IN VITRO-STUDIES RELATING TO HONEY AS AN ALTERNATIVE APPROACH TO WOUND THERAPY

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بسم الله الرحمن الرحيم

Praise be to God

To my father, my mother, my wife and children, and everyone in my family

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ABSTRACT

A comparative study was made between Manuka honey-the main honey used as an antibacterial agent in medicine and some commercially available honeys. Among 22 types of honey, five types were found to be the most effective and these are Rainforest +30, Manuka +20, *Nigella sativa*, Oak and New Zealand Rewarewa. Three types of honey showed negative response to Catalase (an enzyme which breaks hydrogen peroxide), namely New Zealand Rewarewa, Manuka +20 and Rainforest +30.

Exposure of honey to autoclaving generally leads to a reduction in its antibacterial activity. This suggests that hydrogen peroxide and other antibacterial factors are removed/denatured by autoclaving, but that in the case of Manuka against *S. aureus* and Rain forest+30 against *Ps. aeruginosa,* some; unknown, non-destroyed, antibacterial factor remains.

The antibacterial effects of honey on *Fusobacterium nucleatum* (an anaerobic bacterium which is a major cause of mouth abscesses) were studied. Undiluted Manuka honey was again shown to be the most effective antibacterial honey against this pathogen. Three types of bacteria were isolated from honey as contaminants. These were identified using 16SrRNA as: *Lysinibacillus fusiformis*, *Staphylococcus epidermidis* and *Sporosarcina koreensis*; and were respectively isolated from Oak honey, *Nigella sativa* honey and Manuka honey.

Some commercial products containing Manuka honey were tested for their antibacterial efficacy. With the exception of the Manuka Honey Conditioner, all of the products showed antibacterial activity against *S. aureus* such as Body wash, Shampoo, Manuka oil, Kanuka oil, Manuka Body Wash, bee venom with Manuka honey and Vita Complex. Similar results were obtained when *Ps. aeruginosa* tested. However, in all cases, the antibacterial effect on both bacteria was more pronounced

against *S. aureus* than it was against *Ps. aeruginosa.* The antibacterial effect of the three mouth-cleaning products was then tested against some bacterial mouth flora. The Manuka and Aquafresh tooth paste showed inhibition zones for all three bacteria, while the non-honey mouthwash had no effect on bacterial growth.

Four types of honey were diluted from 80% to 5% to determine the minimum biofilm eradication concentration (MBEC). At a concentration of 20% all honeys killed both types of biofilm bacteria. Some pathogenic traits such as pyocyanin production and biofilm formation were found to be influenced by a range of honeys.

Various wound dressings and wound treatments were tested for their antibacterial activity against planktonic and biofilm of *S. aureus* and *Ps. aeruginosa*. Panaderm, a mixture of antibiotics, was the most effective treatment against *S. aureus* and *Ps. aeruginosa*. Activon Manuka exhibited marked activity against *S. aureus* and moderate activity against *Ps. aeruginosa.* Flamazine showed moderate action against *S. aureus* and *Ps. aeruginosa.* The proprietary (non-honey based) hand gel showed weak action on both types of bacteria.

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Chapter1

General Introduction

1 Introduction

The ability of pathogens (bacteria, fungi, viruses, and parasites) to infect a living host is called pathogenicity. Some pathogens possess virulence factors which enable them to initiate infection, including the ability to adhere to hosts cell using special structure like pilli, secrete toxins and enzymes, and finally, the ability to protect themselves from phagocytic cells due to the possession of outer membranes or capsules.

1-1 Some important pathogenic bacteria

Although medically important bacteria vary from severe to weak pathogens the weaker ones may become more dangerous when they acquire new resistance determinants.

1-1-1 *Staphylococcus aureus*

The pathogenic bacterium *Staphylococcus aureus* is a Gram-positive coccus which appears as grape-like clusters when viewed through a microscope. The main pathological features of *S. aureus* include the ability to produce the following toxins and toxic components:

1- Catalase (an enzyme which breaks down hydrogen-peroxide to water).

2- Coagulase (an enzyme which protects it against phagocytes cells by clot formation).

3- Haemolysins (*S. aureus* secrets a haemolysin which lyses red blood cells, including those present on Blood Agar plates).

4- Protein A (a compound which is found on the cell surface of about 95 % of human strains of *S. aureus* and has the ability to bind the Fc portion of

immunoglobulin G (IgG). Protein A gives the bacterial cell antiphagocytic trait.

5- Fibrinolysin which digests fibrin.
6- Leucocidin which kills leucocytes.
7- Hyaluronidase gives the bacteria spaces between host cells by breaking down hyaluronic acid.
8- Lipase which digests lipids.
9- DNAase which hydrolysis DNA.
10- Epidermolytic toxins A and B are responsible for epidermal splitting and exfoliation.
11- Enterotoxins which causes vomiting and diarrhoea.
12- Toxic shock syndrome toxin-1, causing shock, rash and desquamation.

Staphylococcus aureus can be found in wounds, bed sores, and skin infections and can cause hospital acquired or community acquired infections. *S. aureus* is more resistant to antibiotics in the hospital environment and can be transformed to the problematic methicillin resistant *S. aureus* (MRSA) form. Generally, MRSA exhibit resistance to a number of antibiotics, including pencillins, cephalosporins, chloramphenicol, tetracycline, fluroquinolones and others (Zinner, 2007). Vancomycin is now, as the result of necessity, the drug of choice for MRSA infections, although since 1997 bacteria have been developing resistance to this antibiotic (Hiramatsu *et al*., 1997). In addition, many vancomycin resistant *S. aureus* (VRSAs) are being isolated from medical institutions globally (Hiramatsu, 2001, Tiwari and Sen, 2006).

1-1-2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacillus which is an opportunistic pathogen that affects immune compromised individuals and causes life-threatening infections, notably in cystic fibrosis patients and debilitated patients such as those with burns or malignancy. In addition, *Ps. aeruginosa* causes urinary tract infection, wound infection, chronic otitis media and lower respiratory tract infection.

Pseudomonas aeruginosa has some virulence factors which cannot be ignored such as pyocyanin (Molinari *et al* 1993), and exotoxin A which affect protein synthesis in the host cell (Azghani, 1996). Moreover, the bacterium is difficult to eradicate and control with antibiotics or disinfectants.

The main problem with Pseudomonal infection or colonization is that unfortunately, there is only a limited number of antimicrobial agents which possess reliable activity against *Ps. aeruginosa*; these include antipseudomonal penicillins, cephalosporins, carbapenems, and fluoroquinolones, particularly ciprofloxacin (Carmeli *et al*., 1999).

Pseudomonas spp have natural immune characteristics which enable this genus of bacteria to grow and survive in the presence of some types of antibiotics than other Gram negative bacteria. These characteristics are impermeability and efflux or pumping out system which prevent accumulation of antibiotics within the bacterium before they have the opportunity to hit the actual target inside the bacterial cell (Tenover, 2006).

1-2 Antibiotics site of action

Antibiotics in general hit four targets inside the microbial cell or interact with the function of some other targets (Figure1).

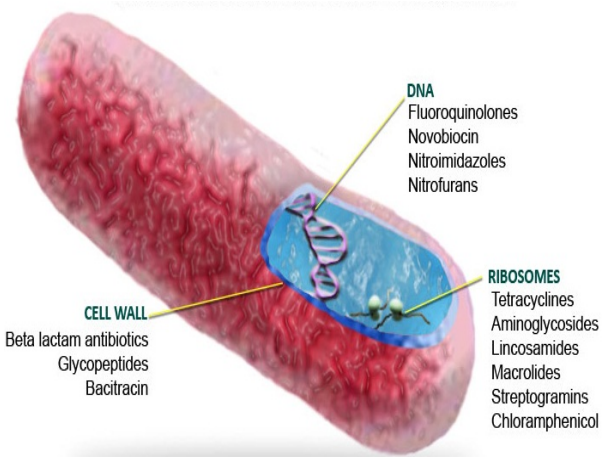


Figure 1. Major Targets of Antimicrobial Agents

(http://amrls.cvm.msu.edu/)

These targets have a unique structure(s) which is different from the host cell and which antibiotics can easily recognise (Table1).

Table 1. Sites of action of antibacterial agents (Finch and Garrod, 2003)

SITE	AGENT	PRINCIPAL TARGET
CELL WALL	Penicillins transpeptidase	Cephalosporins transpeptidase
	Bacitracin isoprenylphosphate	Glycopeptides Acyl-D-alanyl-D-alanine
RIBOSOME	Chloramphenicol peptidyl transferase	Tetracyclines ribosomal A site
	Quinolones DNA gyrase (a subunit)/	topoisomerase IV
NUCLEIC ACID	Quinolones DNA gyrase (a subunit)/	topoisomerase IV
	Novobiocin DNA gyrase (b subunit)	Rifampicin RNA polymerase
CELL MEMBRANE	Polymyxins phospholipids	Ionophores ion transport
	Polymyxins phospholipids	Ionophores ion transport
FOLATE SYNTHESIS	Sulfonamides pteroate synthetase	Diaminopyrimidines dihydrofolate reductase

1-3 Bacterial resistance to antibiotics

Three main mechanisms of antibiotic resistance in bacteria have been found. Bacteria can a) change the target of the antibiotic chemically by enzyme deactivation or destruction, b) they can physically alter the binding site of the antibiotic within the bacterial cell and c) prevent an antibiotic from accessing its target (Mulvey and Simor, 2009). Some examples are shown in Table 2.

TABLE 2. Some examples of method of bacterial resistance to some antibiotic. (www.textbookofbacteriology.net)

ANTIBIOTIC	**METHOD OF RESISTANCE**
Chloramphenicol	reduced uptake into cell
Tetracycline	active efflux from the cell
β-lactams, Erythromycin, Lincomycin	eliminates or reduces binding of antibiotic to cell target
β-lactams, Aminoglycosides, Chloramphenicol	enzymatic cleavage or modification to inactivate antibiotic molecule
Sulfonamides, Trimethoprim	-metabolic bypass of inhibited reaction - overproduction of antibiotic target (titration)

In addition, some bacteria exhibit unique types of resistance, for example, MRSA has the low-affinity penicillin-binding protein (PBP-2a), encoded by mecA gene. The catalytic function of PBP-2a is not affected in the presence of modified synthetic penicillins (Oxacillin – Methicillin – Cloxacillinetc) (Zinner, 2007).

Resistance to antibiotics can be natural or acquired. Natural resistance means that the bacterium has either inherited this resistant or it possesses a naturally occurring structure or system that makes bacteria unaffected by the killing factor.

Some antibiotics are effective against Gram-positive but not against Gram negative bacteria. The differences relating to some structure such as a capsule or to a non-specific permeability barrier present in the outer membrane (Mulvey and Simor, 2009).New determinants or changes in the genetic material which gives the bacteria a novel mechanism of resistance are called acquired resistance.

Acquired resistance results from a mutation or from the acquisition of a new plasmid or transposon formed by conjugation or transduction or transformation.

Mutation can occur in every 10^{-8} to 10^{-9} colony forming units (CFU) when exposed to antibiotics. *Escherichia coli* for example, has developed resistance to streptomycin by mutation (Newcombe and Hawirko, 1949). The new resistance trait is inherited directly by all bacteria in the new generation by what is termed vertical gene transfer (VGT).

The acquisition of antibiotic resistance occurs when a segment of DNA holding a resistance trait is acquired by conjugation or transformation or transduction. This process is known as horizontal gene transfer (HGT) and here, the genetic material can be moved between bacteria from the same species or even between different species (Mulvey and Simor, 2009). Direct contact between donor and recipient cells is required in conjugation, a process which enables small pieces of DNA called plasmids to be transferred between cells (Mazel and Davies, 1999). Conjugative transfer of the genetic determinants has been observed between different bacterial species and even between bacteria and eukaryotic cells such as yeasts (Finch and Garrod, 2003). Transformation occurs when the DNA molecule is acquired from the surrounding

environment and combined with the bacterial genome of the recipient cell. In some types of bacteria transformation occurs readily and without the requirements for any special treatments, as happens in *Campylobacter jejuni* and *Campylobacter coli* (Wang and Taylor, 1990).

DNA or genetic material can also be transferred between bacteria by a bacteriophage which carries its own genome plus a fragment of DNA which was accepted after the bacterial cell was lysed. The bacteriophage then moves to another bacterium and the viral genome becomes integrated into the bacterial gene stream, i.e. transduction a process known to occur in *Staphylococci* (Hawkey, 1998).

1-4 Resistance to antimicrobial agents

The phenomenon of antimicrobial action was largely unknown when Pasteur and Joubert discovered that one type of bacteria could inhibit the growth of another. Heavy metals, dyes, and later the sulfa drugs, were then used in treating some infections before the discovery of the antibiotics. These compounds have limitations such as toxicity, side effects and more importantly-they do not have a direct inhibitory effect on bacterial cells. Selective toxicity, or the ability of a chemical or a drug to kill microorganisms without harming the host is an essential property of antibiotics. Penicillin was the first antibiotic to be developed, but unfortunately, after a short period of the use, bacteria developed resistance to it (it still however, remains an important antibiotic). Nowadays, resistance to antimicrobial agents has developed in parasites (Talisuna *et al*., 2004), fungi (Ghannoum and Rice, 1999), viruses (Gammon *et al* 2008), and more commonly amongst bacteria (Falagas *et al* 2007). Ghannoum and Rice (1999) suggest that the development of bacterial resistance to antibiotics has become more important because:

1- Bacterial infections are responsible for the bulk of community-acquired and nosocomial infections.
2- The large and expanding number of antibacterial classes offers a more diverse range of resistance mechanisms to develop.
3- The ability to move bacterial resistance determinants into standard well-characterized bacterial strain facilitates the development of molecular mechanisms of resistance in bacterial species.

Bacteria have developed resistance to all of the different classes of antibiotics (Alanis, 2005). For example, Sieradzki *et al.* (1999) reported a case of a Vancomycin resistant *S. aureus*, whereas Vancomycin is the drug of choice in case of Methicillin resistant *S. aureus* (MRSA) (Sieradzki *et al* 1999); resistance to Imepenem has also been shown in some strains of *Ps. aeruginosa* (Lepelletier *et al*., 2010, Bahar *et al*., 2010). Bacterial resistance results largely from a) misuse and b) genetic changes occurring in the pathogen. Firstly, excessive and often unnecessary use of antibiotics can significantly contribute to the emergence of bacterial resistance, as can the lack of appropriate implementation of antibiotic policy, lapses in hygiene or poor infection control practise (Rao, 1998). Secondly, as we have seen, bacteria have become resistant to antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via the development of plasmids and transposons. The cost of treatment increases following the development of bacterial resistance due to need of extra healthcare; in the USA for example, a single case of Methicillin resistant *S. aureus* can cost more than $60,000 to treat (Anderson *et al*., 2009), and the estimated cost of antibiotic resistance for hospitals is more than $20 billion (Roberts *et al*., 2009), In one hospital in Netherlands, the cost of very strict anti-resistance measures for a ten year period has reached 2,800,000 Euros (Vriens *et al*., 2002).

Increased antibiotic resistance is significantly associated with increased morbidity and mortality rates in nosocomial infections (Acar, 1997, Goldmann *et al*., 1996). Carmeli *et al* (1999) showed that the prolonged hospitalization of patients resulted in the acquisition of *Ps.aeruginosa*strains, which are highly resistant to antibiotics such as Ceftazidime, Imepenem, Pipracillin and Ciprofloxacin.

1-5 Biofilms

Biofilms are microbial communities (bacteria, archaea, protozoa, fungi and algae) where cells are enclosed within a slime layer attached to living or non-living surfaces, resulting in a small community with an elaborate structure and organization. Biofilms originate with the adhesion of a single cell on a surface via physical attraction of the microbial cell to a substrate and ends with the formation of a biofilm matrix (Figure 2). Some chronic bacterial infections are related to the formation of biofilms which are inherently resistant to antimicrobial agents (Costerton *et al*., 1999). Biofilms are important in bacterial resistance because the minimum inhibitory concentration to kill bacteria within a biofilm is about 100 to 1000 times greater than that seen for the planktonic bacteria (Stewart and Costerton, 2001).

Costerton *et al* (1999) confirmed that this inability of the antimicrobial agent to penetrate the depth of the biofilm contributes to biofilm resistance, a result which may also be due to starvation (Chambless *et al*., 2006), since non-growing cells are generally not susceptible to antimicrobial agents (Costerton *et al*., 1999).

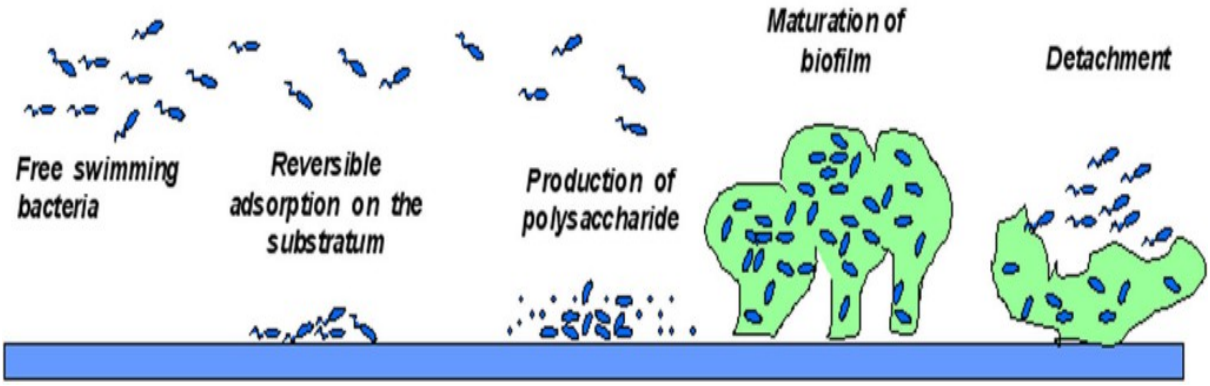


Figure 2.Formation of a biofilm.(http://eng-cs.syr.edu/our-departments/biomedical-and-chemical-engineering/research/control-of-bacterial-biofilm-formation)

1-6 The importance of finding alternatives to antibiotics

Antibiotic use may bring about the following problems:

1- Side effects: as chemical products antibiotics produce many undesirable side effects thereby requiring physicians to take extra care when prescribing; the side effects range from simple to severe.

2- Contribution to cancer: Velicar *et al* (2004) suggested that there is an association between the use of antibiotics and breast cancer.

3- The killing of the normal flora, either inside or outside the body, increases the risk to health. For example, *Clostridium difficile* is inhibited by the normal floral barrier in human and animal guts which prevents infection (Borriello, 1990); the removal of this by antibiotics may allow the pathogen to increase.

4- The development of immune suppression, especially following the long term use of antibiotics (Van Vlem *et al*., 1996).

5- Some factories involved in antibiotics industry discharge their waste directly in the environment, thereby causing pollution problems (Homem and Santos, 2011).

There is therefore a critical need to find new compounds to overcome these problems, notably in relation to antibacterial resistance.

1-7 Alternative approaches to the use of antibiotics

In time, antibiotics will be no longer effective on microorganisms due to significant increases in resistance and to shortages of new antibiotics. Replacement with natural and effective products is therefore extremely important. Alternative medicines might be used; these are generally natural products which were mentioned in the history of ancient civilisations. These products are as diverse from culture to culture and region to region and have been used since antiquity to affect cures. However, the significant shift to antibiotics in the 1940s resulted in the neglect of these treatments, examples of which include apitherapy (i.e. honey therapy), maggot therapy, algae, and herbal therapy.

1-7-1 Apitherapy (Honey therapy)

Although honey has been used as a food and a medicine since antiquity, it has been fully accepted as a therapeutic agent only since 1999. The first two types of honey which were used as medicine were the Australian honey, Jelly bush, and the New Zealand honey, Manuka. The merits of honey have now been well investigated (Table 3).

Table 3. The merits of honey(Al-Waili *et al*., 2011)

Merit	**Component**	**Function**
Supporting the immune system	Antioxidants	Such as flavonoids and aromatic acids which reduce inflammation and cell damage.
	Unknown components	Natural honey contains raw materials which lower the production of prostaglandins which play a role as immunosuppressant and a critical role in cancer development.
	L-arginine	Enhances nitric oxide production which plays role in the immune response.
Killing factors	Hydrogen peroxide	A strong killing factor that is generated by glucose oxidase which is added by bees to nectar.
	Methylglyoxal	Non-peroxide honey such as Manuka honey contains such a unique killing factor.
	Osmolarity	High sugar content ties up water molecules so that microorganisms have insufficient water to grow.
	Acidity	In acidic environment (pH generally between 3.2 and 4.5) most microorganisms cannot grow.
Wounds healing	Physical barrier	When covering wounds with honey, a thick layer is developed which is very difficult to penetrate by environmental contaminants; this creates a moist wound healing environment.
	Nitric oxide	Cell proliferation, wound contraction and collagen formation are regulated by the inducible isoform (iNOS) of Nitric Oxide which is synthesized in the early phase of wound healing by macrophages (Witte and Barbul, 2002).
	Hydrogen peroxide	Vascular endothelial growth factor (VEGF) is an important angiogenic factor for wound healing. H_2O_2 induces the VEGF promoter in macrophage through an oxidant which results in increasing in VEGF production.
Nutrients	Glucose –Fructose – Minerals – Vitamins – Antioxidants – Amino acids	Honey contains such compounds which may play role in its activity and potency

1-7-2 Propolis

Propolis is a product of bees which contains several compounds such as amino acids, steroids, inorganic and phenolic compounds (Mundo *et al*., 2004) which have antimicrobial properties against a wide range of microorganisms (Hegazi *et al*., 2000, Serkedjieva *et al*., 1992, Higashi and de Castro, 1994).

1-7-3 Maggot therapy

Maggot therapy is an old approach which employs the ability of some flies larvae to cause debridement and sloughing of necrotic tissue (Figure 3), as well as the ability to produce antibacterial agents. *Lucillia sericata* is the most common species of blowfly maggot used in medicine. Maggots are particularly effective in treating indolent diabetic wounds (Sherman, 2003). A study has shown that the maggot therapy is more effective when used on wounds infected with Gram-positive bacteria such as *S. aureus* rather than ones caused by Gram-negative bacteria (Jaklic *et al*., 2008). An antimicrobial peptide called Lucifencin can be found in haemolymph, fat body and excretions/secretions (ES) of the maggot (Andersen *et al*., 2010, Cerovsky *et al*., 2010). Cazander *et al* (2009) demonstrated that the excretions/secretions (ES) of maggots can reduce biofilm formation by *Ps. aeruginosa*.

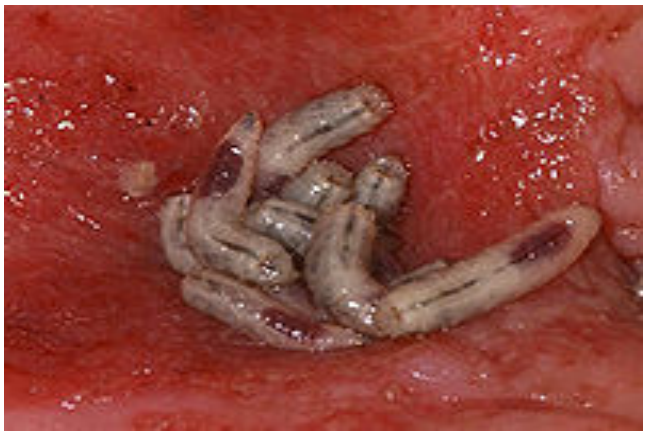


Figure 3. Maggot therapy in action in a wound

Clearly no one would advocate the application of living maggots to infected wounds in place of effective antibiotics, even ignoring the relative effectiveness of the two approaches a move away from "scientific medicine" to a primitive bio-control technique would undoubtedly be seen as an embarrassment by most of the medical profession. The return of maggot therapy highlights the desperate state in which modern medicine finds itself, a situation which is made embarrassing by the fact that it is largely down to its own making due to the mis-prescribing and wasteful way in which antibiotic use has been squandered over the last half century and more. Medical science has, at least for the moment, had to turn away from highly sophisticated approaches to infection control to the use of old fashioned approaches which are based on folklore or even old wives tales. Such a picture is however, misleading as all of the alternative approaches studied in this thesis are backed up by scientific rationales, many of them developed at a time before the introduction of antibiotic therapy. The classic example of this is the scientific rationale behind maggot therapy which was extensively worked out in the 1930s by Baer, his co-workers and followers (Wainwright, 1988). In the same way, all of the necessary methods required to successfully apply maggot therapy in medical practice were well-worked out before 1940, when the method was made redundant. It is interesting to note that while maggot therapy was widely used in the USA in pre-antibiotic days, its use was never sanctioned in the UK, despite its obvious effectiveness. As a result, while US patients who suffered from disgusting, suppurating indolent infections could be relieved of their symptoms, those in the UK, who were denied maggot therapy, largely on the basis of bigotry, had to continue suffering (Wainwright, 1988).

1-7-4 Quorum quenching

Bacteria have the ability to communicate inside some communities such as biofilms using signals i.e. quorum sensing. In order to escape from the immune response of the host it is very important for pathogenic bacteria to coordinate their actions. Interfering with that communication either by degrading or modifying of the signalling molecule affects some pathogenic behaviour such as biofilm formation or interferes with virulence factors i.e. quorum quenching or quorum inhibition (Miller and Bassler, 2001, Nealson *et al*., 1970). It has been proved experimentally that some natural products block quorum sensing in *Ps. aeruginosa* such as garlic (Bjarnsholt *et al*., 2005, Bodini *et al*., 2009), Green tea (*Camellia sinesis*) (Vattem *et al*., 2007, Bodini *et al*., 2009), orange extract (Truchado *et al*., 2012) and honey (Truchado *et al*., 2009a).

1-7-5 Herbal therapy

The use of a plant or a part of a plant to treat disease is known as herbal therapy (Herbalism). Since many drugs have been derived from botanical sources, the medical effects of some herbs or herb oils are well known. Table 4.lists some herbs that have been found to have activity against microorganisms.

Table 4. Some examples of herbal medicines

Common name	**Scientific name**	**Reference**
Onion	***Allium cepa***	Elnima *et al* (1983)
Garlic	***Allium sativum***	Harris *et al* (2001)
Tamarind	***Tamamrindus indica***	Kothari and Seshadri (2010)
Fennel	***Foeniculum vulgare***	La Cantore *et al* (2004)
Dill	***Anethum graveolens***	Kaur and Arorra (2009)
Green tea	***Camillia sinensis***	Gordon and Wareham (2010)
Black Seeds	***Nigella sativa***	Chaieb *et al* (2011)

1-8 Research objectives

The overall aim of this work is to investigate many types of honey, collected from different sources and regions, to determine which are the most active as antibacterial agents and which might offer an effective alternative, in the face of increasing bacterial resistance, to the antibiotics currently in use.

Chapter 2

Studies on the Antibacterial Activities of a Range of Commercially Available Honeys

2-1 Introduction

The curative properties of honey have been known since ancient times, especially as a topical agent for use on wounds. The Quran states that the God inspired the bees to eat from all fruits so that can make a liquid with different colours and a healing effect for people (Chapter fourteen, Surat Al Nahel verse No.69). Honey shows inhibitory activity against a wide range of microorganisms (Al-Waili, 2004, Sheikh *et al*., 1995), including bacteria such as *Bacillus cereus, Staphylococcus aureus, Salmonella,* and *Shigella dysenteriae* (Ceyhan and Ugur, 2001), as well as anaerobic bacteria (Elbagoury and Rasmy, 1993). Molan (1999) reported that the use of honey has recently become more acceptable to both practitioners and the general public. Honey can be used on many types of surface infections (Lusby *et al*., 2005) as well as internal infections, notably intestinal infections (Adebolu, 2005).

Honey is made up of more than 181 components and is a mixture of supersaturated sugars mainly fructose (38%) and glucose (31%). The moisture content is about 17.7%, total acidity 0.08% and ash content 0.18%. Additional components present, at much lower levels, include phenolic acids and flavonoids, enzymes such as glucose oxidase and Catalase, ascorbic acid, carotenoids, organic acids, amino acids, proteins, and α-tocopherol (Viuda-Martos *et al*., 2008).

The diversity of honey depends on the species of bee involved, the botanical source of nectar or exudates collected by the bee, climate, geographical region, the harvesting process, storage conditions and length of storage. The inhibitory activity of honey is not attributable only to the sugar content (Cooper, 2007), however, Mundo *et al* (2004) showed that flavonoids and proteinacious compounds play a role in its antimicrobial activity. The acidity of honey (pH generally between 3.2 and 4.5) also helps to inhibit microbial growth (Iurlina and Fritz, 2005). Moreover, Bogdanov

(1997) suggested that a part of the antimicrobial efficacy of honey might relate to the plant origin of nectar.

There are broadly two types of honey, namely non-peroxide based and peroxide based. The first has the ability to inhibit microorganisms in the presence of Catalase (an enzyme which breaks down hydrogen peroxide) while the second type lacks this ability (Cooper, 2007). Hydrogen peroxide is generated from the oxidation of glucose by glucose oxidase (an enzyme secreted by bees as they deposit nectar into the hive (White *et al*., 1963).

Non-peroxide honey is more useful in wound treatment than the peroxide-based honey which loses its activity in the presence of the Catalase secreted by many types of bacteria, notably *S. aureus.* Manuka honey from New Zealand and Jelly bush honey from Australia are two examples of non-peroxide honeys. Manuka honey is unique and heat stable and contains an antibacterial component, which has been identified as methylglyoxal (Figure 4)(Adams *et al*., 2009).

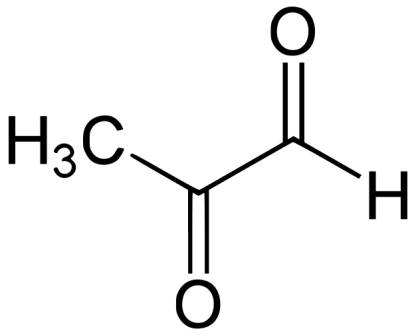


Figure 4. Methylglyoxal (Wikipedia)

Honeys are also effective against biofilms where antibiotic treatment is generally less effective; honey having the ability to penetrate and inhibit biofilms formed by *S. aureus,* Methicillin resistant *S. aureus*, ESBL *Klebsiella pneumonia* and

Ps. aeruginosa (Merckoll *et al*., 2009). Al Andejani *et al.,* (2009) reported that Manuka and Sidr honey have an above average bactericidal rate against previously established biofilms of *S. aureus,* Methicillin resistant *S. aureus*, and *Ps. aeruginosa*. Recently Cooper *et al* (2011) showed that Manuka honey can either interfere with biofilm formation or can kill members of an already established biofilm.

The aim of the work reported in this Chapter was to evaluate the effectiveness of 22 different types of honey on pathogenic bacteria by testing them using a well diffusion assay. The most effective honeys were then subjected to the determination of:

1- Minimum Inhibitory Concentration (MIC).
2- Minimum Bactericidal Concentration (MBC).
3- The effect of Catalase treatment.
4- The effect of exposure to peroxide.
5- The effectiveness of honey when all enzymes have been deactivated.
6- The activity of honey on anaerobic bacteria.
7- The isolation of bacteria and fungi from the most effective antibacterial honeys.
8- The effects of the isolates which have been isolated from honey on the pathogenic bacteria.

2-2 Material and methods

2-2-1 Honey samples

Twenty two honey samples were obtained from various sources. Manuka honey was obtained from Holland and Barrett, Sheffield, UK and *Nigella sativa* honey (*Nigella sativa* seeds with Royal Honey) from a local Arabic shop in Sheffield, UK. Twenty commercial honey samples were also tested. The botanical source and origin is listed in Table 5.

Table 5. The botanical source and the country of origin of the used honey

Honey type	**Botanical Source**	**The origin Country**
Zambezi	Flowering tree	Zambia
Balkan Linden	Lime Flowers	Serbia
New Zealand Rewarewa	Rewarewa tree	New Zealand
New Zealand Rainforest	Wild herbs, flowers and trees	New Zealand
Himalayan Highlands	Forest tree	Kashmir
Balkan Black Locust	Pollen grains of genus *Robinia pseudacacia* (family Fabaceae), rose family (Rosaceae), cabbage family (Brassicaceae) and bean family (Fabaceae) (Natalija Uršulin-Trstenjak *et al*., 2013).	Serbia
Lavender	Lavender	UK
Orange Blossom	Orange Blossom	Spain
Thyme	Thyme	Spain
Oak	Oak	Spain
Chestnut	Chestnut	Italy
Nigella sativa seeds with Royal honey	Grounded *Nigella sativa* with Royal honey	UK
Manuka +20	Manuka (*Leptospermum scoparium*)	New Zealand
Edel	Unknown source	
Rainforest +1	Tineo and Ulmo	Chilli
Rainforest +30	Tineo and Ulmo	Chilli
Bee Bio active	wild herbs, flowers and trees	UK
Famous	Borage herbs	UK
Wald Bluten *honig	Forest flower	Germany
Tannen *honig	Tannen tree	Germany
Bluten*honig	Wild flower	Germany
Wald *honig	Forest tree	Germany

*Honig is German for honey

2-2-2 Test organisms

Two of the most common causing wounds infection bacteria were tested: *Staphylococcus aureus* SH1000 and *Pseudomonas aeruginosa* PA01.

2-2-3 Well-diffusion assay

The bacterial suspension was prepared as described in Appendix (1) and measured in spectrophotometer to equal the standard turbidity obtained previously. Concentrations of 100%, 80%, 40%, 20% and 10% were prepared by dissolving honey with sterile distilled water at 40^{o}C for 30 min for each type of the twenty two honey samples used. The technique of well diffusion method is described in Appendix (2).

2-2-4 Minimum Inhibitory Concentrations (MIC)

The effective antibacterial honeys as determined by well diffusion test were chosen to assess the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) from 80% to 5%.The bacterial suspension was prepared by inoculating 10 ml of nutrient broth with bacteria and calibrated to equalize to 0.5 McFarland turbidity standards. The mixtures were then incubated at 60^{o}C for 10 minutes and mixed using a Vortex mixer for 2 minutes, and the tubes were incubated in 37^{o}C with shaking at 250 rpm for 24hrs. MICs were defined as the lowest concentration of honey, which showed no visible growth.

2-2-5 Minimum Bactericidal Concentrations (MBC)

After determining the MIC, all tubes were inoculated on nutrient agar by taking a loopful (approximately 10 µl) of the mixture and incubating in 37^{o}C overnight. The lowest concentration, which showed no growth on the agar plate, was defined as the minimum bactericidal concentration.

2-2-6 Catalase treatment

The most effective types of honey were tested for Catalase activity, and *Staphylococcus aureus* was chosen for the test. Catalase solution was prepared by dissolving 2 mg of Catalase provided by Sigma Aldrich UK in 10 ml of ultrapure sterile distilled water. Honey was diluted to 50% with distilled water. Diluted honey (1ml) was mixed with 1 ml of bacterial suspension in Catalase solution in sterile Peju tube and 1 ml of the same diluted honey was mixed with bacterial suspension without Catalase as a control in different Peju tube. All tubes were incubated in 37^0C with shaking for 24hrs. Then all tubes were streaked out on Nutrient agar and checked for any growth after 24hrs in 37^0C (Snow and Manley-Harris, 2004); triplicates were used throughout.

2-2-7 Peroxide Test

The most effective types of honey were tested for the presence of peroxide after they were diluted with sterile distilled water at 50%. Instructions provided by QUANTOFIX® Peroxide 25 were applied (Appendix 5). Briefly, a test strip was dipped for one second in honey solution and read after 15 seconds. The resultant colour was compared with the colour scale on the aluminium container and the result was observed and recorded as (Negative or +, ++, +++) depending on colour reaction. Distilled water was used as a negative control and hydrogen peroxide solution (9% for general antiseptic purposes) was used as a positive control.

2-2-8 The effect of dilution and autoclaving on antibacterial effect of honey

Honeys were diluted and autoclaved by the concentrations of 100%, 80%, 40%, 20% and 10%. Sterile Muller-Hinton agar (20ml) plates were used. The surface of the plates was inoculated with previously calibrated inocula of bacteria and allowed to dry before incubation in 37^o C for 30 minutes. Five wells of 8.0 mm in diameter were

cut from the culture media by using a sterile metal cylinder, and then filled with the previous mentioned concentrations. Vancomycin (30µg) was used as a positive inhibition control for *Staphylococcus aureus* and Imipenem (10 µg) for *Pseudomonas aeruginosa*. The plates were incubated at 37^{o}C and checked after 24 hrs for clear, circular inhibition zones around the wells. These zones were then measured. The same steps were performed for raw honey in order to compare the results.

2-2-9 Activity of honey on anaerobic bacteria

Fusobacterium nucleatum, one of the major causes of mouth abscess was tested under anaerobic conditions in order to evaluate the potency of honey under such condition. The same procedures mentioned in well diffusion assay were set up under anaerobic conditions. Anaerobic kit generator and anaerobic indicators supplied by Oxoid® were used. Cultures were set up aerobically and anaerobically as controls.

2-2-10 Isolation of bacteria and fungi from the most active honey

A number of trials were done to isolate microorganisms from honey. Five types of honey, Manuka, Oak, *Nigella sativa*, New Zealand Rewarewa, Rain forest +30 and New Zealand Rainforest were diluted 50% V/W and applied to media supplemented with antimicrobial agents as follows:

2-2-10-1 Isolation of fungi and yeast

Czapek Dox Agar was supplemented with penicillin (5000 units/ml) and streptomycin (5 mg/ml) to exclude the growth of Gram positive and Gram negative bacteria respectively.

2-2-10-2 Isolation of bacteria

Nutrient agar was supplemented with Nystatin (1250 units/ml) (typically as the concentration used in Thayer Martin media) in order to exclude the growth of fungi.

Aspergillus spp and *Staphylococcus aureus* were used as controls for media. Bacterial DNA was purified and identified as described in Appendix (3).

2-2-11 Testing the honey isolates on the pathogenic bacteria

Bacteria and fungi obtained from honey were spread on Muller Hinton agar and incubated for 24 hrs at 37^0C. A bacterial suspension was prepared as mentioned in Appendix (1). The surface of the plates was inoculated with previously calibrated inoculums of the pathogenic bacteria and allowed to dry before incubation in 37^o C for 30 minutes. Three wells of 8.0 mm in diameter were cut from the culture media of the isolated bacteria from honey by using a sterile metal cylinder, each type has three cut and then the piece of agar were applied on the plate on which the pathogenic bacteria suspension had been spread. Vancomycin (30µg) was used as an inhibition control for *S. aureus* and Imipenem (10 µg) for *Ps. aeruginosa*. The plates were incubated at 37^oC and checked after 24 hrs for clear, circular inhibition zones around the piece of agar. These zones were then measured.

2-**2-12 Statistical analysis**

All observations were presented as Mean ± SD (Standard Deviation). The data was analyzed bySigmaPlot® 12.0. $P \leq 0.05$ was considered as the significance level.

2-3 Results and Discussion

2- 3-1 Disc diffusion assay

Most of the tested honeys exhibited moderate activity against *S. aureus* and *Ps. aeruginosa.* Of the 22 types of honey tested five showed marked antibacterial activity (Figure 5). The order of antibacterial activity against *S. aureus* was: Manuka +20,Rainforest +30, Oak, New Zealand Rewarewa and *Nigella sativa*(Table 6), while the order for *Ps. aeruginosa* was Rainforest +30, New Zealand, Rewarewa, *Nigella sativa*, Manuka +20 and Oak (Table 7). The diversity of antimicrobial activity among different types of honey is attributed to factors such as botanical source(which influences its composition), harvesting time, the site of collection, processing and storage conditions (Molan and Cooper, 2000). Dixon (2003) claimed that microbial resistance to honey has never been reported. Lack of antibacterial activity on honeys may however, result from poor storage conditions, where the sugars in honey undergo crystallization and the honey tends to lose its antibacterial activity; this may explain why, when tested here, Bee Bioactive and Famous Honey exhibited no antibacterial activity.

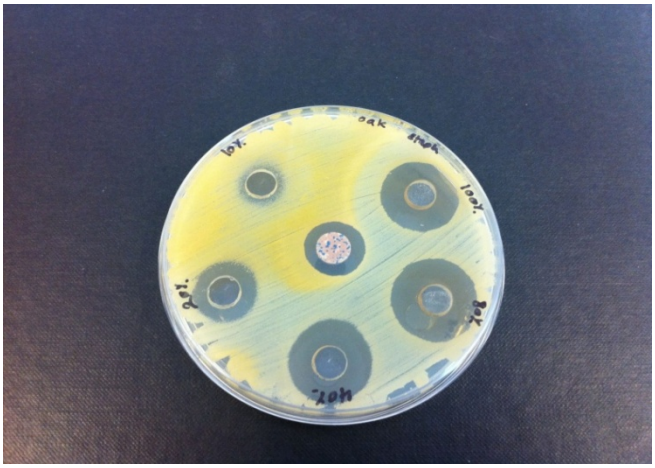


Figure 5. Well diffusion assay (showing clear zones around the wells)

Table 6. The effect of different concentrations of various honeys against *S. aureus* determined by well agar diffusion. The values are means of replicates (well 8.0 mm) ± Standard Deviation. The most effective honeys are highlighted in yellow.

	Honey concentrations				
Types of Honey	**100%**	**80%**	**40%**	**20%**	**10%**
Zambezi Plains(ZP)	**15 ±0**	**14 ±1.5**	**8 ±0**	**8 ±0**	**8 ±0**
Balkan Linden(BL)	**15 ±1.5**	**10 ±0.5**	**8 ±0**	**8 ±0**	**8 ±0**
New Zealand Rewarewa(NR)	**19 ±1**	**18 ±1**	**16 ±0.6**	**8 ±0**	**8 ±0**
New Zealand Rainforest (NRF)	**13 ±0.6**	**11 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
Himalayan Highlands(HH)	**12 ±0.6**	**10 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
Balkan Black Locust(BBL)	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Lavender(L)	**10 ±1.7**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Orange Blossom(OB)	**12 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Thyme(T)	**11 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Oak(OAK)	***20 ±1***	***20 ±1.5***	***19 ±0***	***15 ±0.6***	***9 ±1***
Chestnut(CN)	**15 ±1.5**	**14 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
***Nigella sativa* (NS)**	**19 ±0.6**	**18 ±0.6**	**16 ±1.7**	**8 ±0**	**8 ±0**
Manuka +20	**33 ±1**	**33 ±2**	**25 ±1.5**	**8 ±0**	**8 ±0**
Edel	**20 ±1.2**	**18 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
Rainforest +10	**22 ±0**	**22 ±0.6**	**20 ±0.6**	**8 ±0**	**8 ±0**
Rainforest +30	**27 ±1.5**	**26 ±1**	**26 ±1.2**	**24 ±0.6**	**22 ±1**
Bee Bioactive	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Famous	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Wald Bluten honig	**22 ±0.6**	**19 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
Tanin honig	**24 ±0.6**	**24 ±4**	**19 ±0.6**	**8 ±0**	**8 ±0**
Bluten honig	**21 ±1**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Wald honig	**22 ±1.2**	**19 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**

Table 7. The effect of different concentrations of honey against *Ps. aeruginosa* determined by well agar diffusion. The values are means of replicates (well 8.0 mm) ± Standard Deviation. The most effective honeys are highlighted in yellow.

	Honey concentrations				
Types of Honey	**100%**	**80%**	**40%**	**20%**	**10%**
Zambezi Plains(ZP)	**13 ±1.2**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Balkan Linden(BL)	**10 ±2.3**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
New Zealand Rewarewa(NR)	**14 ±1**	**13 ±1.2**	**8 ±0**	**8 ±0**	**8 ±0**
New Zealand Rainforest (NRF)	**10 ±1.5**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Himalayan Highlands(HH)	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Balkan Black Locust(BBL)	**10 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Lavender(L)	**10 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Orange Blossom(OB)	**10 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Thyme(T)	**11 ±1**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Oak(OAK)	**12 ±0.6**	**10 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
Chestnut(CN)	**11 ±0.6**	**9 ±1**	**8 ±0**	**8 ±0**	**8 ±0**
Nigella sativa (NS)	**14 ±1**	**11 ±1.2**	**8 ±0**	**8 ±0**	**8 ±0**
Manuka +20	**19 ±1.2**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Edel	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Rainforest +1	**20 ±0.6**	**18 ±0.6**	**8 ±0**	**8 ±0**	**8 ±0**
Rainforest +30	**24 ±0.6**	**23 ±0.6**	**21 ±1**	**20 ±0.6**	**8 ±0**
Bee Bio active	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Famous	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Wald Bluten honig	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Tanin honig	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Bluten honig	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**
Wald honig	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**	**8 ±0**

2-3-2 Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC)

Five types of honey: Manuka+20, *Nigella sativa*, Oak, New Zealand Rewarewa and Rainforest +30 were chosen, following recognition of their marked antibacterial activities, to determine their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Figures (5 and 6) show that the minimum inhibitory concentration (MIC) was 10% against *S. aureus* for *Nigella sativa* Oak, New Zealand Rewarewa and Manuka, whereas, the minimum bactericidal concentration (MBC) for all these honeys was 20% (Figure 6). However, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Rainforest +30 against *S. aureus* was 5%.

Manuka +20 showed a MIC of 10% against *Ps. aeruginosa* whereas the MIC was 5% for Rainforest honey (Figure 7). Against *Ps. aeruginosa*, Rainforest +30 was the most effective antibacterial honey type, with a MBC of 10%. The following honeys exhibited MBCs of 20%; Manuka +20, *Nigella sativa*, Oak and New Zealand Rewarewa.

2-3-3 The antibacterial effectiveness of honey after treating with Catalase

In order to determine the degree of involvement of hydrogen peroxide in the antibacterial effects of the honeys, samples were diluted in a Catalase solution to breakdown (and inactivate) the inherent hydrogen peroxide. Three types of honey showed negative response to Catalase, namely New Zealand Rewarewa, Manuka +20 and Rainforest +30. Whereas, Oak Honey and *Nigella sativa* honey were completely deactivated by Catalase (Table 8).

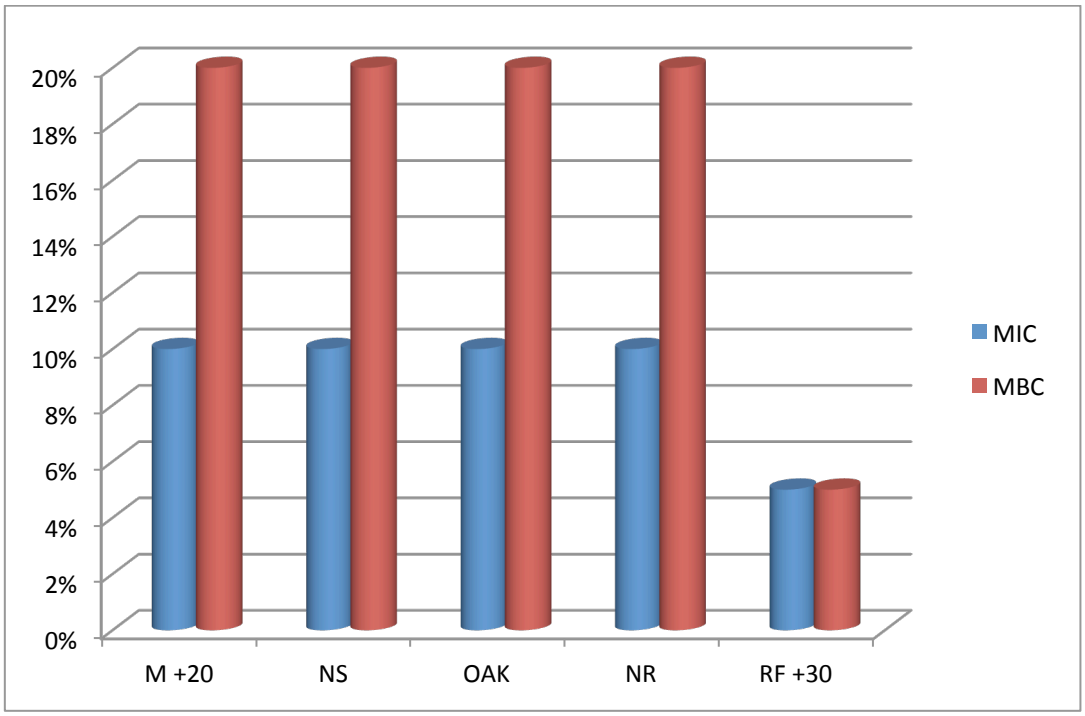


Figure (6): Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different honeys against *S. aureus*

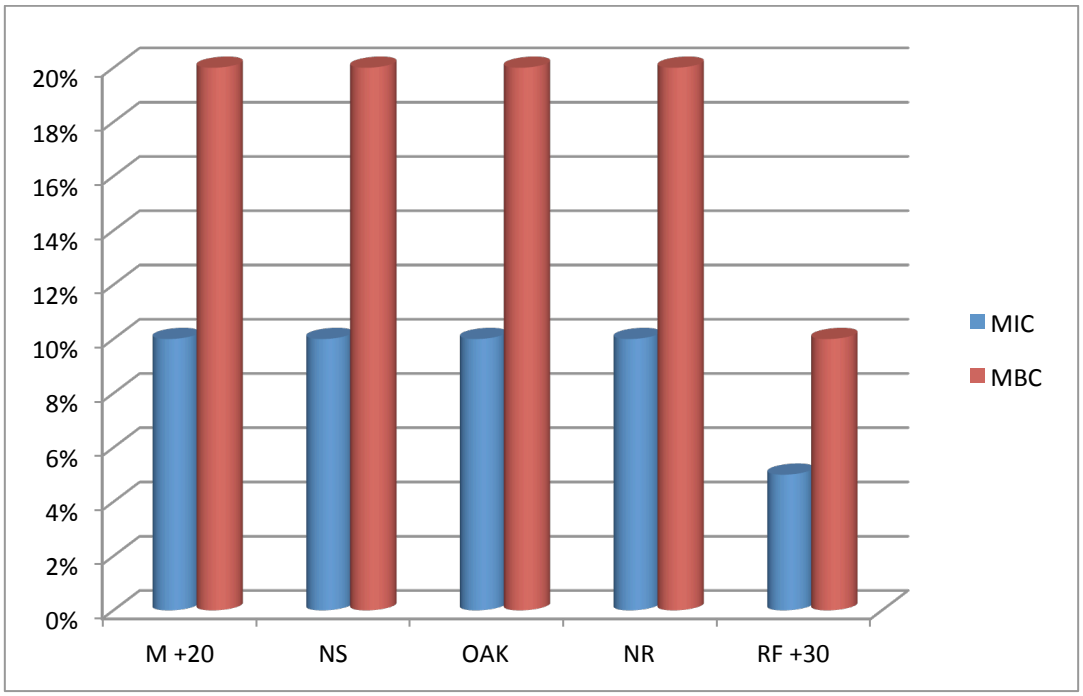


Figure (7): Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different honeys against *Pseudomonas aeruginosa*.

These results show that the first three-named honeys contain complex antibacterial agents, in addition to a small amount hydrogen peroxide; whereas the antibacterial activity of Oak Honey and *Nigella sativa* honey was due only to hydrogen peroxide, and that these honeys contained no complex antibacterial agents; similar conclusions were arrived by Brudzynski (2006).

Table 8.Catalase effect on honey

Honey type	**Control**	**1**	**2**	**3**
Oak	–	+	+	+
Nigella sativa	–	+	+	+
New Zealand Rewarewa	–	–	–	–
Manuka +20	–	–	–	–
Rainforest +30	–	–	–	–

+ = Antibacterial activity lost in presence of Catalase

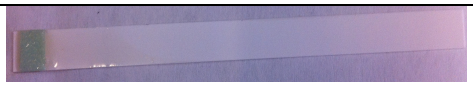
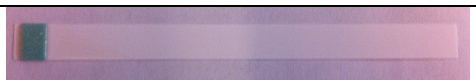
_ = No effect of Catalase on antibacterial activity

2-3-4 Determination of the role of peroxide in the antibacterial activity of honeys using the peroxidase strip test

The results given in Table (9) show that only Manuka honey +20, which is produced from the botanical source *Leptospermum scoparium* was a non-peroxide honey as has been reported byAdams *et al.,*(2009) andPacker *et al*., (2012). Whilst, Rainforest +30 honey shows marked peroxide activity when diluted with water, followed by Oak

honey; New Zealand Rewarewa and *Nigella sativa* honeys showed a low content of peroxide. The antibacterial activity of Rainforest +30 honey is clearly due to its hydrogen peroxide content although, as mentioned above, in other honeys (like Manuka) the antibacterial effect is due not only to hydrogen peroxide, but to more complex antibacterial agents which result from the plant-source of the honey, a conclusion similarly arrived at by Taormina *et al*., (2001).

Table9. Hydrogen peroxide content in honey

Type of Honey	**Result**	**Images**
Negative control	Negative	
New Zealand Rewarewa	+	
Manuka+20	Negative	
Oak,	++	
Nigella sativa	+	
Rainforest +30	+++	
Positive control	+++	

2-3-5 Effect of dilution and autoclaving on antibacterial effect of honey

Hydrogen peroxide and a number of antibacterial enzymes are liberated from some honeys on dilution with water (Bang *et al*., 2003). The honeys shown in Figure (8 and 9) were autoclaved for 15 minutes at 100% (raw honeys) and after dilution to 80% shows that autoclaving of raw honeys reduced antibacterial activity against *S. aureus* of all of the honeys, but only significantly so in the case of Manuka honey. The same

result was seen when the honey were diluted to 80% before autoclaving, showing that while dilution is considered to release antibacterial factors there was no difference seen here between the use of raw and diluted honeys. In the case of *Ps. aeruginosa*, autoclaving had no significant effect on the activities of all honeys except for Rainforest +30 when used raw or diluted (Figure 10 and 11).

The results shown in Figures (8,9, 10 and11) show that autoclaving honey generally leads to a reduction in its antibacterial activity against both *S. aureus* and *Ps. aeruginosa* but this was seen to be significantly different only in the case of Manuka honey for *S. aureus* and Rain Forest honey +30 for *Ps. aeruginosa*. This suggest that hydrogen peroxide and other antibacterial factors are removed/denatured by autoclaving, but that in the case of Manuka against *S. aureus*) and Rain Forest+30 (*Ps. aeruginosa*) some, non-autoclaveable, antibacterial factor remains.

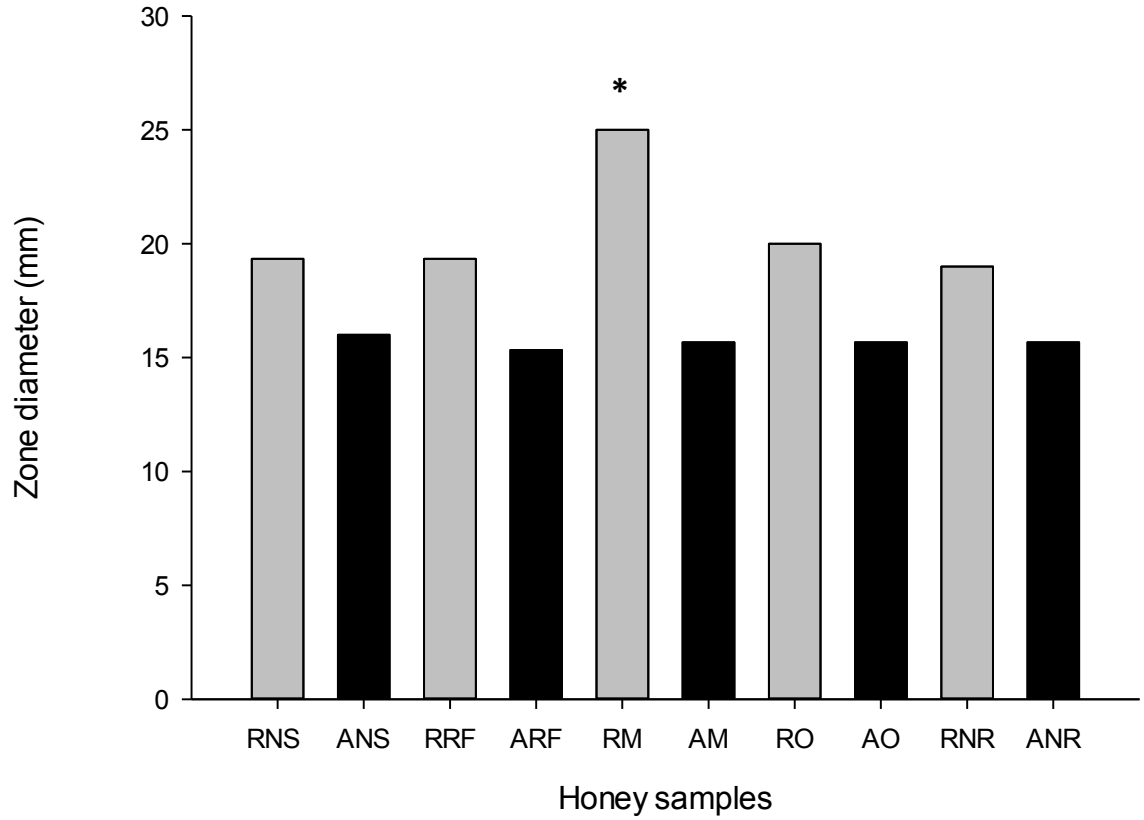


Figure 8. Activity of raw and autoclaved honey against *S. aureus* at 100% (*Significant difference at 0.05)

Raw Honey

Autoclaved Honey

RNS= Raw *Nigella sativa* honey

ANS= Autoclaved *Nigella sativa* honey

RRF= Raw Rainforest honey

ARF= Autoclaved Rainforest honey

RM= Raw Manuka +20 honey

AM=Autoclaved Manuka +20 honey

RO= Raw Oak honey

AO= Autoclaved Oak honey

RNR= Raw New Zealand Rewarewa honey

ANR= Autoclaved New Zealand Rewarewa honey

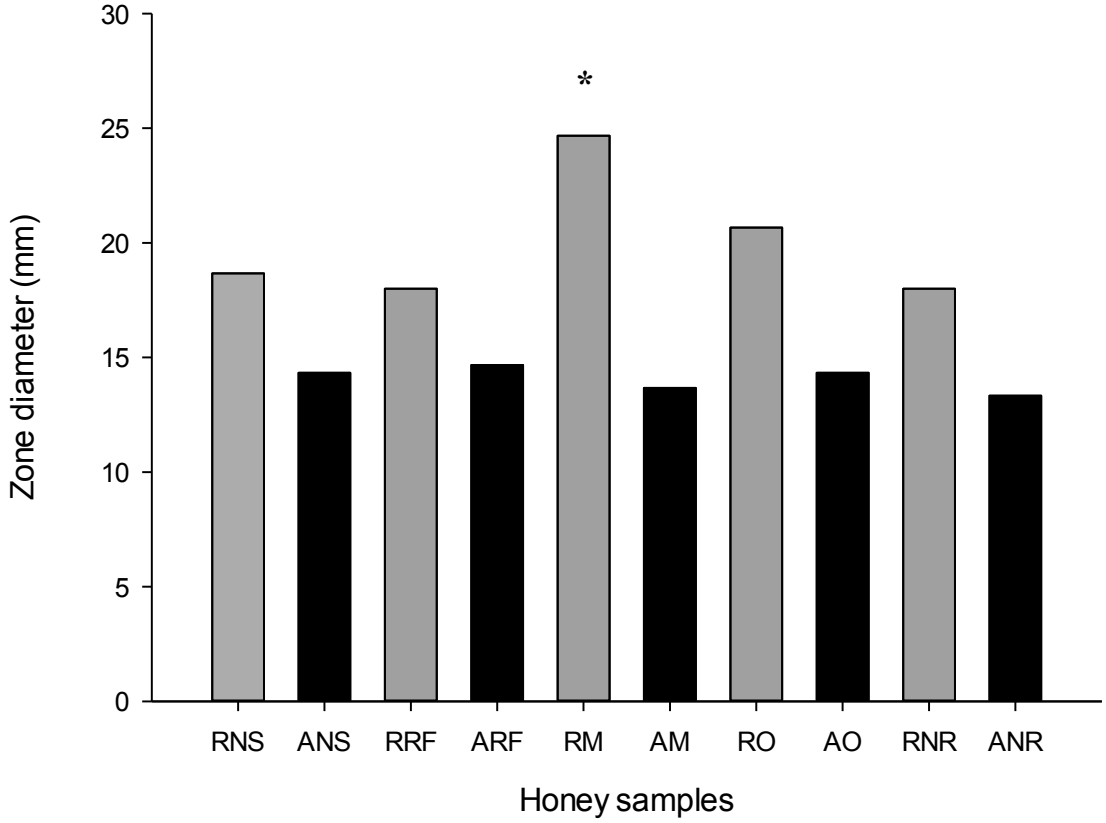


Figure 9. Activity of raw and autoclaved honey against *S. aureus* at 80% (*Significant difference at 0.05)

Raw Honey

Autoclaved Honey

RNS= Raw *Nigella sativa* honey

ANS= Autoclaved *Nigella sativa* honey

RRF= Raw Rainforest honey

ARF= Autoclaved Rainforest honey

RM= Raw Manuka +20 honey

AM=Autoclaved Manuka +20 honey

RO= Raw Oak honey

AO= Autoclaved Oak honey

RNR= Raw New Zealand Rewarewa honey

ANR= Autoclaved New Zealand Rewarewa honey

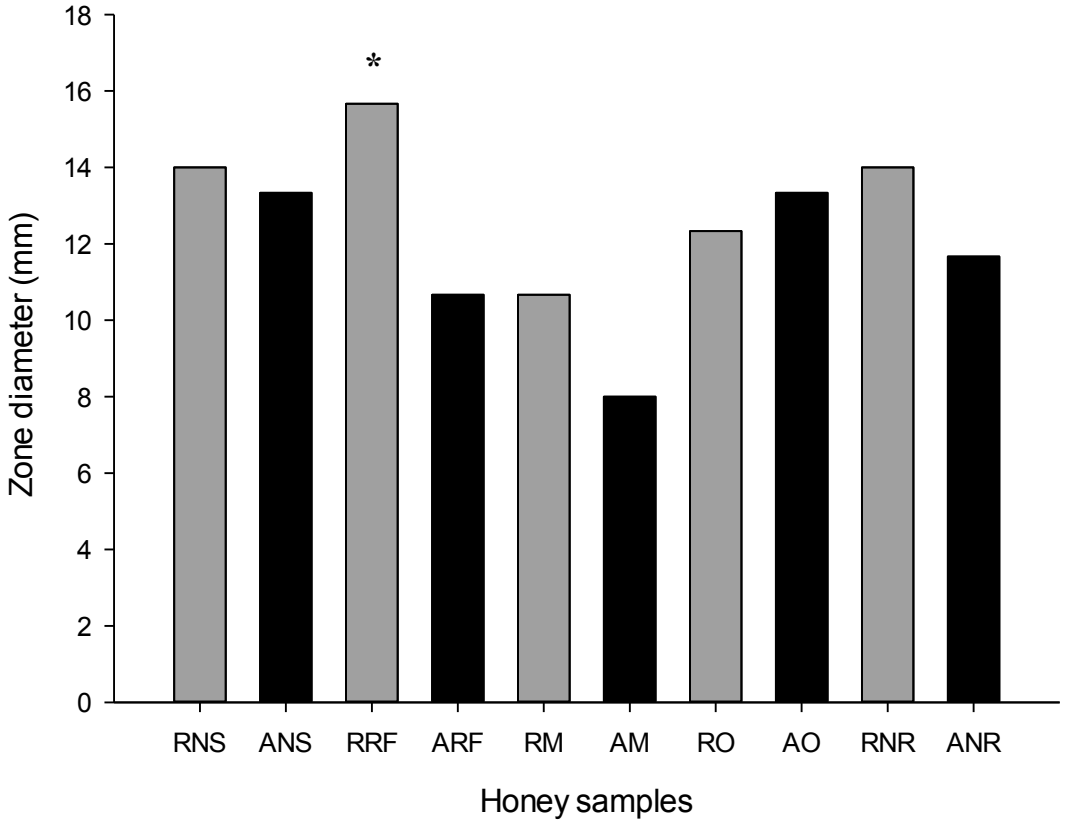


Figure 10. Activity of raw and autoclaved honey against

Pseudomonas aeruginosa at 100% (*Significant difference at 0.05)

Raw Honey

Autoclaved Honey

RNS= Raw *Nigella sativa* honey

ANS= Autoclaved *Nigella sativa* honey

RRF= Raw Rainforest honey

ARF= Autoclaved Rainforest honey

RM= Raw Manuka +20 honey

AM=Autoclaved Manuka +20 honey

RO= Raw Oak honey

AO= Autoclaved Oak honey

RNR= Raw New Zealand Rewarewa honey

ANR= Autoclaved New Zealand Rewarewa honey

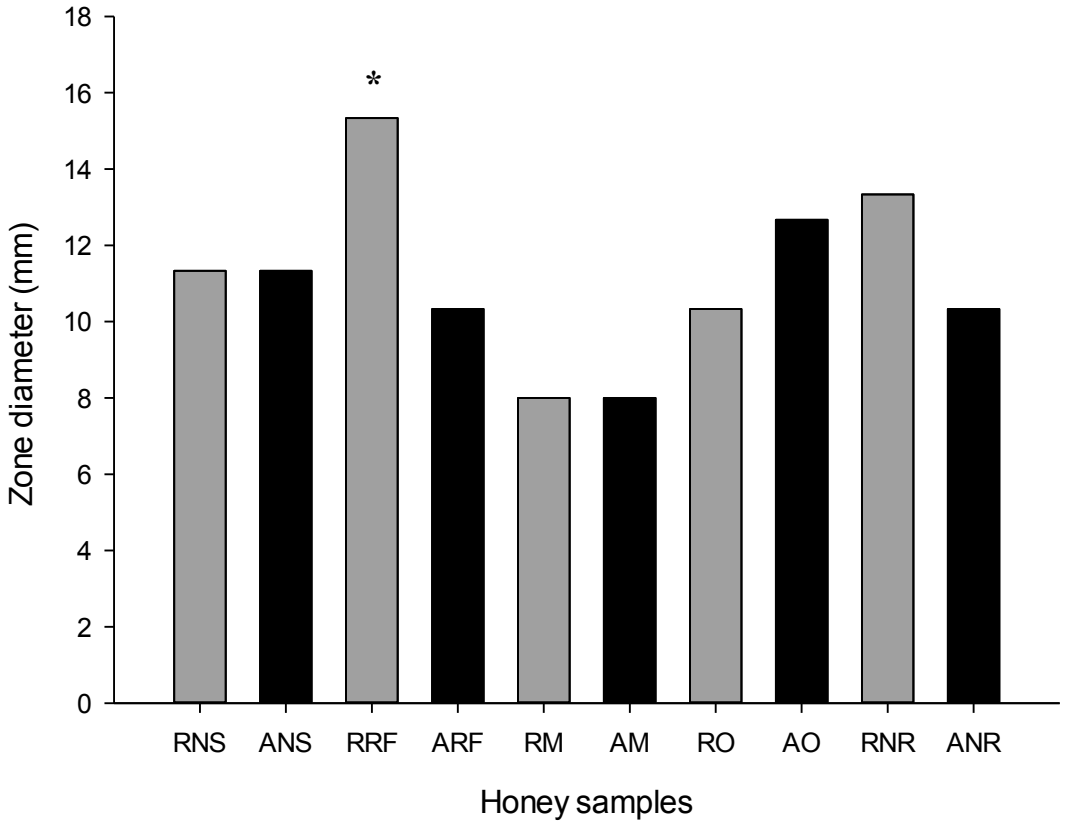


Figure 11. Activity of raw and autoclaved honey against *Pseudomonas aeruginosa* at 80% (*Significant difference at 0.05)

Raw Honey

Autoclaved Honey

RNS= Raw *Nigella sativa* honey

ANS= Autoclaved *Nigella sativa* honey

RRF= Raw Rainforest honey

ARF= Autoclaved Rainforest honey

RM= Raw Manuka +20 honey

AM=Autoclaved Manuka +20 honey

RO= Raw Oak honey

AO= Autoclaved Oak honey

RNR= Raw New Zealand Rewarewa honey

ANR= Autoclaved New Zealand Rewarewa honey

2-3-6 Effect of honeys on anaerobic bacteria

Fusobacterium nucleatum is a major cause of mouth abscess. In this experiment the antibacterial effects of honeys against this bacterium was tested under anaerobic conditions, in order to simulate the anaerobic wound environment. Undiluted Manuka honey was again shown to be the most effective antibacterial honey under these conditions. However, Oak, *Nigella sativa* and New Zealand Rewarewa were more effective than Manuka when diluted to 40% and 20% (Table 10). The results show that certain honeys (again notably Manuka honey) are effective in killing potentially pathogenic, anaerobic bacteria; this is obviously an important factor in the treatment of wounds, many of which become anaerobic.

Table 10. The effect of different types of honey on *Fusobacterium nucleatum* when grown under anaerobic conditions. The values are means of replicates (well 8.0 mm) ± Standard Deviation.

	Zone Diameter(mm)				
Honey Types	**100%**	**80%**	**40%**	**20%**	**10%**
Manuka +20	28±1.2	18±0.58	12±0.58	8	8
Oak	19±0.58	18±1	18±1.2	13±1	8
Nigella Sativa	16±1.5	16±1.2	13±1.2	13±0.58	8
New Zealand Rewarewa	20±2.1	18±1.5	14±0.58	11±1	8

2-3-7 Isolation of microorganisms from honey

During the current studies on honeys it became obvious that some varieties are variously contaminated with both bacteria and fungi. The aim of the work reported here was to isolate any bacteria from honey and identify those using molecular techniques. It is obviously important to determine whether honeys contain contaminating microbes, and in particular to determine if such contaminants are potential pathogens. Similar studies have been reported by Lee *et al*., (2008). Three types of bacteria were isolated as honey contaminants, these were identified using 16SrRNA (Figure 12 and 13) and found to be: *Lysinibacillus fusiformis*, *Staphylococcus epidermidis* and *Sporosarcina koreensis* (see Appendix isolates 1, 2 and 3). They were respectively isolated from Oak honey, *Nigella sativa* honey and Manuka honey.

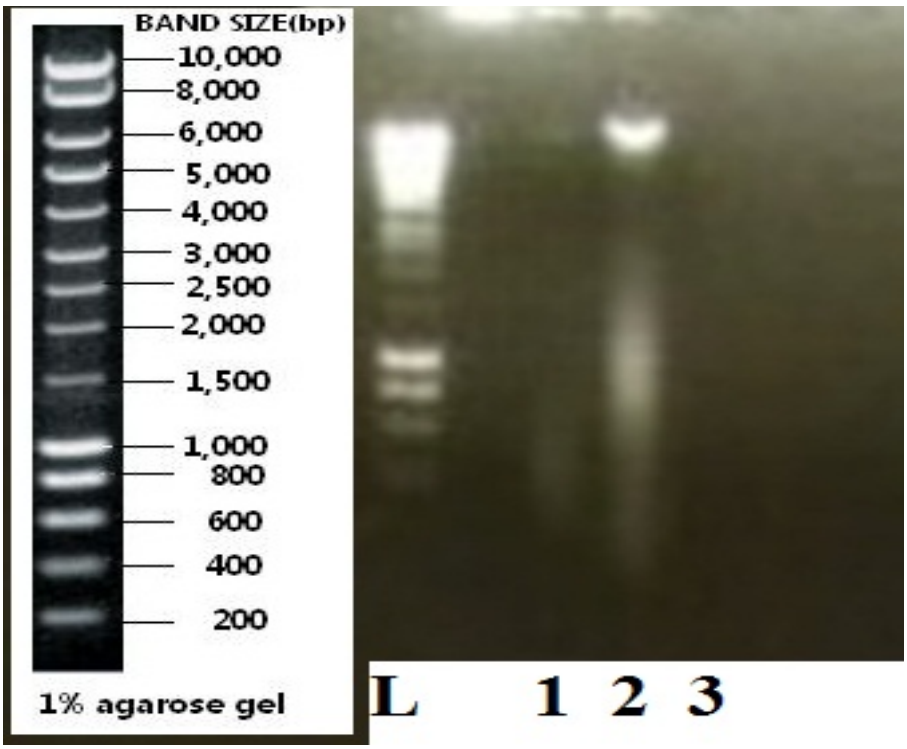


Figure12. Agarose gel electrophoresis showing a successful DNA extraction from unknown isolated bacteria from honey. Lane L, Ladder. 1, 2 are bacterial DNA

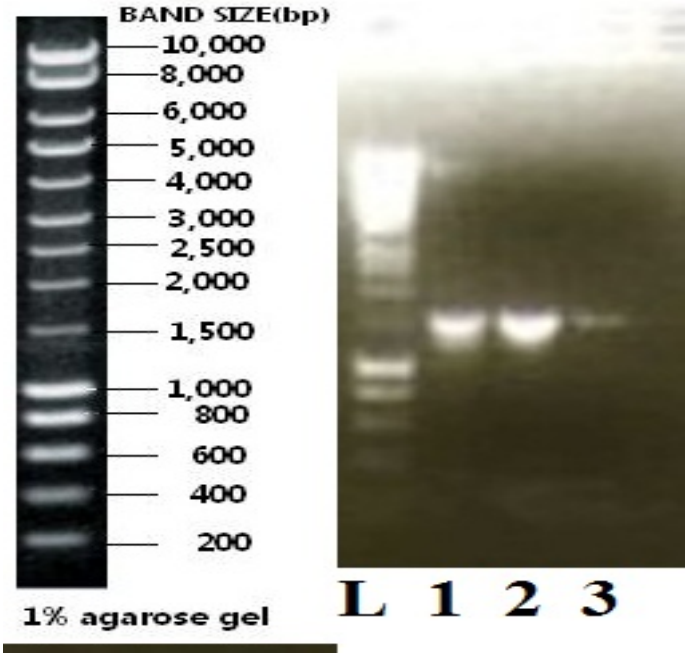


Figure13. Agarose gel electrophoresis showing a successful amplification of 16S rRNA from unknown isolated bacteria from honey. Lane L, Ladder. 1, 2 and 3 are *Lysinibacillus fusiformis*, *Staphylococcus epidermidis* and *Sporosarcina koreensis* respectively

Lysinibacillus fusiformis is a gram-positive rod, none-motile, with an approximate length of 2.5-3.0 micrometers and an approximate width of 0.5-0.9 micrometers. Under adverse conditions, it can generate inactive spherical endospores that are resistant to high temperatures, chemical sterilizing agents damaging chemicals, and ultraviolet light. The developing endospores localize either centrally or terminally within the enlarged sporangia and can remain viable for considerable periods. In terms of pathogenicity, *L. fusiformis* is thought to cause tropical ulcers and dermal and/or respiratory infections .*L. fusiformis* tests positive for oxidase and is an obligate aerobe. *Sporosarcina* as the name suggests, occurs in packages of cells and forms spores. Many species grow at temperatures below 10°C, and are halotolerant and grow in media containing NaCl at a concentration of 3-15%.

Some Staphylococci species, such as *S. epidermidis* is considered as a normal skin flora and it has no risk on healthy people. However, most infections of *S.epidermidis* is associated with immuno-compromised patients.

2-3-8 Effect of isolated microorganisms on pathogenic bacteria

All of the three isolated bacteria referred to above were tested to determine if they exhibit any antibacterial activity against *S. aureus*. None of the isolates showed any anti-Staphylococcal activity (Figure 14, 15 and 16).

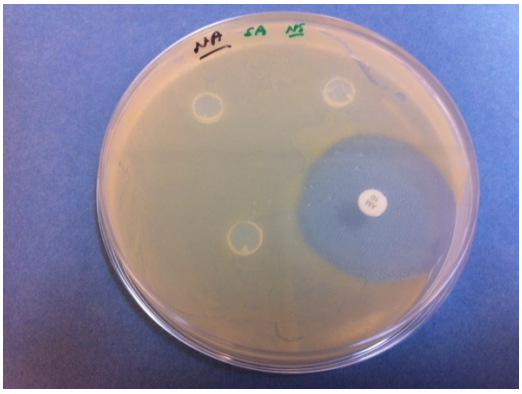


Figure (14): The effect of *Staphylococcus epidermidis* isolated from *Nigella sativa* honey on *S. aureus* (Vancomycin disc used as a positive control)

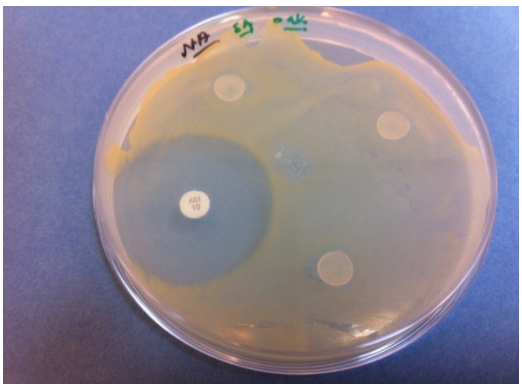


Figure (15): The effect of *Lysinibacillus fusiformis* isolated from Oak honey on *S. aureus* (Vancomycin disc used as a positive control)

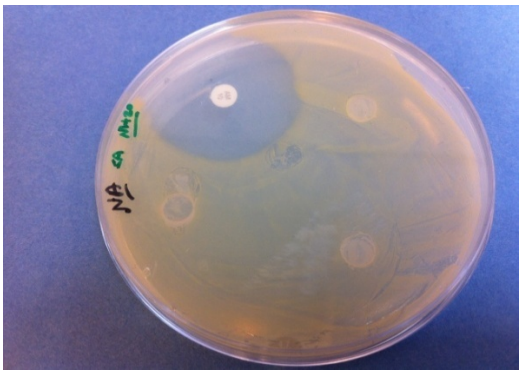


Figure (16): The effect of *Sporosarcina koreensis* isolated from Manuka +20 honey on *S. aureus* (Vancomycin disc used as a positive control)

The antibacterial activity of honey has been reported to vary as much as 100-fold (Molan, 2001). A finding which agrees with the results given in this Chapter where the antibacterial activity of honeys, from around the world, were tested and found to be highly variable. Not all honeys can therefore be used for therapeutic purposes, and care must be taken before a honey is chosen as a wound dressing; such honeys should have a) a high potency level b) a wide spectrum of antibacterial activity, particularly against bacteria commonly associated with wound infections (including anaerobes), c) marked non-peroxide activity. Non-peroxide antibacterial activity being because wound exudates may contain Catalase, which reduces the peroxide-related antibacterial activity when applied to the wound surface; any non-peroxide activity would, in contrast, be still active. Again, the results presented here confirm the marked antibacterial activity of Manuka honey, although the results also show that a small number of other honeys can surpass Manuka in antibacterial activity. Results presented in this Chapter also show that the antibacterial activity of Manuka honey depends on a number of components, notably complex, non-peroxide compounds. Manuka (*Leptospermum scoparium*) honey has been advocated, and found to be

effective, for use in wound treatment in the main for this reason (Molan, 2004; Molan, 2006). Searches for honeys possessing exceptionally high non-peroxide antibacterial activity has been a major aim of a number of a number of studies, including the present one. For example, in a large survey or 345 New Zealand honeys, Allen *et al*., (1991) showed that only 25 samples of Manuka and vipers bugloss honeys gave a significant non-peroxide antimicrobial activity. In another study of Portuguese honeys it was found that only 23% showed measurable non-peroxide activity (Henriques *et al*., 2005). Basson and Grobler (2008) showed that not all Manuka honey samples show exceptionally high antibacterial activity and associated marked non-peroxide activity. The most active Manuka honey appears to be produced from the East Cape region of the North Island of New Zealand (Basson and Grobler, 2008).

As honey dressings, used in wound management, are changed three times daily (Willix *et al*., 1992), an incubation period of 8 h is generally chosen to evaluate the bactericidal concentration of Manuka honey against the most dangerous antibiotic-resistant pathogen, namely MRSA. Molan (1992b) showed that Gram-positive bacteria start to die after 1 hour of honey exposure (Molan, 1992b), so that pathogen-destruction should occur in the usual dressing-exposure period.

The innate properties of honey which include low pH and high sugar content are considered to provide harsh conditions which bacteria cannot tolerate and which induce starvation-based activities, such as sporulation (Snowdon and Cliver 1996), an event known to be associated with degradative enzyme production (Marahier *et al.*, 1993). An antibacterial compound called, bee defensin-1 has also recently been discovered in a medical grade honey (Sherlock *et al*., 2010).

The potential presence of *Clostridium botulinum* spores in honey presents an obvious problem (Molan and Allen, 1996), because it is obviously associated with the risk of

wound infection. Vardi *et al.,* (1998) however, considered this to be a very low risk problem since they could not find a single report of *C. botulinum* wound infection being associated with honey dressings. Simon *et al.,*(2009) similarly could not detect a single case report linking the use of unsterilized honey in wound care and wound infections caused by *C. botulinum.* Local unsterilized honey has also been widely used to treat infected wounds and burns without the appearance of *C. botulinum* wound infections (Postmes *et al.*, 1996; Postmes, 2001).

In conclusion, the results presented in this Chapter confirm the antibacterial effects of honey, (notably Manuka varieties) and suggest that further studies of honeys from around the world are likely to lead to the recognition of active honeys which may surpass Manuka in their wound-healing potential.

Chapter 3

Antibacterial Activity of Some Commercial Products Containing Manuka Honey

3-1 Introduction

The emergence of multi resistant strains of bacteria to commonly used antibiotics has necessitated the search for new approaches. Honey, or apitherapy, is one such approach. Some other approaches are shown in Table 11.

Table 11. The efficacy of some products when combined with honey

Type of Honey	**Combined with**	**Acted On**	**Reference**
Sumra (*Acacai tortilis*)	Propolis	*S. aureus, E. coli* and *Candida albicans*	(Al-Waili *et al*., 2012)
Monofloral honey	Essential oils	*Ps. aeruginosa*	(Abdellah *et al*., 2013)
Tazma	Garlic	*S. aureus, Streptococcus pneumoniaea, Lysteria monocytogenes,* And *Salmonella spp.*	(Andualem, 2013)
West Algerian honey	*Nigella sativa* seeds	*Ps. aeruginosa*	(Abdelmalek *et al*., 2012)
Monofloral	Thymes	*S. aureus, E. coli* and *Ps. aeruginosa*	(Abdellah *et al*., 2012)
Medihoney	Rifampicin	MRSA and *S. aureus*	(Muller *et al*., 2013)

3-1-1 Bee venom

Bee venom is used to relieve pain and treat inflammatory diseases, such as rheumatoid arthritis(Kwon *et al*., 2001) and for the psycho- neurological treatment of autoimmune and nervous system disease (Somerfield *et al*., 1984, Kwon *et al*., 2001). As well as having an anti-inflammatory effect, bee venom exhibits antimicrobial activity against bacteria which cause inflammation such as *Propionibacterium acnes*,

Staphylococcus epidermidis and *Streptococcus pyogenes* (Han *et al*., 2010); bee venom appears to be more active against Gram positive than Gram negative bacteria (Perumal Samy *et al*., 2007).The most active component in bee venom against microorganisms is a compound called melittin (Fennell *et al*., 1968).

3-1-2 Manuka oil

Manuka oil is extracted from the Manuka plant or *Leptospermum scoparium* which occurs in New Zealand. Manuka oil has known antimicrobial properties (Maddocks-Jennings *et al*., 2005). The antimicrobial activity of Manuka oil is attributed to β-triketones, such as leptospermone, flavesone and isoleptospermone (Christoph *et al*., 2000).

3-1-3 Kanuka oil

Kanuka oil is derived from the kanuka plant, *Kunzea ericoides.* Kanuka oil has antimicrobial activity related to the presence of the same components as Manuka oil(Maddocks-Jennings *et al*., 2005).

The aim of the work described in this Chapter was to test some synthetic and natural products combined with Manuka honey including:

1- Cosmetic products containing Manuka honey: (These are proprietary products obtained from Holland and Barrett)
    a. Foaming cleaner.
    b. Foot-Heal cream.
    c. Facial cleaner tonic.
    d. Foaming cream.
    e. Blemish.
2- Washing products containing Manuka honey:
    a. Body wash.

b. Shampoo.

c. Conditioner.

3- A complex of bee venom and Manuka honey.

4- Vita complex (a mix of pollen, royal jelly, honey and propolis).

5- Manuka oil.

6- Kanuka oil.

7- Tooth Paste with Manuka honey compared with conventional toothpaste and mouth wash.

3-2 Materials and Methods

Staphylococcus aureus SH1000 and *Pseudomonas aeruginosa*PA01 were tested; the bacterial inocula were prepared as described in the Appendix 1. Tests were applied according to physical proprieties of the treatment, for example, well diffusion technique (Appendix 2) was used with liquid materials where, with the solid materials the treatment was added to the surface of the media and left for 15 minutes followed by streaking the bacteria out in parallel lines. For the tooth paste experiment, a sterile cotton swab was rubbed in the early morning before mouth washing and cultured on Nutrient Agar supplemented with 5% Horse blood (Obtained from Sigma- Aldrich®) to isolate the normal mouth flora. Plates were incubated overnight at 37^0C. After the growth, 16S r RNA techniques were used as described in Appendix 3 to identify the isolated bacteria.

3-3 Results and discussion

3-3-1 The antibacterial activity of some natural and commercial products and honey products containing Manuka honey

None of the Manuka-containing cosmetics had any effect on the growth of *Ps. aeruginosa* (Table 12). In contrast, the effect of the cosmetics on *S. aureus* was more variable, with the foot-heal cream, facial cleaner and foaming tonic having an inhibitory effect on growth, while the foot-heal cream and blemish cream showed no effect.

Table 12. Effect of various Manuka-containing cosmetics on positive/negative growth of bacteria

Type of cosmetic product	**Results**	
	Staphylococcus aureus SH1000	*Pseudomonas aeruginosa* PA01
Foaming cleaner	**Positive**	**Negative**
Foot-heal cream	**Negative**	**Negative**
Facial cleaner tonic	**Positive**	**Negative**
Foaming cream	**Positive**	**Negative**
Blemish cream	**Negative**	**Negative**

Positive= No growth after 24 hours of the incubation at 37^{o}C.

Negative= Bacterium has grown after 24 hours of the incubation at 37^{o}C.

Table 13. shows the effect of a range of honey products on the growth of *S. aureus*. With the exception of the Manuka Honey Conditioner, all of the products showed antibacterial activity against *S. aureus*. The two oils proved markedly antibacterial, but perhaps surprisingly the Manuka Body Wash was the most active against this bacterium; the bee venom and Vita Complex were also active against *S. aureus.* Again, with the exception of the Manuka Honey Conditioner, all of the products showed antibacterial activity against *Ps. aeruginosa* and again, in all cases, the antibacterial effect on both bacteria was more pronounced against *S. aureus* than it was against *Ps. aeruginosa*.

Table 13. The effect of different types of honey and natural products on *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The values are means of replicates (well 8.0 mm) ± Standard Deviation.

		Zone Diameter(mm)	
Category	**Material**	***S. aureus***	***Pseudomonas aeruginosa***
Washing products with Manuka Honey	Body wash.	24.3±1.2	15.7±10.6
	Shampoo.	18±2	14.7±0.6
	Conditioner.	8	8
	Complex of bee venom and Manuka honey	13.8±0.6	11.7±0.6
	Vita complex (pollen, royal jelly, honey and Propolis)	13.7±0.6	10±0+
Essential oils	Manuka oil.	19.3±0.6	10.3±0.6
	Kanuka oil.	16±1	14.7±1.15

3-3-2 Antibacterial activity of mouth cleansing products on mouth flora

Three types of bacteria were isolated from the mouth, namely *Streptococcus mitis, Streptococcus gordonii* and *Neisseria mucosa* (see Appendix isolates 4, 5 and 6). This isolation compares with the more commonly isolated species, such as *Lactobacillus*, from the mouth. The antibacterial effect of the three mouth-cleaning products was then tested using the well-plate method. The Manuka and Aquafresh tooth paste showed reduced inhibition zones of all three bacteria, while the non-honey mouthwash had no effect on bacterial growth; the mouthwash was then exempted from further study.

Figure (17) shows that both types of toothpaste were effective at killing all of the bacteria studied. The Manuka honey toothpaste was particularly effective against *St. mitis* and *St. gordonii* while being less effective against *N. mucosa* than Aqua Fresh.

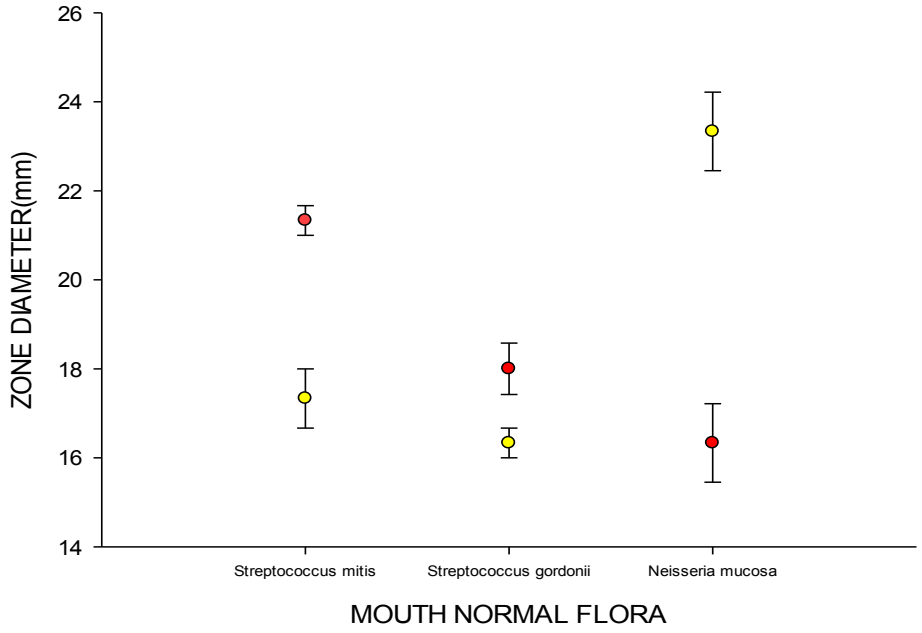


Figure17. The effect of two different toothpastes on mouth normal flora

Toothpaste with Manuka honey

Aqua Fresh

The following comments relating to the marketing of honey products was largely obtained in discussion with Mrs Gloria Havenhand and local honey producer and published expert on the lore associated with honey production and sale. Honey is produced throughout the world and consumed in various floral types by consumers who are seeking choice in flavours texture and claimed health benefits; it can therefore be regarded as a so-called "functional food". Honeys, like many food products are exposed to "aspirational selling", i.e. the sale of a commodity for which no specific claims are made, but which are purchased in the belief that they will

confer some, unspecified improvement in well-being. Since honeys are relatively easy to produce (except where viral and other diseases reduce yields) they are a generally a relatively low cost (Manuka honeys provide an exception in that jars can cost between 40-50 pounds, compared with 3-6 pounds for jars of non-Manuka honey) product which is open to competition from cheap supplies from countries where, because of climate and other economic factors, production costs are very low. As a result honey, producers are always keen to "add value" to their products in order to increase their sales and profit margins. This can be achieved by artful labelling which suggest the countryside, nature, or emotive historical links with the past (such as references to Monks and Abbeys or quaint sounding village names). The use of locality or environment can be another useful approach (such as labelling Yorkshire Honey, Sheffield Honey, Exmoor Honey) or by specifying the presence of specific floral origins, such as Manuka, Wild Blossom, Orange Blossom). An example of the success of such an approach can be seen regarding the sale of Medibee honeys the retail price (and sales) of which was increased following by labelling referring to the established antibacterial properties of these products. Clearly consumers are attracted by any added claims, which can be ascribed to the honeys themselves or related products.

A wide range of health benefits have been claimed for honey in addition to wound healing properties. Clearly, few consumers buy Manuka and other "antibacterial honeys for use as a wound curative or dressing, as wound health is more likely covered by a GP, hospital or nursing home. Consumers are however attracted by other claims such as the ability of honey to improve general health, reduce the symptoms of arthritis, improving arthritis or hay fever and sinus- related problems. Local honey producers often have experience of customers making their own claims for their

products which they pass on to other by word of mouth or written or internet recommendations. Such endorsements are advantageous to producers as they advertise the product without the need for the use of specific health claims to be made; they also lead to profitable return visits and spread the product names far and wide (Internet sales of honey have as a result grown exponentially over recent years). Such medical claims have, in some cases, been backed up by scientific research, while in other cases they remain apocryphal. In the past UK regulatory authorities have allowed vague statements to be put on honey products along the lines of "antibacterial honey, "improves health" etc. Until recently honey producers could make claims to antibacterial activity if these claims were backed up by research data(as was the case with work from this laboratory which validated antibacterial Medibee honeys)but the most recent regulations have prohibited even relatively mild claims from being made on packaging concerning the antibacterial properties, real or imagined of honeys.

One of the major ways by which honey producers can improve their profit base is to add honey to other products thereby producing "added value". This approach is particularly effective in relation to the development of cosmetics like skin care products. Such products attract a high premium by making generalized claims, which attract customers to buy honey-based cosmetics. For example, a range of Manuka based skin care products produced by the New Zealand company Madeleine Ritchie claim that honey's natural antioxidant and anti-microbial properties and its ability to absorb and retain moisture enable it to be used extensively in skin care treatments in order to help protect the skin from the damage of the sun's rays and rejuvenate depleted skin. They claim the treatment give the skin a very 'clean' sensation to the touch, leaving it feeling non-greasy, elasticated, rejuvenated and hydrated. They

further claim that because of its superior antibacterial protective properties, Manuka honey is “a luxurious and prized ingredient in any skin care preparation”.

An examples of the way in which honey products are marketed is given by the following industry statement relating to the bee product-propolis:

“Propolis is a mainly resinous substance that bees collect from trees and plants. Being made up of waxes, resins, fatty acids and even amino acids. Aristotle reputedly coined the name Propolis meaning "defender of the city". This is exactly what Propolis is for the hive; it acts as a bee population’s external immune system keeping the hive sterile and free of microbial invaders. New Zealand propolis is well renowned for its natural purity and high levels of antioxidants. We sell propolis in two forms - capsules and throat spray providing a convenient way to take advantage of the health benefits that propolis can offer. Our propolis capsules are manufactured according to the highest Good Manufacturing Practice (GMP) standards”.

A close examination of this paragraph shows that it links propolis with a long history of use it then unspecifically mentions the immune system, health benefits, antioxidants and purity of manufacture. Mention of amino acids is given to presumably add a scientific veneer to the quote. All of the words are suggestive of health benefits, rather than direct medical claims. They leave the purchaser to link all the words in their own mind and come to the conclusion that propolis, when added to cosmetics, will be beneficial to health.

Amazingly, in the UK alone each year, honey pots are filled with 1800 tons of Manuka and another 10 thousand tons are sold worldwide”. However, the British Food Safety Agency (FSA) has issued an international warning about false and illegal claims on the labels of Manuka honey and has showed that more than 50 percent of the products with that label contain little if any active substances of this esteemed

New Zealand Manuka bush tree (*Leptospermum scoparium*). Additional testing in Hong Kong revealed that 14 of the 55 samples of Manuka honey tested as fake. Although there are counterfeiters and other countries, it turns out that most companies that sell wrongly labelled Manuka honey on the British market comes from New Zealand, so that New Zealand primary producers of bee products face greater scrutiny, not only in the Europe, but also elsewhere.

Chapter 4

Comparative Effects of Honey and Antibiotics on Established Bacterial Biofilms

4-1 Introduction

Bacterial cells can aggregate in biofilms in an elaborate structure enclosed within a self-produced extracellular polymeric matrix, i.e. a biofilm (Costerton *et al*., 1999). In general, bacterial biofilms are formed on wet surfaces and mostly can be seen with the naked eye. Biofilms are present as an individual bacterium or in a combination of different species. The biofilm is highly organised and contain channels allowing the distribution of nutrients (Lawrence *et al*., 1991). Many cases of treatment failure with antibiotics are attributed to bacterial biofilms which are not easily eradicated by conventional therapy (Tart and Wozniak, 2008).

Biofilms present advantages as well as disadvantages. Some biofilms prevent tissue colonization by exogenous pathogens, an ability attributed to the production of hydrogen peroxide, biosurfactants and acids (Kolenbrander, 2000). Some diseases such as gingivitis, peritonitis and caries occur because of the proliferation of biofilms (Kolenbrander, 2000),as some implantable medical devices and impairments to the immune system lead to the formation of biofilm-related infections (Lindsay and von Holy, 2006, Ramsey and Whiteley, 2009).

4-1-1 The mechanism of biofilm formation

Surface conditioning is the first step in biofilm formation, where surfaces are covered with nutrients such as actin, mucin (a glycoprotein) and DNA (Donlan, 2002). The second step is bacterial attachment, where cells adhere reversibly to surfaces by sedimentation, active movement by motile bacteria and by electrostatic interactions (Costerton *et al*., 1978). Cell-bridges are formed by the production of exopolysaccharides, which cement the cells to the surface. An example of an exopolysaccharide is alginate, a polysaccharide produced during the formation of *Ps.*

aeruginosa biofilm (Davies and Geesey, 1995, Davies *et al*.,1993, Stapper *et al*., 2004).

The final stage in biofilm establishment is surface colonization. The attached bacteria proliferate and form micro colonies, which comprise the basic unit of the biofilm community (Figure 18). A wide range of bacterial species can be involved in an individual biofilm and some species have the ability to produce proteins, which inhibit the attachment of other species. Some peripheral cells can be detached from the biofilm thereby returning to the planktonic state to seek a new surface and start a new community (Costerton *et al*., 1999, Davey and O'Toole G, 2000). In *Ps. aeruginosa* biofilms, the enzyme responsible for this process is alginate lyase which dissolves alginate to release bacterial cells (Davies *et al*., 1993). Other mechanisms involved in dispersion are alternation of various biofilm surface components (Neu, 1996) and quorum sensing (cell to cell signalling) which facilitate the release of surface-bound bacterial cells (Fux *et al*., 2005, Davies *et al*., 1998).

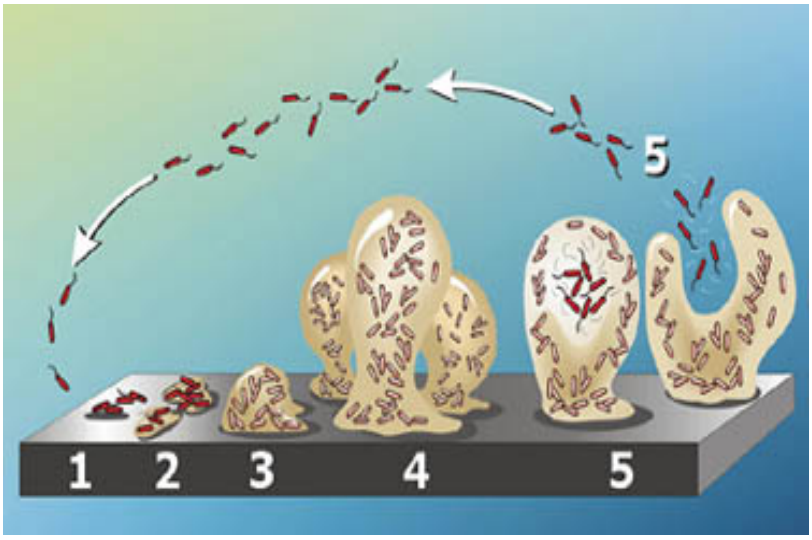


Figure 18. Biofilm formation in *Pseudomonas aeruginosa* (obtained from microbewiki.kenyon.edu)

4-1-2 Antibiotic resistance in bacterial biofilms

Biofilm-related infection symptoms are not readily observed because biofilm growth is normally slow, thereby making it difficult to diagnose such any related infections (Chambless *et al*., 2006). Both antibiotics and host defence mechanisms are effective on planktonic cells but generally not against biofilms (Leid *et al*., 2002).The concentration of antibiotic needed to kill cells within a biofilm is generally about 100 to 1000 times greater than that concentration needed to kill the same bacteria in the free swimming form (Costerton *et al*., 1999).

The resistance of biofilms to antimicrobial agents is attributed to several factors. One proposed factor is that the depth of the structure is impenetrable (Costerton *et al*., 1999, Jefferson *et al*., 2005). Cerca (2005) suggested that polymeric substance in the biofilm matrix impede diffusion of some antibiotics. Another factor which might explain reduced biofilm susceptibility to antimicrobial agent is based on the fact that cells in biofilms are slow-growing bacteria or inert and many antibiotics only affect actively growing cells (Chambless *et al*., 2006, Costerton *et al*., 1999).

4-1-3 Honey and biofilms

Honey has many therapeutic benefits especially in relation to wounds and it is known to inhibit biofilm formation and kill biofilm bacteria (van der Weyden, 2005, Gethin and Cowman, 2009). Honey acts as a broad spectrum antimicrobial agent and is active against multi drug-resistant bacteria (Chambers, 2006); it is also welcomed by patients because it helps reduce both pain and malodour (Dunford and Hanano, 2004). Many other biofilms inhibitors also exist, notably chlorohexidene, garlic, cadexomer, iodine, hydrogen peroxide, lactoferrin, polyhexnide, ocetendine, povidone xylitol and silver (Cooper, 2010). Most types of honey have killing activity which is attributable

to factors such as acidity, osmolarity and the production of hydrogen peroxide. However, some types of honey also contain a unique killing factor called methylglyoxal which is particularly prevalent in Manuka honey. Methylglyoxal (MGO) shows remarkable action *in vitro* when tested against biofilms of methicillin resistant *S. aureus* (MRSA) and *Ps. aeruginosa*; it is also effective against planktonic cells (Kilty *et al*., 2011). Sidr honey from Yemen also has marked biofilm-killing activity when tested against methicillin sensitive *S. aureus* (MSSA), methicillin resistant *S. aureus* (MRSA); the killing factor present in Sidr honey has not however, yet to be identified (Alandejani *et al*., 2009).

The overall aim of the work reported in this Chapter was to study the effect of honey on already established biofilms of *S. aureus* and *Ps. aeruginosa* at different concentrations and to compare their effects with antibiotics.

4-2 Material and Methods

The Minimum Biofilm Eradication Concentration (MBEC) of two types of the most common causing wounds infection bacteria: *Staphylococcus aureus*SH1000 and *Pseudomonas aeruginosa*PA01was detected according to instructions provided by Innovotech Inc. Canada (Appendix3).

4-2-1 Honey treatment

Concentrations from 80% to 5% of the effective honeys were prepared in sterile tubes and then transferred to wells. The vertical well line was used for one type of honey from the third row to the eleventh row. The test for each type of honey was done in duplicate. The twelfth row was used as positive control.

4-2-2 Antibiotic treatment

Two antibiotics were used (Ciprofloxacin for *Ps. aeruginosa* and Erythromycin for *S. aureus*); concentrations were prepared as stated below in sterile tubes and then transferred to wells. Nine serial concentrations of Ciprofloxacin were prepared, started with 40mg/ml and ended with 156µg/ml, and Erythromycin was diluted from 10mg/ml to 39µg/ml. A plate used for an antibiotic and the test was performed in four rows. The first and the second rows used as negative control. The twelfth row used as positive control.

4-3 Results and Discussion

4-3-1 Effect of honey on biofilm

Four types of honey were diluted from 80% to 5% to determine the minimum biofilm eradication concentration (MBEC). Table 14 shows that at a concentration of 20% all honeys killed both biofilm bacteria. The results of this experiment are potentially influenced by the fact that a single cell might survive treatment and grow to give positive result. In studies used by other workers, another protocol was used which depends on the immersion of honey with different concentrations on established biofilm in 96 wells plate and assessing the remaining biofilm residues in the plate (Cooper, 2011). This protocol was not used in the work reported here because of eliminating honey at higher concentration by washing with sterile, distilled water. Surprisingly, Cooper*et al*., (2011) reported that when using this approach Manuka killed a *S. aureus* biofilm at a concentration of 10%.

Table 14.The minimum biofilm eradication concentration (MBEC) of different types of honey for *S. aureus* and *Ps. aeruginosa* biofilms.

	Minimum biofilm eradication concentration (mg/ml)	
Honey Type	***S. aureus* SH10000**	***Ps. aeruginosa* PA01**
Manuka honey +20	20 (Appendix figure 1)	20 (Appendix figure 2)
New Zealand Rewarewa	20 (Appendix figure 3)	20 (Appendix figure 4)
Nigella sativa	20 (Appendix figure 5)	20 (Appendix figure 6)
Oak	20 (Appendix figure 7)	20 (Appendix figure 8)

4-3-2 Effect of antibiotics on biofilm

Table 15 shows that the minimum biofilm eradication concentration (MBEC) for the antibiotics Erythromycin and Ciprofloxacin (tested only against *S. aureus* and *Ps. aeruginosa* respectively), not surprisingly was much lower (1.25mg/ml) than that of any of the honeys. There is however, no dosage comparability between the antibiotics and honeys used here, and the antibiotics, when used in medicine would be taken orally, and reach the wound via a systemic route. In contrast, honey is used directly on the surface of the wound where it directly interacts with wound pathogens.

Table 15. The minimum biofilm eradication concentration (MBEC) for the antibiotics Erythromycin and Ciprofloxacin for *S. aureus* and *Ps. aeruginosa* biofilms.

Antibiotic	**Minimum biofilm eradication concentration**	
	***S. aureus* SH10000**	***Ps. aeruginosa* PA01**
Erythromycin	1.25 mg/ml (Appendix figure 9)	NT*
Ciprofloxacin	NT*	312 µg/ml (Appendix figure10)

*NT= Not Tested

The results of this study show that a range of honeys, including Manuka honey are able to completely inhibit bacterial biofilms at concentrations above 20mg/ml. Since honeys are applied directly to the wound and therefore the associated biofilms, concentrations of this order will be inevitably be achieved, when raw honeys are applied. As a result, it can be concluded that the honeys used here will inhibit

pathogen-biofilms when applied to wounds. Since antibiotics are often ineffective against biofilms the use of undiluted honey provides a potentially effective means of controlling pathogen biofilms infected wounds. This killing effect will be even more pronounced and lasting since it is generally accepted that bacteria, including pathogens, do not develop resistance to honeys. These factors help explaining why honeys can be effective when antibiotics are not, especially in the treatment of indolent ulcers caused by antibiotic-resistant bacteria.

As has already been mentioned, honey has historically been long used as a topical antimicrobial treatment for infected wounds, with medical products officially becoming available from the 1990's. Medical honey is often still used as a last resort when other therapies have proved worthless.

Manuka honey is a broad spectrum agent with potent bactericidal activity which can disrupt the cell cycle in Gram positive bacteria leading to aberrant cell division and weakening of the cell wall; this is combined with an up-regulation of the general stress response (Jenkins *et al*., 2011, Roberts *et al.,* 2012). In Gram negative organisms, particularly *Ps. aeruginosa*, the major targets are integral membrane proteins e.g. OprF which normally stabilise the cell and without which, membrane disruption and eventual lysis occurs (Blair *et al*. 2009).

Stewart (2003) claimed that bacterial resistance to Manuka honey treatment has not been observed empirically yet. This is of particular importance since antimicrobial resistance currently outstrips the rate at which novel antimicrobial approaches can be developed and where the development of resistance to all known antibiotics could potentially occur. Bacterial antibiotic resistance is exacerbated, when microorganisms grow as a biofilm, in which organisms are protected by an extra-polysaccharide layer which can restrict the diffusion of antimicrobials. This results in therapeutic doses not

reaching all of the bacteria within the biofilm, thereby allowing infection to recur. Biofilms, by hindering diffusion, provide an ideal environment in which bacteria are exposed to sub-lethal doses of antibiotic thereby providing a selective pressure for the increased emergence of resistance.

The excessive mis-use of antimicrobial agents has long been recognized as a motivator for the emergence of resistant bacterial strains, leading to a correlation between the availability of an antimicrobial and occurrence of resistance. This process also occurs following the use of topical antiseptics and biocides, including chlorhexidine and silver; short and long term, step-wise resistance training has demonstrated that neither *Ps. aeruginosa* nor *S. aureus* develop resistance to honey and that any increased tolerance to treatment is short-lived.

Small colony variants (SCV) of the original isolates can emerge in populations of bacteria that are exposed to antibiotics (Procter *et al*.,1998) occurring at a rate of 1.8×10^{-8} per cell per generation, and thereby making up a small but persistent proportion of the bacterial population. The ability of SCVs to resist antimicrobial treatment results from their slow growth and impaired electron transport chain; therefore it is not uncommon. Additionally, SCVs characteristically show enhanced biofilm formation (Procter *et al*.,1998) and as a result protection from antibiotics.

Wounds often produce exudates and the high osmolarity of honey enhances this process, with the result that honey is generally diluted *in vivo* and that isolates exposed to Manuka honey within an established biofilm can develop increased resistance, likely the result of the appearance of small colony variants. This problem can be avoided in the treatment of recalcitrant or chronic, infected wounds by ensuring that medical honeys are appropriately applied for a suitable length of time in

combination with other antimicrobials where necessary to ensure that the infection is cured and that any likelihood for resistance is thereby minimised.

Chapter 5

Effect of Honey on Pyocyanin Production by *Ps. aeruginosa* and Biofilm Formation by *Ps. aeruginosa* and *S. aureus*

5-1 Introduction

It is essential that we reduce the prevalence of antibiotic resistant bacteria which are being spread rapidly due to the selection of strong and resistant strains from weak strains within antibiotic treated populations. There is an indirect way by which this reduction can be achieved, namely by altering the ability of bacteria to become pathogenic by disabling or disrupting bacterial communication i.e. by disrupting quorum sensing. When this is achieved, the host is not compromised by inflammation, toxicity, cell damage or any other virulence factors, thereby allowing the body to eradicate the bacteria naturally by employment of the immune system. Quorum sensing (or bacterial cell signalling) describes a natural phenomenon whereby a single bacterium senses the accumulation of a population through signalling molecules. It then communicates with other bacteria inside the population allowing them to work together in order to adapt to changes, which might occur in the immediate environment. Quorum sensing was first observed in *Vibrio fischeri*, a bioluminescent bacteriumthat lives as a mutualistic symbiont in the photophore (light producing organ) of the Hawaiian bobtail squid. It was found that the bacteria do not showluminescence at low concentrations in the free-state. However, when bacteria are present in the photophore at high concentrations (roughly 10^{11}cells/ml) bioluminescence occurs as the transcription of luciferase is induced. Many diverse networks are maintained by quorum sensing including: biofilm formation – sporulation – motility – antibiotic production – conjugation – competence – virulence and symbiosis (Miller and Bassler, 2001).

Two inducing molecules have been identified in bacteria which control quorum sensing, namely, oligopeptides in Gram-positive bacteria (Novick *et al*., 1993), and

acylated homoserine lactones (AHLs) in Gram negative bacteria (Galloway *et al*., 2011).

The accumulation of AHLs occurs because fluctuations in the bacterial population activate the quorum sensing systems. The core of a quorum sensing system has two genes; the LuxI gene, which is responsible for the production of AHL, and the LuxR gene, which is responsible for the production of a protein able to bind AHL with the target gene on the DNA. A definite type of AHL then activates a specific type of R protein (Figure 19).

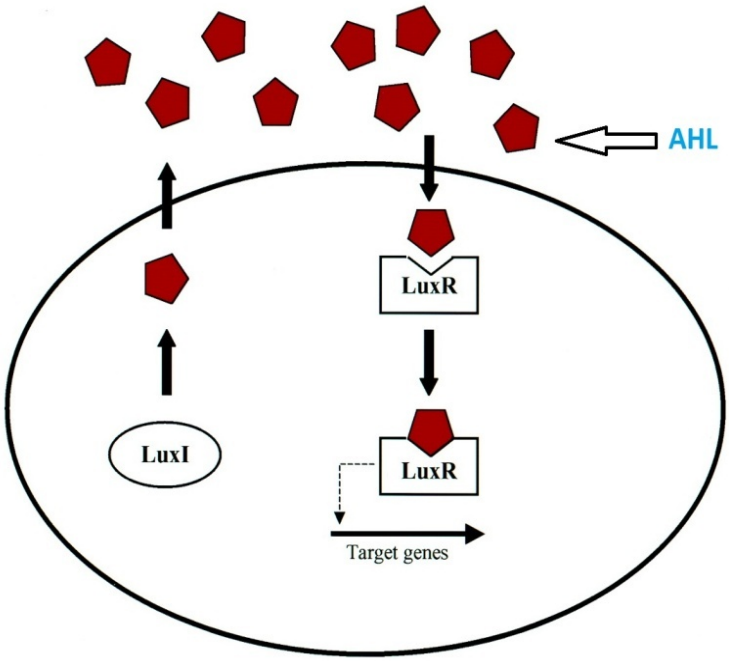


Figure 19. Quorum sensing circuit (Winans and Bassler, 2002)

The AHL signal molecule consists of two parts, a homoserine lactone ring and a fatty acyl side chain which differs in the length; it can be either saturated or unsaturated or consists of an oxo group or a hydroxyl at the third carbon. Different types of AHL molecule can be found in different species as shown in Figure 20.

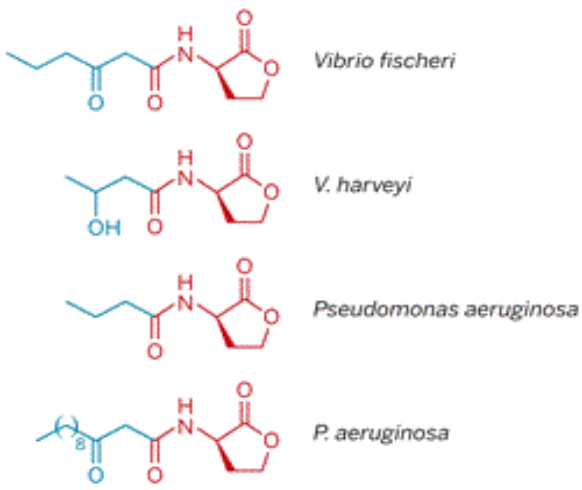


Figure 20. The AHL molecule in some bacterial species (Chemical and Engineering News website)

5-1-1 Quorum sensing in *Ps. aeruginosa*

In *Ps. aeruginosa*, genes encode virulence factors and the synthesis of some important products for bacteria; host interactions are also controlled by quorum sensing system (Van Delden and Iglewski, 1998, Favre-Bonte *et al*., 2003). The following two networks direct quorum-sensing system in *Ps. aeruginosa:*

1- The acyl-homoserine lactone (AHL) or LasR/RhIR network (Fuqua et al., 2001).
2- The 4-hydroxy-2-alkylquinolines (HAQs) or MvfR regulatory network (Gallagher et al., 2002)

Both networks are involved in the regulation of a wide variety of virulence factors and biofilm synthesis (Wang *et al*., 2012). Zhu *et al*. (2002) claim that the level of AHLs production in *Ps. aeruginosa* is associated with the intensity of the infections, which means that AHL production, differs among the same species. Pathogenicity of *Ps. aeruginosa* is attributed to the production of pyocyanin as well as some enzymes. Pyocyanin is a blue soluble dye observed in mucous samples of some patients with

bronchitis; it promotes virulence by interfering with several cellular functions in host cells including cellular respiration, electron transport, gene expression, energy metabolism, and innate immune mechanisms (Rada and Leto, 2013). Pyocyanin exhibits antimicrobial activity against a wide range of microorganisms; however, it cannot be used as an antibiotic because it is toxic to human airway epithelial cells and may cause cancer (Denning *et al*., 1998, Rada et al., 2008).

Some natural products decrease the production of virulence factors by inhibition of quorum sensing. Mihalik *et al*., (2007)suggested that *Camellia sinensis* or green tea significantly inhibits quorum sensing in *Ps. aeruginosa* and subsequently, the inhibition of virulence factors such as pyocyanin production, swarming motility, proteolytic activity observed. It has also been shown that honey at low concentration inhibits the expression of MufR, las, and rH regulons which are associated with virulence factors production such as pyocyanin in *Ps. aeruginosa*(Wang *et al*., 2012).

In addition to virulence factors, honey at low concentration of (0.1 g/ml) has anti-quorum sensing activity, with the inhibition activity increasing with increases in honey concentration (Truchado *et al*., 2009b). Acacia and polyfloral honey also reduces biofilm formation significantly in *E.coli* O157:H7 but not in commensal strains of *E.coli* (Lee *et al*., 2011). Biofilms of *S. aureus*, methicillin resistant *S. aureus* (MRSA), vancomycin resistant *Enterococcus* (VRE) can all be influenced *in vitro* with concentrations of Activon Manuka honey above 10% (W/V) (Cooper *et al*., 2011a), and a Chestnut honey variety was shown to prevent biofilm formation of *Erwinia cartovora*, *Yersinia enterocolitica* and *Aeromonas hydrophilia* through quorum quenching (Truchado *et al*., 2009a).

The overall aim of the work reported in this Chapter was to study the effect of honey at different concentrations on the production of the virulence factor pyocyanin and biofilm formation of *Ps. aeruginosa*.

5-2 Material and Methods

5-2-1 Pyocyanin assay

Five antibacterial types of honey (New Zealand Rewarewa, Manuka +20, Oak, *Nigella sativa* and New Zealand Rainforest +30) were tested for their effectiveness on the pyocyanin production as described by Wang *et al*., (2012). *Ps. aeruginosa* Boston strain ATCC 27853 was grown in *Pseudomonas* broth, which is recommended for the detection of pyocyanin production by *Pseudomonas* species (Appendix 6).

5-2-1-1 Inoculum preparation

More than 20 colonies of the same morphological appearance from a fresh subculture of the bacteria under test were picked off with a sterile loop and dipped into 5 ml sterile phosphate buffer to produce inoculums density equal to McFarland 4 ($12x10^8$ CFU).1 ml was taken with sterile pipette and transfer to 49 ml of double strength media.

5-2-1-2 Honey dilutions

Honey concentrations of 40%, 20% and 10% were prepared in individual sterile tubes. Diluted honey (5ml) was transferred into sterile tube and mixed with 5 ml of the previously prepared bacterial inoculums to give final concentrations of 20%, 10% and 5%. Sterile distilled water was used as a negative control and a positive control was set up at the same time. Tubes were kept overnight in 37^0C aerobic incubator (Baron and Rowe, 1981).

5-2-1-3 Minimum bactericidal concentration

A loopful (approximately 10 µl) of culture was streaked out from each tube on a nutrient agar plate and was incubated for 24 hrs at 37^0C.

5-2-1-4 Pyocyanin extraction

Pyocyanin was extracted from cultures after 24 hrs of incubation. Tubes were centrifuged and 3 ml of supernatant was pipetted into a sterile tube and mixed with 1 ml of chloroform (Figure 21). The chloroform layer was then transferred to a fresh tube and mixed with 1 ml 0.2 M HCL. The top layer was removed and its absorption measured at 520 nm.

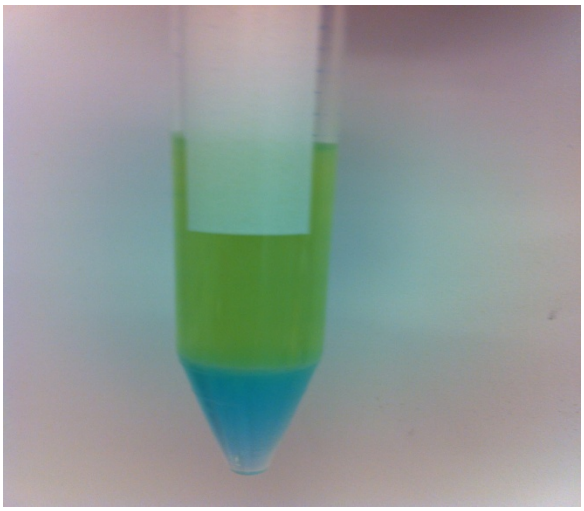


Figure 21. Pyocyanin (in blue) was extracted by mixing with Chloroform

5-2-1-5 Pyocyanin standard curve

Concentrations of 50, 25, 12 and 6 µl/ml of pure pyocyanin (Sigma Aldrich, UK) were prepared in sterile distilled water. Chloroform (1ml) was added to the pyocyanin solution with mixing and left for a minute to allow the pyocyanin layer to separate. The supernatant was discarded and 1 ml 0.2 M HCL was added with mixing. The upper reddish coloured layer was measured in spectrophotometer at 520 nm, and the OD was applied to a pyocyanin calibration curve equation. All experiments were conducted in triplicate.

5-2-2 Effect of honey on non-established biofilms

Polystyrene 96-well assay plates with flat bottoms supplied by CEESTAR® were used and the method described by Cooper *et al*., (2011a) was used .

5-2-2-1 Inocula preparation

More than seven colonies of the same morphological appearance of a fresh subculture of the tested bacteria were picked off with a sterile loop and dipped into 30 ml sterile nutrient broth.

5-2-2-2 Preparation of the honey serial dilutions

Concentrations of 80% of the effective honeys (New Zealand Rewarewa, Manuka +20, Oak, *Nigella sativa* and New Zealand Rainforest +30) were prepared in sterile tubes and then 200 µl was transferred to the third wells in the first five rows respectively. The vertical well line was used for one type of honey from the third row to the eleventh row. The test for each type of honey was done in duplicate. The entire sixth row used as positive control and the first two wells of each column were used as negative controls. The remaining wells were filled with 100 µl of sterile distilled water.By using a multi-channel pipettor; 100 µl was taken from the third row and transferred to the adjacent well with slow pipetting and decanting. The same protocol was applied to all wells to make serial fold dilutions from 80% to 0.32%.

5-2-2-3 Bacterial inoculation

Bacterial suspensions (100 µl) were added to all wells, which had the diluted honey and mixed thoroughly to give dilutions from 40% to 0.16%. The plates were then incubated at 37^0C for 24hrs.

5-2-2-4 Plate reading

After the incubation period, liquid culture was dislodged by pipetting 200 µl from all wells. All wells were then washed with 210 µl of sterile distilled water twice to insure

that most of the planktonic cells were removed; the plates were then left in the oven to dry at 55^0C. In order to stain the biofilm, 50 µl of crystal violet was dispensed inside the wells and left for 5 min, followed by washing with sterile distilled water twice and then drying in the oven. The amount of dye retained reflects the amount of biofilm present. The stained biofilm was extracted with 200 µl of 30% acetic acid, added to all wells and allowed for 30 min to dissolve the adhered protein around the wells. The plate was measured at OD_{595} in the plate reader (Figure 22).

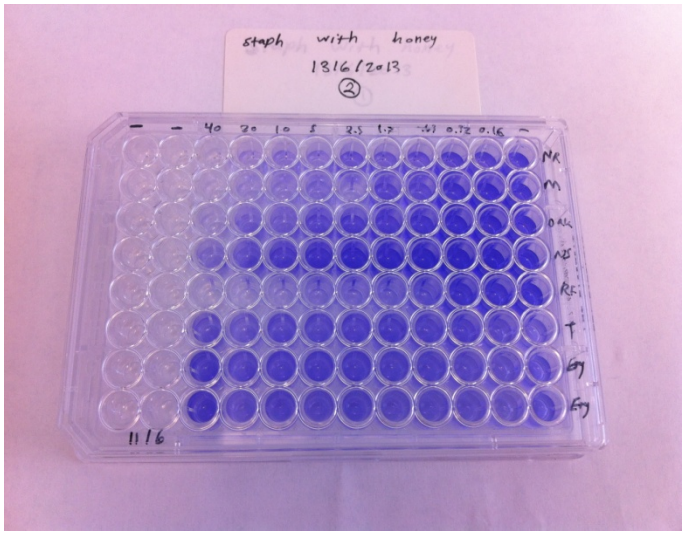


Figure 22. Different concentrations of different types of honey stopped the biofilm formation of *S. aureus*

5-3 Results and Discussion

5-3-1 Effect of honey on Pyocyanin production

Table 16 shows the production of pyocyanin by *Ps. aeruginosa* as influenced by a range of honeys. The table shows minimum bactericidal concentrations for the honeys and pyocyanin inhibition concentrations. When the two values are the same this means that the honey has killed the bacterium without influencing the pyocyanin concentration. When the opposite is found, then the honey has reduced pyocyanin production. This means that a small concentration of honey stopped the production of pyocyanin by the bacterium, a compound that can be toxic to humans. This means that where the concentration of honey does not kill *Ps. aeruginosa* it nevertheless inhibits the production of the human toxicant pyocyanin. Manuka, Oak and Rain Forest honeys were seen to inhibit pyocyanin production without killing bacteria.

Table 16. The minimum concentration of honey that inhibits the growth and production of pyocyanin for *Ps. aeruginosa*

Honey Type	Minimum Bactericidal Concentrationof honey(mg/ml)	Minimum pyocyanin inhibition concentration of honey(mg/ml)	Appendix Figures
Manuka honey +20	20	*10	12
New Zealand Rewarewa	20	20	13
Nigella sativa honey	20	20	14
Oak	20	*10	15
Rainforest +30	10	*5	16

* A honey concentration is considered to be significant when the minimum biofilm inhibiting concentration is less than the minimum bactericidal concentration.

5-3-2 Effect of honey on biofilm formation

Table17 shows an alternative approach to testing honeys on biofilms. In this case different concentrations of honeys where mixed with bacteria and the biofilm was then left to develop. This contrasted with the former approach, where the biofilm was pre-formed and the honey was added. Inhibition of quorum sensing networks by honey is associated with sugar content (Wang *etal*., 2012).

Table 17. The minimum concentration of honey that inhibits the formation of biofilm for *S. aureus* and *Ps. aeruginosa*

	Minimum biofilm inhibiting concentration / **Minimum Bactericidal Concentration (MBC) (mg/ml)**			
Honey Type	***S. aureus* SH10000**	**Appendix Figures**	***Ps. aeruginosa* PA01**	**Appendix Figures**
Manuka honey +20	10/**20***	17	20/**20**	22
New Zealand Rewarewa	20/**20**	18	20/**20**	23
Nigella sativa	20/**20**	19	20/**20**	24
Oak	20/**20**	20	20/**20**	25
Rainforest +30	5/**5***	21	5/**10***	26

* A honey concentration is considered to be significant when the minimum biofilm inhibiting concentration is less than the minimum bactericidal concentration.

The results obtained in this Chapter provide strong evidence to use honey as a prophylaxis agent inside the body since honey consumption is likely to prevent biofilm formation within body tissues and also reduce pyocyanin toxicity. Clearly, honey interacts, at low concentrations, with some pathogenic traits such as biofilm formation and toxin production. In the case of biofilm formation, Manuka honey stops

the formation of biofilms of *S. aureus* at a concentration of 10 mg/ml and Rainforest honey +30 prevents biofilm formation by *S. aureus* and in the case of *Ps. aeruginosa* at 5 mg/ml. Manuka +20, Oak and Rainforest +30 were effective in preventing pyocyanin production by concentrations of 10, 10 and 5 mg/ml respectively. So, it was assumed that the active component in honey reaches the infection or colonized site inside the body, even though, its concentration may be reduced tenfold in the body. However, more investigations are needed to support the possibility of consuming honey as a prophylaxis agent and to test honeys against different types of pathogens possessing different pathogenic traits. Clearly *in vivo* studies will be required here.

It is becoming increasingly obvious that honey impacts on the virulence of bacterial pathogens in addition to affecting both the organism's cellular structure and metabolism. This is a useful attribute for an antimicrobial agent allowing studies devoted to novel ways of attacking bacterial infection to begin to focus on anti-virulence treatments rather than traditional bactericidal or bacteriostatic properties. The major advantage of this approach is that anti-virulence formulations do not bring about the same selective evolutionary survival pressure that encourages the emergence of resistance. Three recent studies have described the mechanism by which honey inhibits quorum sensing and virulence (Lee *et al*., 2011, Wang *et al*., 2012).The concentrations of honey that brought about this effect were far below the MIC. Biofilm formation by *E.coli* O157:H7 was disrupted by sub-lethal doses of honey, which was associated with the repression of quorum sensing genes. Concurrently, a reduction in the expression of genes encoded on the locus of enterocyte effacement and curli genes (*csgBAC*) was noted, both of the operons being known for their ability to play a significant role in the virulence of this bacterium (Lee *et al*., 2011)

Similarly, sub-inhibitory concentrations of honey have been shown to impair quorum sensing in *Ps. aeruginosa* by reducing the expression of the *las* and *rhl* regulons and the transcriptional regulator MvfR (Wang *et al*., 2012). Perturbation of these regulatory networks resulted in a reduction in the expression of associated virulence factors, clearly demonstrating the overall impact that honey exhibits on the bacterial cell at the regulatory level. ***N***-acyl-homoserine lactone production has also been shown to be significantly reduced in *Erwinia carotovora***,** *Yersinia enterocolitica* and *Aeromonas hydrophila*in response to chestnut honey. As was the case for *E. coli*, biofilm formation was also impaired, again emphasizing the close association between these two processes (Truchado *et al*., 2009).Biofilm formation and expression of virulence factors are fundamental to the successful colonization and subsequent pathology of many bacterial infections; thus, by impairing this process honey could prove to be an excellent prophylactic agent.

Another group of virulence factors that are known to be regulated by quorum sensing in numerous pathogenic microorganisms are the siderophores. Such iron-chelating molecules are central to bacterial proliferation in the host environment, providing pathogens with a source of iron; iron acquisition being directly linked to virulence. Pyocyanin, pyochelin and pyoverdin are all utilized by *Ps. aeruginosa*to sequester iron from the human host (pyoverdin and pyochelin are siderophores, whereas pyocyanin appropriates iron from transferring) (Cox 1986). In different strains of *Ps.aeruginosa*, honey treatment was shown to bring about a marked reduction in siderophore production the effect being attributed to sub lethal doses as low as 5% (w/v) which equated to quarter of the MIC (Kronda *et al*., 2011).The reduced production of pyocyanin following honey treatment mirrors a reduction in expression of genes involved in quorum sensing, demonstrating the global impact that

altered gene expression has on the expression of virulence factors (Wang *et al*., 2012). The reduced capacity of pathogenic bacteria to obtain iron from their host will be obviously detrimental to both colonization and to the subsequent sequence of the infection process. These mechanisms show that honey works via two independent mechanisms, i.e. by being both bactericidal and anti-virulent, a combination of properties, which is unlikely to initiate and promote bacterial resistance.

Chapter 6

The Effect of Some Wound Dressings and Treatments on Planktonic Bacteria and Bacterial Biofilm

6-1 Introduction

In relation to wounds, there are many indications, which should be identified before practitioner decides whether the patient needs treatment, or not. Firstly, bacterial contamination may be normal and does not alter the status of the wound or affect the host. Normal colonization is associated with unremarkable symptoms, while critical colonization is associated with moderate pain and a discharge, which is attributed to the presence of a heavy burden of bacteria in the wound. True infection results when pathogens develop inside the wound and leads to clinical infection with increasing cellulites (inflammation and infection of the tissue), bad odour, irresistible pain and heavy discharge. Infections can be focal point of some severe consequences such as bacteraemia – septicaemia – meningitis (Benbow, 2005).

Microbial populations compete for local nutrients, secrete destructive enzymes or cytotoxic metabolites and inhibit both host tissue regeneration and immune responses (Thorn *et al*., 2009).

The main barrier to the normal healing process of the wound is the bio-burden, which includes:

1- Colonizing microorganisms of one or more bacterial species.
2- Devitalized tissue which provides a solid surface for the biofilm to grow on (White and Cutting, 2006).

Antimicrobial agents only effectively affect planktonically growing bacteria and metabolically active bacteria but not bacteria in an inert state (Davey and O'Toole, 2000). The deeper the biofilm, the more inert the population tends to be; therefore, the metabolic activity of the bacteria varies with biofilm depth (Davies, 2003, Sternberg *et al*., 1999).It has been shown that biofilms are the main cause of treatment failure and acute wounds which because of their presence can be turned into chronic, non-

healing wounds (Bjarnsholt *et al*., 2008, Davis *et al*., 2008, James *et al*., 2008); the treatment of such chronic wounds is generally more expensive than acute wounds (James *et al*., 2008).

Biofilms presented in wounds are generally made up of more than one species of bacteria, which make the exchange of drug resistance genes easier and thereby enhance bacterial resistance (Cookson, 2005, Davies, 1994). Hill *et al*. (2010) for example showed that a mixture of *Ps. aeruginosa* and *S. aureus* biofilm was not affected *in vitro* by treating with high concentrations of Ciprofloxacin (5 mg/L) or Flucloxacillin (15mg/L).

6-1-1 Wound dressings

Dressings provide the wound with appropriate protection from environmental contaminants and allow the wound to heal under dry conditions (Benbow, 2005). In addition to antimicrobial compounds, most dressings maintain wound hydration by containing compounds such as alginate, foam, hydrogel and hydrocolloids (Bradshaw, 2011).

Kostenko *et al*.(2010) reported that the type of dressing-base material is correlated with the antimicrobial effectiveness of the dressing. Even though, most dressings used in hospitals have antimicrobial activity,Bradshaw (2011), claimed that an appropriate dressing should be selected after the determination of the bacterial species present within the wound. Some medical dressings are used nowadays with additives such as silver dressings, honey dressings, and iodine.

6-1-2 Silver dressings

Silver has a wide range of actions on bacteria, unlike antibiotics; it affects the respiratory system, cell membrane integrity, transmembranous energy, electrolyte

transport, enzyme activities and cell proliferation (Lansdown, 2002). Moreover, silver decreases bacterial adhesion by compromising intermolecular forces (Chaw *et al*., 2005, Klueh *et al*., 2000). Silver is released effectively in wound environments with a high level of hydration (Lansdown *et al*., 2005), so the more fluids and exudates from the wound, the more effective the silver dressings is (Sibbald *et al*., 2003). Silver dressings achieve marked biofilm inhibition and every type of silver dressings exhibits different activity on bacterial biofilms (Percival *et al*., 2007). The whole bacterial community in the biofilmof *Ps. aeruginosa* and *S. aureus* can be killed after 48 hours when exposed to a silver dressing (Percival *et al*., 2008); this finding did not however, correlate well with*in vivo* observations (Heggers *et al*., 2005). The silver concentration used in the medical dressing is generally insufficient to eradicate chronic wound biofilms (Kostenko *et al*., 2010), the complete eradication of which requires a silver concentration in excess of 10 – 100 times than that used to treat the planktonic bacteria. *In vitro Ps. aeruginosa* biofilm were however shown to be successfully inhibited with silver concentrations of 5-10µg/ml (Bjarnsholt *et al*., 2007).

6-1-3 Honey dressings

The effectiveness of honey is related to many factors:

1. Osmolarity which prevents the bacteria from growing and supports healing process (Moore *et al*., 2001).
2. Acidity making it an unsuitable medium to grow on (Gethin *et al*., 2008)
3. Hydrogen peroxide which is released when the honey is diluted with wound exudates (Kwakman *et al*., 2010).
4. Flavonoids and phenolic acid compounds which support the immune system and kill bacteria (Escriche *et al*., 2013).

5. Some unique compounds which exist only in certain types of honey such as methylglyoxal, which is found in Manuka honey (Kwakman and Zaat, 2012).

Gethin *et al*., (2008) reported that Manuka honey dressings significantly minimized the pH and the extent of the chronic wounds and may thereby contribute to wound healing. In addition, it has been reported that honey dressings increase healing, prevent the formation of scab on burns, sterilise the wound, deodorize wounds, increasing and separating sloughs, reduce pain and finally minimize the formation of scars (Al-Waili *et al*., 2011).Treatment with honey was also reported as a successful therapy for various wounds, such as ulcers, abdominal pain and burns (Sharp, 2009, Topham, 2002).

In a comparison study between honey dressing, ampicillin ointment and saline treatment, honey was shown to lead to the least inflammation, epithelisation and the most active fibroblastic and angioblastic activity (Gupta, 1992). In a similar study honey was found to be more effective than both antiseptics and antibiotics in the treatment of wounds infected or colonized with methicillin resistant *S. aureus* (MRSA) (Blaser *et al*., 2007a). Medihoney dressing have been found to be highly effective therapy especially following the failure (after 12 weeks) of conventional therapies (Dunford and Hanano, 2004). Honey dressings are also more effective at promoting wound healing and less painful than sugar dressing (Mphande *et al*., 2007). According to clinical studies, wounds became sterilize in 3 to 10 days following the use of honey dressings (Van der Weyden, 2003, Mossel, 1980, Tovey, 1991). Moreover, the duration of healing and hospital stay is decreased after wounds are washed with normal saline and the honey is applied with covering by dry gauze (Efem, 1988).

6-1-4 Hand gel (Non honey based)

Hand contact is responsible for the transmission of infections (Zanni, 2008).There are two types of skin flora, a) the resident flora which colonizes deeper skin layers with low potential of pathogenicity, long stay and high resistance to mechanical removal, e.g. both *S. epidermidis* and *Streptococcus viridians*, and b) the transient flora on the other hand colonizes the outer skin layer for short period and with a moderate or high potential of pathogenicity, e.g., *S. aureus* and colifom bacilli (Widmer, 2000). Hand gels (alcohol-based rubs) provide an instant and effective approach for use in hospitals (Marchetti *et al*., 2003), although as Pietsch (2001) pointed out, alcoholic gels are not always effective when used in hospitals as they do not meet the 30 second European standard for hand disinfectants (EN 1500).

6-1-5 Iodine

Iodine is active against proteins and enzymes and works by coupling thiol and sulphydryl groups, and blocks hydrogen bonding which alters phospholipids membrane structures (Leaper and Durani, 2008). Iodine is active on bacteria, fungi and viruses, although its human toxicity can undermine its medical use (Bradshaw, 2011).

The aims of the work reported in this Chapter were to:

1- Evaluate the effectiveness of some types of medically used dressings.
2- Test various dressings on biofilm containing pathogenic bacteria.
3- Test a variety of wound treatments against pathogenic bacteria.
4- Test various wound treatments on biofilm containing pathogenic bacteria.

6-2 Materials and methods

6-2-1 Tested organisms and used media

Two types of the most common causing wounds infection bacteria were tested, namely *Staphylococcus aureus* SH1000 and *Pseudomonas aeruginosa* PA01; the bacterial inoculum was prepared as described in the Appendix 1.

The following media were used:

1- Brain and Heart infusion agar (BHI): the best media for growing biofilms of *S. aureus.*

2- *Pseudomonas* agar base ISO 13720 (ISO): the ideal medium for growing biofilms of *Ps. aeruginosa*.

3- Nutrient agar (NA): used for bacterial plate counts.

4- Nutrient broth: used to enrich bacterial suspensions.

5- Muller Hinton Agar (MHA): for the sensitivity test.

6-2-2 The effect of wound dressings and creams on planktonic bacteria

6-2-2-1 Dressing tests

Four types of wound dressings were tested for their effectiveness as follow:

1. Askina© Calgitrol© Ag.
2. Actilite© (a non-adherent dressing containing Activon Manuka honey).
3. Algivon© (Alginate containing active Manuka honey).
4. Algivon© (Alginate dressing impregnated with 100% Manuka honey).

The dressing were cut into small pieces with size of 12 mm and kept in sterile, empty Petri dishes until used. Muller Hinton agar plates were inoculated with bacteria broth and incubated at 37^0C for 30 minutes. The dressings were then replaced on the plate in three corners with the control placed in the remaining corner (Figure 23). The plates were incubated in 37^0C for 24 hrs.

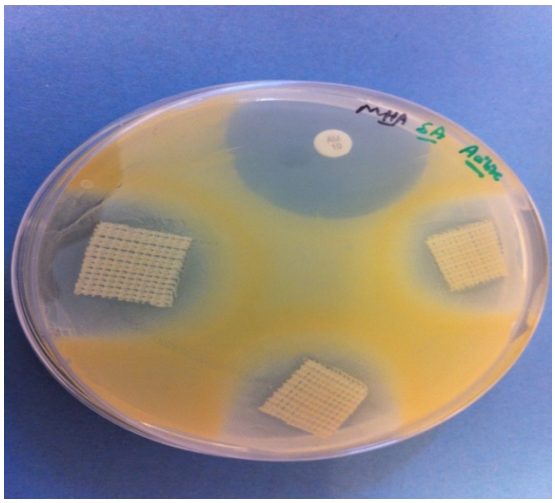


Figure23.The effect of some wound treatments on planktonic bacteria

6-2-2-2 Creams tests

Three types of wound creams, Panaderm*, Activon Manuka honey and Flamazine* and a hand sterilizer* were tested for their antibacterial properties.

Three wells were cut in three corners in the plate to dispense the antibacterial cream inside and left in the incubator after inoculated with bacterial suspension for 24hrs in 37^0C.

*Panaderm is a cream consisting of three types of antibiotics (neomycin sulphate 2.5 mg, Nystatin 100.000 units and gramicidin 0.25 mg).

* Flamazine contains silver as active ingredient.

* The active ingredient in the hand sanitizer is isopropanol.

6-2-3 Effect of wound dressings on the bacterial biofilms

6-2-3-1 Wound dressings

Three types of wound dressings obtained from different companies as detailed below:

1- Silver (Askina© Calgitrol© Ag) referred to here as Ag.

2- Actilite© (a non-adherent dressing with Activon Manuka honey) which is referred to here as NAD.

3-Algivon© (an alginate dressing impregnated with 100% Manuka honey) which is referred to here as M.

All types were tested against the following:

1- Biofilm formation: where the biofilm are not established.

2- Activity on established biofilm, examined after 24 hrs and 48 hrs of dressing application.

6-2-3-2 Nitrocellulose membrane preparation

Nitrocellulose membranes of 25 mm diameter supplemented by Millipore© were used to create the biofilm. All membranes were soaked into phosphate buffer solution (Sigma Aldrich) for 24 hrs before being transferred into bacterial inoculum tubes.

6-2-3-3 Colony biofilm assay

Protocol 3 described by Merritt et al., (2005) was applied. All membranes were soaked into phosphate buffer solution for 24 hrs and then transferred into adjusted bacterial suspension media and left for 15 minutes. By using sterile forceps, membranes were applied to the surface of agar media and incubated down side down in 37^0C for 48 hrs until renewing on new plates for 48 hrs more to enrich the growth of the biofilm.

6-2-3-4 Positive control

After 96 hours of incubation, three membranes were removed from the media and washed in sterile phosphate buffer in order to remove all planktonic cells; these were then transferred into 10 ml separate tubes of sterile phosphate buffer solution and then transferred to a sonicator bath for 1 hour to release all viable cells. The tubes were finally vortexed for 30 seconds to homogenize the suspension. (1 ml) was transferred

to 9 ml phosphate buffer to make dilutions of 10^{-1}, serial dilutions were then made up to 10^{-6}. Aliquots (100 µl) were taken from all tubes and spread on nutrient agar plate by use of a spreader and incubated for 24 hrs to obtain the colony forming unit count (CFU) present in of the main tube. All counts are given as the mean of triplicates.

6-2-3-5 Dressing application

After 96 hrs of incubation, the membranes were removed from the old plates and washed with sterile phosphate buffer solution to remove all planktonic cells then they were transferred to new plates. Distance was standardized by placing them in each edge of the plate (Figure 24). The dressings were cut into square shape with diameter of 30mm to cover all membrane's sides. The plate contained four membranes with three pieces of the same dressing as follows:

1- Negative control: a membrane soaked in phosphate buffer without bacteria.

2- Non established biofilm: this membrane was neutralized in Phosphate buffer for 24 hrs after that it was soaked in bacterial suspension for 15 minutes.

3- Established biofilm: a membrane with established biofilm and this was tested after 24 hrs.

4- Established biofilm: a membrane with established biofilm and this was tested after 48 hrs. All were done in triplicate.

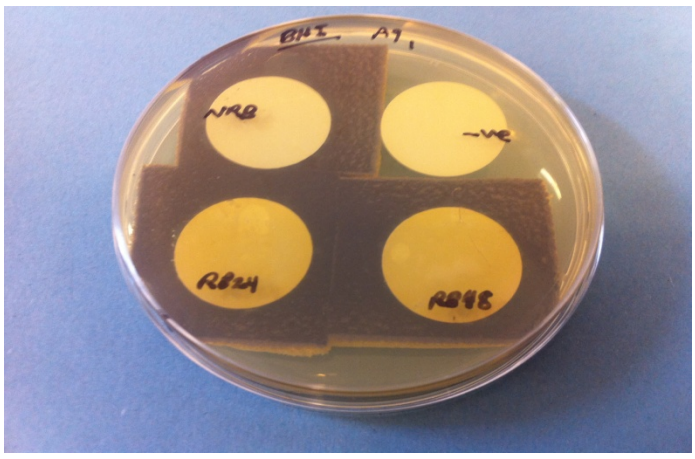


Figure 24. Membranes contain biofilm covered with dressings in different cases

6-2-3-6 Dressing recovery

After 24 hrs of incubation all dressings were removed except one, which examined after 48 hrs. All of the membranes were taken off the media and washed in sterile phosphate buffer to remove all planktonic cells then transferred into 10 ml separate tubes of sterile phosphate buffer solution and transferred to a sonicator bath for 1 hour to release all viable cells. Tubes were vortexed for 30 seconds to homogenize the suspension.1 ml was then transferred to 9 ml phosphate buffer to make a dilution of 10^{-1}, serial dilutions were then made up to 10^{-6}. An aliquot (100 µl) was taken from all tubes and spread on nutrient agar plate using a spreader and incubated for 24 hrs to obtain the colony forming unit (CFU) of the main tube. All counts are the means of triplicates. The same procedure was performed on the 48 hrs dressings.

6-2-4 The effect of wound creams on bacterial biofilms

Four wound treatments were tested as follows:

1- Panaderm© cream: effective against Gram negative and Gram positive bacteria as well as fungi.

2- Flamazine© cream: active component is silver.

3- Activon© cream: active component is Manuka honey.

4- A hand sterilizing agent.

The minimum biofilm eradication concentration (MBEC) was determined using the MBEC™ High-Throughput (HTP) Assay (Innovotech, Canada) (Appendix3).

6-2-4-1 Preparing the antimicrobial challenge plates

The horizontal wells were used for one type of treatment from the ninth column to the twelfth column. The test for each type of treatment was done in the whole column. The fifth and sixth columns were removed by using sterile metal pliers to use these

rows as negative controls afterward whereas the first, second and third columns were used as positive controls (Figure 25).

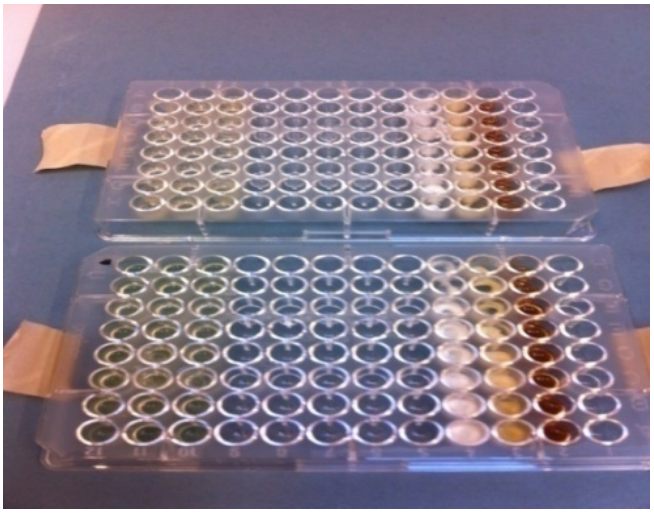


Figure 25. A challenge plate contains some wound treatments

6-3 Results and Discussion

6-3-1 The activity of some dressings on planktonic bacteria

Four types of dressings were tested against *S. aureus* and *Ps. aeruginosa*. Figure 26shows that all of dressings impregnated with Manuka honey exhibited greater antibacterial activity against *S. aureus* than the one containing silver. The most effective type of honey dressing was Actilite© (Non-adherent dressing containing Activon Manuka honey) followed by Algivon© (Alginate with action Manuka honey) and Algivon© (Alginate dressing impregnated with 100% Manuka honey) with zone diameters of 32, 25 and 19 mm respectively. The one silver dressing used on the other hand was more effective than Manuka honey dressings against *Ps. aeruginosa* (zone diameter 24 mm) (Figure 27).

6-3-2 The activity of some wound treatments on planktonic bacteria

A range of wound treatments, such as antibiotics, honey, silver and hand gel were tested on *S. aureus* and *Ps. aeruginosa* (Figure 28, 29). Panaderm, a mixture of antibiotics was the most effective treatment against *S. aureus* and *Ps. aeruginosa* (zone diameter, 25 mm). Activon Manuka exhibited remarkable activity against *S. aureus* (zone diameter 19 mm) and moderate activity against *Ps. aeruginosa* (zone diameter 12 mm). Flamazine showed moderate activity against *S. aureus* and *Ps. aeruginosa* (zone diameters, 14 and 16 mm respectively). The hand gel showed weak activity on both types of bacteria. Al Zahrani and Baghdadi, (2012) claimed that among seven tested types of hand sanitizers only two were effective and suitable for use as anti-bacterial agent.

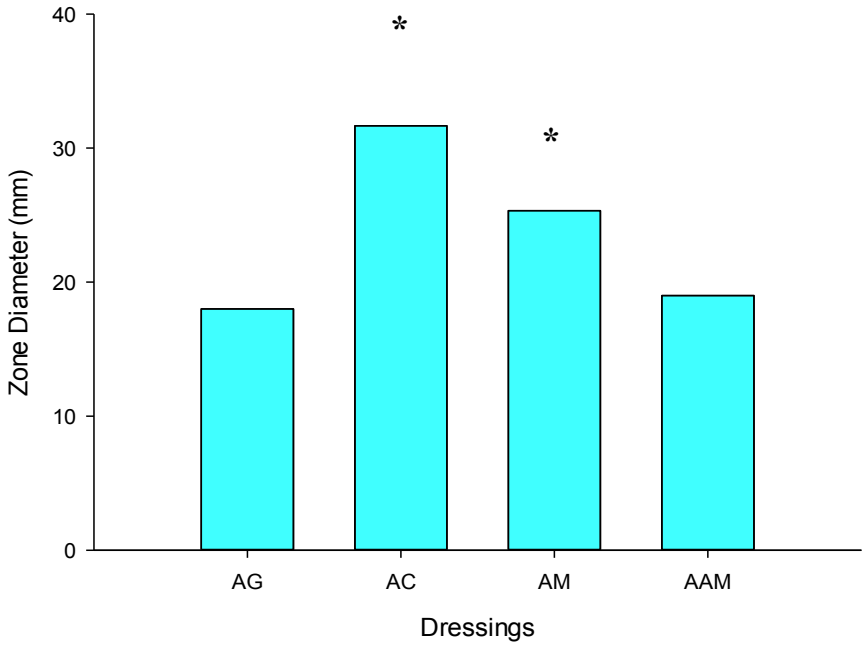


Figure 26. The effect of some wound dressings on the planktonic *Staphylococcus aureus* (* Significant differences)

Ag= Askina© Calgitrol©.

AC= Actilite© (a non-adherent dressing containing Activon Manuka honey).

AM= Algivon© (Alginate containing active Manuka honey).

AAM= Algivon© (Alginate dressing impregnated with 100% Manuka honey).

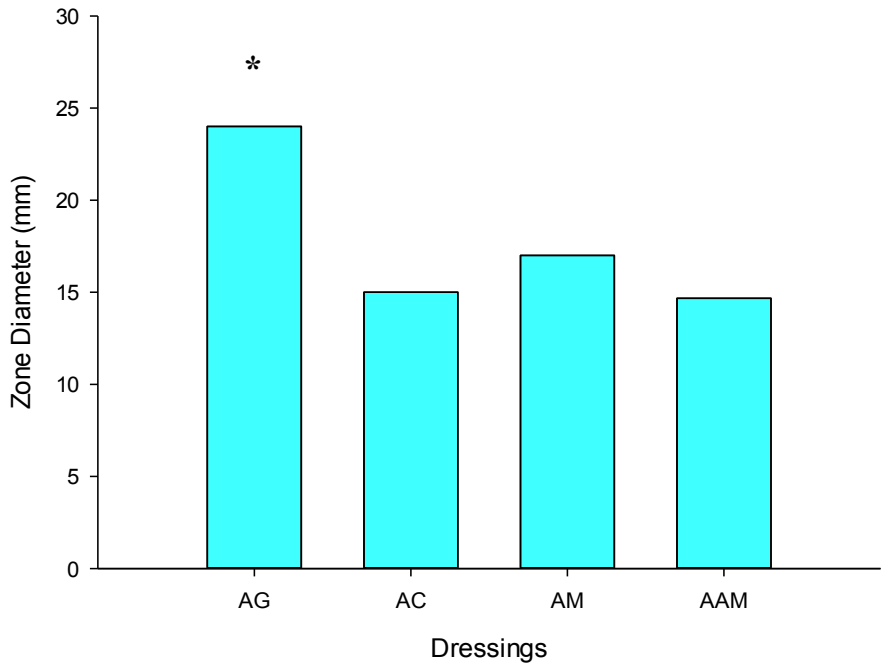


Figure 27. The effect of some wound dressings on the planktonic *Pseudomonas aeruginosa* (* Significant differences)

Ag= Askina© Calgitrol©.

AC= Actilite© (a non-adherent dressing containing Activon Manuka honey).

AM= Algivon© (Alginate containing active Manuka honey).

AAM= Algivon© (Alginate dressing impregnated with 100% Manuka honey).

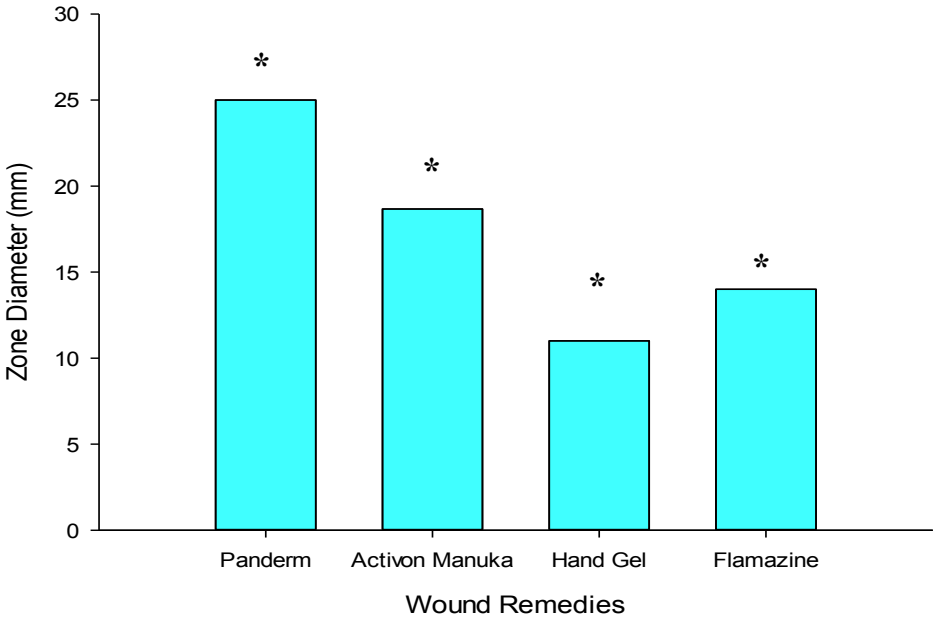


Figure 28. The effect of some wound treatments on the planktonic *Staphylococcus aureus* (* Significant differences)

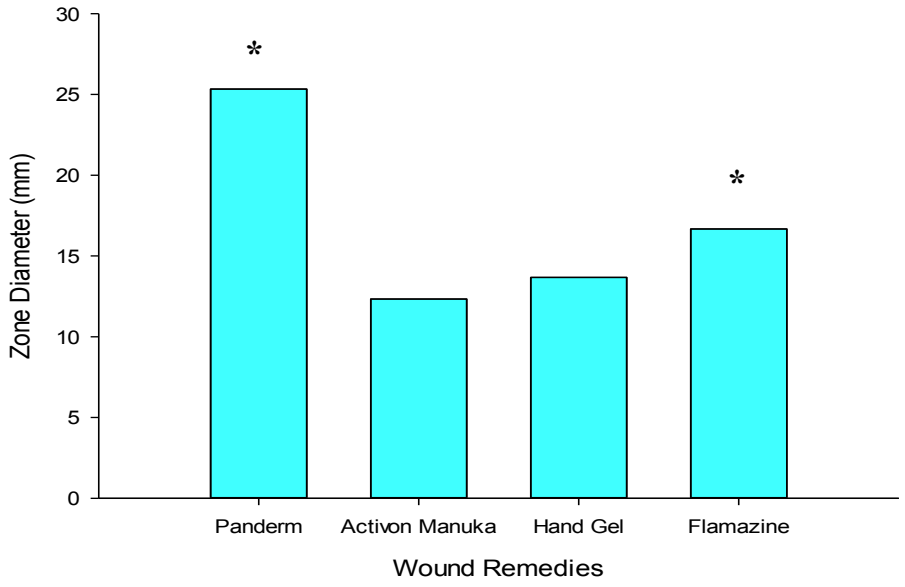


Figure 29. The effect of some wound treatments on the planktonic of *Pseudomonas aeruginosa* (* Significant differences)

6-3-3 Effect of wound dressings on the bacterial biofilm

Three types of dressings were tested in three different types of biofilms of *S. aureus* and *Ps. aeruginosa*. Biofilms were tested: the inocula, at the outset before the biofilm formed and after formation at 24hrs and 48 hrs.

Figure 30shows the effects of a variety of antibacterial dressings on the three types of biofilms. All of the Manuka dressings brought about complete biofilm inhibition after 0, (representing biofilm initiation) 24 and 48 hrs. In contrast the silver dressings achieved only 99% and 86 % inhibition of biofilm formation after 24 and 48 hours. Figure 31 shows that with the exception of the NAD dressing all of the dressings kill all the biofilm *Ps. aeruginosa* at 0, 24 and 48 hours.

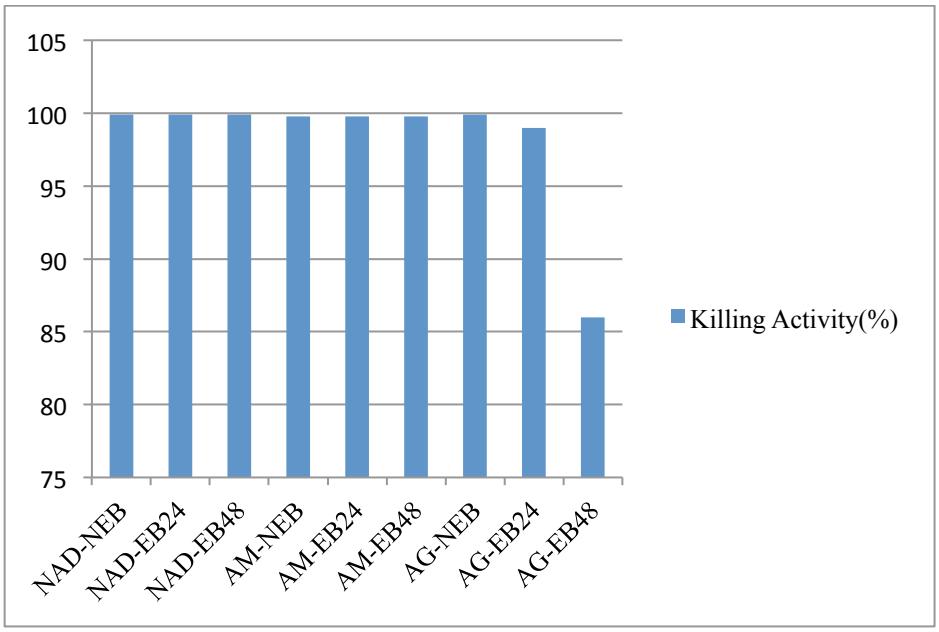


Figure 30. The killing activity of some dressings on the biofilm of *S. aureus*(Appendix figure 27)

Ag = Silver (Askina© Calgitrol© Ag)

NAD = Actilite© (a non-adherent dressing with Activon Manuka honey)

M= Algivon© (an alginate dressing impregnated with 100% Manuka honey)

NEB= Non Established Biofilm

EB24= Established Biofilm for 24 hrs

EB48= Established Biofilm for 48 hrs

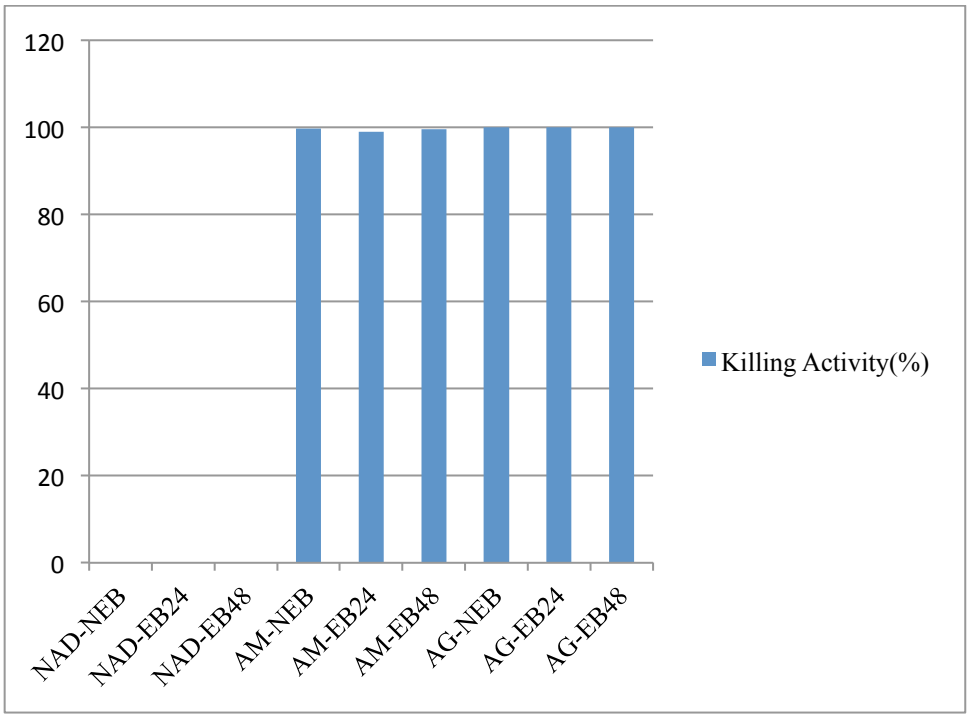


Figure31. The killing activity of some dressings on the biofilm of *Ps. aeruginosa* (Appendix figure 28)

Ag = Silver (Askina© Calgitrol© Ag)

NAD = Actilite© (a non-adherent dressing with Activon Manuka honey)

M= Algivon© (an alginate dressing impregnated with 100% Manuka honey)

NEB= Non Established Biofilm

EB24= Established Biofilm for 24 hrs

EB48= Established Biofilm for 48 hrs

6-3-4 Effect of wound treatments on the bacterial biofilm

All four products inhibited *S. aureus* biofilm formation, with Panaderm showing the most effect, (90% inhibition), while Flamazine Activon and the Hand Gel exhibited around 70% inhibition (Figure 32).The effect of the antibacterial creams and the hand gel on the *Ps. aeruginosa* biofilm is shown in Figure (33). The order of inhibition is seen to be Panaderm, Flamazine and Activon, with the Hand Gel having no effect. In all cases the products used here exhibit differing effects of the bacteria studied.

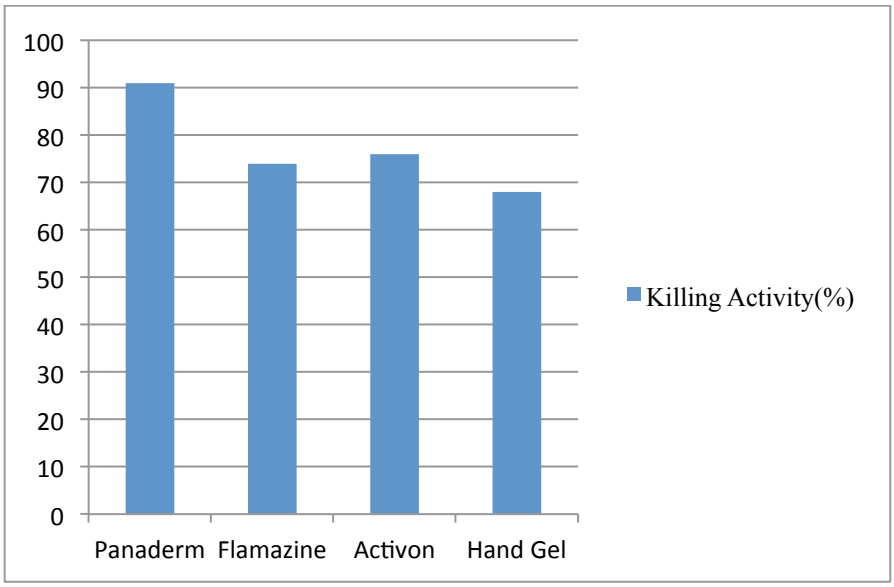


Figure 32.Effect of various cream products and a hand gel on biofilm of *S. aureus* (as measured by minimum biofilm eradication concentration technique).(Appendix figure 29).

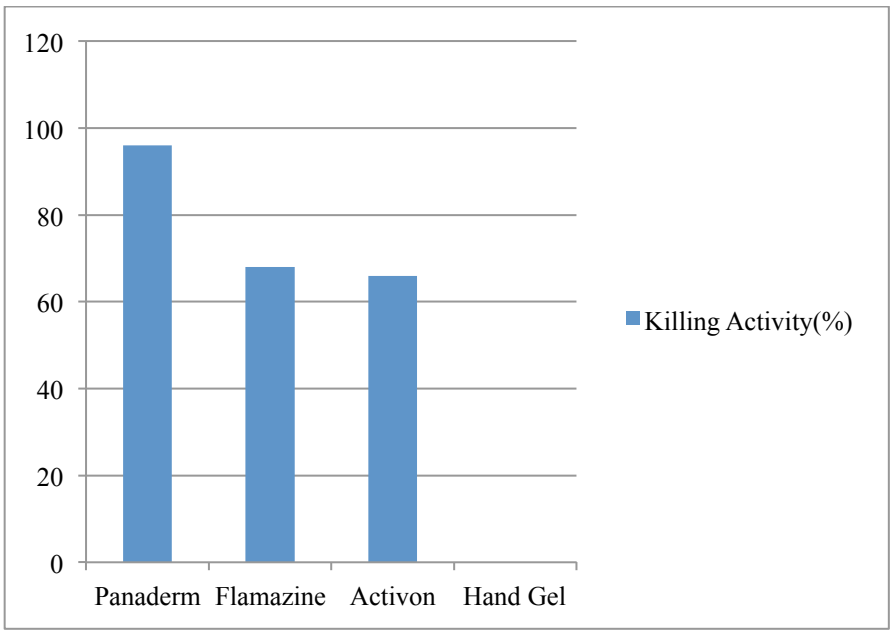


Figure 33.Effect of various cream products and a hand gel on biofilm of *Ps. aeruginosa* (as measured by minimum biofilm eradication concentration technique) (Appendix Figure 30).

Many patients suffer from a profoundly suppressed immune system, especially when caused by leukaemia and chemotherapy which they have to undertake, a problem which frequently results in wound-healing problems (Simon, 2006, Gaur, 2005), such that the patients are susceptible to wound infections which spread rapidly in immunocompromized patients, often leading to potentially life-threatening bloodstream infections (Gaur 2004). Such patients can be treated effectively with honey based dressings. An example of the use of medical honey dressings is provided by the case of a 12-year old patient who had an abdominal lymphoma removed such that it left an open abdominal drainage site (Molan, 2002, 2006). When admitted, the wound was infected with methicillin-resistant *Staphylococcus aureus* (MRSA). The male patient was immediately isolated to avoid nosocomial spread. The wound was then treated with a local antiseptic (octenidin) for 12 days. When no improvement took place an Australian medical honey dressing (Medihoney™), was applied. Two days later, the wound was free of bacteria and chemotherapy against the underlying illness could begin. Medihoney™ became one of the first medically certified honeys licensed as medical product for medical use on wounds, both in Europe and Australia (Molan, 2004, 2006). In addition to having antibacterial properties, medical honey increases the rate at which wounds heal through its anti-inflammatory effects by reducing oedema and the degree of exudation by down-regulating the inflammatory process. Honey dressings also reduce wound pain resulting from nerve endings being sensitized by prostaglandins produced during and from the pressure on tissues caused by oedema. The high sugar content of honey also prevents pain when dressings are changed due to the fact that it keeps the wound surface moist by mobilizing the oedema from the surrounding tissues. The remarkable debriding action of honey is also often observed and this is associated with the sloughing of bacteria-rich material

(Molan, 2002). Finally medical honey successfully prevents malodour from chronic colonized wounds which can cause severe discomfort and social isolation in patient.

Silver dressings are less useful than honey products because they can raise liver enzymes and cause an *argyria*-like syndrome in burns patients (Trop, 2006). As has been shown here, silver dressings often do not exhibit marked antibacterial activity. Iodine dressings have the disadvantage in having severe, harmful effects on the thyroid (Bradshaw, 2011).

For safety reasons, it is recommended that wound with medical honey dressings in all casesshould be supervised by a physician or an experienced wound care nurse in patients with chronic complicated wounds. Medical honey dressings also should keep the honey in contact with the wound for at least 12 h, but preferably for 24 h. Some patients apply the wound dressing overnight, so as not to restrict their mobility throughout the day. The frequency of dressing changes depends on the amount of exudates. In early stages, fresh surgical wounds infected with pathogenic bacteria may require changing twice daily. In stable wound care situations; dressings can be left in place for up to 7 days (Gethin, 2005, White, 2005).

Chapter 7

Final Discussion

7-1.Final Discussion

Russian army medics used honey in World War I to prevent wound infection and to accelerate wound healing and in both World Wars, the Germans used a mixture of cod liver oil and honey to treat ulcers in surface infections (Bansal *etal*., 2005). Honeys have been applied with success to: abscesses, amputation wounds, bed sores, burns, chilblains, cracked nipples, fistulas, varicose wounds, septic and surgical. Honey application to a wound often leads to a stimulation in the rate of healing, essentially because honeys cleanse wounds and stimulate tissue regeneration as well as reducing inflammation. Dressings containing honey thereby provide a useful non adhesive tissue dressing (Efem, 1988). The molecular mechanism by which such wound healing using honey has not yet been determined. Honey both soothes and helps heal infected burns and it can potentially be used in the treatment of gingivitis and periodontal disease. Honey dressings speed healing, sterilizes wounds and reduces pain (Subrahmanyam, 1991). Honey is used with success to treat ulcerations resulting from radical surgery for breast cancer and following radical surgery for carcinoma of the vulva. Similar improvements are observed in following honey treatment of bed sores (Subrahmanyam, 1993). Perhaps, surprisingly, honey can also be used to treat eruptions in measles (Meda *et al*., 2004). Recent research has shown peripheral blood B-lymphocytes and T-lymphocytes proliferate markedly in cell cultures containing honeys; additionally, honey increases phagocyte activity (Olaitan *et al*., 2007). Finally, the rapid healing following the use of honey can be economically beneficial as it leads to a reduction in hospital and surgical costs (Zumla and Lulat, 1989). Honey can be given by mouth to treat and protect against gastrointestinal infection, including gastritis, duodenitis and bacterial and viral gastric ulceration (Tallett *et al*., 1977) largely because honey blocks bacterial adherence to the stomach lining

(Alnaqdy *et al*., 2005). Diarrhoea and gastroenteritis also respond to honey (Bansal *et al*., 2005), and *H. pylori* is also sensitive to honey treatment (al Somal *et al*., 1994). Honey can also be used to treat fungal infections including dermatophytoses and candidiasis (Obaseiki-Ebor and Afonya, 1984) and cutaneous and superficial mycoses like ringworm and athletes foot; it is also effective against seborrheic dermatitis and dandruff (Al-Waili, 2001). Honey also treats recurrent lesions from labial and genital herpes and inhibits the rubella virus. Honey is also used to treat ophthalmological infections such as blepharitis, keratitis, conjunctivitis, corneal injuries, chemical and thermal burns to eyes(Shenoy *et al*., 2009). Honey also decreases venous blood pressure(Rakha *et al*., 2008). Honey is also anticarcinogenic (Bansal *et al*., 2005) and has antineoplastic activity in the experimental bladder cancer (Swellam *et al*., 2003). Finally, honey is relatively free of adverse effects, although topical application can lead to transient stinging sensation; despite this, honey usually relieves pain and is both non-irritating and provides a painless dressing change. Honey allergy is rare although there could be an allergic reaction to the presence of either pollen or bee proteins. Excessive application of honey may lead to tissue dehydration. Risk of wound botulism by *Clostridia* should be eliminated by gamma irradiation of honey without any loss of antibacterial activity (Bansal *et al*., 2005).

Patients suffering from suppressed immune system, due to diseases such as leukaemia and chemotherapy often suffer wound healing problems (Simon *et al.*, 2006, Gaur *et al*., 2005), as well as enhanced susceptibility to wound infections; infections which can cause secondary potentially life-threatening infections of the blood stream (Gaur *et al*., 2004). Such wounds can often be effectively treated with honey (Apitherapy). Honey works by being hygroscopic and drawing moisture out of the wound environment; it thus dehydrates bacteria. Much of this activity is due to its

sugar content, although his alone does not explain honey's antibacterial properties. Although hydrogen peroxide is a major component of honey, Manuka honey also contains other components with antibacterial qualities, notably methylglyoxal (MG); MG is a compound found in most types of honey, but generally in small quantities. Generally, the higher the concentration of MG, the stronger the antibiotic effect. In Manuka honey, MG comes from the conversion of dihydroxyacetone, a compound found in high concentrations in the nectar of Manuka flowers. Medical honey consists of standard mixture of different Manuka (*Leptospermum* spp are known by various common names in both Australia and New Zealand, including Tea Tree, Manuka, Goo Bush and Jelly Bush; it exhibits a standard antibacterial activity as confirmed by appropriate *in vitro* testing methods. The antibacterial properties from *Leptospermum* spp. honeys are both stable to light and heat and activity is not destroyed by sterilization with gamma-irradiation (Molan and Allen, 1996). Some 100 candidates for the antibacterial property of Manuka honey have been suggested (Yaghoobi *et al*., 2013).Even though the active ingredient has yet identified to be methylglyoxal (MG) (Adams *et al*., 2009). Honey producers have developed a scale for rating the potency of Manuka honey called UMF, Unique Manuka Factor. The UMF rating corresponds with the concentration of MG. To be considered potent enough to be therapeutic use, Manuka honey needs a minimum rating of 10 UMF (Molan 1999).

Hydrogen peroxide activity is a major antibacterial component but if this is blocked and the osmotic effect of honey is also curtailed then *Leptospermum* spp. honeys still exhibit antibacterial activity. Non-Manuka honeys vary as much as 100-fold in their antibacterial activity, largely because the main factor involved is hydrogen peroxide (Lusby *et al*., 2005, Cooper *et al*., 1999, Cooper *et al*., 2002).

In addition to being antibacterial, medical honey hastens the healing of wounds by virtue of its anti-inflammatory effects which help reduce oedema and the amount of wound exudates and pain which results from the nerve endings being sensitized by prostaglandins produced during inflammation, as well from the pressure on tissues resulting from oedema. The high sugar content of honey also reduces pain, when dressings are changed, as it keeps the wound surface moist by mobilizing the oedema from the surrounding tissues. Honey also stimulates the rate of angiogenesis, granulation and epithelialisation (Gupta *et al*., 1992), which helps explain the positive results of clinical trials which show that honey speeds up the healing process (Molan, 2006). Honey may also work via its stimulation of an inflammatory response in leukocytes (Tonks *et al*., 2003), as inflammation triggers the cascade of cellular events that produce the growth factors controlling angiogenesis and the proliferation of both fibroblasts and epithelial cells.

Another mechanism may be related to the low pH level of honey (i.e. pH 3.4–5.5; mean 4.4), since it has been shown that acidification of wounds speeds healing (Rendl *et al*., 2001).This was attributed to the low pH increasing the amount of oxygen being off-loaded from capillary haemoglobin. It may also result from suppression of protease activity in wounds by moving the pH away from neutral, i.e. the optimum for their activity (Rushton, 2007).

Honey also shows a marked debriding action which helps to remove slough which is a rich source of bacteria to stimulate an inflammatory response (Molan, 2002). Finally, but very importantly, medical honey successfully reduces the problem of malodour from chronic suppurating wounds.

Honey must be sterilized before it is used on wounds, largely because it can suffer Clostridial contamination, as a result it should not be fed to infants. Such

contamination could lead to spores germinating in anaerobic environments within the wound leading to toxin production and associated paralysis and cardiac arrhythmia. In order to eliminate botulism spores, honey must be heated to 120°C for 10 min, a treatment, which causes adverse changes to the beneficial properties of honey. Since *Clostridium* spores may be found in Manuka honey, for medical purposes it is gamma irradiated, a process, which does not affect its antibacterial activity (Molan and Allen 1996).

Medical honey meets all the criteria expected of a successful wound curative, with the exception that they do not act particularly quickly. For example, medical honeys enhance and accelerate wound healing (debridement, granulation), even when applied for prolonged periods. They also produce no adverse local or systemic effects (e.g. allergy and toxicity problems related to absorption). Importantly, medical honeys are cheap to buy and use and need only be applied twice daily.

Medical honey is particularly effective in the treatment of recalcitrant wounds, notably indolent limb infections (Simon *et al*., 2006). The effectiveness of honey against antibiotic-resistant microorganisms suggests that it could be effectively used as an alternative means of chemoprophylaxis in patients with central venous catheters, and in the treatment of mucositis, a side effect of chemotherapy that attacks the entire gastrointestinal tract from the mouth to the anus and in the treatment of infants with polymicrobial infections (Vardi *et al*., 1998). Topical honey is safe and effective in the management of the signs and symptoms of recurrent lesions from labial and genital herpes (Al-Waili, 2004).According to this experience, children and adults are treated with recurrent herpetic lesions on the lips with medical honey, as soon as a new lesion is developing. In addition, topical medical honey is used in addition to

systemic acyclovir in immune compromized patients with zoster to prevent secondary bacterial skin infection and to accelerate healing of the herpetic lesions.

Medical honey dressings should keep the honey in contact with the wound for at least 12 h, but optimally for 24 h. They can be applied overnight, so as not to restrict. It is generally recommended that the best way to keep honey in a wound is to soak it into a calcium-alginate or hydro fiber dressing, which forms a gel with the honey as it absorbs the wound exudates; prepared medical honey products are available for this purpose. The frequency of dressing changes depends on the amount of exudates produced during treatment. In early stages, this may require a dressing to be change twice a day, although during stable wound care situations honey dressings have been left in place for up to 7 days (Gethin, 2004).

Results have shown a high patient acceptance for honey therapy. Amazingly wound dressings often have to be performed under general anaesthesia to alleviate pain and anxiety. However, shortly after the introduction of medical honey dressings, wounds often improve to such an extent that dressing changes can be performed without analgesic medication. If medical honey dressing is completely moistened with sterile Ringer solution, they can generally be readily and painlessly removed without any troublesome attachment to the wound. Another advantage of honey therapy is that care and ambulance workers, parents and relatives can be quickly educated in the aseptic application of honey dressings, which can be done at home or at work.

Honey can have the disadvantageous effect of causing stinging pain after administration, a problem that can be reduced by pre-treating the wound surface with a sterile anaesthetic cream. However, in some patients who experienced pain after administration, treatment with medical honey has to be stopped (Blaser *et al*., 2007b) or postponed to a later phase of wound healing. Despite this occasional adverse effect

honey treatment has been shown to be both low cost and highly effective (Subrahmanyam 1991, Johnson *et al*., 2005).

The work presented in this Thesis shows that there are a number of potentially effective non-antibiotic honey based treatments which can be used to treat wound infections caused by antibiotic–resistant bacteria, work which provides motivation for studies on other alternative approaches to the control of wound infections. While considerable resources have been directed towards herbal medicine, less attention has been given to other diverse types of alternatives, examples of which include the use of: cod liver oil, chlorophyll, pectin, silicon and clays (Pugh, 1942). Cod liver oil was used in the Second World War to treat infected wounds, either by filling wound-cavities, by using soaked dressings or by using a mixture of 30-50 % in Vaseline. Cod liver oil effectively kills bacteria when it is the oxidized, odorous state, suggesting that vitamin D may be the active ingredient. Highly ground silicon has also been used to treat wounds and can be given by mouth to cure for bleeding ulcers (Pugh, 1942). Stumpf (1906) used clay as a paste to treat indolent infections; it was found, not only to reduce infection, but also to lead to deodorization of the wound; it also stopped irritation and enhanced healing (Pugh, 1942). Kaolin clay has also been used to treat cholera in India and elsewhere and mortality rates can drop from 44% to near zero following its use (Nadkarni, 1906); kaolin combined with morphine was an old remedy used for stomach infections which could be found in the medical cabinets of most of UK households until the 1960s. All of the above named alternatives are clearly worthy of re-evaluation and after modern modification might be used in the future to treat antibiotic–resistant, indolent infections and maybe as in the case of clay-based remedies, for treating cholera.

It may also be possible to combine antibiotics with other drugs in order to control antibiotic resistance. For example, Minocycline, an antibiotic that inhibits protein synthesis in bacteria (and to which bacteria developed resistance) has recently been re-evaluated for medical use when combined with other bioactive drugs. Sixty-nine compounds were, when added to this antibiotic, found to improve its effectiveness, allowing it again to be used as an antibacterial agent. Surprisingly, Imodium, the commonly used anti-diarrheal drug, also improved the antibacterial activity of Minocycline (Ejim *et al*., 2011).

Finally, it is worth considering the commonly held ideas regarding the future, in regard to developments, which might solve the antibiotic resistance problem. Zucca and Savoia (2010)suggest for example that this problem might be solved by the use of antimicrobial peptides, antivirulence factors, inhibitors of pathogen adhesion, inhibitors of pathogen colonization, inhibitors of toxin production and secretion, the development of antibodies, and finally phage therapy. Surprisingly, no mention is made of maggot or honey therapies, despite the fact that these approaches are currently being used in hospitals to treat antibiotic resistant infections. Clearly there exists a noticeable mismatch between the large amounts of research time which is devoted to theoretical applications of sophisticated approaches (e.g. gene therapy) and the current successful application of approaches such as apitherapy and maggot therapy. It would be obviously desirable to move away from these “primitive”, albeit successful approaches, especially since these cannot be used to treat systemic infections. Both intravenous maggot therapy is clearly impossible as presumably is the application of intravenous honey therapy (a German honey extract, referred to as M2 Woelem) was however, developed in the 1950s for use in gynaecology and obstetrics, (De Buman, 1953).

In the absence of effective, more scientific approaches, historical approaches to wound treatment will continue to be used in practical medicine. There exist a range of other potential treatments, such as the use of chlorophyll and clays which have yet to be evaluated in modern Western medicine. While such alternatives to antibiotics can be seen as a desperate measure, and are only provisional, they may have to be used in practical medicine for some time yet until new antibiotics or other effective alternative approaches have been developed.

Suggestions for Further Study

1) ***New-Super honeys***-Although the antibacterial effects of a variety of different honeys, from various parts of the work have been studied here (and in others Theses from this laboratory related to this topic),the potential still exists for a "super honey" to be discovered whose antibacterial properties will exceed those of Manuka honeys. Such honeys may prove to be particularly effective in killing bacteria when applied to wounds in dressings. Much in the way that novel antibiotic-producing organisms are sought, and screened for, from unusual habitats from all over the world so, one could imagine a similar search of honeys produced in remote areas of the world, which could prove successful as novel and highly effective antibacterial agents. It is recommended therefore that searches be made of remote areas in the hope of finding such as yet unknown active honeys. Obviously, care would need to be taken to avoid the possibility of importing into the UK viruses and other microbes which might destroy our native bee population.

2) ***Interactions between "failed antibiotics" and honey.*** It maybe that the use of antibiotics to which bacteria have developed resistance thereby making them of little use in medicine could be "revived" by mixing them with antibacterial honeys. This possibility is based on idea that the honey component would reduce the vitality of the bacterium, which would then be finally killed by the presence of the antibiotic component. Obviously such a combination would likely only be used on external wounds; although the author is not aware of any attempts to intravenously inject dilute honey solutions into human it can be assumed that these would cause anaphylactic shock or other adverse reactions.

3) ***The need for clinical studies.*** This Thesis describes *in vitro* studies. It would obviously be highly desirable (and ultimately essential) to study the effects of the honeys used here on pathogenic bacteria and associated infections *in vivo*. This is obviously particularly important in relation to the effectiveness of the honey-containing and other dressings studied here. The results of such studies may not be always directly related to microbiological effects. For example, it may be that these dressings are not useful because they cause excessive irritation and cannot be tolerated by some patients; such drawbacks would only be detected as the result of *in vivo* studies. Of course such studies obviously require the involvement of medical practitioners and patients, both of which we have no access to at present.

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Appendices

Appendix 1. Preparation of bacterial turbidity-standard suspensions

Sensitivity tests were adjusted to $1.5x10^6$ colony forming units (CFU) which equals 0.5 McFarland. Briefly, two to three isolated colonies of the same morphological type were taken with a sterile loop and inoculated into a tube containing nine ml of nutrient broth and incubated in 37^{o}C for 2 hrs. After incubation, the turbidity was measured in spectrophotometer at 650 nm and the measurement was recorded. The contents of the tube was diluted by taking 1 ml of the broth and transferred to another tube containing 9 ml sterile nutrient broth; this was the first dilution (10^{-1}), the same step was then repeated five times to achieve the dilution of (10^{-5}); 0.1 ml was taken from the last dilution and poured and spread on sterile nutrient agar plate. The plate was incubated for 24 hrs then the total colony count was counted and timed 10^5 and 10 was added to the total to get the actual concentration of 10^6, i.e. the suspension density. If the colony number was too high then it was further diluted with nutrient broth and same steps then should be repeated to obtain 1-2 colonies forming units in dilution 10-^{5}.

Appendix 2. Well diffusion technique

Sterile Muller-Hinton agar (20 ml) plates were used. The surface of the plates was inoculated with previously calibrated inoculums of bacteria and allowed to dry before incubation in 37^o C for 30 minutes. Five wells of 8.0 mm in diameter were cut from the culture media by using a sterile metal cylinder, and then filled with the previous mentioned concentrations. Vancomycin (30 µg) was used as a positive control for *Staphylococcus aureus* and Imipenem (10 µg) for *Pseudomonas aeruginosa*. The plates were incubated at 37^{o}C and checked after 24 hrs for clear, circular inhibition zones around the wells. These zones were then measured.

Appendix 3. Identification of bacterial isolates using the 16s rRNA technique

A bacterial suspension in nutrient broth was prepared and incubated overnight at 37^0C. After the incubation period, 1-3 ml of media was transferred in a sterile Ependorf tube and centrifuged at 6000 x g for 2 min at room temperature and the supernatant was decanted completely. A KeyPrep bacterial DNA extraction kit supplied by ANACHEM® was used and all steps were done as described in the instructions provided by the company. Buffer (RI, 100µl) was added to the pellet and the cells were resuspended completely by pipetting up and down. After full cell homogenising, 20 µl of lysosyme was added and mixed thoroughly and incubated at 37^0C for 20 min. The mixture was then centrifuged at 10,000xg for 3 min and the supernatant was completely decanted. The pellet was resuspended in 180 µl of buffer R2 and 20 µl of proteinase K was added and incubated at 65^0C for 20 min in a water bath with occasional mixing every 5 min. 400 µl of buffer BG was added and mixed thoroughly by inverting the tube several times until a homogeneous solution was obtained and then incubated for 10 min at 65^0C. After the incubation period 200 µl of absolute ethanol was added and mixed thoroughly. The sample was transferred into a column which was assembled in a clean collection tube and centrifuged at 10,000xg for 1 min while the flow was discarded. The column was washed by addition of 750 µl of wash buffer and centrifuged at 10,000xg for 1 min while the flow was discarded. Finally, the column was placed in a clean microcentrifuge tube and 70µl of elution buffer was added and centrifuged at 10,000xg for 2 min to elute DNA. DNA was stored at -20^0C until the next step.

Gelelectrophoresis

Gel electrophoresis was conducted to make sure that the bacterial DNA was well extracted and purified. The following steps were used:

Agarose preparation

Molecular biology grade agarose (0.5 g) was dissolved in 50 ml of 1x TAE (Tris Acetate EDTA) buffer and 40ml distilled water by heating in a microwave oven. The solution was mixed gently and allowed to cool to 55^0C, and 2.5 µl of ethidium bromide was added. After mixing, the solution was poured into a sealed gel rack and a comb was inserted at one side of the gel vertically and left at room temperature for 20 min to solidify. Then the comb was removed and gel was placed into an electrophoresis tank and submerged in 1x TAE buffer.

Sample loading

The DNA sample (10 µl) was mixed with 2 µl of Blue/Orange 6x loading dye with glycerine and loaded into the wells. 6 µl of 1 kb hyper ladder was added into an adjacent well as a reference. The settings were 40 minutes at 80V to allow the DNA to migrate toward the anode. Afterwards the DNA fragments were visualized under a UV transilluminator and the images were captured using a connected digital camera.

Sample amplification

Samples were amplified using Polymerase Chain Reaction (PCR) technique.

A mixture in a sterile Eppendorf tube was prepared as follows:

Master mix, 12 µl, 1 µl of forward primer (5'-CCGAATTCGTCGACAACAGAGGATCCTGGCTCAG-3'), 1 µl of reverse primer(5'- CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3'), 1 µl of the DNA sample, and 35 µl of sterile distilled water. The mixture was then inserted in a PCR machine and the programme was adjusted as follows:

Serial	Temperature	Time(Min)	Passes total
1	94^0C	3	1
2	94^0C	1	35
3	60^0C	1	35
4	72^0C	1	35
5	72^0C	5	1
6	4^0C	∞	1

16SrRNA sequencing and phylogenetic analysis

After PCR, aliquots of 10 µl of each sample with 1µl of forward primer and 1µl of reverse primer in a sterile small size tube were sent to the Medical School Core Genetics Unit (University of Sheffield) to be sequenced. The 16S rRNA gene sequences were adapted using the Finch TV software and then exported into the Basic Local Alignment Search Tool (BLAST), available from the website of the National Centre for Biotechnology Information (NCBI), to identify matches with existing characterized reference sequences.

Appendix 4. Determination of minimum biofilm eradication concentration (MBEC):

Inoculum preparation

More than 7 colonies of the same morphological appearance of a fresh subculture of the tested bacteria were picked off with a sterile loop and dipped into 1.5 ml sterile nutrient broth. 1 ml of prepared inocula transferred into 29 ml of nutrient broth. This 30 fold dilution of the 1.0 McFarland standard serves as the inocula for the MBEC plate (Figure 34). A new MBEC plate was opened and the first row and the second row of pegs were removed by using sterile metal pliers to use these rows as negative controls afterward.

22 ml of the previous prepared fold dilution was added to the MBEC plate and incubated in 37^{o}C for 48 hrs in case of *Staphylococcus aureus* and 96 hrs in case of *Pseudomonas aeruginosa* (Wirtanen et al., 2001).

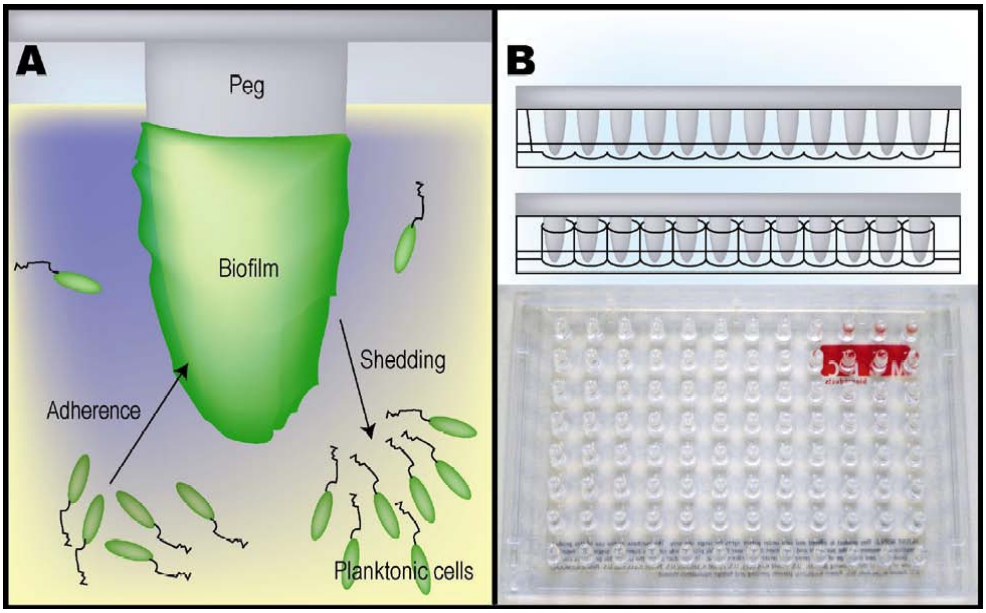

Figure34. The MBEC plate (B) and biofilm formation on the peg (A)

Preparing the antimicrobial challenge plate

Concentrations of the treatment were prepared previously in sterile tubes and then transferred to wells depending on the used treatment. The vertical well line was used for one type of honey from the third row to the eleventh row. The test for each type of honey was done in duplicate. The twelfth row used as positive control.

Preparing a rinse plate

A sterile microtiter plate with 200 µl of physiological saline was setup in every well. This plate was used to rinse the pegs to remove loosely adherent planktonic cells from the biofilm.

Exposure of biofilms

After the incubation period of the MBEC plate, the peg lid from the trough was removed and the pegs submersed in the wells of the rinse plate. The peg lid was let for 1 to 2 minutes. This step was repeated in a new rinse plate.

After washing, the peg lid of the MBEC was inserted into the challenge plate properly and incubated at 37^{o}C for 24hrs.

Neutralization and recovery

After the incubation period, the peg lid of the MBEC plate was washed twice using the same mentioned protocol in two different rinse plates. A 96 wells plate was prepared by adding 200 µl of nutrient broth in all wells and this was the recovery plate. The washed MBEC plate was transferred to the recovery plate and closed tightly to prevent any possibility of the contamination. The plate was transferred onto the tray of the sonicator. The plate was left for 1 hour to allow the vibrations to disrupt the biofilms from the surface of the remaining pegs into the recovery plate. The plate was then incubated for 24 hrs and checked for the visible growth.

Appendix 5. Peroxide test

QUANTOFIX® Peroxide 25

en

Pack content:
1 aluminium container with 100 test strips

Hazard warnings:
This test does not contain hazardous substances that must be labelled.

General indications:
Remove only as many test strips as are required. Close the container immediately after removing a strip. Do not touch the test field.

Additional indications:
QUANTOFIX® Peroxide 25 is also suitable for the detection of peracetic acid, as well as other organic and free inorganic hydroperoxides. When detecting hydroperoxides in organic solvents, moisten the test field with 1 drop of water after evaporation/drying of the solvent.

Instructions for use:
1. Dip the test strip into the test solution for 1 s.
2. Shake off excess liquid.
3. Wait 15 s.
4. Compare with the color scale. If hydrogen peroxide is present, the test field turns blue.

Interferences:
If the sample solution has a pH value of 2–12, the reaction will take place without interferences. Strongly acidic solutions must be buffered with sodium acetate, and alkaline solutions with citric acid to a pH of 5–7. Only the presence of strong oxidants interferes with the determination.

Storage:
Avoid exposing the strips to sunlight and moisture. Keep container cool and dry (storage temperature not above +30 °C).
If correctly stored, the test strips may be used until the use-by-date printed on the packaging.

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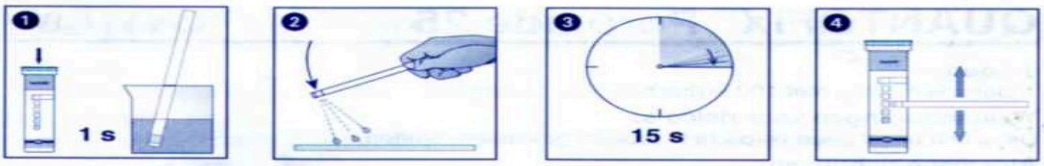

	Haltbarkeitsdatum / Use by / A utiliser jusqu'à / Fecha de vencimiento / Houdbaarheidsdatum / Data di scadenza / Data ważności	LOT	Chargenbezeichnung / Lot number / Numéro de lot / Número de lote / Lotnummer / No del lotto / Oznaczenie szarży
REF	Artikelnummer / Catalog number / Référence / Art. nro. / Referentienummer / Codigo articole / Numer artykułu	Σ	Packungsinhalt / Package content / Contenu de la boîte / Contenido del envase / Verpakkingsinhoud / Contenuto della confezione / Zawartość opakowania
	Packung geschlossen halten / Keep container closed / Refermer la boîte / Mantenga el envase cerrado / Verpakking gesloten houden / Conservare la confezione chiusa / Przechowywać pojemnik szczelnie zamknięty		Vor Feuchtigkeit schützen / Protect from humidity / Protéger de l'humidité / Protéjase de la humedad / Beschermen tegen vocht / Proteggere dall'umidità / Chronić przed wilgocią
	Lagertemperatur / Storage temperature / Température de stockage / Temperatura de almacenaje / Bewaartemperatuur / Temperatura di magazzinaggio / Temperatura przechowywania		Packungsbeilage lesen / Please read instructions / Lire les instructions, svp / Obsérvense las instrucciones de uso / Lees de bijsluiter / Leggere il foglio informativo / Należy przeczytać ulotkę informacyjną
	Sicherheitshinweise in der Packungsbeilage beachten / Observe the safety precautions in instructions / Respecter les précautions de sécurité des instructions / Obsérvense las indicaciones de seguridad / Neem de veiligheidsrichtlijnen in de bijsluiter in acht / Seguire le avvertenze di sicurezza / Należy przestrzegać uwag zawartych w ulotce informacyjnej		

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Appendix 6. *Pseudomonas* broth

HIMEDIA®

Technical Data

Pseudomonas Agar (For Pyocyanin) M119

Pseudomonas Agar (For Pyocyanin) is recommended for the detection of pyocyanin production by *Pseudomonas* species.

Composition**

Ingredients	Gms / Litre
Peptic digest of animal tissue	20.000
Potassium sulphate	10.000
Magnesium chloride	1.400
Agar	15.000
Final pH (at 25°C)	7.0±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 46.4 grams in 1000 ml distilled water containing 10 ml glycerol. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Principle And Interpretation

Pseudomonas Agar is based on the formulation described by King et al (1) and as recommended in U.S. Pharmacopoeia (2) for detecting pyocyanin, a water soluble pigment by *Pseudomonas* species (3). This medium enhances the elaboration of pyocyanin but inhibits the formation of fluorescein pigment. The fluorescein pigment diffuses from the colonies of *Pseudomonas* into the agar and shows blue colouration. Some *Pseudomonas* strains produce small amounts of fluorescein resulting in a blue-green colouration.

Potassium sulphate and magnesium chloride, which enhances the pyocyanin production and suppresses the fluorescein production. A pyocyanin-producing *Pseudomonas* strain will usually also produce fluorescein. It must, therefore, be differentiated from other simple fluorescent pseudomonads by other means. Temperature can be a determining factor as most other fluorescent strains will not grow at 35°C. Rather, they grow at 25-30°C (3).

Quality Control

Appearance
Cream to yellow homogeneous free flowing powder

Gelling
Firm, comparable with 1.5% Agar gel

Colour and Clarity of prepared medium
Yellow coloured clear to slightly opalescent gel forms in Petri plates

Reaction
Reaction of 4.64% w/v aqueous solution containing 1% v/v glycerol at 25°C.pH:-7.0±0.2

pH
6.80-7.20

Cultural Response
Cultural response was observed after an incubation at 35-37°C for 18-48 hours.

Organism	Inoculum (CFU)	Growth	Observed Lot value (CFU)	Recovery	Colour of Medium
Pseudomonas aeruginosa ATCC 9027	50 -100	luxuriant	25 -100	>=50 %	blue-green
Pseudomonas aeruginosa ATCC 27853	50-100	luxuriant	25 -100	>=50 %	blue-green

Storage and Shelf Life

Store below 30°C in tightly closed container and the prepared medium at 2 - 8°C. Use before expiry date on the label.

Reference

1.King, Ward and Raney, 1954, J.Lab. and Clin. Med., 44:301
2.The United States Pharmacopoeia, 2008, The United States Pharmacopoeial Convention, Rockville, MD.
3.MacFaddin J., 1985, Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria, Vol. I, Williams and Wilkins, Baltimore.

Revision : 1 / 2011

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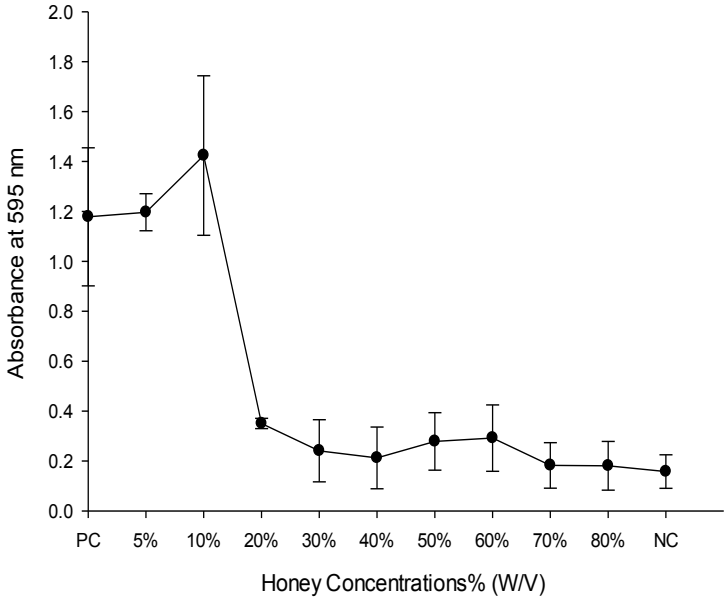
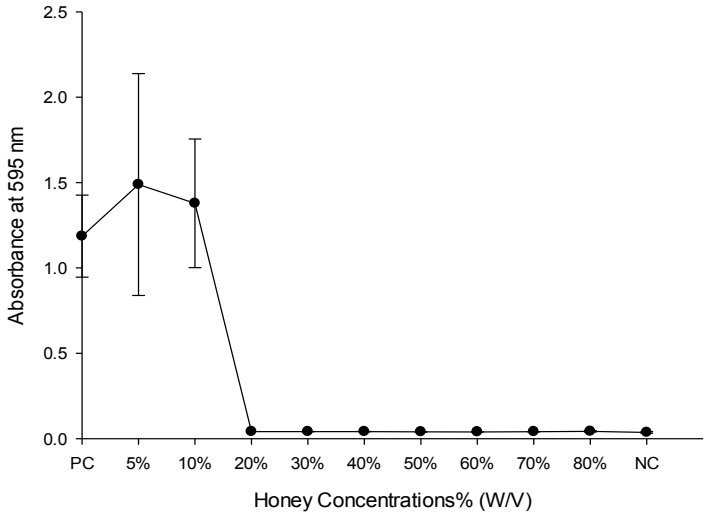
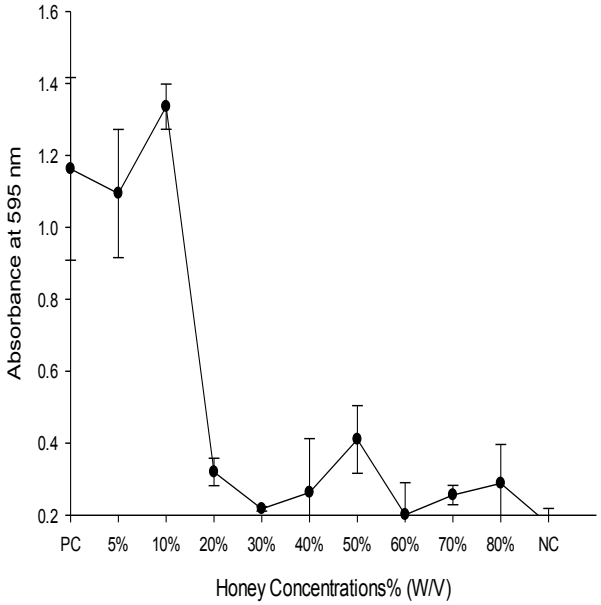
Appendix Figures



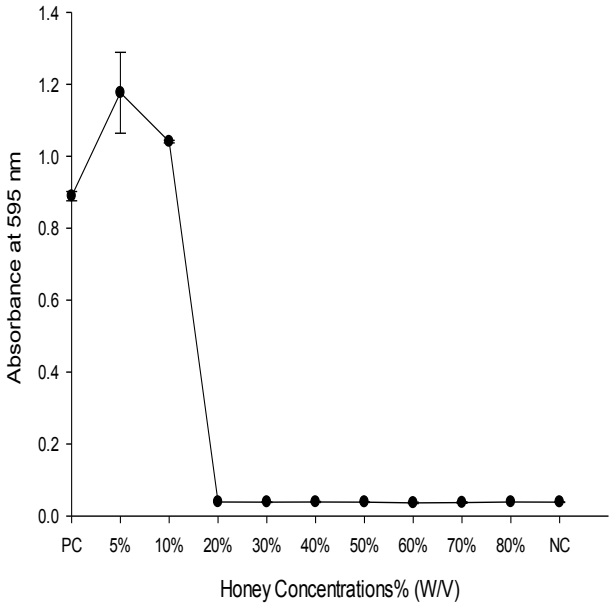
Appendix Figure 1.The effect of different concentrations of Manuka +20 on *S. aureus* Biofilm



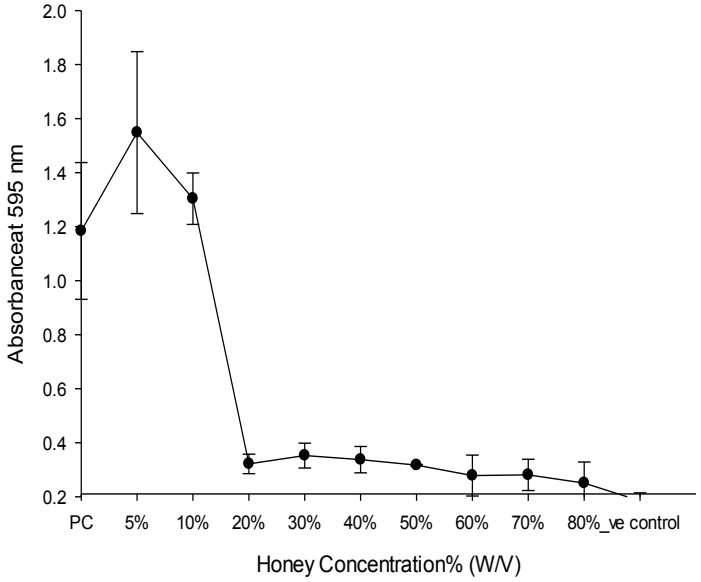
Appendix Figure 2.The effect of different concentrations of Manuka +20 on *Pseudomonas aeruginosa* Biofilm



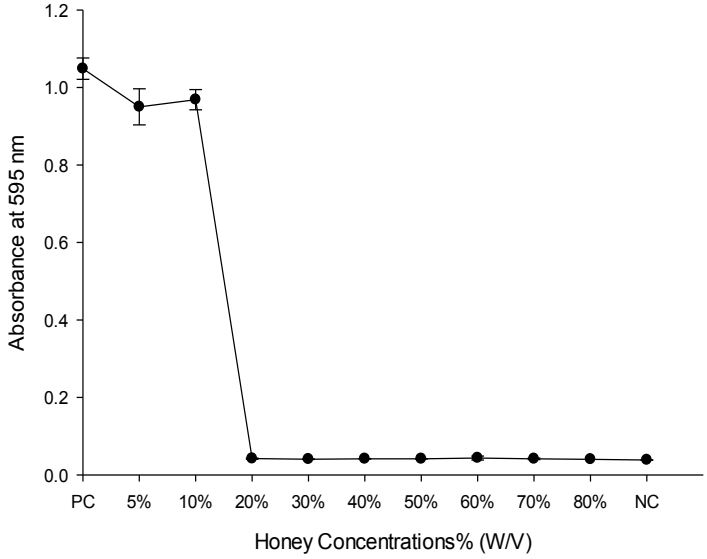
Appendix Figure 3.The effect of different concentrations of New Zealand Rewarewa Honey on *S. aureus* biofilm



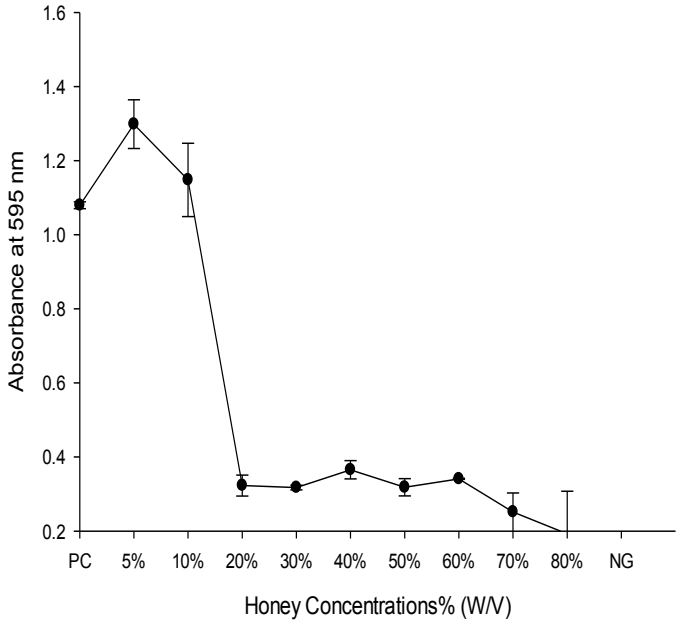
Appendix Figure 4.The effect of different concentrations of New Zealand Rewarewa Honey on *Ps. aeruginosa*biofilm



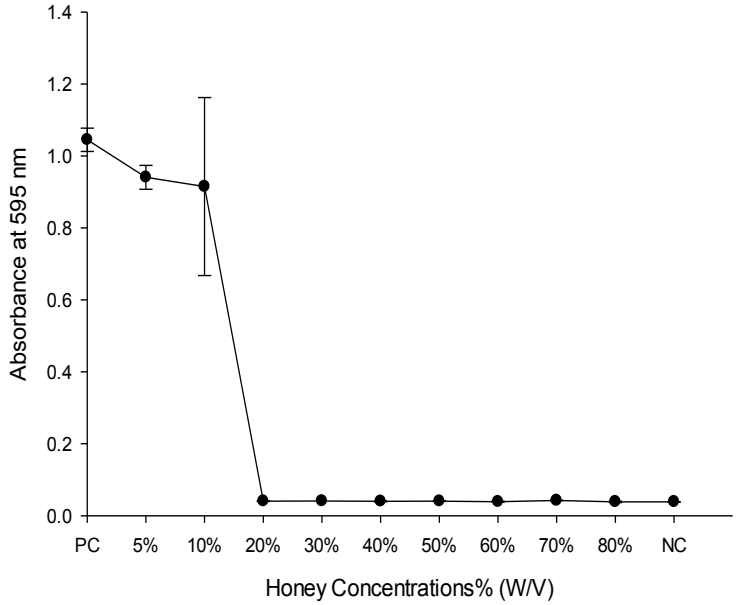
Appendix Figure 5. The effect of *Nigella sativa* honey with *S. aureus* biofilm



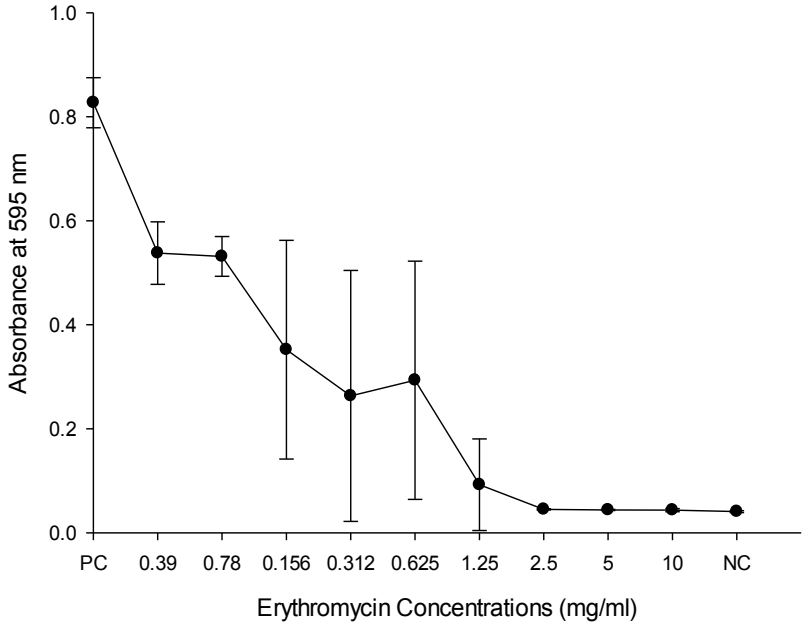
Appendix Figure 6.The effect of different concentrations of *Nigella sativa* honey on *Ps. aeruginosa*biofilm



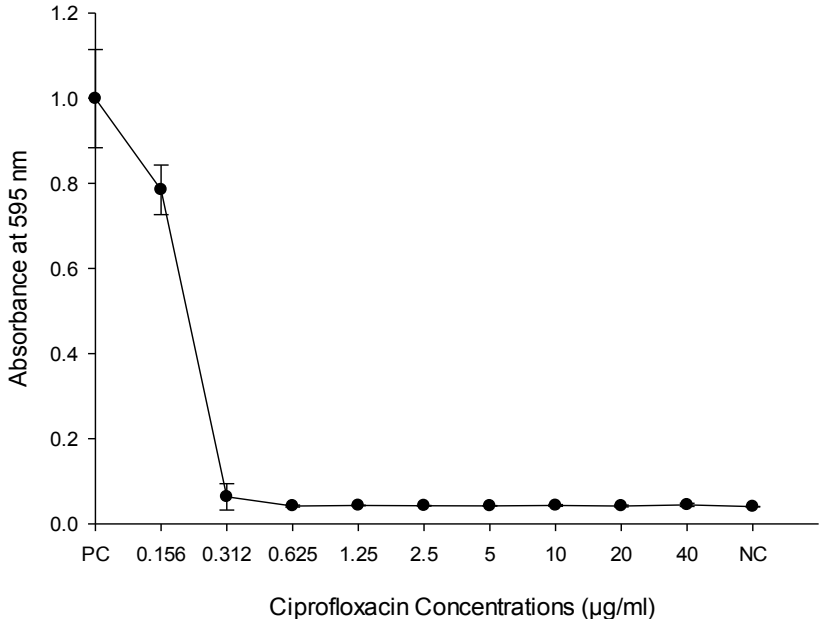
Appendix Figure 7. The effect of different concentrations of oak honey on *S. aureus*biofilm



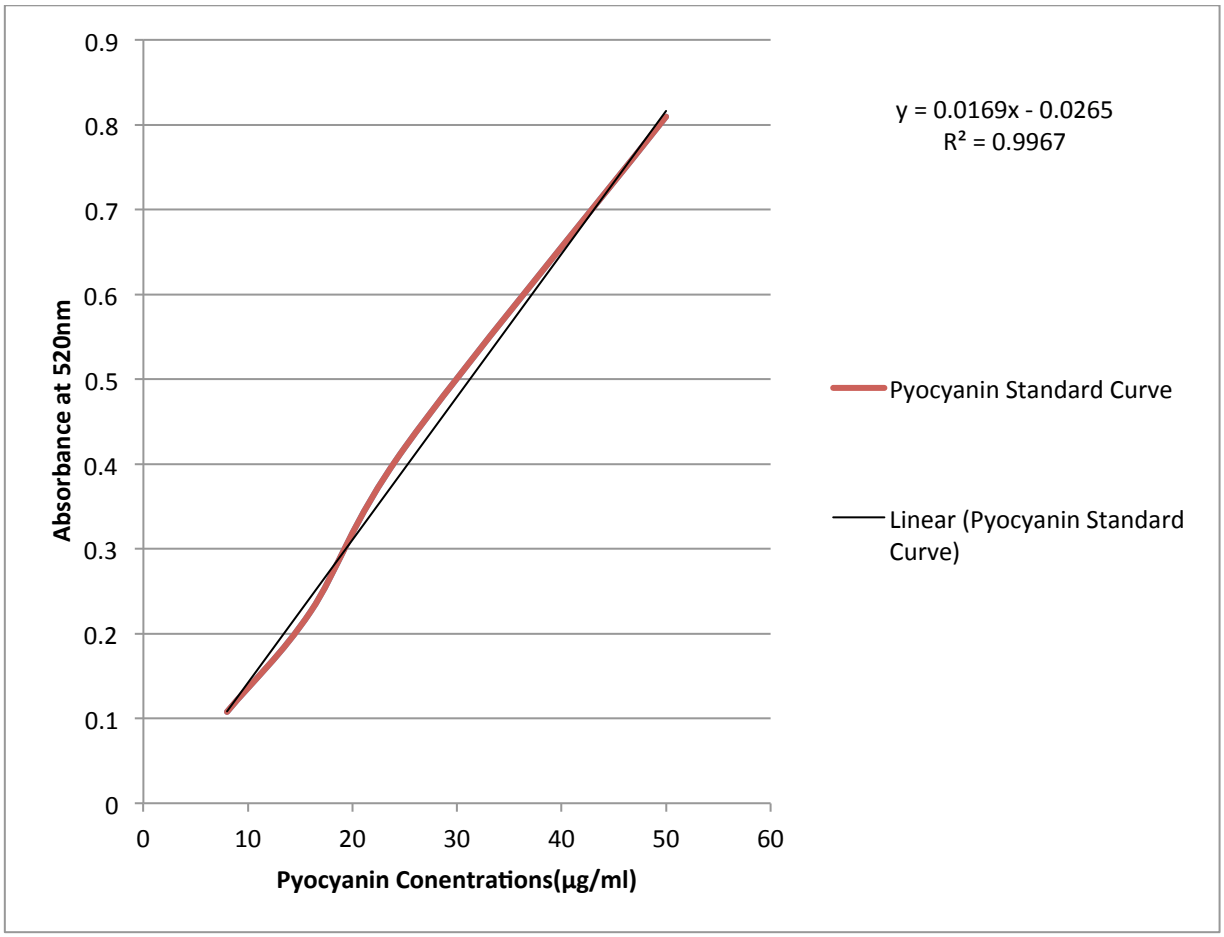
Appendix Figure 8. The effect of different concentrations of Oak honey on *Ps. aeruginosa* biofilm



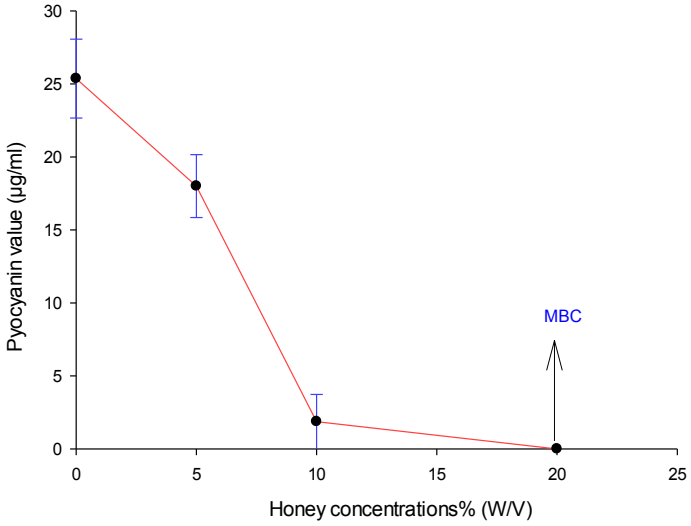
Appendix Figure 9.The effect of different concentrations of Erythromycin on *S. aureus* biofilm



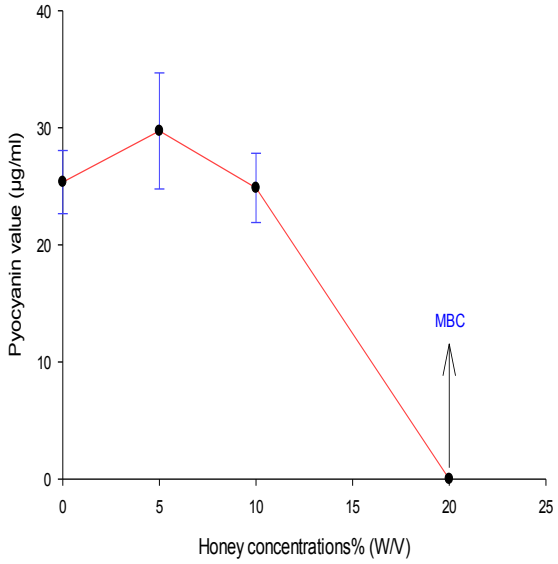
Appendix Figure 10. The effect of different concentrations of Ciprofloxacin on *Ps. aeruginosa* biofilm



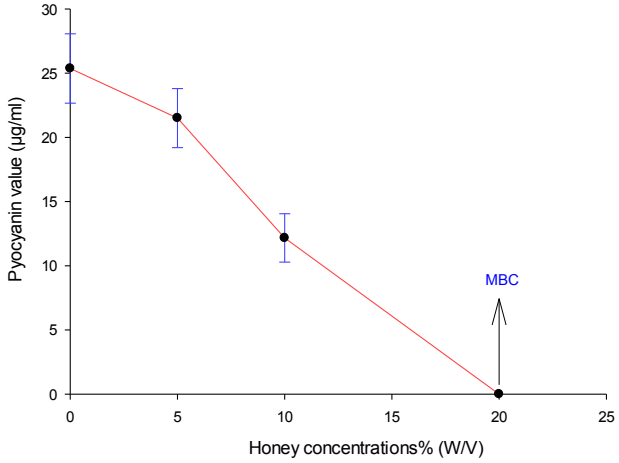
Appendix Figure 11. Pyocyanin standard curve



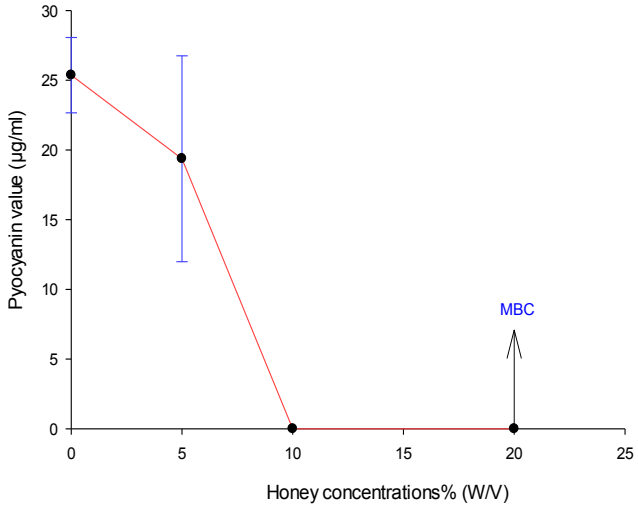
Appendix Figure 12. The effect of Manuka +20 honey on pyocyanin production



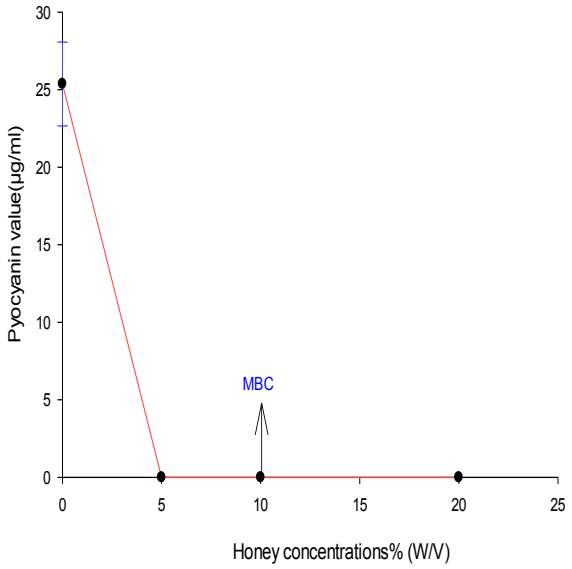
Appendix Figure 13. The effect of New Zealand Rewarewa honey on pyocyanin production



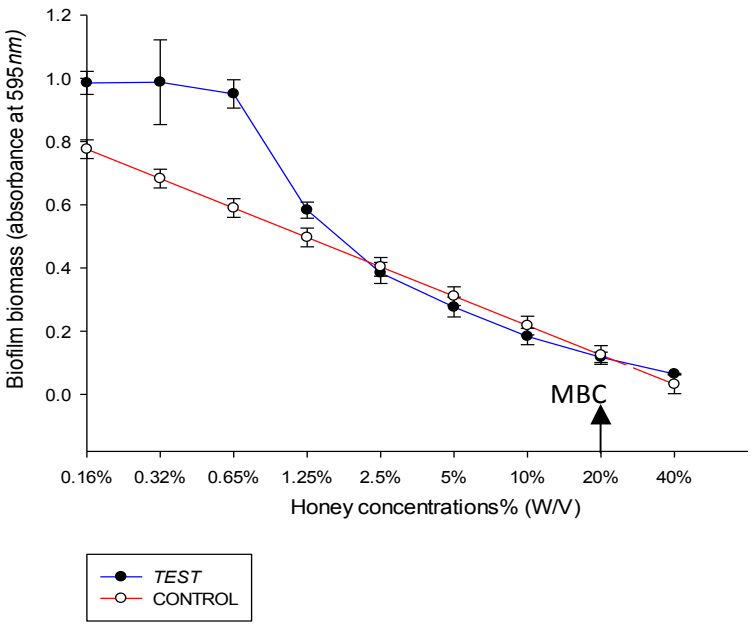
Appendix Figure 14. The effect of *Nigella sativa* honey on pyocyanin production



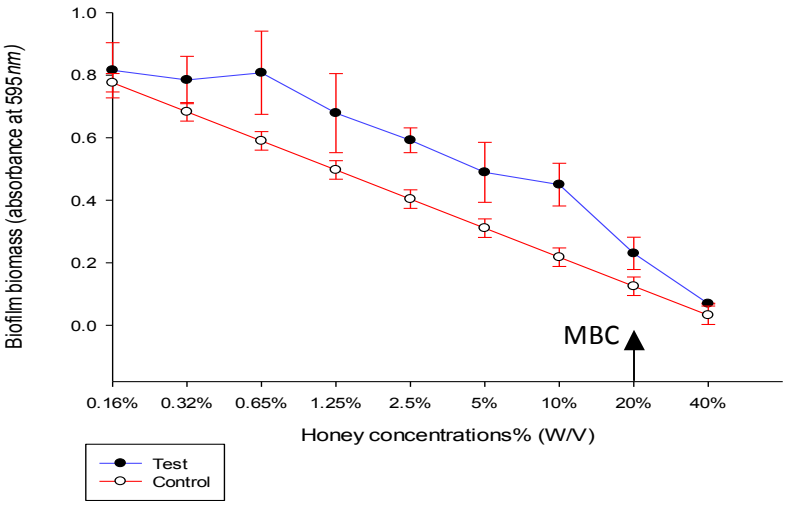
Appendix Figure 15. The effect of oak honey on pyocyanin production



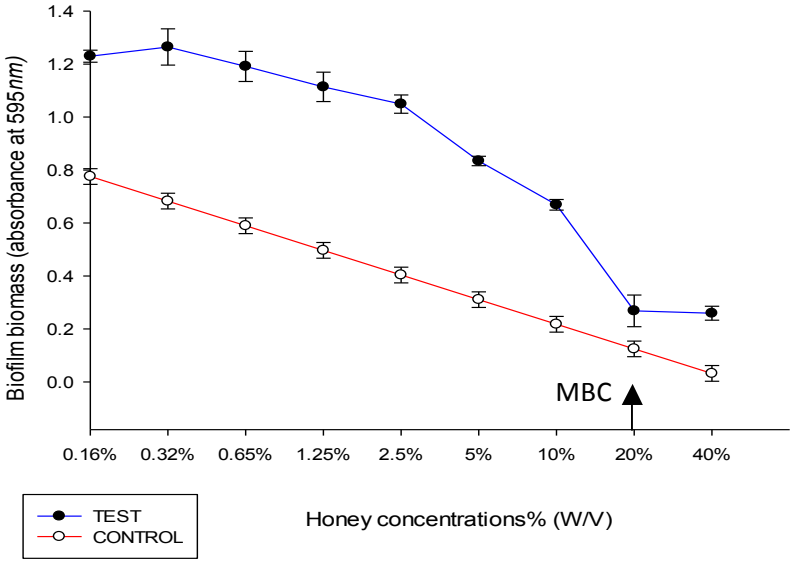
Appendix Figure 16. The effect of Rain Forest +30 honey on pyocyanin production



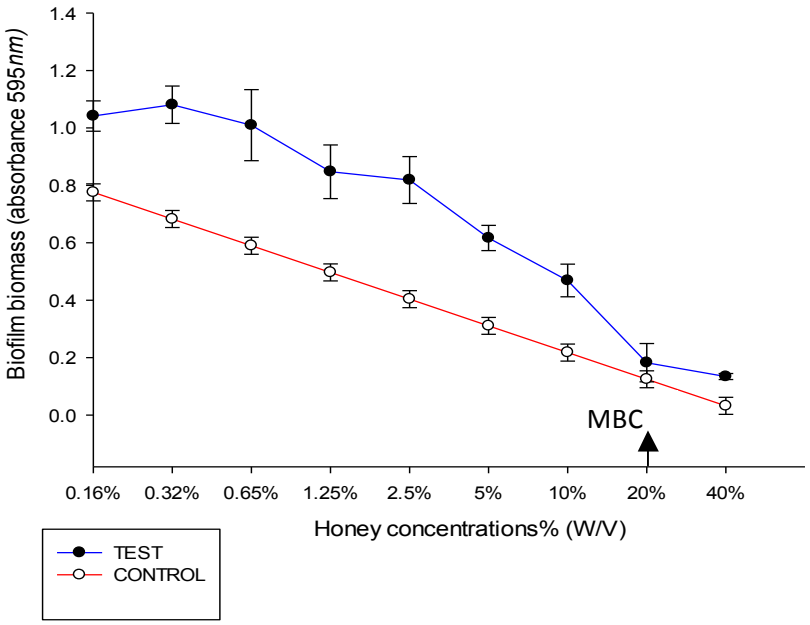
Appendix Figure 17. The effect of Manuka +20 honey on the formation of the biofilm of *S. aureus*



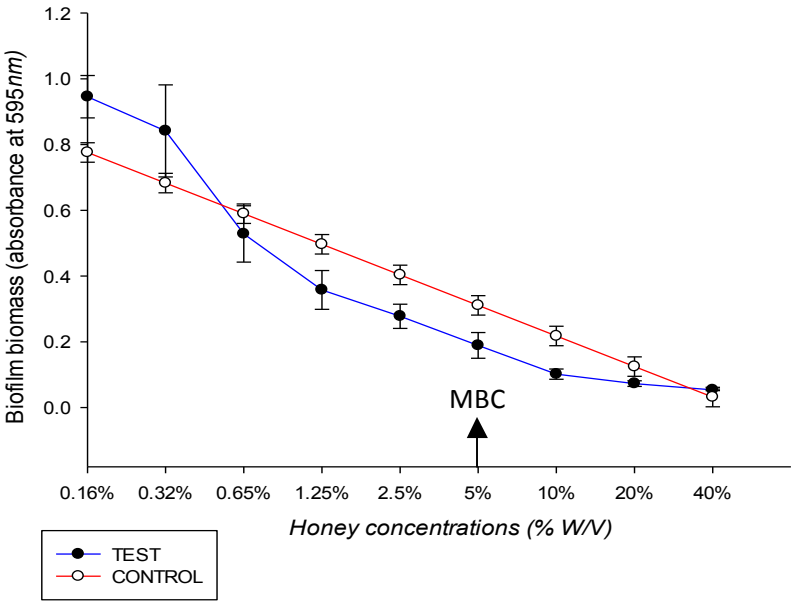
Appendix Figure 18. The effect of New Zealand Rewarewa honey on the formation of the biofilm *S. aureus*



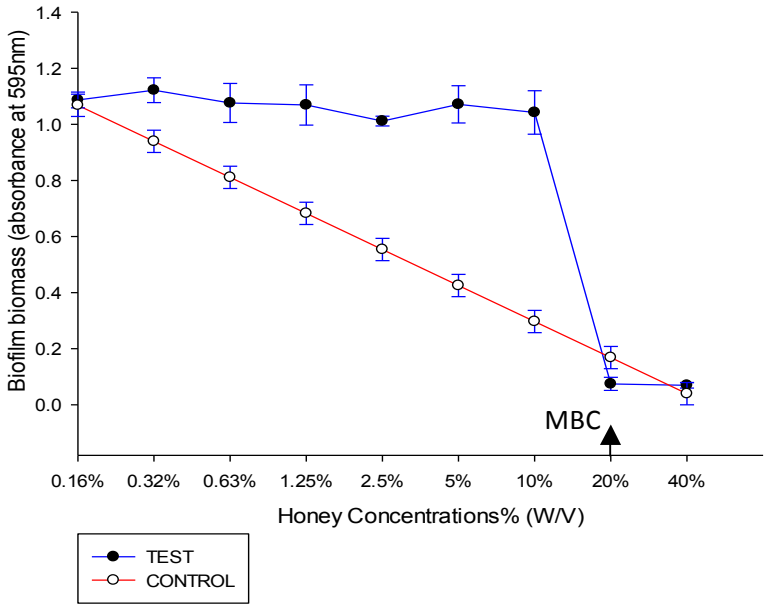
Appendix Figure 19. The effect of *Nigella sativa* honey on the formation of the biofilm of *S. aureus*



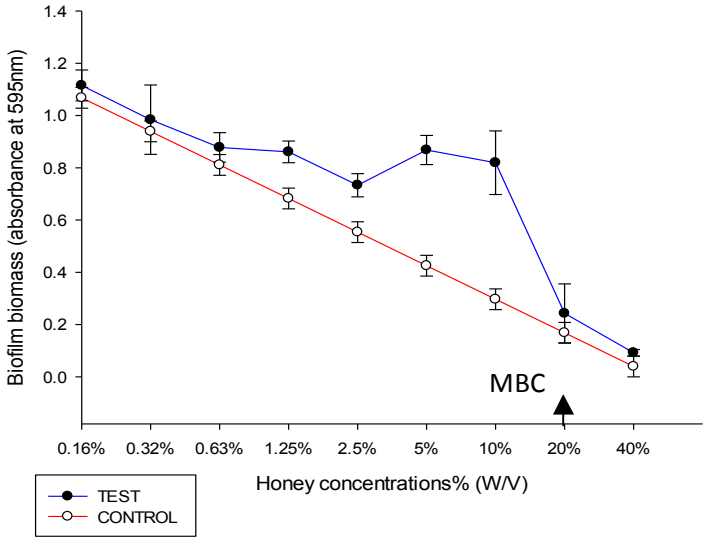
Appendix Figure 20. The effect of oak honey on the formation of the biofilm of *S. aureus*



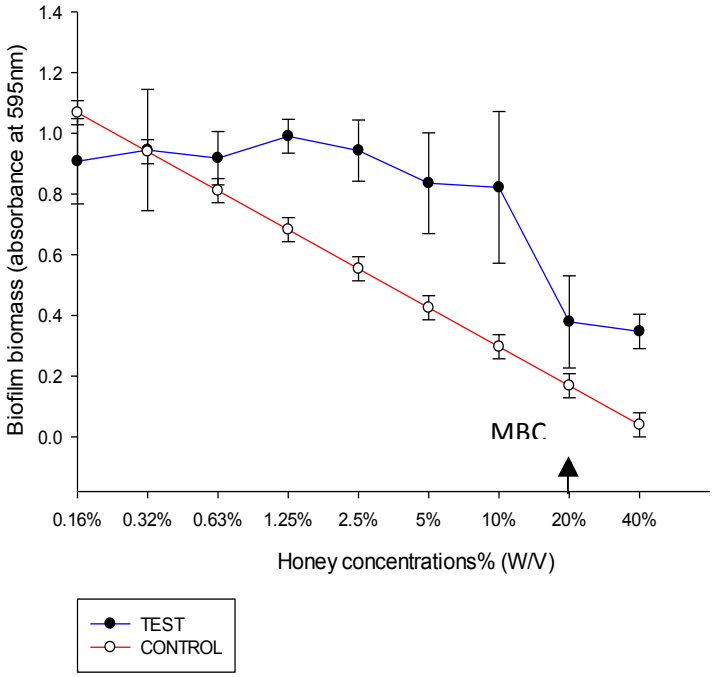
Appendix Figure 21. The effect of Rain forest +30 honey on the formation of the biofilm of *S. aureus*



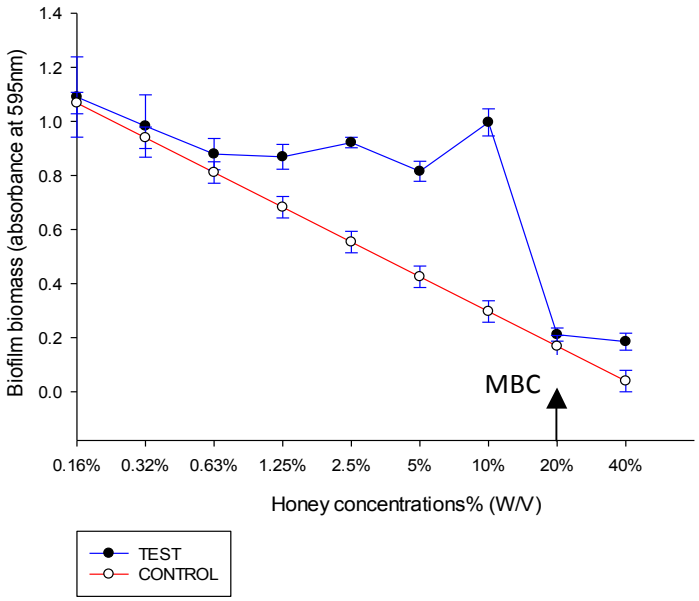
Appendix Figure 22. The effect of Manuka +20 honey on the formation of the biofilm of *Ps. aeruginosa*



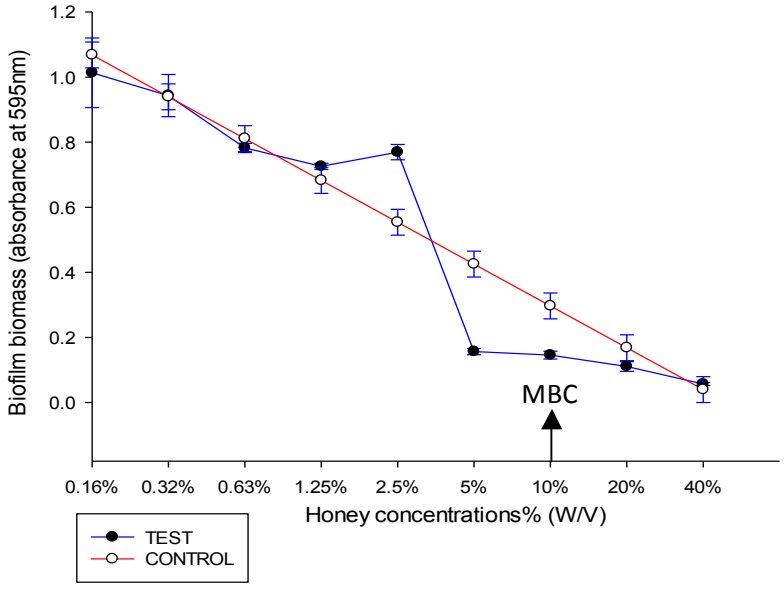
Appendix Figure 23. The effect of New Zealand Rewarewa on the formation of the biofilm of *Ps. aeruginosa*



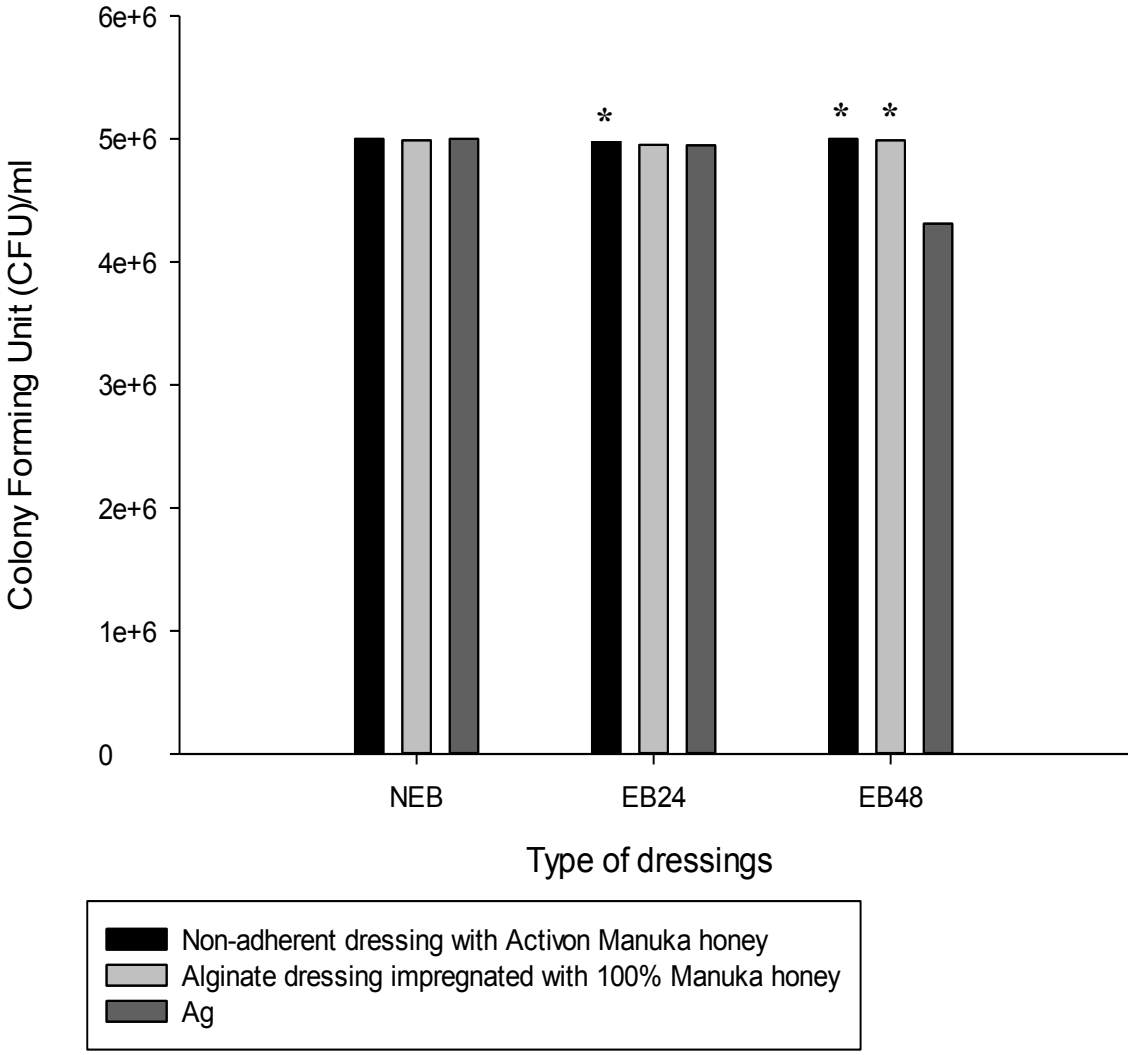
Appendix Figure 24. The effect of *Nigella sativa* on the formation of the biofilm of *Ps. aeruginosa*



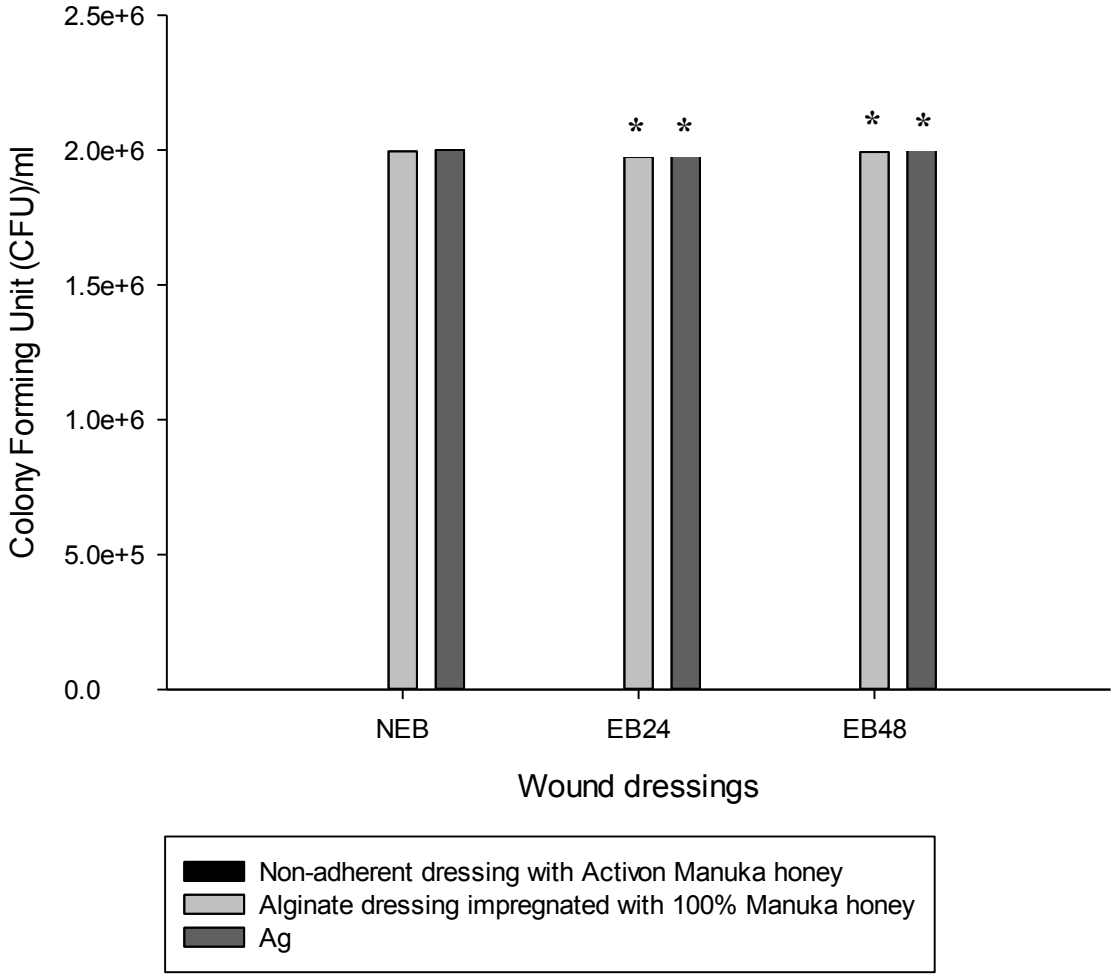
Appendix Figure 25. The effect of oak honey on the formation of the biofilm of *Ps. aeruginosa*



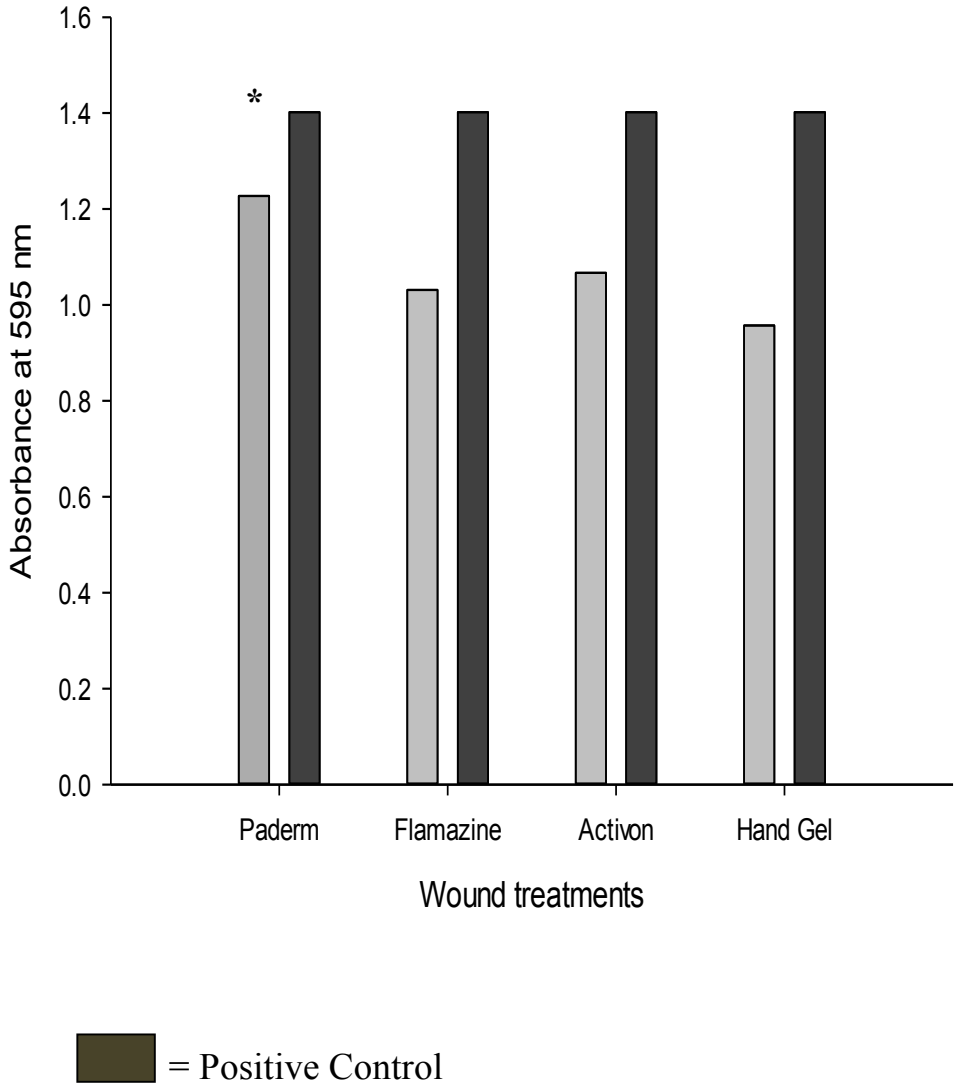
Appendix Figure 26. The effect of Rain Forest +30 honey on the formation of the biofilm of *Ps. aeruginosa*



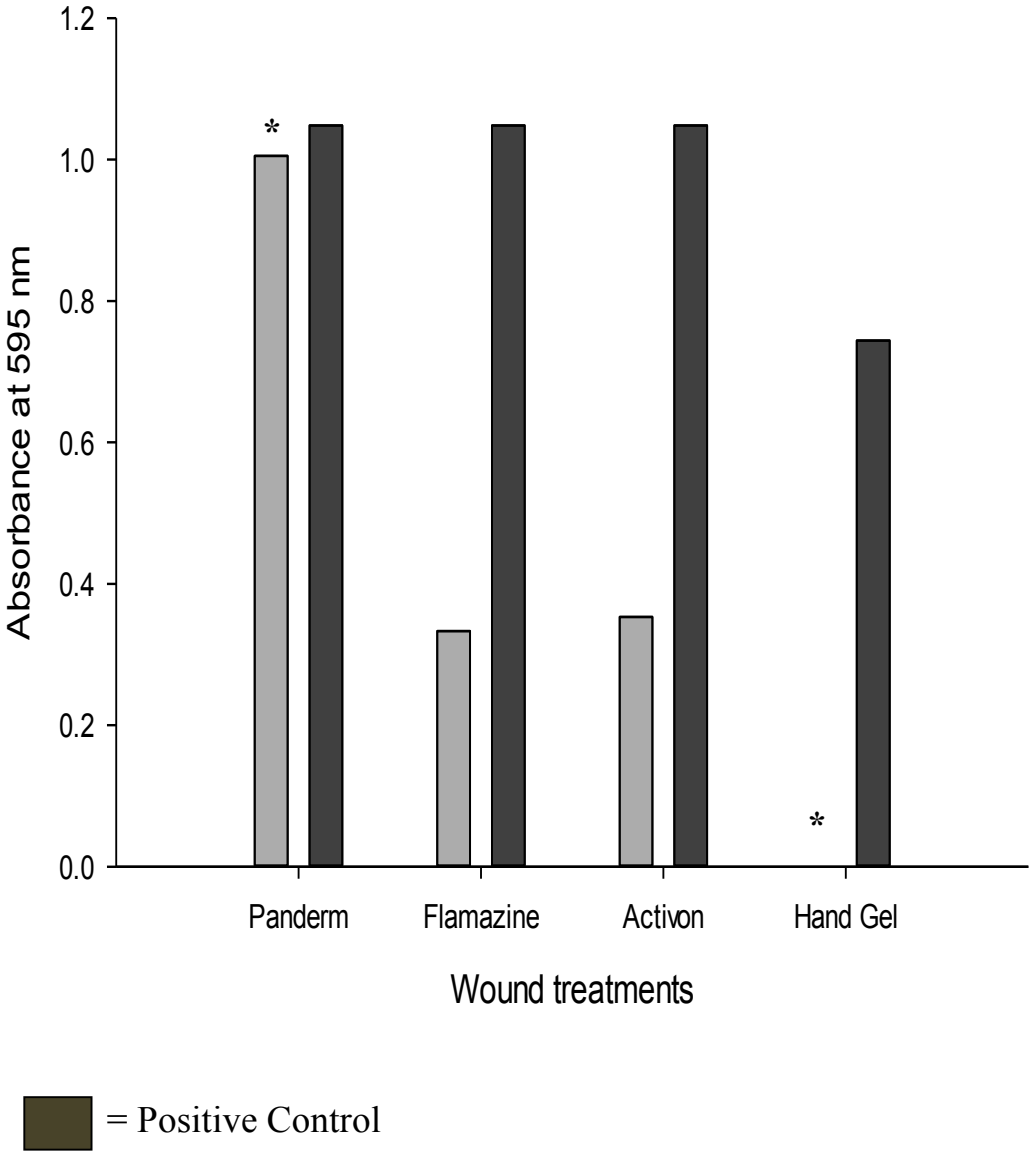
Appendix Figure 27.The activity of some dressings on the biofilm of *S. aureus* at different cases (NEB= Non Established Biofilm) (EB24= Established Biofilm after 24 hrs) (EB48= Established Biofilm after 48 hrs) (* Significant differences)



Appendix Figure 28. The activity of some dressings on the biofilm of *Ps. aeruginosa* at different cases (NEB= Non Established Biofilm) (EB24= Established Biofilm after 24 hrs) (EB48= Established Biofilm after 48 hrs) (* Significant differences)



Appendix figure 29. The activity of some wound treatments on the biofilm of *S. aureus* (* Significant differences)



Appendix Figure 30. The activity of some wound treatments on the biofilm of *Ps. aeruginosa* (* Significant differences)

Appendix statistical analysis.

Appendix statistical analysis 1. Raw and Autoclaved honey at 100% on *S. aureus*

Data source: Data 3 in Raw Honey AND Autoclaved SA 100%

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test

Data source: Data 3 in Raw Honey AND Autoclaved SA 100%

Group	**N**	**Missing**	**Median**	**25%**	**75%**
Col 5	5	0	19.333	19.167	22.500
Col 6	5	0	15.667	15.500	15.833

Mann-Whitney U Statistic= 0.000

T = 40.000 n(small)= 5 n(big)= 5 P(est.)= 0.011 P(exact)= 0.008

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.008)

Appendix statistical analysis 2. The action of Raw Honey AND Autoclavedwith S. aureus at 80%

Data source: Data 3 in Activity OF Raw Honey AND Autoclaved ON S. aureus AT 80%

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test Sunday, February 16, 2014, 15:08:00

Data source: Data 3 in Activity OF Raw Honey AND Autoclaved ON S. aureus AT 80%

Group	N	Missing	Median	25%	75%
Col 6	5	0	18.667	18.000	22.667
Col 7	5	0	14.333	13.500	14.500

Mann-Whitney U Statistic= 0.000

T = 40.000 n(small)= 5 n(big)= 5 P(est.)= 0.012 P(exact)= 0.008

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.008)

Appendix statistical analysis 3. Raw and Autoclaved honey on Ps. aeruginosaat 100%

Data source: Data 3 in Activity OF Raw Honey AND Autoclaved on Ps. aeruginosa 100%

Normality Test (Shapiro-Wilk) Passed (P = 0.412)

Equal Variance Test: Passed (P = 0.799)

Group Name	N	Missing	Mean	Std Dev	SEM
Col 1	5	0	13.333	1.900	0.850
Col 2	5	0	11.400	2.216	0.991

Difference 1.933

t = 1.481 with 8 degrees of freedom. (P = 0.177)

95 percent confidence interval for difference of means: -1.077 to 4.944

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.177).

Power of performed test with alpha = 0.050: 0.154

The power of the performed test (0.154) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Appendix statistical analysis 4. Raw and Autoclaved honey at 80% with Ps.aeruginosa

Data source: Data 3 in Activity OF Raw Honey AND Autoclaved on ***Ps. aeruginosa***at 80%

Normality Test (Shapiro-Wilk) Passed (P = 0.984)

Equal Variance Test: Passed (P = 0.465)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Col 1	5	0	11.667	2.809	1.256
Col 2	5	0	10.533	1.709	0.764

Difference 1.133

t = 0.771 with 8 degrees of freedom. (P = 0.463)

95 percent confidence interval for difference of means: -2.258 to 4.524

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.463).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.

Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Appendix statistical analysis 5.Raw an Autoclaved Manuka +20 at 100% with S. aureus

Data source: Data 4 in Activity OF Raw Honey AND Autoclaved on SA 100%

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	25.000	0.577	0.333
Row 2	3	0	15.667	0.333	0.192

Source of Variation	DF	SS	MS	F	P
Between Groups	1	130.667	130.667	588.000	<0.001
Residual	4	0.889	0.222		
Total	5	131.556			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 1 vs. Row 2	9.333	24.249	<0.001	Yes

Appendix statistical analysis 6. Raw and Autoclaved Manuka +20 honey on *S. aureus* at 80%

Data source: Data 4 in Activity OF Raw Honey AND Autoclaved on *S. aureus* AT 80%

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	24.667	1.202	0.694
Row 2	3	0	13.667	0.882	0.509

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	1	181.500	181.500	163.350	<0.001
Residual	4	4.444	1.111		
Total	5	185.944			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 1 vs. Row 2	11.000	12.781	<0.001	Yes

Appendix statistical analysis 7. The activity of Raw and Autoclaved Rainforest +30 on *Ps. aeruginosa*at 100%

Data source: Data 4 in Activity OF Raw Honey AND Autoclaved on *Ps. aeruginosa*AT 100%

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	15.667	0.333	0.192
Row 2	3	0	10.667	0.333	0.192

Source of Variation	**DF**	**SS**	**MS**	**FP**
Between Groups	1	37.500	37.500	337.500 <0.001
Residual	4	0.444	0.111	
Total	5	37.944		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 1 vs. Row2	5.000	18.371	<0.001	Yes

Appendix statistical analysis 8. Raw and Autoclaved Rainforest +30 honey on *Ps. aeruginosa*at 80%

Data source: Data 4 in Activity OF Raw Honey AND Autoclaved on *Ps. aeruginosa*AT 80%

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	15.333	0.333	0.192
Row 2	3	0	10.333	0.333	0.192

Source of Variation	DF	SS	MS	FP
Between Groups	1	37.500	37.500	337.500 <0.001
Residual	4	0.444	0.111	
Total	5	37.944		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 1 vs. Row 2	5.000	18.371	<0.001	Yes

Appendix statistical analysis 9.The activity of some dressings on the planktonic of *S. aureus*

One Way Analysis of Variance

Data source: Data 2 in the activity of some dressings on *S. aureus*

Group Name	N	Missing	Mean	Std Dev	SEM
Row 1	3	0	18.000	0.000	0.000
Row 2	3	0	31.667	3.786	2.186
Row 3	3	0	25.333	1.155	0.667
Row 4	3	0	19.000	1.000	0.577

Source of Variation	DF	SS	MS	FP
Between Groups	3	361.667	120.556	28.933	<0.001
Residual	8	33.333	4.167		
Total	11	395.000			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 2 vs. Row 1	13.667	8.200	<0.001	Yes
Row 2 vs. Row 4	12.667	7.600	<0.001	Yes
Row 3 vs. Row 1	7.333	4.400	0.009	Yes
Row 2 vs. Row 3	6.333	3.800	0.016	Yes

Row 3 vs. Row 4	6.333	3.800	0.010	Yes
Row 4 vs. Row 1	1.000	0.600	0.565	No

Appendix statistical analysis 10.The effect of some dressings on the planktonic of *Ps. aeruginosa*

One Way Analysis of Variance

Data source: Data 2. The activity of some dressings on ***Ps. aeruginosa***

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	24.000	1.000	0.577
Row 2	3	0	15.000	0.000	0.000
Row 3	3	0	17.000	1.000	0.577
Row 4	3	0	14.667	1.528	0.882

Source of Variation	**DF**	**SS**	**MS**	**FP**
Between Groups	3	170.000	56.667	52.308	<0.001
Residual	8	8.667	1.083		
Total	11	178.667			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 1 vs. Row 4	9.333	10.983	<0.001	Yes
Row 1 vs. Row 2	9.000	10.590	<0.001	Yes
Row 1 vs. Row 3	7.000	8.237	<0.001	Yes
Row 3 vs. Row 4	2.333	2.746	0.074	No
Row 3 vs. Row 2	2.000	2.353	0.091	No
Row 2 vs. Row 4	0.333	0.392	0.705	No

Appendix statistical analysis 11.Comparison between different wound treatments on the planktonic of *S. aureus*

One Way Analysis of Variance

Data source: Data 2. The activity of some wound treatments on *S. aureus*

Group Name	N	Missing	Mean	Std.Dev	SEM
Row 1	3	0	25.000	0.000	0.000
Row 2	3	0	18.667	0.577	0.333
Row 3	3	0	11.000	1.732	1.000
Row 4	3	0	14.000	0.000	0.000

Source of Variation	DF	SS	MS	FP
Between Groups	3	335.000	111.667	134.000	<0.001
Residual	8	6.667	0.833		
Total	11	341.667			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050	
Row 1 vs. Row 3	14.000	18.783	<0.001	Yes	
Row 1 vs. Row 4	11.000	14.758	<0.001	Yes	
Row 2 vs. Row 3	7.667	10.286	<0.001	Yes	
Row 1 vs. Row 2	6.333	8.497	<0.001	Yes	
Row 2 vs. Row 4	4.667	6.261	<0.001	Yes	
Row 4 vs. Row 3	3.000	4.025	0.004	Yes	

Appendix statistical analysis 12.The activity of some wound treatments on the planktonic of *Ps.aeruginosa*

One Way Analysis of Variance

Data source: Data 2 .The activity of some wound treatments on***Ps. aeruginosa***

Group Name	N	Missing	Mean	Std.Dev	SEM
Row 1	3	0	25.333	0.577	0.333
Row 2	3	0	12.333	0.577	0.333
Row 3	3	0	13.667	1.528	0.882
Row 4	3	0	16.667	1.155	0.667

Source of Variation	DF	SS	MS	FP
Between Groups	3	307.333	102.444	94.564	<0.001
Residual	8	8.667	1.083		
Total	11	316.000			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 1 vs. Row 2	13.000	15.297	<0.001	Yes
Row 1 vs. Row 3	11.667	13.728	<0.001	Yes
Row 1 vs. Row 4	8.667	10.198	<0.001	Yes
Row 4 vs. Row 2	4.333	5.099	0.003	Yes
Row 4 vs. Row 3	3.000	3.530	0.015	Yes
Row 3 vs. Row 2	1.333	1.569	0.155	No

Appendix statistical analysis 13.The comparison between different types of dressings on the ready biofilm for 24 hrs of *S. aureus*

One Way Analysis of Variance

Data source: Data 3 in Notebook1

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	4997626.667	323.316	186.667
Row 2	3	0	4951000.000	20223.748	11676.187
Row 3	3	0	4948333.333	9073.772	5238.745

Source of Variation	**DF**	**SS**	**MS**	**FP**
Between Groups	2	4610989876.563	2305494938.281	14.074	0.005

Residual	6	982875731.151	163812621.858
Total	8	5593865607.713	

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005).

Power of performed test with alpha = 0.050: 0.946

All Pair wise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 1 vs. Row 3	49293.333	4.717	0.010	Yes
Row 1 vs. Row 2	46626.667	4.462	0.009	Yes
Row 2 vs. Row 3	2666.667	0.255	0.807	No

Appendix statistical analysis 14.The comparison between different types of dressings on the ready biofilm for 48 hrs of *S. aureus*

One Way Analysis of Variance

Data source: Data 4 in Notebook1

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	4999296.667	300.389	173.429
Row 2	3	0	4988166.667	4517.005	2607.894
Row 3	3	0	4310000.000	65574.385	37859.389

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	2	935163799446.688	467581899723.344	324.673	<0.001
Residual	6	8640987121.871	1440164520.312		
Total	8	943804786568.559			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 1 vs. Row 3	689296.667	22.246	<0.001	Yes
Row 2 vs. Row 3	678166.667	21.886	<0.001	Yes
Row 1 vs. Row 2	11130.000	0.359	0.732	No

Appendix statistical analysis 15.Comparison between different types of dressings on non ready biofilm of *Ps. aeruginosa*

One Way Analysis of Variance

Data source: Data 3 **in *Ps. aeruginosa***biofilm dressings

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	12.000	2.000	1.155
Row 2	3	0	1995000.000	4618.802	2666.667
Row 3	3	0	1999992.000	2.000	1.155

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	2	7.980E+012	3.990E+012	561088.047	<0.001
Residual	6	42666683.525	7111113.921		
Total	8	7.980E+012			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 3 vs. Row1	1999980.000	918.549	<0.001	Yes
Row 2 vs. Row1	1994988.000	916.257	<0.001	Yes
Row 3 vs. Row2	4992.000	2.293	0.062	No

Appendix statistical analysis 16.Comparison between different types of dressings on ready biofilm for 24 hrs of *Ps. aeruginosa*

One Way Analysis of Variance

Data source: Data 4 in ***Ps. aeruginosa*** biofilm dressings

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	15.000	2.000	1.155
Row 2	3	0	1975000.000	5000.000	2886.751
Row 3	3	0	1999997.000	1.000	0.577

Source of Variation	**DF**	**SS**	**MS**	**FP**
Between Groups	2	7.901E+012	3.951E+012	2474067.021 <0.001
Residual	6	50000010.000	8333335.000	
Total	8	7.901E+012		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 3 vs. Row1	1999982.000	848.520	<0.001	Yes
Row 2 vs. Row1	1974985.000	837.915	<0.001	Yes
Row 3 vs. Row2	24997.000	10.605	<0.001	Yes

Appendix statistical analysis 17.Comparison between different types of dressings on ready biofilm for 48 hrs of *Ps. aeruginosa*

One Way Analysis of Variance

Data source: Data 5 in ***Ps. aeruginosa***biofilm dressings

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	11.000	2.000	1.155
Row 2	3	0	1992000.000	2516.612	1452.966
Row 3	3	0	1999991.000	1.500	0.866

Source of Variation	**DF**	**SS**	**MS**	**FP**
Between Groups	2	7.968E+012	3.984E+012	1887156.955 <0.001
Residual	6	12666679.384	2111113.231	
Total	8	7.968E+012		

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 3 vs. Row1	1999980.000	168	5.837	<0.001 Yes
Row 2 vs. Row1	1991989.000	167	9.101	<0.001 Yes
Row 3 vs. Row 2	7991.000	6.736		<0.001 Yes

Appendix statistical analysis 18.Comparison between different wound treatments on the biofilm of *S. aureus*

One Way Analysis of Variance

Data source: Data 3 the effect of some wound treatments on the biofilm of *S. aureus*

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	0.125	0.128	0.0737
Row 2	3	0	0.371	0.0943	0.0545
Row 3	3	0	0.335	0.0118	0.00684
Row 4	3	0	0.445	0.0690	0.0398

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	3	0.170	0.0566	7.523	0.010
Residual	8	0.0602	0.00752		

Total 11 0.230

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.010).

Power of performed test with alpha = 0.050: 0.839

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Row 4 vs. Row 1	0.320	4.524	0.012	Yes
Row 2 vs. Row 1	0.246	3.474	0.041	Yes
Row 3 vs. Row 1	0.211	2.975	0.069	No
Row 4 vs. Row 3	0.110	1.549	0.407	No
Row 4 vs. Row 2	0.0743	1.050	0.544	No
Row 2 vs. Row 3	0.0353	0.499	0.631	No

Appendix statistical analysis 19.Comparison between different wound treatments on the biofilm of *Ps. aeruginosa*

One Way Analysis of Variance

Data source: Data 2 in The effect of some wound treatments on the biofilm of *Pseudomonas aeruginosa*

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Row 1	3	0	1.005	0.00115	0.000667
Row 2	3	0	0.333	0.0156	0.00902
Row 3	3	0	0.353	0.247	0.143
Row 4	3	0	0.000	0.0226	0.0131

Source of Variation	DF	SS	MS	F	P
Between Groups	3	1.592	0.531	34.316	<0.001
Residual	8	0.124	0.0155		
Total	11	1.716			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Row 1 vs. Row 4	1.005	9.898	<0.001	Yes
Row 1 vs. Row 2	0.672	6.619	<0.001	Yes
Row 1 vs. Row 3	0.652	6.422	<0.001	Yes
Row 3 vs. Row 4	0.353	3.477	0.025	Yes
Row 2 vs. Row 4	0.333	3.280	0.022	Yes
Row 3 vs. Row 2	0.0200	0.197	0.849	No

Appendix isolates. 16s rRNA gene sequences

Appendix isolates 1. Isolate of Oak honey:

Description	Max Score	Total Score	Query coverage	E Value	Max ID
Lysinibacillus fusiformis strain DD17 16S ribosomal RNA gene, partial sequence	649	649	100%	0	100%

Lysinibacillus fusiformis strain DD17 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KC689296.1| Length: 787 Number of Matches: 1

Range 1: 43 to 422 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
702 bits(380)	0.0	380/380(100%)	0/380(0%)	Plus/Plus

```
Query  1    GCAAGTCGAGCGAACAGAGAAGGAGCTTGCTCCTTCGACGTTAGCGGCGGACGGGTGAGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  43   GCAAGTCGAGCGAACAGAGAAGGAGCTTGCTCCTTCGACGTTAGCGGCGGACGGGTGAGT  102

Query  61   AACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  103  AACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCG  162

Query  121  AATAATCTGTTTCACCTCATGGTGAAACACTGAAAGACGGTTTCGGCTGTCGCTATAGGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  163  AATAATCTGTTTCACCTCATGGTGAAACACTGAAAGACGGTTTCGGCTGTCGCTATAGGA  222

Query  181  TGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  223  TGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTA  282

Query  241  GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  283  GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG  342

Query  301  AGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  343  AGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAG  402

Query  361  TGAAGAAGGATTTCGGTTCG  380
            ||||||||||||||||||||
Sbjct  403  TGAAGAAGGATTTCGGTTCG  422
```

Appendix isolates 2. Isolate from *Nigella sativa* honey

Description	Max score	Total Score	Query coverage	E Value	Max Id
Staphylococcus epidermidis strain Fussel 16S ribosomal RNA, partial sequence	676	676	100%	0.0	99%

>ref|NR_036904.1| Staphylococcus epidermidis strain Fussel 16S ribosomal RNA, partial sequence
Length=1475

Score = 676 bits (366), Expect = 0.0
Identities = 367/368 (99%), Gaps = 0/368 (0%)
Strand=Plus/Plus

```
Query  1    CCTAATACATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  22   CCTAATACATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGG  81

Query  61   ACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  82   ACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGA  141

Query  121  GCTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  142  GCTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTC  201

Query  181  ACTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  202  ACTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAA  261

Query  241  CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGAC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262  CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGAC  321

Query  301  TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACN  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322  TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACG  381

Query  361  CCGCGTGA  368
            ||||||||
Sbjct  382  CCGCGTGA  389
```

Appendix isolates 3. Isolate from Manuka honey

Description	Max Score	Total Score	Query coverage	E Value	Max ID
Sporosarcina koreensis strain APT41 16S ribosomal RNA gene, partial sequence	342	342	100%	5e-91	100%

Sporosarcina koreensis strain APT41 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KC519413.1| Length: 1437 Number of Matches: 1

Range 1: 1242 to 1426 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
342 bits(185)	5e-91	185/185(100%)	0/185(0%)	Plus/Minus

```
Query  1     CCTTCGGCGGCTGGCTCCCGTAAGGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1426  CCTTCGGCGGCTGGCTCCCGTAAGGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCG  1367

Query  61    TGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTGGCATGCTGATCCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1366  TGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTGGCATGCTGATCCA  1307

Query  121   CGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGGGAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1306  CGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGGGAA  1247

Query  181   CGGTT  185
             |||||
Sbjct  1246  CGGTT  1242
```

Appendix isolates 4. Isolate T1

Description	Max score	Total Score	Query coverage	E Value	Max ID
Streptococcus mitis strain NS51 16S ribosomal RNA, complete sequence.	616	616	100%	1e-176	99%

Streptococcus mitis strain NS51 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_028664.1| Length: 1520 Number of Matches: 1

Range 1: 1111 to 1446 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
616 bits(333)	1e-176	335/336(99%)	0/336(0%)	Plus/Minus

```
Query  1     TACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1446  TACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGC  1387

Query  61    CCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1386  CCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGT  1327

Query  121   AGGCGAGTTGCAGCCTACAATCCGAACTGAGACTGGCTTTAAGAGATTAGCTTGCCGTCA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1326  AGGCGAGTTGCAGCCTACAATCCGAACTGAGACTGGCTTTAAGAGATTAGCTTGCCGTCA  1267

Query  181   CCGGCTTGCGACTCGTTGTACCAGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1266  CCGGCTTGCGACTCGTTGTACCAGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGG  1207

Query  241   CATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1206  CATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAG  1147

Query  301   TGCCCAACTGAATGATGGCAACTAACAATAGGGGTT  336
             ||||||||| ||||||||||||||||||||||||||
Sbjct  1146  TGCCCAACTAAATGATGGCAACTAACAATAGGGGTT  1111
```

Appendix isolates 5. Isolate T2

Description	Max Score	Total Score	Query coverage	E Value	Max ID
Streptococcus gordonii str. Challis substr. CH1 strain Challis 16S ribosomal RNA, complete sequence	785	785	100%	0	100%

Streptococcus gordonii str. Challis substr. CH1 strain Challis 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074516.1| Length: 1510 Number of Matches: 1

Range 1: 1025 to 1449 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
785 bits(425)	0.0	425/425(100%)	0/425(0%)	Plus/Minus

```
Query  1     TACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1449  TACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGC  1390

Query  61    CCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1389  CCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGT  1330

Query  121   AGGCGAGTTGCAGCCTACAATCCGAACTGAGACTGGCTTTAAGAGATTAGCTTGCCGTCA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1329  AGGCGAGTTGCAGCCTACAATCCGAACTGAGACTGGCTTTAAGAGATTAGCTTGCCGTCA  1270

Query  181   CCGACTTGCGACTCGTTGTACCAGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269  CCGACTTGCGACTCGTTGTACCAGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGG  1210

Query  241   CATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1209  CATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAG  1150

Query  301   TGCCCAACTGAATGATGGCAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1149  TGCCCAACTGAATGATGGCAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCA  1090

Query  361   ACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGATGTACCGAAGTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1089  ACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGATGTACCGAAGTA  1030

Query  421   AAACT  425
             |||||
Sbjct  1029  AAACT  1025
```

Appendix isolates 6. Isolate T4

Description	Max Score	Total Score	Query coverage	E Value	Max ID
Neisseria mucosa strain 5567a 16S ribosomal RNA gene, partial sequence	941	941	100%	0	100%

Neisseria mucosa strain 5567a 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KC178491.1| Length: 1406 Number of Matches: 1

Range 1: 19 to 527 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
941 bits(509)	0.0	509/509(100%)	0/509(0%)	Plus/Plus

```
Query  1    CAGAGAAGCTTGCTTCTTGGGTGGCGAGTGGCGAACGGGTGAGTAACATATCGGAACGTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19   CAGAGAAGCTTGCTTCTTGGGTGGCGAGTGGCGAACGGGTGAGTAACATATCGGAACGTA  78

Query  61   CCGAGTAATGGGGGATAACTAATCGAAAGATTAGCTAATACCGCATATTCTCTGAGGAGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   CCGAGTAATGGGGGATAACTAATCGAAAGATTAGCTAATACCGCATATTCTCTGAGGAGG  138

Query  121  AAAGCAGGGGACCTTCGGGCCTTGCGTTATTCGAGCGGCCGATATCTGATTAGCTAGTTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  AAAGCAGGGGACCTTCGGGCCTTGCGTTATTCGAGCGGCCGATATCTGATTAGCTAGTTG  198

Query  181  GTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCGGGTCTGAGAGGATGATCCGCCAC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  GTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCGGGTCTGAGAGGATGATCCGCCAC  258

Query  241  ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAA  318

Query  301  TGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGCCTTCGGGTTGTAAAGG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  TGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTCTGAAGAAGGCCTTCGGGTTGTAAAGG  378

Query  361  ACTTTTGTCAGGGAAGAAAAGGCTGTTGCTAATACCGACAGCTGATGACGGTACCTGAAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  ACTTTTGTCAGGGAAGAAAAGGCTGTTGCTAATACCGACAGCTGATGACGGTACCTGAAG  438

Query  421  AATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439  AATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAA  498

Query  481  TCGGAATTACTGGGCGTAAAGCGAGCGCA  509
            ||||||||||||||||||||||||||||
Sbjct  499  TCGGAATTACTGGGCGTAAAGCGAGCGCA  527
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