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Effects of Selected Malaysian Kelulut Honey on Biofilm Formation and the Gene Expression Profile of Staphylococcus Aureus, Pseudomonas Aeruginosa and Escherichia Coli

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ABSTRACT

Honey is now being renowned as an alternative treatment due to its broad-spectrum antibacterial activity and the inability of bacteria to develop resistance after exposure to it. Honey has been shown to be bactericidal against *E. coli, S. aureus* and *P. aeruginosa* as it destabilizes the bacteria's cell wall. This study was designed to evaluate the effect of a Malaysian *Kelulut* honey on established biofilm, prevention biofilm and on level of gene expression in *S. aureus*, *P. aeruginosa* and *E. coli*. Established biofilm and prevention biofilm assays were conducted on three strains of *S. aureus*, *P. aeruginosa* and *E. coli* using 96-well plates with five different concentrations of honey namely 5%, 10%, 20%, 30%, and 40% (w/v) and the level of gene expression assay was conducted using RT-qPCR. *Kelulut* honey was able to reduce the biofilm mass formation up to 39%, 41% and 37% in *S. auerus*, *P. aeruginosa* and *E. coli* respectively. The lowest concentration of *Kelulut* honey found to prevent biofilm formation in *S. auerus*, *P. aeruginosa* and *E. coli* was 30% (w/v). Approximately fourfold reduction in the total number of viable bacterial cells of *S. auerus*, *P. aeruginosa* and *E. coli* was observed following treatment with 40% (w/v) Kelulut honey. The RT-qPCR showed that twelve genes including *argF, purC, adh, fabG, fliA, fliC oprB, oprH, yjfO (bsmA), ycfR (BhsA), lsrA* and *tnaA* were downregulated, whilst, eight genes including *scdA, pyk, menB, oprC, lasR, algU, rpoS* and *evgA* were upregulated after exposure to *Kelulut* honey. This study showed the efficacy of Kelulut honey against biofilm, and that different concentrations of honey possess different degrees of potential effect on established biofilm. Also, a decreased expression of virulence genes in these bacteria will impact their pathogenicity.

Keywords: *S. aureus*, *P. aeruginosa, E. coli*, Gene expression, Malaysian *Kelulut* honey, Biofilms.

1. INTRODUCTION

All over the world, deaths from infectious diseases amounts to more than 17 million each year and most of these deaths have been linked to bacterial infections [1, 2]. Bacterial infections are generally treated with antibiotics but considering that microorganisms are known to develop numerous mechanisms of resistance [3, 4], antibiotics are losing their effectiveness rapidly [5]. Also, it is more difficult to treat the diseases caused by antibiotic resistant bacteria as opposed to treating the diseases caused by non-resistant ones [6]. Antibiotic resistant bacteria have been spreading worldwide and this has led to the increase in medical costs, hospital stays and death cases [7-9]. For this reason, recent studies are looking into the use of alternative antimicrobial

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strategies including the utilization of plants and honey products in the treatment of bacterial infections [10-13].

Among the oldest and highly reputable traditional medicines is honey, and the long history has seen the application of this substance in treating a number of human diseases [14-16]. The emergence of "apitherapy," which comprises the application of honey and other products associated with bees in treating ailments [17, 18], have preserved the positive reputation of honey. This reputation has continued up to the present day, leading to the emergence of a relatively new branch of alternative medicine, called "apitherapy", which focuses on medical applications [19, 20] of honey and other bee products [21-24]. Nowadays, different types of honey have been used in many countries as an alternative to pharmaceutical products for treating contaminated, infected, and burn wounds [20, 25-27]. This is attributed to the effectiveness of these honeys in inhibiting or killing a broad spectrum of bacteria [28- 30].

Honey comes in various kinds and in many countries, honey has been used as an alternative to pharmaceutical products particularly in the treatment of contamination, infection, and burn wounds [31, 32]. The use of honey has been factored by its ability in inhibiting or destroying a broad spectrum of bacteria [28, 33]. Accordingly, several studies have been carried out in understanding the impacts of honey on bacterial structures [34-40], but most have been focusing on Manuka honey only and mainly on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It is hence necessary to examine other types of honey such as *Kelulut* honey and other microorganisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus Escherichia coli.*

For *Kelulut* honey, its bactericidal effect on *S. aureus*, *P. aeruginosa* and *E. coli* has been investigated *in vitro*. From the electron microscopy, it appears that *Kelulut honey* deformation the cells and the cells appeared curved, distorted and the cell density was markedly decreased [40-42]. The antimicrobial activity of honey may be attributed to several factors, including high osmolarity, acidity, in addition to the presence of hydrogen peroxide (H_2O_2) and non-peroxide components, such as methylglyoxal [42-47]. In addition to exerting direct antimicrobial effects, some honey varieties have been implicated in the differential expression of a number of genes essential for bacterial survival and virulence, including those involved in stress tolerance [48], virulence factor production [49], as well as multicellular behaviors, such as biofilm formation [50], and quorum sensing [51].

Several studies have addressed different aspects of *Kelulut honey* varieties, including their physicochemical properties , their chemical composition [52-55], their antibacterial and antibiofilm activities [40, 56], and their therapeutic usefulness [42, 57, 58]. However, it is not yet known whether these anti-biofilm activities, as well as any possible anti-quorum sensing and anti-virulence activities possessed by this honey could be attributed to alteration of bacterial gene expression. [59-63]. Therefore, The effects of Kelulut honey on gene expression in *S. aureus, P. aeruginosa* and *E. coli* have not been empirically studied in the past, and for this reason, the examined genes were chosen following the published expression profiling studies, whereby the cells of *E. coli, S. aureus* and *P. aeruginosa* have been treated with honeys different from those tested in the present study. As such, these genes were chosen based on the genes involved in the generation of biofilm, quorum sensing, motility and stress survival in these test organisms. Accordingly, six genes of *E. coli* (*yjfO (bsmA), ycfR (BhsA), tnaA, lsrA, evgA*, and *rpoS*), seven genes of *S. aureus* (*argF, purC*, *adh, fabG*, *scdA*, *pyk* and *menB*), seven genes of *P.aeruginosa* (*fliA*, *fliC, oprB*, *oprH*, *oprC, lasR* and *algU*) were chosen for the expression analysis. As shown by past studies, the exposure to honey downregulated and upregulated the differential gene expression involved in biofilm construction, quorum sensing, motility and stress endurance in these test organism [59-66].

MATERIALS AND METHODS

Bacterial strains and culture conditions

Three strains of *S.auerus* (ATCC 25923), *P.aeruginosa* (ATCC 27853) and *E.coli* (ATCC 25922) were used throughout the study. Cultures of bacteria were supplied by Microbiology Laboratory, University of Sultan Zainal Abidin (UniSZA). The supplied bacteria were reconstituted into sterile Mueller Hinton Broth (MHB) (Oxid, UK) and incubated at 37°C. After 24 hours, they were sub-cultured on Mueller Hinton Agar, (MHA) (Oxid, UK) and incubated again at 37°C for another 24 hours before being processed for long storage at -80° in eppendorf tube containing MHB, and 15% glycerol. Working bacterial culture was prepared by inoculating a loopful of primary culture from -20° storage into Erlenmeyer flask containing 15 ml of MHB broth [60-61]. The inoculum was incubated at 37°C for 24 hours. After incubation time, the inoculum was incubated at 37°C for 24 hours. Then, the suspension was adjusted to be equal to 0.5 McFarland standard [62, 67].

Honey samples

Kelulut honey samples were purchased from a bee farm located in Kelantan, a state in Malaysia. All honey samples were stored in airtight amber glass bottles and stored at room temperature until further analysis [52].

Established biofilm reduction assay

Bacterial cultures were adjusted to 0.5 McFarland standard as previously described. Two hundred microliter of the culture was transferred into wells of 96-well plates and the plate was incubated for 48 hours at 37°C without shaking. Wells containing only bacterial culture served as positive control and wells containing honey only served as corresponding negative control. After 48 hours of incubation, planktonic cells were removed and then 200 µl of different concentrations of 40%, 30%, 20%, 10% and 5% (w/v) of honey were added to the wells and then the wells were incubated overnight. After incubation, the plate was washed with PBS. The plate was fixed with 2 ml µl of 2.5% glutaraldehyde for 10 minutes. Then, the plate was washed with PBS. The attached cells or biofilm was then stained with 200 µl of 0.1% crystal violet for 15 minutes and washed two times with PBS. Absorbance was determined at 540 nm wavelength using microtitre plate reader (Tecan Infinite 200 PRO, Austria). This assay was repeated in triplicate [68-71]. The reduction of biofilm mass was calculated following the formula shown below:

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

Biofilm Prevention assay

To determine the concentration of *Kelulut* honey required to prevent a biofilm of *S. aureus, P. aeruginosa* and *E. coli* and forming *in vitro,* a range of concentrations; 40%, 30%, 20%, 10% and 5% (w/v) of *Kelulut* honey were freshly prepared in Muller Hinton Broth (MHB; Oxoid, UK) from a stock solution of 50% (w/v) honey. Approximately 200µl of diluted honey was dispensed into wells of 96-well plates and inoculated with 200µl of a diluted overnight culture of the test organism (population density of 2.5×10^8 colony forming units (cfu/ml). Wells without inoculum served as a corresponding negative control and wells without added honey used as a positive control. The plates were incubated for 48 hours at 37°C and the extent of biofilm formed was evaluated by determining optical density (at 540nm) using microplate reader (Tecan Infinite 200 PRO, Austria) [70, 72]. The experiment was conducted at least three times.

Determination of biofilm viability (by total cell count)

To determine the effect of *Kelulut* honey on biofilm viability, the liquid phase from 48 hours biofilm formed in wells was discarded and contents were washed with 300µl sterile maximum recovery diluent (MRD, Oxoid, UK) to remove planktonic cells. A further 200µl of MRD was added to the washed biofilm and a sterile pipette tip was then used to scrape the bottom of the well to release adherent biofilm. The total viable count of the resulting suspension was determined using the surface drop count [73]. Diluted suspensions were plated onto nutrient agar (NA; Oxoid, UK). The plates

were and incubated for overnight at 37°C and cfu per well was calculated [52, 68, 74].

Extraction of RNA from *S. aureus, P. aeruginosa* and *E. coli* for RT-qPCR

Initially, the inoculum was adjusted as described previously, and 100 µL of inoculum was pipetted into microtiter plate with 100 µL of 20 % (w/v) concentration of honey. Meanwhile, wells with inoculum only without honey was used as a positive control. Then, the plate was incubated for 48 hours at 37ºC. After that, 1 ml of samples, treated and untreated, were re-suspended in PBS and vortexed for 3 minutes and centrifugated at 3,500g for 5 minutes. The supernatant was discarded and the pellet was washed again with PBS. Total RNA from treated and untreated sample was extracted using kit SV Total RNA Isolation System (Promega, UK). Total RNA concentrations was examined using Implen NanoPhotometer® NP80. Total RNA was converted to cDNA following the manufacturer's instructions kit (Promega, UK). For each reaction, qPCR mastermix was prepared by following the manufacturer's instructions (Promega, UK) and PCR primers were used as shown in Tables 1, 2 and 3. The following PCR protocol was used: denaturation at 95ºC for 2 minutes in one cycle, amplification at 95ºC for 15 seconds in 40 cycles and a final elongation annealing at 60ºC for 1 min in 40 cycles. Densitometry was performed using the Applied Biosystems Step One Software v2.3. To determine and calculate the level of gene expression, a modified $2^{-\Delta\Delta}$ Ct method was used [60-62, 70]. The experiment was performed in triplicate.

Statistical analysis

Data were expressed as mean ± standard deviation. Independent student's t-test from SPSS version 20 was used to compare between honey-treated and control groups. The significance level was set at $P<0.05$.

RESULTS

Established biofilm reduction assay

In general, honey samples were able to reduce the mass of established biofilm on *S.auerus*, *P.aeruginosa* and *E.coli*. The data show that different concentrations of honey sample produced different degrees of inhibitory effects on different strains of bacteria. Figure 1 shows that 40% (w/v) concentration of *Kelulut* honey was the most effective to reduce the biofilm mass of *S. auerus* (39%), *P. aeruginosa* (41%) and *E. coli* (37%).

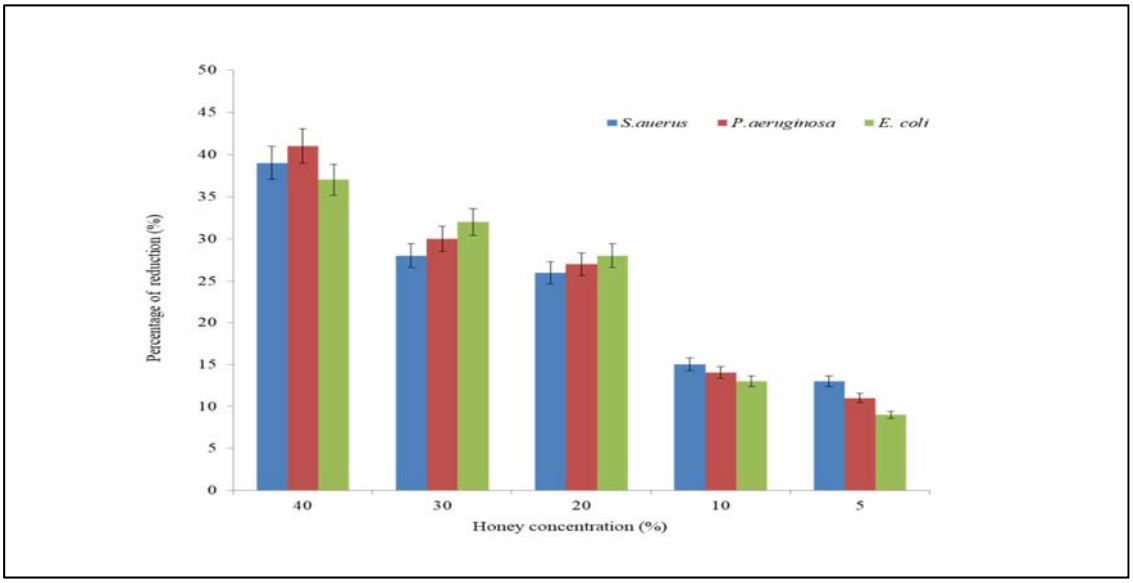

Figure 1: Percentage of reduction of *S.auerus*, *P.aeruginosa* and *E.coli* biofilm mass after exposure to *Kelulut* honey

Table 1: Gene specific primers of *S.auerus* used for RT-qPCR analysis [62]

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
argF	143	Forward	CCAAGCAGAATTCGAAGGA
		Reverse	GGATGCGCACCTAAATCAAT
purC	117	Forward	GAAGCGCATTTTCTCAACAA
		Reverse	CCCTTACCTGCCATTGTGTC
adh	124	Forward	GTTGCCGTTGGTTTACCTGT
		Reverse	TTCAGCAGCAAATTCAAACG
scdA	132	Forward	CGAAAGCAGCGGATATTTTT
		Reverse	GCGAACCTGGTGTATTCGTT
pykA	126	Forward	TGCAGCAAGTTTCGTACGTC
		Reverse	GGGATTTCAACACCCATGTC
menB	109	Forward	CTGGGGAAGGTGATTTAGCA
		Reverse	ACCGCCACCTACAGCATAAC
fabG	122	Forward	CCGGGACAAGCAAACTATGT
		Reverse	CCAAAACGTGCTAACGGAAT
*yqiL**	125	Forward	GACGTGCCAGCCTATGATTT
		Reverse	ATTCGTGCTGGATTTTGTCC

* *yqiL* was used as a reference gene for *S.aerus*

Table 2: Gene specific primers of *P.aeruginosa* used for RT-qPCR analysis [61], [66]

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
oprB	140	Forward	TGACGACGACAAGACAGGAC
		Reverse	GGTCGTTGGAAAGGTTCTTG
oprC	105	Forward	GCCTGAACATCCTCACCAAC
		Reverse	CGGTGAGCTTGTCGTAGGTT
oprH	102	Forward	CTCGACAAGGTGATCGACAA
		Reverse	GGTGTCGGAGATGTTCTCGT
fliA	192	Forward	CTCCAATTGAGCCTCGAAGA
		Reverse	TTCGTTGTGACTGAGGCTGG
fliC	121	Forward	GCTTCGACAACACCATCAAC
		Reverse	AGCACCTGGTTCTTGGTCAG
lasR	129	Forward	CGGTTTTCTTGAGCTGGAAC TCGTAGTCCTGGCTGTCCTT
		Reverse	
algU	113	Forward	GCGAGTTCGAAGGTTTGAGT
		Reverse	CTGCAGAGCTTTGTCGATTG
*rpoD**	146	Forward	GCGACGGTATTCGAACTTGT
		Reverse	CGAAGAAGGAAATGGTCGAG

**rpoD* was used as a reference gene for *P.aeruginosa*

Table 3: Gene specific primers of *E.coli* used for RT-qPCR analysis [60]

Gene name	Amplicon Size (bp)	Direction	Primer sequence (5' → 3')
yjfO (bsmA)	76	Forward Reverse	CGCCAGTAACGGACCATC GTGCTTACGCTACCTATTCG
ycfR (BhsA)	81	Forward Reverse	CGAAGTTCAGTCAACGCCAGAAG TCCAGCGATCCCAGATTTGTCC
tnaA	174	Forward Reverse	CTGGATAGCGAAGATGTG CGGAATGGTGTATTGATAAC
evgA	155	Forward Reverse	TAGCGGAGACGATAATAATAATTC GTTGACTGAAGGCGGAAG
rpoS	199	Forward Reverse	CTCAACATACGCAACCTG GTCATCAACTGGCTTATCC
lsrA	178	Forward Reverse	TACTCATAACCTTCGTGGATTCTG TACTTGCGGCGAGGCTTC
*ftsA**	152	Forward Reverse	GAAGAAGTGACGCAAGAAGATG ACGCCCGAAAGTCCTACC

* *ftsA* was used as a reference gene for *E.coli*

Biofim prevention after treatment with *Kelulut* honey

The lowest concentration of *Kelulut* honey that prevented *S.auerus*, *P.aeruginosa* and *E.coli* forming a biofilm *in vitro* was found to be 30% (w/v). Inhibitory effects are normally expressed as the MIC; here this was determined by assessing optical density. All methods gave similar endpoints, although slighter wider variation was seen by different species (Figure 2).

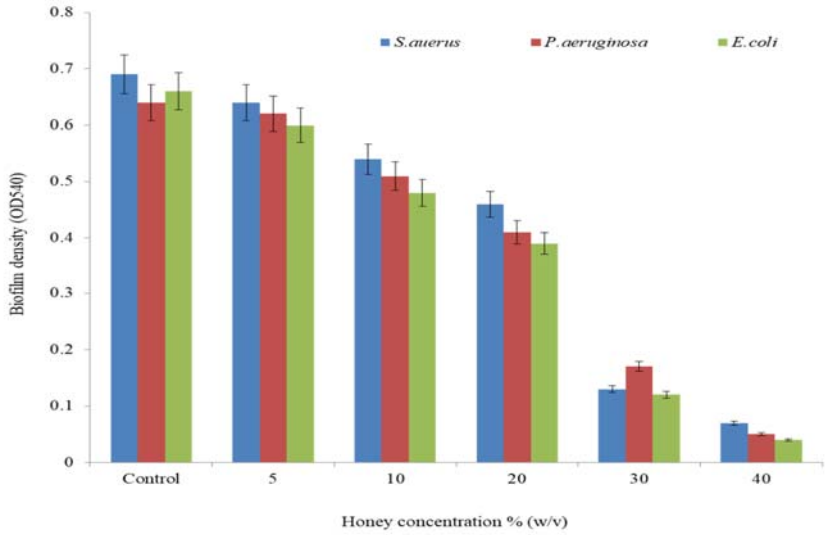

Figure 2: The effect of *Kelulut* honey on biofilm formation. Varying concentrations of honey were incubated with *S.auerus*, *P.aeruginosa* and *E.coli* to determine the lowest concentration required to prevent biofilm formation. The extent of biofilm was assayed by optical density

Determination of biofilm viability (by total cell count)

Treatment of *S.auerus*, *P.aeruginosa* and *E.coli* biofilms established over 48 hours with *Kelulut* honey resulted in up to 4 fold log reductions in TVCs. From biofilm formation in the absence of Kelulut honey, 5.0 x 10^8 c.f.u.m 4.9 x 10^8 and 4.8 x 10^8 of *S.auerus*, *P.aeruginosa* and *E.coli* respectively were recoverable from biofilms. There was an approximately 2.61 log, 2.8 log and 2.78 log reduction in viable cells of *S.auerus*, *P.aeruginosa* and *E.coli* respectively following exposure to 20% (w/v) *Kelulut* honey and 3.1 log, 3.5 log and 3.2 log reduction in viable cells of *S.auerus*, *P.aeruginosa* and *E.coli* respectively after treated with 30% (w/v) *Kelulut* honey compared to untreated biofilm. The reduction in viable cells was even more marked using 40% (w/v) *Kelulut* honey, resulting in a 4.3 log, 4.2 log and 4.4 log reduction of *S.auerus*, *P.aeruginosa* and *E.coli* respectively compared to untreated biofilm (Figure 3).

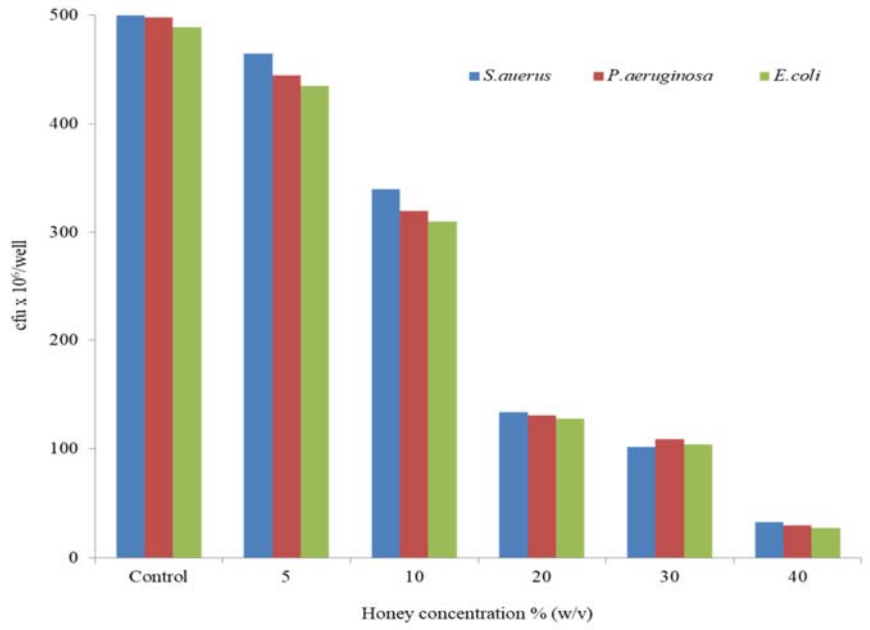

Figure 3: The effect of *Kelulut* honey on biofilm formation of *S.auerus*, *P.aeruginosa* and *E.coli* determined by total cell viability.

Gene expression of *S. aureus* after exposure to *Kelulut* honey

As shown in Figure 4 and Table 4, the expression of seven corresponding genes of *S. aureus* did show the level of different expression (Figure 2). Four genes (*argF*, *purC*, *adh*, and *fabG*) had significantly (P<0.05) decreased levels of expression 1.5-fold, 2.2-fold, 3-fold and 1-fold (P<0.05) respectively, and three genes (*scdA*, *pyk* and *menB*) showed increased expression 5-fold, 7-fold and 3-fold (P<0.05) respectively after honey treatment.

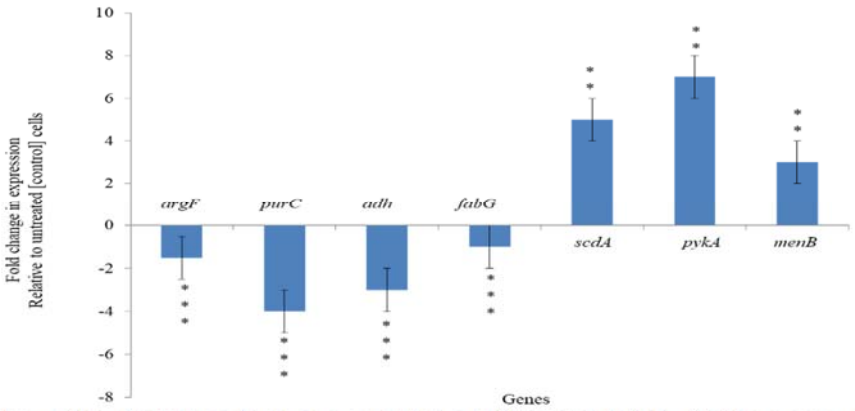

Figure 4: shows the alterations in gene expression profiles associated with the exposure of *S. aureus* to *Kelulut* honey as determined by qPCR. Experiments were run with three technical replicates of each. Mean values of fold changes (± SD) are shown in relation to untreated (control) *S. aureus* cells. Asterisks **$P \leq 0.01$; ***$P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated samples and control.

Table 4: Genes down and up regulated in *S. aureus* detected by RT-qPCR after exposure to *Kelulut* honey

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
argF	0.59	0.66	-1.5	0.04 *	1.2
purC	2.09	0.23	-4	0.04 *	1.3
adh	1.50	0.35	-3	0.04 *	1.1
fabG	0.01	0.99	-1	0.04 *	1.3
scdA	-2.32	5.00	5	0.04 *	1.0
pykA	-2.81	7.00	7	0.04 *	1.2
menB	-1.58	3.00	3	0.04 *	0.8

Gene expression of *P.aeruginosa* after treatment with *Kelulut* honey

Based on Figure 5 and Table 5, seven genes of *P.aeruginosa* showed different degrees of gene expression including two genes (*fliA* and *fliC*) of flagellum-associated genes that were significantly ($P<0.05$) decreased 3-fold and 4.5-fold respectively. Three genes (*oprB*, *oprH* and *oprC)* associated with the outer membrane were decreased 2-fold and 6-fold respectively except *oprC* which was increased 4-fold, and two genes (*lasR* and *algU*) associated with biofilm formation were increased 2.3-fold and 4-fold respectively in the level of gene expression.

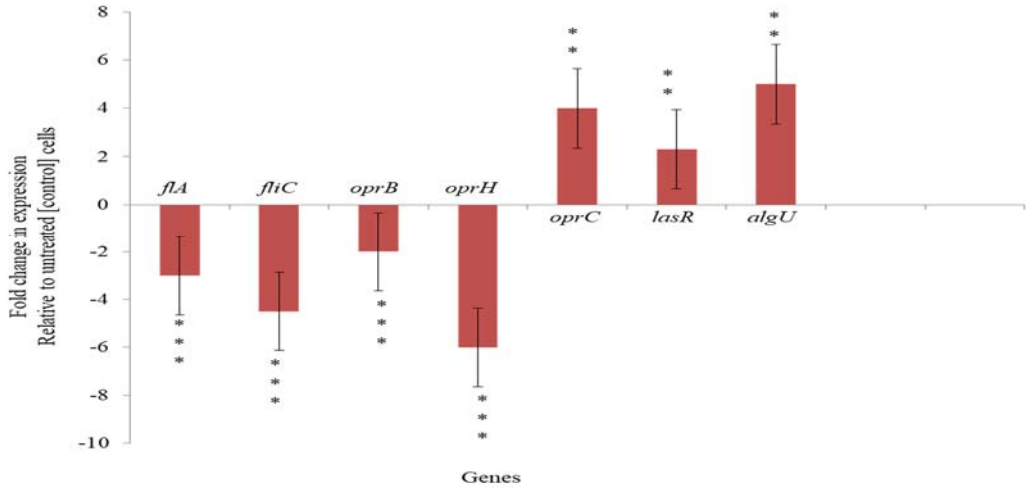

Figure 5: shows the alterations in gene expression profiles associated with the exposure of *P. aeruginosa* to *Kelulut* honey as determined by qPCR. Experiments were run with three technical replicates of each. Mean values of fold changes (± SD) are shown in relation to untreated (control) *P. aeruginosa* cells. Asterisks **$P \leq 0.01$; ***$P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated samples and control.

Table 5 : Genes down and up regulated in *P. aeruginosa* detected by RT-qPCR after being treated with *Kelulut* honey

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
fliA	1.58	0.33	-3	0.04 *	1.8
fliC	2.17	0.22	-4.5	0.04 *	2.1
oprB	1.00	0.50	-2	0.04 *	1.6
oprH	2.58	0.17	-6	0.04 *	2.3
oprC	-2.00	4.00	4	0.04 *	1.1
lasR	-1.02	2.03	2.3	0.04 *	1.0
algU	-2.32	5.00	5	0.04	1.0

Gene expression of *E.coli* after exposure to *Kelulut* honey

According to Figure 6 and Table 6, six genes (*yjfO (bsmA), ycfR (BhsA), tnaA* and *lsr, rpoS* and *evgA*) involved in biofilm formation, quorum sensing and stress survival in *E.coli* were significantly ($P<0.05$) decreased in the level of gene expression, 5-fold, 3-fold, 8-fold and 2-fold respectively except *rpoS* and *evgA* genes which were increased 7-fold and 4-fold respectively after treatment with honey.

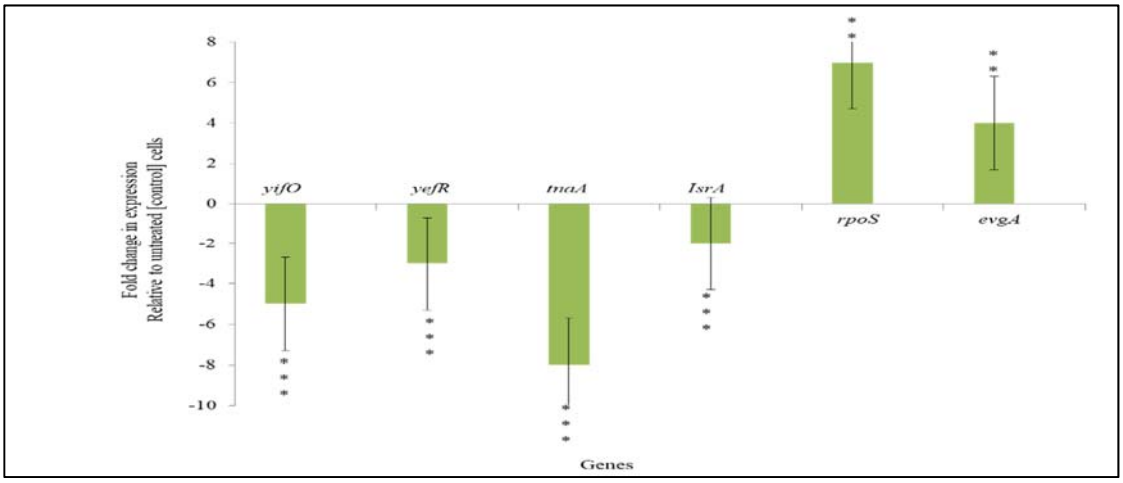

Figure 6: shows the alterations in gene expression profiles associated with the exposure of E. coli **to *Kelulut* honey as determined by qPCR. Experiments were run with three technical replicates of each. Mean values of fold changes (± SD) are shown in relation to untreated (control)** E. coli **cells. Asterisks **$P \leq 0.01$; ***$P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated samples and control.**

Table 6 : Genes down and up regulated in *E. coli* detected by RT-qPCR after treatment with *Kelulut* honey

Gene name	Average ΔΔCt	Expression Fold Change (2^-ΔΔCt)	Expression Fold Change	*P*-value	SD
yjfO (bsmA)	2.32	0.20	-5	0.04 *	2.1
ycfR (BhsA)	1.59	0.33	-3	0.04 *	1.6
tnaA	3.00	0.12	-8	0.04 *	2.3
lsrA	1.00	0.50	-2	0.04 *	1.4
rpoS	-2.81	7.00	7	0.04 *	1.2
evgA	-2.00	4.00	4	0.04 *	0.9

DISCUSSION

In this laboratory study, Kelulut honey was found to prevent the formation of *S.auerus, P.aeruginosa and E.coli* biofilms, as well as inhibiting and disrupting established biofilm. Although determining the density of the bacterial growth in each well yielded information rapidly, the entire contents of the well contributed to turbidity, rather than only the biofilm that was adherent on the walls of each well. Whereas this was appropriate in experiments to estimate the concentration of honey needed to prevent and inhibit a biofilm forming. The results show that different bacterial species has different susceptibility to honey, but the differences in susceptibilities may be found between different strains of the same species. As such, the results obtained for each strain cannot be generalized to the whole bacterial species. Equally, the results demonstrate the ability of honey in preventing and inhibiting the growth of biofilm bacteria, even when the concentrations used are very low. Relevantly in Okhiria et al. (2009), Manuka honey was found to considerably decrease the *P.* aeruginosa biofilm at 40% (w/v) concentration of honey, while 30% (w/v) concentration of honey showed no significant inhibition [75]. In fact, the effectiveness of Manuka honey

against *S. aureus* and *P. aeruginosa* biofilms has been reported [76]. Manuka honey was also found to interrupt the preformed biofilms of *Streptococcus pyogenes* and *Pseudomonas aeruginosa* [39, 61, 70]. Relevantly in Ansari et al. (2013), jujube honey was found to disrupt the pre-formed biofilms of *Candida albicans* [77].

Inhibition of biofilm formation can be explained by the presence of flavonoids, previously reported, which are capable of reducing biofilm synthesis because they can suppress the activity of the autoinducer-2 responsible for cell-to-cell communication [78-80]. 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 [81]. The flavonoid pinocembrin which is believed present in honey is a very unique antibacterial factor [82]. Flavonoid pinocembrin is an antioxidant that is able to kill bacteria and thus might contribute to the reduction of biofilm biomass [82].

As demonstrated by RT-qPCR, the expression of *argF*, *purF*, *adh* and *fabG* genes had decreased, while the genes of *scdA*, *pykA* and *menB* were increased in expression of *S. aureus.* Such results show the ability of *Kelulut* honey in impairing the efficacy of ligand binding that is needed for adherence, demonstrating the improved virulence and biofilm formation in *S. aureus.* Meanwhile, the *agrF* locus comprises a quorum-sensing gene cluster that carries five genes namely *agrB*, *agrD*, agrC, *agrA* and *hla*. These genes ease the generation and the discovery of an autoinducing peptide (AIP) in the regulation of the expression of genes coding for factors of virulence [83-84]. The decreased expression of *argF*, *purF*, *adh* and *fabG* genes in *S. aureus* following the *Kelulut* honey treatment might show that the honey restricts the biofilm formation. Also, the levels of *scdA* gene in *S. aureus* were increased, while changes in the expression of *scdA* will impact the cross-linking of peptidoglycan.

The analysis of gene expression found differential expression of *fliC*, *fliA*, *oprB*, *oprC*, *oprH*, *lasR* and *algU* genes of *P.aeruginosa* when *Kelulut* honey was applied, suggesting the impact of *Kelulut* honey on numerous aspects of the flagellar regulon. In turn, the differential suppression of *fliC* and *fliA* occurred. Hence, the repression of flagella-associated genes allows *Kelulut* honey to mediate the de-flagellation of *P.aeruginosa* which leads to decreased motility, adherence and virulence. Also, it is likely that the reduced expression of the two outer membrane proteins (*oprB* and *oprH*) caused the reduced survivability of *P.aeruginosa*. Furthermore, the exposure to *Kelulut* honey appeared to downregulate the genes of *yjfO (bsmA)* and *ycfR (BhsA)* in *E. coli*. Relevantly, *yjfO (bsmA)* and *ycfR (BhsA)* have been characterized as biofilm-promoting genes in *E. coli* [85-86]. It can therefore be stated that *Kelulut* honey can inhibit or disrupt *E. coli* biofilms.

Genes such as *tnaA* and *lsrA* genes have been shown to greatly affect the quorum-sensing network of *E. coli*, [65]. Following the application of *Kelulut* honey in this study, both genes were downregulated leading to the potential supposition that the tested honey may inhibit quorum-sensing, which means that the honey may reduce the virulence of pathogens such as *E. coli*. Meanwhile, following the *Kelulut* honey treatment, the *evgA* and *rpoS* genes in *E. coli* were upregulated and this finding is in line with that of Blair et al. (2009) who reported the upregulation of *rpoS* and *evgA* genes in *E. coli* after being treated with Manuka honey [64]. Similar to Wasfi et al. 2016, the outcomes of this study also show the downregulation of *rpoS* and *evgA* genes in *E. coli* genes following the honey exposure [60]. It is possible that the similarity in expression pattern denotes the similarity in the phytochemical constituents and/or similarity in the antimicrobial mechanisms of the experimented honeys. Previous study showed that the *sof* and *sfbl* were decreased in the expression of *S. pyogenes* after treated with 20% (w/v) concentration of Manuka honey [70]. Study by Roberts et al., (2014) showed that six genes of *P. aeruginosa* including, *fliA, fliC, flhF, fleN, fleQ* and *fleR*

were reduced in gene expression after treated with 12% (w/v) and 24% (w/v) concentration of Manuka honey [61]. Previous study showed that *tnaA* and *yjfO* (*bsmA*) genes were downregulated in expression of *E. coli* in the range from 12.5 to16.2-fold change after treated with 25% (w/v) concentration of Egyptian honey [60]. Study by Roberts et al., (2012) showed that *algD* of *P. aeruginosa* increased 16-fold in the expression whereas *oprF* decreased 10-fold after treated with 12% (w/v) concentration of Manuka honey [39]. Previous study reported that *ycfR* (*BhsA*) and *evgA* genes of *E. coli* were upregulated in expression in the range from 2.2 to 4.19-fold respectively after treated with 25% (w/v) concentration of Egyptian honey [60]. Study by Al-kafaween et al., (2020) showed that the expression of *sof* and s*fbl* decreased 7.82-fold and 9.23-fold respectively, whereas the expression of *algD* and *oprF* decreased 6.28-fold and 11.11-fold respectively after exposure to 20% (w/v) concentration of Kelulut honey [53].

Honey contains various polyphenols, which differs according to the origin and bee species [87, 88]. Various polyphenols, of which some are also detected in honey, have been proven to curb the development of many diseases. They perform this action via several specific mechanisms such as regulation of a specific gene expression or altering metabolic pathways by means of promoting or blocking specific pathways [87, 89]. However, differences in honey samples may affect the type of polyphenols found in honey. As one type of honey might not contain all of the polyphenols and the protective effects of polyphenols are varied, it is advisable to consume variety of honey samples . The therapeutics effects of stingless bee honey such as antidiabetic, wound healing, anticancer, treatment of eye disease, and effects of fertility as proven by many scientific studies [87, 88]. Among the physicochemical parameters of honey, the acidity and the osmolarity represent the principal factors responsible for the antimicrobial activity of honey. However, there are other factors that are closely related to the antimicrobial capacity of honey such as the hydrogen peroxide content, and other non-peroxide components such as methylglyoxal, the antimicrobial peptide bee defensin-1, polyphenols and other compounds from the bees [69]. Related gene expression in honey was reflected as down-regulation of spaetzle, AMPs abaecin and defensin-1 and up-regulation of lysozyme-2 [90].

CONCLUSION

In this study, we compared patterns of gene expression in *E. coli, S. aureus* and *P. aeruginosa* cells treated with and without *Kelulut* honey. We have also shown that *Kelulut* honey was able to reduce biofilm formation of *E. coli, S. aureus* and *P. aeruginosa*. Differential gene expression in response to honey exposure exhibited downregulation of several genes of *S. aureus, P. aeruginosa* and *E. coli*. The obtained results indicate that *Kelulut* honey may represent promising antibiofilm and anti-virulence agent for treatment and modulation of infections caused by *E. coli, S. aureus* and *P. aeruginosa*.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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تأثير عسل الكيلولوت الماليزي على تكوين الأغشية الحيوية والتعبير الجيني للمكورات العنقودية الذهبية الزائفة الزنجارية والإيشريكيا كولاي

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ملخص

يشتهر العسل الآن كعلاج بديل بسبب نشاطه المضاد للبكتيريا واسع الطيف وعدم قدرة البكتيريا على تطوير المقاومة بعد التعرض له. ثبت أن العسل مبيد للجراثيم ضد المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي لأنه يزعزع استقرار جدار الخلية البكتيرية. صممت هذه الدراسة لتقييم تأثير عسل الكيلولوت الماليزي على الأغشية الحيوية ، والغشاء الحيوي الوقائي ، وعلى مستوى التعبير الجيني في المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي. تم إجراء فحوصات الأغشية الحيوية والغشاء الحيوي الوقائي على ثلاث المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي باستخدام لوحة ميكروتيتر مع خمسة تراكيز مختلفة من العسل وهي 5٪ ، 10٪ ، 20٪ ، 30٪ ، 40٪ ٪ (وزن / حجم). ومستوى مقياس التعبير الجيني باستخدام النسخ العكسي الكمي (**RT-qPCR**). كان عسل الكيلولوت قادرًا على تقليل تكوين كتلة الأغشية الحيوية بنسبة تصل إلى 39٪ و 41٪ و 37٪ في المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي على التوالي. أقل تركيز لعسل الكيلولوت وجد أنه يمنع تكون الأغشية الحيوية في المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي كان 30٪ (وزن / حجم). لوحظ انخفاض في العدد الإجمالي للخلايا البكتيرية القابلة للحياة لكل من المكورات العنقودية الذهبية, الزائفة الزنجارية والإيشريكيا كولاي بعد العلاج بنسبة 40٪ (وزن / حجم) من عسل الكيلولوت .أظهر **RT-qPCR** أن اثني عشر جينًا وتشمل **argF** و **purC** و **adh** و **fabG** و **fliA** و **fliC oprB** و **oprH** و **yjfO (bsmA)** و **ycfR (BhsA)** و **lsrA** و **tnaA** تم انخفاض مستوى التعبير الجيني بعد التعرض لعسل الكيلولوت ، بينما تم ارتفاع مستوى التعبير الجيني لثمانية جينات وتشمل **scdA** و **pyk** و **menB** و **oprC** و **lasR** و **algU** و **rpoS** و **evgA** بعد التعرض لعسل الكيلولوت .أظهرت هذه الدراسة فاعلية عسل الكيلولوت الماليزي فاعليته ضد الأغشية الحيوية ، وأن التراكيز المختلفة من العسل لها درجات مختلفة من التأثير المحتمل على الأغشية الحيوية الراسخة. أيضًا ، سيؤثر انخفاض التعبير عن جينات الفوعة في هذه البكتيريا على قابليتها للأمراض..

الكلمات الدالة: المكورات العنقودية الذهبية، الزائفة الزنجارية والإيشريكيا كولاي، التعبير الجيني ، عسل كيلولوت الماليزي، البيوفيلم.

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