Pseudomonas Aeruginosa and Streptococcus Pyogenes Exposed to Malaysian Trigona Honey In Vitro Demonstrated Downregulation of Virulence Factor

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Background: Honey has been known as a traditional medicine for centuries with its antibacterial properties. It is considered one of the most enduring substances used in wound management.

Objectives: This study aimed to: (i) evaluate the effects of Malaysian Trigona honey on bacterial structure and (ii) assess the anti-virulence potential of this honey by examining their impacts on the expression of selected genes (involved in stress survival and biofilm formation) in a test organism.

Materials and Methods: Trigona honey’s impacts on the bacterial structure (cell morphology) and the expression profiles of select Pseudomonas aeruginosa and Streptococcus pyogenes genes were examined using scanning electron microscopy (SEM) and real-time PCR (RT-qPCR) analysis, respectively.

Results: SEM showed that the decreased cell density deformed, disrupted, and damaged cells for both bacteria. RT-qPCR showed that the expression of fleN, fleQ, and fleR genes of P. aeruginosa were decreased, 4.26-fold, 3.80-fold and 2.66-fold respectively. In addition, scpA, ftsY, and emm13 of S. pyogenes were decreased, 2.87-fold, 3.24-fold, and 4.65-fold respectively.

Conclusion: Our results indicate that Trigona honey may be an effective inhibitor and virulence modulator of P. aeruginosa and S. pyogenes via multiple molecular targets. This deduction needs to be investigated in vivo.

Keywords: Differential expression; Pseudomonas aeruginosa; RT-qPCR; Scanning electron microscope (SEM); Streptococcus pyogenes; Virulence.
flgM (11). Other regulatory Class II genes, under the control of fleQ include flhF, fleR, and fleS. fleR and fleS, form a two-component system, comprised of a response regulator and cytoplasmic sensor kinase, respectively (10). The phosphorylation of fleR by fleS is essential for transcribing and translating Class III genes, specifically the HBB proteins. In addition to forming a pivotal role in flagella production, fleRS, or one of its regulatory products, facilitates the nonpilus adhesion of cells to mucin (12). Class III genes facilitate a regulatory transition from fleQ dependency to flhA by removing the flhA anti-activator: flgM, which is secreted through the completed HBB, reducing flgM’s suppressive effects on flhA (13). Unencumbered flhA is required for the late expression of Class IV genes (such as fliC, motAB, cheAB, flgMN), and completes the hierarchical cascade to result in a fully functioning flagellum (14). A break in this regulatory cascade may impede the cells’ ability to produce a fully functioning flagellum, which may affect the cell’s motility and/or virulence (15). S. pyogenes spectrum of infections can be attributed to its wide range of virulence factors, which lead to adherence, immune system evasion, deliberate stimulation or degradation of host components, and direct cell lysis. Serological specificities between S.pyogenes strains are based on M protein differences (16). S. pyogenes produces a hyaluronic capsule, which provides the bacteria with increased resistance to phagocytosis (17). The repeating units of β1,4-linked glucuronic acid connected via β1,3-linked N-acetylglycosamine form a glycosaminoglycan fiber, which is indistinguishable from those produced in human connective tissue (18, 19). S. pyogenes produces a wide range of virulence factors M protein associated with the cell wall and a major virulence factor of S.pyogenes, which can bind directly to the extracellular matrix components,(20, 21). So far, the best studied adhesions of S.pyogenes and currently 11 different such adhesions have been identified, divided in two types. The first types of proteins are, sof, PrfF2, Fhp54, sfhX, FbaA, FbaB and SflB. The second types of proteins are M1, M13 (emm13) (22), Shr, Sc11, scpA, and fisY. An estimated 60% of initial attachment to cells is realized by streptococcal lipoteichoic acid. Binding of these gene adhesions could result in initial attachment to the planktonic or biofilm production in bacterial internalization (23, 24). 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 (25). Honey’s inherent antibacterial properties are partly conferred by sugars, which account for 80% of its weight, resulting in low water activity and a high osmolarity (26). Trigona stingless bee honey, known as “Kelulut”, is a commercial stingless bee’s species abundant in Malaysia. This bee produces Kelulut honey, a multi floral honey which is stored in clusters of small resin domes of their nests. This study was designed to evaluate the effect of Malaysian Trigona honey on P. aeruginosa and S. pyogenes using SEM and RT-qPCR.

2. Objectives
The aim of this study was (a): to determine the effects of Malaysian Trigona honey on P. aeruginosa and S. pyogenes at the ultrastructural level and (b) to estimate the impacts of this honey on the expressions of virulence-related genes (in other words, to evaluate the anti-virulence potential of this honey) using scanning electron microscopy (SEM) and real-time PCR (RT-qPCR) respectively.

3. Materials and Methods

3.1. Bacteria Strains and Culture Conditions
Reference strains of Paeruginosa ATCC 10145 and S.pyogenes ATCC 19615 were purchased and used for this study. The inoculums were prepared by picking up two to four colonies from stock culture and suspended in 20 mL of sterile in Tryptic soy broth (TSB). Then, the inoculums were incubated for 24 hours at 37 °C (27).

3.2. Honey Samples
Trigona honey samples were purchased from Kelantan, a state in Malaysia. All samples were kept at the room temperature (28, 29).

3.3. Scanning Electron Microscopy (SEM)
The honey sample was diluted with TSB to reach 20% (w/v) concentration of Trigona honey. Bacterial suspension was adjusted to 0.5 McFarland. To form the biofilm, two hundred microliter of the adjusted inoculum was transferred into 96-well plate and incubated for 48 hours at 37 °C without shaking. Wells containing bacterial culture served as a positive control. After 48 hours of incubation, planktonic cells were removed; then 200 µL of 20% (w/v) concentration of honey was added and incubated overnight at 37 °C. Subsequently, all samples were then centrifuged for 10 minutes at 3500 rpm, and the pellets were fixed with 2.5% (v/v) glutaraldehyde in 0.01 M phosphate buffer solution (PBS) overnight. The cultures were then washed with PBS for 15 minutes, followed by deionized water for 20 minutes. All samples were dehydrated with ascending concentrations of ethanol for 10 minutes and subjected to critical point drying. The samples were then coated.
with platinum, placed onto the copper stage holder, and examined by SEM (JEOL 6360LA, Japan) (30).

3.4. Extraction of RNA for RT-qPCR
To perform gene expression analysis using RT-qPCR, total RNA was extracted and converted to cDNA. *P. aeruginosa* and *S. pyogenes* were grown in duplicate in 5 mL TSB medium with 20% of *Trigona* in a sterile universal bottle for 24 hours at 37 °C. After incubation, the samples were re-suspended in 1000 mL PBS and vortexed for 1 min to break up cell aggregates. Honey-treated and untreated cell suspensions were equilibrated (to approximately 2×10⁹ c.f.u.) prior to treatment with mutanolysin (100 mg) and lysozyme (100 mg) (Sigma-Aldrich, USA) for 15 min at 37 °C and immediately processed for RNA extraction. RNA was extracted using the SV total RNA extraction kit (Promega, UK) according the manufacturer’s instructions. The bacterial total RNA integrity was checked by NanoDrop, and each RNA sample was adjusted to give a final concentration of 10 ng. The primers were used for *P. aeruginosa* and *S. pyogenes* as shown in Table 1. Reverse RNA transcription was performed with Oligo (dT)₁₅ primers and Random Primers. Total RNA samples were converted to cDNA using a high capacity RNA to cDNA conversion kit (Promega, UK) and quantitative PCR expression analysis as following the manufacturer’s instructions (Promega, UK). Densitometry was performed using the Applied Biosystems StepOne Software v2.3 to determine the level of relative gene expression in *P. aeruginosa* and *S. pyogenes* samples. A modified 2⁻ΔΔCt method was used. All reactions were carried out in triplicate, and the genes’ expressions were analyzed with reference to the housekeeping gene expression (31, 32, 33, 34, 35, 36, 37).

4. Results
4.1. Deformation and Increased Cell lysis of *P. aeruginosa* and *S. pyogenes* in Response to MTH Treatment.
SEM micrographs of untreated *P. aeruginosa* cells, incubated with only broth, had a regular rod-shaped cell with a smooth surface for the cells, as shown in Figure 1A.

### Table 1. Gene specific primers of *P. aeruginosa* and *S. pyogenes* used for RT-qPCR analysis

| Gene name   | Amplicon Size (bp) | Annealing temp (°C) | Number of cycles | Direction | Primer sequence (5’ → 3’) |
|-------------|--------------------|---------------------|------------------|-----------|--------------------------|
| **1. fleN** | 137                | 56                  | 41               | Forward   | GAGCCGTATACGAGGCATTC     |
|             |                    |                     |                  | Reverse   | GTGTTGGACCAGTCTGTCG      |
| **2. fleQ** | 134                | 54                  | 41               | Forward   | AAGGACTACCTGGCCAACCT     |
|             |                    |                     |                  | Reverse   | CCGTACTTGCACATTTCTC      |
| **3. fleR** | 109                | 55                  | 41               | Forward   | ACAGCCGCAAGATGAACCT      |
|             |                    |                     |                  | Reverse   | TGGATGGCGTTGCGAGTTT      |
| **4. rpoD***| 146                | 53                  | 41               | Forward   | GCGACGGTGATTCGAACTTGT    |
|             |                    |                     |                  | Reverse   | CGAAGAAGGAATGGTCGAG      |
| **5. scpA** | 622                | 55                  | 41               | Forward   | GTCGCGTTACCTACCTGTCC     |
|             |                    |                     |                  | Reverse   | CAAATGCGAAACAAGGTACACC   |
| **6. ftsY** | 97                 | 54                  | 41               | Forward   | TCGAAATCTTTTGCGCTGT      |
|             |                    |                     |                  | Reverse   | ATCAACGTGTGTGGCCAGA      |
| **7. emm13**| 373                | 55                  | 41               | Forward   | CGCCAGGTTTTTCCCGATCAGGAC|
|             |                    |                     |                  | Reverse   | AGCCGATAAAACATTCACAGGA   |
| **8. glr#** | 797                | 54                  | 41               | Forward   | ATGGATACAAAGACAAATTGG    |
|             |                    |                     |                  | Reverse   | TCATAAAGGTACATGCTCCAC    |

*rpoD* was used as a reference gene for *P. aeruginosa* and *glr* was used as a reference gene for *S. pyogenes*.
morphology and covers most of the area, as shown in Figure 1A. The biofilm in the honey treated sample became noticeably shorter than it was in the untreated sample, as shown in Figure 1B. The cell density was decreased, composed of layers of rod shaped cells, and appeared curved and distorted, as shown in Figure 1B. SEM micrographs of *S.pyogenes* control cells, incubated with only broth, were shown to have a cocci, with regular structure and normal size, after 24 hours incubation in liquid media, as shown in Figure 1C. *S.pyogenes* biofilm shows numerous cells and diverse thickness, connected to each other by the extracellular matrix, as shown in Figure 1C. However, cell deformations, and changes in shape and size, were observed after incubation with 20% *Trigona* honey, as shown in Figure 1D. Also, *S.pyogenes* biofilm with honey shows uneven shape the bacteria have uneven shapes; also, the cell surfaces appear rough, with holes and crevices. The cells’ structures were damaged, as shown in Figure 1D.

### 4.2. RT-qPCR of Genes Expression of *P.aeruginosa* and *S.pyogenes*

In the current study, RT-qPCR results showed that all genes were downregulated following exposure to 20% (w/v) of *Trigona* honey, furthermore different degrees of down-regulation were observed. Three genes of *P.aeruginosa* (*fleN*, *fleQ*, and *fleR*) involved

| Gene name | Average ΔΔCt | Expression Fold Change (2^−ΔΔCt) | Expression Fold Change | P-value | SD |
|-----------|--------------|----------------------------------|------------------------|---------|----|
| 1. *fleN* | 2.09         | 0.23                             | -4.26                  | 0.03*   | 1.0|
| 2. *fleQ* | 1.93         | 0.26                             | -3.80                  | 0.04*   | 1.3|
| 3. *fleR* | 1.41         | 0.38                             | -2.66                  | 0.03*   | 1.2|

*Statistically significant change in the level expression compared treated with untreated and reference gene (P<0.05).
in flagellum-associated were showed a statistically significant reduction in level of gene expression after treatment with 20% (w/v) concentration of *Trigona* honey. As shown in Table 2 and Figure 2, the RT-qPCR results demonstrated that the expressions of *fleN*, *fleQ*, and *fleR* genes of *P. aeruginosa* were decreased 4.26-fold (*P*<0.05), 3.80-fold (*P*<0.05), and 2.66-fold (*P*<0.05), respectively. This referred that *Trigona* honey suppresses the level of flagellum gene expression by influence on regulatory *fleN*, *fleQ*, and *fleR*.

Three genes were involved in the surface adhesins, colonization, and biofilm formation of *S. pyogenes*. RT-qPCR results demonstrated that the expressions of *scpA*, *ftsY*, and *emm* of *S. pyogenes* were decreased, 2.87-fold (*P*<0.05), 3.24-fold (*P*<0.05) and 4.65-fold (*P*<0.05) respectively (Table 3 and Fig. 3).

### 5. Discussion

#### 5.1. SEM of *P. aeruginosa* and *S. pyogenes*

Under light microscopy can be observed the shapes of various bacteria, including rods, coccis, cubes or spirals. Electron microscopes development provides new insights into bacterial ultrastructural studies and bacterial organization. Scanning electron microscopy (SEM) provides a three-dimensional aspect of cellular structures and information about their external topography (38). Several reports have been published on the antimicrobial activities of different types of honey against a variety of organisms, including *Staphylococcus aureus, Pseudomonas aeruginosa* and *Streptococcus pyogenes* (33, 39). In this study, SEM was used to determine membrane integrity, morphological cell changes, and evidence of cell division before and after exposure to honey. From the SEM images of *P. aeruginosa* and *S. pyogenes* species undergo morphological changes after exposure to honey; however, the changes were different in each species. Primary research on *Trigona* honey’s action on Gram positive and negative bacteria, using electron microscopy, sequentially identifying the mode of action and the target site was initiated by Al-kafaween et al., (2020) (30). However, *P. aeruginosa* and *S. pyogenes* cells treated with *Trigona* honey had noticeably

![Figure 2](image-url). Altered gene expression profiles associated with exposure of *P. aeruginosa* to *Trigona* honey as determined by RT-qPCR. Mean values of fold changes (±SD) are shown in relation to untreated (control). Error bars denote standard error of the mean from three biological samples.

![Figure 3](image-url). Altered gene expression profiles associated with exposure of *S. pyogenes* to *Trigona* honey as determined by RT-qPCR. Mean values of fold changes (±SD) are shown in relation to untreated (control). Error bars denote standard error of the mean from three biological samples.

| Gene name | Average ΔΔCt | Expression Fold Change (2^-ΔΔCt) | Expression Fold Change | P-value | SD |
|-----------|-------------|----------------------------------|------------------------|---------|----|
| 1. *scpA* | 1.52        | 0.35                             | -2.87                  | 0.03*   | 1.2|
| 2. *ftsY* | 1.70        | 0.31                             | -3.24                  | 0.03*   | 1.2|
| 3. *emm13*| 2.22        | 0.22                             | -4.65                  | 0.02*   | 1.6|

*Statistically significant change in the level expression compared with untreated and reference gene (*P*<0.05).
rougher cell surfaces than untreated cells (Fig. 1). In addition, changes in cells size increased for both types of honey. This suggests that honey might have affected the outer cell membrane. Cell destruction and lysis were observed in *P. aeruginosa* and *S. pyogenes*, which affected the cell wall structure. It was, therefore, documented that both species responded to MIC of *Trigona* honey (30). A previous study using Manuka honey showed that a concentration of 10% honey affected the *Staphylococcus aureus* structure (40). Previous studies showed that stingless bees’ honey and Sider omami honey have disrupted the cell wall and inhibited cell division of *P. aeruginosa*, *S. pyogenes* and *Staphylococcus aureus* (41, 42). The high osmotic effect of honey, due to its high sugar content, also plays a part in decreasing biofilm mass. Besides the osmotic effect of *Trigona* honey, its acidity is expected to have a role in degrading biofilm mass as well. *Trigona* honey’s acidity, which is within the range of pH 3.2 to 4.5, generates an inappropriate environment for bacterial growth, whereas their optimum pH for growth is about pH 7.2 to 7.4 (26).

5.2. RT-qPCR of Genes Expression of *P. aeruginosa* and *S. pyogenes*

Investigations into *Trigona* honey’s impact on gene expression in *P. aeruginosa* and *S. pyogenes* have demonstrated multiple effects on bacterial function. RT-qPCR was used to determine the gene expression level of *P. aeruginosa* and *S. pyogenes* after treatment with *Trigona* honey. Reduced expression was noticeable, with a different expression level in *P. aeruginosa* and *S. pyogenes*. The *fleN*, *fleQ*, and *fleR* of *P. aeruginosa* decreased, 4.26-fold, 3.80-fold, 2.66-fold respectively and *scpA*, *fisY*, and *emm13* of *S. pyogenes* decreased, 2.87-fold, 3.24-fold, and 4.65-fold reduction in expression, respectively, after treatment with 20% of *Trigona* honey. Flagella and pili are important during the initial biofilm formation stages of *P. aeruginosa*, and each represents important virulence factors with respect to binding and signaling for cell–cell communication (43, 44). Suppression of *fleN*, after treated with (20% w/v) concentration of *Trigona* honey should result in the parallel suppression of *fleN*, increasing *fleQ*’s relative activity during formation of biofilm. This suggests a conflict, whereby reduced *fleN* expression should concomitantly result in its increased expression (along with other *fleQ*-dependent genes). However, *Trigona* honey suppresses the expression of *fleQ*. This would cause suppression of *fleN* expression, which is suggested to occur in *Trigona* honey treated samples. It is evident that, following treatment with *Trigona* honey, the decreased expression of *fleN*, *fleQ*, and *fliA*, both of which have critical regulatory effects as flagellar regulon regulators. The resulting effect is a decrease in flagella associated motility. The reduction of flagellated cells could also affect *P. aeruginosa*’s virulence due to its implication with invasive virulence, particularly in burn wound infections (15, 45). The *scpA*, *fisY*, and *emm13* contributed to virulence factors and involved in cell division have been identified in *S. pyogenes*; expression of these genes is usually controlled by global virulence and accessory gene regulators in *S. pyogenes* (46). In current study, a decreased level of gene expression of these genes was found after exposure to *Trigona* honey. A previous study showed that *algD* of *P. aeruginosa* increased 16-fold in expression, whereas *oprF* decreased 10-fold after treated with Manuka honey (33). A study by Al-Kafaween et al., (2019) showed that *algD* and *oprF* of *P. aeruginosa* decreased 6.28-fold and 11.11-fold reduction in expression respectively after treated with *Trigona* honey (30). A previous study showed that the *sof* and *sfb* proteins of *S. pyogenes* decreased in expression after treatment with Manuka honey (32). A study by Roberts et al., (2014) showed that six genes of *P. aeruginosa* (*fleR*, *flhF*, *fliC*, *fleN*, *fleQ* and *fliA*) were reduced in level of gene expression after exposure to Manuka honey. A previous study showed that *tnaA* and *yffO* (*bsmA*) genes were downregulated in expression of *E. coli* in the range of 12.5–16.2-fold after treatment with Egyptian honey (35). A previous study reported that *ycfR* (*BhsA*) and *evgA* genes of *E. coli* were upregulated in expression in the range of 2.2–4.19-fold and 1.09-fold respectively after treatment with Egyptian honey (35). A study by Al-Kafaween et al., (2019) showed that the *sof* and *sfb* of *S. pyogenes* decreased 7.82-fold and 9.23-fold reduction in the expression after treatment with *Trigona* honey (47).

6. Conclusion

The study revealed MTH’s antimicrobial activity against *P. aeruginosa* and *S. pyogenes*. SEM images showed that *P. aeruginosa* and *S. pyogenes* of planktonic and biofilm were lysed and disrupted after treatment with *Trigona* honey. Differential gene expression in response to *Trigona* honey exposure exhibited downregulation of several genes involved in, cell wall, biofilm formation, stress survival in *P. aeruginosa* and *S. pyogenes*. Dysregulation and delocalization of flagellar impaired an adhesion essential for biofilm formation in cyclic adenosine 3′,5′-monophosphate/virulence factor regulator (cAMP/Vf signaling pathway) and bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP signaling pathway) (Fig. 4). A previous study by
Al-Kafaween et al., (2019) showed that Trigona honey appears to prevent P. aeruginosa biofilm via suppressing microcolony-forming genes (30),(47). Mitigation of algD and oprF suggests that alginate biosynthesis enzymes were reduced, and therefore, is promising for cystic fibrosis treatment (Fig. 4). Surface proteins are responsible for S. pyogenes’s ability to survive and multiply in the host. A study by Al-Kafaween et al., (2019) demonstrated that Trigona honey may alter the ECM-receptor interaction signaling pathway, according to the suppression of genes encoding the surface binding protein (sof and sfb1) (Fig. 4) (47). The ftsY, scpA, and emm13 in S. pyogenes were inhibited following exposure to Trigona honey. Trigona honey may reduce the multispansing membrane protein, affecting the signal recognition particle pathway (Fig. 4).

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