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SHORT COMUNICATION

Stimulation effect of probiotic bacteria *Bacillus spp.* and inactivated yeast on the honey bees *Apis mellifera* physiology and honey productivity

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Abstract

In order to find effective and safe ways to prevent the weakening and death of honey bee colonies from various stress factors, it is necessary to focus on the stimulation of physiological processes in the bee's body, activating their own mechanisms of resistance. Bacteria *Bacillus subtilis* and *Bacillus licheniformis* produce important digestive enzymes, which have antimicrobial and detoxification effects, and also stimulate metabolic processes in the organism. The inactivated yeast *Saccaromyces cerevisiae* was used as a protein and vitamin component of the supplement. It was found that the addition of the studied supplement to the bees' feeding increased the activity of proteases 3.32-fold in the gut, non-specific esterases - 2.16-fold in the fat body, glutathione-S-transferases - 2.64-fold in the gut and 1.69-fold in the fat body in the *Apis mellifera*. Application of the supplement in the field has shown that the honey productivity per family increases 1.5-fold compared to the control.

Key Words: probiotics; *Apis mellifera; Bacillus; Saccaromyces cerevisiae;* supplement; detoxification system

Introduction

Over the last twenty years, both technical and scientific progress and the growth of beekeeping have led to a significant increase in honey production, with an average annual growth of 35,000 tonnes since 2000, amounting to a total of 1.8 Mt of honey produced in 2016 worldwide (Pippinato *et al*., 2020). However, in some years, the world production of honey has reduced compared to the previous one (for example, in 2007, 2009, 2018, 2019) (FAOSTAT). High mortality in honey bee colonies has been reported worldwide in recent decades without definitive identification of the causes (Benaets *et al*., 2017). Nevertheless, recent investigations have established some of the most important factors contributing to honey bee losses, in particular, pests and diseases, bee management, including bee keeping practices and breeding, the change in climatic conditions (Potts *et al.*, 2010), agricultural practices, and the use of pesticides (Gill *et al.*, 2012; Hristov *et al*., 2020). The decline in honey bee populations causes serious damage not only to the production of honey, but also to pollination of plants affecting the functioning of natural and agricultural ecosystems (Klein *et al.*, 2007; van Engelsdorp *et al.*, 2008), therefore the attention of the international community is focused on this phenomenon.

In the early spring period, with a meager flow, as well as during wintering, when environmental conditions become unfavorable for the vital activity of bees, they are most susceptible to the influence of various pathogenic factors (Becsi *et al*., 2021). To provide bees with the necessary amount of nutrients and to activate metabolic processes during these periods, beekeepers use various feedings. They can activate the defense systems of bees and increase the amount of obtained honey (Brodschneider and Crailsheim, 2010).

To prevent and protect the honey bees from infections and parasitosis (such as varroatosis, acaripidosis), beekeepers generally use a variety of antibiotics and insecticides, which imposes restrictions on the production of organic beekeeping products (Ruoff and Bogdanov, 2004; Luttikholt, 2007) and their contamination may carry serious human health hazards (Al-Waili *et al.*, 2012). Most importantly, it leads to the accumulation of a stockpile of resistance capabilities in the microbiota of a healthy gut, providing a source of resistance genes for pathogens themselves (Tian *et al.*, 2012). Therefore, in order to find effective and safe ways to prevent the weakening and death of honey bee

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colonies, it is necessary to focus on the stimulation of natural physiological processes in the body of bees, activating their own mechanisms of resistance.

Bacteria *Bacillus subtilis* and *Bacillus licheniformis* are related species of gram-positive bacteria. As antagonists of pathogenic and opportunistic microorganisms (*Staphylococcus* sp, *Salmonella* sp, *Shigella* sp) (Moore, 2013), they stimulate the growth of normal intestinal microbiota (Mazkour *et al.*, 2019). Reproducing in the intestinal lumen, these bacteria produce all the main digestive enzymes (proteases, amylases, lipases, pectinases, cellulases), stimulate metabolic processes in the microorganism (Suva *et al.*, 2016). In addition, there are more than two dozen known antibiotics produced by *B. subtilis* (Kudriashova *et al.*, 2005; Stein, 2005). It should be noted that multi-strain mixture of these microorganisms is able to mutually reinforce and complement each other's biological activity (Cutting, 2011).

Probiotic bacteria are not only antagonists of opportunistic microbiota in the bee's gut, but also a constant source of microbial protein. This is especially important during intensive brood growth - the sensitivity of adult honey bees to pesticides directly depends on the amount and quality of protein consumed in the first 10 days after hatching (Wahl and Ulm, 1983). Because bees fed a high protein diet were better able to survive insult with interacting stressors (Archer *et al.*, 2014) the studied feeding included the inactivated yeast *Saccaromyces cerevicaea* as a protein and vitamin component, since it contains all the essential amino acids (Abbas, 2006) and is easily digested by honey bees (Abbasian and Ebadi, 2002).

Honey bees and other insect pollinators utilize detoxification enzymes such as carboxylesterases and glutathione-S-transferases (GSTs) to mitigate the toxic effects of xenobiotics such as plant defense compounds and pesticides (Panini *et al.*, 2016). Glutathione-S-transferases (GST) are the principal Phase II (conjugation of products of Phase I) enzymes, although they can also function in Phase I during which the toxin structure alters enzymatically (Berenbaum and Johnson, 2015). Indeed, esterase enzyme activity positively correlates with pesticide tolerance in many insect species, including bees of all stages of development (Milone *et al.*, 2020).

Total protease activity in honey bee midgut is also an important parameter related to protein digestion (Li *et al.*, 2012). A decrease in its activity is not only associated with a low level of protein in the diet, it may also be related to inhibitory effects of various infections and parasitosis. The inhibition of the activity of the host's proteolytic enzymes by the parasite often occurs during endoparasitosis (Zółtowska *et al.*, 2005).

Beekeeping is still done mainly to produce honey (Crane, 2009). It was found that honey production is governed by the interaction of three primary factors: average daily brood production, length of worker life and individual productivity of workers (Woyke, 1984). The health and, consequently, the productivity of honey bee colony depends on abiotic factors such as pesticides, management, weather conditions (Abou-Shaara *et al.*, 2017), biotic factors such as mites, viruses, bacteria and fungi as well as on the nutrition profile (Steinhauer *et al.*, 2018).

The aim of this study was to examine the effect of bacteria *B. subtilis* and *B. licheniformis* and inactivated yeast culture on the detoxifying and digestive enzymes of honey bees *Apis mellifera*, as well as on their honey productivity in the field.

Material and methods

Experimental design

Adult worker bees of the Middle Russian race were collected from one hive of a medium-strong family (Novosibirsk region, 54.759912, 82.633827). The bees were taken from the surface of the combs and transported to the laboratory of Novosibirsk state agrarian university.

The bees were kept under laboratory conditions in shaded cages at a temperature of 28 - 30 °C and a relative humidity of 60 – 65 % with approximately 200 bees in each variant. Experimental diet was performed with 60 % sugar syrup with the addition of bacteria with yeast additive (5 g/L of a mixture of *Bacillus subtillis* and *Bacillus licheniformis* and 30 g/L of inactivated yeast). In the control variant, no components were added to the sugar syrup. The syrup was prepared and replaced daily.

Enzymes activity in the fat body and midgut

10 days after the start of the feeding with experimental diet, the honey bees (n = 30) were placed on ice and their fat body and gut were dissected at 4 °C (Carreck *et al.*, 2013). Each organ was homogenized by ultrasound in 100 µl of 0,1 M Na-phosphate buffer pH 7.2 (PBS). The homogenates were centrifuged for 15 min, 10,000 g at 4 °C. The supernatant was used for the analysis of enzyme activity.

The activity of glutathione-S-transferases was determined spectrophotometrically at 340 nm, calculating the rate of increase in the concentration of 5- (2,4-dinitrophenyl)-glutathione, which is a reaction product of dinitrobenzene and reduced glutathione (Habig *et al.*, 1974). Incubation was carried out at a temperature of 28° C for 5 min with the following composition of the reaction mixture: 205 µL of PBS with the addition of 150 mM NaCl, 1 mM glutathione, 1 mM o-Dinitrobenzene, and 5 µL of the supernatant of the studied tissue (Grizanova *et al.*, 2018).

Nonspecific esterase activity was estimated by spectrophotometric analysis of the p-nitrophenylacetate hydrolysis rate (Prabhakaran and Kamble, 1993). Five microliters of the supernatant were incubated with 200 µL phosphate buffer with the addition of 0.54 mM 1-naphthyl acetate in darkness for 5 min at 28 °C, and then the transmission density was measured at 410 nm.

The method for determining the total proteolytic activity was to measure the rate of hydrolysis of 0,3 % azocasein (Sigma) by bee intestinal proteinases with some modifications (Alarcón *et al.*, 2002). 30 µL of intestinal supernatant was added to 210 µL of 0.3 % azocasein in PB, after which the reaction mixture was incubated at 37 °C for 1. The reaction

was stopped by adding 200 µL of a 30 % TCA solution and subsequent incubation at -18 °C for 30 min. Then the resulting mixture was centrifuged for 10 min, 10000 g, and 150 µL of the supernatant was taken from it. After adding 70 µL of 1M NaOH to the resulting mixture, the optical density was measured at 440 nm.

Enzyme's activity was measured in units of optical density (ΔA) of incubation mixture per 1 min and 1 mg of protein.

The total protein concentration in all of the samples was determined according to Bradford (Bradford, 1976). Standard curves to estimate protein concentration in the samples were prepared using bovine serum albumin (BSA).

Microscopic analysis of the fat body

Fat bodies of 30 bees from the control and treated groups were assessed according to the developmental index (a scale from one to five, with five being the best developed structure) proposed by Maurizio (1954), examining the inner surfaces of tergites using a binocular microscope (Fliszkiewicz *et al.*, 2012).

Honey productivity

Three honey bee colonies with one year-old sister queens were selected per variant (one of these colonies was used in laboratory tests of bees enzymes activity in the fatbody and midgut), feeding was carried out three times (every three weeks) during the spring of 2021. Bee colonies of the control variant received sugar syrup without additives, the experimental group of bees received the supplement with the studied additives - 10 g of inactivated yeast and 2 g of a mixture of *B. subtilis* and *B. licheniformis* per colony. The honey was pumped out twice - in July and in September.

Colony honey production was determined by weighing the honey supers before and after extraction, after which the mass of honey per one bee family was calculated (Nelson and Gary, 1983).

Statistic

The data were analyzed using GraphPad Prism® ver. 8 (GraphPad Software, USA). The results are reported as the mean values ± SD. A Kolmogorov-Smirnov normality test was used to check the normal distribution of the data. The data of visual scoring of the fat body of bees was analyzed by a Mann Whitney test. Two-way ANOVA (with Dunnett's multiple comparison test) was used to assess differences between activities of ferments in the insect midguts and fat bodies.

Results and discussion

It was shown that adding bacteria *B. subtilis* and *B. licheniformis* with inactivated yeast to the honey bees' diet significantly increased the activity of GST both in the gut ($p < 0.001$) and in the fat body ($p < b0.05$), and esterases in the fat body ($p < 0.001$) compared to the control after ten days of feeding the diet with supplement (Fig. 1). In addition, the diet with supplement significantly ($p < 0.0001$) increased the activity of proteolytic enzymes in the gut of honey bees compared to the control on the tenth day of the experiment (Fig. 1).

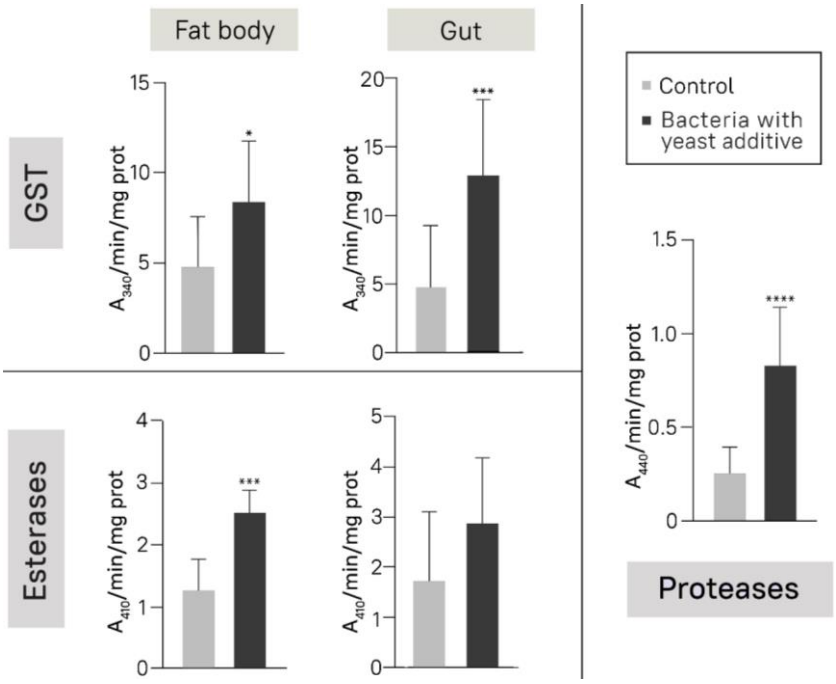

Fig. 1 Increased activity of non-specific esterases and glutathione S-transferases in the fat bodies and glutathione S-transferases and proteases in the guts of honey bees fed with probiotic bacteria and inactivated yeast. The detoxifying enzymes activity was assessed in the fat bodies and the guts of bees. Proteolytic ferments were assessed in the guts. Treatment (bacteria with yeast additive) contained sugar syrup with 5 g/L of *B. subtilis and B. licheniformis* and 30 g/L of inactivated yeast culture; Control contained sugar syrup without additives. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ compared with control group bees)

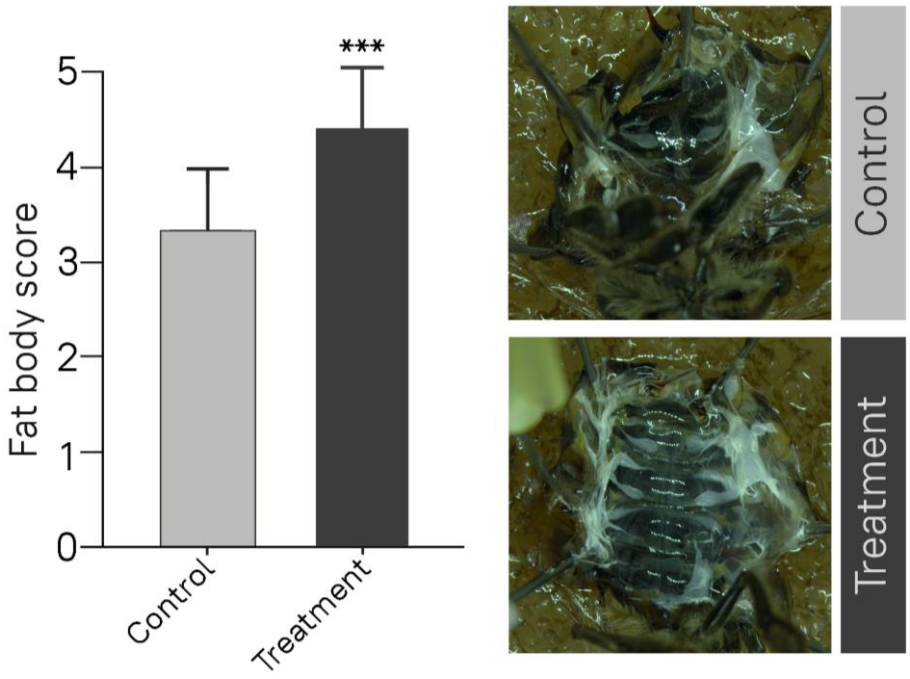

Fig. 2 Increased degree of the fat body development of honey bees fed with probiotic bacteria and inactivated yeast (treatment). Treatment contained sugar syrup with 5 g/L of *B. subtilis and B. licheniformis* and 30 g/ of inactivated yeast culture; Control contained sugar syrup without additives. (***$p < 0.001$ compared with control group bees)

Increasing activity of GST both in the intestine and in the fat body and esterases in the fat body of honey bees indicate the stimulation of the detoxifying system when bacteria *B. subtilis* and *B. licheniformis* and inactivated yeast culture were added to bees' diet. It is important to note that the development of insect resistance to pesticides often has a metabolic basis (Berenbaum and Johnson, 2015; Heckel, 2018) and can be microbe-mediated (Itoh *et al.*, 2018). Therefore, an increase in the activity of detoxifying enzymes can provide protection from the harmful effects of chemical pesticides. An increase in the activity of proteolytic enzymes in the intestine of bees can be caused not only by an increase in the level of protein in the diet (Li *et al.*, 2012), but also directly by proteases produced by *Bacillus* bacteria itself (Connelly *et al.*, 2004).

The fat body of insects is the most metabolically active tissue and plays a crucial role in storing and utilizing energy and in detoxification processes (Skowronek *et al.*, 2021). It is responsible for the synthesis of most of the hemolymph proteins, including antimicrobial peptides (Arrese and Soulages, 2010). Fat body assessment showed that the average value for the group, which diet was supplemented with probiotic bacterial strains and inactivated yeast, was 4.43 ± 0.63 points and was significantly higher (***$p < 0.001$) as compared with the control group - 3,37 ± 0.61 points (Fig. 2).

Well-developed fat body of honey bees fed with receiving *B. subtilis*, *B. licheniformis* and inactivated yeasts suggest a higher level of metabolism and activity of internal defense mechanisms, coinciding with studies of the development of fat body when feeding commercial probiotics with added protein (Kazimierczak-Baryczko and Bozena, 2006). In addition, Evans and Lopez have showed that mix of bacterial spores from species in the genera *Bifidobacterium* and *Lactobacillus* induced as strong immune response (in antimicrobial peptide abaecin level) as a bee pathogen *Paenbacillus larvae* when ingested (Evans and Lopez, 2004).

The average honey productivity for the 2021 season was 27.4 ± 1.04 kg per hive in the control group and 40.1 ± 1.44 kg per hive in the experimental group. Accordingly, the use of inactivated yeast *S. cerevicaea* and bacteria *B. subtilis* and *B. licheniformis* increased the final honey productivity by 1.5 times compared to the control. This can be caused by the normalization of the microbiota of the honey bee (Kaznowski *et al.*, 2005; Vásquez *et al.*, 2012), the suppression of pathogenic microorganisms (Baffoni *et al.*, 2016), the stimulation of the production of antimicrobial peptides in the honey bee's body (Evans and Lopez, 2004), as well as a decrease in the absorption of pesticides by probiotic bacteria (Trinder *et al.*, 2016). In addition, according to many authors, the lack of protein in bee feed negatively affects ovarian activation and brood rearing, which in the long term affects the amount of honey (Herbert *et al.*, 1977; Pirk *et al.*, 2010).

From the presented data it can be assumed, that inactivated yeast mixed with *B. subtilis - B. licheniformis* as a bee supplement has properties of

stimulating the detoxifying and digestive systems of bees, and also affects the honey productivity of honey bee colonies. The use of this feeding option can help to cope with the weakening of bee colonies increasing the quantity of obtained honey. Additional experiments using a larger set of apiaries will be performed to further support the present suggestions. At the same time, honey bee populations of different subspecies from other regions will be tested to determine the supplement's effects on colony performance, changes in the gut microbiome of treated honey bees and resistance to bacterial diseases.

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