sciendo

DOI: 10.2478/fv-2023-0005

FOLIA VETERINARIA, 67, 1: 45—50, 2023

TESTING OF THE EFFICACY OF BEE PROBIOTIC LACTOBACILLI UNDER *IN VIVO* CONDITIONS

Kuzyšinová, K., Mudroňová, D., Toporčák, J., Molnár, L.
Clinic of Birds, Exotic and Free Living Animals, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia

kuzysinova@gmail.com

ABSTRACT

American foulbrood is amongst the most dangerous diseases of the bee-family affecting many honeybee colonies worldwide. In countries of European Union, based on veterinary legislation, the bee colonies tested positive to American foulbrood are eradicated with high economic losses. It is therefore necessary to look for effective prevention, especially by the using of natural ingredients such as probiotics. In this study, we used lactobacilli isolated from digestive tracts of adult healthy honey bees and selected based on their good probiotic properties and ability to inhibit the growth of *Paenibacillus larvae*. These isolates were identified as *Lactobacillus brevis* and *Lactobacillus plantarum*. Night cultures of both strains were used for the preparation of probiotic suspensions and pollen was selected as an appropriate carrier for application of probiotic lactobacilli to the hives. Half a litre of pollen suspension was prepared for each hive. The suspension for experimental hives contained probiotic lactobacilli in concentration of 10^7–10^8 CFU.ml^{-1}. Bees in control hives received pollen suspension without addition of probiotic lactobacilli. The suspensions were supplied to bees three times, once a week. Before and during administration of probiotics, samples of honey bees from each hive were taken every week and numbers of lactobacilli, *P. larvae*, enterobacteria and coliform bacteria were determined in their digestive tracts. Four weeks after the first administration of probiotic-pollen solution the numbers of lactobacilli were increased approximately by 0.5 log. Before starting this experiment, *P. larvae* (approx. 10^7 CFU.ml^{-1}) were detected in digestive tracts of honey bees. In the experimental group after 3 weeks and in the control group after 4 weeks, no viable counts of *P. larvae* were found. The numbers of enterobacteria, coliform bacteria and *Bacillus* sp. decreased in both groups. During the experiment the health and condition of the hives were monitored. In addition, also monitored were: the development of the bee colony, the number of dead bees, the amount of hive debris, the aggressiveness of the bees, and the amount of honey spun; later during the autumn treatment also the fall of *Varroa jacobsoni* was noted. In the experimental and control groups, we recorded a decrease in the amount of hive debris. Only in the experimental group was a slightly above-average development of the bee colony, slightly above-average honey yields and a 70 % lower drop of *Varroa mites*. Based on these results, we assumed that the probiotic-pollen solution had a positive influence on the composition of microbiota in bee digestive tracts and it can increase resistance to *P. larvae*. It also had a positive effect on the health and condition of the bee colony. Probiotic-free pollen solution showed similar but weaker effects.

Key words: administration; condition; microbiota; *Paenibacillus larvae*; probiotics

INTRODUCTION

The American foulbrood is one of the most serious diseases of honey bees, resulting in high mortality rates, being spread worldwide and causing major economic losses. It has been classified as a disease subject to the reporting obligation in terms of the OIE list of transmissible diseases. The disease is caused by the Gram-positive rod-shaped bacterium, *Paenibacillus larvae* [2]. Administration of antibiotics is of minor effect as the antibiotics only affect vegetative forms of *P. larvae*, while the spores continue to spread. Moreover, residues of these drugs remain in bee products, primarily in honey, contributing to the increasing antibiotic resistance of pathogens. These are the reasons why the use of antibiotics against American foulbrood has been prohibited under the European Union legislation. Infection by *P. larvae* is dealt with in a radical way – by eradicating the hives and all the combustible material by burning. Therefore, it is necessary to find an effective method of prevention or therapy of this disease. Excellent possibilities are offered by probiotics. Probiotic bacteria are defined as live microorganisms which, when administered to a host in adequate amounts, confer them health benefits [8]. These bacteria are common commensals of the gut and other body surfaces in humans, animals and insects [10]. Insects have different components to their immune system. It consists of 2 parts, social and individual immunity, both parts are connected [4]. The social defence is based on the cooperation of thousands of individuals to combat parasites or other pathogens (cleaning of colonies and immobilization of pathogens). Individual immunity consists of cellular and humoral immunity [7].

This study deals with the testing of administration of probiotic lactobacilli in bee hives to monitor their impact on the composition of intestinal microbiota of honey bees and condition of hives (the development of the bee colony, the number of dead bees, the amount of hive debris, the aggressiveness of the bees, the amount of honey spun and the drop of *Varroa* mites in autumn).

MATERIALS AND METHODS

Probiotic lactobacilli

Lactobacillus brevis B50 BiocenolTM (CCM 8618) and *Lactobacillus plantarum* P12 used for this experiment were isolated from the digestive tracts of adult healthy honey bees. Both strains were identified by rep-PCR and MALDI TOF-MS method. These strains showed in previous studies positive growth and probiotic properties (good growth properties, ability of autoaggregation, production of organic acids, ability to survive storage at –20 °C for long time) and ability to inhibit the growth of *P. larvae* [12]. The night cultures of both strains were cultured in MRS broth (Merck) at 37 °C under aerobic conditions. These cultures were 3 times washed in physiological solution by centrifugation at 600 × g for 30 min. The sediment was re-suspended in autoclaved tap water and concentration of lactobacilli were adjusted to 10^7–10^8 CFU.ml^{-1}.

Probiotic-pollen suspension

For each application and each experimental hive there was prepared half of a litre of probiotic-pollen suspension composed of 50 g of pollen and probiotic lactobacilli culture. For control hives was prepared half of a litre of pollen suspension composed of 50 g of pollen in autoclaved tap water without addition of probiotic lactobacilli.

Design of the experiment

Six bee colonies (3 experimental and 3 control) were used for the experiment. Probiotic-pollen suspensions were administered to the experimental hives and honey bees in control hives received pollen suspensions without probiotic lactobacilli. The solutions were administered to honey bees three times, once a week (Fig. 1).

Analysis of intestinal microbiota of honey bees

Before and during administration of suspensions, approximately 30 bees from each of colony were taken every week. Digestive tracts (intestine, stomach and rectal sack) were used for the evaluation of microbiota composition. Numbers of bacteria were determined by plating on selective media after ten-fold dilution.

Lactobacilli were cultured in MRS agar (Oxoid, Basingstoke, UK) for 48 h at 37 °C under anaerobic conditions (Gas Pak Plus, BBL). *Paenibacillus larvae* were grown in MYPGP agar (yeast extract 15 g, K_2HPO_4 3 g, sodium

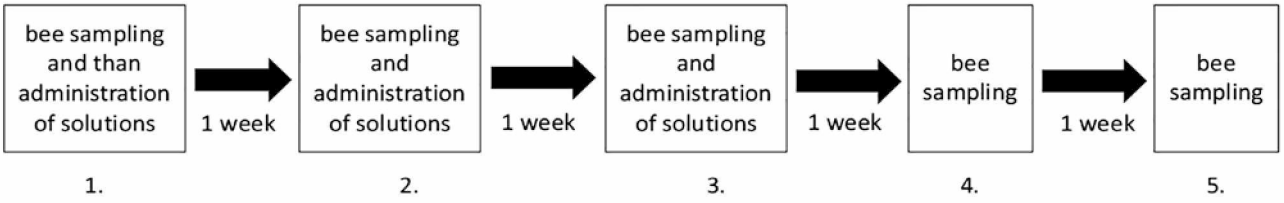

Fig. 1. Experimental design

pyruvate 1 g, D(+) glucose 2 g.l^{-1}, agar 20 g.l^{-1}, pH 7.2) for 48 h at 37 °C. Endoagar (Oxoid, Basingstoke, UK) was used for the cultivation of *Enterobacteriaceae*, Mc Conkey agar (BBL, Becton Dickinson, Cockeysville, USA) for cultivation of coliform bacteria and DCP (dextrose casein peptone) agar (10 g enzymatic casein hydrolysate, 5 g glucose, 0,04 g bromcresol red, 12 g.l^{-1} agar, pH 6.8 ± 0.2) for cultivation of *Bacillus* sp. They were incubated for 24 h at 37 °C.

Clinical examination of honey bee colonies

Throughout the experiment, the health and condition of the bee colonies was monitored, including the development of the bee colony, the number of dead bees, the amount of hive debris, the aggressiveness of the bees, the amount of honey spun, later during the autumn treatment also the drop of *Varroa* mites.

RESULTS

No problems with intake of probiotic-pollen or pollen suspensions were recognized during the study.

Analysis of intestinal microbiota of honey bees

After administration of probiotic-pollen suspension the counts of lactobacilli showed a tendency to increase during the experiment. In two last samplings the counts of lactobacilli were significantly higher in the experimental colonies compared to the control ones (Fig. 2).

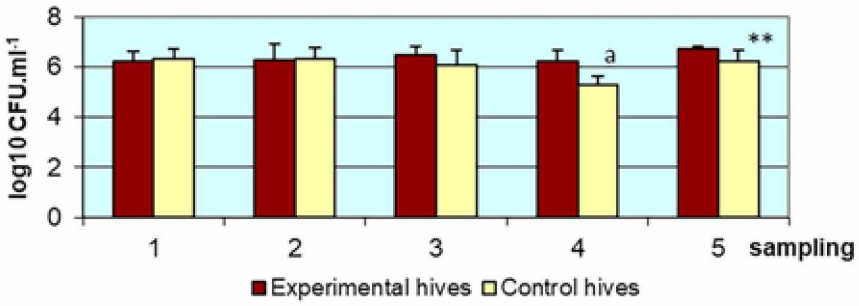

Fig. 2. The counts of lactobacilli expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

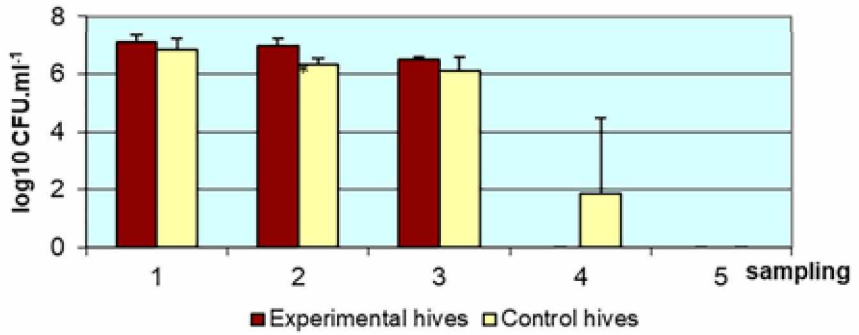

Fig. 3. The counts of *Paenibacillus larvae* expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

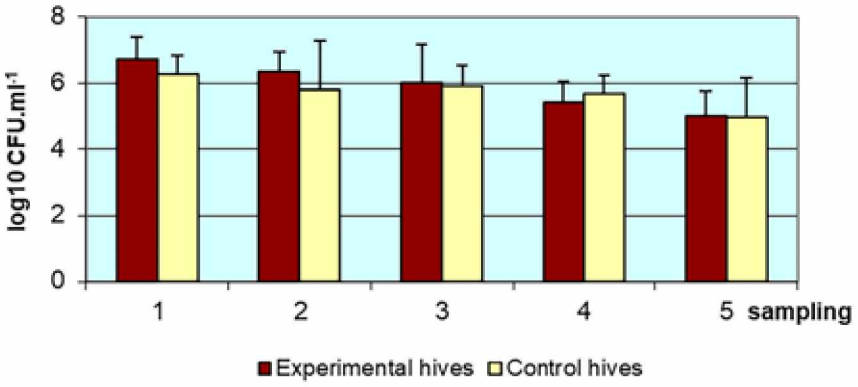

Fig. 4. The counts of *Bacillus* sp. expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

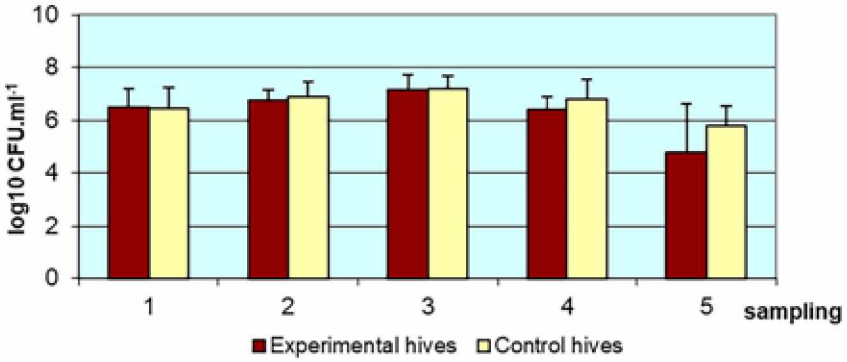

Fig. 5. The counts of coliform bacteria expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

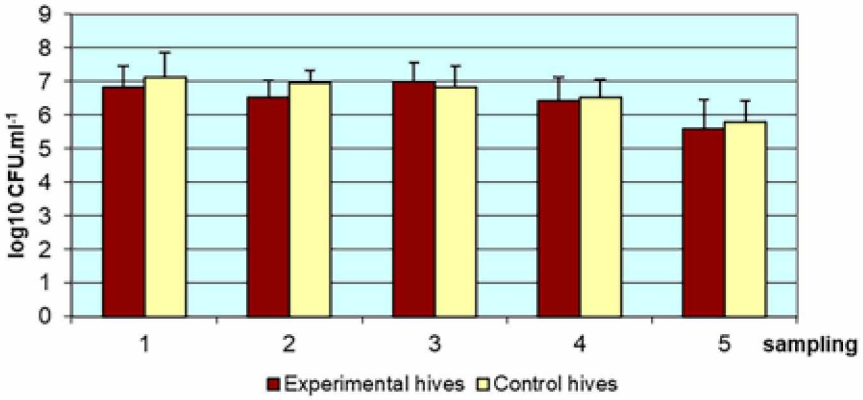

Fig. 6. The counts of Enterobacteriaceae expressed as log10 CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen suspension

Before starting the experiment, *P. larvae* (in concentration approximately 10^7 CFU.ml^{-1}) were found in the digestive tracts of honey bees. After three administrations of probiotic-pollen suspension, *P. larvae* completely disappeared from the digestive tract. After three administrations of pollen suspension to control colonies, the counts of *P. larvae* were significantly reduced ($P < 0.001$) in comparison with the 3rd sampling and at the last sampling there were detected no viable *P. larvae* (Fig. 3).

Before starting the experiment, the counts of *Bacillus* sp. in the digestive tracts of honey bees were between 10^6–10^7 CFU.ml^{-1}. The counts in experimental groups were higher than in control groups, but not significantly. The counts gradually decreased during the experiment,

in experimental groups the decline was more pronounced compared to control groups, but the difference was insignificant (Fig. 4).

During the experiment, the counts of coliform bacteria and *Enterobacteriaceae* decreased by 1–1.5 log after administration of probiotic-pollen and pollen suspensions by 1–1.5 log, but no significant differences between control and experimental groups were observed (Fig. 5 and 6).

Clinical examination of honey bee colonies

Based on the clinical examinations of the hives during the experiment, we concluded that the administered experimental suspensions did not have a negative effect on the hives. On the contrary, a decrease in the amount of hive debris was recorded in both the experimental and control groups, which indicated an intensification of the cleaning instinct of the bees, which was directly proportional to the condition of the bee colony. Only in the experimental group there was noted a slightly above-average development of the bee colony, slightly above-average honey yields and, surprisingly, by up to 70 % lower drop of *Varroa* mites (Table 1).

Table 1. Clinical examination of honey bee colonies

	Control group	Experimental group
Amount of hive debris	below average	almost none
Development of the bee colony	average	above average
Honey yields	average	above average
Drop in *Varroa* mites	average	up to 70 % lower

DISCUSSION

Probiotics represent one of the promising possibilities for replacement of antibiotics in prevention and therapy of many diseases in animals and people. Our results indicated the ability of *L. brevis* B50 BiocenolTM (CCM8618) and *L. plantarum* P12 to inhibit the growth of *P. larvae*. They stabilize the microbiota of the digestive tract of honey bees. Similar conclusions were presented in the study by Canganella and Balsamo [5], who demonstrated the inhibitory effect of lactobacilli against *P. larvae* and *P. alvei* (European foulbrood). Forsgren et al. [9] also observed the ability of lactobacilli and bifidobacteria isolated from the stomach of honey bees to inhibit the growth of *P. larvae*. They administered such lactobacilli to bee larvae through the mother bread and noted significantly lower numbers of infected larvae. A number of authors [1, 3] confirmed the antagonistic action of different bacterial species against *P. larvae*. The gut microbiota of honey bees was shown to have low diversity, and LAB represent an important part of it [11]. The normal bee microbiota is involved in the breakdown and utilization of pollen, degradation of environmental toxic compounds, or activation of bee immune system to prevent colonization of pathogenic microorganisms [6]. Colonisation of the gut by beneficial microbiota can prevent a proliferation of spores *P. larvae* and thus prevent infection. Administration of pure pollen without addition of probiotic lactobacilli also showed a positive effect on the composition of intestinal microbiota of honey bees and contributed to elimination of *P. larvae* from their digestive tract. Pollen can stimulate the immune response of honey bees, because it naturally contains lactobacilli, nutrients which increase the food quality, and some immunostimulating substances (e. g. vitamins, fatty acids. enzymes, etc.). Pollen is given to bee larvae from the third day of life. The greatest content of pollen is in the food given to drones larvae. Therefore, they are more resistant to infection of *P. larvae* than the worker bees larvae. Queen larvae receive the least amount of pollen in their diet and are therefore the most susceptible to disease [13, 14].

CONCLUSIONS

We observed a positive effect of administration of probiotic-pollen or pollen suspension to investigated honey bees reflected in the following:

- honey bees willingly drank the probiotic-pollen and pollen solutions,
- *P. larvae* disappeared from digestive tract of honey bees after administration of the tested solutions,
- the counts of *Enterobacteriaceae*, coliform bacteria and *Bacillus* sp. were reduced by 1–1.5 log,
- a decrease in the amount of hive debris was recorded,
- only in the experimental group there was observed a slightly above-average development of the bee colony, slightly above-average honey yields and 70 % drop in *Varroa jacobsoni*.

ACKNOWLEDGEMENTS

This study was supported by the project VEGA No. 1/0454/22.

REFERENCES

1. **Al-Ghamdi, A., Ali Khan, K., Javed Ansari, M., Almasaudi, S. B., Al-Kahtani, S., 2018:** Effect of gut bacterial isolates from Apis mellifera jemenitica on *Paenibacillus larvae* infected bee larvae. *Saudi J. Biol. Sci.*, 25, 383–387. DOI: 10.1016/j.sjbs.2017.07.005.
2. **Alippi, A. M., Aguilar, O. M., 1998:** Unique DNA fingerprint patterns of *Paenibacillus larvae* subsp. larvae strains. *J. Apicult. Res.*, 37, 4, 273–280. DOI: 10.1080/00218839.1998.11100983.
3. **Alippi, A. M., Reynaldi, F. J., 2006:** Inhibition of the growth of *Paenibacillus larvae*, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore forming bacteria isolated from apiarian sources. *J. Invertebr. Pathol.*, 91, 141–146. DOI: 10.1016/j.jip.2005.12.002.
4. **Bzdil, J., Toporčák, J., Bilíková, K., 2016:** *Immune System of Honey Bees* (In Slovak). University of Veterinary Medicine and Pharmacy in Košice, Slovakia, 88 pp.
5. **Canganella, F., Balsamo, R., 2008:** Isolation and selection of probiotic microorganisms with antagonistic activities against *Paenibacillus larvae* and *Paenibacillus alvei*. In *International Probiotic Conference, Probiotics for the 3rd Millenium,* June 4–7, 2008, the High Tatras, Slovakia, 28–29.
6. **Engel, P., Martinson, V. G., Moran, N. A., 2012:** Functional diversity within the simple gut microbiota of the honey bee. *Proc. Natl. Acad. Sci. U.S.A*, 109, 11002–11007. DOI: 10.1073/pnas.1202970109.
7. **Evans, J. D., Spivak, M., 2010:** Socialized medicine: Individual and communal disease barriers in honey bees. *J. Invertebr. Pathol.*, 103, 62–72. DOI: 10.1016/j.jip.2009.06.019.
8. **FAO/WHO, 2002:** *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food*. FAO/WHO, London, UK, 35–45.
9. **Forsgren, E., Olofsson, T. C., Vasquez, A., Fries, I., 2010:** Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. *Apidologia*, 41, 99–108. DOI: 10.1051/apido/2009065.
10. **Mathialagan, M., Johnson Thangaraj Edward, Y. S., David, P. M. M., Senthilkumar, M., Srinivasan, M. R., Mohankumar, S., 2018:** Isolation, characterization and identification of probiotic lactic acid bacteria (LAB) from honey bees. *Int. J. Curr. Microbiol. App. Sci.*, 7, 894–906. DOI: 10.20546/ijcmas.2018.704.096.
11. **Moran, N. A., Hansen, A. K., Powell, J. E., Sabree, Z. L., 2012:** Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLOS ONE*, 7, 36393. DOI: 10. 1371/journal.pone.0036393.
12. **Mudroňová, D., Rumanovská, K., Toporčák, J., Nemcová, R., Gancarčíková, S., Hajdučková, V., 2011:** Selection of probiotic lactobacilli designed for the prevention of American foulbood. *Folia Veterinaria*, 55, 4, 127–132.
13. **Riessberger-Gallé, U., Von Der Ohe, W., Crailsheim, K., 2001:** Adult honeybee's resistance against *Paenibacillus larvae* larvae, the causative agent of the American foulbrood. *J. Inverteb. Pathol.*, 77, 4, 231–236. DOI: 10.1006/jipa.2001.5032.
14. **Rinderer, T. E., Rothenbuhler, W. C., Gochnauer, T. A., 1974:** The influence of pollen on the susceptibility of honey bee larvae to *Bacillus larvae*. *J. Inverteb. Pathol.*, 23, 3, 347–350. DOI: 10.1016/0022-2011(74)90100-1.

Received November 4, 2022
Accepted January 12, 2023