https://doi.org/10.17221/76/2025-CJFS

Evaluation of antibacterial activities of two types of local Jordanian honey with Manuka honey: A comparative study

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Citation: Al-Kafaween M.A., Al-Qubelat S.A. (2026): Evaluation of antibacterial activities of two types of local Jordanian honey with Manuka honey: A comparative study. Czech J. Food Sci., 44: 16–34.

Abstract: Honey 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. The purpose of this study was to: (*i*) evaluate and compare the antibacterial and antibiofilm activities of Shawkiat and Rabiee honeys with those of Manuka honey against *Escherichia coli* and *Staphylococcus aureus*; (*ii*) assess the anti-virulence potential of these honeys, by examining their impacts on the expression of five selected genes in *E. coli* and seven genes in *S. aureus* that have been previously shown to be involved in the microcolony, biofilm and virulence in the test organism. The antibacterial, antibiofilm and anti-virulence activities of these honeys against both bacteria were investigated by agar well diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), growth curve, time-kill curve, microtiter plate and reverse transcription-quantitative real time polymerase chain reaction (RT-qPCR). The susceptibility tests showed promising antibacterial activities of Shawkiat and Rabiee honeys compared with Manuka honey against *E. coli* and *S. aureus*. The results showed that Manuka honey possessed the lowest MIC value against both bacteria with 20% (w/v) MIC and MBC of 25% (w/v). Slightly higher MIC values were shown by Shawkiat and Rabiee honeys against both bacteria with 25% (w/v) MIC and 50% (w/v) MBC values compared with Manuka honey. Growth curves demonstrated no growth of the two bacteria after treatment with MIC of all the tested honeys. Shawkiat and Rabiee honeys showed that both bacteria lost viability comparably with Manuka honey. The lowest concentration of Shawkiat and Rabiee honeys was able to inhibit and eradicate the biofilm of both bacteria compared with Manuka honey. The qPCR analysis showed that the expression of all the selected genes in both bacteria after treatment with all the tested honeys was downregulated and shared a similar overall pattern of gene expression, with a trend toward reduced expression of the virulence genes of interest. The results of this study indicate that Jordanian honeys possess antibacterial, antibiofilm and anti-virulence activities comparably with Manuka honey. This study revealed that Jordanian honey compared with Manuka honey inhibits *E. coli* and *S. aureus* planktonic cells and biofilm through the downregulation of genes required for growth, biofilm formation and motility.

Keywords: antibiofilm; honey; *Staphylococcus aureus*; *Escherichia coli*; RT-qPCR

Honey is one of the oldest traditional remedies known to humanity, with a history extending over thousands of years across diverse cultures and civilizations (Kuropatnicki et al. 2018). Honey has been used not only as a food source but also as a therapeutic agent; as wound care, skin injuries, gastro and respiratory infections and other various ailments (Nikhat and Fazil 2022). The potential of honey healing was recognised long before the arrival of modern medicine (Kuropatnicki et al. 2018; Al-Kafaween et al. 2022). Honey is a versatile substance with a wide range of uses due to its unique composition including bioactive compounds, sugars, amino acids, enzymes, vitamins and minerals (Tafere 2021; Kumar 2024).

Supported by Al-Zaytoonah University of Jordan (Grant Number: 10/08/2023-2024).

https://doi.org/10.17221/76/2025-CJFS

These components vary based on the flower source, geographic origin and honey processing methods (Wang et al. 2022). The rich composition of bioactive compounds in honey mainly contributes to its diverse health benefits, including antioxidant, antibacterial and anti-inflammatory properties (Al-Kafaween et al. 2022, 2023). Recently, due to the spread of antibiotic resistance and the limitations of conventional antimicrobial therapies, scientific interest in alternative treatments including honey has been increased (Israili 2014). The resistant strains of bacteria have the ability to withstand the effects of antibiotics, complicate the treatment protocols and increase the risk of severe infections and mortality. Biofilm is a complex of single or multiple types of microorganisms which are integrated and attached to the surface and covered with the extracellular polymeric substance (EPS) matrix (Sharma et al. 2014; Dang and Lovell 2016). It acts as a physical barrier to prevent the external conditions and antibiotics from reaching the bacteria and reduce their effectiveness, which complicates the infection management and further contributes to the resistance (Lu et al. 2019; Abu-Sini et al. 2023). Biofilm contributes to the chronic injuries, medical devices-associated infections, and other persistent infections that make serious challenges to medical community (Sharahi et al. 2019; Di Domenico et al. 2022). Honey has various therapeutic applications, but its role in managing biofilm formation and biofilm-related resistance has gained high attention (Gebreyohannes et al. 2019; Jiang et al. 2020; Mishra et al. 2023). Manuka honey is a unique type of monofloral honey, produced primarily in New Zealand and parts of Australia from the nectar of the plant *Leptospermum scoparium*, commonly known as the Manuka tree (Meister et al. 2021). Characterised by its rich, dark colour and slightly bitter earthy flavour the Manuka honey is widely recognised for its exceptional medicinal and nutritional properties (Patel and Cichello 2013; Al-Kafaween et al. 2023). Its global reputation stems from its antibacterial activity, which is primarily attributed to its methylglyoxal (MGO) content, along with other bioactive compounds such as phenols, flavonoids, and hydrogen peroxide (Nolan et al. 2020; Feng 2023). Scientific studies have highlighted its effectiveness in healing wounds, reducing inflammation, soothing sore throats, and inhibiting the growth of antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Nolan et al. 2020; Al-Kafaween et al. 2023; Feng 2023). Manuka honey is used in both traditional remedies and modern medical products, making it a valuable natural resource in functional foods and healthcare. Its quality and effectiveness are often assessed using the Unique Manuka Factor (UMF) or MGO rating systems, ensuring its authenticity and therapeutic efficacy (Jahangir et al. 2020; Sinha et al. 2023). Shawkiat honey, also known as thistle or 'Shawkīyāt' honey in Arabic, is a type of natural honey produced by bees that forage on nectar from wild thorny plants and other thistle-like flora commonly found in regions like southern Jordan (Al-Kafaween et al. 2022, 2023). Shawkiat honey is celebrated for its rich, dark red hue and delightful taste, often accompanied by a refreshing aroma. Shawkiat honey has antioxidants, essential vitamins, minerals, and simple sugars; this honey offers a host of potential health benefits and supports digestion and alleviates gastrointestinal issues (Al-Kafaween et al. 2023; Tanuğur et al. 2024). Rabiee honey is a type of natural honey produced from the nectar of various wildflowers and plants that bloom during the spring season, particularly in regions with rich biodiversity (Tafere 2021; Al-Kafaween et al. 2022). The name 'Rabiee' comes from the Arabic word 'Rabee' (spring), reflecting the season when the bees collect nectar for its production. This honey is typically light to medium amber in colour, with a pleasant floral aroma and a balanced sweet flavour. Due to its floral diversity, Rabiee honey contains a wide range of bioactive compounds, including natural enzymes, antioxidants, vitamins, and minerals, which contribute to its potential health benefits (Ranneh et al. 2021; Afroz et al. 2023). It is valued not only as a nutritious natural sweetener but also for its traditional use in supporting immunity, aiding digestion, and promoting overall wellness. Its freshness and unique flavour profile make it a sought-after honey variety in local markets and among natural health enthusiasts (Tanuğur et al. 2024; Ogwu and Izah 2025). There is an extensive documentation of the antibacterial activity of honey, but there is still a need to understand the efficacy of the local Jordanian honey types (Shawkiat and Rabiee honey) against various bacterial strains, including antibiotic-resistant strains. Therefore, this study aimed to evaluate and compare the antibacterial activity of Shawkiat and Rabiee honey with Manuka honey against *S. aureus* and *Escherichia coli*.

MATERIAL AND METHODS

Honey samples

Shawkiat and Rabiee honeys were purchased from a beekeeper located in Madaba (southwest of the capital of Amman, Jordan) and Manuka honey with the Unique

https://doi.org/10.17221/76/2025-CJFS

Manuka Factor (UMF) of +20 was purchased from a commercial supplier in Amman, Jordan. The stock was labelled and stored at room temperature in the microbiology lab at Al-Zaytoonah University of Jordan. Before each experiment, fresh honey samples were prepared and diluted to achieve specific concentrations.

Bacterial strains and culture conditions

Reference strains of *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538) were obtained from the American Type Culture Collection (ATCC). The bacteria were stored in nutrient broth (NB) medium with 20% (v/v) glycerol at –80 °C. Fresh cultures were prepared from the frozen stock before each assay, by streaking the bacteria on freshly prepared Muller Hinton Agar (MHA) plates using a sterile loop and incubated at 37 °C for 24 h. The bacterial suspension was then adjusted to be 0.5 McFarland.

Antibacterial activity

Agar well diffusion assay. Shawkiat, Rabiee and Manuka honey samples were diluted with sterile distilled water to achieve 75, 50, 25 and 20% (v/v) concentrations. A few colonies of each type of bacteria were selected using the sterilised loop from the fresh culture plates and suspended into 10 mL of phosphate buffer saline (PBS) solution. The optical density (OD) of each bacterial inoculum was adjusted by a spectrophotometer to be within 0.08–0.1 absorbance at 600 nm, which is equivalent to 0.5 McFarland. Bacterial suspension was absorbed with a sterile cotton swab and spread over the surface of Mueller-Hinton agar plate. The plate was rotated approximately 90 degrees four times to ensure the distribution of bacterial suspension. Seven wells were created in each agar plate using a cork borer. The wells were labelled and added 100 µL of the honey concentrations 100, 75, 50, 25 and 20% (v/v). The well with distilled water was used as a negative control and the well with honey was used as a positive control. The plates were incubated at 37 °C for 24 h. The inhibition zones were measured using a digital calliper. The assay was performed in triplicate (Hudzicki 2009; Al-Kafaween and Al-Jamal 2022).

Minimum inhibitory concentration (MIC). The broth microdilution method was used to determine the MIC of Shawkiat, Rabiee and Manuka honeys. A few single identical colonies of each type of bacteria were selected using a sterilised loop from the fresh culture plates and suspended into 10 mL of NB. OD of each inoculum was adjusted as described previously. The 75, 50, 25, 20, 12.5, 10, 6.25, 3.125 and 1.562% (v/v) concentrations of honey samples were freshly prepared with NB. An amount of 100 µL of inoculum and 100 µL of each honey concentration was transferred into the 96-well microtiter plate. Honey was used as a corresponding negative control, inoculum served as a bacterial growth control and the broth was used as a sterility control. The plates were incubated at 37 °C for 24 h. Bacterial growth was assessed by the visual inspection of turbidity in the wells in comparison with the controls. The microtiter plate reader was used to measure the absorbance at 570 nm (Al-Kafaween et al. 2020). The formula mentioned in Equation 1 was used to determine the MIC_{50} and MIC_{90}.

Minimum bactericidal concentration (MBC). The MBC was determined by the streak plate method after obtaining the MIC. From the MIC 96-well plates, 30 µL was taken and plated onto fresh NA. Then, the plates were labelled and incubated at 37 °C for 24 h. The lowest concentration that did not produce any colonies was considered as MBC (Al-Kafaween et al. 2022).

Microcolony formation

The inocula of *E. coli* and *S. aureus* were adjusted as described previously to be equal 0.5 McFarland. An amount of 500 µL of each inoculum was transferred into 24-well plates and incubated for 24 h. Then, 250 µL of the planktonic cells were replaced with 250 µL of MIC of Manuka honey, Shawkiat honey and Rabiee honey and then the plates were incubated at 37 °C for 24 h. The inoculum alone was used as a positive control. After incubation, planktonic cells were removed and the plate was washed with PBS to remove planktonic cells. The attached cells were then stained with 0.1% crystal violet for 5 min and washed twice with PBS. The stained attached cells in the wells were solubilised in 200 µL of absolute ethanol. Visible microcolonies were documented with an attached light microscope using oil immersion. The experiment was performed in triplicate (Zainol et al. 2013; Bouacha et al. 2022).

$$Growth\ inhibition\ (\%) = 1 - \left(\frac{OD\ of\ the\ test - OD\ of\ corresponding\ negative\ control}{OD\ of\ bacterial\ growth - OD\ of\ sterility\ control} \right) \times 100 \quad (1)$$

where: OD – optical density

https://doi.org/10.17221/76/2025-CJFS

Growth kinetics curve

The effects of Shawkiat, Rabiee and Manuka honey on the growth of *E. coli* and *S. aureus* were determined by the growth kinetics curve. Optical density of each inoculum was adjusted as described previously to be equal 0.5 McFarland. Cells were grown and treated in 96-well plates with MIC, half-MIC and quarter-MIC of each honey. The plates were incubated at 37 °C for 24 h. At 60-min intervals, the absorbance of wells was measured at 570 nm using a microtiter plate reader (Bouacha et al. 2022; Al-Kafaween et al. 2023). The experiment was performed in triplicate.

Time-kill curve

The time kill-curve was used to evaluate the effect of honey samples on bacterial growth. The inocula of both bacteria were diluted to be starting with 1×10^6 CFU·mL^{-1} and mixed with MBC of each honey. The inoculum without treatment was used as a positive control. The samples were incubated at 37 °C for 24 h. After the incubation time, samples were collected at different time points every 3 h. For each sample, the mean $\log_{10}$ CFU·mL^{-1} over time were plotted. Then, 30 µL of the mixtures was diluted in NB and streaked onto NA plates. Subsequently, the plates were incubated at 37 °C for 24 h. The total bacterial colonies were counted and the log reduction was determined by subtracting the $\log_{10}$ CFU at the initial time point (0 h) from the $\log_{10}$ CFU at specific time points. The experiment was carried out in triplicate (Akinjogunla et al. 2021; Kumbar et al. 2021; Al-Kafaween et al. 2022; Bouacha et al. 2022).

Antibiofilm activity

Biofilm inhibition assay in microtiter plates. Different concentrations of 90, 80, 60, 40, 20 and 10% (v/v) of Shawkiat, Rabiee and Manuka honeys were prepared in NB. The overnight inoculum of both bacteria was adjusted to 0.5 McFarland using a spectrophotometer at the wavelength of 600 nm. An aliquot of 100 µL of each honey concentration with 100 µL of inoculum was dispensed into 96-well plates. The inoculum served as a positive control and the honey was used as a corresponding negative control. The plates were incubated at 37 °C for 24 h. After incubation, the media were removed and the plates were washed with PBS to remove the planktonic, then the plates were stained using crystal violet for three minutes. The plates were rinsed with distilled water and dried at room temperature. Then, 95% ethanol was used to solubilise the biofilm. The absorbance was measured at 570 nm with a microtiter plate reader. The experiment was conducted in triplicate (Okhiria et al. 2009; Lu et al. 2019; Al-Kafaween et al. 2021, 2021; Huwaitat et al. 2021; Kumbar et al. 2021; Tarawneh et al. 2021, 2022). The percentage of inhibition was calculated by the following formula as mentioned in Equation 2.

Biofilm degradation assay in microtiter plates. Different concentrations of 90, 80, 60, 40, 20 and 10% (v/v) of Shawkiat, Rabiee and Manuka honeys were prepared in NB. The inoculum of both bacteria was adjusted as described previously to be equal 0.5 McFarland. To form the biofilms an aliquot of 200 µL of the inoculum was filled in the 96-well plates and incubated at 37 °C for 48 h. An amount of 100 µL of planktonic cells was removed and replaced with 100 µL of each honey concentration. Then, the plates were incubated at 37 °C for 24 h. The biofilm served as a positive control; the broth was used as a sterility control and the honey was used as a corresponding negative control. After incubation, free-floating planktonic bacteria were removed; the media were discarded and PBS was used to wash the plates. Then, the crystal violet was used to stain the plates for three minutes to quantify the biofilms and the plates were rinsed with distilled water and dried at room temperature. 95% ethanol was used to solubilise the biofilm. The absorbance was measured at 570 nm using a microtiter plate reader. The experiment was performed in triplicate (Sánchez et al. 2016; Al-Kafaween et al. 2019, 2021; Lu et al. 2019; Kot et al. 2020; Kumbar et al. 2021; Tarawneh et al. 2021). The percentage of inhibition was calculated by the following formula as mentioned in Equation 3.

$$Biofilm\ inhibition\,(\%) = \frac{OD\,(positive\ control) - OD\,(treatment)}{OD\,(positive\ control)} \times 100\% \quad (2)$$

$$Biofilm\ degradation\,(\%) = \frac{OD\,(positive\ control) - OD\,(treatment)}{OD\,(positive\ control)} \times 100\% \quad (3)$$

where: OD – optical density

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Gene expression profile

RNA isolation for RT-qPCR. The inoculum for both bacteria was adjusted as described previously to be equal 0.5 McFarland. Then, 200 µL of the culture was dispensed into 96-well plates and incubated at 37 °C for 24 h. After incubation, 200 µL of planktonic cells were carefully removed and replaced with 200 µL of MIC of each honey and incubated at 37 °C for 24 h. Bacteria without honey treatment were used as a positive control. Then the treated and untreated samples were resuspended with 0.5 mL of PBS and centrifuged at 13 000 rpm for one minute. The supernatant was removed and the pellet was washed twice in PBS. Firstly, total RNA was extracted from honey-treated and untreated samples using the SV Total RNA Isolation System (Promega, UK) according to the manufacturer's instructions. After RNA was extracted, the total RNA of samples was collected and quantified using a NanoDrop spectrophotometer. The samples were adjusted to be 100 ng·µL^{-1} and RNA was stored at –80 °C. Secondly, GoScript™ Reverse Transcriptase cDNA Synthesis (Promega, USA) was used to convert total RNA samples to cDNA according to the manufacturer's instructions. Finally, qPCR Master Mix was prepared for each reaction according to the manufacturer's instructions (Promega, USA). The volume of 10 µL from qPCR Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 2 µL of cDNA template, 0.2 µL carboxy-x-rhodamine (CXR) reference dye and nuclease-free water were added to a final volume of 20 µL. The plate was closed with strip caps before centrifuged and it was placed into a real-time PCR instrument. The PCR protocol and cycling conditions were as follows: denaturation in one cycle at 95 °C for two minutes, amplification in 40 cycles at 95 °C for 15 s and annealing in 40 cycles at 60 °C for one minute. Applied Biosystems StepOne Software v 2.3 was used to perform the densitometry (fluorescence). Then, a modified $2^{-\Delta\Delta Ct}$ method was used to calculate and determine the level of gene expression. Sequences of the primers for both bacteria were identified from previous studies as shown in Table 1. The experiments were conducted in triplicate (Jarrar et al. 2019, 2022; Al-Kafaween et al. 2021, 2021, 2022; Javaran 2022).

Statistical methods

All experimental procedures were repeated in triplicate for the statistical analysis. To identify significant difference between treated and untreated samples, all data obtained are presented as mean values ± standard deviation (SD) using an independent *t*-test in SPSS software (v. 26, IBM SPSS Statistics, USA). A difference with a P value of < 0.05 was regarded as statistically significant.

Table 1. Gene specific primers of *E. coli* and *S. aureus* used for RT-qPCR analysis

***Escherichia coli* (ATCC 8739)**				
Gene name	Amplicon size (bp)	Annealing temp. (°C)	Direction	Primer sequence (5'–3')
yjfO (bsmA)	76	53	forward reverse	CGCCAGTAACGGACCATC GTGCTTACGCTACCTATTCG
tnaA	174	54	forward reverse	CTGGATAGCGAAGATGTG CGGAATGGTGTATTGATAAC
evgA	155	53	forward reverse	TAGCGGAGACGATAATAATAATTC GTTGACTGAAGGCGGAAG
rpoS	199	54	forward reverse	CTCAACATACGCAACCTG GTCATCAACTGGCTTATCC
ycfR (BhsA)	81	54	forward reverse	CGAAGTTCAGTCAACGCCAGAAG TCCAGCGATCCCAGATTTGTCC
*16srRNA**	189	55	forward reverse	CCTACGGGAGGCAGCAG GGACTACCAGGGTATCTAAT

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Table 1 to be continued

***Staphylococcus aureus* (ATCC 6538)**				
Gene name	Amplicon size (bp)	Annealing temp. (°C)	Direction	Primer sequence (5'–3')
menB	109	56	forward reverse	CTGGGGAAGGTGATTTAGCA ACCGCCACCTACAGCATAAC
purC	117	62	forward reverse	GAAGCGCATTTTCTCAACAA CCCTTACCTGCCATTGTGTC
fabG	122	54	forward reverse	CCGGGACAAGCAAACTATGT CCAAAACGTGCTAACGGAAT
scdA	132	56	forward reverse	CGAAAGCAGCGGATATTTTT GCGAACCTGGTGTATTCGTT
pykA	126	52	forward reverse	TGCAGCAAGTTTCGTACGTC GGGATTTCAACACCCATGTC
argF	143	52	forward reverse	CCAAGCAGAATTCGAAGGA GGATGCGCACCTAAATCAAT
adh	124	62	forward reverse	GTTGCCGTTGGTTTACCTGT TTCAGCAGCAAATTCAAACG
*yqiL**	125	62	forward reverse	GACGTGCCAGCCTATGATTT ATTCGTGCTGGATTTTGTCC

*reference gene

RESULTS

Agar well diffusion assay

An agar inhibition assay showed Manuka honey to possess an inhibition zone of 12.2 ± 0.15 mm and 12.3 ± 0.22 mm against *E. coli* and *S. aureus*, respectively, at 20% concentration of honey. As compared with Manuka honey, the antibacterial activity of Shawkiat and Rabiee honey was not observed at 20% honey concentration. Also, at 25% concentration Shawkiat honey produced an inhibition zone of 11.5 ± 0.23 mm and 12.1 ± 0.2 mm against *E. coli* and *S. aureus*, respectively. In addition, Rabiee honey sh++owed an inhibition zone of 11.1 ± 0.2 mm and 12.2 ± 0.1 mm against *E. coli* and *S. aureus*, respectively, at 25% concentration of honey (Table 2).

MIC and MBC determination

The MIC, MIC_{50}, MIC_{90} and MBC values of Manuka honey, Shawkiat honey and Rabiee honey against the two tested bacteria are shown in Table 3 and Figure 1. Manuka honey was recorded as the most potent honey against *E. coli* and *S. aureus*, the MIC value for Manuka honey was 20% (w/v) against both bacteria. The MIC value for Shawkiat honey and Rabiee honey was 25% (w/v) against both bacteria. In addition, the respective MIC_{50} and MIC_{90} were 20% (w/v) and 25% (w/v) for Manuka honey against both bacteria. Also, the MIC_{50} and MIC_{90} for Shawkiat honey and Rabiee honey were 25% (w/v) and 50% (w/v) against both bacteria. The MBC value for Manuka honey was 25% (w/v) against both bacteria. The MBC value was 50% (w/v) for Shawkiat honey and Rabiee honey against both bacteria. Interestingly, Shawkiat honey and Rabiee honey demonstrated constant MIC and MBC results at 25% (w/v) and 50% (w/v) for all tests. Shawkiat honey closely resembled Rabiee honey, whereby Manuka honey exerted slightly higher MIC and MBC values against both bacteria than Shawkiat honey and Rabiee honey.

Microcolony formation

The microcolony formation is the early stage of biofilm formation. As shown in Figure 2, the disruption of the microcolony formation of *E. coli* was verified

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Table 2. Agar well diffusion assay for antibacterial activities of Manuka, Shawkiat and Rabiee honey against tested bacteria (mm, mean ± SD, n = 3)

***Escherichia coli* (ATCC 8739)**					
Honey samples	100%	75%	50%	25%	20%
Manuka honey	23.3 ± 0.11	20.6 ± 0.15	18.4 ± 0.2	15.3 ± 0.12	12.2 ± 0.15
Shawkiat honey	19.2 ± 0.21	17.2 ± 0.1	15.5 ± 0.16	11.5 ± 0.23	NIL
Rabiee honey	18.3 ± 0.21	16.5 ± 0.14	14.2 ± 0.15	11.1 ± 0.2	NIL
***Staphylococcus aureus* (ATCC 6538)**					
Honey samples	100%	75%	50%	25%	20%
Manuka honey	23.5 ± 0.15	21.5 ± 0.21	19.2 ± 0.1	15.4 ± 0.2	12.3 ± 0.22
Shawkiat honey	20.4 ± 0.12	18.5 ± 0.2	16.6 ± 0.16	12.1 ± 0.2	NIL
Rabiee honey	18.5 ± 0.2	17.1 ± 0.15	15.3 ± 0.1	12.2 ± 0.1	NIL

NIL – no inhibition zone

by the light microscope after being treated with MIC of Manuka honey, Shawkiat honey and Rabiee honey. When comparing treated cells with untreated ones, Manuka honey was observed to reduce and disrupt the microcolony development in both bacteria slightly more than those treated with Shawkiat honey and Rabiee honey. Light microscopy revealed that both bacteria was easily interrupted following the treatment with all the tested honeys and indicated that the bacterial cells were loosely associated with each other in a background of planktonic cells.

As shown in Figure 3, an increase in optical density that was observed during 24 h for both bacteria without the addition of all honey samples was expected and the exponential phase occurred between 2 to 11 h. This finding of untreated cells was similar to the sample treated with quarter-MIC of all honey samples. Both bacteria exposure to half-MIC of all honey samples resulted in a decreased optical density and decreased lag phase and exponential phase compared to the untreated sample. Also, neither of the two bacteria treated with MIC of all honey samples showed an increase in optical density over 24 h. This suggests that the complete inhibition of both bacteria growth was achieved. But Manuka honey had the highest antibacterial potency against *E. coli* and *S. aureus* while Shawkiat honey and Rabiee honey were the least effective. Overall, the bactericidal activities of Manuka honey were recorded to be one reading higher than the inhibitory effects of Shawkiat honey and Rabiee honey. All bacteria showed dose-response activity to a various extent. Manuka honey appeared most consistent in inhibiting the growth of *E. coli* and *S. aureus.*

As shown in Figure 4, after both bacteria exposure to MBC of all the tested honeys, the total number

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Manuka, Rabiee and Shawkiat honey against both bacteria

***Escherichia coli* (ATCC 8739)**				
Honey samples	MIC	MIC_{50}	MIC_{90}	MBC
Manuka honey	20%	20%	25%	25%
Shawkiat honey	25%	25%	50%	50%
Rabiee honey	25%	25%	50%	50%
***Staphylococcus aureus* (ATCC 6538)**				
Honey samples	MIC	MIC_{50}	MIC_{90}	MBC
Manuka honey	20%	20%	25%	25%
Shawkiat honey	25%	25%	50%	50%
Rabiee honey	25%	25%	50%	50%

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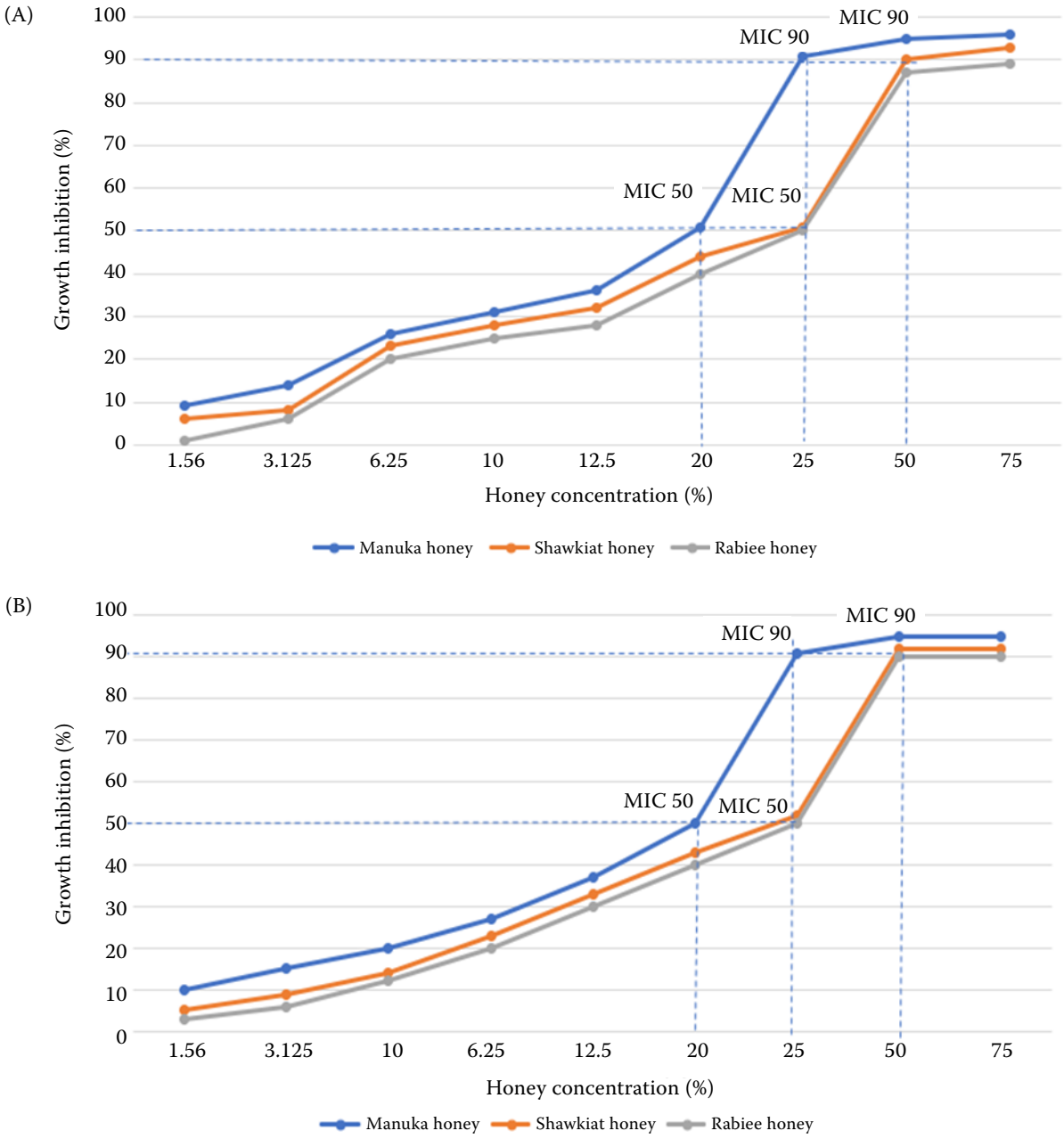

Figure 1. Percentage of bacteria growth inhibition by all honey samples against *E. coli* (A) *and S. aureus* (B)

MIC – minimum inhibitory concentration

of *E. coli* and *S. aureus* cells decreased, the bacteria lost viability over time and could not recover after 24 h compared to untreated cells.

After *E. coli* were treated with MBC of Manuka honey, Shawkiat honey and Rabiee honey for 3 h, the *E. coli* cells decreased in a 1-$\log_{10}$, 0.7-$\log_{10}$ and 0.6-$\log_{10}$ reduction in CFU·mL^{-1}, respectively. When *E. coli* were exposed to MBC of Manuka honey, Shawkiat honey and Rabiee honey for 9 h, the *E. coli* cells decreased in a 2.9-$\log_{10}$, 2.7-$\log_{10}$ and 2.3-$\log_{10}$ reduction in CFU·mL^{-1}, respectively. While, after *E. coli* were treated with Manuka honey, Shawkiat honey and Rabiee honey for 24 h, the *E. coli* cells decreased in a 6-$\log_{10}$, 5.8-$\log_{10}$ and 5.7-$\log_{10}$ reduction in CFU·mL^{-1}, respectively, compared to untreated cells.

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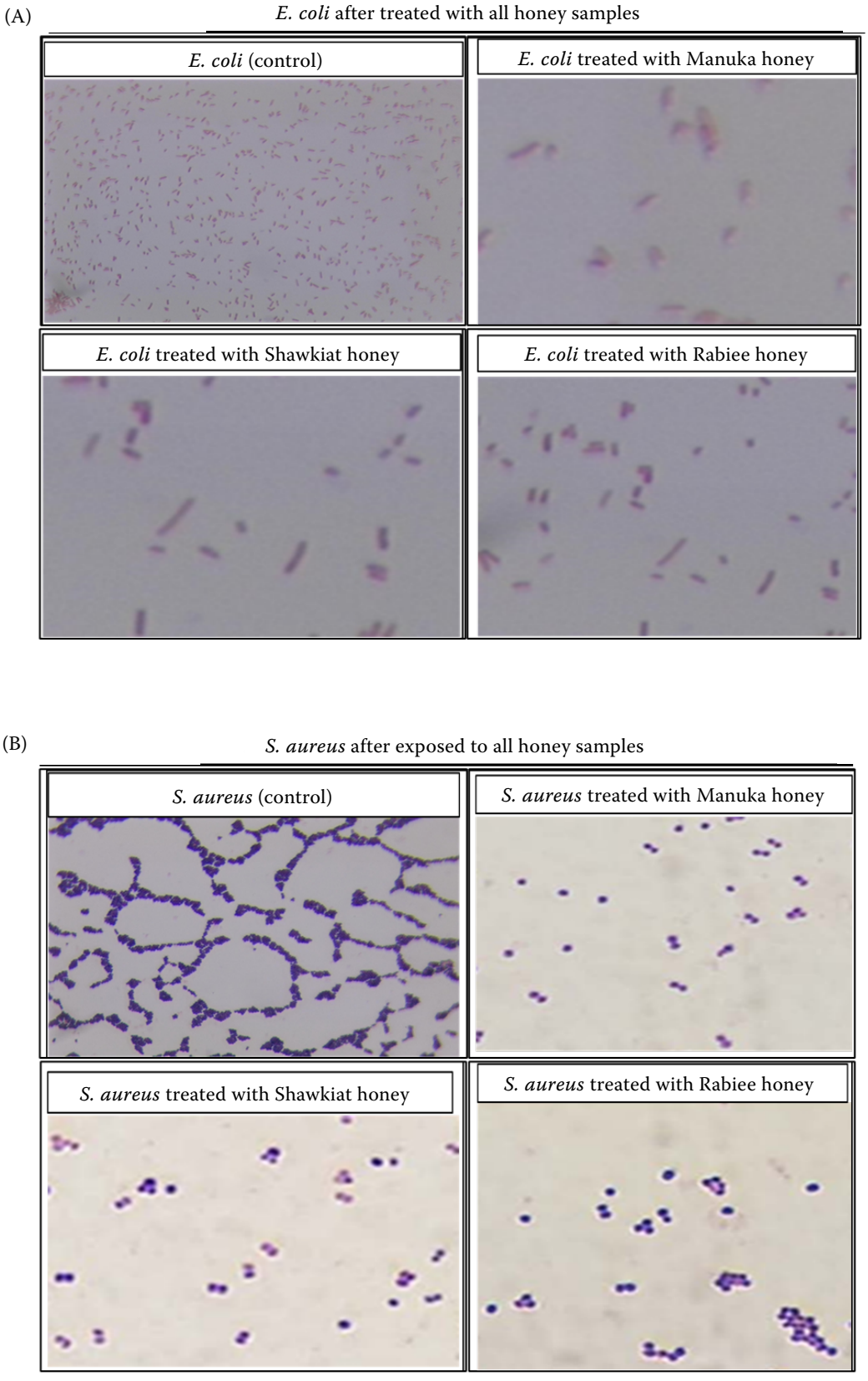

Figure 2. The microcolony of *E. coli* (A) and *S. aureus* (B) after exposed to all honey samples

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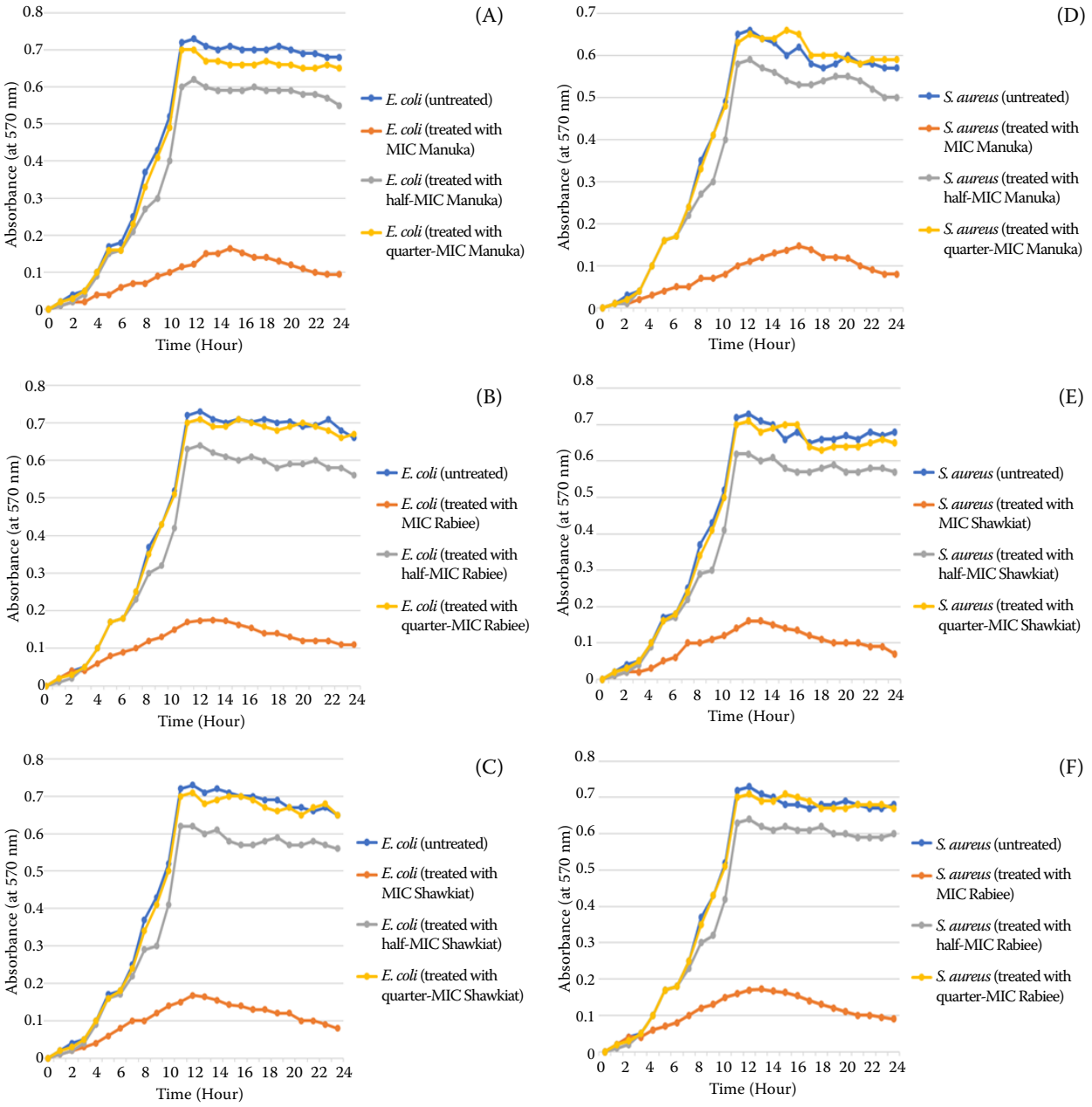

Figure 3. Growth kinetics curve of *E. coli* and *S. aureus* treated with and without all honey samples

MIC – minimum inhibitory concentration

S. aureus cells decreased to a 1-$\log_{10}$, 0.8-$\log_{10}$ and 0.5-$\log_{10}$ reduction in CFU·mL^{-1} after being treated with Manuka honey, Shawkiat honey and Rabiee honey, respectively, at 3 h incubation. After *S. aureus* incubation with Manuka honey, Shawkiat honey and Rabiee honey for 9 h, *S. aureus* cells decreased by 2.9-$\log_{10}$, 2.6-$\log_{10}$ and 2.4-$\log_{10}$ CFU·mL^{-1}, respectively. In addition, *S. aureus* cells decreased by 6-$\log_{10}$, 5.7-$\log_{10}$ and 5.7-$\log_{10}$ CFU·mL^{-1} after being treated with Manuka honey, Shawkiat honey and Rabiee honey, respectively, for 24 h.

Biofilm inhibition after exposed to honey

Manuka, Shawkiat and Rabiee honeys were able to significantly ($P < 0.05$) reduce *E. coli* and *S. aureus* biofilms compared to untreated samples. The 90% concentration of Manuka, Shawkiat and Rabiee honeys demonstrated the greatest reduction of *E. coli*

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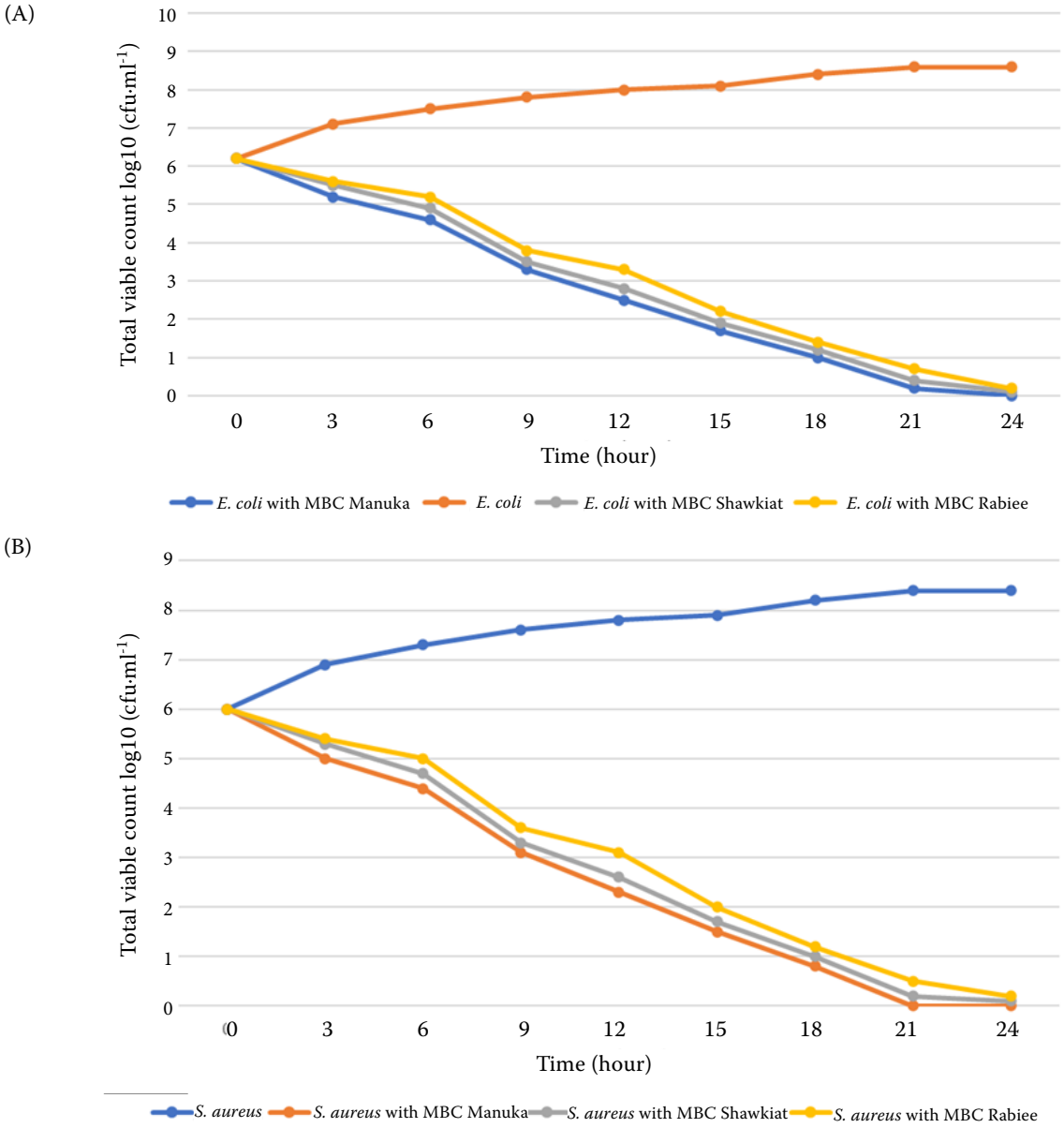

Figure 4. Time–kill curve of *E. coli* (A) and *S. aureus* (B) exposed to all honey samples

MBC – minimum bactericidal concentration

biofilm by 68, 64 and 62%, respectively. The 80% concentration of Manuka, Shawkiat and Rabiee honeys decreased the *E. coli* biofilm by 65, 61 and 60%, respectively. While the lowest inhibition of *E. coli* biofilm was found to be 24, 20 and 17% after treatment with 10% concentration of Manuka, Shawkiat and Rabiee honeys, respectively. Meanwhile, the 90% concentration of Manuka, Shawkiat and Rabiee honeys showed the greatest reduction of *S. aureus* biofilm by 66, 62 and 61%, respectively. The 80% concentration of Manuka, Shawkiat and Rabiee honeys reduced the *S. aureus* biofilm by 64, 59 and 58%, respectively. While the lowest inhibition of *S. aureus* biofilm was found to be 26, 22 and 19% after treatment with 10% concentration of Manuka, Shawkiat and Rabiee honeys, respectively (Figure 5).

Biofilm degradation after treatment with honey

Manuka, Shawkiat and Rabiee honeys significantly ($P < 0.05$) degraded the *E. coli* and *S. aureus* biofilm compared to untreated samples. The greatest degradation of *E. coli* biofilm was at 90%

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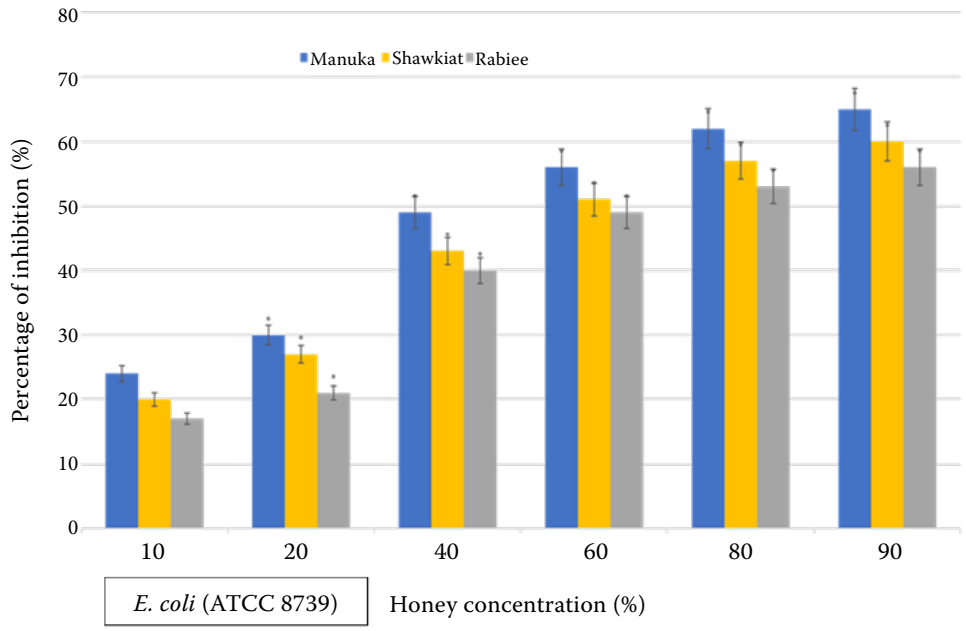
(A)



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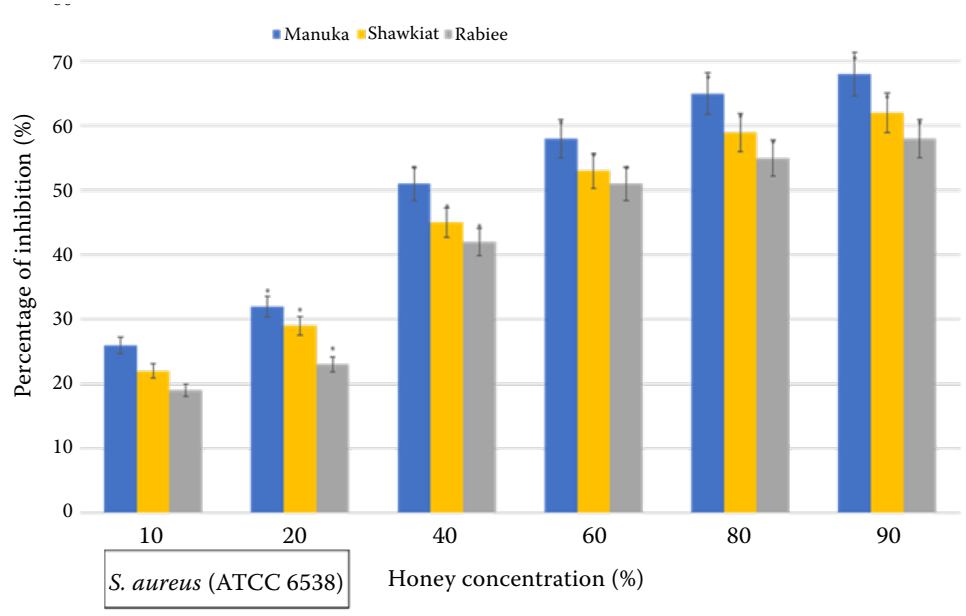

Figure 5. Inhibition of biofilm formation in *E. coli* (ATCC 8739) (A) and *S. aureus* (ATCC 6538) (B) in the presence of manuka, Shawkiat and Rabiee honeys

*significance at $P < 0.05$, difference between treated samples and untreated biofilm

concentration of Manuka, Shawkiat and Rabiee honeys with 64, 60 and 58% degradation, respectively. The 80% concentration of Manuka, Shawkiat and Rabiee honeys decreased the *E. coli* biofilm by 60, 57 and 55%, respectively. While the lowest degradation of *E. coli* biofilm was found to be 19, 16 and 13% after treatment with 10% concentration of Manuka, Shawkiat and Rabiee honeys, respectively. Meanwhile, the 90% concentration of Manuka, Shawkiat and Rabiee honeys showed the greatest degradation of *S. aureus* biofilm by 64, 60 and 57%, respectively. The 80% concentration of Manuka, Shawkiat and Rabiee honeys degraded the *S. aureus* biofilm by 61, 57 and 55%, respectively.

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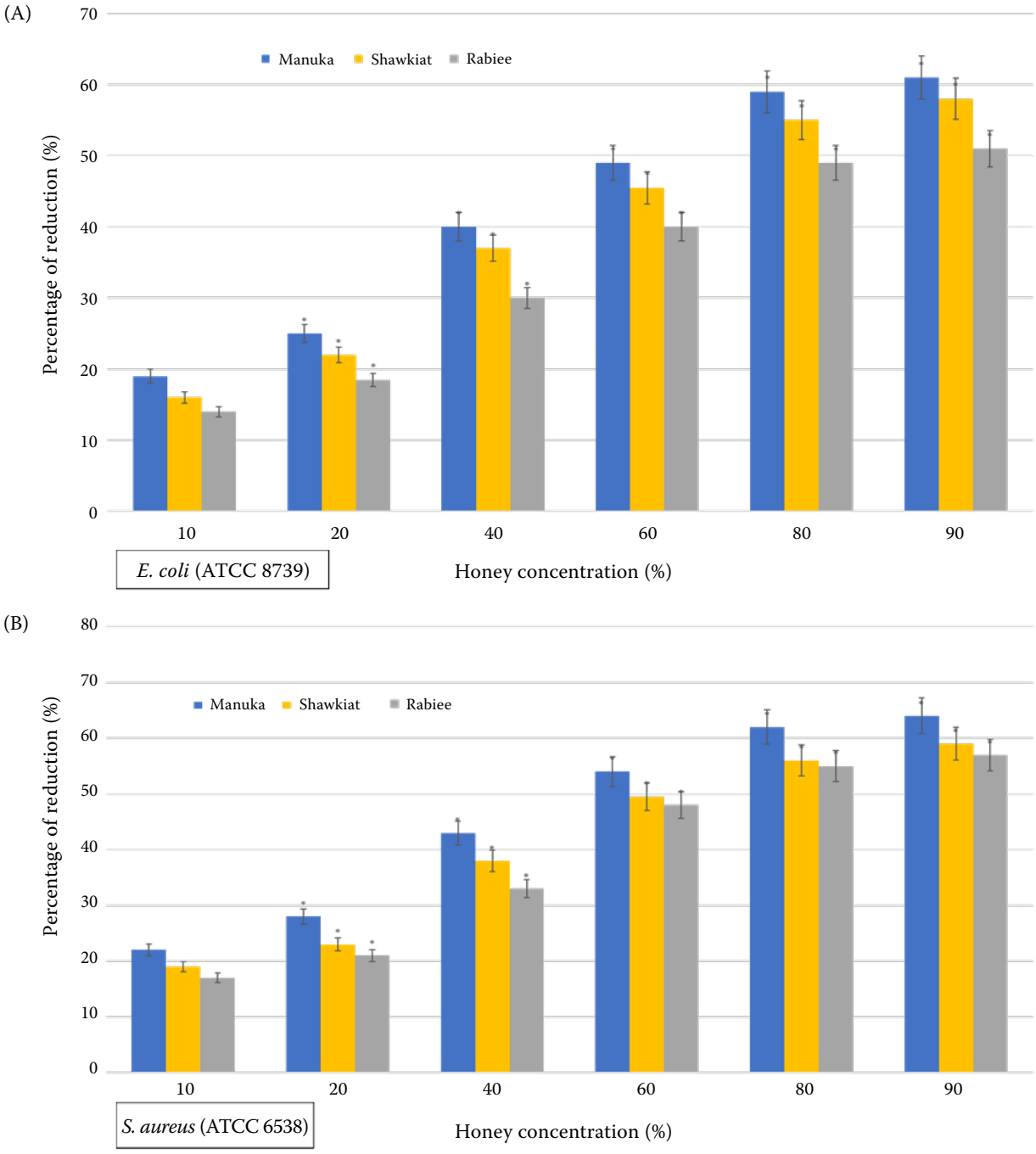

Figure 6. Reduction of *E. coli* (ATCC 8739) (A) and *S. aureus* (ATCC 6538) and (B) biofilm in the presence of manuka, Shawkiat and Rabiee honeys (mean ± SD, $n = 3$)

*significance at $P < 0.05$, difference between treated samples and untreated biofilm

While the lowest degradation of *S. aureus* biofilm was found to be 21, 19 and 15% after treatment with 10% concentration of Manuka, Shawkiat and Rabiee honeys, respectively (Figure 6).

Gene expression profile

In the present study, RT-qPCR was used to evaluate and compare the impacts of the exposure of *E. coli* and *S. aureus* cells to Jordanian honeys and Manuka honey

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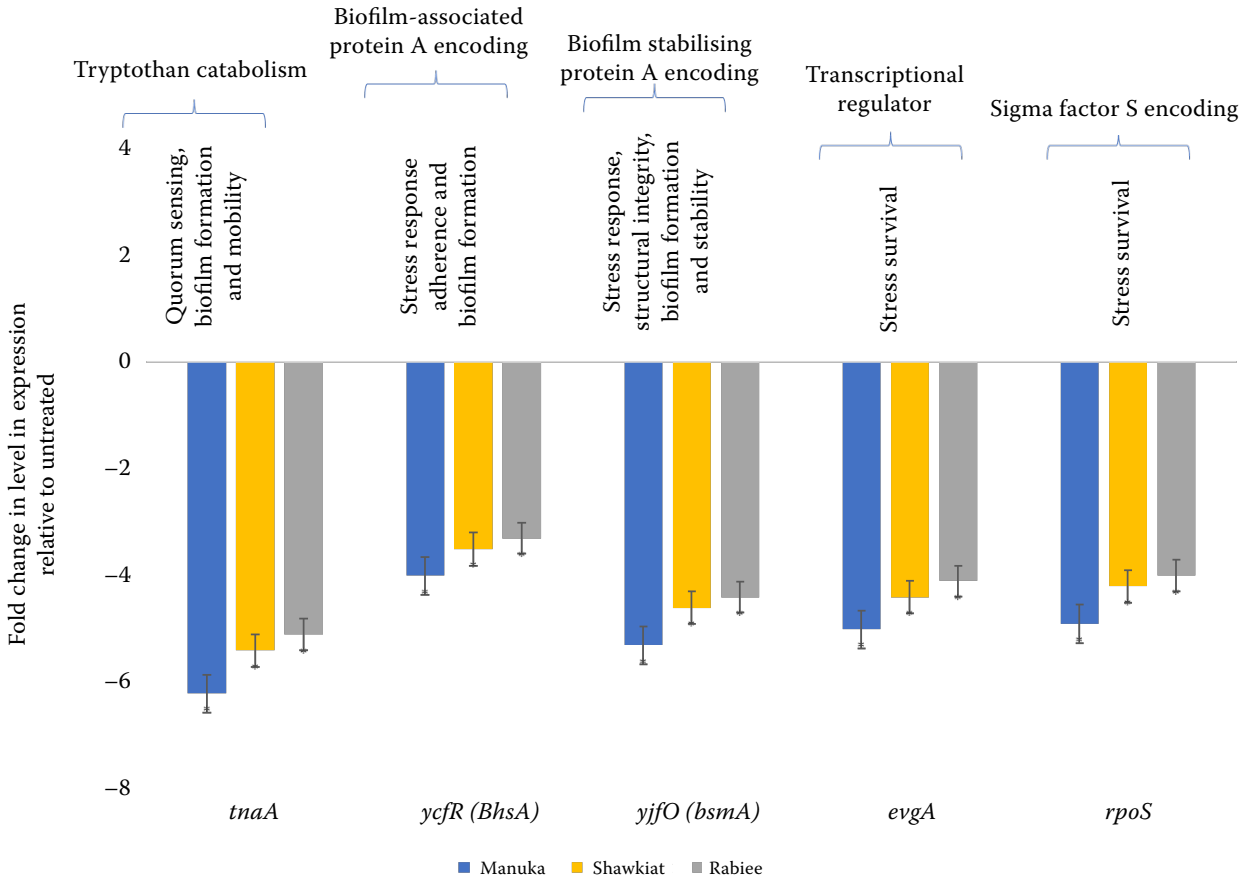

Figure 7. Alteration in level of expression of *E. coli* (ATCC 8739) genes after treated with all the tested honey (mean ± SD, $n = 3$)

*significance at $P < 0.05$, difference between treated and untreated samples

(at MIC) on the expression of five selected genes in *E. coli* and seven genes in *S. aureus* that have previously been shown to be involved in the microcolony, biofilm and virulence of the microorganisms.

Gene expression of E. coli after exposure to honey. The gene expression levels of all genes in *E. coli* were significantly ($P < 0.05$) downregulated after exposure to all the tested honeys compared to the untreated ones. Although different degrees of downregulation were observed following the exposure to the Jordanian honeys and Manuka honey.

The expression of *tnaA, ycfR (BhsA), yjfO (bsmA), evgA* and *rpoS* genes in *E. coli* was decreased 6.2, 4, 5.3, 5 and 4.9-fold, respectively, after exposure to Manuka honey. Whereas the expression of *tnaA, ycfR (BhsA), yjfO (bsmA), evgA* and *rpoS* genes in *E. coli* was decreased 5.4, 3.5, 4.6, 4.4 and 4.2-fold, respectively, after being treated with Shawkiat honey. The expression of *tnaA, ycfR (BhsA), yjfO (bsmA), evgA and rpoS* genes in *E. coli* was decreased 5.1, 3.3, 4.1, 4 and 4.2-fold, respectively, after exposure to Rabiee honey (Figure 7).

Gene expression of S. aureus following treatment with honey. The gene expression levels of all genes in *S. aureus* were significantly ($P < 0.05$) reduced after exposure to the tested honeys compared to the control. The expression of *adh, argF, purC, fabG, pykA, scdA* and *menB* genes in *S. aureus* was decreased 5.4, 4, 5.2, 6, 6.1, 5.8 and 6-fold, respectively, after exposure to Manuka honey. Whereas the expression of *adh, argF, purC, fabG, pykA, scdA* and *menB* genes in *S. aureus* was decreased 4.7, 3.3, 4.4, 5.4, 5.3, 5.1 and 5.2-fold, respectively, after being treated with Shawkiat honey. The expression of *adh, argF, purC, fabG, pykA, scdA* and *menB* genes in *S. aureus* was decreased 0.5, 3.1, 4.2, 5.2, 5.1, 4.8 and 5-fold, respectively, after exposure to Rabiee honey (Figure 8).

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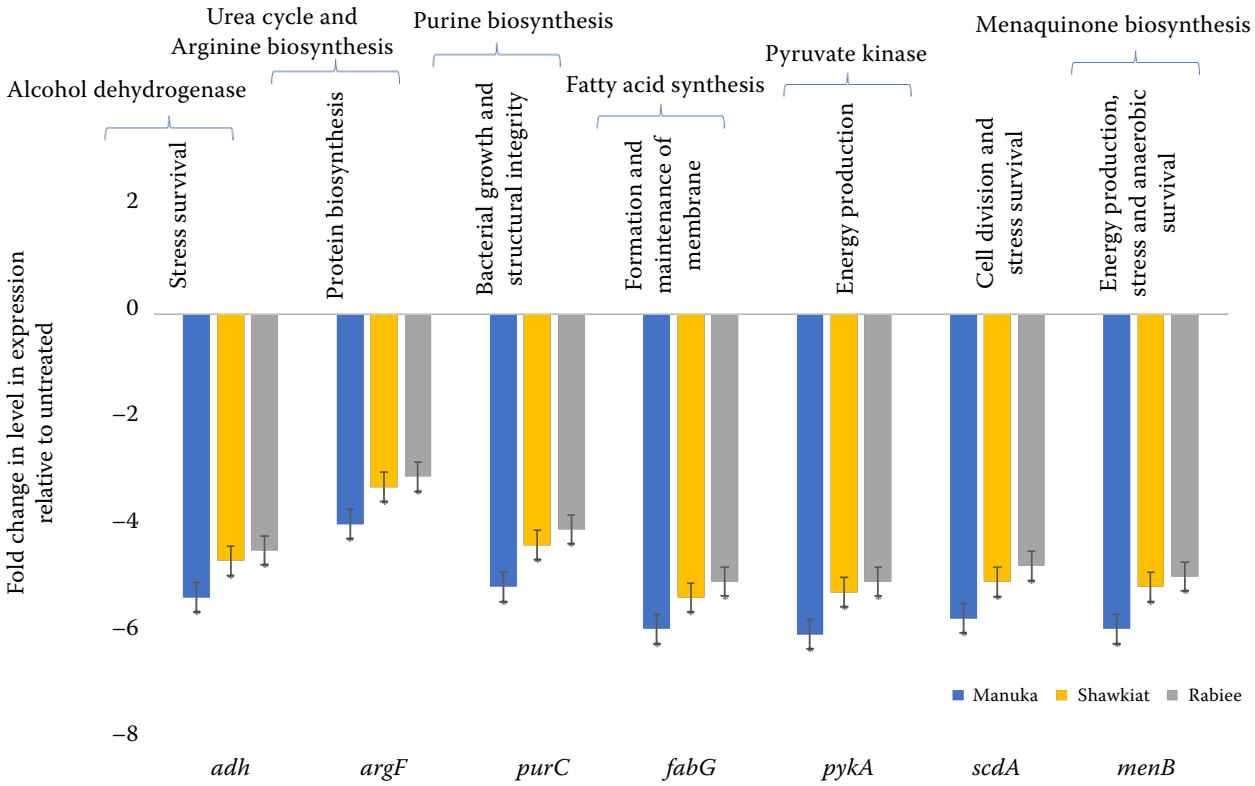

Figure 8. Alteration in level of expression of *S. aureus* (ATCC 6538) genes after treated with all the tested honey (mean ± SD, $n = 3$)

*significance at $P < 0.05$, difference between treated and untreated samples

DISCUSSION

Honey has widely been studied among researchers as a promising complementary and alternative treatment after the prevalence of antibiotics resistance in recent decades (Eteraf-Oskouei and Najafi 2013; Maddocks and Jenkins 2013; Alkhyat and Al-Maqtari 2014). 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 (Wasfi et al. 2016; Al-Kafaween et al. 2023). The second mechanism is mediated through their anti-virulence activity, by downregulating the expression of genes associated with virulence factor production, stress tolerance, and/or multicellular behaviour of the target organism (Wasfi et al. 2016; Al-Kafaween et al. 2023). This latter mechanism will eventually weaken the bacterial coordination, decrease their survival abilities, and interfere with their virulence mechanisms. In the present study, we investigated and compared the antibacterial, antibiofilm and anti-virulence activities of two Jordanian honeys and Manuka honey on *E. coli* and *S. aureus*. To our knowledge, this is the first attempt to assess and compare the impacts of Jordanian honey and Manuka honey varieties on the tested organisms at both antibacterial and molecular levels. A summary of the obtained results is represented in Figure 9.

The antibacterial activity of honey is known to be attributed to multiple factors, including its high concentration, acidity, as well as its content of hydrogen peroxide (H_2O_2) and non-peroxide phytochemical components (Al-Kafaween et al. 2022). The agar well diffusion assay detected the efficiency of honey by measuring the zones of inhibition against the tested bacteria. All the tested honeys, Manuka, Shawkiat and Rabiee honeys, exhibited clear inhibition zones against *E. coli* and *S. aureus* at different concentrations of 20%

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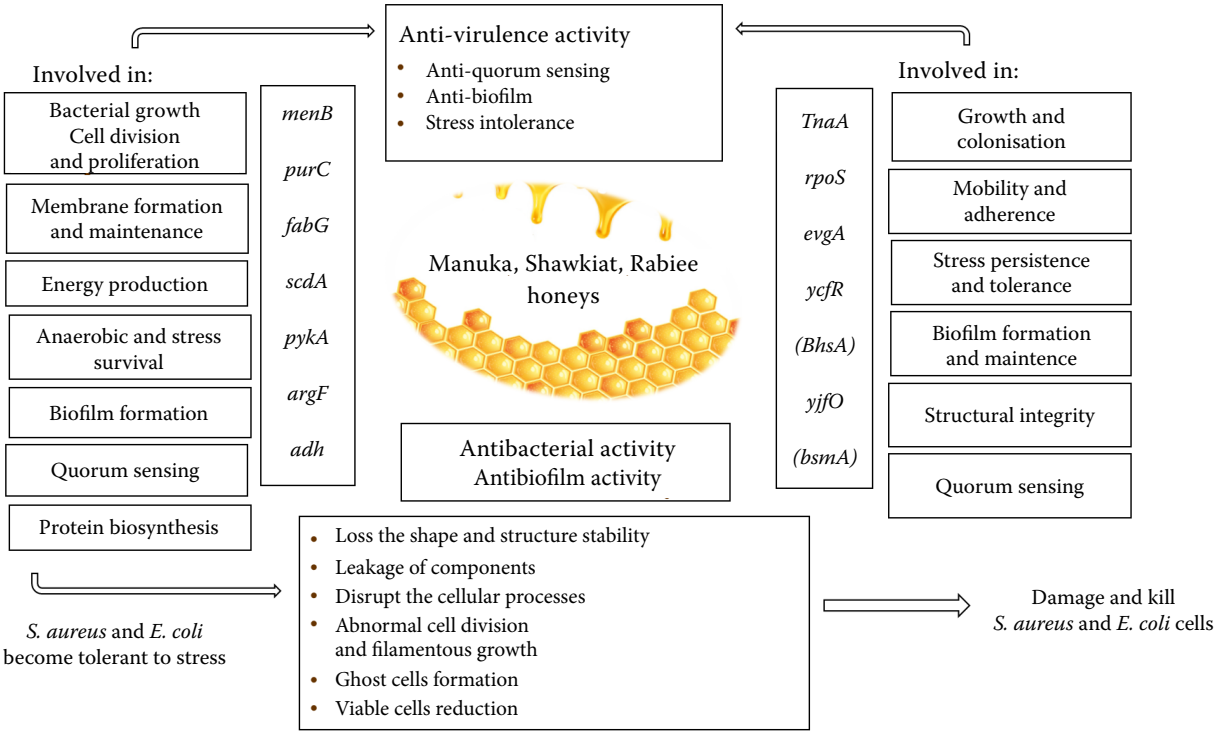

Figure 9. Schematic diagram summarising the effects of manuka, Shawkiat and Rabiee honeys on *E. coli* and *S. auerus* at both planktonic, biofilm and molecular levels

and 25%. Previous studies reported that 20% and 25% concentrations of Manuka honey were the lowest ones that showed the zones of inhibition (Al-Kafaween et al. 2022; Mudenda et al. 2023). The MIC value indicates the lowest concentration required to inhibit the visible bacterial growth by approximately 99%, while the MBC value represents the lowest concentration required to eliminate the bacterial colonies by approximately 99% (Kłodzińska et al. 2018). In the present study, the MIC values were 25% (w/v) for both Shawkiat and Rabiee honey, and 20% (w/v) for Manuka honey against both bacteria *S. aureus* and *E. coli*; the MBCs values were 50% (w/v) for Shawkiat and Rabiee honey and 25% (w/v) for Manuka honey against both bacteria *S. aureus* and *E. coli.* The study by (Al-Kafaween and Al-Jamal 2022) demonstrated that the MBC and MIC values for Manuka honey were 20% and 12.5% against *P. aeruginosa* and *S. pyogenes* (Al-Kafaween and Al-Jamal 2022). MIC_{50} and MIC_{90} represent the lowest concentrations at which 50% and 90% of the bacterial colonies were inhibited, respectively (Al-Kafaween et al. 2022). In the present study, the MIC_{50} and MIC_{90} values were 20% and 25% for Manuka honey, 25% and 50% for Shawkiat honey, 25% and 50% for Rabiee honey against both bacteria *S. aureus* and *E. coli*. The study by Zainol et al. (2013) reported that the MIC_{50} and MIC_{90} values for Tualung honey against *S. aureus*, *P. aeruginosa* and *E. coli* were 10–12% and 15–20%, respectively. Another study revealed that the respective MIC_{50} and MIC_{90} values for Manuka honey were 12.5% and 25% against *P. aeruginosa* (Al-Kafaween et al. 2022). These conflicting results may be attributed to the differences in geographical and seasonal sources, as well as harvesting, processing, and storage conditions of the tested honeys. The growth of *E. coli* and *S. aureus* was inhibited after 24 h with MIC of Shawkiat, Rabiee honey and Manuka honey. The optical density was decreased by the half-MIC of Shawkiat, Rabiee honey and Manuka honey. While in the untreated samples, the optical density value was increased. The growth was not inhibited with the quarter-MIC of Shawkiat, Rabiee honey and Manuka honey and was similar to the control. Previous studies indicated that the growth curves of *E. coli* and *S. aureus* were inhibited after treatment with MIC of Manuka honey and Tualang honey after 24 h (Al-Kafaween et al. 2021, 2021, 2023). The time-kill assay showed that Manuka honey, Shawkiat honey and Rabiee honey significantly

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decreased the total number of *E. coli* and *S. aureus* cells and have a bactericidal effect at MBC concentrations. Previous studies showed that Manuka honey reduced the number of living cells of *S. aureus* after treatment with MBC (Combarros et al. 2019; Al-Kafaween et al. 2023). The biofilm formation is one the bacterial defence and resistance mechanisms (Lu et al. 2019). In this study, a wide range of honey concentrations was selected to investigate their effect on the biofilm formation of *S. aureus* and *E. coli*. The results suggested that all tested honeys were able to inhibit and disrupt the biofilm formation of *E. coli* and *S. aureus*. The study by Al-Kafaween et al. (2021) indicated that 30% (w/v) of honey was the lowest concentration that prevented the formation of *S. aureus* and *E. coli* biofilm (Al-Kafaween et al. 2021). In the present study, the results of the RT-qPCR assay showed that all selected genes in *E. coli* and *S. aureus* were downregulated following the treatment with Manuka, Shawkiat and Rabiee honeys in the range of 3.3-fold to 6.2-fold and 3.1-fold to 6.1-fold, respectively. The genes *ycfR (BhsA)* and *yjfO (bsmA)* in *E. coli* have been characterised as biofilm formation genes and the exposure to the honeys can inhibit and disrupt the biofilm. The *tnaA* gene have been involved in the *E. coli* quorum sensing network and the exposure to the honeys will reduce the bacterial virulence while the genes associated with stress survival were *evgA* and *rpoS* (Wasfi et al. 2016; Al-Kafaween et al. 2021). The results proved the ability of honeys in impairing the activities of *S. aureus* genes. The decrease in the *purC, argF, fabG* and *adh* gene expression shows restriction in the biofilm formation while the impact of honeys on the expression levels of *menB, pykA* and *scdA* may exert the effect on the peptidoglycan cross-linking (Al-Kafaween et al. 2021, 2022). A summary of the obtained results is represented in Figure 9.

CONCLUSION

This study is the first study to compare the effects of Shawkiat honey and Rabiee honey in combination with Manuka honey on *E. coli* and *S. aureus* at planktonic, biofilm and molecular levels. Together, our results revealed that the Jordanian honeys have the potential to be effective inhibitors of *E. coli* and *S. aureus* and their antibacterial activities. Shawkiat honey demonstrated the highest antibacterial activity, higher than Rabiee honey compared with Manuka honey. A reduction of the *E. coli* and *S. aureus* planktonic and biofilms was observed after the exposure to the tested honeys. In this study, Manuka honey exhibited a greater impact, as well as the other results indicated that Shawkiat and Rabiee honeys may show promising antibacterial, antibiofilm and anti-virulence activities for treatment and management of the infections caused by *E. coli* and *S. aureus* compared with Manuka honey. Differential gene expression in response to honey exposure exhibited the downregulation of several genes involved in biofilm formation, quorum sensing, and stress survival in *E. coli* and *S. aureus*. The obtained results indicate that the Shawkiat and Rabiee honeys under study may represent promising antibacterial and anti-virulence agents for treatment and modulation of the infections caused by *E. coli* and *S. aureus*. Future clinical evidence pertaining to the efficacy of Shawkiat and Rabiee honeys in the prevention and treatment of *E. coli* and *S. aureus* induced infections at various tissue/cell types might be required.

Acknowledgement. The authors would like to thank the staff of the Faculty of Pharmacy at Al-Zaytoonah University of Jordan.

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Received: May 9, 025
Accepted: September 8, 2025