

**INHIBITORY EFFECT ON *Staphylococcus aureus* BIOFILM  
THROUGH THE USE OF HONEY**

By

**LOW KA LOK**

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## **ABSTRACT**

### **INHIBITORY EFFECT ON *Staphylococcus aureus* BIOFILM THROUGH THE USE OF HONEY**

**LOW KA LOK**

Honey is known as a traditional medicine for centuries with its antibacterial properties and considered as one of the most enduring-substance used in wound management. However, different types of honey possess different extent of antibacterial property. Thus, this study was designed to evaluate and compare the inhibitory/preventive effect of Malaysian Gelam honey and Manuka honey on established biofilm and biofilm formation. As to accomplish the aim of this study, established biofilm inhibition assay and biofilm prevention assay were carried out. All of these tests were conducted on five strains of *Staphylococcus aureus* (ATCC 6538, ATCC 25923, ATCC 33591, ATCC 33592 and a clinical sample) by using six different concentrations of honey: 0%, 20%, 40%, 60%, 80% and 100% (w/v). For established biofilm inhibition assay, Manuka honey UMF 10 was the most potent with the highest reduction percentage (85.04%). Same for biofilm prevention assay, as Manuka honey UMF 10 was able to reduce the biofilm mass formation up to 98.30% in ATCC 6538 strain. In conclusion, this study showed the efficacy of honey against biofilm and different types of honey possess different degrees of potential effect.

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they had bestowed upon me into the completion of this research project and thesis.

## DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

.....

Low Ka Lok

## APPROVAL SHEET

This project report entitled “**INHIBITORY EFFECT ON *Staphylococcus aureus* BIOFILM THROUGH THE USE OF HONEY**” was prepared by **LOW KA LOK** and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

.....

(Mr. Ng Wen Jie)

Date: .....

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

**FACULTY OF SCIENCE**  
**UNIVERSITI TUNKU ABDUL RAHMAN**

Date: .....

**PERMISSION SHEET**

It is hereby certified that **LOW KA LOK** (ID No: **10ADB01742**) has completed this final year project entitled “**INHIBITORY EFFECT ON *Staphylococcus aureus* BIOFILM THROUGH THE USE OF HONEY**” supervised by Mr. Ng Wen Jie (Supervisor) from the Department of Biomedical Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

Yours truly,

.....

(LOW KA LOK)

## TABLE OF CONTENTS

	<b>Page</b>
<b>ABSTRACT</b>	<b>ii</b>
<b>ACKNOWLEDGEMENTS</b>	<b>iii</b>
<b>DECLARATION</b>	<b>v</b>
<b>APPROVAL SHEET</b>	<b>vi</b>
<b>PERMISSION SHEET</b>	<b>vii</b>
<b>TABLE OF CONTENTS</b>	<b>viii</b>
<b>LIST OF TABLES</b>	<b>x</b>
<b>LIST OF FIGURES</b>	<b>xi</b>
<b>LIST OF ABBREVIATIONS</b>	<b>xii</b>

### CHAPTER

1	INTRODUCTION	1
	1.1 Introduction	1
	1.2 Objectives	3
2	LITERATURE REVIEW	5
	2.1 Honey	5
	2.1.1 Introduction	5
	2.1.2 Antibacterial Activity of Honey	7
	2.2 Gelam Honey	10
	2.3 Manuka Honey	14
	2.4 <i>Staphylococcus aureus</i>	17
	2.4.1 Introduction	17
	2.4.2 Biofilm	18
	2.4.3 Elimination of Biofilm	21
	2.4.4 Potential Effects of Honey on Biofilm	23
3	MATERIALS AND METHODS	25
	3.1 Materials	25



3.1.1	Honey	25
3.1.2	Bacterial Strains	25
3.1.3	Chemicals and Media	26
3.1.4	Equipments and Labwares	27
3.2	Method	28
3.2.1	Experimental Design	28
3.2.2	Kirby-Bauer Assay	29
3.2.3	Established Biofilm Reduction Assay	29
3.2.4	Biofilm Prevention Assay	31
3.2.5	Calculation of Biofilm Mass Reduction	32
4	RESULTS	33
4.1	Kirby-Bauer Assay	33
4.2	Established Biofilm Reduction Assay	35
4.3	Biofilm Prevention Assay	38
5	DISCUSSION	41
5.1	Established Biofilm Reduction Assay	41
5.2	Biofilm Prevention Assay	46
5.3	Future Studies	49
6	CONCLUSION	50
	REFERENCES	51
	APPENDIX	59

## LIST OF TABLES

<b>Table</b>	<b>Title</b>	<b>Page</b>
3.1	Chemicals and media used and their respective manufacturers.	26
3.2	Equipments and labwares used and their respective manufacturers.	27
3.3	Honey sample preparation.	30
4.1	The antibiotics susceptibility and efficacies toward <i>Staphylococcus aureus</i> .	34

## LIST OF FIGURES

<b>Figure</b>	<b>Title</b>	<b>Page</b>
2.1	Three steps of bacterial biofilm formation.	19
3.1	Experimental design.	28
3.2	Illustration of biofilm assay.	32
4.1	Percentage of reduction of <i>S. aureus</i> biofilm mass by using Gelam Honey.	36
4.2	Percentage of reduction of <i>S. aureus</i> biofilm mass by using Manuka Honey UMF 10+.	36
4.3	Percentage of reduction of <i>S. aureus</i> biofilm mass by using Manuka Honey UMF 15+.	37
4.4	Percentage of reduction of <i>S. aureus</i> biofilm mass by using Gelam Honey.	39
4.5	Percentage of reduction of <i>S. aureus</i> biofilm mass by using Manuka Honey UMF 10+.	39
4.6	Percentage of reduction of <i>S. aureus</i> biofilm mass by using Manuka Honey UMF 15+.	40

## LIST OF ABBREVIATIONS

agr	accessory gene regulator
CAPE	caffeic acid phenyl ester
DHA	dihydroxyacetone
EPS	extracellular polymeric substances
MGO	methylglyoxal
MH	Mueller-Hinton
mm	millimeter
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	methicillin-susceptible <i>Staphylococcus aureus</i>
OD	optical density
PBS	phosphate buffered saline
RAP	RNAIII-activating protein
SQS1	staphylococcal quorum sensing 1
SQS2	staphylococcal quorum sensing 2
TSB	tryptone soya broth
UMF	unique manuka factor

## CHAPTER 1

### INTRODUCTION

#### 1.1 Introduction

Honey is known as a traditional medicine for centuries with its antibacterial properties but there are only few brief reviews with little clinical detail, what they know is honey was just known to work (Molan 1998). With the advent of antibiotics, clinical application of honey was abandoned until there is a rise of prevalence of antibiotic-resistant bacteria (Kwakman et al., 2010). Therefore, alternative antimicrobial strategies are urgently needed to overcome this major problem which caused by the abundant use of antibiotics (Kwakman et al., 2010).

Due to the development of antibiotic resistance in bacteria, honey emerges as one of the alternative antimicrobial agent for treatment of ulcers and bed sores, and other surface infections resulting from burns and wounds (Molan 2009; Sharp 2009; Kwakman et al., 2010). After years, scientists and researchers found out that its antibacterial properties are mainly attributed to the combination of hydrogen peroxide, high osmolarity, phenolic compounds and antioxidants (Molan 1998; Lerrer et al., 2007; Molan 2009). Molan (2009)

indicated that honey was very efficient in clearing up infection, promoting healing and even treating bacterial gastroenteritis in infants.

Honey is produced by worker honeybees from floral nectars following by water evaporation and addition of digestive enzyme in the honeycomb (Lerrer et al., 2007). However, so far there are only two well-characterized antibacterial factors identified in honey which including hydrogen peroxide and high osmolarity. Its composition that consists more than 80% of sugars (fructose, glucose, sucrose and others) contributes to the high osmolarity together with hydrogen peroxide play important roles in broad spectrum activity against wide range of bacteria. The antibacterial activity of honey is highly depending on the sources of nectar and also geographical factors like temperature and humidity (Tumin et al., 2005; Lerrer et al., 2007; Okhiria et al., 2009; Wang et al., 2012).

Most of the bacteria are associated with biofilm formation (Olson et al., 2002). Biofilm is generally defined as surface-attached community of cells embedded in a matrix of extracellular matrix, which causes tremendous problems in environment, human and even animals (Boles and Horswill 2008; Archer et al., 2011; Periasamy et al., 2012). According to Monroe (2007), bacterial biofilm occurs naturally within us, such as dental plaque. Based on H änsch (2012)

research, biofilm was first described as water-dwelling bacteria and later being recognized as “biofouling” agent in water-dependent industries.

According to Bordi and Bentzmann (2011), the structure of biofilm allows the bacteria to undergo dormancy and hibernation, as a result, they can survive longer and able to disseminate their genome. Infections that arise from biofilm are characteristically chronic and most of the cases are reported in hospital. Examples of chronic infections due to biofilm including native valve endocarditis, chronic bacterial prostatitis, cystic fibrosis, otitis media, rhinosinusitis, osteomyelitis, periodontitis and chronic wound (Kokare et al., 2009).

## **1.2 Objectives**

The potential effect of honey is varying due to their composition, so different types of honey might have different degrees of antibacterial effect. However, limited published researches about the potential effects of honey on bacterial biofilm. Therefore, the purpose of this study is to evaluate the potential effect of honey against the biofilm of *Staphylococcus aureus*.

Hence, the objectives of this study are:

- 1) To study the inhibitory effect of Malaysian Gelam honey and Manuka honey on established biofilms.
- 2) To study the preventive effect of Malaysian Gelam honey and Manuka honey on the formation of biofilms.
- 3) To determine the differences in potential effect on biofilm between Malaysian Gelam honey and Manuka honey.



## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Honey

##### 2.1.1 Introduction

From years to years, there are several clinical trials have been carried out to show the effectiveness of honey against bacteria. Recently, there was a research showed that honey was more efficient than commonly used antibiotics because it was capable to kill bacteria even in their highly resistant biofilm state such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* without adverse effects. Besides, clinical studies indicated that honey possessed the ability to enhance the effectiveness of healing process and elimination of *in vivo* infection (Aljadi et al., 2000; Agbaje et al., 2006; Wang et al., 2012). Other than wound healing, honey grabs the attention of food industries to play a role in food preservation as an antioxidant due to its ability to combat damage caused by oxidizing agents (Meda et al., 2004).

Honey is the oldest natural sweet substance that produced by honeybees from floral nectar which known for its medicinal uses since ancient times and considered as one of the most enduring-substance used in wound management

(Ferreira et al., 2009; Christinal et al., 2012; Tan et al., 2012; Wang et al., 2012). It is an extremely viscous liquid containing of enzymes which work together with its antibacterial properties to clean and protect the wound from future infection (Zumla & Lulat 1989). The two major elements of honey are sugars (81%) and water (17%) while the remaining 2% including various enzymes, phenolic compounds, antioxidants, aromatic compounds, vitamins and other essential nutrients such as amino acids and proteins (Lerrer et al., 2007; Bogdanov et al., 2008; Ferreira et al., 2009; Sharp 2009; Christinal et al., 2012; Hussein et al., 2012; Jubri et al., 2012; Wang et al., 2012).

According to Jeffrey and Echazarreta (1996) and The National Honey Board (n.d.), enzymes found in honey that derived from hypopharyngeal gland of honeybees including invertase which inverts sucrose to fructose and glucose, glucose oxidase which oxidizes glucose to gluconic acid and hydrogen peroxide in the presence of water and amylase or diastase which breakdown starch into sugars. On the other hand, there are other enzymes which originated from plants such as catalase, acid phosphatase and small portion of amylase. Anyway, the physical and chemical properties of honey are largely depending on floral source of nectar (Cooper et al., 1999; Taormina et al., 2001).

### **2.1.2 Antibacterial Activity of Honey**

According to Molan (1992) and Cooper et al. (2002), there are three explanations for the antibacterial property of honey, which including osmotic effect, acidity and hydrogen peroxide. Based on the study of Al-Waili et al. (2011), osmotic effect, acidity and hydrogen peroxide of honey contribute to wound healing properties including stimulation of tissue growth, enhancing epithelialization and minimizing scar formation. This further proved that the antibacterial activity of honey is related to the wound healing properties of honey. Lusby et al. (2005) stated that antibacterial activity of honey is a factor that helps in promoting the wound healing property of honey. By applying honey on the surface of the wound, it maintains moist wound environment that promotes healing. Besides, the high viscosity of honey helps in providing a protective barrier to prevent the entry of bacteria into the infected area.

The antibacterial property of honey was reported first in 1892 by Dutch scientist van Ketel and being assumed that this property was entirely due to the high osmotic effect of its high sugar contents (Molan 2001). According to Cooper et al. (1999), high osmolarity due to high levels of sugar in honey inhibited microbial growth. However, dilution by wound exudate may reduce the osmolarity to the point where the inhibition action ceases control infection (Cooper et al., 1999; Molan 2001). Nevertheless, Cooper et al. (1999) reported that the antibacterial activity of honey does not depend completely on its high osmolarity but on the release of the hydrogen peroxide.

Ashley (2012) stated that hydrogen peroxide is created from oxygen and hydrogen molecules. Hydrogen peroxide breaks down into water hydrogen and oxygen when it is applied to surface. Oxygen kills germs by combining with other substances to alter the function of the molecules; this process is known as oxidation (PRlog 2009; Ashley 2012). Lynne (2003) has found that high concentration of hydrogen peroxide is disadvantage as it can cause inflammation and damage to human tissue. Straight hydrogen peroxide is relatively unstable and will eventually lose its effectiveness when exposing to air or light so the use of pure hydrogen peroxide is not always encouraged (PRlog 2009). But honey sequesters and inactivates the free iron which catalyzes the formation of oxygen free radicals (Molan 2001). Hydrogen peroxide in the honey is released through the slow-release chemical mechanism, glucose oxidation and this process is catalyzed by glucose oxidase (Jeffrey and Echazarreta 1996; Cooper et al., 1999; Brudzynski et al., 2011).

Glucose oxidase, an enzyme that secreted from the hypopharyngeal gland of the honeybees into nectar to assist in honey formation, will only be activated under right conditions with the pH of honey falls within 5.5 to 8.0 with the presence of certain amount of sodium (Mwipatayi et al., 2004; PRlog 2009). Human skin and body fluids do fulfill these requirements to activate glucose oxidase in order to produce hydrogen peroxide. Mwipatayi et al. (2004) found that glucose oxidase is the enzyme that remains practically inactive in full-strength honey. Thus, honey must be diluted first in order to get rise to

hydrogen peroxide. In other words, when honey comes in contact with wound, glucose oxidase will be activated under right condition, thus break down glucose and producing hydrogen peroxide which contribute to the antibacterial property of honey (PRlog 2009).

Next, acidity is also said to be one of the factors that contributes to the antibacterial property of honey (Tumin et al., 2005). Normally, pH of honey is quite acidic, which is within the range of 3.2 to 4.5 due to the presence of gluconic acid or gluconolactone. Low pH of honey would inhibit many human pathogens which their optimum pH for growth is within the range of 7.2 to 7.4 (Molan 1992; Jeffrey and Echazarreta 1996; Bogdanov 1997; Malika et al., 2004; Sharp 2009). Thus, in undiluted honey, acidity is a significant antibacterial factor. But when honey is diluted, acidity will be neutralized by the medium thus will not cause inhibition to pathogens or will reduce the inhibition power. Since our body fluid has strong buffering capacity, so acidity of honey would most likely being neutralized, which mean the pH will not be too low and so the acidity may not be an effective inhibitor to pathogens (Molan 1992).

However, other than the three factors mentioned above, there is presence of other antibacterial factors (Molan 1992). Some friendly bacteria within honey

including six species of lactobacilli and four species of bifidobacteria can be used to cure sore throat and respiratory conditions that accompany the common cold (The George Mateljan Foundation 2013). In addition, honey is an excellent source of flavonoids, a natural antioxidant which have the ability to protect against allergens, viruses and carcinogens (SoundHealth 2013). According to Mbotto et al. (2009), antibacterial activities of honey can be due to the presence of inhibines including flavonoids, phenolic acids and some unidentified components in honey.

## **2.2 Gelam Honey**

Gelam honey is well-known in Malaysian for their potential health benefits such as wound healing, antioxidant and anti-inflammatory activities (Hussein et al., 2012). It is a local monofloral honey that produced by the honeybee called *Apis mellifera* from the floral source of *Melaleuca cajuputi*, which can be found in the swampy coastal areas of Malaysia (Lee et al., 2011). Gelam honey is reported to have high phenolic compounds such as gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin, hesperetin, luteolin, kaempferol and chrysin (Hussein et al., 2011; Kassim et al., 2012). It is believed that these phenolic compounds contribute to the anti-inflammatory, antibacterial and free radical scavenging activities of Gelam honey (Khalil et al., 2012). Alzahrani et al. (2012) stated that antioxidant compounds may strengthen the host defense and prevent oxidative stress.

According to Tan et al. (2012), Gelam honey accelerated wound healing rate if dressed on excisional wound. Wound treated with Gelam honey can shorten the wound healing time compared to the untreated wound and new epidermis will form and cover the entire wound area to prevent the wound from further injuries. Accelerated wound healing is believed due to the production of hydrogen peroxide, antioxidant, antibacterial properties and high levels of glycine, methionine, arginine and proline which are essential in collagen formation and deposition. In addition, Gelam honey can reduce the formation of hard and intact dark brown scab in wound healing and the scar is thinner due to the viscosity of honey which providing the wound with moist healing environment. This condition further enhances the epithelialization and providing antibacterial barrier protection (Tan et al., 2012).

Moreover, Zohdi et al. (2012) also agreed with Tan et al. (2012) with the application of Gelam honey-based hydrogel on burned wound which proved can accelerate wound closure and re-epithelialization and decrease proinflammatory mediators. The pH of Gelam honey-based hydrogel is 4.3, which is slightly acidic than healthy skin pH 5.5 that creates an unfavorable environment for bacterial growth and enhances maximum release of oxygen to meet the needs of body's tissue repair. High osmolarity of Gelam honey-based hydrogel due to high sugar contents highly capable in absorbing exudates from wound thus can prevent the accumulation of exudates (Zohdi et al., 2012).

Besides, the presence of phenolic contents contribute to the antioxidative and radical scavenging properties of Gelam honey (Zaghloul et al., 2001; Aljadi and Kamaruddin 2004; Hussein et al., 2011). Based on the researches of Zaghloul et al. (2001) and Aljadi and Kamaruddin (2004), ethyl acetate extract of honey was used to evaluate the relation between the extract of phenolic compounds and the antioxidant activity of honey. Results showed that Gelam honey had higher antioxidant activity compared to Coconut honey due to the presence of higher amount of phenolic compounds.

Kassim et al. (2012) has reported that Gelam honey successfully protected host immune cells from inflammation-mediated cytotoxicity and capable to scavenge peroxynitrite. Peroxynitrite is the reaction product of reactive oxygen species and potent inducer of cell death by stimulating the release of mitochondrial apoptosis-inducing factor which triggers DNA fragmentation. Gelam honey acted by inhibiting nitric oxide production, and thus peroxynitrite formation, therefore the effects of these cytotoxic compounds were reduced.

On the other hand, according to Hussein et al. (2012), the treatment of Gelam honey on carrageenan-induced paw edema in rats had shown the paw edema formation can be reduced significantly in a dose-dependent manner. The usage



of carrageenan was to induce inflammation with the release of several proinflammatory mediators, including interleukin 1, interleukin 6, interleukin 12, tumor necrosis factor, interferon, cyclooxygenase, prostaglandin and inducible nitric oxide synthase. After treated with Gelam honey, there was a significant reduction in plasma levels of all these proinflammatory mediators.

In the research done by Lee et al. (2011), phenolic compounds in Gelam honey were able to reduce oxidative damage in rats due to the antioxidant ability to scavenge free radical activity. Gallic acid was one of the phenolic compounds that found on Gelam honey which showed the strongest ability to scavenge free radical activity. The dark color Gelam honey always contains higher amount of antioxidants, in other words, has higher antioxidative and scavenging activities against free radicals or oxygen species.

Study of Jubri et al. (2012) showed the antiproliferative effect of Gelam honey against liver cancer cells (HepG2) due to its high polyphenols content that involved in antioxidant and free radical scavenging activities. Based on the results of the study, Gelam honey inhibited growth of HepG2 cell by activating p53 tumor-suppressor gene and antioxidants. Apigenin and caffeic acid phenyl ester (CAPE) were the two types of phenolic compound found in Gelam honey that contributed to the antiproliferative effect on the cancer cells.

Jubri et al. (2012) concluded that Malaysian Gelam honey inhibited proliferation of liver cancer cells by apoptotic induction without affecting normal liver cells.

Christinal et al. (2012) concluded that Gelam honey capable of suppressing HT 29 colon cancer cells growth by inducing DNA damage and apoptosis as well as suppressing inflammation. PGE<sub>2</sub>, one of the inflammatory markers secreted by HT 29 was reduced due to the presence of flavonoids and phenolic compounds in Gelam honey which activated caspases signaling pathway. Thus, high polyphenols in Gelam honey is said to be one of the factors that contribute to the anti-tumor property.

### **2.3 Manuka Honey**

Manuka honey is made from nectar that collected by bees from the wild Manuka tree, *Leptospermum scoparium* or commonly known as New Zealand tea tree which grows uncultivated throughout the country (The Honey Centre Ltd 2007; Atrott and Henle 2009; Macpherson 2009; Alnaimat et al., 2012; The Honey Farm 2012; Windsor et al., 2012). This monofloral honey is in sticky golden colour and famous with its high antibacterial healing power worldwide (The Honey Centre Ltd 2007; Macpherson 2009).

According to Christensen (2011b), Manuka honey is the only one honey that contains plant-derived factor known as methylglyoxal (MGO), a potent antibacterial agent which acts directly to kill bacteria. The concentration of methylglyoxal is correlated to the non-peroxide activity of Manuka honey (Kirnpal-Kaur et al., 2011). Majtan (2011) and Windsor et al. (2012) both stated that MGO produced by non-enzymatic conversion of dihydroxyacetone (DHA) during honey maturation. The presence of MGO in Manuka honey contributes to its uniqueness and termed as unique manuka factor (UMF). However, only certain Manuka honey contains this unique property and cannot be found in any other honey (Atrott and Henle 2009). However, not all Manuka honey contains equal amount of MGO, thus might not be the best in dealing leg ulcers. Moreover, this unique type of Manuka honey only can be collected from few places in New Zealand. There is an evidence shows that some areas of Manuka bushes do not produce UMF every year, and the concentration of UMF can vary from batch to batch and year to year (SummerGlow Apiaries Ltd 2013).

UMF was first discovered by Dr. Peter Molan, of Waikato University's Honey Research Unit. This UMF Manuka honey has unique extra antimicrobial activity which is not related to its low pH, osmolarity or hydrogen peroxide accumulation, known as non-peroxide activity (Alnaimat et al., 2012). This non-peroxide activity is a phytochemical-derived activity that comes from the nectar of the flower but not added by honeybee activity. UMF and hydrogen

peroxide can work together with synergistic effect that enhancing their antibacterial effect (The Honey Farm 2012). Unlike normal honey, antibacterial properties of Manuka honey are light- and heat- stable (Simon et al., 2009; Mandal and Mandal 2011).

According to Christensen (2011b), UMF number is given to Manuka honey due to their difference in MGO level. This difference is due to natural variation in plant metabolism, manufacturing and processing of the honey. The higher the UMF number, the higher the MGO level the Manuka honey contains and the more potent the antibacterial activity (Hart-Davis 2009). Hart-Davis (2009) indicated that Manuka honey with UMF number 10 or higher is effective in antiviral and antibacterial actions while Manuka honey with UMF below 10 may still achieve good healing results but may not fully eliminate the infection due to the lower level of antibacterial activity (The Honey Centre Ltd 2007).

The Honey Centre Ltd (2007) reported that Manuka honey UMF 15+ or 20+ can draw healing fluid and nutrition to wound surface which create a moisture environment on wound surface if applied externally. So when bandages are removed, new growing tissue will not be damaged and thus enhance the wound healing process. While for internal stomach ulcer type condition,

Manuka honey can inhibit the growth *Helicobacter pylori*, which is one of the causes of stomach ulcers and the healing process is eight times more effective than other normal honey can do. Besides that, a case was reported in 2007 where wounds of methicillin-resistant *Staphylococcus aureus* (MRSA) superbug victims fully healed by using Manuka honey (The Honey Centre Ltd 2007).

## **2.4 *Staphylococcus aureus***

### **2.4.1 Introduction**

*Staphylococcus aureus* is a facultatively anaerobic, Gram-positive cocci bacterium that grows in irregular grape-like cluster. It is often referred to “golden staph” due to its appearance, which is large, round and golden-yellowish colonies (Saravanan and Nanda 2009). This human pathogen normally found on nasal passage, skin and mucous membrane. It causes skin infections, pneumonia, toxic shock syndrome, food poisoning and blood poisoning in both community and hospital (Todar 2008).

Khan et al. (2011) stated that *Staphylococcus aureus* is an adaptable pathogen because it can alter its genotype and/or phenotype to adapt itself to surrounding environment. Example of genotype change is acquisition of  $\beta$ -

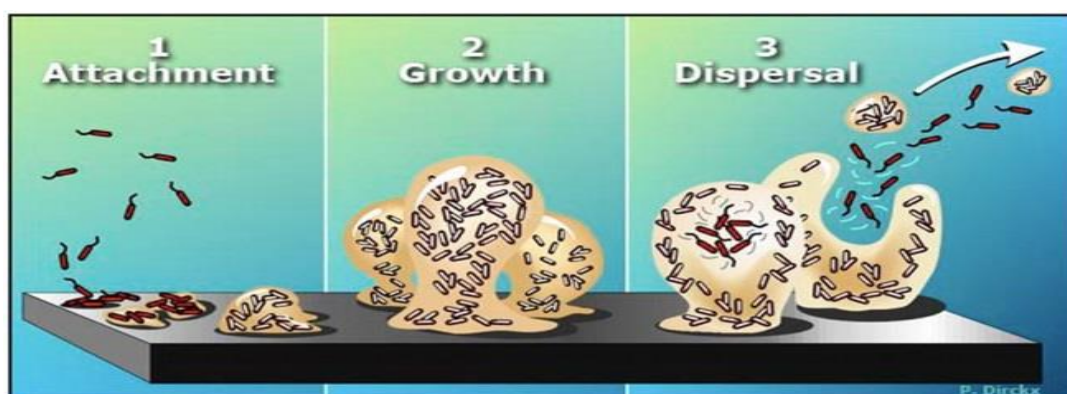
lactamase gene whereas phenotype change is biofilm formation. Yu et al. (2012) stated *Staphylococcus aureus* as an opportunistic human pathogen because it can adhere to many tissues sites and implants in human to form biofilm causing chronic infection.

Some strains of *Staphylococcus aureus* are resistant to antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of staph that evolved an ability to resist against  $\beta$ -lactam antibiotics, including methicillin and penicillin. Since methicillin resistance emerged, MRSA became a causative agent that responsible for life-threatening hospital-acquired or nosocomial infection but has recently emerged in the public known as superbug (Perlroth et al., 2008; Todar 2008). Their ability in causing difficult skin and underlying tissue infections has become a major health concern worldwide (Halc3n and Milkus 2004).

#### **2.4.2 Biofilm**

Formation of biofilm is a three steps sequential process: attachment, growth and dispersal (Fux et al., 2009; Periasamy et al., 2012; Karlsruhe Institute of Technology 2013). Firstly, *Staphylococcus aureus* will attach to the surface of medical device and then grow to give more and more cells on the surface they attached. These cells will further secrete extracellular polymeric substances

(EPS) which anchor them to the surface, creating a multilayer matrix. This matrix is consisting of exopolysaccharide, teichoic acid, protein and DNA from lysed cells. Clusters of cell are then able to spread through blood and body fluid to new infection sites. This step is the critical step in developing biofilm-associated infection (Smith 2005; Beddow 2010; Periasamy et al., 2012).



**Figure 2.1:** Three steps of bacterial biofilm formation (Mittelstadt 2010).

The formation of biofilm always associated with the resistance to multidrug and host immune system (Bordi and Bentzmann 2011). According to Leid et al. (2002), biofilm formation can reduce penetration of leukocytes and efficiency in phagocytosis. As the immune system has been disrupted, thus infection cannot be eliminated from host body. Mah and O'Toole (2001) reported that multidrug resistance is due to inability of antibiotic to penetrate biofilm. This is because exopolysaccharide matrix that produced by biofilm prevents the access of antibiotics. Bordi and Bentzmann (2011) also agreed and stated that bacterial cells embedded in exopolysaccharide matrix which

serves as physical barrier that will limit the diffusion and penetration of antibiotic and antimicrobial agents into the biofilm.

Process of *Staphylococcus aureus* biofilm formation is controlled by quorum-sensing, which is a system used by bacteria for cell-cell communication to regulate gene expression in response to the cell density (Sifri 2008; Fux et al., 2009; Hänsch 2012; Yu et al., 2012). Besides, this intercellular signaling also optimizes the metabolic and behavioral activities of cell population. Quorum-sensing signaling system is mediated by signaling molecules called autoinducers that produced and released by bacteria (Kiran et al., 2008; Sifri 2008; Yu et al., 2012). According to Yarwood and Schlievert (2003), quorum-sensing not only enable cell-cell communication but also regulates colonization and virulence factor. Staphylococcal accessory gene regulator (*agr*) quorum-sensing system decreases the expression of cell surface proteins and increases the expression of virulence factors and this is found to contribute to staphylococcal pathogenesis in several infections (Yarwood and Schlievert 2003).

Two quorum-sensing systems that present in Staphylococci are staphylococcal quorum sensing 1 (SQS1) and staphylococcal quorum sensing 2 (SQS2). SQS1 consists of the autoinducer RNAlII-activating protein (RAP) and its



target molecule (TRAP). SQS 1 also induces the activation of SQS2 which possesses the products of the agr quorum-sensing system and a regulatory mRNA molecule (RNAIII) that induce toxin production (Kiran et al., 2008). According to Balaban et al. (2001), RAP activates RNAIII synthesis via TRAP and TRAP will further interacts with agr quorum-sensing system to upregulate RNAIII and induce the production of virulence factors.

### **2.4.3 Elimination of Biofilm**

Elimination of biofilm is a problem because it is resistant to drug and immune system. Still, antimicrobial therapy is always used as one of the treatments to prevent biofilm from forming or growing (Jorgensen and Ferraro 2009). However, it is not efficient enough, thus it will be used in conjunction with surgical intervention. This combination always involved prolonged antimicrobial therapy in outpatient setting under the guidance of an infectious disease specialist (Archer 2011; Marculescu et al., 2012). However, toxicities and drug interactions need to take into consideration (Archer 2011).

Several host factors must take into consideration in selecting antimicrobial agents, including renal and hepatic function, age, genetic variation, pregnancy and lactation, history of allergy or intolerance and lastly history of recent microbial use. According to Perlroth et al. (2008), rifampin is a broad-

spectrum antimicrobial agent that has the ability to kill metabolically dormant bacteria by penetrating biofilm and concentrates well intracellularly for the treatment of *Staphylococcus aureus* infection. However, results of the study have shown that therapy with rifampin was inconsistent and there was a rapid emergence of resistance. Thus, it has been used in combination with another active antibiotic such as vancomycin or fluroquinolones and seems promising in the treatment of *Staphylococcus aureus* infections (Perlroth et al., 2008; Archer et al., 2011).

Kokare et al. (2009) and Periasamy et al. (2012) indicated that *Staphylococcus aureus* has extraordinary capacity to attach on medical devices such as prosthetic heart valves, urinary catheters, intrauterine devices and implants, through direct interaction by establishing connections to human matrix protein after those proteins have covered those devices. Thus, inclusion of antimicrobial agents at the site of infection such as implanted medical device is a more preventive alternative. Based on Wahlig and Dingeldein (1980) research, this method was first performed by using antibiotic-containing bone cement. Results have proved that it can provide a rapid release of high concentration antimicrobial agents and shown a high efficacy in combating infections. Other than bone cement, antibiotic-containing bead also being selected as one of the treatments as it does not generate heat thus facilitate greater variety of antibiotics.

According to Kent et al., (2006), polymethylmethacrylate antibiotic bead was first introduced 30 years ago as main treatment for osteomyelitis therapy for several advantages including decreased in systemic toxicity, decreased hospitalization duration and overall treatment cost. However, there are limitations too as it needs second surgery to remove those non-biodegradable beads and will further leaves a potential residual soft tissue dead space or osseous defect (Kent et al., 2006; Helgeson et al., 2009). Besides polymethylmethacrylate bead, calcium sulfate can be used as vehicle in carrying antibiotics and it has advantage over polymethylmethacrylate – it is biodegradable so does not require surgical removal (Kent et al., 2006; Cai et al., 2010).

#### **2.4.4 Potential Effects of Honey on Biofilm**

As we know, some infected wounds failed to respond to antimicrobial treatment due to the development of biofilm (Brindley 2012). ScienceDaily (2012) has reported that small concentrations of honey can prevent the formation or inhibit the established bacterial biofilm by killing them up to 85% within two hours. There is a latest study revealed that honey acted by interrupting the interaction between bacteria and human protein fibronectin which displayed on the surface of damaged cells as the bacterial surface molecule can bind to this human protein and lead to development of biofilm (Maddocks et al., 2012).

Giuliano (2012) stated that Manuka honey can dissolve and kill bacterial biofilm in chronic rhinosinusitis that infected by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Manuka honey was also tested on established biofilms of methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* isolates. Results showed that Manuka honey was effective in killing all of the isolates and concluded that it is inexpensive, natural, non-toxic and more effective compared to antibiotics in killing bacterial biofilm (Alandejani et al., 2009). Jervis-Bardy et al. (2011) and Kilty et al. (2011) also said that MGO in Manuka honey is the major antibacterial component in honey. Study showed that effective concentration of MGO against MRSA biofilm ranged from 0.5 mg/mL to 3.6 mg/mL.

Based on the study of Jenkins et al. (2011), growth of MRSA was inhibited by 5%, 10% and 20% (w/v) of Manuka honey and 10% (w/v) of artificial honey containing methylglyoxal. Enlarged MRSA cells containing septa were observed after exposed to Manuka honey indicating that cell division of MRSA was interrupted. These changes were not caused by either sugars or methylglyoxal but the presence of unidentified additional antibacterial components in Manuka honey (Giuliano 2012).

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Honey

Gelam honey was obtained from JL Natural Resources Sdn Bhd, Johor, Malaysia, Manuka honey UMF 10+ and Manuka honey UMF 15+ were obtained from 100% Pure New Zealand Honey Ltd. Honey samples were kept in dark at room temperature.

##### 3.1.2 Bacterial Strains

Five strains of Gram-positive human pathogenic bacteria, *Staphylococcus aureus* including ATCC 6538, ATCC 25923, ATCC 33591, ATCC 33592 were obtained from Faculty of Science, Universiti Tunku Abdul Rahman and a clinical sample was collected from patient were used. Bacteria were cultured and maintained on nutrient agar.

### 3.1.3 Chemicals and Media

**Table 3.1:** Chemicals and media used and their respective manufacturers.

Chemicals / Media	Manufacturers
Nutrient broth and nutrient agar	Merck, Germany
Mueller-Hinton agar	Oxoid Ltd, England
Ethanol 95%	<i>Copens Scientific (M) Sdn. Bhd., Malaysia</i>
Phosphate buffer saline tablets	<i>MP Biomedicals, United States</i>
Absolute ethanol	Prolabo®, England
Acetone	QR ãC, New Zealand
Crystal violet	PC Laboratory Reagent, India
Glutaraldehyde 25%	Unilab, Philippines
Ampicillin (10 µg), bacitracin (10 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), methicillin (5 µg), imipenem (10 µg), penicillin G (10 µg), tetracycline (30 µg), vancomycin (30 µg)	Oxoid Ltd, England

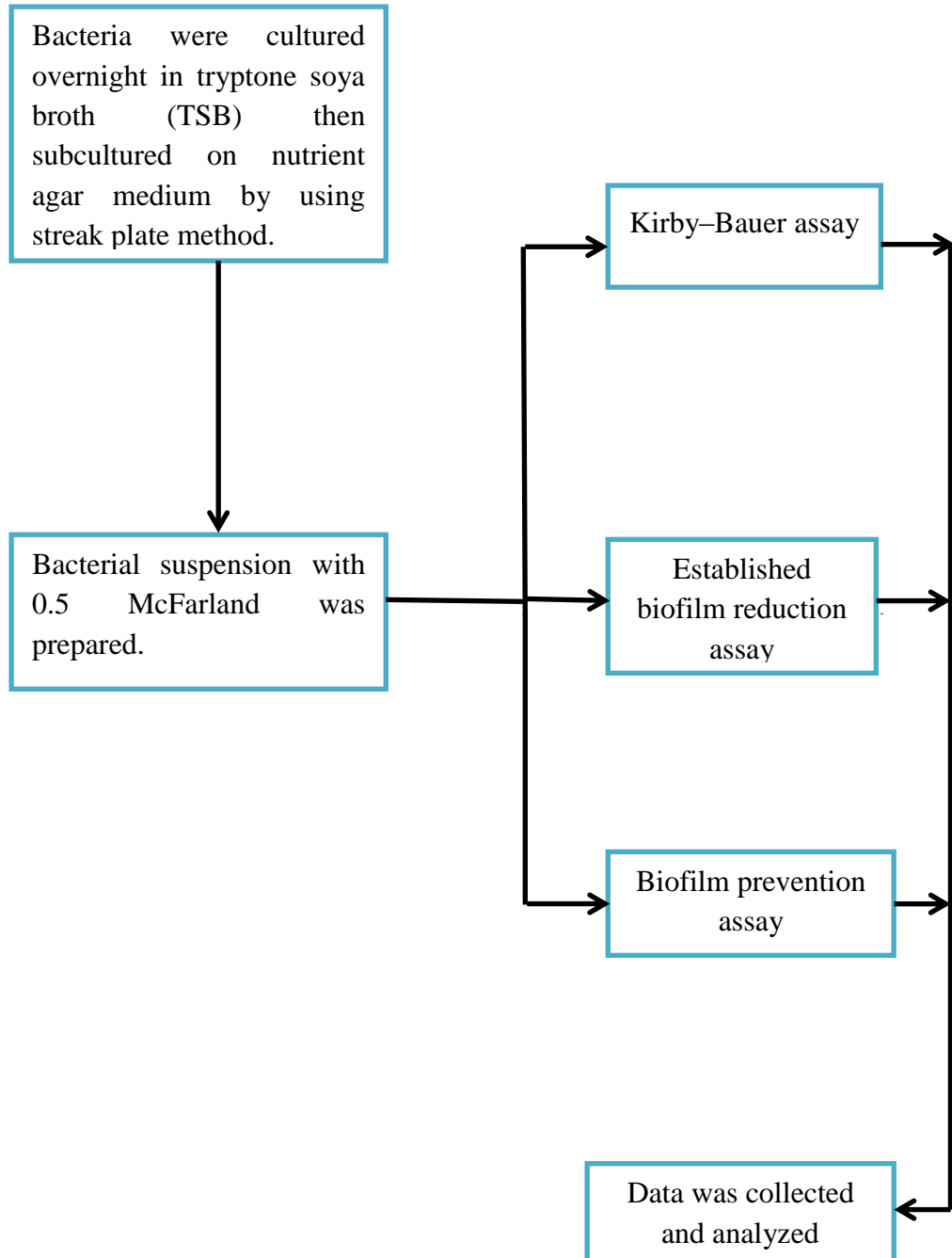
### 3.1.4 Equipments and Labwares

**Table 3.2:** Equipments and labwares used and their respective manufacturers.

Equipments / Labwares	Manufacturers
Laminar flow cabinet model AHC- 4A1	Camfil farr, Malaysia
Incubator	Memmert, Germany
Vortex	VELP® Scientifica, Europe
Spectrophotometer	Thermo Scientific Genesys 20, USA
Microtitre plate reader	TECAN infinite M2000, Switzerland
Multichannel pipette (20-2000 ul)	GILSON, USA
Pipette	ViPR Ω, United Kingdom
Pipette tips for 200 ul and 1000 ul	Axygen® Scientific, USA
15 mL high clarity polypropylene conical tube (17x120mm)	BD Falcon, BD Bioscience, USA
50 mL polypropylene conical tube (30 x 115 mm)	BD Falcon, BD Bioscience, USA
Schott bottle	DURAN , Germany
Parafilm M® all-purpose laboratory film, size: 4" X 125' each	Pechiney Plastic Packaging, USA
Petri dish 90 x 15 mm	Brandon™

## 3.2 Method

### 3.2.1 Experimental Design



**Figure 3.1:** Experimental design.



### **3.2.2 Kirby-Bauer Assay**

Bacteria were cultured in 10 ml of TSB overnight. By using streak plate method, bacteria were streaked across the nutrient agar medium and incubated at 37°C for 24 hours. After that, few colonies of bacteria were picked up and mixed with TSB and the absorbance was measured at 625 nm wavelength using spectrophotometer. Absorbance value was adjusted within 0.08 A to 0.10 A which is equivalent to 0.5 McFarland,  $1.0 \times 10^8$  CFU/ml. Next, a sterile cotton swab was dipped into bacterial suspension and spread evenly on the surface of Mueller-Hinton (MH) agar. Up to 12 commercially-prepared and fixed concentration antibiotic disks were placed on the inoculated agar surface and incubated for 16-24 hours at 37°C. After incubation, zone of inhibition around each antibiotic disk was measured to the nearest millimeter. This test was performed in triplicate. The antibiotic sensitivity of each bacterial strain was determined (Clinical and Laboratory Standards Institute 2007).

### **3.2.3 Established Biofilm Reduction Assay**

Honey sample with six different concentrations was prepared as shown in Table 3.3 below.

**Table 3.3:** Honey sample preparation.

	<b>Honey sample concentrations in % (w/v)</b>					
	<b>0</b>	<b>20</b>	<b>40</b>	<b>60</b>	<b>80</b>	<b>100</b>
<b>Honey (g)</b>	0	20	40	60	80	100
<b>TSB (ml)</b>	(TSB was added until final volume of 100 ml)					

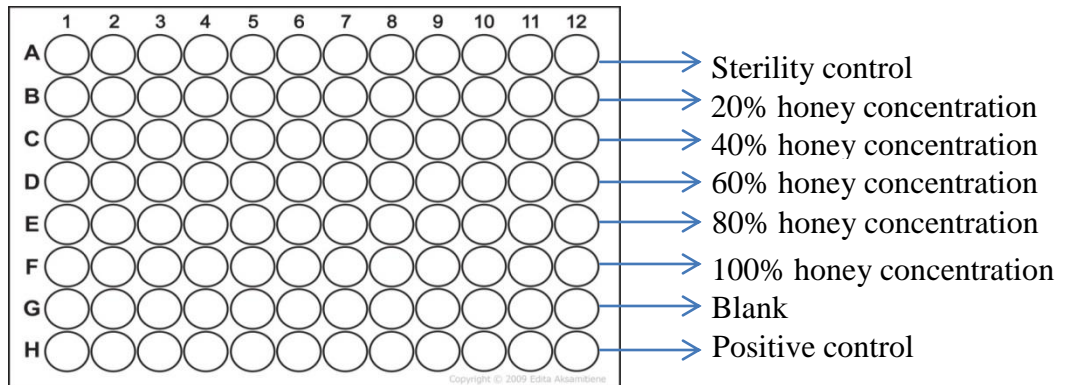
Bacterial suspension with 0.5 McFarland was prepared as described previously. Two hundred microlitre of the culture was dispensed into wells of 96-well, flat-bottomed microtitre plate and incubated for 24 hours at 37°C without shaking to allow establishment of biofilm. Wells containing bacterial culture and TSB but no honey served as positive control; wells containing neither honey nor bacterial culture but TSB alone served as sterility control as shown in Figure 3.2. After 24 hours of incubation, planktonic (unattached) cells were removed, and then 200 µl of different concentrations (0%, 20%, 40%, 60%, 80% and 100% w/v) of honey was added and incubated overnight at 37°C. After incubation, all the honey sample was discarded and the wells were washed with phosphate buffered saline (PBS). The wells were then fixed with 200 µl of 2.5% glutaraldehyde for 10 minutes. After fixation, the fixative was removed and wells were washed again with PBS. The attached cells or biofilm was then stained with 200 µl of 0.25% crystal violet for 10 minutes and gently washed twice with PBS by using a multichannel pipette. The amount of dye retained reflects the intensity of biofilm. Stained microtitre plate was dried overnight, 200 µl of solvent (1:1 acetone:absolute alcohol) was added to extract the dye from stained biofilm. Twenty microlitre of the resulting solution was added to 180 µl of solvent contained in wells of a second

corresponding microtitre plate. Absorbance was determined at 570 nm wavelength using microtitre plate reader to indicate the extent of biofilm biomass. This assay was repeated in triplicate. Average value was determined. The template of 96-well plate is shown in Figure 3.2 (Cooper et al., 2011).

### **3.2.4 Biofilm Prevention Assay**

Six different concentrations of honey sample and 0.5 McFarland bacterial suspension were prepared as described previously. After that, 5 ml of prepared bacterial suspension was inoculated together with 5 ml different concentrations (0%, 20%, 40%, 60%, 80% and 100% w/v) of honey. Then 200 ml of the mixture was then dispensed into wells of 96-well, flat-bottomed microtitre plate and incubated for 24 hours at 37°C without shaking. Wells containing bacterial culture and TSB but no honey served as positive control; wells containing neither honey nor bacterial culture but TSB alone served as sterility control as shown in Figure 3.2. After 24 hours of incubation, planktonic (unattached) cells were removed whereas adherent biofilm (attached cells) was fixed with 200 µl of 2.5% glutaraldehyde for 10 minutes. After fixation, the fixative was discarded and wells were washed with PBS. Biofilm was then stained with 200 µl of 0.25% crystal violet for 10 minutes and gently washed twice with PBS by using a multichannel pipette. Stained microtitre plate was dried overnight, then 200 µl of solvent (1:1 acetone: absolute alcohol) was added to extract the dye from stained biofilm. Twenty microlitre of the resulting solution was added to 180 µl of solvent

contained in wells of a second corresponding microtitre plate. Absorbance was determined at 570 nm wavelength using microtitre plate reader to indicate the extent of biofilm biomass. This assay was repeated in triplicate and the average value was determined (Cooper et al., 2011).



**Figure 3.2:** Illustration of biofilm assay.

Row A to row F represent the treatment of six different concentrations (0%, 20%, 40%, 60%, 80% and 100% w/v) of honey whereas row G is blank. Row H serves as positive control which contains bacterial culture with TSB but without honey sample. Row A which contains only TSB serves as sterility control.

### 3.2.5 Calculation of Biofilm Mass Reduction

The reduction of biofilm mass was calculated by using the formula as shown below:

$$\text{Biofilm mass reduction (\%)} = \frac{\text{OD (positive control)} - \text{OD (treatment)}}{\text{OD (positive control)}} \times 100\%$$

## CHAPTER 4

### RESULTS

#### 4.1 Kirby-Bauer Assay

The results of Kirby-Bauer assay are recorded in Table 4.1. Based on the results, ATCC 33591 and ATCC 33592 were suggested as strains of methicillin-resistant *Staphylococcus aureus* (MRSA) due to their resistance to  $\beta$ -lactam antibiotics, which including methicillin and penicillin. These two strains can also be known as multidrug resistant *Staphylococcus aureus* (MRSA) because they were resistant to most of the antibiotics used in this assay except vancomycin while other tested strains including ATCC 6538, ATCC 25923 and clinical strain were proved to be methicillin-sensitive *Staphylococcus aureus* (MSSA) due to their susceptibility to methicillin. Besides, these three strains were also susceptible to all the antibiotics used in this assay including ampicillin, chloramphenicol, erythromycin, imipenem, penicillin G, tetracycline and vancomycin.

**Table 4.1:** The antibiotics susceptibility and efficacies toward *Staphylococcus aureus*.

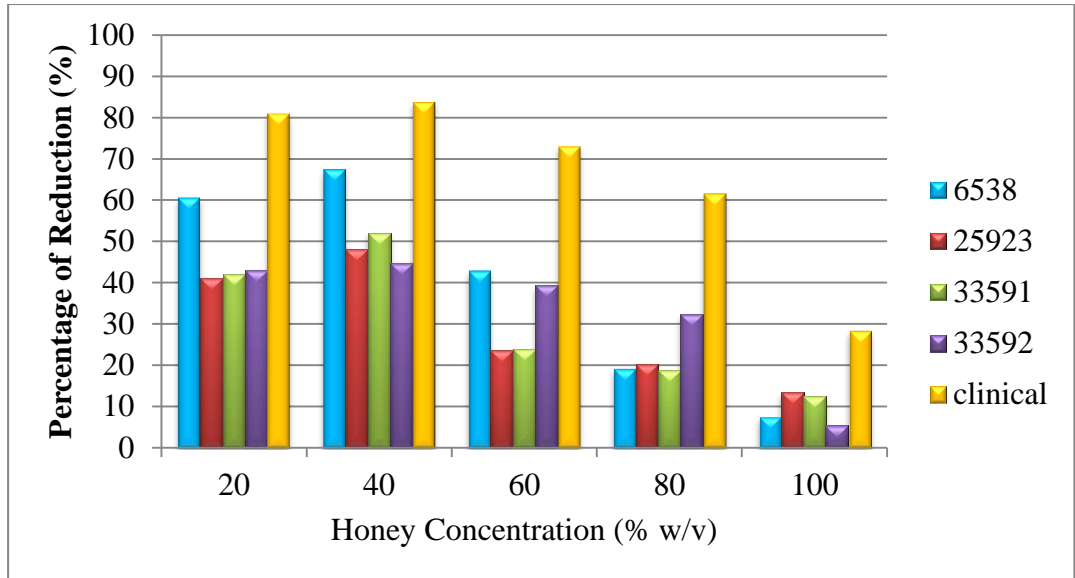
Standard Antibiotics	Concentration (µg)	Diameter of inhibition zone (mm)				
		ATCC 6538	ATCC 25923	ATCC 33591	ATCC 33592	Clinical sample
Ampicillin	10	43 (S)	29 (S)	8 (R)	8 (R)	46 (S)
Chloramphenicol	30	21 (S)	20 (S)	9 (R)	9 (R)	25 (S)
Erythromycin	15	27 (S)	24 (S)	0 (R)	12 (R)	30 (S)
Methicillin	5	31 (S)	20 (S)	0 (R)	7 (R)	23 (S)
Imipenem	10	49 (S)	45 (S)	10 (R)	10 (R)	42 (S)
Penicillin G	10	45 (S)	37 (S)	7 (R)	8 (R)	45 (S)
Tetracycline	30	27 (S)	25 (S)	0 (R)	8 (R)	32 (S)
Vancomycin	30	17 (S)	16 (S)	18 (S)	21 (S)	19 (S)

\*R = Resistant and S = Susceptible

(Clinical and Laboratory Standards Institute 2007)

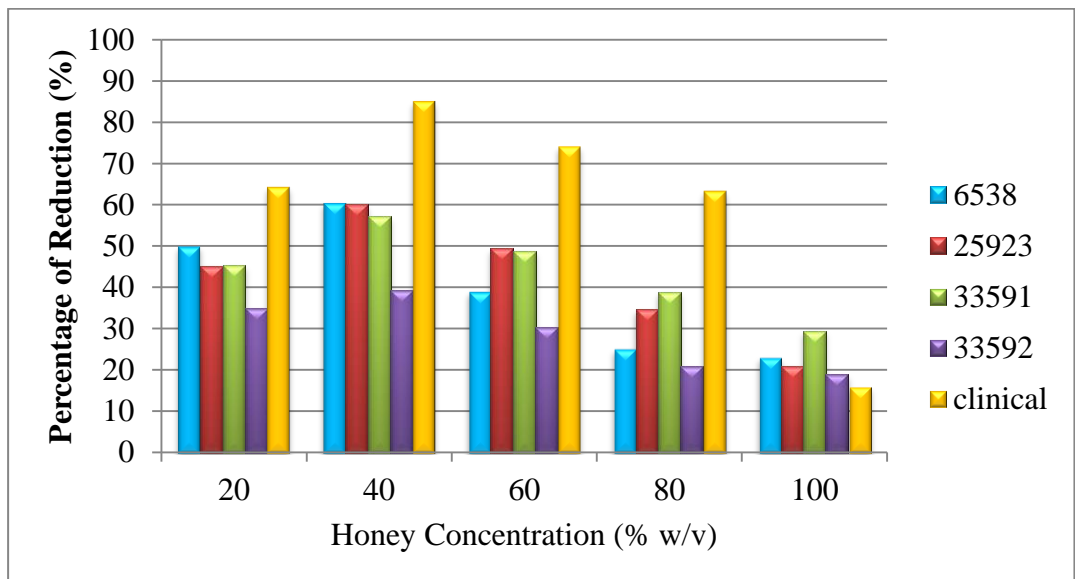
## 4.2 Established Biofilm Reduction Assay

The results of established biofilm reduction assay are summarized in Figure 4.1, 4.2 and 4.3. In general, honey samples were able to reduce the mass of established biofilm on *Staphylococcus aureus* strains (ATCC 6538, ATCC 25923, ATCC 33591, ATCC 33592 and clinical sample). The data shows that different concentrations of honey sample produced different degrees of inhibitory effects on different strains of bacteria. Based on Figure 4.1, 40% (w/v) concentration of Gelam honey was seen to reduce the established biofilm mass the most but the reduction rate was decreased afterwards. The same trend could be observed on other tested strains as well. Gelam honey was able to reduce 83.77% biofilm mass of clinical strain followed by ATCC 6538 (67.36%), ATCC 33591 (52.12%), ATCC 25923 (48.05%) and ATCC 33592 (44.76%). Figure 4.2 shows 40% (w/v) concentration of Manuka honey UMF 10 was the most effective to reduce the biofilm mass of clinical strain (85.04%) followed by ATCC 6538 (60.43%), ATCC 25923 (59.99%), ATCC 33591 (57.10%) and ATCC 33592 (39.33%). Same for Figure 4.3, clinical strain was the most sensitive to Manuka honey UMF 15 in 40% (w/v) concentration where 80.08% of established biofilm mass was reduced followed by ATCC 6538 (65.09%), ATCC 25923 (61.65%), ATCC 33591 (46.24%) and ATCC 33592 (41.07%).



Standard deviation obtained for each reading was not more than 0.0453.

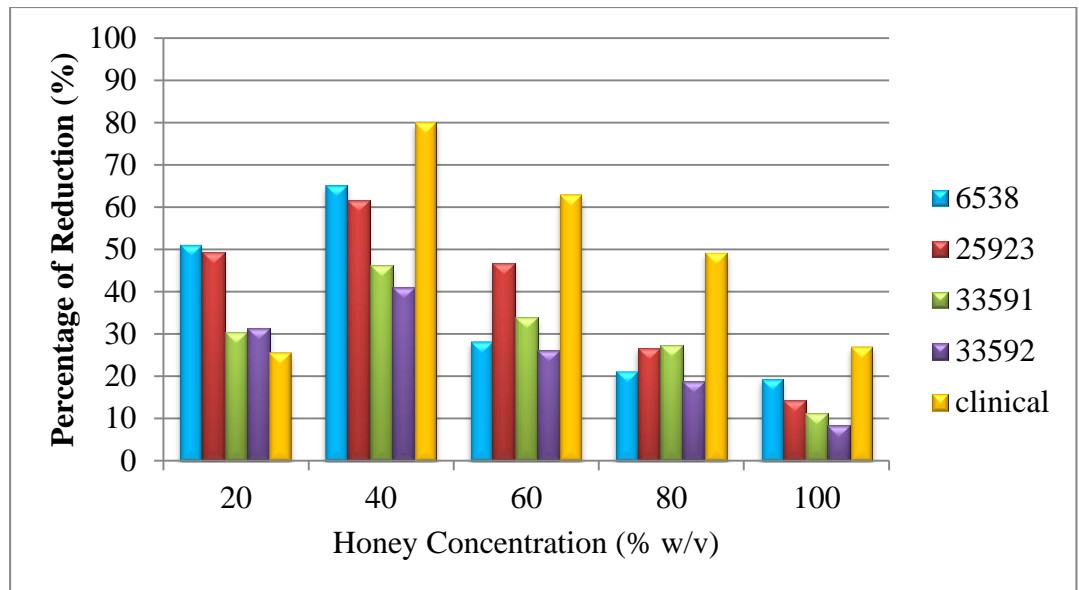
**Figure 4.1:** Percentage of reduction of *S. aureus* biofilm mass by using Gelam Honey.



Standard deviation obtained for each reading was not more than 0.0271.

**Figure 4.2:** Percentage of reduction of *S. aureus* biofilm mass by using Manuka Honey UMF 10+.



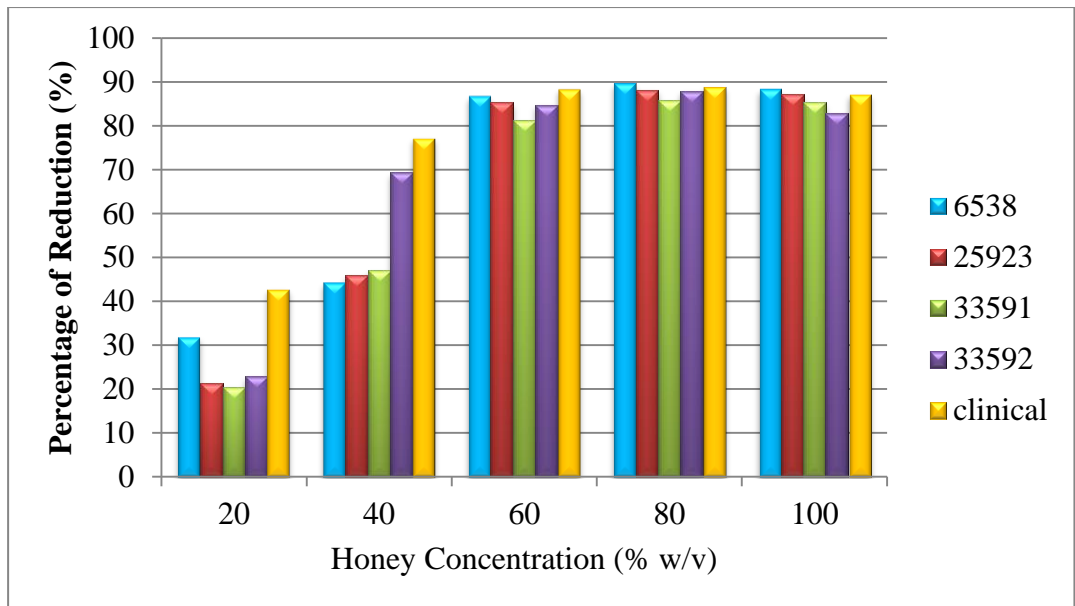


Standard deviation obtained for each reading was not more than 0.0309.

**Figure 4.3:** Percentage of reduction of *S. aureus* biofilm mass by using Manuka Honey UMF 15+.

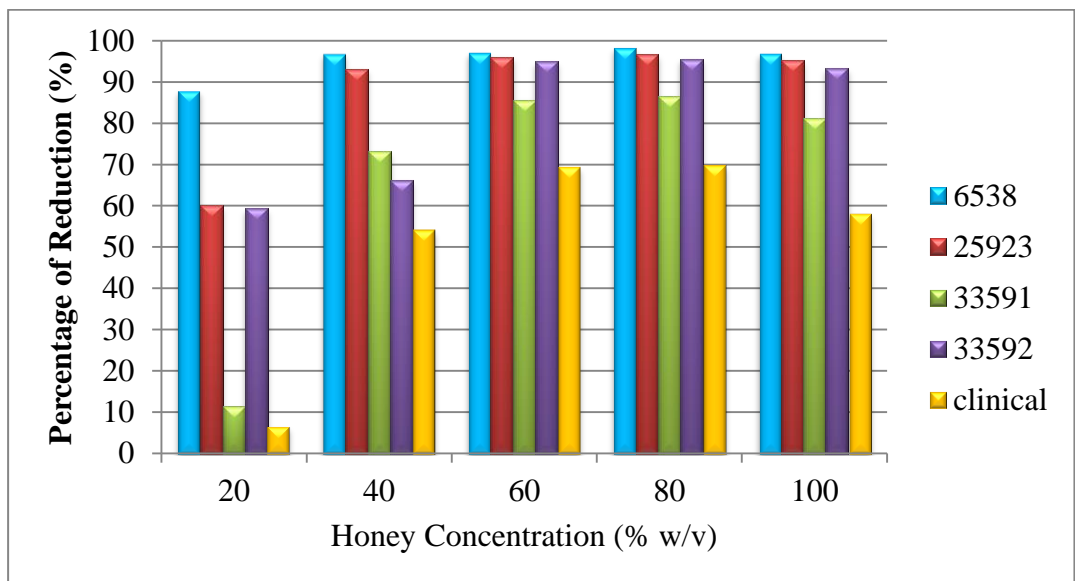
### 4.3 Biofilm Prevention Assay

The results of biofilm prevention assay are shown in Figure 4.4, 4.5 and 4.6. From the data, it shows that the tested honey samples were able to prevent the formation of bacterial biofilm on all tested *Staphylococcus aureus* strains (ATCC 6538, ATCC 25923, ATCC 33591, ATCC 33592 and clinical sample) with different level of inhibitory effects. According to Figure 4.4, 4.5, and 4.6, the result of biofilm prevention assay concluded that all of tested honeys (Gelam honey, Manuka UMF 10 and 15) showed the highest preventive effect on biofilm formation at 80% concentration. Figure 4.4 displays Gelam honey had the highest preventive effect on ATCC 6538 (89.60%) followed by ATCC 25923 (88.25%), clinical strain (88.81%), ATCC 33592 (87.94%) and ATCC 33591 (85.81%). On the other hand, Manuka honey UMF 10 was the most potent in preventing the biofilm formation of ATCC 6538 (98.30%), followed by ATCC 25923 (96.65%), ATCC 33592 (95.56%), ATCC 33591 (86.54%) and clinical sample (69.81%) as shown in Figure 4.5. Lastly, Manuka honey UMF 15 prevented the formation of biofilm the most in ATCC 6538 (98.25%) followed by ATCC 33592 (94.44%), ATCC 33591 (94.25%), ATCC 25923 (92.99%) and clinical sample (88.80%) as shown in Figure 4.6.



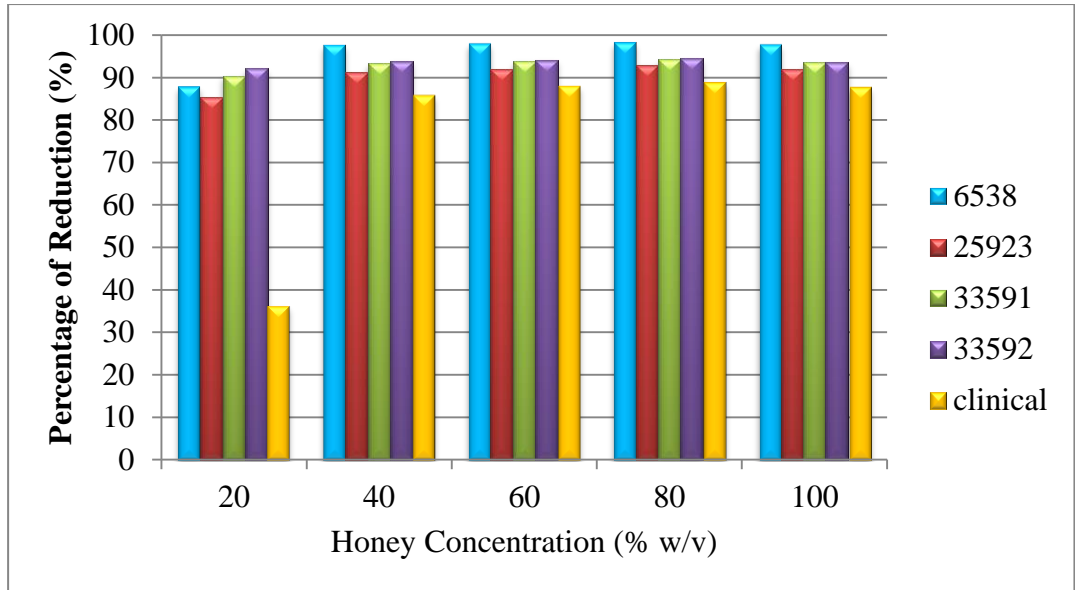
Standard deviation obtained for each reading was not more than 0.0286.

**Figure 4.4:** Percentage of reduction of *S. aureus* biofilm mass by using Gelam Honey.



Standard deviation obtained for each reading was not more than 0.0135.

**Figure 4.5:** Percentage of reduction of *S. aureus* biofilm mass by using Manuka Honey UMF 10+.



Standard deviation obtained for each reading was not more than 0.0176.

**Figure 4.6:** Percentage of reduction of *S. aureus* biofilm mass by using Manuka Honey UMF 15+.

## CHAPTER 5

### DISCUSSION

#### 5.1 Established Biofilm Reduction Assay

Based on the results, Gelam honey at concentration of 40% (w/v) possessed the highest reductive effect on established *Staphylococcus aureus* biofilm but the effect was decreased afterwards and reached the lowest reduction rate at concentration of 100% (w/v). The highest reduction rate of Gelam honey against established *S. aureus* biofilm was believed due to the four major antibacterial properties of honey including acidity, hydrogen peroxide activity, high osmotic effect and the presence of phytochemical components (Molan 1992; Cooper et al., 2002; Al-Waili et al., 2011).

Hydrogen peroxide is known to be the major factor that contributes to the antibacterial activity of honey. However, its amount is strictly controlled by glucose oxidase, an enzyme that produced by honeybees into honey (PRlog 2009). This is because glucose oxidase remains inactive in concentrated honey, in order to activate it and give rise to hydrogen peroxide, honey must be diluted into certain degree to activate the enzyme and produce hydrogen peroxide (Mwipatayi et al., 2004; PRlog 2009). According to the results,

Gelam honey at concentration of 40% (w/v) reduced the established biofilm mass the most, or in other words, gave the best effect of hydrogen peroxide activity. This statement is proven by Mwipatayi et al. (2004), which indicated that diluted honey can activate glucose oxidase and then produce hydrogen peroxide which contributes to the antibacterial property of honey. Besides, dilution of honey can reduce the acidity of honey, which in turn activates glucose oxidase that remained inactivated in full-strength honey. This is because there is a requirement for the pH of honey to fall within the range of 5.5 to 8.0 in order to activate the enzyme (Mwipatayi et al., 2004; PRlog 2009).

High osmotic effect of honey due to the high contents of sugar in honey also plays a role in reducing established biofilm (Molan 2001). Strong interactions between sugar molecules leave no or few water molecules for bacteria thus can inhibit bacterial biofilm in this way (Cooper et al., 1999; Molan 2001; Lusby et al., 2005). Bacteria need certain amount of water to survive. Olaitan et al. (2007) stated this water drawing effect of honey affects most of bacteria because they cannot live in an environment that water contents less than 17.1%. Percentage below this point is too low to support the growth of bacteria. However, this is depending on the bacterial species.

Besides hydrogen peroxide activity and high osmotic effect of honey, acidity of honey is assumed to be a role in reducing biofilm mass as well. Acidity of honey which is within the range of pH 3.2 to 4.5, creates an unfavorable environment for bacterial growth whereas their optimum pH for growth is about pH 7.2 to 7.4 (Molan 1992; Jeffrey and Echazarreta 1996; Bogdanov 1997; Malika et al., 2004; Sharp 2009). The acidity of honey is produced by the reaction of glucose with water and oxygen which give rise to gluconic acid (Olaitan et al., 2007). The presence of phytochemical components within honey known as non-peroxide antibacterial factors including flavonoids, a natural antioxidant, can act against bacteria through their direct antioxidant activity and effects on cell signaling pathways by altering bacterial growth factor at their receptor binding sites (Linus Pauling Institute 2013).

After Gelam honey reached its highest reduction rate at concentration of 40% (w/v), the reduction rate decreased afterwards and the lowest rate was at honey concentration of 100% (w/v). This can be explained by lacking of hydrogen peroxide activity in full-strength honey. In highly concentrated honey samples, glucose oxidase will remain inactive and thus less production of hydrogen peroxide (Mwipatayi et al., 2004; PRlog 2009). In other words, at honey concentrations of 60% (w/v), 80% (w/v) and 100% (w/v), the hydrogen peroxide activity was decreased due to the reduced activities of activated glucose oxidase. It can be said so that the presence of water in 40% (w/v)

concentration of honey was the optimum condition to activate glucose oxidase to generate high level of H<sub>2</sub>O<sub>2</sub>.

On the other hand, Gelam honey at concentration of 100% (w/v), the amount of hydrogen peroxide was the least, so the antibacterial activity was at the lowest point since hydrogen peroxide is the major antibacterial factor but it was not being produced at adequate amount. The presence of only acidity, high osmotic effect and phytochemical components wasn't enough to reduce the established *S. aureus* biofilm. As a result, it is believed that without high level of hydrogen peroxide, other antibacterial factors were not sufficient enough in giving maximum effect in reducing established biofilm. Besides, presence of sugar in honey can enhance the formation of biofilm as well. Sugar will be digested by honey and utilized as fuel for bacterial growth, thus the population of bacteria in biofilm increased (Blueplanet 2013).

For Manuka honey UMF 10 and UMF 15, the same trend of reduction rate was observed as Gelam honey. The highest reduction rate was at 40% (w/v) and it was reduced afterwards then hit the lowest reduction rate at honey concentration of 100% (w/v). As discussed in literature review, the main component that contributes to the antibacterial activity of Manuka honey is methylglyoxal (MGO) but not hydrogen peroxide, which derived from plants



that can inhibit the division cycle of bacterial cells (Christensen 2011b). This unique antibacterial activity is also known as non-peroxide activity (Alnaimat et al., 2012). However, the presence of hydrogen peroxide in Manuka honey can work together with MGO and yield synergistic effect which enhance the effectiveness of antibacterial activity (SummerGlow Apiaries Ltd 2003; The Honey Farm 2012). The presence of this extra unique factor, MGO can double up the antibacterial activity when working together with hydrogen peroxide. But in this study, it showed that as the honey concentration went higher, from concentration of 60% (w/v) to 100% (w/v) which means the honey was getting more concentrated, thus the generation of hydrogen peroxide was reduced so did the synergistic effect. So at high concentration of honey, the presence of MGO with other antibacterial components only was not as effective as when there was adequate amount of hydrogen peroxide to reduce biofilm formation.

On the other hand, Manuka honey UMF 15 has always expected to have higher antibacterial activity than Manuka honey UMF 10 due to their difference in MGO level because Manuka honey with greater UMF number has higher MGO level (The Honey Centre Ltd 2007; Hart-Davis 2009). In Manuka honey UMF 15, the MGO level is higher, thus it is more efficient in reducing biofilm mass and this has been proved in this study in which more established biofilm mass from different strains of *S. aureus* can be reduced by Manuka honey UMF 15 at higher rate compared to Manuka honey UMF 10.

## 5.2 Biofilm Prevention Assay

For the outcome of this assay, the trend of all tested honey (Malaysian Gelam honey, Manuka honey UMF 10 and UMF 15) in preventing biofilm formation is the same, which the reduction rate increased from concentration of 20% (w/v) onwards and reached the highest rate at concentration of 80% (w/v). The reduction rate of biofilm formation at honey concentration of 100% (w/v) was only slightly lower than honey concentrations of 80% (w/v).

For Gelam honey, the increasing reduction rate as the honey increased in concentration was believed due to the effect of honey on quorum-sensing system and also the antibacterial activity of honey including acidity, hydrogen peroxide activity, high osmotic effect and the presence of phytochemical components. According to Lee et al. (2011), honey was found able to reduce the quorum-sensing signaling that regulates the growth of bacterial biofilms. AI-2 importer genes in this signaling pathway had been repressed thus no longer controlling the biofilm formation and virulence of bacteria was suppressed as well.

At the honey concentration of 80% (w/v), low pH value of honey created an acidic environment which did not favor the formation of *S. aureus* biofilm, thus the reduction rate was higher as the concentration increased (Molan 1992;

Jeffrey et al., 1996; Bogdanov 1997; Malika et al., 2004; Sharp 2009). Furthermore, in this assay, bacterial suspension was mixed with the honey sample before establishment of biofilm, so the planktonic cells were more vulnerable or susceptible to acidic environment as compared to the embedded bacterial cells. The level of hydrogen peroxide at this concentration also displayed the optimum effect in reducing the formation of biofilm as the bacteria were rendered harmless by hydrogen peroxide through oxidation (Mwipatayi et al., 2004; PRlog 2009). High osmotic effect of concentrated honey can restrict the availability of free water molecules to the bacterial cells thus their growth was inhibited (Molan 1992; Cooper et al., 2002; Al-Waili et al., 2011). Besides, presence of phytochemical components with direct antioxidant activity was believed to help in reducing the number of bacteria as well (Linus Pauling Institute 2013).

At the honey concentration of 100% (w/v), the reduction rate was only slightly lower which may due to the reduced generation of hydrogen peroxide as compared to the more diluted honey. However, other antibacterial factors still displayed well in reducing formation of biofilm. The lowest reduction rate at 20% (w/v) concentration was believed mainly due to the neutralization of acidity by diluent and the intake of sugar by bacteria, used as fuel. At this concentration, the acidity of honey was neutralized by the medium and also by the bacterial suspension. Furthermore, presence of sugars in honey could be utilized for bacterial growth (Blueplanet 2013).

The similar trend of Gelam honey in reducing the formation of biofilm was also seen on Manuka honey UMF 10 and UMF 15, with the highest prevention rate at honey concentration of 80% (w/v) and the lowest at honey concentration of 20% (w/v). Highest prevention rate was suggested due to the synergistic antibacterial effect between MGO and hydrogen peroxide which further enhanced few fold higher than normal if one of the components working alone whereas the lowest prevention rate was due to the neutralization of the honey's acidity (SummerGlow Apiaries Ltd 2003; The Honey Farm 2012). The acidity was being diluted by media and thus less harsh environment for bacterial survival as compared to higher concentrations of honey.

Anyway, in this study Manuka honey UMF 10 was found to be more potent in reducing the formation of biofilm but not Manuka honey UMF 15. This could be due to the storage of honey along this study that resulted the reduction in antibacterial property. Moisture from the air could be absorbed into the honey and might affected the nature of honey (The George Mateljan Foundation 2013).

### **5.3 Future Studies**

Future studies can be done on the determination of antibiofilm or antibacterial properties of honey such as acidity, sugar contents, hydrogen peroxide, osmolarity and other phytochemical components in order to achieve a better understanding on the mechanisms of action and effects against biofilm or bacteria. As a result, we can determine the optimum condition in which honey works the best as a potential antimicrobial agent. The phytochemical components that present in honey can also be determined to obtain better understanding of what are those unidentified inhibines that contributed to the antibacterial activity of honey other than hydrogen peroxide. Besides, more antibiotics should be tested on bacteria especially in biofilm form to obtain a clear picture of what honey can do but antibiotics cannot do, or vice versa. The outcome is that we can suggest to apply honey in clinical treatment for those diseases and infections that cannot be cured by antibiotics.

## CHAPTER 6

### CONCLUSION

In conclusion, all the tested honey had showed the ability to inhibit/prevent *Staphylococcus aureus* biofilm. Gelam honey and Manuka honey UMF 15 were more effective in reducing established biofilm mass for most of the bacterial strains used in this study as compared to Manuka honey UMF 10. However, Manuka honey UMF 10 gave higher effect in preventing biofilm formation as compared to Manuka honey UMF 15 and Gelam honey. Further screening and studies can be suggested on the antibiofilm activity of honey to expand the usage of honey as an alternative clinical treatment in medical field.

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## APPENDIX

Table of measurement of zone of inhibition (mm) in Kirby-Bauer assay of *S. aureus*.

Standard Antibiotics	Concentration ( $\mu\text{g}$ )	Zone of Inhibition (mm)		
		R	I	S
Ampicillin	10	$\leq 28$	-	$\geq 29$
Chloramphenicol	30	$\leq 12$	13 - 17	$\geq 18$
Erythromycin	15	$\leq 13$	14 - 22	$\geq 23$
Methicillin	5	$\leq 9$	10 - 13	$\geq 14$
Imipenem	10	$\leq 13$	14 - 15	$\geq 16$
Penicillin G	10	$\leq 28$	-	$\geq 29$
Tetracycline	30	$\leq 14$	15 - 18	$\geq 19$
Vancomycin	30	-	-	$\geq 15$

\*R = Resistant; I = Intermediate; S = Susceptible