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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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Honey & antimicrobial properties

- Honey is a **natural complex food** that can be stored for long time at room temperature without the need to add any preservative.

- This is due to a 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)
  - ✓ **antimicrobial peptides** (such as bee defensin-1).

- Many studies have explored the **antimicrobial action and therapeutic uses of manuka honey**, which is native to New Zealand and parts of Australia.

- Manuka honey is currently licensed in many countries as a **topical medical preparation** for the treatment of wounds infection.

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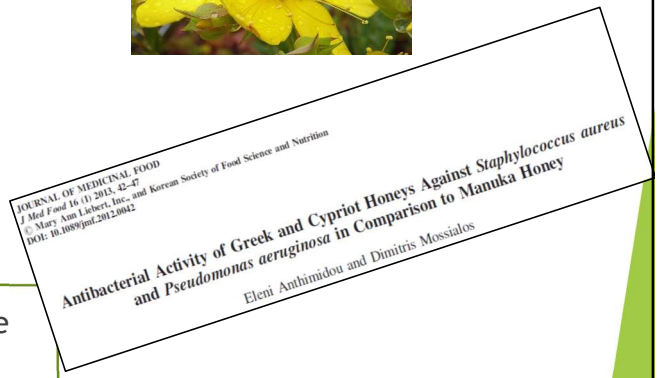
Increasing interest in antimicrobial properties of honey

- **In recent years, there has been increasing interest in the antibacterial properties of honeys produced throughout the word, mainly due to:**

- ✓ the rapid increase of antibiotic resistant bacteria (MDR)
- ✓ the consumers demand for medicinal foods (nutraceuticals)

- **Honey properties and taste vary depending on:**
- ✓ the flora foraged by bees (such as pine, sage, thyme etc.)
- ✓ the geographical foraging area and the local climatic environment (temperature, soil, rainfall, etc.)
- ✓ processing and storage conditions

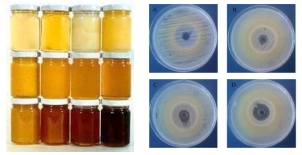


- Studies have revealed that the strong antimicrobial actions of some of the tested honeys may be superior to that of manuka, known for its rich MGO content.

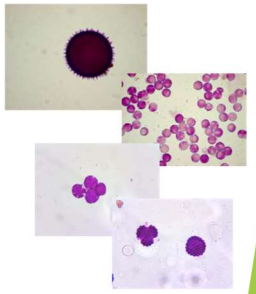
Aim of this work

- **In this study, the antimicrobial actions of eight honeys produced in various locations of the Lemnos island (north-eastern Greece) and that of manuka honey blend (from New Zealand, UMF 30+) were evaluated against ten clinically relevant bacteria, including five Gram-positive and five Gram-negative.**

- To do this, the **agar-well diffusion assay** was applied to measure 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 honey were also calculated against two representative of the bacterial species (S. Typhimurium and S. aureus), following the **broth microdilution and agar spot methods**, respectively.



- The **pH, water activity, and pollen content** of each honey were also determined.

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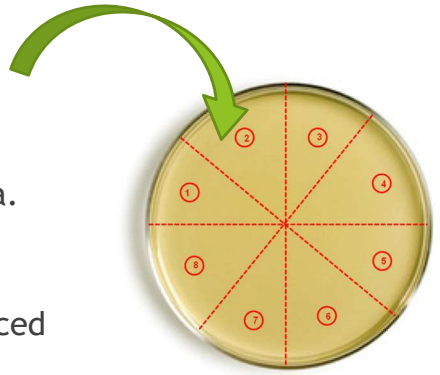
Bacterial strains used as targets

s/n	Bacterial species	Strain	Gram reaction
1	*Salmonella enterica* ser. Enteritidis	P167807	-
2	*Salmonella enterica* ser. Typhimurium	DT193	-
3	*Escherichia coli* O157:H7	ATCC 43888	-
4	*Vibrio parahaemolyticus*	ATCC 17802	-
5	*Pseudomonas aeruginosa*	ATCC 27853	-
6	*Staphylococcus aureus*	DFSN_B26	+
7	*Staphylococcus epidermidis*	FMCC B-202	+
8	*Enterococcus faecalis*	ATCC 29212	+
9	*Listeria monocytogenes*	AAL 20074	+
10	*Bacillus cereus*	ATCC 10876	+

Agar-well diffusion assay

materials and methods

- For each honey, two dilutions (**25 and 12.5 % v/v**) were prepared (using quarter-strength Ringer's solution as the diluent).
- **40 μL** of each dilution were then placed in duplicate in wells (of 5 mm diameter) prepared in soft TSA (0.7% w/v agar) placed in a petri dish.
- Each soft agar medium had also been inoculated with the **target microorganism** (ca. **10^6 CFU/mL**).
- Following the addition of the diluted honey samples into the wells, dishes were 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 halophile *V. parahaemolyticus*.
- Following incubation, the **growth inhibition zones** around each well were measured (mm).
  - **Ampicillin (50 μg/μL)** and **corn glucose syrup (82% v/v)** were used as positive and negative antimicrobial controls, respectively.



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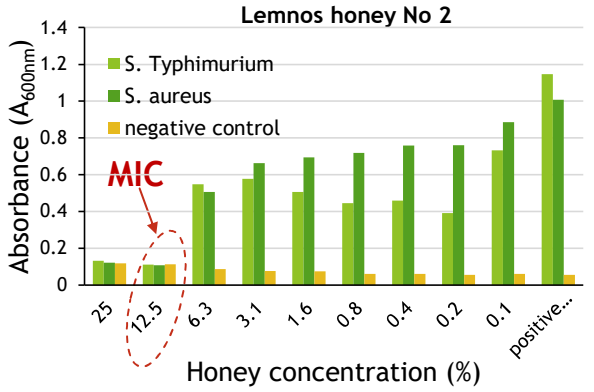
Determination of minimum inhibitory and bactericidal concentrations (MIC, MBC) of each honey

materials and methods

- **MIC:** lowest honey's concentration preventing visible bacterial growth.
- **MBC:** lowest honey's concentration reducing viability of the initial bacterial inoculum by ≥ 99.9%.

- **MIC → Broth microdilution method** (in 96-well PS microtiter plates; **10 concentrations / honey → 25 - 0.1% v/v**).



- **Two strains:** *S.* Typhimurium & *S. aureus* (Gram- & Gram+).
- **Incubation for 24 h at 37 °C in TSB** (optimal growth conditions).
- Absorbance measurements (at 600 nm).

- **MBC → agar spot method**

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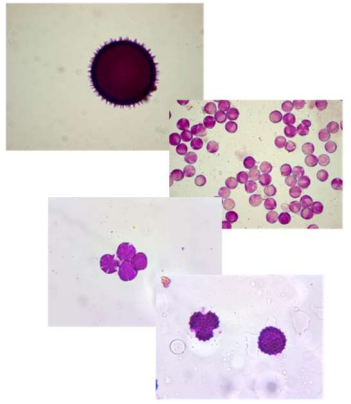
pH, a_w measurements and determination of the botanical origin of honeys

materials and methods

- The **pH** of each honey was measured using the C931P Consort electrochemical analyzer following mixing 10 g of each honey with 75 mL of distilled water.
- The **a_w** of each honey was determined using the LabTouch instrument of Novasina AG.

- **All honeys were also analyzed palynologically using a nonacetalytic technique and according to standard methods <u>to determine their botanical origin</u>.**
- 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.
- These were finally identified with reference to our database pollen grain collection of Lemnos plants, prepared according to standard methods, and results were expressed in percentages.
- For the palynological analysis of the manuka honey blend, literature sources were used to identify the origin of its digitally photographed pollen grains



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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**

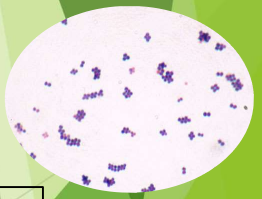
Results

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

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.

Results of the **agar-well diffusion assay**

- 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.

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MIC and MBC of each honey against *S.* Typhimurium and *S. aureus* as there were determined by the **broth microdilution and agar spot methods**

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%

The MIC and MBC of glucose syrup used as negative antimicrobial control are also indicated.

- **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.

pH, a_w values and pollen composition (%) of each honey

Results

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	-

The pH and a_w of glucose syrup are also indicated.

- 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 aw of **glucose syrup** measured 4.85 and 0.731, respectively.
- Pollen composition of the Lemnos honeys were 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

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Summary (main outcomes)

- In recent years, several studies have been published about the antimicrobial actions of honeys collected from various parts of the world.

- 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, 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 manuka.**
- **Such honeys show great potential for the development of natural antimicrobial systems for use in foods and medicine.**

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