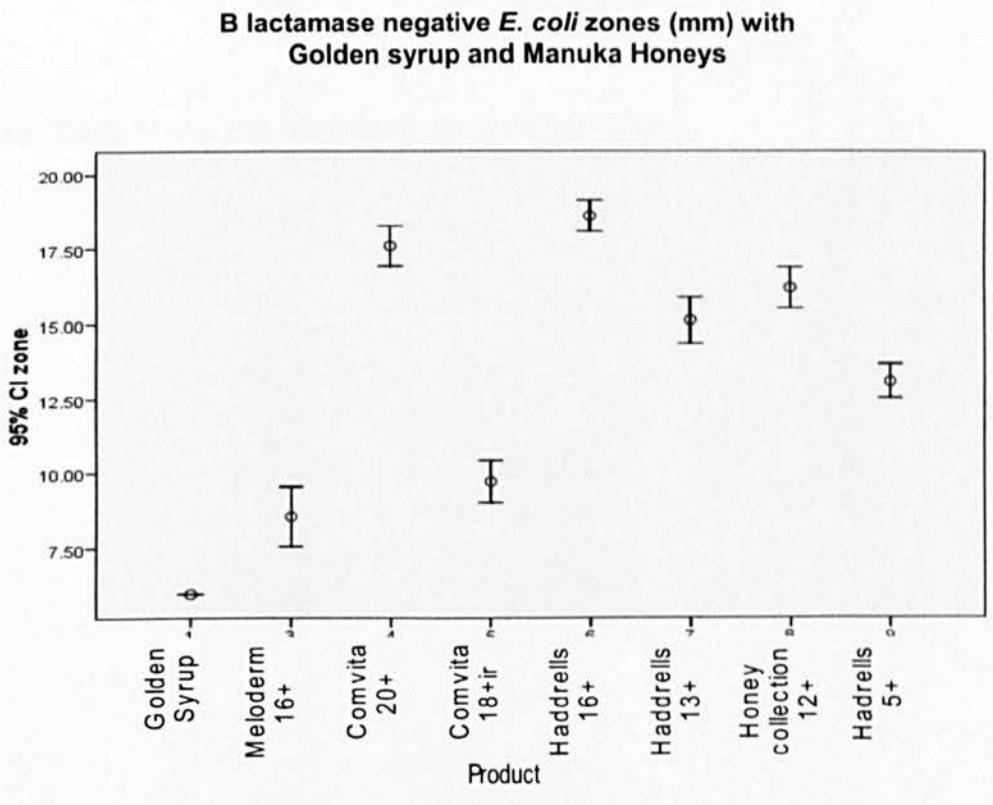
Table A5. McFarland standard number and corresponding cfu /ml

McFarland standard no.	Organisms/ml x 10^8
0.5	1.5
1	3
2	6
3	9
4	12
5	15
6	18
7	21
8	24
9	27
10	30

APPENDIX 6

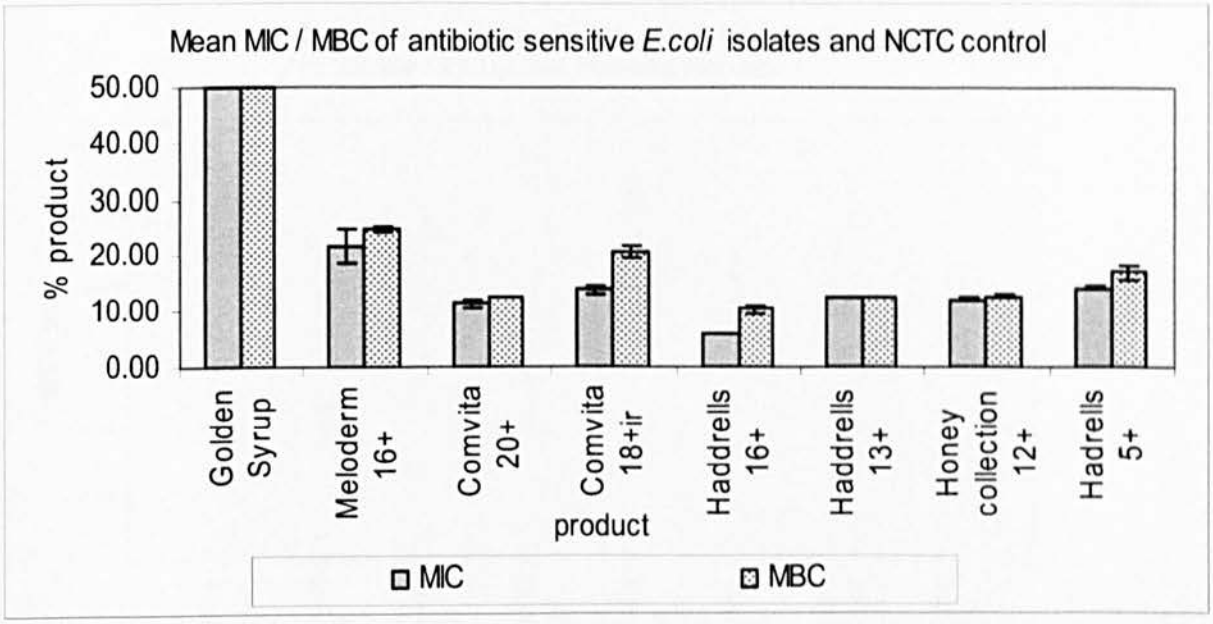
BOX PLOTS AND GRAPHS SHOWING RESULTS OF AGAR DIFFUSION ASSAY AND MIC/MBC ASSAYS OF β-LACTAMSE POSITIVE AND NEGATIVE ISOLATES OF *E.COLI* AND SENSITIVE AND RESISTANT ISOLATES OF *P. AERUGINOSA* ISOLATES WITH GOLDEN SYRUP AND FOUR MANUKA HONEYS

Figure A 6.1 Mean zone sizes produced by Golden Syrup and Manuka honeys with β-lactamase negative *E. coli* isolates



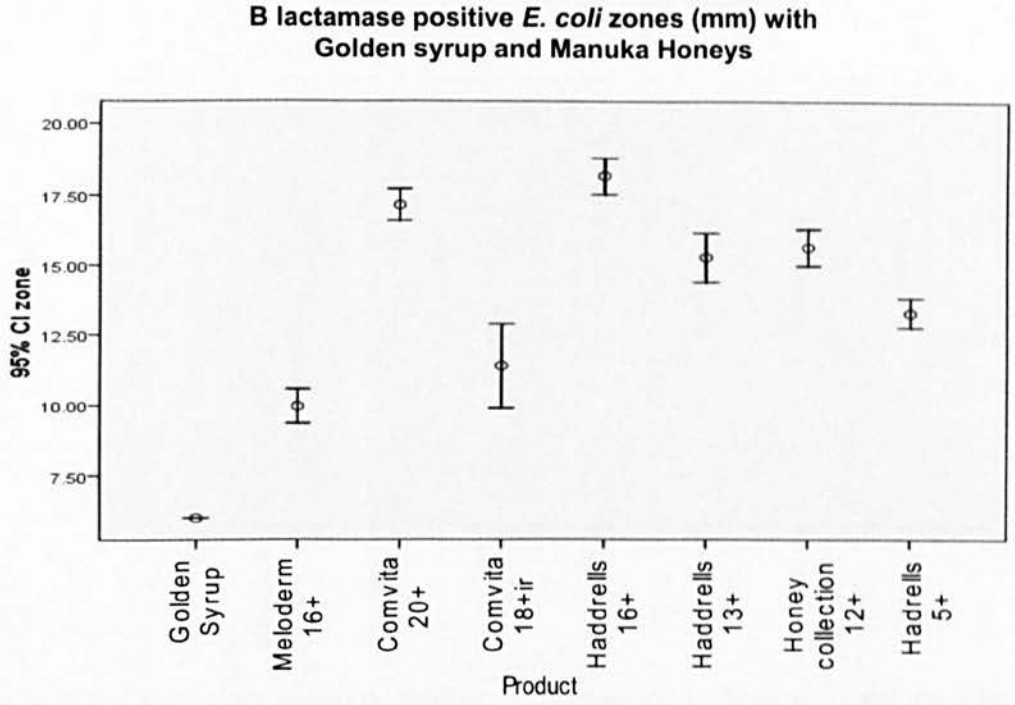
The range of zone sizes achieved with each product are indication by the error bars.

Figure A 6.2 Mean MIC / MBC results of Golden Syrup and Manuka honeys with β-lactamase negative *E. coli* isolates



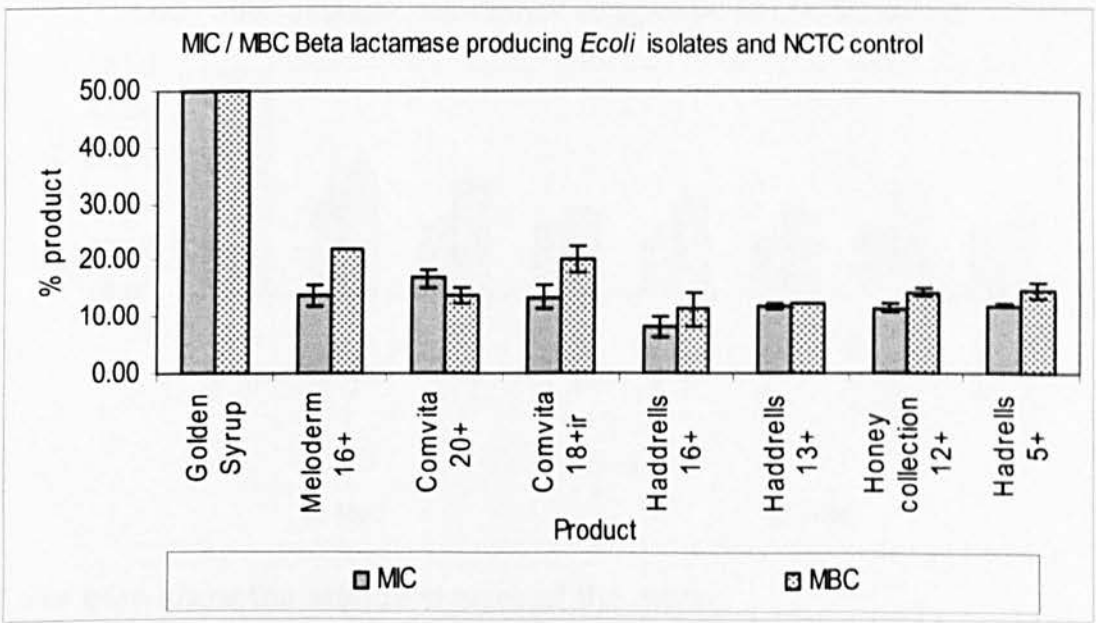
Error bars show the standard error of the mean.

Figure A 6.3 Mean zone sizes produced by Golden Syrup and Manuka honeys with β-lactamase producing *E. coli* isolates



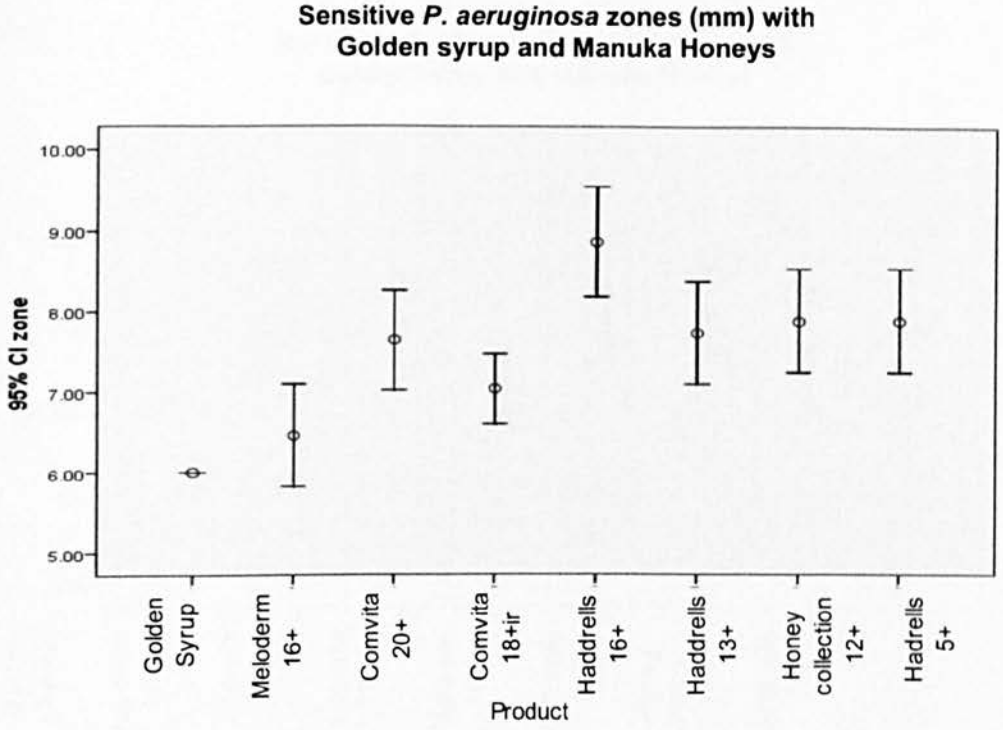
The range of zone sizes achieved with each product are indication by the error bars.

Figure A6.4 Mean MIC / MBC results of Golden Syrup and Manuka honeys with β-lactamase producing *E. coli* isolates



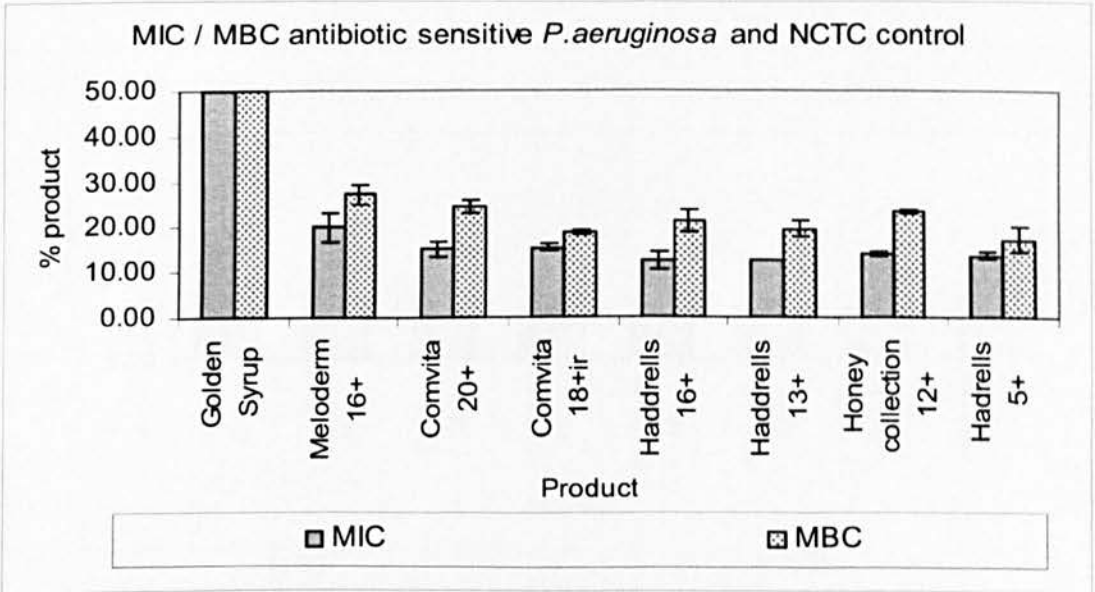
Error bars show the standard error of the mean.

Figure A 6.5 Mean zone sizes produced by Golden Syrup and Manuka honeys with antibiotic sensitive isolates of *P. aeruginosa*



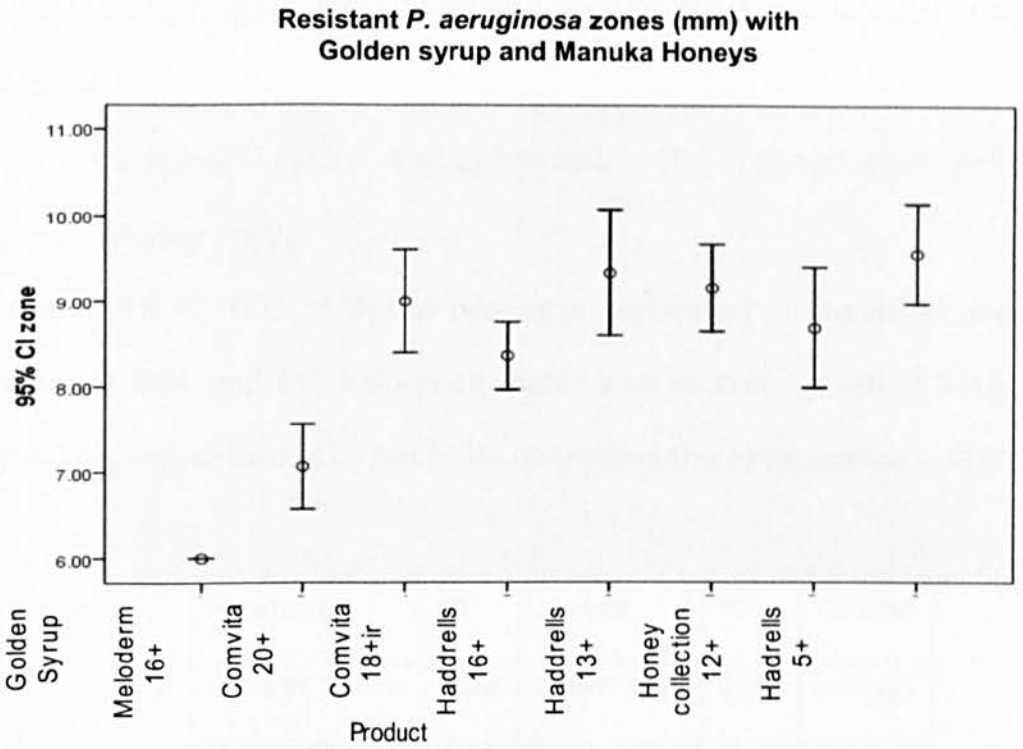
The range of zone sizes achieved with each product are indication by the error bars.

Figure A 6.6 MIC / MBC of Golden Syrup and Manuka honeys with antibiotic sensitive isolates of *P. aeruginosa*



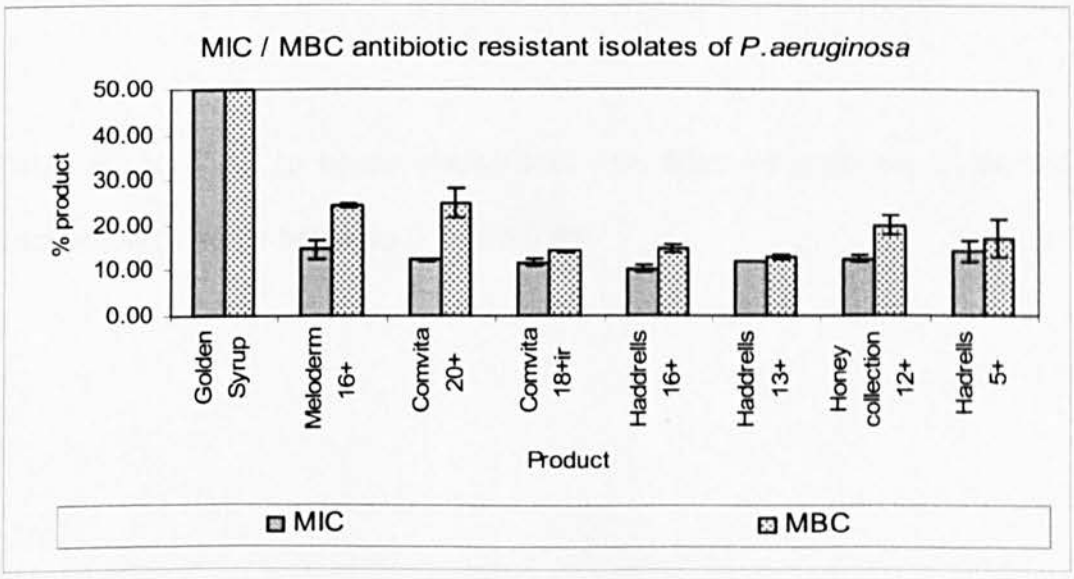
Error bars show the standard error of the mean.

Figure A 6.7 Mean zone sizes produced by Golden Syrup and Manuka honeys with *P. aeruginosa* isolates of increased antibiotic resistance



The range of zone sizes achieved with each product are indication by the error bars.

Figure A 6.8 MIC / MBC of Golden Syrup and Manuka honeys with *P. aeruginosa* isolates of increased antibiotic resistance



Error bars show the standard error of the mean.

APPENDIX 7

RETARDATION FACTORS OBTAINED USING THIN LAYER CHROMATOGRAPHY FOR THE FRACTIONATION OF HONEY AND SYRUP PRODUCTS.

The t ables below show the retatrdation factors (R_f) obtained when performing thin layer chromatography.

Table A7.1a 1D TLC of Syrup products separated using Ethyl acetate 68%, methanol 24% and 8% deionised water and visualised using long wave UV light. The colour stated in the table describes the appearance under UV light.

Product	Rf	colour	Rf	colour
Golden Syrup	0.02	bright blue	0.29	orange
Golden Syrup	0.02	bright blue	_	_
Treacle	0.03	bright blue	_	_
Treacle	0.03	bright blue	_	_

Table A7.1b – A7.1d below shows that only Manuka products contained unique fractions with an Rf between 0.7 and 0.99.

Table A7.1b 1D TLC of Manuka honey products separated using Ethyl acetate 68%, methanol 24% and 8% deionised water and visualised using long wave UV light. The colour stated in the table describes the appearance under UV light

Product	Rf	colour	Rf	colour	Rf	colour			Rf	colour
Meloderm Manuka Honey UMF 16+	0.02	bright blue			0.83	bright green			0.95	faint blue
Meloderm Manuka Honey UMF 16+	0.03	bright blue			0.84	bright green			0.96	faint blue
Comvita Manuka Honey UMF 20+	0.02	bright blue			0.89	bright green			0.99	faint blue
Comvita Manuka Honey UMF 20+	0.03	bright blue			0.85	bright green			0.96	faint blue
Comvita Manuka Honey UMF 18+	0.02	bright blue			0.85	bright green			0.96	faint blue
Comvita Manuka Honey UMF 18+	0.02	bright blue			0.87	bright green			0.95	faint blue
Haddrells Manuka Honey UMF 16+	0.02	bright blue			0.85	bright green			0.96	faint blue
Haddrells Manuka Honey UMF 16+	0.02	bright blue			0.84	bright green			0.97	faint blue
Haddrells Manuka Honey UMF 13+	0.01	bright blue			0.86	bright green			0.96	faint blue
Haddrells Manuka Honey UMF 13+	0.02	bright blue			0.83	bright green			0.96	faint blue
West Coast Manuka Honey UMF 12+	0.02	bright blue			0.83	bright green			0.95	faint blue
West Coast Manuka Honey UMF 12+	0.02	bright blue			0.82	bright green			0.98	faint blue
Haddrells Manuka Honey UMF 5+	0.02	bright blue	0.75	bright green	0.81	bright green	0.9	faint blue	0.97	faint blue
Haddrells Manuka Honey UMF 5+	0.02	bright blue	0.78	bright green	0.83	bright green	0.9	faint blue	0.92	faint blue

Table A7.1c 1D TLC of non Manuka honey products separated using Ethyl acetate 68%, methanol 24% and 8% deionised water and visualised using long wave UV light. The colour stated in the table describes the appearance under UV light

Product	Rf	colour	Rf	colour	Rf	colour			Rf	colour
Lavender Honey	0.02	bright blue								
Lavender Honey	0.02	bright blue								
Eucalyptus Honey	0.02	bright blue								
Eucalyptus Honey	0.02	bright blue								
Acacia Honey	0.02	bright blue								
Acacia Honey	0.02	bright blue								
Blossom Honey	0.02	bright blue								
Blossom Honey	0.02	bright blue								
Basic Honey	0.02	bright blue								
Basic Honey	0.02	bright blue								

As a result of the 1 D TLC only the Manuka honeys and two non Manukas, Eucalyptus honey (K) and the Blossom honey (M) were tested using 2D TLC as these were shown to have the greater antimicrobial activity than the other non Manuka products.

The tables below show the fractions obtained by the first and second phases of the 2D TLC.

Table A7.2a First dimension results of 2D TLC using Ethyl acetate 68%, methanol 24% and 8% deionised water and visualised using long wave UV light

Product	Rf	colour	Rf	colour	Rf	colour
Meloderm Manuka Honey UMF 16+			0.8	bright green	0.99	bright blue
Comvita Manuka Honey UMF 20+			0.8	bright green	0.97	bright blue
Comvita Manuka Honey UMF 18+			0.83	bright green	0.98	bright blue
Haddrells Manuka Honey UMF 16+	0.07	bright green	0.84	bright green	0.98	bright blue
Haddrells Manuka Honey UMF 13+			0.88	bright green	0.95	bright blue
West Coast Manuka Honey UMF 12+	2 x 0.02	bright blue and bright green	0.95	blue	0.9	bright green
Haddrells Manuka Honey UMF 5+	3 x 0.30	2 x bright blue 1 x bright green	2 x 0.80	both green	0.88 0.95	bright green bright blue
Eucalyptus Honey					0.98	bright blue
Blossom Honey						

Table A7.2b Second dimension results of 2D TLC using Ethyl acetate 68%, methanol 24% and 8% deionised water and visualised using long wave UV light

Product	Rf	colour	Rf	colour	Rf	colour	Rf	colour
Meloderm Manuka Honey UMF 16+			0.99	bright green	0.99	bright blue		
Comvita Manuka Honey UMF 20+			0.85	bright green	0.98	bright blue		
Comvita Manuka Honey UMF 18+			0.8	bright green	0.99	bright blue		
Haddrells Manuka Honey UMF 16+	0.79	bright green	0.82	bright green	0.99	bright blue		
Haddrells Manuka Honey UMF 13+			0.91	bright green	0.97	bright blue		
West Coast Manuka Honey UMF 12+	0.78	b. blue	0.95	faint green	0.92	bright blue	0.86	bright green
Haddrells Manuka Honey UMF 5+	0.77 0.84 0.92	bright blue bright green bright blue	0.86 0.88	green green	0.91 0.97	bright green blue		
Eucalyptus Honey					0.97	bright blue		
Blossom Honey								