A CONTRIBUTION TO THE STUDY OF BIOCONTROL AGENTS APITHERAPY AND OTHER POTENTIAL ALTERNATIVES TO ANTIBIOTICS

By

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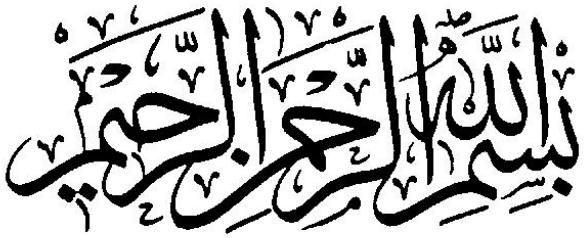

Dedication

This thesis is dedicated to my parents, to the soul of my aunt Malihah, to my wonderful wife Rasha and to my children, Islam and Afnan.

Acknowledgments

Many thanks to Almighty Allah, who blessed me with health, courage, enthusiasm and the determination which enabled me to carry out and conclude this thesis. I am indebted to my supervisor Prof. Milton Wainwright for his invaluable support, comments, encouragement and guidance. I would also like to thank Prof. D. P. Hornby for making the facilities of the Department available to me. My deepest thanks go to Dr. D. J. Gilmour and Prof. Jeff Green, for their advice during my study. Appreciation also goes out to Mr. Chris Hill for his assistance in electron microscopy experiments. Also, I wish to thank all my friends in Prof Wainwright's laboratory, particularly for my best friends Khalid and Reda for our exchanges of knowledge and skills and for their help and encouragement. My special thanks are due to my beloved parents, brothers, and sisters in Jordan and my wife Rasha for their patience, support, continuous encouragement and enthusiasm throughout the period of my study. Finally, I should like to thank my sponsor, Al-Hussein Bin Talal University, for all its financial support that enabled me to carry out this research.

Abstract

We are living in what has been termed the "post antibiotics era' where the emergence of multidrug-resistant pathogen strains is rapidly growing and where the introduction of and the development of novel antimicrobial agents, which are effective against these pathogens has declined significantly. As a result, we urgently need effective alternatives to the use of antibiotics. The overall aim of the study was to add further our knowledge of some of these alternatives to antibiotics, concentrating on maggot therapy, apitherapy and to a lesser extent mycotherapy.

The nature of the exceptionally high non-peroxide antibacterial activity of *Leptospermum scoparium* Manuka honey was further investigated. It was shown that this activity is unlikely to be due to accumulated residual hydrogen peroxide and the microflora of bees and honey probably act as a source of any non–hydrogen peroxide inhibitory components. Tregothnan English Manuka did not show any exceptionally high non-peroxide antibacterial activity compared to New Zealand Manuka.

Studies on the mechanisms of the antibacterial activity of high medical grade New Zealand Manuka (UMF 20+,25+) against methicillin-resistant *Staphylococcus aureus* (MRSA) and (SEM) and TEM studies on the morphological effects on (MRSA) and *Escherichia coli* were conducted. It was observed that Gram-positive and Gram-negative cells were not uniformly affected by Manuka honey, Gram-positive bacteria were more sensitive and had less MICs and MBCs values. Time killing assays confirmed the bactericidal effect of Manuka. 16S rRNA sequencing technique confirmed that that *C. botulinum* spores are not likely to be detectable in tested medical grade New Zealand Manuka honey samples. *Leptospermum scoparium* essential oil showed a marked inhibitory activity against Gram-positive bacteria, including MRSA, *M. phlei* and *B. subtilis;*significant antifungal activity against *Trichophyton terrestre* was also observed.

The *in vitro* antibacterial activity of tamarind (*Tamarindus indica*) was evaluated and compared with 25+ Manuka honey. Data obtained from the agar diffusion, MICs, MBCs values and time to kill study show that tamarind exhibits a stronger antimicrobial effect against the six tested bacteria and *C. albicans* than do high medical grade manuka honeys. It is suggested that an autoclaved, sterilized, commercially available tamarind paste should be clinically evaluated for the treatment of wounds and indolent ulcers which are infected with bacteria, notably MRSA.

In relation to maggot therapy, evidence is provided to support the anti-*Mycobacterium* activity of *Calliphora quadrimaculat*a maggot gases; unfortunately, attempts to isolate these gases were unsuccessful. For first time, *in vitro* evidence (such as antifungal and yeast (*S. cerevisiae* (BY4742) GFP ingestion ability), was provided to support the use of *Lucilia sericata* maggot to control fungi involved in superficial fungal infections, i.e. fungal burn wound infections and dermatomycoses. A study was made to determine if Mycoplasmas can be detected in the hemolymph of maggots of *Calliphora quadrimaculata* and *Lucilia sericata* maggots by using *Mycoplasma*, EZ-PCR Mycoplasma Test Kit. Mycoplasmas were not detected in any of the maggot samples tested. These observations suggest that *Calliphora quadrimaculata* and *Lucilia sericata* larvae are unlikely to act as transmission vectors for Mycoplasmas.

Another antibiotic-alternative called mycotherapy (an approach which as yet has not be reinstated in medicine) was also studied. The results presented here show that mycelium of all *Penicillium* cultures, isolated from foods, and inhibited both MSSA and MRSA. SNAP* MRL Beta-Lactam Kit has been used to test for penicillin and TLC for patulin showed that penicillin was found in culture filtrates obtained from all of the individual fungi, while patulin was produced by only two samples. Clearly then, a pure culture of a *Penicillium*, known to produce penicillin and not patulin or other toxicant (like the two IMI strains used here) could be safely used to treat wound infections in hospitals at the present time.

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Chapter one: Introduction

1. Introduction

From the mid-1940s until relatively recently we have been fortunate to be living through what has been termed "the Golden Age of Antibiotics". During this period all of the major infections caused by bacteria and fungi have been defeated by the use of antibiotics, a period which has also seen dramatic improvements in surgical procedures which have revolutionised medicine and saved countless lives (Wainwright, 1990). Unfortunately this golden age is now at end due to the development of antibiotic resistance amongst pathogens. Such resistance was noticed immediately after the introduction of penicillin and has grown worse over the last half of this century or so to the point where some hospitals have only one antibiotic, vancomycin, available for the treatment of bacterial infections and resistance to this, "last hope" antibiotic, has been reported. The development of antibiotic resistance is particularly problematic in the case of MRSA (Methicillin Resistant *Staphylococcus aureus*), which is endemic in most hospitals worldwide (Boyce *et al*., 2005).

The problem of antibiotic resistance can be mitigated to some extent by better prescribing practises and the banning of the use of these compounds in agriculture. However, the reality is that antibiotic resistance will continue to remain a major problem in future medicine. The obvious way around this problem is to develop new antibiotics which pathogens have not previously seen and therefore have not had time to develop resistance (Mulvey and Simor (2009). Unfortunately the economics and exceptionally high regulatory hurdles presented to pharmaceutical companies wishing to develop new antibiotics are so adverse that no new antibiotics are likely to be produced until the situation becomes so grave that international bodies, such as the UN, demand that new antibiotic compounds be developed for world-wide use. As a result of this quandary clinicians around the world are seeking alternatives to antibiotics for use in the treatment of bacterial and fungal infections. It might be imagined that high-tech

solutions, such as the use of molecular vaccines or gene therapy might have been developed to overcome the problem of antibiotic resistance. In reality, although vast amounts of research monies have been devoted to such sophisticated approaches they are not currently medically useful and clinicians are having to resort to a number of more basic and less sophisticated approaches to pathogen control, especially in relation to the treatment of surface wound infections. Such longstanding, or indolent infections, present a major problem when caused by antibiotic resistant bacteria and may, notably in older patients suffering from diabetes, cause longstanding, painful and long-term suppurating wounds, which if untreated may lead to limb amputation (Boyce *et al*.,2005). The relatively unsophisticated approaches which are now being reintroduced into medicine were in use before the advent of antibiotics and were shown to be useful in medicine, and in some case proved to be highly successful. When antibiotics appeared however, such approaches were naturally seen to be old-fashioned and otherwise undesirable and most became historical oddities which had no place in modern, ”scientific medicine”. Such old-fashioned approaches which are currently being re-evaluated, or are currently being used, in medicine include: Bacteriophage Therapy, Apitherapy (Honey Therapy), Maggot Debridement Therapy and Mycotherapy.

1.1 A brief history of antibiotics

Although microbial antagonism, i.e. the ability of one microorganism to limit the growth, or kill, another, was observed many times during the late Victorian period, the discovery of the first true antibiotic dates to 1928 when Alexander Fleming observed the antibacterial effect of a *Penicillium* mould against *Staphylococcus aureus* (Wainwright, 2008). Despite this discovery, penicillin was not made available as the first true antibiotic until the mid-1940s (Projan and Shlaes, 2004). The true chemotherapeutic era had already begun when protonsil (early synthetic sulphonamide

drug) was discovered in 1932 (Domagk, 1957) and then introduced into medical use in 1935. Sulphonamides were successfully used to cure meningitis, streptococcal infections and infections of the urinary tract, but were less active in the treatment of the Staphylococcal infections (Brumfitt and Hamilton-Miller, 1988). During the 1940s many researchers started to focus on the search for antibiotics produced by soil organisms. One of these researchers was Selman Waksman who, with Albert Schatz, reported the isolation of the first aminoglycoside antibiotics streptomycin (from *Streptomyces griseus*) in 1943. Streptomycin was the first antibiotic to be effective against tuberculosis (TB) and despite producing side effects, including kidney damage and deafness, streptomycin is still being used to treat this disease (McKenna, 1998). Chloramphenicol was subsequently used to treat typhus and subsequently typhoid fever (McKenna, 1998), but its use started to decline in 1960 due to its ability to induce aplastic anaemia, bone marrow suppression and the gray syndrome (Feder, 1986). Based on the detailed examination of the antibacterial substances isolated from a strain of *Cephalosporium acremonium* by Giuseppe Brotzu in the mid 1940s, Florey and his co-workers in Oxford discovered the cephalosporin family of β-lactams which were very active against a wide spectrum of bacterial infections with a very low toxicity (McKenna, 1998; Brown, 1987). Comprehensive investigations during the mid 1950's then led to the recognition and isolation of penicillin 'nucleus' (6-aminopenicillanic acid) (6-APA), a penicillin precursor produced during the fermentation of *P.chrysogenum*. The isolation of (6-APA) paved the way for a large-scale production and marketing of semi-synthetic penicillins during the 1960s, including ampicillin, methicillin, flucloxacillin, amoxycillin, ticarcillin and carbenicillin, followed later by mezlocillin, azlocillin, piperacillin and mecillinam (Brown, 1987; Brumfitt and Hamilton-Miller, 1988). Chlortetracycline was the first member of the tetracycline

group to be isolated by Benjamin M. Duggar in 1947; oxytetracycline then soon followed (McKenna, 1998; Chopra and Roberts, 2001).

Tetracyclines are the second most commonly used antibiotics after the penicillins due their activity against a long list of infections and because they are relatively cheap to produce (McKenna, 1998). The 1970s, which witnessed an increase in the resistance among Gram-negative bacilli, saw the introduction of amikacin, a semisynthetic derivative of kanamycin.(Brumfitt and Hamilton-Miller, 1988; Begg and Barclay, 1995)

In 1962 Lesher and his co-workers recognized nalidixic acid, a by-product of chloroquine production (Barlow, 1963), which became the first quinolone antibiotic to be developed. The first-generation quinolones were active against aerobic Gram-negative bacillary infections, especially those found in the human urinary tract. This limited activity against aerobic Gram-negative bacteria was enhanced (1000-fold) in the 1980s when the second-generation fluoroquinolones were introduced (Dembry *et al*., 1999).

1.2 Commonly occurring sites of antibiotics action

Selective toxicity is considered as the main basis of all antimicrobial chemotherapy, in short the pathogen must be killed but not the human host. (Finch, 2003).The peptidoglycan layer of Gram-positive bacteria is one such selective target, others include compounds which are selective against other bacterial cell macromolecules and which can interfere with protein synthesis, DNA replication and transcription and folic acid metabolism (Figure 1.1) (Lambert, 2005). For example, β-lactams and glycopeptides, such as vancomycin, exert an antibacterial effect by inhibiting formation of the bacterial cell wall peptidoglycan layers. Macrolides, aminoglycosides, chloramphenicol, and tetracyclines interfere with protein synthesis, sulfonamides inhibit DNA synthesis by interfering with the pathway for folic acid synthesis, and

fluoroquinolones target components which are essential for DNA synthesis (Tenover, 2006).

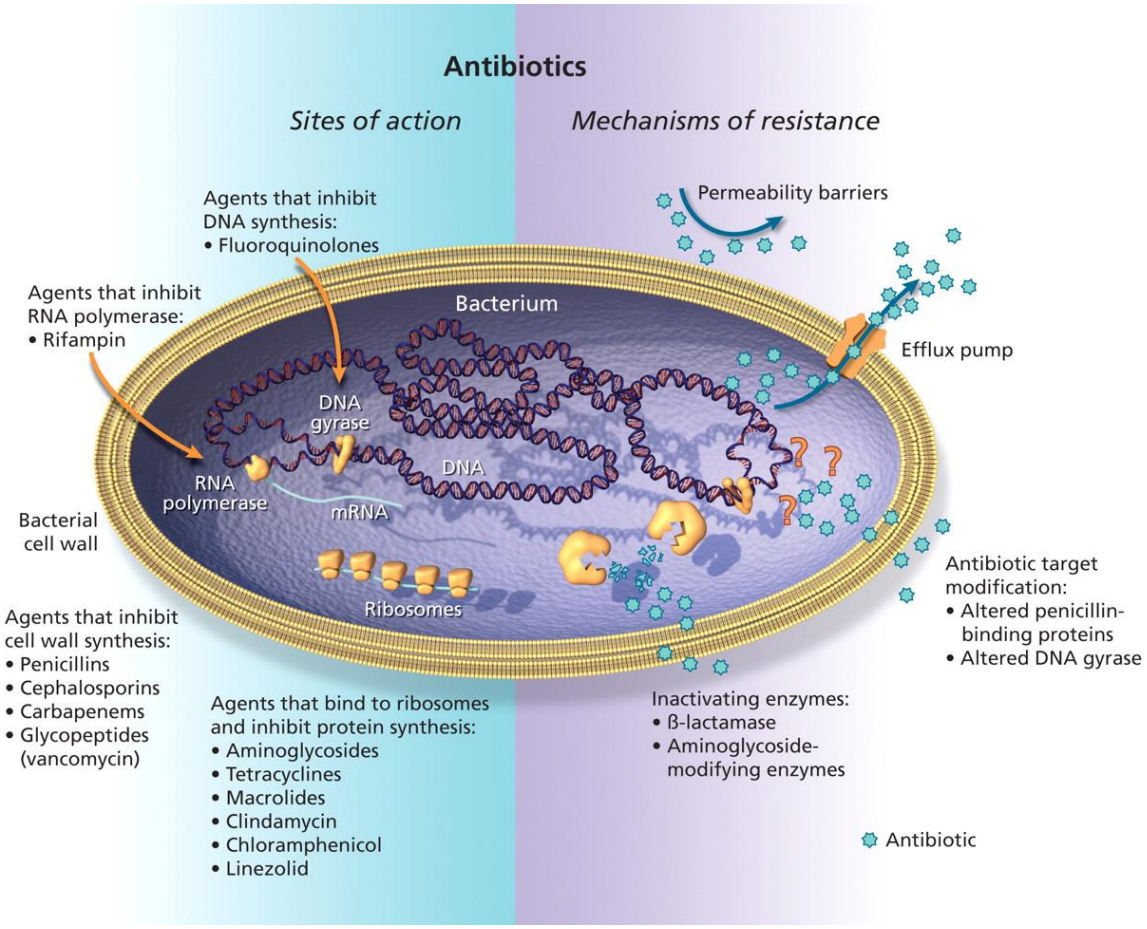

Figure 1.1: Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents (Mulvey and Simor, 2009) Copied under licence from the *Canadian Medical Association* and Access Copyright. Further reproduction prohibited.

1.3 The antibiotic resistance problem.

The resistance of pathogenic bacteria to a wide range of antibiotics is a major public health concern in the 21st century. Resistance of a microorganism to an antibiotic means that a microorganism formerly susceptible to the action of the antibiotic is no longer inhibited by the agent by using normal dosage rates (Finch, 2003).

The unprecedented successes of early antibiotic therapies allowed William H. Stewart, at the time US Surgeon General, to deliver a speech to Congress, in the late

1960s, which shows the general sentiment in the medical community at the time, he said "It's time to close the book on infectious diseases, declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease" (Spellberg *et al*., 2008). This, despite the fact that resistance was detected almost immediately following the introduction of any new antibiotic. For example, penicillin-resistant Staphylococci began to be reported only three years following its introduction as antimicrobial agent into clinical practice and in the subsequent two decades, more than 80% of Staphylococcal isolates from both community and hospitals were found to be resistant to penicillin.

Methicillin, semisynthetic penicillinase resistant penicillins, were introduced in 1961 to treat staphylococcal infections caused by penicillin-resistant strains; methicillin-resistant isolates were however, soon seen after its introduction (Lowy, 2003).

1.4 The genetics basis of antibiotic resistance

1.4.1 Intrinsic resistance

Antibiotic resistance may either be intrinsic or acquired. Intrinsic resistance is due to the innate chromosomal (genetic) makeup of the bacterial strain in question, which can explain the resistance of some bacteria to a certain class of antibiotics by their physiology, biochemistry or morphology that originate by virtue of their inherent genetic makeup (Mulvey and Simor, 2009); this type of resistance is not horizontally transferable (Mathur and Singh, 2005). Generally, Gram-positive bacteria are more sensitive to antibiotics than are Gram-negative bacteria which have more complex cell wall and non- specific permeability barriers in their outer membrane; these prevent the access of antibiotic molecules to their active site (Hammond and Lambert, 1978) (Mulvey and Simor, 2009). *Pseudomonas aeruginosa* has a high degree of intrinsic resistance and was already resistant to many antibiotics even before they were introduced (Finch, 2003).

1.4.2 Acquired resistance

Antibiotic resistance may also be acquired. This occurs when the organism's genetic composition is changed by either a spontaneous mutation in the bacterial DNA genes, or by the acquisition of new genetic material, causing the bacteria to develop new mechanisms of resistance (Finch, 2003; Mathur and Singh, 2005; Mazel and Davies, 1999).

1.4.2.1 Mutational resistance

Mutations usually occur when a single or a few more base pairs of the DNA are deleted, substituted, or added, thereby causing substitution of a single or few more amino acids in an essential peptide. Mutational resistance can significantly affect the genes coding for the antibiotic target and may reduce its affinity for the antibiotics (Finch, 2003). The rate of mutations in bacteria is not particularly high, perhaps occurring at a frequency of one gene mutation per 10^7–10^{10} bacteria, but the large numbers involved in bacterial populations increase the chance of the development of resistance in initially susceptible bacteria (Mulvey and Simor, 2009). Single mutants can cause a high degree of resistance. After a single exposure to streptomycin or erythromycin, for example, up to a thousand-fold resistant increase over the parent strain has been reported (Hammond and Lambert, 1978). High level streptomycin resistant *Mycobacterium tuberculosis* (Mazel and Davies, 1999) and *Enterococcus faecalis* are a common example of acquired resistance caused by a mutation affecting the specific streptomycin–binding protein of the 30S subunit of the bacterial ribosome (Finch, 2003).

1.4.2.2 Transferable resistance

Concern is particularly focussed on the rapid emergence and development of resistance that results from the acquisition of exogenous genetic material from (resistant) species.

In 1959 transferable resistance was first observed, when resistance genes native to *Shigella* were transferred by plasmids to *E. coli* (Hawkey, 1998). Three mechanisms of the horizontal gene transfer (HGT) have been identified :(i) natural transformation, this phenomenon is assigned as the acquisition and incorporation of free"naked" DNA from the extracellular medium (Mathur and Singh, 2005). In bacteria such as *Str. pneamoniae, H. influenza, Helicobacter, Neisseria* and *Moraxella spp,* transformation of resistance factors is a significant mechanism which causes extensive genetic variation (Finch, 2003), (ii) transduction, is a transfer of DNA in a process mediated by bacteriophages (viruses that infect bacteria) which may also participate in the transformation of some resistance genes, this being commonly seen in Staphylococci (Hawkey, 1998): (iii) conjugation, this involves transfer of DNA from one bacterial cell to another and is found to occur in most bacterial genera (Mathur and Singh, 2005). Conjugation plays a vital role in the transfer of resistance across genus and species lines of clinically important bacteria using plasmids and transposons. Plasmids are extra-chromosomal, autonomous, DNA molecules that replicate separately from the bacterial genome and may contain some antibiotic resistance genes (Mulvey and Simor, 2009; Levy, 1997). Conjugative plasmids, which are capable of self-transmission to other bacterial hosts, are common in Gram-negative enteric bacilli and are relativity large; non-conjugative plasmids are common in Gram-positive cocci. In order to increase the probability of their distributing to progeny during cell division, small plasmids are usually present at more than 30 copies per cell, while the large plasmids are usually present at one or two copies per cell, and their replication is closely linked to replication of the bacteria chromosome. Conjugative transfer of plasmids has been observed, however, between distant bacterial groups and even between bacteria and eukaryotic cells such as yeasts (Finch, 2003). Transposons are mobile DNA units, which are generally smaller than plasmids. Beside antibiotic resistance genes, transposons carry

genes required for their transposition, replication and jumping to other regions of the bacterial chromosome or bacterial plasmids (Mulvey and Simor, 2009). Conjugative transposons are circular segments of double-stranded DNA, ranging in size from 18 to over 150 kbp (Salyers and Amabile-Cuevas, 1997), and usually consist of a functional central region flanked by long terminal repeats. Insertion sequences that encode transposases and resolvases are found in the complex transposons which are responsible for their own transposition. Conjugative transposons can transfer independently of plasmid intermediate between chromosomes in different bacterial strains. For example, conjugative transposon Tn416 mediates the spreading of the *tetM* gene from Gram-positive cocci to various bacteria such as *Clostridium* and *Neisseria* (Finch, 2003). Some transposons and plasmids possess genetic elements which help them to acquire new genes; termed integrons which contain a range of gene clusters that often contain multiple determinants of antibiotic resistance (Gold and Moellering, 1996).

1.5 Mechanisms of antibiotic resistance in bacteria

In order to be effective against given bacteria, antibiotics must meet the following: 1) a vital target susceptible to a low concentration of the antibiotic must exist in the bacteria and 2); the antibiotic should directly contact to and bind to its intended bacterial target site in sufficient quantity (Finch, 2003). Whether antibiotic resistance is intrinsic or acquired, bacteria use their genetic determinants of resistance to encode different resistance mechanisms to neutralise the action of antibiotics, these resistance mechanisms can be classified into the following three basic types (Figure 1.1) : (i) enzymatic inactivation by either destruction or chemical modification of the antibiotics; (ii) prevention of antibiotic access to the target site (iii) alteration of the antibiotic target site (Schwarz and Chaslus-Dancla, 2001; Mulvey and Simor, 2009).

Enzymatic inactivation of antibiotics is accomplished when acetyl, adenyl or phosphoric groups are enzymatically transferred to specific sites of the antibiotics (Schwarz and Chaslus-Dancla, 2001), this kind of inactivation is the main mechanism of resistance to beta-lactams. β-lactamases enzymatically cleave the β-lactam ring of penicillins, cephalosporins and carbapenems which leads consequently to the loss of their antibacterial activity. More than 200 different types of β-lactamase have been described (Hawkey, 1998) that may be categorized by their different substrates and activities. The production of β–lactamases commonly mediates penicillin resistance in *S. aureus* and *N. gonorrheae* and ampicillin-resistance in *H. influenzae* (Mulvey and Simor, 2009), whereas the production of chloramphenicol acetyltransferase enzyme mediates chloramphenicol resistance in both Gram-positive and Gram-negative species (Finch, 2003).

For an antibiotic to bind with a bacterial target effectively, an antibiotic must reach a threshold concentration within the bacterium. Therefore, yet another strategy has evolved to neutralize antibiotic activity which prevents an antibiotic from accessing its target site, this can be achieved by either reducing the uptake or increasing the removal of the antibiotics (Croft *et al*., 2007). Efflux pumps, actively pump out antibiotics from the bacterial cytoplasm before they start their action and mediate resistance to the tetracyclines, chloramphenicol and the fluoroquinolones (Levy and Marshall, 2004; Alanis, 2005). In Gram-negative bacteria, the outer membrane of the cell wall can act as a permeability barrier for certain antibiotics. Some essential compounds and antibiotics diffuse into Gram-negative bacteria cell cytoplasm through a cellular membrane protein know as a porin. Porin mutations, which reduce its expression, cause a structural alteration which may prevent or reduce access of antibiotics to their cytoplasmic target active site. This mechanism can be best exemplified by the resistance of *Pseudomonas aeruginosa* and other Gram-negative bacteria to β-lactams and aminoglycosides

(Mulvey and Simor, 2009), *Pseudomonas aeruginosa* switch the charge of their cell wall lipopolysaccharides (LPS) to impede the antibiotics with highly positive charges, like aminoglycosides, from passing the cell wall outer membrane (Schwarz and Chaslus-Dancla, 2001).

Alterations of the antibiotic target site, e.g. by methylation, may make the target active site inaccessible to antibiotics (Schwarz and Chaslus-Dancla, 2001), consequently, this leads to a loss of any antibacterial effect. For example, alterations in the structural conformation of penicillin-binding proteins (PBPs) observed in certain types of penicillin resistance. This occurs in methicillin resistant strains of *S. aureus* (MRSA) which produce an altered penicillin-binding protein (PBP2a) that β-lactam antibiotics fail to bind successfully, therefore, all of the currently available penicillins, cephalosporins and carbapenems are inactive against MRSA (Mulvey and Simor, 2009). In yet another example of resistance, most fluoroquinolones (e.g., ciprofloxacin, levofloxacin and moxifloxacin) resistance stems from alterations to DNA gyrase which are critical for DNA replication in bacteria (Alanis, 2005).

1.6 Selected resistant microorganisms

1.6.1 *Staphylococcus aureus*

Since it was discovered in the 1880s, *Staphylococcus aureus* has been seen as a potential pathogenic Gram-positive bacterium, causing infections, such as post-operative wound infections and minor skin infections. It is regarded as the second most common etiologic agent in bloodstream infections and lower respiratory tract infections. Penicillin-resistant *S. aureus* isolates started to appear in hospitals only two years after the introduction of penicillin in medical use, within a few years later the appearance of penicillin-resistant strains of *S. aureus* in the general community was also reported (Croft *et al.*, 2007; Deurenberg and Stobberingh, 2008). By 1946, around 60%

of hospital isolates in the UK were resistant to penicillin (Lyon and Skurray, 1987). *Staphylococcus aureus* contain mobile genetic islands. DNA from these islands is readily exchanged within the *S. aureus* population. These mobile genetic elements are commonly referred to as *S. aureus* pathogenicity islands and are a possible mechanism for evolution in *S. aureus* (Holden *et al*., 2004).

1.6.1.1 Methicillin Resistant *Staphylococcus aureus* (MRSA)

The introduction of methicillin in 1960, a penicillinase-stable antibiotic, seemed to be the answer to increasing therapeutic problems resulting from antibiotic resistance. However, the relief was short-lived as the first resistant strain was reported in 1961 (Livermore, 2000). Methicillin resistant *Staphylococcus aureus* owe their behaviour to an additional, low-affinity penicillin-binding protein (PBP-2a), encoded by mecA gene which is carried on a cassette chromosome (SCCmec) which is mobile and is an efficient transformer (Zinner, 2007). However, the incidence of hospital infection with multi resistant Staphylococci gradually declined during 1960s and 1970s, but this calm was shattered in the late 1970s and early 1980s, and after the gradual dissemination of new epidemic strains of methicillin-resistant *Staphylococcus aureus* has become endemic in hospitals worldwide (Livermore, 2000; Finch, 2003; Cooper *et al.*, 2003). Epidemic strains of MRSA (EMRSA) were different from those MRSA strains of the 1960s; while most antibiotic resistance in the MRSA strains of the 1960s was plasmid-borne, resistance in EMRSA was carried on the chromosome. The first, EMRSA-1 strain caused outbreaks in London hospitals before spreading beyond (Cooper *et al*., 2003). In the UK, the most widespread strains of MRSA are EMRSA15 and EMRSA16 (Livermore, 2000). MRSA have become resistant to virtually all-available systemic antibiotics, including the β-lactams, aminoglycosides, tetracyclines, macrolides and lincosamides (Finch, 2003). In general, MRSA is currently the most frequently

identified drug-resistant pathogenic bacteria among patients in health-care units (Mulvey and Simor, 2009). *S. aureus*–associated hospitalizations increased more than 60%, and the approximated figure of MRSA-associated hospitalizations more than doubled during the period from 1999 to 2005, thereby suggesting that *S. aureus* and MRSA should be taken into account as a national priority in disease control plans in USA (Klein *et al*., 2007).

Recently, the problem of MRSA has involved two worrying developments. First is the emergence of community-acquired-MRSA (CA-MRSA). Initially MRSA infections were acquired nosocomially. CA-MRSA are becoming the most frequent causes of lung, serious skin and soft tissue infections in previously healthy patients, even when not in contact with hospitals or other health-care facilities (Zinner, 2007). CA-MRSA strains are associated with *Staphylococcal* cassette chromosome (SCCmec types IV-V or VI) and characterized by the presence of exotoxin gene profiles including Panton-Valentine leukocidin (PVL), whereas hospital acquired MRSA (HA-MSRA) generally carry the larger SCCmec types I-III and more resistant phenotypes (Croft *et al.*, 2007; Deurenberg and Stobberingh, 2008; Zinner, 2007; Weber, 2005).

Another critical development is the emergence of vancomycin resistance in *S .aureus* Glycopeptides such as vancomycin have been widely used in the treatment of MRSA infections among hospitalized patients. Unfortunately, MRSA strains with reduced susceptibility to vancomycin have been increasingly reported in the last two decades. In 1997 an MRSA strain with intermediate resistance to vancomycin (MIC 8 µg/mL) was reported in Japan. This strain is known as a Vancomycin-Intermediate *S.aureus* [VISA] (Tenover, 2006). Five years later, in 2002 a Vancomycin- resistant *Staphylococcus aureus* (VRSA) (MIC 32µg/mL) was isolated for the first time in the United States. VRSAs are being isolated in hospitals worldwide (Hiramatsu, 2001; Bataineh, 2006; Tiwari and Sen, 2006). This Vancomycin-resistant *Staphylococcus aureus* achieved

vancomycin resistance by acquiring the *vanA* vancomycin resistance gene from *Enterococcus* spp. (VRE) (Tenover, 2006).

1.6.2 *Escherichia coli*

This is a Gram-negative member of the family Enterobacteriaceae. Although most strains of *E. coli* are not regarded as pathogens, the bacterium plays an important role in nosocomial infections and is considered as a common cause of urinary tract infections (UTIs) (Tenover, 2006; Croft *et al.*, 2007; Guyot *et al.*, 1999; Feng *et al.*, 2002). *E. coli* isolates accounted for 13.6% of nosocomial infections in an USA hospital and responsible for 12.7% of intensive care (ICU) acquired infections in many European countries (Guyot *et al*., 1999).

E.coli has become frequently resistant to aminopenicillins, including amoxicillin or ampicillin and a limited spectrum of cephalosporins. This resistance is facilitated by the acquisition of plasmid-encoded β-lactamases, which degrade and inactivate these antibiotics (Tenover, 2006). The rates of decreased sensitivity to oxyimino-cephalosporins among worldwide clinical isolates ranged from 3% in the United States, 5% in Europe, to 8% in the Western Pacific and 9% in Latin America. This resistance was commonly linked with co-resistance to tetracycline (45-77%), gentamicin (15-75%), ciprofloxacin (14-66%) and co-trimoxazole (6-26%). Fluoroquinolone-resistant *Escherichia coli* is an increasingly common problem in Europe, reaching prevalence rates of 5-l0% in many surveys (Finch, 2003).

1.6.3 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis exists as multi-drug-resistant tuberculosis (MRD-TB) when it is resistant to isoniazid and rifampicin (two key first-line drugs) with or without resistance to other anti-TB drugs (Willcox, 2000; Nations and Walsh, 2006). Rifampicin and isoniazid are the most common first-line anti-TB agents. The action of isoniazid

against *M. tuberculosis* is achieved by an inhibition of the biosynthesis of mycolic acids, which is an essential component of the bacterium cell wall, whereas the action of rifampicin is achieved by it interfering with the synthesis of mRNA by binding to RNA polymerase. Resistance in *Mycobacterium* against these drugs mainly derives from the accumulation of individual mutations in several independent genes resulting in an over production or modification of the drug target (Willcox, 2000).

The development of (MRD-TB) is one of the most common public health problems in the world, especially in the developing countries where deaths from tuberculosis are common. In a rural hospital in South Africa for example, 41% of the confirmed cases of tuberculosis were found to be multidrug resistant (Lawn and Wilkinson, 2006). Only in 2004, it has been estimated that nearly 424,200 cases of MDR TB occurred worldwide (Shah *et al*., 2007). MRD-TB is associated with high mortality rates in all patients, but especially so in people infected with human immunodeficiency virus (HIV), in which the mortality rates can reach to 90 % (Paramasivan, 1998; Finch, 2003).

1.6.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the major causes of nosocomial infections and is highly associated with opportunistic infections among immunocompromised individuals. About 10% of all hospital-acquired infections are caused by *Pseudomonas aeruginosa*; these infections are difficult to treat due to the presence of numerous intrinsic and acquired mechanisms of antibiotic resistance. Multidrug resistance *P. aeruginosa* is increasingly being reported among clinical isolates collected in the USA (Tenover, 2006; Aloush *et al.*, 2006). For example, about 30% of *P. aeruginosa* clinical isolates from intensive care units (ICU) in the USA were found to be resistant to fluoroquinolones and 21% were resistant to carbapenems (Mulvey and Simor, 2009). The situation in Europe and Latin America seemed to be worse, where the results of a

global survey done between 1997 and 1999 showed that no antipseudomonal agent was still effective against >80 % of sampled hospital strains (Finch, 2003).

As mentioned above, *P. aeruginosa* is characterized by intrinsic resistance and some kind of acquired resistance mechanisms. Intrinsically, *P. aeruginosa* is resistant to most β-lactam antibiotics, tetracyclines, chloramphenicol, sulfonamides and aminoglycosides, due to a combination of impermeability with multidrug efflux. This combination prevents the accumulation of antibiotics within the bacterium, pumps the antibiotics out from the cell before they have the opportunity to reach an internal concentration sufficient to react with the site of action (Tenover, 2006). *Pseudomonas aeruginosa* expresses many types of acquired β-lactam resistance, often in combination, these include: acquisition of transposon and plasmid-mediated ESBLs, hyperproduction of AmpC cephalosporinase, oxacillinases, or carbapenemases; mutational loss of porins or upregulation of efflux pumps. In general, acquired resistance in *P. aeruginosa* develops rapidly and can emerge in more than 10% of patients during treatment (Finch, 2003).

1.6.5 *Candida albicans*

The yeast, *Candida albicans* is the most prevalent fungal pathogen of humans and causes oral, vaginal, and systemic infections. These infections are frequently associated with immunocompromised patients and are regularly found in acquired immune deficiency syndrome (AIDS) patients and bone marrow transplant patients (Goldway *et al.*, 1995; White *et al.*, 2002). There are many antifungal agents such as the polyene amphotericin B and the azoles which are available to treat infections caused by *C. albicans*. In the 1990s, azole-resistant isolates of *C. albicans* emerged in HIV-infected patients after receiving extended doses of azole antifungals. Up to one-third of the oral HIV-positive patients' *C. albicans* isolates were documented as azole resistant.

Recently, multidrug resistant *C. albicans* have been also reported in other patient populations, such as those receiving bone marrow transplants (White *et al*., 2002)

1.6.6 Enteroccoci

Enteroccoci are Gram-positive bacteria which possess limited virulence. Although they are not regarded as highly pathogenic as Staphylococci and Streptococci species, Enterococcus species (e.g., *Enterococcus faecalis*, *Enterococcus faecium*) are increasingly encountered in nosocomial infections and may also cause other severe, life-threatening infections (Croft *et al*., 2007; Mulvey and Simor, 2009). *Enterococcus faecalis*, *Enterococcus faecium* are responsible for the majority of human infections caused by enterococcal species, 80 to 90% of all clinical isolates were identified as *E. faecalis* whereas 5 to 15% of such clinical isolates were identified as *E. faecium* , while the remaining (>10) *Enterococcus* species accounted for less than 5% of clinical isolates (Cetinkaya *et al*., 2000).

Enterococci are intrinsically resistant to numerous regularly used antibiotics, such as aminoglycosides, where the uptake of the drug is compromised at the cell wall; this makes the choice of using antimicrobial agents to treat infections caused by Enterococci limited. Intrinsic resistance exists to the β-lactam antibiotics when they are used alone due to the presence of penicillin-binding proteins of the organism having a low affinity for these antibiotics. Intrinsic resistance to the quinolones is due to mutations of the quinolone resistance-determining region. As well as widespread intrinsic resistance, Enterococci are able to readily acquire resistance to all currently existing antibiotics through either mutations or by obtaining foreign genetic materials mediated by mobile DNA including, plasmids and transposons (Cetinkaya *et al*., 2000) (Murray, 1990). For a long time, glycopeptide antibiotics particularly vancomycin, were considered as the most powerful antibiotics to treat infections associated with multidrug-resistant

enterococci. In 1988, the first isolation of vancomycin-resistant *E. faecalis* and *E. faecium* was reported in UK (Cetinkaya *et al*., 2000). Subsequently, Vancomycin Resistant Enterococci (VRE) has been reported worldwide (Croft *et al*., 2007). Enterococci developed this resistance by acquisition of gene clusters carried on plasmid that encode a modified cell-wall precursors which do not bind glycopeptides such as vancomycin (Mulvey and Simor, 2009).

1.7 The impact of antibiotics resistance

Due to the lack of worldwide comprehensive data, there is no accurate estimation as to the total economic cost of the emergence of the antibiotic resistant bacteria. However, it has been documented that infections with antibiotic resistance bacteria lead not always, but usually, to excessive lengths of stay in hospitals, excess mortality rates, increased duration of ICU admission, and to the increased attributable cost of medical care including the use of expensive broad spectrum antibiotics and the development of inappropriate antibiotic therapy complications, such as a necessity to perform surgery (McGowan Jr, 2001; Niederman, 2001; Sipahi, 2008). In 1995 the American Society for Microbiology estimated that the annual healthcare expenses related to the treatment of antibiotic resistant bacterial infections in the USA were more than US$4 billion (Sipahi, 2008). One of the few studies examining the costs of antibiotic resistance showed that methicillin-resistant (MRSA) treatment in New York city cost 22% more than methicillin-susceptible (MSSA) treatment (Levy and Marshall, 2004). The estimated cost of single case treatment (including drugs, procedures, and hospitalization) has increased from $12,000 for a drug-sensitive *M. tuberculosis* to $180,000 for a multidrug-resistant *M. tuberculosis* (Cohen, 1992).

The emergence of multi-antibiotic resistant strains can increase the morbidity and mortality as, for certain infections, the potential efficient treatment is delayed.

Moreover, because antimicrobial susceptibility tests are time consuming, complications or death can result during normally accepted treatment (Cohen, 1992). In a meta-analysis carried out to investigate the effect of methicillin-resistance on the mortality among *S. aureus* bloodstream-infected patients, mortality rates was nearly double in methicillin-resistant *S. aureus* bloodstream infections compared to methicillin-susceptible *S. aureus* bloodstream infections (Mulvey and Simor, 2009).

1.8 The decline of antibiotic research and development

We are now facing two opposing and worrying trends. While the emergence of multidrug-resistant pathogen strains is rapidly growing, the innovation and the development of novel antimicrobial agents which are effective against these pathogens have declined significantly. Moreover, many large pharmaceutical companies as well as biotechnology companies have either reduced, or wholly eliminated, their research efforts in antibacterial agent innovation (Sipahi, 2008; Projan and Shlaes, 2004). Since the 1980s, the world market has witnessed the introduction of only two new classes of antibiotics of novel mechanisms of action (namely linezolid and daptomycin) (Jagusztyn-Krynicka and Wyszynska, 2008).

There are many reasons for this noticeable decline, the most significant one being that the current cost required to bring a new antibiotic agent to the market ranges between $100 million and $350 million in the United States (Gold and Moellering, 1996), and according to the US Department of Health and Human Services it may reach US$1.7 billion (Sipahi, 2008). There are several factors leading to high costs, for example, regulatory agencies have been increasing their requirements in the areas of manufacturing, safety, and efficacy for drug development .In addition, the cost of the clinical trials have increasingly become more expensive. In the 1981–84 period, the

number of patients enrolled in clinical trials averaged 1321, whereas it increased to more than 4237 by 1994–95 (Projan and Shlaes, 2004).

1.9 Alternative methods to antibiotic treatment

The current situation, where antibiotic resistance is becoming increasingly common, and at the same time new antibiotics research and development has declined, has created a therapeutic challenge to the medical community where the development of new approaches and alternative treatments for such infections is becoming increasingly essential.

In this section some of the alternative approaches to the control of microbial infections will be discussed. One of these, is the unorthodox use of biological control that involves the application of living organisms, rather than the application of a metabolite they produce to the human infection; this includes for example, maggot therapy, bacteriophage therapy, the use of probiotics and mycotherapy (Wainwright, 1998; Nicolle, 2006). The procedure basically relies on competition between two species of organism, one the parasite and the other a non-pathogenic saprophyte. The biggest limitation of some of these techniques is that it is mainly restricted to the treatment of surface infections of the skin and vulnerable mucous membranes. In addition to these living alternatives to antibiotics, honey, botanical extracts and essential oils are being used to treat some multi-resistance bacterial infections, notably; wound-associated infections (Bowler *et al.*, 2001; Bowler, 2002).

1.9.1 Maggot therapy

Maggot debridement therapy (MDT), using larvae of certain species of flies to treat antibiotic resistant wounds, is increasingly being used in medicine with considerable success. There are hundreds of reports showing the successful use of maggots in chronic or infected wounds, including diabetic foot ulcers (Sherman, 2003), malignant

adenocarcinoma (Sealby, 2004), and for venous stasis ulcers (Sherman, 2009). It has also been used to combat infections following breast-conserving surgery (Steenvoorde and Oskam, 2005).

Maggot therapy appears to depend upon the production by the maggot of natural antibiotic-like agents, the alkalinisation of the wounds with secreted ammonia, and the consumption of bacteria and the consumption of dead flesh (Whitaker *et al*., 2007).

1.9.2 Bacteriophage therapy

Bacteriophages are bacterial viruses that naturally invade and multiply in bacteria, including pathogens (Nicolle, 2006; Joerger, 2003). They are often very specific to one bacterial species; they are harmless to animals and plants, and destroy their target bacteria (Summers, 2001). Interest in the therapeutic potential of phages dates back to nearly 100 years ago (Nicolle, 2006). According to Sulakvelidze *et al*. 2001, the first reported observation of phages may date as far back as 1896, when Ernest Hankin, a British bacteriologist, isolated a substance that was able to destroy *Vibiro cholerae* from the rivers Ganges and Jumma in India. Hankin assumed that an unknown filterable substance with bactericidal effect was behind this phenomenon and this limited the spread of cholera epidemics. However, the first clear discovery and description of "the bacteriophage phenomenon" was in 1915 by the English bacteriologist, Frederick Twort (Wainwright, 1990; Sulakvelidze *et al.*, 2001; Mathur *et al.*, 2003). The same phenomenon was then independently discovered by Felix d'Herelle, a French-Canadian microbiologist working at the Pasteur Institute in Paris in 1917 (Wainwright, 1990; Summers, 2001). He observed that filtrates from the faeces of dysentery patients stimulated a transmissible lysis of a broth culture of dysentery bacillus. He assumed that a virus was the lytic agent and named it as "Bacteriophage" (Mathur *et al*., 2003).

The efficacy of the bacteriophage was confirmed in 1927, when d'Herelle tried to use bacteriophage to treat Asiatic cholera in India. The phage was given to patients in spoonfuls while patients for whom it was denied acted as controls. D'Herelle claimed that it was a successful treatment, with mortality rates in the treated group being around 8%, compared to over 60 % mortality amongst the controls (Wainwright, 1990). More intensive studies on the therapeutic use of phages were conducted until they were later abandoned, when cheap effective and wide spectrum antibiotics were introduced into clinical use (Mathur *et al.*, 2003). However, a limited bacteriophage therapy continued to be performed in some Eastern European countries, while it was re-evaluated in the United States and Western Europe as a response to the increase in emergence of multi antibiotic-resistant bacteria (Joerger, 2003).

In a comparison with antibiotics, there are several special advantages which make phages potentially attractive therapeutic agents. Firstly, they are highly and specifically lytic against targeted pathogenic bacteria (Sulakvelidze *et al*., 2001); they are self-replicating and keep multiplying as long as local infection as present; bacteriophages are harmless to normal intestinal microflora. In contrast to antibiotics that are well known to have some side effects, there are no reported side effects associated with bacteriophages use, despite their use in millions of patients (Mathur *et al*., 2003). The limited host range can be a problem; for example there is no single bacteriophage that can lyse all *Salmonella serovars* strains (Sulakvelidze *et al.*, 2001; Joerger, 2003). Our poor understanding of the interaction between phage and its host; and low levels of stability of phage preparations also present difficulties (Sulakvelidze *et al*., 2001).

1.9.3 Probiotics

The use of probiotics involves applying live microorganisms, including certain bacteria and yeast species, to deliberately repopulate the harmful composition of intestinal

microflora with beneficial microbes (Nicolle, 2006; Ouwehand *et al.*, 2002; Boehm and Kruis, 2006). Although the use of food, fermented with microorganisms, for the purpose of disease prevention and health protection was documented and described in the classical Roman literature (Boehm and Kruis, 2006), Elie Metchnikoff, the Russian Nobel prize winner, was the first one to suggest the therapeutic use of probiotics. He proposed that a high concentration of lactobacilli in the intestinal microflora plays an important role in the health and longevity of humans (Gionchetti *et al.*, 2006). Heterogeneous lactic acid bacteria are the most commonly used as probiotics; these include Enterococci, *Bifidobacteria* and Lactobacilli (Ouwehand *et al*., 2002). Non-pathogenic yeasts *(Sacchromyces buolardii, Sac. cerevisiae)* and filamentous fungi *(Aspergillus oryzae)* have also been used as potential probiotics (Fuller, 1992). There is considerable evidence, obtained from clinical trials, showing that probiotics could be a promising alternative to antibiotics, particularly in the treatment of inflammatory bowel disease (IBD) and ulcerative colitis (UC) (Gionchetti *et al*., 2006).

1.9.4 Mould therapy

This approach, also termed mycotherapy, is based on folk medicine, where moulds growing on various natural products and foodstuffs have been in use for centuries to treat wounds and cure superficial bacterial infections (Wainwright, 1998). In the early 1940s, when purified penicillin was not widely available and very rare, doctors throughout the world successfully used bandages containing living cultures of *Penicillium notatum* (Fleming's original penicillin-producing mould) to treat some infected wounds, the mould then continued to grow and secrete penicillin and possibly other antibacterial metabolites. A modification of this approach termed Vivicillin, involved the use of a suspension of macerated fungal mycelium which was applied

directly to the infected wound. This probable alternative might deserve re-evaluating, especially as fungal mycelium is also known to aid healing (Wainwright *et al*., 1992).

1.9.5 Antimicrobial peptides (AMPs)

Recently, there is a growing interest in a group of naturally occurring small peptides produced by many tissues and cell types including human neutrophils (Bowler *et al*., 2001). In general, antimicrobial peptides are characterized by their small molecular mass which ranges between 1 to 5 kDa (Joerger, 2003). Their mode of action involves attachment to bacterial cell walls and formation of pores, which induce leakage of the cell contents and resultant death. These types of molecules have a wide spectrum of antimicrobial activity and that can be formulated for use in wound management (Bowler *et al*., 2001). One group of these antimicrobial peptides are gallinacins, purified from chicken leukocytes. In 1994, these successfully exhibited antimicrobial activity against *L. monocytogenes* , *E. coli* and *Candida albicans* in vitro (Joerger, 2003).

1.9.6 Apitherapy (Honey therapy)

Honey is one of these alternative treatments for antibiotic-resistant bacteria that has been gaining an increasingly interest. The antibacterial activity of honey has been extensively researched and is found to contain four main components. The main factor is hydrogen peroxide, which is produced by glucose oxidase (White Jr *et al*., 1963). Two other antibacterial factors are common to honey, namely acidity and osmolarity, a feature which is common to any concentrated sugar solution, the strong interaction between water molecules and the monosaccaride component leaves only a small and insufficient fraction of the water available for the growth of bacteria. Likewise the relatively high acidity of honey is antibacterial: the acidity varies between pH 3.2–4.5 and is beyond the lower limits for growth of most animal pathogens. The fourth component are the phytochemical factors including benzyl alcohol, pinocembrin,

terpenes, 3, methyl 3,5-dimethoxy-4-hydroxybenzoate ,and 5-dimethoxy-4-hydroxybenzoic acid (syringic acid) (Molan, 1997). Hundreds of *in vivo* studies have demonstrated the antibacterial activity of honey (e.g., Manuka honey) against a wide spectrum of bacterial species, particularly those related to wound infections like methicillin-resistant *Staphylococcus aureus* and, Vancomycin–resistant enterococci (Cooper *et al.*, 1999; Cooper *et al.*, 2002; Al-Waili *et al.*, 2005).

Aims of the work presented in this Thesis

The overall aim of the work presented in this thesis was to add new information to our knowledge of some of these alternatives to antibiotics, concentrating on maggot therapy, Apitherapy (honey therapy) and to a lesser extent mycotherapy.

Chapter Two: Studies on the activity of *Leptospermum scoparium* (Manuka) honey

2.1 Introduction

2.1.1 Honey

Honey was used in the medicine of many ancient communities (Molan, 2006), including the ancient Egyptians. The ancient Chinese and Sumerians provided the first written prescriptions relating to the medical use of honey, found as clay tablets, dating back to 2000 B.C. In such prescriptions, honey was used as an ingredient for ointments for use on infected wounds (Jones, 2001). No knowledge of the honey's antibacterial properties was of course known during this early therapeutic use of honey. Molan (2006) has cited that according to Dustmann the modern application of antibacterial honey was first reported by Van Ketel in 1892, followed by Sackett in 1919. Research into the antimicrobial characteristics of honey continued from time to time during the 20th century. However, it was not until the 1980's that interest in the healing properties of honey was revived and clinical studies began again (Davis, 2005).

The antibacterial potency of honey has been attributed to the following; (i) an osmotic effect: honey has a strong osmotic effect since more than 80% of its composition is a mixture of different monosaccharides, thereby making water available at a minimum level and preventing the growth of many microorganisms ; (ii) a naturally low pH:, honey is relatively acidic; its pH ranging from between 3.2 and 4.5, a factor which inhibits many pathogens (Molan, 1997); (iii) production of hydrogen peroxide: hydrogen peroxide plays a key role in the antimicrobial activity of honey. It is created when the hypopharyngeal gland of the bee secretes glucose oxidase into the nectar to help in the synthesis of honey; this reaction produces gluconic acid as well as hydrogen peroxide H_2O_2 (Wahdan, 1998); (iiii) phytochemical factors: many antibacterial phytochemicals have been recognized in honey such as: benzyl alcohol, pinocembrin, terpenes, 3, methyl 3,5-dimethoxy-4-hydroxybenzoate ,and 5-dimethoxy-

4-hydroxybenzoic acid (syringic acid) (Molan, 1997), flavonoids and the phenolic acids (Wahdan, 1998).

Numerous reports and clinical studies have demonstrated the antimicrobial activity of honey against a broad range of microorganisms, including multi-antibiotic resistant strains. A study conducted by Cooper and co-workers showed that more than 50 clinical isolate strains of coagulase-positive *Staphylococcus aureus* were susceptible to less than 5% (v/v) of pasture honey (Cooper *et al.*, 1999). In another study carried out by the same researcher, different honey samples exhibited bactericidal activity against 17 strains of *P. aeruginosa* isolated from infected burns, even when it was diluted more than 10-fold (Cooper *et al.*, 2002). More recently, this anti *P. aeruginosa* activity was confirmed and it was shown to be significantly higher than many antibiotics including amoxicillin/clavulinic acid, sulbactam/ampicillin, and ceftriaxone (Abd-El Aal *et al*., 2007). Honey samples collected from Northern Ireland and France showed a significant ability to inhibit the growth of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) (Maeda *et al*., 2008). Other studies demonstrated the antibacterial activity of honey against: *Escherichia coli*, *Campylobacter jejuni*, *Salmonella enterocolitis, Shigella dysenteriae* (Adebolu, 2005), *Mycobacterium* (Asadi-Pooya *et al*., 2003), methicillin-resistant *Staphylococcus aureus* and, Vancomycin –resistant enterococci (Cooper *et al.*, 1999; Cooper *et al.*, 2002; Al-Waili *et al.*, 2005). The antifungal activity of the honey, especially anti-*Candida* activity (Theunissen *et al.*, 2001; Irish *et al.*, 2006; Koc *et al.*, 2008) has also been reported.

Few studies have been conducted to investigate the antiviral activity of honey one however, was conducted by Zeina *et. al*. (1996) who compared the sensitivity of Rubella virus maintained in kidney cell cultures to a honey solution and to thyme extracts at varying concentrations; honey showed a potent antiviral activity while thyme did not (Zeina *et al.*, 1996). In another work conducted by Al-Waili (2004) the effect of

the topical application of honey on recurrent attacks of herpes lesions was compared to widely-used antiviral drugs (acyclovir cream). The results showed that that application of honey was safe and effective in the management of symptoms associated with recurrent herpes lesions (Al-Waili, 2004).

2.1.2 *Leptospermum scoparium* (Manuka) honey

Results obtained from several studies show that honeys from different geographical locations and different floral origins exhibit different antibacterial activities (Allen *et al.*, 1991; Al-Jabri *et al.*, 2003; Shin, 2005; Küçük *et al.*, 2007). One such famous product is Manuka honey which is derived from the Manuka tree (*Leptospermum scoparium*) a native of New Zealand (Snow and Manley-Harris, 2004). Manuka honey is known to have a unique extra antimicrobial activity which is not related to its low pH, osmolarity or hydrogen peroxide accumulation, termed as a non-peroxide activity (Allen *et al.*, 1991; Molan, 1999b). A general survey of 345 different New Zealand honey samples including Manuka honeys was carried to evaluate the variation in antibacterial activity among them. In order to remove hydrogen peroxide antibacterial activity, catalase was added to the tested diluted honeys. While most of the honeys demonstrated no detectable non-hydrogen peroxide antibacterial activity, Manuka honey exhibited a considerable range of non-peroxide antibacterial activity (Allen *et al*., 1991). Australian jelly-bush honey also has been shown to have a significant non-hydrogen peroxide antibacterial activity (Cooper, 2007). Much research effort has been made to identify the active component responsible for this non-peroxide antibacterial activity, although it was thought that it may be due to plant derived components such as flavonoids and phenolic compounds (Weston *et al*., 2000), recent studies have successfully concluded that this component is methylglyoxal (MG), a highly reactive precursor in the formation of advanced glycation end products (AGEs)

(Adams *et al*., 2008; Mavric *et al*., 2008; Stephens *et al*., 2010). MG concentration is found to be 100-fold higher in Manuka honeys than other conventional honey (Wittmann *et al*., 2008). Atrott and Henle (2009) found a perfect linear correlation between methylglyoxal levels in 61 samples of Manuka honey and their antibacterial activities, which clearly highlights the dominant bioactive compound in Manuka honey is methylglyoxal.

Manuka honeys have been given an industry standard called UMF® (Unique Manuka Factor), which reflects the relative ability of Manuka honey to inhibit the growth of *Staphylococcus aureus.* (e.g., UMF 20 is claimed to have equivalent antibacterial activity to 20% phenol w/v) (Allen *et al*., 1991).

2.1.3 The use of Manuka honey in wound management

The healing properties of honey have been known for thousands of years (Bell, 2007). The beneficial use of honey in wound treatment is not restricted to its well known antimicrobial activities, it can also prevent cross infections and fluid loss of the wound by creating a viscous protective barrier, it has a debriding effect, is anti-inflammatory and stimulates granulation and thereby tissue growth (Davis, 2005; Molan, 1999a; Mathews and Binnington, 2002).

The recognition of the significant non-hydrogen peroxide antimicrobial value of Manuka honey, particularly against strains of antibiotic resistant bacteria isolated from infected wounds, (Cooper, 2004; Davis, 2005; Molan, 1999a; Willix *et al.*, 1992; Al Somal *et al.*, 1994; Mathews and Binnington, 2002) has led to an increase in the use of this honey in wound management. Many reports have shown that Manuka honey gives encouraging results in the treatment of leg ulceration (Gethin and Cowman, 2005), burn wounds and severely infected cutaneous wounds (Visavadia *et al*., 2008). Manuka honey-impregnated dressings have been approved by the regulatory health

authorities in Australia and Europe and been used as an accepted topical treatment for wounds which commonly show a poor response to conventional therapy (Figure 2.1). (Simon *et al*., 2009). One of these medically certified honeys is Medihoney™; Simon and his colleagues (2009) reported the successful use of Medihoney™ to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infected wound in a 12-year old patient.

2.1.4 The incidence of *Clostridium botulinum* in honey

The incidence of *Clostridium botulinum*, a spore-forming anaerobic Gram-positive bacterium (Dodds, 1993), in honey has received widespread attention because of reports linking honey consumption to several infant botulism occurrences (Gilmore *et al.*, 2010; Smith *et al.*, 2010). Generally, the incidence of *C. botulinum* in honey samples is low (Simon *et al*., 2009; Gilmore *et al*., 2010; Schocken Iturrino *et al*., 1999). In a survey carried out in France, among 116 tested samples no botulinum spores were detected (Delmas *et al*., 1994), while 1.0% of Argentinean honey samples were contaminated with botulinum spores (De Centorbi *et al*., 1997).

Figure 2.1: Shows different types of medically certified honeys, Manuka honey-impregnated dressings and Activon medical grade Manuka.

The aim of the work reported in this chapter was to:

1. Investigate the antimicrobial activity of selected honeys from different origins; specifically to evaluate their non-hydrogen peroxide derived activity.

2. Investigate the nature of the non-peroxide antibacterial activity of *Leptospermum scoparium* Manuka honey.

3. Evaluate Tregothnan English Manuka honey antibacterial activity and compare it to the New Zealand Manuka Honey.

4. Conduct studies on the mechanisms of the antibacterial activity of the medical grade New Zealand Manuka against methicillin-resistant *Staphylococcus aureus* (MRSA) and to perform scanning electron microscope (SEM) and transmission electron microscope (TEM) studies on the morphological effects on Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*.

5. To investigate the possibility of the presence of non–hydrogen peroxide inhibitory components in Manuka honey derived from microbial sources.

6. To determine the incidence of *Clostridium botulinum* spores in Manuka honey.

2.2 Materials and methods

2.2.1 Honey samples

Eighteen Honey samples from different origins were obtained from Rowse Honey Ltd, Wallingford, UK. Graded New Zealand Manuka honey samples were obtained from Comvita UK Limited, Berkshire, UK or from Littleover Apiaries Ltd, Derby, UK. Tregothnan English Manuka was obtained from Tregothnan Company, Cornwall, UK.

2.2.2 Determination of pH

The pH of the honey was determined as following; 5 g of honey was dissolved in 5 ml ultrapure MilliQ water, stirred until the solution became homogenous, The pH of the final solution was measured by using a calibrated Jenway 3310 pH meter (Bogdanov, 2002)

2.2.3 Test Organisms

The following test organisms (bacteria) were used: *Escherichia coli*, *Staphylococcus epidermidis, Serratia marcescens, Bacillus sphaericus,* methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis*; unless stated otherwise, the organisms were obtained from the Departmental Culture Collection.

2.2.4 Turbidity standard for inoculum preparation

In order to standardize the inoculum density for all susceptibility tests conducted in this thesis, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent was used. A $BaSO_4$ 0.5 McFarland standards were prepared as follows: a 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v), the absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standard. The barium sulphate suspension was transferred into screw-cap tubes that had the same size and same volume of those used in growing the needed broth culture. Tubes were tightly sealed to prevent any loss by evaporation and stored in the dark at room temperature (Andrews, 2001).

2.2.5 Inoculum preparation was performed as follows

Two to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and inoculated into a tube containing eight to ten ml of Mueller -Hinton broth medium and incubated overnight at 37°C. The turbidity of the actively growing broth culture was adjusted with phosphate buffer saline (PBS) or broth to obtain turbidity optically comparable to that of the $BaSO_4$ turbidity standard (0.5 McFarland standards). This resulted in a suspension containing approximately 1 to 2 x 10^6 CFU/ml of the test bacteria.

2.2.6 Phenol calibration

Different phenol concentrations ranging from 1% to 14 % (w/v) were prepared and their antibacterial activities against *Staphylococcus aureus* were measured using the well diffusion method, The diameter of the clear zone around each well of the phenol references was measured, and plotted against the phenol concentration used, and the standard calibration curve equation shown in Figure 2.2 was used to compare phenol inhibitions with that of the various honey samples (Baltrusaityte and Ceksteryte , 2007).

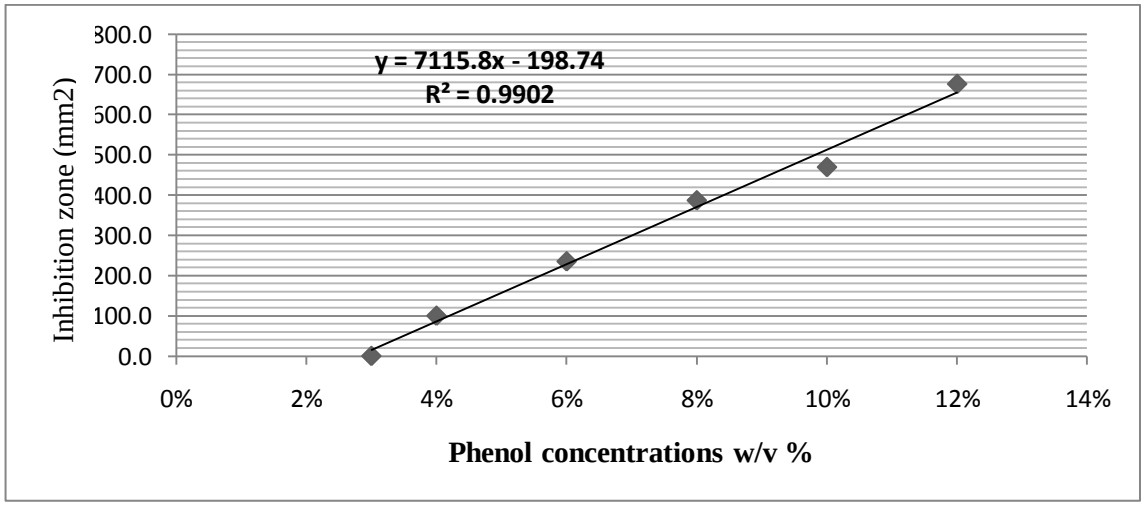

Figure 2.2: Calibration curves for the phenol reference solutions used in the agar well diffusion assay of antibacterial activity against *S. aureus*.

2.2.7 Agar diffusion assay

The plates were prepared using 20 ml of sterile Nutrient Agar. The surface of the plates was inoculated using a 100 µL of 0.5 McFarland standardized inoculum suspension of bacteria and allowed to dry. Wells, 8.0 mm in diameter, were cut from the culture media using a sterile metal cylinder, and then filled with the test honey. The plates were incubated at 37ºC and observed after 24 hours for clear, circular inhibition zones around the wells; these were measured.

2.2.8 Minimum inhibitory concentration (MIC) by tube dilution and dose-response effect

MICs were measured by Broth macro-dilution method. A series of 13 ×100 mm test tubes containing 9 ml Mueller -Hinton broth medium with honey concentrations ranging from 0% to 14% v/v were prepared. Each tube received 1 ml of bacterial suspension adjusted to 0.5 McFarland standards with further 1:100 dilution to get a final density of approximately 5 x 10^5 cfu. The negative control test tubes received only 1 mL of Mueller–Hinton broth. MIC-values were recorded after 18 h of incubation at 37°C with shaking 250 rpm; MICs were defined as the lowest concentration of honey which showed no visible growth. In order to study the dose-response effect of Manuka honey, the initial optical density of the all previous culture tubes were determined at 600 nm prior to incubation (T_1) and measured after the cultures were incubated (T_2). The growth inhibition for the test at each honey concentrations was determined using the equation: Percentage inhibition = 1 - (OD T_2 test- OD T_1 test /OD T_2 control- OD T_1 control) x 100 (Akujobi and Njoku, 2010).

2.2.9 Minimum Bactericidal Concentrations

Bacterial culture (15 µl) was withdrawn from the tubes with no growth and plated onto nutrient agar (Oxoid) then incubated overnight at 37°C to determine MBC. The lowest concentration which showed no growth on the agar was defined as the MBC.

2.2.10 Catalase treatment

Honey samples were tested at a concentration of 50 % (w/v) for antibacterial activity. Catalase solution was made by dissolving 2 mg of catalase (Sigma, 1850 units/mg), in ultrapure distilled water (10 ml). The honey (2.00 g) was dissolved either in distilled water (2.00 ml) or in 2 ml of catalase solution (giving non-peroxide activity).

2.2.11 Time killing curves

The effects of UMF25+ Manuka and 20+ Manuka on the viability of cells were monitored by inoculating 2 mL of an overnight culture of MRSA, adjusted to final density of approximately 5 x 10^6 cfu, into 18 mL NB with and without 12% (v/v) of both Manukas and incubated at 37°C on a shaker (250rpm).100 µL samples were removed at known intervals , diluted serially and 100 µl of the diluted samples were placed on nutrient agar plates and incubated at 37°C for 24 h (Yagi and Zurenko, 2003). Cfu were counted and time kill plots were constructed.

2.2.12 SEM and TEM studies

Overnight cultures of Methicillin-resistant *S. aureus* and *E. coli* (10ml) were centrifuged at 4,000 *g* for 20 min, resultant cells pellets were washed with sterilized distilled water, MRSA cells were re-suspended in 10 ml of phosphate-buffer saline (PBS) pH 7.4 with and without 12 % v/v 25+ Manuka honey and incubated for 4 h at 37°C, whereas *E. coli* cells were re-suspended in 10 ml of phosphate-buffer saline (PBS) pH 7.0 with and without 18 % v/v 25+ Manuka honey and incubated for 6 h at 37°C. After incubation, cells were harvested by centrifuging at 4,000 *g* for 20 min and then washed with sterilized distilled water.

For scanning electron microscopy (SEM), harvested cells were fixed in 3% glutaraldehyde in 0.1M phosphate buffer for 4 hours at 4°C, and then washed twice times with 15 min intervals at 4°C in 0.1M phosphate buffer. Cells were then subjected

to a secondary fixation with 2% osmium tetroxide aqueous for 1 hour at room temperature, and the previous wash step was repeated. After this, cells were serially dehydrated with 75%, 95%, and three times 100 % ethanol, the last one dried over anhydrous copper sulphate for 15 min. For hexamethyldisilazane (HMDS) drying, cells were immersed in 100% hexamethyldisilazane for 30 min after the second 100% ethanol step. Cells were then allowed to air dry overnight. On completion of drying, the cells were mounted on 12.5mm diameter stubs and attached with Sticky Tabs and then coated in an Edwards S150B sputter coater with approximately 25nm of gold. The cells were examined in a Philips XL-20 Scanning Electron Microscope at an accelerating voltage of 20Kv.

For transmission electron microscopy (TEM), harvested cells were fixed in 3% glutaraldehyde in 0.1M phosphate buffer overnight at 4°C, and then washed twice times with 30 min intervals at 4°C in 0.1M phosphate buffer. Cells were then subjected to a secondary fixation with 2% osmium tetroxide aqueous for 2 hour at room temperature. Previous wash step was repeated. After this, cells at room temperature were serially dehydrated with 75%, 95%, and three times 100 % ethanol the last one dried over anhydrous copper sulphate for 15 min. The cells were then placed in an intermediate solvent, propylene oxide, for two changes of 15 min duration. Infiltration was accomplished by placing the cells in a 50/50 mixture of propylene oxide/Araldite resin. The cells were then left in this 50/50 mixture overnight at room temperature. Once this incubation was finished the cells were transferred into full strength Araldite resin and left for 6-8 hours at room temperature after which time they were embedded in fresh Araldite resin for 48-72 hours at 60°C. Semi-thin sections approximately 0.5 µm thick were cut on a Reichert Ultracut E ultramicrotome and stained with 1% toluidine blue in 1% borax. Ultrathin sections, approximately 70-90nm thick, were cut using on a Reichert Ultracut E ultramicrotome and stained for 25 mins with 3% uranyl acetate in

50% ethanol followed by staining with Reynold's lead citrate for 25 mins. The sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80Kv.

2.2.13 Isolation of bacterial and fungal strains from Manuka honey.

In order to isolate bacteria from the honey, 0.1ml of each 5% (v/v) Manuka honey samples (Comvita UMF 25+ Manuka, 20+ Manuka, 15+ Manuka, and 12+ Manuka) solution was spread on nutrient agar plates. The plates were then incubated at 37 °C for 48 h or until the bacterial colonies were of sufficient size to lead to colony replication. The bacterial isolates were streaked onto fresh nutrient agar plates and preserved at 4 °C for further study (Lee *et al*., 2008). In order to isolate fungi 0.1ml of each 5% (v/v) Manuka honey solution was spread on Czapek agar plates supplemented with 0.01% chloramphenicol to inhibit bacterial growth. The plates were incubated at 25 °C for 1-2 weeks, morphologically different colonies were isolated (Pereira *et al*., 1996). For yeast isolation tryptone glucose yeast extract chloramphenicol (TGY) agar was used (100 g glucose, 5 g tryptone, 5 g yeast extract, 0.1g chloramphenicol, 15 g agar per litter (Senses-Ergul and Ozbas, 2006).

2.2.14 Antimicrobial activity tests of bacterial and fungal isolates from honey.

All of the bacterial isolates from honey were streaked onto a Mueller-Hinton agar plate and incubated for 4 days at 25 °C to allow any probable water soluble antimicrobials to diffuse into the agar. All test organisms were streaked at right angles to the honey isolate, starting from the point furthest away from the part on which the honey isolate had grown. Plates were incubated overnight at 37°C and observed for any inhibition of growth of the test organism. The test organisms used were *E .coli, S. marcescens, B. subtilis, S. aureus* and *B. sphaericus*. Fungal isolates were grown in

Czapek broth medium at 25 °C for 2 weeks. Fungal mycelium was filtered and screened for any antibacterial activity by well diffusion assay (Arasu *et al*., 2008).

2.2.15 Identification of bacterial isolates exhibiting antibacterial activity.

Two isolates were identified using 16s rRNA gene sequencing dependant-technique. DNA was extracted using a Anachem Key prep- Bacterial DNA Extraction kit, and then bacterial 16S rRNA gene was amplified and sequenced (as described in section 2.2.16).

2.2.16 The incidence of *Clostridium botulinum* in Manuka honey

2.2.16.1 *Clostridium botulinum* isolation

Dilution centrifugation (DC) (Kuplulu *et al*., 2006) was used to isolate *Clostridium botulinum*; 20 g of honey were diluted in 80 ml sterile distilled water with 1% Tween 80 (Sigma-Aldrich) and shacken until the solution became homogenous. The solution was held at 65 °C water bath for 30 min then centrifuged two times for 30 min at 8700-9000 × *g*. The pellets were suspended in 10 ml of sterile 1% peptone-water and were subject to a heat shock (80 °C for 10 min) (Bianco *et al*., 2008). These suspensions were then transferred into 9 ml TPGY (Trypticase-Peptone-Glucose-Yeast Extract Broth) and incubated in anaerobic conditions at 30°C for up to 10 days, 0.1ml of each tube showing a significant growth was inoculated onto SPS Agar (Sulfite Polymyxin Sulfadizine Agar) plates (Angelotti *et al*. 1962), and incubated under the same conditions for 3 days. Colonies which grew only anaerobically were considered to be pure cultures and were confirmed by 16S rRNA PCR (as described in section 2.2.16).

2.2.17 16SrRNA sequencing

2.2.17.1 Extraction of genomic DNA from pure cultures for PCR

Genomic DNA was extracted from honey isolates using Key Prep-Bacterial DNA Extraction kit the following procedure. 4 ml of culture grown overnight in LB medium was centrifuged at 6,000×g for two minutes at room temperature. The supernatant was decanted completely. The cell pellet was then washed with TE buffer and completely resuspended in 100 µl of buffer R1 by pipetting it up and down. Then10µl of lysozyme (50mg/ml) was added to the cell suspension and mixed thoroughly. After 20 minutes incubation at 37°C, the digested cells were collected by centrifugation at 1000×*g* for 3 minutes and the supernatant was decanted immediately. Collected pellet was then resupended in 180µl of Buffer R2 and 20 µl of Proteinase K, mixed thoroughly and incubated at 65 °C for 20 minutes in a shaking water bath. In order to obtain a RNA-free DNA, 20 µl of RNase A (DNase-Free, 20mg/ml) was added and followed by incubation at 37°C for 5 minutes. Homogeneous solution was obtained by adding 2 volumes of Buffer BG to the previous mixture and mixed thoroughly by inverting tube several times then incubated at 65 °C for a further 10 minutes, immediately 200 µl of absolute ethanol was added and mixed carefully. The samples were transferred into a column assembled in a clean tube and centrifuged for 1 minute at 10,000×g, supernatants were discarded. The column was then washed with 750 µl of the wash buffer and centrifuged twice for 1 minute at 10,000×g in order to remove the residual ethanol, supernatants which were also discarded. Finally, the column was placed into a clean microcentrifuge tube and 100 µl of preheated Elution Buffer, TE buffer or sterile water was added directly onto column membrane and left for 2 minutes which then centrifuged at 10,000×g for 1 minute to elute DNA, to verify its presence and purity, 10 µl of each resultant DNA was mixed with 2 µl of Blue/Orange 6x loading dye and electrophoresed using 1% agarose gel. The checked DNA was stored at -20°C.

2.2.17.2 PCR amplification of 16S rRNA genes and purification.

Amplification of 16S rRNA was performed in a total volume of 50 µl containing 2.0 µl Genomic DNA, 1.0 µl dNTPs, 0.5 µl Taq DNA polymerase (Bioline), 2.5 µl 50mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 5.0 µl 10x buffer and 38µl of sterile distilled water (sdH_2O). The PCR reaction mixtures, after incubation at 94°C for 3 minutes as an initial denaturation, were cycled 30 times through the following temperature profile: denaturation for 1 minute at 94°C; annealing for1 minute at 60°C; and elongation for 5 minutes at 72°C with final incubation for 5 minutes at 75°C, after which 10 µl of each PCR amplification mixture was mixed with 2 µl of Blue/Orange 6x loading dye and analysed by 1% agarose gel electrophoresis. In addition, 6 µl of 1 Kb Hyper ladder loading was used to confirm the correct sized product.

Table 2.1 Oligonucleotide primers used.

Oligonucleotide	Sequence, 5'-3
Forward primer	**5' CCG AAT TCG TCG ACA ACA GAG GAT CCT GGC TCA G 3' (34)**
Reverse primer	**5' CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T 3' (37)**

2.2.18 Quantification and purity of nucleic acid

2.2.18.1 Agarose gel electrophoresis

The presence, size and quantity of the DNA were checked and determined by electrophoresis in 1% agarose gel which was prepared as follow: 0.5g of molecular biology grade agarose was dissolved in 1ml of 50x TAE buffer and 40ml distilled water. The agarose was completely dissolved by heating in a microwave oven. The solution was mixed gently and allowed to cool to 55°C, and then 2.5 µl 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 and allowed to stand in room temperature for 20 minutes. Then the comb and seal were removed and gel was placed into an electrophoresis tank and submerged in 1x TAE buffer. 10 µl of the DNA sample was mixed with 2 µl of

Blue/Orange 6x loading dye and loaded into the wells. 6 µl of Hyper Ladder was loaded as well into one of the wells as a reference. The samples were electrophoresed for 40 minutes at 80V to allow DNA to migrate toward the anode. The DNA fragments were visualized under a UV transilluminator and the images were captured using a connected digital camera.

2.2.18.2 16SrRNA sequencing and phylogenetic analysis

After checking, the purified PCR products were sent to the Medical School Core Genetics Unit (University of Sheffield) to be sequenced. 16SrRNA 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 National Centre for Biotechnology Information (NCBI)http://www.ncbi.nlm.nih.gov, to identify matches with existing characterized reference sequences.

2.2.19 Statistical Analysis

All observations were presented as Mean ± SD. (Standard deviation). The data was analyzed by SigmaPlot© 11.0. $P<0.05$ was considered as significant.

2.3 Results and discussion

2.3.1 Investigation of the antimicrobial activity of selected honeys from different origins.

A total of eighteen honey samples from different origins were evaluated for their antibacterial activity against selected bacteria species representing the Gram-positive species, *Staphylococcus epidermidis Bacillus sphaericus,* and *Bacillus subtilis,* and the Gram negative species, *Serratia marcescens*, and *Escherichia coli. Bacillus subtilis, S.epidermidis, B.sphaericus* and *S.marcescens*; are all opportunistic pathogens, which commonly cause persistent wound infections in immunocompromised patients, while *E.coli* is an enteric organism. In general, as shown in (Table 2.2) all tested honeys, except Kent and Gain Japan honeys, showed a measurable antibacterial activity against all of the tested bacteria with different values. Four of the tested bacteria were most sensitive to Greek pine honey comparable to other tested honeys showed a significant inhibition zone against Gram-negative bacteria, S. *marcescens*, and *E.coli,* 17.0±1.0 and 18.3±1.2 respectively. Kent honey and Gain Japan honey either showed no or limited inhibition to the tested bacteria, especially Gram-negative bacteria. Scottish heather honey displayed a potent activity against *S.epidermidis*, 23.7±1.2 mm, and moderate activity against other bacteria. New Zealand beech honey displayed a potent activity against only *B. subtilis,* 20.7±0.6 mm, and moderate activity against other bacteria. S. *marcescens* displayed the highest resistance for 61% (11 out of 18) of tested honeys, whereas *B. subtilis* was the most sensitive bacteria for 56% (10 out of 18) of tested honeys. These data do not agree with the results reported by El-Sukhon *et al* (1994) who showed that the Gram-positive bacteria are more resistant to the inhibitory action of honey than are Gram negative bacteria (El-Sukhon *et al.*, 1994).

Table 2.2 Antibacterial activity of selected honeys from different origins against five different bacterial species, determined by agar diffusion. The values are means of replicates (well (8.0mm)) ± Standard deviation. NT: not tested.

	Inhibition zone (mm)±SD				
Honey	*E.coli*	*S. marcescens*	*B. sphaericus*	*S. epidermidis*	*B. subtilis*
Greek Pine	17.0 ±1.0	18.3 ±1.2	16.3 ±1.5	17.3 ±2.1	20.3 ±1.2
Yorkshire	15.8 ±2.2	12.6 ±1.1	13.8 ±1.5	13.2 ±1.3	16.2 ±0.8
Chilean ulmo	15.7 ±0.6	15.3 ±0.5	15.3 ±0.5	19.0±0.0	16.0 ±1.0
Australian Eucalyptus	14.3 ±0.5	12.3 ±0.5	14.3 ±0.5	13.6 ±0.5	16.6 ±1.2
Himalayan wild flower	13.8 ±0.3	11.2 ±0.3	14.0 ±1.0	16.2 ±0.8	13.0 ±1.0
Scottish heather	13.7 ±1.2	12.7 ±0.6	15.7±0.6	23.7 ±1.2	17.0 ±0.0
Chilean	13.6 ±0.6	13.5 ±0.7	14.3 ±0.6	14.0 ±0.0	14.3 ± 0.6
New Zealand Clover	13.0 ± 0.6	12.3 ±0.5	16.2 ±0.8	15.2 ± 0.0	14.3 ±0.6
Cuban Comparitan	11.7 ±0.6	12.8 ±1.1	11.7 ±0.6	12.3 ±0.6	13.0 ±1.0
Acacia Hungarian	11.3 ±0.6	12.0 ±0.0	12.0 ±0.0	12.7 ±1.2	11.3 ±0.6
Spanish Orange blossom	11.0 ± 0.5	13.2 ±0.6	11.3 ± 0.6	11.8 ±0.3	10.7 ±0.3
Tasmanian Leatherwood	13.2 ±1.3	12.7 ±0.6	13.7 ± 0.6	13.3 ±0.6	15.7 ±2.5
Organic honey	12.3 ±0.6	11.2 ±0.3	12.3 ±1.2	13.0 ±2.0	15.3 ±0.5
New Zealand beech	12.3 ±0.6	14.3 ±0.5	16.0 ± 1.0	15.7 ±2.5	20.7 ±0.6
Jarrah honey	13.0 ± 1.0	14.0 ±2.0	15.7 ±1.5	18.3±1.2	17.0 ±1.0
Kent honey	0.00	11.3 ±0.6	11.3 ±0.6	11.7 ±1.2	14.0 ±2.6
Gaint Japan	0.00	0.00	11.0 ±0.0	11.3 ±1.5	NT
Troway Hall	13.8 ±0.3	14.3 ±1.5	NT	16.3 ±2.1	16.7 ±1.5

Table 2.2 shows that the majority of tested honeys have broad-spectrum antibacterial activity, particularly Greek Pine, Scottish Heather, Chilean Ulmo, New Zealand Beech and Jarrah Honey**.**

In further attempts to determine if this broad-spectrum antibacterial activity was due to the activity of hydrogen peroxide or due to another factor, comparable to different medical grade Manuka honeys, eighteen different origin honeys (Table 2.3) were evaluated for their total antibacterial activity and non-peroxide activity against methicillin-sensitive *S. aureus* (MSSA), expressed as equivalent phenol concentration (% w/v). In order to measure non-peroxide activity, tested honeys were diluted in a catalase solution to breakdown the inherent hydrogen peroxide. The lowest phenol concentration able to produce a measurable inhibition zone was 3% (w/v) and absence of zones of inhibition indicated activity lower than 3% (w/v) phenol equivalent. The results of the pH of the tested honeys (Table 2.3) indicated that all fell within the ranges normally expected for honey's pH, i.e.between 3.1 and 4.5 (Molan, 1992a).

All of the investigated different origin honeys exhibited some antibacterial activity (total activity, peroxide + non-peroxide activity) as shown in (Table 2.3) but levels were lower than the most of the medical grade Manuka honeys and ranged from 4.4% (w/v) to 8.8% (w/v).

Except for medical grade Manuka honeys that are well known to have unique non-peroxide activity, none of the tested honey had a detectable non-peroxide activity, more than 3.0 w/v phenol. As expected Comvita UMF +25 Manuka had the largest non-peroxide activity equivalent to 10.7% (w/v) phenol while Comvita UMF +20 Manuka had the lowest non-peroxide activity equivalent to 8.1% (w/v) phenol among referenced medical grade Manuka honeys.

Table 2.3 The total antibacterial activity and the non-peroxide activity of selected honeys from different origin against methicillin-sensitive *S. aureus* (MSSA), expressed as equivalent phenol concentration (% w/v), determined by agar diffusion, and the pH of these honeys. (Only the honeys with the highest activity were investigated to determine their non-peroxide activity). NT: not tested

Honey	pH	Total antibacterial activity as phenol equivalent (w/v) % (peroxide + non-peroxide activity	Non-peroxide activity as phenol equivalent (w/v) %
Comvita® UMF® 25+ Manuka	3.6	11.9	10.7
Manuka 250MGO	3.8	10.6	10.3
Comvita® UMF® 15+ Manuka	3.8	9.8	8.9
Littleover Apiary Manuka active 10+	4.1	9.3	8.7
Greek Pine	3.4	8.8	< 3.0
Comvita® UMF® 20+ Manuka	3.2	8.3	8.1
Scottish heather honey	4.5	7.5	< 3.0
Organic honey	NT	7.3	< 3.0
Australian Eucalyptus	3.7	7.2	< 3.0
Yorkshire Honey	3.5	6.9	< 3.0
New Zealand Beech	4.6	6.7	< 3.0
Chilean Honey	4.1	6.5	< 3.0
Himalayan Wild Flower	NT	6.5	< 3.0
Tasmanian Leatherwood	3.8	6.3	< 3.0
Spanish Orange Blossom	3.5	6.1	< 3.0
Chilean Ulmo	4.2	5.9	< 3.0
Troway Hall	NT	5.6	< 3.0
Jarrah Honey	4.5	5.6	NT
New Zealand Cclover	3.4	4.8	< 3.0
Kent Honey	3.5	4.8	NT
Cuban Comparitan	3.5	4.4	< 3.0
Hungarian Acacia	3.3	4.4	< 3.0
Gaint Japan	3.1	< 3.0	NT

Surprisingly, another medical grade Manuka obtained from different company (Littleover Manuka 10+ active) had a non-peroxide activity equivalent to 8.7 % (w/v) phenol which more than Comvita UMF +20 Manuka (Figure 2.3), although both companies use the same standard to evaluated their Manuka honey grades. This means that UMF may be subject to large variations depending on the producing company.

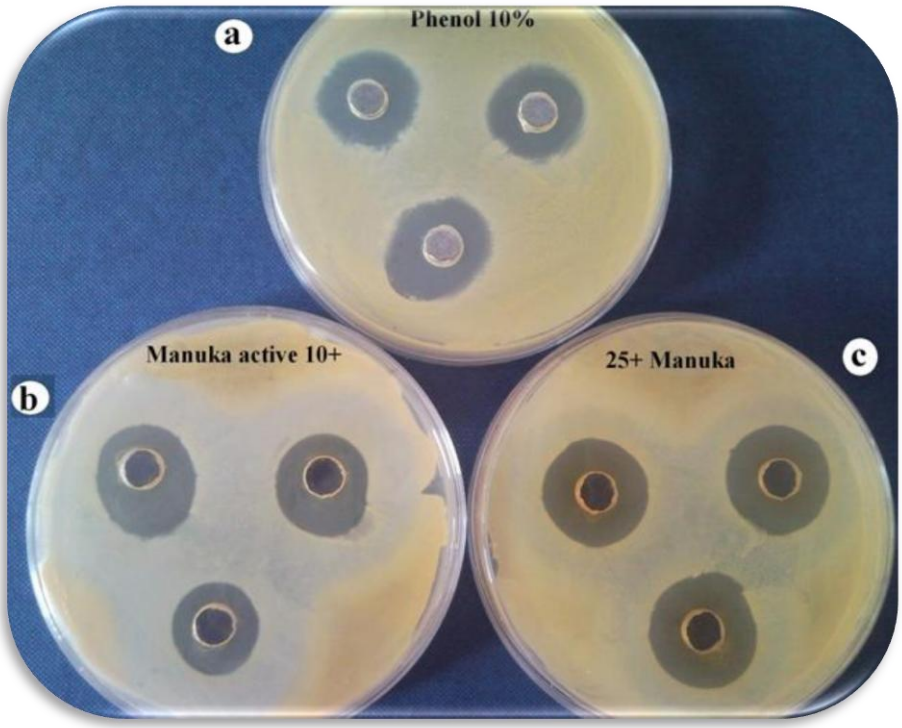

Figure 2.3 Comparison of the results of agar diffusion assay for two medical grade Manuka honeys obtained from two different companies and phenol 10% (w/v) solution. Plates seeded with MSSA: (a) the zone of inhibitions caused by phenol 10 %, (b) the zone of inhibitions caused by Littleover Apiary honey and Manuka active 10+, (c) the zone of inhibitions caused by Comvita® UMF® 25+ Manuka **.**

All tested honeys showed a detectable activity except Gaint Japan Honey which did not exhibit any antibacterial activity against methicillin-sensitive *S. aureus* (MSSA). Ten of these honeys were equivalent to more than 6.0 w/v % phenol; eight types were equivalent to 4.4-5.9 w/v %phenol.

The variation in the honey antibacterial potency has been well reported; it can be vary much as 100-fold (Molan, 2001). Peter Molan, a pioneer researcher in the Waikato Honey Research Unit, concluded that not all honeys can be used for therapeutic purposes, and he recommended that some care must be taken before a honey is chosen as a wound dressing, such honeys should have a high level, and a wide spectrum of antibacterial activity, particularly against bacteria commonly associated with wound infections, and should also have a marked non-peroxide activity. Non-peroxide antibacterial activity is essential due the fact the wound exudate may contains catalase which might destroy to some extent the honey's peroxide activity when applied to the wound surface, while any-peroxide activity would be still active. *Leptospermum scoparium,* Manuka honey has been advocated for wound care for those reasons (Molan, 2004; Molan, 2006).

Searching for honeys with exceptionally high non-peroxide antibacterial activity has been a core aim of many studies. In a large survey for 345 New Zealand honeys, Allen *et al*. (1991) found that only 25 samples of Manuka and vipers bugloss honeys exhibited a detectable non-peroxide activity. Another study of selected Portuguese honeys showed that all of them had a peroxide antibacterial activity while only 23% (7 out of 30) showed a measurable non-peroxide activity (Henriques *et al*., 2005).

The results shown in Table 2.3, show that none of tested honeys exhibited an exceptionally high non-peroxide activity, thereby suggesting that none of tested honeys could achieve medical grade status and act as an alternative to currently used Manuka medical grade honeys. However, the wide spectrum antibacterial activity of some tested honeys, obtained commercially in retail outlets, like Greek Pine, Scottish heather, Chilean ulmo, New Zealand beech and Jarrah honey suggests that they might be used in treating opportunistic infections where expensive Manuka honeys are not

vailable particularly in developing countries; these honeys might also be used in veterinary medicine, where less regard to the safety is required.

2.3.2 Further investigation on the nature of non-peroxide antibacterial activity in *Leptospermum scoparium* Manuka honey.

The data shown in Table 2.3 strongly confirmed the non-peroxide antibacterial activity of the Manuka honey, a fact which has been well documented (Allen *et al.*, 1991; Molan, 1999b), and showed that the use of a catalase concentration up to 100-fold failed to remove this kind of activity. Why *Leptospermum scoparium* Manuka honey should have this unique activity has been extensively investigated. One suggested hypotheses is that Manuka honey has a residual high level of hydrogen peroxide concentration that the normal catalase treatment is not sufficient to eliminate (Weston, 2000). To test this hypothesis the antibacterial assays were repeated using 10, 40, 50, 75 and 100-fold excess of catalase concentration.

The data given in Figure 2.4 shows however, no significant differences between the test performed using the normal amount of catalase and the test performed using a 10, 40, 50, 75 and 100 fold excess of catalase, P values of t-test were 0.988, 0.957, 0.972, 0.980 and 0.949 respectively.

The results presented here, therefore, show that the unusual non-hydrogen peroxide activity of the Manuka honey is unlikely to be due to accumulated residual hydrogen peroxide that cannot be destroyed by the normal catalase concentration.

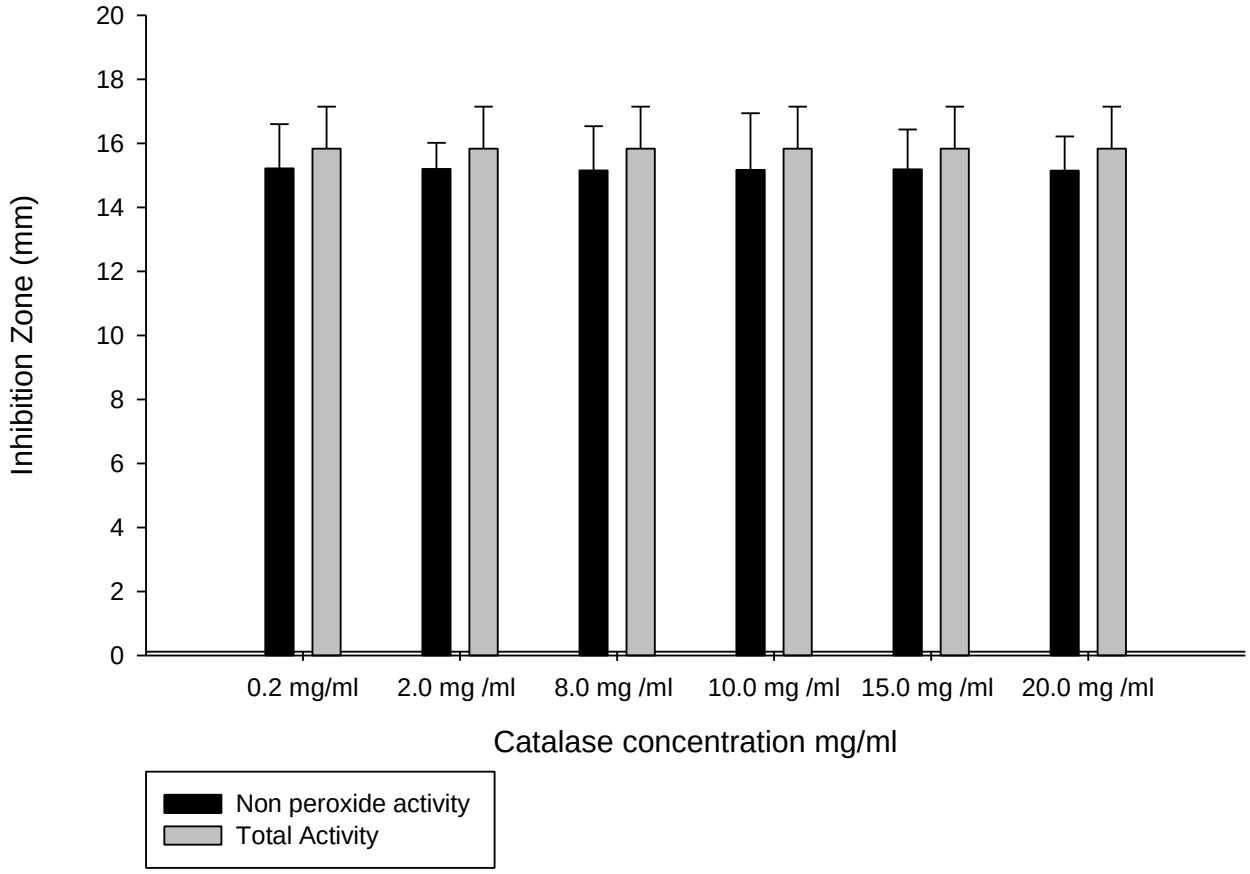

Figure 2.4: Antibacterial assay of 50% (w/v) Manuka honey treated with 10, 40, 50, 75 and 100 fold excess of catalase concentration (non-peroxide activity) compared with non treated 50% (w/v)% Manuka honey (Total activity) , the assay carried out against MSSA, expressed as a mean zone of inhibition of the replicates ± standard deviation.

2.3.3 Evaluation of the antibacterial activity of Tregothnan English Manuka antibacterial activity

In 2008, the Tregothnan Company (Cornwall, UK) introduced the first Manuka honey to be produced in the UK. The following work was aimed at investigating the antibacterial efficiency of Tregothnan English Manuka and compares it with New Zealand Manuka and specifically to evaluate its non-hydrogen peroxide-derived activity. A comparison of the antibacterial activity of Tregothnan English Manuka and New Zealand Manuka was performed by assessing their activity against selected bacterial strains including MRSA and also to determine their non-peroxide activity. Figure 2.5 shows that except for *Serratia marcescens*, all tested bacteria were significantly more resistant to the Tregothnan English Manuka than to New Zealand Manuka. For example, the zone of inhibitions caused by New Zealand Manuka were significantly larger than those caused by Tregothnan English Manuka against MRSA, MSSA and particularly, *B. subtilis* which was 22.0 ±0.8 to New Zealand Manuka and 12.3±1.2 to Tregothnan English Manuka, while there was no statistical significant difference (*P = 0.168*) between the two honeys against *Serratia marcescens*; 13.0±1.2 and 14.7±0.6, respectively. Although Tregothnan English Manuka exhibited an antibacterial activity against all tested bacteria, no exceptionally high activity was seen. It is clear from the data in Figure 2.5 and in the data in Table 2.2 that bacteria were not uniformly affected by the honeys and the Gram-positive bacteria are more susceptible to honey than Gram-negative.

Since New Zealand Manuka honey is well known to have a high non-peroxide activity, the non-peroxide activity of Tregothnan English Manuka was determined. The total antibacterial activity and non-peroxide activity of Tregothnan English Manuka are shown in Figure 2.6, Tregothnan English Manuka lost more than 90% of its antibacterial activity when it treated with catalase enzyme which indicated that the antibacterial activity in Tregothnan English Manuka is mainly to be due to hydrogen peroxide, i.e., Tregothnan English Manuka has not a high non- peroxide activity as does New Zealand Manuka honey.

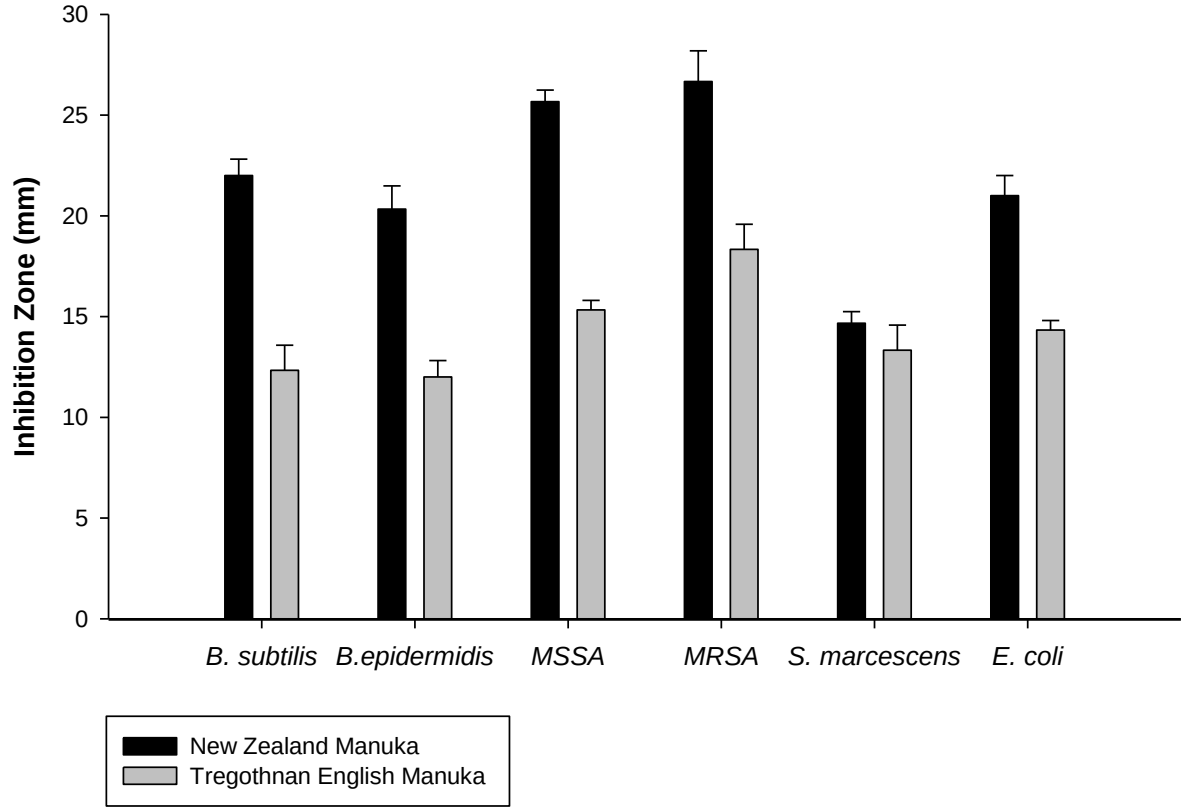

Figure 2.5: A comparison of the sensitivity of some selected bacteria to the antibacterial activity of New Zealand Manuka and Tregothnan English Manuka. Expressed as a mean zone of inhibition (including well (8.0mm)) of the replicates ± standard deviation.

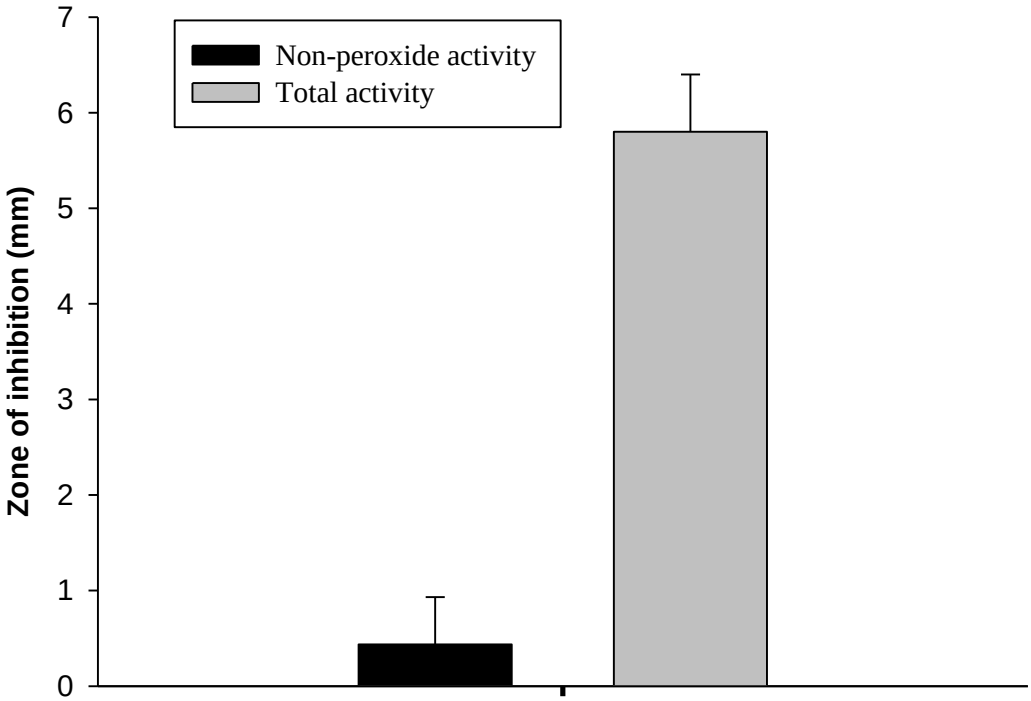

Figure 2.6: Total antibacterial activity and non-peroxide activity of Tregothnan English Manuka. Assay was carried out against MSSA, Expressed as a mean zone of inhibition of the replicates ± standard deviation**.**

However, Basson and Grobler (2008) have reported that not all Manuka honey have exceptionally high antibacterial activity and marked non-peroxide activity, it seems that the most common is Manuka honey produced from the East Cape region of the North Island of New Zealand (Basson and Grobler, 2008).

2.3.4 Studies on the mechanisms of the antibacterial activity of the medical grade New Zealand Manuka.

Although a large body of work exists which describes the antibacterial activity of New Zealand active Manuka honey, the mechanisms of such activity have not been fully explored. In the subsequent experiments, the antibacterial activity of two of the highest UMF medical grade New Zealand Manuka honey (25+ and 20+ Manuka) were further investigated by determining their MICs and the MBCs values against selected seven bacterial species, including MRSA; the MICs values were determined using a broth macrodilution, performing a time kill study on MRSA and showing their surface morphology effects by SEM, TEM studies.

2.3.4.1 Zones of inhibition, MICs and MBCs

The zones of inhibition, the minimum inhibitory concentrations MIC and the minimum bactericidal concentrations (MBCs) values of 20+ Manuka and 25+ Manuka for selected bacteria, including methicillin-resistant *S. aureus* (MRSA) are shown in the Table 2.4.

Generally, and as expected, 25+ Manuka was more powerful than 20+ Manuka, for example, 25+ Manuka displayed larger zones of inhibition against all tested bacteria. For both Manuka honeys *S. marcescens* was the most resistant bacteria, the zones of inhibition were 15.3 ±0.6 for 25+ Manuka and 13.0 ±0.5 20+ Manuka, while methicillin sensitive *S. aureus* (MSSA) bacteria was the most sensitive tested bacteria with zones of inhibition equal to 24.0 ±0.8 for 20+ Manuka and 26.0 ±0.8 for 25+ Manuka. However, these differences between both Manuka were not significant except towards *B. subtilis* where the zone of inhibition was 20.5 ±0.5 for the 20+ Manuka and 25.0 ±0.9 for 20+ Manuka. The results shown previously in Table 2.2 and in Table 2.4 show that both Manuka honeys had an exceptionally wide spectrum of antibacterial activity compared to the eighteen different honeys tested before.

Table 2.4: The zone of inhibition (mean±SD), (diameter mm including well (8.0mm)), the minimum inhibitory concentrations (MICs) (% v/v) and the minimum bactericidal concentrations (MBCs) values of 20+ Manuka and 25+ Manuka for selected bacteria including methicillin-resistant *S.aureus* (MRSA).

Tested bacterial strains	UMF®20+ Manuka			UMF®25+ Manuka		
	Zone of inhibition (mm)	MICs	MBCs	Zone of inhibition (mm)	MICs	MBCs
E.coli	22.2 ±1.0	9%	11%	23.0 ±0.0	8%	10%
S. marcescens	13.0 ±0.5	12%	> 15%	15.3 ±0.6	12%	13%
B. sphaericus	19.0 ±0.9	11%	12%	22.2 ±0.2	8%	9%
S. epidermidis	19.4 ±0.5	11%	12%	21.8 ±0.7	10%	12%
B. subtilis	20.5 ±0.5	10%	12%	25.0 ±0.9	9%	12%
MSSA	24.0 ±0.8	7%	8%	26.0 ±0.8	6%	8%
MRSA	21.3 ±1.2	9%	12%	25.7 ±1.5	6%	10%

In order to obtain more quantitative and precise results the agar well diffusion method was compared with the MIC and MBC values. In Table 2.4, it can be seen that MIC and MBC values of 25+ Manuka against all tested bacteria were somewhat less than from the 20+ Manuka. The MIC and MBC of both Manuka honeys against MSSA were found to be relatively lower than those against other tested bacteria, and ranged from 7 to 8% for 20+Manuka and from 6-8% to 25+Manuka. This result is in agreement with the findings of Cooper (2008) who concluded that generally speaking, *Staphylococci* appear to be the species most sensitive to the antibacterial effects of honey. MICs and MBCs values obtained in this assay are agreed with the results obtained from the agar well diffusion and show that *S. marcescens* was the least sensitive among tested bacteria and had the largest MICs and MBCs. It is worth noting the similarity in susceptibility to 25+ Manuka honey between methicilin-resistant *S. aureus* and methicillin-sensitive *S. aureus* strains where both strains were completely inhibited at 6%.

The results show that both Manuka honeys, at a concentration ranging from 6% to 12% , inhibit the growth of all tested bacteria, which means that the growth of these tested bacteria will be prevented even when New Zealand medical grade Manuka honey is diluted by exudates of the wound up to 9-to 18-fold.

Manuka honey with 25+ UMF showed more inhibitory effects than Manuka honey with 20+ UMF, which emphasises the credibility of the UMF standard and showed the necessity to use the Manuka with the available highest UMF in wound management. According to Cooper *et al.* (1999) the growth of *S. aureus* can be prevented by sugar solution with water activity of 0.864, equivalent to a concentration of 29% (v/v). Our results showed that *S. aureus* was completely inhibited by 6% (v/v) of New Zealand Manuka which showed that the osmolarity of honey does not play a key role in Manuka's antibacterial activity mechanism.

The ratio between the MBC and MIC has been used to distinguish between the bactericidal and bacteriostatic mode of action. If the ratio is less than 4 the antimicrobial agent being tested is considered to have a bactericidal mode of action whilst if it is more than 4 the activity of the antimicrobial agent is considered to be bacteriostatic (Forlenza *et al*., 1981). In this study the ratios between the MBC and MIC of the both Manuka against all tested bacteria were less than 4, indicating that the mode of action for both Manuka honeys is a bactericidal rather than a bacteriostatic. Manuka bactericidal effect could accelerate the wound healing process as it reduces the microbial population density inside the infected wound.

2.3.4.2 Time kill and dose response curves for 25+Manuka and 20+Manuka against methicillin-resistant *Staphylococcus aureus* (MRSA).

Since MRSA is currently the most frequently identified antibiotic-resistant pathogen among hospital patients (Mulvey and Simor, 2009) it was chosen as the organism used to investigate the time kill and dose response curves for 25+Manuka and 20+Manuka.

The dose response curve obtained from plotting the concentration of 25+Manuka and 20+Manuka present against the resultant inhibition of MRSA growth over 20 h incubation is shown in Figure 2.7, it can be seen that the overall performance of 25+ Manuka was significantly more effective against MRSA than +20 Manuka. Manuka +25 achieved complete inhibition of growth at a concentration of 6% (v/v) whereas +20 Manuka accomplished it at 9% (v/v).

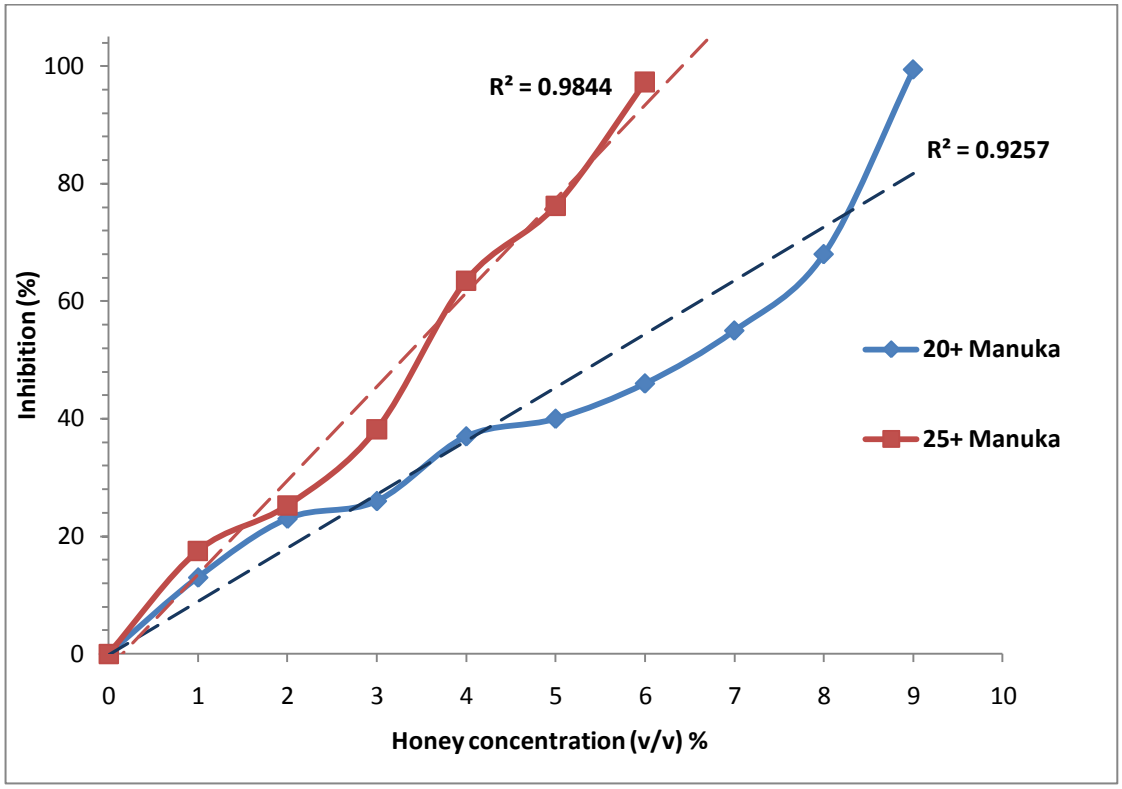

Figure 2.7 : Dose-response curve for the antibacterial activity of 25+ Manuka and 20+ Manuka against methicillin-resistant *S. aureus* (MRSA). The activity is expressed as the percentage inhibition of growth of the bacteria; growth is measured as the increase in the O.D at 600 nm of the culture after 20 hours of incubation.

Both Manuka honeys at low concentrations (1%, 2% and 3%) achieved similar inhibition levels then, as the concentration increased, the inhibition achieved by +25 Manuka gradually becomes significantly more than that achieved by +20 Manuka. It can be concluded that the osmolarity effect does not play a key role in the activity of New Zealand Manuka and that the most effective components of New Zealand Manuka is strongly related to the UMF.

Regarding to the time-to-kill curve of both Manuka honeys against MRSA, shown in Figure 2.8, 12% v/v concentration of both Manuka honeys was used because this concentration is equal to twice the MIC of the 25+ Manuka and greater than its MBC while it is equal 1.5 times of the MIC of the 25+ Manuka and equal to its MBC.

In contrast to the MICs and MBCs values, time-to-kill curve enable us to determine how quickly Manuka honey acts on the MRSA. In the time-kill curves, a gradual decline over 8 h was seen in the number of viable cells of MRSA bacteria treated with 12% v/v of both tested Manuka honeys, but this decline was more rapid in 25+ Manuka than it was in 20+ Manuka. In other words, the total number of MRSA cells did not decrease at the same rate when they treated with 12% v/v of both tested Manuka honeys, after 8 h incubation. Manuka 25+ caused nearly a 3 log reduction in viable MRSA bacteria counts while 20+ Manuka caused less than a 2 log reduction in counts of viable MRSA.

As the fresh dressings of honey that are used in wound management are changed three times daily (Willix *et al*., 1992), an incubation period of 8 h was chosen to evaluate the bactericidal concentration of Manuka honey against the most frequently identified antibiotic-resistant pathogen MRSA. Data shown in Figure 2.8 indicates that MRSA started to die after 2 hours of incubation with 12% New Zealand Manuka honey which somewhat agree with data obtained from a study carried by Molan (1992b) that showed that the Gram-positive bacteria start to die after 1 hour of honey exposure (Molan, 1992b).

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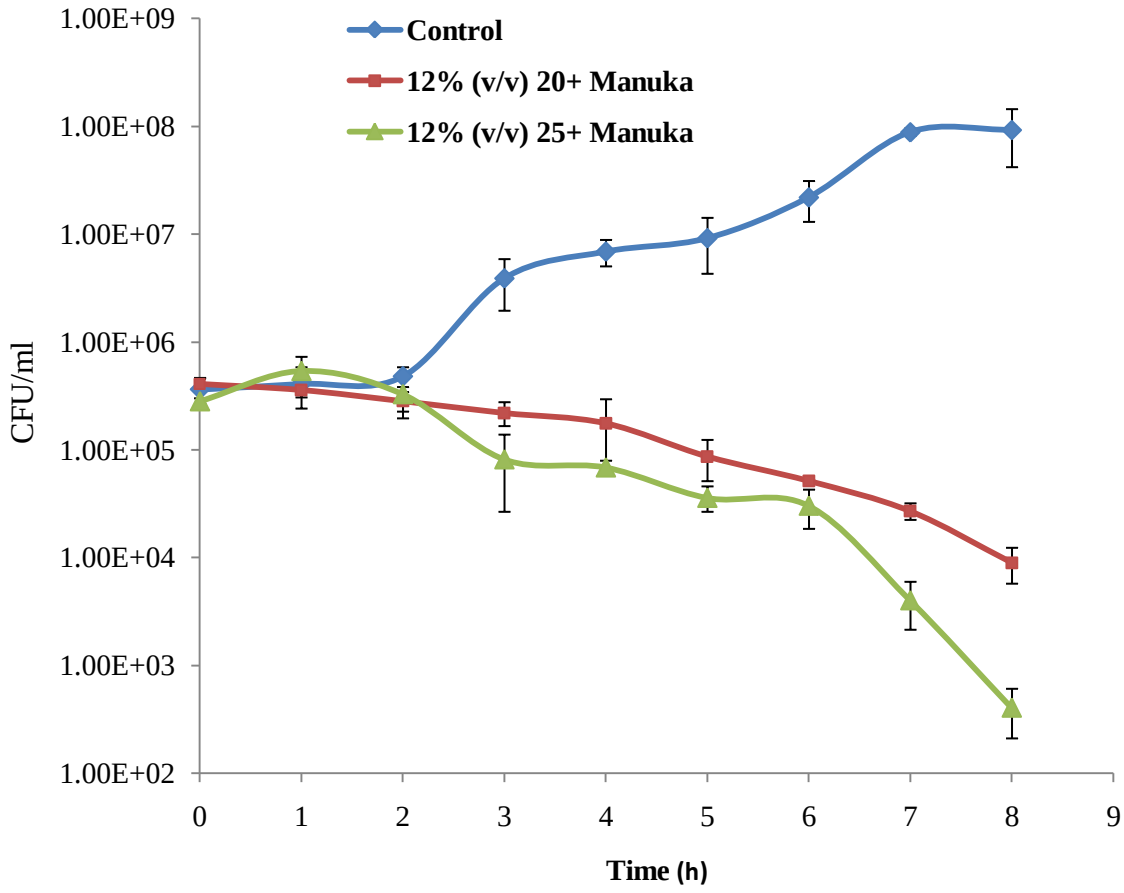


Figure 2.8: Time-kill curves of the effect of 12% (v/v) of 25+ Manuka and 20+ Manuka on methicillin-resistant *S. aureus* (MRSA).

It should be noted however, that although New Zealand Manuka honey is well accepted as an exceptionally effective antibacterial agent with non-peroxide activity, the overall time-to-kill results show that it should not be expected that New Zealand Manuka honey can inhibit or eliminate bacteria within a matter of seconds or minutes. As revealed in the time-to-kill test, New Zealand Manuka honey with the high UMF standard takes at least two hours to begin to exhibit a bactericidal effect on MRSA cells when used at a concentration of 12% and almost certainly takes longer when a lower concentration is used. Nevertheless, results shown in previous experiments support the bactericidal mode of inhibition from New Zealand Manuka honey against the tested bacteria, especially MRSA.

2.3.4.3 Scanning electron microscope (SEM) and transmission electron microscope TEM studies on the morphological effects of 25+Manuka on Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*.

Exploration of the physical effect of the Manuka honey on bacterial cells may allow us to determine the bioactive gradient that causes its exceptional non-peroxide activity; such physical effects can be investigated using electron microscopy. Only Manuka 25+ was chosen for use in these experiments; methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* were used to represent Gram-positive and Gram-negative bacteria respectively.

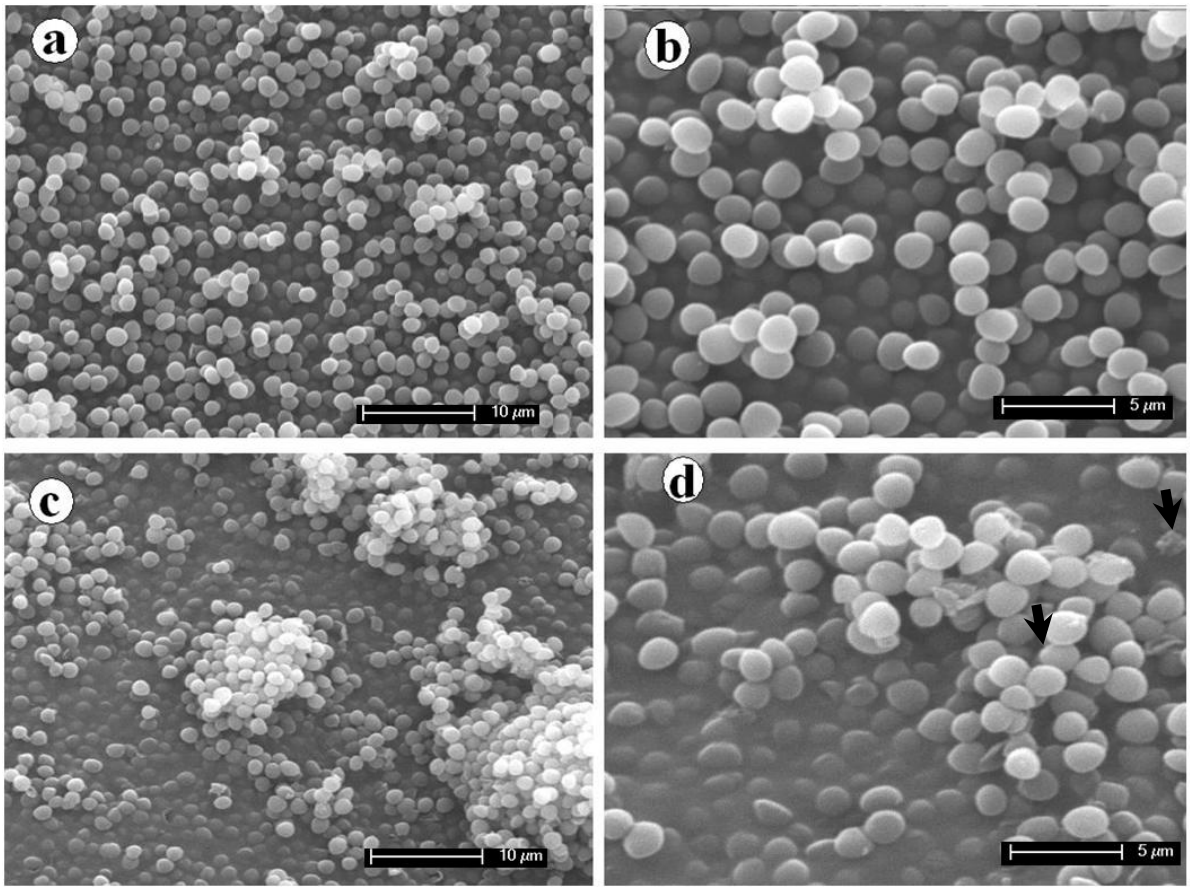


Figure 2.9: External morphology of Methicillin-resistant *Staphylococcus aureus* (MRSA) observed by scanning electron microscopy (SEM).(a) and (b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 4 h at 37°C. (c) and (d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 12% v/v 25+ Manuka honey for 4 h at 37°C. Note black arrows that indicate cells with partially damaged and irregular shape.

The resultant SEM and TEM images are shown in Figure 2.9 and Figure 2.10 which show the effects of 25+ Manuka on the external and internal morphology of MRSA. They show that untreated cells, not exposed to 25+ Manuka, retained their coccal morphology and seemed to be normal while some of the MRSA cells treated with 12 % v/v 25+ Manuka for 4 hours exhibited physical damage (ruptured walls and irregular shape) and some released cellular contents (Figure 2.10, e) . This obviously suggests that some cells underwent lysis and became completely disrupted. It should be noted that the majority of the treated cells were however, still indistinguishable from untreated cells.

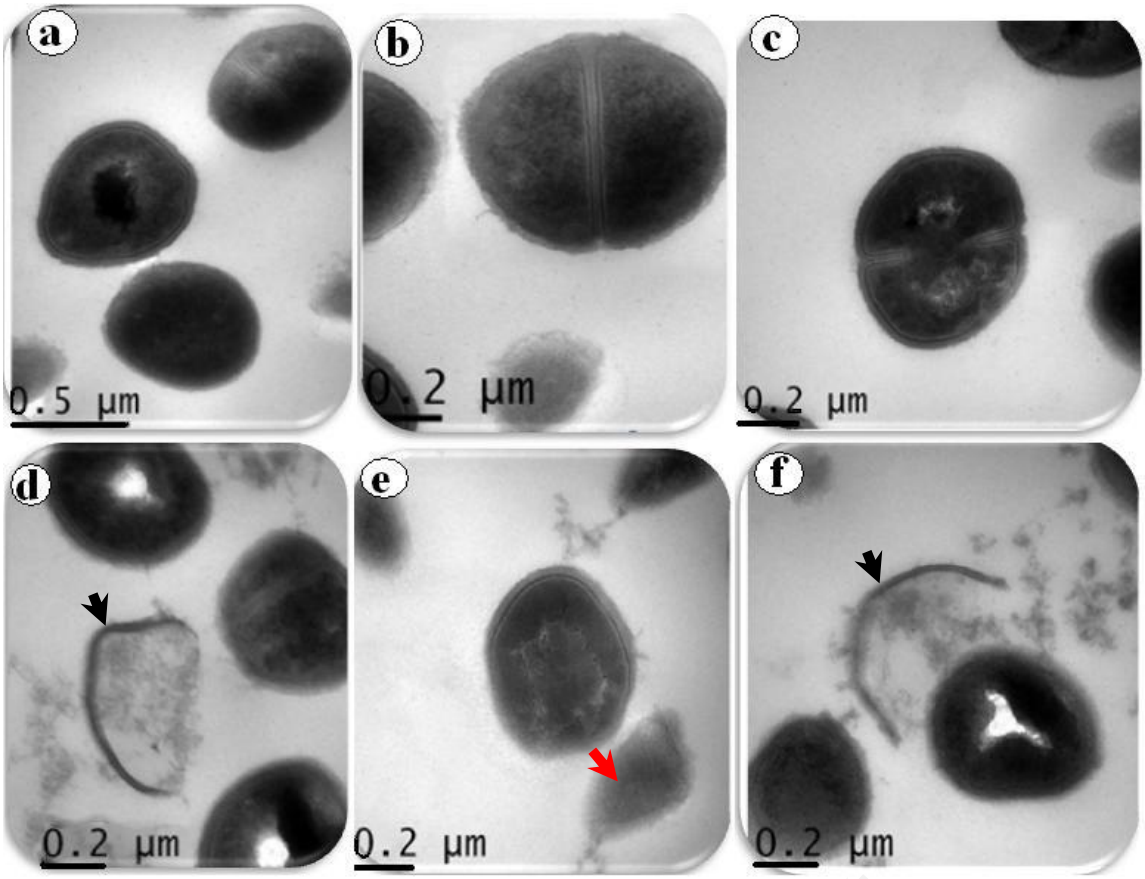


Figure 2.10: internal morphology of Methicillin-resistant *Staphylococcus aureus* (MRSA) observed by transmission electron microscopy (TEM) .(a), (b) and (c) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 4 h at 37°C. .(d), (e) and (f)treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 12% v/v 25+ Manuka honey for 4 h at 37°C. Note the separation of cell membrane from the cell wall (black arrows) and released cellular contents and cell debris (red arrows).

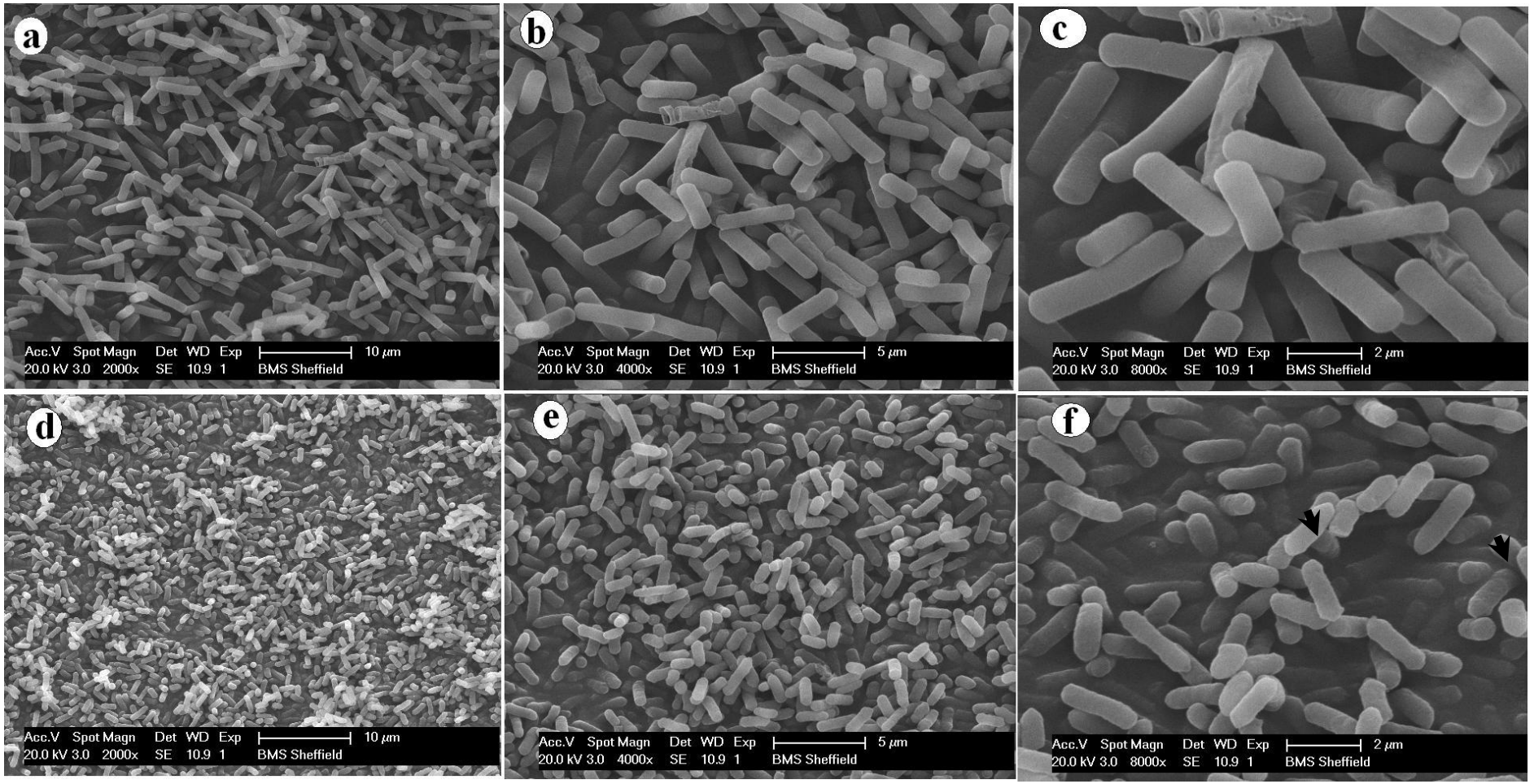


Figure 2.11: External morphology of *Escherichia coli* observed by scanning electron microscopy (SEM) .(a), (b) and (c) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 6 h at 37°C.(d), (e) and (f) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 18% v/v 25+ Manuka honey for 6 h at 37°C. Note abnormal shortened cells with rough surface (black arrows)

SEM and TEM images of *Escherichia coli* (shown in Figure 2.11 and Figure 2.12) show that 18% 25+Manuka honey had little effect on the appearance of the *E. coli* cells, compared to the untreated cells. *Escherichia coli* cells that incubated with 18% 25+Manuka for six hours however, appear shortened and as distorted shapes and some have lost their integrity. Some cells also occur with localized separation of the cell membrane from the cell wall (Figure 2.12, f). In conclusion, generally, both bacteria SEM and TEM images don not show significant morphology changes.

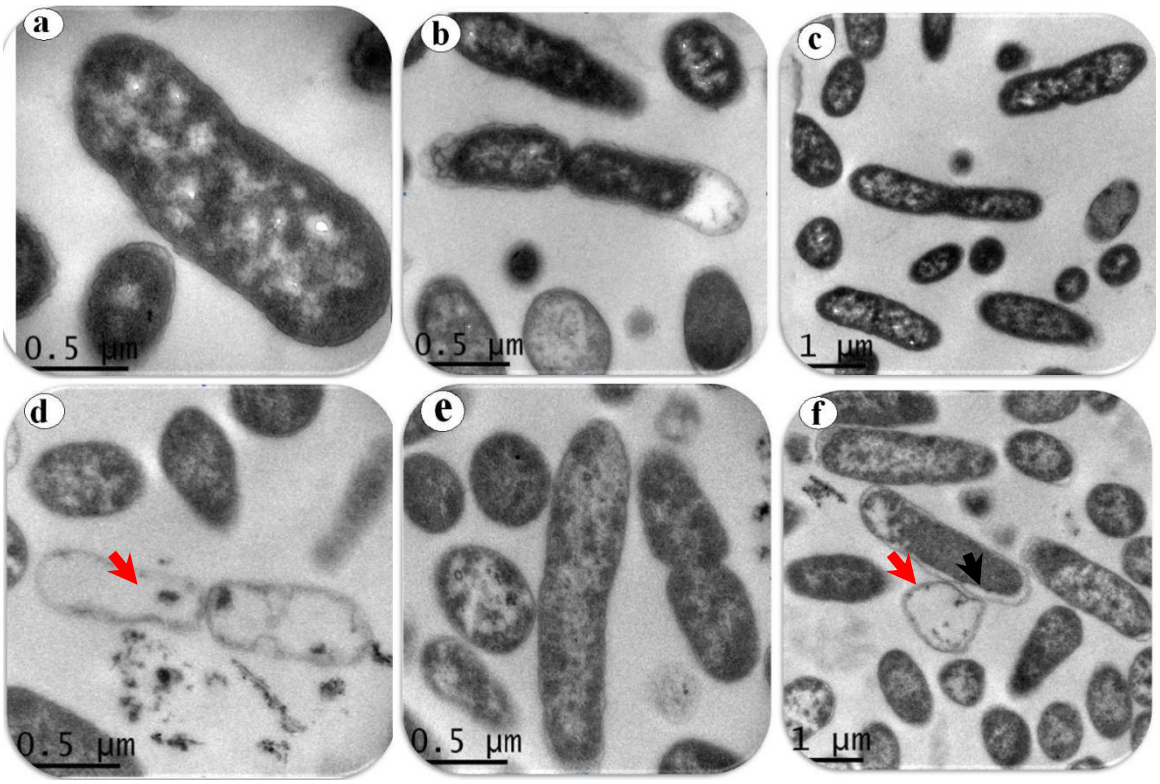


Figure 2.12: Internal morphology of *Escherichia coli* observed by transmission electron microscopy (TEM) .(a), (b) and (c) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 6 h at 37°C. (d), (e) and (f) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 18% v/v 25+ Manuka honey for 6 h at 37°C. Note the separation of cell membrane from the cell wall (black arrows) and cells with released cellular components (red arrows).

2.3.5 Microbial inhibitory components in New Zealand Manuka honey

One hypothesis that has been suggested elsewhere to explain the unidentified antibacterial activity of honey is that some of this unidentified antibacterial activity might be attributed to antibacterial substances produced by honey microflora and even by honeybee- indigenous bacteria prior to honey maturation (Lee *et al*., 2008). In this experiment medical grade New Zealand Manuka honey samples (Comvita UMF 25+ Manuka, 20+ Manuka, 15+ Manuka and 12+ Manuka) were selected to examine the potential of the microorganisms they contain to produce antimicrobial agents.

As shown in Table 2.5 ten bacteria species, three filamentous fungi and two yeasts were isolated from the selected New Zealand Manuka honey samples. Neither isolated yeasts nor isolated filamentous fungi exhibited any antibacterial activity. Only two isolated bacteria showed antibacterial activity; these were subsequently identified using 16S rRNA (Figure 2.13 and Figure 2.14) were found to be *Bacillus pumilus* and *Bacillus atrophaeus.*

Table 2.5: The number of isolated microorganisms and number of bacteria exhibited antibacterial activity

Microorganism	No. of isolates	No. isolates exhibited antibacterial activity
Bacteria	10	2*
Filamentous fungi	3	0
Yeasts	2	0

* These two species were identified as *Bacillus pumilus* and *Bacillus atrophaeus.*

Gram-positivespore-forming bacteria, mainly *Bacillus* genus, and some yeasts are the most commonly found organism in honey due to their ability to survive the harsh conditions present (Olaitan *et al.*, 2007; Snowdon and Cliver, 1996). According to Snowdon and Cliver (1996) primary and secondary contamination sources including

pollen, the digestive tracts of honey bees, dust, air, dirt, flowers, humans, equipment, containers, wind, dust, insects, animals and water; all of these will contribute to the microflora present in honey.

In this study the focus was on the Manuka honey microflora which exhibit antibacterial activity and only isolated bacteria showing antibacterial activity were indentified. Both of the isolated *Bacillus pumilus* and *Bacillus atrophaeus* are Gram-positive spore-forming bacteria. *Bacillus pumilus* is well known to be present in honey (Tolba *et al*., 2007) and is known to produce some substances that could be act as antagonistic factors to fungal and bacterial pathogens, notably a bacteriocin called Pumilicin 4 (Aunpad and Na-Bangchang, 2007), and a surfactin, which is used as an antibiotic (Morikawa *et al*., 1992). Antifungal metabolites (Banerjee *et al*., 2007; Bottone and Peluso, 2003; Munimbazi and Bullerman, 1998), alkaline protease (Kumar, 2002) and endoxylanase are also produced by this bacterium (Panbangred *et al*., 1983). The potential of *Bacillus atrophaeus* to produce bacteriocin subtilosin A has been recognized (Stein *et al*., 2004). This bacteriocin was proven to have antimicrobial activity against a wide spectrum of bacteria (Shelburne *et al*., 2007).

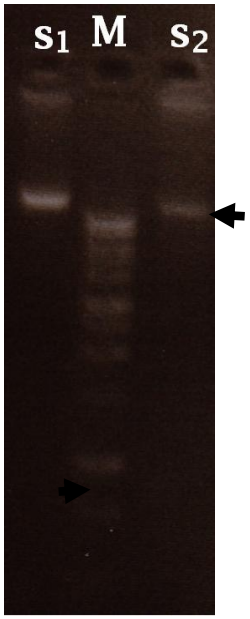


Figure 2.13: Agarose gel electrophoresis revealing a successful DNA extraction from unknown isolated bacteria exhibited antibacterial activity. Lane M, molecular marker. S1 and S2, two bacteria samples.

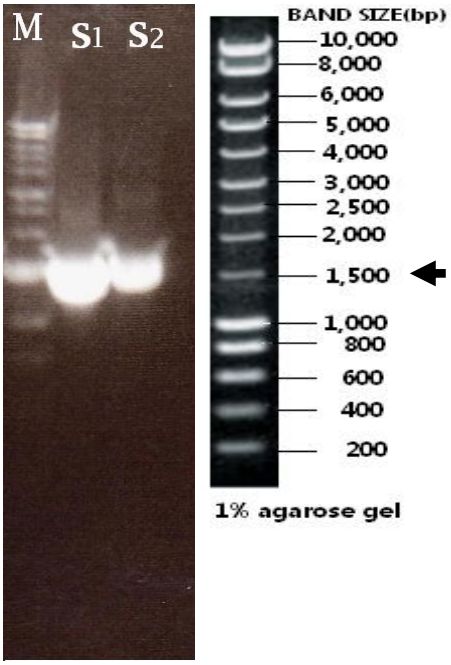


Figure 2.14: Agarose gel electrophoresis revealing a successful amplification of 16S rRNA from unknown isolated bacteria exhibited antibacterial activity. Lane M, molecular marker. S1 and S2, two bacteria samples.

The intrinsic properties of honey including the low pH and high sugar content are considered to provide harsh conditions which bacteria cannot withstand. In addition,

these harsh conditions stimulate bacteria to begin starvation-induced activities such as sporulation (Snowdon and Cliver 1996), which is known to have a link with antibiotic and degradative enzyme production (Marahier *et al.*, 1993; Msadek, 1999; Yan *et al.*, 2003). Recently, a compound called, bee defensin-1 was discovered in a medical-grade honey sample and was shown to make a detectable contribution to the antibacterial activity of tested honey samples (Sherlock *et al*., 2010).

Gram-positive spore-forming bacteria present in honey might provide a source of antibacterial substances produced as a response to the honey-harsh conditions and sporulation process.

2.3.6 Determination of the incidence of *Clostridium botulinum* spores Manuka honey.

Twenty-one samples of seven medical grade New Zealand Manuka honeys obtained from two different companies were analyzed for the presence of *Clostridium botulinum* spores. The results are given in Table 2.6. After being processed according to the dilution centrifugation (DC) method, samples were incubated in TPGY broth media for 10 days. As shown in Table 2.6 in this step 11 out of 21 samples yielded positive results; positive samples were further inoculated onto SPS (Sulfite Polymyxin Sulfadiazine) Agar plates. Only 1 of 11 samples (Littleover Manuka Active 10+) was able to grow anaerobically in SPS Agar. An isolated strain from this positive sample was then identified by 16S rRNA sequencing analysis as *Bacillus licheniformis*. It seems from this study, therefore, that *C. botulinum* spores are not likely to be detectable in tested medical grade New Zealand Manuka honey samples. According to Kuplulu *et al* (2006) the dilution centrifugation (DC) method is more effective at determining the incidence of

Clostridium botulinum spores in honey than other methods such as direct addition (DA) and supernatant filtration (SF). The results presented here show that although

one presumptive *Clostridium* was isolated from honey using both TPGY broth and SPS Agar, this was identified, by 16S rRNA sequencing, as *Bacillus licheniformis* and not *Clostridium botulinum*. Clearly, confirmatory tests should be followed on the isolation of any presumptive *Clostridium botulinum* isolation. Like *Clostridium botulinum, B. licheniformis* is a spore forming Gram-positive bacteria that can grow anaerobically, these shared characterises may explain why *Bacillus licheniformis* gave a false positive when the DC isolation method was used.

Another health issue related to the incidence *Clostridium botulinum* spores in honey was raised by Molan and Allen (1996). They argued that the application of unsterilized honey to wounds could increase the risk of wound infection with *C. botulinum* (Molan and Allen, 1996) and recommended that the honey should be subjected to a commercial sterilization process using gamma-irradiation before using it in wound care. Vardi and colleagues (1998) considered this to be merely as theoretical risk because they did not find a single report in the literature of *C. botulinum* wound infection associated with honey dressings and in their own studies no wound cultures they studied yielded *C. botulinum.*

Recently, Simon *et al* (2009) confirmed that he and his co-workers could not detect a single case report linking the use of unsterilized honey in wound care and C. *botulinum* wound infection. Beside this, local unsterilized honey has widely been used to treat infected wounds and burns without any adverse C. *botulinum* wound infections (Postmes *et al.*, 1996; Postmes, 2001; Subrahmanyam, 1994; Al-Waili and Saloom, 1999).

Table 2.6 Various Medical grade New Zealand Manuka honey samples examined for *C. botulinum* spore incidence

		Step one	Step two	Step three
Manuka honey	No. of tested samples	Positive growth in TPGY broth	Positive growth in SPS Agar (anaerobically)	16S rRNA dependant identification
Comvita UMF 25+ Manuka	3	0	0	
Comvita UMF 20+ Manuka	3	2	0	
Comvita UMF 15+ Manuka	3	2	0	
Comvita UMF 12+ Manuka	3	0	0	
Littleover Manuka Active 15+	3	3	0	
Littleover Manuka Active 10+	3	2	1	*Bacillus licheniformis*
Littleover Manuka Active 5+	3	2	0	

The aforementioned facts, and reports as well as the results obtained in this study (no C. *botulinum* spores were detected in twenty one samples of seven different medical grade New Zealand Manuka honey), suggest that these commercial medical grade New Zealand Manuka honey could be used directly to treat infected wounds in emergency cases, as well as in veterinary practice, when sterilized samples are available. Of course the use of sterilized honey, although an additional expense, is preferable to the use of non-sterilized products.

Chapter Three: The Antimicrobial Activity of Manuka Oil

3.1 Introduction

3.1.1 Essential oils

The essential oils of aromatic plants have long been used in embalming to prevent bacterial growth and avoid decay, a practise generally associated with the Ancient Egyptians (Edris, 2007). The first modern report on the therapeutic use of essential plant oils appeared in 1928, when a French chemist reported that lavender oil facilitated healing of his own hand- burn injury. Many essential oils exhibit antimicrobial activity, including Tea tree oil (derived from the Australian native plant *Melaleuca alternifolia*), and have been recognised for their potential in the treatment of MRSA-related infection as well as an alternative for the treatment of mupirocin-resistant MRSA (Bowler *et al*., 2001). A range of mechanisms of actions by which essential oils can inhibit microorganisms have been proposed. Of particular importance is hydrophobicity, which causes the lipid bilayer to detach from the cell membrane, thereby leading to leakage of whole cell contents. Essential oils may also destroy one, or more, of the bacteria's vital enzyme systems (Edris, 2007).

3.1.2 Manuka oil

Manuka oil from the plant *Leptospermum scoparium* is being investigated as an antimicrobial agent; its activity being based on its high content of triketones (Douglas *et al.*, 2004; Carr, 1998), which inhibits the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and multi-drug-resistant tuberculosis (MDR-TB) (van Klink *et al*., 2005).

A comparative study of the biological activity of different tree oils including Australian Tea tree (*Melaleuca alternifolia*), the New Zealand Manuka (*Leptospermum scoparium*) and Kanuka (*Kunzea ericoides*) carried out by Lis Balchin and his co-workers (2000) showed that Manuka oil has a consistently high antioxidant activity and a marked antifungal effect. In another comparative study, Manuka oil was shown to have a remarkable bactericidal effect against several oral pathogens, and was more active than tea tree oil, eucalyptus oil, lavandula oil, and romarinus oil (Takarada *et al*., 2004).

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

1. Investigate the antimicrobial activity of manuka oil
2. Investigate the Anti-*Trichophyton terrestre* activity of manuka oil

3.2 Materials and methods

3.2.1 Manuka oil

The Manuka oil used was a commercially produced by Phytomed Medicinal Herbs, Auckland, New Zealand.

3.2.2 Test Organisms

The following test organisms were used: *Escherichia coli, Staphylococcus epidermidis, Serratia marcescens,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium phlei* NCIMB8573, *Bacillus subtilis* and *Trichophyton terrestre* (IMI 277732 obtained from the CAB, Kew); unless stated otherwise, the organisms were obtained from the Departmental Culture Collection.

3.2.3 Paper disk diffusion assay

The Kirby Bauer paper disc method was used (Bauer *et al*., 1966). Overnight cultures of the respective test bacteria were standardised (McFarland standardisation), and transferred to MH (Mueller-Hinton) agar plates, which were seeded with 100 µL of each bacterium. Manuka Oil (10 µL) was applied to a sterile filter paper disk (4 mm) placed on the surface of inoculated plates; triplicate plates were used, each one had 3 paper disks and two 30 µg Cefoxitin discs as a positive control. After 24 h incubation at 37°C the zones of inhibition were measured.

3.2.4 Measurement of minimum inhibitory concentration (MIC) using macrodilution:

MICs were measured by broth macro-dilution method. A serial doubling dilution of Manuka oil was prepared in (0.05% Tween 80), with concentrations ranging from 0.002% to 4% v/v. Diluted oil was sterilized and filtered using a 0.20 µm membrane filter, 0.5 McFarland standardised 100 µl of bacteria culture were aseptically added to each dilution. MIC-values were recorded after 18 h of incubation at 37°C with shaking

at 250 rpm; MICs were defined as the lowest concentration of Manuka oil which showed no visible growth.

3.2.5 Determination of minimum bactericidal concentration (MBC).

Diluted broth (15 µl) was transferred into a Petri dish containing MH agar. The plates were incubated overnight at 37°C. The MBC was read as the lowest concentration of manuka oil that did not permit any visible growth on agar plate.

3.2.6 Time killing assay

Inocula for the time kill assays were prepared by inoculating one to two colonies of test bacteria into 8 ml of MH broth and incubating for 18 h at 35°C with shaking, adjusting to a 0.5 McFarland 5 x 10^6 CFU ml^{-1}. 1 ml of the initial inoculum was then added to 9 ml broth containing either 1.5% (v/v) Manuka oil (> 3 MIC) and 0.05% Tween 80 (test) or only 0.05% Tween 80 (control). Treatments were incubated with shaking at 37°C and samples were taken at 0, 1, 2, 4, 6 and 8 h. Viable counts were performed by serially diluting each sample 10-fold in PBS and spreading 100 µL volumes from the appropriate dilutions onto NA in duplicate. After incubation at 37°C, cfu were counted and time to kill plots were constructed.

3.2.7 Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM).

Overnight cultures (10mls) of Methicillin-resistant *S. aureus* and *E. coli* were centrifuged at 4,000 *g* for 20 min, resultant cell pellets were washed with sterilized distilled water; MRSA were cells re-suspended in 10 ml of phosphate-buffer saline (PBS) pH 7.4 with and without 3 % v/v Manuka oil and incubated for 4 h at 37°C, whereas *E. coli* cells were re-suspended in 10 ml of phosphate-buffer saline (PBS) pH 7.0 with and without 6 % v/v Manuka oil and incubated for 6h at 37°C. After

incubation, cells were harvested by centrifuging at 4,000 *g* for 20 min and then washed with sterilized distilled water. SEM and TEM were carried out as described in 2.2.11 section.

3.2.8 The anti-*Trichophyton terrestre* activity of Manuka oil

The *Trichophyton terrestre* inoculum was prepared as follows; a standard-sized inoculum of *T. terrestre* was prepared from 7- to 14-day old cultures grown on PDA at 25°C. Mature colonies were covered with approximately 5 ml of sterile PBS (pH 7.4), PBS was then gently rubbed over the surface with a sterile spreader. The resulting mixture of conidia and hyphal fragments was drawn off with a pipette and transferred to sterile tubes. Heavy particles of the suspension were allowed to settle for 10 to 15 min at room temperature, and the upper homogeneous suspension was used for further testing. The optical densities of the suspensions were read at 530 nm and adjusted to 0.15 to 0.17 to yield 0.6×10^6 to 1.4×10^6 spores/ml of strains. The suspensions containing conidia and hyphal fragments were further diluted to obtain the final desired inoculum size of approximately 0.4×10^4 to 5×10^4 spores/ml. In order to determine the MIC, a standardized culture was mixed with various concentrations (0.008–0.25 % v/v) of manuka oil then 15 µl of this mixture was placed in PDA media plate and incubated at 25°C for 7 days (Koroishi *et al*., 2008). The agar dilution method was used to determine the anti-*Trichophyton terrestre* activity of Manuka oil. *Trichophyton terrestre* was inoculated onto PDA (Potato Dextrose Agar) plates and incubated at 25°C for 7-10 days to obtain young, actively growing cultures consisting of mycelia and conidia. Sterilised diluted Manuka oil with 0.5 Tween 80 was incorporated into PDA sterilised pre-poured medium to give different final concentrations (0.25%, 0.125 and 0.063%). A mycelial disc, 8 mm in diameter, cut from the periphery of the 7-10-day-old cultures, was then aseptically inoculated onto the medium. The inoculated plates were then

incubated at 25 ℃ and the colony diameter measured and recorded after 10 days. The percentage of mycelial inhibition was calculated as follows: % mycelial inhibition = $[(d_c-d_t)/d_c] \times 100$; dc= colony diameter in the control, d_t = colony diameter in treatment, three replicate plates were used for each treatment.

3.3 Results and Discussion.

3.3.1 Antibacterial activity (Disc diffusion assay, MICs and MBCs values)

As listed in Table 3.1, manuka oil effectively inhibited the growth of all tested Gram-positivebacteria and inhibited Gram-negative bacteria to some extent. The inhibition zones were 18.6±1.5 mm against MRSA which as expected was totally resistant to Cefoxitin 30µg antibiotic disk, 19.5±0.6 mm against *M. phlei,* 18.0±1.0 mm against *S. epidermidis* and 19.9± 1.1 mm against *B. subtilis*, whereas manuka oil caused inhibition zone less than 8 mm against both tested Gram-negative bacteria, *S. marcescens* and *E.coli,* 6.0±0.6 mm, 7.6±0.5 mm, respectively. Except *S. epidermidis,* where the minimum inhibitory concentration was 0.25%, the MICs were 0.03% against all tested Gram-positive bacteria including MRSA , *M. phlei* and *B. subtilis* (as shown in Table 3.1). As the results obtained by disk diffusion assay, MICs values of *S. marcescens* and *E.coli* (> 4.0%) confirmed the poor activity of manuka oil against Gram-negative bacteria. After the measurement of minimum inhibitory concentration,15 µl of cultures were taken from tubes showing no bacterial growth, inoculated onto MH agar plates, and cultured for overnight. The concentration that did not permit any visible growth on agar plate was considered as the MBC. Since the ratio between the MICs and MBCs values for MRSA and *B. subtilis* were larger than four, manuka oil seemed to act bacteriostatically against them.

Table 3.1: The zone of inhibition (mean±SD), (diameter mm including disk (4.0mm)), showing the minimum inhibitory concentrations (MICs) (% v/v) and the minimum bactericidal concentrations (MBCs) values of manuka oil for selected bacteria including methicillin-resistant *S. aureus* (MRSA).

Inhibition zone (mm) ±SD				
Bacteria strain	**Cefoxitin 30µg**	**Manuka oil**	**MIC (v/v)**	**MBC (v/v)**
MRSA	0.0	18.6±1.5	0.03%	1.0%
S.marcescens	22.3±2.1	6.0±0.6	> 4.0%	> 4.0%
M. phlei	22.0±2.1	19.5±0.6	0.03%	NT*
S. epidermidis	21.5±2.6	18.0±1.0	0.25%	NT*
E.coli	23.5±0.7	7.6±0.5	> 4.0%	> 4.0%
B.subtilis	24.5±0.7	19.9± 1.1	0.03%	0.50%

*NT: Not tested

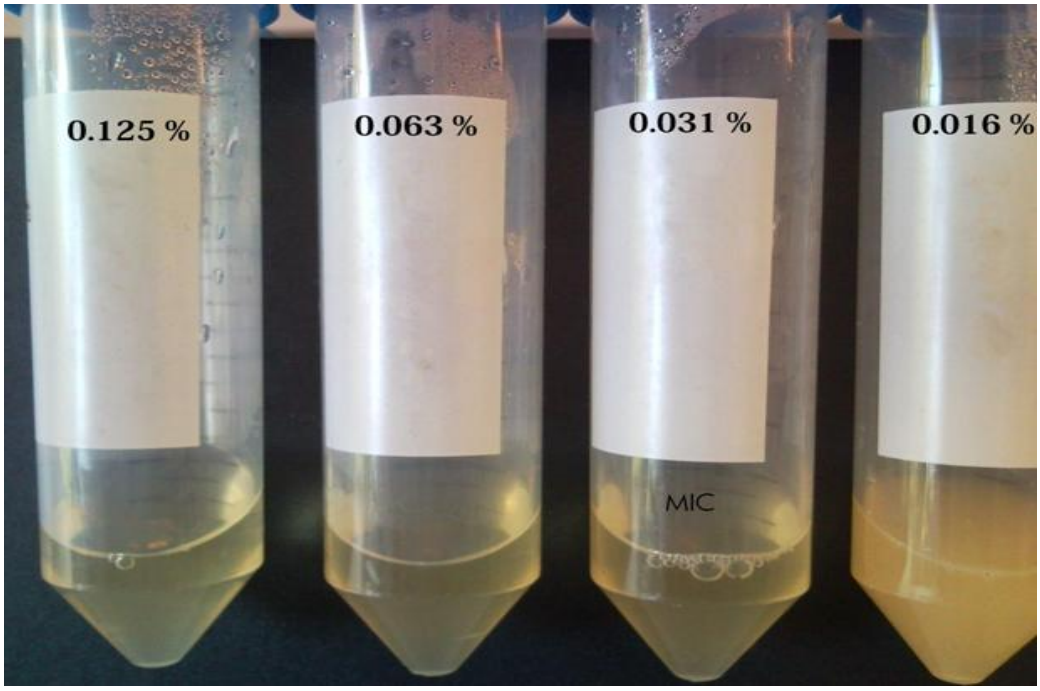


Figure 3.1: A minimal inhibitory concentration (MIC) test determined by macrodilution method. The tube to the right is yellow indicating bacterial growth; the tubes to the left are clear indicating no growth. The MIC is 0.031 % where the bacterial growth halted.

3.3.2 Kill assays.

In order to assess the effect of manuka oil on bacterial cell viability, MRSA and *E. coli* were chosen to represent both Gram-positive (highly susceptible to manuka oil) and Gram-negative bacteria. The (time-kill) curves for MRSA and *E. coli* in MHB at 1.5% v/v concentration of manuka oil are shown in Figure 3.2 and Figure 3.3. As shown in Figure 3.2, treatment of MRSA with manuka oil at a concentration of > MBC (1.5% v/v) was successful in killing $>10^3$ CFU ml $^{-1}$ within 8 hours, while as shown in Figure 3.3 treatment of *E. coli* with manuka oil at this concentration (1.5% v/v), that is less than MIC of tested bacteria did not (as expected) show a lethal effect on *E. coli* cells. This concentration exhibited a limited inhibition, where the cell multiplication process was less than the *E. coli* control.

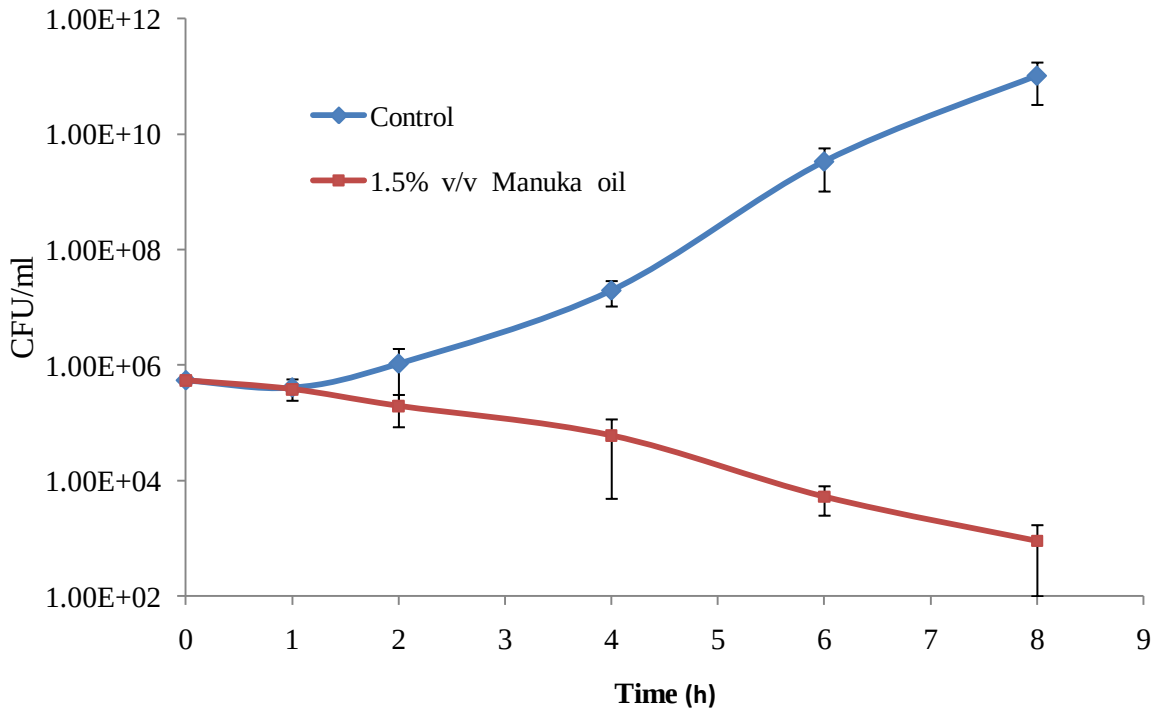


Figure 3.2: Killing of methicillin-resistant *Staphylococcus aureus* (MRSA) by 1.5% (v/v) of manuka oil

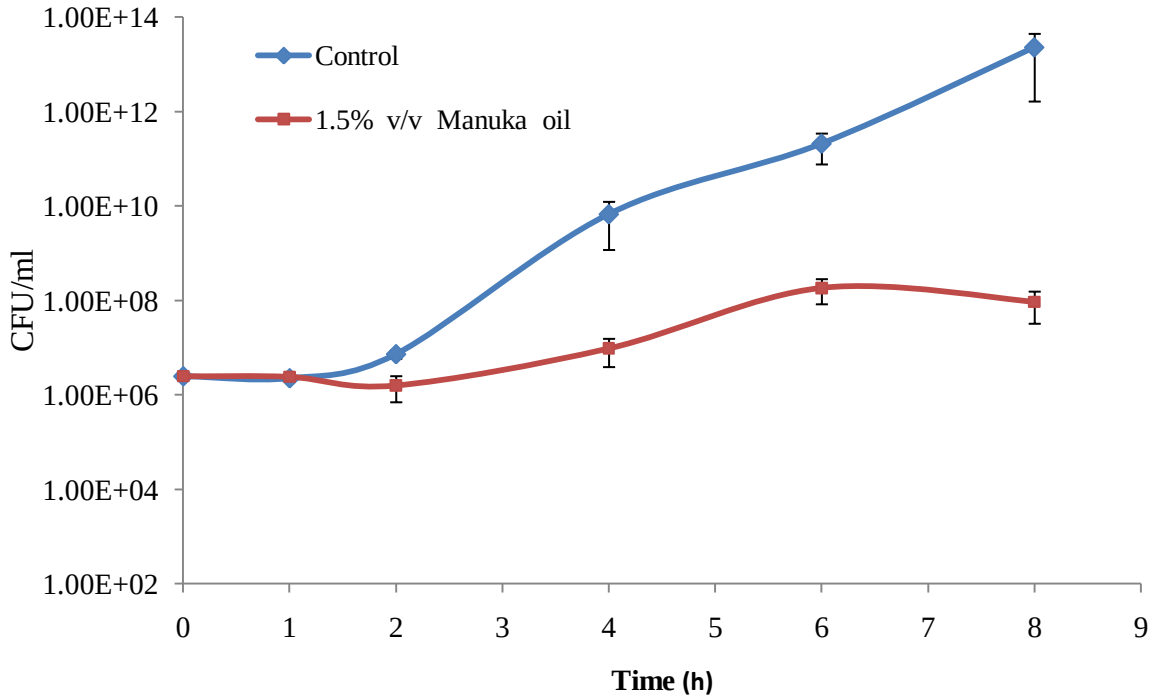


Figure 3.3: The effect of 1.5% (v/v) of manuka oil on *E. coli* growth curve

3.3.3 Scanning electron microscope (SEM) and transmission electron microscope TEM studies showing the morphological effects of manuka oil on Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli.*

The SEM and TEM images in Figure 3.4 and Figure 3.5 show the effects of manuka oil on the external and internal morphology of MRSA, show that untreated cells, which were never exposed to manuka oil, retained their coccal morphology and appeared normal, while MRSA cells treated with 1.5 v/v manuka oil for 4 hours underwent considerable morphological alterations including seriously damaged cells and free cellular contents, Figure 3.5 (c) and (d), shows that a bactericidal concentration of manuka oil induce marked cellular lysis a completely disruption to the MRSA cells.

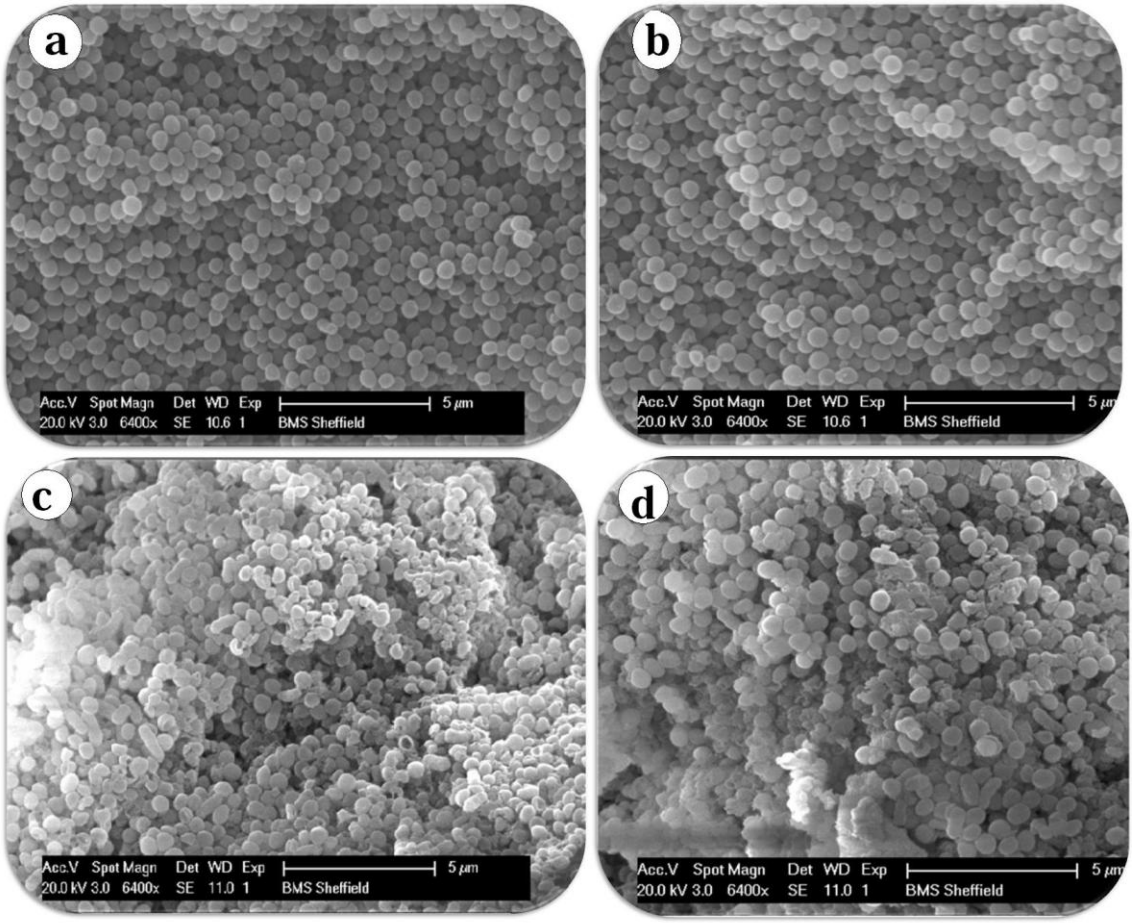


Figure 3.4: External morphology of Methicillin-resistant *Staphylococcus aureus* (MRSA) observed by scanning electron microscopy (SEM) .(a) and (b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 4 h at 37°C. (c) and (d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 3% v/v Manuka oil for 4 h at 37°C.

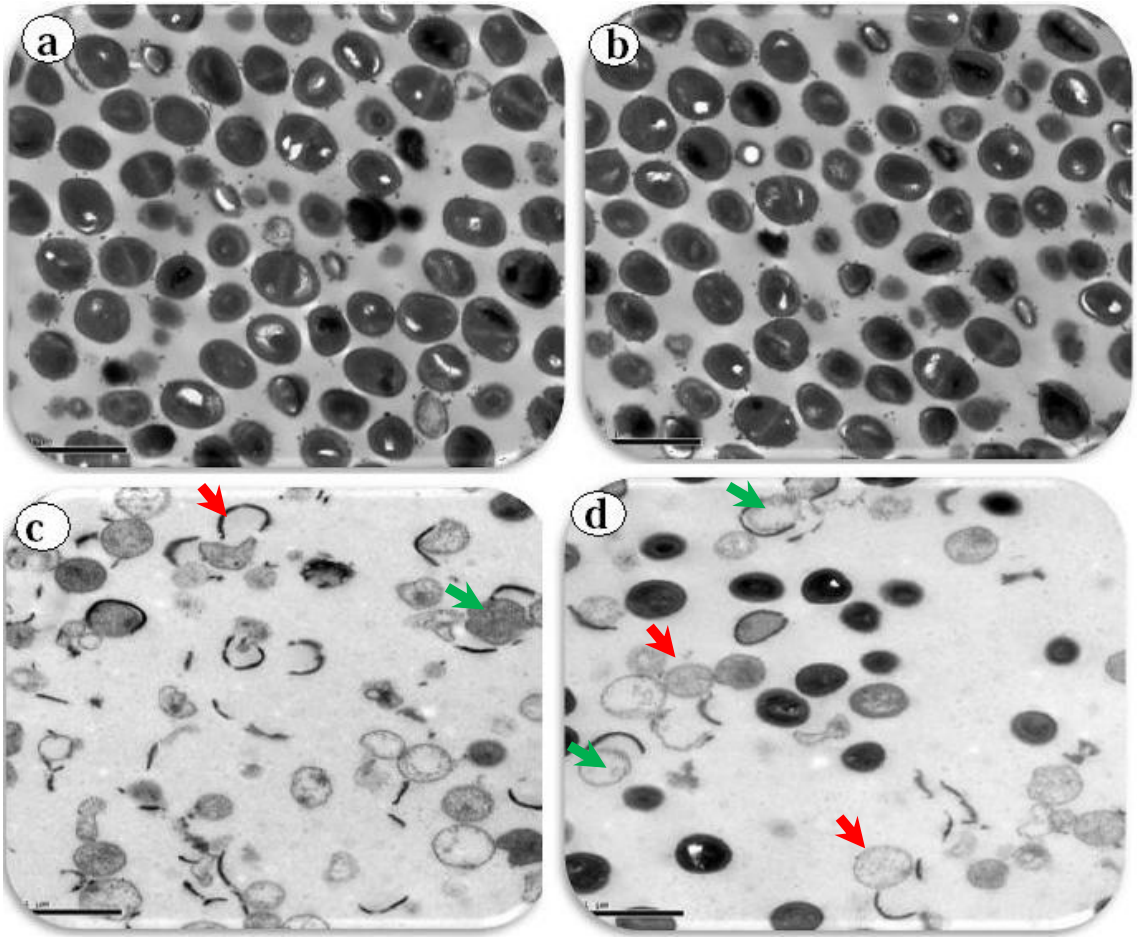


Figure 3.5: Internal morphology of Methicillin-resistant *Staphylococcus aureus* (MRSA) observed by transmission electron microscopy (TEM) .(a) and (b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 4 h at 37°C. (c) and (d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 1.5% v/v Manuka oil for 4 h at 37°C. Note the cell lysis (green arrowheads) and released cellular contents and cell debris (red arrowheads).

The SEM and TEM images shown in Figure 3.6 and Figure 3.7 revealed that 6% v/v manuka oil had little effect on the appearance of the *E. coli* cells, compared to the untreated cells. Cells of *E. coli* cells incubated with 6% v/v manuka oil for six hours appear shortened and distorted shapes and have lost some of their integrity; cells empty of contents and possessing irregular shape were observed (Figure 3.7,(c) and (d)).

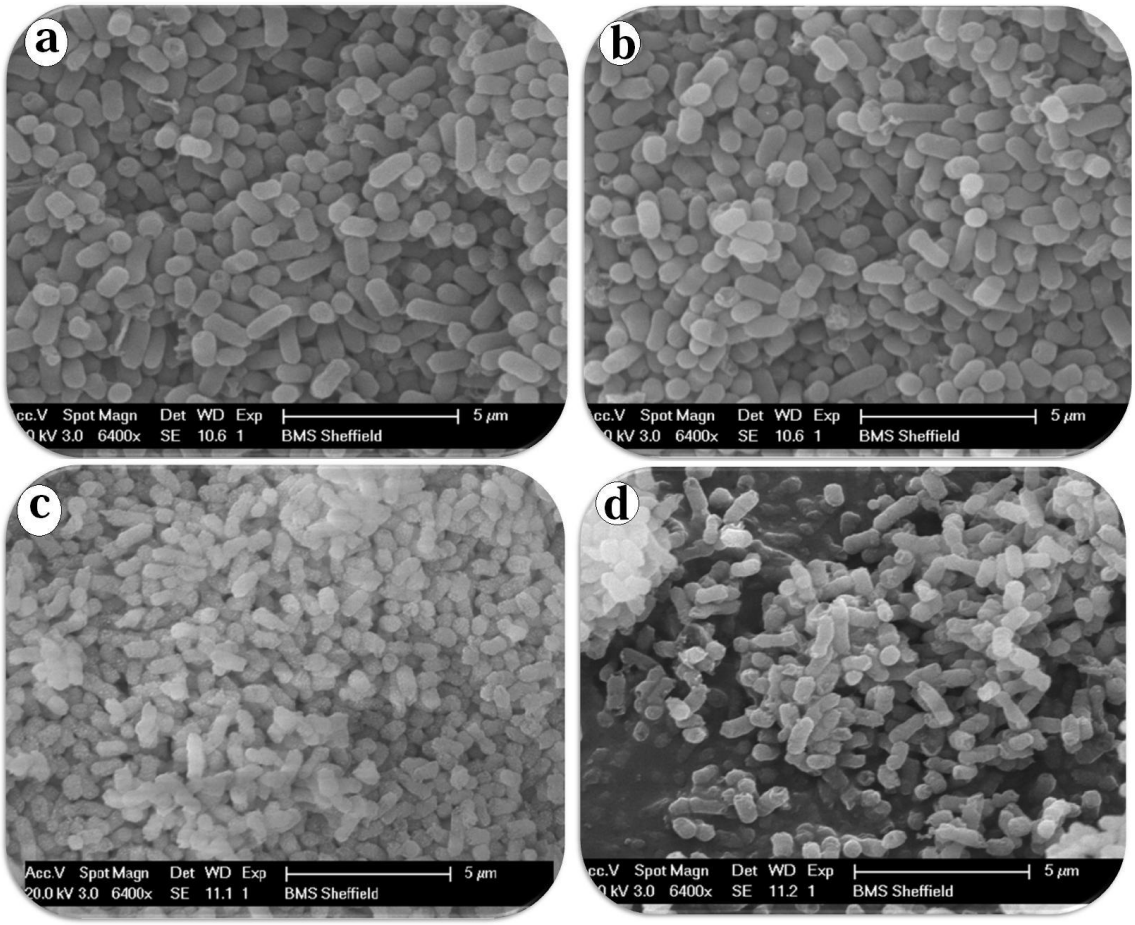


Figure 3.6: External morphology of *Escherichia coli* observed by scanning electron microscopy (SEM) . (a) and (b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 6 h at 37°C. (c) and (d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 6% v/v Manuka oil for 6 h at 37°C. Note abnormal shortened cells with rough surfaces (c) and (d).

A 6% manuka oil solution failed to induce marked cellular lysis in *E. coli* treated cells. Generally, the SEM and TEM observations for both MRSA and *E. coli* confirmed that manuka oil has a potent antibacterial activity against Gram-positive and a moderate effect against Gram-negative bacteria.

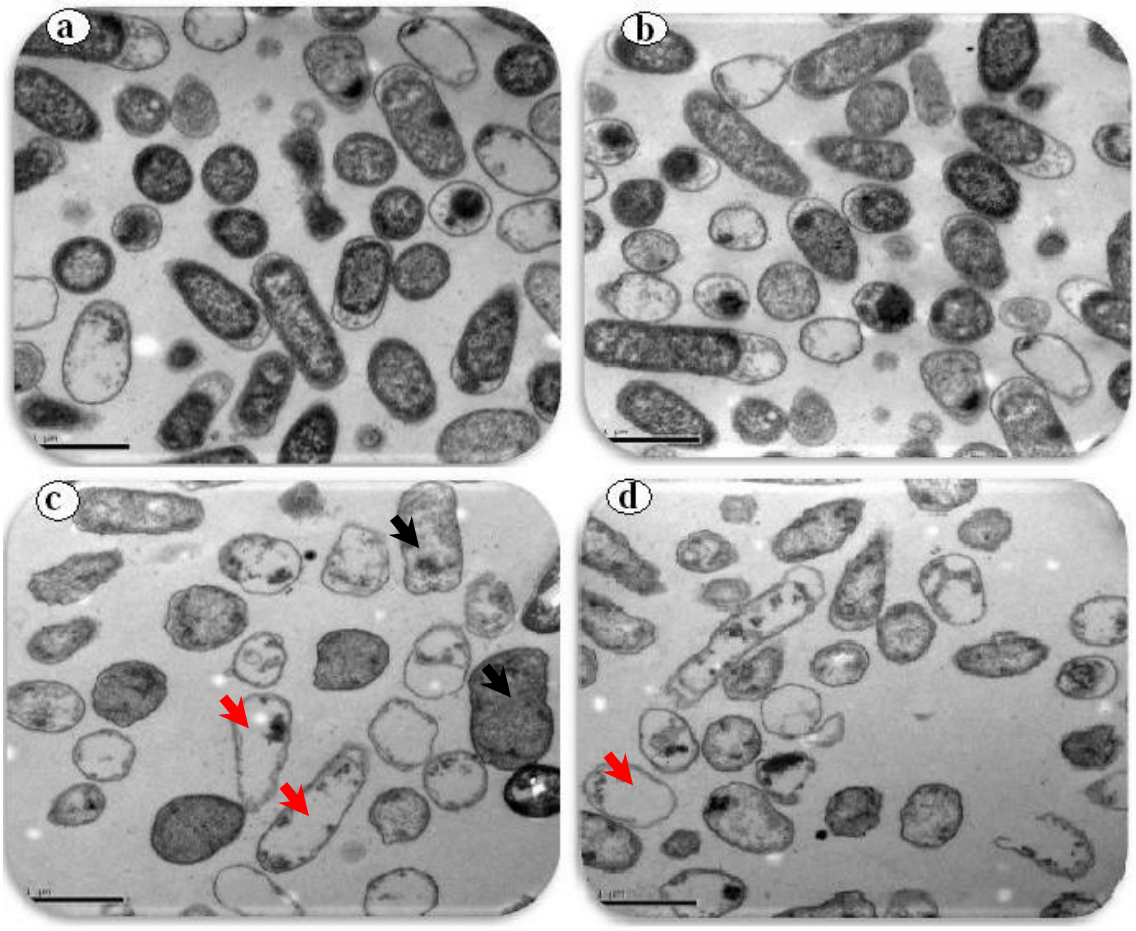


Figure 3.7: Internal morphology of *Escherichia coli* observed by transmission electron microscopy (TEM). (a) and (b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 6 h at 37°C. (c) and (d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 6% v/v Manuka oil for 6 h at 37°C. Note cells without cell contents (red arrowheads) and irregular cell shape (black arrowheads).

The therapeutic properties of plant-essential oils have been known for many thousands of years. Essential oils have been considered as potential sources of novel antimicrobial agents and considerable *in vitro* studies have demonstrated their antimicrobial activity (Prabuseenivasan *et al*., 2006).

In this Chapter, manuka oil was shown to exhibit a strong activity against selected Gram-positive bacterial strains. Limited studies, in the literature, have shown that manuka oil has marked and consistent inhibitory effects on oral pathogens (Takarada *et al*., 2004). Studies have also reported a more marked antimicrobial activity for manuka

oil against Gram negative bacteria than was shown in this thesis (Lis Balchin *et al*., 2000). Earlier studies have suggested that the antibacterial activity of manuka oil is probably due to having high levels of triketones (Carr 1998; Douglas *et al.*,2004; van Klink *et al.*,2005).

In conclusion, a marked inhibitroy activity of manuka oil against Gram-positive bacteria, including MRSA, *M. phlei* and *B. subtilis* was demonstrated where the 0.03% v/v of manuka oil inhibited the growth of these tested bacteria. Such findings could support the use of manuka oil to treat wounds infected with these, and other bacteria; this usage is however best retstricted to Gram-positive bacteria and not to infections caused by Gram-negatives such as *E. coli* and *S. marcescens*, where a high concentration (> 4 %) is required to inhibit their growth. Such a high concentration may produce side effects and could be cytotoxic to the host cells (Takarada *et al*., 2004).

3.3.4 The Anti-*Trichophyton terrestre* activity of manuka oil

Trichophyton terrestre is keratinophilic fungus, which causes dermatophytoses (Bokhari, 2009), i.e. superficial fungal infections of skin, hair, nail, or keratinised tissue in humans and animals (Koroishi *et al.,* 2008).

The anti-*Trichophyton terrestre* activity of manuka oil was assessed by determining the MIC and in the inhibition of radial growth of mycelia. As shown in Figure 3.8, the minimum inhibitory concentration of manuka oil towards *Trichophyton terrestre* is 0.25% v/v.

The results shown in Figure 3.9 show that mycelial growth was significantly inhibited by manuka oil, 47.1±1.0 % of the radial growth of mycelia was inhibited at concentration 0.063 % v/v, 76.2 ± 3.7 % of the radial growth of mycelia was inhibited at concentration 0.123 % v/v and 100% of the radial growth of mycelia was inhibited at concentration 0.25 % v/v, i.e. these results confirm the previous findings showing that MIC is 0.25% v/v.

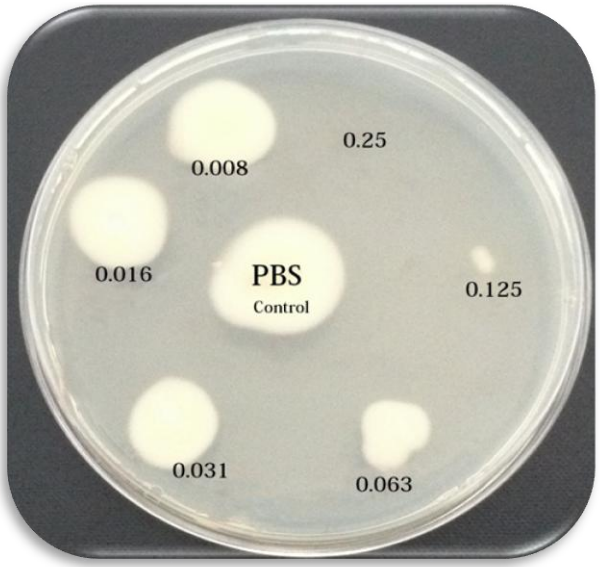


Figure3.8: Growth inhibition test with *Trichophyton terrestre*. Each spot was placed with 15-µl sample containing various concentrations (0.008–0.25 % v/v) of manuka oil. (PBS) Phosphate buffered *saline (control).* The minimal concentration of manuka oil that resulted in inhibition of the *Trichophyton terrestre* was 0.25% v/v.

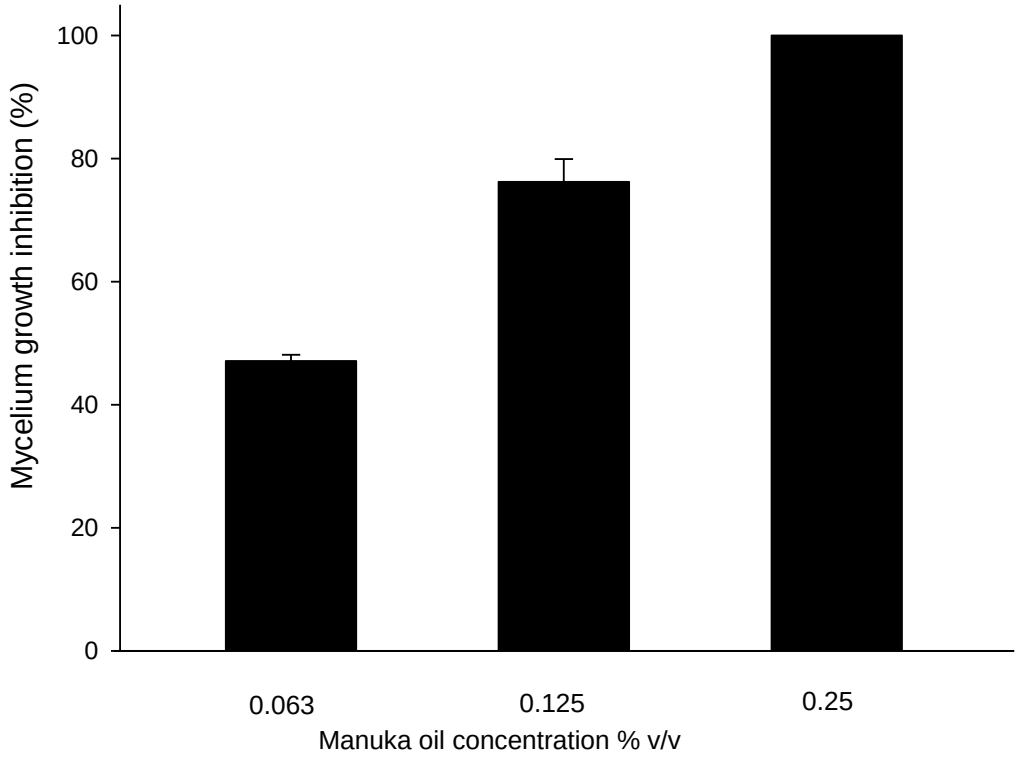


Figure 3.9: Percent inhibition of mycelial growth of *Trichophyton terrestre* in the presence of several concentrations of manuka oil. Percentage of mycelial growth inhibition respective to the control (no oil added). Means of three replicates ±SD.

The observed significant antifungal activity of manuka oil against *Trichophyton terrestre* could indicate its activity in general against dermatophytes. The addition of manuka oil to hair oils used for hair dressings in many developing countries, where dermatophytoses are common (Lakshmipathy and Kannabiran, 2010), e.g. India (Garg and Miller, 1992), could help reduce the incidence and distribution of hair–related dermatophytoses.

Chapter Four: Comparison of the Antimicrobial Activity of Tamarind and High Grade Manuka Honey

4.1 Introduction

Tamarind *(Tamarindus indica* Lit) is a tree belonging to the *Caesalpinaceae* family, it is native to tropical Africa but it has been widely cultivated also in many other regions including subtropical China, Pakistan, Indochina, Philippines, Spain and more commonly in India (Globale, 2010; Biao, 1994). Tamarind has been used for centuries in folk medicine for the treatment of many health problems such as asthma, dysentery, vaginal complaints, gonococci and gastrointestinal disorders (Khanzada *et al.*, 2008; Tayade *et al.*, 2009). The most valuable part of the tamarind is its fruits which are characterized by their reddish-brown colour which can turn black- brown (Biao, 1994). The curative effects of tamarind's fruits have been reported in several pharmacopoeias (Julio et al., 2010).

Tamarind paste is a commercially available product made from the fruit of the leguminous, tamarind tree; it is used widely in the tropical Africa, Asia, and elsewhere, as a refreshing drink and food flavouring. Tamarind is non-toxic (LD_{50}=5000mg per Kg,(Abubakar et al., 2010) and has been used in folk medicine for millennia as a mild laxative and in the treatment of sore throat, dysentery and erysipelas, medical uses which suggest that it possesses antibacterial properties.

Since, as has been detailed above, Manuka honeys are widely used to treat indolent (particularly methicillin resistant, MRSA),it was considered of interest to compare the antimicrobial properties of these two products with a view to using tamarind paste as an alternative to Manuka honey for use in treating infected wounds.

Although the antimicrobial effects of tamarind seeds and other plant components have been previously reported (Doughari, 2006; Nwodo *et al.*, 2010), the antimicrobial activity of commercially available tamarind paste, and the suggestion that it might be used to treat infected wounds, appears to have been overlooked.

The aim of the work reported in this Chapter was to determine the antibacterial and anti-*Candida* properties of tamarind paste and compare these with the antimicrobial activity of a highly antibacterial Manuka (unique Manuka factor, UMF 25) honey.

4.2 Materials and methods

4.2.1 Tamarind Paste

The tamarind used was a commercial paste produced by TRS Foods, Southall, UK.

4.2.2 Test Organisms

The following test organisms were used: *Escherichia coli, Mycobacterium phlei (*NCIMB8573)*, Staphylococcus epidermidis, Serratia marcescens, Bacillus sphaericus, Staphylococcus aureus, Bacillus subtilis* and *Candida albicans* (CA 3153); unless stated otherwise, the organisms were obtained from the Departmental Culture Collection.

4.2.3 Agar well diffusion assay

The antimicrobial activities of tamarind or Manuka were determined using the well diffusion method. The individual test organisms (100 µl of a 0.5 McFarland standardized inoculums) were spread on the surface of Muller Hinton agar using a flame-sterilised glass spreader, and the surface was allowed to dry. Wells (8.0 mm dia.) were cut from the inoculated medium using a flame-sterilized cork borer, and then filled with tamarind. The plates were incubated at 37ºC and observed after 24 hours for the presence of inhibition zones around the wells; these were measured. The same process was repeated for testing the antifungal activity of tamarind, using *Candida albicans* (CA 3153) which was adjusted to a concentration of 10^6 cfu/ml and suspended in PBS **(**phosphate buffer saline), and 100 µL suspension was spread onto the surface of Saboraud Dextrose agar (Oxoid).

4.2.4 Determination of MIC and MBC

The MIC of the tamarind paste (subsequently referred to as tamarind) (using each of the test organisms) was determined using the broth microdilution (microplates) and agar

dilution methods. In broth microdilution, cultures of each tested bacteria was diluted in 2X Muller Hinton and adjusted to a 0.5 McFarland standardized inoculum, while the *Candida* inoculum was diluted in 2 x in Saboraud Dextrose broth and adjusted to 10^6 cfu/ml; standardized 100 µl aliquots were then aseptically dispensed in wells of a 96-well plate. A series of two fold dilutions of tamarind was prepared in sterile distilled water ranging from 5% (v/v) to 0.008 %(v/v) to the bacteria and ranging from 2% (v/v) to 0.02 %(v/v) to the yeast. Diluted tamarind was sterilized and filtered using a 0.20 µm membrane filter, and 100 µl of diluted Tamarind were added to wells and cultured for 24 hr at 37°C. Sterile distilled water with no tamarind was used as a control. The lowest concentration of tamarind which inhibited the growth of each organism was recorded as the MIC. In order to determine the MBC, 15 µl of inoculum was collected from those tubes which did not show any growth and inoculated on sterile nutrient agar (for bacteria) and Saboraud dextrose agar (for *C. albicans*).The plates were then incubated overnight at 37 °C. The MBC was read as the lowest concentration of tamarind or Manuka which did not permit any visible growth on agar plate. In agar dilution, sterilised tamarind was incorporated into sterilised Muller Hinton agar pre-poured medium to give different final concentrations (20%, 19%, 17%, …., 1.0% v/v), the medium poured and the agar in the plates allowed to set. Standardized 10 µl of tested bacteria was placed on the surface of agar plate and then incubated overnight at 37°C.

4.2.5 Determination of time killing curves for *S. aureus*

The effect of tamarind or honey on the viability of cells were monitored by inoculating 2mL of an overnight culture of *Staphylococcus aureus*, adjusted to final density of approximately 5 x 10^6 cfu, into 18 ml Muller Hinton broth with and without 1%(v/v) of tamarind and incubated at 37°C with shaking (250rpm). Samples (100 µl) then were removed at known intervals , diluted serially and 100 µl of the diluted samples were

transferred to Nutrient Agar plates (Oxoid) and incubated at 37°C for 24 h. The number of cfu's was then counted and time kill plots were constructed.

4.2.6 Effect of the autoclaving on the antimicrobial activity of tamarind

Suspensions of tamarind (50% v/v) were autoclaved at 120°C for 15 minutes, and then its antimicrobial activity was assessed against *Staphylococcus aureus* using the well diffusion assay. A non-autoclaved (50% v/v) suspension of tamarind was used as the control.

4.2.7 Statistical Analysis

All observations were presented as Mean ± SD. (Standard deviation). The data was analyzed by SigmaPlot© 11.0. $P<0.05$ was considered as significant.

4.3 Results and discussion

4.3.1 Well diffusion assay

Figure 4.1 shows the antimicrobial activity of undiluted tamarind paste in comparison to 25+ Manuka honey against six bacteria and *Candida albicans*. It is clear that undiluted commercially available tamarind paste significantly inhibited all of the bacteria tested as well as *Candida albicans* and that the effect was more pronounced than that produced by 25+ Manuka honey. Tamarind showed particularly marked inhibitory effect against *C. albicans* and *M. phlei.* Tamarind inhibited *C. albicans* 1.5 times more than 25+ Manuka honey, (zone sizes 44.6±2.5 mm and 29.3±0.6 mm, respectively), whereas tamarind inhibited *M. phlei* nearly twice as much as 25+ Manuka honey (zone sizes, 42.3±2.5 mm and 22.3±3.2 mm, respectively). The fact that tamarind exhibited a greater inhibitory effect on *C. albicans* than did manuka 25 is confirmed visually in Figure 4.2. Generally, Gram-positive bacteria were more sensitive to both of the tested agents than were Gram-negative bacteria but the difference between the susceptibility of both Gram-negative and Gram-positive was more obvious with 25+ Manuka honey.

The marked inhibitory effect of tamarind on *Candida albicans* is confirmed by the data given in Figure 4.3 which shows that tamarind also inhibited the growth of *C. albicans* even when diluted by 10,20 and 50% with water; the inhibitory effect is seen not to be linear, with the inhibition produced by the 10% v/v being approximately 70% of the 100 percent tamarind value, this suggest that an osmotic effect is not involved in tamarind's inhibitory activity.

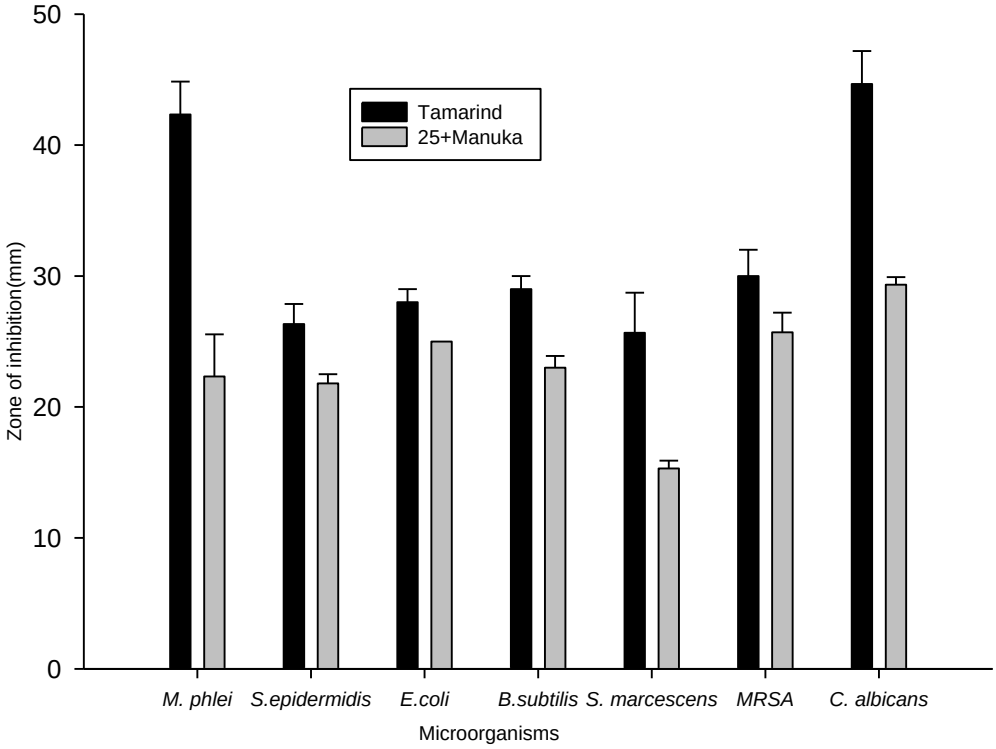


Figure 4.1: The antimicrobial activity of undiluted tamarind paste in comparison to 25+ Manuka honey against six bacteria and *Candida albicans*, determined by the agar well diffusion assay (n = 3). The values are represented as means of the replicates (including the size of the well (8.0mm)) ± Standard deviation.

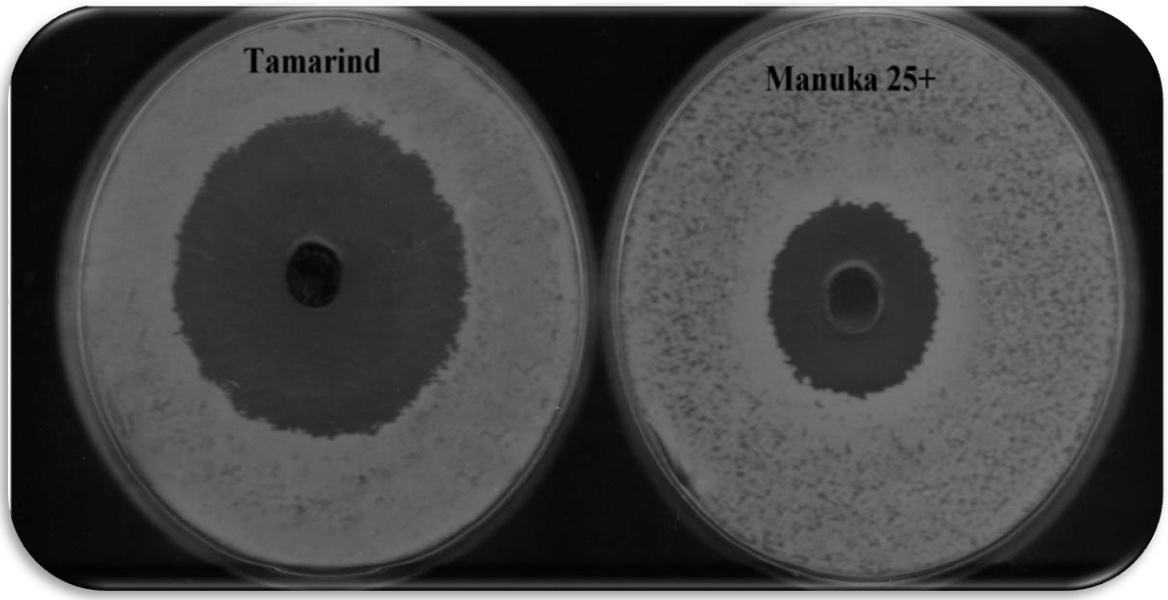


Figure 4.2: Zone of inhibition of the growth of *Candida albicans* formed around wells filled with tamarind and 25+ Manuka, determined by the agar well diffusion method. Note: Marked zone of inhibition caused by tamarind**.**

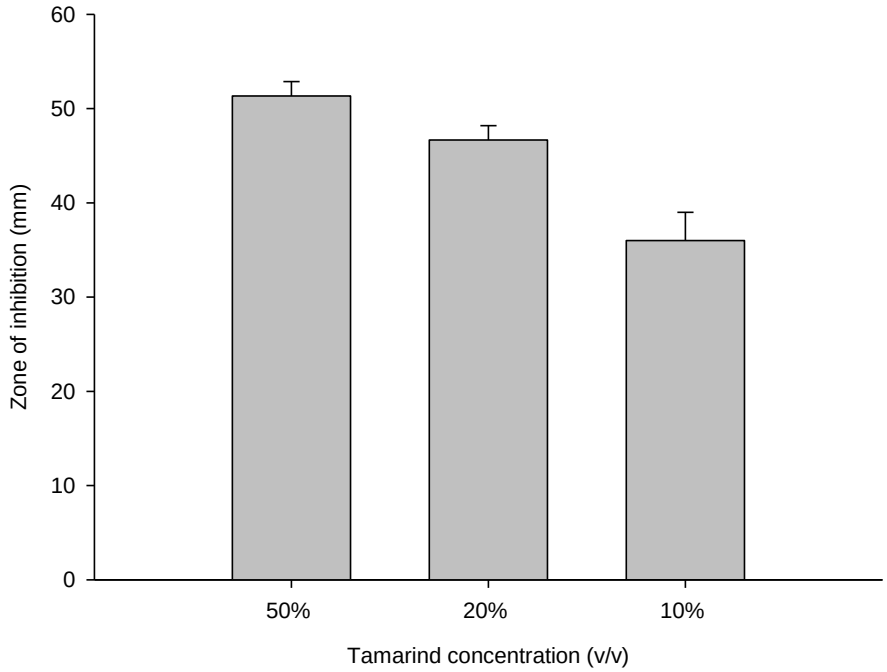


Figure 4.3: Anti-*Candida albicans* activity of different tamarind dilutions (50%, 20% and 10% v/v), determined by the agar well diffusion method, expressed as means of zone of inhibition (including well (8.0mm)) ± standard deviation.

4.3.2 Minimum inhibitory concentration values

Two methods were used to quantify the minimum inhibitory concentrations (MICs), agar dilution and microplate dilution. Agar dilution was performed by incorporation of different concentrations of the antimicrobial agent into agar media followed by adding a standardized inoculum of test microorganisms to the surface of the agar plate; this is the most commonly used methods to quantify the MIC (Wiegand *et al*., 2008), particularly when the antimicrobial agents are available in large volumes. Microplate dilution was carried out using 96-well microtiter plates where a standardized inoculum of test microorganisms were added to liquid medium containing different concentrations of an antimicrobial agent. In contrast to the agar dilution method, the main advantage of the broth dilution method is the ability to determine the MBC or MFC (Minimal Fungicidal Concentration) by making a further liquid subculture from the resultant broth culture. Initially, we performed agar dilution assay as a general screening to explore the range of tamarind's MIC, using agar incorporated with tamarind concentrations ranging from 20% to 1% v/v. Surprisingly, none of tested bacteria showed any growth even in the plates with lowest tamarind concentration (1% v/v), therefore, subsequent microplate dilution assays were started from a concentration of 5% to 0.008 % (v/v).

MICs and MBCs values of tamarind paste against tested bacteria and *C. albicans* are listed in Table 4.1. All MIC values determined by the agar dilution method were less than 1% v/v; more accurate values were given by microplate dilution where only *S. marcescens* showed MIC above 1% v/v, whereas the MIC for other tested bacteria ranged from 0.32% to 0.63% v/v and 0.13% for *C. albicans*. These low MICs value reflect the highly exceptionally antimicrobial activity of tamarind paste comparable to

Table 4.1: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of Tamarind for six bacteria and *Candida albicans*, assessed by agar dilution and microplates dilution methods.

	Agar dilution	Microplates dilution	
Microorganism	MIC (v/v)%	MIC (v/v)%	MBC (v/v)%
M. Phlei	<1	0.63	1.25
S.epidermidis	<1	0.32	0.63
E.coli	<1	0.63	>5
B.subtilis	<1	0.32	>5
S. marcescens	<1	1.25	1.25
MRSA	<1	0.63	1.25
Candida albicans	NT*	0.13	0.13

* NT: Not tested

high grade 25+ manuka honey the MICs values of which, as shown in Chapter Two Table 2.4 ranged from 6% to 12% v/v to the same tested bacteria, for example, MRSA was completely inhibited by 0.63 % v/v of tamarind while 6% v/v of 25+manuka achieved the same level of inhibition by MRSA. Tamarind's inhibition activity was more pronounced against *C. albicans* than against bacteria where the MIC was 0.13%v/v.

There are some marked variations between the MBC /MIC ratios of the tested bacteria, ≤ 2.0 for *M. phlei, S.epidermidis, S. marcescens* and MRSA and > 7.0 for *E. coli* and *B. subtilis,* which suggest that the mechanism of the tamarind's antimicrobial activity may not be uniform against individual bacteria independent of their cell wall structures. In the case of *M. Phlei, S.epidermidis, S. marcescens* and MRSA, tamarind appeared to be bactericidal, while for *E. coli* and *B. subtilis,* it seemed to be bacteriostatic. However, all tamarind's MBCs values were less than 25+ manuka honey MICs and MBCs values. The results obtained here suggest that tamarind paste will still be active against a wide spectrum of bacteria as well as *C, albicans* even when diluted by wound exudates up to 100-fold.

4.3.3 Time kill curve of the effect of tamarind paste against methicillin-resistant *Staphylococcus aureus* (MRSA).

The effect of the above used MIC concentrations of tamarind on the cell counts of MRSA is shown in Figure 4.4. MRSA cells exposed to 1% of tamarind paste were found to lose the ability to multiply and nearly 1 log reduction was achieved after 8 h of incubation, at the same time, the growth control showed at least a 5 log increase in bacterial numbers by 8 h.

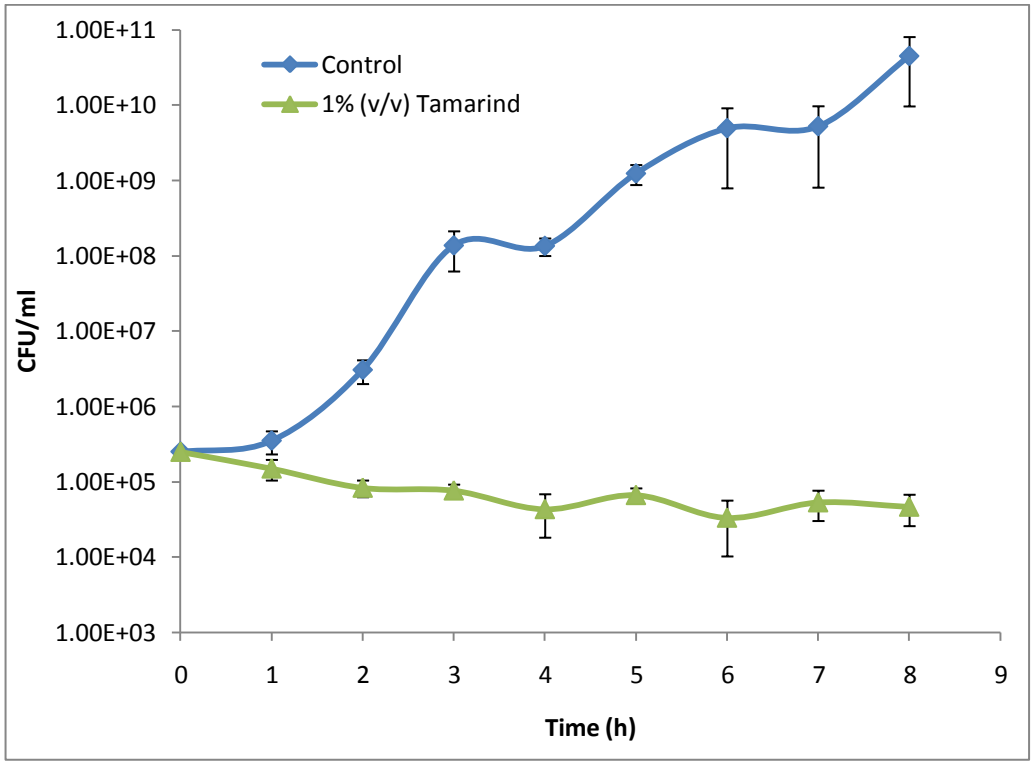


Figure 4.4: The effect of 1% (v/v) Tamarind on the growth of methicillin-resistant *S.aureus* (MRSA).

This data confirms tamarind's pronounced antibacterial activity at 1% v/v, preventing MRSA cells multiplication and causing a reduction in CFU over 8 h of incubation time, whereas the previous time-to-kill results given in Chapter two showed that more than 10% v/v of 25+Manuka is needed to cause a reduction in the MRSA cells.

4.3.4 **The effect of autoclaving on the tamarind's inhibitory activity**.

As shown in Figure 4.5 autoclaved tamarind paste was as equally effective in inhibiting MRSA as non-autoclaved material (there was no statistically significant difference between both activities (P = 0.897). This is particularly important in relation to the possible use of tamarind to treat wounds, since before it could be used for this purpose it would need to be sterilised; autoclaving provides a cheap and readily available means of achieving this sterilization without obviously impairing its antibacterial effects. Medical grade manuka honey in contrast needs to be sterilized using cobalt 60 gamma radiation in order to maintain its activity (Postmes *et al*., 1995) – very expensive process when compared with autoclaving.

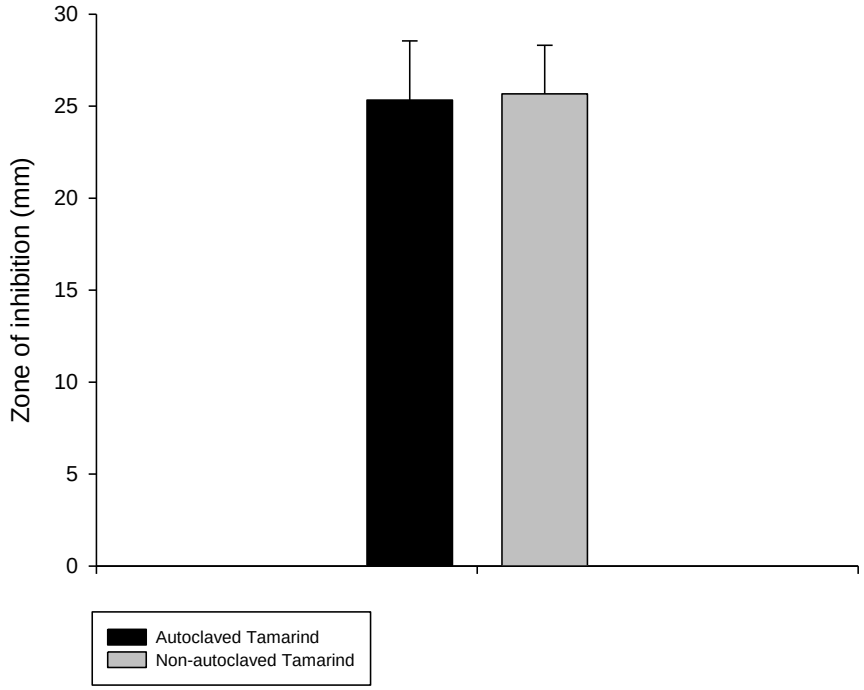


Figure 4.5: The antibacterial activity of 50 % v/v autoclaved tamarind paste and 50 % v/v of non-autoclaved tamarind paste against MRSA, expressed as means of zone of inhibition (including well (8.0mm)) ± standard deviation. There was no statistically significant difference between activities (P = 0.897).

In conclusion, the *in vitro* antibacterial activity of tamarind was evaluated and compared with 25+ Manuka honey. Data obtained from the agar diffusion, MICs, MBCs values and time to kill study shows that tamarind exhibits a stronger antimicrobial effect against the six tested bacteria and *C. albicans* than a high medical grade manuka honey. The marked anti-*Candida* activity of tamarind and antibacterial activity against *M. phlei* and MRSA are particular noticeable. As a result of such marked antibacterial properties, which exceed those of 25+ manuka honey, it is suggested that a autoclaved, sterilized, commercially available tamarind paste should be clinically evaluated for the treatment of wounds and indolent ulcers with are infected with bacteria, notably MRSA, and b) that it might also prove effective in the treatment of mucosal membranes infected with *C. albicans* (i.e.thrush).

Chapter Five: Maggot Debridement Therapy

5.1 Introduction

5.1.1 Biosurgical debridement (maggot therapy)

Biosurgical debridement or "maggot therapy" is defined as the use of live, sterile maggots of certain type of flies to remove the necrotic tissue from non-healing tissue or wounds and thereby promote healing of the remaining healthy tissue (Zacur and Kirsner, 2002; Graninger *et al*., 2002). The approach involves applying sterile larvae of *Lucilia sericata*, commonly called the green bottle fly (Borak, 2008; Zacur and Kirsner, 2002; Chan *et al.*, 2007) to a wound, while a bandage is applied to keep them in place, the number of maggots used varies according to the type and the size of the wound and to the amount of necrotic tissue. The maggots are replaced regularly until the wound is healed (Borak, 2008). In order to obtain sterilized larvae, the surfaces of unhitched fly eggs are soaked in a bichloride solution for one hour, subsequently, the emerging larva remain sterile (Bunkis *et al*., 1985). To avoid physical discomfort to the patient, a new technique, was developed by Fleischmann *et al.* (2004), which involves the use of so-called Biobag, where living larvae are applied to the wound contained in a nylon gauze (Blake *et al.*, 2007; Steenvoorde *et al.*, 2005).

Although the use of larvae to improve wound healing has long been recognized in ancient cultures, including the Chinese, Ambrose Paré, Charles IX's surgeon in chief, was the first to systematically report the beneficial effects of the use of larvae to treat wounds in 1557. In 1829, Baron Larrey reported that while he was working in Napoleon’s army a significant enhancement of granulation formation occurred after maggots were applied to battle wounds (Chan *et al*., 2007; Sherman *et al*., 2000). During the 1930s, maggot therapy became popular In Europe and North America to treat some chronic or infected wounds, especially when William Baer used maggot therapy successfully to treat more than 80 cases of intractable osteomyelitis. Not

surprisingly however, the use of maggot therapy declined immediately after the widespread introduction of penicillin (Chan *et al*., 2007). The increase in the emergence of multi antibiotic resistance in the late 1990s however, has led to a revival in interest in bio-surgical debridement therapy and it is now being used in many countries to treat surface wounds (Sherman *et al*., 2000), including diabetic foot ulcers (Sherman, 2003), malignant adenocarcinoma (Sealby, 2004), and for venous stasis ulcers (Sherman, 2009); it is also used to combat infection after breast-conservation surgery (Church, 2005).

The beneficial effects of maggots on wounds have been attributed to various mechanisms notably the debridement (degradation) of necrotic tissue. It was originally believed that this debriding action of maggots was restricted only to their mechanical wriggling, but recently many proteolytic enzyme classes have been isolated from maggot excretions and secretions (ES) which are able to specifically dissolve the laminin and fibronectin of the extracellular matrix in the necrotic tissue. This liquefies the dead tissues enabling the maggot to take it up by suction (Chan *et al.*, 2007; Graninger *et al.*, 2002); Several recent studies have demonstrated that the maggots ES from aseptically-raised *Lucilia sericata* larvae exhibit antibacterial actions against *both* Gram-positive and Gram-negative bacteria, including MRSA, *Escherichia coli* and *Pseudomonas aeruginosa* (Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Thomas *et al.*, 1999; Jaklic *et al.*, 2008; Jukema *et al.*, 2008). In addition, maggots can ingest bacteria as part of their normal feeding process (Zacur and Kirsner, 2002; Chan *et al*., 2007; Bowler *et al*., 2001). Finally, maggots promote wound healing, stimulate granulation and promote the formation of human fibroblasts (Zacur and Kirsner, 2002).

5.1.2 The potential therapeutic use of maggot fumes

There exists a less well known potential use of maggots in medicine, namely the use of maggot-produced volatiles to treat bacterial lung infections. Wainwright (2007) detailed how gases and volatiles produced by maggots were used during the early 1900s to treat pulmonary tuberculosis and he discussed the claims that consumptives from the Leeds-Bradford area were cured by breathing in the fumes produced by maggots (Wainwright, 2007). Only one subsequent study aimed at investigating the antibacterial activity of *Lucilia cuprina* blowfly gaseous excreta/metabolites (gEMol) has been conducted by (Arora and Baptista, 2010) where it was demonstrated that the gaseous excreta/metabolites (gEM) caused a partial growth inhibition in *Escherichia coli* and methicillin-sensitive Methicillin-resistant *Staphylococcus aureus* (MRSA) (MSSA) (Arora and Baptista, 2010).

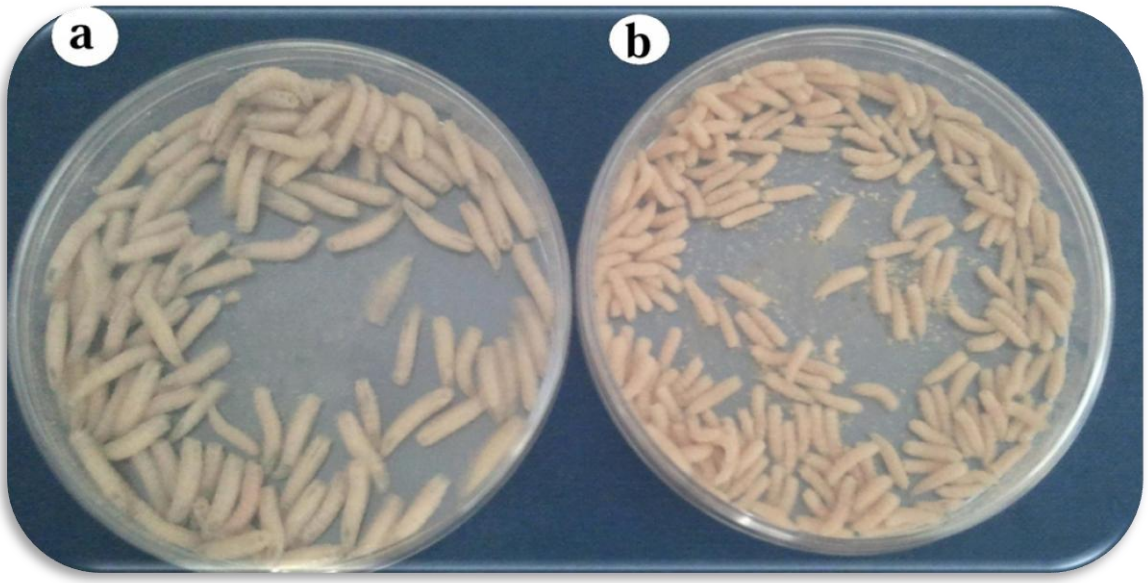


Figure 5.1: (a) Blue blow-fly larva (*Calliphora quadrimaculata*), (b) green bottle fly larva (*Lucilia sericata)*

The aim of the work reported in the Chapter was to:

1. Investigate the antimicrobial activity of *Calliphora quadrimaculata* maggot gases.
2. Attempt to extract the antibacterial agents of *Calliphora quadrimaculata* maggot gases and quantify their activity against *Mycobacterium phlei* and Methicillin-resistant *Staphylococcus aureus* (MRSA).
3. Investigate the anti-MRSA activity of *Lucilia sericata* pupa hemolymph extracts.
4. Determine *in vitro* ability of *Lucilia sericata* maggots to control fungi involved in superficial fungal infections, i.e. fungal burn wound infections and dermatomycoses.
5. Determine the incidence of *Mycoplasma* species in the *Calliphora quadrimaculat*a and *Lucilia sericata* maggot guts.

5.2 Materials and methods

5.2.1 The effect of the Larvae fumes on the bacteria growth

Three plastic containers were prepared, one contained 400 g of the blue blow fly larvae (*Calliphora quadrimaculat*a) (Figure 5.1) fed on beef heart, whereas the control container had 400 g of dead blue blowfly larvae (*Calliphora quadrimaculata*) with the same amount of beef heart. The container had holes in the top to allow for gas exchange. Another container with only 1% ammonium hydroxide vapour was used to check whether the effect of maggot fumes comes as a result of low pH due to ammonia gases that may be produced by maggots. Three glass beakers were placed upside down inside the containers and three inoculated Petri dishes were fixed at the top of the beakers, then incubated overnight at 37°C and the paired cultures examined with the naked eye, comparing individual colony diameters and the number of colonies present.

In order to determine the effect of larvae gases on the viability of *Mycobacterium phlei*, an airtight container containing 600 g of the blue blowfly larvae (*Calliphora quadrimaculata*) fed on beef heart was prepared. Larval fumes were pumped at intervals from the container into a flask containing 200 ml of a *Mycobacterium phlei* suspension. The pumped air was sterilized by inline filters pore size 0.2μm fixed to silicone tube. The flasks were placed and fixed in a water bath at 37°C. Controls were identical except that that the jar contained only beef heart meat. Bacteria (100ml) were removed from the flasks at various time intervals and serial dilutions were plated on agar plates were made and any colonies which developed were counted (Figure 5.2).

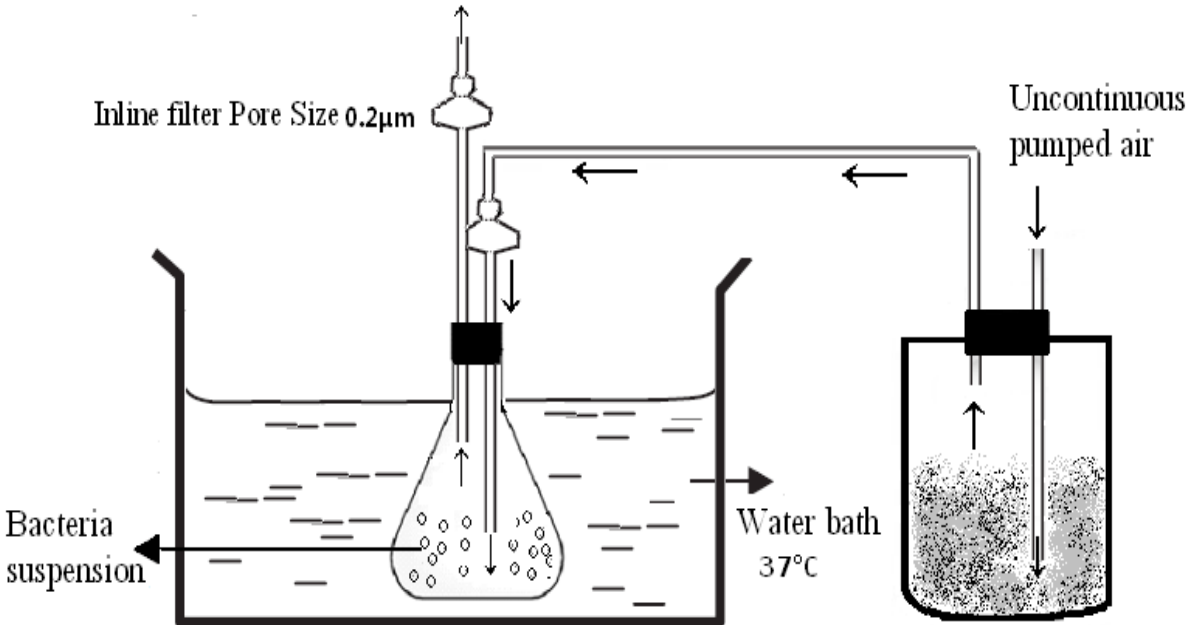


Figure 5.2: Airtight container contains 600 g of the blue blow fly larvae (*Calliphora quadrimaculata*) fed on beef heart; larval fumes were pumped un-continuously from the jar into a flask contained 200 ml of *Mycobacterium phlei* suspension through silicone tube. Pumped air was sterilized by inline filters pore size 0.2µm fixed to silicone tube. The flasks were placed and fixed in a water bath at 37°C for 16 h.

5.2.2 Attempts to extract maggot gases and determine their antibacterial activity.

In order to produce a minimum steady stream of bubbles, the maggot gases were passed through 20 ml of sterilised d H_2O alone (Ertel *et al*., 1991) or with 0.05% Tween 80, and onto activated charcoal, which then was washed with chloroform which was left to evaporate, the precipitate was then re-suspended with sterilised d H_2O with 0.05% Tween 80. All resultant gas-extraction solutions were filtered using 0.2µm vacuum filters in order to sterilize them. The effect of the resultant solutions on the growth of *Mycobacterium phlei* and Methicillin-resistant *Staphylococcus aureus* (MRSA) was

determined by either direct count methods according to (Thomas *et al*., 1999) with some modifications or by measuring the final optical density.

5.2.3 Antibacterial properties of *Lucilia sericata* pupae hemolymph

5.2.3.1 *Lucilia sericata* pupa hemolymph collection

Samples (200g) of 3− 4-day-old larvae were left without food at room temperature until pupation. Hemolymph was collected from pupae by clipping the anterior end near the cephalopharyngeal skeleton. The abdomen often had to be squeezed gently to make the hemolymph flow from the wound. The collected hemolymph was dissolved in 0.1 µ g/mL aprotonin, in 0.1% trifluoroacetic acid (TFA) 1: 10 v/v. The suspension was placed on ice for 6 h and centrifuged four times at 14000 *g* for 30 min. The supernatant was then harvested and kept frozen until assayed (Sahalan *et al*., 2007; Huberman *et al*., 2007).

5.2.3.2 Antibacterial activity assay

Antibacterial activity was determined as follows: Viable counts : the effect of *Lucilia sericata* pupae extracts on the viability of MRSA cells were monitored by inoculating 1mL of an overnight culture of MRSA, adjusted to a final density of approximately 5 x 10^6 cfu, into 9 mL NB with and without both extracts and incubated with shaking at 37°C (250rpm). Samples (100 µL) were removed at intervals, diluted serially and 100 µl of the diluted samples were placed on nutrient agar plates and incubated at 37°C for 24 h. The colonies were counted after overnight incubation and the results expressed as colony forming units (CFU)/ml. Turbidimetric assay: MRSA cells were incubated the extracted hemolymph. The final optical density of the treated and untreated cultures was measured at 600 nm.

5.2.4 Fungal ingestion by Blowfly larvae

5.2.4.1 *Trichophyton terrestre* strain

Trichophyton terrestre (IMI 277732) was maintained on PDA (Potato dextrose agar).

5.2.4.2 Filamentous fungi feeding experiments

Five-ten larvae of *L. sericata* were transferred either onto PDA (Potato dextrose agar) or PDA plates inoculated with *Trichophyton terrestre*. Larvae fed on only PDA (Potato dextrose agar) were used as controls. After being incubated for 60 minutes at 25°C, larvae were removed from the plate and washed twice with PBS. Larvae were then surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed, and then mild pressure was applied at the middle of the larval body to release the entire digestive tract.

5.2.4.3 Filamentous fungi ingestion confirmation

Collected digestive tract were mixed with 50 ml of sterile distilled water; vigorously vortexed for 3 minutes and 100 ul of gut suspension were plated on PDA (Potato dextrose agar) medium supplemented with chloramphenicol (50 mg/l) and cycloheximide (500 mg/l). After incubation for two weeks, plates were checked for any *Trichophyton terrestre* growth (Deshmukh, 2004).

5.2.4.4 Yeast strain

S. cerevisiae (BY4742) GFP was labelled with green fluorescent protein (GFP) using transformation plasmid Pex3p-GFP. *S. cerevisiae* Pex3p-GFP was maintained on YPD Agar (Sigma).

5.2.4.5 Yeast feeding experiments

Ten well washed larvae of *L. sericata* were transferred onto agar plate inoculated with *S. cerevisiae* Pex3p-GFP. Unfed larvae were used as controls. After being incubated for 60 minutes at 25 °C, larvae were removed from the plate and washed twice with PBS. Larvae were then surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed, and then a pressure was applied at the middle of the larval body to release the entire whole digestive tract. (Lerch *et al*., 2000).

5.2.4.6 Detection of fluorescence

To check that the yeast were ingested by the larvae, the digestive tract contents were fixed and then examined using a Nikon Eclipse E400 fluorescent microscope. Results were documented by photography.

5.2.5 Collection of excretion/secretion (ES) from *Lucilia sericata* larvae

In order to collect *Lucilia sericata* larva secretions the following protocol was followed:10 g of Lucilia sericata larvae were placed in individual, sterile 50 ml universal falcon tube containing 4 ml of sterile Milli-Q ultrapure water and incubated over night at 25°C in the dark. The resulting ES was collected from the larvae with a sterile syringe or pipette and centrifuged at 4000rpm for 10 minutes to remove particulate material, after which the supernatant was filter-sterilised (0.20um) and lyophilized. Prior to use, freeze-dried ES was resuspended in sterile Milli-Q ultrapure water at a final concentration of 40 mg/mL (Bexfield *et al.*, 2004).

5.2.6 Preparation of *Trichophyton terrestre* inocula

A standard sized inoculum of *T. terrestre* was prepared from 7- to 14-day old cultures grown on PDA at 25°C. Mature colonies were covered with approximately 5 ml of

sterile PBS (pH 7.4), PBS was then gently rubbed over the surface with a sterile spreader. The resulting mixture of conidia and hyphal fragments was drawn off with a pipette and transferred to sterile tubes. Heavy particles of the suspension were allowed to settle for 10 to 15 min at room temperature, and the upper homogeneous suspension was used for further testing. The optical densities of the suspensions were read at 530 nm and adjusted to 0.15 to 0.17 to yield 0.6×10^6 to 1.4×10^6 to spores/ml of strains. The suspensions containing conidia and hyphal fragments were further diluted to obtain the final desired inoculum size of approximately 0.4×10^4 to 5×10^4 spores/ml (Karaca and Nedret Ko 2004). The spore suspension (5 µl) was mixed with 20 µl of various concentrations of extracts, fractions (or active compounds) and transferred to Petri dishes containing SDA (Sabouraud Dextrose Agar). After incubation at 28 °C for 7 days the plates were photographed.

5.2.7 Anti-*Trichophyton terrestre* activity of *Lucilia sericata* ES

The agar dilution method was used to assess the activity of *L. sericata* ES on *T. terrestre*, *Trichophyton terrestre* was inoculated onto PDA (Potato dextrose agar) plates and incubated at 25°C for 7-10 days to obtain young, actively growing cultures consisting of mycelia and conidia. Sterilised ES was incorporated into PDA sterilised pre-poured medium to give a range of final concentrations (µg $ml^{-1)}$, the medium poured and the agar in the plates allowed to set. A mycelial disc, 8 mm in diameter, cut from the periphery of the 7-10-day-old cultures, was aseptically inoculated onto the medium. The inoculated plates were then incubated at 25 °C and the colony diameter measured and measured after 5, 10, 15 days. The percentage of mycelial inhibition was calculated as follows: % mycelial inhibition = $[(d_c-d_t)/d_c] \times 100$; d_c= colony diameter in control, d_t = colony diameter in treatment, three replicate plates were used for each treatment.

5.2.8 Mycoplasma Detection

Mycoplasma species were detected by using EZ-PCR Mycoplasma Test Kit (Geneflowltd,Cat No.20-70-20) which designed to detect various *Mycoplasma* species *(M. fermentans, M. hyorhinis, M. pulmonis, M. arthritidis, M. arginini, M. orale, M. bovis, M. salivarium, M. hominis, M. pneamoniae, M. pirum and M. capricolum*), as well as *Spiroplasma* and *Acholeplasma* species, with high sensitivity and specificity. EZ-PCR Mycoplasma Test Kit manufacturer's protocol was followed;

5.2.8.1 Test sample preparation:

Larvae were surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed aseptically; the entire larval gut was removed and suspended in sterile distilled water, vortexed for 5 minutes. To pellet cellular debris, 0.5-1.0ml of the entire gut suspension was transferred into a centrifuge tube and centrifuged briefly at 250 x g. The supernatant was then transferred into a fresh sterile tube and centrifuged again for 10 minutes at 15,000-20,000 x g to sediment *Mycoplasma*. The supernatant was carefully decanted and the pellet was re-suspended with 50µl of the Buffer Solution and mixed thoroughly with a micropipette, heated to 95'C for 3 minutes.

5.2.8.2 PCR amplification:

The reaction mixture was prepared in a PCR tube containing the following reagents; 10µl Reaction Mix, 5µl test sample and 35µl of sterile distilled water (sdH2O). 40µl of mineral oil was overlaid to prevent the evaporation of the reaction mixture. All PCR tubes were then placed in a DNA thermal cycle. The PCR reaction mixtures, after incubation at 94°C for 30 seconds as an initial denaturation, were cycled 35 times through the following temperature profile: denaturation for 30 seconds at 94°C;

annealing for 120 seconds at 60°C; and elongation for 60 seconds at 72°C, then one final cycle with the following incubations; 94°C for 30 seconds, 60°C for 120 seconds and 72°C for 5 minutes.

5.2.8.3 Analysis of amplified products by gel electrophoresis*:*

In order to check the amplified products and their size, 2% gel electrophoresis was prepared as previously described 2.2.17.1, 20µl of the amplified PCR product and 1µl of the Positive Template Control were applied to the gel electrophoresis. The size of DNA fragments amplified using the specific primers in this kit was 270bp. After being electrophoresed the DNA fragments were visualized under a UV transilluminator and the images were captured using a connected digital camera.

5.3 Results and discussion

5.3.1 Anti- *Mycobacterium* activity of (*Calliphora quadrimaculata*) maggot gases

As shown in Figure 5.3 the growth of *Mycobacterium phlei,* (a non-pathogenic strain was used as a model to avoid using *M. tuberculosis*) was inhibited by gases from the blue blow-fly (*Calliphora quadrimaculata*) larvae (maggot) grown on beef heart. In contrast, the growth of Methicillin-resistant *Staphylococcus aureus* (MRSA) was not inhibited by maggot gases. Ammonia vapour from (ammonium hydroxide) did not inhibit the growth of *M. phlei* and (MRSA).

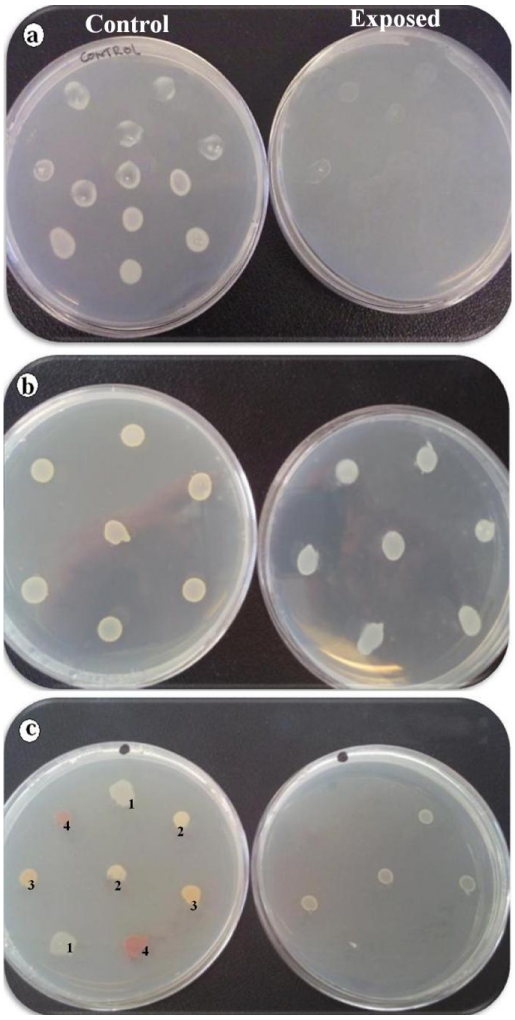


Figure 5.3: The effect (a) of *(Calliphora quadrimaculata)* maggot gases on the growth of *Mycobacterium phlei,* (b) The effect of maggot gases on the growth Methicillin-resistant *Staphylococcus aureus* (MRSA), (c) The effect of ammonium hydroxide vapour on the growth of 1. *E. coli,* 2. *M. phlei,* 3. (MRSA) and 4. *S. marcescens.* Number 2 *(M. phlei)* and 3 (MRSA) were not inhibited.

The ability of the gasses from the *Calliphora quadrimaculata* maggot to inhibit *M. phlei* but not (MRSA) indicated that the germicidal effect of these gases are not general and target a specific spectrum of bacteria. According to Wainwright (2007) , ammonia vapour is one of the main components of maggot gases, results shown in Figure 5.3 revealed that ammonia vapour was unable to suppress *M. phlei* growth which mean that maggot gases have other components cause this anti *M. phlei* activity. Recently, Arora and Baptista (2010) have detected in *Lucilia cuprina* maggot gases several compounds with antibacterial activity including aromatic aliphatic esters of furanone and tetrahydrofuran esters (Arora and Baptista, 2010).

5.3.2 Extraction of maggot gases and evaluation their antibacterial activity.

In order to quantify the *anti- Mycobacterium* activity of *(Calliphora quadrimaculata)* maggot gases, an attempt was made to dissolve these gases into d H_2O, by passing them through 20 ml of sterilised d H_2O and form steady stream of bubbles.

The antibacterial activity of the resultant gas extraction d H_2O solution was determined against *Mycobacterium phlei* and Methicillin-resistant *Staphylococcus aureus* (MRSA). Figure 5.4 and Figure 5.5 shows that the gas-extract failed to inhibit both *Mycobacterium phlei* and Methicillin-resistant *Staphylococcus aureus* (MRSA). It appears therefore that the active ingredients are non-water soluble compounds which do not dissolve in the d H_2O extraction solution. In order to overcome this problem and to enhance the solubility of these expected non-water soluble compounds, Tween 80 was added to d H_2O extraction solution in final 0.05% concentration.

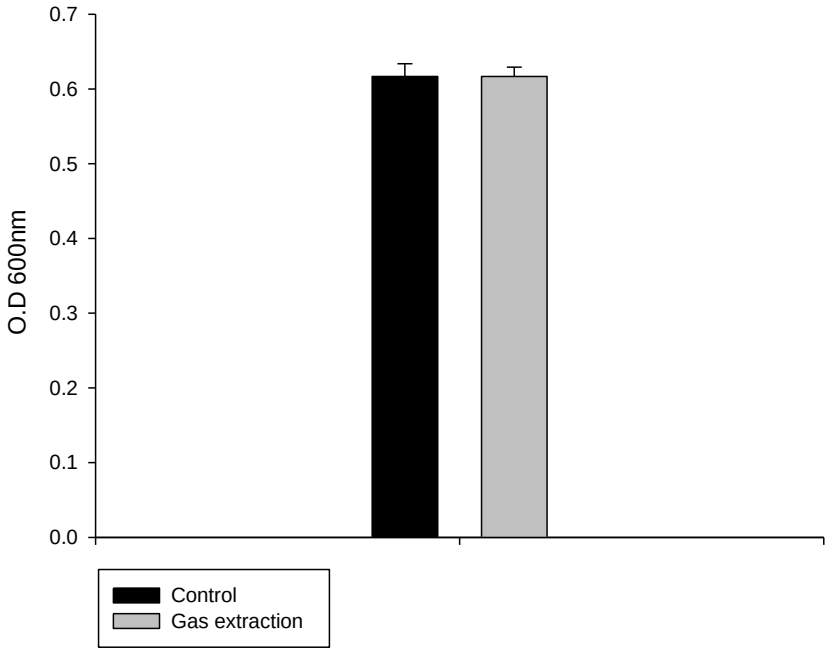


Figure 5.4: The effect of maggot gases extracted by d.H_2O on the growth of *M. phlei*, results are expressed as means of O.D at 600nm ± SD. There is no a statistical significant difference between the input groups (P = 1.000).

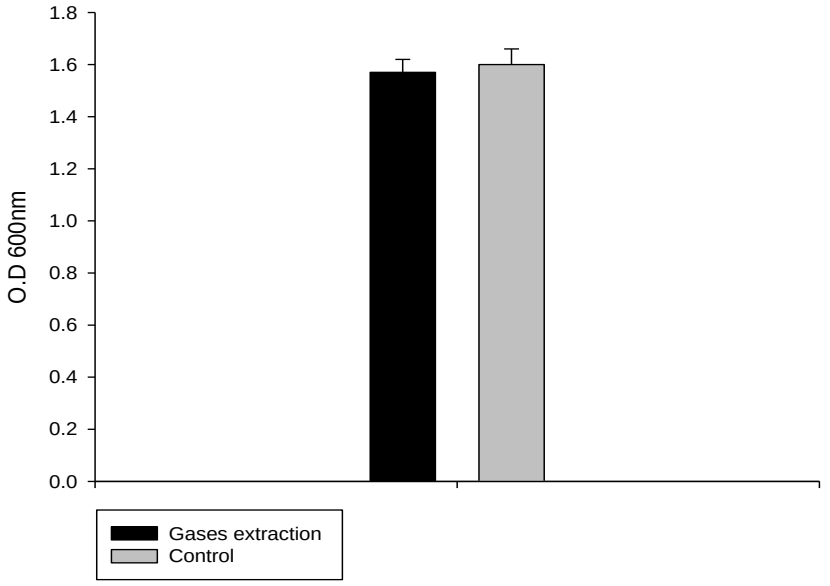


Figure 5.5: The effect of maggot gases extracted by d.H_2O on the growth of Methicillin-resistant *Staphylococcus aureus* (MRSA), results are expressed as means of O.D at 600nm ± SD. There is no statistical significant difference between the input groups (P = 0 .542).

The effect of maggot gases extracted by d.H_2O with 0.05% Tween 80 on the growth of *M. phlei* and MRSA is shown in Figure 5.6 and Figure 5.7, again, no significant inhibitory effect of the gas extraction solution was observed against *M. phlei* and (MRSA).

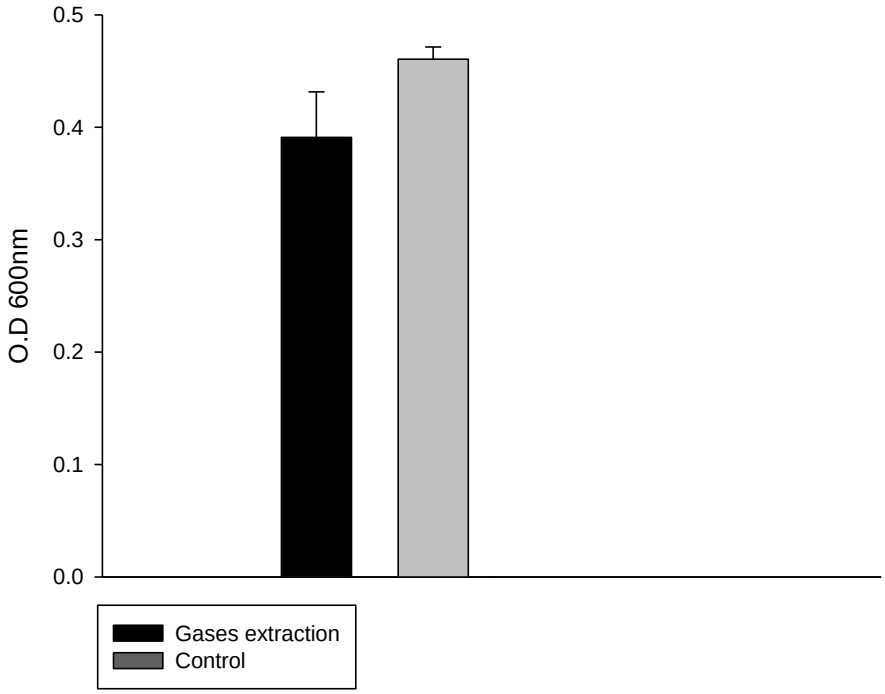


Figure 5.6: The effect of maggot gases extracted by d.H_2O with 0.05% Tween 80 on the growth of *M. phlei*, results are expressed as means of O.D at 600nm ± SD. There is no statistical significant difference between the input groups (P = 0.107).

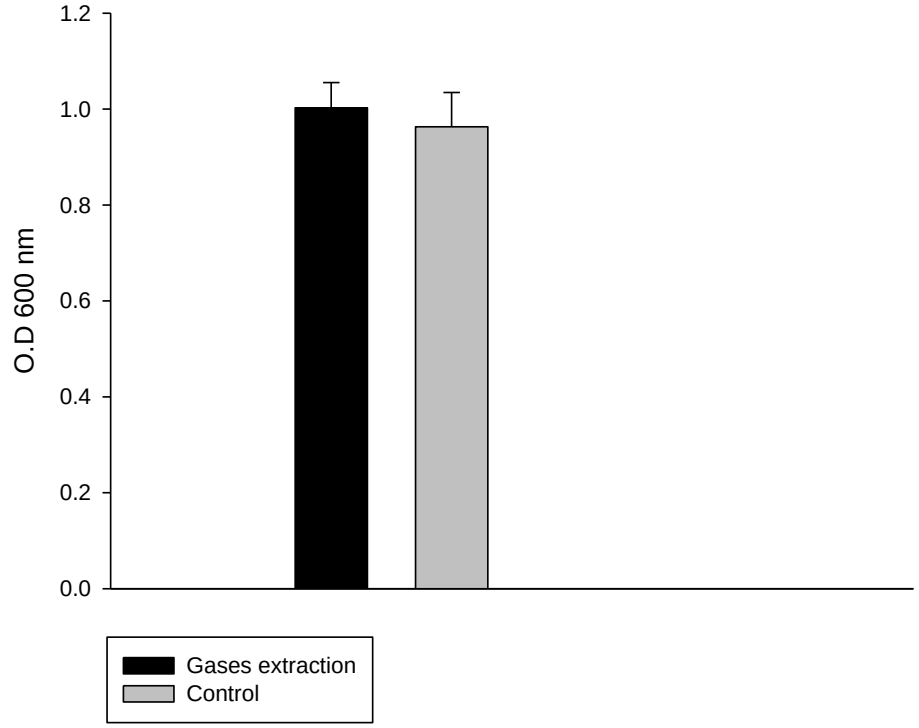


Figure 5.7: The effect of maggot gases extracted by d.H2O with 0.05% Tween 80 on the growth of Methicillin-resistant *Staphylococcus aureus* (MRSA), results are expressed as means of O.D at 600nm ± SD. There is no statistical significant difference between the input groups (P = 0.484).

Activated charcoal was next employed as a potential adsorbent of the antibacterial agent present in maggot gasses. Activated charcoal is an efficient gas absorbent (Ray and Box, 1950) and a gram of activated charcoal has a total surface area in range of 500 to 2000 m^2 (Von Blucher and De Ruiter, 1991). Maggot gases were passed through activated charcoal which was then washed with chloroform and left to evaporate. The precipitate was then re-suspended in sterilised d H_2O with 0.05% Tween 80. Again Figure 5.8 and Figure 5.9 show that no significant antibacterial effect was seen and that no inhibitor was extracted by the use of activated charcoal.

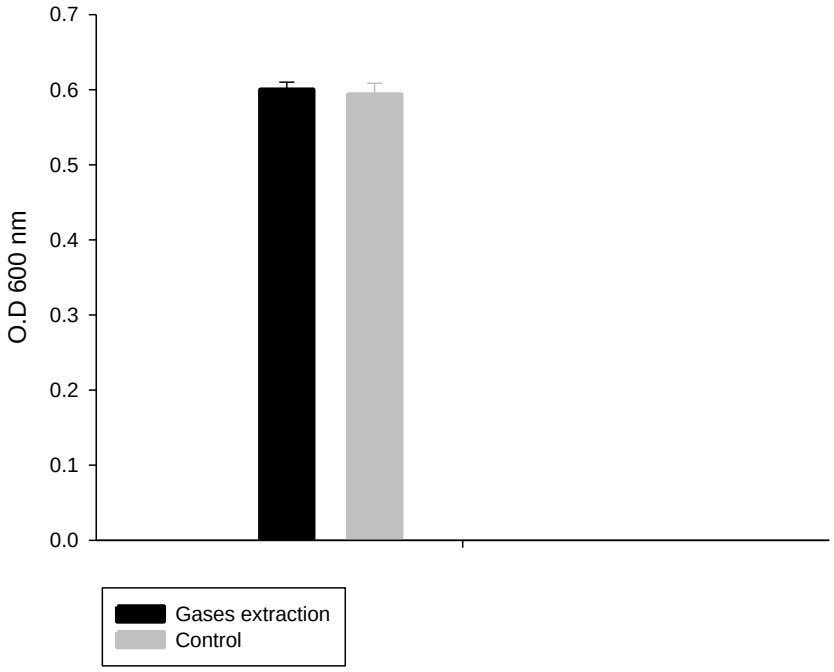


Figure 5.8: The effect of maggot gases extracted by activated charcoal on the growth of *M. phlei*, results are expressed as means of O.D at 600nm ± SD. There is no statistical significant difference between the input groups (P = 0.561).

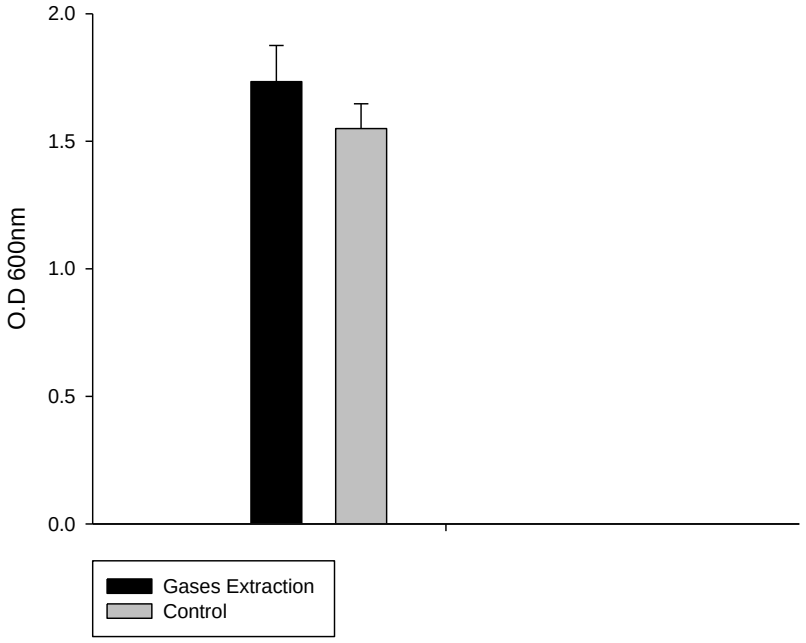


Figure 5.9: The effect of maggot gases extracted by activated charcoal on the growth of Methicillin-resistant *Staphylococcus aureus* (MRSA*)*, results are expressed as means of O.D at 600nm ± SD. There is no statistical significant difference between the input groups (P = 0.136).

As shown in previous three experiments attempts to extract the anti-*Mycobacterium* activity of maggot gases were unsuccessful. One final attempt was made to determine the effect of maggot gases on *M. phlei* cell viability. As shown in Figure 5.10, there was no significant inhibitory effect of the maggot gases exposure on the *M. phlei* cell number. This successive absence of inhibitory influence of gases extraction is hard to explain, but it could be due that the extracted gases were highly diluted in extraction solutions and extracts showing that other extraction techniques need to be applied.

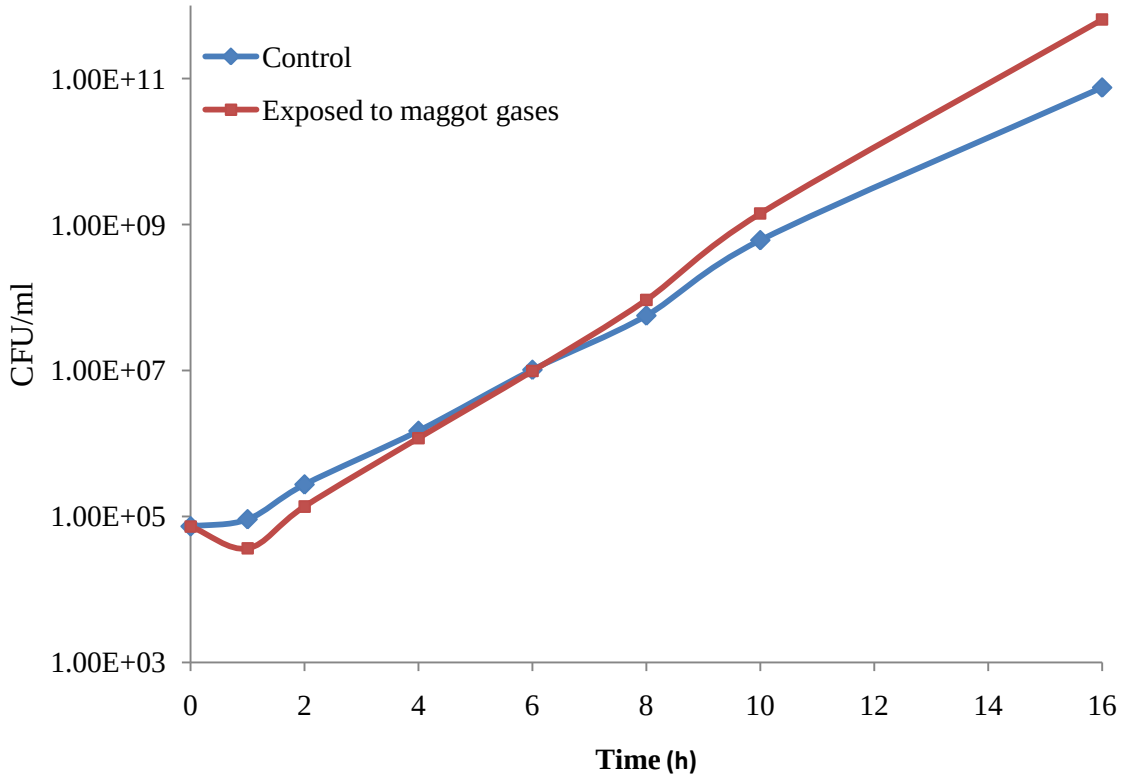


Figure 5.10: Effect of maggot gases on the growth of *M. phlei*, using approach shown in Figure 5.2. Treated cells were exposed to fresh maggot gases.

5.3.3 The anti-MRSA activity of *Lucilia sericata* pupal hemolymph.

Invertebrates, predominantly insects, have been considered as a potential sources of antimicrobial agents, notably peptides (Chernysh *et al*., 2002). In contrast to the higher animals, insects lack immunoglobulin and use antimicrobial peptides to protect themselves from bacterial infections, such peptides are commonly found in the hemolymph of all life stages of the insects (Sahalan *et al*., 2007).

The antibacterial activity of excretions of *Lucilia sericata* maggots has been extensively reported (Arora and Baptista, 2010; Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Thomas *et al.*, 1999; Jaklic *et al.*, 2008; Jukema *et al.*, 2008). Furthermore, whole body extracts and haemolymph of *Lucilia sericata* maggots have been shown to have antibacterial activity (Huberman *et al*., 2007). Since the whole body extracts and haemolymph of *Lucilia sericata* maggots have been reported to have antibacterial activity, we assumed here that the haemolymph of the pupa would have similar antibacterial activity as well. In this section the antibacterial activity of haemolymph extracted from *Lucilia sericata* pupa was investigated.

Effect of hemolymph from pupae of *Lucilia sericata* on the viability of Methicillin-resistant *Staphylococcus aureus* (MRSA) is illustrated in Figure 5.11, whereas the effect of hemolymph from pupae of *Lucilia sericata* on the on the final optical density of MRSA is illustrated in Figure 5.12. As clearly shown in these two figures, hemolymph from the pupae of *Lucilia sericata* does not exhibit any inhibitory effect on MRSA.

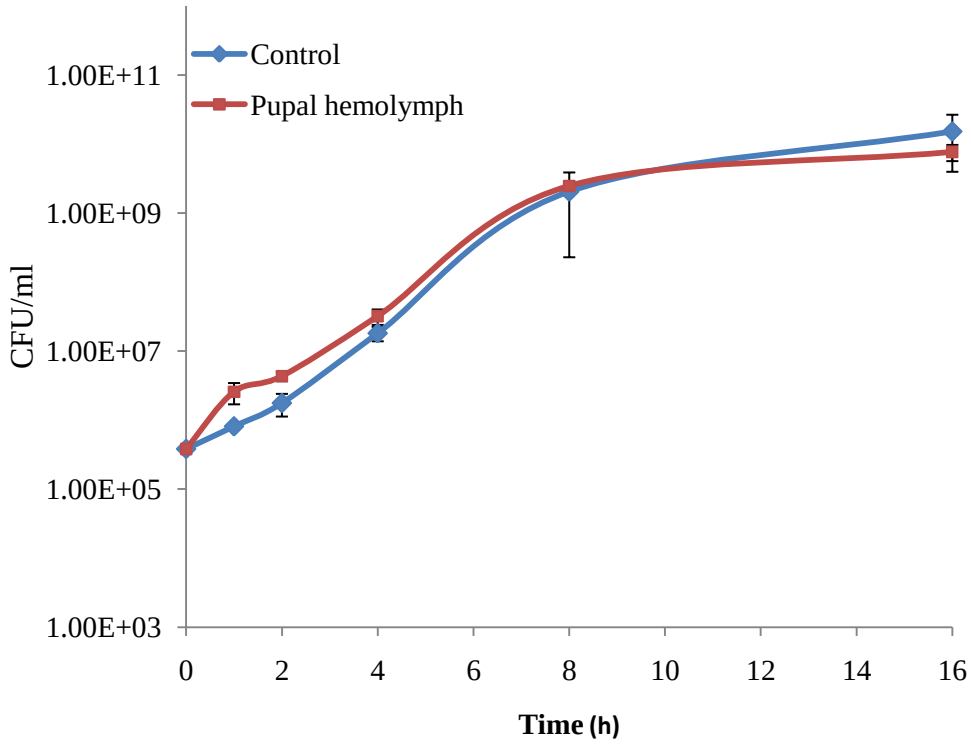


Figure 5.11: Effect of hemolymph from pupae of *Lucilia sericata* on the viability of Methicillin-resistant *Staphylococcus aureus* (MRSA). Values are represented as means of the replicates± standard deviation.

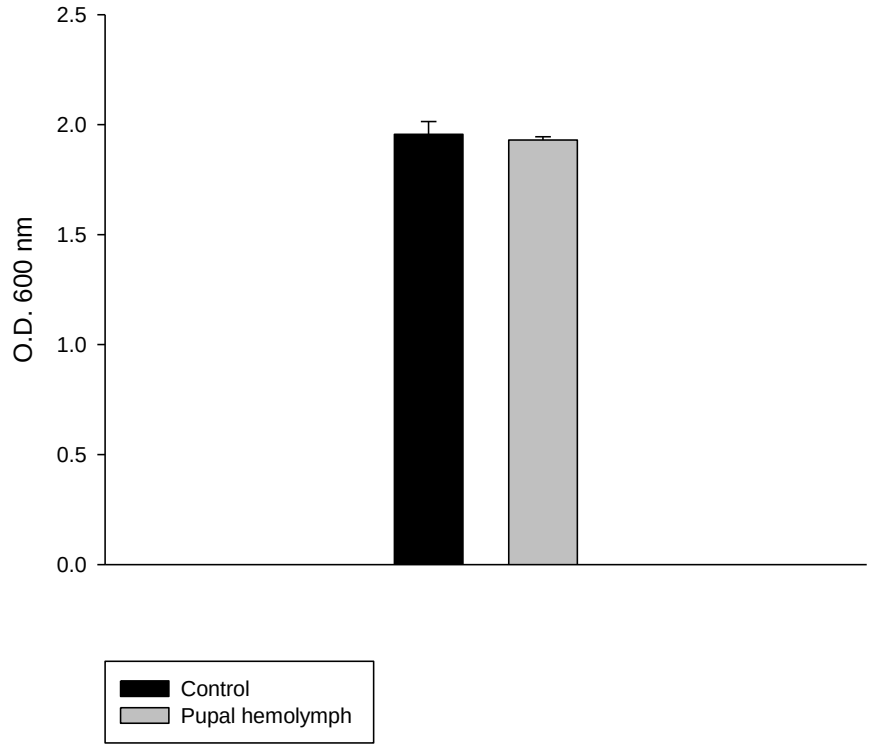


Figure 5.12: Effect of hemolymph from pupae of *Lucilia sericata* on the final optical density of Methicillin-resistant *Staphylococcus aureus* (MRSA) after overnight incubation with Pupal hemolymph. Values are represented as means of the replicates± standard deviation.

5.3.4 Potential use of *Lucilia sericata* maggot to control superficial fungal infections, i.e. fungal burn wound infections and dermatomycoses.

Fungal infections are considered as an important cause of morbidity and death of patients with burn wounds (Horvath *et al*., 2007; Murray *et al*., 2008; Becker *et al*., 1991); according to Rode *et al*., (2008) fungal infections complications that delay the healing process are associated with 30% of burn wounds.

Dermatomycoses, such as ringworm or tinea (Lakshmipathy and Kannabiran, 2010; Weitzman and Summerbell, 1995), are infections of the keratinized layers of skin, hair and nail (Lakshmipathy and Kannabiran, 2010). Dermatomycoses are among the most widespread infectious diseases in the world, mainly in the tropical and subtropical countries (Brasch and Graser, 2005; Lakshmipathy and Kannabiran, 2010). Such infections are difficult to control and expensive to treat (Bokhari, 2009). It has been estimated that the worldwide annual cost of dermatophytosis drug development is over USD $ 0.5 billion (Gräser *et al*., 2008). The prolonged systemic use of antifungal drug treatment of dermatomycoses is highly linked to fungal resistance, drug toxicity and interactions (Koroishi *et al.*, 2008) although positive outcomes in dermatophytosis control are generally associated with topical antifungal therapy (Karaca and Nedret Ko 2004).

The introduction of maggot therapy to treat heavily fungal colonized burn wounds and dermatomycoses such as athlete's foot might prove a viable alternative to the use of conventional antibiotics, especially where antibiotic resistance is found.

In order to evaluate the use of *Lucilia sericata* maggots in fungal wound infections and superficial fungal infections management, two questions need to be answered; firstly, do the *Lucilia sericata* maggot excretions/secretions have antifungal activity ?-

and secondary, can *Lucilia sericata* maggots ingest pathogenic fungi ? To the best of our knowledge there are no previous reports on the antifungal activity of maggots against filamentous fungi, but in regard to the yeast fungi, complete lysis of *Candida albicans* has been shown *in vitro* after 24 h of maggot application (Margolin and Gialanella, 2010), and Jarczyk *et al.* (2008) have reported the elimination of *Candida* spp. from chronic foot ulcerations after treatment with maggots(Jarczyk *et al*., 2008).

In this section attempts were made to, a) investigate the ability of *Lucilia sericata* maggots to ingest filamentous fungi and yeasts, b) investigate the antifungal activity of *Lucilia sericata* maggot excretions/secretions against filamentous fungi only (as it has already been demonstrated against yeasts). *Trichophyton terrestre* was chosen as a model dermatophyte filamentous fungi.

As shown in Figure 5.13, after placing the *Lucilia sericata* maggots into the agar plates inoculated with *S. cerevisiae* (BY4742) GFP for 60 minutes at 25 °C, under fluorescent microscope a significant fluorescence was observed in the gut contents of ten different maggots out of ten examined (i.e.100%), whereas no fluorescence was seen in maggots fed on only agar. This clearly shows that the *Lucilia sericata* maggots successfully ingested yeasts.

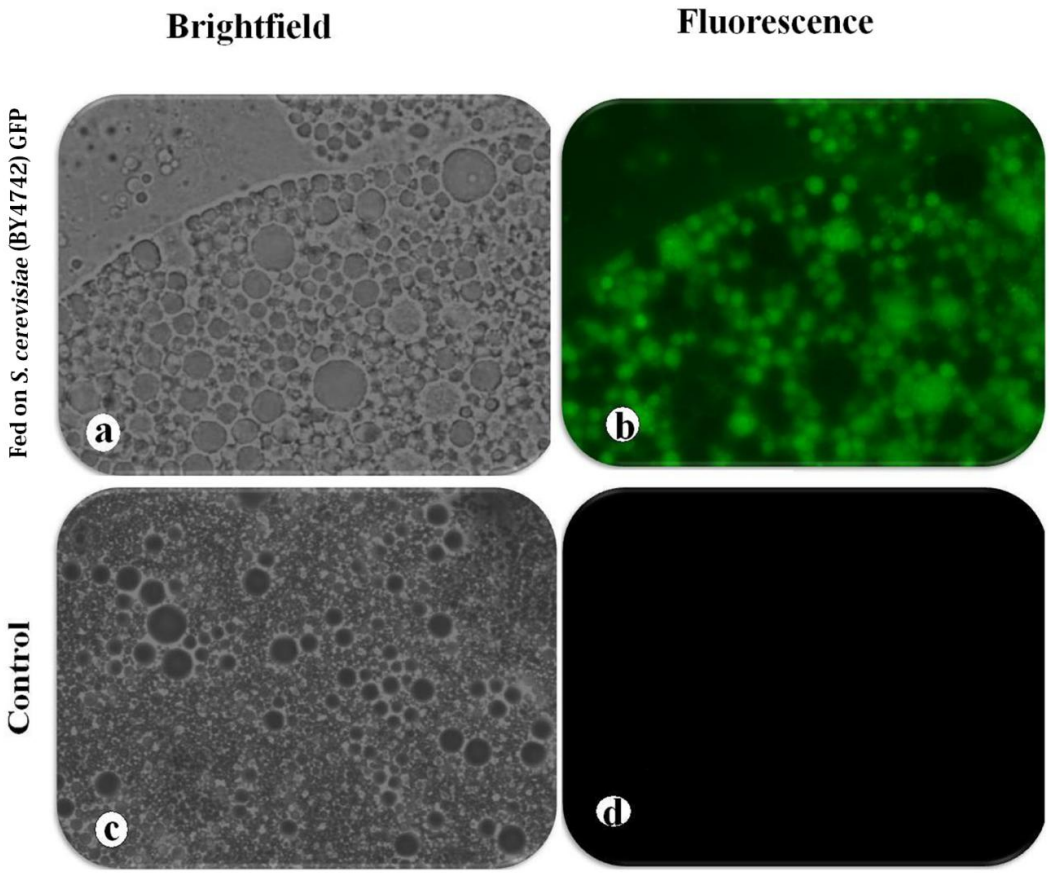


Figure 5.13: Left and Right microscopy images correspond to bright field and GFP fluorescence, respectively. (a) bright field microscopy image of the digestive tract of maggots fed on *S. cerevisiae* (BY4742) GFP (b) GFP fluorescence microscopy image of the gut content of maggots fed on *S. cerevisiae* (BY4742) GFP (c)and (d) bright field and GFP fluorescence microscopy images of the gut content of control maggots

When excretions/secretions (ES) of *Lucilia sericata* maggots were tested against *Trichophyton terrestre* by the agar dilution method, 1mg/ml of ES showed a considerable inhibition of growth of mycelium as illustrated in Figure 5.14. The radial growth inhibition after 10 days of incubation reached 41.2 ±1.8 % in relation to the control. This appears to be the first time that the antifungal activity (i.e filamentous) of maggot's excretions/secretions has been both tested and observed.

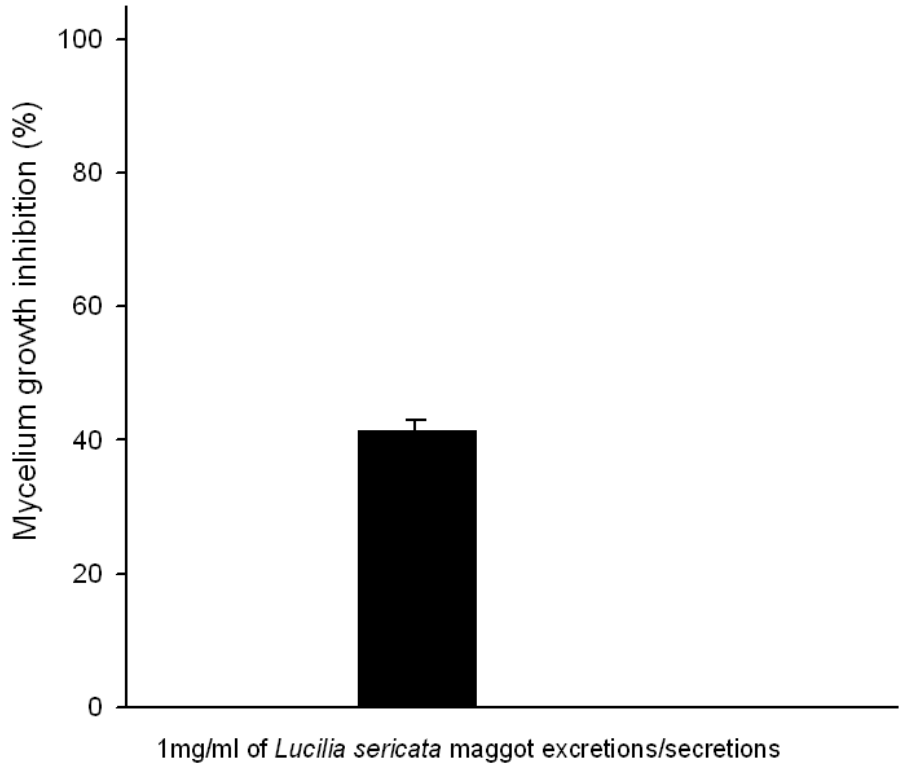


Figure 5.14: Percent inhibition of mycelial growth of *Trichophyton terrestre* in presence of 1mg/ml *Lucilia sericata* maggot excretions/secretions. Percentage of mycelial growth inhibition respective to the control (no ES added). Means of three replicates ±SD.

In investigation of the ability of *L. sericata* maggots to ingest filamentous fungi, no growth of *Trichophyton terrestre* was observed in 16 different samples after 10 days of incubation of a subcultures of the maggot's gut suspensions fed on *Trichophyton terrestre.* This observation could indicate that *L. sericata* maggots are unable to ingest *Trichophyton terrestre* mycelium and spores, or that the fungus is killed as the result of ingestion. Results obtained here show that maggots are able to ingest yeasts and as mentioned above, it has been shown that maggots application led to a completed lysis and elimination of *Candida* spp. (Jarczyk *et al.*, 2008; Margolin and Gialanella, 2010). With regarding to filamentous fungi, the results show that the maggot ES have moderate antifungal activity , but the results failed to provide evidence about

whether the maggots are able to ingest filamentous fungi. However, this does not necessarily mean that maggots are unable to deal with such fungi as maggots are mainly feed through extracorporeal digestion where a mixture of digestive enzymes (such as tryptase, peptidase, and lipase) are continuously produced by larval salivary glands into the surroundings (Lerch *et al.*, 2000; Andersen *et al.*, 2010), the secreted digestive enzymes could lead to the destruction and lysis of the fungal mycelium ,to be subsequently adsorbed by the maggot's powerful suction apparatus (Andersen *et al*., 2010) especially since maggots are known to have an ability to ingest as much as half of their body weight within a few minutes (Fleischmann *et al*., 2004).

The moderate antifungal activity (yeast and mould) of ES of *Lucilia sericata* maggots and the ability of these maggots to ingest yeast and probably to destroy and lyse mould mycelium, could lead to the possible application of maggot therapy in the treatment of wounds undergoing fungal infection and with superficial fungal infections i.e. athlete's foot (Summers and Kaminski, 2003; Namias *et al.*, 2000). Further studies are now needed to help confirm this possibility.

5.3.5 Determine the incidence of Mycoplasma species in the *Calliphora quadrimaculata* and *Lucilia sericata* maggot hemolymph.

Mycoplasma are the smallest prokaryotic organisms and since they lack a cell wall (Greenwood, 1997) are resistant to all antibiotics that target cell wall synthesis, such as penicillin and cephalosporin (Tadesse and Alem, 2006). Mycoplasmas are found in humans, animals, plants, insect, soil and sewage (Greenwood, 1997). Genetically, *Mycoplasma* have a small single circular chromosome containing 0.58 to 2.2 Mbp with a low G+C content (23 to 40 mol%) which drastically reduces their biosynthetic capabilities and explains why they tend to be dependent on a host, phylogeny. Studies

have showed that *Mycoplasmas* are strongly related to the Gram-positive eubacterial subgroup like streptococci and lactobacilli (Waites and Talkington, 2004). As a result of them lacking cell wall, mycoplasma are highly pleomorphic bacteria, their final shape varies depending on environmental conditions and the stage of the growth cycle. Thirty species have been placed in the genus Mycoplasma including: *M. fermentans, M. genitalium, M. hominisa* and *M. pneumonia*, all of which are of medical importance due to their pathogenicity and role in human disease (Greenwood, 1997; Tadesse and Alem, 2006). *Mycoplasma pneumonia*, is a human pathogen which causes atypical pneumonia and other lower respiratory tract infections, while *M. genitalium*, is the presumed cause of pelvic inflammatory disease (Goulet *et al*., 1995).

Insect vectors are among the most important means of spread *Mycoplasma* and rickettsia like organisms in nature(D'Arcy and Nault, 1982). There have been relatively few reports of the isolation of Mycoplasmas from insect and flies larvae; these include isolation from moth larvae, *Leucoma salicis* (Oduori *et al.*, 2005); larvae of *Melolontha melolontha* (L.) (Devauchelle *et al.*, 1970) and flies(Museux *et al.*, 2009).

The aim of the present work was to determine if Mycoplasmas can be detected in the hemolymph of maggots of *Calliphora quadrimaculata* and *Lucilia sericata*.

Table 5.1: The presence or absence of *Mycoplasma* in *Calliphora quadrimaculata* and *Lucilia sericata larvae*

Larvae of	**No. samples**	**Positive results**	**Negative results**
Calliphora quadrimaculata	**3**	**0**	**3**
Lucilia sericata	**3**	**0**	**3**

In order to detect *Mycoplasma*, EZ-PCR Mycoplasma Test Kit was used. In this kit specifically designed primers that target a highly conserved sequences in *Mycoplasma*

DNA only (not bacterial or animal DNA sequences) was used. Generally, this PCR dependant technique is highly sensitive, specific and rapid method compared with conventional direct culture procedures.

The results obtained after conducting a search for Mycoplasmas in 6 different maggots samples are shown in Table 5.1. Agarose gel electrophoresis in Figure 5.15 did not yield a 270bp fragment expected for the positive template kit control. Mycoplasmas were not therefore detected in any of the maggot samples tested. These observations suggest that *Calliphora quadrimaculata* and *Lucilia sericata* larvae are unlikely to act as transmission vectors for Mycoplasmas.

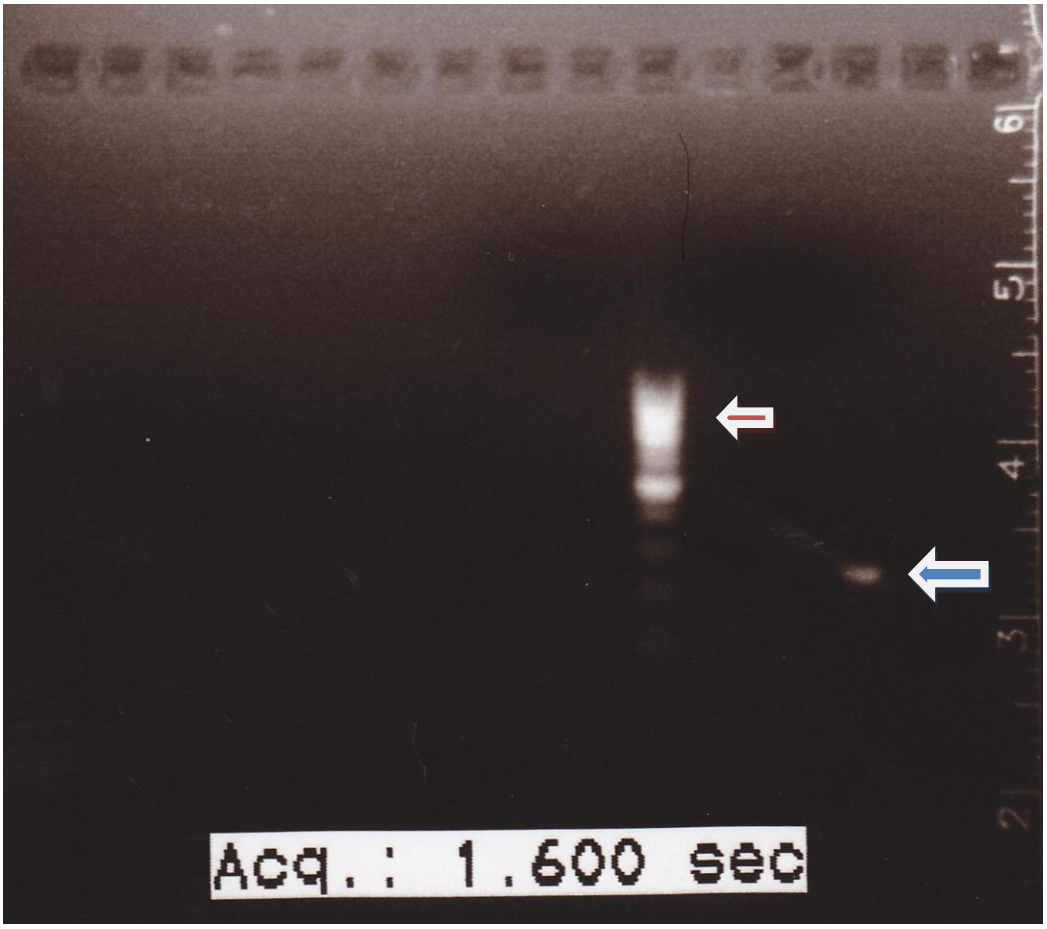


Figure 5.15 : Blue arrow head shows 270bp product of DNA of PCR amplification from positive template kit control and white arrow head shows the ladder type which was used with 16s rRNA. All samples showed no bands (negative results). Sterilized d H_2O sample was used as negative control.

Chapter Six: Fungi isolated from mouldy foods inhibit *Staphylococcus* including MRSA. A Rationale for the Re-introduction of Mycotherapy?

6.1 Introduction

Well before Fleming's famous discovery of penicillin in 1928, moulds had been widely used in all cultures as curative agents. Imhotep, for example, an ancient Egyptian practitioner, used mouldy bread to treat face infections. Literature from more recent folk medicine has documented some other examples of the use of moulds on infections. Mouldy jam and mouldy bread were for example, widely used in Quebec, Devon, Kansas and USA and poultices made from mouldy chewed barley and apple have been used in Asia to cure surface wounds. In 1640, one of London's apothecaries advised that moulds have a curative effect when applied to infections (Wainwright, 1989).

The difficulties facing researchers attempting the large-scale production of penicillin during 1940s encouraged some workers to re-evaluate the therapeutic properties of crude penicillin filtrates for use in hospitals. They developed a number of different methods to apply the crude penicillin topically, such as the application of lint saturated with liquid filtrates or dressings inoculated with the penicillin-producing mould, *P. notatum*. In addition, crude penicillin was applied in agar. Even though many successful cures were achieved using crude penicillin, its unrestrained production and potential purity problems restricted its use and it became completely ignored once purified penicillin became widely available (Wainwright, 1987).

Wainwright and co-workers (Wainwright *et al*.1992) investigated the scientific basis of the mycotherapy; their results suggested that the active ingredient that responsible for this curative effect is not penicillin, but patulin.

The aim of the work reported in this Chapter was to determine if fungi growing as contaminants on mouldy foods produce penicillin and patulin filtrates (when grown on Czapek Medium) which are inhibitory to MRSA.

6.2 Materials and methods

6.2.1 Test organisms

The following test organisms (bacteria) were used: Methicillin-Sensitive *Staphylococcus aureus* (MSSA) and Methicillin-resistant *Staphylococcus aureus* (MRSA); unless stated otherwise, the organisms were obtained from the Departmental Culture Collection.

6.2.2 Sources of the moulds

A variety of naturally mouldy food, vegetables and fruits were collected from local retail outlets; these were: bread, apple, lemons and beetroot. Two penicillin-producing fungi, *Penicillium chrysogenum* IMI 37767 and *Penicillium chrysogenum* IMI 24317, were used as controls.

6.2.3 Mould cultivation

Indigenous fungi were isolated from a variety of collected mouldy foodstuff. Either skin (2 x2 mm) or heavily sporulating colonies were picked off and maintained on Czapek Dox liquid medium (Oxoid) and incubated at 25°C with shaking 150 rpm for two weeks.

6.2.4 Screening of the fungal isolates for antibacterial activity.

Isolated fungal cultures were identified after visulisation under the low power microscope and grown as described above. The mycelia of these cultures were separated by filtering with Whatman filter paper. The antibacterial activity of the harvested mycelia and the filtrates was evaluated directly by using the agar plate diffusion assay as described (2.2.6), but here the wells were filled with either 100 μL of filtered broth or air-dried fungal mycelia. Plates were incubated at 37°C for 24 h. The diameter of the inhibition zone (mm) produced was then measured.

6.2.5 Detection of patulin in fungal liquid cultures.

Pure crystalline patulin (Sigma 5mg) was dissolved in 5ml of chloroform (Fluka) to produce a concentration of 100 μg ml^{-1}. The solution was kept in well-wrapped, aluminium foil bottle and stored at 4°C. The standard was used at room temperature (Gimeno and Martins, 1983).

6.2.5.1 Preparation and development of TLC plates

Sigma-Aldrich® TLC (thin layer chromatography) plates, cellulose matrix, H × W 10 cm × 20 cm with fluorescent indicator were used as a stationary phase. Two 10 μl aliquots of fungal extracts were spotted along a line 3 cm from the lower edge of the plate. 5 μl of patulin standard were spotted as a reference standard solution. The plates were then placed in Latch-lid™ TLC chambers (Aldrich) for one dimensional development for about 2 hours in benzene: methanol: acetic acid - BMA (90:5: 5 v/v/v) (mobile phase); they were left at room temperature until the solvent front had reached a line marked 2 cm from the top of the plate. The plates were then removed and air-dried in a fume cabinet and sprayed with a freshly prepared mixture of 0.5 ml p-anisaldehyde (in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid) and then heated at 110° in a hot-air oven for 10 minutes. Plates were then were visualized by 2UV Transilluminator (300–360 nm) connected to Kodak DC290 camera. After being air-dried and sprayed with a freshly preparation *p*-anisaldehyde (Fluka), developed chromatograms were viewed under visible and UV light for the presence of fluorescent spots. The *p*-anisaldehyde spray allows patulin to be detected as faint brown spots under the visible light and as yellow-orange under UV light. According to Scott *et al*., 1970 using ethanol instead of methanol in the spray reagent will improve the detection of patulin at a detection limit of 0.1 μg (Scott *et al.*, 1970; Scott and Somers, 1968).

6.2.5.2 Extraction of patulin from fungi liquid cultures.

The contents of all fungal liquid culture flasks were filtered through Whatman No.1 filter paper. All extract solutions were then centrifuged (in 50 ml Universal) at 4000 rmp for 15 minutes; supernatants were transferred into 250 ml separating funnel contained tow equal volumes of ethyl acetate. The separating funnel was shaken for 2 minutes and the contents allowed to settle and layers to be separated. The bottom aqueous (fungal culture) layer was removed and discarded, while the top layer (ethyl acetate) was collected and combined with 2 g of anhydrous sodium sulphate(Na_2SO_4), then left to evaporate to dryness.

6.2.6 Beta-Lactam detection in the isolated fungi liquid cultures

The SNAP* MRL Beta-Lactam Kit (IDEXX, USA) is a test for Beta lactams which depends on an enzyme-linked receptor-binding assay, designed to detect most beta-lactam antibiotics, notably penicillin G. The Kit contains : a SNAP Device, a sample tube containing reagent pellet, a pipette, SNAP positive controls and a heater block capable of maintaining an operating temperature of 45°C +5°C .(see Appendix).

6.2.6.1 Test Preparation

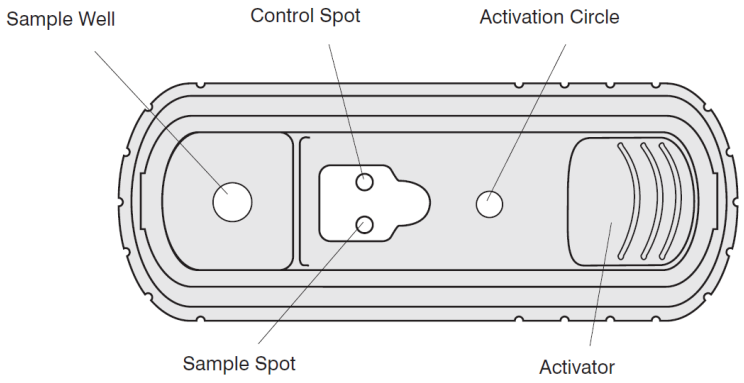
The heater block was preheated to 45°C +5°C, the SNAP device, the pipette and sample tube were removed from the bag and the reagent pellet was checked to be present at the bottom of the sample tube.

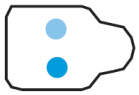
6.2.6.2 Test Procedure

The SNAP device was placed in the heater block; 2ml of the liquid culture(sample) were added, shaken thoroughly, and then the sample tube cap was removed and discarded, 450 µl of the sample was drawn up with the IDEXX pipette, the sample was then added carefully from the pipette to the tube. To dissolve the reagent pellet the

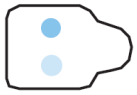
sample tube was shaken. The sample tube was then incubated in the preheated heater block at 45°C for 5 minutes.

The contents of the sample tube was poured into the sample well of the SNAP device, and the tube was discard. The sample flowed across the results window toward the blue activation circle. When the blue activation circle began to disappear, the activator button was pushed firmly until it snapped flush with the body of the SNAP device, during colour development the SNAP device was left in the heater block. After 4 minutes, the result was ready to be read. Before interpreting results, the SNAP device with the sample well was positioned to the left, as shown in the (Figure 6.1).





Negative Result—The sample spot is darker than or equal to the control spot.



Positive Result—The sample spot is lighter than the control spot.

Figure 6.1 Interpreting SNAP* MRL Beta-Lactam Kit test results

6.3 Results and Discussion

The ability of mycelium of *Penicillium* species to inhibit both methicillin-sensitive and methicillin-resistant *S.aureus* is shown in Table 6.1 and Figure 6.2; in the case of the apple isolate, an illustration of the zones produced on the test plate is shown in Figure 6.2. As expected, a paper test disc containing penicillin G inhibited MSSA, but not MRSA. Mycelium from all of the *Penicillium* cultures isolated from foods inhibited both MSSA and MRSA. The obvious question is what antibacterial agent responsible for this inhibition? Tests using SNAP (Figure 6.3) to test for penicillin and TLC (Figure 6.4) for patulin showed that penicillin was found in culture filtrates from all of the individual fungi, while patulin was produced by the apple and beetroot isolates. The isolation of a patulin-producing fungus from apples is however, to be expected since patulin is commonly produced on both apples and in apple juice (Beretta *et al.*, 2000).

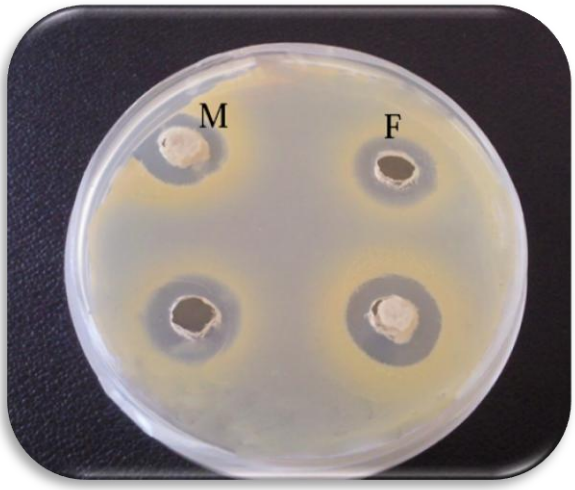


Figure 6.2: The antibacterial activity of mycelia (M) and the filtrates (F) of the fungus isolated from apple liquid culture against MRSA.

Table 6.1: The antibacterial activity of the mycelium of fungi (zone of inhibition, mm) isolated from different foodstuffs.

Test	MSSA	MRSA	Penicillin	Patulin
Penicillin G disk (6µg)	**29**	**0**	**+**	**-**
***Penicillium chrysogenum* IMI 24317**	**25**	**23**	**+**	**-**
***Penicillium chrysogenum* IMI 37767**	**13**	**14**	**+**	**-**
Lemon *Penicillium*	**19**	**19**	**+**	**-**
Beetroot "	**16**	**17**	**+**	**+**
Bread "	**17**	**18**	**+**	**-**
Apple "	**17**	**17**	**+**	**+**

Although penicillin and patulin were tested here it is, of course, possible that other antibacterial agents were also produced which act alone, or together with penicillin or patulin to inhibit the MSSA or MRSA. This would explain how the MRSA was inhibited when this bacterium is known to be resistant to penicillin. In relation to mycotherapy is the nature of the antibacterial agent or agents produced by the mycelium important? Certainly from normal clinical viewpoint, a clinician would be expected to know what agents are being produced by a mould which is being used to treat a wound. This is especially true in the case of *Penicillium* species which are known to produce various potentially dangerous toxic mycotoxins. Patulin, for example is toxic and its long term intake into the body may lead to the formation of cancers. The application to wounds, of a mould which is known to produce penicillin and or patulin, as well as other unknown antibacterial (or otherwise) compounds would obviously be frowned upon by regulatory authorities and would not pass ethical panel scrutiny.

Under what conditions might moulds, isolated from common foods, be used to treat Staph. infected wounds, including those infected with MRSA? During clinical experience gained in the period when *P.notatum* was used as a filtrate, as macerated mycelium in filtrates, or when growing on agar or other substrates applied to wounds exposed no examples of toxic contradictions.

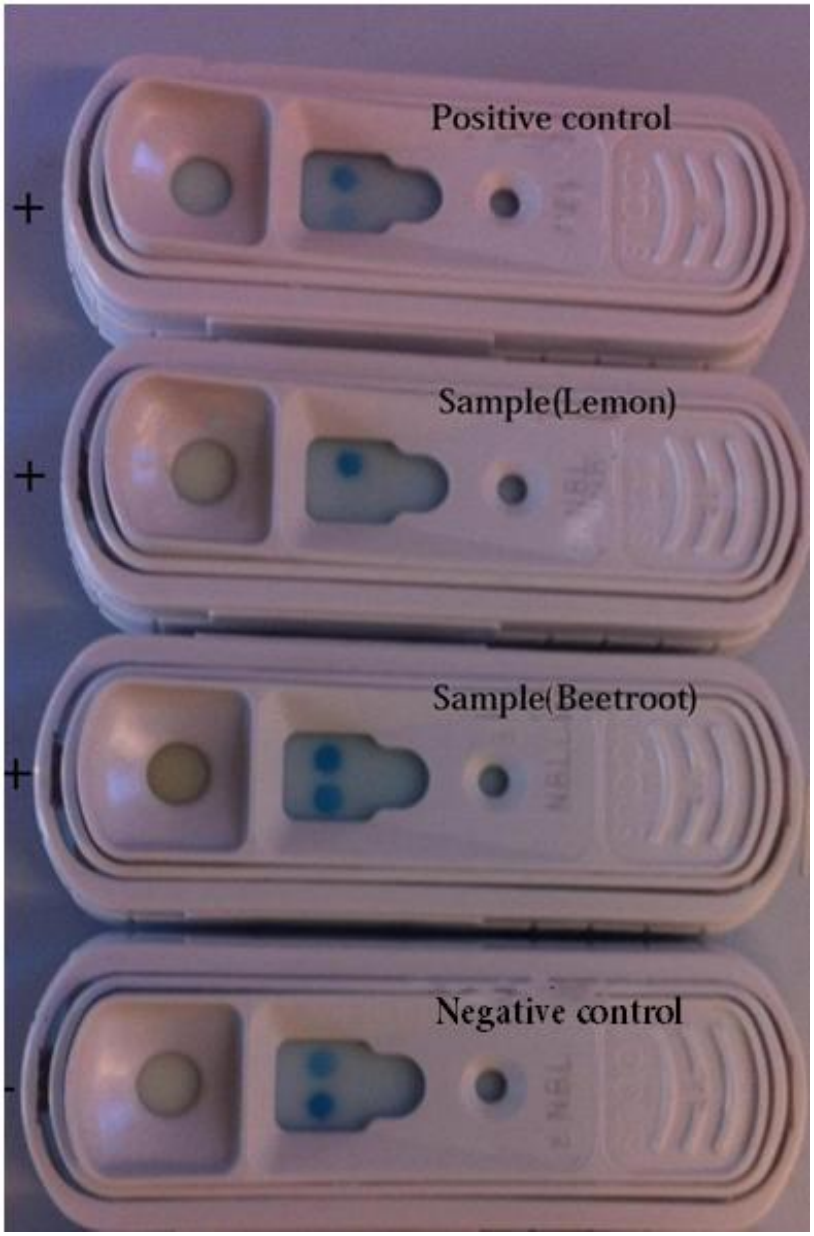


Figure 6.3: SNAP devices showing different results according to samples sources. Note in positive control, lemon and beetroot samples the control spot are darker than the sample spot (positive results), whereas in the negative control (sterilized Czapek Dox liquid medium) control spot was lighter than the sample spot.

Clearly then, a pure culture of a *Penicillium*, known to produce penicillin and not patulin or other toxicant (like the two IMI strains used here) could be safely used to treat wound infections in hospitals at the present time. Is there ever likely then to be situation when the results obtained in this study could be put to practical use? The obvious answer is that *Penicillium* moulds isolated from mouldy foods could be applied

to wounds *in extremis*, that is when no other medical intervention is available to treat an infected wound, e.g. following an event which leads to the total breakdown of society (e.g. following a nuclear war, or during local extreme living conditions such as in a prisoner of war camp. Under these conditions, *Penicillium* cultures could be isolated from mouldy foods (a mixture of such moulds from various foods might prove particularly effective) and could be applied to wounds in the knowledge that they would contain penicillin and or patulin, or other antibacterial metabolites, which could prove the difference between life and death for a patient with an critically infected wound. Alternatively, food-moulds could be applied prophylactic to prevent the infection of an open wound.

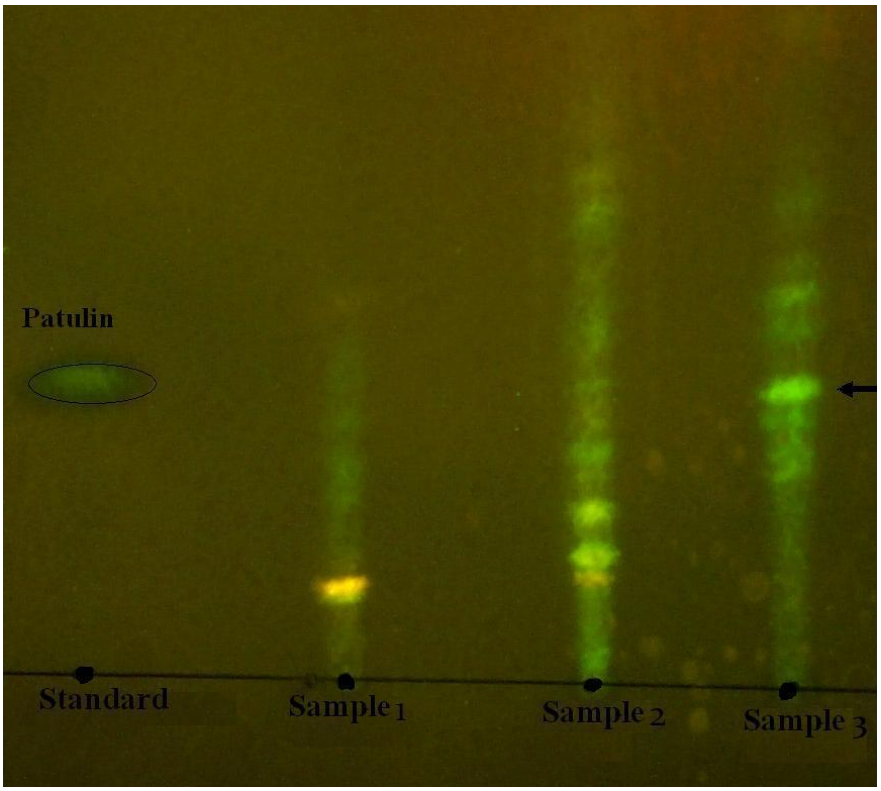


Figure 6.4: TLC plate showing that patulin was detected in sample number 3 (extracted from liquid culture of fungus isolated from apple). TLC plate sprayed with 0.5 ml *p*-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid, heated at 110ºC for 10 min and observed under UV light (300–360 nm). (Left to right) Three samples and one standard are shown.

Chapter Seven: Final Discussion

7.1 Final Discussion

In a review in which the history and potential use of maggot therapy was discussed Wainwright, (1988) stated that "it is unlikely that we will ever see a return to the use of maggot therapy". At the time, this seemed to be a rationale and safe prediction, but as with many similar predictions about the future of medicine and science it turned out to be wrong, much to the subsequent embarrassment of the author! This prediction did not of course take into account the desperate situation in infective disease control which would eventually result from the widespread development of antibiotic resistance to its current highly worrying state. 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 biocontrol 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 largely down to its own making due to the miss-prescribing and wasteful way in which antibiotics have 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 method required to successfully apply maggot therapy in medical practise 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).

The response to the use of honey as a therapeutic agent has been less extreme than that seen in regard to the use of maggot therapy simply because the former approach is obviously seen as inherently less unpleasant, less intrusive and complicated. Simply put, few patients would elect to have living maggots applied to their bodies when more benign and healthy-appearing honey could be used in its place. Again however, as we have seen, a mass of scientific knowledge has accumulated to back up the use of honey in wound therapy.

The most potentially important result of this Thesis is the demonstration that tamarind has marked antibacterial properties. This presents the possibility of using autoclave-sterilized tamarind in the treatment of indolent, antibiotic resistant wounds. It appears that tamarind has not previously been used for this purpose, and so could provide a new approach to wound therapy.

In a similar way the finding that maggot-derived gasses, and volatiles, inhibit *Mycobacterium phlei,* and therefore potentially also *M. tuberculosis*, is of considerable potential significance in relation to the treatment of tuberculosis. While maggot gasses themselves could be used, it is potentially more desirable that the active ingredient of such gas mixtures be isolated and used. Unfortunately it was not possible to find a means of isolating such an active ingredient(s) during the work presented here. In some way this situation is similar to that facing Fleming from 1928 onwards, in that he had

demonstrated the antibacterial properties of penicillin but was unable to chemically purify it in order to convert it into an effective antibiotic for use in medicine.

In relation to maggot therapy,for first time- *in vitro* evidence (such as antifungal and yeast (*S. cerevisiae* (BY4742) GFP) ingestion ability), was provided in support of the use of *Lucilia sericata* maggot to control fungi involved in superficial fungal infections, i.e. fungal burn wound infections and dermatomycoses. A study was made to determine if Mycoplasmas can be detected in the hemolymph of maggots of *Calliphora quadrimaculata* and *Lucilia sericata* maggots. Mycoplasmas were not detected in any of the maggot samples tested. These observations suggest that *Calliphora quadrimaculata* and *Lucilia sericata* larvae are unlikely to act as transmission vectors for Mycoplasmas.

The results presented in this thesis also show that Manuka oil has the potential for use on wounds in order to inhibit Gram-positive, but not Gram-negative bacteria; it could also be used to treat dermatophytes. It is suggested for example, that this oil might be added to shampoos, or used directly, in countries such as India where dermatophytes are common amongst large sections of the population.

The work presented here makes it clear that there are a number of potentially effective non antibiotic treatments which are available for use in treating wound infections caused by antibiotic–resistant bacteria and in the case of manuka oil, against the pathogenic fungus *Trichophyton*. Such work provides motivation for the search for other alternative approaches to wound control. While considerable effort has been directed towards investigating herbal medicine, less attention has been given to other alternatives, examples of which include the use of : cod liver oil, chlorophyll, pectin and silicon and on-clays (Pugh, 1942). Cod liver has been used during war time to treat infected wounds, either by filling wound-cavities, by using soaked dressings by using a mixture of 30-50 % in Vaseline. Cod liver oil is particularly effective in killing bacteria when in

the oxidized, odorous state, suggesting that vitamin D is the active ingredient. Highly ground silicon has been used to treat wounds and can be given by mouth to cure bleeding ulcers (Pugh,1942). In 1906, Stumpf used clay as a paste to treat indolent infections; it was shown not only reduce infection, but also deodorized the wound, stopped irritation and enhanced healing (Pugh,1942) The clay kaolin has traditionally been used to treat cholera in India and elsewhere; in one study mortality rates dropped from 44% to near zero following its use (Nadkarni, 1908) ; kaolin and morphine was an old remedy for stomach infections which could be found in the medical cabinets of most of UK households until the 1960s. Clearly, all of the above mentioned alternatives are worth re-evaluating and following the application of modern scientific approaches could be used in the future to treat antibiotic–resistant, indolent infections and even perhaps, in the case of clay-based remedies, in the treatment of cholera.

Some very new approaches to wound infection control are worth considering and, hopefully, might form the basis for further continuation of the research discussed here. One such approach is to combine non-antibacterial bioactive drugs with antibiotics in order to overcome problems associated with antibiotic resistance. The antibiotic minocycline provides a good example of this approach. Minocycline, an antibiotic which inhibits protein synthesis in bacteria and to which bacteria developed resistance has recently been re-evaluated for its antibacterial activity when combined with other previously approved bioactive drugs. Over 1000 compounds were evaluated and 69, which had never before been used to treat bacterial infections, improved the antibacterial properties of the previously useless antibiotic. Imodium, the commonly used anti-diarrheal drug, also improved the antibacterial activity of minocycline, possibly because it disrupts the membrane potential across bacterial membranes, allowing the antibiotic to become effective again.

Finally, it is worth considering the prevailing view considering the future developments which might solve the problem of antibiotic resistance. An excellent source of such opinion can be found in the review by Zucca and Savio (2010). They suggest the following list of scientific developments which may possibly solve this problem: 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. It is noticeable that no mention is made of the use of maggot or honey therapies, despite the fact that these approaches are currently being successfully used in the treatment of antibiotic resistant infections. There therefore exists a clear mismatch between the dedication of large amounts of research time and effort into the study of the theoretical applications of sophisticated approaches and the actual currently successful application of honey and maggots in the "field" so to speak. It would be obviously desirable to move away from these "primitive", albeit successful methods like apitherapy and maggot therapy, especially since these cannot be used in the treatment of systemic infections (intravenous maggot therapy is clearly impossible as presumably is intravenous honey therapy (note however, that a honey extract called M2 Woelem was developed in the 1950s in Germany for use in gynaecology and obstetrics, (De Buman, 1953)). Nevertheless, in the absence of effective, more scientific, approaches these two historical approaches clearly have their use in practical medicine.

In conclusion, the results presented here suggest that there is considerable scope for the testing of new approaches to the control of infection caused by bacteria and fungi. Alternative approaches such as maggot and honey therapies are already in wide use throughout the world and other treatments such as the use of tamarind could be introduced almost immediately. In addition, other approaches such as mycotherapy are ripe for re-investigation and application in modern medicine. There also remain a range

of other potential treatments, such as the use of chlorophyll and clays which have yet to be investigated using modern scientific approaches. While these alternatives to antibiotics are currently seen as stop gaps for use until newer antibiotics, or other approaches to disease control arrive, it may be that they will remain with us for a long time yet and may even form the centre-point of antimicrobial therapy for decades to come.

7.2 SUGGESTIONS FOR FUTURE WORK

1) It would obviously be desirable to conduct clinical trials to determine the effectiveness of tamarind paste in the treatment of indolent infections and wounds. Such work would require some form of collaboration with medical practitioners who have access to patients having these wounds. Interestingly, the case of Fleming following his discovery of penicillin comes to mind once again, since his ability to demonstrate the effectiveness of un-purified penicillin filtrates was hindered by him not having access to a sufficient number of patients.

2) It is desirable that the active ingredient present in maggot gasses and volatiles be determined to see if this could be used in wound therapy. Again collaboration, this time with an organic chemist, would be desirable.

3) An investigation could be made of other larvae to determine if these exhibit potentially useful antibacterial properties examples of larvae currently studied in this laboratory include those of the black soldier fly and larvae of the Waxworm moth.

4) Finally, it would be of interest to conduct studies on the potential antibacterial agents mentioned above, namely chlorophyll, silicon compounds (including clays) and olive

oil, as well as other materials which have been used in history and/ or folklore to treat wounds infected with bacteria.

5) There are a number of other examples in the literature of biotherapy which may be worth re-examining. One approach which has not been emphasised here is the use of so-called bacteriotherapy, i.e. the use of living bacteria to treat wounds infected with bacteria or fungi; for example, living cultures of *Lactobacillus* have been used to control bacterial infections in wounds (Peral *et al.*, 2009).

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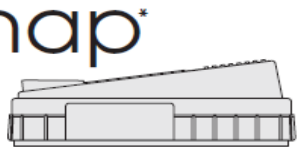
APPENDIX

9.1 Appendix A (SNAP Beta-Lactam test Kit)

New SNAP* Beta-Lactam Test Kit
(penicillin G, amoxicillin, ampicillin,
ceftiofur and cephapirin)
Validated for raw commingled bovine milk

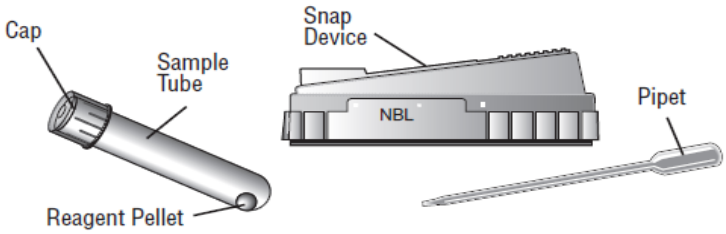


Product and Intended Use

The New SNAP* Beta-Lactam Test is an enzyme-linked receptor-binding assay that detects penicillin G, amoxicillin, ampicillin, ceftiofur and cephapirin residues in raw commingled bovine milk at or below established tolerance and/or safe levels (Refer to the Performance Information section.). This test also detects cloxacillin at a level above the established tolerance and/or safe level (Refer to the Cross-Reactivity section.). The base of the New SNAP Beta-Lactam Test is marked with "NBL."

Kit Components

- SNAP device
- Sample tube and cap
- Reagent pellet
- Pipette



Components Required But Not Provided (Available through IDEXX Laboratories)

- Block heater capable of maintaining an operating temperature of 45°C (113°F) ± 5°C
- A reader supplied by IDEXX capable of reading a SNAP device
  NOTE: The New SNAP Beta-Lactam Test is approved only for use with the SNAPshot* Reader for NCIMS testing.
- Positive and negative controls

rev 6/03

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Performance Information

Sensitivity

Dose Response Information

ppb	Amoxicillin	Ampicillin*	Ceftioflur*†	Cephapirin*	Penicillin*
1					7%
2	0%	0%		0%	37%
3					93%
4	20%	37%		0%	100%
5			87%		100%
6	70%	100%			
8	100%	100%		0%	
10	100%	100%	100%		
12				100%	
20			100%	100%	
30			100%		
50			100%		
Tolerance/Safe Level (ppb)	10	10	50	20	5
90/95% Concentration (ppb)	7.3	5.8	5.4	11.7	3.0

*The drugs indicated with an asterisk (*) have demonstrated a 90/95% sensitivity of this test kit, which is at least 25% less than the tolerance or safe level.

Data presented as percent positive at each concentration.

†Milk from a ceftiofur-treated dairy cow tested with the New SNAP Beta-Lactam Test will test positive (will give a positive test response) to less than 1 ppb ceftiofur.

SENSITIVITY: Based on 30 samples at each milk concentration.

SELECTIVITY: 60 negative control milk samples were evaluated in an independent laboratory and none of these negative control samples tested positive with SNAP.

Cross-Reactivity

The New SNAP Beta-Lactam Test Kit cross-reacts with the following drugs at the levels indicated:

	ppb		
	10	50	100
Cloxacillin	0%	100%	100%
Dicloxacillin	0%	100%	100%
Ticarcillin	0%	100%	100%
Cefadroxyl	100%	100%	100%

The New SNAP Beta-Lactam Test Kit does not cross-react with the following drugs at levels up to 100 ppb: sulfadiazine, sulfanilamide, sulfathiazole, sulfamethazine, sulfapyridine, sulfadimethoxine, tetracycline, oxytetracycline, chlortetracycline, doxycycline, gentamicin, neomycin, streptomycin, ivermectin, erythromycin, novobiocin, furosemide, trichlormethiazide, chlorothiazide, oxytocin, phenylbutazone, dexamethasone, dipyrone, pilimycin, tilmicosin, thiabendazole and p-aminobenzoic acid (PABA).

Operating Instructions

For all NCIMS testing, refer to current 2400 for IDEXX New SNAP Beta-Lactam (Appendix N Bulk Milk Tanker Screening Test Form).

Storage

All materials must be refrigerated at 0°–7°C (32°–45°F). Tests can be kept at room temperature, 18°–29°C (64°–84°F), during the day of use. Remove only the number of tests to be used for the day.

NOTE: Discard unused, unrefrigerated devices at the end of the day.

Sample Information

- Raw commingled bovine milk must be used.
- Samples must be refrigerated and tested within 3 days of collection.
- Thoroughly mix the sample before testing.
- The milk sample must not have been frozen nor thawed at any time before testing.

Precautions and Warnings

- Do not mix sample tubes and devices from different lot numbers.
- Do not use kits past their expiration dates.
- The SNAP device must be run in a horizontal position.
- The New SNAP Beta-Lactam Test is recommended for use by personnel who have received training by an IDEXX representative. In the United States under NCIMS recommendations, trained individuals should maintain proficiency by regular use and/or state-sponsored training/certification programs. Individuals who have not run a SNAP device in the past six months or who desire additional training should contact IDEXX Technical Services at 1-800-321-0207.

Test Preparation

- SNAP devices can be kept at room temperature during the day of use.
- Ensure that the heater block has been preheated and that the temperature has maintained 45°C (113°F) ± 5°C for at least 5 minutes.
- Remove the SNAP device, pipette and sample tube from the bag.
  NOTE: An unused SNAP device should have a light-blue control spot, sample spot and activation circle. If the device does not have light-blue control and/or sample spots, discard the device and open a new SNAP device.
- Verify that the reagent pellet is at the bottom of the sample tube. If not, tap the tube to return the pellet to the bottom.
- Shake the milk sample thoroughly.

Positive and Negative Controls in the United States for NCIMS Testing

- Positive and negative controls must be run daily prior to testing samples, and with each new lot to verify the performance of the reagents and equipment.
- Positive and negative controls must not be frozen.

Negative Control:

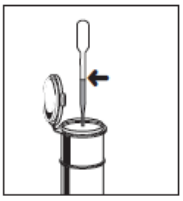
- Use beta-lactam-negative raw milk.

Positive Control:

- Use the IDEXX Penicillin Positive Control, part # 98-06513-00, as directed in the descriptive insert that accompanies the product.

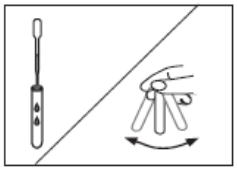
Test Procedure

A. Preparing the Sample



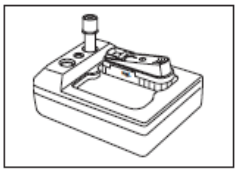
1. Place the SNAP device(s) in the preheated heater block. The device must remain in the heater block for the duration of the test.
2. Shake the milk sample thoroughly.
3. Remove and discard the sample tube cap.
4. With the IDEXX pipette, draw up the milk sample (450 µl ± 50 µl) to the indicator line.

   TIP: When pipetting, take the sample from the middle of the sample container, pipetting away from any bubbles, and slowly draw up the sample to the indicator line on the pipette to avoid air bubbles.

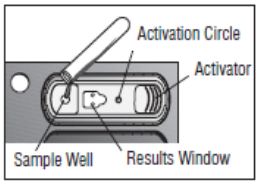


5. Carefully add all of the milk sample from the pipette to the tube.
6. Shake the sample tube to dissolve the reagent pellet.
7. Incubate the sample tube in the heater block at 45°C (113°F) ± 5°C for 5 minutes.

   NOTE: Incubation must occur for a minimum of 5 minutes and no longer than 6 minutes.



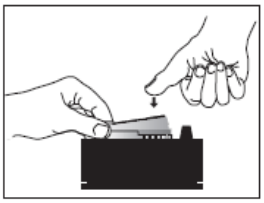
B. Testing the Sample



8. Pour the entire contents of the sample tube into the sample well of the SNAP device and discard the tube.

   NOTE: The sample will flow across the results window toward the blue activation circle.
9. When the blue activation circle BEGINS to disappear, push the activator FIRMLY until it snaps flush with the body of the SNAP device.

   NOTE: When the edge of the activation circle nearest the sample well begins to turn from dark blue to white, activation should occur. Do not let the circle completely disappear.



10. Wait 4 minutes.

    NOTE: The SNAP device must remain in the heater block during color development.
11. Remove the device from the heater block and visually inspect the control spot and sample spots.

    The test is invalid and the same sample should be retested with a new SNAP device if:

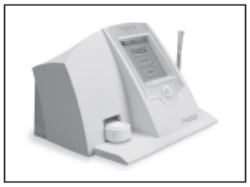
    a. The control spot fails to develop color.

    b. Blue streaking occurs in the background or the background is the same color as the sample or control spots.

    c. The sample or control spots are not uniform in color or exhibit poor spot quality.

    NOTE: Do not put invalid tests into the reader.

C. Reading the Results



Read the results immediately (no longer than 30 seconds in the SNAPshot Reader). Insert the SNAP device and follow the instructions for reading (see the SNAPshot Reader manual for more details).

NOTE: We recommend that the check set devices be used daily to verify the performance of the reader.

Negative Sample

If the ratio is **below or equal to 1.05**, the reader will display a result of "N" or "Negative" and report as "Not Found."

Presumptive Positive Sample

If the ratio is **1.06 or higher**, the reader will display a result of "P" or "Positive."

Example of SNAPshot Reader Printout

Printout		Description
SNAPshot Reader SNAP Test		
Beta-Lactam (5 ppb)		Test Type
7/25/03	12:22 PM	Date and Time
Lot ID:	012345	Lot Number
Tech:	9876	Technician ID Number
Sample:	011	Sample Number
Ratio:	0.79	Ratio
Results:	Negative	Result

In the United States for NCIMS Testing:

- Upon initial screen, a positive result indicates an Initial Positive.

Retest of Initial Positive Samples

- For milk samples yielding an initial positive result in initial testing, promptly retest the SAME sample in duplicate as described in the Test Procedure section.
- Along with these duplicate retest samples, run positive and negative controls prepared as described in the Positive and Negative Controls section.

NOTE: All NCIMS testing must be performed in accordance with Appendix N of the current PMO.

Interpretation of Retest Results

If the controls test correctly, negative results for both retest samples indicate a **Negative Result** (report as **Not Found**).

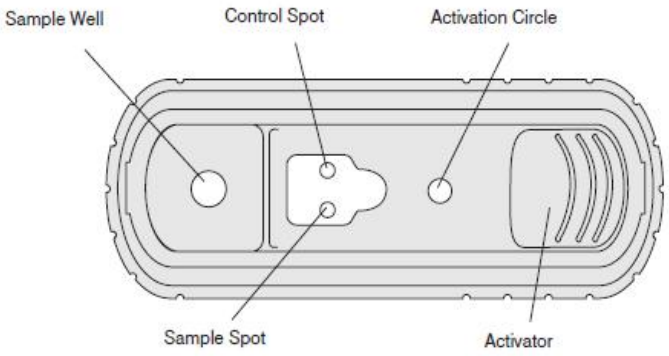
The SNAPshot Reader will print out the following information: the assay identification, date and time, lot number, technician ID, sample ID, ratio and result.

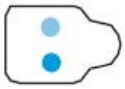
Additional Information

Samples of this test kit model were independently evaluated by the AOAC Research Institute and were found to perform to the producer's specifications as stated in the test kit's descriptive insert. The producer certifies that this kit conforms in all respects to the specifications originally evaluated by the AOAC Research Institute as detailed in the PERFORMANCE TESTED[K] certificate number 030302.

The SNAP test has been approved by AOAC-RI for visual interpretation without a reader. The performance expected from the visual interpretation, and the 90/95% detection level determined by an independent laboratory is reported below. **The Visual Interpretation Procedure for the SNAP device is not acceptable for use in NCIMS milk-monitoring programs.**

Interpreting Test Results





Negative Result—The sample spot is darker than or equal to the control spot.

Positive Result—The sample spot is lighter than the control spot.

Dose Response Visual Interpretation

ppb	Amoxicillin	Ampicillin	Ceftioflur	Cephapirin	Penicillin
1					0%
2	0%	0%		0%	37%
3					97%
4	0%	13%		0%	100%
5			37%		100%
6	53%	93%			
8	100%	100%		0%	
10	100%	100%	100%		
12				100%	
20			100%	100%	
30			100%		
50			100%		
Tolerance/Safe Level (ppb)	10	10	50	20	5
90/95% Concentration (ppb)	6.9	6.2	5.9	11.9	3.1

For technical assistance, call IDEXX Technical Services at 1-800-321-0207.

IDEXX LABORATORIES

One IDEXX Drive, Westbrook, Maine 04092 USA
Tel: 1-207-856-0300 or 1-800-321-0207 Fax: 1-207-856-0630

9.2 16S rRNA partial sequences of isolated strains

MB2

gb|JF836884.1| Bacillus pumilus strain M4 16S ribosomal RNA gene, partial sequence Length=1445

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 Score =  802 bits (434),  Expect = 0.0
 Identities = 434/434 (100%), Gaps = 0/434 (0%)
 Strand=Plus/Minus

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1369

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1129

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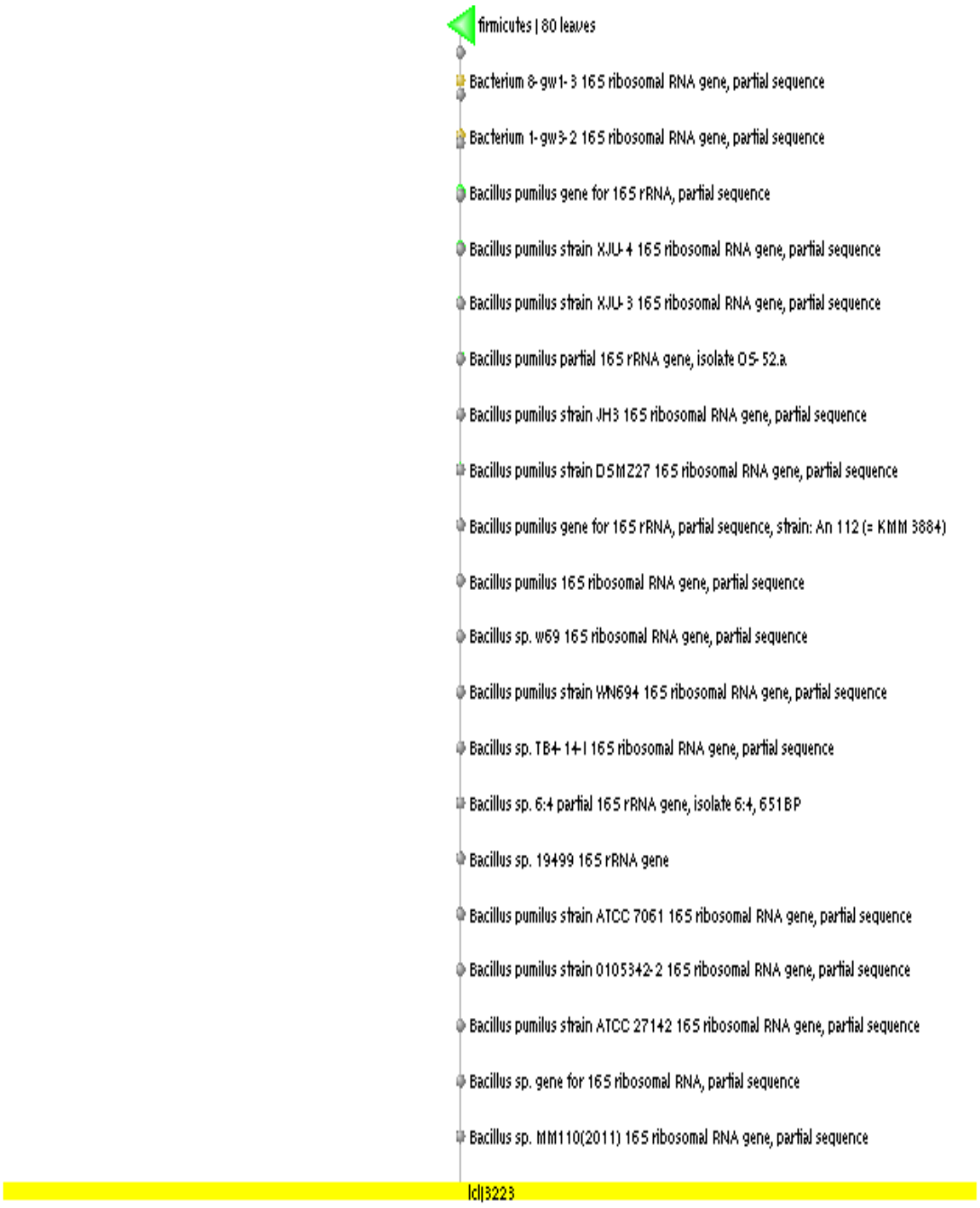
firmicutes | 80 leaves
Bacterium 8-gw1-3 16S ribosomal RNA gene, partial sequence
Bacterium 1-gw3-2 16S ribosomal RNA gene, partial sequence
Bacillus pumilus gene for 16S rRNA, partial sequence
Bacillus pumilus strain XJU-4 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain XJU-3 16S ribosomal RNA gene, partial sequence
Bacillus pumilus partial 16S rRNA gene, isolate OS-52.a
Bacillus pumilus strain JH3 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain DSMZ27 16S ribosomal RNA gene, partial sequence
Bacillus pumilus gene for 16S rRNA, partial sequence, strain: An 112 (= KMM 3884)
Bacillus pumilus 16S ribosomal RNA gene, partial sequence
Bacillus sp. w69 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain WN694 16S ribosomal RNA gene, partial sequence
Bacillus sp. TB4-14-I 16S ribosomal RNA gene, partial sequence
Bacillus sp. 6:4 partial 16S rRNA gene, isolate 6:4, 651 BP
Bacillus sp. 19499 16S rRNA gene
Bacillus pumilus strain ATCC 7061 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain 0105342-2 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain ATCC 27142 16S ribosomal RNA gene, partial sequence
Bacillus sp. gene for 16S ribosomal RNA, partial sequence
Bacillus sp. MM110(2011) 16S ribosomal RNA gene, partial sequence
lcl|3223

MB4

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Strand=Plus/Minus

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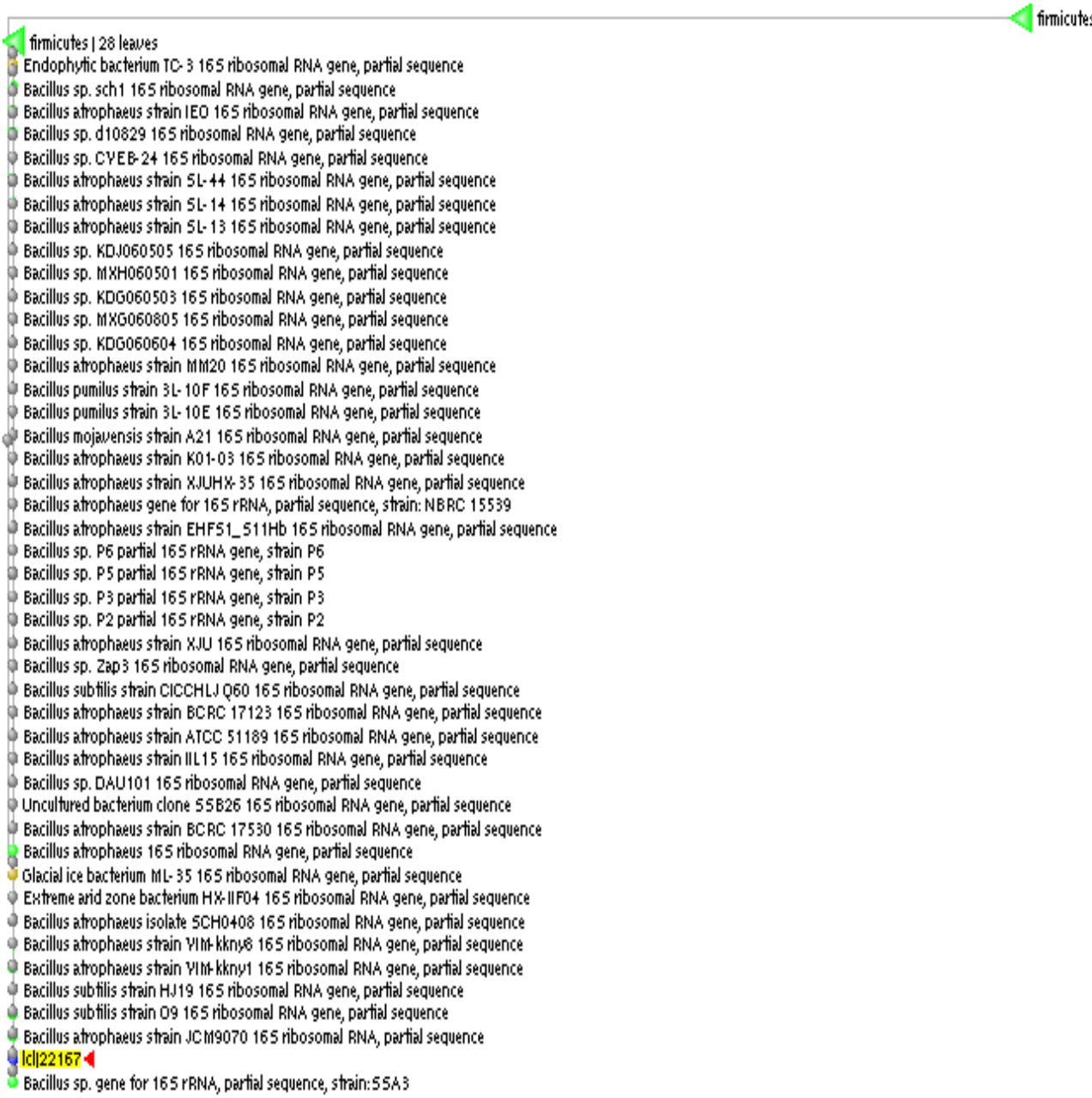
firmicutes
firmicutes | 28 leaves
Endophytic bacterium TC-3 16S ribosomal RNA gene, partial sequence
Bacillus sp. sch1 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain IEO 16S ribosomal RNA gene, partial sequence
Bacillus sp. d10829 16S ribosomal RNA gene, partial sequence
Bacillus sp. CYEB-24 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain SL-44 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain SL-14 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain SL-13 16S ribosomal RNA gene, partial sequence
Bacillus sp. KDJ060505 16S ribosomal RNA gene, partial sequence
Bacillus sp. MXH060501 16S ribosomal RNA gene, partial sequence
Bacillus sp. KDG060503 16S ribosomal RNA gene, partial sequence
Bacillus sp. MXG060805 16S ribosomal RNA gene, partial sequence
Bacillus sp. KDG060604 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain MM20 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain 3L-10F 16S ribosomal RNA gene, partial sequence
Bacillus pumilus strain 3L-10E 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain A21 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain K01-03 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain XJUHX-35 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus gene for 16S rRNA, partial sequence, strain: NBRC 15539
Bacillus atrophaeus strain EHF51_511Hb 16S ribosomal RNA gene, partial sequence
Bacillus sp. P6 partial 16S rRNA gene, strain P6
Bacillus sp. P5 partial 16S rRNA gene, strain P5
Bacillus sp. P3 partial 16S rRNA gene, strain P3
Bacillus sp. P2 partial 16S rRNA gene, strain P2
Bacillus atrophaeus strain XJU 16S ribosomal RNA gene, partial sequence
Bacillus sp. Zap3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CICCHLJ Q60 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain BCRC 17123 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain ATCC 51189 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain IIL15 16S ribosomal RNA gene, partial sequence
Bacillus sp. DAU101 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 55B26 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain BCRC 17530 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus 16S ribosomal RNA gene, partial sequence
Glacial ice bacterium ML-35 16S ribosomal RNA gene, partial sequence
Extreme arid zone bacterium HX-IIF04 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus isolate SCH0408 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain YIM-kkny8 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain YIM-kkny1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain HJ19 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain O9 16S ribosomal RNA gene, partial sequence
Bacillus atrophaeus strain JCM9070 16S ribosomal RNA, partial sequence
lcl|22167
Bacillus sp. gene for 16S rRNA, partial sequence, strain: 55A3

gb|GU339392.1| Bacillus licheniformis strain RUAUMTB001 16S ribosomal RNA gene, partial sequence Length=850

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Strand=Plus/Plus

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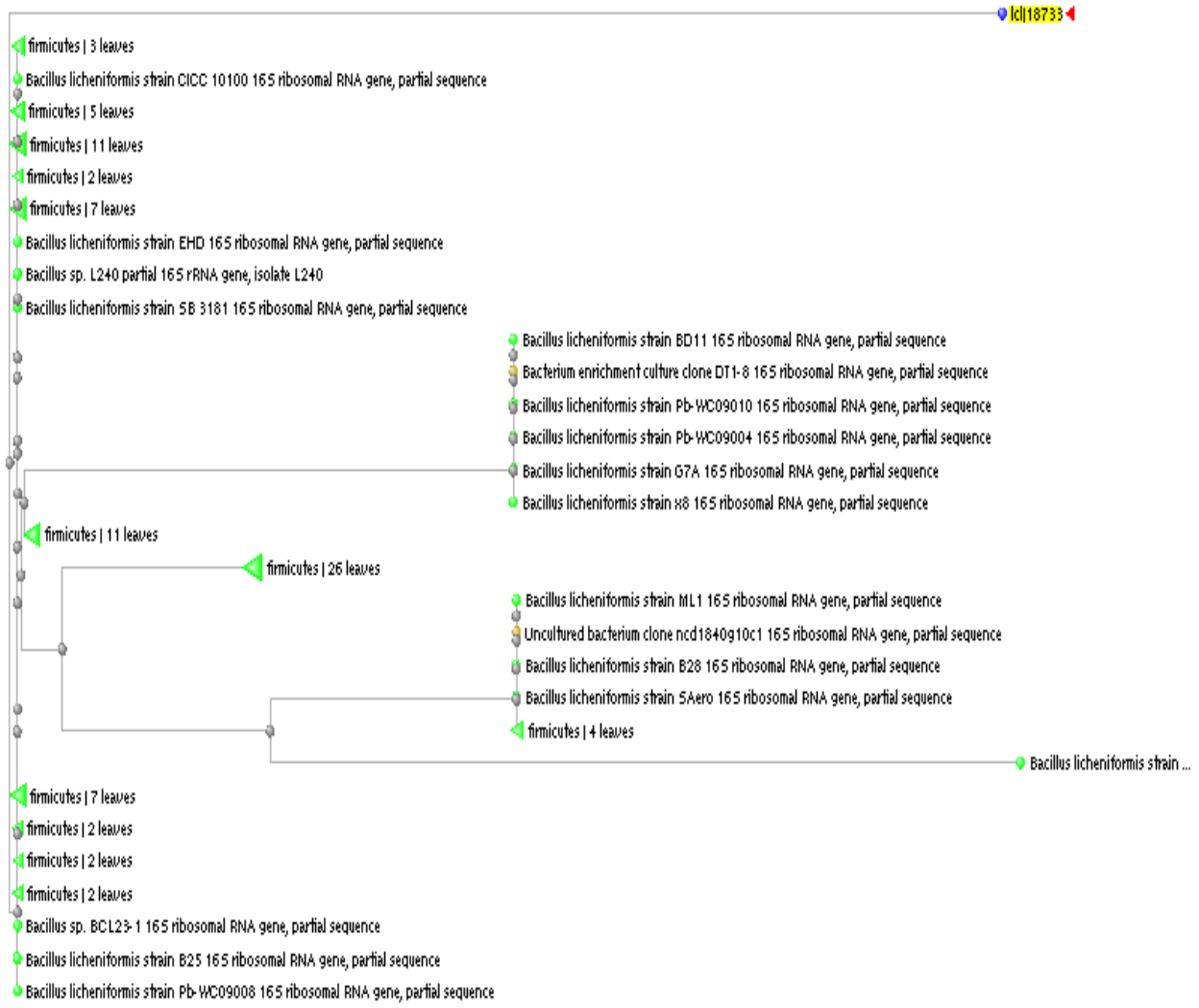

9.3 Appendix B: Third year student poster (Studies on the antibacterial activity of a high grade *Leptospermum scoparium* honey

Studies on the Antibacterial Activity of a High Grade *Leptospermum scoparium* (Manuka) Honey

Sulaiman Alnaimat and Milton Wainwright

Department of Molecular Biology and Biotechnology, Firth Court, Western Bank, University of Sheffield, S10 2TN, England

mbp008sma@sheffield.ac.uk

Introduction

The use of honeys for therapeutic purposes has been common throughout history. One of such product is Manuka honey which is derived from the Manuka tree (*Leptospermum scoparium*) a native of New Zealand [3]. Manuka honey is known to have a unique extra antimicrobial activity which does derive from its low pH, osmolarity or hydrogen peroxide accumulation, termed as a non-peroxide activity [1] [2] . Considerable research has been devoted to attempts to identify the active component responsible for this non-peroxide antibacterial activity, which is generally considered to be due to plant derived components such as flavonoids and phenolic compounds[4], Recent studies have successfully concluded that this component is methylglyoxal (MG), a highly reactive precursor in the formation of advanced glycation end products (AGEs)
Manuka honeys have an industry standard called UMF® (Unique Manuka Factor) which reflects the relative ability of Manuka honey to inhibit the growth of *Staphylococcus aureus*. (e.g.,UMF 20 has equivalent antibacterial activity to 20% phenol w/v). Therefore Manuka honey with a higher UMF® rating has a greater antibacterial effect, and is consequently more desirable for use as a therapeutic agent[1]

Results

1- The nature of Manuka's non-peroxide activity

To measure non-peroxide antibacterial activity of different highly grade manuka , tested honeys were diluted in a catalase solution to breakdown the formed hydrogen peroxide, the formed zones of inhibition were expressed as equivalent phenol concentration (% w/v). Results are shown in table 1.

Table 1: The total antibacterial activity and the non-peroxide activity of selected grade manuka honey against methicillin-sensitive *S. aureus* (MSSA), expressed as equivalent phenol concentration (% w/v), determined by agar diffusion, and the pH of these honeys.

Honey	pH	Total antibacterial activity as phenol equivalent (w/v) % (peroxide + non-peroxide activity	Non-peroxide activity as phenol equivalent (w/v) %
Comvita® UMF® 25 + Manuka	3.6	11.9	10.7
Manuka 250MGO	3.8	10.6	10.3
Comvita® UMF® 15 + Manuka	3.8	9.8	8.9
Littleover Apiary Manuka active 10+	4.1	9.3	8.7
Comvita® UMF® 20+ Manuka	3.2	8.3	8.1

Why *Leptospermum scoparium* Manuka honey should have this unique activity has been extensively investigated. One suggested hypotheses is that Manuka honey has a residual high level of hydrogen peroxide concentration that the normal catalase treatment is not sufficient to eliminate it (Weston, 2000). To test this hypothesis the antibacterial assays were repeated using 10, 40, 50, 75 and 100 fold excess of catalase concentration.

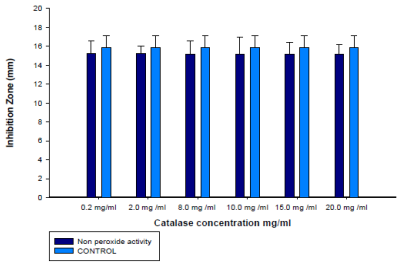

Figure 1: Antibacterial assay of 50% (w/v)% Manuka honey treated with 10, 40, 50, 75 and 100 fold excess of catalase concentration (non-peroxide activity) compared with non treated 50% (w/v)% Manuka honey (Total activity) , the assay carried out against MSSA, expressed as a mean zone of inhibition of the replicates ± standard deviation.

The results presented here therefore show that the unusual non-hydrogen peroxide activity of the Manuka honey is unlikely due to accumulated residual hydrogen peroxide that cannot be destroyed by the normal catalase concentration; in addition.

2- Studies on the mechanism of the antibacterial activity of two highly grade New Zealand Manuka

Antibacterial activity was determined by an agar well diffusion bioassay and MICs and MBC determinants.

Table 2: The zone of inhibition (mean±SD), (diameter mm including well (8.0mm)), the minimum inhibitory concentrations (MICs) (% v/v) and the minimum bactericidal concentrations (MBCs) values of 20+ Manuka and 25+ Manuka for selected bacteria including methicillin-resistant *S.aureus* (MRSA).

Bacteria	UMF®20+ Manuka			UMF®25+ Manuka		
	Zone of inhibition (mm)	MICs	MBCs	Zone of inhibition (mm)	MICs	MBCs
E.coli	22.2 ±1.0	9%	11%	23.0 ±0.0	8%	10%
S. marcescens	13.0 ±0.5	12%	> 15%	15.3 ±0.6	12%	13%
B. sphaericus	19.0 ±0.9	11%	12%	22.2 ±0.2	8%	9%
S. epidermidis	19.4 ±0.5	11%	12%	21.8 ±0.7	10%	12%
B. subtilis	20.5 ±0.5	10%	12%	25.0 ±0.9	9%	12%
MSSA	24.0 ±0.8	7%	8%	26.0 ±0.8	6%	8%
MRSA	21.3 ±1.2	9%	12%	25.7 ±1.5	6%	10%

The results show that both Manuka honeys, at a concentration ranging from 6% to 12% , inhibit the growth of all tested bacteria, which means that the growth of these tested bacteria will be prevented even when New Zealand medical grade Manuka honey is diluted by exudates of the wound up to 9-to 18-fold.

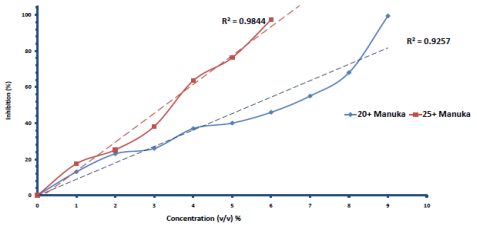

Figure 2: Dose-response curve for the antibacterial activity of 25+ Manuka and 20+ Manuka against methicillin-resistant S. aureus (MRSA). The activity is expressed as the percentage inhibition of growth of the bacteria, growth measured as the increase in the O.D at 600 nm of the culture after 20 hours of incubation.

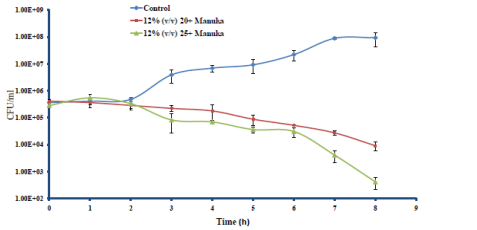

Figure 3: Killing of methicillin-resistant *Staphylococcus aureus* (MRSA) by 12% (v/v) of 25+ Manuka and 20+ Manuka.

3- Scanning electron microscope(SEM) and transmission electron microscope TEM studies on the morphological effects of 25+Manuka on Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*

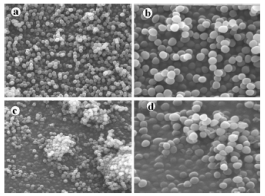

Figure 4: External morphology of Methicillin-resistant *Staphylococcus aureus* (MRSA) observed by scanning electron microscopy (SEM) ,(a) and (b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 4 h at 37°C. (c) and (d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 12% v/v 25+ Manuka honey for 4 h at 37°C.

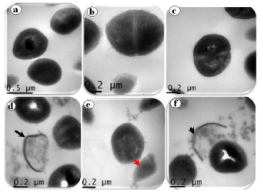

Figure 5: internal morphology of Methicillin-resistant *Staphylococcus aureus* (MRSA) observed by transmission electron microscopy (TEM) ,(a), (b) and (c) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 4 h at 37°C. ,(d), (e) and (f)treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 12% v/v 25+ Manuka honey for 4 h at 37°C. Note the separation of cell membrane from the cell wall (black arrowheads) and released cellular contents and cell debris (red arrowheads).

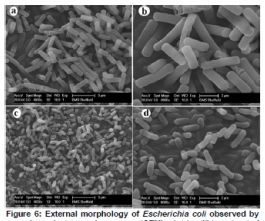

Figure 6: External morphology of *Escherichia coli* observed by scanning electron microscopy (SEM) ,(a)and(b) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 6 h at 37°C.(c) and(d) treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 18% v/v 25+ Manuka honey for 6 h at 37°C

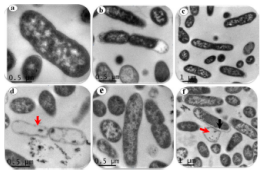

Figure 7: Internal morphology of *Escherichia coli* observed by transmission electron microscopy (TEM) ,(a), (b) and (c) untreated bacteria (control) incubated in phosphate-buffered saline pH 7.4 for 6 h at 37°C. (d), (e) and (f)treated bacteria incubated in phosphate-buffered saline pH 7.4 containing 18% v/v 25+ Manuka honey for 6 h at 37°C. Note the separation of cell membrane from the cell wall (black arrowheads) and cells with released cellular components (red arrowheads).

4- Determination of the incidence *of Clostridium botulinum* spores Manuka honey

Table 3: Various Medical grade New Zealand Manuka honey samples examined for *C. botulinum* spore incidence

Manuka honey	No. of tested samples	Step one Positive growth in TPGY broth	Step two Positive growth in SPS Agar (anaerobically)	Step three 16S rRNA dependant identification
Comvita UMF 25+ Manuka	3	0	0	
Comvita UMF 20+ Manuka	3	2	0	
Comvita UMF 15+ Manuka	3	2	0	
Comvita UMF 12+ Manuka	3	0	0	
Littleover Manuka Active 15+	3	3	0	
Littleover Manuka Active 10+	3	2	1	*Bacillus licheniformis*
Littleover Manuka Active 5+	3	2	0	

Confirming the positive results by using 16SrRNA sequencing

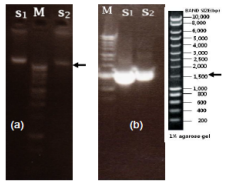

Figure 8: (a) Agarose gel electrophoresis revealing a successful DNA extraction from unknown isolated bacteria exhibited antibacterial activity. Lane M, molecular marker, S1 and S2, two bacteria samples, (b) Agarose gel electrophoresis revealing a successful amplification of 16S rRNA from unknown isolated bacteria exhibited antibacterial activity. Lane M, molecular marker. S1 and S2, two bacteria samples.

After being processed according to the dilution centrifugation (DC) method , samples were incubated TPGY broth media for 10 days. As shown in Table 3 in this step 11 out of 21 samples yielded positive results; positives samples were further inoculated onto SPS Agar plates. Only 1 of 11 samples (Littleover Manuka Active 10+) was able to grow anaerobically in SPS Agar. Isolated strain from this positive sample was then identified by 16S rRNA sequencing analysis as *Bacillus licheniformis*. It seems from this study therefore that *C. botulinum* spores are not likely to be detectable in tested medical grade New Zealand Manuka honey samples.

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