ŻYWNOŚĆ. Nauka. Technologia. Jakość, 2020, 27, 1 (122), 85 – 95

DOI: 10.15193/zntj/2020/122/324

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SELECTED METHODS OF MICROBIOLOGICAL QUALITY ASSESSMENT OF LIME AND ACACIA HONEYS AVAILABLE ON POLISH MARKET

S u m m a r y

In mature honeys small quantities of microorganisms are detected, thus the objective of this research study was to identify the most effective method to microbiologically assess this product. The analyses were carried out using three methods: classical surface, pour plate and spiral culture. Two varietal honey samples were used in the analyses: acacia honey and lime honey. The scope of the research study included the microbiological analyses targeted at the determination of total number of mesophilic aerobic microorganisms, yeast and mould, and mesophilic spore-forming bacteria *Bacillus* spp. Moreover, the following was determined: pH value, water content and water activity. As for the lime honey, the results of the analyses performed using the three above named methods did not differ statistically significantly as regards the total number of mesophilic aerobic microorganisms and spore-forming bacteria *Bacillus* spp. However as for the acacia honey, the total number of mesophilic aerobic microorganisms was statistically significantly higher when obtained using the spiral culturing method of analysis and the count of spore-forming bacteria *Bacillus* spp. – when obtained using the pour plate method. In all the tested samples of acacia honey and the two samples of lime honey (S7 and S5), the count of spore-forming bacteria *Bacillus* spp. exceeded the limit of 5×10^2 cfu/g. In the case of yeast and mould analyses, the results of the analysis obtained by the pour plate method were statistically significantly higher than those obtained using other two methods. In none of the analysed samples of acacia and lime honey, the limit of 5×10^2 cfu/g of the product was exceeded. Based on the analyses performed, it can be concluded that TCAM can be performed using any method, but the pour plate method is recommended for use to analyse yeast and mould and spore-forming bacteria *Bacillus* spp.

Key words: varietal nectar honey, microbiological quality, methods of analysis, microbiological limit

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Introduction

Nectar honeys are obtained from the nectar collected by bees from plant flowers. In the central European countries, broad- and small-leaf lime trees are in bloom at the end of June and in early July. Honey with lime pollen predominating is sweet and has a yellow to light amber colour and a faint aroma of lime blossom, whereas acacia honey obtained from the nectar of the acacia robinia tree is either colourless or light yellow, it has a slight scent of acacia robinia flower and a sweet taste [15]. In their consumer research, Madras-Majewska et al. [11] showed that of the nectar varietal honeys, the most popular is lime honey (31 % of respondents). Multi-flower honey (25 % of respondents) is second and the third is acacia honey (13 % of respondents).

The microbiological contamination of honey is divided into the primary contamination coming from pollen and the digestive tract of bees, dust and soil, and the secondary contamination coming from honey processing, of which people and packaging equipment may be the source [25].

However, owing to the chemical composition of honey, there is a limited amount of microflora. It consists of low water content (17.2 % on average) [25], a high sugar content 76.0 ÷ 80.5 % (average concentration 78.9 %) [23] and a low protein content of 500 ÷ 10,000 mg/kg (average concentration 2,000 mg/kg) [24].

Additional factors to limit the development of microflora are a low pH in the range 3.4 ÷ 4.1 (mean value 3.9), water activity in liquid honey not exceeding 0.61 ÷ 0.62 [9, 26, 28] and the presence of substances with antimicrobial activity (glucose oxidase, lysozyme, flavonoids) [25, 27].

Glucose oxidase oxidizes glucose to gluconic acid with the release of hydrogen peroxide, which contributes to the inactivation of microorganisms [1, 9]. Therefore in mature honey there are mainly osmophilic yeast and fungi, and spores of aerobic and anaerobic bacteria, mainly *Bacillus* spp. and *Clostridium* spp. [3, 4, 5, 16, 22, 26].

Among the yeasts, the most prevalent species are *Hansenula, Nemaiospora, Pichia, Rhodotorula, Saccharomyces, Schizosaccharomyces, Schwanniomyces, Torula* and *Torulopsis.* The presence of yeast at an elevated water content, favourable temperature and factors such as the presence of nitrogen and ash lead to the fermentation with the formation of alcohol and carbon dioxide. Alcohol in the presence of oxygen is converted into acetic acid. The chemical compounds produced as a result of the fermentation change the taste and aroma of honey and this impacts its consumer acceptability. The number of yeasts in the mature honey is highly different and it depends mainly on water activity. It can range from several to several dozen million. Yeasts are determined together with mould because they also show resistance to a high osmotic pressure caused by low water activity in honey. However, there are suggested mechanisms other than the resistance to high osmotic pressure to determine the growth of mould in honey. The following types of fungi can occur: *Atchia, Aspergillus, Coniothecium, Cepha-*

losporium, Chaetomium, Hormiscium, Pencillium, Triposporium. Their number is marked on the level from 0 to several thousand in 1g of the product [25].

In most of the research publications, physicochemical properties of honey are considered in the light of Directive 2014/63/EU of the European Parliament and of the Council [2]. The honey microflora research is mainly focused on the analysis of the occurrence of toxynogenic *Clostridium botulinum* bacteria [4, 10, 12, 13]. A small number of current scientific publications are available on the microbiological and hygienic quality of honeys. Moreover, there is a lack of EU and national legislation in this field [2, 5, 20]. Under the present research study, an analysis was done of mature honey, the largest amounts of which are available on the Polish market and which is willingly chosen by consumers. By reason of a small number of microorganisms determined in honeys, microbiological analyses were carried out using three methods (classical surface, pour plate and spiral culture) in order to demonstrate the most effective method of analysis. As the evaluation criteria, the following was selected: number of yeasts and mould fungi, spores of bacteria *Bacillus* spp., index of the total number of mesophilic aerobic bacteria and the physicochemical parameters affecting the microbiological quality of honey (pH, water activity and water content).

Material and methods

The research material consisted of 15 nectar honeys originating from the Polish apiaries available via one of the retail networks on the Warsaw market. Eight samples of lime honey (samples marked as S1 to S8) and seven samples of acacia honey were selected for analysis (samples marked as S9 to S15). The honey samples were stored in sealed packages at a temperature 20 ± 2 ºC in the dark.

The honey samples were analysed to determine the following: total number of aerobic mesophilic microorganisms and mesophilic spores of the bacteria *Bacillus* spp., genus of yeast and mould. A quantity of 10 g of each sample was homogenized with 90 ml of buffered peptone water (0.1 %) using a Somacher 400 (IUL Instruments, Spain). The buffered peptone water (0.1 %) was utilized to make decimal dilutions. The three successive dilutions of the product were analysed in duplicate. The number of microorganisms was expressed as colony forming units per 1 g of honey (cfu/g). All the honey samples were analysed with the use of microbiological tests and the analyses were done in triplicate. In addition, the following physicochemical analyses were performed to determine: pH value, water activity and percentage of water content.

For the microbiological quality assessment, there were selected a reference plate method (surface and pour plate) and an alternative spiral culture method using a Bentley Instruments WASP device (Bentley Polska, Poland) [7].

WASP is a modern device for automatic plate culturing using the spiral method. Its advantage is that it is not necessary to perform repetitions and dilutions. A spiral

culture consists in spreading the test sample with a micropipette on the surface of the plate with a solidified medium creating an Archimedean spiral running from the centre of the plate to its edges. The counting of the colonies grown was performed using aCOLyte 3 – a completely automatic device for counting bacterial colonies from Petri dishes (Bentley Polska, Poland).

The following microbiological media were used to perform analyses with the use of the reference and alternative methods: nutrient agar (Biokar Diagnostics, France) – analysis of the total count of mesophilic aerobic microorganisms (TCMA) [8, 29], Difco DRBC Agar with chloramphenicol and bengal pink (Becton Dickinson Co., USA) – analysis of the yeast and mould fungi (25 ºC/5 days) [6], agar with bromocresol purple, also known as BCP (Biokar Diagnostics, France) – analysis of the mesophilic spores of bacteria *Bacillus* spp. (37 ºC/5 days).

The pH value was measured by a Lab 860 Schott Instruments pH meter (SI Analytics GmbH, Schott Instruments, Germany). Prior to the measurement, the sample was weighed (10 g) and mixed with distilled water at a ratio of 1:7.5 [18]. The water activity was measured at 25 ± 0.2 ºC by an AquaLab series 4TE' instrument (Decagon Devices, Pullman, Washington, USA) with an electronic dew point measurement. The instrument was in a temperature-stable sampling environment; it was calibrated with saturated salt solutions ranging 0.40 ÷ 0.70. The AquaLab device continued to measure water activity until the difference from three consecutive measurements was less than 0.0005 a_w. The water content was measured in undiluted samples by a PAL-22S refractometer (Conbest, Poland) with a temperature compensation function where the sample temperature was different than 20 ºC. Three or four measurements were taken, the water content was read directly from the refractometer, the average value was given and the arithmetic mean was calculated [18].

The results obtained were statistically analysed using MS Excel (mean and standard deviation). To assess the significance of the differences among the samples, a one-way analysis of variance ($p < 0.05$) and a RIR Tukey's post-hoc test were performed using a Statistica PL ver. 13.1 software package.

Results and discussion

As for the acacia honeys analysed by the spiral method, there were obtained the highest values of the total count of aerobic mesophilic microorganisms in the log range of 2.11 ÷ 2.24 cfu/g of product. The results of the total number of microorganisms obtained by the reference method were statistically significantly lower and in the case of the surface and pour plate method used they were, respectively, log 0.12 ÷ 1.11 cfu/g and log 0.67 ÷ 1.7 cfu/g.

The microbiological contamination of lime honey by aerobic mesophilic microorganisms was at a similar level, as determined by various microbiological culture meth-

ods, and their total number was as follows: acc. to the spiral method – log 1.14 ÷ 2.34 cfu/g; acc. to the classical surface method – log 0.48 ÷ 2.24 cfu/g and acc. to the classical pour plate method – 0.74 ÷ 2.07 cfu/g. There were no statistically significant differences in the number of microorganisms determined by those three methods.

In none of the honey samples analysed did the value of the total number of mesophilic aerobic microorganisms in bee products exceed the level of 5×10^4 cfu/g as specified in Regulation of the Minister of Health on maximum levels of chemical and biological pollution that may be found in food, food ingredients, approved additives, processing aids or on the surface of food [21]. The obtained results of the total number of mesophilic aerobic microorganisms allow to classify the micro-biological quality of honey as satisfactory.

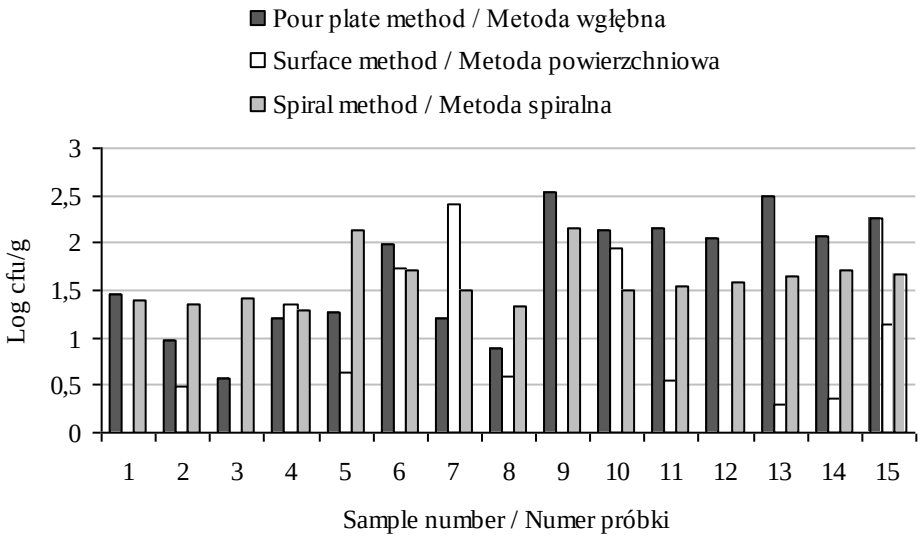

Fig. 1. Microbiological analysis of linden honey (S1 - S8) and acacia honey samples (S9 - S15) to determine mesophilic spore-forming *Bacillus* spp.

Rys. 1. Analiza mikrobiologiczna miodów lipowych (S1 - S8) i akacjowych (S9 - S15) w kierunku bakterii przetrwalnikujących *Bacillus* spp.

There were no statistically significant differences between the results obtained by the three methods in the case of mesophilic spore-forming bacteria of the *Bacillus* spp. genus found in linden honey (Fig. 1). In contrast, in the case of acacia honey, the results obtained in the pour plate method (log 2.0 ÷ 4.53 cfu/g) were statistically significantly different compared to the two other methods (log 0.3 ÷ 1.95 cfu/g by the surface method and log 1.71 ÷ 2.5 cfu/g by the spiral method).

Iurlina et al. [9] found *Bacillus* spp. in 38.5 % of the honey samples analysed, *B. cereus* in 15 samples analysed and *B. pumilus* in 3 samples tested. *Bacillus* spp. detect-

ed under this study showed that the bacterial contamination was at a similar level (log 2.25 cfu/g of *Bacillus* spp.) as that found by the aforementioned authors. The results under this research study are also congruent with those as described by Snadown and Cliver [25].

In the case of lime honey, the microbial contamination by mesophilic spore-forming *Bacillus* spp. was lower than that of acacia honey and their counts determined by the pour plate method ranged between 0.56 ÷ 1.45 log cfu/g, by the surface method – 0.48 ÷ 2.42 log cfu/g and by the spiral method – 1.29 ÷ 2.14 log cfu/g (Fig. 1). The physical-chemical parameters of lime honey did not impact the microbiological quality of honey. The acacia honey pH value was lower (3.14 ÷ 3.35) than the pH value of lime honey (3.37 ÷ 4.09), however the water activity was slightly higher in acacia honey (0.56 ÷ 0.61) compared to that in lime honey (0.53 ÷ 0.57). The content of water was below the acceptable limit of 20 % for all the acacia and lime honey samples (Tab. 1).

Table 1. Selected physical-chemical properties of lime and acacia honey
Tabela 1. Wybrane wyróżniki fizykochemiczne miodów lipowych i akacjowych

Honey Miód	Sample number Numer próbki	pH	Water activity Aktywność wody	Water content Zawartość wody [%]
Lime Lipowy	S1	4,09 ± 0,02	0,57 ± 0,01	18,30 ± 0,03
	S2	3,37 ± 0,01	0,57 ± 0,01	18,83 ± 0,06
	S3	3,78 ± 0,02	0,55 ± 0,01	17,10 ± 0,11
	S4	3,44 ± 0,00	0,55 ± 0,00	18,30 ± 0,06
	S5	3,78 ± 0,01	0,53 ± 0,01	14,90 ± 0,09
	S6	3,77 ± 0,01	0,55 ± 0,00	19,07 ± 0,38
	S7	3,44 ± 0,03	0,55 ± 0,00	16,77 ± 0,06
	S8	3,73 ± 0,00	0,55 ± 0,01	18,10 ± 0,01
Acacia Akacjowy	S9	3,14 ± 0,02	0,56 ± 0,02	18,03 ± 0,32
	S10	3,35 ± 0,04	0,57 ± 0,00	17,00 ± 0,06
	S11	3,20 ± 0,04	0,59 ± 0,02	19,83 ± 0,15
	S12	3,17 ± 0,08	0,61 ± 0,01	19,27 ± 0,06
	S13	3,26 ± 0,06	0,56 ± 0,01	16,10 ± 0,04
	S14	3,14 ± 0,10	0,58 ± 0,00	18,03 ± 0,15
	S15	3,23 ± 0,09	0,61 ± 0,02	18,57 ± 0,08

Accepting the criterion of the permissible count of oxygen *Bacillus cereus* bacilli in 1 g of the product at a level of 100 cells [21], it can be concluded that all the acacia honey samples tested and the two lime honey samples tested (S7 and S5) exceeded the above-mentioned limit. However it should be noted that the limit of permissible count determines the number of potentially pathogenic species of *Bacillus cereus*, while the

limit of the presence of total number of bacteria from the *Bacillus* genus including other bacteria from that genus commonly found in food, e.g. *B. subtilis, B. licheniformis*, could be accepted at a higher level. The majority of bacteria of this type have the GRAS (Generally Recognized As Safe) status awarded by FDA [14].

Only some species like *B. pumilusi, B. meganterium* can cause opportunistic infections. Currently there are no applicable regulations in this area [5], because the quoted Regulation of the Minister of Health was repealed on May 28, 2004 [21]. Therefore the microbiological safety of honey is difficult to assess. The microbiological quality of honey can be a potential health risk, because there is neither any obligation to check it nor any assessment criteria.

On the other hand, the range of available products is very wide, often the products from around the world are connected with each other at various stages of the food chain and sold under a different trade name. According to Regulation (EC) 178/2002 of the European Parliament and of the Council [17], the European law requires system information about the origin of a product or a dangerous component contained therein, but this may not be effective.

The analysis that caused the most difficulties in obtaining a repeatable result was the one performed to detect yeast and mould fungi. Especially in the case of acacia honey it was difficult to obtain a result for all the samples analysed (sample 11 was negative).

In the case of lime honey, one can conclude that the pour plate method proved to be most useful because the highest results were obtained as regards the count of yeast and mould fungi. In mature honey it is the dominant microflora that can be expected to be in the highest concentration. In the case of six samples of acacia honey, the results were obtained by the classical surface method and were within the range of log 0.6 ÷ 1.3 cfu/g. Using the classical pour plate method, the yeast and mould fungi were found in sample S9 and with the spiral method – in samples S10 and S13 - S15 (Fig. 2). Those two methods used to determine the number of yeast and mould fungi can provide false negative results, therefore the classical surface method should be recommended as a useful one.

The results of the analysis obtained by the pour plate method were in the range of 1.49 ÷ 2.21 cfu/g and were statistically significantly higher than the results obtained by the two other methods. The surface and spiral method gave log 0.22 ÷ 1.53 cfu/g and log 0.12 ÷ 0.78 cfu/g, respectively (Fig. 2). In none of the analyzed samples of acacia and lime honey was the limit of 500 cfu/g of product exceeded [21].

According to various authors, the pH value of nectar honey is in the range of 3.1 ÷ 4.9 [5]. Analyzing the pH values of the honeys examined, it was found that the pH value of acacia honey was lower than that of the lime honey and it was in the range as described in the reference literature (Tab. 1).

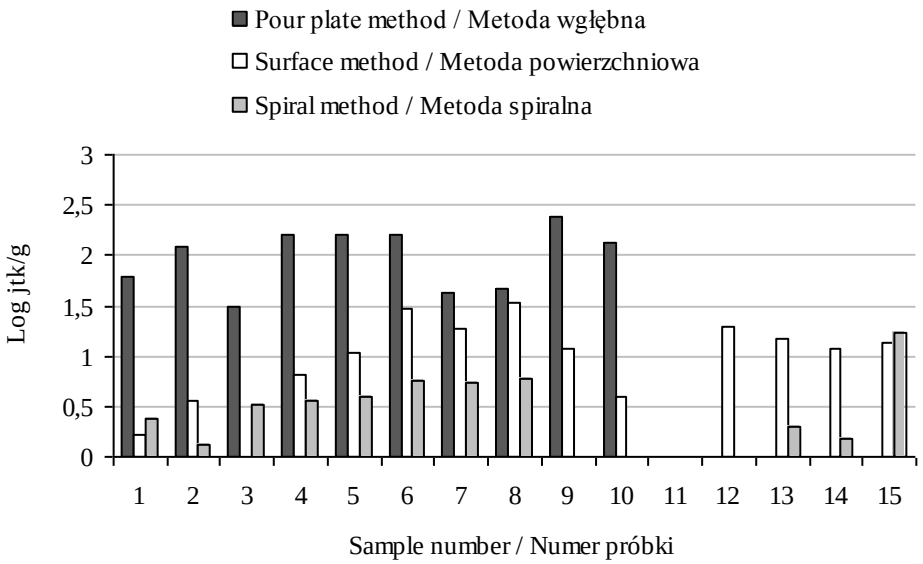

Fig. 2. Microbiological analysis of lime honey samples (S1 - S8) and acacia honey samples (S9 - S15) to determine yeast and mold fungi

Rys. 2. Analiza próbek miodów lipowych (S1 - S8) i akacjowych (S9 - S15) w kierunku drożdży i grzybów pleśniowych

In the case of acacia honey, the water activity was within the limits of 0.56 ÷ 0.61, whereas in the case of lime honey, lower values were found – 0.53 ÷ 0.57 (Tab. 1). None of the analyzed honeys exceeded the permitted percentage of water content as set in Regulations of the Minister of Agriculture and Rural Development and in Directive 2014/63/EU of the European Parliament and of the Council of 15 may 2014 amending Council Directive 2001/110/EC relating to honey [2, 15, 19].

Conclusions

1. The conducted microbiological analyses showed statistically significantly higher contamination by mesophilic aerobic microorganisms only in the case of acacia honey. This was determined by the spiral method. A similar relationship was found when determining the count of spore-forming *Bacillus* spp. bacteria.
2. As regards lime honey, there were no statistically significant differences between the results obtained by the three methods, whereas in the case of acacia honeys analysed by the pour plate method, there were obtained statistically higher results than those obtained by the surface method.
3. The results obtained make it possible to state that in the case of TAMC analysis, all the tested methods can be suitable for use, while in the case of spore-forming bac-

teria *Bacillus* spp., yeasts and mould, the classical pour plate method turns out to be better.

4. The lack of legal regulations regarding the microbiological contamination limit of honey may pose a potential hazard to the health of consumers, especially from spore forming bacteria *Bacillus* spp.

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WYBRANE METODY OCENY JAKOŚCI MIKROBIOLOGICZNEJ MIODÓW LIPOWYCH I AKACJOWYCH DOSTĘPNYCH NA POLSKIM RYNKU

S t r e s z c z e n i e

W dojrzałym miodzie wykrywa się niewielką liczbę mikroorganizmów, stąd celem pracy było określenie najskuteczniejszej metody oceny mikrobiologicznej tego produktu. Analizy przeprowadzono trzema metodami: klasyczną powierzchniową i klasyczną wgłębną oraz metodą posiewu spiralnego. Do badań użyto dwóch odmian miodu: akacjowego i lipowego. Zakres prac obejmował wykonanie analiz mikrobiologicznych w kierunku ogólnej liczby mezofilnych drobnoustrojów tlenowych, drożdży i pleśni oraz mezofilnych przetrwalników bakterii *Bacillus* spp. Ponadto oznaczono wartość pH, zawartość wody

i aktywność wody. W przypadku miodu lipowego wyniki uzyskane trzema metodami nie różniły się statystycznie istotnie pod względem ogólnej liczby mezofilnych drobnoustrojów tlenowych i bakterii przetrwalnikujących *Bacillus* spp. Natomiast w przypadku miodu akacjowego uzyskano statystycznie istotnie wyższe wyniki ogólnej liczby mezofilnych drobnoustrojów tlenowych metodą posiewu spiralnego, a bakterii przetrwalnikujących *Bacillus* spp. – metodą posiewu wgłębnego. Wszystkie badane próbki miodu akacjowego i dwie próbki miodu lipowego (S7 i S5) przekroczyły limit 5×10^2 jtk/g w przypadku bakterii przetrwalnikujących *Bacillus* spp. Wyniki uzyskane metodą wgłębną były statystycznie istotnie wyższe niż wyniki uzyskane metodami posiewu powierzchniowego w przypadku analizy drożdży i pleśni. W żadnej z analizowanych próbek miodu akacjowego i lipowego nie został przekroczony limit 5×10^2 jtk/g produktu. Na podstawie przeprowadzonych badań można stwierdzić, że ocenę jakości mikrobiologicznej miodów w kierunku ogólnej liczby drobnoustrojów mezofilnych tlenowych można przeprowadzać dowolną metodą, natomiast do analizy drożdży i pleśni oraz bakterii *Bacillus* spp. zaleca się stosowanie metody posiewu wgłębnego.

Słowa kluczowe: miód nektarowy odmianowy, jakość mikrobiologiczna, metody analizy, limit mikrobiologiczny