

Biotechnological Evaluation of Bee Ethology During Honey Collection and Probiotic Properties of Honey

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Abstract: The influence of biotechnological factors, such as purebred families and honey base on the ethology of bees in the process of nectar collection and the species composition of the association of probiotic cultures in freshly pumped honey to bees was found out. The research was conducted using Ukrainian steppe bees. The following most typical external features of their breed, namely the length of the proboscis from 6.34 to 6.44 mm, cubital index in the range of 2.17 ± 0.04 - 2.61 ± 0.05 , discoidal displacement 76 - 100 %, the shape of the edge of the wax mirror 68 - 96% of convex cases. The species composition of honey plants in typical conditions of the Forest-Steppe zone of Ukraine, their contribution to the general honey harvest during the whole beekeeping season and the formation of antibacterial defense mechanism of bees based on the association of probiotic cultures in their intestines and freshly pumped honey are analyzed. Data on differentiation of selected cultures from bee honey by cultural-morphological and biochemical properties are given. Probiotic cultures were identified by cultivation on differential media. Thus, cultures of *Lactobacillus plantarum* and *Bifidobacterium bifidum* were accumulated on MRS medium (solid and liquid), and *Enterococcus faecium* was accumulated on tryptone soy medium. It is shown that the quantitative characteristics of honey accumulation in the bee nest depend on a number of biotechnological factors. The main indicators that affect the ethology of bees when collecting nectar are purebred and species composition of the honey base. The results of the research serve as a basis for further studies of the ethology of the Ukrainian steppe breed of bees during honey collection, its popularization and use in large apiaries (100 and more families) according to the breed zoning plan in the Forest-Steppe zone of Ukraine and for the development of new antibacterial, specific for bees, organic products based on probiotic cultures, isolated from honey.

Keywords: Biotechnological Factors, Ethology of Bees, Ukrainian Steppe Breed, Honey Base, Honey Collection, Freshly Pumped Honey, Probiotic Cultures

1. Introduction

The economic importance of biotechnology is to increase production efficiency, improve product quality based on environmentally friendly science-intensive technologies. Ukraine occupies one of the first places in the world in honey exports [9, 17–20]. The urgent task is to maintain this position by increasing the production of quality and safe beekeeping products [7, 10, 16].

It is known that the behavioral response of bees during honey collection largely depends on a number of biotechnological factors, including the species composition of honeybees, purebred families, and others. [1, 2, 5, 7, 8, 14]. Statistical data on honey productivity by regions and breed affiliation show that the largest share of sweet product in Ukraine is obtained from the Ukrainian steppe breed of bees [17, 19]. Recently, the media advertises breeds from the mountains of the European continent, which have "overproduction" in different climatic zones of Ukraine, but

over time, their effectiveness decreases, aggression appears and very often death occurs during the winter [12, 21]. Uncontrolled and unsystematic use of non-zoned breeds of bees within the natural range, aboriginal breeds, to a large extent not only destroys centuries-old heritage of Ukrainian beekeepers, but over time reduces their productivity in future generations [12, 13, 15]. Taking into account these changes, there is a need to adjust the breed zoning plan and select the breed of bees that will provide high productivity for future generations. This breed is represented by the Ukrainian steppe bee, which we propose to study comprehensively for breeding improvement and effective use in its natural habitat. Given that these bees are a breed of strong families with high productivity and adaptability to industrial maintenance in multi-hull hives, we consider it appropriate to study this category of bees [15, 16]. Every year more and more tangible climate changes are manifested in the cultivation of thermophilic plants further north and the increase in the length of the bribe-free period, which affects on the verge of comfort of aboriginal breeds of bees [4, 11].

It is also known from the literature that during honey collection bees can become infected with a number of agents of various etiologies, including bacterial. However, in the course of evolution, honey bees have developed protective mechanisms against infection during this process. These include the presence in bees of the probiotic cultures association, the formation of which depends on the species composition of honey cultures, the breed of families, technological factors of their keeping, the state of the environment and others [22, 23]. Thus, 13 lactic acid bacteria were found in freshly pumped honey [22], as well as bifidobacteria and enterococci, which have probiotic properties [23]. This biotechnological field of research is of great importance for the development and implementation in beekeeping of specific organic products based on probiotic cultures isolated from honey. These drugs can be successfully used to obtain environmentally friendly bee products (eg, honey without antibiotics), stimulate reproduction and increase resistance of bees to pathogens of bacterial diseases and adverse environmental factors.

In connection with the above, the development of biotechnological approaches to increase honey productivity of families based on finding out and practical use of the influence of breed and species composition of honey plants on bee ethology and their resistance to infectious factors during nectar collection is an important area of research [14, 23]. The *aim* of the work is to study the influence of biotechnological factors (species composition of honey plants, purebred families) on the ethology and association of probiotic cultures in bees in the process of collecting nectar.

2. Materials and Methods of Research

The work was performed on the basis of Bila Tserkva National Agrarian University during 2016-2020. During ethological research in the process of collecting nectar on flowers purebred of bees was studied visually, on the basis of

the behavior of bees, color and features of the body hair. Visual assessment of qualitative physiological characteristics - honey seal, malice, diligence was performed according to the method of GD Bilash [2]. To confirm the affiliation of families to the Ukrainian breed, an external assessment of bees in 10 apiaries within the Bila Tserkva district of Kyiv region, enterprises of various forms of ownership the size of more than 100 families, was conducted. Selection of material for the study and determination of biomorphometric parameters was performed in accordance with the guidelines [2, 6]. To do this in August after completion main honey collection, from 10 families in each apiary on honeycombs with sealed brood were selected 50-60 young bees that just came out of the cells. Samples were preserved with ethyl alcohol (70%). To measure from each sample were taken 25 bees, dissected them and selected proboscis, right front wing and fifth sternite with wax mirror. Micrometric studies were performed according to the method of V. V. Alpatov [1]. The amount of marketable honey obtained was determined by weighing the samples taken from the nest honeycombs before and after pumping honey from them [6]. The daily honey collection was recorded by the control family method, weighing the hives on the scales after the flight of bees [2].

Freshly pumped honey was used to isolate probiotic cultures. In the first stage of research, selective nutrient media were produced MRS-broth of 5 ml in test tubes, MRS-agar, endo agar from dry commercial concentrates of Himedia (India), bifidobacterium - agar Him Labs Inc (India). The media were made according to the instructions on the package. The agar medium was poured into Petri dishes. Preparation of honey samples was carried out in accordance with DSTU 8684: 2016. A suspension of freshly pumped honey cultured in MRS broth was sown on the Petri dishes with agar media. Only three test tubes and three cups of each honey sample for each separate agar medium. The exposition of test tubes and cups lasted 24-48 hours. in a thermostat at a temperature of 30 °C. Probiotic bacteria were identified and their biochemical properties were studied according to known methods (Lyaskovsky et al., 2008; Tsisaryk et al., 2012).

Research materials were processed by the method of variation statistics based on the calculation of arithmetic mean (\bar{X}), root mean square error (S_x) [3]. The probability of the obtained results and the difference between the indicators were calculated according to Student's t-test.

3. Research Results and Their Discussion

Visual assessment and ethological studies have shown that in terms of color, body hair and qualitative physiological characteristics - honey seal, malice, fertility - worker bees belong to the Ukrainian steppe breed. Analysis of the obtained exterior data showed that the length proboscis bees of all families meet the norms of the morphological standard of the Ukrainian steppe breed. Fluctuations in this indicator ranged from 6.34 to 6.44 mm and did not approach its limit (Table 1). According to the

measurements of the cubital index, which is considered an important feature for assessing the purity of Ukrainian steppe bees, we conclude that there is a sufficient level of

compliance of families with the requirements of the typical characteristics of the breed. No significant deviations outside the standard were detected.

Table 1. Selective evaluation of experimental bee colonies by exterior characteristics, $\bar{X} \pm Sx$, $n=250$.

№ apiary	Length proboscis, mm	Cubital index	Discoïd offset, number of positive cases, %	Wax edge shape mirrors, number convex cases, %	Conformity behind the complex signs
1	6,38±0,02	2,19±0,06	100	76	+
2	6,35±0,02	2,20±0,04	96	92	+
3	6,38±0,02	2,42±0,05	88	96	++
4	6,35±0,02	2,31±0,05	92	96	+
5	6,34±0,03	2,43±0,06	76	88	+
6	6,44±0,02	2,40±0,03	100	84	++
7	6,42±0,03	2,61±0,05	96	84	++
8	6,43±0,02	2,22±0,04	100	88	++
9	6,40±0,02	2,17±0,04	100	68	+
10	6,34±0,03	2,43±0,05	88	84	+

Notes: corresponds to / + /; does not meet / - / standard; ($P \geq 0.95$).

Discoïd displacement, as well as signs of the shape of the posterior edge of the wax mirror of the fifth sternite, testify to the characteristic features of the Ukrainian breed of bees.

According to the results of a comprehensive assessment of the external features of the studied material, we can note the most typical features of bees of the Ukrainian steppe breed of forty families with the desired indicators for reproduction. At the same time, the tendency of the twenty two families of breed to approach the lower limit, whose individual bees have atypical traits in relation to the native breed, was revealed.

Thus, the data obtained in the experiments indicate a sufficient level of compliance with the general background of families belonging to the regional breed of Ukrainian steppe bees. The honey harvest activity of bees is inextricably linked with the presence of nectar-bearing plants in the natural environment and the period of their flowering. Our research was conducted in typical conditions of medical harvests of the Forest-Steppe zone. It is shown that the conveyor flowering of honey plants begins with forest grasses, willows, horticultural crops, winter rape. In summer, the main collection of honey is white acacia, buckwheat, linden, sunflower.

White acacia in this area under favorable weather conditions secretes nectar from the middle of the third decade of May for two weeks. During the flowering of white acacia in such honey-harvesting conditions, strong bee families collected 3-5 kg of nectar, and at the end of the honey-harvest they received up to 20 kg of marketable honey. After honey collection from white acacia, nectar collection weakens, which encourages bee colonies to swarm.

From the third decade of June to mid-July, bees collect nectar from broad-leaved linden, and later - heart-shaped. During the mass flowering, bees brought 5-6 kg of nectar to the hive, which makes it possible to receive 15-17 kg of honey from the family in some years. Our research was also conducted during the migration of the apiary in the field. During the honey harvest of winter rape (flowering peak occurred in mid-May with the introduction of 2-3 kg of nectar

to the hive per day) and buckwheat (began to produce nectar from the third decade of June for 20-30 days). From these honeycombs, bee families collected 9-10 and 12-15 kg of marketable honey, respectively. Bees from sunflower brought 2-3 kg of nectar to the hive. At the end of the honey harvest, an average of 11-13 kg of marketable honey was pumped out. Observe the feasibility of choosing sunflower, honey value of which is increasing, so it began to grow even in the southern regions of Polissya. Increasing the production of quality and safe beekeeping products is possible through the development of modern technologies based on understanding the breed characteristics of bee colony biology, studying the various relationships of bees with ecosystem elements and their rational use of existing honey base [14]. Therefore, elucidation of the influence of a number of biotechnological factors, such as the purity of families and the species composition of honeybees on the productivity of bees during their nectar collection is important [2, 5, 7, 8, 14].

The obtained results made it possible to establish the most typical features of bees of the Ukrainian steppe breed and their behavior during nectar collection in the Forest-Steppe of Ukraine (on the example of Bila Tserkva district). It is shown that bees of the Ukrainian steppe breed, which are aboriginal for this area, use biological reserves of nectar with a high degree of efficiency.

The results of the research will serve as a basis for further studies of the ethology of the Ukrainian steppe breed of bees during honey harvest, its popularization and use in large apiaries (over 100 families) according to the plan of breed zoning in the forest-steppe zone of Ukraine.

Thus, a study of the honey base of Bila Tserkva district of Kyiv region was conducted. The relief landscape of this area is very diverse, and the vegetation of the forest area is represented mainly by deciduous trees. In the first tier there are oak, linden, maple, white acacia, in the second - field maple, Tatar maple, leafy elm, various species of willow, alder. Hazelnuts and weeds grow in the undergrowth. Among the herbaceous honeysuckles, corydalis, dandelion, lamium galeobdolon for a long time. No less important for the honey

base of the forest-park zone are also ornamental and garden trees, which include white and yellow acacias, amur velvet, chestnut, apple, pear, cherry, sweet cherry and more. Together, these honeybees, secreting nectar and pollen, under favorable weather conditions provide a continuous increase in feed reserves by an average of 200-400 g per day. This number stimulates the bees to active flight work, and the queen bee to increase egg production, which allows to increase the strength of the family to the main first honey harvest. The analysis of the obtained data on the characteristics of the honey harvesting conditions of the Forest-Steppe of Ukraine, the period of their flowering makes it possible to determine the optimal periods of

intensive honey harvesting and to develop scientifically sound honey conveyors in further research.

In order to isolate probiotic cultures from freshly pumped honey by culturing in MRS broth, cell inoculum was obtained in the form of turbidity. One day after sowing, a characteristic growth of colonies was observed on differential media: on MRS agar - lactobacilli, on agar endo - enterococci, on bifidobacterium agar - bifidobacteria.

When microscoping smears under immersion from the substrate of cultivation of existing lactobacilli, enterobacteria and bifidobacteria, which were stained by the Gram method, it was observed that on all differential media cells were stained as gram-positive (Figure 1).

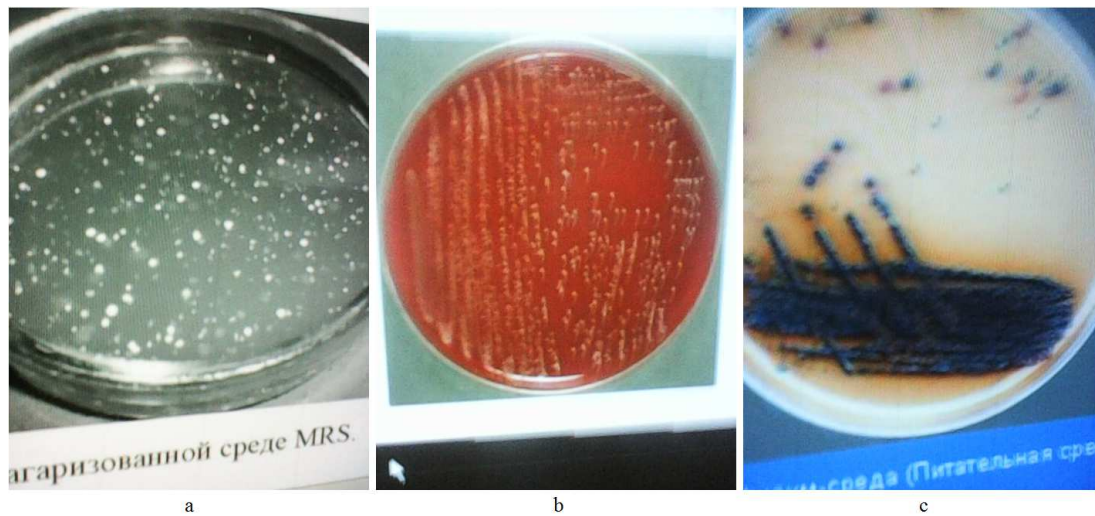


Figure 1. Growth of colonies on differential media: a - *L. plantarum* on MRS agar; b - *Bif. bifidum* on bifidobacterium agar Him Labs Inc; c - *E. faecium* on Endo agar.

Lactobacilli are rod-shaped straight, but some cells have been slightly curved, an optional anaerob. During the aging of the culture, the shape of the cells changed to coccoid; also observed the formation of filamentous forms, increasing the diameter of the colonies to 7 mm. Bifidobacteria are severe anaerobes, small rods 2–5 mkm, which eventually form branched forms, do not form spores. When cultivated on the liver differential media of Biorock make white colored colonies in the form of "comets". Enterobacteria are facultative anaerobes, oval small cells 0.6–2.0 mkm (streptococci). During cultivation, the formation of spores

and capsules, fermentation-type metabolism, in which carbohydrates are fermented with the formation of lactic acid, reducing the acidity to 4.2 ± 0.1 , but without the appearance of gas, was not observed. When cultured in chromogenic broth, the affiliation of culture cells to *Enterococcus faecium* was confirmed.

Conducted biochemical studies (Table 2) of cultures isolated from freshly pumped honey showed the characteristic assimilation of His sugars, which is characteristic of lactobacilli, namely: ferment well glucose, lactose and sucrose; weaker - maltose with the formation of acids, mainly lactic.

Table 2. Fermentolysis of His sugars by probiotic cultures isolated from freshly pumped honey.

Name of the strain	Glucose	Lactose	Sucrose	Maltose	Indol	Lactic acid	Gas formation
<i>Lactobacillus plantarum</i>	++++	++++	++++	++	yes	yes	no
<i>Enterococcus faecium</i>	++++	++++	++++	++++	yes	no	no
<i>Bifidobacterium bifidum</i>	++++	++++	++++	++++	no	p/p	no

Notes: ++++ - ferments sugar well; ++ - ferments sugar less;
yes - a positive reaction to indole, the presence of lactic acid, gas formation;
p/p - partially positive reaction to indole, the presence of lactic acid, gas formation;
no - a negative reaction.

This culture also gives a positive reaction to indole. Bifidobacteria fermented glucose, sucrose, lactose and mannose. They did not form indole, weakly formed lactic

acid and gas. Enterobacteria fermented sucrose, lactose, glucose, mannose and formed indole, lactic acid and gas. It is due to the characteristic assimilation of His sugars was

founded that it corresponds to lactobacilli, enterobacteria, bifidobacteria. According to the set of determined cultural-morphological and biochemical features, it was found that the selected cultures from samples of freshly pumped honey belong to *Lactobacillus plantarum*, *Enterococcus faecium* and *Bifidobacterium bifidum*.

The evolutionary relationships of honey bees with agro- and biocenoses have recently undergone significant changes under the pressure of various anthropogenic factors. Risks that adversely affect bee health and behavior include biological, chemical and physical factors. They usually have a complex effect, contribute to a number of infectious diseases, adversely affect the overall health of bees and, consequently, their adaptive defense mechanisms, behavior, productivity, quality and safety of products [23, 24].

Under these conditions, the search for and development of complex organic products - drugs to stimulate the development and productivity of bee colonies, prevention and treatment of infectious diseases, including bacterial, is a priority for modern beekeeping. Such drugs include probiotics. These are microbial drugs, the main active ingredients of which are probiotic cultures, which exhibit antagonistic properties to pathogenic microflora [23]. One of the main requirements for them is the specificity of action, which means probiotic cultures must meet the normal flora of the intestines of animals for which they are intended [22, 23].

Therefore, the first part of research on the development of probiotics for bees is the isolation, identification and characterization of probiotic cultures from their intestines or freshly pumped honey. It is known that the species composition of the association of microorganisms of the normoflora of bees can vary depending on the breed of bees, the technology of their content and the conditions prevailing in certain areas [22, 23]. In this work, we surveyed a number of apiaries of the Forest-Steppe of Ukraine from Kyiv region, isolated and characterized the association of probiotic crops from freshly pumped honey. The obtained results are considered in the context of construction in the following researches of new probiotic preparations specific to bees.

4. Conclusions

The breed of bees and the availability of honey harvest are one of the main biotechnological factors in increasing honey production. Bees of aboriginal Ukrainian steppe breed with high productivity use biological reserves of nectar of the Forest-Steppe of Ukraine. The honey base of Bila Tserkva district of Kyiv region is characterized and it is established that under favorable weather conditions it provides a continuous increase in feed stocks on average 200-400 g per day.

The species composition of the association of probiotic crops in freshly pumped bee honey selected from apiaries of Kyiv region was studied. According to the combination of cultural-morphological and biochemical features, it was found that the selected cultures from samples of freshly

pumped honey belong to *Lactobacillus plantarum*, *Enterococcus faecium* and *Bifidobacterium bifidum*.

5. Recommendations for Future Research

The obtained results serve as a basis for further research of ethology of Ukrainian steppe breed of bees during honey collection, its popularization and use in large apiaries (over 100 families) according to the plan of breed zoning in the Forest-Steppe zone of Ukraine, as well as for development of new antibacterial organic drugs on the basis of probiotic cultures isolated from honey.

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