Changes in the antibacterial activity of Hungarian varietal honeys as a function of storage

PhD thesis

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Pécs, 2024

1. Introduction

Antibiotic therapy is currently the most effective solution for severe and prolonged respiratory tract bacterial infections. Regarding the resistance of the most important bacteria causing upper respiratory tract infections, it can generally be said that macrolide and trimethoprim/sulfamethoxazole resistance is significant and the number of penicillin-resistant or moderately sensitive bacterial strains is increasing. The unfounded and irresponsible use of antibiotics increased the occurrence of resistant bacterial strains, so even today antibiotic resistance represents one of the biggest patient safety risks (Wei et al., 2023). Knowing these facts makes it even more relevant to learn about the antibacterial effect of natural substances.

Potentially infectious, biofilm-forming bacteria such as *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* or *Haemophilus influenzae* can also be found in the microbiota of the upper respiratory tract of a healthy person. Extracellular polysaccharides, which play an important role in the formation of biofilms, can prevent the antibiotic from reaching the target molecule, thereby providing a high degree of resistance to the bacteria living in the biofilm. The antibiofilm effect of honey has been proven in several clinical studies, so its use as an adjunctive therapy may also be significant in the case of upper respiratory tract infections (Krishnakumar et al., 2020). The higher the H_2O_2 and total polyphenol content of each type of honey, the more significant their antibacterial activity (Bucekova et al., 2019). However, H_2O_2 breaks down into water and oxygen over time, so its quantity decreases thanks to the catalase enzyme. Based on these, it can be assumed that honeys stored for years lose their antibacterial effect due to the decreasing H_2O_2 content. As a result, monitoring the decrease in honey's activity is absolutely necessary.

The justification for the therapeutic use of honeys is their complex composition of active ingredients. In contrast to other natural substances, the antibacterial property of honey is not due to a specific compound or active ingredient, but to their combination, so different strains of bacteria develop resistance to it to a different extent.

The basic question of our research is to what extent the botanical origin and storage time of four Hungarian varieties of honey (black locust, goldenrod, linden, sunflower) influence their antibacterial effect, and against which biofilm-forming bacterial strains is the antibacterial effect of each honey sample the most effective. Our research series reveals the quality and effectiveness of Hungarian honeys suitable for medicinal purposes, and our results provide a suitable basis for the use of these honeys as antibacterial agents.

2. Aims

At the beginning of our research, the following goals were formulated:

- Verification of the botanical origin of the black locust, goldenrod, linden and sunflower honeys included in the study and purchased from Hungarian apiaries, by means of melissopalynological analysis.
- Monitoring the color, pH value and electrical conductivity of honey samples from three consecutive years (2020, 2021, 2022) over the years.
- Determination of the minimum inhibitory concentration (MIC) of honeys with different storage times for respiratory bacteria (*P. aeruginosa*, *S. pneumoniae*, *H. influenzae*, *H. parainfluenzae*) using the microdilution method in accordance with CLSI (Clinical & Laboratory Standards Institute) guidelines.
- Determination of the minimum bactericidal concentration (MBC) of honey samples obtained in different years.
- Study of the antibiofilm formation effect of honeys on 96-cell microtiter plates (crystal violet test) as a function of storage time.
- Exploration of the mechanism of action behind the antibiofilm activity of honey (membrane degradation test) involving Gram-negative *P. aeruginosa* and Gram-positive *S. pneumoniae* bacteria.
- Capturing scanning electron microscopic (SEM) images in order to visualize the effect of inhibiting biofilm formation.

3. Material and methods

3.1. Honey samples

For our tests, we used black locust/acacia (*Robinia pseudo-acacia*), goldenrod (*Solidago gigantea*), linden (*Tilia* spp.) and sunflower (*Helianthus annuus*) honeys purchased from the same Hungarian apiaries in three consecutive years (2020, 2021, 2022). During the selection of the samples, we took into account the results of our previous experiments with regard to the antibacterial effect, and we chose varieties of honey that are easily available for customers. By analyzing the honey of the invasive goldenrod, our goal was to explore the therapeutic potential of this product obtained form the plant. The examination of the physicochemical parameters of honey and the testing of its antibacterial effect took place in 2022, not only in 2022 samples, but also in the case of samples from 2020 and 2021. The honey samples came from the southern

Transdanubian region and were stored at room temperature (20–21 °C) and in the dark until used.

3.2. Melissopalynological analysis

In order to establish the true type of honeys, it is crucial to clarify their exact botanical origin, so we performed a melissopalynological analysis following the method of Von der Ohe et al. (2004). Ten grams of our honey samples were added to centrifuge tubes and mixed with 20 mL of distilled water using a Combi-spin FVL-2400N Vortex (Biocenter Kft.). The subsequent centrifugation lasted for 10 min at a speed of 8753×g with a Neofuge 15R centrifuge (Lab-Ex Ltd.), after which the supernatant was poured off. This step was followed by another centrifugation (5 min, 8753×g) and decantation, then 10 mL of distilled water was measured to the sediment left from the first centrifugation. Then 250 µl of distilled water was added to the sediment remaining in the centrifuge tube, and after vortexing, 20 µl of the thus prepared pollen suspension was pipetted onto a microscope slide which was then placed on a heating plate set to 40 °C (OTS 40, Tiba Kft.). The water was evaporated, and then a small piece of Kaiser's glycerin jelly with fuchsine (Merck Life Science Ltd.) was added to each pollen sample. When the jelly melted, the preparations were covered with a coverslip. Pollen preparations were analyzed with Nikon Eclipse E200 microscope equipped with a Michrome 20MP CMOS digital camera (Auro-Science Consulting Kft.), and photomicrographs were taken at 400 × magnification using 4.3.0.605 version of TCapture software. At least 500 pollen grains were counted per honey sample, indicating how many pollen grains belong to a given plant species, genus or family. The relative frequency of pollen types was given as a percentage of all pollen grains.

3.3. Physicochemical parameters

The color intensity of the varietal honeys used in the tests was determined according to Beretta et al. (2005). The 50% (w/w) honey solutions prepared were placed in an ultrasonic water bath (water temperature: 45-50 °C) for 5 min and then filtered (0.45 µm pore size, Agilent Technologies). Color intensity results calculated as the difference in absorbance values measured at 450 and 720 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Schweiz GmbH) were expressed in milliabsorbance units (mAU).

A DSZ-708 Multiparameter analyzer (Simex Ltd.) was used to examine pH values and electrical conductivity. The pH value of the honey samples was determined according to the Codex Alimentarius and the pH meter was calibrated each time with buffer solutions at values of 4 and 9 (Codex Alimentarius, 2009). For the measurement, 10 g honey was dissolved in 75

ml freshly distilled carbon dioxide-free water, and in the case of electrical conductivity, a 20% honey solution was prepared with distilled water. The electrical conductivity of honey means the conductivity of the honey solution measured at 20 °C, the results were expressed in milli-Siemens per centimeter (mS/cm) (Bogdanov et al., 1997).

3.4. Microbiological tests

3.4.1. Culture bacteria

The antibacterial effect of varietal honeys was investigated in case of both Gram-negative and Gram-positive bacteria. *Haemophilus influenzae* (DSM 4690) and *H. parainfluenzae* (DSM 8978) strains were grown in a supplemented Mueller Hinton broth special. 500 µl Haemophilus supplement B (Diagon Kft.) and 750 µl (1 mg/ml) NAD were added to 3750 µl of Mueller–Hinton II Broth (MHB, Oxoid Ltd.). For *Pseudomonas aeruginosa* (ATCC 27853) and *Streptococcus pneumoniae* (DSM 20566), 100 ml of sterile MHB medium was used. The incubation time of bacterial suspensions was 12 h at 37 °C in a shaker incubator at 60 rpm (C25 Incubator Shaker, New Brunswick Scientific) (Hindler & Jorgensen, 2011).

3.4.2. Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentrations (MIC) were determined by broth microdilution test, which is commonly used in microbiological laboratories in accordance with CLSI guidelines (CLSI Document, 2012) . The procedure was performed on 96-cell microtiter plates. Three dilution series were used, as we expected - based on preliminary experiments - that the antibacterial activity would decrease with increasing storage time. The following dilution series were prepared (using Mueller–Hinton broth): 57.5, 55, 52.5, 50, 47.5, 45, 42.5% (w/w) (2020 honeys), 40, 37.5, 35, 32.5, 30, 27.5, 25% (w/w) (2021 honeys) and 25, 22.5, 20, 17.5, 15, 12.5, 10% (w/w) (2022 honeys). Then 100 µL of the honey solutions and bacterial suspensions (10^5 CFU/mL) were measured into one cell of the microtiter plate, followed by incubation at 37 °C for 24 h. We performed our tests with six repetitions and considered the lowest honey concentration as the MIC, in which case there was no visible bacterial growth in the cells.

After the MIC determination, 10 µL of culture medium was taken from each cell of the microtiter plate, where no visible growth was observed, to test the minimum bactericidal concentration (MBC). In this case, the samples were cultured on BA plates and the incubation at 37 °C lasted for 24 h. Finally, we read the lowest concentration where no bacterial growth was observed on the plates (Tan et al., 2009).

3.4.3. Biofilm inhibition study

The biofilms were formed on 96-cell microtiter plates applying the crystal violet (CV) assay, thereby testing the antibiofilm effect of the honey samples (Peeters et al., 2008). The treatments were performed with honey samples with a concentration of MIC/2 and a bacterial suspension nutrient solution with a cell count of 10^8 CFU/mL was used as a positive control, and a cell-free honey nutrient solution was used as a negative control. As a first step, 200 μL of bacterial suspension was measured into one cell of the microtiter plate and the subsequent incubation at 37 °C lasted for four hours, thereby promoting the adhesion of the bacterial cells. The non-adherent cells were removed with physiological saline and only then were the honey solutions pipetted into the cells. In this case, the incubation was 24 h at 37 °C, and then the non-adherent cells were washed again with physiological saline. In order to fix the cells, 200 μL of methanol was added to each well for 15 min at room temperature (RT). In order to stain the bacterial biofilm, 200 μL of 0.1% crystal violet dye was applied after removing the methanol. After 20 min (RT), the excess dye was removed with water, and the crystal violet bound to the biofilm was dissolved with 200 μL of 33% acetic acid per cell. The absorbance values were determined at 590 nm using a plate reader (BMG Labtech SPECTROstar Nano). Among other things, the crystal violet dye binds to extracellular polysaccharides (EPS) which play an important role in the formation of biofilms, therefore enabling the estimation of the total biomass of the biofilm in the cell of the microtiter plate. The inhibition rate was determined based on the following formula: $(1 - S/C) \times 100\%$ (C and S were defined as the average absorbance of control and sample groups, respectively) (Yanwei et al., 2018).

3.4.4. Membrane degradation assay

During the study of cell material release, each bacterial suspension (10^8 CFU/mL) was prepared in PBS (phosphate buffer saline) and bacterial cells without honey treatment were used as a control. The bacterial cells were treated with honey samples of 20, 40, 60 and 90% (w/w) concentration for one hour. In addition, the time dependence of membrane degradation was also investigated, in this test the bacterial cells were suspended in PBS containing 60% (w/w) honey. The treatments lasted 0, 20, 40, 60 and 90 min. After the treatments, the bacterial cells were centrifuged in both cases (Neofuge 15R, Lab-Ex Ltd.) at 12,000×g for 2 min. The absorbance of the supernatant containing nucleic acid was measured at 260 nm with a Metertech SP-8001 (Abl&e-Jasco Ltd.) spectrophotometer. The results were expressed as a percentage, compared to the control (Bennis et al., 2004).

3.4.5. Scanning electron microscopy (SEM)

In order to visualize the inhibitory effect of honey on biofilm and to illustrate the structural changes, SEM images were taken. Both Gram-positive (*S. pneumoniae*) and Gram-negative (*P. aeruginosa*) bacteria were included in the study, and the samples were treated with linden honey which has an effective antibiofilm effect. During the procedure, biofilms were formed on degreased and sterilized coverslips after the plates were incubated for 4 h at 37 °C in 5 mL of bacterial suspension (10^5 CFU/mL). After the adhesion, washing with physiological saline followed, and then linden honey from three different years was applied at a concentration of MIC/2 (5 mL). Untreated coverslips were used as controls. After 24 h of incubation (37 °C), non-adherent cells were washed again, and this was followed by 2 h (RT) incubation in 2.5% glutaraldehyde to fix the biofilm. The samples were dehydrated in ascending ethanol series (50%, 70%, 80%, 90% and absolute ethanol) for 2 × 15 min (RT). As a next step, the coverslips were placed in a 1:2, 1:1, 2:1 mixture of t-butyl alcohol and absolute ethanol. The samples were then transferred to absolute t-butyl alcohol for 1-1 h (RT) followed by overnight freeze-drying. The examination of biofilms coated with a gold membrane was performed with a JEOL JSM IT500-HR scanning electron microscope (Jeol Ltd.) (Kerekes et al., 2013).

3.4.6. Statistical analysis

Statistical analyses were carried out using Excel® (Microsoft Corp.) and the PAST software package version 3.1152 after normality checking with the Shapiro-Wilk test. Data were expressed as means ± standard deviations (SD) in case of honey color intensity (Table 2) and membrane degradation results (Tables 5, 6). The honey types had been compared with each other based on a given parameter using one-way ANOVA. If the null hypothesis of the ANOVA was rejected, we used Student t-test to establish a difference between the two group pairs (honey types). The *p*-values at 1% ($p \leq 0.01$) or 5% ($p \leq 0.05$) were considered significant.

4. Results and conclusions

4.1. Melissopalynological analysis and physicochemical parameters of honey samples

In order to determine the true type of honey samples, it is crucial to clarify their exact botanical origin, which limits both the honey's market-value and its use in medicine. Based on the pollen analysis and examination of the physicochemical properties of the honeys included in the research, all honey samples can be considered unequivocally as varietal honey (Tables 1 and 2). The pollen content of honey is of great importance, as its quantity determines the nature of the honey. In black locust honeys, *R. pseudoacacia* pollen was the dominant pollen type in

accordance with the honey type declared by the beekeeper, similarly to goldenrod, linden and sunflower honeys which also contained high amounts of *Solidago*, *Tilia* and *Helianthus* pollen, respectively.

The color of honey is one of the most variable parameters, which can range from white, through pale yellow and red, to black. In this case, the determining factor is the plant origin and the storage time. The color of fresh black locust honey was pale, yellowish green, but the stored samples were darker (44.4 ± 1.7 vs. 103.1 ± 3.0 mAU), approaching the color of light amber colored linden honey. We observed a similar color change in the case of the other honey samples. The most obvious contrast between fresh and stored honey colors was experienced in sunflower honeys (116.6 ± 1.5 vs. 422.8 ± 2.0 mAU). The highest difference in color intensity was measured between honey samples stored for two vs. one year, afterwards we did not observe any significant difference in smell and texture in the next 2 years.

The pH of honey mainly depends on dissociated acids, which affects both the development of microorganisms and enzyme activity. The pH value of the varietal honeys varied between 3.22 and 4.32. Regarding the average of 3 years, the highest values were measured in linden honeys (4.26 ± 0.07), the lowest values in black locust honeys (3.26 ± 0.04). Also electrical conductivity was the lowest in black locust honey (0.126 ± 0.01 mS/cm), followed by sunflower (0.222 ± 0.002 mS/cm), linden (0.589 ± 0.02 mS/cm) and goldenrod honey (0.616 ± 7.2 mS/cm). The pH and electrical conductivity did not change with storage time, so similar values were obtained for honeys of the same variety from different years.

Table 1. Relative frequency of pollen types in the studied honeys

Honey Samples		**Pollen type – relative frequency (%)**						
		Robinia	*Solidago*	*Helianthus*	*Tilia*	*Brassica*	Asteraceae	Other
Black locust	2020	**56.1**	2.4	3.8	16.2	13.1	3.6	4.8
	2021	**51.8**	-	8.8	14.5	8.3	9.1	7.5
	2022	**46.6**	3.7	6.5	10.3	20.6	1.9	10.4
Goldenrod	2020	1.7	**63.2**	2.3	0.6	7.6	9.8	14.8
	2021	4.8	**73.4**	3.6	1.9	6.7	8.4	1.2
	2022	0.8	**60.6**	6.3	2.6	10.2	7.8	11.7
Linden	2020	18.8	0.7	16.3	**47.2**	1.7	4.4	10.9
	2021	20.3	1.5	10.2	**53.6**	2.1	5.6	6.7
	2022	22.6	2.2	11.2	**45.5**	3.2	5.9	9.4
Sunflower	2020	13.2	3.4	**66.5**	8.4	-	3.6	4.9
	2021	16.4	2.7	**58.7**	13.8	0.5	1.8	6.1
	2022	5.5	3.2	**69.6**	4.2	-	7.6	9.9

Table 2. Sensory characteristics and physicochemical parameters of honey samples

Honey Type, Plant Name	Year	Sensory Characteristics (Color, Odor and Consistency)	$ABS_{450–720}$ (mAU)	Electrical conductivity (mS/cm)	pH
Black locust, *Robinia pseudoacacia*	2020	Light amber, weak odor, liquid, viscous	103.1 ± 3.0 [a]	0.132 ± 0.01	3.30 ± 0.02
	2021	Light amber, weak odor, liquid, viscous	99.3 ± 3.3 [a]	0.121 ± 0.01	3.22 ± 0.03
	2022	Pale, yellowish green, weak odor, liquid, viscous	44.4 ± 1.7 [b]	0.126 ±0.01	3.27 ± 0.06
Goldenrod, *Solidago gigantea*	2020	Dark amber, moderately intense odor, semisolid, fine granulated	280.7 ± 1.5 [a]	0.624 ± 0.01	3.56 ± 0.03
	2021	Dark amber, moderately intense odor, semisolid, fine granulated	279.4 ± 1.9 [a]	0.609 ± 0.01	3.52 ± 0.04
	2022	Amber, moderately intense odor, semisolid, fine granulated	236.9 ± 3.2 [b]	0.615 ± 0.00	3.59 ± 0.03
Linden, *Tilia* spp.	2020	Amber, strong odor, semisolid fine granulated	221.9 ± 1.7 [a]	0.567 ± 0.03	4.27 ± 0.04
	2021	Amber, strong odor, semisolid fine granulated	211.1 ± 3.4 [b]	0.607 ± 0.02	4.32 ± 0.03
	2022	Light amber, strong odor, semisolid, fine granulated	166.3 ± 4.0 [c]	0.592 ± 0.02	4.19 ± 0.03
Sunflower, *Helianthus annuus*	2020	Dark golden yellow, weak odor, semisolid, coarse granulated	422.8 ± 2.0 [a]	0.221 ± 0.01	3.68 ± 0.04
	2021	Dark golden yellow, weak odor, semisolid, coarse granulated	413.5 ± 4.2 [a]	0.224 ± 0.01	3.61 ± 0.05
	2022	Golden yellow, weak odor, semisolid, coarse granulated	116.6 ± 1.5 [b]	0.223 ± 0.01	3.66 ± 0.04

$ABS_{450–720}$: absorbance of diluted honey samples referring to their color. Data are means ± standard deviations of three independent determinations (n = 3). Different lowercase letters indicate a significant difference among years of the given honey samples according to Student's *t*-test ($p \leq 0.05$).

4.2. MIC and MBC values of honey samples

The MIC and MBC values of the investigated honey samples showed variation in terms of the storage time, the type of honey, and the bacterial strains included in the study (Tables 3 and 4). Based on our results, it can be observed that as the storage time increased, higher concentrations of honey solutions were needed to achieve the appropriate inhibitory effect. In the case of the 2020 honeys, the MIC values ranged between 42.5 and 50%, while for the 2022 samples, between 10 and 17.5%. A difference of a similar magnitude was also experienced for the MBC values.

Linden and sunflower honey exerted the most significant inhibitory effect, while black locust honey was the least active. *Haemophilus* strains were the most sensitive, while *P. aeruginosa* proved to be the most resistant bacterium. In the case of Hungarian varietal honeys, we were the first to carry out a comparative study covering several years, thereby proving the importance of storage time (Nagy-Radványi et al., 2024).

Table 3. MIC values of honey samples (measured in 2022)

MIC values (%)		*H. influenzae*	*H. parainfluenzae*	*P. aeruginosa*	*S. pneumoniae*
Black locust	2020	50	50	50	50
	2021	30	30	35	32.5
	2022	12.5	12.5	17.5	12.5
Goldenrod	2020	47.5	47.5	50	50
	2021	27.5	27.5	35	30
	2022	12.5	12.5	17.5	12.5
Linden	2020	42.5	42.5	47.5	45
	2021	25	25	32.5	27.5
	2022	10	10	12.5	10
Sunflower	2020	42.5	42.5	47.5	45
	2021	25	25	32.5	30
	2022	10	10	12.5	10

Table 4. MBC values of honey samples (measured in 2022)

MBC values (%)		*H. influenzae*	*H. parainfluenzae*	*P. aeruginosa*	*S. pneumoniae*
Black locust	2020	55	55	57.5	57.5
	2021	37.5	37.5	40	37.5
	2022	20	20	25	20
Goldenrod	2020	55	55	57.5	55
	2021	35	35	40	35
	2022	20	20	25	20
Linden	2020	47.5	47.5	52.5	50
	2021	30	30	35	32.5
	2022	15	15	20	15
Sunflower	2020	50	50	55	50
	2021	32.5	30	37.5	35
	2022	15	15	20	15

4.3. The results of biofilm inhibition tests of honey samples

When examining the inhibitory effect of honey on biofilm formation, the individual honeys were used at a concentration of MIC/2. The different monofloral honeys had a distinct effect on the biofilm formation of the four bacterial strains included in the study, and the storage time also significantly influenced this ability of the honey samples. Based on our observations, the antibiofilm effect of longer-stored honeys is lower in comparison to freshly examined samples. Compared to the 2022 varietal honeys, the antibiofilm effect of the 2020 samples was almost halved. In the case of the 2020 honey samples, the average inhibition rate varied between 34.7-53.4%, while for the 2022 honeys we measured values between 67.9-83.2%. The 2021 samples inhibited bacterial biofilm formation by 45.3-66.8%. The antibiofilm effect of linden and

sunflower honey was the highest, with the inhibition rate reaching 80% against *Haemophilus* strains in the 2022 samples. Black locust and goldenrod honeys showed lower activity, but the fresh samples from 2022 also inhibited biofilm formation by an average of 70% in the selected bacterial strains. Considering all years, the honey samples were able to exert the most significant antibiofilm effect against *Haemophilus* strains, while their activity was lower against *P. aeruginosa* and *S. pneumoniae* strains (Nagy-Radványi et al., 2024).

It should be noted that we were the first to describe the biological activity of honey against *H. parainfluenzae* in our previous study, and we were the first to demonstrate the antibacterial and antibiofilm activity of black locust, linden, and sunflower honeys against *Haemophilus* strains (Balázs et al., 2021). In addition, these properties of goldenrod honey were reported for the first time in the case of respiratory pathogens *H. influenzae* and *H. parainfluenzae* (Nagy-Radványi et al., 2024). In our previous publication, we were the first to publish data on the biofilm formation inhibiting effect of black locust, linden and sunflower honey against *S. pneumoniae* bacteria (Balázs et al., 2021), and we were also the first to describe the antibiofilm activity of goldenrod honey on this bacterium (Nagy-Radványi et al., 2024). Our research group was the first to explore the reduction of the antibiofilm effect of four different types of Hungarian honey against both Gram-positive and Gram-negative respiratory bacteria related to their storage time.

4.4. Membrane degrading properties of honey samples

To demonstrate one of the mechanisms of action of honey, we chose linden honey, which showed high activity in previous studies. In this series of experiments, we also worked with samples from three consecutive years and studied the membrane-degrading effect of honey solutions of different concentrations (20, 40, 60, 90%) in the case of Gram-negative (*P. aeruginosa*) and Gram-positive (*S. pneumoniae*) bacteria (Table 5). In the 2020 linden honey treatment, the loss of integrity of the bacterial membrane was observed at concentrations of 60% and above, while when the 2021 sample was used, membrane degradation occurred already at a concentration of 40%. The highest release of DNA was measured when 2022 linden honey was used, here some activity occurred even at the low concentration of 20%. Using the 2022, 60% honey treatment, the amount of DNA released by the *S. pneumoniae* bacterium reached 43.7%.

In order to investigate the kinetics of DNA release, the 60% solution of the samples was measured at different time intervals (20, 40, 60, 90 minutes). This experiment showed how many minutes it took for the bacterial membrane to start breaking down after the treatment with

linden honey from different years (Table 6). The release of DNA from the bacterial cells started at 20 and 60 minutes in case of 2022 and 2020 linden honey, respectively. Based on our results, Gram-negative *P. aeruginosa* proved to be a more resistant bacterium in this case as well (Tables 5 and 6) (Nagy-Radványi et al., 2024).

In our test series, 2022 linden honey successfully degraded the membranes of both test bacteria, thus achieving the release of the bacterial genetic material and ensuring the death of the bacterial cell. Although the activity of the 2020 and 2021 samples was lower, significant destruction can still be achieved by increasing the honey concentration. Honey as an additional treatment can contribute to effective antibiotic therapy and the suppression of resistance.

Table 5. Effect of linden honeys on the release of DNA in Gram-negative (*P. aeruginosa*) and Gram-positive (*S. pneumoniae*) bacteria

Linden Honey		**DNA release from bacterial cells (%)**	
Year	**Concentrations (%)**	***P. aeruginosa***	***S. pneumoniae***
2020	0	0	0
	20	0	0
	40	0	0
	60	7.6 ± 0.9^{a}	9.7 ± 1.3^{b}
	90	45.2 ± 2.5^{a}	49.9 ± 3.0^{b}
2021	0	0	0
	20	0	0
	40	8.7 ± 1.3^{a}	11.3 ± 2.1^{b}
	60	18.8 ± 2.3^{a}	21.6 ± 2.6^{a}
	90	56.2 ± 2.5^{a}	60.1 ± 2.9^{b}
2022	0	0	0
	20	10.9 ± 2.1^{a}	15.2 ± 2.4^{b}
	40	28.7 ± 2.1^{a}	38.6 ± 1.2^{b}
	60	39.2 ± 1.7^{a}	43.7 ± 2.8^{b}
	90	100	100

Data are means ± standard deviations of six independent determinations (n = 6). Different lower case letters in the same row for each year indicate significant differences among the two bacteria according to Student's *t*-test ($p \leq 0.05$).

Table 6. Kinetics of 260 nm absorbing nucleic acid released from Gram-negative (*P. aeruginosa*) and Gram-positive (*S. pneumoniae*) bacteria treated with 60% (w/w) linden honeys

Linden Honey		**DNA release from bacterial cells (%)**	
Year	**Time (min)**	***P. aeruginosa***	***S. pneumoniae***
2020	0	0	0
	20	0	0
	40	0	0
	60	7.6 ± 0.9^{a}	9.7 ± 1.3^{b}
	90	9.8 ± 1.3^{a}	10.5 ± 2.2^{a}
2021	0	0	0
	20	0	0
	40	12.4 ± 2.3^{a}	14.2 ± 2.3^{a}
	60	18.8 ± 2.3^{a}	21.6 ± 2.6^{a}
	90	23.2 ± 1.8^{a}	2.3 ± 3.1^{b}
2022	0	0	0
	20	22.5 ± 2.5^{a}	26.5 ± 1.8^{b}
	40	35.1 ± 2.5^{a}	40.5 ± 2.2^{b}
	60	39.2 ± 1.7^{a}	43.7 ± 2.8^{b}
	90	65.1 ± 3.0^{a}	69.8 ± 2.2^{b}

Data are means ± standard deviations of six independent determinations (n = 6). Different lower case letters in the same row for each year indicate significant differences among the two bacteria according to Student's *t*-test ($p \leq 0.05$).

4.5. SEM results

The inhibitory effect of highly active linden honey on the biofilm formation of Gram-negative (*P. aeruginosa*) and Gram-positive (*S. pneumoniae*) bacteria was illustrated in SEM images. In the case of untreated samples, the three-dimensional structure of bacterial biofilms was formed, while in the case of treated samples, biofilm formation was inhibited to an extent corresponding to the age of the honeys. The 2020 and 2021 linden honey reduced biofilm formation to a lesser extent than the 2022, similar to previous results. In the case of the 2020 and 2021 honey samples, the bacterial cells adhered to the surface and the microcolonies formed, but significantly fewer bacterial cells made up the biofilm. *S. pneumoniae* bacterial cells treated with 2022 linden honey were crushed and their cell material burst out. In addition, it can be observed in the images of the untreated samples that *P. aeruginosa* produces a larger amount of biofilm than *S. pneumoniae* during the same time, thus contributing to the increase of its resistance (Nagy-Radványi et al., 2024). This test clearly illustrates, similarly to the membrane degradation experiments, that the unique properties of each bacterial strain (e.g. the production of alginate spores by *P. aeruginosa*) determine the resistance and not the

classification into the Gram-negative or Gram-positive group. The unique characteristics of varietal honeys also contribute to the fact that honey samples are able to reduce the formation of biofilm with different degrees of effectiveness.

5. Summary

When searching for alternative solutions to reduce antibiotic resistance, it is important to get to know new, even natural, materials more thoroughly, and to reveal the advantages and disadvantages of their use. Honey has a strong antibacterial effect, however, in order for it to be a complementary treatment to an antibiotic therapy, several aspects must be taken into account. Our study highlights the importance of honey's botanical origin, as some types of honey are able to exert a significant antibacterial effect, and it is also important that the treatment is carried out against the bacterial strain most sensitive to the given honey. We found that among the respiratory pathogens causing respiratory infections included in the study (Gram-negative *H. influenzae*, *H. parainfluenzae*, *P. aeruginosa* and Gram-positive *S. pneumoniae*), *P. aeruginosa* was the bacterium most resistant to honey in the *in vitro* microbiological tests. Among our honey samples, linden and sunflower honey had the most significant antibacterial activity, mainly against the two *Haemophilus* strains, similarly to the other types of honey included in the study, where these bacteria also proved to be the most sensitive. Based on our results, the inhibitory effect of black locust honey and goldenrod honey from *S. gigantea*, which is considered an invasive flood plant, is also not negligible. Our research also answered the question of how much the age of honey can influence the antibacterial effect, and to what extent the antibiofilm activity decreases depending on the storage time. In addition to the freshly spun honeys, we explored the biofilm inhibiting effect of honey samples stored for one and two years, as well as one of the mechanisms behind this, membrane degradation and its kinetics. The results obtained during these tests were illustrated with SEM images. Based on our research, honeys suitable for medicinal purposes (as complementary therapy) should be used as fresh as possible during the various treatments, since in this case they exert the most significant antibacterial activity. In acute respiratory infections of viral origin, reducing the use of antibiotics is essential, as it is unjustified and harmful. After the acute phase, there is a particularly high risk of bacterial superinfection, in which case the consumption of honey can be a first-rate, excellent means of preventing superinfection, thus promoting the unnecessary use of antibiotics.

Our new scientific findings are summarized below:

1. In the case of Hungarian varietal honeys, we were the first to implement a series of comparative experiments spanning several years, thereby proving the importance of storage time in terms of honey's antibacterial activity. The inhibitory effect of the honeys was almost halved after two years.
2. We found that the physicochemical parameters of the honey (pH and electrical conductivity) did not change depending on the storage time, but the color of the samples darkened. The process of color change mostly took place in the first year.
3. We were the first to reveal the antibacterial and antibiofilm activity of honey derived from goldenrod, which causes serious nature conservation damage, against the Gram-negative respiratory pathogens *H. influenzae* and *H. parainfluenzae* (the inhibition rate for a fresh sample of 2022: 78.1 and 78.3%).
4. We successfully demonstrated the biofilm inhibiting effect of goldenrod honey (inhibition rate: 74.2% for the 2022 sample) in the case of the Gram-positive *S. pneumoniae* bacterium.
5. We were the first to describe how much honey concentration (min. 40% after one year, min. 60% after two years) is needed to degrade the bacterial membrane depending on the storage time in the case of linden honey.

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7. Acknowledgment

I would like to express my gratitude to all those who provided the conditions for my work and contributed to the realization of the goals of the research with their cooperation.

Special thanks to my supervisors, Dr. Ágnes Farkas and Dr. Marianna Kocsis, who supported, encouraged and provided me with a lot of useful professional advice over the years. They always turned to me with patience and understanding, and stood by me in case of any questions or problems.

I owe a debt of gratitude to Dr. Viktória Lilla Balázs, who helped me throughout my work, I could always count on her for the planning and implementation of microbiological tests. With her encouraging attitude as a friend, she always helped me overcome difficulties and contributed to the success of my work.

I thank Dr. Béla Kocsis for his help and professional guidance in the preparation of the microbiological tests.

I owe a debt of gratitude to Dr. Péter Szabó, who I relied on for the preparation of the SEM images.

I would also like to thank all the employees of the Pharmacognosy Institute of the University of Pécs for their far-reaching support.

I am grateful to my parents, who help me achieve my goals, and to my husband and daughter for their patience and perseverance.

8. List of publications

8.1. Articles related to the thesis

Nagy-Radványi L., Balázs V.L., Kocsis B., Csikós E., Ángyán V.D., Szabó P., Biró V., Kocsis M., Farkas Á. (2024): Antibacterial activity of Hungarian varietal honeys against respiratory pathogens as a function of storage time. ***Scientific Reports***. 14:10200. **[IF: 4,996; Q1; D1]**

Balázs V.L., **Nagy-Radványi L.**, Bencsik-Kerekes E., Koloh R., Szabó D., Kocsis B., Kocsis M., Farkas Á. (2023): Antibacterial and Antibiofilm Effect of Unifloral Honeys against Bacteria Isolated from Chronic Wound Infections. ***Microorganisms***. 11:509-516. **[IF: 4,5; Q2]**

Balázs V.L.*, **Nagy-Radványi L.***, Filep R., Kerekes E., Kocsis B., Kocsis M., Farkas Á. (2021): *In vitro* antibacterial and antibiofilm activity of Hungarian honeys against respiratory tract bacteria. ***Foods***. 10:16-32. **[IF: 5,561; Q1]**

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8.2. Other publications

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8.3. Abstracts

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