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The Antibacterial Activity of Honey

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A B S T R A C T

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*. Honey is a supersaturated sugar solution with a high osmolarity; 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). Hence honey has a broad spectrum antimicrobial activity. Honey contains trace amount of vitamins, flavonoids, antioxidant components and unidentified plant derived elements (phytochemical components). 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. 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. 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. Further research into the mechanism of action of manuka honey on relevant wound infecting pathogens is considered necessary.

Introduction

Honey, an ancient remedy rediscovered during the 1990s, is now being utilized for wound care in Australia (Johnston *et al.*, 2003), New Zealand (Molan and Betts, 2003) and in the UX (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 Par (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.

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. 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 (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 (Tamehiro *et al.*, 2002).

It is recognized that honeys from different floral sources and geographical locations vary considerably not only in their antibacterial activity but also in their color (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 (Inoue *et al.*,2005). 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-I or tumour necrosis factor, or by neutrophils and macrophages (production by the NADPH-oxidase located in plasma membrane) (Burdon, 1995).

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 have 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). *S. 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 *S. aureus* have increased in incidence, both in hospital and the community, one of the most worrying being methicillin-resistant *S. aureus* or MRSA, which have increased in incidence in the last decade (Howell-Jones *et al.*, 2005), the treatment of which is difficult due to the fact that some strains can be resistant to more that one antimicrobial agent. Another common wound pathogen known for its resistanceis *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, 2003).

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, Iyophilised or in frozen stocks. This has been described for many different microorganisms ranging from Escherichia coli (Acha *et al.*, 2005), to Clostridium dificile (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).

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 *S.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 cultivable.

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 (Oliver,2005). 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 is 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. 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).

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 (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 (Crane, 2001).

The characteristics of honey

Honey is the product of the honeybees 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 characteristicst o nectar honey as it already contains enzymes added by the insect.

Chemical characteristics

Honey is a complex substance, made up of at least 181 different substances known at present, (Jones,2001); with some researchers believing that the number is

actually closer to 600 different substances (Bogdanov *et al.,* 2004). Honey is a saturated or supersaturated sugar solution, meaning that it possesses a high concentration of sugar (with approximately 17% water on average). 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 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 (Mato *et al.,* 2003). The organic acids present in honey are believed to contribute to the organoleptic properties such as flavour and color as well contributing to physical and chemical characteristics such as pH, acidity and electrical conductivity (Mato *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 Rud), 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 cash 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 RudA 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 hydroxyrnethylfurfural (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 foranged, however as the composition of honeys is further characterisedc hemically, other markers, like specific proteins or organic acids are being used for honey classification (Mato *et al.,* 2003). Unifloral honeys are largely derivecd 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).

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 (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 thennal conductivity (helps distinguish between floral and honeydew honeys) and hygroscopicity (the capacity of each honey to absorb humidity) (Crane 1975).

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 benzy1penicillin followed after by methicillin in 1959 but this was soon followed (1980s) by reports of methicillin resistance (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). 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).

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-resistant 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 *Pseudomonasa eruginosa*. 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); 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.

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 and around the world (Schmidt 2004). 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 *Staphylococcus aureus* (VRSA), so that in addition to being resistant to *vancomycin Propcqlion* of MRSA igolates in participatirg ocuntries in 2004

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 debridement 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.

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, ischaernia, 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 polyrnicrobial 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, 2005).

Cutting and White (2005) have described the symptoms required for the diagnosis of an infection as being: localised erythrema, localised pain, localised heat, cellulitis,

oederna, abscessed, ischarge, delayed healing, discoloration, friable and bleeding tissue and bad odor. 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-haernolytic 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.*, 2002). 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 2005).

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 sterilized 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.

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 (Snow and Manley-Harris 2004). The complexity of the honey composition has been recognised as one of the factors preventing a full characterization 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).

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 seems 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 (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 in vitro (Wheat,2004).

In order to better understand the mechanism of action of honey, in particular honey, as an antimicrobial agent in wound management, its mechanism of action in two important wound pathogens, Staphylococcus aureus and 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 *S.aureus* and *P.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 .The susceptibility of *S. 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.

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 remains a useful wound remedy for another 4000 years.

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 characterization 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 characterized could 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).

Further studies on the kinetics of death of species other than *S. aureus* and *P. aeruginosa* are necessary for the characterization of honey's antimicrobial activity as well as long term studies on the possibility of resistance arising. Furthermore, the characterization 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 *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 utilization of methods such as MALDI-TOF for the identification of specific up regulated or down regulated 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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