THE ANTIBACTERIAL ACTIVITY OF HONEY

Thesis submitted in candidature for the degree of

DOCTOR OF PHILOSOPHY

by

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Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Statement 1

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I would like to dedicate this thesis to my grandfathers, Adelino Frutuoso and Francisco Henriques, who I wish could be here today to see me fulfill my dream.
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A Henriques, N F Burton, and RA Cooper “The physiological effects of manuka honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa*” *In preparation*
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Honey is an old remedy recently rediscovered as a possible alternative to modern antibiotics in wound management but its mode of action is not fully understood. The antibacterial activity of honey can be divided into hydrogen peroxide and non-hydrogen peroxide-derived activity. This later type of activity is characteristic of honeys from Australasia (e.g. manuka honey) and preferred for wound management, although historically local honeys have been used. The main aim of this study was to investigate the mechanisms of antibacterial action of manuka honey. The stability of antibacterial action of manuka honey under different conditions was determined, it was observed that manuka honey lost its antibacterial activity when pH was increased and that it remained the same with heating. Storage seemed to increase the potency of manuka honey. The effects of honey on Staph. aureus and Pseud. aeruginosa, were investigated using MIC/MBC determinations, time-kill studies, commitment to death, resistance training, electron microscopy, effects on respiration rates, leakage of intracellular material, and for Staph. aureus the proteome of treated and non-treated cells were compared. It was observed that the effect of manuka honey on Gram positive and Gram-negative cells is different. Gram-positive bacteria had a lower MIC than Gram-negative bacteria, but the time-kill experiments and the commitment-to-death showed that Gram negative were inhibited more rapidly. Clinical strains of both bacteria showed different time-kill profiles to type strains. The methodology used for MIC determination was found to affect to the results obtained. No honey-resistant Gram-positive bacteria were recovered, but Gram-negative bacteria were found to be able to become phenotypically resistant to manuka honey. Electron microscopy showed that honey inflicted physical damages in both types of cells, and in Gram-positive bacteria led to an increase in the proportion of population of cells with a complete septum. Gram-positive cells incubated in honey increased their endogenous respiration rate whilst this was decreased in Gram-negative, major leakage was observed in Gram-negative bacteria whilst only minor leakage was observed in Gram-positive bacteria, which is consistent with the amount of damage observed with electron microscopy. The proteome analysis of Staph aureus, revealed a general down regulation of protein synthesis. Thirty Portuguese honeys were assayed for their antibacterial activity and honeys derived from Lavandula stoechas (lavender) were found to possess non-peroxide activity. A selection of manuka honeys was screened for antimicrobial producing bacteria. In total 106 bacteria were recovered (85% were identified as Bacillus sp.) and of those, 76 were capable of inhibiting the growth of at least one strain of bacteria tested, meaning that some of the antibacterial activity in manuka honey could be due to the presence of antimicrobial agents of bacterial origin. The antibacterial activity of manuka honey has previously been claimed to be due to hydrogen peroxide production and not to a non-peroxide source of activity. A study of free radical production and antioxidant potential demonstrated that manuka honey did not produce any hydroxyl radicals via the Fenton reaction. Thus hydrogen peroxide could not be present. It was also observed that even free radical-producing honeys were able to quench radical production in vitro. In conclusion this study has demonstrated that the non-peroxide activity of manuka honey is not exclusive to Australasia honeys, that it is not derived from hydrogen peroxide generation and may have a microbial origin. Furthermore the action of manuka honey on Gram-negative bacteria seems to be more physical than in Gram positive where it appears to interfere with the cell physiology, perhaps by stopping the cell cycle before cytokinesis.
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List of abbreviations

ATP: Adenosine Tri-Phosphate

BSAC: British Society of Antimicrobial Chemotherapy

CCCP: Carbonyl Cyanide m-Chlorophenylhydrazone

CLSI: Clinical and Laboratory Standards Institute

DMPO: 5,5-Dimethyl-1-Pyrroline-N-Oxide

DNA: Deoxyribonucleic Acid

EARSS: European Antimicrobial Resistance Surveillance System

EDTA: Ethylenediaminetetraacetic acid

EPR: Electron Paramagnetic Resonance

FACS: Fluorescence-activated cell sorter

HMF: Hydroxymethylfurfural

IBRA: International Bee Research Association

IEF: Isoelectric focusing

MBC: Minimum Bactericidal Concentration

MIC: Minimum Inhibitory Concentration

MOPS: 3-(N-Morpholino)-propanesulfonic Acid

RLU: Relative light units

SDS-PAGE: Sodium Dodecyl (lauryl) Sulfate-Polyacrylamide Gel Electrophoresis

SEM: Scanning Electron Microscopy

TEM: Transmission Electron Microscopy

TNFα: Tumor necrosis factor alpha

TVC: Total Viable Count

VBNCl: Viable but non culturable count
I Introduction
1. Honey and healing

1.1 History of the use of honey in Medicine

Today honey is mainly known for its sweetening capacity and as a desirable natural food product, however it has not always been so. In ancient communities it was regarded as an important medical treatment for all kinds of health problems (Molan 1992; Zaghloul et al. 2001).

Honey and other bee products have been important to Mankind for many millennia, where it has been used by man either for food, or for medical purposes. Consequently bees gained a near sacred place in some religions. It is difficult to be accurate about when the relation between Man and bee first started, but since Man started to express himself through cave paintings, bees are pictured and the act of honey hunting depicted (Figure 1). The Bible, the Hebraic religion, the Koran and the Hindu sacred texts refer to honey as the life nectar, and the bees that make it are sacred and must be protected (Apimondia 2001; Crane 2001).

Figure 1. Honey hunters (Crane 2001), reproduced here with the permission from International Bee Research Association.
In the Hebrew religion the Promised Land is described as “a country, which abounds in olive oils and in honey”, while in the Koran it states that “… your Lord inspired the bee (…) it brings out from its entrails a liquid of various colours, where remedy for Man is (…)”. The Christian Bible contains at least 21 references to honey or bees. In the Hindu religion there is the belief that life fed on honey and milk can be extended by 500 years (Apimondia 2001)

1.1.2 Egyptians/ Sumerians

For many centuries, honey was the only sweetening agent available in Europe and although most people regard honey as merely a food product and think of its medicinal application as a relatively new concept, the reality is that references to its uses in Medicine began at least 4000 years ago (Jones 2001).

The first written records referring to the use of honey in medicine date back to 2000 B.C to Sumerian clay tablets where there was a description of the use of honey as an ingredient for ointments to be used in wound infections (Jones 2001). References to honey’s healing properties and its use in Medicine are found in Old Egyptian papyrus, the best known being the Smith Papyrus, found in Ebers, that dates to 1800 B.C, a document describing diagnosis and treatment procedures for several ailments (Molan 1992). Honey used to be added to other ingredients, like olive oil, animal fat, herbs and essential oils, or used on its own for the treatment of many different types of ailments that ranged from stomach problems, infected wounds, ophthalmologic problems to energy tonic and beauty ingredient, among many others (Figure 2).
Honey and the honeybees were of such importance to the ancient Egyptians that one of the symbols of the kingdom of Lower Egypt was a honeybee, signifying the importance that this society gave to this animal (Ransome 1986).

Figure 2. Bee-keeping in Ancient Egypt. Relief shows cylindrical hives made of clay. (Tomb of Pabasa) in Hamad Medical Corporation website (www.hmc.org.qa).

Its use is also mentioned in ancient Babylonian texts for rubbing on skin, ophthalmic remedies, illnesses of the ear and in gynaecology. Its use has also been described in African tribes as a laxative, to help lower fever, as a remedy against snakebites, and to combat inflammation and infections. Furthermore, the Masai tribe warriors recognised its powers to increase strength, most likely due to its sugar content (Apimondia 2001).

1.1.3 Greeks and Romans

References to the use of honey are also found in literature dating back to ancient Greece and the Roman Empire (Zumla and Lulat 1989).
Beekeeping dates back to at least 3400 B.C. in the Greek civilization, with archaeological remains of hives discovered in Phaistos. Several well-known physicians and philosophers like Democrit of Abderos, Zenos and Pythagoras claimed that their longevity was due to their consumption of honey and honey drinks, a fact that is also claimed nowadays when in a Russian census the majority of Russia’s oldest people were found to be beekeepers that consumed products from the beehive on a regular basis (Norris 1970). Furthermore, Hippocrates, today considered the father of Modern Medicine, recommended it for the treatment of a number of conditions, including stomach and ophthalmologic problems. Honey was also one of the ingredients of the food of the Gods (ambrosia) and was the preferred energy tonic of the athletes that competed in the Olympic games (Apimondia 2001).

In the Roman Empire, beekeeping was continued as done in Ancient Greece, with records of it being described by Virgil and by Pliny the Elder. Again honey was recommended for the treatment of wounds, but as a topical agent in conjunction with fish liver oil (Apimondia 2001). It also remained a revered product for use in religious ceremonies (Apimondia 2001).

1.1.4 Middle Ages and Renaissance

All of these uses of honey continued through the Middle Ages (Figure 3), not only in human medicine, but also in veterinary medicine to treat wounds in farm animals (Jones 2001; Clewlow 2004).
Paulus Aegineta (607-690) described in his work “Epitome” (summarized medical knowledge after Galen) the use of cooked honey as an astringent, and raw honey was used to clean wounds (Forrest 1982). Ambroise Paré (1510-1590) a French surgeon to four successive kings and a famous military surgeon described several different treatments for wounds, among which he advised the use of a dressing impregnated in a salve composed among other things of rose honey (Dealey 2004).

1.1.5 Modern Times

Honey was used in this way until the beginning of the 20th century, e.g. in World War I honey was part of the combat gear (Bergman et al. 1983). With the advent of Sir Alexander Fleming’s discovery of the existence of antibiotics, honey was gradually relegated to Folk Medicine, and was no longer seen as suitable for use in modern medicine. The dismissal of honey from mainstream medicine was mainly due to the fact that its application was based on empirical knowledge, people knew that it worked but they did not know how or what were the mechanisms involved in its
effectiveness (Molan 2001b). Nurses that underwent training in British hospitals in the 1970s still used honey regularly for its debriding action rather than as an antimicrobial agent (Robson 2005).

The first record of antibacterial activity in honey dates back to Van Ketel in 1892 (Dustman 1978) and research into the characteristics continued intermittently during the 20th century. However it was not until the 1980’s that interest in the healing properties of honey was rekindled and clinical research began again.

1.2 The characteristics of honey

Honey is the product of the honeybee’s processing of the nectar or honeydew from flowering plants. Nectar is a sugar solution produced by the glands of flowers (the nectaries) that has functions in the attraction of insects and birds to visit the flower to allow cross-pollination. Once the honeybee collects the nectar it is transported back to the hive in the bee’s nectar sack where it undergoes several transformations. When the bee arrives in the hive the nectar is transferred to the honey sac of a worker bee that will be responsible for taking it into the honeycombs. Here there will be a series of regurgitations and ingestions, whereby the bee will add some enzymes to the nectar and moisture content is reduced. The honey produced from honeydew (a sugar solution excreted by insects from the order Rhynchota) (Crane 1975) has different characteristics to nectar honey as it already contains enzymes added by the insect.
1.2.2 Chemical characteristics

Honey is a complex substance, made up of at least 181 different substances known at present, (Jones 2001; White, Jr. et al. 1963); with some researchers believing that the number is actually closer to 600 different substances (Bogdanov et al. 2004).

![Average composition of honey (%)]

**Figure 4.** Average chemical composition of honey (% by weight).

Honey is a saturated or supersaturated sugar solution, meaning that it possesses a high concentration of sugar (with approximately 17% water on average) (Fig. 4). There are 15 different sugars in honey. The main sugars present in honey are fructose (an average of 38%), glucose (~31%) and disaccharides like maltose (7.3%) and sucrose (1.3%), and higher sugars (1.5%) (White, Jr. 1979). The level of sucrose can be increased by over-feeding the bees with sugar during spring (Anklam 1998). The presence of fructose and glucose in honey is due to the action of the bee enzyme invertase on the sucrose molecules contained in nectar, producing a ratio between glucose and fructose of 1.2:1 (Anklam 1998). Starch has also been recovered from...
honey, and this is solely a product of the processing of the nectar, as it is not present in the raw nectar.

Honey is also rich in organic acids, with at least 30 different organic acids being recovered from this product, among the most common are gluconic acid, acetic acid, citric acid, lactic acid, succinic acid and formic acid (Mato et al. 2003). These acids are the results of the action of enzymes like glucose oxidase on the sugars present in honey and makeup an average of 0.50% of the honey by weight (Crane 1975; Mato et al. 2003). The organic acids present in honey are believed to contribute to the organoleptic properties such as flavour and colour as well contributing to physical and chemical characteristics such as pH, acidity and electrical conductivity (Mato et al. 2003; Suarez-Luque et al. 2003).

In the 1930s citric and malic acid were thought to be the predominant acids in honey, but in 1960 gluconic acid was shown to be the predominant form. This organic acid is derived from two sources, the action of the enzyme glucose oxidase and the metabolic activity of certain Glucobacter spp bacteria (present in the bee's gut). The concentration of this organic acid depends on the time needed for the manufacture of the honey, strength of the bee colony and the quality of the nectar to be transformed. Other organic acids are either intermediates of the Krebs cycle or products of enzymatic pathways, and as such can differ significantly from honey to honey. Hence variations in the organic acids present in honey, can be used as indicators of deterioration, authenticity and purity (Mato et al. 2003).
Another important characteristic of honey is its low pH, the normal pH ranges between 3-6. This acidity is thought to be caused by the presence of the different organic acids in the honey, and is one of the factors limiting the growth of microorganisms (Ceyhan and Ugur 2001).

Honey possesses a low amount of nitrogen (0.041% w/v) that is part of proteins, enzymes and free amino acids. The amount of nitrogenous compounds present in honey may affect its characteristics; the high protein concentration (2% w/v) in heather honey, for example is responsible for its viscous characteristics. Estimation of protein in honey by the volume of precipitate with tannin has been used in the past to distinguish between honey and artificial blends (White, Jr. and Rudyj 1978). The presence of enzymes in honey is important, as these aid the transformation of the nectar into honey, some of the most common enzymes recovered in honey are invertase, catalase, phosphatase, glucose oxidase and diastase (Crane 1975).

The mineral component of honey is referred to as the ash portion and makes up about 0.1% of all the components in honey. Ash is more abundant in darker honeys and monofloral honeys tend to have lower ash content (Crane 1975). Potassium makes up around half of the total ash content in honey, and other minerals found are calcium, copper, sodium, magnesium, manganese and chlorine salts. Another 30 different mineral complexes may be used to determine floral origin of the honeys as they are characteristic for each plant (Anklam 1998).

Honey contains trace amount of vitamins, flavonoids, antioxidant components and unidentified plant derived elements (phytochemical components) (Sato and Miyata
2000). It also possesses trace amounts of other beehive products like propolis, royal jelly and wax of which the first two are recognised as antimicrobial agents as well (Anklam 1998).

The actual chemical profile varies considerably from honey to honey, depending upon the floral origin of the nectar and even on the year and time of year in which the honey is collected (White, Jr. and Ruddy 1978). The type of bee producing the honey also changes its chemical characteristics (DeMera and Angert 2004), and there are certain chemical markers that are used to determine if the honey is authentic (that is if it has been altered in any way, like the addition of extra sugar or water), like protein, moisture, sugar, and hydroxymethylfurfural (HMF) content (Mateo and Bosch-Reig 1998). HMF is a product of sugar breakdown in honey as a result of heating or storage, so this substance is useful to determine if the honey was heat processed or aged.

Usually honeys used for medical purposes, since the first recordings, tended to be local honeys that were produced mainly from the nectar collected from one predominant flower source. Analysis of the pollen content of honey is mainly used to determine the predominant floral species that the bee has foraged, however as the composition of honeys is further characterised chemically, other markers, like specific proteins or organic acids are being used for honey classification (Ferreres et al. 1996; Mato et al. 2003; White, Jr. and Ruddy 1978). Unifloral honeys are largely derived from a single floral source and useful because their characteristics can be better defined than those of honeys that come from more than one floral source (multifloral do not demonstrate a predominant floral source when the pollen is analysed).
1.2.1 Physical characteristics

Honey is a supersaturated sugar solution with a high osmolarity (Wahdan 1998); it will exert a high osmotic pressure on bacteria because water molecules will be largely bound to sugar molecules, making them unavailable for the growth of most microorganisms (one of the reasons why honey is not easily spoiled) (Ceyhan and Ugur 2001). Hence honey has a broad spectrum antimicrobial activity.

Honey is usually a viscous substance (viscosity ranges from 3.10 poise for alfalfa honey to 4.11 poise for sumac honey), varying with the temperature at which it is measured (Crane 1975; White, Jr. et al. 1963). This is due to its high sugar concentration and protein profile (Apimondia 2001), which gives it properties that make it desirable for use in the topical treatment of infections. It will act as a barrier when applied to wounds (Molan 2001a), protecting the wound from external contamination, as well as limiting the release of microbes.

Other physical characteristics that can help in the identification of the different honey types are colour (colours range from white to almost black), the thermal conductivity (helps distinguish between floral and honeydew honeys) and hygroscopicity (the capacity of each honey to absorb humidity) (Crane 1975).

1.3 Manuka honey, a unique product

Throughout history, physicians have selected certain honeys for medicinal purposes from those locally available. Honeys from different geographical locations and
different floral origins are now known to have different antibacterial activities (Aljabri et al. 2003a; Allen et al. 1991; Ceyhan and Ugur 2001; DeMera and Angert 2004; Molan et al. 1988; Taormina et al. 2001). Manuka honey (from Leptospermum scoparium) is one such product.

Today it is understood that the antibacterial activity of honey is multi-factorial, as it depends on the action of different components present in the honey, and that these vary from honey to honey. High sugar concentration, low water content and low pH prevents the growth of many bacteria, but does not fully account for the total antimicrobial activity seen in some honeys. Additional activity may be due to the formation of hydrogen peroxide by the breakdown of the glucose present in the honey by the action of the enzyme glucose oxidase (present in bees’ guts). This enzyme is introduced into honey by the bee during the nectar processing and converts glucose into gluconic acid and hydrogen peroxide upon dilution of the honey (Bang et al. 2003). When honey is undiluted, little or no hydrogen peroxide production occurs; this seems to be due to the inhibition of the glucose oxidase activity, and has been suggested not to be due to components in the honey but to the low pH, (Bang et al. 2003) or to high substrate concentration. When honey is diluted, levels of hydrogen peroxide released depend on the concentration of catalase in honey (enzyme capable of destroying hydrogen peroxide) and the amount of time, since kinetics of hydrogen peroxide production have been shown to reach a peak and decline with time (Bang et al. 2003).

It had been observed that some honeys possessed an antibacterial compound sensitive to light and heat, and this compound was termed inhibine. This was later identified as
hydrogen peroxide, and the activity derived for the action of this substance on microbial cells was termed hydrogen peroxide-derived activity. Slow release of hydrogen peroxide at low concentration when some honeys are diluted inhibits microorganisms without damaging human cells and tissues (White, Jr. et al. 1963).

A survey of 345 New Zealand honeys (which included manuka honeys from *Leptospermum scoparium*) used an agar diffusion assay for measuring antimicrobial activity in diluted honeys (diluted in water for determination of hydrogen peroxide-derived activity and in catalase solution for non-hydrogen peroxide-derived activity) and comparing them to known phenol standards. Most honeys in this study exhibited some type of antibacterial activity and 38% of manuka samples and 25% of viper's bugloss samples demonstrated non-hydrogen peroxide-derived activity (Allen et al. 1991; Jones 2001). The inference from these observations is that certain honeys contain antimicrobial factors in addition to sugar content, low pH and hydrogen peroxide generation.

It is thought that non-hydrogen peroxide activity may be due to plant derived components such as flavonoids and phenolic compounds, but at the moment no identification has been achieved, although some researchers have been able to define one of its characteristics: it seems to remain in the acidic fraction of the honey and be destroyed by an increase in pH (Bogdanov 1997).

In order for honey to be accepted as an alternative to antibiotics, it is necessary to characterise the components that are responsible for its activity. Although several of the major components of the antibacterial activity of honey are known, like the sugar
concentration, pH and hydrogen peroxide, these do not account for the total antibacterial activity observed in many honeys. Part of the problem with the identification of antibacterial components is the complexity of honey itself and the possible interaction between the different substances that comprise it. Also, each honey type possesses different characteristics due to the differences in composition. At present non-hydrogen peroxide honeys derived from Leptospermum species (such as manuka in New Zealand and jellybush in Australia) are being developed for clinical use (Cooper 2005b; Cooper et al. 1999; Cooper et al. 2002c).

Today, there are already 3 honey-based products on drug tariff in the UK. The first was licensed in March 2004, Activon Tulle®, from Advancis Medical (Nottingham, UK). It is a tulle dressing impregnated with manyka (Leptospermum scoparium) honey. Since the introduction of this product two more have been introduced into the UK: Mesitran® from Medlock Medical (Oldham, UK) which consists of a range of products that contain at least 20% of medical grade honey and Medihoney Wound Gel® from Medihoney (Reading UK). Medihoney contains predominatly Australian jellybush (Leptospermum polygalifolium) honey. Yet within Europe and the world many more honey-based products are being developed for clinical use like HoneySoft® (MediProf), Melmax® (Dermagenics, Netherlands), ApiNate® and Manuka Care 18+® from Comvita (New Zealand). A range of products from Bfactory (Wageningen, Netherlands) which include Revamil® (hydrophilic gel for chronic and infected wounds), Hypogeen® (ointment for superficial wounds), Vetranyl® (Honey based ointment for veterinary use), Honeyfeel® (range of honey based products for dry or irritated skin), Dermasil® (honey based cream for irritation and minor wounds caused by eczema) and Revamil fem® (pure honey product to restore the vaginal pH.
and the natural vagina flora). Besides the products currently available in the UK on
drug tariff it is also possible to buy honey for medical purposes in supermarkets or
over the counter in health food shops in the form of tubes of sterilised honey and raw
honey.

The majority of the medical products based on honey available in the UK use
*Leptospermum* sp. honeys, therefore this thesis concentrates on manuka honey derived
from *Leptospermum scoparium*. This honey has been demonstrated to possess a wide
range of antimicrobial activity, being effective against Gram positive and negative
bacteria (Al-Jabri *et al.* 2005; Al-Waili 2005; Aljadi and Yusoff 2003; Alnaqdy *et al.*
2005; Badawy *et al.* 2004; DeMera and Angert 2004; French *et al.* 2005; Gulati *et al.*
Al Waili and Saloom 1999; Alsomal *et al.* 1994; Cooper and Molan 1999; Cooper *et al.*
1999; Cooper *et al.* 2000; Cooper *et al.* 2002a; Cooper *et al.* 2002c; English *et al.*
2004; McGovern *et al.* 1999; Natarajan *et al.* 2001), yeasts and dermatophytes (Al
Waili 2004a; Brady *et al.* 1996; Al-Waili 2005), viruses (Al Waili 2004b) and
parasites (Zeina *et al.* 1997).

1.4 Honey and the search for new antimicrobial agents

When antibiotics were first discovered, they were regarded as the cure for all
infections. For some time this seemed to be the case, with deaths from infection
drastically being reduced, but early on there were some danger signs with the first
penicillin resistant bacteria, more precisely *Staphylococcus aureus*, being isolated in
1944 by Kirby and by the 1950's penicillin resistance was already common in UK hospitals (Gosbell 2003). In order to fight the emergence of penicillin resistance, semisynthetic penicillins were introduced in the UK, the first being benzylpenicillin followed after by methicillin in 1959 but this was soon followed (1980s) by reports of methicillin resistance (Greenwood 2000; Perez-Roth et al. 2001) and when vancomycin was introduced to everyday practice, as an antibiotic of last resort, reports soon followed about the emergence of resistance to this as well in 1998 (Gosbell 2003; Schmitz et al. 2000). Within one year of the approval of oxazolidinones (a new class of synthetic antimicrobials) by the FDA resistance has emerged, making treatment of *Staphylococcus* infections difficult (Schmitz et al. 2000).

The increasing prevalence of different antibiotic resistant bacteria in both hospital and community, and the decreased rate of discovery/development of new antibiotics (Greenwood 1995), makes the discovery of alternatives treatments necessary to combat resistant bacteria (Dzidic and Bedekovic 2003; Greenwood 1995; Martinez and Baquero 2002).

### 1.4.1 Multiresistant strains or superbugs

“Superbugs” is how the media refers to antibiotic-resistant bacteria. Although it has become a great concern recently to the general public, with media titles like “Superbug hits the healthy” (Le Page 2003) or “Superbug crackdown is launched” (Collignon 2002), the truth is that antibiotic-resisitant bacteria always existed in nature
but that they were discovered soon after the introduction of antibiotics into clinical practice (Gosbell 2003).

Some of the bacterial species with antibiotic resistance causing the greatest concerns are methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-resistant *Pseudomonas aeruginosa*.

There is a natural variance among all communities of living organisms, and this means that in a population of bacteria there will always be those which are more resistant to some antibiotics than others (Felmingham 2002; Normark and Normark 2002); this phenomenon is innate resistance. When bacteria are faced with an antimicrobial agent, the ones that are more resistant tend to survive better than susceptible cells and are able to pass their characteristics onward to their progeny. Hence later populations of bacteria appear to have an increased resistance in comparison to the initial population. The process of natural selection is particularly effective in bacteria, and because they have short generation times, the results can be observed in only relative short time (Ang et al. 2004a).

Another important factor is that bacterial cells have a high spontaneous mutation rate (about $10^{-7}$ per cell division) (Schmitz et al. 2000). This means that they can change their characteristics rapidly, thus providing a greater variation on which natural selection can act, which helps them to survive in ever-changing environmental conditions.
There are also mechanisms through which bacteria can acquire antimicrobial resistance by gene transfer from resistant cells during transduction, transformation and conjugation (Dzidic and Bedekovic 2003).

The indiscriminate use of antimicrobial agents, that is their use for conditions that do not require their prescription, and the lack of compliance with proper treatment regimes, has led to the emergence by natural selection of resistant bacteria in Europe (Fig. 5) and around the world (Schmidt 2004).

![Proportion of MRSA isolates in participating countries in 2004](Fig. 5. Incidence of MRSA in bacteraemias Europe during 2004 (EARSS, downloaded on 18-01-06)).

One of the main problems faced in the treatment of antibiotic resistant bacteria is the fact that some have become multi-resistant, for example vancomycin-resistant \textit{Staphylococcus aureus} (VRSA), so that in addition to being resistant to vancomycin
resistance to methicillin, penicillin, teicoplanin, and others has been acquired (Schmidt 2004). Infections with these organisms are difficult to treat because the majority of the antibiotics available are not effective, leaving limited choice, which can be a problem when the tolerance of the patient has to be considered (Gosbell 2003).

1.4.2 Decrease in new antibiotics

After Sir Alexander Fleming discovered penicillin in 1928, there was a pause until the 1940s when the discovery of new antibiotics was made at a steady pace until the 1960’s. Agents such as streptomycin, tetracyclines, chloramphenicol, vancomycin, erythromycin and cephalosporins were discovered (Schmidt 2004). After this initial burst of new antimicrobial agents, came the idea that the chemical configuration of known antimicrobial agents could be altered in order to make them more effective and more tolerated by the human body. This lead to the introduction of antibiotics like methicillin, oxacillin, and flucloxacillin, which were derived from the penicillin nucleus (Rolinson 1998).

The next step in the discovery of new antimicrobial agents was thought to be the analysis of bacterial genomes to identify possible targets for the design of new antimicrobial agents. This has so far not led to the development of new antibiotics directed at new microbial targets, and finding targets that are suitable for more than one species of bacteria is proving difficult both in time and financial terms (Lowy 2003; Varaldo 2002).
Hence few new antibiotics, like linezolid, have been developed in the past decade. Much of the money and research time that was initially allocated by pharmaceutical companies to the research into new antimicrobial agents has been withdrawn or seriously decreased as it is no longer economically viable (Lowy 2003; Varaldo 2002). The sequencing of bacterial pathogen genomes that heralded a new age of antimicrobial discovery has, as yet, not bore fruit and the amount of money involved in the research of molecular techniques is not proving a cost-effective investment for pharmaceutical companies (Lowy 2003).

The increasing development of resistant strains seems to be fuelled as well by the increase in global travel, urbanization that leads to overcrowding, the use of inappropriate antimicrobial therapies and the use of antibiotics on animal feeds as growth accelerators (Schmidt 2004). All these facts have lead to a decrease in options for the treatment of infections caused by resistant bacteria, and thus an increase in mortality and morbidity. Traditional medicine might offer an alternative source of treatments.

1.5 Wound care problems

Wounds can simply be divided into two categories: acute or chronic. Acute wounds tend to heal with minimal medical intervention, while chronic wounds will not heal within predicted time frames. With an ageing population, especially in developed countries, there is a need to focus on the problems that this kind of population entails (Howell-Jones et al. 2005). One such problem is the increase in the incidence of chronic wounds due to a variety of factors: bad circulation, diabetes and other types of insufficiencies (Collier 2004).
One of the biggest problems in wound care is how to treat wounds infected with antibiotic resistant bacteria in patients that are usually fragile. The options at the moment are the use of antibiotics and radical surgery either for the debridment of the wound or amputation of the affected area. However in patients that have a fragile health state like the elderly these might not be viable options, thus alternatives are required (Eron et al. 2003). However aggressive therapy is not always well received and compliance may be an issue.

Undeveloped countries also face wound management problems as a result of poor living conditions and wars. In some these of countries where health resources are limited, and AIDS prevalence high, mortality due to infected wounds is high and the main medical resources used are those from traditional medicine as the cost of treatments is high and the distribution of drugs is poor (Ryan 2000). This is made more serious by the mobility of bacterial resistance, which has resulted in increased incidence of antibiotic resistance in countries where antibiotic use is not widespread (Schmidt 2004). These two aspects of wound care combined show the need for effective, cheap alternative antimicrobial agents.

1.5.1 Factors influencing wound healing

There are many reasons why a wound might not progress to complete healing within the expected time. Conditions that affect healing times are nutritional status, diabetes, cardiac or respiratory insufficiency, ischaemia, infections, antimicrobial therapy, mobility, hydration, age, underlying illnesses and previous immunosuppressive
therapies, such as chemotherapy and radiotherapy. These factors will all contribute to the way in which the patient's body responds to a wound and will influence its capability for healing (Collier 2004).

Although some may believe that if a wound has bacteria it will not heal, most wounds support polymicrobial communities (Bowler et al. 2001). The actual presence of bacteria may contribute to the stimulation of the immune response for a rapid wound healing (Edward-Jones and Greenwood 2003). Furthermore, nowadays there is an increasing understanding of the difference between colonization and infection. A wound may be colonized but not infected. Infection status will depend on many factors, not only on the microbiological results but also the actual symptoms presented, as there are species of bacteria that can exist in wounds and still allow it to heal (Cooper 2005c).

Cutting and White (2005) have described the symptoms required for the diagnosis of an infection as being: localised erythema, localised pain, localised heat, cellulitis, oedema, abscess, discharge, delayed healing, discoloration, friable and bleeding tissue and bad odour.

To assess whether a wound is infected or not, besides the visual analysis samples are collected either by swabs or by wound biopsy and the bacteria present identified and quantified (Bowler et al. 2001). The interpretation of the microbiological results requires caution, as the species/strains of bacteria need to be identified, numbers, virulence and possible interactions between them need to be determined in order to
assess the need for antimicrobial therapy, and to be able to provide the right type of therapy for the situation at hand (Howell-Jones et al. 2005; O'Meara et al. 2000).

The range of organisms that can be recovered from an infected wound is large and may include any number of the following: beta-haemolytic streptococci (like *Streptococcus pyogenes*), enterococci (like *Enterococcus faecium* or *Enterococcus faecalis*), staphylococci (like *Staphylococcus aureus*, MRSA or *Staphylococcus epidermidis*), Gram negative aerobic (like *Pseudomonas aeruginosa*) and facultative rods (like *Acinetobacter*, *Enterobacter*, *Escherichia coli*, *Klebsiella*, *Proteus* and *Serratia* species). Anaerobes are also found (usually in deeper wounds) as well as yeasts and fungi (Cooper et al. 2002b). This means that in most cases broad-spectrum antimicrobial agents are required to resolve the infection.

Infected wounds tend to have a complex mixed population of microorganisms. This population is capable of forming communities, also known as biofilms, which allow them to thrive in the wound environment. Biofilms are three-dimensional structures of bacteria attached to a surface (in this case a wound bed) and encapsulated in slime that communicate among each other using chemical signals (quorum sensing). This arrangement protects them from phagocytosis and the action of antimicrobial agents, making their eradication more difficult (Cooper 2005c).

1.5.3 Cost

Since the problem with antibiotic resistance first arose, there have been increasing limitations on the materials that can be used for wound management. Alternatives
have consisted of combination therapy whereby more than one type of antibiotic is used so that the therapy has a broader range of action, and can effectively kill the maximum number of microorganisms, but this has increased costs.

Another approach is the use of new types of wound dressing with added antimicrobial agents like silver, or iodine (Cooper 2004; Stewart 2002). This kind of approach has shown some efficacy, nevertheless, as with all new developments these products are expensive and not easily accessible to physicians in less developed countries, and even in the developed countries it requires significant cost to be supported either by the patient or by the health care system.

There is an increase in the costs relating to hospital stays, as patients with chronic wounds or with wounds infected with antibiotic resistant bacteria tend to be hospitalised for longer periods (Cooper et al. 2002c). The application of new approaches to wound healing requires more specialised training and increased nursing time until the wound heals. All of these approaches require substantial financial commitment by the health system which must be balanced against the possible savings resulting from the use of new treatments, which in the case of less developed countries is not always possible.

1.6 Alternative antimicrobial wound management.

Besides honey there are other alternative antimicrobial therapies being used in wound management with varying success like, maggots, bacteriophage, tea tree oil, and antimicrobial peptides, as well as surgical debridment.
Maggots are an ancient form of wound management that besides allowing for the physical debridment of the wounds, by removing the necrotic tissue, are reported to promote wound healing by decreasing the microbial levels (by ingesting bacteria), increasing wound pH, promoting granulation and stimulating human fibroblasts. Nevertheless the acceptance of this therapy is sometimes an issue as some patients feel discomfort (Leach 2004).

Bacteriophages are viruses that specifically target bacteria. Since the emergence of antibiotics the idea that they could be used as antimicrobials was ignored in most places except in Georgia (Carson and Riley 2003). The idea of targeting specific pathogens with their naturally existing “enemies” is appealing as resistance to conventional antimicrobial therapy increases. Nevertheless, there are concerns of the possible development of antiphage antibodies during therapy. The bioavailability of the phages may be limited during therapies as they are quickly cleared from the circulation by the reticuloendothelial system. The emergence of mutant phages is possible, and the fact that for example MRSA seems to be less susceptible to bacteriophage attack than the antibiotic-susceptible S. aureus may restrict application (Carson and Riley 2003).

The use of tea tree oil and other essential oils extracted from plants (aromatherapy) has been recognised for a long time. Tea tree oil has been recognised for its capacity to eliminate MRSA and has been considered an alternative for the treatment of mupirocin-resistant MRSA (Bowler et al. 2001). It has also been demonstrated to have a broad-spectrum of action and its mechanisms of action in vitro has been
elucidated for different types of bacteria like *Escherichia coli, Staphylococcus aureus* and *Candida albicans* (Cox et al. 1998; Cox et al. 2000; Cox et al. 2001; Gustafson et al. 1998; Hada et al. 2003)

Antimicrobial peptides are molecules that occur naturally in many types of cells, including human neutrophils. These molecules attach themselves to bacterial cell walls and form pores inducing leakage of the cell contents and death. These types of molecules are the target of research into compounds that have a wide spectrum of activity and that can be formulated for use in wound management (Bowler et al. 2001).

Surgical debridment is also considered as an option for the elimination of infection from a wound. It consists in the removal of the devitalised and contaminated tissue in the wounds and exposing healthy tissue that can proceed to healing. This can be done using surgery, speciality dressings or maggots (Bowler et al. 2001).

Today conventional antibiotic therapy is no longer the solution for all cases of infection, and this is especially true in the case of wound management. As such alternatives to modern treatment options (antibiotics) may be found in traditional medicine remedies such as honey. It has been used for many millennia as a potent and effective antimicrobial agent and has recently been rediscovered for use in modern medicine. It is a complex chemical and physical substance that is known for its broad-spectrum antimicrobial action and wound healing properties.
Some attempts have been made to analyse the composition of honey in an attempt to identify the most potent antimicrobial components. Little success has been achieved, and to this day there is limited knowledge of the identity of components responsible for antimicrobial activity. Manuka honey is one of the honeys that has had been the object of much of the research in wound management, mainly due to its high antimicrobial activity. As a result many of the recently introduced wound management products, available on drug tariff in the UK are based on this honey.

Although there has been more research into honey in the last 20 years that in the last two centuries, there are still many questions unanswered. Studies often have been conducted using different methodologies, making results difficult to compare; they usually concentrate on the effectiveness of honey, or its spectrum of antimicrobial action and not on the way that it is able to produce such an effect. Furthermore, studies with local honeys are few and once again, they tend to be done with many different methodologies.

This work attempts to fill some of these gaps in our knowledge of the antimicrobial activity of honey. It concentrates on manuka honey, which is the base of most honey-based medical products currently available but includes a search for honeys in Europe with potential for medical use. The possibility of the inhibitory activity of manuka honey being derived from a microbial source, and the relation between its antioxidant potential and antimicrobial activity are examined.

The need to understand the way in which manuka honey inhibits bacteria led to the study of the kinetics of death of common wound infecting bacteria treated with
manuka honey and its capacity for selecting resistant strains in the short-term. In order to better understand its mode of action, studies on the effects of manuka honey on bacterial morphology and physiology were also conducted. Despite the large numbers of studies that report the antimicrobial activity of honey, few have included these aspects.

Therefore the general aims and objectives for each chapter can be summarized as:

Chapter 2. To investigate the antimicrobial activity of selected Portuguese honeys
   1. To collect and characterise physicochemically selected Portuguese honeys
   2. To determine the antimicrobial potential of these Portuguese honeys
   3. To assess the non-hydrogen peroxide-derived antimicrobial activity and relate it to the floral origin.

Chapter 3. To investigate the possibility of the presence of inhibitory components in honey derived from bacterial sources.
   1. To isolate bacteria from a range of manuka and jellybush honeys
   2. To characterise and identify isolates recovered from a range of different honeys
   3. To determine the potential for organisms isolated from honey samples to produce substances that inhibit other bacteria in liquid and solid media assays.
Chapter 4. To study of the antioxidant potential of selected honeys

1. To determine the superoxide quenching capabilities of a range of hydrogen peroxide producing and non-hydrogen peroxide producing honeys
2. To detect the formation of hydroxyl radicals in peroxide and non-peroxide honeys
3. To assess the hydroxyl radical quenching potential of a range of honeys
4. To relate the antimicrobial activity and colour of a range of honeys to their free radical quenching potential.

Chapter 5. To investigate the kinetics of death of *Staphylococcus aureus* and *Pseudomonas aeruginosa* exposed to manuka honey.

1. To characterise the antibacterial activity of a manuka honey in response to storage, pH, temperature and equivalence to penicillin.
2. To determine the Minimum Inhibitory Concentrations of honeys, using a range of media and methods.
3. To determine whether manuka honey possessed bactericidal activity in time-kill studies.
4. To determine if bacterial cells lyse after treatment with manuka honey by performing total cell counts of honey-treated bacterial cultures.
5. To determine whether honey selects for resistant bacteria in short-term.
6. To determine the time for commitment to death of bacteria in the presence of honey.
Chapter 6. To observe the effects of manuka honey on the morphology of bacterial cells (*Staphylococcus aureus* and *Pseudomonas aeruginosa*)

1. To determine changes to the surface morphology of bacterial cells using scanning electron microscopy
2. To determine changes to the interior of bacterial cells using transmission electron microscopy
3. To assess the difference in damage observed between exponential phase cells and stationary phase cells

Chapter 7. To evaluate the physiological effects of honey on bacterial cells (*Staphylococcus aureus* and *Pseudomonas aeruginosa*)

1. To assess the sensitivity of stress response and cytokinesis mutants to manuka honey
2. To determine the effects of honey on the respiration rates of bacteria
3. To assess whether bacterial energy conservation becomes uncoupled from electron transport processes by incubation in honey
4. To investigate the possibility of materials being leaked from bacterial cells incubated with manuka honey.
5. To characterise the changes in protein synthesis of cells incubated in honey.
II Methods and Experiments
2. A survey of the antimicrobial activity of some European honeys
2.1 Introduction

The data presented in this chapter was published in: A Henriques, N F Burton, and RA Cooper "Antibacterial activity of selected Portuguese honeys" Journal of Apicultural Research, 44(3): 119-123, 2005

Honey, an ancient remedy rediscovered during the 1990s, is now being utilised for wound care in Australia (Johnston et al. 2003), New Zealand (Molan and Betts 2003) and in the U.K (Dunford and Hanano 2004; Stephen-Haynes 2004). A range of wound dressings, ointments and sterile products has been developed, but most employ honeys with proven antibacterial activity such as manuka and jellybush, which are produced in Australia and New Zealand. Yet bees have been introduced in Australasia only relatively recently. Because recorded evidence to the use of honey as medicine dates back to at least 4000 years these types of honey could not be the ones being referred to in the Smith papyrus for example.

Evidence from historical documents indicates that ancient people carefully selected honey for medicinal purposes from locally available honeys, for example Ambroise Paré (1510-1590) specifically advocated the use of rose honey for the production of a debriding agent for wounds (Dealey 2004), Dioscorides advised the use of pale yellow honey from Attica for the treatment for “rotten and hollow ulcers”, and Aristoles refers to pale honey as particularly useful for the preparation of salves for “sore eyes and wounds” (Molan 2000). Even today in folk medicine some honeys are of more value than other, like strawberry honey in Sardinia, lotus honey in India (for
the treatment of eye problems) and honey from the Jirdin valley in Yemen for their high therapeutic usefulness (Molan 2000).

It seems probable that most countries should have honeys suitable for use as a medicine, whose selection for medical purposes is possible. This is a common practice in some undeveloped countries, but many developed countries do not utilise their sources.

Previously in UWIC a survey of 139 Welsh honeys was performed for the determination of antibacterial potency and healing stimulation potential (by assaying the release of TNFα from monocytic cell line, Monomac-6). This study demonstrated limited antimicrobial activity, and the activity was found to be hydrogen peroxide-based. The honeys studied did, however, demonstrate a strong potential for stimulation of wound healing (Wheat 2004).

The aim of this study was to quantify the antibacterial activity of 30 Portuguese honeys with specific attention to the non-hydrogen peroxide derived activity, with a view to evaluating their antimicrobial potential for wound care. Wound healing stimulation was not assessed because it was outside the scope of this project, which focuses on the antimicrobial action of honey.
2.2 Methods

2.2.1 Honey sample collection

With the support of the Portuguese beekeepers society (mostly amateurs) (S.A.P, Sociedade dos Apicultores de Portugal), 30 samples of the most common types of honey produced in Portugal were collected between the summer of 2003 and the summer of 2004. These were representative of the major sources of honey being produced in Portugal and were collected from all geographical areas of the country.

All the honeys were raw, unprocessed honeys, that were stored at 4°C in the dark, once they had been received in the laboratory.

The suspected floral source of each honey was reported by the beekeepers, but this was determined by pollen analysis in the laboratory.

2.2.2 Physicochemical characterization

2.2.2.1 Determination of pH

The pH of the honey was determined following the method described by the International Honey Commission (Bogdanov 2002). Ten grams of honey that had been equilibrated to 20°C for 24 hours in an air-conditioned laboratory was dissolved in 10 ml ultrapure water (<15ppb TOC), making a 50% (w/v) solution and mixed using a magnetic stirrer. The pH of the final solution was measured by using a Chemlab instrument pH 1000 meter previously calibrated using buffers of known pH (Fisher).
2.2.2.2 Colour

The colour of all samples was determined using the optical density method recommended by the National Honey Board, where the absorbance of a 50% (w/v) honey solution was determined at 560 nm in a Cecil spectrophotometer (10 millimetres path length) and absorbance was multiplied by 2, to give the absorbance value of the undiluted solution. Honeys were then categorised according to a previously established reference table (USDA 1985).

2.2.2.3 Protein content

Protein content was determined using the DC Protein assay kit (BioRad) that is based on the Lowry method for the determination of protein content in honey (White, Jr. and Rudyj 1978). In short a 50% (w/v) solution of honey in sterile deionised water was prepared as well as a 1/10 dilution, 5 μl of these solutions were dispensed into the wells of a 96-well microtiter plate, then 25 μl of Reagent A (alkaline copper tartrate) were added followed by 200 μl of Reagent B, (dilute Folin reagent). The plate was incubated at room temperature for 15 minutes and then read in a microtiter plate reader (Anthos Reader 2001, Anthos Labteco Instruments) at 750 nm. Suitable standard solutions of bovine serum albumin (Sigma) (range 0.2-2 mg/ml) were used and a calibration curve obtained, to which a best-fit line was added and the equation of this line was used to obtain the concentration of proteins in the honey samples. Each sample was run in triplicate.
2.2.2.4 Moisture content

Water content was determined using an Atago HHR-2N refractometer. The samples were prepared according to the International Honey Commission guidelines (Bogdanov 2002). Representative samples of each honey were transferred to sterile universal containers, sealed and incubated in a shaking waterbath at 50°C for 30 minutes. After incubation the samples were allowed to cool to 20 °C in an air-conditioned laboratory. Before testing the sample was thoroughly mixed. A drop of honey was placed on the lens of the refractometer, and the lid closed carefully to ensure an even spread of the sample with no air bubbles on the lens. Then the refractometer was held towards the light and the position of the interface was recorded. Between each sample, the refractometer was cleaned and dried.

2.2.2.5 Sugar content

Sugar content was determined using a Bellingham & Stanley 40-85% sugar refractometer. The undiluted honey samples were allowed to equilibrate to 20°C for 24 hours then thoroughly mixed before testing. A drop of honey was placed on the lens of the refractometer, and the lid closed carefully to ensure an even spread to the sample with no air bubbles on the lens. Then the refractometer was held towards the light and the position of the interface was recorded, which gives % readout, equivalent to the % sugars present in the sample. Between each sample, the refractometer was cleaned and dried.
2.2.2.6 Hydroxymethylfurfural (HMF) concentration

The hydroxymethylfurfural (HMF) in honey was determined using the method described by White, Jr. (1979). Five grams of each of the honey samples were weighed into 50 ml volumetric flasks with a total of 25 ml of water and 0.50 ml of Carrez Solution I (15 g potassium ferrocyanide in 100 ml of water) was added to each. The solution was mixed and 0.50 ml of Carrez solution II (30 g zinc acetate in 100 ml of water) was added to each of the volumetric flasks, and the solution diluted to volume with water. The solutions were filtered through paper rejecting the first 10 ml. Of the remaining filtrate 5 ml were added to each of two 18x150 mm test tubes and 5 ml of water were added to tube one (sample), and 5 ml of 0.20% sodium bisulphite (breaks down HMF) into tube two (blank). The solutions were mixed well in a vortex mixer and the absorbance was read at 284 and 336 nm in a Cecil spectrophotometer and the HMF calculated using the following formula:

\[
\text{HMF (mg/Kg honey)} = (A_{284} - A_{336}) \times 149.7 \times \frac{5}{\text{weight of sample in grams}}
\]

2.2.2.7 Antibacterial activity

The method used was as described by Allen et al. (1991), except that the test organism used was *Staphylococcus aureus* NCTC 6571. This method is used to determine the amount and type of antibacterial activity present in the honey and compare it to a phenol (disinfectant) standard. Each sample was assayed in quadruplicate and activity compared to phenol, and a New Zealand manuka honey (M109) was also tested as a control for high activity. Each sample was diluted in
distilled water for the determination of total antibacterial activity, in order to bring the antibacterial activity within the range covered by the phenol standards. Each sample was also diluted in catalase solution (1 mg/ml) to destroy any peroxide present so that residual non-peroxide antibacterial activity could be determined. Honeys were assayed as 50% and 25% (w/v) solutions to allow for a range of antibacterial activity.

2.2.2.8 Pollen analysis

The pollen analysis was performed according to the method described by Loveaux et al. (1978). Ten grams of honey were dissolved in 20ml of water and centrifuged for 10 minutes, 2500 rpm at room temperature (Sanyo Harrier 15/18 centrifuge). Supernatant was decanted; the sediment was resuspended in liquefied glycerine-gelatine, transferred to a glass slide and mounted with a coverslip. At least 100 pollen grains in each sample were counted and identified using light microscopy. Samples were classified according to the predominant pollen (>45% of pollen grains), except for monofloral honeys normally with under-represented pollen (such as lavender) where 20% was used (Loveaux et al. 1978).
2.3 Results

2.3.1 Physicochemical characterisation

The results of the chemical and physical analysis of the honeys (Table 1) indicated that all fell within the ranges normally expected for pH, water, sugar and protein content of blossom honeys. However, HMF levels of seven samples were above the 40mg/Kg standard in draft EU 96/0114 (EC 2005; EC 2005), and two exceeded the 60 mg/Kg standard of the draft CL 1998/12-S of the Codex Alimentarius (FAO and WHO 1993). It should be noted that the summers of 2003 and 2004 were particularly hot in Portugal (UNEP 2004), possibly resulting in the elevated levels of HMF.

2.3.2 Pollen analysis and antibacterial activity

Floral sources reported by beekeepers were not reliable and corresponded to confirmed identities on 13 occasions (Table 2). Only 4 samples were multifloral and *Lavandula sp.* was the most frequent type at 11 samples. Although reported as orange blossom, sample 26 did not contain the low percentage of *Citrus sinensis* pollen grains normally expected (Loveaux *et al.* 1978).
Table 1. Physicochemical characterization results of 30 Portuguese honeys

<table>
<thead>
<tr>
<th>Honey number</th>
<th>pH</th>
<th>Water content % (w/v)</th>
<th>Sugar content % (w/v)</th>
<th>Protein content (mg/g)</th>
<th>HMF (mg/Kg)</th>
<th>Colour</th>
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<td>81</td>
<td>15.78</td>
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<td>80</td>
<td>12.83</td>
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<td>24.6</td>
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<td>Mean</td>
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<td>80.4%</td>
<td>15.16</td>
<td>28.8</td>
<td>Light amber</td>
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<tr>
<td>S.D.</td>
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<td>0.59</td>
<td>1.2</td>
<td>5.35</td>
<td>22.3</td>
<td>NA</td>
</tr>
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</table>

S.D = Standard deviation, NA = not applicable
The bioassay method compares inhibition zones created by the honey to zones of inhibition produced by phenol standards (Fig. 6).

**Figure 6.** Example of a honey bioassay plate. The diameter of the inhibition zones is proportional to the potency of the honeys tested.

The diameter of the zones of inhibition of the phenol standards were measured and plotted against the phenol concentration used, and the trendline equation of the graph (Fig. 7) was used to calculate the potency relating to phenol of the honey samples.

**Figure 7.** Example of a phenol calibration curve in a honey bioassay experiment.
The bioassay used in this study allows for the distinction between the peroxide (the honey is diluted in sterile water and hydrogen peroxide generation is allowed to occur) and non-peroxide antibacterial activity (the honey is diluted in a catalase solution that will breakdown the hydrogen peroxide formed upon dilution) in the honeys and compares it to a phenol standard. The lowest phenol standard able to produce an inhibition zone in this assay is 2% (w/v), and absence of zones of inhibition indicated activity lower than 2% (w/v) phenol equivalent.

All of the Portuguese honeys tested possessed some antibacterial activity (total activity, peroxide + non-peroxide activity) as shown in Table 2, but levels were lower than the reference manuka sample and ranged from 4.3% (w/v) to 13.2% (w/v). Manuka had non-peroxide activity equivalent to 18% (w/v) phenol, this type of activity was found in only 7 Portuguese honeys (of which 5 were *Lavandula stoechas*), with a maximum of 11.5% (w/v) phenol equivalent.

Of the honeys that showed non-peroxide antibacterial activity, only one had an elevated HMF value (honey 28, 49 mg/kg), nevertheless this value was still within the tolerated values for warm countries of 60 mg/kg, and its non-peroxide antimicrobial potency was one of the lowest recorded in this study, 5.8% (w/v) phenol equivalent.
### Table 2. The antibacterial activity and floral source of 30 selected Portuguese honeys.

<table>
<thead>
<tr>
<th>Honey Sample</th>
<th>Total antibacterial activity as phenol equivalent (w/v) %</th>
<th>Non-peroxide activity as phenol equivalent (w/v) %</th>
<th>Presumed floral source</th>
<th>Confirmed floral source</th>
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</thead>
<tbody>
<tr>
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<td>Calluna sp.</td>
<td>Multifloral</td>
</tr>
<tr>
<td>2</td>
<td>4.4</td>
<td>ND</td>
<td>Eucalyptus sp.</td>
<td>Rubus sp.</td>
</tr>
<tr>
<td>3</td>
<td>7.1</td>
<td>ND</td>
<td>Multifloral</td>
<td>Calluna sp.</td>
</tr>
<tr>
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<td>Rosmarinus sp.</td>
</tr>
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<td>6.6</td>
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<td>Rosmarinus sp.</td>
</tr>
<tr>
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<td>7.5</td>
<td>ND</td>
<td>Rosmarinus sp., Eucalyptus sp</td>
<td>Lavandula sp.</td>
</tr>
<tr>
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<td>Calluna sp.</td>
<td>Calluna sp.</td>
</tr>
<tr>
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<td>7.6</td>
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<td>Echium sp.</td>
</tr>
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<td>6.8</td>
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<td>Lavandula sp.</td>
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<td>Eucalyptus sp.</td>
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<td>Rubus sp.</td>
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<tr>
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<td>4.3</td>
<td>ND</td>
<td>Rubus sp., Origanum sp.</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td>17</td>
<td>7</td>
<td>ND</td>
<td>Eucalyptus sp.</td>
<td>Eucalyptus sp.</td>
</tr>
<tr>
<td>18</td>
<td>5.8</td>
<td>ND</td>
<td>Multifloral</td>
<td>Rubus sp.</td>
</tr>
<tr>
<td>19</td>
<td>11</td>
<td>ND</td>
<td>Lavandula sp.</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eucalyptus sp.</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.8</td>
<td>ND</td>
<td>Multifloral</td>
<td>Multifloral</td>
</tr>
<tr>
<td>21</td>
<td>7.3</td>
<td>ND</td>
<td>Eucalyptus sp.</td>
<td>Eucalyptus sp.</td>
</tr>
<tr>
<td>22</td>
<td>7.8</td>
<td>ND</td>
<td>Calluna sp.</td>
<td>Calluna sp.</td>
</tr>
<tr>
<td>23</td>
<td>6.9</td>
<td>5.6</td>
<td>Eucalyptus sp.</td>
<td>Multifloral</td>
</tr>
<tr>
<td>24</td>
<td>5.3</td>
<td>ND</td>
<td>Not known</td>
<td>Rubus sp.</td>
</tr>
<tr>
<td>25</td>
<td>10.5</td>
<td>8.9</td>
<td>Lavandula sp.</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td>26</td>
<td>5.6</td>
<td>ND</td>
<td>Citrus sinesis</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td>27</td>
<td>9.8</td>
<td>ND</td>
<td>Echium sp.</td>
<td>Echium sp.</td>
</tr>
<tr>
<td>28</td>
<td>6.7</td>
<td>5.8</td>
<td>Lavandula sp.</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td>29</td>
<td>7.7</td>
<td>ND</td>
<td>Lavandula sp.</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td>30</td>
<td>10.9</td>
<td>6.2</td>
<td>Lavandula sp.</td>
<td>Lavandula sp.</td>
</tr>
<tr>
<td>Median</td>
<td>7.2</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND: Not detectable
3.4 Discussion

The bioassay showed that all honeys tested possessed detectable antibacterial activity. More than half of them demonstrated antibacterial activity between 7 and 12% (w/v) phenol equivalent. When tested for non-peroxide antibacterial activity by incubation with catalase, 76% of the honeys showed no detectable activity; i.e. all of their antibacterial activity was due to the production of hydrogen peroxide. Most of the honeys that demonstrated non-peroxide antibacterial activity were *Lavandula stoechas* honeys (5 out of 7), one was *Echium* sp., and the other was multifloral.

In testing 345 New Zealand honeys, 25 honey samples were shown to be non-peroxide honeys, these identified as manuka (*Leptospermum scoparium*) and viper’s bugloss (*Echium vulgare*) honeys (Allen *et al.* 1991). A similar study of Welsh honeys, showed that all possessed some total antimicrobial activity, but no non-peroxide activity was detected (Wheat 2004). Most of the honeys used in that study were presumed multifloral, although pollen analysis was not performed (Cooper, personal communication)

Honeys from *Lavandula stoechas* possess higher concentrations of coumarin than other *Lavandula* sp honeys, this being one of the identifying features of this honey (Guyot-Declereck *et al.* 2002; Molan *et al.* 1988). Coumarin is a product of phenylalanine metabolism that also has as an intermediate cinnamic acid. Both these components (coumarin and cinnamic acid) have been shown to possess antibacterial activity (Aljadi and Yusoff 2003; Sanchez *et al.* 1986) and it is possible that they contribute to the measured non-peroxide activity of these honeys.
One of the reasons that manuka honey has been advocated for wound care is its non-peroxide antibacterial activity (Molan and Betts 2004). Most wounds produce some exudate that could be expected to contain catalase (5-8 μmoles/ml), because it is present in plasma. This might cause the inactivation of a peroxide honey with low potency if applied to the surface of a wound, whereas a non-peroxide honey would not be affected in this way (Molan 2000).

According to Molan (Molan 2000) honey to be used in wound care should have a UMF (Unique Manuka Factor) rating of non-peroxide activity of at least 10 (i.e. non-peroxide activity equivalent to 10% (w/v) phenol) and in the present study two of the Lavandula stoechas honeys possessed ratings of 11.5 and 9 that could indicate suitability for use in wound care.

Although there have been many studies on the effectiveness of honey as an antibacterial agent for wound management, many of the studies do not state the type of honey used (Molan 1999). Of the studies that evaluate the antibacterial activity of specific honeys, most show manuka as the most potent. The honeys used in this study do not show an activity as high as manuka honey, but nevertheless it is possible that other honeys with higher activity might be found if larger and more extensive studies were conducted.

The results obtained in this study suggest that selected Portuguese honeys could be used as antimicrobial agents in wound care, especially for emergency use in the home for the treatment of superficial burns and minor injuries. Another application might arise in veterinary medicine, where regulations regarding the use of antibiotics
commonly used for human treatments limit the range of antimicrobial agents available for treating infected wounds.

Local wild unprocessed honey is already used in many countries for wound care (Al-Jabri et al. 2003b; Lev 2003; Meda et al. 2004; Sanchez et al. 1986; Subrahmaniam and Ugane 2004). The potential exists for a country like Portugal to develop sterilised dressings and other honey-based products for wound care with local honey as a novel alternative for the treatment of infected wounds.

It is probable that a larger study with a wider variety of honeys derived from all of the different regions that produce honey in Portugal would yield a better indication as to which honeys are the most suitable for wound care. As would a more comprehensive study of the chemical and physical properties of Lavender honeys produced in Europe, for the understanding and possible identification of new antimicrobial compounds.

Of the Portuguese honeys studied here, a median level of antibacterial activity was observed in 20% of the samples tested. Further studies are needed into the chemical and antibacterial activity characterisation of both unifloral and multifloral defined European honeys, together with the determination of its wound healing potential through the testing of its effects on cell lines, as there is the potential to find a high activity honey that would be an ideal alternative antimicrobial for wound management.
3. Bacterial inhibitory components in honey
3.1 Introduction

All honeys are non-sterile with a natural bacterial flora (total viable count ranging between 0 and 5000 cfu/g) mainly composed of Gram-positive sporing bacteria, such as *Bacillus* spp. which accounts for an average of 60% of bacteria recovered, depending on the amount of processing of the honey (Snowdon and Cliver 1996). The bacteria present in honey may have several origins, as both the production of honey by the bee and the handling of the final product by the beekeeper may introduce microorganisms other than those present in the honey’s raw material, the nectar. This means that organisms found in the environment around honey (air, dust, flowers, soil) are likely to be identified in honey (primary contamination).

Some of the bacteria most commonly found in the beehive environment and honey are *Actinomyces, Bacillus, Clostridium, Corynebacterium, Pseudomonas, Micrococcus* and many species of yeasts and fungi, which are capable of living in the presence of high sugar concentrations. The handling of the honey is the source of secondary contamination and is introduced by humans, equipment, containers, insects and animals (Snowdon and Cliver 1996).

The main focus of is on the antibacterial activity of manuka honey (from *Leptospermum scoparium*), this honey was chosen as the focus for this research as most of the medical products developed and available for drugs tariff in the UK are based on this type of honey. Although honeys share some general antibacterial characteristics, like the high sugar concentration and low pH, manuka honey has been found to possess other characteristics that make it very useful to medicine.
Manuka honey possesses strong non-hydrogen peroxide derived antibacterial activity whose nature is not as yet known (Cooper 2005a). Although the honey-based products used on drug tariff are sterilised using gamma radiation, many of the studies on the antibacterial activity of honey were done using non-sterile honey (French et al. 2005; Al Waili and Saloom 1999; Postmes et al. 1996; Subrahmanyam 1994; Cooper et al. 2002c), so there is the possibility that the non-hydrogen peroxide activity is derived from a microbial source in the honey. Furthermore, even if the bacteria in the honey are inactivated through gamma irradiation, it is still possible that their antibacterial metabolites would remain active in the honey after sterilisation, and could be the source of the as yet unexplained antibacterial activity or at least contribute to it in part.

Although it is generally considered that all manuka honeys are useful as medical products, there is a wide range in the potency of honeys derived from the same floral origin, in this case the manuka shrub (Allen et al. 1991). This suggests that the antibacterial potency is not exclusively derived from plant components; as if this was the case all honeys derived from manuka shrub would possess similar potency. There is, therefore, a need to investigate “variable” sources of activity and microbial contamination might be one of these sources.

The majority of the bacteria found in honey are Gram positive spore-forming bacteria, that are able to survive in honey by forming spore that can resist the extreme condition of pH and high osmolarity present in honey until the conditions change and the spores can germinate into vegetative cells (Snowdon and Cliver 1996). It is
known that there is a link between sporulation and the switching on of secondary metabolism in bacteria that can lead to the production of antimicrobial substances (Demain and Fang 2000; Tamehiro et al. 2002; Yan et al. 2003). Many of the organisms reported to be present in honey in the form of endospores, like Bacillus spp., are known to produce antimicrobial products (Demain and Fang 2000; Leifert et al. 1995; Takahashi et al. 1986; Tamehiro et al. 2002).

American foul brood is a disease cause by Paenibacillus larvae subsp larvae, and it affects the larvae of bees in the hive. It is the cause of many problems in beekeeping and because of the close proximity of honey to the bee larvae inside the hive, honey is many times contaminated with the causative organism of this disease (Alippi et al. 2002; Chantawannakul and Dancer 2001). This bacterium has been reported to be capable of producing a broad-spectrum antimicrobial substance (Glinski and Jarosz 1992; Holst 1945). It is possible that honey collected from infected hives might be contaminated with substances, unlike honey from uninfected hives.

The aim of this work was therefore to determine the potential for microorganisms present in manuka honey to produce antimicrobial agents.
3.2 Methods

In this study 13 New Zealand manuka honeys and 1 Australian jellybush honey were tested from a stock of honeys held at UWIC, and kindly given by Professor Peter Molan. Selection of honeys was blind, the honeys were given by the supervisor and labelled with letters; they included a range of potencies, and included manuka honey M109 (used as a control in chapter 2 and more extensively in chapters 5, 6 and 7).

3.2.1 Isolation and identification of the bacteria present in honey

Five grams of honey was diluted in sterile deionised water in a volumetric flask to give a 50% (w/v) solution which was then diluted down to $10^{-2}$ using $\frac{1}{4}$ strength Ringer's solution and 100 $\mu$l of each solution was spread onto a plate of Tryptone Soya Agar (TSA) (Oxoid) in duplicate. After incubation for 48 hours at 30°C (to allow slower growing aerobes to produce visible colonies), the total viable number of organisms was determined. Aerobic counts were also determined by spreading 100$\mu$l of the neat solution (50%) onto selective agar: MacConkey's Agar (Oxoid) and Blood Agar (Oxoid), which were incubated overnight, and Paenibacillus larvae Agar (PLA) (Schuch et al. 2001), which was incubated for 4 days at 37°C. An anaerobic total viable count (TVC) was also determined by spreading 100 $\mu$l of the 50% solution onto TSA and Blood Agar that were incubated anaerobically at 37°C for 48 hours in an anaerobic workstation (Modular Atmosphere Controlled System Workstation, DW Scientific).
After incubation, colonies were counted and TVC determined. Pure cultures were obtained from each colony type and identified using Gram stain, catalase production, BBL CRYSTAL Gram-positive identification kit (Becton, Dickinson) and in some cases API Coryne, API Staph and API 50 CHB kits (BioMérieux).

3.2.2 Screening for antimicrobial activity of isolates after cultivation in liquid medium

Initially all isolates were cultivated in Tryptone Soya Broth (TSB) (Oxoid) for 5 days at 30°C to obtain culture supernatant that was tested for antimicrobial activity by a bioassay. After the incubation two different methods were used. In the first method, 1ml of the culture was centrifuged in a microcentrifuge (Sanyo Centaur) at 13225 x g for 10 minutes in order to pellet out the cells, and 100 µl of supernatant was added to each well in a bioassay plate (243 mm x 243 mm x 18 mm). Bioassay plates each contained 150 ml BBL Nutrient agar (Becton, Dickinson) that was seeded with 100µl of an overnight culture of one of several test organisms (*Staphylococcus aureus* NCTC 6571, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NCTC 10418 or *Candida albicans*). In this last case Sabouraud Dextrose Agar (Oxoid) was used instead of nutrient agar. Wells were cut with a sterile cork borer (size 4) and phenol standards (100 µl) ranging from 2% (w/v) up to 7% (w/v) were inoculated into some of the wells in a quasi Latin square. The bioassay plate was incubated overnight at 37°C and the inhibition zones recorded. Each sample was tested in duplicate.

Cultures that presented some possible inhibition in this method were used for further experiments, using a second methodology. Briefly, five millilitres of a TSB overnight
culture was taken and centrifuged for 20 min at 9600 g in a bench top centrifuge (Sanyo Harrier 15/18 centrifuge). The resulting supernatant was removed and filtered through a 0.2 µm filter (Cellulose Nitrate Membrane Filter, Whatman International Ltd, England) to remove bacterial cells in order to determine if the inhibition was due to soluble factors in the culture supernatant. 100 µl of the filtrate (in duplicate) was inoculated into another bioassay plate, together with the phenol standards. The bioassay plate was incubated overnight at 37°C and the diameter of inhibition zones recorded. Because of low potency of the culture supernatants and filtrate that was observed, the procedures were repeated using Bailey's Liquid medium to cultivate all isolated organisms. This medium has been described as the ideal medium to grow Paenibacillus larvae subsp larvae and stimulate secondary metabolite production (Bailey and Lee 1962).

3.2.3 Screening for antimicrobial activity of isolates after cultivation in solid medium

In order to screen for the production of antimicrobial agents in solid medium, all of the isolates were streaked onto a segment of TSA plate and incubated for 5 days at 30°C (as shown in Figures 8 and 9) to allow possible water soluble antimicrobials to diffuse into the agar. After incubation overnight cultures of the test organisms grown as before were streaked at right angles to the honey isolate, starting from the point furthest away from the segment on which the honey isolate had grown. Plates were incubated overnight at 37°C and observed for inhibition of growth of the test organism.
The test organisms used were 4 type cultures (Oxford strain *Staphylococcus aureus* NCTC 6571, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NCTC 10418 and *Staph. aureus* NCTC 10017) and 9 clinical isolates recovered from infected wounds (*Enterococcus faecalis, Klebsiella oxytoca, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Enterobacter cloacae, Streptococcus spp., Staphylococcus epidermidis* and *Streptococcus spp.*) from the collection of clinical wound isolates held by Dr. Rose Cooper at UWIC. Each isolate was tested in duplicate.
3.3 Results

3.3.1 Isolation and identification of the bacteria present in honey

The spread plates prepared from 14 honey samples yielded 106 isolates. The TVC (Table 3) for honeys ranged from $1.4 \times 10^4$ to $2 \times 10^1$ cfu/g with a mean of $2.18 \times 10^3$ cfu/g (SD 4.84 x 10^3) for the aerobes in TSA, and between $6.80 \times 10^2$ and $2 \times 10^1$ cfu/g with a mean of $8.14 \times 10^2$ cfu/g (SD 9.56 x 10^1) in the case of the anaerobes in TSA. It was not possible to identify all isolates to species level.

### Table 3. Aerobic and anaerobic total viable count for the honey samples tested using various growth media.

<table>
<thead>
<tr>
<th>Honey</th>
<th>Aerobic Incubation</th>
<th>Anaerobic Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSA (cfu/g)</td>
<td>BA (cfu/g)</td>
</tr>
<tr>
<td>A</td>
<td>$1.41 \times 10^4$</td>
<td>TNTC</td>
</tr>
<tr>
<td>B</td>
<td>$1.20 \times 10^3$</td>
<td>$2 \times 10^2$</td>
</tr>
<tr>
<td>C</td>
<td>$2 \times 10^2$</td>
<td>$3.2 \times 10^2$</td>
</tr>
<tr>
<td>D</td>
<td>$8.9 \times 10^2$</td>
<td>$8.4 \times 10^2$</td>
</tr>
<tr>
<td>E</td>
<td>$7 \times 10^1$</td>
<td>$2 \times 10^2$</td>
</tr>
<tr>
<td>F</td>
<td>$4 \times 10^1$</td>
<td>NG</td>
</tr>
<tr>
<td>G</td>
<td>$1.3 \times 10^4$</td>
<td>TNTC</td>
</tr>
<tr>
<td>H</td>
<td>$2 \times 10^1$</td>
<td>$8 \times 10^1$</td>
</tr>
<tr>
<td>I</td>
<td>$2 \times 10^1$</td>
<td>$4 \times 10^1$</td>
</tr>
<tr>
<td>J</td>
<td>$4 \times 10^1$</td>
<td>$3.8 \times 10^2$</td>
</tr>
<tr>
<td>K</td>
<td>$2 \times 10^2$</td>
<td>TNTC</td>
</tr>
<tr>
<td>L</td>
<td>$1.75 \times 10^2$</td>
<td>$1.4 \times 10^1$</td>
</tr>
<tr>
<td>M</td>
<td>$2.5 \times 10^2$</td>
<td>$2.6 \times 10^2$</td>
</tr>
<tr>
<td>N</td>
<td>$2.5 \times 10^2$</td>
<td>$4 \times 10^1$</td>
</tr>
<tr>
<td>Average</td>
<td>$2.18 \times 10^3$</td>
<td>$\geq 3.3 \times 10^3$</td>
</tr>
<tr>
<td>Stand. Dev</td>
<td>$4.84 \times 10^3$</td>
<td>$6.3 \times 10^3$</td>
</tr>
</tbody>
</table>

Legend: TSA = Tryptone Soya Agar, BA = Blood Agar, MAC = MacConkey’s Agar, PLA = Paenibacillus larvae agar, NG = no growth (<20cfu/g), TNTC = $> 1.5 \times 10^4$ cfu/g.
The isolates were selected for characterization because they exhibited different morphological characteristics on the primary isolation plates, like colony shape, size or colour. However, it must be emphasised that the identification techniques used did not allow discrimination between strains or subspecies and so although 4 isolates from honey A had different colonial morphologies and may have represented different strains of the same species, all were reported as *B. subtilis*. The bacteria that are listed as “other” in Table 4 were *Lactococcus* spp., *Corynebacterium* spp., *Enterococcus* spp., *Micrococcus* spp. and *Pediococcus* spp.

Sample I and H had the lowest bacterial counts while samples A and G had the highest aerobic count and K the highest anaerobic count. It was possible to observe that isolates that yielded the same identification did not always have the same colony morphology, this might have been due to strain variation (methodology used in this study did not characterise isolates according to strain), for example for honey A (Table 4) where initially 7 different colony types were identified, but characterised to only 3 distinct species.

Of the cultures recovered *Bacillus* spp. were most commonly observed (Table 4), from this table it is also possible to see that there was a difference in the types of organisms recovered in aerobic conditions and those recovered in anaerobic condition of incubation.
Table 4. Number of isolates recovered in each honey for the most common genera.

<table>
<thead>
<tr>
<th>Honey</th>
<th>Number of aerobic isolates</th>
<th>Number of anaerobic isolates</th>
<th>Identification of the Bacillus spp. isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>isolates</td>
<td>Number of Bacillus spp.</td>
<td>Number of Staph. spp.</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>K</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

A total of 106 isolates were recovered from the 14 honey samples tested. The majority of the isolates recovered were from the Bacillus genus (65%), but 29% of these could not be identified to species. The most common species identified were Bacillus subtilis and Bacillus pumilus.

4.3.2 Screening for antimicrobial activity of isolates after cultivation in liquid medium

Attempts to identify antibacterial activity in the culture supernatants by growing the isolates recovered from the 14 types of honey in liquid medium were unsuccessful.

4.3.3 Screening for antimicrobial activity of isolates after cultivation in solid medium

Eleven of the thirteen test cultures were inhibited by one or more of the honey isolates and of the test bacteria exposed to the honey isolates, only two clinical isolates
(Pseudomonas aeruginosa and Enterobacter cloacae) were not inhibited by any isolate. Staphylococcus aureus NCTC 6571 showed the greatest inhibition and the other isolates were variously inhibited (Fig. 8 and 9).

**Figure 8.** Example of no inhibition of growth of test organisms by bacterial strain isolated from honey (vertical= test organisms; horizontal= isolate from honey).

![Figure 8](image)

**Figure 9.** Example of inhibition of growth of test organisms by bacterial strain isolated from honey (shown by arrows) (vertical= test organisms; horizontal= isolate from honey).

![Figure 9](image)
Of the 106 isolates recovered from the honey samples, 76 inhibited one or more species and 30 did not cause any inhibition (Table 5). The isolates that failed to inhibit any of the test bacteria were *Staphylococcus capitis*, *Bacillus circulans*, *Staphylococcus warneri*, *Lactococcus lactis* spp. *lactis*, *Staphylococcus aureus*, *Micrococcus* sp., *Bacillus* spp., *Enterococcus durans* and *Bacillus sphericus*. Of the isolates causing inhibition 85% were *Bacillus* sp.

**Table 5.** Results for the antimicrobial screening in solid medium (x shows inhibition observed).

<table>
<thead>
<tr>
<th>Honey</th>
<th>Isolate no</th>
<th>Test strains</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2 3 4 5 6 7 8 9 10 11 12 13</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>X</td>
<td>X X X X X X X X X</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>X X X</td>
<td>X X X X X X X X X</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>X X X</td>
<td>X X X X X X X X X</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>X</td>
<td>X X X X X X X</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>X X X</td>
<td>X X X X X X X X</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>X X X X X X</td>
<td>X X X Bacillus subtilis</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>X X X X X</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>X</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>X X X X X X X X X</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>X X X X X X X X X</td>
<td>Bacillus pumilus</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>X X X X X X X X X</td>
<td>Bacillus pumilus</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>X</td>
<td>Corynebacterium spp.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>X X X X X X x</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>X X X X X X X X X</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>X</td>
<td>Bacillus brevis</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>X</td>
<td>Staphylococcus epidermis</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>X X X X X X X X</td>
<td>Corynebacterium spp.</td>
</tr>
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87
Table 5 (cont.). Results for the antimicrobial screening in solid medium.

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Table 5 (cont.). Results for the antimicrobial screening in solid medium.

<table>
<thead>
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<th>Identification</th>
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<td>X</td>
<td>Bacillus sublilis</td>
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<td>N 4</td>
<td></td>
<td>X</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>N 5</td>
<td>x</td>
<td>X</td>
<td>Doubtful B. brevis / pumilus</td>
</tr>
<tr>
<td>N 7</td>
<td>x x x</td>
<td>X</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>N 9</td>
<td>x x x</td>
<td>X x x x</td>
<td>Bacillus subtilis</td>
</tr>
</tbody>
</table>

Legend:
- X: Inhibition observed

Test Strains
- 1: Oxford Staph. aureus NCTC 6571
- 2: Enterococcus faecalis (clinical) 6633
- 3: Klebsiella oxytoeca (clinical) 8: E. coli (clinical)

4: Pseud. aeruginosa (clinical)
5: Staph aureus NCTC 10017
6: E. coli NCTC 10418
7: B. subtilis ATCC 6633
8: E. coli (clinical)
9: Staph. aureus (clinical)
10: Enterobacter cloacae (clinical)
11: Streptococcus spp. (clinical)
12: Staph. epidermis (clinical)
13: Streptococcus spp. (clinical)

* Isolates with no inhibition were not shown on this table.
3.4 Discussion

According to Snowdon and Cliver (1996) the majority of the microorganisms able to survive the harsh conditions present in honey are Gram positive spore-forming bacteria, especially of the *Bacillus* genus, and some yeasts. In this study neither filamentous fungi nor yeasts were sought. From the plate counts on various media it is evident that the bacterial loads in the different honey samples varied. Differences were probably due to differences in the origins of honey and the way that each of them has been handled. For example, honeys C, I, J, and L contained *Staphylococcus epidermidis* or *Enterococcus durans*, which are bacteria that are usually associated with the skin microbial flora, and as such might be indicators of contamination during handling.

Usually the level of bacteria found in commercial honey is between 1 and 5000 cfu/g (Snowdon and Cliver 1996). Most of the honey samples tested here had a bacterial load between these two values and only honey A and honey G had greater than 5000 cfu/ml. Although above the average, this does not mean that the honey is not fit for consumption, as that decision also depends on the type of microbial flora found in the honey. In general the majority of the bacterial flora in honey is aerobic, but it may also contain some anaerobic bacterial spores, such as *Clostridium* spp. Among the members of this group that are important in the clinical context is *Clostridium botulinum*, and cases of infant botulism have been linked to honey consumption but with low incidence (Eaton 2001).
It is worthwhile to note that in this work no *Clostridium* species were found. This is very important as if it was present and raw, unsterilised honey were to be used in wound management, there is the potential to cause wound botulism (Eaton 2001).

Aerobic bacteria are important in wounds; nevertheless anaerobic infections may develop (Brook and Frazier 1999; Brook 2001) and the existence of bacteria in honey able to grow under these conditions should be taken into account. In this case none of the bacteria recovered from the anaerobic incubation were known pathogens. In order to prevent any potentially dangerous bacteria infecting wounds through clinical use of honey it can be sterilised by gamma-radiation without significant loss of potency (Molan and Allen 1996).

It is likely that the some of the bacteria recovered from the honey samples would have been present as endospores and germinated once media without honey was presented to them. The survival of vegetative bacterial cells in honey is usually not more than a few weeks (Snowdon and Cliver 1996). It is important to note that of the honeys used, only one was a processed honey, and that all of the others were raw honeys.

Identification of isolates proved problematic using commercial test kits, particularly for *Bacillus* sp where the same isolate could be identified differently by the two kits used. Routine DNA methods are not yet available for the identification of the species found in this study. A technique such as RAPD (Randomly Amplified Polymorphic DNA) could give some idea of similarities and differences between strains, although it would not give a definite identification. One of the solutions could possibly be the use of sequencing analysis; nevertheless that technique is not available in UWIC.
Bacteria of the *Bacillus* genus are part of the normal bacterial flora found in soils. These have been found to contribute to the management of plant diseases (Milner *et al.* 1996) by producing antimicrobial agents that eliminate fungi and bacteria that otherwise would attack the plants. *Bacillus subtilis* is known to produce bacteriocins (microbially produced peptides that are active against bacteria of closely related bacterial species) (Zheng and Slavik 1999) as well as ribosomal (e.g. TasA and subtilorin) and non-ribosomal antibiotics (e.g. Surfactin, fengycin, etc) that have antifungal and antibacterial activities (Tamehiro *et al.* 2002). *B. licheniformis*, is known to produce bacitracin (Ishihara *et al.* 2002). Other *Bacillus* spp. isolates recovered also are known to produce different types of antimicrobial agents: *B. cereus* is known to produce kanosamine and zwittermicin A (Milner *et al.* 1996), *B. pumilus* is known to produce antifungal metabolites (Bottone and Peluso 2003; Munimbazi and Bullerman 1998), *B. brevis* is known to produce the antimicrobial agent gramicidin S (Leifert *et al.* 1995) and *B. circulans* was shown to produce nucleoside antibiotic (bagougeramines A and B) which demonstrate a broad range of antimicrobial activity (Takahashi *et al.* 1986). The possible presence and contribution of these to the antimicrobial activity of certain honeys would have to be addressed by using chemical analysis to try and detect the presence and concentration of these compounds. Techniques for detection of antibiotics in honey are mainly based on chromatography, like high-performance liquid chromatography, or mass spectroscopy (Thompson *et al.* 2005; Vivekanandandan *et al.* 2005), but new methods involving biosensors (Caldow *et al.* 2005) and immunochemical methods (Heering *et al.* 1998) are being developed.
The production of antimicrobial substances by bacteria is often associated with environmental triggers, as starvation, or stress originated by the growth of other bacteria (Robertson et al. 1989; Marahiel et al. 1993). Another factor that can also affect the stimulation or inhibition of the production of antimicrobial substance is the physical characteristics of the growth medium, if it is solid or liquid (Madigan et al. 2000).

The use of PLA and Bailey’s medium in this work was to detect the presence of *Paenibacillus larvae* subsp *larvae*. This bacterium is a pathogen of bees that causes American foulbrood disease, it might hold the potential for the production of antimicrobial agents (Holst 1945; Glinski and Jarosz 1992). However no *Paenibacillus larvae* subsp *larvae* was recovered.

The production of any antimicrobial agent might be facilitated by the extreme conditions that the microorganisms face in the honey, which can induce sporulation and switch on secondary bacterial metabolism. Sporulation might be induced in solid medium rather than in liquid medium (Codon et al. 1995; Haggblom et al. 2002) and this might explain why no antibiotic activity was detected from isolates grown in liquid medium.

The inhibition in the solid medium was widespread; most of the *Bacillus* spp. demonstrated some capacity to inhibit the test organisms in the solid media assay. The positive results obtained with the use of clinical strains as test organisms enabled the observation that the antimicrobial being produced in each case seems to be a broad
spectrum agent as it inhibits both Gram positive and negative bacteria as well as type cultures and clinical strains.

The fact that *Pseud. aeruginosa* and *Enterobacter cloacae* were not inhibited by any isolates might be due to the fact that both of them were clinical isolates. Also *Pseud. aeruginosa* is known for its resistance to antimicrobial agents (Larsen *et al.* 1993; Salunkhe *et al.* 2005). Clinical isolates do not behave the same way as type cultures of the same species would, because they have been exposed to different environmental pressures, forcing them to find mechanisms for adaptation that type cultures do not need (Rutala *et al.* 1997).

These results raise the issue of what should be the permitted level of antibiotics in honey. Hives can be treated with antibiotics for the treatment of diseases like American foul brood disease. At the moment the level of antibiotics allowed in honey is 0 mg/kg (FAO and WHO 1993), as an indication of no-treatment of the honey. Nevertheless recently this level has been brought into question when low levels of streptomycin (3 to 16 ppb) were detected in Zambian honeys (Zambia Bee Products Association 2005). Honey from a wide area was affected and it is not likely that hives had been treated with streptomycin, because of its prohibitive cost. Since *Streptomyces* growing in the environment is capable of producing this antibiotic, and it has been reported to be present in soils (Huddleston *et al.* 1997), it is possible that these low levels of streptomycin have occurred by natural contamination of soil in water sacs of foraging bees (Zambia Bee Products Association 2005).
With the development of more sensitive techniques for the quantification of antibiotics in complex substances such as honey it is possible that honey which had previously been considered to be free of antibiotics actually possesses low levels and that this is part of the natural production of honey. This work has shown that in the honey there are bacteria capable of producing antimicrobial agents. Analytical techniques are now needed to show if these antimicrobial agents are present and at sufficiently high concentrations to have an effect on wound pathogens.
4. A study of the antioxidant potential of selected honey
4.1 Introduction

It is recognised that honeys from different floral sources and geographical locations vary considerably not only in their antibacterial activity but also in their colour (Molan 1992) and wound healing potential (Wheat 2004). These properties change on exposure to light, heat and time (Dustman 1978). Antioxidants are present in many vegetables, fruits, and food products, such as black tea, coffee and honey. These agents have been characterised as flavonoids, and phenolic acids (Gheldof et al. 2002), which may also function as antimicrobial agents.

Antioxidants are known for their cytoprotective effects by scavenging free radicals. Free radicals are reactive molecules that arise from the products of the oxidation/reduction of oxygen and hydrogen. The high oxidative/reductive capability of these radicals can damage cell membranes by altering the oxidative state of certain components such as DNA and proteins that results in altered conformation (Heim et al. 2002; Inoue et al. 2005; Sabu and Kuttan 2002).

Two of the most physiologically important free radical species are hydroxyl and superoxide radicals. These are produced by human immune cells to counteract microbial infections, for example from human fibroblasts in response to cytokines like interleukin-1 or tumour necrosis factor, or by neutrophils and macrophages (production by the NADPH-oxidase located in plasma membrane) (Burdon 1995). They are also produced by bacterial cells, to allow them easier access to human cells for example Pseudomonas aeruginosa, secretes pyocyanin and pyochelin (toxins) that act in synergy with hydroxyl radical to damage the endothelial cell wall (Britigan et
or in the case of *Cryptococcus neoformans* that has in its pigments a stable free-radical population that makes it more resistant to antibody-mediated phagocytosis (Wang et al. 1995).

One of the most common pathways for the production of hydroxyl radicals is through the Fenton reaction:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\bullet + \text{OH}^-
\]

The \( \text{Fe}^{3+} \) can then react further with \( \text{H}_2\text{O}_2 \) to produce superoxide:

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{O}_2^\bullet + 2\text{H}^+
\]

Superoxide radicals are also generated from the reduction of molecular oxygen as occurs in the phagocyte respiratory burst:

\[
\text{O}_2 + e^\bullet + \text{H}^+ \rightarrow \text{O}_2^\bullet + \text{H}^+
\]

Honeys vary in their antioxidant potential (Gheldof et al. 2002; Meda et al. 2005) and there is anecdotal evidence that darker honeys possess higher antioxidant potentials than lighter coloured honeys (Gheldof et al. 2002).

Manuka honey is used in commercially available medical products, this honey is thought by most researchers to have most of its antimicrobial activity derived from sources other than the hydrogen peroxide generation, although it may produces
hydrogen peroxide but in a minute concentration as demonstrated by the difference (small) between the peroxide and non-peroxide activity when using a bioassay. Nevertheless there are those that claim that the level of hydrogen peroxide production in the manuka honey is higher than generally accepted and that the assays currently used to test the type of antimicrobial activity not due to the generation of hydrogen peroxide are not taking this into consideration. It is suggested that these experiments were using a concentration of catalase (breaks down hydrogen peroxide) which was too low to inactivate this activity in manuka honey (Weston 2000).

The aim of this study was to determine the antioxidant potential of three natural honeys of differing floral and geographical origins, by the determination of the free radical quenching potential of honey, and on the other hand the determination of the production of free radicals via the Fenton reaction using electron paramagnetic resonance (EPR) (Fig. 10) spectroscopy. The antimicrobial activity of each honey was also determined and relationships between these parameters were investigated.
Figure 10. Diagram of EPR spectroscopy method.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^\cdot
\]

Add relevant spin trap (molecule with high affinity that stabilizes the free radical)

This adduct (free radical + spin trap) will have a particular, non-random spin that can be detected by
4.2 Methods

Five samples of honey were used. An artificial honey that reflected the main components of honey was prepared by dissolving 1.5 g of sucrose, 7.5 g of maltose, 40.5 g of fructose and 33.5 g of glucose in 17 ml of deionised water. This solution was included in the study to evaluate the contribution of the predominant sugars and water to the antibacterial and antioxidant characteristics of honey. A clear, runny honey (Gale's) obtained from a supermarket was included to represent a heat-processed honey. A peroxide generating honey derived from mixed floral source (pasture PS9), a non-peroxide, generating honey, and monofloral manuka (M109) honey was also used, supplied by Professor Peter Molan of the University of Waikato, New Zealand and a local honey obtained from West Wales were also used.

4.2.1 The determination of the colour of honeys

As described in section 2.2.2.2.

4.2.2 Determination of antimicrobial activity

The method used was that of Allen et al. (1991), described in section 2.2.3.

4.2.3 Superoxide radical enzymatic quenching assays
Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system following a method previously described (Valentao et al. 2002a; Valentao et al. 2002b).

\[
\text{Xanthine oxidase} \\
x + \text{uric acid} + \text{superoxide} \\
+ \text{Lucigenin} = \text{Generates light}
\]

A master mixture for each sample was prepared in two 1.5 ml eppendorf tubes each containing 290 µl of 5 mM xanthine (sodium salt), 100 µl of the test solution (positive control, 50% honey solution or negative control), and 100 µl of 1mM lucigenin. As a positive control, ascorbic acid (1 mM) was used, and for a negative control, the antioxidant compound was replaced by sterile deionised water.

After mixing, 150 µl of this mixture were transferred to a well in a 96 well microtitre plate using at least 5 repeats. To each well was added 10 µl of xanthine oxidase (0.5 units/ml) and the plate was incubated for 15 minutes in the dark. The plate was read in the luminometer (MLX Microtiter plate Luminometer, Dynex Technologies).

4.2.4 Electron Paramagnetic Resonance (EPR) spectroscopy

This work was undertaken in the Medical Microbiology Department of the University Hospital of Wales, with the assistance of Dr. Simon Jackson.

EPR spectroscopy was employed in two experiments: detection of hydroxyl radical (OH•) formation from hydrogen peroxide that may be present in the diluted honey
samples and in a second experiment to test for any antioxidant activity in the honey by quenching free radicals introduced into diluted honey samples. To detect highly reactive radicals, such as OH•, the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Sigma) was used. This compound reacts with OH• to form a stable paramagnetic species (the ‘spin-adduct’ DMPO-OH•) that gives a characteristic 1:2:2:1 quartet of lines in the EPR spectrum.

To determine the potential for OH• production by the different honey solutions, Fe2+ was added to the honey to generate OH• by Fenton chemistry from any peroxide present. As a positive control for trapping this radical, Fe2+ was used to generate the hydroxyl radical from H2O2.

In typical experiments 40 μL of 0.1 M DMPO was added to 100 μL of a fresh 50% (w/v) solution of honey in an eppendorf tube and mixed gently by pipetting. To initiate the reaction, 20 μL of 0.05 M FeSO4 was added and the solution immediately drawn into a glass capillary tube that had been sealed at one end. This tube was placed in the cavity of an EPR spectrometer (Varian E104 operating at 9.2 GHz microwave frequency and 20 mW power) and EPR spectra recorded. Typical spectrometer conditions were 100 Gauss (G) scan range, 120-second scan time and 1.0 G field modulation.

In some experiments, the honey samples were also ‘spiked’ with 30 μL of 30% (v/v) H2O2 prior to addition of the FeSO4 and the decay time of the resultant EPR spectrum was used to determine the OH• radical ‘quenching’ or antioxidant potential of each honey.
4.3 Results

4.3.1 Determination of the colour and antimicrobial activity of honeys

The results of the colour determination assay ranged from extra white to amber and are shown in table 6.

Table 6. Results of the colour determination of honeys tested (absorbance at 560 nm and its correspondent colour).

<table>
<thead>
<tr>
<th>Honey Type</th>
<th>Absorbance @ 560 nm</th>
<th>Colour Description</th>
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</thead>
<tbody>
<tr>
<td>Artificial Honey</td>
<td>0.068</td>
<td>Extra white</td>
</tr>
<tr>
<td>Commercial Honey</td>
<td>0.507</td>
<td>Light amber</td>
</tr>
<tr>
<td>Pasture Honey (PS9)</td>
<td>1.024</td>
<td>Amber</td>
</tr>
<tr>
<td>Manuka Honey (M109)</td>
<td>1.166</td>
<td>Amber</td>
</tr>
<tr>
<td>West Wales</td>
<td>1.016</td>
<td>Amber</td>
</tr>
</tbody>
</table>

The antimicrobial activity of each honey is shown in Table 7. Only manuka honey demonstrated significant non-peroxide activity. Antibacterial activity in both pasture and local Welsh honey was largely due to the generation of hydrogen peroxide, whereas artificial and Gales honey had no detectable activity in this assay (therefore equivalent to less than 2% (w/v)).
Table 7. Antimicrobial activity of selected honeys determined using the bioassay method.

<table>
<thead>
<tr>
<th>Honey type</th>
<th>Phenol equivalent % (w/v)</th>
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<tr>
<td></td>
<td>Total activity</td>
</tr>
<tr>
<td>Artificial Honey</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Commercial Honey</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Pasture Honey (PS9)</td>
<td>19</td>
</tr>
<tr>
<td>Manuka Honey (M109)</td>
<td>18.1</td>
</tr>
<tr>
<td>Welsh Honey</td>
<td>6.6</td>
</tr>
</tbody>
</table>

4.3.3 Superoxide radical enzymatic quenching assays

In the superoxide radical quenching assay, the greater the amount of superoxide radical present in the reaction mixture, the more light is detected. Each honey sample exhibited different abilities to quench superoxide radical (Figure 11). As expected, the greatest light emission was observed with the negative control (sterile deionised water) and the least with manuka honey. With the natural honeys, greatest quenching activity was observed in manuka (M109) honey followed by the pasture honey (PS9) and the supermarket honey. The artificial honey was less effective than all of the experimental honeys, but did show some quenching activity compared to deionised water.
Figure 11. Superoxide radical concentrations detected in the xanthine/xanthine oxidase assay. The lower the peak the stronger the quenching capacity of the honey tested.
4.3.4 Electron Paramagnetic Resonance (EPR) Spectroscopy

In total 58 EPR spectra were collected from the tested samples. The control spectrum is shown in Figure 12. The only honey observed to generate radicals via the Fenton reaction (Fig. 13) was the pasture honey (PS9). Although the profile was not a typical hydroxyl radical profile, it was possible to deduce that this was due to hydroxyl radical generation because the signal disappeared when catalase (Fig. 13) was added to the reaction mixture. Interaction between hydrogen peroxide and some of the components in honey may give rise to unusual profiles. No radicals were detected with any of the other honey samples in the presence of iron.

When hydrogen peroxide was added to each of the honey solutions the expected profile for hydroxyl radical was immediately observed and quenched with time. Manuka honey profiles are illustrated in Fig. 14, (a) with no hydrogen peroxide addition, (b) with added hydrogen peroxide and (c) after incubation, similar profiles were obtained for West Wales, commercial and artificial honey. Speed of signal quenching of hydroxyl radicals varied between honeys, with manuka being the faster quencher, followed by the West Wales honey then by the commercial honey, the artificial honey and finally by pasture honey. It was possible to observe that pasture honey radical profile disappeared with time (Fig. 15b) to levels similar to those verified when catalase was added to the honey solution (Fig. 13b).
Figure 12. (a) EPR spectrum of the OH• radical trapped with DMPO from the Fenton reaction (positive control). (b) The same as in (a) but without Fe²⁺ or H₂O₂ (negative control).

Figure 13. (a) typical EPR spectra obtained with a 50% (w/v) solution of pasture honey in the presence of FeSO₄ and DMPO. (b) same as (a) but with the addition of 1 mg/ml catalase.
Figure 14. Antioxidant capacity of manuka honey. (a) Typical EPR spectra obtained for 50% (w/v) manuka honey, and for 50% (w/v) manuka honey is incubated with H\textsubscript{2}O\textsubscript{2} + FeSO\textsubscript{4} and DMPO, measured at (b) at time 0 and (c) after 5 minutes following addition of H\textsubscript{2}O\textsubscript{2}.

![EPR spectra for manuka honey](image)

Figure 15. Antioxidant capacity of pasture honey. (a) Typical EPR spectra obtained for 50% (w/v) pasture honey, measured at (b) at time 0 and (c) after 1 hour.

![EPR spectra for pasture honey](image)
4.4 Discussion

As discussed in the introduction, one source of antibacterial activity in honey is the production of hydrogen peroxide by the enzyme glucose oxidase present in honey. The results obtained in this section show that honeys of high antibacterial capability, like manuka honey, do not necessarily have a high hydrogen peroxide generation capability, and that even the honeys that generate hydrogen peroxide and free radicals through the Fenton reaction such as pasture honey also have an antioxidant activity. The EPR spectroscopy allowed the detection of any free radicals produced via the Fenton reaction in the presence of hydrogen peroxide produced by honey, and the results obtained demonstrate that manuka honey did not produce any detectable levels of hydrogen peroxide using EPR spectroscopy, but still showed a high antibacterial activity, equivalent to 18% (w/v) phenol. Furthermore, the quenching assays, both using the EPR spectroscopy and the xanthine oxidase method demonstrated that different honey possesses different antioxidant potentials.

Honey has been shown to elicit the spontaneous synthesis of cytokines by monocytes that have the potential to mediate the immune response (Tonks et al. 2001; Tonks et al. 2003). Some immunological cells, like neutrophils and macrophages, utilise the presence of free radicals like the superoxide and hydroxyl radicals to modulate the activity of other cells such as monocytes and platelets, when responding to challenges (Burdon 1995). Nevertheless in dealing with a prolonged insult, like a chronic infection, such a stimulus may give rise to an excessive response. The ability to dampen free radicals may therefore contribute to the complex interaction that helps to resolve the state of chronic inflammation that typifies chronic wounds.
When *Pseudomonas aeruginosa* invades human tissue it induces free radical formation as a means to enhance invasion and this can lead to cellular damage (Britigan *et al.* 1992; Nunoshiba *et al.* 1995; Wang *et al.* 1995). Honey has already been shown *in vitro* to be bactericidal for *Pseudomonas aeruginosa* (Cooper *et al.* 2002a) and has been suggested as a topical treatment for wounds infected with this bacterium. This study provides evidence that manuka honey especially has the ability to quench free radicals, which could minimise cellular effects of bacterial infection as well as chronic inflammation.

In a previous study of the antioxidant potential of honey, phenolic acid concentration was measured (Al-Mamary *et al.* 2002). A more recent study has indicated that the consumption of honey can provide protection to healthy humans by increasing antioxidant activity of plasma (Schramm *et al.* 2003). This study demonstrates that different honeys possess varying capabilities to quench superoxide and hydroxyl radicals *in vitro*. This is an important factor when considering using honey in a clinical context because both antibacterial activity and antioxidant potential should be evaluated. It is important to note that the honey with the highest antibacterial activity tested here (manuka honey) did not produce detectable free radicals in the presence of the components of the Fenton reaction using this sensitive EPR technique.

The nature of the antibacterial activity of manuka is unknown. Although activity has been demonstrated *in vitro* in the presence of catalase (Al-Mamary *et al.* 2002; Allen *et al.* 1991), and the activity therefore been called non-peroxide activity, this activity has paradoxically been attributed to hydrogen peroxide on the basis that the levels of
catalase utilised in the bioassay were insufficient to inactivate hydrogen peroxide (Weston 2000). This idea has since been refuted by (Snow and Manley-Harris 2004) using increased amount of catalase (10-fold increase) in relation to the normal concentration used and no statistically significant difference was observed.

It has been shown that the optimal dilutions at which honeys will produce the maximum amount of hydrogen peroxide is between 40 and 60% (w/v) (Bang et al. 2003). In this study the failure to detect hydroxyl radicals using EPR in the presence of the reagents needed for the Fenton reaction to take place, indicates that hydrogen peroxide was not generated when manuka was diluted by a factor of at least 2 in vitro. The results presented here, therefore, confirm previous suggestions that antibacterial activity in this honey is caused by components other than hydrogen peroxide. Furthermore the production of radicals was observed in pasture honey, a hydrogen peroxide generating honey.

Pasture honey produces hydrogen peroxide upon dilution, and the addition of the reagents for the Fenton reaction enabled the detection of a profile in the EPR, although it was not the traditional hydroxyl radical profile. It is possible to say that the radicals formed are the product of the Fenton reaction because when catalase was added this profile disappeared. Probably the hydroxyl radicals, being very reactive substances, reacted with organic materials in the honey and created a different profile to the one usually attributed to hydroxyl radicals. Artificial and commercial honey could not produce any radicals because the first never possessed the enzyme needed for its production, and the second had the enzyme inactivated (due to the heat

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treatment that it had received prior to commercialisation). Hence these honeys, together with manuka honey did not show the presence of any radicals.

When the results of the quenching potential of these different types of honey are related to the results obtained for the colour determination, it is possible to verify that darker honeys like manuka, West Wales and pasture honeys (amber colour) are more effective antioxidants than the lighter honeys, artificial and commercial (extra white and light amber, respectively). These results confirm data obtained by Taormina et al. (2001) that demonstrated that darker honeys have a higher antioxidant and antibacterial potential. Perhaps one of the ways through which honey was selected locally for use in Medicine was through the selection of darker honeys. In order for the relationship between colour and antioxidant potential to be explored, a greater number of honey samples need to be tested. Although sugars alone do not possess as high antimicrobial activity as honey, they demonstrate radical quenching potential in these assays. This is not uncommon and other researchers have demonstrated this (Morelli et al. 2003; Park et al. 2003)

Honeys with a hydrogen-peroxide-derived antibacterial activity are useful for medical purposes for their effective action against infection, nevertheless this work has demonstrated that these honeys also possess the capacity of producing free radicals, as is the case of Pasture honey. This honey has also shown a high free radical quenching capacity in the superoxide enzymatic assay (Fig.11) and also in the quenching of the hydroxyl radicals observed through EPR spectroscopy (Fig.15). It seems that this honey has a modulatory effect on its own production of free radicals as the amount detectable decreased with incubation time. It is possible to speculate that this can have
a benefit *in vivo* by acting as an early signal for the recruitment of macrophages and neutrophils to the site of infection and as an antibacterial agent at the same time, because bacterial cell walls are also susceptible to attack by free radicals (Nunoshiba *et al.* 1995). These results suggest that the use of hydrogen peroxide-generating honeys, such as pasture honey, that produce some free radicals, may not be detrimental to the wound healing process, as they seem to possess a self modulatory capacity that decreases the activity of these radicals.

This research has investigated the relationship between antioxidant potential and different types of honey, as well as the relation between the antibacterial activity and the production of hydrogen peroxide *in vitro*. This is only one of the steps necessary to understand the complex action of honey on wounds and other types of infection, and there is a need to continue this work with *in vivo* studies that are vital for the full understanding and acceptance of honey as a real alternative to modern antibacterial agents.
5. An investigation into the kinetics of death of *Staphylococcus aureus* and *Pseudomonas aeruginosa*
5.1 Introduction

Understanding the mode of action of a new antimicrobial agent is necessary for it to be used appropriately and safely. Although honey is not a new antimicrobial agent, its use in conventional medicine is recent, with the introduction of the first manuka honey impregnated wound dressing on to drug tariff in the UK in March 2004. Further research into the mechanism of action of manuka honey on relevant wound-infecting pathogens is considered necessary, if honey is to be accepted by healthcare practitioners (Molan and Betts 2004). Appropriate evidence on the kinetics of death, such as time-to-kill studies, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration values has been suggested (Stratton 2003).

*Staphylococcus aureus* and *Pseudomonas aeruginosa*, as representative species of common wound infecting bacteria were selected for antimicrobial studies because they are among the bacterial species that incur the biggest problems to control and eradicate (Howell-Jones et al. 2005). *Staph. aureus* is the most common wound infecting pathogen; it is ubiquitously associated with human skin and may become an opportunistic infectious agent when there is a break in the barrier provided by the skin (Lowy 2003). With the increase in the use of antimicrobial agents, antibiotic-resistant *Staph. aureus* have increased in incidence, both in hospital and the community, one of the most worrying being methicillin-resistant *Staph. aureus* or MRSA, which have increased in incidence in the last decade (Ang et al. 2004b; Howell-Jones et al. 2005), the treatment of which is difficult due to the fact that some strains can be resistant to more than one antimicrobial agent. Another common wound pathogen known for its resistance is *Pseud. aeruginosa*, due to its ability to form biofilms in wounds that lead
to persistent infection due to the difficulty in eliminating all the bacterial cells from the wound, and also for its natural resistance to antimicrobial agents both in wounds and in nature (Gilbert and McBain 2003b).

It is important to test new antimicrobial agents against both laboratory reference and clinical strains (Cooper et al. 2002a). Strains of bacteria collected from environmental sources, in this case wounds, have been subjected to environmental pressures that may have altered their susceptibility to antimicrobials, and bacteria collected from different wounds may respond differently, as they may have been subjected to different environmental conditions.

Organisms held in stock cultures, as is the case of laboratory strains, can lose their virulence and/or viability during long term storage, be it at room temperature, lyophilised or in frozen stocks. This has been described for many different microorganisms ranging from *Escherichia coli* (Acha et al. 2005), to *Clostridium difficile* (Freeman and Wilcox 2003), *Mycobacterium* sp. (Nascimento and Leite 2005) and amoebae (Gupta and Das 1999).

Accurate antimicrobial testing is important for the correct determination of the usefulness of an antimicrobial agent, in the early years there was no standardization between laboratories and the results for the antimicrobial agent differed greatly as different concentrations of inocula and antimicrobial agents were used (Piddock 1990). Since this problem was first recognised in 1952 standardised techniques have been developed for susceptibility testing among them are the methods developed by the Clinical Laboratory Standards Institute, CLSI (formerly known as NCCLS) in the
USA, which publishes revised methods for antimicrobial susceptibility testing every 3 years (Piddock 1990), and the British Society for Antimicrobials and Chemotherapy (BSAC). These methods thoroughly describe inocula size, media and antimicrobial dilutions, as well as provide reference tables to assess resistance or susceptibility to a particular antimicrobial agent and also quality control protocols.

The use of a liquid media based antimicrobial susceptibility assay is more practical in relation to the solid media tests as it allows for the determination of the MBC, and allows the possibility of scale down to a microtiter plate, which in turn allows for a bigger number of replicates and assays to be performed with a minimum amount of antimicrobial agent (Piddock 1990). If resistance is observed in vitro it is likely to be present in vivo, although susceptibility does not have such a clear cut interpretation, as in vivo there are more interfering substances than in the standardized laboratory assays (Varaldo 2002).

Time-to-kill, MIC/MBC, time for commitment to death and total cell counts were determined here for manuka honey, acting on Pseud. aeruginosa and Staph. aureus. An investigation was also undertaken to assess the ability of the bacteria to develop resistance to manuka honey, since the rapid emergence of honey-resistant microbes would seriously limit the effectiveness of this alternative antimicrobial.

Recently there has been some debate among the scientific community regarding what happens to cells when they cease to be culturable. Cells that could not be cultured have been considered to be non-viable, nevertheless it seems that some bacteria can enter a dormant state in which they retain metabolic activity but cannot be cultured,
and this state has been termed the viable but non-culturable (VBNC) state (Barer and Harwood 1999; Byrd et al. 1991; Heim et al. 2002b; Kell et al. 1998; Oliver 2005; Roszak and Colwell 1987). The triggers behind the transition from culturable to non-culturable are not fully understood. In many bacteria this state is a response to starvation, as is the case with *Escherichia coli* (Nystrom 2003), whilst in others it is a response to adverse environmental conditions, such as low temperature, as is the case of *Vibrio vulnificus* (Barer and Harwood 1999). One of the main characteristics of this state is that although the culturable counts obtained by plating techniques (previously regarded as viable counts) decrease significantly over the course of time, the total cell count decreased very slowly over time. The number of viable cells are predicted to decrease at a rate between that of the total cell counts and that the culturable counts (Kell et al. 1998).

In order to determine if the honey could be inducing a VBNC state in *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the total counts were determined and compared with the culturable counts.
5.2 Methods

5.2.1 Cultures and honeys used

Three type cultures were used *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* NCTC10017 and *Staphylococcus aureus* NCTC 6571, also 10 clinical isolates (five MRSA and five *Pseud. aeruginosa*) from a cohort of bacteria collected from wounds and held at UWIC. MRSA 18 was isolated from a patient successfully treated with manuka honey at a local hospital (Natarajan et al. 2001)

Three types of honey were used: a hydrogen peroxide-generating honey (pasture honey, PS9) and two non-hydrogen peroxide generating honeys (manuka honeys), one sterile (SH) (kindly supplied by the New Zealand natural Food Company) and the other non-sterile (M109). Manuka honey provided by Prof. Molan.

5.2.2 Antibacterial activity of honey

As described in section 2.2.3.

The antibacterial activity of M109 was assayed at the start of this project and at different time points during the course of this research because anecdotal evidence suggested that it changed with time. The activity of M109 was compared to penicillin (Sodium salt, Sigma, 1650 Units/mg) by the same method. This bioassay was also used to determine the effect of pH and heat on manuka honey. Aliquots of a 50% (w/v) solution of M109 manuka honey were adjusted to pH 1, 7, and 10 through the
addition of HCL (1 M) or NaOH (1 M) before using in the bioassay pH was measured using a Chemlab instrument pH 1000 meter previously calibrated using buffers of known pH (Fisher). To determine the effect of heat, aliquots of a 50% (w/v) solution of M109 manuka honey was heated at 80°C for 30, 60 and 120 minutes before using in the bioassay.

5.2.2 Minimum Inhibitory Concentration (MIC) according to the CLSI methods and BSAC methods.

The Minimum Inhibitory Concentration is the lowest concentration of an antimicrobial agent (in this case honey) that prevents the growth of a microorganism. The CLSI and BSAC method are standards methods for susceptibility testing of antimicrobial agents commonly used in hospital laboratories around the world. The CLSI microdilution method of determining MIC was described in (Ferraro et al. 2003). The BSAC method was is based on the CLSI method except that iso-Sensitest Broth (Oxoid) was used, BSAC does not possess a defined method for broth microdilution assays, nevertheless it uses iso-sensitest agar for use in their antibiotic susceptibility test method, which is also similar to the CLSI method. The use of the isosensitest broth with a method based on the CLSI method has been described by (Koeth et al. 2000).
5.2.3 Minimum Inhibitory Concentration (MIC) by tube dilution

Khan tubes containing 2 ml in total were used. To 1 ml of double strength nutrient broth, was added 1 ml of double the desired final concentration of honey made up in the appropriate diluent. The diluents were sterile deionised water to determine total activity, 1 mg/ml catalase solution (Sigma) for non-hydrogen peroxide derived antibacterial activity, and 90% (v/v) Foetal Calf Serum to determine the effect of proteins on the antibacterial activity of honey (Bryant 1972). An overnight broth culture of the test organism was used as an inoculum without dilution; each tube was inoculated with 10 μl and incubated at 37°C for 24 hours. TVC of the inocula were routinely performed after the tubes were inoculated to determine the population size of inocula, to check retrospectively that each tube had been inoculated with approximately 10⁶ cells. Growth or no growth was recorded by noting turbidity and appropriate positive (single strength broth and inoculum) and negative controls (broth and honey) were included to aid interpretation. Tubes with the lowest concentration without growth were recorded as the MIC.

5.2.4 Minimum Inhibitory Concentration (MIC) in Microtiter plates

As described above in section 5.2.3 but in a 96-well microtiter plate with flat bottom and lid, with a total volume of 200 μl and an inoculum of 1 μl for each well. The plate was incubated at 37°C for 24 hours before measuring turbidity at 400 nm in a plate reader (Anthos Labtec Instruments). TVCs were performed to determine the population size of inocula. Appropriate positive (broth and inoculum) and negative
controls (broth and honey) were included. Wells with the lowest concentration without growth were regarded as the MIC.

5.2.5 Minimum Bactericidal Concentration (MBC)

From the plate wells and the tubes with no growth, 20 µl was removed and plated onto nutrient agar (Oxoid) that was then incubated at 37°C for 24 hours to determine MBC. The lowest concentration to show no growth was the MBC.

5.2.6 Time-Kill Curves

A 40 µl volume of an overnight culture in nutrient broth (NB) (Oxoid) of the bacteria was incubated at 37°C with shaking in 20 ml of nutrient broth alone or nutrient broth containing 10% (w/v) or 20% (w/v) M109 manuka honey for Staph. aureus and Pseud. aeruginosa respectively. Samples were removed at known time intervals (30 minutes) usually up to 6 hours and total culturable counts were determined using the Miles and Misra surface drop count method (Drabu and Blakemore 1990; Levett 1991; Tweats et al. 1981). Time-kill curves were plotted and trendlines used to extrapolate estimated times required to achieve 3 decimal log reductions, which demonstrates 99.9% reduction in total number and which is considered to demonstrate bactericidal activity (Duarte et al. 2003; Montero et al. 2002).

5.2.7 Total cell and culturable counts

A time-to-kill experiment was performed as described in section 5.2.6.
Briefly, 40 µl volume of an overnight culture in nutrient broth (NB) (Oxoid) of the bacteria was incubated at 37°C with shaking in 20 ml nutrient broth alone or nutrient broth containing 10% (w/v) or 20% (w/v) M109 manuka honey for *Staph. aureus* and *Pseud. aeruginosa* respectively. Samples of 5 ml were removed at known time intervals (1 hour) up to 6 hours. The samples were filtered through a reusable 0.22 µm syringe filter (Millex GV, Fisher Scientific, UK). Using flame sterilised forceps the membrane filter was removed and added to 5 ml of sterile nutrient broth (Oxoid), and mixed in a vortex mixer at top speed for 60 seconds.

For the total culturable counts the method used was the Miles and Misra surface drop method. To determine the total cell count a bacterial counting kit (Molecular Probes, USA) that allowed quantification of the total number of bacteria present using flow cytometry was used. In this method 1 ml of the cell suspension was used and 1 µl of SYTO green dye (Molecular Probes, USA) was added and incubated at room temperature for 15 minutes. After this incubation 10 µl of the fluorescent beads (Molecular Probes, USA) was added and the solution vortexed at full strength for 1 minutes to disrupt any clumps. This solution was used for flow cytometry using the FACS machine (Cytomics FC 500 MPL, Becton-Dickinson). In order to detect which signals were bacteria and which signals were beads, solutions of beads in media (no cells) and cells in media (no beads) were used and gates used to limit the areas were each type of signal would appear, so that when the test samples were applied the signals due to the fluorescence of the beads would appear in the gate determined to be from the beads and the cells in the remaining gate. At the end of each run 5000 events (or 5000 signals) were recorded, the total number of cells was determined by dividing
the total number of events in the bacteria gate by the number of events in the beads
gate. This number was then multiplied by $10^6$ to give the number of cells per ml.

5.2.8 Resistance training

Resistance training was attempted by two methods: single concentration exposure and
rising concentration exposure (Thomas et al. 2000). For resistance training by single
concentration exposure 20 ml cultures were set up at 37° in the shaking water bath
using a sub lethal concentration of 2.5% (w/v) sterile honey in nutrient broth (NB) for
Staph aureus NCTC 10017 and 8% (w/v) for Pseud. aeruginosa ATCC 27853. These
were then sequentially subcultured 10 times by taking 40 μl of 24-hour culture into
fresh 20 ml honey suspension.

For resistance training by increasing concentration, cultures were set up as above,
using a starting concentration of 1% (w/ v) sterile honey in NB for Staph. aureus and
5% (w/v) in NB for Pseudomonas aeruginosa, and this was increased stepwise by
0.5% (w/ v) intervals for Staph. aureus and 1% intervals for Pseud. aeruginosa for 10
subcultures with incubation interval of 24 hours at 37°C in a shaking water bath.
Throughout the resistance training, the identity of each resistant culture was
confirmed by BBL crystal kits (BD).

MIC and MBCs of the final isolates obtained from both methods were determined and
compared with initial MIC and MBCs. Any shown to have an increased resistance
was subcultured a further 6 times into fresh nutrient broth without honey and the MIC
and MBC were determined again. The difference between the initial and the final
MIC and MBC was analysed using MiniTab 4.0 as the tool for statistical analysis and performing the Mann-Whitney statistical test on the results obtained for the MIC and MBCs.

5.2.9 Commitment to death

Time-to-kill experiments were performed for Staph. aureus NCTC 10017 and Pseud. aeruginosa ATCC 27853 as described in section 6.2.6, but instead of total viable counts being determined samples of the honey-treated cells (100 μl) were collected in 1 hour intervals for 8 hours and then a further sample at 24 hours and added to 10 ml of single strength nutrient broth (Oxoid) and incubated over night. Growth (turbidity) or no growth (no turbidity) was recorded and time for commitment to death was considered the first time point where no growth was detected.
5.3 Results

5.3.1 Antibacterial activity of honey

PS9 had an average phenol equivalent for total activity of 16.4% (w/v) and no detectable non-hydrogen peroxide activity, M109 had an average total activity (Table 8) equivalent to 18.45% (w/v) and non-hydrogen peroxide-derived activity equivalent to 18.36% (w/v) and for sterile honey the average values were 17.3% (w/v) for total activity and 16.8% (w/v) for non-hydrogen peroxide-derived activity.

M109 was received in UWIC in January 2003 and was stored in a cold room in the dark kept at 4°C and its potency did seem to change with time (Table 8). Using the Mann-Whitney test there is a statistically significant difference between all the time points (p<0.05).

Table 8. Increase in the potency of manuka honey M109 over time as determined using the bioassay method.

<table>
<thead>
<tr>
<th>Activity related to phenol % (w/v)</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity</td>
<td>17.6 ± 1.9</td>
<td>16.8 ± 1.1</td>
<td>20.9 ± 3.7</td>
</tr>
<tr>
<td>Non-peroxide activity</td>
<td>17.2 ± 2.5</td>
<td>16.7 ± 1.1</td>
<td>21.2 ± 1.4</td>
</tr>
</tbody>
</table>

Undiluted manuka honey showed an activity equivalent to 1.1 mg/ml of penicillin solution using the same bioassay method developed for the comparison of honey to phenol standards. The potency of M109 manuka honey did not seem to be affected by
heating at 80°C however activity became undetectable on increasing the pH to 10 (Table 9).

Table 9. The effect of temperature and pH on the total (peroxide and non-peroxide) antibacterial activity of M109 manuka honey.

<table>
<thead>
<tr>
<th>No treatment</th>
<th>Effects of temperature</th>
<th>Effects of pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80°C for 30min</td>
<td>80°C for 60min</td>
</tr>
<tr>
<td>Total activity related to phenol</td>
<td>18.7</td>
<td>17.1</td>
</tr>
<tr>
<td>ND: Not detectable</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3.2 Minimum Inhibitory Concentration (MIC) according to the CLSI methods and BSAC methods.

Determining MIC of M109 against *Staph. aureus* and *Pseud. aeruginosa* was not consistent for all of the methods used (Table 10). There were statistically significant differences (p<0.05) between the different methods used.
Table 10. MIC values for M109 against *Staph. aureus* and *Pseud. aeruginosa* determined by different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th><em>Staph. aureus</em></th>
<th></th>
<th><em>Pseud. aeruginosa</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median % (w/v)</td>
<td>n</td>
<td>Range</td>
<td>Median%</td>
</tr>
<tr>
<td>Microtiter plate</td>
<td>2.9</td>
<td>10</td>
<td>2.5-3.4</td>
<td>9.5</td>
</tr>
<tr>
<td>Tubes</td>
<td>2.2</td>
<td>4</td>
<td>2.2-2.4</td>
<td>10</td>
</tr>
<tr>
<td>CLSI</td>
<td>3.5</td>
<td>8</td>
<td>3-4</td>
<td>12</td>
</tr>
<tr>
<td>BSAC</td>
<td>4.5</td>
<td>8</td>
<td>4.5-5</td>
<td>12</td>
</tr>
</tbody>
</table>

5.3.3 MIC and MBC in tubes and microtiter plates

The results of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination in tubes and in microtiter plates are shown below in Table 11 and 12 respectively.
Table 11. Median MIC and MBC values determined in tubes with three honey samples (M109, SH and PS9) and two type cultures (*Staph. aureus* and *Pseud. aeruginosa*).

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas aeruginosa ATCC 27853</th>
<th>Staph. aureus NCTC 10017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (%w/v)</td>
<td>Range</td>
</tr>
<tr>
<td>M109</td>
<td>10</td>
<td>10-12</td>
</tr>
<tr>
<td>M109 + catalase</td>
<td>15</td>
<td>12-20</td>
</tr>
<tr>
<td>M109 + serum</td>
<td>18</td>
<td>15-20</td>
</tr>
<tr>
<td>PS9</td>
<td>22</td>
<td>20-30</td>
</tr>
<tr>
<td>PS9 + catalase</td>
<td>32</td>
<td>30-32</td>
</tr>
<tr>
<td>PS9 + serum</td>
<td>30</td>
<td>30-35</td>
</tr>
<tr>
<td>SH</td>
<td>18</td>
<td>14-26</td>
</tr>
<tr>
<td>SH + catalase</td>
<td>30</td>
<td>28-30</td>
</tr>
<tr>
<td>SH + serum</td>
<td>22</td>
<td>20-25</td>
</tr>
</tbody>
</table>

Cells with the symbol (*): result could not be determined due to endospore germination during the test period.

n = number of replicates

SD = standard deviation

M109 = manuka honey (non-sterile)

PS9 = pasture honey (non-sterile)

SH = manuka honey (sterile)
Table 12. Mean MIC and MBC values determined in microtitre with three honey samples (M109, SH and PS9) and two type cultures (Staph. aureus and Pseud. aeruginosa).

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas aeruginosa ATCC 27853</th>
<th>Staph. aureus NCTC 10017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (w/v)</td>
<td>Range</td>
</tr>
<tr>
<td>M109</td>
<td>9.5</td>
<td>9-11</td>
</tr>
<tr>
<td>M109 + catalase</td>
<td>16</td>
<td>16-24</td>
</tr>
<tr>
<td>M109 + serum</td>
<td>15</td>
<td>15-15</td>
</tr>
<tr>
<td>PS9</td>
<td>19</td>
<td>14-28</td>
</tr>
<tr>
<td>PS9 + catalase</td>
<td>30</td>
<td>22-30</td>
</tr>
<tr>
<td>PS9 + serum</td>
<td>25</td>
<td>20-25</td>
</tr>
<tr>
<td>SH</td>
<td>16</td>
<td>12-18</td>
</tr>
<tr>
<td>SH + catalase</td>
<td>28</td>
<td>22-28</td>
</tr>
</tbody>
</table>

Cells with the symbol (*): result could not be determined due to endospore germination during the test period.

n = number of replicates
SD = standard deviation
M109 = manuka honey (non-sterile)
PS9 = pasture honey (non-sterile)
SH = manuka honey (sterile)

As the ratios between the MBC and MIC in all respective pairs were <4, a bactericidal mode of action was indicated. Sterile manuka honey (from the New Zealand Natural Food Company) was used in the assay of the effect of serum in the antibacterial activity of honey because the presence of serum seemed to promote the growth of the survival and subsequent growth of the natural microbial flora in pasture and manuka honeys. To obviate this difficulty sterile honey was utilised and MBC values were successfully obtained.
5.3.4 Time-kill studies

For time-to-kill studies bactericidal concentrations of honey at least twice the the value of the calculated MIC values were selected, so that lethal concentrations were present. For *Staphylococcus aureus* 10% (w/v) M109 was used and for *Pseudomonas aeruginosa* 20% (w/v) was used. Although for staphylococci 6 clinical isolates and one type culture were tested at least twice, data in Fig.15 shows representative time-kill profiles of only 3 organisms. Similarly Fig. 16 shows representative data of 3 of the 7 pseudomonads tested.

**Figure 15.** Representative curves of the results obtained in the time-to-kill experiment in *Staph. aureus* strains in nutrient broth with and without 10% (w/v) manuka honey.
Figure 16. Representative curves of the results obtained in the time-to-kill experiment with *Pseud. aeruginosa* strains in nutrient broth with and without 20% (w/v) manuka honey.

Using trendlines, 3 log reduction times and the number of survivors at 24 hours, were estimated (Table 13). All strains tested, except MRSA 18 achieved a $10^3$ decimal reduction in the number of survivors at 24 hours, but sensitivities among the clinical strains varied with estimated sterilization times ranging between 1530 and 391 minutes for *Staph. aureus* and between 321 and 92 minutes for *Pseud. aeruginosa* strains. Nevertheless, these observations support a deduction of bactericidal mode of inhibition from manuka honey against the bacteria tested.
Table 13. Estimated time for 3 log reduction in viable bacterial counts in the presence of honey for the strains of *Staph aureus* and *Pseud. aeruginosa* tested in this study.

<table>
<thead>
<tr>
<th><em>Staph aureus</em> in 10% (w/v) manuka honey</th>
<th><em>Pseud. aeruginosa</em> in 20% (w/v) manuka honey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolate Number</strong></td>
<td><strong>Time for 3 log reduction</strong></td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------</td>
</tr>
<tr>
<td><em>Staph. aureus</em> NCTC 10017</td>
<td>427 minutes</td>
</tr>
<tr>
<td>MRSA 2</td>
<td>573 minutes</td>
</tr>
<tr>
<td>MRSA 5</td>
<td>391 minutes</td>
</tr>
<tr>
<td>MRSA 8</td>
<td>1012 minutes</td>
</tr>
<tr>
<td>MRSA 15</td>
<td>689 minutes</td>
</tr>
<tr>
<td>MRSA 18</td>
<td>1530 minutes</td>
</tr>
<tr>
<td>mean</td>
<td>770 minutes</td>
</tr>
</tbody>
</table>

*Note:* The numbers given to the clinical strain tested refers to the stock number given by Dr. Rose Cooper when the isolates were received.

### 5.3.5 Total and culturable counts

When time-kill experiments were monitored simultaneously by total culturable counts or TVC and total cell counts it was realised that trends did not concur. Where as the culturability of *Staph. aureus* and *Pseud. aeruginosa* in the presence of lethal concentrations of M109 (10% and 20% respectively) decreased the total number of cells did not reduce at the same rate (Figure 18 and 19). In cultures without M109 both total and culturable number of cells increased with time (Figure 17 and 18).
Figure 17. *Staph. aureus* total and culturable cell count in nutrient broth alone and nutrient broth with 10% (w/v) manuka honey (boxes = total counts and line = culturable counts).

![Graph showing cell count over time for Staph. aureus](image)

Figure 18. *Pseud. aeruginosa* total and culturable cell count in nutrient broth alone and nutrient broth with 20% (w/v) manuka honey (boxes = total counts and line = culturable counts).

![Graph showing cell count over time for Pseud. aeruginosa](image)
5.3.6 Resistance training

No honey-resistant cultures of either bacterium were recovered by resistance training with exposure to stepwise increasing concentrations of manuka honey. Neither bacterium was recovered above their MIC values (4% (w/v) and 10% (w/v) manuka honey for *Staph. aureus* and *Pseudomonas aeruginosa* repectively). The results obtained in the training by repeated exposure to a sub-lethal concentration of M109 are shown in Table 14. The MIC for *Pseudomonas aeruginosa* MIC was significantly different from the original value, and this apparently resistant strain was grown for 6 sub-cultures in honey-free medium and the MIC re-tested. The MIC and MBC after this period were not significantly different from the original MIC and MBC (p>0.05).

**Table 14.** Resistance training MIC and MBC determination of the test strain before and after training.

<table>
<thead>
<tr>
<th></th>
<th><em>Staph. aureus NCTC 10017</em></th>
<th></th>
<th><em>Pseudomonas aeruginosa ATCC 27853</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before training</td>
<td>After training</td>
<td>Before training</td>
</tr>
<tr>
<td></td>
<td>Mean n SD</td>
<td>Mean n SD</td>
<td>Mean n SD</td>
</tr>
<tr>
<td>MIC</td>
<td>2.8 5 0.4 3.8</td>
<td>5 0.8 0.09</td>
<td>15.7 8 2.0</td>
</tr>
<tr>
<td>MBC</td>
<td>7 5 2.8 7.4</td>
<td>5 1.5 1</td>
<td>20.6 6 2.4</td>
</tr>
<tr>
<td>SH</td>
<td>5.2 5 1.8 5.2</td>
<td>5 1 0.91</td>
<td>25 10 3.2</td>
</tr>
<tr>
<td>MBC</td>
<td>13.6 5 0.9 13.6</td>
<td>5 0.9 1</td>
<td>29.3 3 1.1</td>
</tr>
</tbody>
</table>

* this was found to be significant using the Mann-Whitney statistical test

SH = sterile honey, and SHC = sterile honey and catalase.

5.3.6 Commitment to death
The commitment to death experiment showed that *Staph aureus* did not survive after 8 hours of exposure to 10% (w/v) manuka honey and that *Pseud. aeruginosa* after 4 hours exposure to 20% (w/v) manuka honey did not grow.
5.4 Discussion

From the results of the bioassay it is possible to note that the potency of this honey has increased with time during storage. Others have commented on the fact that antibacterial characteristics of honey change with time, although in some cases it increases, whilst in other it decreases in potency (Al Waili 2004c; Bogdanov 1997). Honey is an ever-transforming substance; with enzymes present that are able to effect transformations such as increase in HMF content with time or heating and constant chemical reactions taking place like sugar crystallization that change the honey’s characteristics (Khismatullina 2005; Gleiter et al. 2006).

Although honey had been discussed and treated as the alternative to antibiotics, when quantifying its antibacterial activity it is generally compared to phenol. Although phenol is an effective antiseptic it is not commonly used in the treatment of infected wounds. Also the diffusion curves of phenol and honey from wells in bioassay plates are likely to differ, and phenol may not be the best reference standard.

In order to have a better understanding of the potential of honey for the treatment of infected wounds, it was compared to a penicillin solution. The results showed that the neat honey had a potency equivalent to 1.1 mg/ml of penicillin. When taking the MIC for penicillin–sensitive Staph. aureus into account (0.12 μg/ml, (Ferraro et al. 2003), a 3% solution (MIC calculated in microtiter plates, Table 12) has a potency of 0.033 mg/ml (equivalence to penicillin) which is still at least 275 times stronger than the MIC reported for sensitive species. This is a result to which practitioners might be able to relate easier than to phenol value.
Recently a new method has been put forward on the testing of the antibacterial activity of honey, that uses a spectrophotometric method (Patton et al. 2006) instead of the traditional agar diffusion assay described in this thesis. In this new method the MIC are determined using a microtiter plate. This new method has the advantage of using less materials, being less expensive and allowing full automation and real-time monitoring (Patton et al. 2006).

There was a significant difference between the MIC when different methods were used ($p<0.05$). In the methods described by CLSI and BSAC defined media such as Iso-Sensitest broth and Mueller-Hinton broth are used, these are defined media that are advised for the testing of the susceptibility of different organisms to conventional antibiotics. The use of defined media in these cases is preferred because some ions such as calcium can decrease the activity of some antibiotics, and other increase it (Ferraro et al. 2003). In the present study nutrient broth was used. This can be justified because the wound environment is not defined and contains many products that could interfere with the action of an antimicrobial agent; furthermore, honey itself is not a defined substance like penicillin, and different honeys possess different compositions. Therefore using a defined medium would not necessarily reflect the environment in which the honey would to be used clinically.

The MIC/MBC determination showed that the results obtained using the microtitre plate and the tube dilution methods were not significantly different ($p>0.05$). In this case the MICs and MBCs calculated with the microtitre plate method were higher for both bacterial strains than the values calculated in the control, because growth or no
growth in the microtiter plate method is determined by comparing readings of optical density it can be more sensitive than the visual observation of turbidity in tubes using the naked eye. In this work the microtiter plate method was the one of choice due to the combined convenience of using less antimicrobial agent and allowing for more repeats under the same conditions (Table 10).

Antimicrobial agents with bactericidal activity are generally preferable to agents with bacteriostatic activity. By killing bacteria a barrier to the progression of healing might be removed, whilst the inhibition of growth without death has the disadvantage that once antimicrobial treatment is stopped the bacteria might grow again (Stratton 2003; Dzidic and Bedekovic 2003).

The MBC/ MIC ratio has previously been used as an indicator of mode of inhibition (bactericidal or bacteriostatic activity of antimicrobial agents) (Andreoni et al. 1985; Perez et al. 1999). When it is less than 4 the antimicrobial agent being tested is considered to have a bactericidal mode of action whilst if this ration is >4 the activity of the antimicrobial agent is considered to be bacteriostatic. All of the honeys tested had an MBC/MIC ratio <4, and were therefore considered to have a bactericidal mode of action, confirming the results of the time-kill studies.

Serum proteins are also known to interfere with the action of many antimicrobial agents, and so it was important to determine whether honey maintained bactericidal activity at achievable concentration in the clinical context (Polasa and Krishnaswamy 1987). The presence of serum increased the MIC values for all the honeys and bacteria tested, but honey still remains effective at clinically achievable
concentrations and remained bactericidal as demonstrated by the MIC/MBC ratios (Andreoni et al. 1985; Perez et al. 1999).

From the determination of the MIC/MBC in the presence of 90% Foetal Calf Serum, it was seen that M109 retained bactericidal activity (Bryant 1972) (Table 12), also the results showed that the MIC against Staph. aureus was much more affected by the action of the serum proteins in the honey than for Pseud. aeruginosa. This suggests that the mode of action of manuka honey on these two bacteria might be different (Table 12).

Wound exudates contain serum that has high concentrations of enzymes, such as catalase that can destroy the peroxide activity of a honey, thus honeys with high non-peroxide activity are more suitable for wound treatment as they retain their activity even after dilution with catalase containing fluids.

Although there was variation in rates of inhibition between each isolate (clinical and type cultures) (Figures 15 and 16), bactericidal mode of action was confirmed in most cultures tested, with type cultures being more sensitive than clinical strains, as expected, as the clinical isolates have been subjected to environmental stress that can switch on resistance mechanisms.

MICs obtained in this study were similar but not identical to values previously reported (Cooper et al. 1999; Cooper et al. 2002a; Cooper et al. 2002c). This is not unexpected as honey is a natural product and samples are never consistent, furthermore the honeys used in these studies had a slightly lower potency (from 16 to
17% w/v). Both MBC and time to kill studies confirmed that manuka honey was bactericidal for *Staph. aureus* and *Pseud. aeruginosa*. Although MICs indicated that *Staph. aureus* were more susceptible to manuka honey than *Pseud. aeruginosa*, time-kill curves showed that *Staph. aureus* at manuka concentrations approximately three times MIC were killed at a slower rate than *Pseud. aeruginosa* at concentrations twice the MIC. This difference between the rates of kill for the two bacterial species tested could be an indication of a different mode of action for manuka honey with Gram positive bacteria when compared to Gram negative bacteria. Further tests are needed with a wider range of bacteria.

Unexpectedly, the total number of cells did not decrease at the same rate as the number of culturable cells of *Staph. aureus* and *Pseud. aeruginosa* when exposed to 10% (w/v) and 20% (w/v) manuka respectively. Similar total and viable count profiles can be obtained for cells in the Viable-but-non-culturable (VBNC) where cells are alive but cannot be cultured (Roszak and Colwell 1987). This is thought to be a physiological adaptation of non-sporo forming cells so they can cope with environmental stress (starvation, temperature, osmotic concentration, oxygen concentration or exposure to light) and it was first identified in *Vibrio cholerae* and *Escherichia coli* (Xu *et al.* 1982). Since its characterization, about 60 different bacteria have been reported to enter this state (Oliver 2005) among them are some human pathogens like *Enterococcus faecalis* and *Pseudomonas aeruginosa*.

Training experiments showed that *Staph. aureus* developed no resistance to manuka honey, but it is possible that a longer period of adaptation may be needed for resistance to develop. For these experiments 10 sub culturing steps were chosen to
mimic 10 wound dressing changes, as the average treatment time for sterilization of infected wounds has been reported to be between 7 and 9 days (Natarajan et al. 2001). On the other hand *Pseud. aeruginosa* showed a significant increase in the MIC for total honey activity when the single exposure to a sub-lethal concentration of honey was used. This resistance was thought to be due to a phenotypic adaptation and not a genetic mutation, as the susceptibility levels returned to normal after culturing in media without honey. This has been observed in *Bacillus* sp. in response to environmental factors and in bacteria that live in biofilms in response to disinfectants (Haque and Russell 2004; Russell 1999).

Honey is a complex substance and microorganisms might be inhibited by a combination of antimicrobial factors present in honey, if so, the probability of resistance arising, would be expected to be reduced because microorganisms would have to develop simultaneous resistance to more than one antimicrobial factor. Nevertheless this does not mean that honey should be used indiscriminately, honey should still be utilised carefully to avoid the risk of developing resistance. Further work is needed to the test the possibility of resistance arising in these and other bacteria relevant to wound infection. Furthermore, there is a need for accurate *in vivo* studies that aim to verify all of this *in vitro* data.

The commitment to death experiment showed that cells exposed to manuka honey reached a point at which viability was irreversibly lost at, sooner in *Pseud. aeruginosa* than in *Staph. aureus*. This might have been due to the concentration difference of honey used, or to the difference in susceptibility of the bacteria themselves. Further combinations of concentrations need to be tested.
6. A study of the morphological effects of honey on bacterial cells

(Staphylococcus aureus and Pseudomonas aeruginosa)
Different antimicrobial agents act in different ways. Beta-lactam antibiotics, like penicillin and cephalosporin, interfere with bacterial growth by inhibiting the formation of cross-linkages in peptidoglycan molecules within bacterial cell walls. The aminoglycosides (eg. gentamicin) act by interfering with protein synthesis, often by binding to a ribosomal subunit or interfering with peptide bond formation. Quinolones inhibit DNA synthesis by binding to DNA gyrase, which is an enzyme needed for unwinding supercoiled DNA prior to replication (O’Meara et al. 2000). In all of these cases mode of action of the antimicrobial molecule was discovered after the identification and characterization of the active compound.

For most antibiotics the action of the antimicrobial agent is usually specific, by affecting a narrow range of substrates or enzymes, which in turn are only available in a limited number of target sites within a bacterial cell. For example, cephalosporin, a broad spectrum antibiotic effective against Gram positive and negative bacteria, is only capable of incapacitating the cells by interfering with cell wall synthesis, by interfering with peptidoglycan synthesis in the final transpeptidation needed for the cross-linking of the peptidoglycan molecules (O’Meara et al. 2000).

In some honeys, an important source of the antibacterial action has been identified as the generation of hydrogen peroxide, where it supplements the physical action of osmotic pressure (Cooper 2005c). Nevertheless taking into account the complexity of honey, it is possible that many other components have a role to play in the antibacterial activity of honey. Those honeys, whose activity is reduced by the
addition of catalase, are termed peroxide honeys, because the generation of hydrogen peroxide contributes to their activity. In the case of honeys that retain high activity in the presence of catalase the honey is a non-peroxide honey. Manuka honey is an example of such a honey. Furthermore, the non-hydrogen peroxide-derived activity in honey might be due to the combined action of some of the many different antibacterial components present in honey (acting in synergy) upon the bacterial cells.

Most of the attempts to identify the antibacterial factors in honey have been concentrated in the analysis of the chemical composition of honey (Bogdanov 1997; Snow and Manley-Harris 2004). Several attempts have been made at fractionating honey to try to isolate the extra antimicrobial factors (Bogdanov 1997; Colombara and Clark 2001; Wahdan 1998; Weston et al. 2000; Zaghloul et al. 2001). A completely different approach is to use electron microscopy to observe the type of physical damage inflicted on the bacterial cells treated an agent, in the hope that changes to structure might allow a more informed search for the antimicrobial substance responsible for such effects.

Electron microscopy has previously been used to determine the physical effect of antimicrobial agents on bacterial cells. Silva and colleagues (Silva et al. 1979) used electron microscopy to determine the effect of phenethyl alcohol on the morphology of Bacillus sp. and Streptococcus sp. This approach has also been used for the determination of the effects of biocides on bacterial cells (Hugo and Longworth 1965; Johnston et al. 2003). Recently it was also used for determining the effects of tea tree oil on Escherichia coli (Gustafson et al. 1998). In these cases electron microscopy was mainly used to determine membrane integrity and electron density of the
cytoplasmic space because loss of electron dense material was used to indicate loss of membrane constituents (Gustafson et al. 1998).

Depending on the targets of the antimicrobial agents in question, stationary and exponential phase cells can have different sensitivities to the antimicrobial agent. This is because during the exponential phase of growth there is a rapid cellular growth, which is accompanied by the synthesis of many proteins and enzymes that are targets for the antimicrobial agent. These biosynthetic routes are of lesser significance in stationary phase, when the cells are growing slowly or not at all and secondary metabolisms starts producing a different range of molecules that can act as potential targets. Hence stationary phase cells tend to be more resistant to antimicrobial action.

Eukaryotic cells possess different phases in their cell cycle that include checkpoints at the end of each phase of growth in which the cells verify if growth and division is possible and should continue which are well understood, but the bacterial cell cycle is poorly understood. Bacteria mainly divide through binary fission (Fig. 19). Following the daughter cells separation, the cells enlarge in what is called the I-period, and mature. After this period the chromosome replication starts in the a period called the C-period, and after the replication of the chromosome is complete the next period is termed the D-period and it is the time between chromosome replication and cell division or cytokinesis (Edwards 1981).
In bacterial cell cycle one point that is of special interest to many scientists is the regulation of cytokinesis, as bacterial cells are diverse in shape and possess their DNA in the cytoplasm, and not inside a nucleus like eukaryotic cells. After DNA replication there are two types of signals that initiate the DNA prior to cell division: the Min system (the family of proteins, including the MinD protein produced by the cell are negative regulator of the formation of the division plate) and the nucleoid occlusion (the division plate will only form in areas that do not contain nucleic acids). These two systems form “inhibition” zones in the cells, areas where the DNA will not segregate, this allows for an empty zone in the cells where the new cell wall for division during cytokinesis is going to be formed (Errington et al. 2003) (Fig. 20). There is more information about the formation of the division plane for rod-shaped bacteria like Bacillus subtilis, than for cocci-shaped bacteria like Staphylococcus aureus, although it is thought that the processes are similar (Koch 2000).
The new cell wall is formed with the help of the action of the FtsZ protein, this is a cytosolic protein that when DNA is segregated to the poles of the cells, forms between them and through its polymerization it forms a ring in the cell which acts as the division plane for the formation of the two new daughter cells. Once this ring is formed it acts as the scaffold for the new cell wall and it is thickened by the deposition of cell wall components like peptidoglycan. Also murosomes (vesicles containing enzymes capable of separating the two daughter cells, autolysins) are secreted into this ring. The actual regulation of all these steps is of great interest to microbiologists, as is the activity of different antimicrobial agents upon the cell cycle of bacteria (Anderson et al. 2004; Errington et al. 2003; Gueiros-Filho and Losick 2002; Sun and Margolin 2001).

The aim of this study was to understand the physical effects of exposure to honey on bacterial morphology, and to differentiate between changes induced by honey treatment in Gram positive and Gram negative bacteria in both exponential and stationary phases of growth. Therefore, *Staph. aureus* and *Pseud. aeruginosa* in both these phases of growth were studied.
6.2 Methods

Cultures of *Staph. aureus* and *Pseud. aeruginosa* in different phases of growth (exponential and stationary) were incubated in manuka honey contained in buffer solutions between 4 to 8 hours, and morphology was compared to untreated cells using scanning and transmission electron microscopy. Throughout the rest of this thesis Tris buffer (0.05 mM, pH 7.2) was used for suspension of *Staphylococcus aureus* and MOPS buffer (0.2 M, pH 7.2) for suspension of *Pseudomonas aeruginosa* unless otherwise stated.

6.2.1 Growth curves

Growth curves for each of the two type cultures used in this experiment (*Staph. aureus* NCTC 10017 and *Pseud. aeruginosa* ATCC 27853) were performed by inoculating 20 ml of Isosensitest broth with 100 μl of an overnight culture and removing 2 ml of culture at known time intervals to determine absorbance at 520 nm Cecil spectrophotometer. Optical density versus time was plotted and exponential and stationary phases were identified so that cells at appropriate phases of growth were collected in the following experiments.

6.2.2 Collection of stationary phase cells

Cultures were grown overnight in two 20 ml volumes of Isosensitest broth (Oxoid) in a shaking (100 revs/min) water bath at 37°C to achieve stationary phase cells. The total volume of culture was spun down at 3000 g for 30 minutes (MSE harrier 15/80
centrifuge, Sanyo) and re-suspended in 20 ml of 0.05 mM Tris buffer (pH 7.2) with and without 10 % (w/v) manuka honey (in the case of *Staph. aureus*) for 4 hours, or MOPS buffer (pH 7.2) with and without 20 % (w/v) manuka honey for 8 hours in the case of *Pseud. aeruginosa*.

For scanning electron microscopy (SEM), 4.5 ml of cell suspension were harvested, washed and resuspended in 1 ml of buffer, whilst for transmission electron microscopy (TEM) 3 ml of cell suspension was similarly concentrated into 1 ml of buffer. Washed cells were fixed in 750 μl of 3% glutaraldehyde (Fluka) for one hour at 4°C, then washed twice with 1.5 ml of sodium phosphate buffer (0.1 M, pH 7.4) and resuspended in 1 ml of the same buffer. The solutions were stirred in the dark and at 4°C overnight.

6.2.3 Collection of exponential phase cells

Stationary phase cultures (1 ml) were used to inoculate 100 ml of Isosensitest broth (Oxoid) and incubated for 3 hours in a shaking water bath at 37°C at 100 rpm. The samples were then processed as described in section 6.2.2

6.2.4 Preparation of cells for electron microscopy

Cells were examined by scanning and transmission electron microscopy following the method described by Lemar *et al.* (2002) except that TEM cells were embedded in Araldite resin and not Spurr. A brief description of the method follows.
Briefly, gluteraldehyde fixed cells were resuspended in buffered 1% (v/v) osmium tetroxide (OsO₄) in phosphate buffer (0.1 M, pH 7.2) for 1 hour at 4°C. This additional fixation stage prepared the lipids in the bacterial membranes for electron microscopy.

After fixation the cells were spun down in a microcentrifuge (Eppendorff 5414) and osmium tetroxide was decanted. This was replaced with 30 % (v/v) ethanol, and incubated for 5 minutes. The cells were once again spun down, the 30% ethanol removed and re-suspended in 50% (v/v) ethanol. This solution was incubated for 5 minutes. The same procedure was repeated for 50%, 70%, 90% and 100% (v/v) ethanol. If the samples could not be fully processed on the same day they would be left in 70% (v/v) ethanol and kept at 4°C.

6.2.5 Scanning electron microscopy

The samples were added to small plastic containers (labelled by cutting a roman numeral into it) that were fitted with a 0.4 μm filter membrane (Nucleopore Track-Etch membrane 13 mm, Whatman) attached to blotting paper to trap cells for critical point drying (Fig 21). Any ethanol in the sample was allowed to dissipate to the air and into the blotting paper, and 100% ethanol was added and allowed once again to evaporate taking care not to let the sample completely dry (this procedure was repeated twice). After the final wash a small volume of ethanol was added to the containers (this will be displaced in the critical point dryer).

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The samples are added to the critical point drying machine (CPD 030; Balzers, Lerchtenstein) and dried to critical point. Then the samples were mounted using double sided adhesive tape onto an aluminium slab (10 x 10 mm Jeol type, Agar Scientific). Sputter coating (gold coating) was performed in a sputter coater (S150B; Edwards, West Sussex, UK) and the images were viewed using a scanning electron microscope (20.0 kV, XL 20, XL series, Philips).

In total 8 samples were prepared for Staph. aureus and 10 for Pseud. aeruginosa. For each sample a minimum of 6 images were collected (2 high magnification and 4 low magnification) and a maximum of 14 (4 high magnification and 8 low magnification). At least 600 different cells in randomly selected fields of view were examined for each treatment and the number of damaged (holes in the cell, debris, distorted morphology) and undamaged (normal cells shape) cells was recorded.

**6.2.6 Transmission electron microscopy**

Following ethanol replacement (the suspension steps in increasing concentrations of ethanol) was complete, pellets were released into a fresh 10 ml glass container using a squirt of 100% ethanol and a cocktail stick. The pellet was allowed to stand 15 minutes in 100% ethanol, and then transferred to fresh 100% ethanol for a further 5
minutes. Then the pellet was transferred to 5 ml propylene oxide and left for 15 minutes in order to displace the ethanol inside the pellet of cells.

The pellets were transferred to Araldite resin (5 g of araldite CY212 and 5 g of DDSA are preheated, mixed together and 0.3 g of BDMA, Agar Scientific) / propylene oxide (50:50), about 9 ml per sample and left overnight in the orbitator (Agar Scientific) that rotated the samples at an angle for better infiltration of the resin into the pellet and for the propylene oxide to evaporate. The infiltrated pellets were then transferred into 5 ml of fresh resin (no propylene oxide), and left for 12 hours in the orbitator (Agar Scientific) Once this incubation was finished the pellets were transferred into moulds previously filled with full strength resin taking care to place the pellets near to the edge. The moulds were then incubated in the oven for 3 days at 60°C.

For the ultra-thin sectioning glass knives were used for the trimming and for the sectioning and an Ultratome III (LKB, Stockholm, Sweden). Before the samples could be sectioned they had to be trimmed in order to remove the excess resin around the samples and expose the pellet of cells. The sections were then mounted (placed) onto an uncoated 3.00 mm copper grid (Agar Scientific).

A measure of Nesco film was added to the tile of the designated staining area and labelled on the top with the reference names of the samples to be stained. A drop of uranyl acetate was then added on top of the film underneath the respective labels and 2 grids were added to the drop with the side containing the ultra-thin section facing down, and were incubated in the dark for 10 minutes. A drop of lead citrate was added to the film for each of the samples and the grids were transferred to the lead citrate
and incubated for 5 minutes. After this staining the grids were washed 3 times using the same procedure, but with drops of filter sterilised water.

For TEM 10 resin blocks with *Pseud. aeruginosa* were prepared and from each block at least 5 grids were prepared. Two were stained, and examined by TEM and photographed. In the case of *Staph. aureus*, 8 resin blocks were prepared and from each block a minimum of 6 grids were prepared, of which 2 were stained for visualization with TEM. The microscopy was performed in a transmission electron microscope (1210; Jeol).

Throughout these experiments cells that were not treated with honey, are referred as control cells and cells treated with honey are test cells. Electron micrographs were examined for structural changes; usually more than 600 cells were observed each for normal and abnormal appearance (such as holes, different shape, debris and septa) in both SEM and TEM images. Also, numbers of cells with complete septa were recorded in TEMs of *Staph. aureus*. Results were analysed for statistically significant differences using the Mann-Whitney test in MiniTab Statistical computing package (Version 14).
6.3 Results

6.3.1 Growth curves

The exponential cells were collected after 3 hours incubation at 37 °C in nutrient broth as indicated by the arrows in Figure 22 and 23.

**Figure 22.** *Pseud. aeruginosa* growth curve in nutrient broth.

![Figure 22](image)

**Figure 23.** *Staph. aureus* growth curve in nutrient broth.

![Figure 23](image)
6.3.2 *Staph. aureus* electron microscopy

Using the scanning electron microscope control cells (Figure 24) showed cells with normal morphology (regular cocci) whilst *Staph. aureus* samples incubated with 10% w/v manuka honey for 4 hours cells, some physical damage (ruptured walls and irregular shape) were observed (Figure 25 and 26). The most interesting changes were observed in thin sections by transmission electron microscopy, where an increased number of septated cells (Figure 28) and cells with electron dense material (Figure 29) in honey treated samples were noted in comparison to control cells (Figure 27).

For *Staph. aureus* an average of 6 TEM micrographs with a magnification of 10 000 times (for septum count) and 80 000 times for detail view for each of the 8 (4 exponential and 4 stationary) samples. For SEM an average of 8 micrographs were taken with a magnification of 10 000x (for the assessment of damage) on each of the 8 samples (4 exponential and 4 stationary) for analysis. The amount of cellular structural damage (debris, ruptured cell walls and irregular shape), observed by SEM and the number of septated cells (cells with a complete septum present), observed by TEM was determined (Table 15). The criteria for including cells in the analysis were as follows:

1) The field of view in the photo should contain the lowest possible number of clumped of cells (when clumps were present the cells in the clumps were disregarded).

2) Only whole cells (cells that were entirely in the micrograph) were counted.

3) For the septum determination, cells with a complete septum were counted and compared to those with partial or no septa.
4) To assess structural damage, cells with debris, irregular shape and rupture walls were recorded, and compared to numbers of cells without these changes.

5) The fields selected for capture were selected on the basis of medium cell density. An image with an average of about 400 cells was used (too many cells were difficult to count and few cells would have made it necessary to take more photos)

The amount of damaged cells present in Table 15 was determined using SEM and the amount of cells with fully formed septa was assessed using TEM.

Table 15. *Staph. aureus* electron microscopy results (damage and full septa).

| Treatment          | Exponential phase cells | | Stationary phase cells | | |
|--------------------|-------------------------| | | | |
|                    | % Damage | % Septa | | % Damage | % Septa | |
| No honey           | 1 (n = 612) | 20 (n = 1025) | | 1 (n = 599) | 10 (n = 1178) | |
| 10% (w/v) honey    | 10 (n = 610) | 30 (n = 1135) | | 10 (n = 634) | 20 (n = 1235) | |
| Stat. Sign.        | p = 0.014 | | | p = 0.05 | |

n = number of cells counted

It was not possible to perform statistics on the amount of damaged cell counts were accumulative, and not separated into fields of view, as in the septum formation.
Figure 24. SEM micrograph of *Staph aureus* cells after 4 hours incubation in Tris buffer (50K x magnification).

Figure 25. SEM micrograph of *Staph aureus* cells after 4 hours incubation in Tris buffer containing 10% (w/v) manuka honey (10K x magnification).
**Figure 26.** SEM micrograph of *Staph aureus* cells after 4 hours incubation in Tris buffer containing 10% (w/v) manuka honey (detail) (80K x magnification).

![SEM micrograph](image)

**Figure 27.** TEM micrograph of *Staph aureus* cells after 4 hours incubation in Tris buffer (60K x magnification).

![TEM micrograph](image)
Figure 28. TEM micrograph of *Staph aureus* cells after 4 hours incubation in Tris buffer containing 10% (w/v) manuka honey (20K x magnification).

Figure 29. TEM micrograph of *Staph aureus* cells after 4 hours incubation in Tris buffer containing 10% (w/v) manuka honey (electron dense cells) (60K x magnification).
6.3.3 *Pseud. aeruginosa* electron microscopy

For *Pseud. aeruginosa* an average of 12 TEM micrographs of each of the 10 samples (5 exponential and 5 stationary) with magnification ranging from (10 000 to 40 000x), and an average of 8 SEM micrographs with a magnification of 10 000x of the 10 samples (5 exponential and 5 stationary) were analysed.

In this study two different buffers were used. Initial attempts to analyse structural changes in *Pseudomonas* sp. employed cells suspended in Tris buffer (0.05 mM, pH 7.2), but then it was realised that Tris buffer, has been shown to alter membrane permeability in Gram negative bacteria (Irvin et al. 1981). Therefore this was replaced by MOPS buffer (0.2 M, pH 7.2). Electron micrographs obtained by TEM and SEM for *Pseud. aeruginosa* suspended in Tris buffer can be seen observed in figures 30-35.

In SEM and TEM controls there did not appear to be obvious damage nevertheless SEM of honey treated cells showed cells with holes and irregular shape (Fig. 34), TEM of treated cells present gaps in the cytoplasmic space (Fig. 31) and both control and test samples are less electron dense (Fig 31 and 32). As there was some damage observed with TEM (less electron dense) the rest of the work was carried out using MOPS which is not a buffer known to induce membrane permeability in Gram negative bacteria (which can also be confirmed by the absence of membrane damage in the electron microscopy micrographs of MOPS treated cells).
Figure 30. TEM micrograph of *Pseud. aeruginosa* cells in Tris buffer (20K x magnification).

Figure 31. TEM micrograph of *Pseud. aeruginosa* cells in Tris buffer containing 20% (w/v) manuka honey for 4 hours (20K x magnification).
Figure 32. TEM micrograph of *Pseud. aeruginosa* cells in Tris buffer for 4 hours (20K x magnification).

Figure 33. SEM micrograph of *Pseud. aeruginosa* cells in Tris buffer (80K x magnification).
Figure 34. SEM micrograph of *Pseud. aeruginosa* cells in Tris buffer containing 20% (w/v) manuka honey for 4 hours (65K x magnification).

Figure 35. SEM micrograph of *Pseud. aeruginosa* cells in Tris buffer for 4 hours (50K x magnification).
In the case of the *Pseud. aeruginosa* suspended in and visualised by SEM regular rod shaped cells for the controls with no surface changes observed (Fig. 36) (cells incubated in MOPS buffer for 8 hours) were observed. After exposure to MOPS buffer with 20% (w/v) manuka honey the number of cells presenting irregular shape (Fig. 37 and 38), blebs (membrane damage) (Fig. 38) and debris around them (membrane rupture) (Fig 37 and 38) increased. The statistical analysis of the damages observed after exposure to honey are summarized in Table 16.

Using transmission electron microscopy, control cells presented some electron dense material and the occasional mineral deposit. Electron micrograph of honey-treated cells presented electron dense deposits, possibly minerals, and some empty vacuoles inside the cells (Fig. 40 and 41) were observed in cells exposed to honey. Some empty cells (shells of cells that had a membrane rupture) were also observed (Fig. 40 and 41). It is possible that the empty spaces inside the cells could have been mineral deposits that were lost during ultra-thin sectioning. Another characteristic of honey treated cells was the fact that they appeared to have a darker cytoplasm, which is indicative of an increase in electron dense material. Septated cells were not observed using TEM in *Pseud. aeruginosa*.

**Table 16.** *Pseud. aeruginosa* damaged cells observed by SEM at 8 hours incubation MOPS buffer with or without 20% (w/v) manuka honey.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exponential phase cells</th>
<th>Stationary phase cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>No honey</em></td>
<td>2% (n =1568)</td>
<td>2% (n =2414)</td>
</tr>
<tr>
<td><em>20% (w/v) honey</em></td>
<td>80% (n =1100)</td>
<td>60% (n=283)</td>
</tr>
<tr>
<td>Stat sig.</td>
<td>p=0.0003</td>
<td>p=0.0001</td>
</tr>
</tbody>
</table>

n=number of cells observed
**Figure 36.** SEM micrograph of untreated *Pseud. aeruginosa* cells (10K x magnification).

**Figure 37.** SEM micrograph of *Pseud. aeruginosa* cells after 4 hours incubation in MOPS buffer containing 20% (w/v) manuka honey. A) debris, b) irregular cells shape c) short rods (10K x magnification).
Figure 38. SEM micrograph of *Pseud. aeruginosa* cells after 8 hours incubation in MOPS buffer containing 20% (w/v) manuka honey. a) debris, b) irregular cells shape, c) cells blebbing (10K x magnification).

![SEM micrograph of *Pseud. aeruginosa* cells](image1)

Figure 39. TEM micrograph of untreated *Pseud. aeruginosa* cells (10K x magnification, size bar = 5 μm).

![TEM micrograph of untreated *Pseud. aeruginosa* cells](image2)
Figure 40. TEM micrograph of *Pseud. aeruginosa* cells after 8 hours incubation in MOPS buffer and 20% (w/v) manuka honey (20K x magnification, size bar = 2.5 μm).

Figure 41. TEM micrograph of *Pseud. aeruginosa* cells after 8 hours incubation in MOPS buffer and 20% (w/v) manuka honey (25K x magnification, size bar = 2 μm).
The results for the EM of *Staph. aureus* (damage and septa formation) and *Pseud. aeruginosa* (damage) in the different phases of growth, stationary and exponential phases of growth (Table 15 and 16) were significantly different (*p*=0.0003 for *Pseud. aeruginosa* and *p*=0.028 for *Staph. aureus*). Exponential cells presented significantly more damage and/or septa that stationary phase cells.
Analysis of the images obtained by electron microscopy showed that both of the bacteria underwent morphological changes in the presence of honey, and that the type of damage observed was different in each species. The criteria for counting damaged cells excluded cells in clumps because the whole cell was difficult to visualize, and it was impossible to accurately estimate the extent of cell damage. For example, one edge of the cell might show no damage, but the other, which was inside a clump of cells, might have been damaged. Also only cells with fully formed septa were considered in this study as cells with a partial septum were rare it was impossible to judge whether these septa would develop later or not and lead to cytokinesis.

Using SEM with *Staph aureus* it was observed that honey induced physical changes in cells (Table 14), with an increase in the number of cells demonstrating irregular shape and increased incidence of cellular debris (Fig 26). However, the most striking change was an increase in the number of septated cells (Fig. 28) present in the honey treated cultures that was observed using TEM. In both phases of growth (*p*=0.014 for exponential phase cells and *p*=0.05 for stationary phase cells), increased septation was significant. Septa are formed immediately before cell division therefore, if the cells were healthy, this could be an indication that the cells were about to divide. If this true there would be an increase in the number of cells present, nevertheless in chapter 6 it was shown that the culturable cell count decreased almost immediately after exposure to honey. Contrary to expectation from time-kill curves (Fig. 15 and 16) the total number of cells did not decrease in parallel with the number of culturable cells (Fig. 18 and 19). It is possible to speculate that the cell division stage of the bacterial cell
cycle was interrupted just prior to cytokinesis, or that the cells were prompted to form cross wall too soon in the cell cycle and could not finish the cell division process for lack of maturity.

It is possible that the cells could have entered a viable-but-non-culturable (VBNC). In this state, cells are normally alive but cannot be cultured. This could be of importance in a clinical context because cells would be unable to multiply in the wound allowing time for the immune system to act upon an infected wound effectively. Attempts were made to determine whether the *Staph. aureus* cells increased in size, but this was not achievable. *Staph. aureus* are spherical so an increase in volume is difficult to identify because it translates into small changes in cell diameter. The presence of electron dense material is one of the characteristic of starvation responses in bacteria (Watson *et al.* 1998) and this can lead to a VBNC – type of response (Oliver 2005).

In the case of *Pseud. aeruginosa*, SEM images showed an increase in the number of cells with damage (Table 16) to their cells walls, as illustrated by the irregular shape, blebs and increase in debris (Figs 37 and 38). When considering some of the components in honey that are responsible for its antibacterial activity, such as the sugar concentration (increased osmotic pressure) (Vijaranakul *et al.* 1995) and pH (Tuttle *et al.* 1977), one would expect membrane damage to become apparent in the first instance due to the efflux of water from the inside of the cells (leading to decrease in turgor pressure, plasmolysis and cells shrinkage) (Vijaranakul *et al.* 1995) and in the case of pH inactivation of enzymes essential in the cells wall turnover (Tuttle *et al.* 1977). This would lead to a decrease in the cell volume which is observed in the SEM micrographs, by comparing the length of the rods in figure 37
with the length of the rods in Figure 39, in the latter the rods are visibly shorter, denoting a decrease in cellular volume.

A reduction of the honey-treated cell size could be observed with SEM in *Pseud. aeruginosa*, in this case the honey-treated cells have assumed an almost coccal shape, which is an indication of a stress response (Oliver 2005).

The TEM images of *Pseud. aeruginosa*, showed an increase in electron dense (dark colour) materials within the cytoplasm of honey treated cells (Fig. 40), compared with the control (Fig. 39). Also rough cell walls (Fig. 41, letter a) and vacuoles within cells were observed (Fig. 40 and 41, letter b) and these could have been sites of mineral deposits that were lost during ultra-thin sectioning. This is supported by the presence in some cells of black circular deposits inside the cells (Fig. 40, letter c), which could represent minerals that were lost during sectioning from the cells with empty pockets inside.

Increased septation was not observed in honey-treated *Pseud. aeruginosa*, but this does not mean that it was not present, because this is a rod-shaped cell, there are more planes of sectioning decreasing the chances of sectioning exactly where the septum has formed. Since this study was completed, further work has been conducted by a research assistant in UWIC that has demonstrated that at 24 hours the proportion of septated MRSA cells increased to almost 100% (Cooper, personal communication).

These observations of morphological changes in both the *Pseud. aeruginosa* and *Staph. aureus* suggest a possible entry into the VBNC state. In order to verify this an
accurate estimate of the number of viable cells would have to be obtained, this is usually obtained through flow cytometry analysis of cultures with specific viability dyes like oxonols. Several attempts have been made to quantify the number of viable cells in honey treated cultures with using DiBAC₄ (3) (Sigma) and SYTO 3 dyes by flow cytometry (Molecular Probes). To date limited success has been achieved, as the cells seemed to become impermeable to these dyes and inconsistent results have been obtained. Previous studies at UWIC (Cooper, personal communication) have failed to stain honey-treated cells with propidium iodide and ongoing student projects are investigating this problem. It appears that dead cells do not accumulate in cultures exposed to honey. It is possible that lysis quickly follows loss of viability.

Although fluorescent dyes might possibly be used to explain the difference between the total and culturable cells counts, they are unlikely to help to explain the increase in the number of septated cells observed in the honey treated Staph. aureus cultures. It seems that manuka honey induces interruption of the bacterial cell cycle just prior to division. One way of investigating this phenomena further would be to investigate the effect of honey on the expression, synthesis or activity of the enzymes murein hydrolase and peptidoglycan hydrolase, which are both, coded for in the atl gene. These enzymes are responsible for the separation of daughter cells, as well as the cells wall turnover; if they were incapacitated the cells would not be able to divide (Foster 1995; Takahashi et al. 2002).

The main conclusion to be drawn from these experiments is that Gram negative and Gram positive bacteria respond in different ways to honey treatment. In Pseud. aeruginosa the damage seems more physical in nature, whilst in Staph. aureus there
seems to be a physiological response to the exposure to honey that leads to its loss of viability.
7. A study of the physiological effects of honey on bacterial cells

(Staphylococcus aureus and Pseudomonas aeruginosa)
Electron microscopy (Chapter 6) showed that following incubation of *Staph. aureus* with honey for 4 hours, the proportion of cells with a complete septum increased. It is possible that an interruption in the daughter cell separation step of the cell cycle that in bacteria is mediated by the products of the *atl* gene (Takahashi *et al.* 2002; Foster 1995) and one way this hypothesis could be verified would be to screen genetic mutants for changed sensitivity to manuka honey. Using the data collected through electron microscopy it seems that the effects on *Pseud. aeruginosa* seem to be more of a physical nature, whilst the effects in *Staph. aureus* seem to be more of a physiological nature.

In order to better understand the effects of honey upon the cytokinesis process and upon the stress response of the bacterial cells, which is responsible for the survival of cells in response to starvation, two *Staph. aureus* mutant strains deficient in the *atl* gene (responsible for the precursor that generates the enzymes responsible for cytokinesis) and *sigB* gene (responsible for the expression of the Sigma factor B which protects cells from stress, by helping in the correct folding and translocation of proteins, among other tasks), will be assayed for changes in susceptibility to honey in comparison to the parental strains.

The antimicrobial activity of honey has different effects in Gram positive and Gram negative bacteria. In order to have a better understanding of the physiological changes that led *Staph. aureus* to an increase in the number of septated cells a better understanding of how the cellular protein profile (proteome) changes in the presence
of honey. Furthermore, there is a need to further understand the physiological changes that are brought about in Gram negative bacteria, like *Pseud. aeruginosa*, in order to compare to those observed in Gram positive bacteria. This information would allow a better understanding of the manuka honey mode of action.

Biocidal agents can be divided into 6 classes: oxidizing agents (eg. hydrogen peroxide and hypochlorite), alkylating agents (eg. formaldehyde and glutaraldehyde), metal ion binding agents (eg. EDTA), nucleic acid binding agents (eg. acridine dyes), protein denaturing agents (eg. phenols and alcohol) and agents that interact with proteins (eg. cationic detergents). Their potency is dependent upon their capacity to reach their intracellular target, so uptake of a biocide is vital. In order to study the mechanism of action of biocides different methods can be employed, these can include analysis of the surface binding and uptake characteristics, detection of membrane damage by leakage of cytoplasmic constituents and inhibition of key metabolic functions like respiration (Lambert 2004).

When bacteria are exposed to antibacterial agents bacteria can respond by altering their physiological activity (Gilbert and McBain 2003), by activating previously unexpressed genes (Gilbert and McBain 2003; Martinez and Baquero 2002), and in the case of physical damage, by leaking of molecules into the surrounding media (Tuttle *et al.* 1977; Gilbert and McBain 2003). If the cells incur gross membrane damage, large holes may be formed allowing ATP and even large molecules like proteins, to be leaked into the surrounding media, whereas of minor membrane damage, only small molecules will be able to exit the cell, such as inorganic phosphate and potassium ions (Johnston *et al.* 2003). In order to study the effects of
manuka honey on membrane integrity, leakage of materials such as inorganic phosphate, protein and ATP will be studied by assaying their concentration in the cell free supernatant after honey treatment.

The electron transport chain, because of its central metabolic role is a suitable target for many antimicrobial agents, and as such it is considered important when trying to understand the action of honey on microbial cells to investigate the effect of inhibitory agents on respiration and ATP synthesis (Cabral 1993; Murray and Murro 1991). An oxygen electrode will used to determine the capacity of honey treated cells to respond to nutrient stimuli, to assess their rate of endogenous oxygen consumption and determine whether energy conservation is coupled to the electron transport chain, by using the uncoupling agent CCCP.

An investigation into the effects of Medihoney on *Escherichia coli* K12 (Blair 2004) using microarray technology, revealed that honey produces a general down regulation of genes involved in protein synthesis. No further work has been developed into using this technology to study the effects of honey on Gram positive bacteria. In UWIC microarray technology is not available so a different approach was used to the effects of manuka honey on Gram positive bacteria protein profile (proteome).

Whilst the microarray technology allows for the study to protein profile by an analysis of the translation of the genes coding for them, Two-dimensional protein electrophoresis allows for the study of the final protein complement of the cell at any one time. This technique combines two steps of protein separation, the first of which separates the proteins based on a pH gradient in special strips, which are then inserted
into polyacrylamide gels where they are separated once more along an electric gradient. This allows for the proteins to be separated and a profile or proteome obtained. This can then be compared with proteomes for the same bacteria under different condition to determine the effects of an antimicrobial agent. One of the main advantages that two-dimensional protein electrophoresis besides the lower cost when compared with microarray technology, is that any bacteria can be tested using this technique (including clinical strains), whilst with the microarray technology any assay is dependent on the commercial availability of microarrays for the specific bacteria to be studied or will involve the development of in-house microarrays that can take up a great amount of time and cost.

The aim of this work is to try to elucidate the physiological changes that occur in *Pseud. aeruginosa* and *Staph. aureus* when exposed to manuka honey.
7.2 Methods

Throughout this chapter Tris buffer (0.05mM, pH7.2) and MOPS buffer (0.2M, pH 7.4) were used as well as *Staphylococcus aureus* NTCC 10017 and *Pseudomonas aeruginosa* ATCC 27853.

7.2.1 Mutant screening altered sensitivity to manuka honey

Two *Staph. aureus* mutants tested for a change in the sensitivity to manuka honey were a SigB mutant (in SH 1000, TET 5, reference number 1028) and its parental strain (SH 1000, reference number 682) and an atl mutant (in 8325-4, ERY 5, reference number 187) and its parental strain (8325-4, reference number 57), which were created in Professor Simon Foster’s laboratory in Sheffield University. The cultures were kindly provided by Professor Foster for this study.

For these 4 cultures the MIC and the MBC against M109 was tested using the microtiter plate method described in section 3.2. Statistical analysis was performed using the Mann-Whitney test in Mini-Tab 4.0, to determine whether the differences were significant.

7.2.2 The effect of manuka honey on the respiration of bacteria

Cultures of 50 ml of *Staph. aureus* and *Pseud. aeruginosa* were incubated overnight in Iso-sensitest broth and the total culturable count determined using the method described in section 6.2.6 before harvesting. Aliquots with 20 ml of the overnight
culture are spun down (15 minutes at 3 000 g) and re-suspended in either Tris/ MOPS buffer (control) or Tris/ MOPS buffer and 10 or 20 % honey (manuka, Gale’s or artificial, previously described in chapter 5) respectively.

At known time intervals 3 aliquots of 1 ml of samples (of both the test and control suspensions) were collected by centrifugation at 13 000 g and cells were washed twice in 1 ml of the appropriate buffer, Tris (0.05 M, pH 7.2) for Staph. aureus and MOPS (0.2M, pH 7.4) for Pseud. aeruginosa. After washing, 1 ml of washed cells suspended in buffer were added to a Clarke type oxygen electrode chamber (teflon O₂ permeable membrane, silver anode/platinum cathode with magnetic stirring) (Rank Brothers oxygen electrode) with mixing.

In order to determine the effect of honey incubation on the endogenous rate of respiration after the cell suspensions were added to the chamber the rate of oxygen consumption was recorded for a minimum of 2 minutes, both for cells incubated in buffer alone and in buffer with honey. After this time 40 µl (for Staph. aureus) or 100 µl (for Pseud. aeruginosa) of Isosensitest broth was added to stimulate respiration and the rate was recorded for 10 minutes (Tincu et al. 2003) in order to observe the effects of honey incubation on the response to the media stimuli.

The effect of manuka honey on cells which had not been incubated in honey was tested by recording the oxygen consumption rate after the addition of medium for 5 minutes and by adding 100 µl of a neat manuka (M109) solution (made less viscous by incubation at 50°C for 30 minutes). Changes in oxygen consumption were recorded for a further 8 minutes (total of 15 minutes).
The bacterial cultures were also treated with uncoupling agent CCCP (Sigma) (stock concentration of 0.1 mg/ml, 500 μM) to determine if the electron transport chain was coupled to ATP synthesis. Briefly, a titration curve was made to determine the optimum amount of uncoupling agent to add to the chamber in order to get the maximum stimulation of respiration (Fig. 42). For this the cells incubated in buffer were added to the oxygen electrode chamber and after recording the endogenous rate of oxygen consumption, the appropriate volume of isosensitest broth was added (40 μl for Staph aureus and 100 μl for Pseud aeruginosa). The respiration rate of the cultures was recorded for up to 2 minutes, after which different volumes of the uncoupling agent CCCP was added to the chamber. Once this concentration was determined, the endogenous and respiration rates were recorded both for cells incubated in buffer alone and in buffer with manuka honey for different periods of time and the uncoupling agent was added and the effect recorded.

Figure 42. Determination of the optimal concentration of CCCP uncoupler in Staph. aureus culture grown in isosensitest broth.
An average of 10 repeats per time point were recorded, using a data recorder. This recorded the data as percentage saturation (arbitrary units) of oxygen. In order to estimate the oxygen respiration rate in each experiment, a calibration curve was produced as follows. Maximum O₂ saturation at 37°C was taken to be 10.56 μ mol/ml (Gnaiger 2004) the respiration rates corresponding to a series of angles of electrode trace (Fig. 43) were calculated, and used to construct a calibration curve (Fig. 44). The angle, and therefore the respiration rate, for any individual experiment could then be read off the graph or calculated from the equation.

**Figure 43.** In order to calculate the rate of respiration, the angle of the respiration curve was determined as shown in this figure and used in the equation shown in Fig.44.
Figure 44. Calibration curve for determination of oxygen consumption rates based on
the measurement of the angle of the respiration curves.

\[ y = 0.0174x + 0.4259 \]

7.2.3 Leakage studies

Initially these studies were performed with cells grown in broth alone and in broth
containing manuka honey, and cell-free supernatants were collected to test for the
leakage of inorganic phosphate, ATP and proteins. However it was found that it was
not possible to detect changes even between the different concentrations of standards.
The next approach was to use suspended cells in buffer alone or buffer with honey,
and to test the culture supernatants for leaked metabolites, but it was found that honey
was the interfering substance. Therefore, it was decided to use cells that were treated
in buffer alone or in buffer with honey for known intervals before cells were
resuspended in honey free buffer, and supernatants from these cell suspensions were
used for the determination of leaked metabolites (Cox et al. 1998).
Hence two litre cultures of either *Staph. aureus* NCTC 10017 or *Pseud. aeruginosa* ATCC 27853 in Isosensitest broth (Oxoid) were incubated overnight at 37°C with shaking (100 rpm). Cells were harvested by centrifugation for 5 minutes at 10 000 g (Sorval RC-5b Refrigerated Superspeed Centrifuge, Du Pont Instruments) and resuspended in 1 litre of Tris buffer (0.05 mM, pH 7.2) alone or Tris buffer with 10% (w/v) manuka honey in the case of *Staph. aureus*, or MOPS alone or MOPS (0.2M, pH 7.2) with 20% (w/v) manuka honey for *Pseud. aeruginosa*.

The suspensions were incubated at 37°C with shaking and every 30 minutes (up to 2 hours), 200 ml of cells from either the cells in buffer or cells in buffer and honey were collected and spun down for 2 minutes at 10 000 g (Sorval RC-5b Refrigerated Superspeed Centrifuge, Du Pont Instruments). Pellets were resuspended in 9 ml of buffer (Tris for *Staph. aureus* and MOPS for *Pseud. aeruginosa*) and aliquotted in 1.5 ml portions into 6 eppendorf tubes. Each pellet was washed twice in 1.5 ml of buffer and washed cells were resuspended in 1.5 ml of their respective buffers. For 3 of the tubes the cell suspension was immediately spun down and the supernatant removed to a fresh eppendorf (labelled time 0 minutes) and the remaining 3 tubes were allowed to stand at room temperature for 15 minutes (time 15 minutes). After the 15 minutes incubation the cells were spun down and the supernatant transferred to a fresh eppendorf. Once all of the supernatants had been collected assays for inorganic phosphate, protein and ATP leaked to the buffer were performed. Summary of this method can be seen in Figure 45.
7.2.3.1 Determination of inorganic phosphate in the cell free supernatant of cells with and without manuka honey treatment

For the determination of inorganic phosphate the method of Fiske and Subbarow (1925) was used. Briefly a standard solution of KH$_2$PO$_4$ containing 1 µmole/ml that was used to prepare a range of dilutions between 0.1 and 1.0 µmole phosphate in 1 ml. To each dilution was added 1.0 ml of 10 M H$_2$SO$_4$ followed by 1.0 ml of 2.5% (w/v) ammonium molybdate solution. After mixing, 0.1 ml of a freshly prepared solution of reducing agent (0.2% w/v 1-amino-2-naphthol sulphonic acid, 1.2% w/v
sodium metabisulphite and 1.2% w/v sodium sulphite) was added and the mixture allowed to stand at room temperature for 30 minutes. Extinction at 660 nm was measured against a blank reagent containing 1.0 ml of water instead of diluted phosphate. One ml samples of culture supernatant were processed in the same way.

7.2.3.2 Determination of protein in the cell free supernatant of cells with and without manuka honey treatment

Culture supernatants were tested for protein as described in section 2.2.2.3.

7.2.3.3 Determination of ATP cell free supernatant of cells with and without manuka honey treatment

For ATP determination the UniLite® kit from Biotrace Ltd (Bridgend) was used. Reagent A was reconstituted in Reagent B immediately before use (to provide a stabilised luciferase solution). Samples (100 µl) were added to ATP free tubes, 40 µl of the swabbing solution (a releasing agent) was added and then 40 µl of the luciferase solution. The tube was mixed for 5 seconds and the resulting light measured using the BioTrace portable luminometer. ATP (Sigma) standards were diluted and measured immediately with Biotrace luminometer and a calibration curve was plotted to read the results from the test samples (Fig. 46). This method is based on the manufacturers manual.
Figure 46. Example of a calibration curve for ATP standard curve in Biotrace luminometer (RLU = relative light units).

7.2.4 2D protein electrophoresis protocol for Staph. aureus cells

When Staph. aureus cells were treated with manuka honey it was found that they leaked protein. The optimal time to extract maximum levels of protein for both test and control samples was determined with a timed extraction of proteins from Staph. aureus.

Two litres of overnight culture of Staph. aureus NCTC 10017 cells in Isosensitest broth (Oxoid) were prepared by overnight incubation at 37°C and shaking (100 rpm). The overnight culture was divided in two equal portions and harvested at 10000 g for 5 minutes (Sorval RC-5b Refrigerated Superspeed Centrifuge, Du Pont Instruments). One of the portions was resuspended in Tris buffer (0.05mM, pH 7.2) alone and the other in 10% (w/v) manuka honey in Tris buffer. Both were incubated at 37°C with shaking (100 rpm). One hundred ml of each sample were collected every 10 minutes.
for the first hour and every 30 minutes up to 2 hours and spun down at 10 000 g for 2 minutes. Once the cells were collected they were processed for protein extraction

Due to time constraints this experiment was only performed in *Staph. aureus*.

7.2.4.1 Protein extraction from *Staph aureus* cells incubated with and without manuka honey.

This method was based on the method by Kohler (2003). The pelleted cells from 7.2.4 were washed twice in 2.5 ml of ultrapure water and then resuspended in the same volume of tris EDTA buffer with 2 mM phenylmethylsulfonyl fluoride (protease inhibitor). A volume of 250 µl of 1 mg/ml lysostaphin (Sigma) was added and the solution incubated for 60 minutes at room temperature.

After the incubation the cells were sonicated (MSE Soniprep 150) at full strength for 3 periods each of one minute (note that the cells were kept in disposable 25ml centrifuge tube inside a beaker with ice to prevent overheating). Lysis was verified under a light microscope (Olympus CH-2). The resulting liquid was spun down at 21,000 g for 30 minutes at 4°C (Sorval RC-5b Refrigerated Superspeed Centrifuge, Du Pount Instruments) to remove insoluble and aggregated proteins that would interfere with the first step of two-dimensional protein electrophoresis, which is the IsoElectric Focusing (IEF).

Protein content was determined using the method described in section 2.2.2.3 and the cell free extract was either used immediately for IEF or frozen at -20°C until used.
7.2.4.2 Protein sample preparation for two dimensional protein electrophoresis

The sample rehydration buffer (1.2x) was prepared by adding 14.4 g of urea (Sigma), 0.6 g CHAPS (Sigma), 150 µl of 3-10 ampholytes (Invitrogen) and 0.6 ml of 0.1% (w/v) bromophenol blue solution to 15 ml of deionised water. The volume was adjusted to 25 ml and the resulting solution was separated in 1ml aliquots and frozen for future use. Prior to using this buffer, dithiothreitol (DTT) (Sigma) was added to a final concentration of 20 mM.

The solution for rehydration of the strips consisted of 85 µg of protein (maximum of 35 µl volume), was added to 120 µl of 1.2x rehydration buffer to give a total volume of 155 µl.

7.2.4.3 Rehydrating ZOOM® Strips

The ZOOM® IPGRunner cassette (Invitrogen) was set on a level surface with the sample loading wells facing upwards and 155 µl of sample rehydration buffer containing the protein sample loaded into each sample loading well located at the rounded edge of the ZOOM® IPGRunner cassette. The ZOOM® strip card (pH 3-10 Non-linear) (Invitrogen) was removed from its pouch and a strip was peeled away from the card backing. The protective strip cover remained on the card. Each strip had a gel side and a side with printed marking on it. The ZOOM® strip was held at the basic end (-) using forceps with the printed side facing down. Then using the fingers to guide the strip, the acidic end (+) of the strip was inserted into the enclosed channel through the sample loading well at the curved end of the ZOOM® IPGRunner cassette until acidic end (+) of the strip touched the end of the channel slot (IMPORTANT:
avoid large air bubbles, small bubbles will not affect rehydration). This procedure was repeated for as many strips as the number of samples to be run.

All sample loading wells, including unused wells, were sealed using the sealing tape provided making sure that the tape fully seals the wells and the ZOOM® IPGRunner cassette was incubated for 8-16 hours (overnight) at room temperature.

After the overnight incubation the sealing tape and the two sample loading devices were removed from the ZOOM® IPGRunner cassette. An electrode wick was placed at each end of the ZOOM® IPGRunner cassette over the adhesive and 750 µl of deionised water was evenly applied to each of the electrode wicks, any excess water was blotted off using filter paper. The ZOOM® IPGRunner Core and the IPGRunner cassette containing the rehydrated strips were placed on a flat surface in the upright position and the ZOOM® IPGRunner cassette was pushed towards the electrodes of the ZOOM® IPGRunner Core until the electrode wicks of the ZOOM® IPGRunner cassette were in contact with the electrodes of the IPGRunner core. If two cassettes were used the procedure was repeated, if not the buffer dam was used in place of the second IPGRunner cassette.

The sandwich containing the ZOOM® IPGRunner core and ZOOM® IPGRunner cassettes (or buffer dam) was held tightly together and inserted into the mini-cell chamber of the ZOOM® IPGRunner, followed by the gel tension wedge. The negative electrode must fit into the opening in the gold plate on the Mini-cell chamber.
The gel tension lever was pulled toward the front of the ZOOM® IPGRunner mini-cell until lever came to a firm stop and the ZOOM® IPGRunner sandwich is fixed. The outer chamber was then filled with 600 ml of deionised water by pouring the water through the gap between the gel tension wedge and the back of the outer chamber and taking care that water does not spill into the inner chamber.

The ZOOM® IPGRunner cell lid was placed on the ZOOM® IPGRunner core, and the system was connected to the power supply. Isoelectric focusing (IEF) was performed by running the electrophoresis using the PowerEase 500 power pack (Invitrogen) at 500 V for 5 hours (settings per strip: 500 V, 1 mA and 0.5 W).

Once IEF was finished, the lid was removed and the water from the outer chamber poured off. The gel tension wedge was unlocked and the IPGRunner sandwich removed from the mini cell and laid flat on the bench (note: the cassettes can be stored at -80°C until required for second dimension electrophoresis).

7.2.4.4 Performing SDS-PAGE using ZOOM® gels

The film cover from the cassette was peeled off and the ZOOM® strip removed from the ZOOM® IPGRunner cassette using forceps and prepared for SDS-PAGE by equilibrating the strips.

A solution of 1X NuPAGE LDS (Invitrogen) sample buffer was prepared by diluting 4X NuPAGE LDS stock solution with deionised water (10 ml for each sample). To 4.5 ml of the 1X NuPAGE LDS sample buffer 500 µl of the NuPAGE sample
reducing agent (10x) (Invitrogen) was added in a 15 ml conical tube and the ZOOM® Strip was placed in this tube and incubated for 15 minutes at room temperature (the solution was decanted when incubation finished).

An alkylating solution (125 mM) was prepared by dissolving 116 mg of fresh iodoacetamide in 5 ml of 1XNuPAGE LDS sample buffer (need 5 ml of this solution per strip) and incubating the ZOOM® strip in it for 15 minutes at room temperature (this solution was decanted when incubation finished).

7.2.4.5 SDS-PAGE

Before starting the SDS-PAGE a 0.5% (w/v) agarose (Sigma) solution was prepared in the appropriate running buffer and kept warm (55-65°C). The ZOOM® Bis-Tris 8-12% (Invitrogen) gel cassette was removed from the pouch and the tape covering peeled off.

The plastic ends of the IPG strip were cut and the strip inserted into the ZOOM® gel well. The IPG strip was aligned in the ZOOM® Gel using a thin plastic ruler or a weighing spatula avoiding air bubbles. Once the strip was in place ~400 µl of 0.5% (w/v) molten agarose solution was added into the ZOOM® Gel well containing the IPG strip taking care not to allow it to overflow into the molecular weight marker well and the agarose was allowed to solidify. The size marker Magic western marker (2.5 µl) (Invitrogen) was added to the marker well.
The gel cassette/Buffer core sandwich/ gel tension wedge was assembled, once this was firmly in place the lower buffer chamber and upper buffer chamber was filled with the 1 L of MOPS running buffer (Invitrogen). The electrophoresis was then run at 200 V for 55 minutes.

7.2.4.6 Staining of polyacrylamide gels from two dimensional protein electrophoresis

When the electrophoresis was completed the sandwich containing the ZOOM® Gel was disassembled and the gel removed from its protective case in order to be stained. In this case two consecutive stains were used: SimplyBlue (a Coomassie blue based stain from Invitrogen) and silver stain.

For the SimplyBlue stain the gel was washed 3 times for 5 minutes in ultra-pure water and then left in 20 ml of Simplyblue stain overnight. After the overnight stain, the stain was removed by washing with ultra-pure water for 2 hours, changing the water every 15 minutes. Photographs of the results were taken using the UVP AutoChemi system (UVP).

The gel was then washed for a further 30 minutes in ultra-pure water, after which it was stained with a silver stain method based on Morrisey (1980). Briefly, the gel was incubated at room temperature in a 50% methanol - 50% acetic acid solution for 30 minutes, after which it was incubated for a further 30 minutes in a 5% methanol and 7% acetic acid solution. At the end of this it was rinsed thoroughly with ultra-pure water. The gel was then fixed for 30 minutes in 5% glutaraldehyde solution and rinsed
3 times with ultra-pure water (15 minutes washes). The gel was then incubated in a 5 μg/ml solution of DTT for 30 minutes followed by 30 minutes incubation in 1% silver nitrate. The gel was then rinsed once in ultra-pure water and then in developer (1.5 g of sodium carbonate in 50 ml of ultra-pure water + 25 μl of formaldehyde) solution with no formaldehyde. Finally, the gel was developed by adding the developer with formaldehyde, the gel was agitated gently until the bands started to appear (5 to 10 minutes) and the reaction stopped at an appropriate time by the addition of 1 g of solid citric acid.

Photographs of the gels were then taken using the UVP AutoChemi imaging system (UVP) using the LabWorks UVP software, and the results were analysed by Nonlinear dynamics using the software tool Progenesis PG200. This software detected the number of different spots in each gel photograph and their intensity, and compared the profiles between the different gels. Spots were identified as those that were either upregulated or downregulated compared with control. A possible identity for some spots was proposed based on size determination and literature searches, but definitive identifications were not possible without further analytical techniques on isolated protein spots.
7.3 Results

7.3.1 Mutant screening altered sensitivity to manuka honey

The MIC and MBC determination for the mutant strains showed that the honey had bactericidal activity on all strains tested, but the atl mutant seems to be more sensitive to M109 (Table 17).

Table 17. MIC for the mutant and parental strains (autolysin and stress mutants) in the presence of manuka honey.

<table>
<thead>
<tr>
<th>Parental atl</th>
<th>atl mutant</th>
<th>Parental SigB</th>
<th>SigB mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>1.4</td>
<td>0.6</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>1.8</td>
<td>0.2</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>1.8</td>
<td>0.2</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td>1.6</td>
<td>0.8</td>
<td>2</td>
<td>1.8</td>
</tr>
<tr>
<td>1.6</td>
<td>0.6</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>1.4</td>
<td>0.4</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>1</td>
<td>0.4</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>Mean</td>
<td>1.45</td>
<td>1.825</td>
<td>1.675</td>
</tr>
<tr>
<td>SD</td>
<td>0.316228</td>
<td>0.483292</td>
<td>0.260494</td>
</tr>
</tbody>
</table>

Statistical analysis was performed on this data using the Mann-Whintey test. This showed that the difference between the parental and mutant atl strains was significant (p = 0.0009) whilst the difference between the parental and the mutant SigB mutant is not significant (p = 0.4524).
7.3.2 The effect of manuka honey on the respiration of bacteria

Cells kept in buffer alone (control cells) always demonstrated an increased rate of oxygen consumption on addition of isosensitest broth with time of incubation in honey and still responded to the addition of the uncoupling agent (Fig. 47). *Staph. aureus* cells treated with honey showed an increase in the endogenous oxygen consumption rate with time (Fig. 47). This was found not to be due to the uncoupling as the cells still responded to the addition of uncoupler CCCP (40 μM) (Fig. 42). However in the case of *Pseud. aeruginosa* the endogenous oxygen consumption rate of cells incubated in honey decreased, and the cells were also remained coupled (Fig. 49).

**Figure 47.** Example of an oxygen electrode profile (y axis = % saturation of oxygen, x axis = time in minutes).
Figure 48. Effect of incubation in 10% (w/v) manuka honey on *Staph. aureus* respiration (respiration rate minus endogenous rate). Error bars indicate range of results obtained in an average of 4 independent experiments.

Figure 49. Effect of incubation in 20% (w/v) manuka honey on *Pseud. aeruginosa* respiration (respiration rate minus endogenous rate). Error bars indicate range of results obtained in an average of 4 independent experiments.
The attempts to determine the effect of adding manuka honey to control cells in the oxygen electrode failed, as the honey added directly to the electrode settled at the bottom of the oxygen electrode chamber, even with the magnetic flea at full speed, and blocked the electrode. When other types of honey were used such as Gale’s and artificial honey, both Staph. aureus and Pseud. aeruginosa responded in the same way as with manuka honey. For Staph. aureus treated with any type of honey the endogenous rate of oxygen consumption increased, whilst oxygen consumption rates for non-honey treated cells decreased (Fig. 50). Pseud. aeruginosa incubated with any of the honeys the endogenous rate of oxygen consumption was decreased, whilst non-honey treated cells maintained a stable oxygen consumption rate (Fig. 51).

**Figure 50.** Endogenous respiration rates of Staph. aureus cells incubated in buffer containing 10% (w/v) manuka, artificial or Gale’s honey.
Figure 51. Endogenous respiration rates of *Pseud. aeruginosa* in buffer containing 20% (w/v) manuka, artificial or Gale’s honey.

7.3.3 Leakage studies

After the cells were exposed to either buffer alone or buffer with honey, the cells were washed and resuspended in buffer alone, measurements were taken of the initial levels of ATP, proteins and inorganic phosphate (represented by the columns labelled 0 minutes in figures 52-63) and levels of these products were once again determined after 15 minutes of incubation at room temperature in the buffer alone (represented by the columns labelled as 15 minutes in figures 52-63).

For *Staph. aureus* there did not seem to be any leakage of any of the metabolites studied in the control cells (Fig. 52, 54 and 56), in the treated cells there seemed to be leakage of ATP (Fig. 57) (at 120 minutes), but not of proteins (Fig. 55). There seems to be some leakage of inorganic phosphate when the profile obtained for control cells (Fig. 52) is compared to that obtained for honey-treated cells (Fig. 53).
**Figure 52.** Inorganic phosphate leakage from *Staph. aureus* cells incubated in Tris buffer at 37°C with shaking (100 rpm) (error bars show the range of results).

![Graph showing inorganic phosphate leakage](attachment:graph1.png)

**Figure 53.** Inorganic phosphate leakage in *Staph. aureus* cells in Tris buffer with 10% (w/v) manuka honey at 37°C with shaking (100 rpm) (error bars show the range of results).

![Graph showing inorganic phosphate leakage with manuka honey](attachment:graph2.png)
**Figure 54.** Protein leakage from *Staph. aureus* cells incubated in Tris buffer at 37°C with shaking (100 rpm) (error bars show the range of results).

![Graph showing protein leakage from Staph. aureus cells in Tris buffer.](image)

**Figure 55.** Protein leakage from *Staph. aureus* cells incubated in Tris buffer with 10\% (w/v) manuka honey at 37°C with shaking (100 rpm) (error bars show the range of results).

![Graph showing protein leakage from Staph. aureus cells in Tris buffer with manuka honey.](image)

There was no apparent difference between the protein released from the cells kept in buffer (Fig. 54) in comparison with the cells treated with honey (Fig. 55), as the
maximum values were found to be higher for the non-treated cells (Fig. 54) than for treated cells.

**Figure 56.** ATP leakage from *Staph. aureus* cells incubated in Tris buffer at 37°C with shaking (100rpm) (error bars show the range of results).

![Figure 56](image)

**Figure 57.** ATP leakage from *Staph. aureus* cells incubated in Tris buffer with 10% (w/v) manuka honey at 37°C with shaking (100rpm) (error bars show the range of results).

![Figure 57](image)
The profiles of ATP release in treated and non-treated cells were similar, nevertheless the maximum amount of ATP released from the non-treated cells (Fig. 56) was lower than in the treated cell group (Fig. 57), which suggested cellular damage had allowed leakage of ATP.

Although the inorganic phosphate recorded for Pseud. aeruginosa cells exposed to honey treatment showed a trend to increase (Fig. 59) the total values did not appear different from those obtained in cells incubated in buffer alone (Fig. 58). In the case of Pseud. aeruginosa, there seemed to be release of proteins into the media (Fig. 61) with at least a two-fold increase in the amount of recorded protein for the honey-treated cells in comparison with control cells (Fig. 60). There is also an increase in the amount of recorded ATP in the buffer for treated cells, with an increase of more than 10-fold between the cells kept in buffer alone (Fig. 62) and the cells kept in buffer and honey (Fig. 63).

Figure 58. Inorganic phosphate leakage from Pseud. aeruginosa cells incubated in MOPS buffer at 37°C with shaking (100 rpm) (error bars show the range of results).
The honey-treated cells showed an increasing trend in the amounts of released inorganic phosphate (Fig. 59), although the levels of phosphate detected were similar to those recorded for non-treated cells (Fig. 58). The maximum levels of inorganic phosphate recorded in the honey-treated cells was actually lower than the highest concentration of phosphate recorded in the non-treated cells. This observation together to the results obtained for inorganic phosphate leakage in Staph. aureus cells might indicate that the technique was not sufficiently sensitive for the detection of the concentrations of phosphate being leaked of the cells. For Pseud. aeruginosa, especially, electron microscopy had demonstrated major structural damage in honey-treated cells and leakage of Pi, ATP and protein was expected.
**Figure 60.** Protein leakage from *Pseud. aeruginosa* cells incubated in MOPS buffer at 37°C with shaking (100 rpm) (error bars show the range of results).

![Graph showing protein leakage from Pseud. aeruginosa cells incubated in MOPS buffer at 37°C with shaking (100 rpm).](image)

**Figure 61.** Protein leakage from *Pseud. aeruginosa* cells incubated in MOPS buffer with 20% (w/v) manuka honey at 37°C with shaking (100 rpm) (error bars show the range of results).

![Graph showing protein leakage from Pseud. aeruginosa cells incubated in MOPS buffer with 20% (w/v) manuka honey at 37°C with shaking (100 rpm).](image)

The concentration of protein recovered from supernatants of honey-treated *Pseud. aeruginosa* (Fig. 61) was almost 3 times higher than the concentration of protein
leaked in non-treated cells (Fig. 60), indicating an increase in protein leakage in the presence of honey.

**Figure 62.** ATP leakage from *Pseud. aeruginosa* cells incubated in MOPS buffer at 37°C with shaking (100 rpm) (error bars show the range of results).

![Figure 62](image)

**Figure 63.** ATP leakage from *Pseud. aeruginosa* cells incubated in MOPS buffer with 20% (w/v) manuka honey at 37°C with shaking (100 rpm) (error bars show the range of results).

![Figure 63](image)
The concentration of ATP detected in supernatants of honey treated *Pseud. aeruginosa* (Fig. 63) was 10 times higher than the concentration of ATP leaked from non-treated cells (Fig. 62) indicating leaking caused by the presence of honey.

**7.3.4 Two dimensional protein electrophoresis of *Staph aureus* cells incubated with and without honey**

The results of the time-course for cellular protein extraction (not released protein) from *Staph aureus*, showed that the amount of protein extracted from honey-treated cells decreased with time (Fig. 64), this was a consistent result for 3 extraction experiments. Although initially the aim was to use protein collected from cells exposed to buffer with 10% (w/v) manuka honey after 4 hours incubation in honey for 2-D electrophoresis, the protein concentration extracted at that time point was too low to efficiently rehydrate the IEF strips.

**Figure 64.** Collated data of the yield of protein from *Staph aureus* cells kept incubated in Tris buffer containing 10% (w/v) manuka honey (180 minutes) (arrow indicates final collection point).
It can be seen (Fig. 64 and 65) that the time point when the most protein was extracted from the treated samples was after 50 minutes incubation, so this time point was used to achieve the maximum concentration of protein to load the IEF strips.

**Figure 65.** Time-course extraction of proteins from *Staph. aureus* incubated in buffer alone (control) and buffer with 10% (w/v) manuka honey (120 minutes) (arrow indicates the final collection point).

The proteome profiles obtained with the use of 2-D protein electrophoresis showed that all times points assayed (control at 0 minutes, control after 50 minutes incubation in buffer or 50 minutes honey-treated cellular protein) had different profiles of proteins. The cellular protein collected from cells at the start of this study (cells at time 0) yielded 79 different spots (Fig. 66), whilst the cellular protein profile of the cells that were incubated in buffer alone for 50 minutes yielded 85 different spots (Fig. 67). This suggests the switching on of protein synthesis during incubation in buffer. The 2-D profile of the cellular proteins of the cells incubated in buffer with 10% (w/v) manuka honey presented 65 different spots (Fig. 68), thus demonstrating a possible down regulation of the protein synthesis. In total, 123 different spots were detected.
**Figure 66.** Two-dimensional protein electrophoresis profile of the cellular proteins of *Staph. aureus* cells incubated in buffer for 0 minutes.

**Figure 67.** Two-dimensional protein electrophoresis profile of the cellular proteins of *Staph. aureus* cells incubated in buffer for 50 minutes.
Figure 68. Two-dimensional protein electrophoresis profile of the cellular proteins of *Staph. aureus* cells incubated in buffer with 10% (w/v) manuka honey for 50 minutes.

This experiment was repeated once to ensure reproducibility. The results were similar to those shown in Figure 69, although the picture quality was not as good. A different image-capturing device (BioRad, GelDoc) was used for the second experiment, because the UVP system used in the first experiment was not available.
Figure 69. Example of matching of some of the major spots between the two repeats of gels for cellular protein profile of Staph. aureus a) is the analysed photograph and b) is the repeat of the same gel using the GelDoc system.

The analysis of the protein profile between the different gels was kindly performed by Nonlinear Dynamics using the software tool Progenesis PG200. The analysis of the photographs proved difficult as the quality of the images was poor due to the limitations of the imaging capture system used. One of the problems was encryption of the photo. The capture device used only 12 bits (8 bits in the BioRad system) and this had to be transformed into 16 bits, which led to loss of quality. The limitations of the capture device also led to an increase in the background noise (Figure 70), which made spot detecting difficult.

Figure 70. Example of background noise of the photos taken, to illustrate that it was rough when it should have been smooth.
As a baseline the spots identified in the control at time 0 minutes were used, as these were extracted from cells growing in media alone, with all the conditions necessary to thrive. Nine proteins were upregulated in the controls held in buffer without honey for 50 minutes (yellow circles in Figure 71), 21 proteins were downregulated (green in Figure 71), 5 proteins remained unchanged (light blue in Figure 71) and 50 proteins were un-matched (dark blue in Figure 71). An inference was that these could be new proteins.

**Figure 71.** Proteome profile of the cellular proteins of *Staph. aureus* after 50 minutes of incubation in buffer compared with the profile at 0 minutes incubation.

The profile of the cellular protein of honey-treated *Staph. aureus* was compared to both control at 0 minutes and to control at 50 minutes. When comparing the treated
Sample profile to the control it was possible to deduce that 6 proteins were upregulated in the honey-treated cells (yellow circles in Figure 72), 11 proteins were downregulated (green in Figure 72), 2 proteins remained unchanged (light blue in Figure 72) and 13 proteins were un-matched (dark blue in Figure 72).

**Figure 72.** Proteome profile of the cellular proteins of *Staph. aureus* after 50 minutes of incubation in buffer with 10% (w/v) manuka honey compared with the profile at 0 minutes incubation.

Although the comparison between the treated cells protein profile and the non-treated cells at 0 minutes indicated the presence of 13 “new” proteins and hence suggested the switching on of protein synthesis due (unmatched, dark blue in Figure 73), comparison with non-treated cells incubated for the same period of time (50 minutes) but in the absence of honey, suggested a different interpretation. It was possible to detect 10 upregulated proteins in the honey-treated cells (yellow circles in Figure 73) in comparison to the control incubated for the same period of time but in the absence
of honey, 13 proteins were downregulated (green in Figure 73), 15 proteins remained unchanged (light blue in Figure 73) and 27 proteins were un-matched (dark blue in Figure 73).

**Figure 73.** Proteome profile of the cellular proteins of *Staph. aureus* after 50 minutes of incubation in buffer with 10% (w/v) manuka honey compared with the profile of non-treated cells at 50 minutes incubation.

The most common method for the identification of the specific proteins present in each spot would be to perform matrix-assisted laser desorption ionisation-time of flight mass spectroscopy (MALDI-TOF MS), nevertheless due to time constraints and the unavailability of this methodology at UWIC another approach has been used as an indication of a possible identity of some of the spots. Because of time constraints 3 groups of spots were randomly selected for an indication of a possible identity through literature review shown in Figure 74 with the numbers 1, 2 and 3.
Spot 1 is a protein with an approximate molecular size of 120 Kda (Fig. 74), the literature search of proteins characterised in *Staph aureus* with this size yielded a paper referring to a range of surface proteins (with variable size, from 120 to 33 KDa) that are associated with siderophore production (Courcol *et al.* 1997), or a collagen adhesion exoprotein (with a molecular size of 129 KDa) (Nakano *et al.* 2002). This spot was increased 1.5 fold more than the non-treated cells incubated for 50 minutes in comparison with the non-treated cells that were not incubated in buffer (Fig. 74a). If cells that were treated with honey for 50 minutes, when comparing with cells that were not incubated, there was no difference (Fig. 74b), but if the spot was compared with the same spot in the gel for non-treated cells incubated in buffer for the same
amount of time (Fig. 74c) there was a downregulation of the expression of this protein as there is more than a 1.5-fold decrease in the intensity of the spot when compared with the control cells exposed to the same conditions, but without the honey.

Spot 2 is a protein with an approximate molecular size of 60 KDa (Fig. 74). The literature search for the identification of proteins with this size yielded a variety of papers that referred to two groups of proteins: heat-shock protein 60 or HSP60 (Dziewanowska et al. 2000; Kwok et al. 1999) and surface binding proteins (Boden and Flock 1992; Heilmann et al. 1997; Hussain et al. 2002; McGavin et al. 1993; Tompkins et al. 1992). This spot was upregulated in the cells incubated both with and without honey, when compared with non-treated, non-incubated cells (Fig. 74a and b), but when the intensity of the spot incubated in honey, is compared with the spot of the cells incubated in buffer alone (Fig. 74c) the intensity was reduced by at least a factor of 1.5-fold.

Spot 3 has an approximate molecular size between 30 and 40KDa (Fig. 74). According to the literature this size range can include proteins from the autolysin-adhesin family (Heilmann et al. 2005; Nakano et al. 2002), alpha and beta-hemolysin (Nakano et al. 2002) and extracellular binding proteins (Hussain et al. 2001). This spot had an increased expression when the cells were incubated in buffer alone, when compared with the protein profile of non-incubated cells (Fig. 74a) but had no difference between the non-incubated cells and the cells that were honey-treated (Fig 74 b and c).
7.4 Discussion

The determination of the susceptibility of the *Staph. aureus* mutants to manuka honey along with their parental strains showed that only the *atl* mutant had a significantly higher susceptibility (Table 17).

The *sigB* gene is responsible for the regulation of *Irg* and *cid* genes that are in turn responsible for the regulation of murein hydrolase (one of the enzymes necessary for cytokinesis) and involved in sensitivity to penicillin (Rice *et al.* 2004). Sigma factor $\sigma^B$ has also been associated with the regulation of transcription of virulence-associated loci in *Staph. aureus*, as well as controlling general stress response in response to several environmental stresses. Also it might indirectly control the production of biofilms in *Staph. epidermidis* (Horsburgh *et al.* 2002). A mutant with a defect in this regulator factor will be expected to be deficient in a number of stress responses. A lack of difference in susceptibility between the parental strain and the strain deficient in *sigB* therefore indicated that this is not a target site of the antibacterial activity of manuka honey.

The *atl* gene codes for the AtI molecule (137 KDa) that is the precursor of the enzymes murein hydrolase and peptidoglycan hydrolase, which are 2 enzymes that are involved in the processes of cell wall turnover and cytokinesis. Mutants for this gene will have an incomplete complement of autolysins, and will generate giant clumps of undivided daughter cells (Foster 1995). Their inhibition does not lead to cell death, suggesting that cell division is not essential for cell survival (Foster 1995). The increased sensitivity of the *atl* mutant strain to manuka honey might mean that this is
actually a defence mechanism of the cell, possibly through its role in cell wall turnover. These results seem to lead to the idea that the antimicrobial activity of honey may be due to interference in peptidoglycan biosynthesis, as in this case the Atl products would be responsible for overcoming the deficiency in peptidoglycan. In this case normal cells exposed to honey might recruit most of their Atl for the production of peptidoglycan leaving the actual cytokinesis process on hold. In cells that do not have this gene, this mechanism of counteracting the action of the honey would not be present and thus they would be more sensitive to inhibition by honey.

The generation of energy is essential for the growth and division of bacterial cells. Energy production takes place in the cell membrane and is one of the prime targets for antimicrobial agents, especially those with the ability to disrupt the cell membrane. The effects of honey on bacterial respiration is therefore an important piece of information when trying to understand the mode of action of honey as an antibacterial agent. The observation that the endogenous rate of oxygen consumption for Staph. aureus, increased in the presence of honey (manuka, Gale’s and artificial), might be an indication of increased metabolism (Fig. 47 and 49). This in turn could lead to depletion of the internal nutrient reserves and lead to either cell death, or entry to a dormant state, like Viable But Non-Culturablc (VBNC).

Initially the rapid rate of endogenous oxygen consumption in Staph aureus was thought to be due to uncoupling of the electron transport chain. The activity of autolysins seems to be regulated by the proton motive force (Calamita et al. 2001; Kemper et al. 1993) and if the cells were uncoupled, then the proton motive force would be absent and the autolysins non-functional. However, when the uncoupling
agent CCCP was added, the cells showed an increased rate of oxygen consumption, and this meant that they were still coupled. If they were not coupled the addition of uncoupling agent would have made no difference. The possibility of *Staph. aureus* being in a VBNC state following exposure to manuka honey is supported by the data collected in chapter 5, where the total counts of bacteria remained high whilst the culturable counts decreased (Fig. 18).

It is possible that because the experiments with the oxygen electrode were done using cells suspended in buffer, that a VBNC state could have been brought about because limited nutrient availability, and lead to starvation (one of the signals for induction of the VBNC state). Yet total and culturable counts were performed in the presence of nutrients, and demonstrated that the total counts remained constant whilst the culturable counts decreased. At the moment it is not possible to conclude that these observations prove that the honey treated *Staph aureus* enter the VBNC state. More studies are needed to assess the viability of cells exposed to honey, before a definitive conclusion can be made.

In the case of the *Pseud. aeruginosa* the endogenous oxygen consumption rate decreased in the presence of all of the honeys tested (Fig. 48 and 50). The addition of uncoupling agent to honey-treated *Pseud. aeruginosa* cells also elicited a response, which meant that the cells were coupled. From the electron microscopy (Chapter 7) it was possible to observe that the damage to this Gram negative bacterium was more extensive than that observed in the Gram positive *Staph. aureus*. The disruptions observed in the membranes of *Pseud. aeruginosa* cells might explain the decreases in respiration, by affecting the normal functioning of the electron transport chain and
consequently of the cellular respiration. Honey-treated *Pseud. aeruginosa* still remained coupled as they continued to respond to the addition of the uncoupler to the oxygen electrode chamber.

Since the effects of manuka honey on the respiration rates of both *Staph. aureus* and *Pseud. aeruginosa* seemed to occur in the presence of other two honeys (Gale's and artificial), it is possible to suggest a non-specific common factor (like sugar) rather than an influence specific to manuka honey.

The leakage studies of *Staph. aureus* showed that there was leakage of inorganic phosphate (Fig. 53) and ATP (Fig. 57) from honey treated cells compared with non-treated cells (Fig. 52 and 56 respectively). There did not seem to be any leakage of proteins (Fig. 54 and 55). This does not tie up with the time-course extraction of proteins from *Staph. aureus* cells for the 2D protein gel electrophoresis, where a decrease in extracted protein with time was seen (Fig. 62 and 63). There is not enough information to draw a conclusion about the significance of protein release.

This type of leakage (ATP and inorganic phosphate) is indicative of membrane damage (Johnston *et al.* 2003), and concurs with the electron microscopy observations previously described in chapter 6 (Fig. 26), where there was a significant increase in the number of observable damaged cells using scanning electron microscopy, with exposure to honey.

For *Pseud. aeruginosa*, leakage studies revealed a marked release of protein (Fig. 59) and ATP (Fig. 61), with a 10-fold increase in the recorded concentrations of these
substances in comparison to control cells (Fig. 58 and 60). In the case of inorganic phosphate there was a steady trend towards the increase of leaked inorganic phosphate in honey-treated cells (Fig. 57), but the range of detected phosphate was similar to the control cells (Fig. 56). ATP and proteins are larger molecules than phosphate, and could only be present in the buffer by passing through major holes in the cell wall of Pseud. aeruginosa (Johnston et al. 2003). In fact these results are once more supported by the data collected with electron microscopy (Chapter 6) where physical damage to the cell wall of Pseud. aeruginosa was observed with both SEM and TEM.

Initially, potassium leakage was to have been assayed in this study, but it was found that the methodology available (selective potassium electrode) was not sensitive enough to detect the amounts of potassium expected to be leaked from the bacterial cells. For inorganic phosphate the same applied. In the case of Pseud. aeruginosa, where electron microscopy (shown in chapter 6) demonstrated altered cell morphology and cell wall damage, and leakage of protein and ATP were detected, it is likely that phosphate leakage also occurred because phosphate is a smaller molecule. This was not observed. It is possible to speculate that the method for determination of inorganic phosphate was not sensitive enough to detect the small quantities of ion that might have leaked into the buffer.

The proteome analysis of Staph. aureus (using 2D gel electrophoresis) was performed in order to better understand the effects of honey on the expression of proteins in this bacterium. It was performed only in Staph. aureus because the previous results suggested that the mechanism of action of honey on these bacteria was not limited to physical damage. Increased incidence of cell walls in cells incubated in honey
suggested physiological changes which might have been reflected by differences in the proteome profile between honey-treated cells and non-treated cells.

Initially the aim was to use cells maintained in buffer and honey for 4 hours, but a time-course extraction of protein from honey-treated Staph. aureus, showed a marked decrease in the concentration of extracted proteins, which were not enough to rehydrate the IEF strips and perform 2D gel electrophoresis. This was another indication of the down-regulation of protein synthesis.

The time point chosen for this experiment was 50 minutes incubation in either buffer alone and buffer and honey, as this was the time point when the most proteins could be recovered from honey-treated cells. At this point the concentration of protein extracted from cells was too low for rehydration of strips with the minimum allowable volume of protein 10 μl. therefore concentration of buffer was increased to allow the use of a greater volume of protein solution, to ensure correct protein loading of strips.

The pH gradient chosen was the 3-10 NL (non-linear) because it was the widest range available. As the aim was to determine the type of changes that could occur in the proteome profile of Staph. aureus, this would include all extractable proteins, although the compromise was on separation of the proteins, because they would have a smaller area to separate in.

The quality of the gels was not ideal, as there was a lot of streaking (non-specific bands in the gels, fig 66-68), this can have been due to high salt concentration in the protein sample used to rehydrate the IEF strips. In this study, care was taken to use
ultrapure water at all stages, so as to limit the concentration of salts in the final extracts, but in the future additional cleaning up (removal of salts) might yield a better image. Furthermore, the limitation of the imaging apparatus, in terms of quality of the photographs, led to problems in the detection of small spots, this could be overcome with the use of more powerful imaging tools that include for example a 16-bit camera. Another step that could improve the results obtained would be the use of larger IEF strips, or strips with a narrower pH range which would give better separation of proteins.

The 3 spots that were selected for an indication of possible identity using literature search, seem to be possibly responsible for the adhesion of cells to the extracellular matrix. Spot 1 (~120 KDa) might possibly code for a collagen adhesion protein (Nakano et al. 2002) or for an extracellular protein that is involved in siderophore production. Iron is an essential nutrient for the survival of bacterial; in order to overcome the low levels of iron uptake in vivo, bacteria produce siderophores (low molecular weight iron chelators) and iron-regulated membrane proteins. These mechanisms are thought to be important virulence factors in Staph aureus. Recent work by Courcol et al. (1997) has demonstrated that proteins with the size 120 KDa, could be repressible in the absence of iron. Because this protein is downregulated after incubation in honey, when compared with control cells that were incubated but not exposed to honey (Fig. 74) it is possible that honey might be acting on the available iron chelating it, and making the iron unavailable to these proteins, thus downregulating their expression, and ultimately leading to cells death due to the lack of this nutrient.
Spot 2 is a protein with the approximate molecular size of 60KDa; this spot is upregulated in cells incubated in the presence or absence of honey when compared with cells that were not incubated (Fig. 74a and b), but downregulated in cells incubated in honey when compared with cells incubated in buffer alone (Fig. 74c). Proteins with an approximate size of 60 KDa in *Staph. aureus* can belong to the heat-shock protein family and regulate attachment to surfaces. It is possible to observe when comparing the image of the gel containing the cellular proteins from the treated cells (Fig. 68) that the density of spots around the 60-80 KDa size was higher than in the cells that were not incubated (Fig.66).

Heat shock proteins are a family of molecular chaperone proteins responsible for the cellular response to not only heat but to many other environmental stresses. They act by helping in the folding and translocation of other proteins across membranes (Qoronfleh *et al.* 1998). Their role in pathogenesis is still poorly understood, but there are virulence factors, which are influenced by temperature, so Hsp can possibly have a role here. These proteins are known to be present in the cytoplasm, periplasm, associated with membrane proteins and in the membrane surface (when the bacteria are intracellular) (Qoronfleh *et al.* 1998). The fact that there are not many proteins expressed with the size of HSP60 in the cells that were not honey-treated or incubated (Fig. 67 and 68), when compared with incubated cells (Fig. 66) is not surprising, as the cells were collected from an environment that provided the nutrients to grow (isosensitest broth) and incubated in buffer which does not contain any nutrients for the bacteria to growth, thus increasing the cellular stress. Nevertheless, this response seems to be decreased in the honey treated cells (Fig. 74c) when compared with cells
incubated in buffer alone, this could be the result of down-regulation of protein expression or of inhibition of protein synthesis.

Spot 3 contained a protein with an approximate molecular size of 30-40 KDa, whose expression increased in cells incubated in buffer alone (Fig. 74a), but not in the honey treated cells (Fig. 74b and c). This spot could possibly be an autolysin protein (Heilmann et al. 2005; Nakano et al. 2002). Autolysins, as previously described in chapter 6, are proteins that are responsible for the cell wall turnover, cytokinesis and cell growth. If spot 3 is indeed an autolysin, the fact that it did not increase in expression upon incubation when resuspended in buffer with 10% (w/v) honey, like the control cells resuspended in buffer alone could explain the increase in septated cells observed using TEM in chapter 6 (Fig. 28). If the control cells require an increase in expression of autolysins in response to incubation in buffer and when observed through the TEM, there was no significant increase in the number of septated cells (Table 15), the increase in septation might mean that the honey-treated cells were stimulated to divide (possibly by the incubation in buffer), but as the autolysins did not increase in expression the cells remained septated and could not divide.

Furthermore, all of the protein sizes used for the literature search yielded as possible identifications a range of proteins responsible for adhesion, which were down regulated in the honey treated cells. One of the mechanisms for bacteria to cause persistent infections in wounds is the formation of biofilms, and for that they require the possibility of attachment to the surface. Honey has been used many times as an effective treatment of persistent infections (Cooper et al. 2001; Dunford and Hanano
2004; Cooper et al. 2001; Dunford and Hanano 2004; Natarajan et al. 2001). It is possible that one of its mechanisms of action on these wounds could be the inhibition of bacterial adhesion formation that makes debridement more effective.

The main conclusion from this work is that the antimicrobial action of honey is different in Gram negative and Gram positive bacteria. In Gram negative it seems to have a more physical action of disrupting cells walls, whilst in Gram positive bacteria although it also acts by disrupting cell walls, perhaps by inhibiting peptidoglycan biosynthesis, as β-lactams do, it also seems to affect the cell physiology. More work needs to be done on the differences between the activity of honey on Gram negative and Gram positive bacterial physiology so that a better understanding of the mechanism of action of honey is achieved.
III Conclusions
Traditionally medicinal honey would have been of local origin, but from carefully selected floral sources (Molan 2000). Nowadays, one of the most popular honeys for wound management is manuka honey, derived from the manuka shrub (*Leptospermum scoparium*), as it has been shown to possess a high antibacterial activity. Today, honeys can be divided into two main types: those with hydrogen peroxide derived antimicrobial activity, and those with non-hydrogen peroxide derived antimicrobial activity. All honeys possess high sugar content, low water content and low pH which helps to limit the growth of microorganisms.

Since the recent publicity of the non-hydrogen peroxide activity of manuka honey, it has been exported from New Zealand to many countries and formulated into several medical products for use in wound management. These include non-adherent impregnated dressings, alginate and honey wound dressings, ointments and sterilised honey in tubes for use with individual patients. Honeys for medical purposes have been recommended to possess at least a non hydrogen peroxide activity equivalent to 10% (w/v) phenol equivalent (Unique manuka Factor, or UMF 10). This standard has been proposed because some wound environments possess large volumes of exudate, that among other components possesses hydrogen peroxide-degrading enzymes, so in order to assure a high antibacterial activity on wounds of this type, honeys with a high non-hydrogen peroxide activity are preferred as the antibacterial activity is guaranteed (Molan, 2000).
The work presented in this thesis has demonstrated that honeys with non-hydrogen peroxide derived antimicrobial activity above UMF 10 have been found in Europe. Although a survey of 139 Welsh honeys did not find any honeys with non-hydrogen peroxide derived antimicrobial activity (Wheat 2004), here a pilot study of 30 Portuguese honeys found some honeys (*Lavandula stoechas*) with activity in high enough potency to be used in the treatment of infected wounds. This result suggests that the potential exists to find honeys with a similar antimicrobial activity in Europe.

Some researchers have suggested that the non-hydrogen peroxide derived antimicrobial activity of manuka honey is unreal and that if more catalase was used to "knock out" the production of hydrogen peroxide during the bioassay, that all honeys would show a hydrogen peroxide derived antimicrobial activity (Weston 2000). EPR spectroscopy used during this work has shown that there is no detectable generation of hydrogen peroxide in manuka honey, thus disproving this theory, and confirming the findings of (Snow and Manley-Harris 2004), who found non-peroxide activity in the presence of high catalase concentrations.

The nature of the non-hydrogen peroxide derived antimicrobial activity of manuka honey is still unknown. Attempts have been made at fractionation, and active fractions were found, the compositions of which were never fully determined (Bogdanov 1997; Snow and Manley-Harris 2004). The complexity of the honey composition has been recognised as one of the factors preventing a full characterisation of its chemical composition. There are many components present at low concentrations that can interfere with assays for chemical definitions and an increasing number of new more sensitive techniques that are able to identify new components. The way in which these
components interact in honey to create the antibacterial activity that can be observed
in vitro and in vivo is also not well understood.

The production of honey is not a sterile process. In the hive environment there are
bacteria, yeasts and mould that readily contaminate honey but which do not generally
grow on honey, due to its sugar concentration and pH (Snowdon and Cliver 1996).

Organisms commonly known to be present in soil and other environmental samples
have been known to produce antimicrobial agents (Takahashi et al. 1986a; Zheng and
Slavik 1999; Huddleston et al. 1997). Their presence in the hive allows their possible
presence in honey. This work has determined that some of the organisms that are
present in non-sterile honeys have the potential to produce antimicrobial agents. 106
isolates were recovered from the 14 honeys tested, all Gram positive organisms and
the majority were from the Bacillus genus. Not every strain of a bacterial species that
is known to produce an antimicrobial substance will do so, nevertheless some of the
strains isolated from the manuka honeys tested in this work showed the potential to
inhibit different strains of wound infecting bacteria in a solid medium assay. Assays
in liquid medium were also performed with reduced success, leading to the conclusion
that the production of the antimicrobial agent(s) by the strains isolated from honey is
stimulated by growth in a solid medium, which has previously been reported to
stimulate production of antimicrobial agents in other microorganisms (Codon et al.
1995).

It has been reported that a bee-pathogen Paenibacillus larvae subsp larvae could
produce an antimicrobial substance (Holst 1945). This is a bacterium which is specific
to hives and can be transferred to honey (Alippi 1995). In the manuka honey samples tested this bacterium was not detected; however, it must be acknowledge that it is a fastidious organism and difficult to identify.

Problems were encountered in identifying to species level some of the isolated bacilli. This was probably because Bacillus species were closely related (Bottone et al. 2003; Chen and Tsen 2002; Manzano et al. 2003) and the identification kits available have been developed for use with clinical or type culture isolates, rather than environmental isolates.

Honey-based products available for wound management are generally made of gamma irradiated honey after manufacture and before use, so as to be safe for use in all patients, including immuno-compromised patients, nevertheless there are reports of the use of non-sterile honey being as effective (Molan and Allen 1996). It is possible that the bacteria that are present in the raw honey (non-sterilised) can secrete some of the antimicrobial compounds that they produced in order to elicit the inhibition of the growth of the wound pathogens tested in this work, thus contributing to the overall antimicrobial activity of the honey.

The benefit of honey for wound management does not seem to be limited to its antimicrobial properties, as it has been described as a stimulator of the immune response in wounds, leading to an improved wound healing (Snowdon and Cliver 1996; Tonks et al. 2001; Tonks et al. 2003). This activity seems to be distinct from the honey's antimicrobial activity, as honey with a weak antibacterial activity has
been shown to stimulate the release of cytokines important in the wound healing process \textit{in vitro} (Snowdon and Cliver 1996; Wheat 2004).

Another recognised benefit of honey is its antioxidant potential, and its ability to quench radical production. The observations presented in Chapter 4 are in agreement with previously published work which found that darker honey possess a higher antioxidant potential than lighter honeys (Taormina \textit{et al.} 2001). Also it has revealed that even honeys that produce hydrogen peroxide with the potential to give rise to free radicals like hydroxyl radicals (as is the case with pasture honey) have a self-limiting production of these radicals, as the free radical signal detected using EPR spectroscopy was quenched with time. Thus pasture honey has a self-modulatory capacity for the production of free radicals (meaning that it is able to produce free radicals, but at the same time it possesses antioxidant activity that modulates its effects). The presence of radicals might be desirable for the recruitment of some immune cells, but this needs to be self limiting so as not to damage healthy cells through oxidation/reduction reactions.

In order to better understand the mechanism of action of honey, in particular manuka honey, as an antimicrobial agent in wound management, its mechanism of action in two important wound pathogens, \textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa}, was investigated, with the use of kinetics studies, resistance training, electron microscopy, leakage, oxygen consumption and mutant and proteome analysis.
All the assays have revealed that the mechanism of action of honey on Gram positive bacteria is different to that on Gram negative. In the kinetic studies honey was shown to be bactericidal for both Staph. aureus and Pseud. aeruginosa in the presence or absence of catalase and also in the presence of serum, whose proteins are known to interfere with the activity of some antimicrobial agents (Craig and Ebert 1989). The susceptibility of Staph. aureus and Pseud. aeruginosa to honey was decreased in the presence of serum, but bactericidal activity was still recorded at concentrations that could be achievable in a clinical setting (Table 18).
Table 18. Summary of investigation of the antibacterial action of manuka honey on bacterial cells

<table>
<thead>
<tr>
<th></th>
<th>Staph. aureus (Gram positive)</th>
<th>Pseud. aeruginosa (Gram negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>Lower MIC than Gram negative but slower time-kill curves</td>
<td>Higher MIC than Gram positive but faster time-kill curves</td>
</tr>
<tr>
<td><strong>Commitment to death</strong></td>
<td>8 hours</td>
<td>4 hours</td>
</tr>
<tr>
<td><strong>Resistance training</strong></td>
<td>No resistance observed</td>
<td>Resistant strain recovered in single concentration exposure (phenotypic adaptation)</td>
</tr>
<tr>
<td><strong>Electron microscopy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Damage</strong></td>
<td>Increase in surface damage in honey treated cells</td>
<td>Increase in surface damage in honey treated cells</td>
</tr>
<tr>
<td><strong>Septa</strong></td>
<td>Increase of fully septated cells in honey treated cultures</td>
<td>No septa observed</td>
</tr>
<tr>
<td><strong>Stationary Vs Exponential</strong></td>
<td>Exponential cells significantly more susceptible to honey</td>
<td>Exponential cells significantly more susceptible to honey</td>
</tr>
<tr>
<td><strong>Leakage</strong></td>
<td>Some leakage</td>
<td>Major leakage</td>
</tr>
<tr>
<td>  <em>ATP</em></td>
<td>Some leakage</td>
<td>Major leakage</td>
</tr>
<tr>
<td>  Inorganic phosphate</td>
<td>Perhaps some leakage</td>
<td>Some leakage</td>
</tr>
<tr>
<td>  Protein</td>
<td>Some leakage</td>
<td>Major leakage</td>
</tr>
<tr>
<td><strong>Respiration rates</strong></td>
<td>Increased rate</td>
<td>Decrease rate</td>
</tr>
<tr>
<td>  <em>Endogenous</em></td>
<td>Respond to media</td>
<td>Respond to media</td>
</tr>
<tr>
<td>  <em>Respiration</em></td>
<td>Coupled</td>
<td>Coupled</td>
</tr>
<tr>
<td><strong>Mutant sensitivity</strong></td>
<td>Mutant more sensitive than parental strain</td>
<td>Not done</td>
</tr>
<tr>
<td>  * atl*</td>
<td>No difference</td>
<td>Not done</td>
</tr>
<tr>
<td>  * sigB*</td>
<td></td>
<td>Not done</td>
</tr>
<tr>
<td>  2D protein electrophoresis</td>
<td>Down regulation of protein synthesis, maybe of stress proteins and autolysins</td>
<td>Not done</td>
</tr>
</tbody>
</table>
Time-to-kill studies, have demonstrated that susceptibility to honey, not only varies among bacterial species, but also among strains of the same species. Clinical strains have shown a variety of sensitivity to manuka honey. These results suggest that susceptibility should not be assumed and that the kinetics/ sensitivity of a strain causing wound infection, for which treatment with honey is going to be used, should be verified, so that the appropriate treatment regime is achieved. For example if the bacteria has a lower susceptibility and the wound to be treated has a lot of exudate, which will dilute the honey, in order to maintain honey concentrations, the dressing should be changed more often, whilst if the bacteria has a high susceptibility less changes may be used, plus a honey with a higher non-peroxide activity should be used to overcome any breakdown of hydrogen peroxide by the wound fluid.

Resistance to antimicrobial agents is a concern, even with new antimicrobial agents like linezolid, one of the newest agents used to combat MRSA infection. Linezolid has recently been introduced in general use (introduced in 2001), resistance has been reported both in MRSA and Staph aureus (Peeters and Sarria 2005). This work has shown that, in the short-term, phenotyping resistance is possible to arise especially in Pseud. aeruginosa. This result stresses the point that with honey, as with any other antimicrobial agent, care must be taken in its use and surveillance of resistance patterns should be done.

There are bacteria capable of entering a dormant state where they remain viable but become sterile and as such cannot be cultured. Some researchers believe that these sterile cultures have entered a recently recognised stress response state, named Viable
but non culturable (VBNC), in which they are still metabolically active but are not able to divide in order to form colonies in an agar plate (Barer and Harwood 1999). Therefore these bacteria will have a different count for viability and culturability whilst maintaining a relatively constant total cell count (Barer and Harwood 1999). In the observations made in this thesis although the culturable counts of the bacteria decreased with time in the presence of honey the total numbers remained constant for both Staph. aureus and Pseud. aeruginosa, which could be an indication that the honey might be leading these bacteria into this VBNC state. In order to verify this an accurate viable count (usually done with fluorescent dyes and flow cytometry) needs to be performed, which was not possible in this study.

Recently the VBNC state existance has been questioned (Nystrom, 2003). The main question that is raised is: Is this state of sterility a stress coping response or just one step in programmed cell death? The VBNC cell formation is said to be a response to environmental stresses which is genetically controlled and reversible, but none of these claims have been proved. Another theory for cell sterility is the theory of programmed cell death. If the balance between toxin and anti-toxin production by the bacterial cells is tipped towards a decrease in production of anti-toxin, this may lead to cell death, which is claimed to be a system of altruistic cell death during environmental stresses such as starvation. Once this balance is changed, the cells lose their ability to form colonies but retain viability. The last theory to explain the loss of culturability in some cultures is the theory of stochastic deterioration. This claims that the loss of culturability of these cells is due to the increase in the demand for oxidation management of starved cells, leading to the depletion of the SigB factor, that reaches a point where it cannot cope with the damages inflicted by the oxidation
reactions occurring during starvation and so cells lose the ability to be cultured before eventually dying (Nystrom, 2003).

Regardless of what this sterility state is leading to (survival or death) it seems that honey is inducing this response in *Staph aureus* and *Pseud aeruginosa*.

Electron microscopy results have shown that there is an increase in visible damage in both bacterial species used, with exponentially growing cells demonstrating a greater sensitivity than stationary phase cells, and in *Staph. aureus* there was also a significant increase in the number of cells with a complete septum. The increase in septation could be due to an arrest in the cell cycle just before cytokinesis. In *Pseud aeruginosa* an increase in external damage could be observed but no septa were observed. This confirms that the activity of honey upon Gram negative bacteria is different from the activity upon Gram positive cells.

Honey is a broad spectrum antimicrobial agent. Usually broad spectrum antimicrobial agents, like tetracycline, target the same targets in Gram positive and Gram negative cells (Madigan *et al.* 2000). Honey seems to possess two different modes of action, perhaps with different substances in its composition acting as the antibacterial agents in different bacteria.

The assay of the susceptibility to honey of mutant *Staph. aureus* strains and their parental strains, led to the conclusion that the stress response gene $\sigma^B$ (Horsburgh *et al.* 2002) and its products were not targets for the manuka honey as the susceptibility of the knock-out mutant was the same as the parent strain. So any effects of honey on
the stress response of *Staph aureus* are the result of other damages and not the direct targeting of the *SigB* gene or gene product. The results for the screening of the *atl* mutant on the other hand showed an increased susceptibility of the mutant to the antimicrobial action of honey, this means that the gene and its products are implicated in the activity of honey. As the *atl* gene codes for the major autolysins in *Staph. aureus*, which are involved in the separation of the daughter cells and cell wall turnover (Foster 1995), if the honey was interfering with peptidoglycan biosynthesis, the *atl* gene products would be needed to try and counteract this activity. Possibly all or most of the autolysins produced would be recruited for this resistance mechanism, leaving less available to continue the cells cycle and cleave the two daughter cells at the point of cytokinesis.

In *Staph aureus* exposure to honey (not only manuka, but also Gale’s and artificial honey) led to an increase in the endogenous respiration rate, not due to the uncoupling of the electron transport chain. This increase in respiration might be a response from the cells to the interference of honey with the cell cycle, as some responses may need to be activated that consume more oxygen. Nevertheless protein synthesis did not seem to be one of the upregulated responses as the proteome analysis and time-course extraction of protein from treated *Staph. aureus* cells seemed to indicate an inhibition of protein synthesis. It is possible to speculate that incubation of *Staph aureus* in honey either stimulates respiration that leads to starvation or that this stimulation is a response to overcome damages in the cell. Also an increase in the O₂ levels introduced in the cells would favour a metabolic increase and an increase of the cellular concentration of reactive oxygen species that could damage the cells and bring about the sterility state observed in chapter 5, through the of stochastic
deterioration theory. On the other hand *Pseud. aeruginosa* oxygen consumption studies showed a decrease in the endogenous rate of oxygen consumption after honey treatment. This can be due to the fact that these cells present more clear membrane damage and thus the site where respiration needs to occur is damaged and it cannot take place normally.

Leakage studies demonstrated that in both bacteria honey causes membrane damage as the level of release ATP, protein and inorganic phosphate were elevated, with a bigger increased in the amount of leaked material noted in the *Pseud. aeruginosa* in comparison to *Staph. aureus*. This was expected, as with electron microscopy the damaged observed in *Pseud. aeruginosa* was more extensive than in *Staph. aureus*, leading to an increase in the leakage of intracellular materials into the surrounding media. Further studies are required to investigate different aspects of the changes in physiology, perhaps with studies of differences in membrane potential, lipid profile in membranes and gene expression. Also more assays on the leakage of different metabolites from honey treated cells, not only *Staph. aureus* and *Pseud aeruginosa* but other clinically relevant bacteria such as *Enterococci* sp. and yeasts such as *Candida* sp., are required.

The 2D electrophoresis of the extractable cellular proteins of *Staph. aureus* demonstrated that there was a general downregulation of protein synthesis in honey treated cells in comparison with control, possibly their stress response as well. Indicating a possible mechanism of action: by reducing the stress response the honey makes the bacteria more vulnerable to antimicrobial agents, which would otherwise have been counteracted by the bacterial stress response. The definite identification of
some of the downregulated spots on the proteome profile of honey-treated *Staph. aureus* cells would allow a more accurate analysis of the effects of honey upon bacterial cells. It would be expected that the differences in proteome profile in *Pseud. aeruginosa* as all the other observations made during this thesis would indicate a different mode of action in the two species.

It is possible to conclude that there are many areas relating to the use of honey in wound management that require further investigation, nevertheless this work has attempted to shed some light into some of these. The main conclusion to be drawn from this work, is that honey's antimicrobial activity is likely as complex as its own composition, and that its activity is due to a combination and synergistic action of more than one antimicrobial component, rather than a single substance. Most of all, although this is a broad spectrum antimicrobial agent, the mechanisms of antibacterial action among Gram positive and negative bacteria are different. Care must be taken in its use for medical purposes just as for any other agent, so that it remain a useful wound remedy for another 4000 years.
8.2 Future work

The pilot study of the Portuguese honeys demonstrated the potential for medical grade honey to exist in Europe. This work needs to be further developed with the collection, analysis and characterisation of a bigger number of Portuguese honeys. Also a European Network for honey research, where ideas could be shared and a bigger volume of samples tested and characterised, would be valuable.

The development of the results obtained in chapter 4, the production of antimicrobial agents by the bacteria present in honey, should include an accurate identification of the bacterial strains isolated from the honeys tested, possibly using sequencing or other molecular techniques such as RAPD analysis (Chen and Tsen 2002) and also the identification of the products that these strains were producing that led to the inhibition of the clinical isolates tested, perhaps with the use of chromatography techniques (Leifert et al. 1995).

The EPR and antioxidant potential of honeys should be further investigated not only for its potential in wound healing, but also because antioxidants have been shown to possess some antimicrobial activity (Alcaraz et al. 2000; Basile et al. 1999; Mori et al. 1987; Ng et al. 1996; Sato et al. 2000). Hence the assay of a bigger number of honeys for their antioxidant potential and the characterization of the components responsible for this activity, with the use of HPLC, GC-MS or LC-mass spectroscopy (Inoue et al. 2005) for their identification is possible.
Further studies on the kinetics of death of species other than \textit{Staph. aureus} and \textit{Pseud. aeruginosa} are necessary for the characterisation of honey's antimicrobial activity as well as long term studies on the possibility of resistance arising. Furthermore, the characterisation antimicrobial activity of manuka honey through the use of electron microscopy should include more time points of exposure to honey, different concentration of honey and the use of different types of honey to determine if the activity observed is characteristic of the action of manuka honey or general to all honeys.

Bigger leakage studies including more molecules such as DNA and potassium ions (Johnston \textit{et al.} 2003) would provide more information about mode of action. A better understanding of the intracellular signalling in honey treated cells is also necessary and bigger proteome and RNA transcription studies could provide such information. A study of the kinetics of RNA and protein synthesis, with the use of radioactive isotopes incorporation (Clements and Foster 1998), would also provide valuable information for the better understanding of the mechanism of action of honey in bacterial cells. More studies on the effect of honey on bacterial proteomes is also required, using higher definition image capturing devices, larger IEF strips and a wider range of bacteria and times of exposure, in order to better understand the effects of honey on protein expression. Further work should also include the utilisation of methods such as MALDI-TOF for the identification of specific upregulated or downregulated proteins in the proteome profiles obtained with two-dimensional electrophoresis.
Further investigation into the effects of honey on the stress response of cells should be studied with techniques like western blotting which would allow for the collection of specific data into the effects of honey upon the expression of specific stress proteins. One of the main problems in this case is that commercially available antibodies for use in western blots are usually not tested or developed for use with bacterial samples, and further work to verify the usefulness of these products should be considered.

Finally, more *in vivo* data is required on the action and effectiveness of the action of honey on wound management for it to be accepted by the medical community as a real alternative to the use of topical antibiotics.
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