In vitro Screening of Honey against Enterococcus spp. Biofilm

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***Abstract*—Honey is known widely as a remedial agent for its wound healing, antibacterial, antioxidant and anti-inflammatory properties. Enterococci, on the contrary are associated with biofilm formation on medical devices which lead to devastating infections. This study was conducted to investigate the inhibitory effect of honey on established biofilm and prevention of biofilm formation. The biofilms of *Enterococcus* spp. (ATCC 19433, ATCC 29212, LMG 16192 and LMG 16216) were cultivated in microtitre plates with the treatment of different types of honey (Malaysian Gelam honey and Manuka honey [UMF 10 and 15]). The estimation of biofilm biomass extension was determined by measuring absorbance at 570 nm wavelength. It was found that Manuka honey UMF 15 was the most effective in reducing established biofilm biomass as compared to Malaysian Gelam honey. Nevertheless, Malaysian Gelam honey was found to be effective in preventing biofilm formation of *Enterococcus* spp. as compared to Manuka honey. In brief, Malaysian Gelam honey is effective to prevent enterococcal biofilm fomation whereas Manuka honey can be recommended as a potential therapeutic agent for biofilm related enterococcal infections.**

***Index Terms*—honey, gelam, manuka, *enterococcus* spp., biofilm**

I. Introduction

Honey is defined as a natural sweet substance which is produced from the floral nectar by honeybees. Honey is well-known for its antimicrobial activities and it has been reported to have antibacterial effect to about 60 species of bacteria and antifungal properties as well [1]. Besides, honey has many medicinal properties as reported which is applied as wound dressings and to clear infections by boosting the immune system, stimulating cell growth and possessing anti-inflammatory and antioxidant activities [2].

The most general source of Manuka honey is derived from *Leptospernum scoparium* and the honey is dark in colour and highly thixotropic. Manuka honey is popular with its antibacterial activities and this property is termed as Active Manuka. The outstanding antibacterial properties of Manuka honey are believed due to the synergistic effect between hydrogen peroxide and non-peroxide activity [3]. According to Reference [4], Manuka honey is regarded as the best natural antibiotic in the world. While the notable Gelam honey in Malaysia is originated from the floral source of *Melaleuca* spp. which is also commonly known as Gelam tree [5]. Gelam honey is noteworthy it's antibacterial, antioxidant, anti-inflammatory and wound healing activities [6].

Biofilm is defined as a bacterial community living within self-produced extracellular polysaccharide (EPS) matrix [4]. The EPS provide protection to the bacterial community from antibacterial and phagocytic onslaught. Biofilm is formed when adherence of bacteria to surfaces in aqueous environment starts to secrete slimy, glue-like substance that anchor them to all kinds of material namely medical implant materials, plastics, metals and animal or human tissue. Bacterial colonists that formed initially interact with each other by van der waals forces and when cell adhesion occurs, they anchor permanently on surfaces. Next, the biofilm will undergo maturation by providing more diverse adhesion sites and start to spread to the other surfaces [7].

Enterococci have been associated with biofilms on different medical devices such as artificial hip prostheses, prostetic heart valves, central venous catheters, intrauterine devices and urinary catheters [8]. The ability to form biofilm of Enterococci has become one of its virulence factors that cause nosocomial infections [9]. Among the enterococci, the ability to form biofilm for *Enterococcus faecalis* was greater than *Enterococcus faecium* [10]. Anyhow, the ability of forming biofilm would probably depend on the origin of strain and cultivation conditions [9].

Honey is known as an effective agent to prevent the formation of biofilm [4]. Reference [11] also reported that honey was able to reduce biofilm formation of enterohemorrhagic *Escherichia coli* 0157:H7. Furthermore, studies also showed the biofilms of methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA),

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vancomycin-resistant enterococci (VRE), *Streptococcus pyogenes* and *Streptococcus mutans* can be prevented and inhibited by Manuka honey [12]-[14]. Previous reports showed honey can be one of the potential biofilm inhibitors however up-to-date there is no scientific data regarding the effect of Malaysian Gelam honey on *Enterococcus* spp. biofilm so this study was conducted to compare its effectiveness to reduce the establish biofilm mass and prevent the biofilm formation with Manuka honey.

II. Materials and Methods

A. Test Materials

Manuka honey (UMF® 10 and 15) from New Zealand and Malaysian Gelam honey were used throughout this study. Honey samples were kept in the dark at room temperature. The test organism used in the study, *Enterococcus faecalis* (ATCC 19433, ATCC 29212 and LMG 16216) and *Enterococcus faecium* (LMG 16192) were provided by Faculty of Science, Universiti Tunku Abdul Rahman. Bacteria were cultured and maintained on Mueller-Hinton agar.

B. Effect on Established Biofilm

Establishment of 24-hour biofilm: 200 µl of 0.5 McFarland bacterial suspension was added into a flat-bottomed 96-well microtitre plate. It was incubated for 24 hours at 37℃ without shaking to allow biofilm establishment. Then, the planktonic cells (unattached cells) were removed carefully by pipetting without touching the wall and the bottom of the well.

Exposure of biofilm to honey: a range of concentrations of honey (20-100% w/v) was prepared and wrapped to prevent light reactions which may lead to inaccuracy [15]. Then, 200 µl of each honey concentration was added to the biofilm (attached cells) in the corresponding row of wells and incubated for 24 hours at 37℃.

Determination of biofilm mass: the honey sample was removed and washed gently with 200 µl of phosphate buffer solution (PBS). Then, the attached cells were fixed with 200 µl of glutaraldehyde (2.5%) for 10 minutes and washed again with 200 µl of PBS. It was stained with 200 µl of crystal violet (0.25%) for 10 minutes and washed for five times with PBS. The stained biofilm was dried overnight at room temperature. Then, the dye was solubilized with 200 µl of solvent (1:1 acetone: absolute ethanol) for 10 minutes. Finally, 20 µl of the resulting solution was added to 180 µl of solvent (1:1 acetone: absolute ethanol) contained in wells of the second corresponding microtitre plate. The absorbance was determined at 570 nm wavelength by using Tecan Infinite M 200 microtitre plate reader to determine the extent of biofilm biomass. The experiments were performed in triplicates.

C. Prevention of Biofilm Formation

Establishment of 24-hour biofilm with the treatment of honey: 5 ml of honey sample with different concentrations was added into 5 ml of 0.5 McFarland bacterial suspension. Then, 200 µl of the mixture was added into a flat-bottomed 96-well microtitre plate. It was then incubated for 24 hours at 37℃ without shaking to allow biofilm establishment.

Determination of biofilm mass: the mixture was removed carefully and the remaining attached cells were fixed with 200 µl of glutaraldehyde (2.5%) for 10 minutes and washed again with 200 µl of PBS. Then, it was stained with 200 µl of crystal violet (0.25%) for 10 minutes and washed for five times with PBS. The stained biofilm was dried overnight at room temperature and the dye was solubilized with 200 µl of solvent (1:1 acetone: absolute ethanol). Lastly, 20 µl of the resulting solution was added to 180 µl of solvent contained in wells of the second corresponding microtitre plate. The absorbance was determined at 570 nm wavelength by using Tecan Infinite M 200 microtitre plate reader to determine the extent of biofilm biomass. The experiments were performed in triplicates.

III. Results

A. Effect on Established Biofilm

The results for the inhibitory effect of honey on established biofilm are summarized into Table I to III accordingly. The outcome is shown in the reduction percentage of biofilm biomass after treatment of honey. For the effect of Gelam honey on established biofilm, the result is compiled into Table I. As shown in the table, the highest reduction of biofilm biomass for all the strains were 66.09% for ATCC 19433, 70.30% for ATCC 29212, 74.96% for LMG 16192 and 82.63% for LMG 16216 at 80% (w/v) of honey. Gelam honey at 80% (w/v) was found to be the most effective on LMG 16216 and least effective on ATCC 19433. It was also found that at 20% (w/v), Gelam honey was able to reduce 61.94% of the biofilm biomass of LMG 16216 as compared to other bacterial strains.

TABLE I. Effect of Gelam Honey on Established Biofilm

Honey Concentration (% w/v)	Reduction of Biofilm Biomass (%)			
	ATCC 19433	ATCC 29212	LMG 16192	LMG 16216
0	0	0	0	0
20	16.48	40.11	37.43	61.94
40	45.96	51.61	68.95	73.46
60	59.41	66.79	71.25	77.87
80	66.09	70.30	74.96	82.63
100	57.54	43.75	7.02	16.28

The result for the effect of Manuka honey UMF 10 on established biofilm is shown in Table II, which shows the highest reduction percentage of biofilm biomass at 100% (w/v) for ATCC 29212, LMG 16192 and LMG 16216 which were 83.26%, 62.02% and 86.54% respectively. For ATCC 19433, the highest reduction percentage of biofilm biomass was 74.69% at 80% (w/v) of honey. From the result, Manuka honey UMF 10 was found to be the most effective on LMG 16216 and least effective on LMG 16192.

As shown in Table III, the highest biofilm biomass reduction percentage of Manuka honey UMF 15 was at 100% (w/v) for ATCC 29212, LMG 16192 and LMG 16216 and at 80% (w/v) for ATCC 19433. For ATCC 29212, LMG 16192 and LMG 16216, the reduction of biofilm biomass were 90.28%, 62.11% and 89.52% whereas for ATCC 19433 was 77.18%. Manuka honey UMF 15 was found to be the most effective on LMG 16216 and the least on LMG 16192. It was found that at concentration of 20% (w/v), the percentage of reduction of LMG 16216 was very high as compared to other bacterial strains, which was 70.99%.

TABLE II. EFFECT OF MANUKA HONEY UMF 10 ON ESTABLISHED BIOFILM

Honey Concentration (% w/v)	Reduction of Biofilm Biomass (%)			
	ATCC 19433	ATCC 29212	LMG 16192	LMG 16216
0	0	0	0	0
20	49.95	27.16	34.21	46.37
40	57.00	70.97	49.97	77.72
60	69.84	74.42	55.30	79.34
80	74.69	77.63	57.63	82.76
100	63.35	83.26	62.02	86.54

TABLE III. EFFECT OF MANUKA HONEY UMF 15 ON ESTABLISHED BIOFILM

Honey Concentration (% w/v)	Reduction of Biofilm Biomass (%)			
	ATCC 19433	ATCC 29212	LMG 16192	LMG 16216
0	0	0	0	0
20	54.54	49.00	37.92	70.99
40	71.31	78.33	47.99	85.46
60	72.08	84.04	58.44	87.53
80	77.18	87.97	60.97	88.17
100	63.96	90.28	62.11	89.52

B. Prevention of Biofilm Formation

The results for the effect of honey in preventing biofilm formation are summated in Table IV to VI accordingly. From Table IV which shows the effect of Gelam honey in preventing the formation of biofilm, the highest reduction of biofilm biomass was at 100% (w/v) for all the bacterial strains which were 97.26% for ATCC 19433, 95.24% for ATCC 29212, 81.49% for LMG 16192 and 91.65% for LMG 16216. Gelam honey was found to be the most effective in preventing biofilm formation of ATCC 19433 and least effective on LMG 16192.

According to Table V which summarizes the effect of Manuka honey UMF 10 in preventing biofilm formation, the concentration of honey at 100% (w/v) was found to be the most effective in reducing biofilm biomass for all the bacterial strains. At 100% (w/v), the reduction percentage of biofilm biomass for ATCC 19433 was 97.38%, ATCC 29212 was 92.42%, LMG 16192 was 97.21% and LMG 16216 was 93.93%. Manuka honey UMF 10 was found to be very effective on ATCC 19433 and least effective on ATCC 29212. For LMG 16192, the honey concentration at 20% (w/v) was found to be very effective in preventing biofilm formation as it managed to reduce 91.63% of biofilm biomass which was very high as compared to other bacterial strains.

The result for the effect of Manuka Honey UMF 15 in preventing biofilm formation is summed up in Table 6. From the table, the highest percentage of biofilm biomass reduction was at 100% (w/v) for all the bacterial strains. The values were 97.47% for ATCC 19433, 90.98% for ATCC 29212, 78.64% for LMG 16192 and 76.49% for LMG 16216. It was found that Manuka honey UMF 15 managed to reduce the highest percentage of biofilm biomass for ATCC 19433 and the least for LMG 16216. From the result, it was observed that at 20% (w/v) concentration, the reduction percentage of biofilm biomass of ATCC 29212 was very high as compared to the rest, which was 81.94%. It was also observed that the reduction percentage of biofilm biomass was very low at 20% (w/v) against LMG 16216, which was only 12.14%.

TABLE IV. EFFECT OF GELAM HONEY IN PREVENTING BIOFILM FORMATION

Honey Concentration (% w/v)	Reduction of Biofilm Biomass (%)			
	ATCC 19433	ATCC 29212	LMG 16192	LMG 16216
0	0	0	0	0
20	28.55	41.44	13.83	34.38
40	63.31	59.73	16.59	89.43
60	89.05	79.75	57.26	90.24
80	96.17	93.61	79.91	91.30
100	97.96	95.24	81.49	91.65

TABLE V. EFFECT OF MANUKA HONEY UMF 10 IN PREVENTING BIOFILM FORMATION

Honey Concentration (% w/v)	Reduction of Biofilm Biomass (%)			
	ATCC 19433	ATCC 29212	LMG 16192	LMG 16216
0	0	0	0	0
20	10.51	58.75	91.63	65.83
40	74.63	80.07	93.98	79.02
60	95.43	89.23	95.76	90.45
80	97.21	91.52	97.00	93.30
100	97.38	92.42	97.21	93.93

TABLE VI. EFFECT OF MANUKA HONEY UMF 15 IN PREVENTING BIOFILM FORMATION

Honey Concentration (% w/v)	Reduction of Biofilm Biomass (%)			
	ATCC 19433	ATCC 29212	LMG 16192	LMG 16216
0	0	0	0	0
20	13.81	81.94	56.21	12.14
40	96.28	88.83	73.13	52.69
60	97.38	89.63	74.17	75.07
80	97.40	90.06	75.42	76.46
100	97.47	90.98	78.64	76.49

IV. DISCUSSION

A. Effect on Established Biofilm

Effect of Gelam honey on established biofilm: from Table I, the highest reduction percentage of biofilm biomass for all the strains was found to be at concentration of 80% (w/v). However, at the concentration of 100% (w/v), the percentage of reduction of biofilm biomass decreased for all the strains. This might due to few reasons in regards to the properties of honey. Most of the honey requires water to carry out reaction for antibacterial activities such as the production of hydrogen peroxide [16]. Hydrogen peroxide is produced in a reaction involving the enzyme, glucose oxidase which requires water for activation [4]. At concentration of 100% (w/v), the honey sample was too concentrated and the volume of tryptic soy broth (TSB) which was used as a diluent, was very low. Thus, the honey could not act efficiently to inhibit and reduce the biofilm biomass due to insufficient volume of water for the activation of glucose oxidase.

Although the volume of TSB, the diluent was high at honey concentration of 20% (w/v), the reduction of biofilm biomass observed was not high. This might due to the diluted honey did not have prolonged antibacterial activity. Diluted honey has the ability to prevent the accumulation of hydrogen peroxide, this can cause the reduction of hydrogen peroxide for the antibacterial activities [16]. At concentration of 80% (w/v), honey acted efficiently as compared to honey sample of 100% (w/v) due to sufficient volume of TSB. As reported, the interaction between water and sugar molecules in high concentration of honey decreases the water availability for bacterial survival and this interaction causes an osmotic effect against the bacterial biofilm which reduce biofilm biomass [16]. Even at concentrations below 80% (w/v), reduction of biofilm biomass still occurred due to the osmotic effect.

Acidity of honey is also believed to play a role in reducing biofilm biomass. As observed in the study, honey sample at concentrations of 20-100% (w/v) was able to reduce biofilm biomass in all bacterial strains. This is because of the natural acidity of honey which plays a role in antibacterial activities. Although the acidity of honey does not contribute much in antibacterial activity especially in diluted form [16], it did help in reducing the biofilm biomass of bacterial strains. Besides, there are also other antibacterial factors such as lysozyme and flavonoid pinocembrin which may also present in honey [16]. This statement can be applied in this study which supports the effectiveness of honey in reducing biofilm biomass. The presence of lysozyme is able to breakdown the established biofilm by digesting the bacteria [17]. The flavonoid pinocembrin which is believed present in honey is a very unique antibacterial factor [18]. Flavonoid pinocembrin is an antioxidant that is able to kill bacteria and thus might contribute to the reduction of biofilm biomass.

Effect of Manuka honey on established biofilm: as shown in Table II and III, the most effective reduction of biofilm biomass for Manuka honey (UMF 10 and UMF 15) was at concentration of 100% (w/v) for ATCC 29212, LMG 16192 and LMG 16216. However, the highest reduction percentage of biofilm biomass for ATCC 19433 was not at 100% (w/v) but at 80% (w/v). For Manuka honey, the highest reduction percentage of biofilm biomass occurred at concentration 100% (w/v) due to the presence of non-peroxide activity in Manuka honey [16]. The antibacterial activity was observed in concentrated honey sample as it did not require water for the activation of enzyme glucose oxidase to produce hydrogen peroxide. That is the possible reason why the reduction of biofilm biomass increased along with the increment of honey concentration.

Manuka honey UMF 15 was found to be more effective in reducing biofilm biomass as compared to Manuka honey UMF 10. This is most likely due to the UMF rating, the rating indicates the antibacterial efficacy or the percentage of phenol in water, the lowest number indicates the lowest efficacy in non-peroxide activity and vice versa [3]. Therefore, in this study Manuka honey UMF 15 showed better antibacterial efficacy than Manuka honey UMF 10 in reducing biofilm biomass. Other than that, methylglyoxal (MGO) which is normally present in Manuka honey, was suggested in playing a role in reducing biofilm biomass. MGO is an active component of non-peroxide antibacterial activity in Manuka honey and it is also a very reactive precursor to produce advanced glycation end products (AGEs) which are toxic and able to kill bacteria, therefore it might involved in assisting to reduce biofilm biomass [19]. Besides MGO targets protein and DNA synthesis which may cause the death of bacteria due to insufficient capacity for DNA repair and detoxification enzymes [20]. The death of bacteria causes the reduction of biofilm biomass. Thus, it can be explained the non-peroxide antibacterial factor of Manuka honey. Apart from that, other properties such as acidity, osmotic effect and hydrogen peroxide activity also believed to contribute to the reduction of biofilm biomass.

B. Prevention of Biofilm Formation

Effect of Gelam honey in preventing biofilm formation: from Table 4, the highest reduction of biofilm biomass was obtained at 100% (w/v) for all the bacterial strains. The results also showed that the reduction of biofilm biomass increases as the concentration of honey increases. The effectiveness of reduction of biofilm biomass was also believed due to antibacterial properties of honey as mentioned earlier. Other than that, it was believed that the prevention of biofilm formation due to the inhibition of bacterial quorum sensing [11]. Reference [11] reported honey has the ability to repress quorum sensing signaling. He mentioned chestnut honey was able to inhibit quorum sensing *N*-acyl-*L*-homoserine lactones (AHLs) and biofilm formation in *Erwinia carotovora*, *Yersinia enterocolitica* and *Aeromonas hydrophilia.* So, it is possible to believe that Gelam honey has the ability to inhibit quorum sensing which further prevent the formation of biofilm.

Phenolic compounds which present in Gelam honey is believed to have antibacterial effects [21]. The phenolic compounds of Gelam honey manifest free radical

scavenging activities which are believed to reduce biofilm biomass as a result of oxidative damage produced. Up to date, phenolic compounds such as gallic acid and ferulic acid are found only in Gelam honey [11]. Therefore, Gelam honey has very unique antibacterial properties that do not present in other types of honey. Gallic acid is a very strong free radical scavenger which is able to decrease or chelate the divalent ions. It acts as catalyst towards lipid peroxidation process [11]. Ferulic acid, is a monohydroxylated phenolic compound which plays a role as peroxynitrite scavengers by nitration. These processes are believed in reducing biofilm biomass formation by bacteria [22].

Effect of Manuka honey in preventing biofilm formation: low concentration of honey is also able to reduce biofilm formation by inhibiting the expression of biofilm-related curli genes, quorum sensing genes and virulence genes in bacteria without inhibiting the cell growth [11]. For example, the Manuka honey UMF 10 at concentration of 20% (w/v) was found to be very effective in preventing biofilm formation of LMG 16192 as it managed to reduce 91.63% of biofilm biomass. Therefore, from the factors mentioned in previous statement, it explained the effectiveness of Manuka honey UMF 10 against LMG 16192.

As shown in Table 5 and 6, the highest reduction of biofilm biomass for Manuka honey (UMF 10 and UMF 15) was at concentration of 100% (w/v). Honey at high concentrations is able to inhibit biofilm formation and adhesion of bacteria via its antibacterial properties [11]. Apart from the antibacterial properties mentioned earlier, it was also believed that Manuka honey was able to prevent biofilm formation due to bee defensin-1 which is an antibacterial peptide was found against bacterial spoilage and involved in neutralization reaction which reduced the bacterial activity [23]. Presence of this peptide indirectly prevents the biofilm formation of bacteria.

C. The Overall Outcome of Biofilm Assays

From the results, all tested honey was found to be able to reduce biofilm biomass of all tested strains from 20% (w/v) concentration onwards. The inhibitory effect of honey on established biofilm and in preventing biofilm formation are believed due to the properties of honey itself namely acidity, osmotic effect, hydrogen peroxide activity as well as the additional antibacterial factors such as non-peroxide activities and activities of phenolic compounds [16].

From the study, the most effective honey in inhibiting and preventing biofilm formation of all tested strains of was determined and it was summarized in Table VII. It was found that Manuka honey UMF 15 was the most effective in reducing established biofilm biomass of ATCC 19433, ATCC 29212 and LMG 16216. Gelam honey on the other hand, was found to be effective in reducing established biofilm biomass of LMG 16192. For the prevention of biofilm formation, Gelam honey was found to be the most effective against ATCC 19433 and ATCC 29212 whereas Manuka honey UMF 10 was found to be effective against LMG 16192 and LMG 16216.

TABLE VII. THE MOST EFFECTIVE HONEY FOR EACH BACTERIAL STRAIN

***Enterococcus* spp.**	**Types of honey**
Effect on established biofilm	
ATCC 19433	Manuka honey UMF 15
ATCC 29212	Manuka honey UMF 15
LMG 16192	Gelam honey
LMG 16216	Manuka honey UMF 15
Effect in preventing biofilm formation	
ATCC 19433	Gelam honey
ATCC 29212	Gelam honey
LMG 16192	Manuka honey UMF 10
LMG 16216	Manuka honey UMF 10

D. Conclusions

In summary, all tested honey was found to be able to reduce biofilm biomass of all tested bacterial strains even at the lowest concentration. Manuka honey UMF 15 was found to be effective in reducing established biofilm biomass for most strains than Malaysian Gelam honey while both Malaysian Gelam honey and Manuka honey UMF 10 were effective in preventing biofilm formation.

The mechanism of the antibacterial effect of honey is not fully understood [14]. Thus, it will be beneficial if further research is carried out to detail out the specific mechanisms of honey in inhibiting and preventing biofilm formation. By understanding the true mechanism of antibacterial effect of honey, it can therefore be applied for the *in vivo* studies. These can help to fight against nosocomial infections due to bacterial biofilms. The use of honey as therapeutic agent could help to save cost and reduce chemical drug toxicities or side effects.

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Kit-Yin Lim, Ju-Yee Chong and **Ka-Lok Low** are the UTAR biomedical science undergraduate students. They were involved in the projects of bioactivities of honey against biofilm of various bacterial strains.

