

Effect of Application of Probiotic Pollen Suspension on Immune Response and Gut Microbiota of Honey Bees (*Apis mellifera*)

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Abstract

Although the use of probiotic bacteria in invertebrates is still rare, scientists have begun to look into their usage in honey bees. The probiotic preparation, based on the autochthonous strain *Lactobacillus brevis* B50 BiocenoI™ (CCM 8618), which was isolated from the digestive tracts of healthy bees, was applied to the bee colonies in the form of a pollen suspension. Its influence on the immune response was determined by monitoring the expression of genes encoding immunologically important molecules in the honey bee intestines. Changes in the intestinal microbiota composition were also studied. The results showed that the probiotic *Lact. brevis* B50, on a pollen carrier, significantly increased the expression of genes encoding antimicrobial peptides (abaecin, defensin-1) as well as pattern recognition receptors (toll-like receptor, peptidoglycan recognition proteins). Gene expression for the other tested molecules included in Toll and Imd signaling pathways (dorsal, cactus, kenny, relish) significantly changed during the experiment. The positive effect on intestinal microbiota was manifested mainly by a significant increase in the ratio of lactic acid bacteria to enterobacteria. These findings confirm the potential of the tested probiotic preparation to enhance immunity in bee colonies and thus increase their resistance to infectious diseases and stress conditions.

Keywords Probiotic lactobacilli · Honey bee intestine · Immune signaling pathways · Intestinal bacteria

Introduction

Recently, dramatic declines of managed colonies have been noted all over the world (e.g., in the USA for the last 5 years losses have reached from 28 to 45%) [1]. Several causes of these large-scale losses have been reported, including bee pathogens (*Paenibacillus larvae*, *Varroa destructor*, *Acarapis woodi*, *Nosema* spp., bee viruses, etc.), pesticides, contaminated water, use of antibiotics, poor nutrition, and incorrect breeding management [2, 3]. Since there is zero tolerance of antibiotic residues in bee products in the EU, in

particular in honey, their use in the treatment or prevention of these infectious diseases is associated with high economic losses or is totally prohibited (such as in the case of American foulbrood). Moreover, antibiotic therapy is often insufficiently effective, leads to the development of resistant bacterial strains, and disturbs honey bee microbiome. Probiotics represent an effective alternative to antibiotics used for the therapy and prevention of bacterial diseases. Probiotic bacteria are commonly used in vertebrates, but their effect on invertebrates is poorly studied, and above all their influence on the immunity [4]. As well as the fact that honey bees are economically the most important for pollination, they are also important producers of bee products (honey, royal jelly, wax, poison, etc.), therefore a need for the use of probiotics in bees is all the more urgent; especially because the use of antibiotics leaves residues in bee products, and the genes for antibiotic resistance can be spread in the environment. It was shown that like in mammals [5], healthy gut microbiota plays a crucial role in maintaining honey bee health. Gut microbiota stimulates the immune system and inhibits pathogens, and thus, it is critical for disease prevention [6, 7]. Well-studied and confirmed mechanisms of probiotic action include: (a) alteration of the intestinal microbiota, (b) competitive exclusion, (c)

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modulation of the mucosal immune system, (d) enhanced barrier function, (e) production of antimicrobials, and (f) organic acid production [8]. However, insects have different components to their immune system. The immune system of bees consists of 2 parts: social and individual immunity [9]. The social defense is based on the cooperation of thousands of individuals to combat parasites or other pathogens. Such behavioral strategies include guarding and cleaning of colonies and immobilization of pathogens [10]. Individual immunity consists of physical and chemical components and is divided into the cellular and humoral immunity. Both parts of immunity are closely interconnected [9].

Lactobacilli play a first-rate role in immunomodulation in the intestine [11]. Four signaling pathways have been confirmed within honey bee's immunity – Toll, Imd, JAK/STAT, and JNK. These pathways are mutually interconnected. One signaling pathway can activate part of another. The Toll signaling pathway is activated by Gram-positive bacteria. After the recognition of peptidoglycans by peptidoglycan recognition proteins (PGRPs), a cascade of serine protease splits pro-spätzle into spätzle which matures and binds to membrane toll receptor. In this signaling pathway, regulation proteins are also involved, such as Myd88, Pelle, and Tube. The subsequent degradation of cactus (an analogue to the mammalian I- κ B) and translocation of dorsal (an analogue to the mammalian NF- κ B) into the nucleus results in the stimulation of gene expression of antimicrobial peptides (AMPs) [12]. In honey bees, the Imd pathway is more conserved [13]. Activation of Imd signaling pathway is similar to Toll signaling pathway activation, both start after recognition of bacteria by PGRPs. This results in Imd activation, and the degradation of inhibitor κ B (Kenny) by the caspase Dredd. Relish, corresponding to NF- κ B, is translocated into the nucleus, and the end of the cascade is the production of AMPs [12]. AMPs are necessary to combat invading microbial pathogens. Evans and Lopez [10] confirmed a higher gene expression of AMP abaecin after feeding honey bee larvae a mixture of probiotic lactobacilli and bifidobacteria as well as pathogenic *Paenibacillus larvae*, but precise mechanisms of the stimulation and the reaction of other AMPs are still unclear. Therefore, the aim of our experiment was to study the effect of probiotic lactobacilli, administered on a pollen carrier on: (1) the immune response, determined by the expression of genes encoding immunologically important molecules in the honey bee intestines and (2) changes in intestinal microbiota.

Material and Methods

Probiotic Strain

Autochthonous *Lactobacillus brevis* B50 BiocenoI™ (CCM 8618) used in the experiment was isolated from the healthy

adult bee's digestive tracts at the Department of Microbiology and Immunology of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia. The strain was tested for selected probiotic properties and showed good growth properties, strong antibacterial activity against *Paenibacillus larvae*, high resistance to long-term storage ($-20\text{ }^{\circ}\text{C}$ for 5 years), auto-aggregation ability, and high production of organic acids [4].

Preparation of Diet Supplements

For the preparation of probiotic supplement, *Lact. brevis* B50 was cultivated in MRS broth (Merck, Darmstadt, Germany) at $37\text{ }^{\circ}\text{C}$ overnight. Subsequently, night cultures of lactobacilli were centrifuged for 20 min at 700 g. The resulting sediment was washed twice in sterile saline solution by centrifugation (15 min at 700 g). The prepared pellet was resuspended in autoclaved tap water to reach a final lactobacilli concentration of 10^8 – 10^9 cfu (colony forming units) in 1 mL of pollen suspension containing 50 g of dried pollen per 500 mL. Pure pollen suspension was prepared from 50 g of dried pollen and 450 mL of autoclaved tap water. The application doses for one colony was 500 mL of pollen suspension with or without probiotic lactobacilli.

Experiment Design and Sampling

Bee hives ($n = 30$) were divided into 3 groups. The first group was administered probiotic *Lact. brevis* B50 BiocenoI™ (CCM 8618) in pollen suspension ($n = 10$), the second group was fed pure pollen suspension ($n = 10$), and the control group that did not receive any supplements ($n = 10$). During the next 3 weeks, bees in the first two groups were once a week given the pollen suspension with *Lact. brevis* B50 or pure pollen, respectively. The samples were collected before the first application of probiotic or pure pollen suspension (zero sampling – 0th sampling) and then again 1 week (1st sampling), 3 weeks (2nd sampling), and 5 weeks (3rd sampling) after the first application. Samples consisted of approximately 30 honey bees from each colony. Bees' digestive tracts (0.5 g) were extracted for microbiological cultivation, and 10 guts were washed with PBS to remove their contents. Washed guts were immediately placed into RNA later (Ambion, Austin, TX, USA) and used for the isolation of mRNA.

Microbiological Analysis

Bacteria from the homogenized samples of bees' digestive tracts were diluted by tenfold dilution in isotonic saline solution and plated onto MRS agar (Merck) to determine counts of lactic acid bacteria (LAB), onto Endo agar (Himedia, Mumbai, Maharashtra, India) to count enterobacteria (ENT), and blood agar, composed of Columbia agar (Oxoid,

Basingstoke, Hampshire, UK) with 5% defibrinated sheep blood was used to count the total aerobes. After inoculation, the MRS plates were incubated in an anaerobic atmosphere using the GasPak system (Becton Dickinson, Franklin Lakes, NJ, USA) for 48 h at 37 °C. Endo agar and blood agar plates were incubated for 24 h at 37 °C aerobically. The potential presence of *Paenibacillus larvae* was controlled on MYPGP agar (10 g Müller-Hinton agar, 15 g yeast extract, 3 g K₂HPO₄, 2 g D-glucose, 1 g sodium pyruvate, and 20 g agar/L; pH 7.4 with additional nalidixic acid in a final concentration of 18 µg/mL). The bacterial counts were expressed in log of colony forming units per gram of intestinal content (log₁₀ cfu/g) ± standard deviation.

RNA Extraction and cDNA Synthesis

The total RNA of guts was extracted using Purezol™ reagent (BioRad, Hercules, CA, USA). Contaminating DNA was digested with DNA Rapid Removal Kit (Thermo Scientific, Waltham, MA, USA), and total RNA purity and quantity was determined at 260/280 nm using NanoDrop 8000 (Thermo Scientific). First strand cDNA was constructed using Revert Aid Premium Reverse Transcriptase and oligo (dT)18 and random hexamer primers (Thermo Scientific).

Gene Expression Analysis (qPCR)

Primers for gene expression analysis of β-actin, abaecin, defensin-1, toll, and relish genes were designed according to Khongphinitbunjong et al. [14] (Table 1), peptidoglycan recognition protein (PGRP SC4300), dorsal-1 genes according to Cizelj et al. [15], cactus-1 gene according to Evans [13]. Primer for the application of kenny gene was designed using the gene sequences of kenny (XM_001120619.4) from the GenBank database and using Primer-Blast software. RT-qPCR was performed using CFX96Manager Software (BioRad) in 10 µL reaction volume containing 1 xiQ™ SYBR® Green Supermix (BioRad), 0.5 µM forward and reverse primers and 40 ng/µL of cDNA. β-actin was used as a

reference gene for internal control. Each assay included a negative control without a cDNA template. All reactions were performed in triplicates. The experimental protocol consisted of the initial denaturation at 95 °C for 5 min, followed by amplification including 40 cycles of 4 steps: denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 15 min followed by melting curve analysis to confirm amplification of a specific product. Each assay included a reaction efficiency calculation (100 ± 5%). Relative normalized expression was calculated by the 2^{-ΔΔCT} method. Results of the gene expression experiment conducted in triplicates were expressed as mean ± standard deviation (SD).

Statistical Analysis

The results were analyzed in the GraphPad Prism version 3.00 statistical program using two-way ANOVA. In the case of a statistically significant difference between groups or over time, the differences between groups and samplings were subsequently analyzed using a post-hoc Tukey's test. Statistically significant differences between the groups are presented in graphs, significant differences between samplings are described in the text, for better clarity.

Results

Microbiological Screening

The influence of the administration of the probiotic preparation and pure pollen mash on the intestinal microbiota of honey bees was evaluated on the basis of counts of LAB representing beneficial microbiota, counts of ENT, and total aerobic bacteria representing symbionts as well as many enteropathogens. In the probiotic group, we have noted a significant increase in the number of LAB in all three post-application collections compared to the control and pollen groups (Fig. 1a). In this group, we also observed a significant

Table 1 List of primers used in RT-qPCR for immune-related genes

Genes	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product length (bp)	Reference
β-actin	TTGTATGCCAACACTGTCCTTT	TGGCGGATGATCTTAATTT	120	[14]
Abaecin	CAGCATTCGCGTATGTACCA	GACCAGGAAACGTTGGAAAC	72	[14]
Defensin-1	TGTCGGCCTTCTTTCATGG	TGACCTCCAGCTTTACCCAAA	102	[14]
PGRPSC4300	GAGGCTGGTACGACATTGGT	TTATAACCAGGTGCGTGTGC	103	[15]
Toll	TAGAGTGGCGCATTGTCAAG	ATCGCAATTTGTCCAAAAC	167	[14]
Cactus-1	CACAAGATCTGGAGCAACGA	GCATTCTTGAAGGAGGAACG	203	[13]
Dorsal-1	AGAGATGGAACGCAGGAAAC	TGACAGGATATAGGACGAGGTAA	104	[15]
Kenny	TTGGTATCATGCATCAAAGTGTGG	TCCGGGTACAATCGGATACAG	260	This study
Relish	GCAGTGTGAAGGAGCTGAA	CCAATTCTGAAAAGCGTCCA	244	[14]

increase of LAB counts between zero and first sampling ($P < 0.001$) and such high numbers of LAB were maintained until the end of the experiment. The number of ENT was significantly lower in the probiotic group than in the control group, and this trend was also observed in the 2nd collection (Fig. 1a). The proportions of ENT had the opposite tendency as LAB, thus in the probiotic group ENT counts decreased significantly between zero and first sampling ($P < 0.05$). The numbers of total aerobic bacteria showed a decreasing tendency in the 1st and 2nd samplings in all groups in comparison with zero sampling (Fig. 1a). As with ENT, in the probiotic and pollen-fed groups, there was a more pronounced decline compared to the control group, but without statistical significance.

The LAB and ENT ratio is a widely recognized indicator of intestinal microbial health. A significantly higher ratio was observed in the first and second sampling in the probiotic group in comparison with control and pollen groups. This confirmed the positive effect that the probiotic pollen suspension has on gut microbiota of honey bees (Fig. 1b). No *P. larvae* were detected in bee digestive tracts throughout the experiment.

Effect on Immune System

The immunomodulatory effect of lactobacilli was evaluated based on the study of two activation pathways, Toll and Imd, resulting in the production of antimicrobial peptides. In the first stage, we focused on pattern recognition receptors recognizing pathogen-associated molecular patterns on pathogens – toll-like receptor for Toll pathway and PGRP for Imd pathway. The gene expression of PGRP was increased in both treated groups in the 1st and 2nd samplings. A statistically significant lower expression was recorded only in the last sampling from the probiotic group in comparison with the control and pollen groups (Fig. 2a). We recorded a significant increase in the gene expression of toll-like receptor in the first sampling, and subsequently, its expression decreased in next samplings (Fig. 2b). Subsequently, we studied the expression of genes for regulatory molecules of both pathways. Again, we could see an increase of cactus gene expression in the probiotic group in the first sampling and its gradual decline to the third sampling. Significantly, the highest gene expression for cactus was observed in the pollen group in the 2nd and 3rd samplings in comparison with the control and probiotic groups (Fig. 2c). The gene expression of dorsal declined in the probiotic group as well as in the pollen group in the 1st and 2nd samplings; it means that the trend was the opposite when compared with other studied molecules (Fig. 2d). Kenny gene expression significantly increased in the probiotic group between the 1st and 2nd sampling (Fig. 2e), and relish gene expression was almost on the same level in the probiotic group in the first three

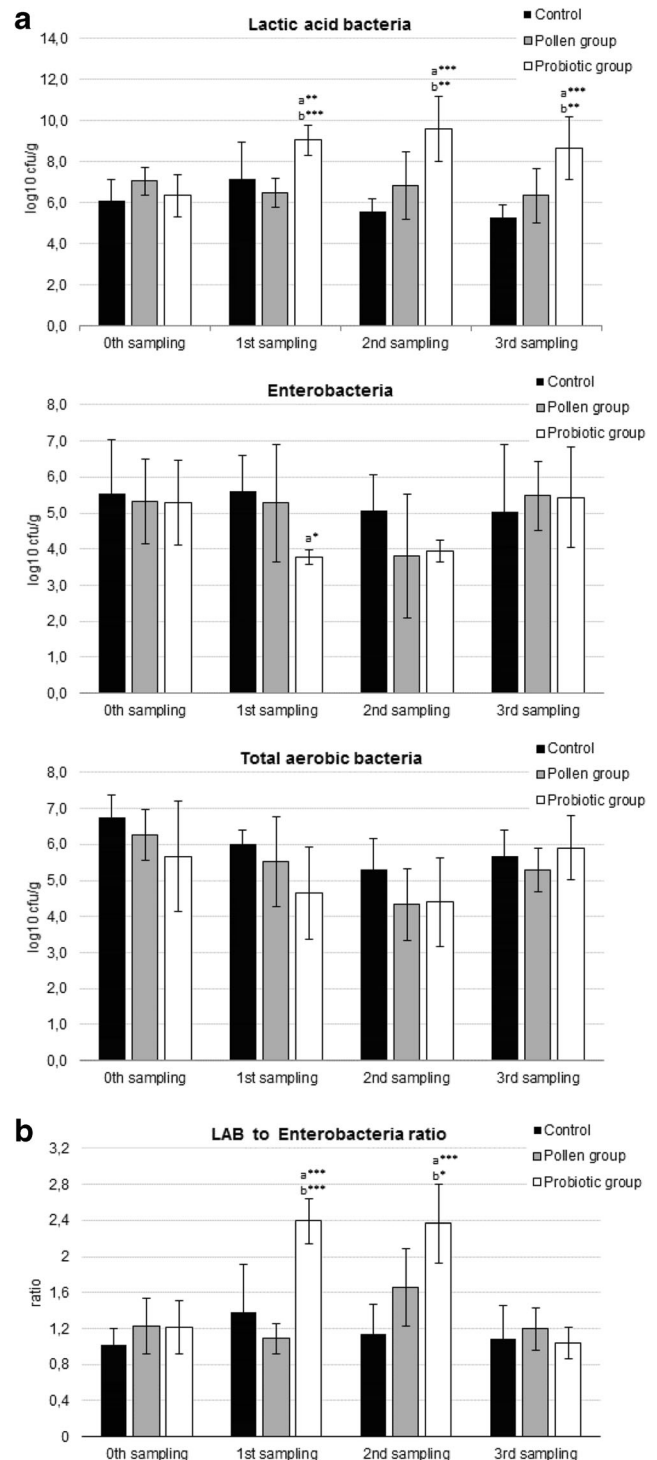


Fig. 1 Number of bacteria in control, pollen and probiotic groups expressed as cfu/g: a) Lactic acid bacteria, Enterobacteria, Total aerobic bacteria, and b) LAB to ENT ratio. a - significantly different from control; b - significantly different from pollen group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

samplings, and slightly decreased in the last sampling ($P > 0.05$; Fig. 2f). We assumed that activation of both pathways has been decreased. Concerning AMP, we recorded the same trend as we did for PGRP, toll, and cactus, an increase

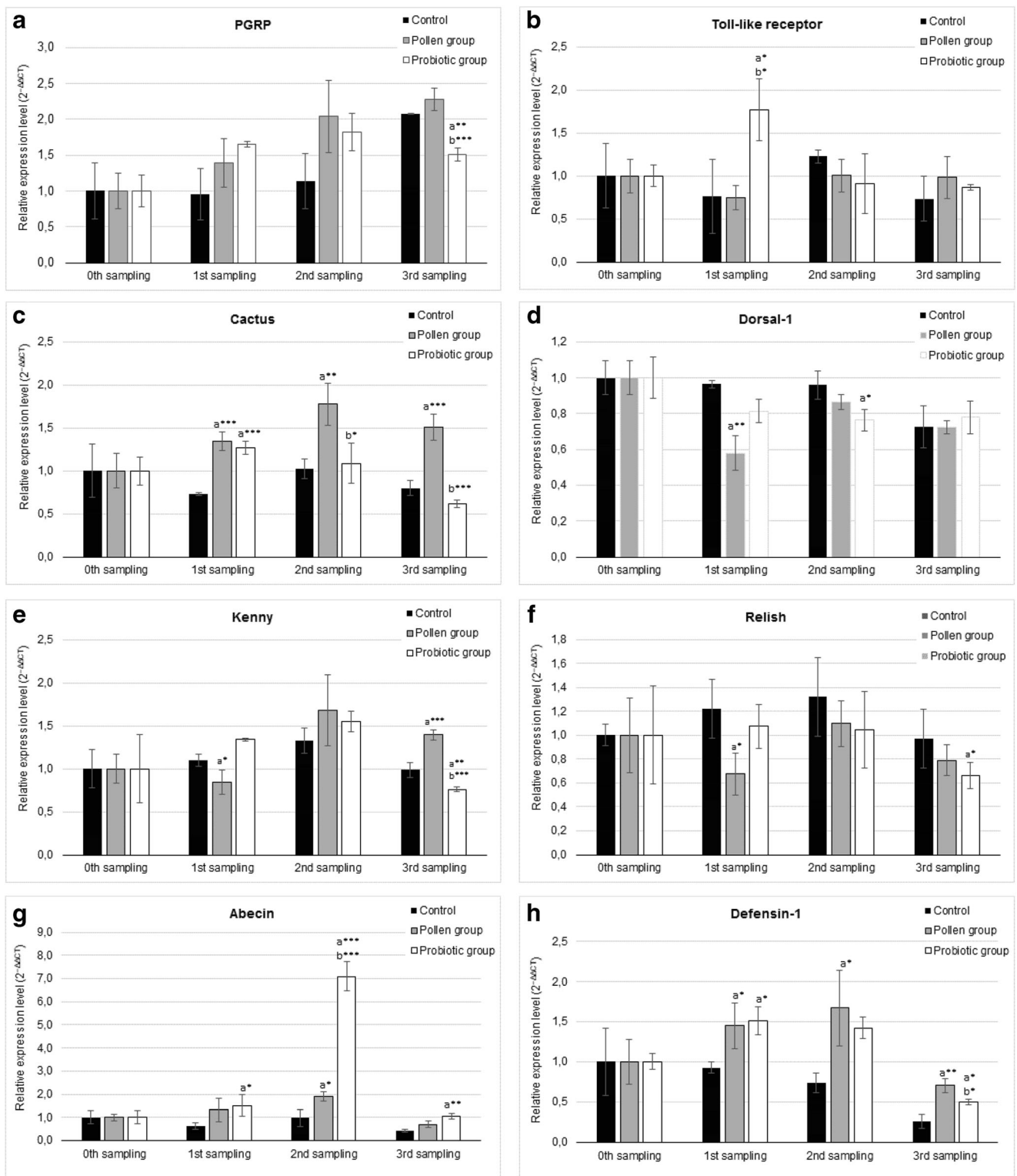


Fig. 2 Effect of pollen suspension and probiotic preparation on the gene expression for bee immunologically important molecules: a) PGRP, b) Toll-like receptor, c) Cactus, d) Dorsal-1, e) Kenny, f) Relish, g) Abecin,

h) Defensin-1. a - significantly different from control; b - significantly different from pollen group; *P < 0.05; ** P < 0.01; *** P < 0.001.

of defensin-1 gene expression in the 1st sampling in both treated groups and subsequently a decline to the last sampling (Fig. 2h). A similar trend was recorded in the gene

expression for abecin, with the difference being that the maximum expression was reached in the 2nd sampling (Fig. 2g).

Discussion

Nowadays, alternative methods of prevention of infection diseases using natural substances, such as probiotics, prebiotics, plant extracts, or fatty acids, which neither adversely affect the bee products nor put load on the environment, are in center of interest. Using probiotics is possible to influence the composition of bee gut microbiota as well as the immune response, similar to in mammals. The gut microbiota of honey bees is shown to have low diversity, and LAB represent an important part of it [16]. 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 [17]. In our study, we confirmed a positive effect of the probiotic preparation on intestinal bee microbiota, which was manifested by an increase in LAB representation and a reduction of ENT. Moreover, even 3 weeks after discontinuing the administration of the probiotic preparation, lactobacilli in significantly increased numbers were present in the intestines of the bees, providing better protection for the entire colony, since the lactobacilli are maintained in the hive environment and transmitted to the next generations by bee feeders. This fact also confirms the colonization ability of the *Lact. brevis* B50. In the case of a group that received only pollen, there was also a slight increase in LAB numbers in the gut, which attributed to the fact that pollen naturally contains LAB and represents a suitable environment for their multiplication [18].

Within invertebrates, modulation of the expression of immunologically important molecules has been studied on model organisms (e.g., *Drosophila melanogaster*), but only a few studies have been carried out on honey bees (*Apis mellifera*) [13]. Similarly, to in other animals, endogenous gut microbiota can stimulate immunity in bees [7]. LAB has a positive effect on the immune system by increasing synthesis of antimicrobial substances [10]. The effect of probiotic organisms on bee immune signaling pathways has not yet been analyzed in detail. Janashia and Alaux [19] used 5 different LAB strains (*Fructobacillus fructosus* 49a, *Fructobacillus pseudoficulneus* 57, *Fructobacillus tropaeoli* 46, *Bifidobacterium asteroides* 26p, *Lactobacillus kunkeei* 14p) in their study. Each bee diet contained one of these bacterial suspensions which were fed to larvae. *Bifidobacterium asteroides* 26p and *Fructobacillus pseudoficulneus* 57 stimulated immunity by up-regulating the expression of apidaecin. In another study, 9 different LAB strains (*Enterococcus* sp., *Weissella* sp., *Lactobacillus* sp.) in a feeding experiment significantly increased the expression of genes encoding abaecin, hymenoptaecin, and defensin. Probiotic bacteria can stimulate the immune system, but this function is species- and strain-specific [20]. In our study, we were focused on two signaling pathways – Toll and Imd signaling pathways. Concerning the Toll signaling pathway, our results showed increased gene

expression for PGRP in the probiotic group in the first and second sampling corresponding with the presence of peptidoglycan in the cell wall of *Lact. brevis*. PGRPs are the most important pattern recognition receptors for insects [21]. In an experiment by Yoshiyama et al. [20], the Toll pathway was activated by 5 LAB strains (*Fructobacillus fructosus* 49a, *Fructobacillus pseudoficulneus* 57, *Fructobacillus tropaeoli* 46, *Bifidobacterium asteroides* 26p, *Lactobacillus kunkeei* 14p). *Frischella perrara* activated the immune system by up-regulating genes coding pattern recognition receptors and antimicrobial peptides [22]. Immunity reflects bacterial colonization and maintains homeostasis between endogenous and pathogenic bacteria and the host. PGRPs have evolved mechanisms for regulating the endogenous microbiota composition [23]. The Toll signaling pathway continued with the activation of Toll-like receptors, the expression level of which was significantly higher in the probiotic group 7 days after the probiotic preparation was first applied. Surprisingly, the gene expression for cactus was higher in the pollen group. Pollen containing dietary protein is necessary to synthesize the effector peptides of signaling pathways [24]. In a study by Lourenço et al. [25], newly emerged bees were infected with 2 different bacteria *Serratia marcescens* and *Micrococcus luteus*. Abdominal carcasses from these bees were used to study Toll and Imd signaling pathways (PGRP-S3, B-gluc-2, transferrin-1, cactus-2, dorsal-1B, relish, abaecin, defensin-1, hymenoptaecin). Both bacteria activated Toll and Imd pathways. We assume that we did not detect the activation of the signaling pathway, as the 1st sampling was done up to 7 days after the first application of probiotics. In this sampling, we have probably measured only the final activation products – antimicrobial peptides. Sampling schedules differ significantly among experiments. In comparison with our design of samplings, Lourenço et al. [25] collected bees 0.5, 1, 3, 6, and 12 h after infecting with *S. marcescens* and 3 and 6 h after infecting with *M. luteus*. Janashia and Alaux [19] used bee larvae to study immune stimulation 72 h after first feeding endogenous bacteria. In a study by Erler et al. [26] samples were taken at 0.5, 1, 2, 4, 8, 12, and 24 h, and they used drones to study the expression of 16 genes encoding molecules included in immune system pathways and AMPs (basket, cactus, dorsal, hem, kenny, myd88, prophenoloxidase, relish, Tak 1, TEP A, toll 1, toll 6, abaecin, defensin-1, hymenoptaecin). Bees were infected with *Escherichia coli* and autoclaved standard bee ringer solution. They noted up-regulation of genes encoding AMPs in both groups, and they also found out that relish was up-regulated only within the first hour after the bacterial challenge, but decreased quickly afterwards. We considered that the Toll and Imd pathways that were studied could be activated; however, the 1st sampling was performed too late to record the increase of NF- κ B (dorsal). We only noted the up-regulation of I- κ B (cactus), and the up-regulation corresponded with the termination of AMP production. So

far, it is known that the production of AMPs can be regulated by different signaling pathways, e.g., defensin-1 is regulated by Toll pathway and abaecin by Toll and Imd pathways [27]. Both signaling pathways are based on cascades, which result in the up-regulation of AMP's gene expression. On the other hand, it is possible that gene expression for AMPs was activated through another signaling pathway, probably JNK signaling pathway. AMPs play a primary role in the fight against pathogens; however, some pathogens are able to down-regulate molecules of signaling pathways. Parasitic mite infestation together with wing deformity virus decreases the expression of genes encoding AMPs [28]. Likewise, AMPs were down-regulated 3 and 6 days after inoculation with *Nosema ceranae* [29]. Our results showed an up-regulation of AMPs. It is an important point in immune stimulation after the application of probiotic preparation in stress periods.

Conclusion

This study illustrates the effect of probiotic preparations on bee's immune system and gut microbiota. Our results indicate a positive influence on modulating gut microbiota composition as well as on the immune response. The probiotic preparation had a direct effect on immunity responding by up-regulating the gene expression for antimicrobial peptides. There is also an indirect effect by increasing LAB numbers and reducing ENT in the bee intestines. The preparation based on autochthonous strain *Lact. brevis* B50 Biocenol™ is recommended for preventive use during critical periods (e.g., before and after winter, full harvesting period, transportation). In the future, we are planning to study the effect of probiotic preparation on bee immune system after infection with different pathogens.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict interest.

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