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THE STUDY CORRELATION BETWEEN PHYSICOCHEMICAL PROPERTIES, BOTANICAL ORIGIN AND MICROBIAL CONTAMINATION OF HONEY FROM THE SOUTH OF UKRAINE

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ABSTRACT

The honey production is one of the most important agriculture branches of Ukraine. Since the beginning of 2000 year, honey becomes one of the first products of agriculture production that was allowed to export from Ukraine to the EU countries. The present study aims to evaluate microbial composition of honey of higher and first grades from south of Ukraine, which differ in water, sugar content and acidity. The correlation beetween physicochemical properties, botanical origin and microbial composition of honey were investigated. Total aerobic count (TAC), total count of fungi (TFC) and yeast (TYC), number of sporeforming anaerobes (SFA), Bacillus cereus and Clostridium botulinus were determined. Plate dilution method with specific cultural mediums and incubation conditions was used for indication and identification of microorganisms as well for quantitative CFU count determination in 1g of honey. The results indicated that the value of TAC was from 1.4 ±0.1 x 10^2 to 3.7 ±0.5 x 10^2 CFU.g^{-1} in higher grade honey and from 2.1 ±0.3 x 10^2 to 4.6 ±0.4 x 10^2 CFU.g^{-1} in first grade honey. Average amounts of fungi and yest in the first grade honey were more than in higher grade honey and amounted 15.±0.9 CFU.g^{-1}, 6.48 ±0.7 CFU.g^{-1} and 17.98 ±1.2 CFU.g^{-1}, 8.04 ±0.8 CFU.g^{-1} respectively. Also it was found that, 25.8% and 37.1% of higher and first grade honey samples had sporeforming anaerobes. The more of samples with Bacillus cereus and Clostridium botulinum were among samples of first-grade honey samples. In addition, we have determined that, samples of buckwheat honey and honey from fodder crops of both grades were more contaminated with all microorganisms than samples of sunflower honey and honey from fruit and non-fruit trees.Results of this work has revealed the relationship between the number of different groups and species of microorganisms in higher and first grade honey from south of Ukraine and its physicochemical properties and botanical origin. Our research could help to substantiate the sources of contamination of honey and confirm the existing findings of scientists about the direct proportional dependence of the content of microorganisms in honey, depending on its moisture content, acidity and sugars.

Keywords: *honey; total aerobic count; fungi; yeast; sporeforming anaerobes; Bacillus cereus; Clostridium botulinum*

INTRODUCTION

At the thought of experts from the World Health Organization, microbiological risks, related to the quality of raw materials and food products are one of the most important biological threats at the present stage. Outbreaks of diseases caused by the consumption of hazardous foods are reported worldwide. In the case of honey, scientists are reporting that it can be dangerous for children because of botulism, which is caused by *Clostridium botulinum*. The presence of *Clostridium botulinum* in honey is a rare phenomenon, but contamination with this microorganism depends on total microbial contamination of honey. If the total number of microorganisms in honey is higher, that the more likely the presence in the honey of this dangerous microorganism **(Grenda et al., 2018; Grabowski and Klein, 2017)**.

Honey is natural product, produced by honeybees and has viscid fluid state, sweet flavor and specific aroma. Well known, that due to contain of significant amounts of minerals, vitamins, simple sugars, organic acids, antioxidants and enzymes, honey is considered as good product with nutritional and therapeutic properties **(Da Silva et al., 2016; Alvarez-Suarez et al., 2018)**. Therefore, this product is very popular among people, including children due to its consumptional properties. In addition, much is known about antiinflammatory and antimicrobial characteristics of honey and effective using it for increase the state of immunity, wound healing, gastrointestinal disorders, skin diseases and even cancer **(Rao et al., 2016; Kateel et al., 2018; Mabrouka, Ayed and Grara, 2018; Pasias et al., 2018)**.

The honey production is one of the most important agriculture branches of Ukraine. Since the beginning of

2000 year, honey becomes one of the first goods of agriculture production that was allowed to export from Ukraine to the EU countries. According to official data, Ukraine is the largest in Europe and the third largest in world producer of honey **(Dankevych, Dankevych and Pyvovar, 2018)**.

Council Directive 2001/110/EC and **Regulation (EC) No. 470/2009** specify key common criteria as quality norms for honey of all EU countries and those countries that export this product. According to these regulations, honey must be natural and free from foreign and harmful components.

However, honey can be associated with some adverse health effects of human, especially when contaminated with hazardous chemicals or microorganisms **(Laredj and Waffa, 2017; Silva et al., 2017; Kostić et al., 2019)**.

In recent years there has been growing interest in studying the presence of microorganisms in honey and bee products and bee products as potential sources of pathogenic microorganisms. A number of studies have been carried out and reported about bacterial species found in honey samples produced in different country. Among the isolated microorganisms were the following: *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Streptococcus epidermidis*, *Corynebacterium sp.*, *Klebsiella pneumonia*, *Escherichia coli*, *Salmonella sp.* and *Proteus spp.* **(Saba, Shakeel and Kamran, 2015; Adadi and Obeng, 2017)**.

In contrast to the physicochemical properties, microbial contamination of honey is not regulated because of the lack of legislation in the European Union and other countries.

Microorganisms get in the honey from digestive tract of honeybees, which have natural microorganisms, from nectar, pollen and flowers, from environment of beehive, and during processing **(Sinacori et al., 2014; Bentabol Manzanares et al., 2017)**.

It was found that moisture content, low acidity, presence of sugars (glucose, fructose and sucrose) and natural antimicrobial substances are influenced on microbial composition of the honey **(Fernández et al., 2017; Alvarez-Suarez et al., 2018; Pomastowski et al., 2019)**. The microbial spectrum of honey can include pathogenic bacteria especially those which capable to sporoforming, fungi and yeasts **(Kunová et al., 2015; Silva et al., 2017)**. These microorganisms can survive in honey and causing of undesirable changes by reducing the shelf-life of the product. But more important is that such honey can be harmful to the health of the consumer. Therefore, it is very important to know the diversity of microorganisms, which can be present in honey, especially due to disseminate pathogenic for human microorganisms in the international traded marketing. In addition, to the best of our knowledge, this is the first study that discribe the microbiological quality of honey from Ukraine.

Scientific hypothesis

The main scientific hypothesis of this study was to examine the differences in microbiological composition of two grades of honey of different botanical origin from south of Ukraine. In this context we tried to study correlation beetween physicochemical properties, botanical origin of honey and presence of microorganisms. We believe that quantitive and qualitive presence of microorganisms in honey depends on its geographical and botanical origin.

MATERIAL AND METHODOLOGY

Samples collection

The work was performed in the microbiology laboratory of Public Helth Department of Sumy State University and Odessa Regional State Laboratory of the Civil Service of Ukraine on the quality of food safety and consumers` protection. A total of 124 samples of honey were investigated. Samples were gathered from 9 apiaries located in Odessa region (southern part of Ukraine) and their studies were conducted during years 2016 – 2018.

Fresh honey samples were examined – no more than one month after selection, and kept in good hygienic conditions on apiaries. By origin, honey was from the following honey melliferous plants: sunflower, buckwheat, fruit trees (cherry, apple, pear, cherry, peach, cherry-plum), non-fruit trees (forest trees: false acacia, linden), fodder crops (alfalfa, clover, rape, sweet clover).

Before conducting microbiological studies of honey, we tested it for physicochemical indicators in an accredited according to the requirements of the international standard **ISO 17025:2005** Odessa Regional State Laboratory.

Physicochemical studies of honey were conducted to determine the honey grade according to the requirements of the state standard of Ukraine **(DSTU 4497:2005)**. Requirements for physicochemical indicators of honey according to **DSTU 4497:2005** are shown in Table 1.

The honey samples were categorized on the basis of their physicochemical indicators into two groups for further microbiological investigation. In order to better comparison of microbiological research results, we selected an equal number of honey samples; 62 samples of higher grade honey and 62 samples of first grade honey.

Methods of microbiological analysis of honey samples

Total aerobic count (TAC), total count of fungi (TFC) and yeast (TYC), number of sporeforming anaerobes (SFA), *Bacillus cereus* and *Clostridium botulinus* were determined. Plate dilution method with specific cultural mediums and incubation conditions was used for indication and identification of microorganisms as well for quantitative CFU count determination in 1 g of honey. Serial dilutions were prepared using peptone water as diluent by weighing 10 g of honey into 90 mL of sterile diluents. The 1.0 mL of the dilution was inoculated into sterile plates with specific cultural mediums using the surface spread plate technique and incubated.

Determination of TAC was carried out with using nutrient agar (Oxoid, UK) and surface spread plate technique acording to **DSTU ISO 7218:2007** and aerobicaly incubated at 37 ±1 °C for 48 h. After incubation period colonies were calculated.

For determination of TFC and TYC all dilution of honey samples were inoculated onto Dicloran-Glicerol selective medium (Himedia, India) according to the standard method of **ISO 21527-2:2008**. The plates were incubated at 25 ±1 ºC for 5 – 7 days, to growth of moulds and yeasts colonies. For identification of fungi from yeasts we used macroscopic examination and studied cultural characteristics (colony size, form, consistency, surface and pigmentation) and microscopic examination of morphological characters under microscope (observation of vegetative cells).

The number of sporeforming anaerobes was determined by classical method. Seeding on iron sulfite agar (Oxoid, UK) was performed according to **ISO 15213:2003**. After anaerobicaly incubation of samples at 37 ±1 °C for 48 h cultural, microscopic and biochemical identification of anaerobs was made.

Statistic analysis

All experiments were carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations. Student t-test was used for the statistical analysis of the obtained results. Data are presented as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Common knowledge that, honey is a natural product with a large amount of sugars (fructose, glucose and sucrose), enzymes, macro- and microelements. The presence of these components in honey causes a low content of water in it. This all makes honey an unfavorable environment for most microorganisms. In addition, honey also has antimicrobial compounds that fall into it from the flowers or through the process of their transformation into a hive **(Pasias et al., 2018)**. Given these conditions, few types of microorganisms have the ability to develop or remain in honey. Due to this honey is product, that has less number of microorganisms and even demonstrate antimicrobial characteristics against some pathogenic bacteria **(Rao et al., 2016; Kateel et al., 2018; Mabrouka, Ayed and Grara, 2018)**. Despite all these numerous inhibiting factors of honey, some types of microorganisms which enter the honey during its production and storage they still remain viable and can even multiply, causing its spoiling and can be harmful for consumers health **(Silva et al., 2017; Pasias et al., 2018)**. Microorganisms in the honey may arise from the nectar and different parts of plant flower, as well as during of processing **(Adadi and Obeng, 2017)**.

In recent literature, there are no information about microbiological contamination honey from Ukraine. Thereby, in this work, we report results of microbiological analysis of two grades of honey from south of Ukraine. The honey is an important source of income for farmers in the area. The Odessa region lies in the steppe zone of Ukraine, where the main agricultural plants are sunflower, buckwheat, rape, white and yellow sweet clovers, alfalfa, clover. Other plants as dandelion, linden and false acacia also play important role as melliferous plants. Of particular importance for beekeeping in the Odessa region are plantations of fruit trees (apple, pear, cherry-plum, cherry, peach, cherry).

According to the requirements of the State Standard of Ukraine **(DSTU 4497:2005)** and in accordance with the physico-chemical parameters of honey determine its grade – higher or first grade (Table 1). Higher grade honey is characterized by low water content (up to 18.5%), while for first grade honey this prameter should not exceed 21%. According to the **Council Directive 2001/110/EC**, the water content in honey should be no more than 20%. Water content of honey is a major indicator that affects the survival and reproduction of bacteria in honey.

Other indicators of honey that affect on quantitative and qualitative microbial composition are its acidity and sugar content. Both of these indicators are higher for first-grade honey. Therefore, based on the results of physico-chemical indicators of honey, we have formed two groups of honey samples (1st group – the highest grade honey and 2nd group – the first grade honey) for microbiological research and examined the total number of microorganisms, the number of molds and yeast, and the spore-forming microorganisms. The results of these studies are shown in Table 2.

Our results in Table 2 show, that the maximum number of microorganisms (total bacterial counter) in the honey samples was not more than 3.7 ±0.5 x 10^2 CFU.g^{-1} for the higher grade honey and not more than 4.6 ±0.4 x 10^2 CFU.g^{-1} for the first grade honey. And it averaged 2.62 ±0.1 x 10^2 CFU.g^{-1} and 3.67 ±0.3 x 10^2 CFU.g^{-1} in each grade respectively.

According to **Kunová et al. (2015)** these parameters was lower than our data in most samples of honey samples from Slovakia and ranged from 8.9 x 10^1 to 2.18 x 10^1 CFU.g^{-1}. In honey samples from the Czech Republic – 2.0 x 10^2 – 1.38 x 10^3 CFU.g^{-1} and Germany – 7.55 x 10^1 – 4.77 x 10^2 CFU.g^{-1}.

At the same time **Adjaloo et al. (2017)** assessed of Honey Quality in Ghana and noticed that Total aerobic count (TAC) was maximum 7.9 × 10^3 CFU.g^{-1}, which was much more than the maximum value of this indicator for Ukrainian higher grade honey and did not exceed the mean value of the both grades.

Adadi and Obeng (2017) assessed of bacterial quality of honey producedin Tamale metropolis (Ghana) and recorded the least mean bacterial count of 6.0 x 10^4 CFU.g^{-1} in location B and the highest, 1.1 x 10^5 CFU.g^{-1}, samples taken from location D. At the same time, these authors did not found bacteria in samples from location E and F. The variation in bacterial species at the various production location and the absence of bacteria growth in two locations is an indication of the differences in production practices, as well as hygienic conditions.

Also we found, that the most amount of microorganisms were in buckwheat honey and honey from fodder crops, the less was in honey obtained from sunflower, non-fruit and fruit trees.

Although our results on the total number of microorganisms in honey differ slightly from others researchers, it can nevertheless be argued that there is a relationship between bacteria counts in honey and its physicochemical components and geographical origin **(Kunová et al., 2015; Saba, Shakeel and Kamran, 2015; Pasias et al., 2018; Pomastowski et al., 2019)**.

In addition, our data is consistent with result of **Sinacori et al. (2014)**, who suggested that the highest microbial diversity was found in multifloral honeys. However, the same author belived that no correlation among the microbial species and the botanical/geographical origin of honey, but some strains of microorganisms were highly adapted as they were found in several samples of different origin honey.

Table 1 Physicochemical indicators of higher and first grades of honey in Ukraine according to National Standard of Ukraine **(DSTU 4497:2005)**.

Name of indicators	Higher grade	First grade
Water Content (moisture),%, not more	18.5	21.0
Mass fraction of reducing sugars (to anhydrous substance),%, not less	80.0	70.0
Mass fraction of sucrose (to anhydrous substance),% not more	3.5	6.0
Diastase number (to anhydrous substance), units Goth, not less	15.0	10.0
Hydroxymethyl-furfural (HMF) content, mg per kg, not more	10.0	25.0
Acidity, milliequivalents of hydroxide (0.1 mol.dm^{-1}) per kg, not more than	40.0	50.0
Proline content, mg per 1 kg, not less	300	300

Table 2 The level of contamination two grades of honey by anaerobic bacteria, fungi, yeast, (n = 62 samples of honey of higher grade and n = 62 samples of honey of first grade).

Honey produced from different melliferous plants species	The level of contamination honey samples, CFU.g^{-1}±SD					
	Honey of higher grade			Honey of first grade		
	APC x 10^2	TFC	TYC	APC x 10^2	TFC	TYC
Sunflower honey	2.1 ±0.3	14.1 ±0.9	5.4 ±0.5	3.9 ±0.2	17.2 ±1.3	7.1 ±0.5
Honey from buckwheat	3.1 ±0.2	16.4 ±1.4	6.8 ±0.4	4.4 ±0.4	18.9 ±1.9	8.6 ±0.2
Honey from fruit trees*	2.8 ±0.3	14.2 ±1.1	6.5 ±0.6	3.2 ±0.6	17.6 ±0.9	7.9 ±0.3
Honey from non-fruit trees**	1.4 ±0.1	15.4 ±0.7	6.1 ±0.2	2.1 ±0.3	17.1 ±0.8	7.4 ±0.5
Honey from fodder crops***	3.7 ±0.5	17.2 ±0.9	7.6 ±0.4	4.6 ±0.4	19.1 ±1.1	9.2 ±0.8
Average value	**2.62 ±0.1**	**15.46 ±0.9**	**6.48 ±0.7**	**3.64 ±0.3**	**17.98 ±1.2**	**8.04 ±0.8**

Note: APC – Aerobic Plate Count, TFC – total fungal count, TYC – total yeast count; * Fruit trees: cherry, apple, pear, cherry, peach, cherry-plum; **Non- fruit trees (forest trees): false acacia, linden; ***Fodder crops: alfalfa, clover, rape, sweet clover.

Table 3 Results of honey sample studies by contamination of sporeforming anaerobes, include *Bacillus cereus* and *Clostridium botulinum*.

Honey produced from different melliferous plants species	Honey of higher grade				Honey of first grade			
	No. investigated samples	No. positive samples/percentage			No. investigated samples	No. positive samples/percentage		
		SFA	Bacillus cereus	Clostridium botulinum		SFA	Bacillus cereus	Clostridium botulinum
Sunflower honey	13	3/23.1	3/23.1	0	13	5/38.5	4/30.8	1/7.7
Honey from buckwheat	12	5/41.7	4/33.3	1/8.3	12	6/50.0	4/33.3	2/16.7
Honey from fruit trees*	14	2/14.3	2/14.3	0	14	4/28.6	4/28.6	0
Honey from non-fruit trees**	11	2/18.2	2/18.2	0	11	3/27.3	3/27.3	0
Honey from fodder crops***	12	4/33.3	3/25.0	1/8.3	12	5/41.7	4/33.3	1/8.3
Total	62	16/25.8	14/22.6	2/3.2	62	23/37.1	19/30.6	4/6.5

Note: SFA – Total number of sporeforming anaerobes; * Fruit trees: cherry, apple, pear, cherry, peach, cherry-plum; **Non-fruit trees (forest trees): false acacia, linden; ***Fodder crops: alfalfa, clover, rape, sweet clover.

The next important groups of microorganisms in honey that maintain its physicochemical parameters are microscopic fungi and yeast. We found some correlation between of number of these groups of microorganisms with grade of honey and physicochemical components respectively. It was found that the higher grade of honey had the lower the number of microorganisms than first grade in 1.12 – 1.2 for fungi and in 1.2 – 1.3 times for yeast.

Besides, in the first grade honey amount of yeast and fungi was the more than in higher grade honey. Thus, the number of fungi in the first grade honey was on average 17.98 ±1.2 CFU.g^{-1}, which is on average 1.2 – 1.5 times higher than in the higher grade honey. Yeast in samples of first grade honey was within 7.1 ±0.5 and 9.2 ±0.8 CFU.g^{-1}, and in samples of higher grade honey – 5.4 ±0,5 – 7.6 ±0.4 CFU.g^{-1}. In the first grade honey, the increase in the amount of yeast was in 1.2 – 1.3 times.

The least amount of fungi and yeast was in honey samples from sunflower, fruit and non-fruit trees comparatively to honey samples from buckwheat and fodder crops. This can be explained by the fact that flowers of buckwheat and fodder crops are more contaminated by microorganisms from soil as these plants are undersized than trees and sunflowers.

In contrast to our study **Sinacori et al. (2014)** reported wide range in quantity of yeasts or moulds with maximum concentrations of 18,600 and 27 CFU.g^{-1}, respectively. The author attributes the high concentration of fungi in honey samples to the high moisture content.

As reported **Kunová et al. (2015)**, four samples of honey from 3 different EC country were negative for microscopic fungi count. The maximum value of microscopic fungi in one sample of honey was 1.5 x 10^{2} CFU.g^{-1} (from Slovakia). In research performed by **Sinacori et al. (2014)**, with honey samples of different types of melliferous plants, 17 of filamentous fungi of different species were isolated.

The next part of our study was to assess presence of total count of sporeforming anaerobes and in particular *Bacillus cereus* and *Clostridium botulinum*. The results of the studies are shown in Table 3.

The data in Table 3 indicate the intensity of contamination of honey with sporeforming microorganisms, and also characterize the incidence of honey samples detection which were contaminated with this type of microorganisms. As shown in Table 3, the higher grade honey samples had less anaerobic bacteria than first-grade honey samples. It was found that, 25.8% of the 62 studied samples of hight grade honey had sporeforming anaerobes, in 22.6% and 3.2% of cases had *Bacillus cereus* and *Clostridium botulinum* respectively. In the first-grade honey samples, these microorganisms were slightly higher at 37.1% for sporoform bacteria, 30.6% for *Bacillus cereus*, and 6.5% for *Clostridium botulinum.*

As mentioned by **Maikanov et al. (2019)** among 197 honey samples from Kazakhstan, *C. botulinum* was noticed in only one (0.5%). This result is lower than our result and results which were obtained in the Europe countries **(Matovic et al. 2015; Wojtacka et al., 2017; Grenda et al., 2018)**.

C. botulinum spores in 5 honey samples (8.47%) originating from different regions of the Republic of Serbia were found by **Matovic et al. (2015)**. The highest number of anaerobic isolates were detected in the samples of buckwheat Polish honey, at 10/26 (38%) **(Rózanska, 2011)**.

It is well known, that the main sources of sporeforming anaerobes in honey are the conditions of honey harvest and hygienic practices on the apiaries, type of melliferous plants and soil, climate.

Sporeforming microorganisms were found with higher frequency in samples of buckwheat honey and in honey from fodder crops in both grades – in higher grade honey and in first grade honey.

In samples of higher grade honey from buckwheat and fodder crops *Clostridium botulinum* were found in 8.3% of cases. In the first grade honey samples, *Clostridium botulinum* was found in the 1 sample of sunflower honey, in the 1 sample of honey from fodder crops and in two buckwheat honey samples. But more often than *Clostridium botulinum*, *Bacillus cereus* was found in honey samples. *Bacillus cereus* were isolated in 14 samples of higher grade honey and 19 samples of the first grade honey, 22.6% and 30.6%, respectively. In contrast of it, the presence of *Clostridium botulinum* spores in honey has been reported extensively **(Abdulla et al., 2012; Grant, McLauchlin and Amar, 2013)**. **Saba, Shakeel and Kamran (2015)** could not find spors of *C. botulism* during their study. **Sinacori et al. (2014)** reported that the highest microbial diversity was found in multifloral honeys. But this author also suggest, that no correlation among the microbial species and the botanical/geographical origin.

The results of this study clearly showed that the honey samples collected from apiaries of from south of Ukraine contained different amounts of microorganisms, that depended on the botanical origin of the honey as well as on the honey grade.

CONCLUSION

By our research we established that honey from the south of Ukraine, which according to the state standard and its physicochemical indicators belongs to the higher and first grades, has low microbial contamination.

In addition to the above, our research found that there was a relationship between microbial contamination of honey and its botanical origin. Samples of buckwheat honey and honey from fodder crops of both grades were more contaminated with all microorganisms than samples of sunflower honey and honey from fruit and non-fruit trees.

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