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# Physicochemical parameters, microbiological quality, and antibacterial activity of honey from the Bucovina region of Romania

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**In this study, the physicochemical properties (electrical conductivity (EC), pH, free acidity, moisture content, DPPH radical scavenging activity, total polyphenols content (TPC), flavonoids content (FC), individual polyphenols, carbohydrates, and organic acids), microbiological quality, and antibacterial activity of honey (raspberry, rosehip, alfalfa, hawthorn, and honeydew honey) from Bucovina were evaluated. Along with melissopalynological analysis, the physicochemical parameters were determined for the honey samples to characterize the samples and to assess their applicability in classifying honey based on its botanical origin. Another objective of the study was the evaluation of the microbiological quality and antibacterial activity of honey samples. The antibacterial activity was examined against the growth of four pathogenic bacterial strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella enterica* serovar Typhimurium ATCC 14028) by the diffusion test in the agar well and by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The analyzed honey samples were within the safe limits, except for three samples in which *Bacillus cereus* was detected and two other samples that had values above the acceptable limit for yeasts. Thus, 40.46% of all honey samples had bactericidal activity that was superior or at least comparable to manuka honey MGO550 against *Staphylococcus aureus*, 57.69% against *Salmonella enterica* serovar Typhimurium, 74.15% against *Pseudomonas aeruginosa*, and 75% against *Escherichia coli*. Of all types of honey analyzed in this study, honeydew honey had the highest bactericidal activity, followed by polyfloral honey.**

**Keywords** *Honey*, Physicochemical parameters, Honey microbiota, Antimicrobial activity

Honey is a natural product used since ancient times due to its taste, aroma, and therapeutic properties (antibacterial, antiviral, anti-inflammatory, and antioxidant activity)[1]. Honey is a complex substance that comes in a wide variety of forms based on bee species, production techniques, and its botanical and geographic origins. The different levels and consistency of antimicrobial efficacy are caused by this variation[2]. Therefore, research into the bioactivity of various types of honey harvested in regions with high plant biodiversity is of great interest[3].

Physicochemical properties of honey (low water activity, high sugar content, low pH, etc.) influence the microbial communities[3]. Honey contains antimicrobial compounds (e.g. methylglyoxal, antioxidants, hydrogen peroxide, antimicrobial peptides etc.) that kill or suppress the growth and proliferation of an extensive number of microorganisms, including multidrug resistant pathogens[4]. It is also believed that the content of secondary plant metabolites such as flavonoids, polyphenols and volatile compounds influences the antibacterial effect of honey[4].

Honey is considered a functional food a so it is especially necessary to be free of contaminants that are harmful to human health. Still, despite its chemical properties, honey may have a varied microbial population. The microbiota, which includes bacteria, yeasts, and molds, may derive from a variety of vegetal components, the digestive tract of the honeybee, and the environment (including the hive, and honeycomb structures where the honey is harvested and stored)[5]. Microbiological quality can be determined by performing microbiological tests for groups of microorganisms that act as hygienic indicators (such as: total number of microorganisms, number of molds and yeasts, number of *Bacillus* spp., number of fecal coliforms) and microbiological tests for

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detection of microorganisms that act as consumer safety indicators (for example *Salmonella* spp., anaerobic spore-forming bacilli, *Bacillus cereus*, etc.).

Regulations of the European Union eliminated national microbiological standards or guidelines for evaluating the microbiological quality of honey and bee products, so these are absent in European countries. The European Parliament Regulation (EC) No 178/2002 (Council, E. U.)[6]and the Codex Alimentarius code[7]are used to assess the microbiological quality of honey. These regulations cover all phases of food and feed production, processing, and distribution. The microbiological properties of honey are related to the specifications set forth in compliance with the rules and regulations for the identification and use of food-related microbiological and sanitary standards[7]. The quality of honey is established by physical and chemical characteristics, well compiled by European Directive 2014/63/EU[8], while the microbiological field is ignored[9].

Romania produces approximately 18,000 tons of honey per year because the climatic conditions and bees are favorable for beekeeping. Bucovina is a mountainous area in the north-east of Romania famous for the honey produced here[10].

The aim of this study was to conduct a comparison between honey from Bucovina area (honeydew honey, monofloral honey, and polyfloral honey from the mountain area) and manuka honey. To be able to compare the floral and honeydew honey samples from Bucovina with manuka honey, the samples were characterized from a physical, chemical, and microbiological point of view. Also, the study was fully planned to determine the antibacterial activity against 4 pathogens (*Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *Escherichia coli*), and to compare the action of honey with that of antibiotics.

## Materials and methods

### Honey samples

Fifty-five samples of honey were collected in 2022 and 2023 from beekeepers or beekeeping associations in the mountainous regions of northeastern Romania. Before being analyzed, honey samples were stored at room temperature and shielded from sunlight. The honey types investigated in this study were hawthorn honey (*Crataegus* spp.), alfalfa (*Medicago sativa*), rosehip (*Rosa canina*), raspberry (*Rubus idaeus*), honeydew honey, manuka (*Leptospermum scoparium*), and polyfloral honey from the mountainous area. Manuka honey was purchased from the market with 3 types of methylglyoxal concentrations: manuka 100, manuka 250 and manuka 550 (Manuka Health New Zeeland, local supplier Dante International SA).

### Melissopalynological analysis, chemical composition, and content of bioactive compounds of honey

This analysis was performed in accordance with the International Commission for Bee Botany[11], as described previously[12].

Moisture content was determined based on refractive index, according to the method developed by[13]. pH was determined with a pH meter, electrical conductivity of honey samples was measured with a portable conductometer, and free acidity was determined by the tritimetric method[14]. Total phenolic content (TPC) and flavonoids content were calculated using the procedure described by[15].

DPPH assay was performed according to[16]. According to Brand-Williams et al. (1995), the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of honey samples was measured on samples prepared, as follows: a magnetic stirrer was used for 15 min to mix 1 g of honey sample that was dissolved in 5 mL of methanol 40% (v/v) with acidified water. Next, 250 µL of DPPH was combined with 35 µL of honey sample. A QE65000 spectrometer (Ocean Optics, USA) was used to measure the solution's absorbance at 515 nm and the results were expressed as % DPPH using the formula in Eq. 1:

$$\%\,\mathrm{DPPH} = \left[A_0 - \frac{A_1}{A_0}\right] \times 100, \tag{1}$$

$A_0$ = DPPH absorbance, $A_1$ = the sample absorbance.

Carbohydrate composition was determined by HPLC according to Oroian et al.[17], and polyphenols composition was analyzed by the method proposed by[18]. Organic acids composition was also determined by HPLC as described previously by Özcelik et al.[19]. Honey samples were also investigated based on FTIR spectra collected between 4000 and 650 $cm^{-1}$ with a resolution of 4 $cm^{-1}$. The spectra of honey samples were captured using a Nicolet iS-20 spectrometer (Thermo Scientific, Karlsruhe, Dieselstraße, Germany) in the mid-infrared range of 4000 –650 $cm^{-1}$ with a resolution of 4 $cm^{-1}$. Every sample was placed on the ATR surface, and using OMNIC software (version 32, Thermo Scientific), duplicate spectra were collected at 25 °C. The collected spectra were further processed and displayed using the SpectraGryph software (version 1.2.11, Dr. Friedrich Menges, Germany, www.effemm2.de). The samples were analyzed without being liquefied. All samples were analyzed under the same conditions.

### Microbiological analysis

The determination of the microbial quality of honey (yeasts and molds, *Enterobacteriacee*, *Bacillus* spp., aerobic mesophilic bacteria and *Salmonella* spp.) was made according to ISO 21527-1:2008[20], ISO 21528-1:2017[21], ISO 6579-1:2017[22], ISO 4833-1:2013[23], and respectively ISO 7932:2004[24]. The antimicrobial activity of honey was tested against *Staphylococcus aureus* ATCC 2921, *Escherichia coli* ATCC 25,922, *Pseudomonas aeruginosa* ATCC 27,853 and *Salmonella enterica* serovar Typhimurium ATCC 14,028 using the well-diffusion method and assays were made in three replicates. Distilled water was used as a negative control and antibiotics (Oxoid gentamicin (30 µg), Oxoid cefuroxime, Oxoid ciprofloxacin, Oxoid tetracycline, Oxoid amoxycillin/clavulanate acid, Oxoid piperacillin/tazobactam and Oxoid ceftriaxone) served as a positive control. Antibiotic discs were obtained from

AMS 2000 Trading (Bucharest, Romania) The antimicrobial activity of honey was also studied using the broth microdilution method against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica* serovar Typhimurium.

### Statistical analysis

Results were submitted to analysis of variance (ANOVA) using IBM SPSS Statistics 26 trial version (IBM Corp., Armonk, NY, USA). Unscrambler X version 10.1 software (Camo, Norway) was used to perform principal component analysis (PCA).

## Results and discussion

### Pollen analysis

The pollen sources that the bees visit is identified through this analysis, and it helps identify the floral and geographic origins of honey. Although the minimum percentage needed for classification varies depending on the botanical species, monofloral honey must contain at least 45% of the pollen grains from a single plant species[25]. The samples were categorized as follows based on pollen analysis: 4 samples of raspberry honey, 5 samples of hawthorn honey, 3 samples of alfalfa, 4 samples of rosehip, 19 samples of honeydew honey, 3 samples of manuka, and 17 samples of polyfloral honey from the mountainous area.

In the manuka honey samples, a percentage greater than 70% of grains of *Leptospermum scoparium* was identified. The percentage of *Leptospermum scoparium*pollen grains in 30 manuka honey samples that were analyzed by[26] ranged from 45 to 90%. 47% of the honey tested showed low manuka tree pollen content, below the Moar limit (70%) required to classify honey as manuka honey. The samples analyzed in this study met the Moar limit.

Honeydew honey contains honeydew elements (such as wax tubes, algae, and fungal spores), and honeydew honey is characterized by a very low content of pollen grains and a higher conductivity value (more than 0,8 mS/cm)[27]. If the ratio of honeydew honey elements to pollen grains is greater than 3, the sample is honeydew honey. Honey with a ratio between one and three is classified as mixed honey, whereas floral honey is defined as those with a ratio of less than one between the elements of honeydew honey and the number of pollen grains in nectariferous species. The honeydew honey samples analyzed in this study were low in pollen sediments, and the ratio between honeydew elements and pollen grains was greater than 3[28]. The most frequent fungal elements found in honeydew honey samples according to the pollen analysis, were *Cladosporium*, *Aspergillus*, and *Alternaria*.

### Physicochemical analysis

*Moisture content*

The second-largest component of honey is water, and the amount of this component is correlated with the maturity of the product[29]. Because the stability of honey is determined by the amount of water it contains, moisture is a parameter that offers information on the quality of honey. Since it controls the stability of honey and resistance to deterioration due to yeast fermentation, moisture is a crucial quality parameter for the shelf life of honey[30]. In this study, the moisture varied between 16.71 and 19.29% (Table 1) and these values were in agreement with the legislative provisions that specify that honey must not exceed moisture content of 20%[7]. According to[29], the moisture content of pine honeydew honey from Greece ranged from 10.5 to 20.5%, whereas the moisture content of honeydew honey from Spain varied from 16.4 to 16.5%. Nguyen et al.[31] analyzed 2 samples of manuka honey and alfalfa honey and reported that the moisture content varied between 18.2 and 19.1%.

*pH and free acidity*

The free acidity of honey is the marker of fermentation processes and the microbiological contamination.

Honey typically has pH values in the range of 3.5 to 5.5 and this is because inorganic ions like phosphate and chloride as well as organic acids, especially gluconic acid, are present. For the honey samples examined in this study, the average pH ranged from 3.61 for hawthorn honey to a maximum of 4.14 for honeydew honey (Table 1). The botanical origin of honey determined differences in this parameter ($p < 0.05$). Similar results were obtained by[32]who reported that the pH values for the honey samples studied varied from 4.2 (alfalfa honey) to 5.1 (lotus honey). Six Nigerian honey samples that were examined by[33]had pH values ranging from 3.49 to 6.71. According to[17], the acid content of honey affects its texture, stability, and shelf life.

One crucial feature that may suggest microbial spoiling in honey is its free acidity[34]. The highest acidity level set by the Codex Alimentarius[7]is 50 milliequivalents of acid per 1000 g. Acetic acid is produced by alcoholic hydrolysis and contributes to sugar fermentation when free acidity values surpass the maximum permitted limit[35]. In our study, the lowest acidity was determined in rosehip honey (21.02 meq/kg) and the highest (33.42 meq/kg) in honeydew honey (Table 1)[29]. consulted various studies carried out on honey samples from Serbia, Romania, and Croatia and reported that for honeydew honey samples, the values for free acidity varied between 15.1 and 49.4 meq/kg, indicating acceptable quality.

*Electrical conductivity*

In routine honey quality control, electrical conductivity is used to distinguish between honeydew and blossom honey. The electrical conductivity of honey is influenced by its mineral content, acidity, and botanical origin[36]. The monofloral honey samples (hawthorn, alfalfa, rosehip, and raspberry) that were analyzed had an electrical conductivity lower than 500 µS/cm, as indicated by the values in Table 1, which allowed them to be categorized as pure floral honey. With an electrical conductivity of 990.15 µS/cm, honeydew honey had the highest value;

| Parameter | Origin | | | | | | | F value |
|---|---|---|---|---|---|---|---|---|
| | Hawthorn | Alfalfa | Rosehip | Honeydew | Manuka | Polyfloral | Raspberry | |
| **Chemical composition of honey** | | | | | | | | |
| pH | 3.61 (0.26)$^{ab}$ | 3.78 (0.52)$^{ab}$ | 3.89 (0.30)$^{ab}$ | 4.14 (0.21)$^{b}$ | 3.73 (0.12)$^{ab}$ | 4.04 (0.40)$^{ab}$ | 3.50 (0.11)$^{a}$ | 4.25*** |
| Free acidity (meq/kg) | 29.22 (6.64)$^{a}$ | 32.23 (3.41)$^{a}$ | 21.02 (10.38)$^{a}$ | 33.42 (8.87)$^{a}$ | 28.56 (4.07)$^{a}$ | 27.68 (7.86)$^{a}$ | 32.87 (7.53)$^{a}$ | 1.77ns |
| Electrical conductivity (µS/cm) | 323.78 (125.31)$^{a}$ | 395.66 (132.46)$^{ab}$ | 463 (124.42)$^{ab}$ | 990.15 (84.29)$^{c}$ | 482.33 (44.43)$^{ab}$ | 578.52 (136.69)$^{b}$ | 384.25 (193.97)$^{ab}$ | 40.84*** |
| Moisture (%) | 19.03 (2.04)$^{a}$ | 18.73 (2.30)$^{a}$ | 17.99 (2.12)$^{a}$ | 16.71 (1.26)$^{a}$ | 19.29 (0.95)$^{a}$ | 18.15 (1.58)$^{a}$ | 18.61 (2.22)$^{a}$ | 2.81* |
| Fructose (%) | 37.43 (2.89)$^{b}$ | 35.84 (1.96)$^{b}$ | 34.38 (3.51)$^{ab}$ | 30.46 (1.89)$^{a}$ | 38.43 (1.45)$^{b}$ | 34.36 (2.02)$^{ab}$ | 38.11 (3.82)$^{b}$ | 13.65*** |
| Glucose (%) | 29.19 (2.75)$^{a}$ | 29.16 (3.30)$^{a}$ | 30.12 (6.25)$^{a}$ | 27.29 (2.49)$^{a}$ | 32.04 (0.82)$^{a}$ | 28.83 (2.56)$^{a}$ | 32.27 (4.54)$^{a}$ | 2.33* |
| Sucrose (%) | 0.05 (0.11)$^{a}$ | 0.5 (0.68)a | 1.67 (0.80)$^{b}$ | 0.67 (0.33)$^{a}$ | 0.18 (0.02)$^{a}$ | 0.34 (0.35)$^{a}$ | 0.49 (0.25)$^{a}$ | 8.95*** |
| Ribose (%) | 0 $^{a}$ | 0 $^{a}$ | 0 $^{a}$ | 0.005 (0.01)$^{a}$ | 0 $^{a}$ | 0 $^{a}$ | 0 $^{a}$ | 0.982ns |
| Turanose (%) | 3.43 (0.78)$^{b}$ | 2.45 (1.14)$^{ab}$ | 1.95 (0.72)$^{ab}$ | 2.30 (0.71)$^{ab}$ | 1.88 (0.59)$^{a}$ | 2.84 (0.85)$^{ab}$ | 2.17 (0.17)$^{ab}$ | 2.79* |
| Maltose (%) | 2.76 (1.57)$^{a}$ | 4.16 (1.96)$^{a}$ | 2.56 (0.62)$^{a}$ | 3.56 (0.65)$^{a}$ | 2.30 (0.19)$^{a}$ | 3.14 (0.87)$^{a}$ | 3.47 (1.50)$^{a}$ | 1.84ns |
| Trehalose + Erlose (%) | 1.07 (0.52)$^{ab}$ | 0.87 (0.41)$^{ab}$ | 0.65 (0.49)$^{ab}$ | 2.72 (0.36)$^{c}$ | 0.48 (0.22)$^{a}$ | 1.45 (0.46)$^{b}$ | 0.85 (0.61)$^{ab}$ | 32.04*** |
| Melibiose (%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Melezitose (%) | 0.19 (0.37)$^{a}$ | 1.41 (0.81)$^{ab}$ | 0.74 (0.67)$^{ab}$ | 1.96 (0.37)$^{b}$ | 0.13 (0.05)$^{a}$ | 1.43 (1.34)$^{ab}$ | 0.56 (0.69)$^{ab}$ | 4.98*** |
| Raffinose (%) | 0$^{a}$ | 0.05 (0.04)$^{a}$ | 0.07 (0.14)$^{a}$ | 0.53 (0.20)$^{b}$ | 0.06 (0.10)$^{a}$ | 0.18 (0.17)$^{a}$ | 0.08 (0.16)$^{a}$ | 13.87*** |
| F/G | 1.29 (0.17)$^{a}$ | 1.24 (0.07)$^{a}$ | 1.17 (0.20)$^{a}$ | 1.13 (0.15)$^{a}$ | 1.20 (0.05)$^{a}$ | 1.20 (0.10)$^{a}$ | 1.19 (0.17)$^{a}$ | 1.18ns |
| Gluconic acid (g/kg) | 7.68 (0.95)$^{b}$ | 8.29 (1.17)$^{b}$ | 6.58 (1.20)$^{ab}$ | 6.04 (2.69)$^{ab}$ | 3.72 (0.89)$^{a}$ | 7.66 (1.77)$^{b}$ | 6.88 (0.83)$^{ab}$ | 2.23* |
| Formic acid (g/kg) | 0.40 (0.90)$^{a}$ | 0$^{a}$ | 0.63 (0.71)$^{a}$ | 0.47 (0.56)$^{a}$ | 0.59 (0.48)$^{a}$ | 0.46 (0.67)$^{a}$ | 0.03 (0.04)$^{a}$ | 0.65 ns |
| Acetic acid (g/kg) | 2.00 (1.41)$^{a}$ | 1.32 (0.07)$^{a}$ | 1.78 (0.63)$^{a}$ | 1.80 (1.43)$^{a}$ | 0.67 (0.47)$^{a}$ | 2.30 (2.70)$^{a}$ | 1.60 (0.92)$^{a}$ | 0.42 ns |
| Propionic acid (g/kg) | 0.23 (0.14)$^{a}$ | 0.21 (0.09)$^{a}$ | 0.01 (0.01)$^{a}$ | 0.09 (0.25)$^{a}$ | 0$^{a}$ | 0.12 (0.15)$^{a}$ | 0.10 (0.10)$^{a}$ | 0.92 ns |
| Lactic acid (g/kg) | 0$^{a}$ | 0.06 (0.11)$^{a}$ | 0.02 (0.05)$^{a}$ | 0.03 (0.10)$^{a}$ | 0.13 (0.23)$^{a}$ | 0.01 (0.03)$^{a}$ | 0$^{a}$ | 1.16 ns |
| Butyric acid (g/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Succinic acid (g/kg) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Content of bioactive compounds** | | | | | | | | |
| Total phenolic content (mg GAE/100 g) | 32.24 (11.78)$^{a}$ | 30.77 (9.03)$^{a}$ | 24.32 (7.22)$^{a}$ | 40.57 (7.50)$^{ab}$ | 54.16 (9.31)$^{b}$ | 36.80 (11.10)$^{ab}$ | 23.83 (5.14)$^{a}$ | 5.20*** |
| Flavonoids content (mg QE/100 g) | 50.71 (21.22)$^{a}$ | 47.36 (10.90)$^{a}$ | 58.57 (23.46)$^{a}$ | 69.71 (24.69)$^{a}$ | 73.08 (10.16)$^{a}$ | 49.67 (9.97)$^{a}$ | 45.22 (16.10)$^{a}$ | 2.65* |
| DPPH (%) | 47.30 (6.30)$^{a}$ | 45.59 (7.44)$^{a}$ | 45.44 (7.48)$^{a}$ | 52.89 (8.59)$^{a}$ | 57.50 (4.55)$^{a}$ | 46.21 (8.14)$^{a}$ | 43.61 (5.54)$^{a}$ | 2.30* |

**Table 1**. Physicochemical properties, carbohydrates content, and organic acids content of different types of honey (hawthorn, alfalfa, rosehip, honeydew, manuka, polyfloral, and raspberry). Mean values and standard deviation in brackets. ns – not significant ($p > 0.05$), * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$. $^{a–c}$ – different letters in the same row indicate significant differences between samples ($p < 0.001$).

any values above 800 µS/cm are unique to honeydew and should not be used with floral honey. Polyfloral honey had a value of electrical conductivity of 578.52 µS/cm and manuka honey had a value of electrical conductivity of 482.33 µS/cm. The variation of this parameter was significantly ($p < 0.001$) influenced by botanical origin[37]. reported for pine honey produced in Greece values for electrical conductivity between 0.91 and 1.31 mS/cm. It was reported by[38] that the electrical conductivity values of honey samples (pine, sunflower, cotton, and flower honey) ranged from 351 µS/cm to 1447 µS/cm (pine honey).

*Total phenolic content*

Phenolic compounds have an aromatic or aliphatic structure and phenol is included in their structure. They are regarded as the primary class of plant secondary metabolites[39]. A greater variation in the content of phenolic compounds was observed in polyfloral honey than in monofloral honey because flowering diversity for polyfloral honey production could increase the values of phenolic compounds[39].

The total phenolic content (TPC) of samples of manuka honey, alfalfa, rosehip, hawthorn, honeydew and polyfloral is shown in Table 1. For monofloral honey, TPC ranged from 23.83 mg GAE/100 g (raspberry honey) to 32.24 mg GAE/100 g (hawthorn honey). Polyfloral honey had a TPC content of 36.80 mg GAE/100 g, while honeydew honey presented an average TPC value of 40.57 mg GAE/100 g. The highest polyphenol content was identified in manuka honey (54.16 mg GAE/100 g). The variance of this parameter was significantly ($p < 0.001$) influenced by botanical origin. According to[40], manuka monofloral honey (*Leptospermum scoparium*) from Australia or New Zealand has the highest concentration of phenolic compounds (203–217 mg GAE/100 g).

*Flavonoids content*

Flavonoids are the main functional compounds that contribute significantly to the antioxidant activity of honey, bringing beneficial effects to the health of consumers[41]. The highest flavonoid content was found in manuka honey (73.08 mg/100 g, QE), followed by honeydew honey (69.71 mg/100 g, QE), rosehip honey (58.57 mg/100 g, QE), and hawthorn honey (50.71 mg/100 g, QE). Table 1displays the values determined for flavonoid content in function of botanical origin[42]. analyzed Chinese honey and found that pomegranate honey had the highest total

flavonoid content (48.88 mg/100 g, QE), while manuka honey displayed values between 50.15 mg/100 g, QE and 60.72 mg/100 g, QE[43]. analyzed honey from Romania and reported that the flavonoid content of the analyzed samples varied from 9.29 mg QE/100 g (wild cherry honey) to 263.86 mg QE/100 g (tilia honey).

*DPPH assay*

The antioxidant activity of honey is regarded as a crucial quality factor. Antioxidant activity is closely associated with phenolic substances, which are derived from nectar and pollen and are effective scavengers of oxygen radicals[44]. Due to their phenolic compositions, some studies have shown that honey with a dark amber color has a higher antioxidant capacity[45].

In this study the highest DPPH value (Table 1) was identified in manuka honey (57.50%) and honeydew honey (52.89%) and the lowest in raspberry honey (43.61%)[37]. reported for Greek pine honey an average antioxidant activity value of 60.11[44]. reported an antioxidant capacity of 23% for tilia honey samples collected from beehives located in rural areas. The anti-radical activity in 31 samples of Algerian honey ranged from 4.4 to 84%, according to[46].

*Carbohydrates content*

The primary constituents of honey are carbohydrates, which also serve as the body's energy source and determine the nutritional value of honey[47,48]. Some of the most important functional qualities of honey, such as its antibacterial activity, ability to hold onto moisture, longer shelf life, and ability to maintain a particular color and taste, are due to the sugars it contains[49]. Important variables affecting the crystallization of honey include fructose and glucose, fructose/glucose (F/G), and glucose/water (G/W)[48]. Honeydew honey is characterized by many authors as having a lower content of fructose and glucose[50].

The fructose content of the studied honey samples (Table 1) was found to be highest in manuka honey (38.43%) and lowest in honeydew honey (30.46%). The analysis of the samples revealed a significant variation of fructose content ($p < 0.01$) based on their botanical origin. The glucose content of raspberry honey was the highest (32.27%), while honeydew honey had the lowest (27.29%). The amount of sucrose in every sample of honey that was examined was less than 5% which is the limit allowed by European legislation. Ribose was identified only in honeydew honey. Also, honeydew honey had the highest content of maltose (3.56%), trehalose + erlose (2.72%), melezitose (1.96%) and raffinose (0.53%) of all the analyzed honey samples. Compared to flower honey, honeydew honey had a higher concentration of oligosaccharides, primarily trisaccharides (melezitose, raffinose, and maltose) and other larger oligosaccharides[51]. It was already suggested to use melezitose to distinguish between blossom and honeydew honey[5252]. analyzed 156 honey samples and raffinose was mainly detected in honeydew honey at levels of 5.5 mg/g, while it was not detected in floral honey[53]. analyzed manuka honey and Sologne summer forest honey and reported for manuka honey a content of 40% fructose, 31% glucose, and sucrose was not detected. In addition, the fructose/glucose ratio was calculated for all 55 honey samples (Table 1). In cases when the F/G ratio is less than 1.11, honey crystallizes quickly[54]. Results obtained in this study show that the F/G ratio was greater than 1.11 for each sample, indicating a slow rate of honey crystallization.

*Organic acids content*

A percentage < 0.5% of the composition of honey is represented by organic acids. They have an important role in defining the pH, antioxidant, and antimicrobial activities of honey[55]but also have a substantial impact on the sensory characteristics, such as taste and aroma, as well as the preservation and stability of the product[56]. The main non-aromatic organic acid in honey is gluconic acid, however, thirty non-aromatic organic acids may be present in honey (e.g., citric acid, malic acid, lactic acid, succinic acid)[57]. As it can be seen in Table 1, gluconic acid predominated in all of the examined honey samples. It was found that manuka honey had the lowest gluconic acid content (3.72 g/kg) and alfalfa honey had the highest (8.29 g/kg). The results obtained in this study were in agreement with those reported by[58]and[56], who found that the main organic acid in every sample of honey they examined was gluconic acid. Both honeydew and floral honey contain a significant amount of glutonic acid, which varies based on the plant source and the pollen and nectar that bees collect from flowers[57]. After gluconic acid, a high concentration was determined for acetic acid. Low concentrations of formic, propionic, and lactic acids were found in all honey samples; butyric and succinic acids were not detected. The lactic acid content of honey could result from lactic acid bacteria in the digestive tract of bees and can be transferred to honey, causing a natural fermentation of the product in the honeycomb[59].

*Polyphenols composition*

Polyphenols, which are classified as either non-flavonoids (mainly phenolic acids, stilbenes, and lignans) or flavonoids (flavonols, flavones, anthocyanidins, flavanones, and isoflavones), are a very diverse class of compounds with great importance from a functional standpoint[60].

Twelve polyphenols were examined in the samples, and the majority was present in all honey samples in varying amounts (Table 2). The highest concentrations of gallic acid, vanillic acid, caffeic acid, luteolin, quercitin, rosmarinic acid, chlorogenic acid, and kaempferol were found in manuka honey, which was in line with the high antioxidant activity of these samples. Protocatechuic acid and 4-hydroxybenzoic acid had the highest concentration in polyfloral honey (2.41 mg/100 g and 0.71 mg/100 g). Raspberry honey had the highest content of *p*-coumaric acid (0.43 mg/100 g), and the highest concentration of myricitin (0.33 mg/100 g) was identified in honeydew honey. The content of luteolin and quercitin varied significantly depending on the botanical origin ($p < 0.01$). According to[61], seven phenolic acids: p-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic, benzoic, and cinnamic were found in six different varieties of honey from Poland (polyfloral, tilia, rape, buckwheat, acacia, and honeydew).

| Polyphenols mg/100 g | Origin | | | | | | | F value |
|---|---|---|---|---|---|---|---|---|
| | Hawthorn | Alfalfa | Rosehip | Honeydew | Manuka | Polyfloral | Raspberry | |
| Galic acid | 0.27 (0.43)$^{a}$ | 0.81 (1.40)$^{ab}$ | 0$^{a}$ | 0.23 (0.43)$^{a}$ | 1.25 (1.11)$^{b}$ | 0.13 (0.16)$^{a}$ | 0$^{a}$ | 3.43** |
| Protocatechuic acid | 1.08 (1.99)$^{a}$ | 0.63 (0.41)$^{a}$ | 0.62 (0.63)$^{a}$ | 1.47 (0.70)$^{a}$ | 2.29 (1.25)$^{a}$ | 2.41 (6.45)$^{a}$ | 1.56 (2.49)$^{a}$ | 0.23ns |
| 4- Hydroxybenzoic acid | 0.24 (0.21)$^{a}$ | 0.24 (0.22)$^{a}$ | 0.23 (0.19)$^{a}$ | 0.30 (0.38)$^{a}$ | 0.40 (0.26)$^{a}$ | 0.71 (1.39)$^{a}$ | 0.14 (0.19)$^{a}$ | 0.55ns |
| Vanillic acid | 0.04 (0.05)$^{a}$ | 0.05 (0.03)$^{a}$ | 0.06 (0.05)$^{a}$ | 0.03 (0.05)$^{a}$ | 3.21 (5.20)$^{b}$ | 0.05 (0.05)$^{a}$ | 0.03 (0.02)$^{a}$ | 4.21** |
| Caffeic acid | 0.29 (0.14)$^{a}$ | 0.28 (0.33)$^{a}$ | 0.13 (0.07)$^{a}$ | 0.45 (0.25)$^{a}$ | 1.70 (1.29)$^{a}$ | 1.27 (4.17)$^{a}$ | 0.45 (0.70)$^{a}$ | 0.35ns |
| Chlorogenic acid | 0.01 (0.01)$^{a}$ | 0.01 (0.01)$^{a}$ | 0.01 (0.02)$^{a}$ | 0.02 (0.04)$^{a}$ | 0.19 (0.26)$^{b}$ | 0.01 (0.01)$^{a}$ | 0.01 (0.02)$^{a}$ | 4.08** |
| *p*-coumaric acid | 0.23 (0.26)$^{a}$ | 0.07 (0.06)$^{a}$ | 0.04 (0.01)$^{a}$ | 0.07 (0.03)$^{a}$ | 0.02 (0.01)$^{a}$ | 0.32 (0.89)$^{a}$ | 0.43 (0.51)$^{a}$ | 0.60ns |
| Rosmarinic acid | 0.04 (0.02)$^{ab}$ | 0.06 (0.02)$^{ab}$ | 0.09 (0.03)$^{ab}$ | 0.03 (0.01)$^{a}$ | 0.12 (0.16)$^{b}$ | 0.04 (0.03)$^{ab}$ | 0.06 (0.05)$^{ab}$ | 2.94* |
| Myricetin | 0.21 (0.14)$^{a}$ | 0.19 (0.04)$^{a}$ | 0.24 (0.21)$^{a}$ | 0.33 (0.91)$^{a}$ | 0.30 (0.06)$^{a}$ | 0.14 (0.13)$^{a}$ | 0.14 (0.11)$^{a}$ | 0.19ns |
| Luteolin | 0.01 (0.01)$^{a}$ | 0.01 (0.02)$^{a}$ | 0.02 (0.02)$^{a}$ | 0.01 (0.01)$^{a}$ | 0.37 (0.14)$^{b}$ | 0.01 (0.02)$^{a}$ | 0.01 (0.01)$^{a}$ | 56.42*** |
| Quercitin | 0.06 (0.01)$^{a}$ | 0.02 (0.03)$^{a}$ | 0.08 (0.07)$^{a}$ | 0.05 (0.04)$^{a}$ | 0.50 (0.42)$^{b}$ | 0.12 (0.12)$^{a}$ | 0.11 (0.17)$^{a}$ | 6.14*** |
| Kaempferol | 0.07 (0.03)$^{a}$ | 0.06 (0.03)$^{a}$ | 0.02 (0.02)$^{a}$ | 0.02 (0.03)$^{a}$ | 0.35 (0.09)$^{a}$ | 0.13 (0.47)$^{a}$ | 0.05 (0.01)$^{a}$ | 0.76ns |

**Table 2**. Polyphenols content for different types of honey (hawthorn, alfalfa, rosehip, honeydew, manuka, polyfloral, and raspberry). Mean values and standard deviation in brackets. ns – not significant ($p > 0.05$), * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$. $^{a–b}$ – different letters in the same row indicate significant differences between samples ($p < 0.001$).

## FTIR

Analysis of honey samples by ATR-FTIR was performed to gather useful information on the chemical structure and particular functional groups that make up the composition of honey. The ATR-FTIR spectra of honey samples were collected from 4000–650 $cm^{-1}$ and are presented in Fig. 1 as overlay spectra of honey samples (Fig. 1A) and spectra according to honey botanical origin limited to 1500–650 $cm^{-1}$ (Fig. 1B), which is the region of interest for the identification of carbohydrates in honey. The broad band observed in the spectral region of 3600–3000 $cm^{-1}$was assigned to stretching and bending vibrations of the O–H functional group in water, carbohydrates, organic acids, and phenols contained by honey[62]. Between 3000 and 2800 $cm^{-1}$ all samples had two common peaks, at 2932 $cm^{-1}$ and 2870 $cm^{-1}$, which were attributed to asymmetrical and symmetrical C–H stretching vibrations in carboxylic acids and stretching vibrations of the $NH_3$group in free amino acids. The presence of these two peaks in the FTIR spectra of honey was also reported by other authors[63,64]. The peak around 1650 $cm^{-1}$was due to stretching or bending vibrations of the O–H group in water[14,65].

The region between 1500 and 650 $cm^{-1}$ (Fig. 1B) was assigned to vibrations of the functional groups of honey carbohydrates. The peak at around 1419 $cm^{-1}$ was correlated to C–O–H, $–CH_2$ and C–H stretching in glucose, the peak at 1343 $cm^{-1}$ was due to O–H bending in the C–OH groups of fructose, while 1245 $cm^{-1}$ and 1147 $cm^{-1}$were attributed to bending and stretching vibrations of C–C in fructose, and respectively C–O and C–O–C of all carbohydrates contained by the samples[64]. The two prominent peaks at 1049 and 1028 $cm^{-1}$ were due to C–O stretching vibrations in fructose and glucose; the high fructose and glucose content of manuka honey, hawthorn and alfalfa honey, and the high F/G ratio of these samples was therefore corroborated by the ATR-FTIR spectra.

Furthermore, honeydew honey samples displayed in this region a distinct profile by comparison to the rest of the samples, most likely because of the lower fructose and glucose content and low F/G ratio. The peak at 916 $cm^{-1}$ was assigned to C–H bending vibrations mostly in glucose, and the following three peaks were characteristic to fructose: C–C bending at 865 $cm^{-1}$, and C–C–H stretching (816 $cm^{-1}$) and bending vibrations (775 $cm^{-1}$)[9].

## Microbial quality of honey

The most important parameters for determining the quality and safety of honey are those that define its microbiological characteristics. Microorganisms found in honey are primarily bacteria, molds, and yeasts. Nectar, pollen, honeybee intestines, equipment, humans, containers, winds and dust are all potential sources of contamination with microbial organisms. Bee intestines, for example, contain approximately 70% Gram-negative bacteria (e.g., *Klebsiella*, *Escherichia coli*, *Flavobacterium*, *Pseudomonas*, *Proteus*), 27% Gram-positive bacteria (e.g., *Streptococcus*, *Bacillus*, *Clostridium*spp.), and approximately 1% yeast[66]. According to Yupanqui Mieles et al.[67], the microbiological composition depends on the surrounding environment of the hive but also on the metabolic activity of bees.

The microbiological quality of honey samples was initially determined by performing microbiological tests for groups of microorganisms that act as safety indicators, such as the absence of bacteria *Salmonella* spp. and *Enterobacteriaceae*. In the current study, the *Enterobacteriaceae* or *Salmonella*spp. was not detected in any of the tested honey samples, which was in accordance with Regulation (EC) No 2073/2005[68]. Other authors obtained the same results by examining honey samples[69,70].

70.91% of honey samples tested negative for mesophilic aerobic bacteria, while 29.09% showed growth (20% had a count of 10 cfu/g of aerobic mesophilic bacteria, 3.64% had $7 \times 10^1$ cfu/g, and 5.45% had a higher contamination of $5 \times 10^2$cfu/g). Contamination of honey with mesophilic aerobic flora may has its origin from

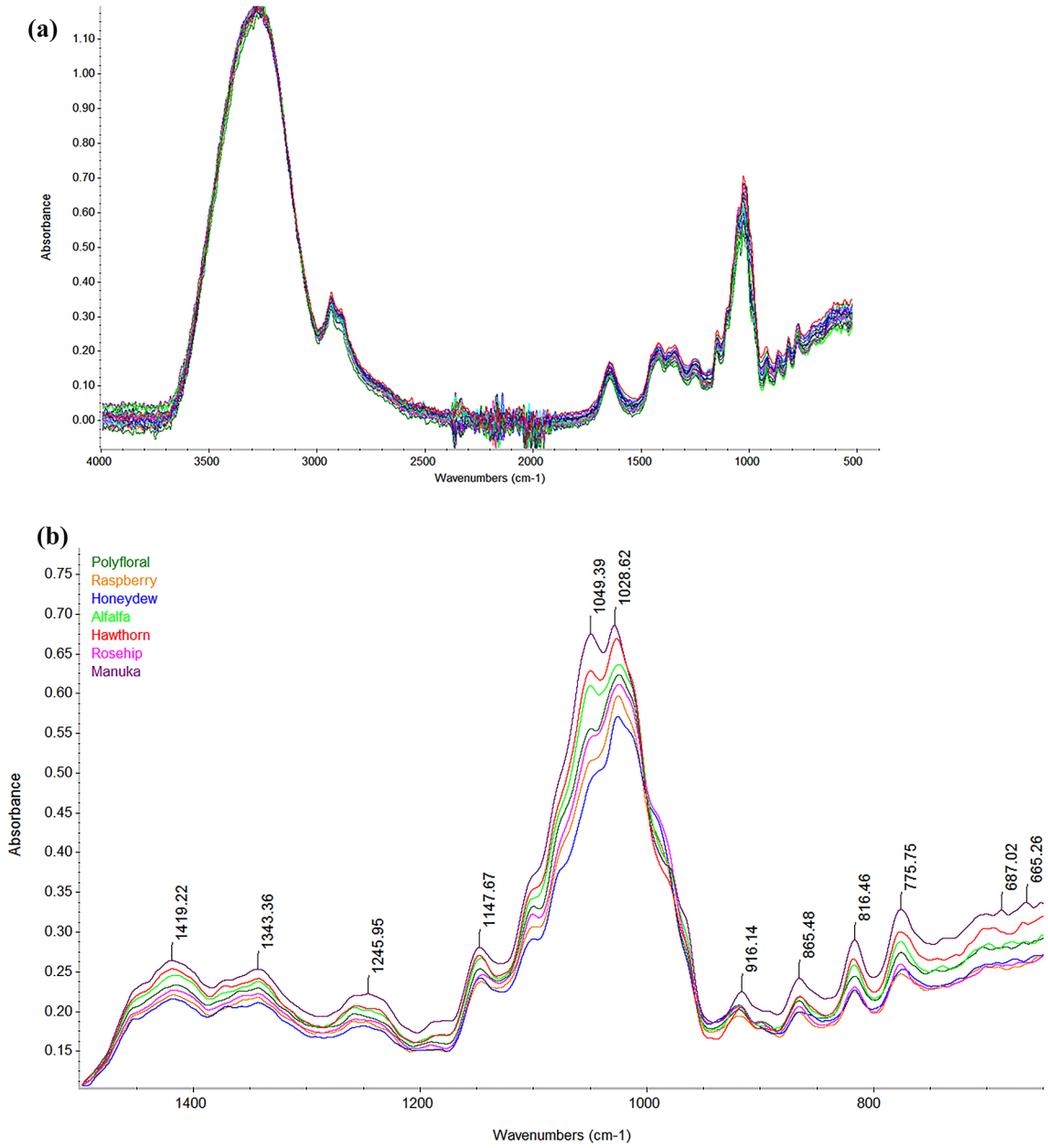


**Fig. 1**. (**A**). Overlay ATR-FTIR spectra of honey samples: polyfloral (dark green), raspberry (yellow), honeydew (blue), alfalfa (light green), hawthorn (red), rosehip (pink), manuka (purple). (**B**). ATR-FTIR spectra according to honey botanical origin: polyfloral (dark green), raspberry (yellow), honeydew (blue), alfalfa (light green), hawthorn (red), rosehip (pink), manuka (purple).

handling, processing and storage or from normal flora of the gastrointestinal tract of bees[71]. The low levels of aerobic mesophilic bacteria in our analyzed honey samples (94.55%) suggested that honey storage and beekeeping practices were performed in good conditions. The contaminated samples were polyfloral, monofloral and two of the manuka samples (manuka honey 250 – 10 cfu/g and manuka 100–7 × 10 cfu/g). 2 polyfloral honey samples and 1 hawthorn honey sample belong to the 5.45% group. Regarding the honeydew samples, only one sample had a population of 10 cfu/g. Other authors reported similar results[72]. For example[72], found in the analyzed honey samples values of mesophilic bacteria between 27 and 229 cfu/g. They also stated that many other authors had results between $1.0 \times 10^3$ to $5.0 \times 10^3$cfu/g[73]. found in the investigated samples values between 180 and 290 cfu/g, while[74] found between 10 and 370 cfu/g.

*Bacillus* spp. was found in 27.27% of the 55 samples tested, of which 12.73% were polyfloral honey samples (7 samples). The highest contamination ($9 \times 10^1$ cfu/g and $4 \times 10^3$ cfu/g) was found in three samples, where *Bacillus cereus* was also identified; the other four polyfloral samples with growth had values ranging from 10 to 20 cfu/g, with *Bacillus cereus* not present. In the monofloral samples, it was found that a single sample of manuka (manuka 100) showed bacterial growth ($1.3 \times 10^2$ cfu/g) with *Bacillus* spp., two samples of hawthorn

(10–180 cfu/g), two samples of raspberry (10 cfu/g), and two samples of rosehip (10–30 cfu/g). From all the honeydew samples tested, only one sample had *Bacillus*spp. (30 cfu/g) which is the same sample where the growth of aerobic mesophilic bacteria took place[75]. discovered *Bacillus cereus* in 55.55% of polyfloral honey samples, much more than it was found in this study. The outcomes achieved of microbiological analyses of honeys for *Bacillus* sp. in the present study cannot be referred to the EU legislation, because official permitted levels of bacterial contamination for this product have not been established. Therefore, the presence of *Bacillus cereus* in honey should be strictly controlled.

The presence of yeasts was detected in 43.64% of the total samples and the presence of molds in 25.45% of the total samples. Except for two honeydew samples, which had values of $1.4 \times 10^2$ and $2.0 \times 10^2$cfu/g, the rest of the samples had values for yeasts between 10 and 90 cfu/g. These values correspond to the standard specifications. For molds, the values found also fell within the legislative specifications with values between 10 and 30 cfu/g. No yeast or mold growth was detected in manuka honey[76]. found yeasts and molds in 35.3% of 17 honey samples ($1 \times 10^2 - 3 \times 10^3$cfu/g)[77], found mold yeast levels in the range of 10 to 160 cfu/g in three out of the 35 samples tested.

## Analysis of the antimicrobial activity of honey

*The antimicrobial activity using the well-diffusion method*

The agar well diffusion test was used to perform a first assessment of the antibacterial activity of honey. The antibacterial activity of the honey samples was determined by measuring the zone of inhibition of the tested bacterial growth around the wells on the agar medium. The inhibition zone diameters of honey samples against *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *Escherichia coli* are shown in Table 3.

The antibacterial effects of the tested honey types (including manuka honey) were more efficient against *Staphylococcus aureus*, as showed by larger inhibition zones, than the effects against *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *Escherichia coli*. The research results showed that 80% of the honey samples had inhibitory activity against *Staphylococcus aureus*, 76.36% against *Pseudomonas aeruginosa*, 74.55% against *Escherichia coli*, and 58.18% against *Salmonella enterica* serovar Typhimurium. Furthermore, 54.55% of the analyzed honey samples had antimicrobial activity for all pathogens studied, 18.8% had activity for three pathogens, 5.45% for two pathogens, 5.45% for one pathogen, and the remaining 16.36% (9 samples) of the samples did not show antimicrobial activity (for the concentrations of honey analyzed – 12.5%, 25%, 50%). The diameters of honey inhibition zones increased in a dose-dependent manner as concentration increased. The lowest antibacterial effect was found in the case of *Salmonella enterica* serovar Typhimurium, whose development was inhibited to a smaller degree by honey treatments in comparison to other bacteria.

*Analysis of the antimicrobial activity using the well diffusion method of Bucovina honey versus manuka honey*

The zones of inhibition of honeydew at concentrations ranging from 25 to 50% were greater than those of manuka 550 honey for *Staphylococcus aureus*, except for two samples at 50% concentration (honeydew 1 and honeydew 3) and seven samples at 25%. The results also showed that *Staphylococcus aureus* showed sensitivity to 25% of the honey samples but not to manuka 100 and manuka 250 honey samples at the concentration of 12.5%. Moreover, at the concentration of 25% honey, *Staphylococcus aureus*was susceptible to 44.23% of the samples but not to manuka 100 and 250 samples. The results obtained by[78] indicated smaller or equal diameters for ZOI (9.6–11.9 mm) compared to many of those obtained in the present study at concentrations of honey of 50% for *Staphylococcus aureus* and *Escherichia coli*.

Regarding the strain of *Escherichia coli*, 4 honeydew samples had the same activity as manuka 550 honey and 58% (11 samples) of all honeydew samples had a better effect than manuka 250, and 65.38% of the samples had a greater efficiency than manuka 100. Also, our results correspond with a study published by[79], who investigated Yemeni Sidr honey against *Escherichia coli* at 50% and 80% concentration. 27% of all samples investigated in this study showed antimicrobial activity against *Escherichia coli* at the concentration of 25% compared to manuka honey (manuka 100, manuka 250 and manuka 550) which had no activity. The same was observed in the case of the *Pseudomonas aeruginosa*, which showed susceptibility to 27% of the honey samples, but not to manuka honey (manuka 100, manuka 250, manuka 550) at a concentration of 25%. Similarly[80], obtained ZOI values between 10 and 14 mm for the tested honey samples of 25% concentration and ZOI values of 17–19 mm for honey samples tested for 50% concentration against *Pseudomonas aeruginosa*. Manuka 550 had a larger diameter than all the samples studied at 50% for the *Salmonella enterica* serovar Typhimurium strain. This was no longer found at the concentration of 25% because 7.69% of all analyzed samples had a diameter greater than manuka 550. Moreover, at a concentration of 25%, *Salmonella enterica* serovar Typhimurium showed sensitivity to 7.69% of the samples but not to manuka 100 or manuka 250.

It should be noted that the honey varieties tested were less active against the Gram-negative bacteria used in the experiments than the Gram-positive bacteria. The difference in the sensitivity of honey between Gram-positive and Gram-negative bacteria was likely due to the differences in cell wall composition. Gram-positive bacteria, unlike Gram-negative bacteria, lack an outer membrane that protects the peptidoglycan layer, making it easier for antimicrobial agents to penetrate and cause damage[81].

*Honey susceptibility vs. antibiotic susceptibility*

The current increase in the number of microbial species resistant to antibiotics highlights the need to find antibacterial alternatives. In the conducted study it was found that honeydew honey had antibacterial effect on pathogens very close to the effect of antibiotics or even better in some cases.

*Susceptibility to honey vs. susceptibility to antibiotics of the gram-positive ATCC strain.*

| Samples | Staphylococcus aureus | | | Salmonella enterica serovar Typhimurium | | Pseudomonas aeruginosa | | Escherichia coli | |
|---|---|---|---|---|---|---|---|---|---|
| | 12.50% | 25% | 50% | 25% | 50% | 25% | 50% | 25% | 50% |
| | ZOI / mm | | | | | | | | |
| H1 | 0 | 0 | 17.00 ± 1.41 | 0 | 1.33 ± 0.47 | 0 | 14.33 ± 0.47 | 0 | 12.00 ± 0.00 |
| H 2 | 0 | 27.00 ± 0.00 | 33.00 ± 0.00 | 15.00 ± 0.00 | 17.00 ± 0.00 | 18.33 ± 0.47 | 23.00 ± 0.82 | 12.67 ± 1.24 | 17.00 ± 0.82 |
| H 3 | 0 | 0 | 09.33 ± 0.47 | 0 | 14.33 ± 0.47 | 0 | 12.00 ± 1.63 | 0 | 11.33 ± 0.94 |
| H 4 | 0 | 17.00 ± 0.00 | 25.33 ± 0.47 | 0 | 0 | 0 | 11.00 ± 0.00 | 0 | 11.33 ± 1.25 |
| H 5 | 20.00 ± 0.82 | 27.67 ± 0.47 | 32.34 ± 0.47 | 0 | 15.33 ± 0.47 | 0 | 15.33 ± 0.47 | 0 | 15.00 ± 0.82 |
| H 6 | 0 | 12.00 ± 0.00 | 26.67 ± 0.47 | 0 | 12.67 ± 0.47 | 0 | 1.33 ± 0.47 | 0 | 11.67 ± 0.47 |
| H 7 | 14.00 ± 0.00 | 20.00 ± 0.00 | 28.67 ± 0.47 | 0 | 11.00 ± 0.00 | 11.00 ± 0.00 | 20.00 ± 0.82 | 11.33 ± 0.47 | 12.67 ± 0.94 |
| H 8 | 14.33 ± 0.47 | 19.67 ± 0.47 | 25.67 ± 0.47 | 0 | 12.67 ± 0.67 | 0 | 1.33 ± 0.47 | 0 | 12.66 ± 0.94 |
| H 9 | 17.33 ± 0.47 | 24.67 ± 0.47 | 29.00 ± 0.82 | 0 | 1.67 ± 0.94 | 0 | 15.00 ± 0.00 | 11.33 ± 0.47 | 1.33 ± 0.47 |
| H 10 | 17.00 ± 0.00 | 23.67 ± 0.41 | 30.00 ± 0.82 | 0 | 16.33 ± 0.47 | 12.67 ± 0.47 | 16.67 ± 0.47 | 0 | 15.00 ± 0.82 |
| H 11 | 14.00 ± 0.00 | 20.33 ± 0.47 | 26.00 ± 0.00 | 0 | 16.00 ± 1.41 | 1.67 ± 0.47 | 18.00 ± 0.82 | 0 | 14.33 ± 0.47 |
| H 12 | 15.00 ± 0.00 | 25.33 ± 0.47 | 29.00 ± 0.82 | 0 | 16.67 ± 1.25 | 12.67 ± 0.47 | 16.00 ± 0.00 | 12.67 ± 1.25 | 16.33 ± 0.47 |
| H 13 | 1.00 ± 0.00 | 20.00 ± 0.47 | 26.00 ± 0.00 | 0 | 12.67 ± 0.94 | 1.67 ± 0.47 | 16.00 ± 0.82 | 12.00 ± 0.00 | 16.33 ± 0.49 |
| H 14 | 1.67 ± 0.47 | 20.33 ± 0.47 | 25.33 ± 0.47 | 0 | 1.33 ± 0.47 | 0 | 15.33 ± 0.47 | 0 | 1.67 ± 0.47 |
| H 15 | 0 | 18.00 ± 0.00 | 28.33 ± 0.47 | 0 | 12.33 ± 0.47 | 1.33 ± 0.473 | 14.67 ± 0.94 | 0 | 14.67 ± 0.94 |
| H 16 | 15.00 ± 0.00 | 20.00 ± 0.00 | 25.67 ± 0.47 | 0 | 1.33 ± 0.47 | 1.67 ± 0.47 | 17.67 ± 0.47 | 0 | 14.33 ± 0.47 |
| H 17 | 0 | 0 | 26.33 ± 0.47 | 0 | 12.67 ± 0.94 | 12.00 ± 0.00 | 16.00 ± 0.00 | 0 | 16.67 ± 0.47 |
| H 18 | 0 | 25.33 ± 0.47 | 34.00 ± 0.82 | 14.67 ± 0.47 | 18.00 ± 0.82 | 0 | 18.67 ± 0.47 | 0 | 12.67 ± 0.94 |
| H 19 | 12.00 ± 0.00 | 27.33 ± 0.47 | 35.00 ± 0.82 | 12.67 ± 0.47 | 16.67 ± 0.94 | 0 | 1.33 ± 0.47 | 0 | 11.00 ± 0.82 |
| P 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P 2 | 0 | 24.33 ± 0.47 | 29.67 ± 0.47 | 0 | 11.00 ± 0.47 | 0 | 1.33 ± 0.47 | 0 | 0 |
| P 3 | 0 | 16.33 ± 0.47 | 29.00 ± 0.82 | 0 | 0 | 0 | 12.67 ± 0.47 | 0 | 0 |
| P 4 | 0 | 12.67 ± 0.47 | 15.33 ± 0.47 | 0 | 15.67 ± 0.94 | 12.67 ± 0.47 | 1.67 ± 0.47 | 0 | 15.00 ± 0.00 |
| P 5 | 0 | 0 | 12.33 ± 0.47 | 0 | 0 | 0 | 12.67 ± 0.47 | 0 | 12.67 ± 0.47 |
| P 6 | 0 | 0 | 31.33 ± 0.47 | 0 | 14.33 ± 0.47 | 0 | 0 | 0 | 16.00 ± 0.82 |
| P 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P 8 | 0 | 0 | 28.33 ± 0.47 | 0 | 1.00 ± 0.82 | 11.00 ± 0.00 | 1.67 ± 0.47 | 0 | 12.67 ± 0.94 |
| P 9 | 0 | 0 | 30.00 ± 0.82 | 0 | 14.33 ± 0.47 | 1.00 ± 0.82 | 18.33 ± 0.47 | 0 | 16.67 ± 0.47 |
| P 10 | 0 | 0 | 12.33 ± 0.47 | 0 | 0 | 0 | 11.00 ± 0.00 | 0 | 12.00 ± 0.00 |
| P 11 | 0 | 20.33 ± 0.47 | 28.33 ± 0.47 | 1.33 ± 0.47 | 16.00 ± 0.82 | 11.00 ± 0.00 | 16.00 ± 0.00 | 0 | 14.33 ± 0.47 |
| P 12 | 0 | 0 | 1.33 ± 0.47 | 0 | 0 | 0 | 12.00 ± 0.00 | 0 | 1.33 ± 0.47 |
| P 13 | 0 | 0 | 1.67 ± 0.47 | 0 | 11.67 ± 0.47 | 0 | 1.33 ± 0.47 | 0 | 11.67 ± 0.47 |
| P 14 | 0 | 0 | 1.67 ± 0.47 | 0 | 11.33 ± 0.47 | 0 | 12.67 ± 0.47 | 0 | 1.00 ± 0.82 |
| P 15 | 0 | 0 | 14.00 ± 0.00 | 0 | 09.00 ± 0.00 | 0 | 1.00 ± 0.00 | 0 | 11.33 ± 0.47 |
| P 16 | 15.00 ± 0.00 | 26.33 ± 0.47 | 30.33 ± 0.47 | 0 | 0 | 0 | 12.67 ± 0.94 | 0 | 10.33 ± 0.47 |
| P 17 | 0 | 0 | 15.33 ± 0.47 | 0 | 17.00 ± 0.82 | 0 | 16.00 ± 0.82 | 0 | 16.00 ± 0.82 |
| HA 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HA 2 | 0 | 0 | 12.33 ± 0.47 | 0 | 0 | 0 | 0 | 0 | 0 |
| HA 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| HA 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11.33 ± 0.47 |
| HA 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| A 1 | 0 | 0 | 0 | 0 | 0 | 0 | 11.00 ± 0.00 | 0 | 12.67 ± 0.94 |
| A 2 | 0 | 0 | 14.33 ± 0.47 | 0 | 0 | 0 | 1.00 ± 0.82 | 0 | 0 |
| A 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RA 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RA 2 | 0 | 0 | 14.33 ± 0.47 | 0 | 0 | 0 | 1.33 ± 0.47 | 0 | 1.00 ± 0.82 |
| RA 3 | 0 | 0 | 0 | 0 | 0 | 0 | 11.00 ± 0.00 | 0 | 0 |
| RA 4 | 0 | 0 | 16.33 ± 0.47 | 0 | 11.33 ± 0.47 | 0 | 12.00 ± 0.00 | 0 | 1.00 ± 0.82 |
| RA 1 | 0 | 26.33 ± 0.47 | 32.33 ± 0.47 | 0 | 1.00 ± 0.82 | 0 | 1.33 ± 0.47 | 1.33 ± 0.74 | 16.00 ± 0.81 |
| R 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| R 3 | 0 | 0 | 11.00 ± 0.00 | 0 | 0 | 0 | 0 | 0 | 0 |
| R 4 | 1.00 ± 0.00 | 19.33 ± 0.47 | 29.33 ± 0.47 | 0 | 0 | 12.00 ± 0.00 | 16.00 ± 0.00 | 11.00 ± 0.82 | 1.00 ± 0.82 |
| M 100 | 0 | 0 | 1.67 ± 0.47 | 0 | 0 | 0 | 1.33 ± 0.47 | 0 | 12.00 ± 0.00 |

Continued

| Samples | Staphylococcus aureus | | | Salmonella enterica serovar Typhimurium | | Pseudomonas aeruginosa | | Escherichia coli | |
|---|---|---|---|---|---|---|---|---|---|
| | 12.50% | 25% | 50% | 25% | 50% | 25% | 50% | 25% | 50% |
| | ZOI / mm | | | | | | | | |
| M 250 | 0 | 0 | 20.00 ± 0.00 | 0 | 11.33 ± 0.47 | 0 | 0 | 0 | 1.67 ± 0.47 |
| M 550 | 11.67 ± 0.47 | 20.67 ± 0.47 | 23.00 ± 0.00 | 12.67 ± 0.47 | 20.00 ± 0.82 | 0 | 12.67 ± 0.47 | 1.33 ± 0.47 | 16.67 ± 1.24 |

**Table 3**. Antibacterial activity (Inhibition Zone (mm ± SD) of all tested honeys at different concentrations against bacteria (H - honeydew honey, P - polyfloral; HA- hawthorn, A - alfalfa, RA - raspberry, R - rosehip, M - manuka honey).

A percentage of 43.64% of the total 50% concentration honey samples had an antibacterial effect (ZOI = 25–33 mm) against *Staphylococcus aureus* like most antibiotics tested according to CLSI and EUCAST. These results showed that they have superior effect to manuka 550 honey of the same concentration (50%). Thus, 89.47% of the total honeydew samples had a diameter of the inhibition zone of more than 25 mm for *Staphylococcus aureus*. These results show that they have an antibacterial effect close to the effect of the antibiotics ceftriaxone (22–28 mm), ciprofloxacin (22–30 mm), tetracycline (24–30 mm) and gentamicin (19–27 mm). Of these honeydew samples, 10 samples had ZOI diameters above 27 mm, which compares them with cefuroxime (27–37 mm) and piperacillin/tazobactam (27–36 mm) and 9 samples had the effect comparable to Amoxicillin/Clavulanic acid (28–36 mm) on *Staphylococcus aureus*. The results showed that these honeydew honey samples had superior effect to the manuka honey of 50% concentration, which had ZOI = 23 mm against *Staphylococcus aureus*. Among the samples of monofloral honey, 2 of them also had superior effect to that of manuka on *Staphylococcus aureus* with a ZOI of 29 mm and 32 mm respectively at the same concentration of 50%. 41.18% of the samples of polyfloral honey also showed antibacterial effect with a ZOI between 28 and 31 mm against *Staphylococcus aureus* at the same concentration of 50%. At the concentration of 25%, it was observed that 43.64% of the total honey samples investigated in this study showed bactericidal activity for *Staphylococcus aureus* with a ZOI between 12 mm and 26 mm. Of these, 16.36% showed activity similar to that of the antibiotics with a ZOI > 24 (two polyfloral honey samples, one monofloral sample, and the rest being honeydew samples). Manuka honey in 25% concentration had a ZOI of 21 mm for *Staphylococcus aureus* which is below the values of antibiotics. 12.5% of honey samples tested did not show antibacterial activity for *Staphylococcus aureus*, same as antibiotics. Based on research published in the literature[82,83] it is possible to obtain an enhanced antimicrobial activity from the combination of honey with antibiotics.

*Susceptibility to honey vs. susceptibility to antibiotics of the gram-negative ATCC strain*

The susceptibility of *Salmonella enterica* serovar Typhimurium and *Escherichia coli*at 12.73% and respectively at 18.18% of the total honey samples tested was very close to the susceptibility of these bacteria to some antibiotics (tetracycline, amoxicillin/clavulanic acid), but this susceptibility was not in accordance with the CLSI or EUCAST guidelines. In a study conducted by[1], Gentamicin and three types of pure honey from Ibadan and Abeokuta, south west Nigeria, were tested against *Pseudomonas aeruginosa* and *Escherichia coli*. The study used undiluted and fresh aqueous dilutions of 1:2, 1:4, and 1:6 in an agar diffusion method[1]. Honey and its 1:2 to 1:6 aqueous dilutions demonstrated 100% and 96.4% activity against *Pseudomonas aeruginosa* and *Escherichia coli*, while Gentamicin had lower antibacterial activity at concentrations of 8.0 and 4.0 µg/mL (Albaridi, 2019)[79]. obtained inhibition zone diameters comparable to those obtained in the current study for *Escherichia coli*.

The results of the analyses indicated that 27.27% of the total honey samples had antibacterial activity for *Pseudomonas aeruginosa* like Gentamicin or even better than gentamicin. 12.73% of all honey samples had antibacterial activity like ceftriaxone. 31.58% of the 50% honeydew honey samples had antibacterial activity for *Pseudomonas aeruginosa* like ceftriaxone and 57.89% had antibacterial activity for *Pseudomonas aeruginosa* similar to gentamicin. From the total samples of polyfloral honey, three samples had antibacterial activity for *Pseudomonas aeruginosa* like Gentamicin and one similar to Ceftriaxone.

*The antimicrobial activity using the broth microdilution method*

In the beginning, the agar well diffusion test revealed that all the tested honey types could have antibacterial activity against the four ATCC strains. The antibacterial effects exerted by the tested honey types (including manuka honey) were more potent against Gram-positive bacteria, as demonstrated by larger inhibition zones, compared to the effects against Gram-negative bacteria.

The minimum inhibitory concentration (MIC) of honey As previously stated, the honey samples were further tested to determine their minimum inhibitory and bactericidal concentrations against four pathogenic bacterial strains of significant importance. The MIC values of the tested honey types against the four pathogens studied were variable (Table 4).

### *Minimum inhibitory concentration (MIC) of honey for Staphylococcus aureus*
### *Minimum inhibitory concentration (MIC) of honey for Escherichia coli*

The study showed that some of the examined honeys had surprisingly high antibacterial, especially anti-staphylococcal potential. Therefore, the MIC value of the tested honey types against *Staphylococcus aureus* ranged from 1.56 to 50% (v/v). In comparison, the MIC value for *Staphylococcus aureus* of each type of manuka

| Samples | Staphylococcus aureus | | Salmonella enterica serovar Typhimurium | | Pseudomonas aeruginosa | | Escherichia coli | |
|---|---|---|---|---|---|---|---|---|
| | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC |
| H 4 | 3.13 | 1.56 | 12.50 | 6.25–12.50 | 6.25 | 3.13–6.25 | 12.50 | 6.25–12.50 |
| H 12 | 3.13 | 1.56 | 12.50 | 6.25–12.50 | 6.25 | 3.13–6.25 | 12.50 | 6.25–12.50 |
| H 14 | 3.13 | 1.56 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 3 | 3.13 | 1.56–3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 |
| H 5 | 3.13 | 1.56–3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 16 | 3.13 | 1.56–3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 7 | 6.25 | 1.56 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 |
| H 13 | 6.25 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 25.00 | 12.50 |
| H 15 | 6.25 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 17 | 6.25 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 1 | 6.25 | 3.13 | >75.00 | 12.50 | 25.00 | 6.25 | 12.50 | 12.50 |
| H 2 | 12.50 | 3.13 | 12.50 | 6.25 | 25.00 | 6.25 | 25.00 | 12.50 |
| H 8 | 12.50 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 9 | 12.50 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 10 | 12.50 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 11 | 12.50 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 19 | 12.50 | 6.25 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| H 18 | 25.00 | 3.13 | 25.00 | 12.50 | 25.00 | 12.50 | 25.00 | 12.50–25.00 |
| H 6 | 25.00 | 6.25 | 25.00 | 12.50 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 |
| P 4 | 6.25 | 1.56 | 6.25 | 3.13–6.25 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| P 2 | 6.25 | 3.13 | 12.50 | 6.25–12.50 | 25.00 | 12.5–25 | 12.50 | 6.25–12.50 |
| P 16 | 6.25 | 3.13 | 25.00 | 12.50–25.00 | 25.00 | 12.5–25 | 25.00 | 12.50–25.00 |
| P 11 | 12.50 | 1.56 | 50.00 | 25.00 | 25.00 | 12.50 | 25.00 | 12.50 |
| P 8 | 12.50 | 3.13 | 12.50 | 6.25–12.5 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 |
| P 3 | 12.50 | 3.13 | 25.00 | 12.50–25.00 | 12.50 | 6.25–12.50 | 25.00 | 12.50–25.00 |
| P 5 | 25.00 | 3.13 | 75.00 | 50.00 | 50.00 | 25.00 | 12.50 | 6.25–12.50 |
| P 6 | 25.00 | 3.13 | 25.00 | 12.50 | 25.00 | 12.50 | 25.00 | 12.50 |
| P 10 | 25.00 | 6.25 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 |
| P 1 | 25.00 | 6.25 | >75.00 | 50.00 | >75.00 | 50.00 | 25.00 | 12.50–25.00 |
| P 17 | 50.00 | 3.13 | 50.00 | 12.50 | 12.50 | 3.13–6.25 | 12.50 | 6.25–12.50 |
| P 9 | 50.00 | 6.25 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 |
| P 13 | 75.00 | 6.25 | >75.00 | 25.00 | 25.00 | 12.50 | 75.00 | 25.00 |
| P 14 | 75.00 | 12.50 | >75.00 | 25.00 | >75.00 | 25.00 | >75.00 | 25.00 |
| P 7 | 75.00 | 50.00 | 75.00 | 25.00 | 75.00 | 50.00 | 75.00 | 25.00 |
| P 12 | >75.00 | 50.00 | >75.00 | 50.00 | >75.00 | 50.00 | 75.00 | 50.00 |
| P 15 | >75.00 | 25.00 | >75.00 | 25.00 | >75.00 | 25.00 | >75.00 | 25.00 |
| HA 1 | 6.25 | 3.12–6.25 | 25.00 | 12.50–25.00 | 12.50 | 6.25–12.50 | 25.00 | 12.50–25.00 |
| HA 2 | 6.25 | 3.12–6.26 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 |
| HA 5 | 50.00 | 6.25 | 75.00 | 12.50 | 25.00 | 6.25–12.50 | 25.00 | 6.25–12.50 |
| HA 3 | >75.00 | 50.00 | >75.00 | 50.00 | >75.00 | 50.00 | >75 | 50.00 |
| HA 4 | >75.00 | 50.00 | >75.00 | 50.00 | >75.00 | 50.00 | >75 | 50.00 |
| R 1 | 12.50 | 3.13 | 25.00 | 6.25–12.50 | 12.50 | 6.25–12.50 | 12.50 | 6.25–12.50 |
| R 4 | 12.50 | 3.13 | 12.50 | 6.25–12.50 | 12.50 | 6.25 | 12.50 | 6.25–12.50 |
| R 2 | >75.00 | 50.00 | >75.00 | 25.00 | >75.00 | 50.00 | >75.00 | 25.00 |
| R 3 | >75.00 | 12.50 | >75.00 | 25.00 | >75.00 | 25.00 | >75.00 | 25.00 |
| A 2 | 25.00 | 6.25 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 |
| A 1 | >75.00 | 75.00 | >75.00 | 75.00 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 |
| A 3 | >75.00 | 50.00 | 75.00 | 50.00 | >75.00 | 25.00 | 75.00 | 25.00 |
| RA 1 | >75.00 | 50.00 | 75.00 | 25.00 | >75.00 | 50.00 | 75.00 | 25.00 |
| RA 2 | >75.00 | 25.00 | >75.00 | 25.00 | >75.00 | 12.50 | 75.00 | 25.00 |

Continued

| Samples | Staphylococcus aureus | | Salmonella enterica serovar Typhimurium | | Pseudomonas aeruginosa | | Escherichia coli | |
|---|---|---|---|---|---|---|---|---|
| | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC |
| RA 3 | >75.00 | 25.00 | >75.00 | 50.00 | >75.00 | 50.00 | 75.00 | 50.00 |
| RA 4 | 75.00 | 6.25 | 75.00 | 25.00 | 25.00 | 12.50 | 25.00 | 12.50–25.00 |
| M 100 | 75.00 | 6.25 | >75.00 | 12.5–25 | 25.00 | 12.50–25.00 | 25.00 | 12.50–25.00 |
| M 250 | 50.00 | 6.25 | 50.00 | 12.50 | 25.00 | 12.50 | 25.00 | 12.50 |
| M 550 | 12.50 | 6.25 | 25.00 | 12.50 | 25.00 | 6.25–12.50 | 12.50 | 6.25–12.50 |

**Table 4**. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of honey samples (H - honeydew honey, P - polyfloral; HA- hawthorn, A - alfalfa, RA - raspberry, R - rosehip, M - manuka honey).

honey (100, 250, and 550) was found at 6.25% (v/v). Among all analyzed honey samples, honeydew honey (all samples) had the highest antibacterial activity against the *Staphylococcus aureus* strain with MIC values between 1.56% and 6.25%. Except for four honey samples, all of the polyfloral honey samples in the study had MIC values for *Staphylococcus aureus* ranging from 1.56 to 6.25%. In terms of the monofloral honey samples analyzed, there were also samples from each type that had MIC values for *Staphylococcus aureus* better or equal to those obtained from the analysis of manuka honey (Table 4). It is important to note that 53.85% of all samples had MIC values for *Staphylococcus aureus* between 1.56% and 3.1%. These honey samples were four respectively two times more effective against *Staphylococcus aureus* in comparison to reference samples of manuka honey (manuka 100, manuka 250, manuka 550).

### *Minimum inhibitory concentration (MIC) of honey for Escherichia coli*

Gram-negative bacteria, particularly *Escherichia coli*, were less sensitive to all the honey samples tested. The same observation was made by[42]. None of the honey samples could inhibit the growth of these microorganisms at concentrations lower than 6.25% (g/v); the lowest level of MIC activity had values between 6.25% and 12.5%. The MIC values of manuka 550 honey for *Escherichia coli* strain were also between 6.25% and 12.5% (g/v), and the exact same activity was detected for 46.15% of all honey samples in the current study. 73.7% of the honeydew honey samples (that is 27% of the total samples) presented MIC values for *Escherichia coli* between 6.125 and 12.5. These honeydew samples were two times more effective against *Escherichia coli* strains in comparison to the reference sample of manuka 100 honey or manuka 250, and as effective as the manuka honey sample with a high concentration of methylglyoxal (550 mg/kg).

### *Minimum inhibitory concentration (MIC) of honey for Salmonella enterica serovar Typhimurium*

Unlike *Escherichia coli*, the *Salmonella enterica* serovar Typhimurium strain was a little more susceptible to the analyzed honey samples, especially compared to honeydew honey, which presented MIC values for *Salmonella enterica* serovar Typhimurium between 6.25 and 12.50. Compared to manuka honey to which *Salmonella enterica*serovar Typhimurium was susceptible at MIC values of 12.50% (manuka 550 and manuka 250) and 12.50–25.00% (manuka 100), honeydew honey (84.21% of the total honeydew samples) proved to be much more efficient with MIC values between 6.25 and 12.50. 15.80% of the honeydew samples had the same effectiveness as the manuka 250 and 550 samples[84]. also obtained similar results. The susceptibility of the *Salmonella enterica* serovar Typhimurium strain to monofloral honey samples was comparable to that of the *Escherichia coli* strain, and the MIC values were between 6.25 and 50% (one sample – alfalfa 1 had MIC = 75%).

### *Minimum inhibitory concentration (MIC) of honey for Pseudomonas aeruginosa*

The current study found that of all the Gram-negative bacteria tested, the *Pseudomonas aeruginosa* strain was the most susceptible to honey. It is very important to note that of all the honey samples, 65% showed antibacterial activity two or even three times higher than manuka 100 (MIC value between 3.125 and 12.5%); 52.00% had MIC values better than manuka 250 (MIC between 3.125 and 12.5%); 35.00% had MIC values better than manuka 550 (MIC between 3.125 and 6.25%), and the rest of the samples had MIC for *Pseudomonas aeruginosa*between 25% and 50%[85]. discovered that the MIC values for *Pseudomonas aeruginosa* of the analyzed samples ranged from 8 to 40%. The research discovered that 90% of the honeydew samples tested had surprisingly high antimicrobial activity against *Pseudomonas aeruginosa*. Two honeydew samples and one polyfloral were able to inhibit the growth of *Pseudomonas aeruginosa* reference strains at concentrations ranging from 3.125 to 6.25% (v/v). *Pseudomonas aeruginosa* were more susceptible to 65.00% (47.00% and respectively 30.00%) of the polyfloral samples than manuka honey (manuka 250 and manuka 550) with MIC values of honey ranged between 3.125 and 25.00%.

## The minimum bactericidal concentration (MBC) of honey

The minimum inhibitory and bactericidal concentrations were performed against the 4 pathogens of clinical importance at different concentrations of honey samples ranging from 75 to 1.56%. The obtained results showed that the ratio between the two concentrations varied between 1 and 4 for most of the honey samples. This demonstrated that 84.62% of the honey samples, at the concentrations studied, had antibacterial activity for *Staphylococcus aureus*, of which 16.46 had bacteriostatic activity and 71.15% had bactericidal activity. 75% of

the samples had bactericidal activity for *Salmonella enterica* serovar Typhimurium, 78.85% for *Pseudomonas aeruginosa* and 88.46% for *Escherichia coli*. In comparison, manuka honey 100 and 250 had an MBC/MIC ratio of 12 and 8, respectively, which indicated bacteriostatic activity for Gram-positive bacteria; manuka 550 showed bactericidal activity (the ratio between the two MBC/MIC concentrations was equal to 2). The results of the study showed that for Gram-negative bacteria all the manuka honey samples had a bactericidal activity (the ratio between the two MBC/MIC concentrations varied between 1 and 4).

## Principal component analysis (PCA)

In this study, PCA was applied to analyze and identify the honey samples that share similar characteristics, the parameters were. The first principal component (PC-1) accounted for 49% of the variance, while the second principal component (PC-2) accounted for 18% of the variance; together, the first two principal components accounted for 67% of the initial variability.

The separation of honey samples according to physicochemical parameters and antimicrobial action is presented in Fig. 2. Honeydew honey is perfectly grouped, while manuka honey was also grouped according to the results obtained. The polyfloral honey, alfalfa, rosehip, raspberry and hawthorn honey samples did not form well-defined groups because there were differences regarding their antimicrobial action.

In Fig. 3, the parameters which are in the outer ellipse had greater contribution to the variability than the parameters located in the inner ellipse. The honeydew honey samples were correlated with electrical conductivity, trehalose + erlose content, ribose content, melezitose content, raffinose content and with antimicrobial activity of 12.5%, 25% and, 50% honey against *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *Escherichia coli*. Manuka honey samples were correlated with total polyphenols content, quercitin, vanillic acid, kaemferol, caffeic acid, chlorogenic acid, succinic acid and turanose content.

Regarding the physicochemical parameters, it seems that the total flavonoids content was in opposition with the rest of the parameters. A greater contribution to the variability presented the minimum bactericidal concentrations (MBC) against *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli*, and minimum inhibitory concentrations against *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa* and *Escherichia coli*.

According to the Pearson correlation (Table 5) (* - means correlation is significant at the level 0.05 level), the antimicrobial action of a 12.5%, 25% and 50% concentration of honey against *Staphylococcus aureus* was positively correlated with electrical conductivity ($r = 0.588^*$, $r = 0.594^*$ and $r = 0.699^*$), TFC ($r = 0.428^*$, $r = 0.304^*$ and $r = 0.292^*$), DPPH ($r = 0.313^*$, $r = 0.293^*$, $r = 0$), trehalose + erlose (Table 6) ($r = 0.506^*$, $r = 531^*$ and $r = 0.592^*$), melezitose content ($r = 0.366^*$, $r = 0.533^*$ and $r = 0.535^*$), raffinose content ($r = 0.518^*$, $r = 0.538^*$ and $r = 0.560^*$) and negatively correlated with moisture ($r = -0.438^*$, $r = -0.372^*$ and $r = -0.446^*$), fructose ($r = -0.411^*$ and $r = -0.496^*$ and $r = -0.567^*$) and glucose content ($r = -0.324^*$, $r = -0.402^*$ and $r = -0.459^*$). The antimicrobial action of a 12.5% concentration of honey against *S. aureus* was also positively correlated with TPC ($r = 0{,}272^*$) and negatively correlated with succinic acid ($r = -0.278^*$). The antimicrobial action of a 50% concentration of honey against *S. aureus* was also positively correlated with pH ($r = 0.560^*$).

The minimum inhibitory concentration (MIC) against *Staphylococcus aureus* was negatively correlated with electrical conductivity ($r = -0.413^*$), TFC ($r = -0.274^*$) and trehalose + erlose ($r = -0.393^*$) and positively

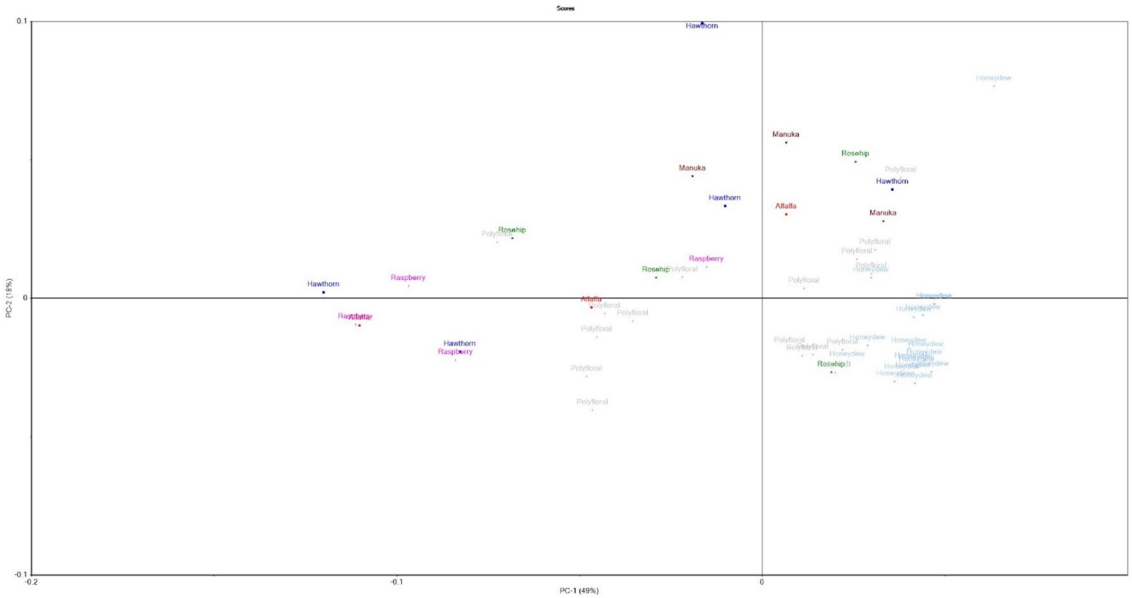


**Fig. 2**. Principal component analysis - scores: honeydew honey (light blue rhombus), polyfloral (grey rhombus), raspberry (pink line), alfalfa (red cercle), rosehip (green triangle), hawthorn (blue square) and, manuka honey (brown triangle).

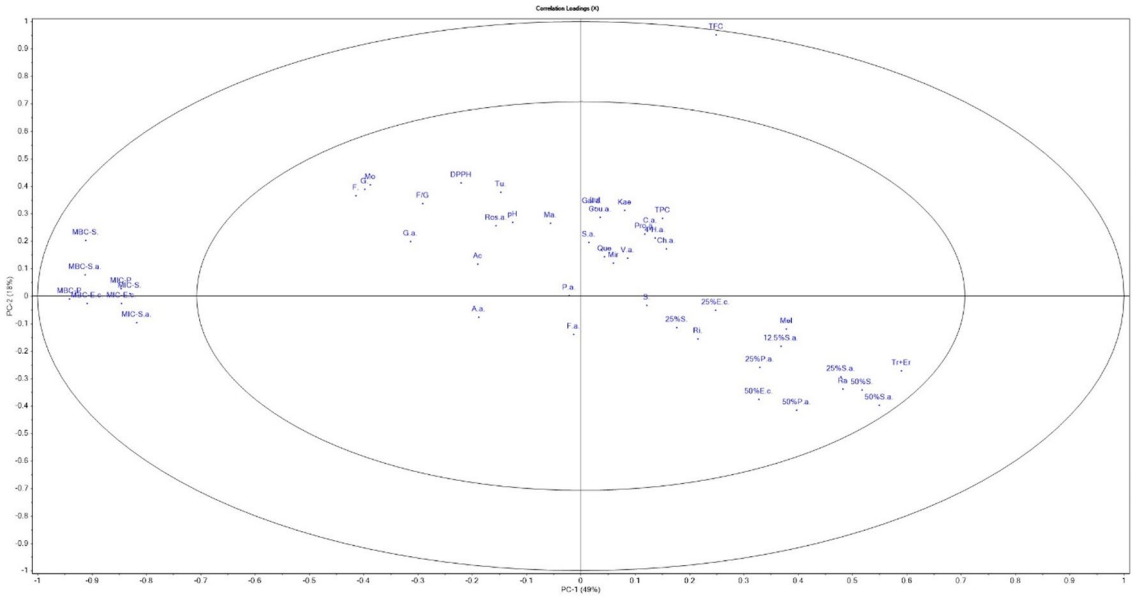


**Fig. 3**. Principal component analysis - loadings: pH, Ac. - free acidity, EC - electrical conductivity, Mo - moisture, TPC - total polyphenols content, TFC - total flavonoids content, DPPH, G.a. - gallic acid, P.a. - protocatechuic acid, 4-hA – 4- hydroxybenzoic acid, V.a. - vanillic acid, Ch.a. - chlorogenic acid, C.a. - caffeic acid, Cou.a. - p-coumaric acid, R.a. - rosmarinic acid, My - miricetin, Que - quercitin, Lu - Luteolin, Ka - Kaempferol, F - fructose content, G - glucose content, S - sucrose content, Tu - turanose content, Ma - manose content, Tr - trehalose content, Mel - melezitose content, Ra - raffinose content, Ri- ribose content, Tr + Er - Trehalose + Erlose content, G.a. - gluconic acid, F.a. - formic acid, A.a. - acetic acid, P.a. - propionic acid, L.a. - lactic acid, B.a. - butyric acid, S.a. - succinic acid, 12.50% S.a. – 12.50% honey against *Staphylococcus aureus*, *25%* S.a. – *25%* honey against *Staphylococcus aureus*, *50%* S.a. – *50%* honey against *Staphylococcus aureus*, *25% S. – 25%* honey against *Salmonella enterica* serovar Typhimurium, *50% S. – 50%* honey against *Salmonella enterica* serovar Typhimurium, *25%* P.a. – *25%* honey against *Pseudomonas aeruginosa*, *50%* P.a. – *50%* honey against *Pseudomonas aeruginosa*, *25% E.c. – 25%* honey against *Escherichia coli*, *50% E.c. – 50%* honey against *Escherichia coli*, MBC S.a. - minimum bactericidal concentrations for *Staphylococcus aureus*, MIC S.a. -minimum inhibitory concentrations for *Staphylococcus aureus*, MBC S. - minimum bactericidal concentrations for *Salmonella enterica* serovar Typhimurium, MIC S. - minimum inhibitory concentrations for *Salmonella enterica* serovar Typhimurium, MBC P.a. - minimum bactericidal concentrations for *Pseudomonas aeruginosa*, MIC P.a. - minimum inhibitory concentrations for *Pseudomonas aeruginosa*, MBC E.c. - minimum bactericidal concentrations for *Escherichia coli*, MIC E.c. - minimum inhibitory concentrations for *Escherichia coli*.

correlated with fructose content ($r = 0.340^*$). The minimum bactericidal concentration (MBC) against *Staphylococcus aureus* was negatively correlated with pH ($r = -0.402^*$), electrical conductivity ($r = -0.481^*$), trehalose + erlose ($r = -0.460^*$), melezitose content ($r = -0.410^*$) and raffinose content ($r = -0.312^*$). The minimum bactericidal concentration (MBC) against *Staphylococcus aureus* was positively correlated with fructose ($r = 0.428^*$) and glucose content ($r = 0.335^*$).

The antimicrobial action of a 25% concentration of honey against *Salmonella enterica* serovar Typhimurium was positively correlated only with succinic acid ($r = 0.380^*$). The antimicrobial action of a 50% concentration of honey against *Salmonella enterica* serovar Typhimurium was positively correlated with pH ($r = 0.476^*$), electrical conductivity ($r = 0.649^*$), TPC ($r = 0.396^*$), DPPH ($r = 0.353^*$), trehalose + erlose ($r = 0.575^*$), melezitose content ($r = 0.293^*$) and raffinose content ($r = 0.542^*$) and, negatively correlated with moisture ($r = -0.435^*$) and fructose content ($r = -0.471^*$).

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of honey was not correlated with polyphenols content (Table 7).

The minimum inhibitory concentration (MIC) against *Salmonella enterica* serovar Typhimurium was positively correlated with fructose content ($r = 0.433^*$) and negatively correlated with pH ($r = -0.307^*$), electrical conductivity ($r = -0.508^*$), TFC ($r = -0.317^*$), trehalose + erlose ($r = -0.424^*$), melezitose content ($r = -0.306^*$) and raffinose content ($r = -0.321^*$). The minimum bactericidal concentration (MBC) and against *Salmonella enterica* serovar Typhimurium was positively correlated with fructose content ($r = 0.450^*$) and moisture content ($r = 0.283^*$) and negatively correlated with the same parameters as in the case of MIC.

The antimicrobial action of a 25% and 50% concentration of honey against *Pseudomonas aeruginosa* and against *Escherichia coli* shows positive and negative correlations with the same parameters as in the case of pathogenic agents *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium (Tables 8 and 9). This demonstrates that the antimicrobial activity of honey depends on its physico-chemical characteristics.

| Variables | pH | Acidity | Conductivity | Moisture | Polyphenols content | Flavonoids content | DPPH | Gluconic acid | Formic acid | Acetic acid | Succinic acid | Propionic acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MBC *Salmonella* | **−0,433** | 0,185 | **−0,537** | **0,283** | −0,132 | −0,144 | −0,118 | 0,061 | 0,090 | 0,097 | −0,098 | −0,044 |
| MIC *Salmonella* | **−0,307** | 0,045 | **−0,508** | 0,207 | −0,206 | **−0,317** | −0,185 | 0,094 | 0,090 | 0,233 | −0,092 | −0,005 |
| MBC *P. aeruginosa* | **−0,423** | 0,232 | **−0,503** | **0,332** | −0,143 | **−0,266** | −0,098 | 0,132 | 0,112 | 0,206 | −0,155 | 0,022 |
| MIC *P. aeruginosa* | **−0,394** | 0,067 | **−0,500** | **0,300** | −0,164 | **−0,293** | −0,043 | 0,115 | 0,160 | **0,298** | −0,094 | −0,085 |
| MBC *E. coli* | **−0,345** | 0,233 | **−0,427** | 0,248 | −0,087 | −0,214 | −0,032 | 0,170 | 0,053 | −0,027 | −0,112 | 0,030 |
| MIC *E. coli* | **−0,326** | 0,095 | **−0,395** | 0,182 | −0,101 | −0,253 | −0,074 | 0,082 | 0,121 | 0,017 | −0,052 | −0,064 |

**Table 5**. Pearson correlations for MIC (Minimum Inhibitory concentration) and MBC (Minimum Bactericidal Concentration) of honey and its physicochemical parameters and organic acids content. *Values in bold are different from 0 with a significance level alpha = 0.05.*

| Variables | Fructose | Glucose | Sucrose | Ribose | Turanose | Maltose | Trehalose + Erlose | Melezitose | Raffinose | F/G |
|---|---|---|---|---|---|---|---|---|---|---|
| MBC *Salmonella* | **0,450** | 0,261 | −0,191 | −0,227 | 0,006 | 0,004 | **−0,450** | **−0,372** | **−0,335** | 0,128 |
| MIC *Salmonella* | **0,433** | 0,258 | −0,105 | −0,188 | −0,079 | −0,087 | **−0,424** | **−0,306** | **−0,321** | 0,128 |
| MBC *P. aeruginosa* | **0,416** | 0,258 | −0,192 | −0,223 | −0,006 | −0,194 | **−0,445** | **−0,297** | −0,250 | 0,115 |
| MIC *P. aeruginosa* | **0,378** | **0,276** | −0,193 | −0,182 | 0,052 | −0,144 | **−0,428** | −0,239 | **−0,268** | 0,074 |
| MBC *E. coli* | **0,353** | 0,254 | −0,161 | −0,178 | 0,010 | −0,112 | **−0,361** | **−0,315** | −0,218 | 0,063 |
| MIC *E. coli* | **0,311** | **0,280** | −0,191 | −0,151 | 0,032 | −0,113 | **−0,330** | **−0,292** | −0,181 | 0,014 |

**Table 6**. Pearson correlations for MIC (Minimum Inhibitory concentration) and MBC (Minimum Bactericidal Concentration) of honey and carbohydrate content. *Values in bold are different from 0 with a significance level alpha = 0.05*.

## Conclusions

The physicochemical parameters of raspberry, rosehip, alfalfa, hawthorn, polyfloral and honeydew honey samples from Bucovina, Romania, and of manuka honey samples were analyzed to characterize the honey samples and verify their usefulness in classifying honey depending on the botanical origin. Individual and total phenolic compounds, flavonoid content and antioxidant activity showed the highest values in manuka honey, but honeydew honey showed very close values for these parameters. Electrical conductivity proved to be an important parameter in the characterization of honey samples because, together with pollen analysis, it led to the identification of the botanical origin of honey.

The present study also investigated microbial quality, and antimicrobial activities of the honey types from the Bucovina region of Romania. The research confirmed that the microbial loads of all honey samples fall within the safety limits for use, with the exception of five samples. An impressively large number of the honey types had higher bactericidal activity than manuka honey. Thus, the antibacterial and physicochemical properties of honey from Bucovina, particularly those that are superior or even comparable to manuka honey, can be investigated, as this may lead to applications in food industry. 40.46% of all honey samples had bactericidal activity that was superior or at least comparable to manuka honey MGO 550 against *Staphylococcus aureus*, 57.69% against *Salmonella enterica* serovar Typhimurium, 74.15% against *Pseudomonas aeruginosa*, and 75% against *Escherichia coli*. Of all types of honey analyzed in this study, honeydew honey had the highest bactericidal activity, followed by polyfloral honey.

This study is important because it provides valuable insights into the physicochemical and antimicrobial properties of honey types from Bucovina, Romania, and compares them to well-known honey, such as manuka honey. Additionally, the study highlights the strong antibacterial properties of Bucovina honey, showing that many of the honey types exhibited bactericidal activity that was equal to or better than manuka honey against common pathogens. Furthermore, the study assures that the honey from this region is generally safe for consumption, with only a few samples falling outside the safety limits, making it a reliable product for both health and commercial purposes.

| Variables | Galic acid | Protocatechuic acid | 4- Hydroxybenzoic acid | Vanillic acid | Caffeic acid | Chlorogenic acid | *p*-coumaric acid | Rosmarinic acid | Miricetin | Luteolin | Quercitin | Kaempferol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MBC *Salmonella* | 0,046 | −0,135 | −0,193 | −0,062 | −0,165 | −0,114 | −0,052 | 0,064 | −0,088 | 0,095 | −0,014 | −0,108 |
| MIC *Salmonella* | −0,061 | −0,129 | −0,151 | −0,052 | −0,150 | −0,129 | −0,112 | −0,030 | −0,077 | −0,044 | −0,099 | −0,125 |
| MBC *P. aeruginosa* | −0,060 | −0,128 | −0,193 | −0,033 | −0,153 | −0,109 | −0,104 | 0,126 | −0,056 | −0,092 | −0,010 | −0,112 |
| MIC *P. aeruginosa* | −0,039 | −0,113 | −0,164 | −0,090 | −0,150 | −0,158 | −0,078 | 0,107 | −0,035 | −0,134 | −0,109 | −0,109 |
| MBC *E. coli* | −0,051 | −0,104 | −0,208 | −0,096 | −0,160 | −0,167 | −0,117 | 0,125 | −0,061 | −0,116 | −0,044 | −0,115 |
| MIC *E. coli* | −0,021 | −0,035 | −0,131 | −0,110 | −0,090 | −0,185 | −0,078 | 0,085 | −0,050 | −0,141 | −0,118 | −0,054 |

**Table 7**. Pearson correlations for MIC (Minimum Inhibitory concentration) and MBC (Minimum Bactericidal Concentration) of honey and polyphenols content. *Values in bold are different from 0 with a significance level alpha = 0.05.*

| Variables | 12.5% S. aureus | 25% S. aureus | 50% S. aureus | 25% Salmonella | 50% Salmonella | 25% *P.* aeruginosa | 50% *P.* aeruginosa | 25% E. coli | 50% E. coli |
|---|---|---|---|---|---|---|---|---|---|
| MBC *Salmonella* | −0,258 | **−0,411** | **−0,442** | −0,137 | **−0,386** | −0,234 | **−0,279** | −0,240 | **−0,269** |
| MIC *Salmonella* | −0,200 | **−0,342** | **−0,424** | −0,152 | **−0,461** | **−0,270** | **−0,276** | −0,206 | **−0,369** |
| MBC *P. aeruginosa* | −0,168 | **−0,327** | **−0,406** | −0,165 | **−0,425** | −0,257 | **−0,281** | −0,156 | **−0,278** |
| MIC *P. aeruginosa* | −0,215 | **−0,321** | **−0,432** | −0,178 | **−0,516** | **−0,310** | **−0,375** | −0,227 | **−0,407** |
| MBC *E. coli* | −0,175 | **−0,341** | **−0,373** | −0,172 | **−0,354** | −0,256 | −0,256 | −0,164 | −0,234 |
| MIC *E. coli* | −0,125 | −0,219 | **−0,336** | −0,085 | **−0,367** | −0,236 | −0,239 | −0,211 | **−0,299** |

**Table 8**. Pearson correlations for MIC (Minimum Inhibitory concentration) and MBC (Minimum Bactericidal Concentration) of honey and its antimicrobial action against pathogens (concentrations of 12.5%, 25%, and 50% honey). *Values in bold are different from 0 with a significance level alpha = 0.05.*

| Variables | MBC S. aureus | MIC S. aureus | MBC Salmonella | MIC Salmonella | MBC *P.* aeruginosa | MIC *P.* aeruginosa | MBC E. coli | MIC E. coli |
|---|---|---|---|---|---|---|---|---|
| MBC *Salmonella* | **0,862** | **0,636** | **1** | **0,777** | **0,805** | **0,680** | **0,753** | **0,662** |
| MIC *Salmonella* | **0,683** | **0,759** | **0,777** | **1** | **0,707** | **0,687** | **0,599** | **0,727** |
| MBC *P. aeruginosa* | **0,775** | **0,705** | **0,805** | **0,707** | **1** | **0,874** | **0,893** | **0,780** |
| MIC *P. aeruginosa* | **0,642** | **0,715** | **0,680** | **0,687** | **0,874** | **1** | **0,767** | **0,784** |
| MBC *E. coli* | **0,841** | **0,719** | **0,753** | **0,599** | **0,893** | **0,767** | **1** | **0,834** |
| MIC *E. coli* | **0,719** | **0,742** | **0,662** | **0,727** | **0,780** | **0,784** | **0,834** | **1** |

**Table 9**. Pearson correlations between MIC (Minimum Inhibitory concentration) and MBC (Minimum Bactericidal Concentration) of honey for each pathogen. *Values in bold are different from 0 with a significance level alpha = 0.05.*

## Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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## Author contributions

L.L. and D.P. wrote the main manuscript text. L.L., D.P., M.O., and F.U. analyzed the samples and interpreted the results. All authors reviewed the manuscript.

## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

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