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Original article

The antimicrobial mechanism of Greek thyme honeys against methicillin-resistant *Staphylococcus aureus* clinical isolates: a case study of comparison with Manuka honey

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Summary The antibacterial potential of honey has been of great scientific interest. Understanding the underlying mechanism is essential to explore its potential as therapeutic alternative against a range of (non)-pathogenic microorganisms. The floral origin of honey is critical for its antibacterial activity and Greek thyme honeys have been of increasing interest due to their chemical composition and bioactivity. In this study, the antimicrobial effect of six Greek honeys, with different percentages of thyme pollen grains and two Manuka honeys were tested against four MRSA clinical isolates (ATCC 43300, 0791, 28965, 01322). Agar-well diffusion assay and total viable counts were used to examine the antimicrobial strength of honeys, while the effect thereof on cellular redox state and cell membrane was tested by flow cytometry. Thyme honeys had superior or equal antimicrobial strength compared to this of Manuka, while thermal processing did not significantly affect this activity. Acidity and the high H_2O_2 content, common features for all thyme honeys, caused cellular oxidative damage and cell death unlike observed in Manuka-treated populations. The activity of the monofloral thyme honey (74% thyme pollen) was higher than the other indigenous polyfloral samples, which confirms the medicinal importance of this medicinal plant.

Keywords Antimicrobial, flow cytometry, Manuka, methicillin-resistant *S. aureus*, oxidative stress, thyme.

Introduction

Honey has been acknowledged in modern medicine for the significant antimicrobial effect towards (non)-pathogenic species and important clinical isolates. The antimicrobial activity of honey varies greatly with its origin and is also dependent on the natural vegetative flowers blooming in different seasons and places. Greece has a long history of beekeeping dating back to 5500 BCE and ranks number two worldwide, with apicultural density; about 11.1 beehives per sq. km (Henein, 2015). The Mediterranean climate, the wide biodiversity (5800 species and 1893 subspecies) and endemism (22.2% of all species present with 1278 species and 452 subspecies) favour the production of honeys of high nutritional profile, filled with vitamins and antioxidants (Georghiou & Delipetrou, 2010).

There are only a few systematic studies regarding the classification of honeys based on their origin, compositional profile and antimicrobial strength (Alissandrakis *et al.*, 2009; Aliferis *et al.*, 2010; Melliou & Chinou, 2011). However, most honeys harvested form beehives in Greek mainland and islands showed abundance in phenolics (flavonoids and phenolic acids), volatile compounds and trace metals that were seen to enhance its antimicrobial potency (Spilioti *et al.*, 2014). For instance, thyme, fir, pine, citrus and orange blossom honey species showed the highest phenolic content (up to 300 mg kg^{-1}), compared to other indigenous honeys (Lawag *et al.*, 2022). The pH thereof was found to range between 3.4 (orange blossom) and 5.31 (fir honey), while the free acidity ranged between 15.44 meq kg^{-1} (thyme) and 50.75 meq kg^{-1} (fir and pine). The lactonic/free acidity (L/FA) ratio ranged from 0.04 to 0.33 (Karabagias *et al.*, 2014). As regards their antimicrobial activity, citrus, bean herb (*Satureja* spp.), oregano and sage honeys, originated from Epirus Greece, were significantly antimicrobial towards *S. aureus* ATCC 12600, *S. mutans and F. nucleatum*. (Voidarou *et al.*, 2021a). Greek pine honey (pH 3.4) was also highly antibacterial against *S. marcescens* and *E. coli* comparing to other European

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(Spanish, British, New Zealand), Cuban, Chilean and Australian honeys (Alnaimat *et al.,* 2012; Serin *et al.,* 2018). Thyme and acacia honeys (Mountain Olympus) showed high antimicrobial activity towards *C. freundii*, *S. infantis* and *S. typhimurium* due to the H_2O_2 content, the antimicrobial proteins and the polyphenols (Tsavea & Mossialos, 2019). Also, thyme honey originated from Kos, Kea, Kithira, Rhodes, Mani showed high antibacterial and antifungal activity towards *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 25922), *E. cloacae* (ATCC 13047), *K. pneumoniae* (ATCC 13883) and *P. aeruginosa* (ATCC 227853), *C. albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801) and *C. glabrata* (ATCC 28838) (Melliou & Chinou, 2011). Chestnut, and heather (Peloponnese), pine (Thasos Island) and other polyfloral honeys (northern Greece) were significantly effective against *P. aeruginosa* and *S. aureus* (Anthimidou & Mossialos, 2013). The presence of oregano essential oils such as rosmarinic acid, thymol and carvacrol and other flavonoids, triterpenoids, sterols, vitamin C and vitamin A, in oregano honey (pH 3.9, Northern Greece, Epirus) made these honeys significantly antimicrobial towards fourteen *H. pylori* strains (Voidarou *et al.*, 2021b). Likewise, strawberry honey (Arbutus unedo honey) demonstrated high antibacterial and antifungal activity due to non-isoprenoid compounds as isophorone and abscisic acid and the phenolic secondary metabolite homogenistic acid (HGA) (Graikou *et al.,* 2022). Interestingly, no organism was seen so far to be honey-resistant (Maddocks & Jenkins, 2013), while subinhibitory honey concentrations were proved to restore oxacillin susceptibility in methicillin-resistant *S. aureus* (MRSA) (Jenkins & Cooper, 2012). Of note, Greek H_2O_2-producing honeys, were seen to have substantial antimicrobial activity higher to this of the acclaimed Manuka rich in methylglyoxal (MGO) (Johnston *et al.,* 2018).

Lemnos, a Greek island in the northern part of the Aegean Sea, is known for the rich wild medicinal flora with the *Compositae*, *Laminaceae*, *Apiaceae* and *Rosaceae* being the most abundant families. Most of the 976 identified plant taxa were known for their medicinal properties (e.g. digestive, respiratory, endocrine, cardiovascular system, skin, eyes, etc.) and their attraction to pollinators (Papageorgiou *et al.,* 2020). In particular, thyme honey (*Laminaceae* family) is one of the species thriving in Greek mainland and islands such as Lemnos (Karabagias *et al.,* 2017; Papageorgiou *et al.,* 2020). Among the thyme honey floral marker compounds, 3-hydroxy-4-phenyl-2-butanone and 3-hydroxy-1-phenyl-2-butanone are the most important with a concentration up to 35 mg/kg of honey (Alissandrakis *et al.,* 2009). Phenols are involved in the generation of substantial amounts of H_2O_2, while interacting with other honey components during the oxidation of glucose oxidase (Gox), thus, the phytochemical profile of thyme honey is important for its activity. However, thyme is just part of the Lemnos local flora and the contribution of thyme in antimicrobial activity of Greek honeys possibly results from the synergy with other co-existing floral species available in island (i.e. *Compositae*, *Apiaceae* and *Rosaceae*), which have been studied less.

Besides the increasing realisation that physicochemical properties of honey and the mechanism of colloid formation affect the production of H_2O_2 by Gox, the cascade of reactions and/or the role of phytochemicals compounds are still elusive (Brudzynski, 2020). Thus, we still consider the efficiency of the enzymatic reaction as the main determinant of honey's antimicrobial activity until new evidence become available. Towards this direction, many studies have well demonstrated that Greek H_2O_2-producing honeys have a medium to high antimicrobial activity that is usually positively correlated with the gluconic acid and the H_2O_2 levels accumulated upon honey dilution (Masoura *et al.,* 2020). The concentration of accumulated H_2O_2 ranges between 0.04 and 4 mM and for most honeys the maximum yield is achieved at concentrations 30%–50% (v/v). The major determinant of honey acidity (pH 3.4–4.5), gluconic acid, is accumulated in a range between 8.6 and 60 mM. The synergy of oxidative, acid and osmotic stress was seen to cause multiple lesions on cell wall integrity and to affect multiple pathways involved in cellular homeostasis and redox activity (Masoura *et al.*, 2022). However, this mechanism seems to be dependent on honey composition (i.e. phytochemical compounds, metals, sugars, polyphenols, acidity, H_2O_2, etc.).

In this study, the antibacterial activity of six honeys produced in Lemnos Island and that of two Manuka honeys (New Zealand; UMF 10+ and UMF 15+) were evaluated against four MRSA isolates (i.e. ATCC 43300, 0791, 28 965, 01322), isolated from hospitalised individuals. The pH and H_2O_2 accumulation, were determined for each honey in a range of dilutions (i.e. 12%, 25%, 50% v/v) that was previously shown to cause the highest antibacterial effect. Oxidative stress and cell membrane damage were monitored by flow cytometry (FC) at single cell level. From a commercial point of view, studies like this aim to highlight the benefits of local Greek honeys and make them market competitive with added value as functional foods or medicinal products.

Material and methods

Growth media and chemicals

Tryptic Soy Broth, Tryptic Soy Agar and PBS were purchased from Oxoid (Hampshire, UK). Propidium

iodide (PI), 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) and the Fluorimetric H_2O_2 assay kit were purchased from Sigma-Aldrich, Gillingham, UK.

Honey samples and pollen composition

Six natural and (un)processed honeys, denoted as 'h1-h6', were provided from Honey Hasapis apiaries located in different sites of Lemnos Island. Sample h1 was identical to h2 and sample h5 was identical to h6; however, samples h2 and h6 were thermally processed at 45 °C for 24 h to examine the effect of processing on the antimicrobial mechanism thereof. The pollen analysis was conducted by accredited method. The pollen grains, identified in honey samples, were grouped in four frequency classes (Louveaux *et al.,* 1978). Samples varied in percentage of thymus pollen concentration from 19% to 74% (Table 1). Two samples of medical-grade Manuka honey (UMF 10+ and UMF 15+) purchased from local retailer in the UK, were tested. Upon arrival, honey samples were stored in their original packaging, at room temperature (22 °C) in the dark.

Bacterial strains and culture preparations

Four clinical isolated MRSA strains (ATCC 43300, 0791, 28 965, 01322), denoted as S1–S4, were provided from the Institute of Microbiology and Infection (IMI; University of Birmingham) and used as the target organisms. Working cultures were prepared by inoculating one colony into 10 mL Tryptic Soy Broth and incubating overnight at 37 °C under aeration (150 rpm). Overnight cultures were pelleted (3900 ***g*** for 3 min in an Eppendorf Centrifuge 5810), washed twice in PBS and resuspended in PBS to a final absorbance of OD_{600} 0.5.

Disc-diffusion assay on agar

Cultures (0.25 mL) of each MRSA strain grown in TSB at 37 °C for 24 h were surface spread on Tryptic Soy Agar plates (TSB containing 1.2% w/v agar) in petri dishes. Once solidified, sterile paper discs (2.5 cm in diameter) were immersed in different concentrations (50%, 25%, 12% v/v) of freshly prepared diluted honey solutions and placed on the surface of inoculated TSA. One disc was applied to each plate. The undiluted H_2O_2 (3 mM) was used as a negative control. After incubation at 37 °C for 24 h, zones of inhibition surrounding discs were measured with a ruler. The diameter of zones, including the diameter of the disc, were recorded. The experiment was repeated three times using independently grown bacterial cultures.

Total viable counts – contact method in a liquid medium

This method involves incubating of stationary phase grown MRSA cultures in different concentrations of Greek and Manuka honeys. Each honey (at the respective concentration) was inoculated with the working culture at a 1:1 (v/v) ratio (200 μL total volume). Samples were incubated at room temperature (22 °C), for 24 h. Post-incubation, each sample was serially diluted in sterile PBS and plated on TSA plates using the Miles and Misra technique (Miles *et al.,* 1938) and incubated at 37 °C for 24 h. The viable bacterial counts (CFU mL^{-1}) were determined.

Flow cytometry

A BD Accuri C6 flow cytometer (Becton Dickinson Biosciences, 310 Oxford) was used for FC analysis. Before staining, samples were pelleted (3900 ***g*** for 3 min in an Eppendorf Centrifuge 5810), washed twice in PBS and

Table 1 Pollen composition (%) is given for each Lemnos honey

	pH			H_2O_2			
Concentration **Sample**	**50% (v/v)**	**25% (v/v)**	**12% (v/v)**	**50% (v/v)**	**25% (v/v)**	**12% (v/v)**	**Dominant pollen grains composition (%)**
h1	3.5	3.5	3.5	4.8	4	2.4	Lamiaceace (Thymus 64%, Ballota type) 17%,
h2	3.5	3.5	3.5	4.7	4.2	2.5	Leguminosae 5%, Bracicaceae 4%
h3	3.6	3.6	3.5	4.7	4	2.5	Lamiaceace (Thymus 74%)
h4	3.7	3.7	3.7	4.5	4	2.4	Lamiaceace (Thymus 19%), Loranthaceae, Asparagaceae, Brasicaceae 15%
h5	3.5	3.5	3.5	4.2	3.6	2.2	Lamiaceace (Thymus 28%, Ballota type 27%),
h6	3.6	3.6	3.4	4.5	3.6	2.5	Fabaceae (Trifolium 13%, Anthyllis 4%), Asparagaceae 8%, Brassicaceae (6%)
M15	4.4	4.4	4.5	0.08	ND	ND	NA
M10	4.2	4.2	4.3	0.1	ND	ND	

pH and H_2O_2 (mM) concentration is given for each honey at the three consecutive dilutions (50%, 25% and 12%). The 'not detectable' H_2O_2 is denoted by 'ND'.

resuspended in equal volume of PBS. For analysis of membrane permeability, samples were stained with 4 μg/mL Propidium iodide (PI) and for the detection of intracellular ROS accumulation samples were stained with 2 μg/mL 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) and incubated for 10 min and 1 h, respectively, in the dark. Untreated bacteria (kept in PBS) and bacteria treated with 3 M H_2O_2 for 30 min, served as 'healthy' and 'dead' or 'membrane compromised' controls, respectively. Post-incubation, samples were washed, and the pellet was resuspended in fresh PBS. Fluorescence was measured using a 670 LP and 533/30 BP filter for the detection of PI and DCF, respectively.

Hydrogen peroxide assay

Hydrogen peroxide concentration was determined for each honey sample using the Fluorimetric H_2O_2 assay kit, according to the manufacturer's instructions. The red fluorescence formed after the reaction of peroxidase and H_2O_2 was measured at 540 nm excitation and at 590 nm emission using the CLARIOstar (BMG LABTECH) multi-detection microplate reader. Dose–response curves were generated using the MARS software (BMG LABTECH). To calculate the H_2O_2 concentrations in different honeys, a standard curve was generated using dilutions of a fresh 20 mM H_2O_2 stock solution. All determinations were performed in triplicates.

pH measurement

The pH of each honey was measured using a Mettler Toledo (Scientific Lab Supplies, Nottingham, UK). Before the measurements, honeys were heated up to 30 °C and were mixed with sterile water to reach the respective concentrations of 12%, 25% and 50%. Following the manufacturers' guidelines, for calibration, three independent measurements were taken for each sample.

Statistical analysis

The statistical analysis and graphical display were performed in GraphPad Prism. Data are presented as mean ± standard deviation (SD). The average inhibition activity of each honey sample, against the four MRSA strains, was compared with two-way ANOVA (Tukey's multiple comparisons test). FC data, within different bacteria treatments, were compared with two-way ANOVA (Sidak's multiple comparisons test). All *P*-values and significance levels are indicated in the figures and figures legends.

Results and discussion

The disc-diffusion assay, plate counts and FC were combined to investigate the antibacterial potential of each honey and understand the underlying mechanism, as regards the effect of honeys on cellular integrity and cell viability.

The disc-diffusion assay showed the dilution factor as the main determinant of the antibacterial strength of honeys, while no differences on the strain susceptibility were observed by using this method (Table 2). All the six honey samples (h1–h6) showed higher inhibition (4.3–4.6 cm) when diluted at 50% v/v, and this declined at higher honey dilutions; 25% v/v (3.3–3.9 cm) and 12% v/v (2.5–3.6 cm). This agrees with a general premise that antimicrobial potential of H_2O_2-producing honey maximises at 30%–50% v/v and then declines at higher dilutions (Masoura *et al.*, 2020). Also, this has been explained by the phase transition and the conformational changes of the honey matrix, that upon dilution is separated in two-phase system; nano- and micro-size particles. At a range of 30%–50% v/v honey concentration, reaction between the sugars and other macromolecules is favoured; therefore, the efficiency of Gox reaction and the production

Table 2 Diameters of the zones of inhibition (cm) of 4 MRSA strains (S1–S4) in the presence of Greek honeys (h1–h6) and Manuka (UMF10+, 15+), each applied at three concentrations (50%, 25% and 12% v/v)

Conc.	50% (v/v)					25% (v/v)					12% (v/v)				
Strain/Sample	S1	S2	S3	S4	Aver.	S1	S2	S3	S4	Aver.	S1	S2	S3	S4	Aver.
h1	4.6	5.0	4.6	4.2	4.6^{a}	4.0	4.2	3.5	4.0	3.9^{a}	3.3	3.5	3.0	3	3.2^{a}
h2	4.5	4.8	4.5	4.5	4.6^{a}	4.0	4.0	3.4	3.5	3.7^{a}	3.0	3.5	3.5	3.8	3.5^{a}
h3	4.8	4.5	4.5	4.2	4.5^{a}	3.7	3.7	3.5	3.2	3.5^{a,b}	2.6	2.8	2.5	2.9	2.7^{b}
h4	4.5	4.1	4.5	4.5	4.4^{a}	3.5	4.0	3.0	3.5	3.5^{a,b}	2.5	2.7	2.5	2.5	2.6^{b}
h5	4.6	4.2	4.0	4.4	4.3^{a}	3.6	3.2	3.4	3.0	3.3^{a,b}	2.5	2.5	2.5	2.5	2.5^{b}
h6	4.7	4.5	4.5	4.3	4.5^{a}	3.8	3.7	3.3	3.3	3.5^{a,b}	2.6	2.7	3.0	2.5	2.7^{b}
M15	4.7	4.7	4.5	4.0	4.5^{a}	3.2	3.0	3.5	3.5	3.3^{a,b}	2.5	2.5	2.5	2.5	2.5^{b}
M10	3.5	3.5	3.7	3.0	3.4^{b}	2.5	3.0	3.0	3.0	2.9^{b}	2.5	2.5	2.5	2.5	2.5^{b}

Each value comprises the inhibition zone added to the disc diameter (2.5 cm). The average inhibition activity, of each honey towards the four MRSA strains, was analysed using two-way ANOVA (Tukey's multiple comparisons test, *P* value <= 0.05) for each of the dilutions. Values showing the same letter are not significantly different.

of H_2O_2 and gluconic acid is optimal (Brudzynski *et al.,* 2017). As regards the pollen composition, at 50% v/v honey concentration, no significant differences were seen between Greek honeys and Manuka (UMF15+) honeys; all of them showed significantly higher activity than the Manuka (UMF10+). At 25% v/v, h1 and h2 only were significantly more antibacterial than UMF10+. However, at 12% v/v of honey concentration, h1 and h2 showed significantly higher average activity compared to the other Greek honeys and the Manuka (Table 2).

Overall, at all concentrations tested, h1 and h2 composed of thyme (64%) and other pollen grains were seen as the most antibacterial honeys. Manuka honeys were more inhibitory at 50% dilution most probably due to higher concentration of methylglyoxal (MGO), one of the main antimicrobials thereof and/or other phytochemicals (Johnston *et al.,* 2018). No differences on the antimicrobial strength were shown due to thermal processing of h2 and h6 honeys. As was expected Manuka UMF15+ caused higher bacterial inhibition than the UMF10+ and lower or equal to this of the six Greek honeys. For this reason, only Manuka UMF15+ was considered for further investigation.

While disc-diffusion method provides rather a categorical classification, CFU counts showed differences in the antibacterial strength of the tested honeys and the susceptibility of MRSA strains. To define the antibacterial strength of each honey, thresholds were set to cluster the effects into three groups/phenotypes; (a) 'weak' for the strains showing equal/less than 2 logs reduction, (b) 'intermediate' for those showing 2–4 logs reduction and (c) 'strong' those with higher than 4 logs reduction. Figure 1 shows that, in agreement with the zone inhibition assay, the antibacterial strength of all honeys declined at higher honey dilutions (50% > 25% > 12% v/v). At 50% v/v dilution, the activity of all natural honeys was higher than or equal to this of UMF15+ (50% v/v). In case of Manuka, this is possibly due to presence of MGO (and H_2O_2), even in low concentrations (Johnston *et al.,* 2018). The six Greek honeys and the Manuka showed a 'strong' effect towards MRSA1, MRSA3 and MRSA4 by causing bacterial inhibition below the detection limits (20 CFU mL^{-1}), and intermediate effect on MRSA2 by causing a reduction of 3–4 logs (Fig. 1a–d). At 25% v/v dilution, MRSA1, MRSA3 and MRSA4 were completed eradicated by h1–h6 only, while the effect on MRSA2 was equal to this caused by honeys diluted at 50%, showing a resistant phenotype of this strain. Manuka honey, diluted at 25% v/v in water, induced a 'weak effect' of 2 logs reduction to MRSA3, MRSA4 while no effect was observed towards MRSA1 and 2 (Fig. 1e–h), that proves the superior activity of honeys h1–h6 comparing to Manuka tested here.

At 12% of honey concentration a high variability on antibacterial activity was observed. MRSA3 was completely eradicated by all honeys, except Manuka that caused only 2 logs reduction in the population. MRSA4 was more susceptible to Greek honeys than to Manuka, while MRSA1 and 2 were equally susceptible to all honeys that caused up to 2–3 logs reduction (intermediate to weak effect) (Fig. 1i–l). Among the Greek honeys, only h3 was more effective (strong to intermediate effect), even when diluted at 12%, while causing 4 and 3 logs reduction to MRSA1 and MRSA4, respectively, and complete eradication to MRSA3, respectively (Fig. 1k–l).

Overall, the antibacterial activity of all Greek honeys (h1–h6) declined at higher dilutions, except for h3 that holds high activity even when diluted at 12% v/v. At higher dilutions, the antibacterial effect is not identical across all the strains (MRSA2 had been the most resistant while MRSA3 is susceptible to all honeys even at 12% dilution). As for Manuka the antibacterial effect did not correlate with the dilution, instead, it fluctuated among the tested strains (e.g. UMF15+ diluted at 50% caused a strong effect to all strains except for MRSA2, while at 25% caused a weak effect to all strains. At 12% dilution, UMF15+ caused intermediate effect to strains MRSA1, 2 and 3 and a weak effect on MRSA4) (Fig. 1; Table 2). Thermal processing did not affect the activity of h2 and h6 as was also seen by zone inhibition assay.

The importance of the acid and oxidative stress, that occur upon the oxidation of glucose by Gox and the accumulation of gluconic acid and H_2O_2 has been previously acknowledged (Brudzynski, 2020; Brudzynski *et al.,* 2017; Masoura *et al.,* 2020). Thus, the concentration thereof and the pH were measured for each honey and the respective dilutions (Table 1). The pH of Greek samples varied between 3.5 to 3.7 while of the respective Manuka UMF15+ varied between 4.2 to 4.5. The Greek samples also accumulated significantly higher H_2O_2 concentration than other honey varieties (up to 3.4–4 mM) (Brudzynski, 2020). At 50% honey concentration, H_2O_2 ranged between 4.2–4.8 mM, and this range declined to 3.6–3 mM, and 2.2–2.5 mM by increasing the dilution 25% v/v and 12% v/v, respectively. Of note, honeys accumulating <3 mM H_2O_2 still, were seen to exert antimicrobial effect and were considered as powerful antimicrobials (Brudzynski, 2020). As was expected from the literature (Majtan *et al.,* 2014) H_2O_2 content in Manuka honey, was very low or no detectable (ND) (Table 1).

We have shown previously that low pH enhances the deleterious effects of H_2O_2, towards bacterial homeostatic mechanism and the synergy of acid-oxidative-osmotic stress cause cell membrane lesions and intracellular ROS accumulation (Masoura *et al.,* 2020). Dual staining with PI and H_2DCFDA,

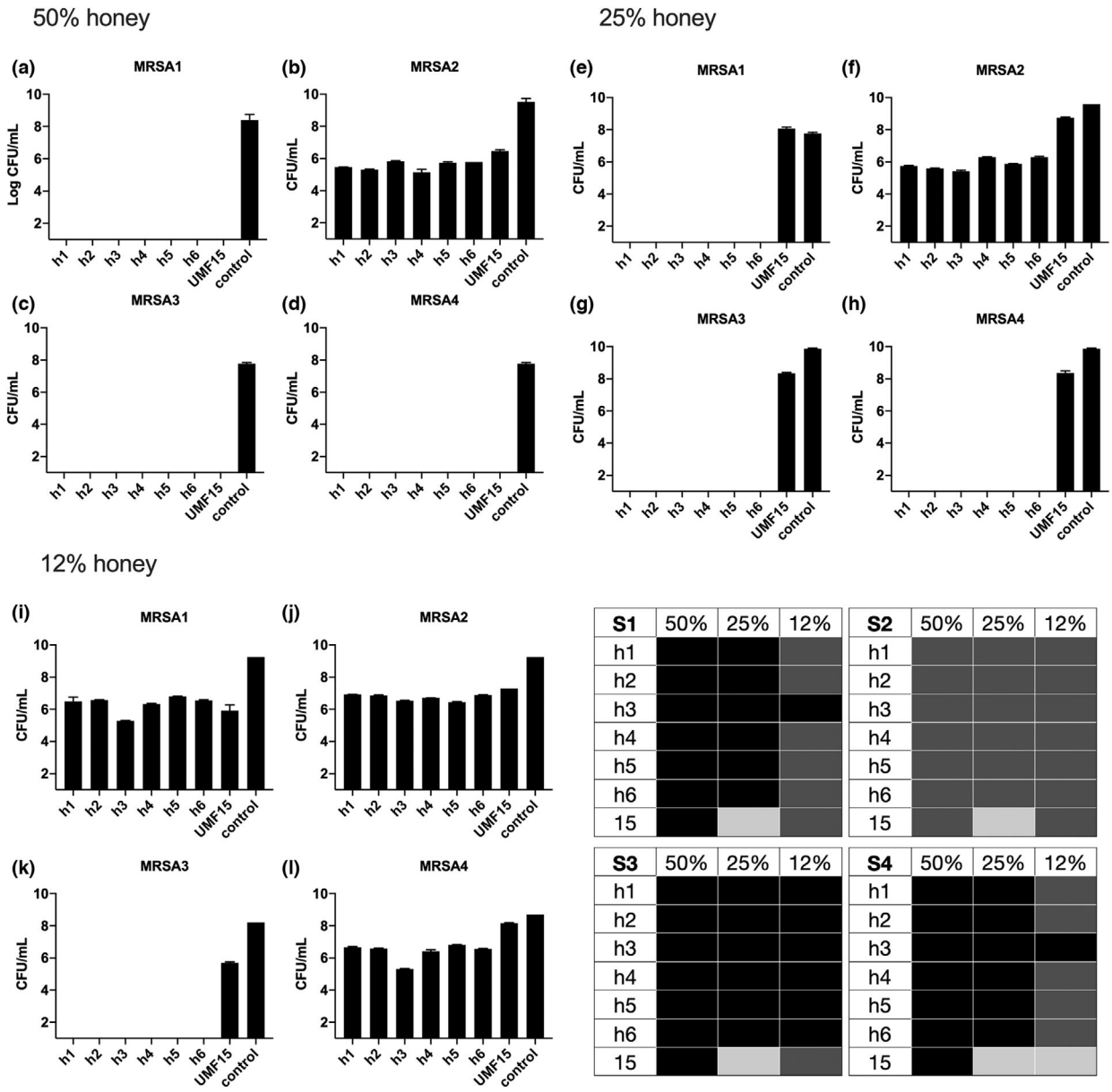

Figure 1 Antibacterial effect of Greek (h1–h6) and Manuka (UMF15+) honeys on MRSA strains (S1–S4). Colony counts show the cell survival post-exposure to honey at concentration 50% (a–d), 25% (e–h) and 12% (i–l) v/v of honey in water. Error bars show ± standard deviations, (n = 3; biological replicates). The table (down-right side) summarises the antibacterial effect of all honeys, towards the four strains (S1; ATCC 43300, S2; 0791, S3; 28 965, S4; 01322), in black, grey and light grey colour corresponding to 'strong', 'intermediate' and 'weak' effect, respectively.

indicative of cell membrane damage and ROS accumulation, respectively, was used to investigate whether h1–h6 and/or Manuka honeys induce such an effect to MRSA strains. The raw data are given in Figure S1 and the average of the three individual measurements are given in Tables S1 and S2. For Greek honeys the effect on cell wall integrity and the ROS accumulation differed among the MRSA strains and was affected by the honey type and the concentration thereof. Honeys diluted at 50% and 25% v/v caused membrane lesions (PI^+) up to 89.08% and 96.03% of the treated bacteria (Table S1) and ROS (DCF^+) were detected to a maximum of 80.1% and 70.8% of the population, respectively (Table S2). In successive dilutions of Greek honey samples, the ROS detection within treated populations declined, possibly due to the decline of H_2O_2

concentration (due to oxidation). Only a low percentage (2.7%–28.3%) of MRSA population was PI^+ (indicative of cell membrane damage), and ROS accumulation was detected in just 2.97% of the MRSA exposed to 12% of natural honeys. This confirms that oxidative stress is one of the key factors in the synergistic antimicrobial mechanism of H_2O_2-producing honeys (Masoura *et al*., 2022).

Of note, both cell membrane lesions and ROS accumulation fluctuated within MRSA strains. For instance, ROS accumulation in resistant MRSA2, was lower comparing to this of other strains (20%–38% vs. 46%–80%) and agrees with resistant phenotype of the strain as showed by CFU data. Although no resistance to honey has been reported, it is possible that certain strains survive better than others by resistant mechanisms towards honey targets (i.e. efflux pumps activity and oxidative stress response, quorum sensing and biofilm formation, metabolic activity, membrane potential, structural changes such as membrane injury, flagellum mobility, cell lysis) (Combarros-Fuertes *et al*., 2020). Both MRSA1 and MRSA3, susceptible to all honeys, were seen with higher percentage of DCF (oxidised form of H_2DCFDA), indicator of ROS accumulation (Tables S1 and S2). The average effect on cell membrane lesion and ROS accumulation, in MRSA strains, is shown in Fig. 2. Regardless the variability within MRSA phenotypes, its shown that honeys h1–h6, at concentrations 50% and 25% v/v, cause significantly higher membrane lesion and ROS accumulation than the respective 12% v/v honey.

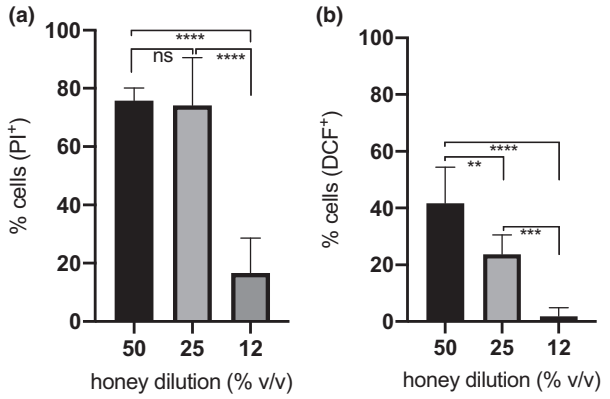

Figure 2 The effect of honey concentration on the bacterial cell damage and the ROS accumulation. Flow cytometry analysis of MRSA strains exposed to natural and Manuka honeys at three levels of concentration (50%, 25% and 12% v/v). The mean percentage of cells that were PI^+ and DCF^+, indicative for cell membrane destruction/permeability and ROS accumulation, respectively, is shown for all the four MRSA strains tested. Data were analysed using two-way ANOVA: asterisks show significance levels of Sidak's multiple comparisons test (****P <= 0.0001, ***P <= 0.0005, **P <= 0.005); all bars without asterisks are not significant (ns; P > 0.05).

As for Manuka honeys tested here, although the H_2O_2 was not detectable, both cell membrane damage and ROS accumulation was observed in flow cytometry (FC). Manuka promoted efflux pumps blockade in dose-dependent way, that further caused remarkable metabolic disruption by compromising the cell membrane potential and integrity. This has a subsequent effect on cell morphological changes (shrinkage or enlargement) that might result to cell death due to the mismatch in surface-to-volume ratio (Moghadam & Khaledi, 2021). MRSA strains treated with 50% of Manuka UMF15+ were PI^+ at a range of 68%–91%, with a few exceptions that might be related to a strain resistance. For instance, membrane integrity of strains MRSA2 and MRSA4 was less affected post-exposure to 25% and 12% of Manuka and the respective intracellular ROS accumulation was also lower comparing to this of the strains MRSA1 and MRSA3 (Tables S1 and S2). These data agree with the CFU counts showing that Manuka UMF15+ had a weak effect on both resistant strains MRSA2, MRSA4. Also, at 12% v/v, whereas the effect of Greek honeys on membrane integrity declined, Manuka showed destructive effect. This effect does not seem to be due to oxidative damage, since the respective percentage of DCF^+ cells is low.

These results demonstrated that the Greek honeys have remarkable antibacterial potential towards clinically important isolates while in general demonstrate higher than or equal activity to this of the tested Manuka. Although honeys h1–h6 accumulated remarkable H_2O_2, and no major differences in pH were seen, the differences in antimicrobial potential could also link to their pollen composition. No correlation between the thermal process and the antimicrobial strength of the respective honeys was found. However, pollen composition substantially affected the antimicrobial potency of honeys. The most antimicrobial thereof, h3, is composed of 74% of thyme pollen grains, followed by h1 and h2 being composed of thyme (64%) and other pollen grains (Table 1). The medicinal importance of thyme plant is mostly related to high concentration of terpenes and their corresponding acetates or even thymol, carvacrol, geraniol and trans-sabinene (Wiese *et al.,* 2018). Thyme honeys of various geographical origins have shown significant antimicrobial activity against a range of (wound) bacteria (Mahmoodi-Khaledi *et al.,* 2015; Bendahbia *et al*., 2020) and bee-disease-associated microbes (Wiese *et al.,* 2018). Therefore, it possible that higher concentration of thyme pollen grain increases the antimicrobial activity of honeys.

Conclusion

Manuka honey is massively applied for uses in the cosmetic and pharmaceutical industry due to its touted and well-evidenced activity against microbes. Although the

antimicrobial activity of other honeys, originating from various places around the world, was proved to be better than this of Manuka, their studies lack depth in establishing the mechanism of action instead of simple realising their antimicrobial activity. Regardless the significant antimicrobial potential of Greek honeys, the incomplete knowledge of the active components and the antimicrobial mechanism are major limitations. Our study focused on 6 Greek honeys, produced in Lemnos Island, known for its biodiversity in plants of high medicinal properties. The antimicrobial activity thereof was equal to or higher than this of renowned Manuka. The antimicrobial effect of all tested samples was seen to be linked to their high H_2O_2 activity (higher than this of other studied species) and low acidity, two perquisites in the antibacterial activity of honey. The synergy of oxidative/acid stress caused membrane lesions and intracellular ROS accumulation similar to the mechanism of other antibacterial honey species. Monofloral thyme honey was effective towards resistant MRSA phenotypes even at higher dilutions, whereas usually the antimicrobial strength usually declines. This implicates that terpene (and their acetates) of thyme pollen grain might have a particular (synergistic) role in the antimicrobial mechanism of honey or even contribute to the sustainable release of H_2O_2 and gluconic acid. However, a thorough characterisation of its composition is required to better understand the underlying mechanism. Future studies should focus on the compositional analysis of honeys to investigate the antimicrobial potential of multi- and monofloral species.

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Author contributions

Maria Masoura: Data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); resources (supporting); software (equal); supervision (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Konstantinos Gkatzionis:** Conceptualization (supporting); funding acquisition (lead); methodology (supporting); project administration (supporting); supervision (equal).

Conflict of interest

The authors confirm that this is an original research article, and no conflict of interests associated with this publication. All authors have read, approved the MS and are aware of its submission to JFST.

Peer review

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Data availability statement

All data generated or analysed during this study are included in this published article (and its supplementary information files).

References

Aliferis, K.A., Tarantilis, P.A., Harizanis, P.C. & Alissandrakis, E. (2010). Botanical discrimination and classification of honey samples applying gas chromatography/mass spectrometry fingerprinting of headspace volatile compounds. *Food Chemistry*, **121**, 856–862.

Alissandrakis, E., Tarantilis, P.A., Pappas, C., Harizanis, P.C. & Polissiou, M. (2009). Ultrasound-assisted extraction gas chromatography–mass spectrometry analysis of volatile compounds in unifloral thyme honey from Greece. *European Food Research and Technology*, **229**, 365–373.

Alnaimat, S., Wainwright, M. & Al'Abri, K. (2012). Antibacterial potential of honey from different origins: a comparsion with manuka honey. *Journal of Microbiology, Biotechnology and Food Sciences*, **1**, 1328–1338.

Anthimidou, E. & Mossialos, D. (2013). Antibacterial activity of Greek and Cypriot honeys against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in comparison to manuka honey. *Journal of Medicinal Food*, **16**, 42–47.

Bendahbia, K., Abidli, Z., Zaazaa, L. *et al.* (2020). Antimicrobial activity of thyme honey from morocco against pathogenic bacteria. *Journal of Hygienic Engineering and Design*, **32**, 23–27.

Brudzynski, K. (2020). A current perspective on hydrogen peroxide production in honey. A review. *Food Chemistry*, **332**, 127229. https://doi.org/10.1016/j.foodchem.2020.127229

Brudzynski, K., Miotto, D., Kim, L., Sjaarda, C., Maldonado-Alvarez, L. & Fukś, H. (2017). Active macromolecules of honey form colloidal particles essential for honey antibacterial activity and hydrogen peroxide production. *Scientific Reports*, **7**, 1–15.

Combarros-Fuertes, P., Fresno, J.M., Estevinho, M.M., Sousa-Pimenta, M., Tornadijo, M.E. & Estevinho, L.M. (2020). Honey: another alternative in the fight against antibiotic-resistant bacteria? *Antibiotics*, **9**, 774. https://doi.org/10.3390/antibiotics9110774

Georghiou, K. & Delipetrou, P. (2010). Patterns and traits of the endemic plants of Greece. *Botanical Journal of the Linnean Society*, **162**, 130–153.

Graikou, K., Andreou, A. & Chinou, I. (2022). Chemical profile of Greek Arbutus unedo honey: biological properties. *Journal of Apicultural Research*, **61**, 100–106.

Henein, M. (2015). Beeculture.com. https://www.beeculture.com/what-is-killing-the-bees-in-greece/

Jenkins, R.E. & Cooper, R. (2012). Synergy between oxacillin and manuka honey sensitizes methicillin-resistant *Staphylococcus aureus* to oxacillin. *Journal of Antimicrobial Chemotherapy*, **67**, 1405–1407.

Johnston, M., McBride, M., Dahiya, D., Owusu-Apenten, R. & Nigam, P.S. (2018). Antibacterial activity of manuka honey and its components: an overview. *AIMS Microbiology*, **4**, 655–664.

Karabagias, I.K., Vavoura, M.V., Nikolaou, C., Badeka, A.V., Kontakos, S. & Kontominas, M.G. (2014). Floral authentication of

Greek unifloral honeys based on the combination of phenolic compounds, physicochemical parameters and chemometrics. *Food Research International*, **62**, 753–760.

Karabagias, I.K., Louppis, A.P., Kontakos, S., Papastephanou, C. & Kontominas, M.G. (2017). Characterization and geographical discrimination of Greek pine and thyme honeys based on their mineral content, using chemometrics. *European Food Research and Technology*, **243**, 101–113.

Lawag, I.L., Lim, L.Y., Joshi, R., Hammer, K.A. & Locher, C. (2022). A comprehensive survey of phenolic constituents reported in Monofloral honeys around the globe. *Food*, **11**, 1152.

Louveaux, J., Maurizio, A. & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World*, **59**, 139–157.

Maddocks, S.E. & Jenkins, R.E. (2013). Honey: a sweet solution to the growing problem of antimicrobial resistance? *Future Microbiology*, **8**, 1419–1429.

Mahmoodi-Khaledi, E., Kashef, N., Habibi-Rezaei, M. & Moosavi-Movahedi, A.A. (2015). In vitro characterization of antibacterial potential of Iranian honey samples against wound bacteria. *European Food Research and Technology*, **241**, 329–339.

Majtan, J., Bohova, J., Prochazka, E. & Klaudiny, J. (2014). Methylglyoxal may affect hydrogen peroxide accumulation in manuka honey through the inhibition of glucose oxidase. *Journal of Medicinal Food*, **17**, 290–293.

Masoura, M., Passaretti, P., Overton, T.W., Lund, P.A. & Gkatzionis, K. (2020). Use of a model to understand the synergies underlying the antibacterial mechanism of H_2O_2-producing honeys. *Scientific Reports*, **10**, 1–14.

This study showed the fundamentals of the antimicrobial mechanism of H_2O_2-producing honeys, which is based on enzymatic production of gluconic acid and H_2O_2 upon honey dilution. The rationale of this study is used as reference and/or confirmation for this study.

Masoura, M., Milner, M., Overton, T., Gkatzionis, K. & Lund, P. (2022). Use of TraDIS to probe the antibacterial mechanism of a model honey on *E. coli* K-12. *Frontiers in Microbiology*, **12**, 803307.

Melliou, E. & Chinou, I. (2011). Chemical constituents of selected unifloral Greek bee-honeys with antimicrobial activity. *Food Chemistry*, **129**, 284–290.

This study showed that (±)-3-Hydroxy-4-phenyl-2-butanone isolated from monofloral thyme honey, is an important antimicrobial with significant activity against both Gram-positive and -negative bacteria and fungi.

Miles, A.A., Misra, S.S. & Irwin, J.O. (1938). The estimation of the bactericidal power of the blood. *Epidemiology & Infection*, **38**, 732–749.

Moghadam, M.N. & Khaledi, E.M. (2021). Antibacterial activity and mechanism of action of some Iranian honeys compared to manuka honey against multidrug-resistant respiratory and urinary infections. *Food Bioscience*, **41**, 101003.

Papageorgiou, D., Bebeli, P.J., Panitsa, M. & Schunko, C. (2020). Local knowledge about sustainable harvesting and availability of wild medicinal plant species in Lemnos Island, Greece. *Journal of Ethnobiology and Ethnomedicine*, **16**, 1–23.

This study refers to the sustainability of harvesting practices used in Lemnos island while identifying the most common plant taxa and their medicinal applications. The families of *Compositae*, *Lamiaceae*, *Apiaceae* and *Rosaceae* are of the most represented island's medicinal plant populations and biodiversity and this is an informative starting point for development of systematic apiculture. It also a reference point regarding the honey types used in this study, which are considered highly antimicrobial.

Serin, S., Turhan, K.N. & Turhan, M. (2018). Correlation between water activity and moisture content of Turkish flower and pine honeys. *Food Science and Technology*, **38**, 238–243.

Spilioti, E., Jaakkola, M., Tolonen, T. *et al.* (2014). Phenolic acid composition, antiatherogenic and anticancer potential of honeys derived from various regions in Greece. *PLoS One*, **9**, e94860.

Tsavea, E. & Mossialos, D. (2019). Antibacterial activity of honeys produced in Mount Olympus area against nosocomial and foodborne pathogens is mainly attributed to hydrogen peroxide and proteinaceous compounds. *Journal of Apicultural Research*, **58**, 756–763.

Voidarou, C.C., Rozos, G., Alexopoulos, A. *et al.* (2021b). In vitro screening potential antibacterial properties of the Greek oregano honey against clinical isolates of *Helicobacter pylori*. *Food*, **10**, 1568.

Voidarou, C.C., Antoniadou, M., Rozos, G. *et al.* (2021a). An in vitro study of different types of Greek honey as potential natural antimicrobials against dental caries and other oral pathogenic microorganisms. Case study simulation of oral cavity conditions. *Applied Sciences*, **11**, 6318.

Wiese, N., Fischer, J., Heidler, J., Lewkowski, O., Degenhardt, J. & Erler, S. (2018). The terpenes of leaves, pollen, and nectar of thyme (*Thymus vulgaris*) inhibit growth of bee disease-associated microbes. *Scientific Reports*, **8**, 1–12.

This study showed that antimicrobial activity of thyme honey is mostly due to carvacrol and thymol, while depending on their concentration terpenes and their acetates cause also bacterial growth inhibition. This is in agreement with our results that showed increased antibacterial activity of thyme honeys.

These studies, referred to thyme honey antimicrobial markers and the general antimicrobial mechanism of H_2O_2-producing honeys are considered important references for the explanation of our findings and suggestions for further research.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Dot plots representing DCF (FL3; *x* axis) and PI (FL1; *y* axis) fluorescence of the cFDA/PI double-stained MRSA cells.

Table S1. Percentage of PI positive cells of MRSA strains (S1–S4) post-exposure to 50%, 25%, and 12% dilutions of natural honeys (h1–h6) and Manuka (UMF15+) honey.

Table S2. Percentage of DCF positive cells of MRSA strains (S1–S4) post-exposure to 50%, 25%, and 12% dilutions of natural honeys (h1–h6) and Manuka (UMF15+ honey).