UDC 579.62 638.154.3

THE FEATURES OF MICROBIOTA ISOLATED FROM THE HONEYCOMBS WITH AFFECTED BEE BROOD

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Received on October 21, 2015

Aim. To investigate the microbiota of honeycombs with affected bee brood. **Methods.** Visual, immunochromatographic, cultural-morphological, biochemical, electron-microscopic methods were used to isolate and previously identify a number of microorganisms. **Results.** 10 samples of honeycombs with sealed brood were studied. The following agents of bee diseases were isolated and previously identified: *Paenibacillus larvae* – American foulbrood; *Basillus paraalvei* – parafoulbrood; *Melissococcus pluton* – European foulbrood. **Conclusions.** The number of cocci, spore-forming coli and yeasts, found in the samples of honeycombs, commonly represent normal microflora of bees, but their number increases significantly in case of viral and bacterial infections.

Keywords: bacterial diseases of bees, microbiota from the combs with affected bee brood, *Paenibacillus larvae, Bacillus paraalvei, Melissococcus pluton.*

DOI: 10.15407/agrisp2.03.009

INTRODUCTION

The diseases of honey bees are the violation of normal life of bees caused by changes of morphofunctional processes in the individuals or in the groups, exposed to adverse external and internal factors. The knowledge of these factors and the ability to manage them is the basis for maintaining strong and highly productive bee colonies [1, 2].

Microorganisms cause significant harm to beekeeping. Among the bacterial diseases of bees the most dangerous is American foulbrood (malignant foulbrood) – infection of bee colonies, accompanied by death of larvae and adult propupae. In the infected combs the spores remain virulent for 35 years, in the hives and in the wax – 20, in the honey extractor – 5 years. The causative agent of the disease is *Paenibacillus larvae* (*Bacillus larvae*). The representatives of genera *Bacillus* and *Paenibacillus* are also pathogens of other bacterial diseases of bees: European foul brood (*Paenibacillus alvei* (*Bacillus alvei*); *Brevibacillus laterosporus* (*Bacillus laterosporus* = *Bacillus orpheus*); *Paenibacillus apiarius* (*Bacillus apiarius*); parafoul brood (*Basillus paraalvei*), powdery brood (*Paenibacillus larvae pulvifaciens*) [3–7].

It is shown that the European foulbrood agents can be the following microorganisms: *Melissococcus pluton*, *Enterococcus faecalis*. The agents of salmonellosis (paratyphoid) – an infectious disease of bee colonies, accompanied by loss of adult bees – are *Salmonella thyphimurium*, *S. pullorum, S. gallinarum*. The agent of colibacteriosis – an infectious disease of bees, accompanied by loss of adult bees – is *Escherichia coli*, and spiroplasmosis (the May disease, pollen toxemia) is caused by *Spiroplasma apis* and other kinds of spiroplasma [8, 9].

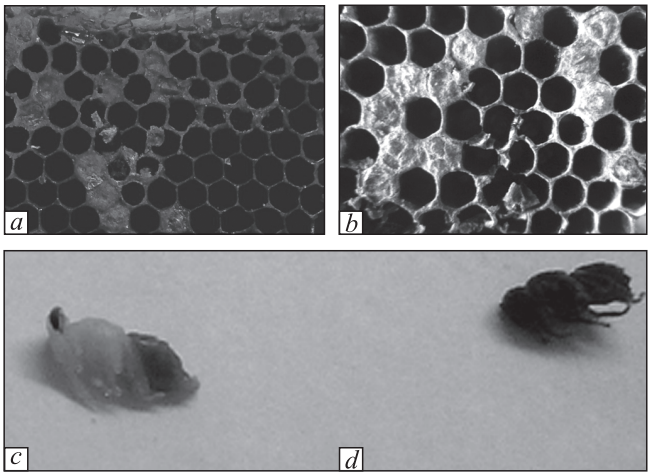

Fig. 1. The sample of comb No. 4 (*a*) and No. 5 (*b*) with brood and removed from the sealed cells propupae (*c*, *d*)

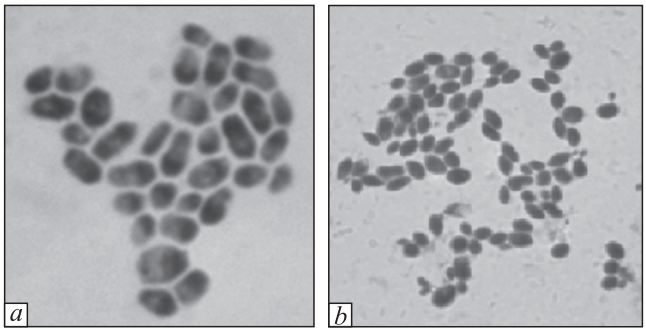

Fig. 2. The cells of yeasts isolated from the sample No. 1 (*a*) and No. 4 (*b*). (Magnification × 480)

The aim of our study was to examine the comb microbiota with affected enclosed brood of bees.

MATERIALS AND METHODS

The work was carried out on the basis of the agreement on creative collaboration between the Laboratory of Bees Pathology, NSC "Institute of Beekeeping named after P. I. Prokopovych", NAAS of Ukraine and Scientific Research Laboratory (SRL) of Microbiological and Immunological Problems of Biotechnology, NSC "Institute of Biology" of Taras Shevchenko National University of Kyiv.

The objects of study were 10 samples of enclosed brood cells selected in May-August 2012–2014 in Kyiv and Zhytomyr regions. The visual examination of samples of pathological material involved the examination of the brood status (open or closed), the presence of motley brood, sick and dead larvae, their location in the cell, texture and smell [10].

The immunochromatographic study of samples was carried out using a commercial set of test systems BioTest (RF) following the instructions.

A diagnostic washout was prepared for the bacteriological studies of pathological material from the cells with sick brood. A sterile saline solution was poured with a sterile pipette to selected 15–20 cells which contained dead larvae or their remains, and retained for 20–30 minutes. The contents of cells was carefully mixed and transferred to sterile tubes.

In order to select spore bacteria for suspected mixed forms of brood diseases, the material in a test tube was heated to 70 °C for 3–4 minutes and filtered through filter paper. The resulting filtrate was centrifuged at 5,000 rpm for 10 minutes or suspended for 30–40 minutes, and heated up again to 70 °C for not more than 3 minutes. 1–2 ml were taken from the bottom of the tube and sown on the prepared media. The second part of tubes with the washout was heated in a water bath at the temperature of 96–98 °C for 3–5 minutes [4, 11].

The prepared washouts were sown on culture media: Tomashets, Cherepov, Bailey, MPA and MPA with 10 % normal horse serum, thioglycollate medium. The sowings were grown in the incubator at 35–37 °C.

The microscopy of Gram-stained cells was conducted using the light microscope of Micromed company (Italy); the equipment of the mentioned company was used to photograph the micropreparations; TSView program was used to view photos.

Structural peculiarities and morphology of the spore-forming microorganisms were studied with transmission electron microscope JEM 1400 (Jeol, Japan) at the voltage of 80 kV and instrumental magnification of ×600–×10000. The formvar (Serva, Germany) covered copper grids (Sigma, USA) were used. 2 % uranyl acetate solution served as a contrastor.

The determination of cultural-morphological and physiologal-biochemical characteristics of isolated cultures was conducted according to the standard methods [4, 10, 11].

RESULTS AND DISCUSSION

Ten samples of enclosed brood cells, kindly provided by the Laboratory of Pathology of Bees, NSC "Institute of Beekeeping named after P. I. Prokopovych" NAAS of Ukraine, were analyzed.

In the samples Nos. 1, 2, 4, 5–8, 10 the dead brood was mainly in enclosed cells, in the samples Nos. 3 and 9 – in the open cells. The lids were of a dark color with a cone-like hole in the center. The brood was a dough-like, viscous mass with the smell of rot and yellowish-

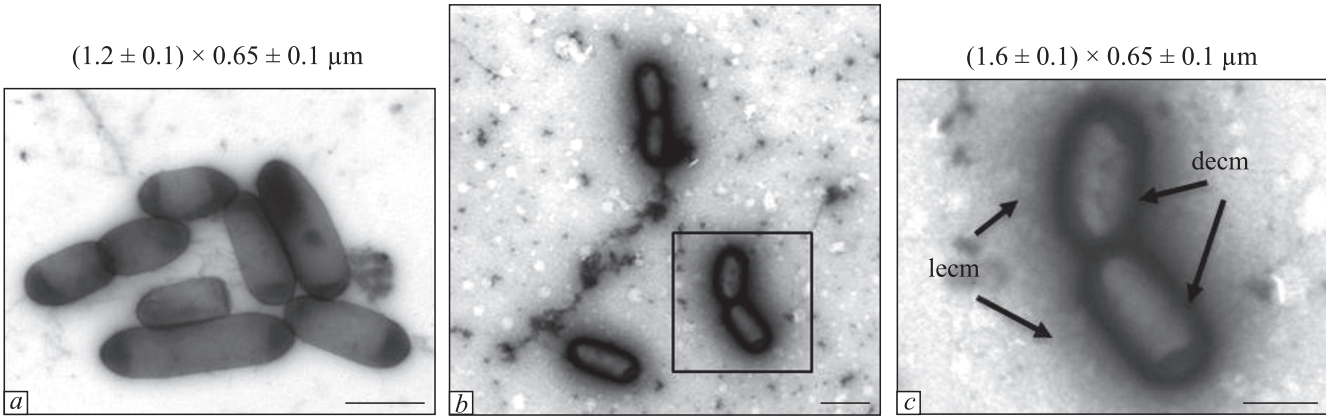

Fig. 3. The morphology of the bacterial strains isolated from the sample No. 1: (*a*) – strain 1.1; (*b* and *c*) – strain 1.2, where (*c*) is a detailed figure of the cell marked in the (*b*). Symbols: "lecm" – low density extracellular matrix, "decm" – dense extracellular matrix. The numbers on the (*a*) and (*c*) are the means ± Sd of the length and width of the cells within the populations of bacteria

gray liquid (Fig. 1, *b*, *d*). In the sample No. 4 the affected pupae were white, softened and had a putrefactive smell (Fig. 1 *a*, *c*). The content of pupae was a milky white liquid.

The studies have shown that the composition of the microbiota of different samples of combs differed among themselves. Yeasts were discovered only in two comb samples No.1 and No.4 (Fig. 2). The colonies were milky white with smooth edges, 1–5 mm in size, they neither fermented sucrose, mannitol, lactose, nor contained catalase.

It is known that yeasts can serve as producers of vitamin B in the intestines of bees [12].

At the same time, yeasts are often found in the bees that had monotonous diets based on artificial feed, or the presence of a monoculture of plants or ate nectar from plants, treated with pesticides, or in the bees, treated with antibiotics. So, the presence of yeasts in the intestines of bees can be an indicator of stress conditions from which the bees have suffered. There are the following representatives of the yeasts of bees: *Torulopsis magnoliae*, *T. glabrata, Candida parapsilopsis, Hansenula anomala* [13].

Eleven strains of aerobic bacilli-shaped spore-forming bacteria were isolated from nine samples. Most cultures on the same type of MPA medium formed colonies on the first day of cultivation – small whitish colonies, with straight edges, about 1 to 3 mm in size. Further cultivation for 7 days resulted in the formation of large flattened rough colonies with irregular edges, 8–10 mm in size. Some surface growth in the form of a thick film is observed on meat-peptone broth (MPB); eventually the film falls to the bottom.

The investigated spore-forming bacteria differed in size, nature and location after the division and by their biochemical characteristics. The cells had rounded ends, were placed separately, in pairs (strains isolated from samples No. 1, 2, 4, 9, 10) or formed chains (strains isolated from samples No. 5, 6). The size of the cells varied from (0.6–0.9) × 1–2 μm to (1.0–1.2) × 2–4 μm. The cultures were movable; they produced catalase, did not assimilate maltose, lactose, sucrose, and mannitol. The spores in all the selected cultures were oval, centrally placed and did not exceed the size of vegetative cells.

The data of electronic microscopy demonstrate that strain 1.2, isolated from sample 1, differs from other cultures by the presence of a clearly visible capsule around the cellular wall and active production of exometabolites (Fig. 3, *a*, *b*).

The results of light and electron microscopy, morphological features of the colonies, and biochemical research suggest that the strains, isolated from the samples No. 1, 2, 4–6, 9, 10 are representatives of the genus *Bacillus*.

Different types of spore-forming bacteria were detected in the intestines of bees, namely, *B. megaterium, B. subtilis, B. pumilus, B. licheniformis, B. circulans, B. alvei, B. coagulans, B. brevis, B. cereus, B. sphaericus, B. firmus, B. laterosporus, B. polymyxa*, which are their normal microbiota. We know that microbes can act as antagonists of *Ascosphaera apis*, which is the causative agent of chalk brood. Bacilli are the producers of antibiotic substances, fatty acids, enzymes and help to digest starch, proteins, sugars, and cellulose [14].

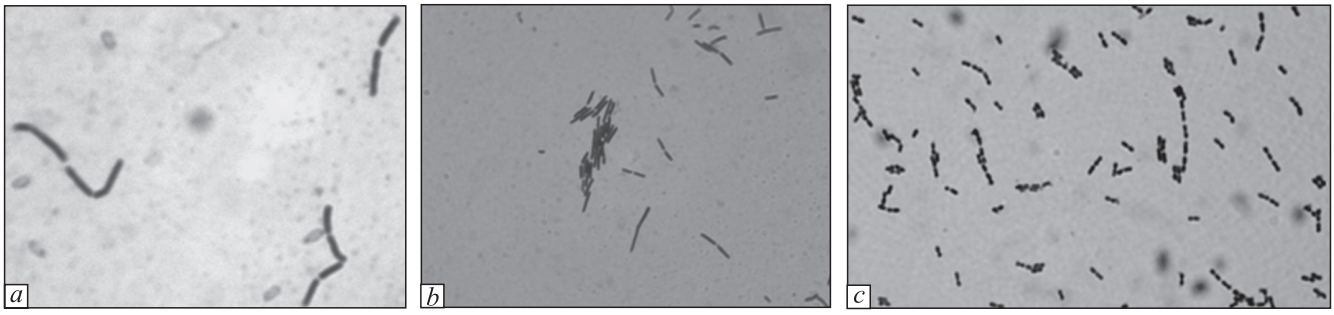

Fig. 4. The cultures of bacteria isolated from the samples: No. 3 (*a*), No. 7 (*b*), No. 8 (*c*). (Magnification × 1200)

Gram-positive spore-forming bacteria with a number of specific features were isolated from sample No. 3. The growth of microorganisms was observed after cultivating the washouts, heated to 98 °C, on the MPA medium, with the addition of serum and Tomashetsa. The culture did not grow on MPA medium only. On the third day of cultivation the formation of rough gray and yellow colonies with irregular edges was observed. The growth of culture was accompanied with the turbidity of culture medium with subsequent formation of the sediment on the MPB with the serum.

The microscopy of colonies, which grew up, demonstrated that isolated bacteria cell were (0.5–0.8) × × 2–3 μm in size and located in chains on the fixed slides (Fig. 4, *a*).

The culture fermented glucose with the release of the acid, did not ferment lactose, sucrose, galactose, arabinose, maltose, xylose, mannitol, sorbitol, dulcitol, and inositol.

The dilution of gelatin, peptonization of milk, absence of hemolytic and catalase activity were observed.

The results of immunochromatographic research confirmed our assumption about the presence of the pathogen of American foulbrood – *Paenibacillus larvae* – in sample No. 3.

Spore-forming bacteria, forming small colonies that grew slowly, were isolated from sample No. 7 on MPA medium with serum. Rough colonies with a gray tint, characterized by creeping growth, were observed on Tomashetsa medium and on MPA with serum. The precipitate was formed on the bottom of the tube on MPB culture.

The isolated microorganism was a Gram-positive bacillus with the size of (0.5–0.7) × 2.4–3.5 μm. The cells were placed separately or in pairs (Fig. 4, *b*). This microorganism did not ferment glucose, raffinose, mannitol, did not contain catalase and cysteine desulfhydrase, was unable to break down aromatic amino acids, was movable and had proteolytic activity, contained caseinase, collagenase, elastase, gelatynase, did not form zones of hemolysis on blood agar.

This microorganism is most likely to be *Bacillus paraalvei* – the pathogen of parafoulbrood of bees.

Gram-negative bacilli-shaped bacteria with pointed ends were separated from sample No. 8. The culture on agar medium formed yellow colonies (4–5 mm) with straight edges. The culture did not form any spores without assimilating lactose, sucrose, mannitol, did not contain cysteine desulfhydrase and did not break down aromatic amino acids.

In addition to bacilli-shaped bacteria and yeasts, cocci were also isolated from four infected combs on MPA medium. Gram-positive cocci were found in samples No. 3, 5, and 6. In fixed preparations they were located in clusters and resembled bunches of grapes. The bacteria were Gram-positive, assimilated maltose, lactose, sucrose, and mannitol, contained cysteine desulfhydrase, were motionless and contained elastase.

It is known that cocci are found in healthy bees. The representatives of such genera as *Staphylococcus*, *Stomatococcus, Micrococcus, Deinococcus* may be found in the intestines of bees [14].

Gram-positive cocci, growing only on the media Cherepova and Bailey, were isolated from sample No. 8. On these media the selected strain formed small (1 mm) white or transparent colonies with smooth edges. In the liquid medium the culture grew in the form of even turbidity and a wall ring. Gram-positive microorganisms were the lanceolate cocci with the size of 0.7–1.5 μm, which were placed separately, in chains of different length, or in clusters (Fig. 4, *c*).

The culture assimilated glucose and fructose with the formation of acid without gas. It did not ferment lactose, sucrose, galactose, maltose, raffinose, mannitol,

sorbitol, and inositol. It did not contain cysteine desulfhydrase, catalase, caseinase, collagenase, elastase, gelatinase, did not break down aromatic amino acids. The data suggest that the isolated strain may be the causative agent of European foulbrood, namely, *Melissococcus pluton*.

Therefore, visual, cultural-morphological, electron microscopic, and biochemical methods were used to isolate and previously identify microorganisms, namely, *Paenibacillus larvae* – pathogen of American foulbrood; *Bacillus paraalvei* – parafoulbrood pathogen; *Melissococcus pluton* – European foulbrood pathogen.

Most isolated cultures were represented with the aerobic spore-forming bacilli-form bacteria, which are not the pathogens of bees, but can occur as secondary microflora of viral and bacterial infections. Cocci were found in three samples of pathological material, which present normal microflora of bees, but in sick insects their number is growing significantly.

Особливості мікробіоти, виділеної зі стільників з ураженим розплодом бджіл

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Мета. Дослідити мікробіоту стільників з ураженим бджолиним розплодом. **Методи.** Використано візуальні, імунохроматографічні, культурально-морфологічні, біохімічні, електронно-мікроскопічні методи для виділення та попередньої ідентифікації мікроорганізмів. **Результати.** Проаналізовано 10 зразків стільників з запечатаним розплодом. Виділено та попередньо ідентифіковано збудників хвороб бджіл: *Paenibacillus larvae* − американського гнильця, *Bacillus paraalvei* – парагнильця, *Melissococcus pluton* − європейського гнильця. **Висновки.** Кількість виявлених у зразках стільників коків, спороутворювальних паличок і дріжджів, які зазвичай є нормальною мікрофлорою бджіл, достовірно збільшується при вірусних і бактерійних інфекціях.

Ключові слова: бактеріальні хвороби бджіл, мікробіота зі стільників з ураженим розплодом бджіл, *Paenibacillus larvae*, *Bacillus paraalvei*, *Melissococcus pluton.*

Особенности микробиоты, выделенной из сот с пораженным расплодом пчел

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Цель. Исследовать микробиоту сотов с пораженным пчелиным расплодом. **Методы.** Использованы визуальные, иммунохроматографические, культурально-морфологические, биохимические, электронно-микроскопические методы для выделения и предварительной идентификации микроорганизмов. **Результаты.** Проанализированы 10 образцов сотов с запечатанным расплодом. Выделены и предварительно идентифицированы возбудители болезней пчел: *Paenibacillus larvae* − американского гнильца, *Bacillus paraalvei* − парагнильца, *Melissococcus pluton* − европейского гнильца. **Выводы.** Количество выявленных в образцах сотов кокков, спорообразующих палочек и дрожжей, представляющих обычно нормальную микрофлору пчел, достоверно увеличивается при вирусных и бактериальных инфекциях.

Ключевые слова: бактериальные болезни пчел, микробиота из сотов с пораженным расплодом пчел, *Paenibacillus larvae, Bacillus paraalvei, Melissococcus pluton*.

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