Antibacterial activity of selected varieties of Malaysian honey against *Escherichia coli*: A comparative study

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Abstract

Background: The purpose of this study was to investigate the antibacterial activity of three varieties of Malaysian honeybees; Tualang honey (TH), Gelam honey (GH), and Acacia honey (AH) against *Escherichia coli*.

Methods: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the honey samples against *E. coli* were determined by the broth microdilution assay in the presence and absence of catalase enzyme. The mode of inhibition of honey samples against *E. coli* was investigated by the effect of time on viability. Impacts of the honey samples on the expression profiles of the selected genes of *E. coli* were examined using RT-qPCR analysis.

Results: The results showed that TH and GH honey possessed lowest MIC and MBC values against *E. coli* with 20% and 25% (w/v) respectively. Highest MIC and MBC values were observed by AH honey against *E. coli* with 25% (w/v) and 50% (w/v) values, respectively. Among the tested honey samples, TH and GH exhibited the highest total antibacterial activity and the highest levels of peroxide-dependent activity. Time-kill curve demonstrated a bactericidal rather than a bacteriostatic effect; with a 2-log reduction estimated within 540 min. Viable cells were not recovered after 9 hours exposure to MIC of all honey-treated samples. The RT-qPCR analysis showed that all honey-treated cells share a similar overall pattern of gene expression, with a trend toward reduced expression of the virulence genes of interest.

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Conclusion: This study demonstrates that Malaysian honey have the potential to be effective inhibitor and virulence modulator of *E. coli* via multiple molecular targets.

Keywords

Antibacterial activity, *Escherichia coli*, Minimum inhibitory concentration, Minimum bactericidal concentration, Malaysian honey, RT-qPCR Time-kill curve.

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Introduction

Honeybees is one of the oldest traditional medicines that has been highly reputed and widely used for the treatment of several human diseases for thousands of years [1]. 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 of honey and other bee products [2-3]. Nowadays, different types of honey have been used in many countries as an alternative to pharmaceutical products for treating contaminated, infected, and burn wounds [4-5]. This is attributed to the effectiveness of these honeybees in inhibiting or killing a broad spectrum of bacteria [6-7]. *E. coli* is particularly interesting because it has been recognized as one of the most frequently isolated bacteria in nosocomial and surgical-site infections [8]. Although some studies have examined the effects of honeybees on bacterial structures [9, 10], the majority of these studies were conducted on one type of honey, known as Manuka honey, and were mostly focused on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The antimicrobial activity of honey may be attributed to several factors, including high osmolality, acidity, in addition to the presence of hydrogen peroxide (H_2O_2) [11] and non-peroxide components, such as methylglyoxal [12]. 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 virulence factor production [13], stress tolerance [14], as well as multicellular behaviours, such as biofilm formation [15], and quorum sensing [16]. Honey's composition (and hence its antimicrobial activity) is dependent on the environmental and geographical locations from which the original nectar was collected [17]. This is attributed to natural variations in floral sources and climatic conditions at different locations [17]. Several studies have addressed different aspects of Malaysian honey varieties, including their physicochemical properties, their chemical composition, their antibacterial and antibiofilm activities and their therapeutic usefulness [18-20]. 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 these honeys could be attributed to alteration of bacterial gene expression. To the best of our knowledge, only three published studies to date have focused on the honey-induced expression patterns in *E. coli* [15-17, 21-22]. Therefore, the objectives of the present study were threefold: (a) to evaluate the direct antimicrobial activity of three varieties of the Malaysia honey against *E. coli* in vitro; (b) to

estimate the impacts of these honey varieties on the expressions of virulence-related genes, with focus on genes involved in biofilm formation, quorum sensing, and stress survival.

Materials and Methods

Honey sample

Three Malaysian honey samples of different floral origins, namely, Tualang honey (TH), Gelam honey (GH), and Acacia honey (AH) were obtained from the apiarists and all honey samples were stored at 4°C in the dark until further analysis. The Three types of honey used were: (a) Tualang; a wild polyfloral honey produced by *Apis dorsata* located on one of the tallest tropical rainforest trees from species *Koompassia excelsa*. (b) Gelam; honey derived from mangrove swamp in Johore state known as *Melaleuca cajuputi* powell, and (c) Acacia; honey derived from a plant widely used in the forest plantation industry from Sarawak state of Malaysia known as tropical acacia species or *Acacia mangium* [21, 23].

Free, lactonic, and total acidity of honey

Honey acidity was determined by a titrimetric method [24-25]. Briefly, 10 g of each honey samples were dissolved in 100 ml of distilled water and the pH was assessed by means of a digital pH meter. The honey solutions were then titrated with 0.05 N Sodium hydroxide (NaOH) solution until pH 8.5 was achieved (to determine the free acidity). This was followed by an immediate back titration with 0.05 N Hydrochloric acid (HCl) until the pH reached 8.3 (to determine the lactonic acidity). Total acidity was obtained by adding free and lactonic acidities. Results were expressed as mille-equivalent of acid per kilogram (meq kg^{-1}) of honey. The procedure was repeated three times in order to achieve accurate result.

Total and non-peroxide antibacterial activity of honey

Honey samples were tested for antimicrobial activity against *E. coli* ATCC 25922 as the bacterial model strain, obtained from the American Type Culture Collection. Using sterile 96-well plates, the minimum inhibitory concentrations (MICs) were determined by the broth microdilution assay, as described by [26] with minor modifications. All honey solutions were freshly prepared before each assay. All assays were performed in triplicate samples and were repeated three times to obtain reliable results. To determine the total (peroxide and non-peroxide) antibacterial activity, a series of concentrations of each type of honey was prepared in Nutrient broth (Oxoid, England) to obtain concentrations of 75%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%. In addition, 20% (w/v) was prepared. These honey solutions were filtered through 0.45µm filters and 150 µl of the filtrate was placed in each of the test wells of 96-well plates. The working bacterial culture was prepared by adjusting an overnight culture of the test organism to be 0.5 McFarland standard and further diluting it in NB to yield a working bacterial suspension of approximately 5×10^6 CFU/ml. The 96-well plates were then inoculated with 50 µl of the working bacterial suspension to achieve a final test concentration of bacteria of approximately 5×10^8 CFU/ml in each well. The plates were incubated at 37°C for 24 hours in a shaker incubator (100 rpm). Positive (growth) controls (consisting of inoculated broth), and negative (sterility) controls (containing uninoculated broth and honey) were included. Wells with the lowest concentration of honey that showed no visible growth were regarded as the MIC [8, 18]. In addition to their visual determination, MICs were confirmed by measuring the absorbance at 570 nm using a plate reader (Tecan Infinite 200 PRO, Austria). The non-peroxide antibacterial activity of the honey samples was evaluated as described above, with the exception

that catalase (Sigma; at a final concentration of 1% w/v) was added to each honey dilution to remove its content of H_2O_2 . Positive (growth) control wells (consisting of inoculated broth and catalase), and negative (sterility) control wells (containing uninoculated broth, the corresponding honey, and catalase) were processed along with each honey sample subjected to the assay [8].

The minimum bactericidal concentration (MBC) was determined by taking a loopful of the culture medium from each test well (from the broth MIC assay) that showed no apparent growth and subculturing on fresh nutrient agar (NA) plates. After incubation at 37°C for 24 hours, the MBC was read as the least concentration showing no growth on the NA plates [23, 27].

Time-kill curve

The minimum inhibitory concentration of TH, GH and AH that was chosen for subsequent experiments. Working bacteria culture was adjusted to be 1×10^5 cfu/ml).

The effect of TH, GH and AH on the viability of cells was monitored by inoculating 40 μ L of an overnight culture of *E. coli* into 20 mL NB with and without TH, GH and AH and incubated at 37°C in a shaking water bath (100 rpm). Samples were removed at known intervals and the TVCs determined as above [23, 27].

Reversibility of inhibitory effects

Working bacteria culture was prepared as previously described, adjusted to be equal to 1×10^5 cfu/ml). Cultures of *E. coli* with and without MIC TH, GH and AH in NB were set up as in the time-kill studies and incubated at 37°C in a shaking water bath. At time 0 and at hourly intervals, 100 μ L samples were removed from each of the flasks, transferred to 10 mL NB, incubated overnight at 37°C and the viability deduced by the presence of turbidity [28].

RNA isolation and cDNA synthesis

A volume of 20 ml of each of the tested honeys was prepared at inhibitory concentrations (MIC) in NB broth medium and filter sterilized. An overnight culture of *E. coli* was diluted 1:100 and cells were grown in NB broth to mid-exponential phase ($OD_{600} = 0.3-0.5$). Aliquots of this culture (500 μ L) were used to inoculate the honey solutions, which were then incubated with shaking at 37°C for 9 h. At the end of incubation, samples were washed twice with cold phosphate-buffered saline. The supernatant was discarded and the pellet was washed with PBS. The pelleted cells were resuspended in 50 μ L of lysozyme (Sigma-Aldrich, UK), and 50 μ L mutanolysin (Sigma-Aldrich, UK) and incubated at room temperature for 10 minutes and total RNA was isolated using kit SV Total RNA Isolation System (Promega, UK) according to the recommendations of the manufacturer. The concentration and purity of the isolated total RNA was determined by the ND-1000 spectrophotometer (NanoDrop, USA), and the integrity of the RNA samples was verified by agarose gel electrophoresis. Finally, 1 μ g of total RNA from each sample was reverse-transcribed to cDNA using cDNA Synthesis Kit (Promega, UK). For each reaction, qPCR mastermix was prepared by following the manufacturer's instructions (Promega, UK) as per the manufacturer's instructions. In parallel, RNA was isolated from both untreated bacterial cells and TH, GH and AH honey-treated ones, and subjected to cDNA synthesis, using the same procedures [8, 23].

Quantitative real-time polymerase chain reaction (RT-qPCR) analysis

The effects of honeys on the expression levels of eight target genes [*yjfo* (*bsmA*), *csgA*, *ycfR* (*BhsA*), *tnaA*, *lsrA*, *evgA*, *rpoS*, and *H-NS*] involved in bio-film formation, quorum sensing, and stress survival in the tested organism, were examined using qPCR. Primers for the qPCR used in the current study were selected from previous studies as shown in **Table 1**.

Table 1. Gene specific primers of *E. coli* used for RT-qPCR analysis [21, 30].

Gene name*	Amplicon Size	Annealing temperature	Direction	Primer sequence
	bp	°C		5' → 3'
<i>yjfO (bsmA)</i>	76	53	Forward Reverse	CGCCAGTAACGGACCATC GTGCTTACGCTACCTATTCC
<i>csgA</i>	191	56	Forward Reverse	ATGGCGGCGGTAATGGTG GTTGACGGAGGAGTTAGATGC
<i>ycfR (BhsA)</i>	81	54	Forward Reverse	CGAAGTTCAGTCAACGCCAGAAG TCCAGCGATCCCAGATTGTCC
<i>tnaA</i>	174	54	Forward Reverse	CTGGATAGCGAAGATGTG CGGAATGGTGTATTGATAAC
<i>lsrA</i>	178	55	Forward Reverse	TACTCATAACCTTCGTGGATTCTG TACTTGCGGCGAGGCTTC
<i>evgA</i>	155	53	Forward Reverse	TAGCGGAGACGATAATAATAATTC GTTGACTGAAGGCGGAAG
<i>rpoS</i>	199	54	Forward Reverse	CTCAACATACGCAACCTG GTCATCAACTGGCTTATCC
<i>H-NS</i>	170	54	Forward Reverse	CCGAACGAACTGCTGAATAG TTACCTTGCTCATCCATTGC
<i>ftsA</i>	152	55	Forward Reverse	GAAGAAGTGACGCAAGAAGATG ACGCCCCGAAAGTCCTACC
<i>16S rRNA</i>	189	55	Forward Reverse	CACACTGGAAGTGAAGACAC CTTCTTCTGCGGGTAACG

The qPCR mastermix for each reaction was prepared based on the manufacturer's instructions (Promega, UK). All reactions (20 µl) were performed using three technical replicates, with 100 ng cDNA and 400 nM primers per reaction. The PCR cycling conditions were as follows: one cycle with 95°C for 5 min (hot start); then 40 cycles of denaturation at 95°C for 30s, annealing and fluorescent data collection at 53-56°C (depending on primers used) for 30s, and extension at 72°C for 30s. On completion of the reaction, a dissociation curve was generated to verify that a single product was amplified. In all qPCR runs, negative controls (lacking the template or the reverse transcription step) were run in parallel. The *16S rRNA* and the *ftsA* genes were selected as internal controls, and the stability of their expression among honey samples was assessed using the Best Keeper tool [29]. The relative mRNA levels of

genes of interest were determined and normalized to the expression of the housekeeping genes using the Applied Biosystems StepOne Software v2.3. The qPCR data were expressed as the changes in expression levels of genes in honey-treated *E. coli* as compared to their levels in the untreated bacteria [8, 18]. Experiments were run with three technical replicates of each. **(Table 1)**

Statistical analysis

All data obtained including quantitative qPCR were presented as mean values ± standard deviation (SD). To determine significant differences between control and treated samples, independent student t-test from (SPSS version 20) was used. A difference with a *P* value of <0.05 was considered to be statistically significant.

Results

Acidity of honey

Among the analyzed honey varieties, TH showed the highest total acidity (30.9 ± 0.59 meq/kg), followed by GH (26.8 ± 0.79 meq/kg), and AH (24.95 ± 0.82 meq/kg). The pH values of the honeys ranged from 3.45 to 4.00, which increased in the following order: AH (pH 3.45) < GH (pH 3.64) < TH (pH 4.00) (**Table 2**). Interestingly, when the lactic acid was calculated, the three honeys showed comparable levels of this acidity, in the range of 9-11 meq/kg.

Table 2. Acidity of the TH, GH and AH (expressed as the mean of triplicate samples $\pm$ standard deviation).

Honey samples	pH	Free acidity	Lactonic acidity	Total acidity
		meq/kg	meq/kg	meq/kg
TH	4.00 ± 0.02	15.9 ± 0.23	11 ± 0.34	30.9 ± 0.59
GH	3.64 ± 0.03	13.2 ± 0.31	10 ± 0.45	26.8 ± 0.79
AH	3.45 ± 0.02	12.5 ± 0.26	9 ± 0.53	24.95 ± 0.82

Antibacterial activities

The MIC values of the three tested Malaysian honeys against *E. coli* are shown in **Table 3**. TH and GH were recorded as the most potent honey against the test organism, which both showed MIC value of 20% (w/v) was required to inhibit the bacterial growth. In the case of AH, a concentration of 25% (w/v) was the lowest concentration required to inhibit the growth of the tested organism. To examine the influence of H_2O_2 on the antibacterial activity of the tested honeys, samples were pre-treated with catalase prior to the incubation with *E. coli*, which was followed by evaluation of their MIC values. The removal of H_2O_2 from the three tested honeys reduced their antibacterial activity against *E. coli* with different degrees. The highest reduction in antibacterial activity was observed in the case of AH honey, in which the removal of H_2O_2 caused an approxi-

mate three-fold increase in the MIC against *E. coli* (from 25 to 75% w/v) (**Table 3**). The results showed that both TH and GH honey possessed lowest MBC value against *E. coli* with 25% (w/v). Highest MBC value was shown by AH honey against *E. coli* with 50% (w/v). The close proximity of the MIC and MBC values (**Table 3**) indicated a bactericidal mode of action for TH, GH, and AH with *E. coli*.

Table 3. MICs of TH, GH, and AH on *E. coli* were assessed by the broth microdilution assay in the presence and absence of catalase enzyme.

Honey samples	Absence of catalase MIC %	Presence of catalase MIC %	MBC %
	w/v	w/v	w/v
GH	20	50	25
AH	20	50	25
AH	25	75	50

Time-kill studies

The time-kill curve clearly shows an increase in number of *E. coli* cells without TH, GH and AH treatment (**Figure 1**). From figures below, TH was more effective for *E. coli* tested up to 8 hours than GH and AH. However, TH, GH, AH honey resulted in 2.2-log, 1.9-log, and 1.6-log reduction in cfu/ml of *E. coli* cells respectively compared to the starting inoculum at 9 hours incubation (**Figure 1**). Loss of viability was observed when bacteria were incubated with MIC (w/v) TH, GH and AH in NB with time compared to untreated cells (**Figure 1**). Extrapolation of viable bacterial population sizes in the presence of TH, GH and AH estimated that the mean time to achieve a 2-log reduction was 540 min.

Reversibility of inhibitory effects

Removing samples from cultures incubated with MIC TH, GH and AH in NB to NB alone demonstrated that viable cells were not recovered after 9 hours (**Figure 2**). This suggested that the inhibitory

Figure 1: The effects of TH, GH, and AH on the viability of *E. coli*.

effect of TH, GH and AH was irreversible. The MIC and MBC were confirmed by growth kinetics curve and time-kill studies, where cells exposed to TH, GH and AH were found to lose viability with time, yet the numbers of untreated cells increased (**Figure 2**).

Figure 2: Growth analysis of *E. coli* treated with and without MIC TH, GH and AH.

RT-qPCR analyses

In the current study, qPCR was used to evaluate and compare the impacts of exposure of *E. coli* cells to three Malaysian honeys (at MIC; for 9 h) on the ex-

pression of eight genes that have been previously shown to be involved in the fitness and virulence of the microorganism. In this study, MIC (TH; MIC 20% (w/v), GH; 20% (w/v) and AH; MIC 25% (w/v) was used for gene expression. Because MIC is defined as the lowest concentration of honey that prevents at least 99% of bacterial growth. The selected genes included three genes involved in biofilm formation [*yjfo* (*bsmA*), *csgA*, and *ycfR* (*BhsA*)], two genes involved in quorum sensing (*tnaA* and *lsrA*), and three genes associated with stress survival (*evgA*, *rpoS*, and *H-NS*). Ct values between biological replicas were standardized against the reference gene and changes in relative expression to untreated cells were analysed. As revealed by the independent student t-test from (SPSS version 20), there was a significant overall difference ($P < 0.05$) in the expression of each of the tested genes among the different groups. The P values for each individual were as follows: *yjfo* (*bsmA*) ($P = 0.0006$); *csgA* ($P = 0.0003$); *ycfR* (*BhsA*) ($P = 0.0015$); *tnaA* ($P < 0.01$); *lsrA* ($P < 0.01$); *evgA* ($P < 0.05$); *rpoS* ($P < 0.05$); and *H-NS* ($P < 0.05$). These results were included in **Figure 3**. All genes, with the exception of only two genes [*ycfR* (*BhsA*) and *evgA*] were downregulated following exposure to TH, GH and AH under study (**Figure 3**). Although different degrees of downregulation were observed following exposure to the TH, GH and AH, all the downregulated genes showed less than six-fold change, except for the *tnaA* gene that was downregulated in the range of 11.3-14.4 fold (**Figure 3**). In the case of *ycfR* (*BhsA*) and *evgA* genes, its expression was upregulated following exposure to the three tested Malaysian honeys. The fold increase in expression of the *ycfR* (*BhsA*) gene was in the range of 2.9-7.2 fold, while *evgA* was upregulated in the range of 0.54-0.78 fold following exposure to Malaysian honeys (**Figure 3**). In the case of *H-NS* gene, its expression was downregulated following exposure to TH and GH, while remained unaltered after exposure to AH (**Figure 3**). The fold decrease in expression of the *H-NS* gene was in the range of 1.12-1.31 fold.

Figure 3: Alterations in gene expression profiles associated with exposure of *E. coli* to the TH, GH, and AH as determined by qPCR.

Mean values of fold changes ($\pm$ SD) are shown in relation to untreated (control) *E. coli* cells. Asterisks indicate statistically significant differences in the expression of each gene between treated samples and control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

To evaluate the contribution of osmolarity and acidity on the level of expression of the target genes, this assay was also performed on cDNA synthesized from RNA extracted following exposure of the cells to an TH, GH and AH solution. In the presence of honeys, downregulation in the expression of all the tested quorum-sensing genes, and upregulation in the expression of *evgA* involved in stress survival was observed with *E. coli* (Figure 3). In the case of biofilm-forming genes, exposure of *E. coli* cells to Malaysian honeys caused a decreased expression of *yjfO (bsmA)* and *csgA*, while it caused an increased expression of *ycfR (BhsA)* (Figure 3).

Discussion

The ability of different types of honey to combat infections may be attributed to at least two

complementary mechanisms. The first mechanism is attributed to their direct biocidal activity, owing to the presence of multiple factors that can damage susceptible organisms [16]. The second mechanism is mediated through their anti-virulence activity, by down regulating the expression of genes associated with virulence factor production, stress tolerance, and/or multicellular behaviours of the target organism (such as biofilm formation and quorum sensing) [16]. This latter mechanism will eventually weaken bacterial coordination, decrease their survival abilities, and interfere with their virulence mechanisms. It is well known that the total antibacterial activity of honey is attributed to multiple factors, including its high osmolarity, acidity, in addition to its content of hydrogen peroxide (H_2O_2) and non-peroxide phytochemical components [31]. Therefore, the current stu-

dy assessed these individual antibacterial factors and evaluated their relative contributions to the overall antibacterial activities of the honeys under study. The antibacterial activity of honey has been assayed using various methods across the globe with special attention devoted to minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In this study, MIC was performed using 96-well microtitre plate and data were collected by means of a spectrophotometric endpoints evaluation. MIC is defined as the lowest concentration of honey that prevents at least 99% of bacterial growth while MBC is defined as the lowest concentration of honey required to kill at least 99% of the bacteria [27]. To examine the effect of peroxide, the MIC values were determined in the presence and absence of catalase enzyme, which is known to break down H_2O_2 . The possible contribution of pH and acidities (free, lactic and total) in solutions of the tested honeys were investigated. In the absence of catalase, the MIC values of the TH and GH were 1.2 fold lower than that observed with the AH was 2 fold. This may indicate that the phytochemical components exerted a more specific growth-inhibitory activity against *E. coli* than the osmotic effects of sugars. In this respect, several reports have shown the high antimicrobial activity of Trigona, Tualang, Gelam and Acacia honeys against both Gram-positive and Gram-negative bacterial strains [27, 32, 33]. Previous findings by Tan *et al.*, (2009) reported that honey tualang was superior in their antimicrobial activity against *E. coli* [34]. Our findings support and confirm those mentioned earlier, since among the honeys investigated, that TH and GH exhibited the highest total antibacterial activity against *E. coli*, as indicated by having the lowest MIC values in the absence of catalase (20% w/v). These types of honeys also showed the highest levels of hydrogen peroxide dependent activity, as the removal of its H_2O_2 (by catalase enzyme) caused the highest fold increase in MIC, resulting in an

approximately three fold-shift. In the current study, both TH and GH honey varieties showed equal MIC values of 20% (w/v) and AH honey MIC value 25% (w/v). These MICs were comparable, to some extent, with those obtained by Zainol *et al.*, (2013), who reported antibacterial activities of Malaysian Tualang, Gelam and Acacia honeys against *E. coli*, with the MIC values of 20%, 12.5%, and 25% (w/v) respectively [27]. However, in agreement to our results previous study reported that Tualang honey possessed higher antimicrobial activity against *E. coli* with the MIC value of 22.5 % (w/v) [34]. These similarity and conflicting results may be attributed to the differences in geographical and seasonal sources, as well as harvesting, processing, and storage conditions of the honeys tested. Reductions in viable cells seen in the time-kill experiments (**Figure 1**) confirmed a bactericidal mode of action, with a time to achieve 2-log reductions estimated to be 540 min. Failure to recover viable bacteria after 9 hours in the reversibility experiment confirmed that the inhibition was irreversible (**Figure 2**). Turning to evaluation of acidity, the three tested honeys were acidic in nature, with pH values ranging from 3.45 to 4.00, which are likely to be low enough to inhibit the growth of many microorganisms [31]. The obtained values were in line with those of previous studies that reported a pH ranging from 3.8 to 5.2 among different Malaysian Tualang, Gelam and Acacia honey varieties [20, 35, 36]. Interestingly, although the total and free acidity values in AH honey were both less than the corresponding values of TH and GH honeys, the antibacterial activity of the TH and GH honeys was higher than the AH honey. This observation is consistent with that of Badawy *et al.*, (2004), who found no correlation between free acidity of honey and its antibacterial activity against tested *E. coli* [37]. The findings suggest that each honey has different effects on the tested bacteria. This could be due to the presence of different organic antibacterial factors contributed by honey [38, 39].

A number of genes have been shown to be involved in the fitness and virulence of *E. coli*, and thus modulating the expression of these genes can add to the effectiveness of antimicrobial therapy. Herein, eight of these genes playing important roles in biofilm formation, quorum sensing, and stress survival in *E. coli* were selected, and their differential gene expression profiles in response to exposure to the tested honeys were determined using qPCR.

A number of genes [including *yjfO* (*bsmA*), *csgA*, and *ycfR* (*BhsA*)] have been previously shown to be involved in the process of biofilm formation in *E. coli* [40-42]. The current results showed that both *yjfO* (*bsmA*) and *csgA* were downregulated after honey treatment, while *ycfR* (*BhsA*) was upregulated. This pattern of expression was the same regardless of the tested honey. The importance of this expression pattern becomes clearer when it is taken into account that *yjfO* (*bsmA*) and *csgA* have been previously characterized as biofilm-promoting genes in *E. coli* [40, 41], while *ycfR* (*BhsA*) has been shown to act as a biofilm repressor gene [42]. Therefore, the current findings may suggest that the honeys under study can prevent or disrupt *E. coli* biofilms. In a study conducted by Weber *et al.*, (2010) mutation of the *yjfO* gene in *E. coli* has been shown to cause alteration of cell motility, increased sensitivity to pH and oxidative stresses, and reduction of viability, rather than only affecting the biofilm formation [41].

A set of genes have been previously shown to play an important role in the quorum-sensing network of *E. coli*, such as the *tnaA* and *lsrA* genes [43]. The present results showed that both genes were down regulated in response to all the tested honeys. It is tempting to speculate that the tested honeys may act as quorum-sensing inhibitors, and thus may have the potential to decrease the virulence of pathogens like *E. coli*, by interrupting their cellular communication system. The current results are in agreement with those of Lee *et al.*, (2011), who reported down regulation of multiple genes

involved in biofilm formation and quorum sensing in the pathogenic *E. coli* strain following exposure to honey samples from Korean and American origins [44].

Since bacterial cells are exposed to various stressful conditions, these cells are equipped with stress survival mechanisms, being encoded in *E. coli* by a number of genes, including the *evgA*, *rpoS*, and *H-NS* [45-47]. Among these three tested genes, *rpoS* and *H-NS* were downregulated after exposure to TH and GH while remained unaltered after exposure to AH. The expression profile of *evgA* was upregulated and has approximately similar pattern in expression after exposure to all the tested honeys. Given the various stresses caused by honey exposure (including acid, osmotic, and oxidative stresses triggered by H₂O₂), it is likely that the downregulation of the above-mentioned genes will render bacterial cells less protected against stresses and damage caused by honey, which may eventually lead to loss of viability once the damage is beyond repair (**Figure 4**).

The current results are in agreement with those of Wasfi1et al., (2016), who reported downregulation of *rpoS*, *H-NS*, and *evgA* genes following treatment of *E. coli* to Egyptian honeys [8]. Also, our results are in contrast to those of Blair *et al.* [22], who showed marked upregulation in *rpoS*, *H-NS*, and *evgA* genes following exposure of *E. coli* to Manuka honey. This difference in expression pattern may reflect differences in the phytochemical constituents and/or differences in the antimicrobial mechanisms of the tested honeys in both studies [31],

Contrary to the effect of most of the tested honeys, exposure of *E. coli* to Malaysian honey varieties resulted in downregulation of all the tested stress survival genes (*rpoS* and *H-NS*) except *evgA* (**Figure 3**). This indicates that the honey-induced changes in expression of this group of genes are most probably due to specific molecules contained in these honeys and not simply due to their sugar content. This suggests that the antiquorum sensing activities

Figure 4: Schematic diagram summarizing the effects of the tested honeys on *E. coli* ATCC 8739 at both structural and molecular levels.

In this figure, thin lines with pointed arrows indicate induction of gene expression or stimulation of a phenotype, while bar-headed lines indicate repression of gene expression or repression of a phenotype. Adapted from Wasfi et al., (2016) with a modification according to our study.

of the tested honeys are, at least partially, attributed to their sugar content, which is in accordance to the results of a previous study [8, 16].

Conclusion

The results of this study demonstrated that the tested Malaysian honeys have the potential to be effective inhibitors of *E. coli*. Malaysian honey, namely Tualang and Gelam honey have high antibacterial potency of total and non-peroxide activities implying that peroxide and other constituents are mutually important as contributing factors to the antibacterial system of honey. The correlations between MIC, MBC and osmotic and total acidity values of Malaysian honey were proven to be dependent on honey origin. TH honey showed the highest antibacterial activity, followed by GH and AH ones.

Differential gene expression in response to honey exposure exhibited downregulation of several genes involved in biofilm formation, quorum sensing, and stress survival in *E. coli*. The obtained results demonstrate that the honeys under study may represent promising antibacterial and anti-virulence agents for treatment and modulation of infections caused by *E. coli*. Future clinical evidence pertaining to the efficacy of the tested honeys in the prevention and treatment of *E. coli*-induced infections at various tissue/cell types might be required.

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Declaration of Competing Interest

The authors declare that there is no competing interests.

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