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ORIGINAL ARTICLE

A comparative study of antibacterial and antivirulence activities of four selected honeys to Manuka honey

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ABSTRACT

Background and Objectives: Honey has excellent antibacterial properties against various microorganisms of several different species. To date, there is no comparative evaluation of the antibacterial activity of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (MH). The purpose of this study was to conduct such study and to compare the antibacterial activity of JH, KMH, GH, and AH with that of MH against *Pseudomonas aeruginosa* and *Streptococcus pyogenes.*

Materials and Methods: Activity was assessed using broth microdilution, time kill viability, microtiter plate, scanning electron microscope (SEM) and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR).

Results: The susceptibility tests revealed promising antibacterial activities of all honeys against both bacteria. The MICs of JH, KMH, GH, and AH ranged from 20% to 25% compared to MH (12.5%) against both bacteria. The MBCs of JH, KMH, GH, and AH ranged from 20% to 50% compared to MH (20%) against both bacteria. Treatment of both bacteria with 2× MIC (Minimum inhibitory concentration) of MH, JH, KMH, GH, and AH for 9 hours resulted in reduction in colony-forming unit (CFU/ml). SEM images showed that the morphological changes, cell destruction, cell lysis and biofilm disruption in both bacteria after exposure to all honeys. RT-qPCR analysis revealed that the expression of all genes in both bacteria were downregulated following treatment with all honeys. Among the all-tested honeys, MH showed the highest total antibacterial and antivirulence activities.

Conclusion: Our results indicate that all honeys activity included inhibition of both bacteria due to a decrease in expression of essential genes associated with both bacteria, suggesting that all honeys could potentially be used as an alternative therapeutic agent against certain microorganisms particularly against *P. aeruginosa* and *S. pyogenes.*

Keywords: Honey; *Pseudomonas aeruginosa*; *Streptococcus pyogenes*; Gene expression profiling; Real-time polymerase chain reaction

INTRODUCTION

The biofilm trait of high antimicrobial resistance to antibiotics and disinfectants is a multifactorial and is attributed to slow antibiotic penetration, reduced microbial growth rates, persisters and unique physiology (1). Bacterial biofilms are normally pathogenic and can cause nosocomial infections. The National Institutes of Health (NIH) reported that among all microbial and chronic infections, 65% and 80%, re-

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spectively, are associated with biofilm development (2). Bacterial infections are becoming more difficult to treat due to higher numbers of patients with multiple underlying conditions and the rise in pathogens, which are resistant to modern antimicrobial treatments (3). This is difficult with a rarity of new antibiotics in development and has urged renewed interest in several novel antimicrobial therapeutics. Part of the challenge in treating bacterial infections is biofilm formation. When bacteria exist as a biofilm, they are significantly less sensitive to antibiotics; this is a result of metabolic changes to cells within the biofilm and structural features influencing drug permeability (4). The development and range of antibiotic resistance are an alarming threat to the effective treatment and inhibition of bacterial infections in humans and animals (4). Solving this problem requires searching for natural antimicrobial alternatives (5). Presently, more researchers are turning their attention to conventional medicines as a possible source of antimicrobial agents (6). Honey is one of the oldest traditional remedies that has been extremely reputed and extensively utilised for the treatment of various human infections for over 2000 years ago (7). Nowadays, different kinds of honey have been used in several nations as an alternative to pharmaceutical products for treating infected, burn wounds and contaminated. The antimicrobial properties of honey may be attributed to many factors, including high osmolarity, acidity, in addition to the presence of hydrogen peroxide (H_2O_2) and non-peroxide components, such as methylglyoxal (8). Honey's composition is reliant on the environmental and geographical areas from which the original nectar was collected (8). This is attributed to natural variations in floral sources and climatic conditions at different locations (8). Therefore, several researchers have investigated the therapeutic effects of kinds of honey obtained from different geographical areas worldwide (9, 10). In addition, 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, virulence factor production, as well as multicellular behaviors, such as biofilm formation, and quorum sensing (11, 12). The present study aimed to investigate the effects of five kinds of honey on *P. aeruginosa* and *S. pyogenes* with a view to better understanding its potential to impact virulence and to compare the antibacterial activity of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (UMF +10 (MH).

MATERIALS AND METHODS

Honey samples. Manuka honey (UMF +10 (MH), Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) were purchased from commercial supplier. The samples were packed and sealed in amber glass bottles and stored at 4ºC in the dark until processed (13).

Microorganisms and culture conditions. A reference strains of *P. aeruginosa* ATCC 15692 and *S. pyogenes* ATCC 49399 were obtained from the American Type Culture Collection (ATCC). *P. aeruginosa* and *S. pyogenes* were stored at -80ºC in nutrient broth (NB) medium (Oxoid, UK) with 20% (v/v) glycerol. Prior to each assay, *P. aeruginosa* and *S. pyogenes* strains were sub-cultured from the frozen stock preparations onto nutrient agar (NA) plates (Oxoid, UK). The plates were incubated at 37ºC for 24 hours. Pure liquid cultures (pre-inocula) of *P. aeruginosa* and *S. pyogenes* were maintained in NB (13, 14).

Agar well diffusion assay. The inoculum density of *P. aeruginosa* and *S. pyogenes* was adjusted to be 0.5 McFarland. A sterile cotton swab was dipped into the bacterial suspension and was rotated onto the tube with firm pressure to remove excess fluid. The swab was streaked over the entire surface of Muller Hinton agar plate (Oxoid, UK) for three times and each time the plate was rotated approximately 90ºC to ensure even distribution. A sterile 9 mm cork borer was used to create six wells of agar plate. The wells of agar plate were added with 150 µL of 100%, 75%, 50%, and 25% (w/v) concentrations of MH, JH, KMH, GH and AH. Distilled water was used as a negative control. The plates were incubated at 37ºC for 24 hours. Digital venire calliper was used to measure the zones of inhibition (13, 14).

Minimum inhibitory concentration (MIC). The concentrations of MH, JH, KMH, GH and AH; 50%, 25%, 12.5%, 6.25%, 3.125% and 1.562% (w/v) were freshly prepared with NB broth. The minimum inhibitory concentration (MIC) value was determined using broth microdilution method. Briefly, the cell density for both bacteria was adjusted to be 1×10^8

CFU/mL. A 100 µL was transferred into microtiter plate with 100 µL of each concentration of MH, JH, KMH, GH and AH. Broth medium only was used as negative controls and inoculum without honey was served as positive controls. The plates were incubated overnight at 37°C. Absorbance was measured by using the microtiter plate reader (Tecan Infinite 200 PRO, Austria) at 540 nm. The MIC_{50} and MIC_{90} were determined by using the following formula as mentioned below (13, 14).

$$\text{Growth inhibition (\%)} = \frac{\text{1-OD of the test well – OD of corresponding negative control}}{\text{OD of bacterial growth control−OD of sterility control}} \times 100$$

Minimum bactericidal concentration (MBC). MBC test was performed after MIC assay via streak plate method. A 20 µL from each well of the microdilution method was taken and plated onto NA plates. Subsequently, the plates were incubated for 24 hours at 37°C. MBC was considered as the lowest antimicrobial concentration that produced no colony growth (13, 14).

Time-kill studies. The inhibitory concentration (2×MIC) of MH, JH, KMH, GH and AH that was chosen for subsequent experiments was 2×MIC, because it was two times the MIC. The effect of MH, JH, KMH, GH and AH on the viability of the cells was determined by time-kill curve studies. By inoculating 100 µL of 1×10^6 CFU/mL of both bacteria into 10 mL NB with and without 2×MIC of each honey. Then, the samples were incubated at 37°C in a shaking water bath (100 rpm) for 9 hours. After incubation time, the samples were collected every 3 hours up to 9 hours. Then, the mean of Log10 CFU/ml over time were plotted for each sample. Subsequently, the log reduction (LR) was calculated for each sample by subtracting the Log_{10} CFU at zero time and the Log_{10} CFU at 9 hours of incubation to determine the TVCs (13, 14).

Biofilm assessment. Different concentrations of MH, JH, KMH, GH and AH; 15%, 30%, 45%, and 60% (w/v) in NB were freshly prepared from a stock solution of 100% (w/v). Both bacteria were adjusted to be 0.5McFarland within 0.05 to 0.10 at 600 nm wavelength using spectrophotometer. Then, 200 µl of the culture was dispensed into wells of microtiter plate and incubated at 37°C for 48 hours. After biofilms were formed, 100 µL of planktonic cells were removed and replaced with 100 µL of each honey concentrations. Then, the plates were incubated for overnight (18 hours). Biofilm without honey treatment was served as a positive control, broth only was employed as a sterility control, and honey with broth was served as a corresponding negative control. Finally, after incubation time was done, the media were then removed by invertip the plate and tapping the plate. The plate was washed three times with PBS to remove free-floating planktonic bacteria and was then drained inverted for drying. The plates were stained with 200 µl of 0.1% crystal violet for 5 minutes. Then, the plates were rinsed under running tap water to remove excess stain and were dried at room temperature before solubilizing the biofilm with 95% of ethanol. Microplate reader (Tecan Infinite 200 PRO, Austria) was used to measure the optical density at 595 nm wavelength. Percentage of biofilm degradation was calculated by following formulas as described below (13, 15).

$$\text{Biofilm (\%)} = \frac{(OD_{595}\text{ of positive control})-(OD_{595}\text{ of individual or (combined) antimicrobial})}{(OD_{595}\text{ of positive control})} \times 100\%$$

Scanning electron microscope (SEM) of single-species biofilm. *P. aeruginosa* and *S. pyogenes* were cultivated in NB for 24 hours at 37°C and adjusted to be equal 0.5McFarland. Centrifugation at 3,500g for 10 min at room temperature was used to collect cells and suspended in NB with MICs of MH, JH, KMH, GH and AH for 24 hours. Inoculums without adding honey were used as a positive control. Pellets were collected, fixed overnight with 2.5% (v/v) glutaraldehyde in 0.01 M phosphate buffer solution (PBS). 0.1 M sodium cacodylate buffer were used to rinse the samples. After that, 1% osmium tetroxide in 0.1 M sodium cacodylate buffer were used to rinse the samples. 0.1 M sodium cacodylate were used again to rinse the samples. Subsequently, 0.01 PBS was used to wash the samples and underwent serial dehydration with ascending concentrations of ethanol and subjected to critical point drying. The samples were coated with platinum, placed onto the copper stage holder and examined by scanning electron microscope (SEM) (JEOL 6360LA, Japan) (13).

Scanning electron microscope (SEM) of mixed-species biofilm. The effects of MH, JH, KMH, GH and AH on mixed-species biofilm was determined using SEM. Briefly, *P. aeruginosa* and *S.*

pyogenes cell suspensions were adjusted to be equal to 0.5 McFarland, 1:1 mixed-species were prepared in sterile NB and 200 µL of this standard, cell suspension was added into microtiter plate and then incubated for 24 hours at 37°C. After incubation time was done, the liquid phase was replaced by 200 µL of MIC of each honey. Biofilm mixed-species without honey treatment was used as a positive control. Then, the plates were incubated for 24 hours at 37°C. Subsequently, all samples were then centrifuged at 3500 rpm for 5 minutes. SEM procedure was followed as described earlier. The samples were then viewed by SEM (13).

RNA extraction for RT-qPCR. A0.5 McFarland of *P. aeruginosa* and *S. pyogenes* cells were treated with MIC of MH, JH, KMH, GH and AH. Meanwhile, positive control was included inoculum without honey. Then, the incubation time was performed at 37ºC for 8 hours in a shaking (100 rpm). Subsequently, one ml of treated and untreated cells was separated and centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded and the pellet was washed with PBS. Total RNA extracted using kit SV Total RNA Isolation System (Promega, UK) according to the manufacturer's instructions. Total RNA concentrations were examined by ImplenNanoPhotometer® NP80. Total RNA samples were converted to cDNA according to the manufacturer's instructions (Promega, UK). Samples were diluted to 100ng/µl using ultra-pure water. 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 and 2. 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 StepOne Software v2.3. To determine and calculate the level of gene expression, a modified $2^{-\Delta\Delta}$ Ct method was used (13, 16-18).

Statistical analysis. For all assays, all experiments were carried out in triplicate. All data were expressed as mean ± standard deviation. Independent student t-test from (SPSS version 20) was used to compare between treated and untreated groups. The statistical analyses performed were considered significant when $P< 0.05$.

RESULTS

Agar well diffusion assay. Inhibition zone for MH, JH, KMH, GH and AH against *P. aeruginosa* and *S. pyogenes* is mentioned in Tables 3 and 4. All tested honeys were observed to have antibacterial activity against both bacteria. In general, all tested honeys showed a measurable antibacterial activity on both bacteria with different values. MH, JH, KMH, GH and AH showed a significant inhibition zone against both bacteria at 100%, 75%, 50% and 25% concentrations.

Determination of MICs, $MICs_{90}$, $MICs_{50}$ and MBCs. As shown in Table 5, the MIC value for MH, JH, KMH, GH and AH against planktonically grown *P. aeruginosa* was 12.5%, 25%, 20%, 20% and 20% (w/v) respectively. The MBC value for MH, JH, KMH, GH and AH against planktonically grown *P. aeruginosa* was 20%, 50%, 25%, 25% and 50% (w/v) respectively. In addition, the MIC_{90} for MH, JH, KMH, GH and AH against planktonically grown *P. aeruginosa* was 20%, 50%, 25-50%, 25% and 50% (w/v) respectively. The MIC_{50} for MTH against planktonically grown *P. aeruginosa* was 12.5%, 20%, 20-25%, 20% and 25% (w/v) respectively.

From Table 6, the MIC value for MH, JH, KMH, GH and AH against planktonically grown *S. pyogenes* was 12.5%, 25%, 20%, 20% and 20% (w/v) respectively. The MBC value for MH, JH, KMH, GH and AH against planktonically grown *S. pyogenes* was 20%, 50%, 25%, 50% and 50% (w/v) respectively. In addition, the MIC_{90} for MH, JH, KMH, GH and AH against planktonically grown *S. pyogenes* was 25%, 50%, 25-50%, 25-50% and 50% (w/v) respectively. The MIC_{50} for MTH against planktonically grown *S. pyogenes* was 20%, 20%, 20-25%, 20% and 25% (w/v) respectively.

Time-kill studies. The total number of *P. aeruginosa* cells significantly decreased when exposed to 2×MIC MH, JH, KMH, GH, and AH. However, *P. aeruginosa* incubated with 2×MIC MH, JH, KMH, GH, and AH demonstrated rapid loss of viability. Therefore, after exposure to 2×MIC of MH, JH, KMH, GH and AH, *P. aeruginosa* resulted in 1.7-$\log_{10}$, 1.3-$\log_{10}$, 1.5-$\log_{10}$, 1.4-$\log_{10}$ and 1.2-$\log_{10}$ reduction in CFU/ml compared to untreated cells at 6 hours incubation ($P<0.05$) respectively. *P. aeruginosa* incubated with 2×MIC MH, JH, KMH, GH, and AH demonstrated that the greatest bactericidal activ-

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

Gene name	Amplicon Size (bp)	Annealing temp (C°)	Direction	Primer sequence (5' → 3')	References
*fli*A	132	55	Forward	CTCCAATTGAGCCTCGAAGA	(13, 19)
			Reverse	TTCGTTGTGACTGAGGCTGG	
*fli*C	121	55	Forward	GCTTCGACAACACCATCAAC	(13, 19)
			Reverse	AGCACCTGGTTCTTGGTCAG	
*flh*F	127	54	Forward	CGAGCCTGAACGTGAAGAAT	(13, 19)
			Reverse	GCCTCGTCCAGCTTAGTCA	
*fle*N	137	56	Forward	GAGCCGTATACGAGGCATTC	(13, 19)
			Reverse	GTGTTGGACCAGTCGTTCG	
*fle*Q	134	54	Forward	AAGGACTACCTGGCCAACCT	(13, 19)
			Reverse	CCGTACTTGCGCATCTTCTC	
*fle*R	109	55	Forward	ACAGCCGCAAGATGAACCT	(13, 19)
			Reverse	TGGATGGCGTTGTCGAGTT	
*rpo*D*	146	53	Forward	GCGACGGTATTCGAACTTGT	(13, 19)
			Reverse	CGAAGAAGGAAATGGTCGAG	

*Reference gene

Table 2. Gene specific primers of *S. pyogenes* used for RT-qPCR analysis

Gene name	Amplicon Size (bp)	Annealing temp (C°)	Direction	Primer sequence (5' → 3')	References
Sof	873	57	Forward	ACTTAGAAAGTTATCTGTAGGG	(13, 19)
			Reverse	TCTCTCGAGCTTTATGGATAG	
*sfb*I	960	55	Forward	AACTGCTTTAGGAACAGCTTC	(13, 19)
			Reverse	CCACCATAGCCACAATGCT	
*scp*A	622	55	Forward	GCTCGGTTACCTCACTTGTCC	(13, 19)
			Reverse	CAATAGCAGCAAACAAGTCACC	
*fts*Y	97	54	Forward	TCGAAAATTCTTTGGCCTGT AT-	(13, 19)
			Reverse	CAAACGTGTTGTGCCAGA	
*glr**	797	54	Forward	ATGGATACAAGACCAATTGG	(13, 19)
			Reverse	TCATAAGGTGACATGCTCCAC	

*Reference gene

Table 3. Antibacterial activity (Inhibition Zone (mm) ± SD) of all tested honeys at different concentrations against *P. aeruginosa*

Honey samples	100%	75%	50%	25%
MH	25.3 ± 0.6	21.4 ± 0.1	18.6 ± 0.2	14.6 ± 0.3
JH	19.2 ± 0.4	17.2 ± 0.4	14.1 ± 0.6	13.0 ± 1.0
KMH	25.1 ± 0.6	21.3 ± 0.5	17.1 ± 0.4	11.6 ± 0.3
GH	20.2 ± 0.4	16.0 ± 0.6	12.1 ± 0.5	11.5 ± 0.2
AH	19.7 ± 0.5	18.0 ± 0.4	12.1 ± 0.6	11.4 ± 0.1

Table 4. Antibacterial activity (Inhibition Zone (mm) ± SD) of all tested honeys at different concentrations against *S. pyogenes*

Honey samples	100%	75%	50%	25%
MH	25.1 ± 0.5	21.2 ± 0.1	18.4 ± 0.3	14.1 ± 0.4
JH	18.4 ± 0.5	16.1 ± 0.2	13.1 ± 0.5	12.0 ± 0.8
KMH	24.1 ± 0.2	20.1 ± 0.3	16.7 ± 0.3	10.8 ± 0.7
GH	19.4 ± 0.2	15.4 ± 0.1	11.7 ± 0.4	10.6 ± 0.5
AH	18.6 ± 0.7	17.2 ± 0.2	11.6 ± 0.3	10.3 ± 0.3

Table 5. MIC, MIC_{90}, MIC_{50} and MBC of all tested honeys against *P. aeruginosa*

Honey samples	MIC % (w/v)	MIC_{90} % (w/v)	MIC_{50} % (w/v)	MBC % (w/v)
MH	12.5%	20%	12.5%	20%
JH	25%	50%	20%	50%
KMH	20%	25-50%	20-25%	25%
GH	20%	25%	20%	25%
AH	20%	50%	25%	50%

Table 6. MIC, MIC_{90}, MIC_{50} and MBC of all tested honeys against *S. pyogenes*

Honey samples	MIC % (w/v)	MIC_{90} % (w/v)	MIC_{50} % (w/v)	MBC % (w/v)
MH	12.5%	25%	20%	20%
JH	25%	50%	20%	50%
KMH	20%	25-50%	20-25%	25%
GH	20%	25-50%	20%	50%
AH	20%	50%	25%	50%

ity at 9 h incubation with >3-log_{10} killing unit for MH and JH and with >2.5-log_{10} for KMH, GH and AH. The change in cell count in *P. aeruginosa* treated and untreated cells was statistically significant (Fig. 1).

The number of *S. pyogenes* cells decreased after following treatment with 2×MIC MH, JH, KMH, GH, and AH with 1.7-log_{10}, 1.3-log_{10}, 1.3-log_{10}, 1.5-log_{10}, and 1.3-log_{10}, reduction in CFU/ml (≈99% killing) respectively at 6 h. The mean difference between treated and untreated *S. pyogenes* cells was statistically significant (P <0.05). However, 2×MIC MH, JH, KMH, GH, and AH achieved a 2.5-log_{10}, 2.3-log_{10}, 2.2-log_{10}, 2.4-log_{10}, and 2.7-log_{10} reduction (≈99% killing) in *S. pyogenes* population at 9 h (Fig. 1).

Sub-inhibitory concentrations of all tested honeys decreased the biofilm biomass. The average of optical density (OD) for control sample and tested sample of biofilm mass was calculated. The effect of MH, JH, KMH, GH and AH on biofilm biomass varied depending on the MH, JH, KMH, GH and AH concentrations. A statistically significant (P<0.05). When MH, JH, KMH, GH and AH were used at the 15% (w/v), the optical density of *P. aeruginosa* biofilm biomass was reduced to 1.8, 2.1, 2.2, 2.3, and 2.3 respectively compared to untreated biofilm. However, at 30% (w/v) MH, JH, KMH, GH and AH, the optical density of *P. aeruginosa* biofilm biomass was reduced to 0.9, 1.5, 1.6, 1.7, and 1.8 respectively compared to untreated biofilm. Meanwhile, the optical density of *P. aeruginosa* biofilm biomass was reduced to 0.5, 1.0, 1.1, 1.3, and 1.4 respectively compared to untreated biofilm at 45% (w/v). At 60% (w/v) MH, JH, KMH, GH and AH was more effective at reducing the optical density of *P. aeruginosa* biofilm biomass by 0.3, 0.5, 0.6, 0.8 and 0.8 respectively compared to untreated biofilm (Fig. 2).

In the presence of 15%, 30%, 45% and 60% (w/v) MH, JH, KMH, GH and AH concentrations, the optical density of established *S. pyogenes* biofilms was significantly (P < 0.05) decreased compared to untreated biofilm. After MH, JH, KMH, GH and AH was used at the 15% (w/v), the optical density of established *S. pyogenes* biofilms was reduced to 1.7, 1.9, 2.1, 2.2, and 2.2 respectively compared to untreated biofilm. However, at 30% (w/v) MH, JH, KMH, GH and AH, the optical density of established *S. pyogenes* biofilms was reduced to 0.8, 1.4, 1.5, 1.6, and 1.7 respectively compared to untreated biofilm. Meanwhile, at 45% (w/v) MH, JH, KMH, GH and AH, the optical density of established *S. pyogenes* biofilms was reduced to 0.4, 0.9, 1.0, 1.2, and 1.3 respectively compared to untreated biofilm. In addition, at 60% (w/v) MH, JH, KMH, GH and AH was more effective at reducing the optical density of established *S. pyogenes* biofilms by 0.2, 0.4, 0.5, 0.7, and 0.7 respectively compared to untreated biofilm. It was observed that the lowest concentration of MH, JH, KMH, GH and AH prevented *S. pyogenes* to establish biofilm was found to be 15% (w/v). Remarkably, MH was the most effective in preventing formation of *S. pyogenes* and *P. aeruginosa* biofilm. The inhibiting effect of MH, at low concentrations (15%), on the formation of *S. pyogenes* and *P. aeruginosa* biofilm was greater than that of the other honeys (Fig. 2).

Scanning electron microscope (SEM) of single-species biofilm. SEM micrographs of untreated *P. aeruginosa* cells showed that the cells appeared to be rod-shaped with regular structure and hundreds of bacterial cells are connected by a substantial amount of extracellular matrix. Extensive structural changes in biofilms were seen following treatment with all honeys and loss of viability was found, in addition to loss of biofilm structure. SEM images provided reasonable evidence of damage and disruption of

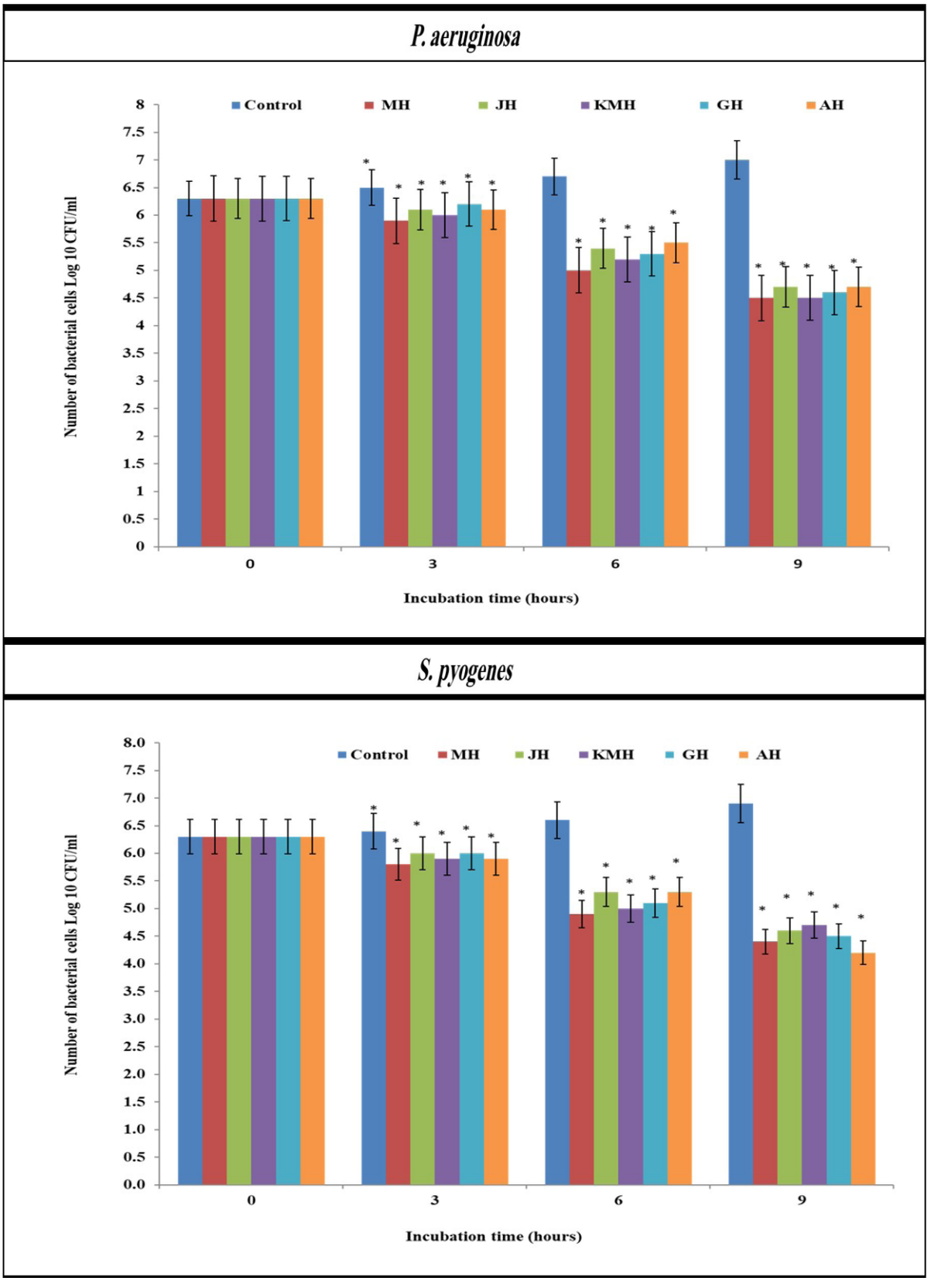


Fig. 1. Time-kill studies of *P. aeruginosa* and *S. pyogenes* after exposed to all honeys. Asterisks; *P<0.05 indicate statistically significant difference between treated and control samples.

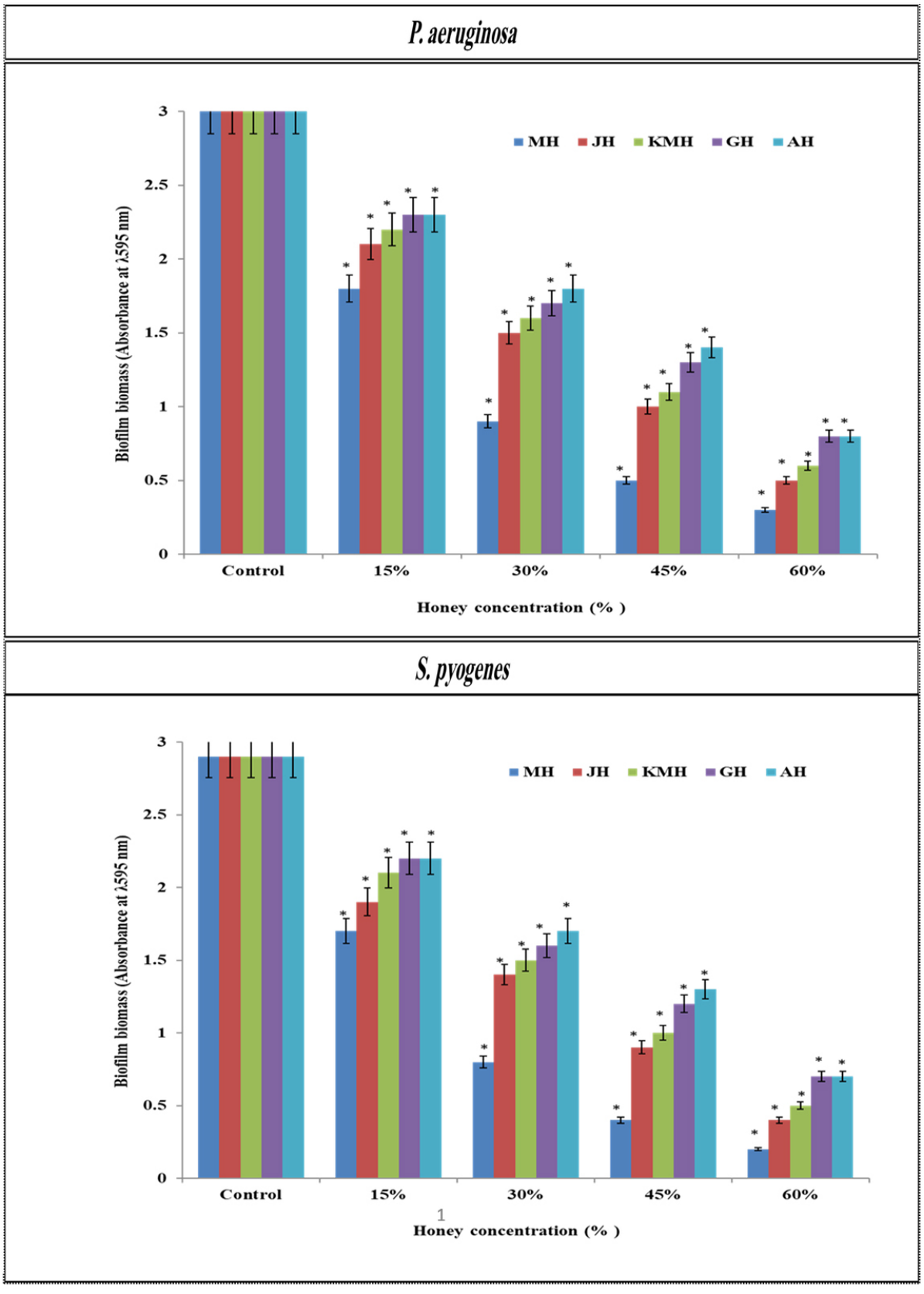


Fig. 2. Biofilm formation of *P. aeruginosa* and *S. pyogenes* grown with and without all honeys. Asterisks; * $P<0.05$ indicate statistically significant difference between treated and control samples.

the integrity of biofilm after exposure to all honeys. In addition, rough cell surfaces were observed after treated with all honeys (Fig. 3).

SEM micrographs of untreated *S. pyogenes* demonstrated the regular cocci with chain structure and *S. pyogenes* biofilm shows numerous cocci cells and diverse thickness connected each other. When *S. pyogenes* following treatment with all honeys the density of biofilm formed were reduced compared to untreated. In addition, several morphological changes, such as changes of cocci shape, abnormal cell division and ruptured cell structure were observed after exposed to all honeys (Fig. 3).

Scanning electron microscope (SEM) of mixed-species biofilm. SEM showed that the surface structure and morphology of mixed-species biofilms formed by *P. aeruginosa* and *S. pyogenes* without honey treatment. The control group of mixed-species biofilms showed the typical multilayer growth of bacterial biofilms, while the group treated with MH, JH, KMH, GH and AH demonstrated that exhibited a reduction of mixed-species biofilm, reduction of cell density, and decrease extracellular matrix compared to control cells for both bacteria. Altogether, the findings provide evidence that MH, JH, KMH, GH and AH have a potent antibiofilm action against the mixed-species biofilm (Fig. 3).

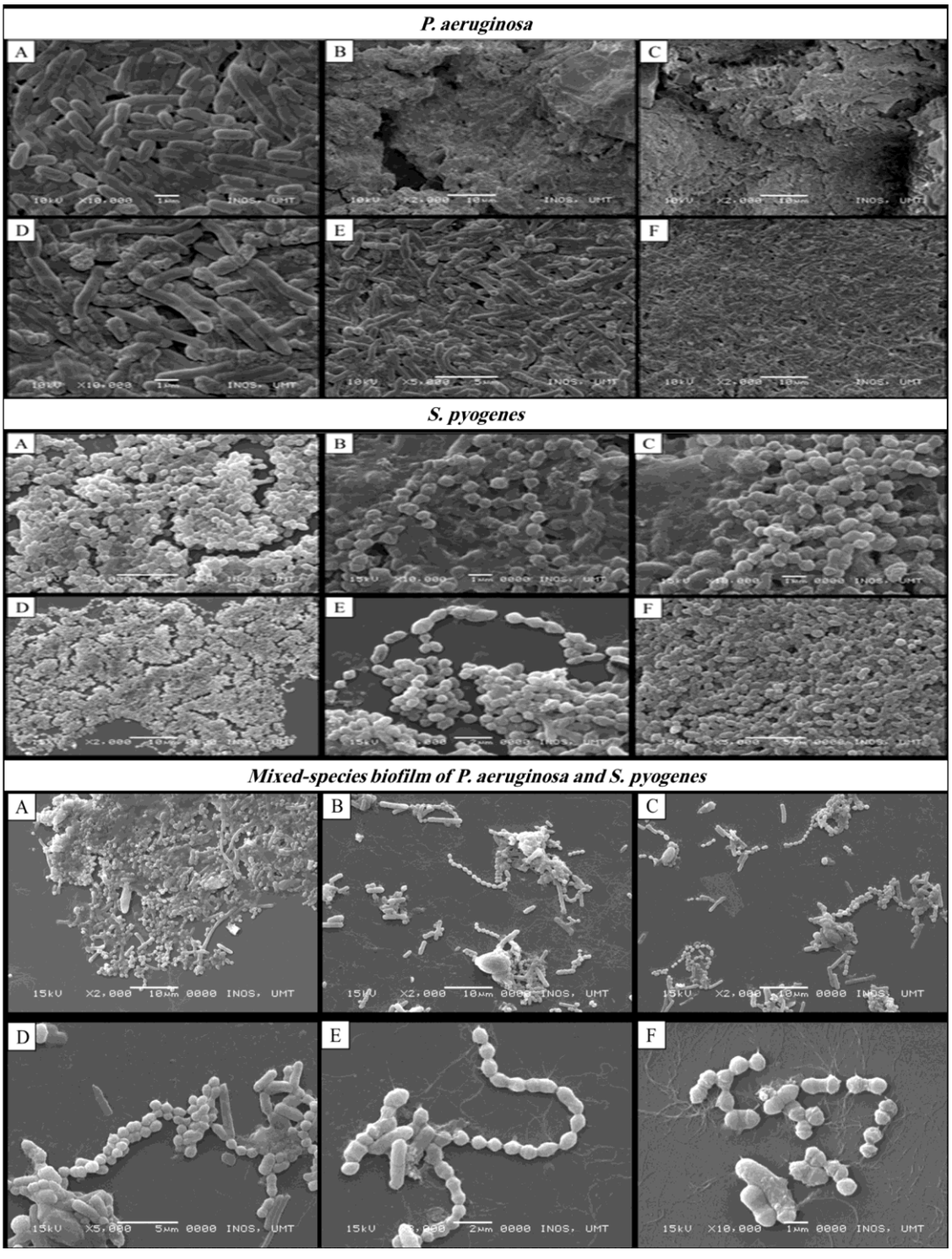
Characterization of virulence factor activity indicated that honeys were able to reduce activity of several key virulence factors. In the present study, RT-qPCR was used to assess and compare the expression of six genes in *P. aeruginosa* that have been previously shown to be involved in the flagella regulon proteins, biofilm formation, motility and virulence of the microorganism and four genes in *S. pyogenes* that have been previously shown to be involved in the fibronectin binding proteins, surface adhesins and biofilm formation after exposure to all honeys.

Effects of five tested honeys on the mRNA expression of *P. aeruginosa*. Following treatment of *P. aeruginosa* with MIC of MH, JH, KMH, GH and AH there were significant reductions ($P<0.05$, $P\leq 0.01$ and $P\leq 0.001$) in the relative abundance of mRNA for *fli*A, *fli*C, *flh*F, *fle*N, *fle*Q and *fle*R when compared to untreated cells. When *P. aeruginosa* treated with MH, the fold change ranged from 3.2-fold to 6.3-fold (for *fle*R, *fli*A, *flh*F, *fle*Q, *fli*C and *fle*N respectively). Also, when *P. aeruginosa* treated with JH, the fold change ranged from 2.7-fold to 4.5-fold (for *fli*A, *fle*R, *flh*F, *fle*Q, *fli*C and *fle*N respectively). In addition, when *P. aeruginosa* treated with KMH, the fold change ranged from 1.9-fold to 4.1-fold (for *fle*R, *fli*A, *fle*Q, *flh*F, *fle*N, and *fli*C respectively). Furthermore, when *P. aeruginosa* treated with GH, the fold change ranged from 1.5-fold to 3.8-fold (for *fle*R, *fli*A, *fle*Q, *flh*F, *fle*N, and *fli*C respectively). When *P. aeruginosa* treated with AH, the fold change ranged from 1.3-fold to 2.8-fold (for *fle*R, *fli*A, *fle*Q, *flh*F, *fle*N, and *fli*C respectively). Comparing MH honey treated *P. aeruginosa* cell samples with JH, KMH, GH and AH, the expression of mRNA transcripts for each gene tested with MH was decreased more than other honeys (Fig. 4).

Effects of five tested honeys on the mRNA expression of *S. pyogenes*. The major genes encoding the surface adhesins for *scpA, ftsY, sfbI,* and *sof* in *S. pyogenes* were downregulated after exposure to MIC of MH, JH, KMH, GH and AH. Following treatment of *S. pyogenes* with MIC of MH, JH, KMH, GH and AH there were significant reductions ($P<0.05$, $P\leq 0.01$ and $P\leq 0.001$) in the relative abundance of mRNA for *scpA, ftsY, sfbI,* and *sof* when compared to untreated cells. When *S. pyogenes* treated with MH, the fold change ranged from 4.4-fold to 6.8-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). Also, when *S. pyogenes* treated with JH, the fold change ranged from 3.6-fold to 5-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). In addition, when *S. pyogenes* treated with KMH, the fold change ranged from 3.7-fold to 6.1-fold (for *scpA, ftsY, sof* and *sfbI* respectively). When *S. pyogenes* treated with GH, the fold change ranged from 3.4-fold to 4.8-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). Whereas, when *S. pyogenes* treated with GH, the fold change ranged from 2.8-fold to 4.3-fold (for *scpA, ftsY, sfbI,* and *sof* respectively). Comparing MH honey treated *S. pyogenes* cell samples with JH, KMH, GH and AH, the expression of mRNA transcripts for each gene tested with MH was decreased more than other honeys (Fig. 4).

DISCUSSION

Antibacterial activity of honey has been broadly discussed among researchers worldwide. It is postulated to be closely on several factors such as, os-



Single-species biofilm of *P. aeruginosa*: Control (A), *P. aeruginosa* treated with MH (B), JH (C), KMH (D), GH (E), and AH (F). Viewed at 10,000×, 5,000× and 2,000× magnification. Scale bar 1µm, 5µm and 10µm.

Single-species biofilm of *S. pyogenes:* Control (A), *S. pyogenes* treated with MH (B), JH (C), KMH (D), GH (E), and AH (F). Viewed at 10,000×, 5,000x and 2,000× magnification. Scale bar 1µm, 5µm and 10µm.

Mixed-species biofilm of *P. aeruginosa* and *S. pyogenes*: Control (A), *P. aeruginosa* and *S. pyogenes* exposure to MH (B), JH(C), KMH (D), GH (E) and AH (F). Viewed at 10,000×, 5,000× and 2,000× magnification. Scale bar 1µm, 2µm, 5µm and 10µm.

Fig. 3. SEM of single and mixed-species biofilm of *P. aeruginosa* and *S. pyogenes* after exposure to all honeys

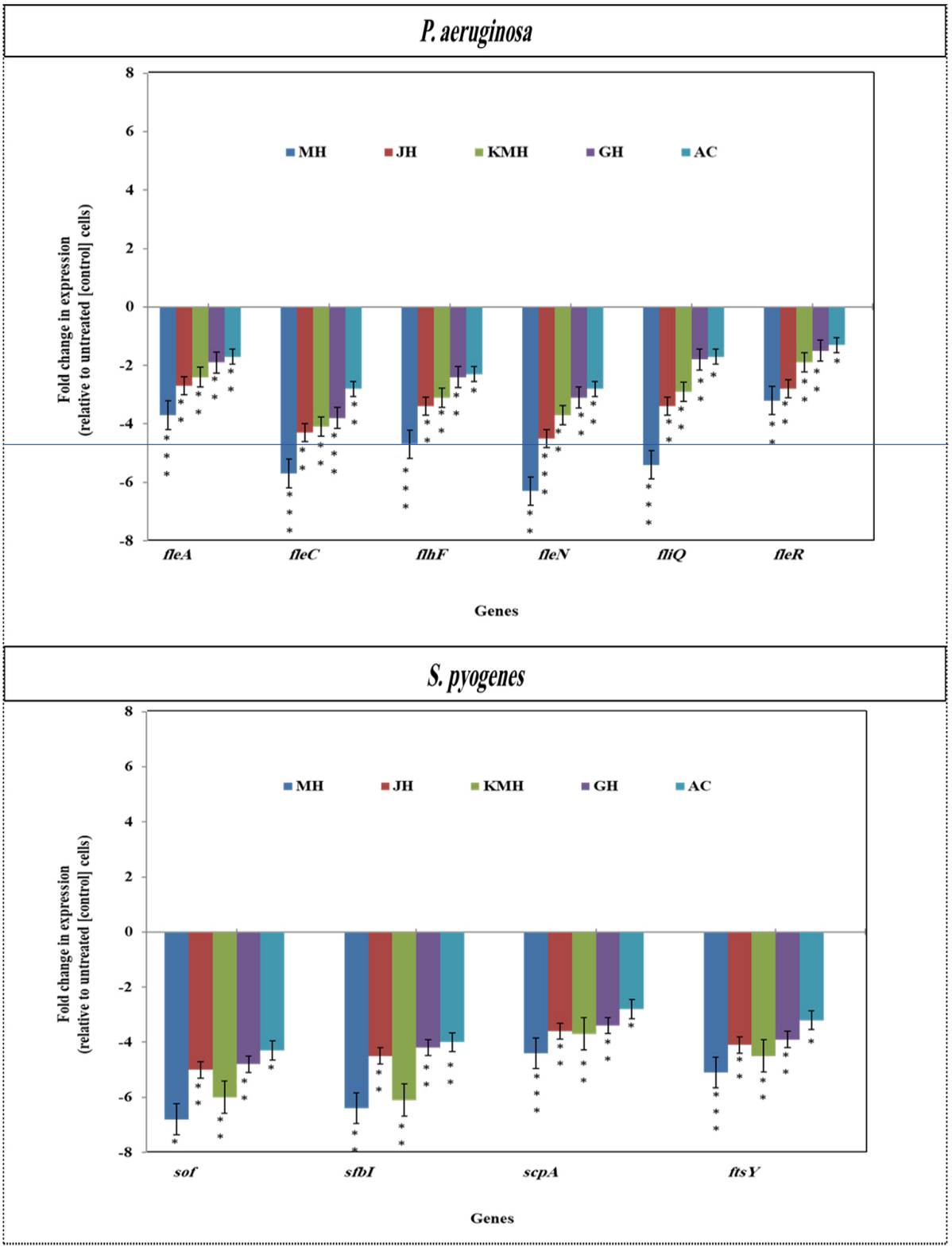


Fig. 4. Changes in gene expression profiles of *P. aeruginosa* and *S. pyogenes* after treated with all tested honeys as determined by RT-qPCR. Mean values of fold changes (± SD) are shown in relation to untreated *S. pyogenes* cells. Asterisks; * $P<0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$ indicate statistically significant difference in the expression of each gene between treated and untreated samples.

molarity, pH and other major constituents such as phenolic acids and flavonoids (20). Previous study showed that 25% concentration of honey exhibited lower antibacterial action (21). The similarly or divergence of results might be due to several reasons such contain different level of active compounds including phenolic acids and flavonoids (22). Limitations of some antibacterial assay such as agar well-diffusion test were reported including the insensitivity in detecting low level of antimicrobial activity, variation in the experimental conditions and permeability of non-polar components (23, 24). Agar well-diffusion test may not be the most appropriate method to evaluate the antibacterial activity of honey. Micro-broth dilution was performed to determine the MIC for antibacterial activity of honey toward all the tested bacteria (25). The lowest concentration of honey solution needed to inhibit 99% of bacterial growth is considered to be MIC. The lowest concentration of honey required to kill at least 99% of the tested bacterial strains is defined as MBC (25). In the current study, the MIC values ranging from 12.5% to 20% against both bacteria and the MBC values ranging from 20% to 50% against both bacteria. Previous studies showed that the MIC for Algerian, Manuka and Egyptian clover honeys against *P. aeruginosa* was at 20% and MBC was at 25% (25-28). Recently, other studies revealed that MIC for Manuka honey on *S. pyogenes* was at 20% and MBC was at 25% (29, 30). A previous study showed that the MIC for Manuka honey against *P. aeruginosa* was at 12% and MBC was at 16% (31). Time-kill studies were used to determine the bactericidal or bacteriostatic actions of antimicrobials (32). It is investigated by plotting $\log_{10}$ CFU/mL over time (13). The $\log_{10}$ CFU/ml for *P. aeruginosa* and *S. pyogenes* exposure to all honeys were seen at 9 hours which is about >3-$\log_{10}$ of both bacteria were killed. All tested honeys were able to decrease biofilm biomass in both bacteria. However, this study found that the higher concentration of all honeys was necessary to complete elimination of established biofilm. Regarding to the results obtained for *P. aeruginosa* and *S. pyogenes* biofilms, a significant reduction was observed after 24 hour's exposure to all honey at all concentrations were used. In the current study, SEM revealed that the morphological changes of cells, cells destruction, cells lysis and biofilm disruption in both bacteria following treatment with all honeys. In addition, SEM images showed that the treated mixed-species biofilm presented several damaged cells in both bacteria compared to untreated mixed-species biofilm. The previous study demonstrated that the structure of *P. aeruginosa* was influenced using Manuka honey (33). A study by Enany et al. (2015) pointed that Sidr honey disrupted the cell of *S. aureus* (34). As demonstrated by RT-qPCR, a number of genes *fli*A, *fli*C, *flh*F, *fle*N, *fle*Q and *fle*R have been previously shown to be involved in the process of microcolony, biofilm formation and motility in *P. aeruginosa* (13, 16). Also, a set of genes have been previously shown to play an important role in the adhesion and biofilm formation and quorum-sensing network of *S. pyogenes*, such as the *sof, sfbl, emm13, scpA* and *ftsY* genes (29). The current results revealed that all selected genes in both bacteria were downregulated following exposure to all honeys. Our results are in agreement with those of (16, 31), who reported downregulation of multiple genes involved in microcolony, motility and biofilm formation in *P. aeruginosa* strain following exposure to manuka honey. Study by Maddocks et al. (2012) reported that downregulation of *sof,* and *sfbl* genes in *S. pyogenes* after exposure to Manuka honey (29). Previous study showed that five genes; *fleN, fleQ, fleR, fliA* and *fliC* in *P. aeruginosa* and five genes; *sof, sfbl, scpA, ftsY* and *emm13* in *S. pyogenes* were reduced in gene expression following treatment with Tualang honey (13). Another 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 treated with 25% concentration of Egyptian honey (6). Study by Roberts et al. (2014) showed that *fliA, fliC, flhF, fleN, fleQ* and *fleR* genes in *P. aeruginosa* were reduced in gene expression after treated with manuka honey (16). 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 treated with 25% concentration of Egyptian honey (6). It was noticed that all these studies that mentioned above are in agreement with our results. This indicates that the honey-induced alterations in the expression of this group of genes are most probably due to particular molecules contained in honey and not only due to their sugar content. Previous study suggested that the osmotic action of sugar combined with hydrogen peroxide and bee-derived antibacterial peptide defensin-1 is crucial for the antibiofilm activity of honey (35). In addition, this change in expression pattern may indicate variations in the phytochemical

components and/or differences in the antimicrobial mechanisms of all honey on both bacteria (30). It is evident that honey is effective at inhibiting the growth of both bacteria, causing abnormal cell by reducing structural integrity to the point of cell lysis as mentioned in SEM results. To our knowledge, this is the first attempt to compare the impacts of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (MH) on the tested organisms at both structural and molecular levels.

CONCLUSION

This is the first attempt study to compare the impacts of Jarrah honey (JH), Kelulut Madu honey (KMH), Gelam honey (GH), and Acacia honey (AH) with that of Manuka honey (MH) on the tested organisms at both structural and molecular levels. A reduction of *P. aeruginosa* and *S. pyogenes* cell growth in both planktonic and biofilm state was observed with all honey treatment. Comparing all honeys tested, for planktonic and biofilm cultures, Manuka honey (MH) had a higher effect on both bacteria. In this study, results indicate that the JH, KMH, GH, and AH may represent promising antibacterial, antibiofilm and anti-virulence agents for treatment and modulation of infections caused by *P. aeruginosa* and *S. pyogenes* compared with MH. Antibacterial and antibiofilm activities of all tested honeys against both bacteria, which were further supported by the morphological and structural investigations. However, understanding the behavior of *P. aeruginosa* and *S. pyogenes* species in polymicrobial biofilms is an important step in the clinical context and for the selection of the most efficient treatment. Because of this, the effect of all honeys was assessed on structure of mixed *P. aeruginosa* and *S. pyogenes* biofilms. The honeys were able to reduce both species in the mixed biofilm and were demonstrated to be a promising alternative for the treatment of infections caused by mixed species biofilms. The use of a natural product such as honey may be used in clinical practice, to prevent or even treat *P. aeruginosa* and *S. pyogenes* infections. This study, suggest that each honey could have a crucial derivatives compound that have the ability to effectively inhibit the biofilms of *P. aeruginosa* and *S. pyogenes.*

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