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Influence of a probiotic on the mortality, sugar syrup ingestion and infection of honeybees with Nosema spp. under laboratory assessment

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After the withdrawal of fumagillin, there is no effective drug against Nosema disease in the EU. Therefore, intensive research is conducted in order to find new nosemacides. Probiotic microorganisms compete with pathogenic microbes in the gastrointestinal tract. This competition involves adhesion to the intestinal epithelium, which leads to greater availability and utilisation of nutrients.

The aim of this study was to determine the effect of a probiotic supplement in the apian diet on the mortality and food ingestion of honeybees, and especially on the course of Nosema spp. infestation.

In experiments 1 and 2, the addition of the probiotic caused an increase in the Nosema spp. infection in the summer and winter honeybees. A special probiotic dedicated to the apian diet should be developed.

Keywords: probiotic, laboratory test, sugar syrup, bee mortality, Nosema disease, Apis mellifera

After the withdrawal of fumagillin, there is no effective drug against Nosema disease in the European Union (EU). Therefore, intensive research is conducted in order to find new nosemacides. Two *Nosema* species that infest honeybees (*Apis mellifera*), i.e. *Nosema apis* and *Nosema ceranae*, belong to the phylum microsporidia in the kingdom fungi. *Nosema* spp. spores can be identified and distinguishes by molecular analyses or under electron microscopy (6, 8, 12, 15, 17, 20). *Nosema* spp. are also regarded as one of the causative agents of Colony Collapse Disorder (CCD) (6, 10).

Since 2006, the use of antibiotics as inhibitors has been prohibited in the EU husbandry. Therefore, natural substances have been recommended for improvement of animal vitality and health. Probiotics are representatives of such agents. The World Health Organization defines probiotics as "live microorganisms that exert a beneficial health effect on the host organism when applied in appropriate amounts" (11). Health-promoting properties of fermented dairy products were already known in antiquity. Later, probiotic bacteria were shown to help seal intestinal walls (4). Probiotic microorganisms compete with pathogenic microbes in the gastrointestinal tract. This competition involves adhesion to the intestinal epithelium, which leads to greater availability and utilisation of nutrients. In addition, probiotic microorganisms produce substances that inactivate pathogens, e.g. organic acids and antibiotics. These substances reduce the pH of gastric contents, which inhibits the development of some pathogens, e.g. bacteria and fungi (5, 18). Probiotic microorganisms are represented by bacteria from the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, etc. (13).

Apidological investigations showed that the addition of a probiotic to pollen substitutes stimulated the development of pharyngeal glands and the fat body, as well as increased protein utilisation (26). Szymaś et al. (27) did not find harmful effects after the application of a probiotic in bees. They observed that honeybees ingested higher amounts of food when it was supplemented with the probiotic. This suggests that probiotics may prove useful in the treatment of Nosema disease and could become very important for veterinary practice in apiculture.

The honeybee has become a useful model for investigations of the physiological/genetic effects of food supplementation in both insects and mammals (14, 21-26). Therefore, we decided to use *A. mellifera* in this study, as well.

The aim of the study was to determine the effect of a probiotic supplement in the apian diet on the mortality and food ingestion of honeybees, and especially on the course of *Nosema* spp. infestation.

Material and methods

The experiments were conducted on worker bees (*A. mellifera*) originating from one mother queen. They populated one colony free of *Nosema* spp., which was confirmed by the PCR technique. The primers selected to differentiate between the two *Nosema* species were 321-APIS for *N. apis* and 218-MITOC for *N. ceranae* (12, 15, 16).

Summer bees were collected in May, and winter bees, at the end of August. The following protocol was used: a single comb with brood on the 20th day of development was placed in an air-conditioned chamber and kept at a constant temperature and humidity (36°C, 65% RH) for 1 day. The just emerged, 1-day-old workers were sampled from the comb and placed in wooden cages, as described by Borsuk (1, 2). All bees were feed with sugar syrup (1 : 1) until the second day of the experiment.

In experiment 1, performed on winter bees in one replicate, three groups were formed:

- control (C) – the bees were fed with pure sugar water syrup at the proportion of one part of sugar to one part of water (1 : 1);
- infested and untreated (Inf UT) – from the 3rd to the 4th day of the experiment, the bees were fed with sugar water syrup (1 : 1) prepared with water containing 8×10^6 Nosema spp. spores in one litre of the syrup. After the 4th day, the bees were fed with pure sugar water syrup (1 : 1);
- infested and treated with 0.5 µl probiotic (Inf TP 0.5) – from the 3rd to the 4th day of the experiment, the bees were fed with sugar water syrup (1 : 1) prepared with water containing 8×10^6 Nosema spp. spores in one litre of the syrup. After the 4th day, the bees were fed with sugar water syrup (1 : 1) with the addition of 0.5 µl probiotic/1 ml of the syrup.

In experiment 2, performed on both summer and winter bees in one replicate, four groups were created:

- control (C) – the bees were fed with sugar water syrup (1 : 1);
- infested and untreated (Inf UT) – from the 3rd to the 4th day of the experiment, the bees were fed with sugar water syrup (1 : 1) prepared with water containing 8×10^6 Nosema spp. spores in one litre of the syrup. After the 4th day, the bees were fed with pure sugar water syrup (1 : 1);
- infested and treated with 0.5 µl probiotic (Inf TP 0.5) – from the 3rd to the 4th day of the experiment, the bees were fed with sugar water syrup (1 : 1) prepared with water containing 8×10^6 Nosema spp. spores in one litre of the syrup. After the 4th day, the bees were fed with sugar water syrup (1 : 1) with the addition of 0,5 µl probiotic/1 ml of the syrup;
- infested and treated with 1.5 µl probiotic (Inf TP 1.5) – from the 3rd to the 4th day of the experiment, the bees were fed with sugar water syrup (1: 1) prepared with water containing 8×10^6 *Nosema* spp. spores in one litre of the syrup. After the 4th day, the bees were fed with pure sugar water syrup (1 : 1) with the addition of 1.5 µl probiotic/1 ml of the syrup.

The probiotic used in the experiments was approved for sale under the veterinary identification number αPL 0614002p. It was composed of *Lactobacillus casei*, *Lactobacillus plantarum* – 5.0×10^6 units/ml; *Saccharomyces cerevisiae* – 5.0×10^6 units/ml; *Rhodopseudomonas palustris* – abundant in 1 ml (cane molasses). This probiotic is recommended for animals.

Each experimental group consisted of 12 cages with 50 workers per cage. Dead workers were removed daily from each cage. Microscopic samples were made from dead worker bees in order to count *Nosema* ssp. spores in five vision fields of the Bürker chamber (7, 9).

The results were statistically analysed with the SAS software (SAS Institute 2002-2003 SAS/STAT User's Guide Version 9.13, Cary, NC, Statistical Analysis System Institute). The one-way ANOVA (a group effect was the experimental factor) and Tukey's HSD (honestly significant difference) test (19).

Results and discussion

In experiment 1, winter bees infested with *Nosema* spp. and ingesting the probiotic at a dose of 0.5 µl/1ml (Inf TP 0.5) of the syrup exhibited a slightly increased mortality rate compared with the bees in the control group (Fig. 1). The sugar syrup supplemented with the probiotic was consumed by the bees more willingly, which was consistent with the results obtained by Szymaś (27).

In experiment 2, winter bees infested with *Nosema* spp. and ingesting the probiotic at doses of 0.5 and 1.5 µl/1 ml (Inf TP 0.5 and Inf TP 1.5) of the syrup exhibited the lowest mortality rate. The addition of the probiotic at a dose of 1.5 µl/1 ml syrup resulted in a decline in syrup consumption by winter bees, which contradicts the results obtained by Chorbiński and Szymaś (3, 27).

In experiments 1 and 2, the addition of the probiotic caused an increase in *Nosema* spp. infection in summer and winter honeybees (Tab. 1). The increase in *Nosema* spp. infection was probably related to the reduced pH in the midgut of the bees, which resulted from consumption of the probiotic (5, 18). Probiotic substances reduce the pH of gastric contents, which inhibits the development of some pathogens (5, 18), but promotes the development of *Nosema* spp. (15).

Tab. 1. Number of *Nosema* spp. spores in infested bees [mln]

Group	Experiment 1 – winter bees	Experiment 2 – summer bees	Experiment 2 – winter bees
Control (C)	0[a]	0[a]	0[a]
Infested with *Nosema* spp. and untreated (Inf UT)	16[b]	9[b]	25[b]
Infested with *Nosema* spp. and treated with 0.5 µl probiotic (Inf TP 0.5)	36[c]	63[c]	35[c]
Infested with *Nosema* spp. and treated with 1.5 µl probiotic (Inf TP 1.5)	–	67[d]	43[d]

Explanation: a, b, c, d – different letters in columns indicate statistically significant differences between the groups ($p < 0.05$)

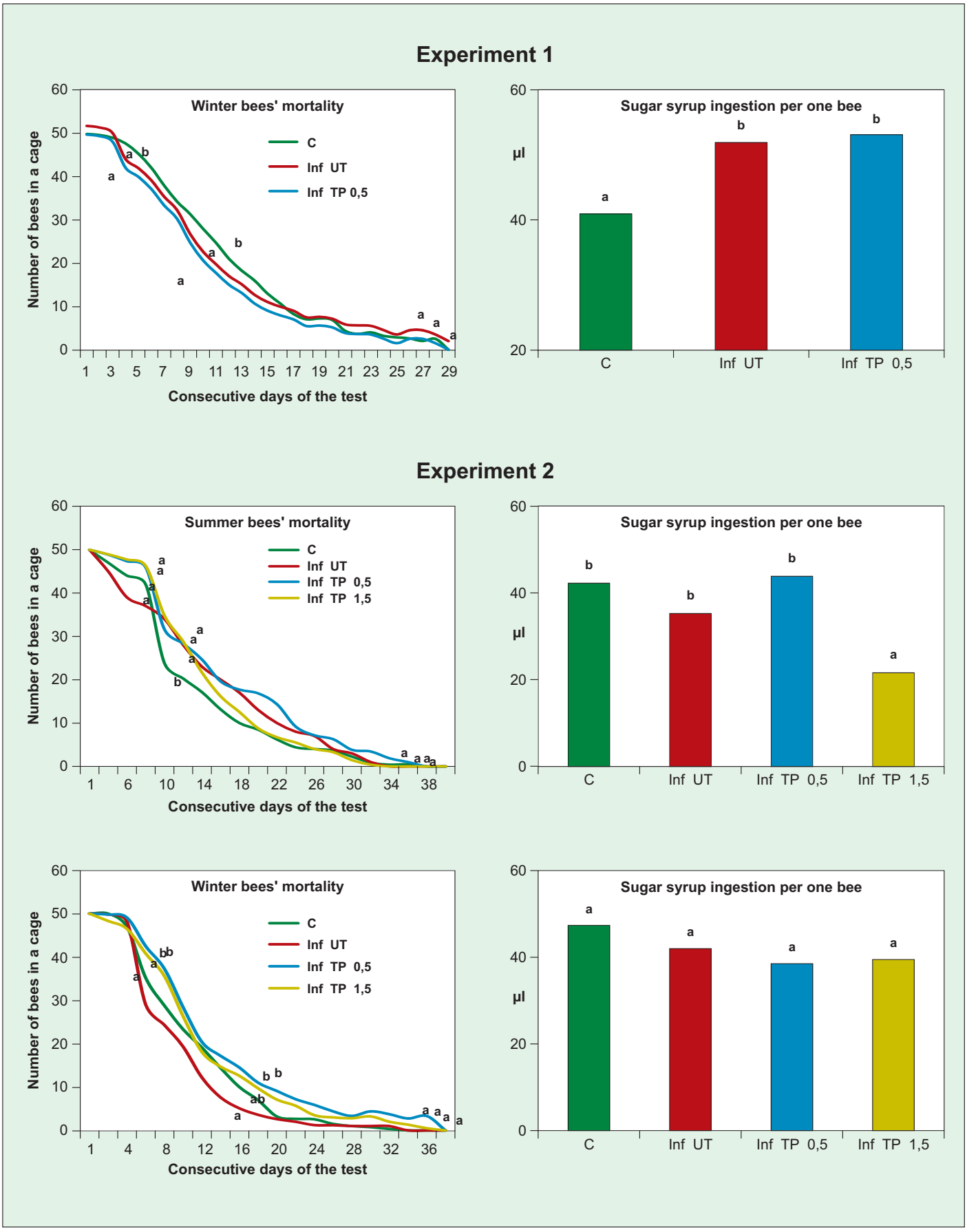

Fig. 1. Mortality and sugar syrup ingestion in laboratory cage tests
Explanation: a, b – different letters indicate statistically significant differences between the groups ($p < 0.05$); C – control, Inf UT – infested with *Nosema* spp. and untreated, Inf TP 0,5 – infested with *Nosema* spp. and treated with 0.5 µl probiotic, Inf TP 1.5 – infested with *Nosema* spp. and treated with 1.5 µl probiotic

The probiotic used in this study, recommended for animal feeding, was unsuitable for nosemosis treatment in bees. A probiotic preparation similar to the natural bacterial flora of healthy bees should be developed.

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