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Research article

Molecular identification of major bacteria in honey and the effect of microwave treatment on its microbial quality and antibacterial activity

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Abstract: The objectives of this study were to assess the microbial quality of honey, evaluate the effect of microwaves on microbial survival, and assess the antibacterial activity of honey. Bacteria, yeast and mold were evaluated in samples before and after microwave treatment. Dominant bacterial contaminants were also identified. The antibacterial activity of honey was assessed against nine pathogens using an agar well diffusion assay. The minimum inhibitory concentration was determined for four honey samples that exhibited the highest antibacterial activity. In addition, one sample of Manuka honey was tested to compare its microbial load as well as its antibacterial activity to local honey samples. Sequencing using 16S rRNA gene was used for the identification of dominant bacteria. The average standard plate count, yeasts and molds were 286.5, 161.0 and 25.5 CFU/g, respectively. Microwave treatment decreased microbial populations gradually with increasing power levels and exposure times. The present study indicated that raw honey had a significant antibacterial activity which decreased following microwave treatment. The identity of 125 isolates was confirmed with *Bacillus* being most frequently isolated.

Keywords: honey; microwave treatment; microbial quality; antibacterial activity; 16S rRNA gene

1. Introduction

Honey is mainly composed of (%): monosaccharides including fructose (38.19), glucose (31.28), and disaccharides such as maltose (7.3), sucrose (1.3) and water (17.2). In addition, it contains trace amounts of enzymes (α-amylase, α-glucosidase, glucose oxidase and catalase), proteins, amino acids, phenolic compounds, carotenoids, minerals, vitamins, colloids, sugar alcohols, pigments, flavor and aromatic substances [1,2]. Honey components vary from one type to another because of botanical origins, geographical regions, bee species, entomological sources and other factors such as processing methods, storage conditions, climatic changes, and maturity [3–5]. It is characterized by sensory, physicochemical, and microbiological characteristics which help determine its quality [6,7]. The water activity (a_w) of honey ranges between 0.5 and 0.65, the higher a_w results in microbial spoilage [8]. The majority of the antibacterial activity and medicinal properties of honey against a broad spectrum of bacteria, including multidrug-resistant, is likely due to various components, such as hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin-1, as well as phenolic compounds (flavonoids and phenolic acids) [9,10]. Furthermore, the hygroscopic nature of honey is a unique property that is expressed as the honey's ability to absorb and retain moisture or expel water molecules into or out of the surrounding environment, which adds to its antibacterial activity [11].

Microbial and chemical contamination of honey is due to several sources. Primary sources are those present in pollen, such contaminants originating from dust, other insects, and from the GIT of the honeybee itself [11]. Secondary sources include chemicals such as pesticides, industrial pollutants, radioactive pollutants, and heavy metals that might occasionally contaminate honey during collection or processing [12].

Most microbes present on or in honeybee GIT reproduce under aerobic conditions, while a few reproduce under anaerobic conditions [13]. Various bacteria have been isolated from honey including *Bacillus, Clostridium, Acinetobacter and Micrococcus* [12,14–16]. Additionally, yeast and mold have been isolated. Mold growth may lead to the production and accumulation of mycotoxins such as aflatoxin B1 which can not only affect the entire bee colony but can also render it dangerous to consume [17]. Moreover, honey may contain *Clostridium botulinum* spores that may lead to food poisoning and is considered a possible cause of sudden infant death. It is for this reason that incorporating honey in food prepared for infants under one year old is not encouraged [18,19]. Due to the propensity of bacterial contamination, it may be necessary to treat honey to increase both shelf life and safety especially if given to neonates and the immunocompromised [20]. At present, several methods have been proposed to decontaminate honey including high-temperature short-time pasteurization (HTST) [21], ultrasound [22], and microwave [20].

Microwave technology emerged in the mid-1980s as a quick method of cooking and heating of food [23]. Microwave is a term expressing the electromagnetic spectrum, with wavelengths ranging from 1mm to 1m and frequencies between 0.3 and 300 GHz [24,25]. Two frequencies are normally used in microwave heating; 0.915 and 2.45 GHz which are reserved by the US Federal Communications Commission (FCC) for industrial, scientific, and medical (ISM) purposes [26]. However, lower frequency microwaves are better suited for penetration [27]. Domestic microwaves use a 2.45 GHz frequency, while industry uses either a 0.915 or 2.45GHz frequency. Domestic microwave is primarily used for heating and reheating food. At the industrial level, microwave heating has been applied to food processing practices such as drying, heating, or cooking, pasteurization, sterilization and preservation of food [27,28]. Microwave heating of food creates molecular motion between food molecules, leading to heat generation, which is a function of time and power. However,

pasteurization or sterilization of food via microwave treatment can be understood with various mechanisms, such as selective heating, where microbial bodies absorb more heat than the surrounding, leading to their destruction [25]. Other mechanisms involve electroporation where the electrical potential throughout the cell membrane can create pores in cells, resulting in the leakage of cellular material. A third mechanism involves magnetic field coupling, where vital components of the cell, such as protein or DNA coupled in the magnetic field can be destroyed [29,30]. This leads to irreversible denaturation of enzymes, proteins, nucleic acids, disruption of membranes and eventually cell death [31,32].

The efficiency of microwave sterilization depends on food characteristics as well as the frequency and length of application [33]. Microwaves are differently absorbed by water, fatty substances, and sugars and thus the food composition determines the efficacy of treatment [34,35]; overall, in-food heating increases efficacy with increasing moisture content and dissolved sugars [20]. However, microwave processing has been reported to change the flavor and nutritional characteristics of some food although to a lesser degree than conventional heating [36].

Honey is a supersaturated viscose sugar solution with the length of sugar chains determining its viscosity and palatability [37,38]. Occasionally, honey crystalizes, a phenomenon, usually is not desired by honey consumers, although is naturally and commonly occurring in honey. In order to get rid of the crystallization, they expose it to mild heat treatment to dissolve the crystals. Glucose oxidase, diastase, invertase and other protective enzymes that act as antioxidants and antibacterial agents or purported to possess healing power are also degraded because of the heat generated by this treatment [39–44]. Furthermore, excessive microwave treatment decreases bioactive components, including antioxidant and anti-inflammatory activities in honey, denaturation of proteins, decreases vitamin content and decreases the freshness of honey [45,46].

This study investigated the feasibility of using microwave treatments, based on time and power levels, to eliminate or minimize standard plate count (SPC) bacteria, yeasts and molds in honey and to investigate the effect of microwave heating on the antibacterial activity of honey. Also, to identify major bacteria in the honey samples.

2. Materials and methods

2.1. Honey samples

Honey samples were obtained from local apiarists (n = 20) from April to November in 2019. All samples were collected from various regions in the northern and southern Jordan Valley. Some samples were collected from nectar of plants growing on reclaimed water areas near wastewater treatment plans (9 samples) while others from natural forests areas (11 samples). Beekeepers placed samples into a sterile tightly closed glass containers with minimal handling and stored at 25 ℃ in the dark until used (six months during lab tests). The Manuka honey sample contains at least 515 mg/kg of methylglyoxal and 15^{+} UMF as appeared on the label. Its pH was 4.2. It is originated from New Zealand and was purchased from the local market.

2.2. Determination of honey water activity

Water activity (a_w) was measured at 25 ℃ (±0.2 ℃) using an electronic water activity meter (Aqualab Series, Hygrolab, Rotronic Instr. Corp, Huntington, NY, US) [47]. Measurements were performed in

triplicates for each sample, and the average was reported.

2.3. Microwave treatment

Honey samples (15 g) capped in 100 mL Teflon tubes, were microwaved using MAXI-14 (MILESTONE ETHOS EASY, Italy). Advanced microwave digestion system (closed system conditions) was used in this study at different power settings (400 and 800 watts). After the internal temperature of the samples reached 80 ℃, which was maintained throughout treatment, the samples were held for 10, 30, and 60 sec at either 400 or 800 watts as per [48] with minor modification. The treated samples were cooled within the microwave system. Samples were analyzed for SPC, yeast and mold. Untreated honey samples were used as controls.

2.4. Enumeration and presumptive identification of SPC, yeast and mold

Microbial enumeration was conducted according to the method described by Breslin et al. [49] with minor modification. Briefly, samples (4 g) were diluted 5-fold in sterile saline (0.9% NaCl) and centrifuged (29,220 x g at 4 ℃; 20 min). Resulting pellets were resuspended by vortex mixing for 1 min in 4 ml sterile saline. The pour plate method was used: 100 µL of resuspended cells were added to Tryptic Soy Agar (20 mL, TSA; Oxoid, UK) and incubated (37 ℃, 24 h) under aerobic conditions. This protocol was repeated using MacConkey (MAC) agar (37 ℃ for 48 h; Oxoid, UK) Baird-parker (BP) agar (37 ℃ for 72 h; Oxoid, UK) Salmonella-Shigella (SS) agar (37 ℃, 48 h; Oxoid, UK) and Potato Dextrose agar (PDA; 28 ℃, 5–7 d Oxoid, UK). In addition, 100 µL were streaked onto de Man Rogosa and Sharpe (MRS agar; Oxoid, UK) and incubated anaerobically (30 ℃, 4 d) using an Oxoid AnaeroGen system. Plates were inoculated in duplicate and average numbers of colonies was reported. Presumptive isolates were stored by mixing 1ml of fresh culture broth with 666 µL of 50% glycerol and stored at −80 ℃.

2.5. Molecular identification

Presumptive isolates were streaked onto TSA plates and incubated (37 ℃, 24 h) under aerobic conditions. Single colonies were cultured in TSB broth aerobically (37 ℃, 6 h). Broth cultures were subsequently used for genomic DNA extraction using phenol-chloroform extraction [50].

In the present study, a set of universal primers 27F (5'-AGAGTTTGATCCTGG CTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify a specific region of the bacterial 16S rRNA genes, using appropriate amplification conditions as described by Miyoshi et al. [51]. PCR amplifications were analyzed using 1% agarose gel electrophoresis. Sequencing of PCR amplicons was performed by Macrogen Inc (Korea), using forward primers. All resulting 16S sequences were determined using a BLASTN program. The identified *Acinetobacter* spp. was performed by PCR using species-specific primers. PCR partial amplification of $bla_{OXA\text{-}51}$ like gene was performed as previously described by Turton et al. [52]. *A. baumannii* (ATCC 19606) DNA was used as a control.

2.6. Bacterial strains used for testing antibacterial power of honey

To assess the effect of microwave treatment on the antibacterial activity of honey, 9 bacteria

(Table 1) were challenged with treated, untreated raw honey and Manuka honey. All bacteria were obtained from the Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology. Bacterial cultures used in the study were stored at −80 ℃.

Table 1. Targeted Bacteria.

Genus spp.	ATCC no.	Resistance phenotype
Escherichia coli	BAA-2452	Colistin disk 10μg
Klebsiella pneumoniae	BAA-2146	Colistin disk 10μg
Pseudomonas aeruginosa	BAA-2114	Colistin disk 10μg
Acinetobacter baumannii (S)	19606	Colistin disk 10μg
Acinetobacter baumannii (MDR)	BAA- 1605A	Colistin disk 10μg
Listeria ivanovii	19119	Meropenem disk 10μg
Enterococcus faecium	BAA-2316	Meropenem disk 10μg
Staphylococcus aureus MRSA	33591	Vancomycin disk 30μg
Staphylococcus aureus MRSA	43300	Vancomycin disk 30μg

Note: S: sensitive, MDR: multi-drug resistant, MRSA: methicillin resistant *S. aureus*.

Honey samples which exhibited higher antibacterial activities were evaluated using AWD and MIC protocols. Samples: 1, 7, 14 and 20 were chosen.

2.7. Agar well diffusion

AWD was performed as per Mama et al. [53] with slight modification. Upon thawing, each organism was streaked onto Luria-Bertani (LB) agar plates and incubated at 37 ℃, for 16–20 h. Single colonies were cultured in Mueller Hinton broth (2.0 ml, MHB; Oxoid, UK). Suspensions were adjusted to a 0.5 McFarland standard ($OD_{625\ nm}$).

Mueller Hinton (25 mL; Oxoid, UK) agar plates were surface streaked using sterile cotton swabs, previously dipped in the microbial suspensions, over the entire surface of the plates in order to obtain uniform bacterial densities. Wells (6 mm) were cut into each agar plate using a sterile glass cork borer. Five wells (for each sample) were filled with honey (50 μL) previously diluted using sterile distilled water (w/v, %): 25, 50, 75 and 100. A negative control consisting of a well filled with sterile distilled water (50 μL) and a positive control consisting of a standard commercial antibiotic disc, Oxoid, UK (Table 1) were included in each plate. Plates were placed in a laminar flow cabinet at ambient temperature for 15 min to allow for diffusion and subsequently incubated aerobically (37 ℃, 18–24 h). Antibacterial activities were assessed by measuring the diameters of the inhibition zones. Each assay was carried out in duplicates and the means and standard deviations of the zones were calculated.

2.8. Minimum inhibitory concentrations

Four samples yielding higher antibacterial activities assessed by AWD and the Manuka honey sample were chosen for MIC determination. Experiments were performed in triplicates by microdilution using 96-well plates before and after microwave treatment as described by Wiegand et al. [54]. Each sample (%): 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.0976 and 0.0488 was assessed against each of the chosen microorganisms.

Bacteria were first grown on Luria-Bertani (LB) agar and incubated at 37 ℃, for 16–20 h. Each microorganism was suspended in MHB, adjusted to 1×10^8 CFU/ ml (0.5 McFarland) and diluted by a factor of 1:100 by adding 200 µL bacterial suspension to 19.8 mL sterile MHB in sterile 50 mL conical tubes. Resultant suspensions (10^6 CFU/mL) of 100 µL were added to wells containing 100 µL of diluted honey in order to obtain a final concentration of 5.0×10^5 CFU/well. Sterile MHB was added in one well for sterility assessment; bacterial solutions in broth were included as a positive control while honey mixed with broth were included as negative controls. Plates were incubated at 37 ℃, for 18–24 h and the $OD_{600\ nm}$ determined using a microtiter plate reader (BioTek Epoch).

2.9. Statistical analysis

All data were presented as means ± standard deviations and were analyzed by Statistical Package for Social Sciences (SPSS) software version 26.0 (IBM Corporation; Armonk, New York, USA). The Nonparametric Wilcoxon signed-rank test was used to compare two related samples in order to assess whether their population mean ranks differed and used with data that violates the assumption of normality. Mean values using the Wilcoxon signed-rank test were conducted to determine the differences between honey samples before and after microwave treatments and among variable treated groups. For all analyses, differences were considered significant at p-value < 0.05. Linear regression analyses were used to describe the effects of water activity on microbial counts.

3. Results

3.1. Microbial counts of raw honey

The average SPC of the honey samples was 286.5 CFU/g with a range from 50 to 660 CFU/g while the yeast count ranged from 10 to 710 CFU/g with an average 161 CFU/g; mold counts were the lowest ranging from 0 to 110 CFU/g with an average of 25.5 CFU/g (Figure 1). Manuka honey samples contained no detectable SPC. Seven samples (35%) contained SPC greater than 470 CFU/g; four of these sample contained yeasts > 320 CFU/g, while only one sample contained mold counts greater than 100 CFU/g.

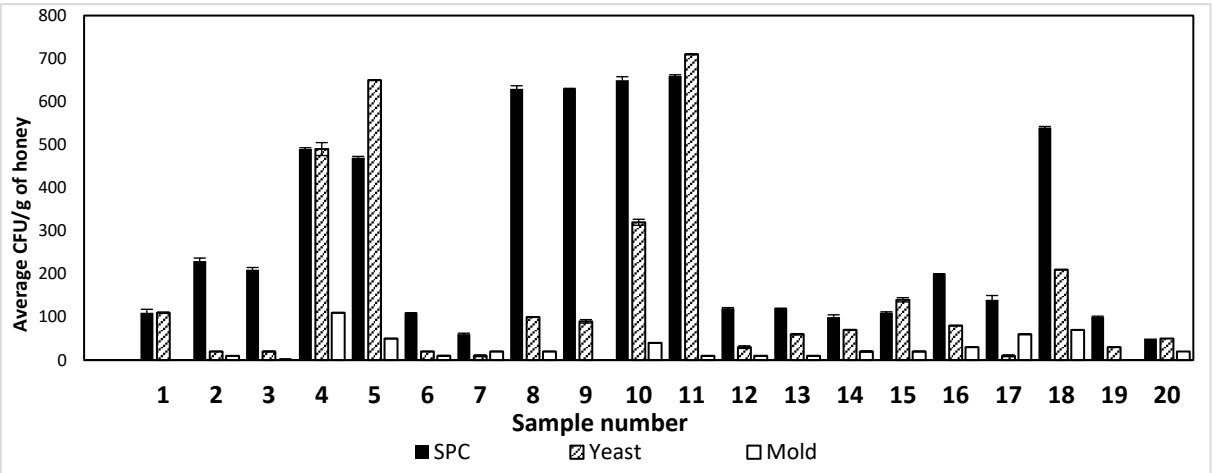

Figure 1. Microbial assessment of honey samples.

3.2. Water activity (aw) in raw honey

The a_w for the local samples ranged from 0.58 to 0.72; while for Manuka honey was 0.595. Regression equations showed no significant association ($r^2 < 1$) between a_w and SPC, yeast or mold; r^2 values ranged from 0.0012 to 0.1673. Due to the high microbial load in some samples collected from hives close to the reclaimed water.

3.3. Effect of microwave treatment on SPC, yeast and mold.

Microwave treatment of samples was effective in reducing SPC. Increasing microwave from 400 to 800 W concomitant with an increase in the exposure time decreased the SPC. Microwave heating at 400 W reduced SPC from 286.5 to 82.5, 54.5 and 37.0 CFU/g after 10, 30 and 60 sec, respectively (Figure 2). At 800 W, reductions in SPC increased: 48.5, 28.5 and 19.5 CFU/g after 10, 30 and 60 sec of exposure, respectively.

Microwave heating at 400 W reduced yeast from 161.0 to 39.5, 15.5 and 14.0 CFU/g, after 10, 30 and 60 sec, respectively. At 800 W, yeasts were reduced from 161.0 to 28.5, 15.5 and 11.5 CFU/g, after 10, 30 and 60 sec, respectively.

The samples contained lower mold levels compared to SPC and yeasts. Similarly, mold populations gradually decreased with an increase in microwave power as time of treatment was increased. Microwave heating of honey samples at 400W reduced mold from 25.5 to 11.0, 8.0 and 6.0 CFU/g after 10, 30 and 60 sec, respectively. Microwave heating at 800W resulted in enhanced reductions of mold from 25.5 to 5.5, 2.5 and 1.5 CFU/g after 10, 30 and 60 sec, respectively (Figure 2).

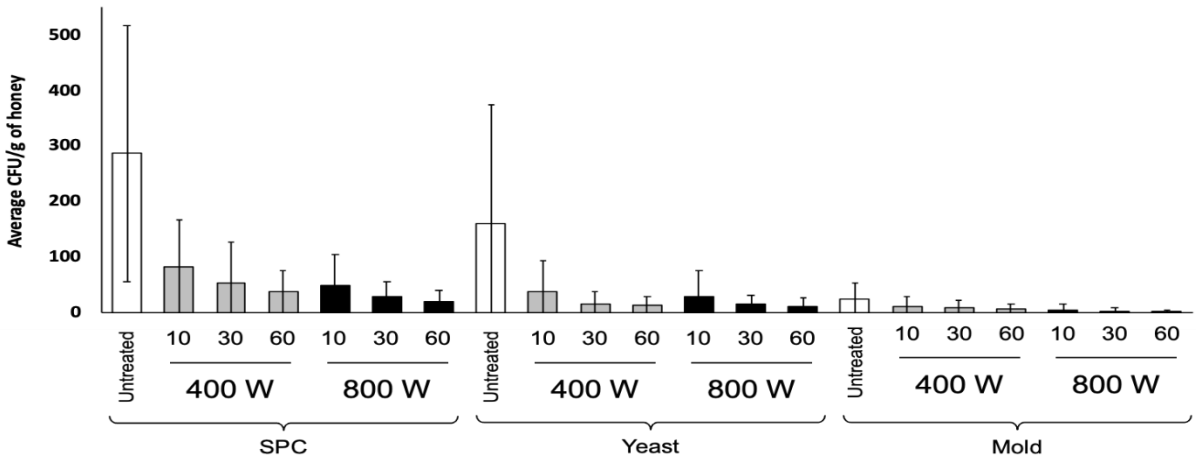

Figure 2. The effects of microwave treatments on SPC, yeast and mold at 400 and 800 W at 10, 30 and 60 sec. The data represent averages of CFU/g for each sample ± standard deviations.

3.4. Identification of bacterial isolates

Sequencing results of the 16S rRNA gene for the identified 125 bacterial isolates are presented in Table 2.

Table 2. Distribution of bacteria identified in the 16S rRNA gene of bacteria isolated from honey.

Bacteria	No of species (relative abundance)	% Identity
Bacillus (total)	109/125 (87.2%)	
Bacillus (spp)	52/109 (47.7%)	93.70–99.48
B. subtilis	22/109 (20.2%)	93.23–99.14
B. licheniformis	10/109 (9.2%)	91.43–99.31
B. safensis	6/109 (5.5%)	96.92–98.29
B. amyloliquefaciens	5/109 (4.6%)	94.14–97.49
B. pumilus	5/109 (4.6%)	98.24–96.73
B. velezensis	4/109 (3.7%)	96.50–99.47
B. cereus	3/109 (2.8%)	95.93–97.29
B. kochii	1/109 (0.92%)	96.32
B. tequilensis	1/109 (0.92%)	98.06
Acinetobacter baumannii	8/125 (6.4%)	94.87–98.85
Staphylococcus spp.	3/125 (2.4%)	
S. epidermidis	2/3 (66.7)	99.45
S. haemolyticus	1/3 (33.3)	98.03
Pseudomonas spp.	3/125 (2.4%)	95.56–98.33
Enterococcus faecium	2/125 (1.6%)	97.21–100

The 8 *Acinetobacter* isolates identified as *A. baumannii* (positive for *bla*$_{\text{OXA-51-like}}$ gene specific for *A. baumannii*) (Figure 3).

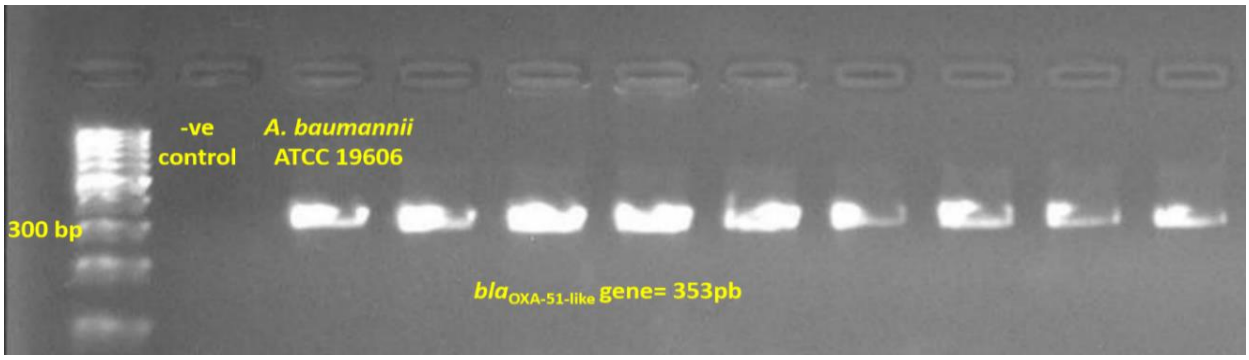

Figure 3. Agarose gel electrophoresis of *bla*$_{\text{OXA-51-like}}$ gene in *A. baumannii*.

3.5. Antibacterial activity

All samples exhibited varying degrees of antibacterial activity against all selected strains. Diluted Manuka honey showed no antibacterial activity when tested by AWD. The antibacterial activity of honey increased with the increase in honey concentration, as shown in Table 3.

Table 3. Antibacterial activity of raw honey evaluated by AWD.

Bacteria	Diameter of inhibition zone (mm) (n = 20)				
Raw honey concentration	Manuka honey (100%)	100%	75%	50%	25%
Escherichia coli	12	7.6 ± 7	0	0	0
Klebsiella pneumoniae	12	8.55 ± 8.2	0.5 ± 0.3	0	0
Pseudomonas aeruginosa	0	9 ± 8.5	0.8± 0.5	0	0
Acinetobacter baumannii 19606	11	10.2 ± 5.6	0.8 ± 1.8	0.4 ± 1.2	0
Acinetobacter baumannii BAA-1605A	15	10 ± 5.2	2 ± 3	0.2 ± 0.8	0
Listeria ivanovii	12	8.5 ± 5.5	0	0	0
Enterococcus faecium	15	10.3 ± 10	0.25 ± 0.01	0	0
Staphylococcus aureus 33591	15	12 ± 11.8	1.1 ± 1.2	0.2 ± 0.01	0
Staphylococcus aureus 43300	16	11.1 ± 10.8	0.9 ± 0.4	0.3 ± 0.02	0

Initial screening by AWD demonstrated that all undiluted honey samples exhibited bacterial inhibition. Moreover, at lower concentrations 75% and 50% (w/v), some honey samples continued to exert an antibacterial effect against all tested bacterial strains except for *E. coli* and *L. ivanovii*. However, at 25% no detectable antibacterial activity was observed with all samples. It should be noted that the antibacterial activity of some samples was more than the positive control value (antibiotic discs) except against *L. ivanovii*.

Honey samples treated by microwave heating.

The antibacterial activity of honey was divided into three groups according to the inhibition zone diameter (high, medium, and no-effect; Table 4). All treatments, depending on the times of exposure and power levels, for undiluted honey samples were significant ($p < 0.05$) in reducing the inhibition of all bacteria compared to the untreated samples.

Table 4. Antibacterial activity assessed by AWD assay before and after microwave heating.

	Diameter of Inhibition Zone (mm) (Mean ± SD) (n = 20)								
Anti-bacterial activity	Treatment Bacterial Strain	Un-treated	400 watts			800 watts			Positive control
			10 s	30 s	60 s	10 s	30 s	60	
(a) High	*E. coli* BAA-2452	13.3 ± 2.5	4.5 ± 0.5	6.8 ± 3.2	4.3 ± 3.3	2.3 ± 2.6	6 ± 4.2	4.8 ± 4.1	11
(n = 4)	*K. pneumonia* BAA-2146	14.3 ± 2.2	6.3 ± 4.8	3.3 ± 4.3	5.8 ± 2.9	6.8 ± 3.1	6 ± 2.3	5.5 ± 2.4	11
	P. aeruginosa BAA-2114	13.3 ± 1.7	5 ± 2.2	8.8 ± 3.2	8.8 ± 0.5	3.3 ± 2.2	4.8 ± 1.5	3 ± 3.8	13.5 ± 1.7
	A. baumannii 19606	14 ± 3.6	5.3 ± 1	6.8 ± 3.5	3.8 ± 5.2	3.8 ± 5.2	6 ± 4.5	4.8 ± 0.5	11
	A. baumannii BAA-1605	14.5 ± 2	2 ± 2.3	5.5 ± 3	8.5 ± 2.4	3.3 ± 2.2	5.3 ± 1.9	6.8 ± 2.6	15.3 ± 0.8
	L. ivanovii 19119	13.9 ± 3.1	4.5 ± 3.3	6.5 ± 1.9	9.8 ± 1.3	6.5 ± 4.4	9.3 ± 1	8.5 ± 2.4	29.4 ± 2.1
	E. faecium BAA-2316	13 ± 2.4	3.3 ± 2.2	4.5 ± 3.3	7.3 ± 2.2	3.5 ± 2.5	4 ± 4.6	0	14.1 ± 0.9
	S. aureus 33591	16.8 ± 1	4	6 ± 4.7	4.8 ± 4.1	2.3 ± 2.6	6 ± 4.2	4.8 ± 4.1	22.9 ± 1.5
	S. aureus 43300	17.8 ± 1	11.3 ± 3	13 ± 4.7	10.3 ± 3	9.5 ± 0.6	11 ± 1.4	11 ± 5.3	18.1 ± 0.6
(b) Medium	*E. coli* BAA-2452	9 ± 3	2.3 ± 2.2*g	2.7 ± 2.2*g	3.5 ± 3.9*g	2.4 ± 2.9*g	2.4 ± 2.9*g	3 ± 3.6*g	11.2 ± 0.4
(n = 11)	*K. pneumonia* BAA-2146	10.7 ± 3.8	2.1 ± 3.3*g	2.8 ± 3.2*g	6.2 ± 3.7*b, g	5.2 ± 3*a, g	5.1 ± 3.1*g	6 ± 3.4*g	10.6 ± 0.9
	P. aeruginosa BAA-2114	9.6 ± 3.9	4.7 ± 3.7*g	5.6 ± 3.7*g	3.9 ± 3.2*g	2.3 ± 3*a, g	1.2 ± 2*g, b	2.2 ± 3.3*g	13.9 ± 1
	A. baumannii 19606	12.5 ± 2.8	5 ± 3.4*g	4 ± 4.4*g	4.7 ± 4.4*g	6.1 ± 2.6*g	4.2 ± 3.3*g	4.4 ± 2.7*g	11.1 ± 0.8
	A. baumannii BAA-1605	11 ± 4.2	2 ± 3*g	3.8 ± 3.6*g	4.6 ± 3.5*g	3.5 ± 2.5*g	2.2 ± 2.8*g	5.1 ± 4*e, g	15.4 ± 0.6
	L. ivanovii 19119	10.2 ± 2.6	4.2 ± 3.2*g	3.2 ± 2.8*g	6.2 ± 3.8*b, g	4.4 ± 2.5*g	8.5 ± 2*b, d, g	6.9 ± 3.3*g	29.6 ± 2.6
	E. faecium BAA-2316	12.31.5	2.3 ± 2.8*g	2.9 ± 3.2*g	3.2 ± 3.4*g	2.6 ± 2.9*g	2.5 ± 3.2*g	3.4 ± 3.6*g	14.5 ± 1
	S. aureus 33591	13.6 ± 3.6	1.5 ± 2*g	2 ± 3.8*g	1.7 ± 2.5*g	3.8 ± 4.5*g	3.6 ± 5.9*g	4.5 ± 5.7*g	22.7 ± 1.3
	S. aureus 43300	12.8 ± 4.9	6.4 ± 4.2*g	6.6 ± 4.6*g	5 ± 6*g	1.1 ± 1.9*g	0.9 ± 2*g	1.2 ± 2.7*g	18.1 ± 0.6
(c) No antibacterial activity samples (n = 5)									

Experiments were performed in duplicate; activity is shown as the diameter (mm) ± SD.

a: trt 400W-10s, b: trt 400W-30s, c: trt 400 W-60s, d: trt 800W-10s, e: trt 800W-30s, f: trt 800W-60s, g: Un-trt. Trt: treatment. *Significant at ($p < 0.05$).

Minimum Inhibitory Concentrations.

MIC values of honey samples that gave the highest antibacterial activities according to AWD before and after microwave treatment were evaluated. However, statistical analysis of the MIC data did not show a significant ($p > 0.05$) decrease in antibacterial activity (Table 5).

Table 5. Antibacterial activity of honey assessed by MIC before and after microwave heating.

MIC (%w/v) (Mean ± SD) (n = 4)							
Treatment	Untreated	400 watts			800 watts		
Bacterial Strain		10 s	30 s	60 s	10 s	30 s	60 s
E. coli BAA-2452	12.5	25*g	25*g	25*g	25*g	25*g	25*g
K. pneumonia BAA-2146	12.5	25*g	25*g	25*g	25*g	25*g	25*g
P. aeruginosa BAA-2114	12.5	25*g	25*g	25*g	25*g	25*g	25*g
A. baumannii 19606	9.37 ± 3.6	21.8 ± 6.25	25	25	25	25	25
A. baumannii BAA-1605	6.25	21.8 ± 6.25	25*g	25*g	25*g	25*g	25*g
L. ivanovii 19119	9.37 ± 3.6	25	25	25	25	25	25
E. faecium BAA-2316	18.75 ± 7	25	25	25	25	25	25
S. aureus 33591	4.68 ± 1.8	21.8 ± 6.25	25	25	25	21.8 ± 6.25	25
S. aureus 43300	6.25 ± 4.4	21.8 ± 6.25	21.8 ± 6.25	21.8 ± 6.25	25	25	25

The experiments were performed in triplicate, and the data are expressed in the form of mean ± SD. (n = 4).

a: trt 400W-10s, b: trt 400W-30s, c: trt 400 W-60s, d: trt 800W-10s, e: trt 800W-30s, f: trt 800W-60s, g: Un-trt. Trt: treatment. *Significant at ($p < 0.05$).

Antibacterial activity of Manuka honey.

Compared with local honey samples, the negative effect of microwave treatments on Manuka honey was less, as shown in Table 6.

Table 6. Antibacterial activity of Manuka honey assessed by AWD and MIC before and after microwave heating.

Treatment	Untreated	400 watts			800 watts		
Bacterial Strain		10 s	30 s	60 s	10 s	30 s	60 s
(A) Diameter of Inhibition Zone (mm) (n = 1)							
E. coli BAA-2452	16	10	13	12	12	11	11
K. pneumonia BAA-2146	12	8	8	8	8	8	8
P. aeruginosa BAA-2114	0	0	0	0	0	0	0
A. baumannii 19606	16	9	9	9	9	9	9
A. baumannii BAA-1605	15	11	11	11	11	11	11
L. ivanovii 19119	12	11	10	10	10	10	10
E. faecium BAA-2316	15	15	14	14	14	14	14
S. aureus 33591	15	14	13	10	10	10	10
S. aureus 43300	16	15	15	14	14	12	12

Continued on the next page

Treatment	Untreated	400 watts			800 watts		
Bacterial Strain		10 s	30 s	60 s	10 s	30 s	60 s
(B) MIC (%w/v) (Mean ± SD) (n = 1)							
E. coli BAA-2452	12.5	25	25	25	25	25	25
Diameter of Inhibition	12.5	25	25	25	25	25	25
Zone (mm) (n = 1)							
P. aeruginosa BAA-2114	12.5	25	25	25	25	25	25
A. baumannii 19606	6.25	12.5	12.5	12.5	12.5	12.5	12.5
A. baumannii BAA-1605	6.25	12.5	12.5	12.5	12.5	12.5	12.5
L. ivanovii 19119	6.25	12.5	12.5	12.5	12.5	12.5	12.5
E. faecium BAA-2316	12.5	25	25	25	25	25	25
S. aureus 33591	6.25	12.5	12.5	12.5	12.5	12.5	12.5
S. aureus 43300	3.125	12.5	12.5	12.5	12.5	12.5	12.5

4. Discussion

Honey samples contained a mean SPC of 286.5 CFU/g. Lower numbers were observed for yeast and mold, with an average of 161 and 25.5 CFU/g, respectively. Similar to our results, it was reported that the SPC in honey ranged from 0 to several thousand CFU per gram [12,55]. In this respect, Tysset and Rousseau. [56] tested 175 honey samples from France and reported SPC yeast and mold mean of 227 and 90 CFU/g, respectively. Likewise, Iurlina and Fritz. [55] examined 70 honey samples originating in Argentina with some samples being imported. Their results indicated that honey from commercial sources, apiaries and bulk containers, exhibited SPC means of 244, 500 and 223 CFU/g, respectively. While yeast and mold from commercial, apiary, and bulk honey storage were 34, 164, and 100 CFU/g, respectively. In contrast, Nakano and Sakagucki, [15] reported a SPC of 83 CFU/g in 270 samples collected from Japan; a lower count of 24 CFU/g was recorded for imported samples.

SPC, yeast, and mold counts are well known to vary from country to country. This could be due to the different practices used during honey harvesting and packaging. Floral types and the environment where beehives are located may also contribute to the natural microflora of honey. For instance, beehives located near reclaimed water or other types of contaminants may be reflected in the microflora of the honey [12,57–59]. The microbial content in honey depends on many factors that affect honeybee, some of which are biotic, including bacteria, mold, parasites and viruses. Abiotic factors that stress honeybees, including environmental stresses as extreme temperatures, seasonal variation, and environmental pollutants from industry as well as urbanization activities are also important [60]. Nonetheless, this requires further studies to correlate the relationship between microbial contaminants in honey versus environmental stresses.

The 16s rRNA was used to help identify bacteria in the samples. *Bacillus* accounted for 87.2% of the isolates. This result agrees with studies reported by Malika et al. [61], Pomastowski et al. [62], and Tsadila et al. [63]. These researchers reported that *Bacillus* was the most abundant species in honey. This occurrence in honey ostensibly reflects the common presence of *Bacillus* in soil. Dust or contaminated water on flora could thereby contaminate pollinating honeybees [64].

This is the first report for the isolation of *A. baumannii* from honey samples. Veress et al. [16] isolated *A. lwoffii* while *A. pollinis* sp. nov., *A. baretiae* sp. nov., *A. rathckeae* sp. nov. were recently isolated from floral nectar and honeybees by Alvarez-Perez et al. [65]. In addition, Alvarez-Perez et

al. [66] isolated *A. nectaris* sp. nov., *A. boissieri* sp. nov., from the floral nectar of Mediterranean wild plants. Kim et al. [67] isolated *A. apis* sp. nov., from the intestinal tract of a honeybee. The isolation of *A. baumannii* from honey can be attributed to the close proximity of some honeybee hives to reclaimed water since *A. baumannii* is abundant in soil and reclaimed water [68,69]. These results stress the importance that soil, and vegetation might be a reservoir for *Acinetobacter* spp. [70]. As a result of environmental conditions, *A. baumannii* may have contaminated the samples during harvesting and packaging [65]. Similar to previous studies *Staphylococcus*, *Pseudomonas* and *Enterococcus faecium* were isolated. These microorganisms are also common in soil and reclaimed water [61–63,70,71].

The presence of pathogenic bacteria in honey is rare [12]. Contamination with pathogens due to improper hygienic practices while harvesting, handling and packaging is possible. Further, some bacteria that are associated with honeybee microbiota, such as lactic acid bacterial, *Streptococcus* and *Enterococcus* spp., have been isolated from honey [72]. Therefore, microwave heating could be one method used to address post-harvest contamination without affecting nutritional and sensory quality.

Results in the current study showed that microwave treatment reduced SPC, yeast, and mold in all honey samples depending on power and exposure times. As expected, the effect of increasing the power from 400 to 800 W was more pronounced than the effect of increasing the time, which is concordant with results obtained by Benlloch, Tinoco et al. [73]. This can be attributed to the increased movement of particles by increasing the microwave energy, which excites water molecules and thereby increasing the lethality of heat [74].

Honey, which is normally contaminated with certain bacteria and mold, can be treated with microwave to decrease the microbial load and render it safe for consumption by infants and immunocompromised individuals [61,75]. De La Paz Moliné et al. [76] used microwave heating to inactivate SPC, yeast, and mold counts in honey at different times and power levels (800W for 45 and 90 sec). They reported a decrease in all microbes proportional to the increase in time and power. Earlier, Hebbar et al. [20] reported similar results, where yeast counts decreased substantially upon microwave treatment of honey.

In addition to therapeutic benefits, honey has been reported to exert antibacterial effects; the most prominent of which is the high concentration of sugars. Average CHO of honey is 85%. This high osmotic fluid draws water from microbial cells leading to death via dehydration or inactivation by lysis [77,78]. Honey also contains antibacterial agents such as hydrogen peroxide, methylglyoxal, bee defensin-1, and phenolic compounds that help in killing bacterial cells [10,77,79,80].

In the current study, 25% honey did not exert any inhibition as shown by AWD. In contrast, this concentration did show effectiveness when evaluated using MIC. One limitation in evaluating the antibacterial activities by the AWD is the need for the active components to diffuse into the agar. Volatile and phenolic compounds (non-polar compounds) which might not diffuse easily may account for lower inhibition zones [81,82]. Furthermore, lack of hydrogen peroxide production in undiluted honey at high concentrations is also manifested in the observed low inhibition zones [82]. Therefore, it is difficult to compare the results with those of other studies, and the inability to distinguish between bacteriostatic and bactericidal activity [83].

MGO is the principal effective antibacterial agent in Manuka honey (mono-floral) from the Manuka myrtle tree in New Zealand and Eastern Australia. Uniquely, Manuka honey has a little hydrogen peroxide activity, unlike other honey types that contains hydrogen peroxide as the significant antibacterial agent [84,85]. Testing antibacterial activity of Manuka honey using well diffusion assay

pose a challenge since MGO diffusion through the agar is hindered and requires limited moisture to facilitate the diffusion mechanism despite it is low molecular weight [86]. Thus, it is likely that, inconsistency of results in Manuka honey between AWD and MIC methods is reported.

Two strains of MRSA and one *A. baumannii* strain were observed to be the most sensitive to honey. These results appear in agreement with studies by Gobin et al. [87] who found that *A. baumannii,* as well as *S. aureus,* were the most sensitive to honey. This effect could be due to the physiology or morphology of these bacteria *per se* that makes them more sensitive.

Raw honey contains unique compounds and bacteria secreted from the GIT of bees that act as antimicrobial agents and have probiotic properties, which might impart some antibacterial activity [72,88–92]. Further, Ibarguren et al. [93] tested several *Enterococcus faecium* isolates from six different samples of Argentinian honey against different strains of *Listeria monocytogenes* and found that there was an inhibitory effect.

In the current study, all honey samples were shown to exert antibacterial activity against nine pathogenic bacteria; however, the activity decreased after microwave treatment. This could be due to the inactivation of beneficial bacteria [94] as well as the inactivation of antibacterial compounds found in honey [48]. Furthermore, microwave heating has been reported to inactivate glucose oxidase, diastase (α-amylase), and invertase (α-glucosidase) enzymes which have antibacterial activity [43,48,95].

Methylglyoxal (MGO) is the main antimicrobial component in Manuka honey, [96] and its antibacterial activity is negatively affected by heat treatments, as well as the MGO content decreases with increasing temperature [97].

5. Conclusions

In the present study, 125 bacteria were isolated from twenty honey samples originating from diverse geographical regions in Jordan. *Bacillus* spp. represented the most prevalent bacteria. Also, the presence of *Acinetobacter baumannii* in honey has been reported for the first time.

Further, this is the first study to use a closed microwave system in honey treatment. Microbial populations decreased by increasing microwave power and time of exposure. In addition, raw honey exhibited antibacterial activity. However, microwave treatment reduced antibacterial activity. Therefore, it is not recommended to treat honey in the microwave, and there is a need to study other treatment methods to reduce the microbial load without negatively affecting the antibacterial activity of honey.

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Conflict of interest

All authors declare no conflict of interest in this paper.

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