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*preliminary communication*

# Antibacterial Activity of Honey and Beebread of Different Origin Against *S. aureus* and *S. epidermidis*

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**Summary**

The study is aimed at the evaluation of antimicrobial properties of honey and beebread products of different origin. The inhibitory action of 34 honey and 4 beebread samples was tested against *Staphylococcus aureus* and *Staphylococcus epidermidis* by the agar well diffusion method. Total antibacterial activity was evaluated by measuring the clear zone around the well, and expressed in phenol concentration possessing equivalent activity. Honey samples were tested after dilution to 50, 25 and 10 % (by mass per volume). The solutions containing 10 % (by mass per volume) of honey did not have any effect on the growth of bacteria; some honey samples had no inhibitory activity on any of the concentrations used. The contribution of catalase and neutralization to the antimicrobial activity of honey was also assessed. It was found that the antibacterial activity of the tested honey samples was dependent on hydrogen peroxide formation, while such dependence was not observed for the beebread samples. Floral source of honey and bacterial culture were other two factors related to the antibacterial activity. However, the possible contribution of phytochemicals, which may be transferred to honey, should be assessed by using other methods.

*Key words:* honey, beebread, antibacterial activity, *Staphylococcus aureus*, *Staphylococcus epidermidis*

## Introduction

Honey is an important and unique food product containing bioactive compounds derived from bees and plants. Numerous studies demonstrate that honey possesses antimicrobial activity (*1–7*); it destroys and/or inhibits the growth of some pathogenic vegetative microorganisms (*8*). The unique composition of honey contributes to its antimicrobial properties; however, its antibacterial effect is not completely understood. Some researchers believe that the main antimicrobial activity comes from bee-origin, the others attribute antimicrobial honey properties to the components derived from flora (*9*). For instance, Bogdanov (*9*) determined that antibacterial substances of honeydew honey samples are of bee-origin. Allen *et al.* (*1*) and Molan (*2,3*) found that antimicrobial activity of nectar honey and the mechanism of its action are mostly dependent on the floral source from which nectar honey has been collected. Mundo *et al.* (*10*) reported that honey inhibited bacteria due to a high sugar concentration (lowering water activity), hydrogen peroxide, and proteinaceous compounds present in honey.

The enzymatic production of hydrogen peroxide is considered to be a major factor of antimicrobial honey effects (*1–3,6,11*). The amount of this bactericidal compound in honey depends on the amount of catalase, with different types of plants contributing to different concentrations of catalase (*12*). Allen *et al.* (*1*) reported

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that the sensitivity of glucose oxidase to denaturation by light could be influenced by unidentified substances present in some floral sources (*1*). Therefore, the properties of honey of multifloral origin, collected from different types of flowers, differ from those of monofloral honey samples.

It is thought that light, temperature, oxygen, processing, and storage may also affect the antibacterial activity of honey. Allen *et al.* (*1*) surveyed 345 samples of New Zealand honey and found that the age does not affect their antibacterial activity. Honey with high hydrogen peroxide activity is sensitive to heat and light (*2,3,9*) because the main enzyme, which generates hydrogen peroxide, is inactivated.

Other factors, such as high osmotic pressure/low water activity ($a_w$), low pH/acidic environment, low protein content, high carbon to nitrogen ratio, low redox potential due to the high content of reducing sugars, viscosity/anaerobic environment and other chemical agents/phytochemicals are also likely to play some role in defining antibacterial activity of honey (*11*).

The composition of active components in plants depends on various factors, particularly plant cultivar and chemotype, and climatic conditions. Consequently, it can be reasonably expected that honey properties from different locations should be different. Honey production in Lithuania has a very long tradition tracing to ancient times; however, its bioactive properties have not been studied more comprehensively. The major purpose of this work is to evaluate the antimicrobial activity of various honey samples from Lithuania and some other honey products. Such data would assist in more focused application of honey as a possible natural remedy and/or functional ingredient for the control of the most trivial human pathogens in the food processing.

## Materials and Methods

### *Melissopalynological analysis*

The floral source of honey samples was determined by the melissopalynological method (*13,14*). Determination of botanical origin of honey is based on the relative frequency of the pollen from nectar secretion plants. Different opinions exist regarding the use of pollen present in honey for the indication of its botanical origin (*15*), however until the present date, this method has been frequently used for this purpose. Pollen was identified by using previously published data (*16,17*) and pollen collection of well-known plants, which was prepared for microscoping at the Apicultural Department of the Lithuanian Institute of Agriculture.

The prepared slides were examined using a microscope with magnification of 400 for the identification of pollen in honey and counting honeydew elements. At least 500 pollen grains (PG) and honeydew elements (HDE) were counted in 100 fields. All plant elements were observed separately.

After the identification of PG and HDE in honey samples, the pollen of nectarless plants and the HDE were deducted from the total sum. The percentage of pollen from nectar plants in botanical composition of honey was calculated. HDE were calculated as percentage from total sum of PG and HDE. Unifloral spring honey from willow (K02, K03, K04, K05), polyfloral spring honey (K01, K06, K24), summer honey from spring rape (K07–K23) and polyfloral summer honey K25 were selected for analysis. Unifloral honey met the botanical and chemical composition requirements established by the International Commission for Bee Botany, presently called International Commission for Plant-Bee Relationships (*13*) and recommended methods and standards (*18–20*). It should be noted that the main honey plant in Lithuania is spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) and the pollen in honey from this plant is over-represented.

Botanical origin of samples is listed in Table 1.

### *Honey samples and their preparation*

Honey samples were obtained from apiarists throughout Lithuania. All samples were collected during the 2003 flowering season. Some honey samples were made when bees were fed with pine (*Pinus silvestris*), birch (*Betula pendula*) and stinging nettle (*Urtica dioica*) extract additives (the detailed botanical composition was not tested). The samples of beebread were also tested. Beebread is the mixture of pollen with honey to stick it together. The commercial samples of beebread were used during the analysis; detailed composition of beebread samples was unknown. The only commercially available sample of thermally processed beebread was also tested. The sources and detailed characterization of honey samples are listed in Table 1.

All samples were prepared aseptically and were handled protected from direct sunlight. Honey solutions were prepared in three fractions: 50, 25 and 10 % (by mass per volume). The samples of each honey (10 g) and sterile water were stored at 37 °C for 30 min before mixing, to facilitate homogenization. The 50 % (by mass per volume) solutions thus prepared were diluted to 25 and 10 %. The samples were assayed immediately after dilution.

Beebread is not homogenous mixture of honey and pollen, because pollen is not soluble in honey and water. Therefore, the suspensions of 50, 25 and 10 % (by mass per volume), which were produced by diluting the beebread with distilled water, were tested for the antibacterial activity.

Seventy five percent of all honey samples were neutralized with 0.1 M NaOH to pH=(7.0±0.1) (measured with potentiometer) and were diluted to 50 % (if it was necessary).

### *Chemicals*

The concentration of 2 mg/mL of catalase (2000 units/mg, Sigma-Aldrich, Steinheim, Switzerland) from bovine liver in pure distilled water was freshly prepared every day. Honey samples were diluted to 50 and 25 % with this catalase solution and assayed in the same way as other honey samples. The final concentration of catalase corresponded to 0.1 and 0.15 %, respectively.

Reference solutions of phenol (99 %, Novomoskovskij zavod, Moscow, Russia) of 8, 10, 12, 14, 16, 18 and 20 % (by mass per volume) were prepared in purified water. The antibacterial activity of each solution was then tested five times by adding 100 μL of phenol solution to the well.

Table 1. Characterization of the tested honey samples and beebread

| Sample code | Collection dates | Botanical composition/% | Municipality | Location |
|---|---|---|---|---|
| K01 | 03. 06. 2003 | fruit tree 35.6, willow (*Salix alba* L., *Salix caprea* L.) 35.5, spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 18.8, dandelion (*Taraxacum officinale* L.) 6.7, chestnut (*Aesculus hippocastanum* L.) 3.4 | Kedainiai | Akademija |
| K02 | 09. 06. 2003 | willow (*Salix alba* L., *Salix caprea* L.) 76.1, fruit tree 10.3, mustard (*Sinapis arvensis* L.) 7.1, dandelion (*Taraxacum officinale* L.) 3.7, alder (*Frangula* L.) 2.8 | Kedainiai | Krakes, Janiunai |
| K03 | 09. 06. 2003 | willow (*Salix alba* L., *Salix caprea* L.) 68.9, fruit tree 24.6, dandelion (*Taraxacum officinale* L.) 6.5 | Kedainiai | Uzupe, Lazai |
| K04 | 08. 06. 2003 | willow (*Salix alba* L., *Salix caprea* L.) 67.4, fruit tree 21.7, dandelion (*Taraxacum officinale* L.) 10.9 | Kedainiai | Voluciai |
| K05 | 14. 06. 2003 | willow (*Salix alba* L., *Salix caprea* L.) 55.3, fruit tree 28.3, wild mustard (*Sinapis alba*) 8.0, dandelion (*Taraxacum officinale*) 8.4 | Kedainiai | Medininkai and Spitole |
| K06 | 13. 06. 2003 | fruit tree 33.3, willow (*Salix alba* L., *Salix caprea* L.) 32.5, dandelion (*Taraxacum officinale* L.) 24.4, spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 4.9, hawthorn (*Crataegus* L.) 4.9 | Kedainiai | Degesiai |
| K07 | 30. 06. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 96.8, other plants* 3.2, honeydew 3.1 | Kedainiai | Voluciai |
| K08 | 07. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 96.6, other plants* 3.4, honeydew 3.3 | Kedainiai | Gudziunai |
| K09 | 09. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 92.0, fruit tree 3.5, willow (*Salix alba* L., *Salix caprea* L.) 2.5, dandelion (*Taraxacum officinale*) 2.0 | Kedainiai | Degesiai |
| K10 | 22. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 48.5, willow (*Salix alba* L., *Salix caprea* L.) 30.0, cornflower (*Centaurea cyanus* L.) 7.2, red clover (*Trifolium pratense* L.) 7.0, wild mustard (*Sinapis alba* L.) 5.3, bird's foot trefoil (*Lotus corniculatus* L.) 2.0 | Kedainiai | Paberze |
| K11 | 24. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 45.9, red clover (*Trifolium pratense* L.) 24.7, fruit tree 8.8, white clover (*Trifolium repens* L.) 6.5, willow (*Salix alba* L., *Salix caprea* L.) 5.3, buckwheat (*Fagopyrum esculentum* Moench) 4.7, cornflower (*Centaurea cyanus* L.) 4.1, honeydew 27.0 | Kedainiai | Daumantai |
| K12 | 28. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 62.5, wild mustard (*Sinapis alba* L.) 17.5, cornflower (*Centaurea cyanus* L.) 7.6, willow (*Salix alba* L., *Salix caprea* L.) 6.8, dandelion (*Taraxacum officinale* L.) 3.6, fruit tree 2.0 | Kedainiai | Medininkai |
| K13 | 29. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 77.5, thistle (*Cirsium* L.) 8.5, willow (*Salix alba* L., *Salix caprea* L.) 5.4, dandelion (*Taraxacum officinale* L.) 2.7, linden (*Tilia cordata* L.) 3.2, fruit tree 2.7 | Kedainiai | Spitole |
| K14 | 30. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 63.7, willow (*Salix alba* L., *Salix caprea* L.) 8.8, cornflower (*Centaurea cyanus* L.) 7.6, wild mustard (*Sinapis alba* L.) 7.2, caraway (*Carum carvi*. L.) 5.0, fruit tree 4.8, linden (*Tilia* L.) 2.9 | Kedainiai | Uzupe |
| K15 | Jul. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 84.7, wild mustard (*Sinapis alba* L.) 10.5, cornflower (*Centaurea cyanus* L.) 3.5, other plants* 1.3, honeydew 2.7 | Marijampole | unknown |
| K16 | 25. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 89.2, linden (*Tilia cordata* L.) 7.7, beans (*Vicia faba* L.) 1.7, other plants* 1.4 | Kedainiai | Slapaberze |
| K17 | 05. 08. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 88.0, wild mustard (*Sinapis alba* L.) 6.9, cornflower (*Centaurea cyanus* L.) 2.2, other plants* 2.9 | Kedainiai | Degesiai |
| K18 | 06. 08. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 54.8, wild mustard (*Sinapis alba* L.) 24.2, cornflower (*Centaurea cyanus* L.) 6.7, willow (*Salix alba* L., *Salix caprea* L.) 4.0, raspberry (*Rubus idaeus* L.) 4.0, fruit tree 3.2, other plants* 3.1 | Kedainiai | Krakes, Janiunai |
| K19 | 07. 08. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 71.5, wild mustard (*Sinapis alba* L.) 14.7, willow (*Salix alba* L., *Salix caprea* L.) 4.1, fruit tree 4.1, cornflower (*Centaurea cyanus* L.) 2.6, other plants* 0.4 | Kedainiai | Lazai |
| K20 | 22. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 78.5, willow (*Salix alba* L., *Salix caprea* L.) 6.2, fruit tree 6.2, red clover (*Trifolium pratense* L.) 3.8, cornflower (*Centaurea cyanus* L.) 2.4, wild mustard (*Sinapis alba* L.) 2.9 | Kedainiai | Terespolis |
| K21 | 08. 08. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 74.8, fruit tree 6.9, wild mustard (*Sinapis alba* L.) 5.7, raspberry (*Rubus idaeus L.*) 4.0, caraway (*Carum carvi* L.) 3.3, willow (*Salix alba* L., *Salix caprea* L.) 3.0, other plants* 2.3 | Kedainiai | Siaudyne |

Table 1. – continued

| Sample code | Collection dates | Botanical composition/% | Municipality | Location |
|---|---|---|---|---|
| K22 | 12. 08. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 73.4, cornflower (*Centaurea cyanus* L.) 7.3, red clover (*Trifolium pratense* L.) 6.5, willow (*Salix alba* L., *Salix caprea* L.) 5.6, wild mustard (*Sinapis alba* L.) 6.2, fruit tree 1.0 | Kedainiai | Gudziunai |
| K23 | 20. 08. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 71.4, wild mustard (*Sinapis alba* L.) 13.9, willow (*Salix alba* L., *Salix caprea* L.) 9.5, dandelion (*Taraxacum officinale* L.) 5.2 | Kedainiai | Medininkai |
| K24 | 26. 08. 2003 | willow (*Salix alba* L., *Salix caprea* L.) 38.5, fruit tree 13.6, raspberry (*Rubus idaeus* L.) 12.4, dandelion (*Taraxacum officinale* L.) 11.2, chestnut (*Aesculus hippocastanum* L.) 10.7, spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 7.7, honeydew 6.1, thistle (*Cirsium arvense* Scop.) 5.9 | Neringa | Pervalka |
| K25 | 24. 07. 2003 | spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 42.5, white clover (*Trifolium repens* L.) 18.1, bird's foot trefoil (*Lotus corniculatus* L.) 16.6, honeydew 9.4, linden (*Tilia cordata* L.) 9.8, raspberry (*Rubus idaeus L.*) 8.0, willow (*Salix alba* L., *Salix caprea* L.) 3.0, beans (*Vicia faba* L.) 2.0 | Taurage | Pasesuvys |
| E26 | Jul. 2003 | honey with pine (*Pinus silvestris*) extract: thistle (*Cirsium arvense* Scop.) 37.8, spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 40.6, honeydew 42.2, raspberry (*Rubus idaeus L.*) 21.6 | unknown | unknown |
| E27 | Jul. 2003 | honey with birch (*Betula pendula*) extract: thistle (*Cirsium arvense* Scop.) 29.4, spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 41.2, honeydew 34.6, raspberry (*Rubus idaeus L.*) 20.6, wild mustard (*Sinapis alba* L.) 8.8 | unknown | unknown |
| E28 | Jul. 2003 | honey with stinging nettle (*Urtica dioica*) extract: thistle (*Cirsium arvense* Scop.) 32.0, spring rape (*Brassica napus* L. ssp. *oleifera annua* Metzg.) 24.0, honeydew 32.0, raspberry (*Rubus idaeus* L.) 20.0, white clover (*Trifolium repens* L.) 12.0, wild mustard (*Sinapis alba* L.) 12.0 | unknown | unknown |
| C29 | Aug. 2003 | unknown (honey from local apiarist) | Vilkaviskis | Svitrunai |
| C30 | Jul. 2003 | unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania) | unknown | commercial sample |
| C31 | Aug. 2003 | unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania) | unknown | commercial sample |
| C32 | Jul. 2003 | unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania) | unknown | commercial sample |
| C33 | May 2003 | unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania) | unknown | commercial sample |
| C34 | Jul. 2003 | unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania) | unknown | commercial sample |
| C35 | 2003 | unknown (beebread after thermal processing, EKO agros, R. Maciene, Lithuania) | Pakruojis | commercial sample |
| C36 | 17. 09. 2003 | unknown (beebread with honey and honeycomb, EKO agros, R. Maciene, Rockaiciai, Lithuania) | Pakruojis | commercial sample |
| C37 | Sept. 2003 | unknown (beebread with honey and honeycomb, EKO agros, R. Maciene, Rockaiciai, Lithuania) | Pakruojis | commercial sample |
| C38 | 17. 09. 2003 | unknown (beebread with honey and honeycomb, EKO agros, R. Maciene, Isdagieciai, Lithuania) | Pakruojis | commercial sample |

*percentage of pollen in the honey was less than 1.0 %

### *Assessment of antibacterial activity*

Antibacterial activity of honey samples and reference solutions was tested by an agar diffusion test (*1*). Two undesirable species in food products, namely *S. aureus* and *S. epidermidis*, were used as test cultures. Bacteria were grown in nutrient broth (Merck KGaA, Darmstadt, Germany) at 37 °C for 24 h. After cultivation, 100 μL of the culture were poured into 150 mL of sterilized nutrient agar (Oxoid, CM3, Merck KGaA, Darmstadt, Germany) cooled to 50 °C. The agar was mixed and poured in four 90-mm diameter Petri dishes immediately after mixing and stored at 4–6 °C overnight before being used the next day.

Five wells of 6 mm in diameter were punched in the solid agar with the mouthpiece of a sterile Pasteur pipette (five in each plate). The bottom of each well was covered with a drop of melted nutrient agar in order to prevent infiltration of the sample between the culture medium and the bottom of the plate, which would interfere with the test. The wells were numbered at random.

Each sample was tested in quadruplicate by adding 100 μL of the test solution to four wells. Total activity, non-peroxide activity and the activity of neutralized honey were evaluated. The blank sample containing sterile distilled water was tested in the same way as the honey samples.

The plates were incubated overnight at 37 °C and afterwards were placed over the black template to measure the clear zone of inhibition diameter (mm). The diameter was measured along the horizontal and vertical lines on the template. The mean radius of the clear zone around each honey sample was calculated (diameter of the well was 6 mm) and measured in mm.

The mean radius of the clear zone around each well of phenol reference solution was measured simultaneously and a graph of phenol concentration against the mean radius of the clear zone was plotted. After linear approximation of the obtained curve, the equation was deduced; this was used to determine the activity of each honey sample from the mean radius around the clear zone. The activity was expressed as the equivalent phenol fraction (in %). Calibration curves were prepared for each culture.

It was found that the relationship between antimicrobial agent concentration and clear zone radius is linear over the full range tested. Most likely, the calibration curves gave straight lines because small wells in the agar were used; thus the antibacterial substances in them became easily depleted as diffusion into the surrounding agar occurred. Calibration curves and the equation obtained with phenol solutions are provided in Fig. 1.

### *Statistical analysis*

All values are expressed as the mean±standard deviation. Standard deviations were calculated using spreadsheet software (Excel®). Correlation coefficients to determine the relationship between antimicrobial activity and the amount of one plant were calculated using MS Excel® software (CORREL statistical function).

## Results

In the first series of experiments honey samples were tested after their dilution to 50, 25 and 10 % (by mass per volume). The results revealed remarkable variations in antibacterial activity of the tested honey samples. However, some of the 38 tested honey samples at the fractions applied did not have any antimicrobial activity against the tested microorganisms (Table 2); for instance, 15 samples did not inhibit *S. aureus* and 11 samples had no effect against *S. epidermidis*. Five honey samples lost their inhibitory activity against both tested bacteria after dilution to 25 %.

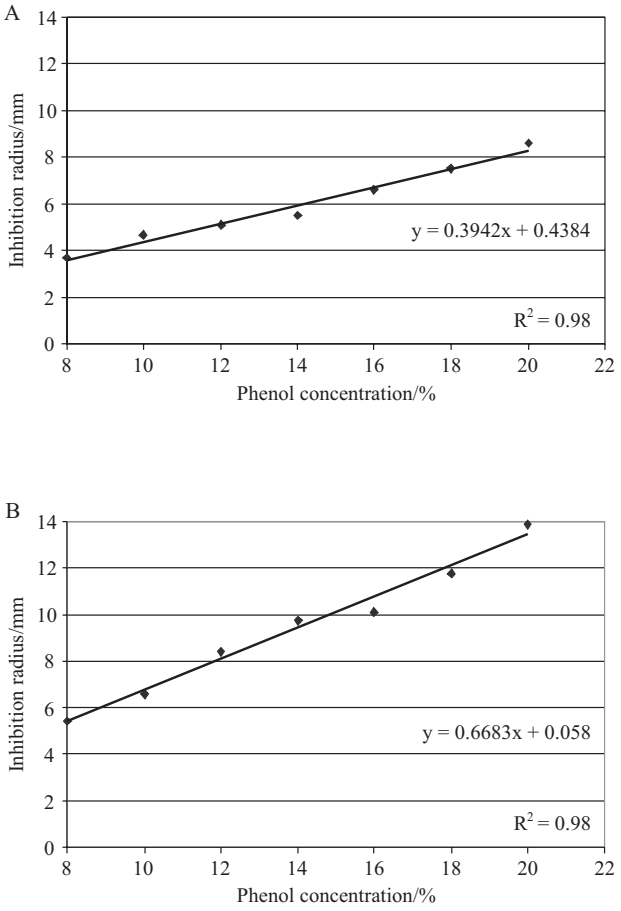


**Fig. 1.** Calibration curves for the phenol reference solutions used in the agar well diffusion assay of antibacterial activity (the value of each point is the mean of five determinations); A – inhibition of *S. aureus*, B – inhibition of *S. epidermidis*

The results of the assay of the total antimicrobial activity of untreated honey and beebread are summarized in Table 3. No values are shown for 10 % (by mass per

Table 2. Number of samples that inhibited the growth of the tested bacteria at 50 and 25 % (by mass per volume) solutions

| Plant source | Total no. of samples | Total antibacterial activity/% | | | | Non-peroxide activity | After neutralization/% | |
|---|---|---|---|---|---|---|---|---|
| | | *S. aureus* | | *S. epidermidis* | | Both tested bacteria | *S. aureus* | *S. epidermidis* |
| | | 50 | 25 | 50 | 25 | | 50 | 50 |
| spring rape (more than 45 %) | 17 | 14 | 11 | 16 | 13 | 0 | 16 | 17 |
| willow (more than 45 %) | 4 | 2 | 1 | 4 | 3 | 0 | 4 | 4 |
| multifloral | 10 | 3 | 2 | 3 | 2 | 0 | 6 | 4 |
| honey with birch extract | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| honey with pine extract | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
| honey with stinging nettle extract | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| beebread | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |

Table 3. Antibacterial activity of 50 and 25 % natural and 50 % neutralized honey solutions (only the samples of each type with the highest activity, as well as medium activity of each group are shown)

| Plant source | Maximum total activity/% phenol | | | | Maximum activity after neutralization/% phenol | |
|---|---|---|---|---|---|---|
| | *S. aureus* | | *S. epidermidis* | | *S. aureus* | *S. epidermidis* |
| | 50 | 25 | 50 | 25 | 50 | 50 |
| spring rape (more than 45 %) | 6.97±0.80 | 4.28±0.26 | 4.12±0.58 | 3.14±0.71 | 11.18±0.95 | 7.16±0.56 |
| willow (more than 45 %) | 3.96±0.45 | 2.14±0.48 | 3.28±0.79 | 1.88±0.58 | 9.83±1.20 | 6.37±0.83 |
| multifloral | 6.97±0.66 | 3.80±0.80 | 5.62±0.62 | 3.70±0.58 | 12.13±0.70 | 6.93±0.32 |
| honey with birch extract | – | – | – | – | – | – |
| honey with pine extract | 1.11±0.86 | – | 2.67±0.28 | – | 2.30±0.48 | 3.09±0.15 |
| honey with nettle extract | – | – | – | – | 4.76±0.83 | 2.81±0.39 |
| beebread | 6.02±0.61 | 4.44±0.61 | 4.31±0.62 | 3.42±0.41 | 8.64±0.48 | 4.50±0.76 |
| | Medium total activity/% phenol | | | | Medium activity after neutralization/% phenol | |
| spring rape (more than 45 %) | 4.76 ±0.62 | 3.08±0.58 | 2.83±0.49 | 1.89±0.51 | 5.90±0.68 | 4.05±0.64 |
| willow (more than 45 %) | 3.68±0.42 | 2.14±0.48 | 2.89±0.60 | 1.74±0.52 | 6.44±1.35 | 3.96±0.77 |
| multifloral | 4.12±0.64 | 2.49±0.75 | 3.70±0.59 | 2.62±0.55 | 7.44±0.71 | 4.59±0.36 |
| honey with birch extract | – | – | – | – | – | – |
| honey with pine extract | 1.11±0.86 | – | 2.67±0.28 | – | 2.30±0.48 | 3.09±0.15 |
| honey with nettle extract | – | – | – | – | 4.76±0.83 | 2.81±0.39 |
| beebread | 5.57±0.66 | 3.57±0.67 | 3.98±0.52 | 2.51±0.65 | 7.55±0.61 | 3.79±0.63 |

The values represent average±standard deviation of four replicates

volume) solutions since both tested bacteria were resistant to all honey and beebread samples at this fraction. The inhibition effect considerably decreased when honey samples were diluted from 50 to 25 % (by mass per volume). The inhibition activity of 50 % honey and beebread solutions was higher than that of 25 % solutions. The antimicrobial activity of 50 and 25 % honey solutions was equivalent to the inhibitory effect of phenol solutions of 1.73–6.97 and 0.59–4.44 % fractions, respectively. The solutions of beebread were active in both tested cultures at 50 and 25 % fractions. In general, the samples of beebread showed stronger inhibition of the growth of bacteria than honey solutions (Table 3).

Non-peroxide antimicrobial activity was evaluated by the addition of catalase to the honey and beebread solutions. Phenol equivalent coefficients for beebread samples after the addition of catalase varied from (2.20±0.65) to (5.78±0.70) % (by mass per volume, $N$=4) and were only slightly lower compared with the same coefficients of the untreated beebread samples (Table 3).

The antibacterial activity of neutralized honey was equivalent up to (12.13±0.70) % (by mass per volume, $N$=4) phenol solution (C29, undefined honey), while the highest activity of untreated honey (K21, rape honey) was equivalent to the phenol solution of (6.97±0.80) % (by mass per volume, $N$=4).

The antimicrobial effect of honey samples against *S. aureus* and *S. epidermidis* was different, indicating that the sensitivity of these bacteria to the antimicrobial activity of honey is different. The correlation coefficients between the amount of rape and willows in honey and their antibacterial activity against *S. aureus* were 0.08 and 0.14, respectively. This indicates that there was no correlation between these two parameters. The correlation coefficients between equivalent concentration of phenol solution of antibacterial activity against *S. epidermidis* and the amount of rape and willow in honey were –0.08 and –0.435, respectively.

## Discussion

### *Total antimicrobial activity of untreated honey and beebread*

Literature sources indicate that antibacterial activity of honey considerably depends on the floral source (*1*); consequently the honeys could be distinguished by their predominant plant composition. It is interesting to note that botanical composition of some honeys (*e.g.* K02 and K03, K07 and K08) was quite similar (Table 1), however their antimicrobial activity was different. For instance, the sample K02 did not inhibit the growth of *S. aureus*, while the sample K08 was not active against both cultures. It is worth noting that the colour of these honey samples was different; K02 and K07 honey samples were darker than K03 and K08. This suggests that botanical source of honey might be less important for their antibacterial activity; consequently, something else, most likely bee-origin metabolism products, defines antimicrobial properties of honey. Possible deviations in the identification of the floral source of these honey samples should also be considered (*15*). It was well established that the hydrogen peroxide activity in honey correlates with floral sources (*3*).

Only three of the ten tested multifloral honey samples (K24, K25 and C29) inhibited the growth of *S. aureus* and *S. epidermidis*. It is interesting that commercially

available honey samples did not exhibit antibacterial properties. Most likely, processing and/or storage conditions had negative influence on the antimicrobial properties of these honey samples. It is known that honey for sale can be heated from 45 to 80 °C. Therefore, the loss of antibacterial activity in heat processed commercial honey samples could be accounted for denaturation of glucose oxidase.

### *Non-peroxide antimicrobial activity*

Non-peroxide activity can be detected by adding catalase to honey solution. Catalase destroys hydrogen peroxide, which is slowly formed in honey as a result of the glucose oxidase action. All tested samples, except beebread, lost their antibacterial properties after the addition of catalase (compared with total antibacterial activity), indicating that antibacterial activity of the tested honey samples was due to the hydrogen peroxide formation, while non-peroxide activity can be attributed to the beebread samples. According to the previously published data, in some honey samples from New Zealand, other than peroxide-based mechanisms were prevalent in honey antibacterial properties (*1,6*).

### *Effect of pH on antibacterial activity of honey and beebread*

The effect of pH on the antimicrobial activity of honey and beebread samples was assessed by neutralizing the products with 0.1 M NaOH. The pH of the honey solutions under study (50 %, by mass per volume) was from 3.9 to 4.8; some literature sources indicate that the average pH value for honey is 3.9 (*8,12*). It should be noted that the pH of the samples increased slightly upon dilution. Dilution may have a complex effect on honey, since it may increase glucose oxidase activity, leading to the formation of $H_2O_2$ and gluconic acid as well as diluting the other organic acids present in honey. Neutralization of honey and beebread samples was aimed at establishing possible effect of low pH of Lithuanian honey samples on their antibacterial properties. For instance, it was reported that the pH of *S. aureus* growth varies from 4.0 to 9.8 (*21*); consequently pH of the neutralized honey should be favourable for bacterial growth. It is probable that neutralization of honey will affect the activity of glucose oxidase, since the optimal pH for this enzyme is in the range of 5.0–8.0. Therefore, neutralization of honey to pH=7.0 should promote the activity of glucose oxidase, resulting in the increase of hydrogen peroxide production. Most likely, it is the main factor explaining the fact that antibacterial activity of the tested neutralized honey samples was higher by 1.5–2.5 times as compared to honey total activity before neutralization. Furthermore, after their neutralization, eight samples became active against *S. aureus* and three against *S. epidermidis*. Published data on the activity of the neutralized honey is rather scarce. Snow and Manley-Harris (*22*) investigated the stability of antibacterial activity of honey to alkaline solutions. They found that at pH=11 antibacterial activity of manuka (*Leptospermum scoparium*) honey was irreversibly lost. In our case, neutralized honey acquired higher antibacterial activity.

The results obtained suggest that stronger antibacterial activity of neutralized Lithuanian honey samples is due to the hydrogen peroxide formation by glucose oxidase. The increase of enzyme activity in the neutral media promotes the production of hydrogen peroxide, which inhibits the growth of bacteria. This assumption is supported by the fact that the results obtained are in agreement with those obtained after the addition of catalase.

Thermally processed beebread (C35) retained uniform activity after catalase addition and product neutralization. This indicates that inhibition properties of beebread samples did not depend on hydrogen peroxide; most likely the activity of thermally processed beebread was due to the presence of other, thermally stable compounds. Neutralization had less significant effect on the increase of antibacterial properties of other beebread solutions (with honey and honeycomb) as compared to honey solutions. It was reported that formation of hydrogen peroxide in beebread was lower than in rape, clover and heath origin honey samples by 3.20, 3.17 and 2.10 times, respectively (*23*).

## Conclusions

Assessment of antimicrobial activity of different Lithuanian honey samples and beebread against *S. epidermidis* and *S. aureus* showed that inhibitory effects are not inherent to all the selected honey samples; to achieve the inhibition of bacterial growth, the concentration of honey should be sufficiently high, usually higher than 25 % (by mass per volume). *S. epidermidis* was more resistant to the antimicrobial effects of honey than *S. aureus*. The results obtained after product neutralization and its treatment with catalase revealed that antibacterial activity of the tested honey samples is mainly due to the enzymatic formation of hydrogen peroxide. However, the samples of beebread possessed residual non-peroxide activity as well. The study indicates that the use of honey and beebread in food formulations can help to control some food pathogens, however, further investigations are needed to establish possible effects of honey origin on the inhibitory properties against food microorganisms.

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## References

1. K.L. Allen, P.C. Molan, G.M. Reid, A survey of the antibacterial activity of some New Zealand honeys, *J. Pharm. Pharmacol. 43* (1991) 817–822.
2. P.C. Molan, The antibacterial activity of honey: 1. The nature of the antibacterial activity, *Bee World, 73* (1992) 5–28.
3. P.C. Molan, The antibacterial activity of honey: 2. Variation in the potency of the antibacterial activity, *Bee World, 73* (1992) 59–76.
4. P.C. Molan, M. Brett, Honey has potential as a dressing for wounds infected with MRSA, *The Second Australian Wound Management Association Conference*, Brisbane, Australia (1998).
5. R. Cooper, P.C. Molan, The use of honey as an antiseptic in managing *Pseudomonas* infection, *J. Wound Care, 8* (1999) 161–164.

*6.* R.J. Weston, K.R. Mitchell, K.L. Allen, Antibacterial phenolic components of New Zealand manuka honey, *Food Chem. 64* (1999) 295–301.

*7.* B.C. Nzeako, J. Hamdi, Antimicrobial potential of honey on some microbial isolates, *Med. Sci. 2* (2000) 75–79.

*8.* H. Chick, H.S. Shin, Z. Ustunol, Growth and acid production by lactic acid bacteria and bifidobacteria grown in skim milk containing honey, *J. Food Sci. 66* (2001) 478–481.

*9.* S. Bogdanov, Nature and origin of the antibacterial substances in honey, *Z. Lebensm. Wiss. Unters. Technol. 30* (1997) 748–753.

*10.* M.A. Mundo, O.I. Padilla-Zakour, R.W. Worobo, Growth inhibition of food borne pathogens and food spoilage organisms by select raw honeys, *Int. J. Food Microbiol. 97* (2004) 1–8.

*11.* *Honey – Health and Therapeutic Qualities*, The National Honey Board, Longmont, CO, USA (2002) (http://www.nhb.org).

*12.* R.J. Weston, The contribution of catalase and other natural products to the antibacterial activity of honey: A review, *Food Chem. 71* (2000) 235–239.

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

*14.* L. Persano Oddo, M.G. Piazza, A.G. Sabatini, M. Accorti, Characterization of unifloral honeys, *Apidologie*, *26* (1995) 453–465.

*15.* P.C. Molan, The limitations of the methods of identifying the floral source of honeys, *Bee World*, *79* (1998) 59–68.

*16.* H. Straka: *Pollen and Spores*, Gustav Fisher, Stuttgart, Germany (1975) pp. 30–65 (in German).

*17.* A.N. Burmistrov, V.A. Nikitina: *Melliferous Plants and Their Pollen*, Rosagropromizdat, Moscow, Russia (1990) pp. 15–189 (in Russian).

*18.* W. Von der Ohe, L. Persano Oddo, M.L. Piana, M. Morlot, P. Martin, Harmonized method of melissopalynology, *Apidologie* (Suppl. 1), *35* (2004) 18–25.

*19.* L. Persano Oddo, R. Piro, Main European unifloral honeys: Descriptive sheet, *Apidologie* (Suppl. 1), *35* (2004) 38–51.

*20.* L. Persano Oddo, M.L. Piana, S. Bogdanov, A. Bentabol, P. Gotsiou, J. Kerkvliet, P. Martin, M. Morlot, A. Ortiz Valbuena, K. Ruoff, K. Von der Ohe, Botanical species giving unifloral honey of Europe, *Apidologie* (Suppl. 1), *35* (2004) 82–93.

*21.* M.R. Adams, M.O. Moss: *Food Microbiology*, Royal Society of Chemistry, Cambridge, UK (2000) p. 479.

*22.* M.J. Snow, M. Manley-Harris, On the nature of non-peroxide antibacterial activity in New Zealand manuka honey, *Food Chem. 84* (2004) 145–147.

*23.* V. Čeksterytė, Effect of temperature and storage time on hydrogen peroxide content in honey of different biological origin, *Biologija, 2* (2000) 296–298 (in Lithuanian).