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Comparing the Antimicrobial Actions of Greek Honeys from the Island of Lemnos and Manuka Honey from New Zealand against Clinically Important Bacteria †

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Abstract: Honey is a natural food that has a long history as a traditional medicine because of its many biological characteristics, including antimicrobial, antioxidant, anti-tumor and anti-inflammatory properties. In this study, the antimicrobial actions of eight different honeys from Lemnos island (north-eastern Greece) plus manuka honey (from New Zealand, UMF 30+, licensed in many countries as topical medical preparation) were evaluated against ten clinically relevant bacteria, including five Gram-positive (*Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Bacillus cereus*) and five Gram-negative (*Salmonella enterica* serovars Enteritidis and Typhimurium, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*). To achieve this, an agar-well diffusion assay measured the diameter of inhibition zones (mm) of two selected concentrations for each honey (25 and 12.5% *v*/*v*). The minimum inhibitory and bactericidal concentrations (MIC and MBC) of each sample were also calculated and compared against two representative bacterial species (*S.* Typhimurium and *S. aureus*) using microdilution and agar spot methods, respectively. The pH, water activity, and pollen-grain content of each honey were also determined. Results revealed that all the Lemnos honeys presented antibacterial action, which for some samples was superior to that of manuka, highlighting their potential for exploitation as natural antimicrobial systems for use in foods and medicine.

Keywords: honey; antimicrobial; Lemnos Greece; manuka; bacterial pathogens; minimum inhibitory and bactericidal concentrations

1. Introduction

Honey is a natural complex food that can be stored for a long time at room temperature without the need to add any preservative. This quality results from the synergistic combination of its low water activity (a_w < 0.6), low pH (ca. 3.2–4.5) and its antimicrobial compounds, such as hydrogen peroxide (H_2O_2), phenolic compounds (such as flavonoids), methylglyoxal (MGO), and antimicrobial peptides (such as bee defensin-1) [1]. Many studies have explored the widely reported antimicrobial action and therapeutic uses of manuka honey, which is native to New Zealand and parts of Australia and is currently licensed in many countries, as a topical medical preparation for the treatment of

wounds infection [2,3]. In recent years, there has been increasing interest in the antibacterial properties of honeys produced throughout the world, often in response to the rapid increase of antibiotic-resistant bacteria [4] and consumers' demand for medicinal foods (nutraceuticals) [5]. Honey properties and taste vary depending on the flora foraged by bees (such as pine, sage, thyme), the geographical foraging area and the local climatic environment (including temperature, soil, rainfall), as well as processing and storage conditions [1]. Studies have revealed that the strong antimicrobial actions of some of the tested honeys may be superior to that of manuka [6–8], known for its rich MGO content [2]. In this study, the antimicrobial actions of eight honeys produced in different locations of Lemnos Island (north-eastern Greece) and that of a manuka honey blend (from New Zealand, UMF 30+) were evaluated against ten clinically relevant bacteria, including five Gram-positive and five Gram-negative. The pH, water activity, and pollen content of each honey were also determined.

2. Materials and Methods

2.1. Honey Samples and Bacterial Strains

Eight freshly produced honeys harvested from beekeepers with apiaries in various locations of Lemnos island, and one sample of a medical-grade manuka honey blend (UMF 30+; Manuka Health, New Zealand) purchased from a local pharmacy, were tested. On arrival at the laboratory, all samples were stored in the dark in a refrigerator, and were analyzed within two months of receipt. Ten clinically relevant bacterial strains were used as the target microorganisms. Five were Gram-positive (Staphylococcus aureus str. DFSN_B26, S. epidermidis str. FMCC_B202, Enterococcus faecalis str. ATCC 29212, Listeria monocytogenes str. AAL 20074, and Bacillus cereus str. ATCC 10876), and five were Gram-negative (Salmonella enterica Enteritidis str. P167807, S. enterica Typhimurium str. DT193, Escherichia coli O157:H7 str. ATCC 43888, Vibrio parahaemolyticus str. ATCC 17802, and Pseudomonas aeruginosa str. ATCC 27853). The long-term cryostorage of the strains and the preparation of their working cultures were done following standard microbiolgical procedures.

2.2. Agar-Well Diffusion Assay

For each honey, two dilutions (25 and 12.5 % *v*/*v*) were prepared using quarter-strength Ringer's solution (Lab M) as the diluent. 40 μL of each dilution were then placed in duplicate in wells (of 5 mm diameter) prepared in soft TSA (i.e., TSB also containing 0.7% *w*/*v* agar) in a petri dish (of 90 mm diameter). In each petri dish, eight wells had been created with the help of an inverted Pasteur glass pipette. Before the creation of the wells, each soft agar medium had also been inoculated with the target microorganism (ca. 10^6 CFU/mL) and left to solidify in the dishes. Following the addition of the diluted honey samples to the wells, dishes were left for 2 h at room temperature and were then placed at 37 °C for 24 h (except for B. cereus, which was incubated at 30 °C). Soft TSA also contained 3% (*w*/*v*) NaCl in the case of alophile *V. parahaemolyticus*. Following incubation, the growth inhibition zones around each well were measured with the help of a ruler. Ampicillin (50 μg/μL) and corn glucose syrup (82% *v*/*v*; Haitoglou Bros SA, Kalochori, Thessaloniki, Greece) were used as positive and negative antimicrobial controls, respectively. The last one was selected because it has the average sugar content of honey. The experiment was repeated three times using independently grown bacterial cultures.

2.3. Determination of Minimum Inhibitory and Bactericidal Concentrations (MIC, MBC) of Each Honey

The MIC of each honey was determined against both *S*. Typhimurium and *S. aureus*, representing Gram-negative and Gram-positive bacteria, using the classical broth microdilution method. To do this, ten successive binary dilutions (i.e., 25–0.1% *v*/*v*) of each honey were prepared using TSB as the diluent. Subsequently, 180 μL of each dilution were transferred to a well (in duplicate) of a sterile flat-bottomed 96-well polystyrene (PS) cell culture plate (transparent, Ref 30096; SPL Life Sciences, Gyeonggi-do, Korea) and 20 μL of a 100-fold dilution of the appropriate bacterial working culture were then added, giving an initial bacterial concentration in each well of ca. 10^5

CFU/mL. Wells without bacteria and wells without added honey served as negative and positive growth controls, respectively. The plates were sealed with parafilm and statically incubated at 37 °C for 24 h. The growth in each well was turbidimetrically assessed by the naked eye to calculate the MIC value to give the lowest concentration of each honey that totally inhibited the visible bacterial growth. To calculate MBC, from all the wells showing no visible growth, 10 μL were aspirated and spotted on TSA and the number of colonies was counted following incubation at 37 °C for 24 h. MBC for each honey was defined as its lowest concentration reducing the initial inoculum by at least 3 logs (i.e., no appearance of colonies). The experiment was repeated three times using independently grown bacterial cultures.

2.4. pH, a_w Measurements and Determination of the Botanical Origin of Honeys

The pH of each honey was measured using the C931P Consort electrochemical analyzer (Turnhout, Belgium) following mixing 10 g of honey with 75 mL of distilled water, while its a_w was determined using the LabTouch instrument of Novasina AG (Lachen, Switzerland). Before all measurements were taken, honeys were left outside refrigerator for sufficient time to reach room temperature. All honeys were also analyzed palynologically using a nonacetalytic technique to determine their botanical origin and according to standard methods [9]. Thus, for each honey, more than 800 pollen grains were counted and digitally photographed using Motic Compound Microscope B3-223 ASC equipped with a CCD color camera (MoticEurope, S.L.U.; Cabrera de Mar Barcelona, Spain). These were finally identified with reference to our database pollen grain collection of Lemnos plants, prepared according to standard palynological methods [9], and results were expressed in percentages. For the palynological analysis of the manuka honey blend, literature sources [10] were used to identify the origin of its digitally photographed pollen grains.

3. Results and Discussion

The results of the agar-well diffusion assay are presented in Table 1. In general, the Gram-positive bacteria were more resistant compared to the Gram-negative ones, apart from *P. aeruginosa* for which no inhibition was observed for any of the tested honeys. Similarly, none of the honeys at either tested concentration (i.e., 25 and 12.5% *v*/*v*) could inhibit the growth of *S. epidermidis*, *L. monocytogenes* and *B. cereus*, while *E. faecalis* was found susceptible only to the action of manuka honey. The two *Salmonella* serovars (i.e., Enteritidis and Typhimurium), *E. coli* O157:H7 and *V. parahaemolyticus*, were inhibited by all nine tested honeys at both concentrations (except for Lemnos honey No 6 applied at 12.5% against *S*. Enteritidis). *S. aureus* was found susceptible to only two Lemnos honeys (samples 7 and 8) and to manuka. These two local honey samples, and in particular sample 7, were found to present the strongest antibacterial actions, being able to inhibit five of the ten tested strains. This inhibition against most of the susceptible strains was superior to that of manuka, which was still able to inhibit six of the ten strains. Glucose syrup, used here as a negative antimicrobial control to test for any inhibition due to possible osmotic effects, was able to inhibit only the four Gram-negative *S*. Enteritidis, *S*. Typhimurium, *E. coli* O157:H7 and *V. parahaemolyticus*. However, except against the last halophilic species, the growth inhibition zones of glucose syrup were significantly lower than those recorded following the application of the honey samples. As expected, kanamycin (50 μg/μL), used here as a positive antimicrobial control, was quite effective against all the tested strains, displaying the strongest action against *S*. Enteritidis and the lowest against *P. aeruginosa* (with recorded inhibition zones equal to 35.8 ± 3.2 and 13.3 ± 2.1, respectively).

Table 1. Diameters (mm) of inhibition zones of honeys, each applied at two concentrations (25 and 12.5% *v*/*v*) against the target bacteria as there were determined by the agar-well diffusion assay. Each value also comprises the diameter of the well (5 mm). The inhibition zones of glucose syrup and kanamycin, used as negative and positive antimicrobial controls, are also indicated.

s/n	Sample	Conc.	Gram−					Gram+				
			***S.* Enterit.**	***S.* Typhim.**	***E. coli***	***V. parah.***	***P. aerugin.***	***S. aureus***	***S. epiderm.***	***E. faecal.***	***L. monoc.***	***B. cereus***
1	**Lemnos honey No 1**	25% (*v*/*v*)	22.0 ± 2.0	18.0 ± 0.0	22.0 ± 0.0	22.0 ± 1.6	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	17.0 ± 4.2	19.3 ± 5.8	19.3 ± 1.2	19.5 ± 0.7	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
2	**Lemnos honey No 2**	25% (*v*/*v*)	21.3 ± 3.1	19.0 ± 1.4	21.3 ± 3.1	20.7 ± 3.1	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	19.0 ± 1.4	14.5 ± 5.5	18.7 ± 3.1	19.3 ± 3.2	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
3	**Lemnos honey No 3**	25% (*v*/*v*)	20.7 ± 1.2	25.0 ± 4.2	21.3 ± 1.2	23.0 ± 2.6	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	17.5 ± 3.5	9.5 ± 3.5	19.3 ± 1.2	18.3 ± 2.1	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
4	**Lemnos honey No 4**	25% (*v*/*v*)	24.0 ± 3.5	20.0 ± 2.8	21.3 ± 4.2	20.0 ± 2.8	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	19.7 ± 4.5	18.3 ± 2.9	18.3 ± 3.5	18.0 ± 2.8	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
5	**Lemnos honey No 5**	25% (*v*/*v*)	23.0 ± 1.4	22.0 ± 2.0	21.3 ± 4.6	23.0 ± 1.4	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	20.0 ± 0.0	12.0 ± 3.0	21.0 ± 1.4	21.0 ± 1.4	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
6	**Lemnos honey No 6**	25% (*v*/*v*)	20.0 ± 2.0	23.0 ± 4.2	22.0 ± 2.0	21.0 ± 1.4	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	5.0 ± 0.0	12.0 ± 4.2	20.0 ± 2.0	18.0 ± 2.8	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
7	**Lemnos honey No 7**	25% (*v*/*v*)	27.3 ± 1.2	22.0 ± 2.0	28.7 ± 1.2	26.0 ± 2.0	5.0 ± 0.0	30.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	22.7 ± 2.3	21.0 ± 1.4	26.0 ± 0.0	21.7 ± 5.9	5.0 ± 0.0	24.0 ± 3.5	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
8	**Lemnos honey No 8**	25% (*v*/*v*)	22.0 ± 2.0	24.7 ± 1.2	28.0 ± 0.0	26.0 ± 2.0	5.0 ± 0.0	30.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	20.0 ± 4.0	20.0 ± 0.0	24.7 ± 1.2	23.3 ± 3.1	5.0 ± 0.0	26.7 ± 2.3	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
9	**Manuka honey**	25% (*v*/*v*)	24.0 ± 2.0	22.0 ± 0.0	28.0 ± 0.0	22.0 ± 0.0	5.0 ± 0.0	30.0 ± 3.5	5.0 ± 0.0	32.0 ± 5.7	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	21.0 ± 1.4	20.0 ± 0.0	25.0 ± 1.4	20.0 ± 0.0	5.0 ± 0.0	25.3 ± 4.2	5.0 ± 0.0	29.0 ± 4.2	5.0 ± 0.0	5.0 ± 0.0
10	**Glucose syrup (82% *v*/*v*)**	25% (*v*/*v*)	11.5 ± 6.4	12.0 ± 4.2	16.5 ± 2.1	21.3 ± 1.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
		12.5% (*v*/*v*)	10.0 ± 0.0	5.0 ± 0.0	14.0 ± 3.5	18.7 ± 3.2	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.0 ± 0.0
11	**Kanamycin**	50 μg/μL	35.8 ± 3.2	33.9 ± 2.9	35.7 ± 3.2	32.9 ± 3.1	13.3 ± 2.1	24.5 ± 2.1	33.7 ± 2.0	21.6 ± 1.0	25.1 ± 2.4	27.7 ± 1.6

The MIC and MBC of each honey against *S.* Typhimurium and *S. aureus*, as they were determined by the broth microdilution and agar spot methods, are presented in Table 2. Lemnos honey No 2 was found to present the strongest antibacterial action displaying MIC and MBC against both bacterial species equal to 12.5% (*v*/*v*). For all the other tested honeys, MIC and MBC were either 25% (*v*/*v*) or even higher. Glucose syrup could not inhibit neither bacteria at the concentrations this was tested (i.e., 25–0.1% *v*/*v*). No clear correlation between the antimicrobial results of the two tested methods, i.e., agar-well diffusion and broth microdilution, could be established. The pH, a_w values and pollen composition (%) of each honey are shown in Table 3. As expected, the pH values varied between 3.6 (for almost all Lemnos honeys) to 4.3 (for manuka honey). Water activity was found to vary from 0.551 (Lemnos honey No 6) to 0.627 (manuka honey). The pH and a_w of glucose syrup measured 4.85 and 0.731, respectively. Pollen composition of the Lemnos honeys was multifloral, containing pollens from a variety of plant species including myrrh (*Anthillis hermanniae*) and thyme (*Thymus capitatus*)-dominant pollen grains-, burdock (*Arctium lappa*), thistle (*Silybum marianum*) etc., thus highlighting the rich plant biodiversity encountered in the island of Lemnos.

Table 2. MIC and MBC of each honey against *S.* Typhimurium and *S. aureus* as there were determined by the broth microdilution and agar spot methods. The MIC and MBC of glucose syrup, used as negative antimicrobial control, are also indicated.

s/n	Sample	MIC		MBC	
		S. Typhimurium	***S. aureus***	***S. Typhimurium***	***S. aureus***
1	**Lemnos honey No 1**	>25%	>25%	>25%	>25%
2	**Lemnos honey No 2**	12.50%	12.50%	12.50%	12.50%
3	**Lemnos honey No 3**	>25%	>25%	>25%	>25%
4	**Lemnos honey No 4**	25%	25%	25%	25%
5	**Lemnos honey No 5**	>25%	>25%	>25%	>25%
6	**Lemnos honey No 6**	>25%	25%	>25%	25%
7	**Lemnos honey No 7**	25%	25%	25%	25%
8	**Lemnos honey No 8**	25%	25%	25%	25%
9	**Manuka honey**	>25%	25%	>25%	25%
10	**Glucose syrup (82% *v*/*v*)**	>25%	>25%	>25%	>25%

Table 3. pH, a_w values and pollen composition (%) of each honey. The pH and a_w of glucose syrup are also indicated.

s/n	Sample	pH	a_w	Dominant Pollen Grains Composition (%)
1	**Lemnos honey No 1**	3.55 ± 0.00	0.574	***Antillis hermanniae* 48.3%**; *Sinapis arvensis* 12.1%; *Melia azedarah* 8.7%; *Thymus capitatus* 2.5%
2	**Lemnos honey No 2**	3.61 ± 0.02	0.587	***Antillis hermanniae* 29.1%**; *Arctium lappa* 13.7%; *Thymus capitatus* 4.2%; *Melia azedarah* 4.2%; *Ferula communis* 1/3%
3	**Lemnos honey No 3**	3.60 ± 0.03	0.568	***Echium vulgare* 33.0%**; ***Antillis hermanniae* 23.0%**; *Pyrus amigdaliformis* 11.0%; *Melia azedarah* 8.0; *Arctium lappa* 7.5%; *Thymus capitatus* 1.5%
4	**Lemnos honey No 4**	3.62 ± 0.02	0.574	***Antillis hermanniae* 25.3%**; *Echium vulgare* 18.4%; *Sinapis arvensis* 16.3%; *Melia azedarah* 8.6%; *Arctium lappa* 5.3%; *Thymus capitatus* 2.5%
5	**Lemnos honey No 5**	3.60 ± 0.02	0.597	***Rubus fruticosus* 11.9%**; *Pyrus amigdaliformis* 8.6%; *Thymus capitatus* 4.8%; *Echium vulgare* 3.3%; *Melia azedarah* 1.9%; *Antillis hermanniae* 1.0%
6	**Lemnos honey No 6**	3.67 ± 0.01	0.551	***Echium vulgare* 18.3%**; *Antillis hermanniae* 10.2%; *Pyrus amigdaliformis* 8.8%; *Arctium lappa* 7.3%; *Rubus fruticosus* 6.8%; *Thymus capitatus* 6.8%; *Melia azedarah* 5.9%; *Silybum marianum* 3.1%
7	**Lemnos honey No 7**	3.62 ± 0.03	0.570	***Thymus capitatus* 23.3%**; *Melia azedarah* 7.0%; *Rubus fruticosus* 7.0%; *Antillis hermanniae* 5.8%; *Silybum marianum* 3.5%; *Hypericum perforatum* 3.5%
8	**Lemnos honey No 8**	3.63 ± 0.02	0.604	***Echium vulgare* 19.5%**; *Antillis hermanniae* 13.7%; *Rubus fruticosus* 12.7%. *Thymus capitatus* 10.2%; *Pyrus amigdaliformis* 9.3%

9	**Manuka honey**	4.26 ± 0.03	0.627	***Leptospermum scoparium* 75.8%**; *Trifolium repens* 14.2%; Lotus type 9.2%
10	**Glucose syrup**	4.85 ± 0.03	0.731	-

4. Conclusions

In recent years, several studies have been published about the antimicrobial actions of honeys collected from various parts of the world [11–13]. These have revealed promising antimicrobial activity of some honeys even against multidrug-resistant bacterial pathogens, such as the methicillin resistant *S. aureus* (MRSA). These have also emphasized the variability in the antimicrobial effect of honeys depending on the sample and target microorganism and pointed to the need for further research. Our study, focusing on honeys produced in a Greek island of the north Aegean region (i.e., Lemnos), known for its biodiversity and containing wild plants of medicinal importance, such as thyme and myrrh [14], complements all these other studies. In summary, our results revealed that all the Lemnos honeys presented antibacterial action which, for some samples, was superior to that of well-publicized manuka. Such honeys show great potential for the development of natural antimicrobial systems for use in foods and medicine.

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