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Research Article

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 *emm*13 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.

1. Background

P. aeruginosa is a pathogen of humans, plants, and animals, with colonies as diverse as the isolation site, and is able to cause devastating infections because of the strong attachment potential with pili (1, 2) and production multiple virulence genes (3, 4). Many virulence factors of *P. aeruginosa* are involved in motility and biofilm formation and were regulated by complex interactions with and between quorum-sensing (QS) molecules (5, 6). The flagellar regulon is comprised of over 40 different genes (7). The highest regulatory class (Class I) includes the master regulators *fleQ* and *fliA* (encoded by *fleQ* and *fliA*, respectively), which respond to stimuli from outside the flagellar regulon. The positive and negative regulation of *fleQ* is achieved through external factors σ70 and *Vfr*, respectively, while the regulation of *fliA* still remains unknown. *fleQ* is capable of binding bis- (3′ –5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), modulating the transition between surface translocation and surface attachment (8). Additionally, *fleQ* demonstrates hierarchical control over *fliA*, regulating most Class II and III genes, while *fliA* regulates the genes grouped as Class IV (9). *fleQ* positively regulates its own anti-activator (*fleN*) which is encoded by the Class II gene *fleN* on the *fleN* and *flhF* operon. This negative feedback loop is crucial for *P.aeruginosa* to remain mono-flagellated through the effects of *flhF* (10). Additionally, *fleQ* positively regulates the *fliA* antisigma factor: *flgM*, encoded by the Class II gene

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 *fliA* by removing the *fliA* anti-activator: *flgM*, which is secreted through the completed HBB, reducing *flgM'*s suppressive effects on *fliA* (13). Unencumbered *fliA* 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 glycosaminogly can 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*, *PrtF2, Fbp54*, s*fbX*, *FbaA*, *FbaB* and *SfbI*. The second types of proteins are M1, M13 (emm13) (22), *Shr*, *Scl1*, s*cpA*, and *ftsY*. 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 *P.aeruginosa* 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) glutaraldehdye 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 2×10^9 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 $2^{-\Delta\Delta}$ Ct 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**. *P.aeruginosa* biofilm shows hundreds of bacterial cells are connected by a substantial amount of extracellular matrix, which produces stringy

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	GTGTTGGACCAGTCGTTCG
2. *fleQ*	134	54	41	Forward	AAGGACTACCTGGCCAACCT
				Reverse	CCGTACTTGCGCATCTTCTC
3. *fleR*	109	55	41	Forward	ACAGCCGCAAGATGAACCT
				Reverse	TGGATGGCGTTGTCGAGTT
4. *rpoD**	146	53	41	Forward	GCGACGGTATTCGAACTTGT
				Reverse	CGAAGAAGGAAATGGTCGAG
5. *scpA*	622	55	41	Forward	GCTCGGTTACCTCACTTGTCC
				Reverse	CAATAGCAGCAAACAAGTCACC
6. *ftsY*	97	54	41	Forward	TCGAAAATTCTTTGGCCTGT
				Reverse	ATCAAACGTGTTGTGCCAGA
7. *emm13*	373	55	41	Forward	CGCCAGGGTTTTCCCAGTCACGAC
				Reverse	AGCGGATAACAATTTCACACAGGA
8. *glr#*	797	54	41	Forward	ATGGATACAAGACCAATTGG
				Reverse	TCATAAGGTGACATGCTCCAC

*****_rpoD_ was used as a reference gene for *P.aeruginosa* and [#]_glr_ was used as a reference gene for *S.pyogenes*

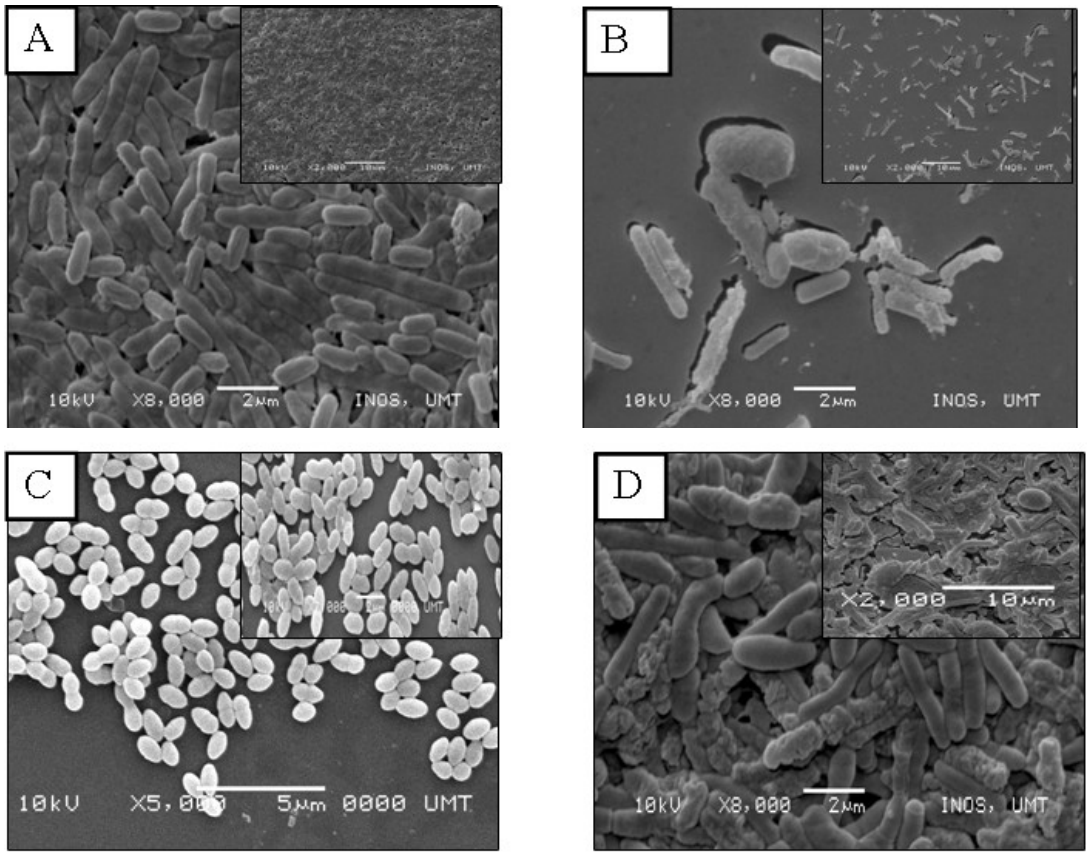

Figure 1. SEM of *P.aeruginosa* and *S.pyogenes* with and without *Trigona* honey. (A) *P.aeruginosa* (control). (B) *P.aeruginosa* treated with *Trigona* honey. (C) *S.pyogenes* (control). (D) *S.pyogenes* treated with *Trigona* honey. Viewed at 2000x and 80000x magnification. Scale bar 5µm.

Table 2. Genes down regulated in *P.aeruginosa*, detected by RT-qPCR.

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 ($P<0.05$).

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

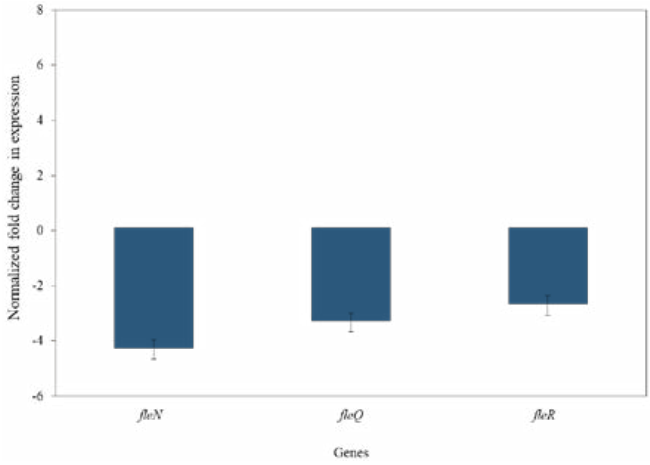

Figure 2. Altered gene expression profiles associated with exposure of *P.aeruginosa* to *Trigona* honey as determined by RT-qPCR. Mean values of fold changes (±SD) are shown in relation to untreated (control). Error bars denote standard error of the mean from three biological samples.

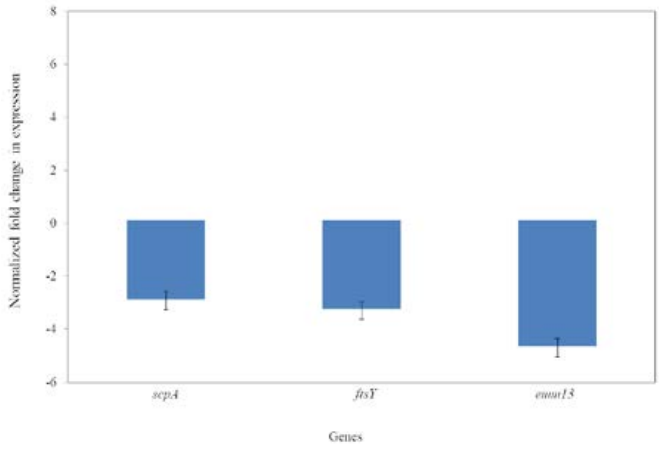

Figure 3. Altered gene expression profiles associated with exposure of *S.pyogenes* to *Trigona* honey as determined by RT-qPCR. Mean values of fold changes (±SD) are shown in relation to untreated (control). Error bars denote standard error of the mean from three biological samples.

Table 3. Genes down regulated *S.pyogenes*, detected by RT-qPCR.

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. *emm*13	2.22	0.22	-4.65	0.02*	1.6

*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 emm13 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, cocci, cubes or spirals. Electron microscopes development provides new insights into bacterial ultrastructural studies and bacterial organization. Scanning electron microscope (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 aerus*, *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

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 omani 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*, *ftsY,* 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*, *ftsY,* 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 *sfbl* 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 *yjfO* (*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 *sfbl* 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/Vfr signaling pathway) and bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP signaling pathway) (**Fig. 4**). A previous study by

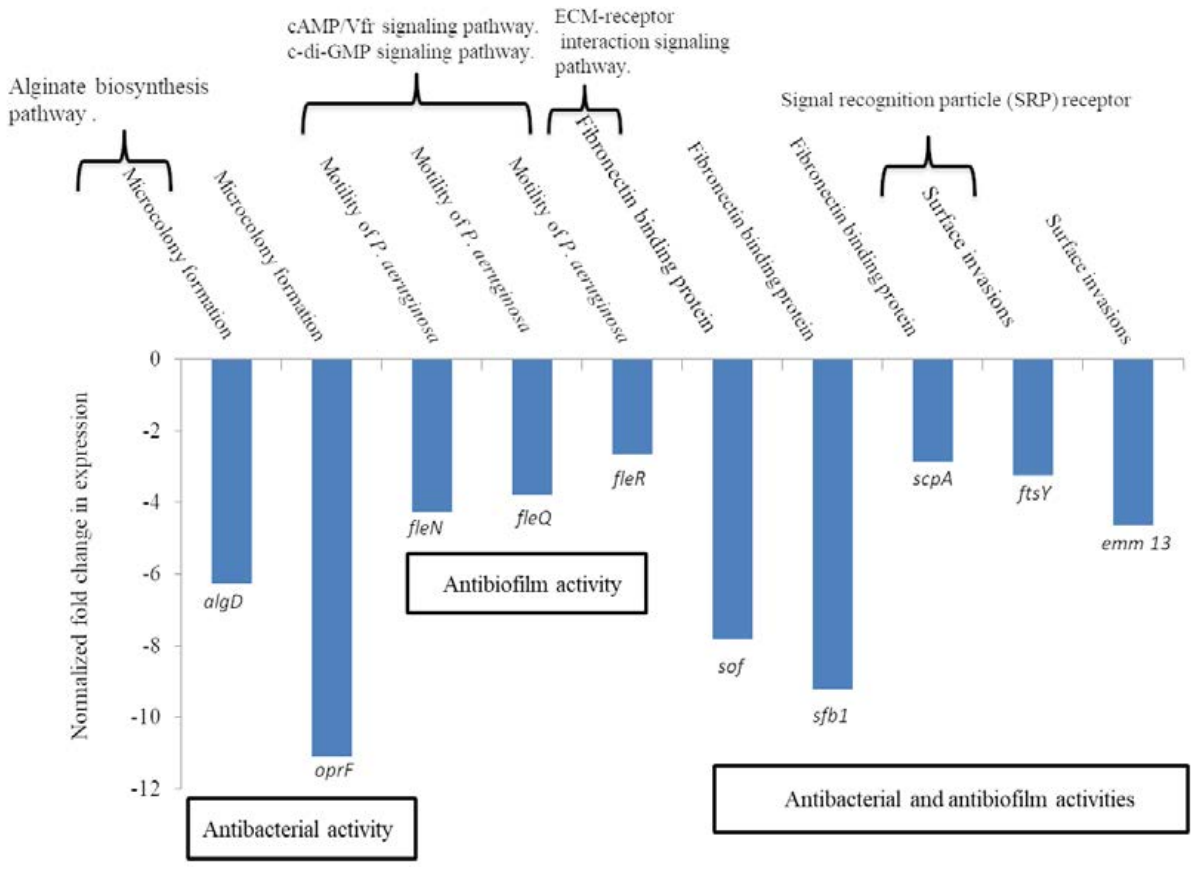

Figure 4. Gene expression profile in *P. aeruginosa* and *S.pyogenes* following *Trigona* honey treatment.

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 *sfbl*) (**Fig. 4**) (47). The *ftsY, scpA,* and *emm*13 in *S. pyogenes* were inhibited following exposure to *Trigona* honey. *Trigona* honey may reduce the multispanning membrane protein, affecting the signal recognition particle pathway (**Fig. 4**).

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