Bulletin of Insectology **70** *(1): 31-38, 2017*
ISSN 1721-8861

Plant alkaloid sanguinarine and novel potential probiotic strains *Lactobacillus apis*, *Lactobacillus melliventris* and *Gilliamella apicola* promote resistance of honey bees to nematobacterial infection

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Abstract

Entomopathogenic nematodes (EPNs) are obligate insect parasites symbiotically associated with entomopathogenic bacteria. They can be used as a natural infection model combining bacterial infection with infection by multicellular parasite and as such, can be used in biological control and also offer a powerful tool to study insect immunity. Both mix of non-pathogenic bacteria and even nectar alkaloids were previously hypothesized to positively modulate honey bee health. We used EPNs for evaluating the overall immune resistance of honey bee larvae treated with potentially immuno-modulating substances - a plant alkaloid sanguinarine (extracted from *Macleaya cordata*) and non-pathogenic strains of *Lactobacillus apis*, *Lactobacillus melliventris* and *Gilliamella apicola*, native to honey bee gut isolates. Honey bee 5th instar larvae (L5) and white pupae were infected with *Heterorhabditis bacteriophora* or *Steinernema feltiae*, both carrying their symbiotic bacteria. In comparison to untreated honey bee larvae we observed an increase in survival of 13.5 ± 6.43 or 11.25 ± 5.77% in case of sanguinarine and *S. feltiae* or *H. bacteriophora*, respectively. Similarly, mix of above mentioned bacteria inoculated at the same time increased survivorship to 23.25 ± 1.53 or 11.0 ± 6.0% for *S. feltiae* or *H. bacteriophora*. This is the first record of use of nematobacterial infection for evaluating the immune status of a beneficial insect. Addition of low doses of both sanguinarine and non-pathogenic strains of selected bacteria had a positive impact on the resistance of honey bee to pathogen. This method can serve as a valuable tool for immunological tests in honey bees.

Key words: entomopathogenic nematode, honey bee, immunity, non-pathogenic bacteria, plant alkaloid.

Introduction

Honey bees are used by humans for several thousand years, but the immune system of bees is still far from being fully understood. Moreover, we still don't have a clear idea about all immune mechanisms, which mediate honey bees' response to the pathogens. These pathogens negatively influence life of the honey bees and very often even their viability, causing direct impact on agriculture and industry. Therefore, a detailed knowledge of bee immunity is crucial for successful treatment and prevention against bee diseases.

As other insect, honey bees use variety of innate cellular and humoral immune reactions which can differ between developmental stages (Wilson-Rich *et al*., 2008; Laughton *et al*., 2011) and during senescence (Roberts and Hughes, 2014). Several antibacterial peptides functioning against bacterial infection were described (in honey bees specially apidaecin and royalysin) and also other parts of immune system can be involved (phagocytosis by haemocytes, coagulation or phenoloxidase activity) (Lourenco *et al*., 2013).

Honey bees are social insects where the so called "social immunity" was developed (Cremer *et al*., 2007). It is reported that because of their social life and behaviour honey bees lost many immune genes which are present in other insects, e.g. well studied Lepidoptera or Diptera (Evans *et al*., 2006).

Honey bees can be naturally infected by a broad spectrum of pathogens (bacteria and viruses mainly) causing many diseases (reviewed by Evans and Schwarz, 2011). Their immune system based on non-specific recognition of pathogen associated molecular patterns by pattern recognition receptors can be experimentally challenged with many other pathogens under laboratory conditions (e.g. entomopathogenic nematodes; EPNs), even if their contact in the nature is very limited. EPNs *Heterorhabditis bacteriophora* and *Steinernema feltiae* are obligate and lethal insect parasites. These EPNs are symbiotically associated with entomopathogenic bacteria *Photorhabdus luminescens* (producing red pigments and bioluminescence) or *Xenorhabdus bovienii* (producing yellow pigments) respectively, creating the highly pathogenic nematobacterial complex that is able to kill its host within 24 to 48 hours. The infective juveniles (IJs) with their bacterial symbionts are able to infect a broad spectrum of insect species. The bacterial symbionts are essential to kill the host and digest its tissues to provide nutrients for themselves and for developing nematodes. In last decades they have been mass produced and used increasingly as biological control agents of insect pests (Ehlers, 2001). EPNs natural infection

model is widely used in *Drosophila melanogaster* Meigen and *Galleria mellonella* (L.) research to test their immunocompetence (Hallem *et al*., 2007; Wang, 2010; Hyrsl *et al*., 2011; Dobes *et al*., 2012; Arefin *et al*., 2013). In honey bees several studies reported their non-susceptibility to nematode infection under natural conditions (Kaya *et al*., 1982; Baur *et al*., 1995). In this study, our effort was to prove that honey bee larvae and pupae can host nematobacterial complexes under laboratory conditions and exploit the interaction between the three organisms (honey bee hosts, nematodes and bacteria) for overall evaluation of efficiency of honey bee immunity influenced by potential modulators (plant alkaloid and non-pathogenic bacteria).

The gut of adult honey bee hosts up to 10^9 bacterial cells (Martinson *et al*., 2012), consisting of 8 abundant phylotypes making up to 95% of the total bacteria that appear to be specific to social bees (Jeyaprakash *et al*., 2003). Some of these bacteria (lactobacilli and proteobacteria) have been linked to immunity and defence against pathogens (Evans and Lopez, 2004; Forsgren *et al*., 2010; Audisio and Benitez-Ahrendts, 2011; Endo and Salminen, 2013; Cariveau *et al*., 2014). Prophylactic effect of probiotic bacteria, mainly lactobacilli, is well known from vertebrates (Ouwehand *et al*., 2002), but even in invertebrates the administration of live or dead bacteria can lead to an increase in resistance, an effect referred to as immune priming (Milutinovic and Kurtz, 2016). Also other factors such as plant alkaloids can modulate immunity of floral visitors. One hypothesized function is antimicrobial properties, which may benefit insect pollinators by reducing the intensity of pathogen infections (Manson *et al*., 2010). Alkaloids are also studied for therapeutic self-medication for invertebrates with complex social structure to reduce or probably even prevent diseases (Gherman *et al*., 2014). Moreover, in insect particularly honey bees the promoting effect of plant alkaloids, such as caffeine, on immunity and lifespan was observed previously (Strachecka *et al*., 2014) making them a promising group of potential immunomodulators. In our study we tested sanguinarine, the plant alkaloid extracted from *Macleaya cordata* (Papaveraceae), and a mix of three non-pathogenic species of probiotic bacteria previously isolated from honey bee gut.

Materials and methods

Honey bees and experimental design

Experimental beehives were arranged in apiary at Kyvalka near Brno, Czech Republic in two consecutive years. To collect honey bee larvae and pupae at the same developmental stage from all experimental groups (control, sanguinarine or bacteria treatment), new bee colonies for experiment were made from original colonies as four frame nuclei into warm insulated brood boxes. Each nucleus was equipped with one comb with eggs and young larvae in the middle, and two combs with hatching young bees on both sides. Honey comb and bees shaken off from the next three combs as well as ripe queen cell were added. To avoid flux between nuclei, they were localized several meters from each other. After 24 hours stabilization, sanguinarine was added as a part of sucrose syrup (1:1 sucrose, water) using glass feeders on the top of frames; while tested bacteria were sprayed on experimental comb. We suppose that alkaloid or bacteria were transferred by honey bees into stores and circulated inside the hive, the experimental larvae were treated from eggs or early larval stage. Control group obtained sucrose syrup only. Brood combs with honey bee L5-LS larvae (8th-9th day post eclosion) and Pw pupae were collected and transferred vertically into laboratory at 25 °C (L5 - fifth instar larva before sealing, LS - fifth instar larva after sealing, Pw - first pupal stage with white pigmentation of compound eye in accordance to Rembold *et al*., 1980). Isolated larvae and pupae (0.155 ± 0.015 g) were collected on moist tissue paper and subjected to infection assay. Experiments with dose-dependence of infection included control larvae or pupae only (without any alkaloid or bacterial treatment). The entire experiment was repeated three times independently in two consecutive years (control, sanguinarine and bacteria treated nuclei in first year and two other replicates of all three groups in second year).

Sanguinarine and mix of non-pathogenic bacteria

Sanguinarine, a powdered extract of *M. cordata* (Naturalin Bio-Resources Co., Ltd., China) was mixed with fructose syrup to obtain a concentrated stock solution. Concentration of sanguinarine in powder extract is 40%, the other major alkaloid present is chelerythrine constituting nearly 20%. Sanguinarine powder was analysed after dilution in 60% MeOH using HPLC-DAD on a system consisting of a Dionex P680 pump and UVD340 detector. Separation was performed under a linear gradient using 30 mM formic acid and acetonitril on a Phenomenex Gemini column (5 μm C18 110 Å, LC Column 250 × 4.6 mm), according to a slightly modified method previously published by Chen *et al*., 2009. Finally, concentrated stock solution was diluted to 1 g of sanguinarine per 1 litre of sucrose syrup. This dose proved to be safe and effective for honey bees in a previous study (Flesar *et al*., 2010).

Lactobacillus apis (NCBI accession: KM068134), *Lactobacillus melliventris* (KM068135) and *Gilliamella apicola* (KM068136) were isolated from honey bee digestive tract in our previous study (Hroncova *et al*., 2015) originally characterized by Killer et al. (2013), Kwong and Moran (2013), Olofsson *et al*. (2014), respectively. Bacteria were cultured in 30 ml Erlenmeyer flasks filled with MRS broth (Oxoid) for 24 h at 37 °C with the exception of *G. apicola*, which was cultured in the same medium for 48 h. Following this, they were combined in equal ratios in total volume of 90 mL and 12 mL of MRS medium was sprayed on experimental comb which responds to the dose of 1.1×10^7 bacteria of *L. melliventris* per mL, 2.2×10^8 *L. apis* per mL and 1.4×10^6 *G. apicola* per mL.

Infection assay

Isolated honey bee larvae or pupae were collected on moist tissue paper. For each experimental nucleus, a group of 20 individuals was collected and used for ex-

perimental infection. Nematodes *H. bacteriophora* (H222, isolated from Pouzdrany, Czech Republic) and *S. feltiae* (isolated from Prosenice, Czech Republic), were multiplied on *G. mellonella* larvae. Infective juveniles were applied on tissue paper inside Petri dish with 10 cm diameter at a multiplicity of 1-20 nematodes per larva or pupa. After 48 hours incubation at 25 °C larvae or pupae were scored for mortality. For sanguinarine or bacteria treated honey bees, the dose of 10 nematodes per larva was selected and mortality after 24, 48 and 72 hrs was recorded. Negative control without EPNs was tested with 100% survival for 72 hrs at 25 °C in Petri dishes with moist filter paper. *H. bacteriophora* harbouring green fluorescent protein (GFP) labelled *P. luminescens* was used to monitor the infection during optimizing experiments (similarly as shown previously in *Drosophila* by Dobes *et al*., 2012) as well as bioluminescence of host cadavers which was determined by LM01-T luminometer (Immunotech, Czech Republic).

Statistical analysis

Honey bee larval and pupal mortality was analysed using general linear models in Statistica 12 software (StatSoft, USA). Normality and homogeneity of data was tested using Shapiro-Wilk W test and Levene's test. Dunnett's test or non-parametric Kruskal-Wallis ANOVA with Dunn's multiple comparisons test were used to identify significant effects of treatment (bacteria and sanguinarine) in comparison to the control. Significant differences ($p < 0.05$) among tested groups are marked in graphs with different letters. Comparison of honey bee larval and pupal susceptibility to EPNs was done using Student's T-test. Significant differences are marked by asterisk ($p < 0.05$) or two asterisks ($p < 0.01$) in graphs.

Results

Larvae as well as pupae were successfully infected by two entomopathogenic nematode species. We optimized the infection for EPN *H. bacteriophora* and *S. feltiae*; both species cause typical coloration of cadavers due to pigments produced by their symbiotic bacteria (red or yellow, respectively, figure 1), which develop and multiply in honey bee larvae and release new generations of IJs (figure 2).

Successful infection with *H. bacteriophora* was further verified by detection of bioluminescence of infected cadavers. Symbiotic bacterium *P. luminescens* multiplies in cadaver and mean bioluminescence signal (10000 ± 150 RLU, n = 10) was detected using luminometer. We also visualized undergoing infection using GFP labelled *P. luminescens*, infected larvae and pupae showed bright GFP signal in whole cadaver under fluorescence light (figure 3). To keep our model widely accessible and natural, we used wild-type *Heterorhabditis*-*Photorhabdus* complex in subsequent experiments.

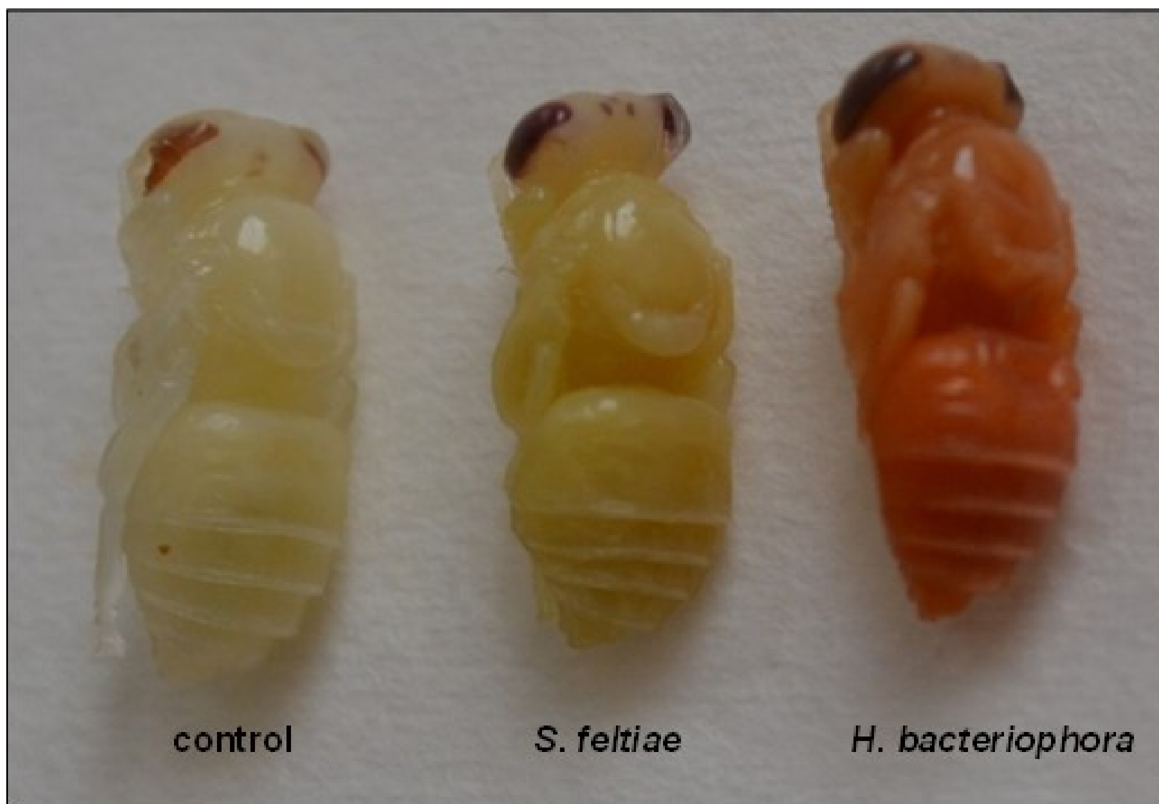

Figure 1. Honey bee pupae 48 hours after infection by *S. feltiae* (middle) or *H. bacteriophora* (right) with their typical coloration caused by their symbiotic bacteria *X. bovienii* or *P. luminescens*. Typical coloration of honey bee pupae is visible at uninfected control pupa (left).
(In colour at www.bulletinofinsectology.org)

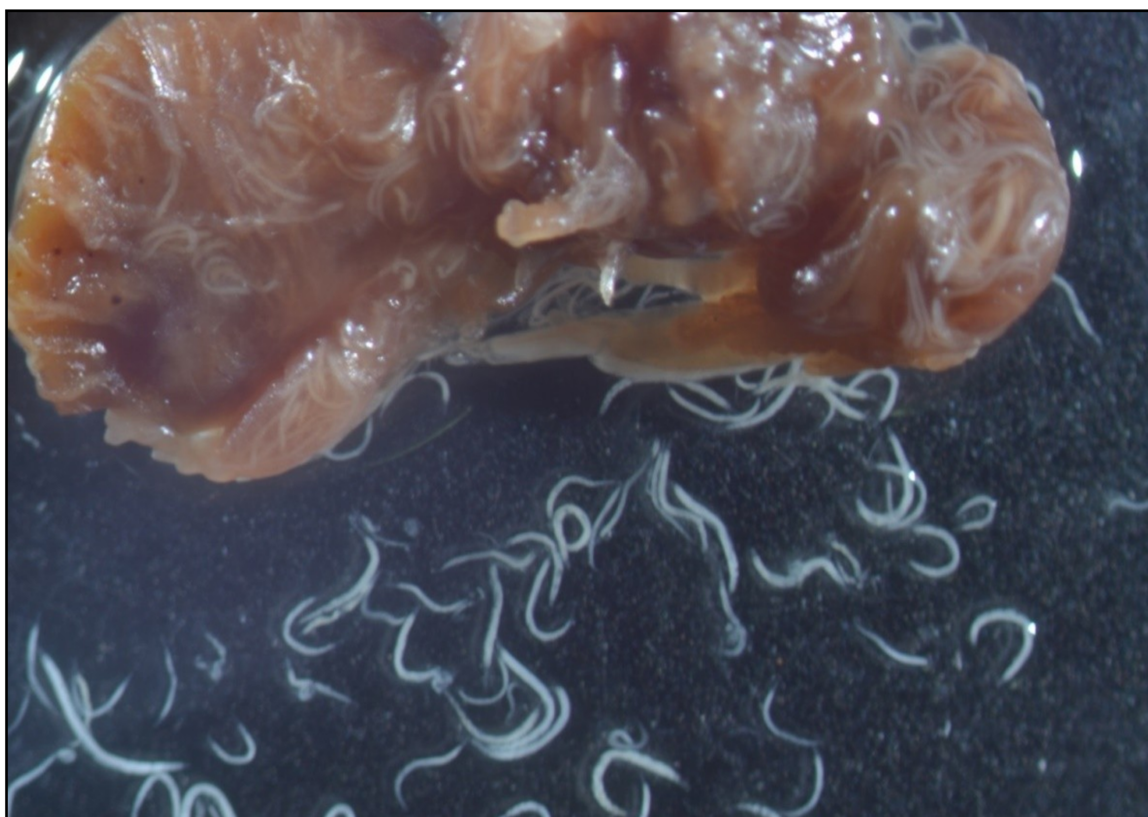

Figure 2. Honey bee pupa infected by nematobacterial complex *Heterorhabditis–Photorhabdus*. New generation of IJs is released from cadaver after 7 days.
(In colour at www.bulletinofinsectology.org)

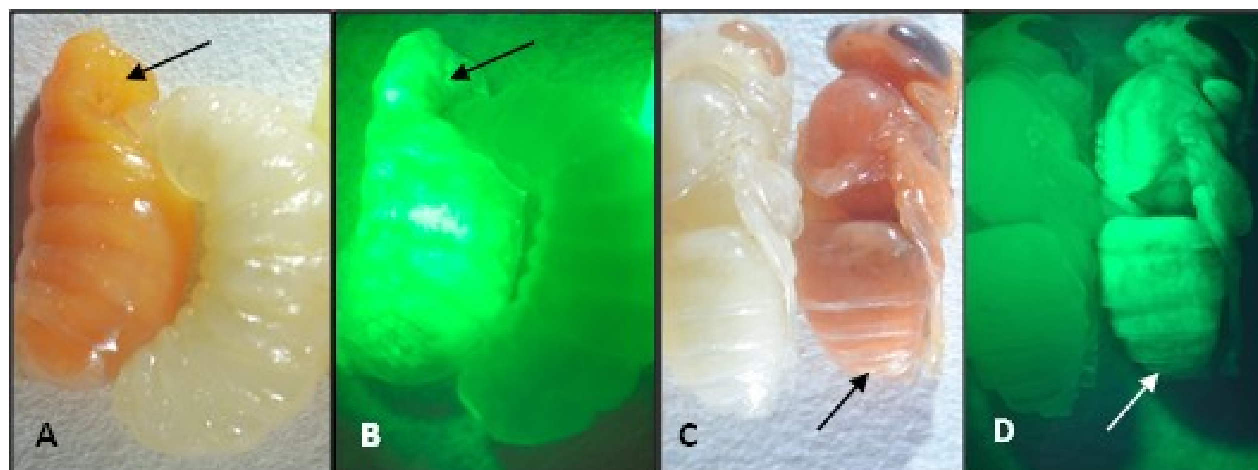

Figure 3. To demonstrate the role of symbiotic bacteria of EPN, the natural symbiont *P. luminescens* was replaced with GFP expressing strain. The bacteria are localized in the gut of IJs and cause septicemia after release into the insect hemocoel. Pictures shows uninfected and infected (arrows) larvae and pupae under day light (A, C) and fluorescence (B, D).
(In colour at www.bulletinofinsectology.org)

Dose dependence of mortality on number of IJs per honey bee larva or pupa was clearly demonstrated as shown in figure 4. Both nematode species caused similar mortality of bee larvae. Even dose of one IJ of *H. bacteriophora* per larva was able to kill 30-60% of hosts demonstrating high susceptibility of honey bees. Larvae were more susceptible to the infection by 10 IJs of *S. feltiae* than pupae (F = 1.75; df = 4; T-test P = 0.022) probably due their thinner cuticle which normally acts as physical barrier. The susceptibility of larvae and pupae to *H. bacteriophora* infection was comparable (F = 1.057; df = 5; T-test P = 0.331). The dose of 10 IJs/host was selected as a standard sub-lethal dose of EPNs for subsequent experiments.

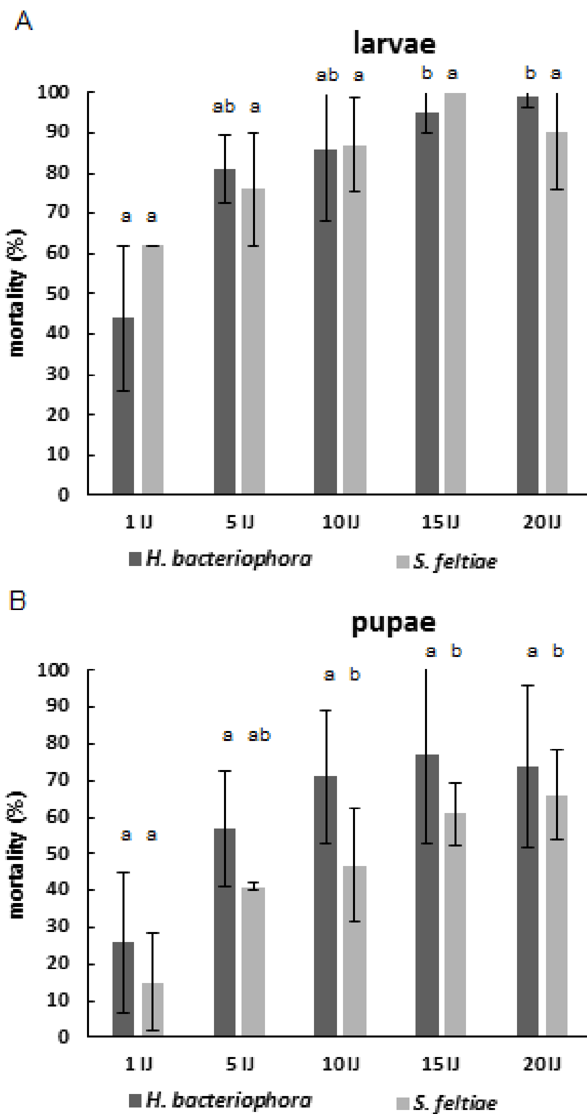

Figure 4. Mortality of honey bee larvae (A) and pupae (B) 48 hours after infection is dependent on dose of IJs used for infection. Honey bees were infected with nematobacterial complex *Heterorhabditis-Photorhabdus* and *Steinernema-Xenorhabdus* (mean ± SD). Significant differences are indicated by different letters above the columns.

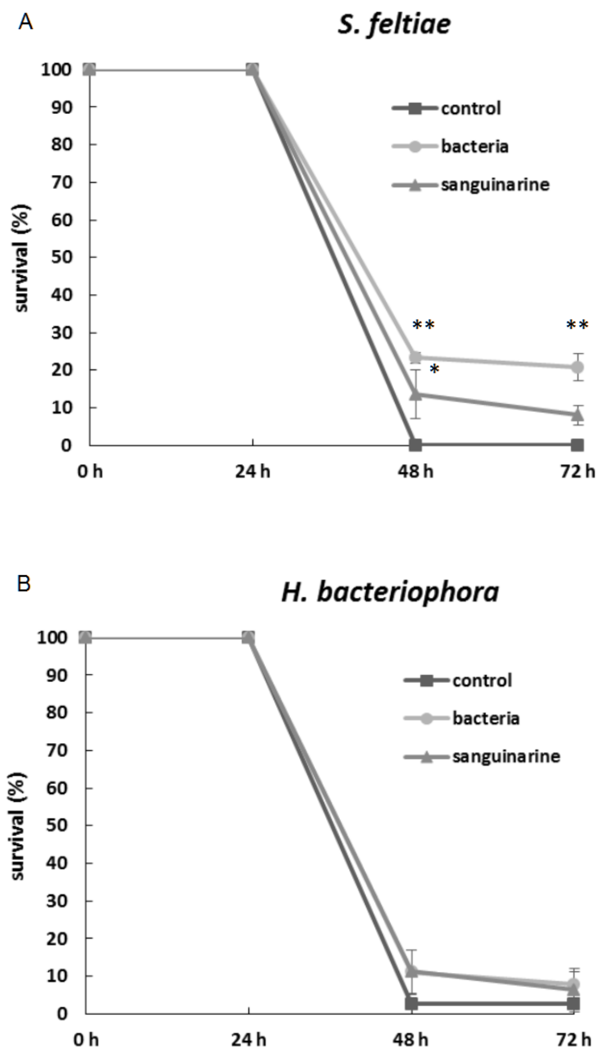

Figure 5. Immuno-stimulating effect of plant alkaloid sanguinarine and mix of non-pathogenic bacteria (*L. apis*, *L. melliventris*, *G. apicola*) on honey bee larvae infected with 10 IJs of *S. feltiae* (A) and *H. bacteriophora* (B) per larva. Data are expressed as percentage of survivorship (mean ± SD, * = $P < 0.01$, ** = $P < 0.05$).

Application of selected non-pathogenic bacteria and sanguinarine led to enhanced survival of honey bee larvae after nematobacterial infection compared to control, figure 5. A mix of three non-pathogenic bacteria increased survivorship to about 23.25 ± 1.53 and 11.0 ± 6.0% for *S. feltiae* and *H. bacteriophora*, respectively over control. Similarly, survival in sanguinarine group after 48 hours post infection increased to about 13.5 ± 6.43 and 11.25 ± 5.77% in case of *S. feltiae* and *H. bacteriophora*, respectively, whereas most of control larvae succumbed to the infection. Decreased mortality after nematobacterial infection was significant in *S. feltiae* infection after non-pathogenic bacteria treatment (df = 9; Dunnett's test $P = 0.001$ and $P < 0.001$ for 48 and 72 hours after infection respectively) and statistically significant at first time point for sanguinarine (df = 9; Dunnett's test $P = 0.022$ and $P = 0,074$ for 48 and 72 hours after infection respectively). Similar but non-significant trend was observed also in case of *H. bacteriophora* infection in both experimental groups.

Discussion and conclusions

Over the past several years, governments, beekeepers, and the general public have become concerned by increased losses of honey bee colonies, calling for more research on how to keep colonies healthy while still employing them extensively in agriculture. The immunocompetence of honey bees relies largely on the

quality and diversity of nutrients available (Alaux *et al*., 2010). However, it is unclear what protects honey bees against pathogens and chemicals in the natural environment. Suitable prophylaxis could balance at least partly the negative effects of environment on honey bee health as e.g. recently discussed neonicotinoid pressure (Porrini *et al*., 2014; Pistorius *et al*., 2015). As social immunity is present in honey bee colonies, one has to think also about the effect of potential immunostimulants on social behaviour and chemical communication inside the hive (Richard *et al*., 2012).

The plant *M. cordata* is traditionally used in Chinese medicine. It contains several isoquinolone alkaloids and sanguinarine and chelerythrine are considered to be responsible for the plant's pharmacological effects (Zdarilova *et al*., 2008). These active substances are used as natural feed substances and were successfully tested for toxicity in mammals (Kosina *et al*., 2004; Psotova *et al*., 2006; Zdarilova *et al*., 2006). Moreover, sanguinarine has high antimicrobial effect against growth of *Paenibacillus larvae*, the etiological agent of the American foulbrood, one the most important diseases of honey bees. Sanguinarine has low oral toxicity to honey bees (Flesar *et al*., 2010). Our study shows, that sanguinarine may help to increase the protection of honey bee larvae against EPN experimental infection.

In addition to the host's immune system, vertically transmitted microbial symbionts are sometimes suspected to play a role in insect defence against infection by viruses (Hedges *et al*., 2008), bacteria (Dillon *et al*., 2005), or eukaryotic parasites (Jaenike *et al*., 2010). Microbial symbionts of honey bees offer a promising tool to improve honey bee health (reviewed by Crotti *et al*., 2013). A part of our objective was to test the role of naturally occurring non-pathogenic bacteria from digestive tract of honey bees on their health, with major implications for research on bee decline and sustainable pollinator management. For this experiment we have selected species from our previous study (Hroncova *et al*., 2015). Lactobacilli have been proposed as probiotics of honey bee with the goal to protect them against the common pathogen *P. larvae* and *Melissococcus plutonius* (Evans and Lopez, 2004; Audisio and Benitez-Ahrendts, 2011; Endo and Salminen, 2013; Gaggia *et al*., 2015). Forsgren *et al*. (2010) demonstrated a strong inhibitory effect of the combined honey bee stomach flora of lactic acid bacteria and of two lactobacilli phylotypes on the *in vitro* growth of *P. larvae*. Their results clearly demonstrate that addition of lactic acid bacteria to young honey bee larvae exposed to spores of pathogen decreases the proportion of larvae. However, the mechanism of action is still unknown; microbiota can benefit their host in multiple ways including metabolising food and toxins, nutrient supplementation, and can lead to increased immunocompetence and resistance of honey bee larvae and other developmental stages to pathogens (Evans and Lopez, 2004). Also, our results strongly suggest that selected non-pathogenic bacteria linked to the honey bee gut have important implications for nematobacterial infection in particular and for honey bee pathology in general.

Nematobacterial infection combines in itself the infection caused by bacteria and the influence of multicellular parasite (nematode) which invades insect host and serves as the vector of bacteria. It is of note that not only bacteria are able to influence defences and immunity of host, but also EPNs produce a number of proteases and virulence factors affecting the invaded insect (Hao *et al*., 2012). We used EPNs infection to study immunity of *D. melanogaster* in our previous studies (Hyrsl *et al*., 2011; Dobes *et al*., 2012, Arefin *et al*., 2013) and here we show that honey bee larvae and pupae *in vitro* conditions are also suitable hosts for nematobacterial complex. We optimized the natural infection of honey bee larvae and pupae for EPNs species *H. bacteriophora* and *S. feltiae*. There is only a single report recorded for *in vitro* infections of honey bee larvae by *S. feltiae* and *S. affinis* (Zoltowska *et al*., 2003a; 2003b). Zoltowska *et al*., (2003a) showed higher susceptibility of honey bee larvae in Petri dishes than in isolated combs and that worker larvae are more susceptible than drone larvae. Upon direct application of *S. feltiae* at the dose of 10 IJ applied on honey bee larvae, Zoltowska *et al*. (2003b) observed 62.5% successfully invaded individuals after 48 hours and a decrease of host protein level. This high susceptibility is in accordance with our results and observed differences in pathogenicity can result from specific conditions of infection assay or depend on the particular nematode strain used. Our results thus verify the fact that isolated honey bee larvae and pupae can be infected by entomopathogenic nematodes; it is of note that GFP labelled symbiotic bacteria harboured in EPN can be used for tracking the early-stage infection. Laboratory setting with 25 °C temperature and moist filter paper is suitable for honey bees, nematodes as well as their symbiotic bacteria. Mortality of honey bee larvae depends on EPNs dose and was comparable to Lepidopteran larvae such as *G. mellonella* (Hyrsl, 2011). Honey bee larvae are more susceptible to the infection than pupae probably because of the thickness of the cuticle and open digestive tract which is preferred as the site of entrance for infective juveniles.

Under natural conditions, honey bee larvae and adults are unreachable to nematode infection (Kaya *et al*., 1982; Baur *et al*., 1995) because of sticky wax and honey present in honeycombs as well as higher temperature affecting survival of EPNs in the hive (even using high-temperature-tolerant nematode species as shown by Baur *et al*., 1995). Nematode infection is possible only with caged adult honey bee workers; Shamseldean *et al*., 2004 tested six EPN species against honey bee workers and showed that they are more susceptible to steinernematid species than to heterorhabditids.

In summary, we observed a positive effect on survival rate after EPNs infection of honey bee larvae following application of sanguinarine and non-pathogenic bacteria, which can act as a novel potential probiotic. The infection by EPNs can serve as unique model of combined infection applicable in tests of host immunocompetence.

Acknowledgements

We thank Zdenek Mracek for wild type isolates of EPN and Todd Ciche for the GFP-expressing symbiotic strain of *Photorhabdus luminescens*. Thanks also to Dalibor Titera, Jaroslav Havlik, Martin Kamler, Jana Hurychova, Jakub Berka and Oldrich Veverka for their help with field experiments and Natraj Krishnan for reading the manuscript. Our research is supported by grant from Ministry of Agriculture of Czech Republic (NAZV-KUS QJ1210047) and the Internal Grant Agency of the Czech University of Life Sciences Prague (CIGA 20132013).

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Received December 15, 2015. Accepted November 14, 2016.