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# FACTORS OF NON-SPECIFIC RESISTANCE OF BEE HEMOLYMPH WHEN FEEDING PROBIOTIC DRUG 'BILAKT'

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Summary. The paper presents the results of the feeding the 'Bilakt' probiotic on factors of non-specific resistance in bees. Hemolymph samples were taken on the 7th, 14th, and 21st days, and the activity of lysozyme and phagocytosis, bactericidal activity were determined. According to the research results, the lysozyme activity in the hemolymph of 3–5-day-old larvae and nurse bees of the experimental groups after 21 days was reliably 1.5 times higher than the control group and before feeding. The increase in the hemolymph bactericidal factor in 3–5-day-old larvae, and in nurse bees — threefold on the 21st day after the end of feeding with 'Bilakt' drug. Phagocytic activity before the beginning of the experiment in the hemolymph of 3–5-day-old larvae was 39.5%, on the 1st day after the end of feeding with 'Bilakt' it increased and exceeded this indicator by 29%. The phagocytic index before feeding was 2.04 ± 0.11. On the 1st day after the end of feeding, it increased by 18.4%, respectively. The phagocytic activity of hemolymph of nurse bees was 45.8%. Accordingly, the phagocytic index was 2.2 ± 0.12. On the 1st day after the end of 'Bilakt' feeding, phagocytic activity increased by 40.9%, the phagocytic index was 3.24 ± 0.1, which was 32.1% higher than the initial level. Research results indicate that the use of 'Bilakt' helps to improve the general physiological condition of sick bees by stimulating the cellular and humoral mechanisms of protection of insects from pathogens, i.e. increasing the non-specific protective properties of both the body of 3–5-day-old larvae and bee adults

Keywords: prevention, bactericidal activity, phagocytic activity

Introduction. Among the urgent tasks of veterinary support of beekeeping, the use of ecologically safe means for bee disease prevention, harmonized with EU requirements, is gaining importance. The search for effective biological agents that do not harm the bees and the quality of the products produced by them is the primary task of a veterinary specialist (Bugera, 2008; Fedoruk and Kovalchuk, 2013; Tran et. al., 2013).

In this regard, the use of probiotics deserves special attention in the system of bee disease prevention (Dvylyuk, 2013; Collins and Gibson, 1999; Galatiuk et. al, 2020). In honeybees, the gut contains a microbiome composed of various bacterial taxa that influence the stimulation of immune and metabolic pathways, digestion or detoxification of food, and defense against pathogens and parasites. Stressors, including toxins and poor nutrition, disrupt the microbiome and increase susceptibility to opportunistic pathogens (Motta et. al, 2022; Anderson et. al, 2013; Audisio and Benítez-Ahrendts, 2011; Corby-Harris et.al, 2014; Endo and Salminen, 2013).

The participation of the bee organism in limiting the spread of infectious agents depends not only on the efficiency of the immune response. It is also determined by non-specific resistance factors, which are functionally based on phagocytosis, stimulation of humoral defense mechanisms and are the most effective first barrier in the fight against the causative agent of the disease. Among humoral factors of natural resistance, an important role is played by the enzyme lysozyme, which, being adsorbed on the mucopeptide cell wall of a microorganism, splits them (Fedoruk et al., 2009). Lysozyme increases its activity in a short period of time after the disease-causing agent enters the body of insects and is one of the starters of other protective factors. Lysozyme is a relatively small protein molecule (about 15 kDa). Its concentration in the hemolymph of larvae and adults ranges from 5 to 25 mg/ml. The antibacterial response is carried out by increasing the activity of lysozyme. Thus, the life products of lactobacilli, representatives of the beneficial microflora of the bees' intestines, contribute to the increase of a complex of factors of non-specific resistance: the content of lysozyme, the phagocytic and bactericidal activity of the hemolymph of bees (Fedoruk et al., 2009; Glynski and Jarosz, 1993; Boman, 1982).

The purpose of the study. In this regard, this work aimed to determine the effect of the probiotic 'Bilakt', which includes lactic acid bacteria (LAB) from the genus *Lactobacillus*, as well as bacteria from the genus *Bifidobacterium*, on factors of non-specific resistance of bee hemolymph: bactericidal and phagocytic activity in general, and in particular lysozyme activity.

Materials and methods. The experiments were conducted in the Sector of Bee Disease Study and the research apiary of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. Research period: 3rd decade of May. One experimental and one control group of similar bee families-analogs (three in each) were formed. By the time the control and experimental groups were formed, the bee families had

4.0 kg of bees, 5.0 kg of fodder honey, a two-year-old queen, sealed brood on 3 frames (240 squares) and 2 frames with open brood.

The first group of bee families was the control. For feeding, these bee colonies were given sugar syrup (1:2), prepared in boiled water, at the rate of 50 ml per seam of bees (on average 2,000 adults), with an interval of 2 days, 4 times, using a feeder. The second group was an experimental one, feeding was carried out at the same time as in the control group, with the same frequency, with sugar syrup, but with the addition of 'Bilakt' at the rate of 9 $cm^3$ per 2.5 liters of syrup. 'Bilakt' drug contains microorganisms *Lactobacillus plantarum* and *Bifidobacterium* sp. in the amount of $1\times10^8$ microbial cells/$cm^3$, as well as the nutrient medium on which they were cultivated. The total duration of the experiment was 30 days from the moment of the start of feeding.

Bee viability monitoring (death, food activity, behavior) was mainly carried out visually. From each group of bees, 100–150 specimens from frames with open brood with the largest number of 5–15-day-old nurse bees and 3–5-day-old larvae were selected. Selection and examination of collective samples of hemolymph from each group of bees was carried out before the beginning of feeding the experimental concentrations, as well as 7, 14, and 21 days after the end of 'Bilakt' feeding.

Determination of lysozyme activity in hemolymph was carried out by the turbidimetric method. The *Micrococcus lysodeikticus* culture was prepared in a phosphate buffer, the collected samples of hemolymph were diluted 10 times with physiological solution. The activity of lysozyme in the hemolymph sample was calculated according to the calibration curve in μg/ml (Labynskaya, 1978).

The bactericidal activity of hemolymph was studied by the method of diffusion in agar (Labynskaya, 1978). Pathogenic for bees microorganisms were used as test cultures — foulbrood causative agents: American (*Paenibacillus larvae*), European (*Melissococcus pluton*), para foulbrood (*Paenibacillus alvei*). A culture of microorganisms at a concentration of 1 billion cells/$cm^3$ was placed on the surface of the agar in a Petri dish. The dishes were kept in a thermostat at a temperature of 37 °C for 2 hours. As a marker, wells with a diameter of 3–4 mm were made, into which samples of hemolymph were introduced. The results were calculated after 24, 48, 72 hours (Labynskaya, 1978).

The indicator of phagocytosis activity of hemolymph cells was determined according to the Berman method (Labynskaya, 1978). To assess completed phagocytosis, we determined the number of cells that successfully completed the process (including fragments of destroyed cells) per 100 cells involved in the phagocytosis process.

We processed the results of the experimental studies statistically using the MS Excel 2010 computer program. To determine the arithmetic mean (M), its error (m), and the level of probability (p), we referred to the Student's *t*-test table by Melnychenko et al. (2006).

This research adheres to bioethical norms and complies with various guidelines. Specifically, it was conducted in accordance with the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006).

Results and discussion. In the process of studying the effect of 'Bilakt' feeding on the body of bees, it was established that the hemolymph lysozyme activity indicators in the experimental group reliably increased in all individuals (Tabs 1, 2).

Table 1 — Hemolymph lysozyme activity of 3–5-day-old larvae (n = 30)

| Bee groups | Selection period, day | Lysozyme activity, μg/ml |
|---|---|---|
| Control | Before feeding | 42.20 ± 1.52 |
| | 7 | 41.97 ± 0.03 |
| | 14 | 41.67 ± 0.39 |
| | 21 | 41.83 ± 0.37 |
| Experimental | Before feeding | 48.39 ± 1.92 |
| | 7 | 64.00 ± 1.00* |
| | 14 | 60.70 ± 4.11* |
| | 21 | 64.00 ± 4.08* |

Note: * — $p < 0.05$ reliably the indicators of the control group and prior to the start of the feeding process.

Table 2 — Hemolymph lysozyme activity of nurse bees (n = 30)

| Bee groups | Selection period, day | Lysozyme activity, μg/ml |
|---|---|---|
| Control | Before feeding | 44.80 ± 1.90 |
| | 7 | 44.43 ± 0.47 |
| | 14 | 43.29 ± 0.89 |
| | 21 | 43.60 ± 1.30 |
| Experimental | Before feeding | 43.30 ± 0.79 |
| | 7 | 62.59 ± 2.40* |
| | 14 | 63.18 ± 2.40* |
| | 21 | 66.51 ± 3.10* |

Note: * — $p < 0.05$ reliably the indicators of the control group and prior to the start of the feeding process.

Based on the information provided in Table 1, it appears that the activity of lysozyme in the hemolymph of 3–5-day-old larvae in the experimental group increased by reliably 1.5 times after 7 days of consuming a mixture of 'Bilakt' and sugar syrup. This increase was also observed to be 1.4 times higher after 14 days and

1.5 times higher after 21 days, compared to the control group and the pre-feeding period.

According to the information presented in Table 2, it appears that the nurse bees in the experimental group exhibited a hemolymph lysozyme activity level that was reliably 1.4 times higher after being fed a mixture of 'Bilakt' and sugar syrup for 7 and 14 days, and 1.5 times higher after 21 days, in comparison to the control group and the pre-feeding period.

In the experimental group, it appears that the activity of lysozyme in the hemolymph of 3–5-day-old larvae increased by 34% after 7 days of not feeding, followed by a 31.4% increase after 14 days and a 34.7% increase after 21 days. Nurse bees also showed an increase in lysozyme activity, with a 29.1% increase on the 7th day, 31.5% — on the 14th day, and 34.5% — on the 21st day. However, there was no increase in lysozyme activity observed in bees from the control groups during the experiment.

The bactericidal activity of bee hemolymph was determined in the samples taken before the start of the experiment and 21 days after the end of feeding, it was established that the experimental parameters reliably increased in all individuals (Table 3).

Table 3 — Bactericidal activity of hemolymph of bees

| Bee groups (n=30) | Bactericidal factor, h | | | | | |
|---|---|---|---|---|---|---|
| | *P. larvae* | | *M. pluton* | | *P. alvei* | |
| | larvae | nurse bees | larvae | nurse bees | larvae | nurse bees |
| Pre-feeding period | 6 | 4 | 6 | 4 | 6 | 4 |
| Control | 6 | 4 | 6 | 4 | 6 | 4 |
| Experimental, 21st day | 12 | 12 | 12 | 12 | 12 | 12 |

From the data in Table 3, it can be seen that the bactericidal factor in the hemolymph of 3–5-day-old larvae doubled, and in nurse bees, it tripled on the 21st day after the end of feeding with 'Bilakt'.

Indicators of phagocytic activity of hemocytes of the hemolymph of 3–5-day-old larvae changed after feeding 'Bilakt'. The results are presented in Table 4.

From the data in Table 4, it can be seen that the phagocytic activity before the beginning of the experiment was 39.5%, on the 1st day after the end of feeding with 'Bilakt', it increased and exceeded this indicator by 29%, on the 7th day — 30.5%, on the 14th day — 27.8%, and on the 21st day — 27%.

The phagocytic index before feeding was 2.04 ± 0.11, on the 1st day after the end of feeding it increased by 18.4%, on the 7th, 14th, and 21st days it decreased by 2.0%, 2,8%, and 4.0%, respectively.

The dynamics of indicators of phagocytic activity and the phagocytic index of the hemolymph of nurse bees are presented in Table 5.

Table 4 — Indicators of phagocytic activity of hemolymph of 3–5-day-old larvae (n=30)

| Period | Indicators | | | | |
|---|---|---|---|---|---|
| | Total number of hemocytes, pcs. | Hemocytes that phagocytized microbes, pcs. | Number of microbial cells, pcs. | Phagocytic activity,% | Phagocytic index |
| Pre-feeding | 253 | 100 | 204 | 39.5 | 2.04 ± 0.11 |
| After feeding with the drug 'Bilakt' after days: | | | | | |
| 1 | 180 | 100 | 250 | 55.6 | 2.50 ± 0.14 |
| 7 | 176 | 100 | 240 | 56.8 | 2.45 ± 0.14 |
| 14 | 183 | 100 | 245 | 54.6 | 2.43 ± 0.12 |
| 21 | 185 | 100 | 243 | 54.0 | 2.40 ± 0.12 |

Table 5 — Indicators of phagocytic activity of the hemolymph of nurse bees (n = 30)

| Period | Indicators | | | | |
|---|---|---|---|---|---|
| | Total number of hemocytes, pcs. | Hemocytes that phagocytized microbes, pcs. | Number of microbial cells, pcs. | Phagocytic activity,% | Phagocytic index |
| Pre-feeding | 218 | 100 | 220 | 45.8 | 2.2 ± 0.12 |
| After feeding with the drug 'Bilakt' after days: | | | | | |
| 1 | 129 | 100 | 324 | 77.5 | 3.24 ± 0.10 |
| 7 | 133 | 100 | 307 | 75.2 | 3.07 ± 0.11 |
| 14 | 150 | 100 | 290 | 66.7 | 2.90 ± 0.12 |
| 21 | 175 | 100 | 282 | 57.1 | 2.82 ± 0.12 |

At the beginning of the experiment, phagocytic activity was 45.8%. Accordingly, the phagocytic index was 2.2 ± 0.12. On the 1st day after the end of 'Bilakt' feeding, phagocytic activity increased by 40.9%, the phagocytic index was 3.24 ± 0.1, which is 32.1% higher than the initial level. On the 21st day, these indicators decreased by 26.3% and 13%, respectively.

Our research findings suggest that 'Bilakt' positively impacts the overall physiological state of ill bees by enhancing their cellular and humoral defense mechanisms against pathogens, thereby in an increase in the non-specific protective features of both adult bees and 3–5-day-old larvae.

Conclusions. It was established that the use of the drug 'Bilakt' contributed to the stimulation of non-specific resistance of 3–5-day-old larvae and nurse bees. According to the research results, the activity of lysozyme in the hemolymph of 3–5-day-old larvae and nurse bees

of the experimental groups after 21 days was reliably 1.5 times higher compared to the control group and the pre-feeding period.

Bactericidal factor of the hemolymph of 3–5-day-old larvae doubled, and in nurse bees — tripled on the 21st day after the end of feeding with 'Bilakt'. Phagocytic activity before the beginning of the experiment in the hemolymph of 3–5-day-old larvae was 39.5%, on the 1st day after the end of feeding with 'Bilakt', it increased and exceeded this indicator by 29%.

The phagocytic index before feeding was 2.04 ± 0.11, on the 1st day after the end of feeding it increased by 18.4%, respectively. The phagocytic activity of hemolymph of nurse bees was 45.8%. Accordingly, the phagocytic index was 2.2 ± 0.12. On the 1st day after the end of 'Bilakt' feeding, phagocytic activity increased by 40.9%, the phagocytic index was 3.24 ± 0.1, which is 32.1% higher than the initial level.

Prospects for further research. It is promising to conduct further tests on the drug 'Bilakt' due to its high physiological activity. Additionally, its use in beekeeping during the spring growth and development of bee families shows potential in preventing bacterial and fungal diseases among bees.

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