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Faculty of Agrobiology, Food and Natural Resources

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Microbiota of the digestive tract of bees and related insects and the influencing factors

Doctoral thesis

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Declaration

I declare that given Ph.D. thesis is solely my own work unless otherwise referenced or acknowledged.

Prague, 24th October 2016

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Proverb

This thesis is submitted in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE and consists of six research chapters, out of which, five have already been published in research journals, 1 is in the process of submission and the last chapter presents original unpublished confidential data in the process of manuscript preparation.

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Content

1

General Introduction

Bees are important crop pollinators and thus they are important for agriculture and food production. When the sudden collapse of several bee colonies in the Europe, USA and Asia began without any apparent reason around ten years ago, the focus was set on bee health. It is important to preserve the bee colonies healthy and viable, but the Colony collapse disorder renders the bee colonies to collapse due to the massive loss of adult foraging bees. Normally some bee colonies also die due to seasonal variation during the winter-spring transition, however, these losses also seem to be rising in the last few years without any apparent reason. Many different factors seem to play a role, as for instance; use of pesticides and antibiotics, nutrition abundance and variance, bee pathogens; including parasites, viruses, pathogenic fungi and bacteria. A new paradigm has been set during the last few decades, that health can be linked to gut microbiota, which exists in the vast majority of animals, and often form complex symbioses with their hosts that affect their host's biology in numerous ways. To date, the majority of studies of these complex interactions have focused on the nutritional benefits provided by the microbiota; however, the natural microbiota can also influence development, immunity, and the metabolism of its host. *Apis mellifera*, the honey bee, harbours a distinctive bacterial community that is present in individuals from distant locations around the world; however, the basis of the bee-microbiota association is unknown. *Apis* and *Bombus* species share a set of distinct bacterial communities that are not present in other bees and wasps. Additionally, environmental factors seem to be changing the microbial composition in various ways. Diet has been shown to shape microbial communities in vertebrates but also in insects. Moreover, emerging hypotheses show that insects might be attracted by nectar of plant containing high concentrations of small molecular weight bioactive metabolites, e.g. plant alkaloids, many of them exhibiting effect in speculated self-healing nutritional attitudes. Thus, there seems to be a complex of reason for bee colony deaths, a reasonable approach to gut microbiota would be to address set it as a complex community, and to investigate the bacteria in their normal habitat.

The aim of this thesis is to explore the complex mechanisms of bees and wasps gut microbial populations, their possible role in the immune response and ways of its manipulation.

1.1 The role of bees and wasps in the ecosystem

Plant–pollinator relationships may be one of the most ecologically important classes of animal–plant interaction: pollinators are essential for the reproduction of at least two thirds of flowering plant species, including many which are now endangered (Ollerton et al., 2011,

Knight et al., 2005) and without pollinators, many plants could not set seed and reproduce (Kearns et al., 1998). Pollinators are of great ecological and economic importance. They pollinate a wide variety of crops with an estimated global value of €153 billion, which represented 9.5% of the value of the world agricultural production used for human food. Vegetables and fruits are the leading crop categories in value of insect pollination with about €50 billion each, followed by edible oil crops, stimulants, nuts and spices (Gallai et al., 2009). Although much attention has focused on managed populations of honey bees, pollinators include a diversity of insects, with the main groups in temperate areas being bumble bees, social wasps, hoverflies and solitary bees (Evison et al., 2012). With regard to the more than 20 000 species of bees (Michener, 2000), it has been recognized that all but the parasitic ones play a role in pollination. Bees are a monophyletic group of Hymenoptera that transitioned to a completely herbivorous diet from the carnivorous diet of their wasp ancestors. Bees may be solitary or may live in various types of communities. The most advanced forms of these are eusocial colonies found among the honey bees, bumble bees, and stingless bees.

The honey bees represent highly advanced social insects, which live in colonies of about 10,000–50,000 females and a few hundred males (Wilson, 1971). Each colony has one reproductive queen, which lays eggs and produces several pheromones, and most individuals are non-reproductive females (workers) that provision and rear young within large colonies. *Apis mellifera*, is domesticated around the world for honey production and is a key link in the human food supply because yields of some fruit, seed and nut crops decrease by more than 90% without these pollinators (Watanabe, 1994, Southwick and Southwick, 1992). When wild bees do not visit agricultural fields, managed honey bee hives are often the only solution for farmers to ensure crop pollination. Managed bees are highly social, frequent a multitude of environmental niches, and continually share food, thus, these conditions promote the transmission of parasites and pathogens. The hive of the honey bee may be best characterized as an extended organism that not only houses developing young and nutrient rich food stores, but also serves as a niche for symbiotic microbial communities that aid in nutrition and defence against pathogens (Anderson et al., 2011). Since 2006, high annual losses of honey bee colonies in North America and Europe have profound ecological and economic implications (van der Zee et al., 2012).

The bumble bees (*Bombus terrestris* and *B. pascuorum)* are common European bumble bees, but *B. terrestris* has large colonies and short-tongued workers that visit a general range of flowers, whereas *B. pascuorum* has smaller colonies and long-tongued workers that specialize on visiting flowers with deep corollae (Prys-Jones and Corbet, 1991). Bumble bees are

integral wild pollinators, which also occur throughout the Americas and much of Asia and pollinate individual species, floral communities, and agricultural crops (Goulson, 2010). Recent domestication has boosted their economic importance in crop pollination to a level surpassed only by the honey bee (Delaplane et al., 2000). The overall value of bumble bee pollination services to natural ecosystems has not been estimated such as in honey bees, but it is known, that the persistence of many or all of the 250 or so bumble bee species world-wide is required (Williams and Osborne, 2009). Bumble bees now represent a €55 million industry (Velthuis and Van Doorn, 2006). The main agricultural crop that bumble bees pollinate is the greenhouse tomato (*Lycopersicon esculentum*). Worldwide, this involves about 95% of all bumble bee sales. Only the value of these bumble bee pollinating tomato crops is estimated to be €12 billion per year (Velthuis and Van Doorn, 2006). It seems probable that reductions in the abundance and species richness of bumble bees may lead to widespread changes in plant communities (Corbet et al., 1991). These changes may have further knock-on effects for associated herbivores and other animals dependent on plant resources and may therefore reduce the efficiency of this vital ecosystem function to the detriment of plant communities and associated invertebrates (Daily, 1997). Also urban habitats are potentially important for bumble bee conservation because of the presence of flower-rich gardens and parks (Goulson et al., 2002). Due to dramatic declines in abundance and geographic range of bumble bee species mainly scientists from Europe and North America begun to study bumble bees and to publish their results (Buchmann and Nabhan, 1997). A series of recent initiatives, including the International Pollinator Initiative (Williams, 2003), have been aimed at the conservation of this important group. The concerns about pollinator decline worldwide over recent decades have now been acknowledged internationally at the highest level.

The common wasp, *Vespula vulgaris*, is a eusocial vespid found in throughout the Northern Hemisphere and has been introduced to Australia and New Zealand. This species as well as other wasps are known in the United States as yellowjackets. The main economic importance of the wasps lies in the damage they cause to fruit and negative impact on bee populations which, in turn, reduces the pollination of clover. On the other hand, wasps are beneficial in that they kill and eat many harmful insects, especially blowflies and caterpillars to feed to their young (Thomas, 1960). In particular, *V. germanica* wasps obtain carbohydrates from nectar, sap, and fruits, while proteins are obtained by arthropod predation and scavenging on vertebrate and invertebrate carrion (Akre and MacDonald, 1986). However, in periods of food scarcities and also in colder weather adult wasps and old queens die.

The biggest losses of all above mentioned insect species (except of common wasp, where losses have not been reported) are probably caused by a combination of different factors including environmental stresses, such as diverse of food resources and the use of insecticides, also management practices and biotic stresses, such as parasites and infectious diseases (Engel and Moran, 2013b).

1.2 Environmental pressures on pollinators

The biology and health of the bees and wasps has been of interest to human societies for centuries. Research on honey bee health is surging, in part due to colony losses which have been attributed to multiple environmental factors including numerous pathogens, including viruses, fungi, bacteria, and protozoa. Also one of the principal factors is likely to have been the loss and degradation of habitats and critical food resources due to changes in land-use and agricultural practices (Williams, 2005, Goulson et al., 2005).

1.2.1 Diseases

Pollinators face a diverse pathosphere and their ability to resist these threats depends upon commensals, nutritional status, the accumulation of toxic compounds, and genetically based resistance and tolerance mechanisms. Although honey bee pathology has been a field of study since ancient Greece (Aristotle referred to contagious brood disease as a 'wildness' in colonies), many questions remain regarding the impacts of microbes upon bee health (Evans and Schwarz, 2011). Modern sequencing techniques have identified several novel viruses and microbes in bees (Cox-Foster et al., 2007, Runckel et al., 2011). Pollinators face viruses, microbes or parasites spanning several kingdoms, although the most damaging threats and hence the most researched groups are viruses, bacteria, parasites and fungi from which the most detrimental effect on pollinators have a mite *Varroa destructor,* microsporidium *Nosema* spp., bacterium *Paenibacillus larvae* and some viruses.

Varroa

The ectoparasitic mite, *Varroa destructor* (formerly known as *Varroa jacobsoni*), is the most detrimental honey bee parasite in the world today (Rosenkranz et al., 2010). It was originally known only from *Apis cerana* (which is found in southern and eastern Asia), but expanded its host range to include *A. mellifera* during the first half of the 20th century, spreading rapidly around the world, and is currently considered the single greatest threat to apiculture. Because *Varroa* and honey bees have not co-evolved for a long period of time, they do not exhibit an

adapted host-parasite relationship, resulting in *Varroa* often killing its host (Oldroyd, 1999). *Varroa* mites have been considered a problem for beekeeping since around the late 1960s; by the 1970s, they had reached Western Europe and South America and by the 1980s they had reached the United States. Today, it can safely be assumed that all honey bee colonies within the mite's range harbour *varroa* mites. As a consequence of mite infestation, dramatic colony losses have repeatedly occurred in affected countries (Vanengelsdorp et al., 2007).

Many physical and physiological detrimental effects of the *Varroa* mite have been described at the individual bee and colony levels. Repeated *Varroa* feeding on adult bee and brood haemolymph injures the bees physically, reduces their protein content and wet and dry body weights, and interferes with organ development (Bowen-Walker and Gunn, 2001). The parasitic mite and the viruses they vector contribute to the flight behaviour of forager bees, to the effect that foragers might not return to the colony. This is interpreted as an adaptive behaviour of the bees to remove the parasites or pathogens from the colony (Kralj and Fuchs, 2006). The mite is relatively small, 1.5mm by 1mm and its metabolic rates range 1.1% to 2.4% to that of the bee pupa depending on the infestation level. But the nutritional demand of the mites is very high, owing to their inefficient metabolic machinery, utilizing up to 25% of the nutritional reserves of the pupae accumulated in tissue during the larval stage. The feeding of the mites contributes to the malformation and weakening of the bees and eventually of the colony (Garedew et al., 2004). Although mites have a strong (on average eightfold) preference for parasitizing drone brood (Martin, 1995), they successfully reproduce in worker cells, especially when drone brood is absent or heavily parasitized (Boot et al., 1995). Reproduction on worker brood leads to an exponential increase in mite numbers (Martin, 1995, Martin and Kemp, 1997, Fries et al., 1994). Mite reproduction on worker brood is debilitating to the emerging worker. Moreover, mites are transmitting as vectors most of the common viral disease of honey bees. The mite's feeding helps spread viral infections among bees. Consequently, mite kills the colony within six months to two years of mite infestation (Oldroyd, 1999). The length of the time span does not only depend on the mites' potential to multiply in bee brood but also depends on the *Varroa* invasion pressure from nearby colonies. A high bee density combined with severe *Varroa* infestation accelerates bee death. When adult bees are infected prior to overwintering, they survive a shorter time compared to mite-free workers and they do not transition from summer to winter bees. As a consequence, they do not live as long as winter bees and are less able to contribute to the build-up of colony strength in the early spring. When high mite levels are present, parasitized colonies collapse during the winter (Kovac and Crailsheim, 1988, Boecking and Genersch, 2008).

Nowadays, beekeepers utilize a wide range of different chemical substances, application techniques and methods to keep mite populations under control. The following agents are currently permitted for the control of *Varroa* mites: Flumethrin (in many locations it is ineffective due to mite resistance (Lodesani et al., 1995) and Coumaphos. There is no chemical treatment with 100% effectiveness. A multitude of other miticides are no longer permitted because these can still be found as residues in bees wax (Wallner, 1999, Bogdanov, 2006). Residues in bee products must be avoided, which is why it is forbidden to implement any type of *Varroa* treatment in a colony from which honey will be harvested in the same year. Natural substances such as formic, oxalic acid and thymol have not yet resulted in resistant mite populations, but while they reduce mite populations, they are not consistently highly effective in all situations. Only formic acid has a miticide effect on *Varroa* on adult bees as well as mites inside capped brood. For this reason, formic acid can be used effectively immediately after the last honey harvest, when still brood exists in the bee colonies. All other miticides should only be applied when the colony is free of brood (Boecking and Genersch, 2008). However, probiotic microorganisms originally isolated from digestive tract of host do not have negative effect on any stages of honey bees and can be used in the fight against *Varroa.* The most powerful strains appear to be Actinobacteria (Maddaloni and Pascual, 2015) and *Bacillus subtilis* (Sabaté et al., 2012).

Nosematosis

Nosema disease is one of the most prevalent adult honey bee diseases (Bailey and Ball, 2013) and is caused by two described species of microsporidia, *Nosema apis* and *Nosema ceranae*. *Nosema ceranae* is a microsporidian parasite originally known to infect the Asian honey bee, *Apis cerana*, while *Nosema apis* was discovered in the European honey bee, *Apis mellifera* (Fries et al., 1996). However, this is no longer true and *N. ceranae* remains the overwhelming species across the whole Europe and America (Klee et al., 2007, Paxton et al., 2007). *N. ceranae* was first described from the Asian honey bee (*A. cerana*) in samples from the Bee Institute of the Chinese Academy of Agricultural Sciences outside Beijing, China (Fries et al., 1996), while *N. apis* has been known since 1909 (Zander, 1909). Discrimination between the two species became possible since the development of molecular biological detection methods (Klee et al., 2007). Cross-infection experiments using both *N. ceranae* and *Nosema apis* in both *A. cerana* and *A. mellifera* demonstrated that both parasites were cross-infective across hosts, but that *N. ceranae* developed better in *A. mellifera* compared to *N. apis* in *A. cerana* (Fries and Feng). The outcome of the experiments indicates minor differences in

infectious dose and multiplication rate between the two species. Moreover, the mortality caused by *N. ceranae* was not significantly higher than for *N. apis* and *N. ceranae* appeared to have no competitive advantage within host (Forsgren and Fries, 2010). Honey bee colonies are frequently infected, and this infection targets all colony members, including adult worker bees, drones, and queens. Nosema infection occurs mostly through ingestion of spores with food or water. The physical and chemical conditions of the midgut trigger the germination of spores and the vegetative stage of Nosema begins to grow and multiply inside midgut cells. Bailey and Ball (1991) showed that 30–50 million spores could be found inside a bee's midgut within 2 weeks after initial infection. Eventually the spores pass out of the bee in its feces, providing new sources of the infection through cleaning and feeding activities in the colonies (Chen et al., 2009). Symptoms of nosematosis caused by *N. apis* are more easily observed in honey bee colonies which show large numbers of dead bees and diarrhoea spotting at hive entrances evidencing digestive disorders of adults. Symptoms of *N. ceranae* infestations are more nebulous, consisting primarily of poor colony growth and dwindling. Whereas, infected bees do not exhibit obvious external disease symptoms, infection of *Nosema* causes digestive disorders, shortens bee life span (Wang and Mofller, 1970), decreases population size of honey bee colonies (Malone et al., 1995) and can induce queen supersedure (Webster et al., 2004). As a result, this leads to a reduction of honey production, crop pollination and thus, economical agricultural losses (Anderson and Giacon, 1992). The role of *Nosema* infection in recent bee losses is unclear (Evans et al., 2009) but several studies suggest that Nosema infestation is an important factor contributing to colony collapse unless the infections are controlled (Higes et al., 2008, Martín-Hernández et al., 2007). However, most published data on colony losses linked to *N. ceranae* infections are correlations and fail to provide evidence of cause and effect, suggesting a multifactorial cause. Also *Nosema bombi* can have large effects on individual bees. Infected animals may have crippled wings, and queens may have distended abdomens and be unable to mate (Otti and Schmid-Hempel, 2007). However, transmission most likely occurs when spores are fed to larvae (Rutrecht et al., 2007). Consequently, prevalence of *N. bombi* in the field at the individual and colony levels is considerably lower than prevalence of the much more easily transmitted *Crithidia bombi* (Baer and Schmid-Hempel, 1999). *N. bombi* is a generalist parasite; there is no evidence of host-species-specific strains in Europe (Tay et al., 2005) but colonies of *Bombus terrestris* experimentally infected with *N. bombi* are smaller and produce fewer reproductive offspring, which are often crippled (males) or incapable of mating (gynes), than uninfected colonies (Otti and Schmid-Hempel, 2007, Otti and Schmid-Hempel, 2008). In the field, colony

infection may occur through drift of infected workers into non-natal colonies (with subsequent infection of larvae and adults); infection of adults by spores during foraging; or infection of larvae that are fed contaminated pollen (Rutrecht et al., 2007).

The major commercial medication available, based on the antibiotic fumagillin, is effective but in contrast to some other parts of the world where *Nosema* infections may be controlled using fumagillin, antibiotic treatments of honey bee colonies are not legal in most parts of Europe. Other practices such as wax renewal, acetic acid fumigation of stored comb are being applied (Williams et al., 2008). However, the goal of the current study is to assess the effects of the oral administration of the metabolites produced by probiotic microorganisms. These are organic acids which could act as an organic tool to reduce individual loads of *Nosema* without fumagillin application and keep controlled the reinfestation when antibiotic control is used, improving its efficacy (Maggi et al., 2013, Porrini et al., 2010).

American Foulbrood

Paenibacillus larvae subspecies *larvae* (Heyndrickx et al., 1996), a spore-forming bacterium, are the causative agent of the American foulbrood (AFB), one of the most serious and destructive brood diseases of honey bees. A colony is known to be diseased when larvae are observed with the clinical symptoms of American foulbrood. Individuals become infected by swallowing spores of *P. larvae larvae* that contaminate their food at a very young age. After spores germinate in the larval midgut, the vegetative forms penetrate the tissue of the intestine and multiply, which finally kills the larva (Gregorc and Bowen, 1998). This infective stage of the disease organism is transmitted by adult bees directly to other larval bees (Bamrick, 1967). Newly hatched larva can become infected by as few as 10 spores (Woodrow, 1942), but the dosage-mortality relationship is greatly influenced by larval age (mortality decreases sharply as the age at inoculation increases), genetic constitution, bacterial strain (Genersch et al., 2005) and also larvae from different lines of honey bees showed different mortality rates following inoculation (Rothenbuhler and Thompson, 1956). However, it seems that the infected larva has no means to successfully combat infection and to escape being killed by *P. larvae*. Hence, individual immune responses though elicited by infection (Evans, 2004) are obviously not effective leaving it to the social immune response to deal with the infection at colony level (Wilson-Rich et al., 2009, Rauch et al., 2009).

Since AFB is a notifiable disease in many countries, measures against and treatment of AFB are often regulated by law and include the destruction of clinically infected hives. In some countries, antibiotics are allowed for the treatment of infected colonies and common strategy

for the prevention and treatment is the use of oxytetracycline hydrochloride (OTC), sulfathiazole or streptomycin. However, in most European countries, the use of antibiotics in the treatment of bee diseases is not permitted (to avoid contaminating honey). Drifting foragers can spread the disease very rapidly (Pfeiffer and Crailsheim, 1998) and therefore The European Union Commission was taken Decisions 2003/881/EG and 2005/60/EG, regulating the imports *Bombus* spp., colonies and honey bees in order to prevent the import of *P. larvae.* But none of the potentially virulent bee parasites are covered under this legislation; thus, veterinary screening is likely to be ineffective in controlling the spread of bee parasites (Meeus et al., 2011). Hence, there is an urge to develop new alternative treatment strategies also for bees, which so far follow three different directions: (1) Breeding for honey bee genetic stock showing an increased individual or social immune response against AFB (Evans and Lopez, 2004, Spivak and Reuter, 2001, Wedenig et al., 2003); (2) treatment with natural antibacterial substances like essential oils of various plants (Fuselli et al., 2008a, Fuselli et al., 2008b, Gende et al., 2009) or propolis (Antúnez et al., 2008, Bastos et al., 2008) and (3) biocontrol through probiotic antagonistic bacteria, what according to recent studies it appears the best solution (Alippi and Reynaldi, 2006, D Evans and Armstrong, 2005, Olofsson and Vasquez, 2008).

Viruses

A very crucial aspect of the dynamics of virus infections and evolution of host–pathogen interactions is the mode of transmission. In general, transmission of a virus can occur horizontally or vertically, or both. In horizontal transmission, viruses are transmitted among individuals of the same generation (air-borne infection, food-borne infection and sexual infection). In vertical transmission, viruses are passed vertically from mother to offspring via egg (Clayton and Tompkins, 1994, Ewald, 1993).

Over the past years at least 18 virus types and strains have been recorded as disease pathogens of adult bees and bee brood, although RNA viruses predominate in honey bees, DNA viruses have occasionally been reported (Clark, 1978). Viruses that cause the most damage of honey bees are Chronic bee paralysis virus (CBP), Acute bee paralysis virus (ABPV), Israeli acute paralysis virus (ICPV), Kashmir bee virus (KBV), Black queen cell virus (BQCV), Cloudy wing virus (CWV), Sacbrood virus (SBV), Deformed wing virus (DWV) and Slow bee paralysis virus (SBPV). These viruses in bees can remain asymptomatic, or cause changes in physiology, behaviour, morphology and are associated with weak and dying colonies (Genersch and Aubert, 2010, van Engelsdorp et al., 2009, Chen and Siede, 2007). Moreover,

bee colonies can be attacked by more than one virus simultaneously and multiple viral infections and the most of these viruses may exist and even co-exist in honey bee workers, queens (Chen et al., 2005) or colonies without provoking apparent symptoms.

Viral disease of bumble bees cause Entomopox virus (Schmid-Hempel, 1998) and viruses (Black queen cell virus, Deformed wing virus, Acute bee paralysis virus, Slow bee paralysis virus and Sacbrood virus) which were originally thought to only affect honey bees, and incidentally are all named after their effects in honey bees, also occur in wild bumble bees. Researchers found that levels of these viral diseases were much higher in bumble bees, which may suggest that those viruses may even rely on bumble bees to spread amongst other hosts (Genersch et al., 2006, Evison et al., 2012, Fürst et al., 2014, Niu et al., 2014). The fact that bumble bees and honey bees are able to share nectar and pollen resources in the same field suggests that geographical proximity of two host species could play a role in host range breadth of viruses (Peng et al., 2011). Studies of the effects of interspecific transfer of pollinator viruses are especially important for bumble bees, since bumble bee diversity is diminishing rapidly in many regions of the world (Biesmeijer et al., 2006, Cameron et al., 2011, Potts et al., 2010).

Viruses infect not only honey bees but also all developmental stages of the bee including eggs, brood, and adults of wasps. Species of *Vespula* have been also observed to be infected with Deformed wing virus (Evison et al., 2012), which has been considered as the main suspect behind unexplained honey bee colonies collapsing worldwide (Schroeder and Martin, 2012). Kashmir bee virus has previously been observed in common wasps in New Zealand (Rose et al., 1999). Elsewhere Vespula sp. wasps have been known to be infected with a range of viruses commonly found in honey bees, including the Israeli acute paralysis virus, Deformed wing virus, Kashmir bee virus, Black queen cell virus, and Sacbrood virus (Rose et al., 1999). The transmission of the viruses between species has been observed to occur via foraging in the same environment on flowers or pollen (Singh et al., 2010). This sharing of pathogens can result in a correlated prevalence of viruses between hymenopteran species such as bumble bees and honey bees (Fürst et al., 2014).

The damage caused to colonies by viral infection varies considerably according to a number of factors, which include the type and strain of virus involved, the strength of the colony, weather conditions, season and food availability. Basically, bees and wasps are well-protected against infection with their chitin body shell and gut coating (Ritter and Akratanakul, 2006). However, parasitic mites sucking the blood of the bees, however, can penetrate their protection of body. Therefore, increased infestation by parasites is often accompanied by

increased virus infection mainly Deformed wing virus. Therefore, the global spread of *Varroa* it to become one of the most widely distributed and contagious insect viruses on the planet (Martin et al., 2012) and it is increasingly of interest to both insect researchers and beekeepers, primarily because of their association with the parasitic mite *Varroa jacobsoni*.

Repeating previously observed scenarios, the dramatic increase in emerging virus diseases may still be worsened by the continuing development of international exchanges and the potential dissemination of still undiscovered viruses or other agents that may favour their active multiplication (Genersch and Aubert, 2010). Recently only efficient acaricide treatments have existed but now, simple and economically acceptable treatment against virus infections is in view. Several stressors like infestations by viruses can contribute to the occurrence of dysbiosis phenomena, resulting in a perturbation of the microbiocenosis established in the organism. Therefore supplementation of microbial commensals appears to be the best solution which can help to renew homeostasis.

1.2.2 Pesticides

Modern agriculture increasingly depends on the use of chemical substances to control weeds (herbicides), fungi (fungicides), rodents (rodenticides) and arthropod pests (insecticides) to ensure high yields. Bees may frequently become exposed to environmental chemicals (Thompson, 2003). Bee poisoning, the accidental killing of bees through the use of insecticides, first became a problem during 1870s. The problems remained restricted and localized until the surge in modern agriculture methods following World War II. Highly effective synthetic organic insecticides became feasible for use not only on all types of crops but also on tremendous acreages of rangelands and forests. Development of efficient herbicides led to serve reduction in bee forage plants on both cultivated and wild lands (Johansen, 1977).

There are several classes of insecticides; the most widely used are the cholinesterase inhibitors (organophosphates), followed by other more recently developed compounds, such the neonicotinoids (abbreviated as neonics) (Costa et al., 2008). Organophosphate pesticides (OP) were developed in the early 1940s and nowadays, they are used extensively worldwide. OP insecticides have high acute toxicity which varies not only with the route and extent of exposure, but also the chemical structure of the agent. However, their major effect is direct mortality. The mechanism of toxicity is the inhibition of acetylcholinesterase, resulting in an accumulation of the neurotransmitter acetylcholine and the continued stimulation of

acetylcholine receptors (Kwong, 2002). Neonics (e.g. imidacloprid), new classes of systemic insecticides have been developed by chemical modifications of nicotine and other nicotinic agonists. Since the introduction of imidacloprid in the early 1990s, the use of different neonicotinoid insecticides has grown considerably. These are an important group of neurotoxins specifically acting as antagonists of the insect nicotinic acetylcholine receptors (Matsuda et al., 2001).

Effect on honey bees

These insecticides may not only affect pest insects but also non-target organisms such as pollinators. Sensitivity to pesticide exposure is affected by age of individuals (older bees are more susceptible due to foraging activity (Wahl and Ulm, 1983, Rortais et al., 2005)) and nutrition (honey bees fed high quality pollen are less susceptible to pesticide exposure than bees fed protein-deficient pollen or pollen substitutes). Migratory commercial beekeepers typically provide pollen substitute to colonies during transport and seasonal dearth to maximize brood production prior to and during pollination services (Wahl and Ulm, 1983). Another sensitivity supporting factor is management in the fight against infection. Beekeepers purposely expose bees to pesticides commonly used in hive to control *Varroa destructor* mites. Residues of varroacides, substances used to kill *varroa* mites, increasingly appear to be of major importance in the discussion of sub-lethal pesticide loads in honey bee colonies (Frazier et al., 2008, Martel et al., 2007). Residues of these systemic or acute insecticides can be present at 'trace' levels in the plant pollen and nectar or bees can be at risk also by guttation - a natural plant phenomenon causing the excretion of xylem fluid at leaf margins. When bees consume guttation drops, collected from plants grown from neonicotinoid-coated seeds, they encounter death within few minutes (Girolami et al., 2009).

However, insecticides do not only kill the bees directly but also can change their behaviour. Impacts of OP pesticide on memory have been investigated early through the study of the effect of ethyl-parathion, an organophosphate insecticide, on time memory in bees trained to visit a feeding site (Schricker and Stephen, 1970). At a sublethal dose, parathion induced a shift of the visiting time to the early hours of the day. This shift can be explained either by a change in the circardian clock or by the adoption of new feeding schedules learned by associating time with food presence (Bloch, 2010). Many studies have examined potential correlations between change behaviour and pesticide exposure, particularly, the class of neonicotinoid insecticides. Results demonstrate that after acute intoxication by imidacloprid or its metabolites, early symptoms of neurotoxicity were observed. They include

hyperresponsiveness, hyperactivity, and trembling. After several hours, these symptoms gradually disappeared, and the worker bees became hyporesponsive and hypoactive (Suchail et al., 2001). Sublethal dosages of imidacloprid were able to affect foraging behaviour of honey bees. They showed abnormalities in revisiting the feeding site. Some of them went missing, and some were present again at the feeding site the next day. Returning bees also showed delay in their return trips (Yang et al., 2008). Henry et al. (2012) tested neonicotinoid systemic pesticide thiamethoxam which caused high mortality due to homing failure at levels that could put a colony at risk of collapse. On the other hand 3-yr field survey in France studied honey bee (*Apis mellifera*) colony health in relation to pesticide residues found in the colonies, but no statistical relationship was found between colony mortality and pesticide residues (Chauzat et al., 2009). The mentioned studies demonstrated no lethal effect of pesticides on bees but they increases the sensitivity of individuals or colony to pesticide exposure and can further reduce their ability to fight bacterial or viral infections (Suchail et al., 2001, Pettis et al., 2012, Wu et al., 2012).

Effect on bumble bees

Pesticides could affect also bumble bee populations either directly, as insecticides that kill bumble bees (Williams, 1986, Thompson, 2001), or indirectly, as herbicides that kill their food plants (Williams and Carreck, 2014). The impact of insecticides was observed in colonies of the bumble bee *Bombus terrestris* exposed laboratory to field-realistic levels of the neonicotinoid imidacloprid, and then allowed them to develop naturally under field conditions. Treated colonies had a significantly reduced growth rate and suffered 85% reduction in production of new queens compared with control colonies. Given the scale of use of neonicotinoids they may be having a considerable negative impact on wild bumble bee populations across the developed world (Whitehorn et al., 2012).

Effect on wasps

Nowadays, the importance of the wasps lies in killing many harmful insects (blowflies and caterpillars to feed to their young) and they are consider as pests because of damages they cause to fruit in orchards and vineyards (Thomas, 1960). Therefore present studies not deal the impact of pesticides on health of wasps. Contrary, researchers develop the product to their disposal (Sackmann and Corley, 2007, Hanna et al., 2012, San Martin, 2014).

Based on the facts of mentioned studies suggesting the negative impact of insecticides in most countries, a legal framework is in place to protect honey bees and other pollinator insects

from the negative effects of pesticides and other agrochemicals. The relevant decrees are the European Council Directive 91/414 in Europe, and the Federal Insecticide Fungicide and Rodenticide Act in the US. As a consequence of the protection by laws and decrees, direct poisoning of honey bees by pesticides in the field is now a comparatively infrequent event in most countries of Europe and North America.

1.2.3 Quality of nutrition

By ensuring reproduction of many plants, pollinators are essential to the functioning of natural and agricultural ecosystems (Klein et al., 2007, Gallai et al., 2009). In turn, pollinators benefit from this pollination service by harvesting nectar (the adult bees convert into honey and store in beeswax cells) and pollen, which provides most of the protein, amino acids, fats, vitamins and mineral requirements of a bee and wasp diet (T'ai and Buchmann, 2000). However, nectar and pollen also contain secondary compounds such as a non-protein amino acids, alkaloids, phenolics, glycosides and terpenoids (Baker and Baker, 1983, Detzel and Wink, 1993), which can have antimicrobial activity (Hagler and Buchmann, 1993, Manson et al., 2010, Richardson et al., 2015).

Social insect nutrition can be investigated on three levels – colony nutrition, adult nutrition and larval nutrition – with increasing complexity, because disorders in prior stages affect subsequent stages and vice versa. Pollen is important at the colony level, since it enables the production of jelly by young workers that is used to feed larvae, the queen, drones and older workers (Crailsheim et al., 1992). Therefore, a direct consequence of pollen deficiency is a decrease in the colony population (Keller et al., 2005) and likely could result in immunocompromised individuals that are more susceptible when exposed to pathogens (Naug, 2009, Alaux et al., 2010). However, not only shortage is important but also nutritional value of pollen, which is primarily defined by its absolute and relative content of essential amino acids. Thus, the most studies analysed pollen are focused on the protein content (Roulston and Cane, 2000). Pollen from different plants has a different composition of amino acids (Crailsheim, 1990) and leading to different survival rates in honey bees when fed only one type of pollen. Similarly, when pollen quality was tested for bumble bees, it was found that rearing success is better with pollen containing a high protein content (e.g., from *Brassica* and *Prunus*) than with pollen from other plants species (e.g., *Helianthus* and *Taraxacum*) (Day et al., 1990, Roulston and Cane, 2000, Aupinel et al.). Corn (*Zea mays*) pollen, a wind-pollinated species, has been found to have a low nutritional value. This has also been described for another wind pollinated species, *Typha latifolia* (Schmidt et al., 1989).

So, pollen intake and composition are known for influencing the physiological metabolism (Alaux et al., 2011, Ament et al., 2011), level of ovary development (Hoover et al., 2006), the tolerance to pathogens like bacteria (Rinderer et al., 1974), virus (DeGrandi-Hoffman et al., 2010), parasite (Huang, 2012), microsporidia (Rinderer and Elliott, 1977, Huang, 2012, Foley et al., 2012) and reducing the sensitivity of bees to pesticides (Wahl and Ulm, 1983). However, bees rarely face a total lack of pollen in their environment but are rather confronted with variability in time and space of pollen resource abundance, type and diversity because of modern intensive agricultural practices, characterised by monocultures and simplification in crop rotations (Decourtye et al., 2010). Moreover, habitat fragmentation and loss of buffer zones of wild and semi-wild habitats in intensively farmed areas not only leave bee colonies short of food resources during times of dearth, but also lack the natural forage diversity that may be required for optimum nutrition (Kremen et al., 2002, Di Pasquale et al., 2013). One of the most stressful times for bee colonies in temperate climates is the overwintering period, when foraging opportunities are absent. Accordingly bees need to feed on high-quality pollen in fall in order to produce long-lived bees that can survive winter (Amdam and Omholt, 2002). Although beekeepers mitigate this stress by providing pollen supplements and monitoring for treatable diseases, most colony losses occur during or soon after winter (Vanengelsdorp et al., 2011). On the other hand one of the reasons for colony losses is wrong feeding practices of beekeepers (van Engelsdorp et al., 2008). The problem is feeding by some sugars, like mannose (Staudenmayer, 1939), galactose, arabinose, xylose, melibiose, raffinose, stachyose and lactose (Barker, 1977, Barker and Lehner, 1974), which are toxic to bees as well as sucrose solution, invert sugars, high fructose corn syrup (HFCS) or various fruit syrups (Neupane and Thapa, 2005). Also grape syrup causes dysentery and reduces longevity, and its use is therefore not recommended (Bees, 1978). Risk in the feeding is another toxic substance hydroxymethylfurfural formed in honey as a result of heat treatment or storage by beekeepers.

Amongst the factors adversely affecting the bees anthropogenic factors appear to be of greater importance than natural factors (Kosior et al., 2007). To halt population declines and species extinctions it will be necessary to preserve aspects of traditional farming practices and to be afforded legal protection in all countries of the region. The implementation of the European Union's Common Agricultural Policy is likely to have the greatest single impact upon pollinators in the near future.

1.2.4 Secondary metabolites as part of the honey bee nutrition

Nowadays it is becoming increasingly accepted that honey bee colonies require a quality diet that is nutritionally balanced. Honey bees forage for floral nectar as their main carbohydrate source and for pollen as their main source of amino acids, fatty acids, and micronutrients. Also insect pollinators regularly feed from flowers because of content of metabolite-rich nectar. Secondary metabolites, including tannins, phenols, alkaloids and terpenes, have been found in floral nectar across 21 angiosperm families (Adler, 2000). However, the consequences of such nectar for pollinators and plants remain unclear. One of several adaptive hypotheses is that several secondary metabolites reduce the severity of gut infections in pollinators. This pressure is enhanced in insect societies due to a suite of traits, including the high number of individuals living in high densities, relatively low genetic variability, and the relatively stable, high levels of humidity and temperatures of their nests (Schmid-Hempel, 1998). Honey bees take advantage of plant-derived compounds that are foraged by the workers to boost their immune defence by self-medication (Simone et al., 2009, Baracchi et al., 2015, Gherman et al., 2014). Self-medications as type of "group defence" behaviour represent a special case of "social immunity" that includes mechanisms to reduce host exposure to parasites and reduce infection risk through behavioural and/or physiological defence (de Roode and Lefèvre, 2012). Recent research (Manson et al., 2010, Richardson et al., 2015) has shown that several alkaloids (including nicotine) and their glycosides reduce the *Crithidia bombi* load after being consumed by bumblebees, suggesting that these pollinators might exploit nectar toxins or other metabolites to self-medicate. A daily diet containing nicotine, lasting more than two months, reduced the life expectancy of bumblebees, and this effect was stronger in smaller bees. Also alkaloid gelsemine appears to have little effect on the fitness or physiology of bees (Elliott et al., 2008, Manson and Thomson, 2009). Although gelsemine-rich nectar can be distasteful and deterrent to pollinators (Adler and Irwin, 2005, Gegear et al., 2007), *Gelsemium sempervirens* consistently attracts a number of floral visitors, including the bumble bees.

Secondary metabolites could affect bee immune function, including by upregulating bee immune response (Schmid-Hempel, 2005), simply by inducing diuresis (Tadmor-Melamed et al., 2004), causing physical or chemical changes to gut lining (Kollien and Schaub, 2000), or stimulating bees' endosymbiotic gut bacteria (Koch and Schmid-Hempel, 2011). In many animals, the gut microbial community, in particular, confers functions related to nutrition and

susceptibility to disease and thus might also play an important role in the health and resilience of honey bees.

1.3 Gut microbiota

A microbiota is defined as a collection of microorganisms living in a limited region or habitat. For example, all microbes inhabiting the gastrointestinal (GI)-tract of an organism are referred to as a gut microbiota. These bacterial communities vary immensely in total size, in composition, and in locations and functions within the gut (Rangberg et al., 2012). All insect species are known to harbour a rich and complex community of microorganisms participated in many types of interactions ranging from pathogenesis to obligate mutualism (Dillon and Dillon, 2004). One reason for the microbial diversity is that different groups of insects have different feeding habits; this results in different gut structures and functions and promotes the establishment of different phylotypes. Microbiota is known to upgrade nutrient-poor diets; aid digestion of recalcitrant food components; protect from predators, parasites, and pathogens; contribute to inter and intraspecific communication; affect efficiency as disease vectors; and governs mating and reproductive systems. However, gut bacteria also can contribute to nutrition of insects. Bacteria passing through the gut can simply be digested and used per se as nutrients (nutritional bacteria). This indicates that bacteria passing through the gut, ingested with fermented food, might be an important nutrient source (Engel and Moran, 2013b). In the 1980s, the principal microbiota of this insect was described as Gram-negative, Gram-positive, and Gram-variable bacteria, and moulds and yeast (Gilliam, 1997). Up until the turn of the century, however, almost all identifications and quantifications of the insect gut microbial assemblage were determined by cultivation-dependent techniques (Gilliam, 1997, Rada et al., 1997). Over the past decade, some new molecular methods have increased our ability to correctly describe microbial assemblages by utilizing 16S rDNA. In recent years there has been renewed interest in the understanding of the composition and function of insect gut microbiota for manipulating these microbial symbionts is thought to be an effective strategy for controlling the spread of pathogens that use insects as hosts (Dillon et al., 2005). Despite good reasons for knowing more about insect gut communities and despite the recent massive increase in studies of microorganisms living in insect guts, broad rules about how these communities are organized are just beginning to emerge. The last decade has seen the publication of many relevant studies ranging from community diversity surveys to molecular studies on how gut bacteria interact with host immune systems. However, some insect species, including cockroaches, termites, ants, and some wasps and bees, show gregarious or

social behaviour, including oral trophallaxis, which can enable direct or indirect social transmission, thus promoting the evolution of specialized host-dependent symbionts (Hongoh et al., 2005, Hongoh et al., 2006, Martinson et al., 2012).

Microbiota of honey bee (*Apis mellifera*)

The European adult honey bee, *Apis mellifera*, has been reported to contain up to 10^9 bacterial cells, 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). Bacterial symbionts confined to the hindguts of adults are acquired in the first few days following emergence of adults from the pupal stage, through social interactions with other adult worker bees in the colony (Martinson et al., 2012). PCR-based methods in the larval and adult intestine and rectum revealed three new species within the Gram negative bacterial phylum Proteobacteria;, these are *Gilliamella apicola* and *Frischella perrara* from Gammaproteobacteria (Kwong and Moran, 2013, Mohr and Tebbe, 2006) and Betaproteobacteria *Snodgrassella alvi* (Martinson et al., 2012, Engel and Moran, 2013a, Corby-Harris et al., 2014a, Kwong and Moran, 2013, Moran et al., 2012, Koch et al., 2013, Engel et al., 2014). Two other species clusters are from distantly related clusters of Alphaproteobacteria, initially called Alpha1 and Alpha2 (Babendreier et al., 2007). Alpha1 is a close relative of *Bartonella* species, which is often abundant in about half of sampled workers (Moran et al., 2012). Alpha2 consists of numerous strains of Acetobacteraceae, including Alpha 2.1, which is a gut specialist, and Alpha 2.2 (*Parasaccharibacter apium*) (Corby-Harris et al., 2014b). The presence of members of the genus *Lactobacillus* appears to be rather random; however in larval stages were detected (Mohr and Tebbe, 2006, Ahn et al., 2012). Between 3 and 4 distinct classes of lactobacilli have been identified and were recently characterised: *Lactobacillus apinorum*, which is phylogenetically similar to *Lactobacillus kunkeei*; Firmicutes (Firm) 4 clade represented by *Lactobacillus mellifer* and *Lactobacillus mellis* and the Firm5 clade represented by recently identified phylogenetically close species of *Lactobacillus melliventris*, *Lactobacillus kimbladii*, *Lactobacillus helsingborgensis*, *Lactobacillus kullabergensis* (Olofsson et al., 2014) and *Lactobacillus apis* (Killer et al., 2013). Bifidobacteria are present in relatively low abundance in honey bees. *Bifidobacterium* spp. cluster within the phylum Actinobacteria corresponds to species including *Bifidobacterium asteroides* from honey bees (Bottacini et al., 2012) and several species described from bumble bees (Killer et al., 2011, Killer et al., 2009, Wu et al., 2013).

Microbiota of bumble bee (*Bombus terrestris*)

Recently documented declines in bumble bee populations have motivated studies of the function and community structure of the microbiota associated with these hosts. The guts of bumble bees (*Bombus* species) have recently been shown to possess simple and highly specific microbiota that is closely related to some of the honey bee associates. Recent studies suggest the *Bombus* gut bacterial community is predominately comprised of members from: Orbaceae (Gammaproteobacteria), Lactobacillaceae (Firmicutes e.g. *Lactobacillus apis, Lactobacillus bombi, Lactobacillus kunkeei, Lactobacillus johnsonii, Fructobacillus fructosus* and *Enterococcus faecium*), Neisseriaceae (Betaproteobacteria), Acetobacteraceae (Alphaproteobacteria), Bacteroidetes and Actinobacteria (*Bifidobacterium actinocoloniiforme, Bifidobacterium bohemicum and Bifidobacterium bombi*) (Audisio and Benitez-Ahrendts, 2011, Killer et al., 2009, Killer et al., 2011, Killer et al., 2013, Endo and Salminen, 2013, Koch et al., 2013, Kwong and Moran, 2013, Cariveau et al., 2014). Several other bacteria like Bacteroidetes have been found at low abundance (<1%) in some bee guts (Babendreier et al., 2007, Sabree et al., 2012). While much of the evidence suggests that the gut microbiota of bumble bees are highly conserved and of relatively low diversity (Martinson et al., 2011, Koch and Schmid-Hempel, 2011a), it has been shown that detectable shifts in bumble bee gut bacterial diversity may occur in response to infection (Cariveau et al., 2014, Koch et al., 2012). Also diversity and composition of the bacterial communities are affected by nutritional status of the host (Dillon et al., 2010), colony identity and colony age (Koch et al., 2012) and the activation of the immune system upon parasitic infections (Lazzaro and Rolff, 2011).

Microbiota of wasp (*Vespula vulgaris*)

Apis and *Bombus* species share a distinct bacterial microbiota that is not present in other bees and wasps. *Vespula* have a broad diet, including insects, carrion, fruit and nectar (Edwards, 1980, Harris, 1991) so they are likely to be exposed to a wide range of microorganisms. However, *Vespula germanica* has not characteristic gut bacterial profile what shows a great variety of bacterial profiles, and the fact that *V. germanica* is not dependent on a particular mutualistic microflora for its nutrition. A number of bacterial strains appeared widespread, but community composition varied between nests (Reeson et al., 2003). Furthermore, the comparison with other bees and wasps suggests that changes in social lifestyle may have had a stronger effect on the evolution of the gut microbiota than the dietary shift from predatory

ancestors to pollen feeding (i.e. herbivorous) species (Kaltenpoth, 2011). In fact, digestive tract of *Vespula* sp. harbours about 50 fungal species which include *Actinoplanes* spp., *Fusarium* spp., *Nosema* spp., *Pseudozyma* spp., and *Rhodotorula* spp., which have been associated with hymenoptera. Other fungal genera (e.g. *Emericella* spp., *Wallemia* spp.) have been previously identified from soil and may represent contaminant from wasp nesting material (Lester et al., 2015, Rose et al., 1999). Also five to seven nematodes, four protozoans, two viral species and 12 bacterial such as species within the genera *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pseudomonas*, and *Yersina*, and genera within the class *Actinobactera*. Other microbial species, including *Neorickettsia risticii*, have never been observed in the Hymenoptera, but only in other insect groups (Singh et al., 2010, Evison et al., 2012, Rose et al., 1999, Lester et al., 2015). The wasp gut was also found to contain the lactic acid bacteria *Lactococcus*, *Lactobacillus* and *Enterococcus*, which ferment sugar to produce lactic acid, and in some cases other products including ethanol and acetate (Reeson et al., 2003). The proteome analysis indicated the presence of Actinobacteria which are also present in floral nectar, suggesting a horizontal transmission between bees (Anderson et al., 2013) and other nectar foraging species including wasps.

1.4 Possible role of microbiota in bee health

Losses of honey bee colonies have brought attention to the need for understanding the microbial associations of this species, including both symbiotic and pathogenic interactions. Bees are an emerging system for the study of gut microbial communities, having the advantages of a small, well-defined microbiota, as well as possessing the stability and social transmission routes of more complex (e.g., mammalian) systems. Its relative simplicity and the ability to cultivate all major members *in vitro* (Engel et al., 2013) make the bee gut a powerful model for investigating fundamental questions in microbial ecology, such as the origins, maintenance, and functions of strain-level variation and the dynamics of heterogeneous gut community assembly (Kwong et al., 2014). Over the past several years, governments, beekeepers, and the general public in the United States and Europe have become concerned by increased losses of bee colonies, calling for more research on how to keep colonies healthy while still employing them extensively in agriculture. However, what protects bees against pathogens? 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). Commensal bacteria consisting of different *Lactobacillus* and

Bifidobacterium sp. have been proposed as probiotics of bee with the goal to protect them against the common pathogen *Paenibacillus larvae* and *Melissococcus plutonius* (Evans and Lopez, 2004, Forsgren et al., 2010, Audisio and Benitez-Ahrendts, 2011, Endo and Salminen, 2013). Also four bacterial taxa isolated from bee larvae (*Stenotrophomonas maltophilia, Acinetobacter sp., Brevibacillus formosus* and *Bacillus fusiformis*) completely inhibited *P. l. larvae* growth in pairwise plate assays (D Evans and Armstrong, 2005, Evans and Armstrong, 2006). Honey bees and bumble bees also can be parasitized by *Crithidia* species as well as by other parasites and pathogens. The residential gut microbiota of European bumble bees protects against the common trypanosomatid pathogen *Crithidia bombi* (Koch and Schmid-Hempel, 2011b). Community analyses of gut microbiota in three species of wild bumble bees in eastern North America revealed higher *Crithidia* incidence in individuals with low colonization by the core gut bacterial species *S. alvi* and *G. apicola*, consistent with a protective role by one or both of these bacterial species (Cariveau et al., 2014) what is in agreement with increase in diversity of the microbiota in individuals naturally infected with either *Crithidia. bombi* or *Nosema bombi* (Koch et al., 2012).

Moreover, gut bacteria may also show antagonistic activity against pathogens by producing various antimicrobial substances such as antimicrobial peptides/molecules, fatty acids and H_2O_2 (Klaenhammer, 1993, Servin, 2004). Hence, organic acids produced by a *L. johnsonii* strain could contribute as an organic tool to reduce individual loads of *Nosema* without fumagillin application and keep controlled the reinfestation when antibiotic control is used, improving its efficacy (Maggi et al., 2013). Also some isolates of Actinobacteria known as a commensal of the honey bee are oxalotrophic, may carry ramifications into the use of oxalic acid to control the number of phoretic mites in the managed colonies of honey bees (Maddaloni and Pascual, 2015). This finding may have ramifications in the use of oxalic acid as a control agent against pests.

Honey bees are vulnerable not only to various pathogens such as bacteria, protozoa, fungi, and parasitic mites which cause heavy damages every year. There are many types of viruses and pesticides that threaten honey bee colonies. There is no study confirmed direct positive correlation between microflora and pesticides or viruses. However, microbiota can benefit their host in multiple ways including metabolising food and toxins, nutrient supplementation, and can lead to increase immunocompetence and resistance of bee larvae and other life stages to viruses and pesticides (Evans and Lopez, 2004).

Another benefits of mutualistic microbes are provide nutrients and vitamins. Honey bees require relatively high levels of vitamins, including the vitamin B complex, and gut bacteria

represent a likely source of B vitamins (Snyder et al., 2010). A metagenomics study of the worker gut community (Engel et al., 2012) contained an excess of carbohydrate-processing genes, particularly in strains of *G. apicola*. This indicates strain diversity in ability to use different dietary components, suggesting that the particular set of strains present in individual workers or in colonies might affect nutritional ecology of the bees or might act to neutralize dietary toxins. The *Lactobacillus* and *Bifidobacterium* species also promote the digestion of carbohydrates and thus in nutrition of their hosts (Lee et al., 2015, Engel et al., 2012).

Supplementation of colonies with these naturally occurring bacteria or their antagonistic products can provide a novel way of controlling some diseases. Hence, these bacteria will likely have future implications in the realm of applied sciences, such as in the engineering and application of probiotics. Also might assist the beekeeper both in colony management and the creation of some "bee packages". However, many questions concerning the natural state of a healthy microbial community will be difficult to address due to the putatively facultative nature of most honey bee symbioses, and the complexity and plasticity of the social system. Linking the microbiota to its functional role is critical because functional stability may be achieved despite a large variation in the microbial population size or composition.

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Aims and Objectives of this thesis

The aim of this thesis is to explore the complex mechanisms of bees and related species gut bacterial populations, their links to insect immunity and investigate the opportunities for an intervention.

Part of our aim was to characterise the bumble bee and wasp gut microbiota using 16S RNA sequencing in a search for potentially novel bacterial species.

- We aimed to characterise of microbiota of the digestive tract of bees.
- We focused on effect of ontogenetic stage, age and geographic location of bees and their impact on the development of microbiota.
- We aimed to investigate the changes in honey bee microbiota under different management conditions and dietary supplements.

We have developed following testable hypotheses based on the literature review or upon our preliminary research:

- As eusocial insects with genetic and morphological similarities shared with honey bees, wasps might be harbouring bifidobacteria in their digestive tract such as honey bees.
- Honey bee microbiota responds to the developmental stage (gender) and location of the colony and there are previously undescribed differences among individuals within one a single hive.
- Honey bees are preferentially feeding nectar rich in other alkaloids than nicotine or caffeine.
- Honey bee microbiota can be modulated by feeding of native bacterial isolates, leading to responses in colony fitness.

3

Vagococcus entomophilus sp. nov., from the digestive tract of a wasp (*Vespula vulgaris*)

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Abstract

Three unknown Gram-stain-positive, catalase-negative, facultatively anaerobic and coccus-shaped strains of bacteria were isolated from the digestive tracts of wasps (*Vespula vulgaris*). Analysis of 16S rRNA gene sequences revealed that these strains had identical sequences and showed that *Vagococcus salmoninarum*, with 96.2% sequence similarity, was the closest phylogenetic neighbour. Further analyses based on *hsp*60 and *phe*S gene sequences of representatives of the family *Enteroccocaceae* and genotypic and phenotypic characterization using $(GTG)_5$-PCR fingerprintings, *Eco*RI ribotyping, DNA G+C content, whole-cell protein profiling, cellular fatty acid profiles analysis and extensive biotyping confirmed that the investigated strains were representatives of a novel bacterial species within the genus *Vagoccocus* for which the name *Vagoccocus entomophilus* sp. nov. is proposed. The type strain is VOSTP2^T (=DSM 24756T=CCM 7946^T).

Keywords: *Vagococcus entomophilus* sp. nov., *Vespula vulgaris*, digestive tract, multilocus sequence analysis

Representatives of the genus *Vagococcus* were separated from related genera within the order *Lactobacillales* in 1990 and are classified as members of the family *Enterococcaceae* (Collins et al., 1989; Collins, 2009). Vagococci represent bacteria that have been isolated from different environments. Different species have been found in ground beef (Shewmaker et al., 2004), an acidogenic fermentation bioreactor (Wang et al., 2011), a swine-manure storage pit (Lawson et al., 2007), a dead seal and a harbour porpoise (Hoyles et al., 2000), human clinical sources and pigs (Teixeira et al., 1997), common otter (*Lutra lutra*) (Lawson et al., 1999), the spoilage microbiota of cooked shrimp (Jaffre`s et al., 2010) and diseased salmonid fish (Wallbanks et al., 1990). The ecological importance of vagococci has not been explored. Some strains may act as potential pathogens (Ruiz-Zarzuela et al., 2005). However, there are some studies indicating that *Vagococcus fluvialis* could be a probiotic bacterium for economically important marine fish (Roman et al., 2012; Sorroza et al., 2012).

In this study, we describe three novel strains of bacteria isolated from a wasp (*Vespula vulgaris*) and representing a novel species of the genus *Vagococcus*; the strains were distinct from all other species of bacteria isolated from the digestive tracts of insects.

Individual wasps (*Vespula vulgaris*) had been caught in the locality of Modřany (Prague, Czech Republic) in 2010 and transported to the microbiological laboratory immediately after capture. The body surfaces of wasps were treated with 70% ethanol to remove potential contamination. After decapitation, digestive tracts were removed aseptically and immediately transported to Hungate tubes containing sterile anaerobic tryptone–phytone–yeast extract (TPY) broth (Scardovi, 1986) and glass beads. Samples were then weighed, homogenized in a common lab vortex mixer and serially diluted. The range of dilutions from 1022 to 1027 w/v was used for the experiments. The 0.5 ml aliquots of the extracts were plated on modified TPY (MTPY) agar (Rada & Petr, 2000) and incubated under anaerobic conditions (Anaerobic jars, Oxoid) at 37°C for 72 h. Individual bacterial colonies were picked up, transferred into tubes containing anaerobic TPY broth and cultivated at 37 °C for 24 h. Investigated strains designated as VOSTP2^{T}, VOSTP5 and VOSTP6 were isolated from three different wasps.

Reference type strains of species of the genus *Vagococcus V. salmoninarum* CCM 4305^{T}; *V. penaei* CCM 8416T; *V. fluvialis* CCM 4304T; *V. carniphilus* CCM 8414^{T}; *V. lutrae* CCM 4937^{T}; *V. fessus* CCM 8413^{T}; *V. acidifermentans* CCM 8417^{T} and *V. elongatus* CCM 8415^{T} were obtained from the Czech Collection of Microorganisms (CCM, Masaryk University, Brno, www.sci.muni.cz/ccm).

Chromosomal DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen). Then, almost complete 16S rRNA gene sequences (1490 bp) were then amplified by PCR using the forward primer fD1 (5′-CCGAATTCGTCGAC-AACAGAGTTTGATCCTGGCTCAG-39) and reverse primer rP2 (5′-CCCGGGATCCAAGCTTACGGCTACCTTG-TTACGACTT-39) (Weisburg et al., 1991). The PCR was performed under the following conditions: 92 °C for 5 min; 35 cycles of 92 °C for 1 min, 52.5 °C for 90 s and 72 °C for 2 min; 72 °C for 5 min. Checked and purified DNA fragments were sequenced using an automatic gene-tic analyser ABI PRISM 3130xl (Applied Biosystems). Sequences of 16S rRNA genes were then edited and compared with the sequences from the most closely related species as described previously (Killer et al., 2011). Strains VOSTP2^T, VOSTP5 and VOSTP6 (lengths of sequences 1430 bp, 1410 bp and 1409 bp, respectively) revealed identical 16S rRNA gene sequences. The 16S rRNA sequences of the most closely related strains and other phylogenetic markers were searched using the BLAST algorithm freely available on the website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence similarities of phylogenetic markers with those of the most closely related strains were calculated using the jPHYDIT program (Jeon et al., 2005). Only the sequences of the type strains of most closely related species have been used for calculation. *V. salmoninarum* CCUG 33394^T (GenBank accession number Y18097) was the closest phylogenetic relative with a validly published name on the basis of the 16S rRNA gene, with the similarity value of 96.2%. However, the closest relative based on 16S rRNA gene sequence similarity is the unspecified *Enterococcaceae* bacterium AaMG2 (GenBank accession number GQ915078) isolated from the digestive tract of mosquito (*Aedes albopictus*; Diptera: Culicidae) with a similarity value of 99.8%. Multilocus Sequence Analysis (MLSA) is a suitable tool to confirm the status of novel bacterial species (Ventura et al., 2006). Partial sequences of hsp60, pheS, rpoA and atpA genes were amplified by PCR methods according to the protocols of Goh et al. (2000), Naser et al. (2005b) and Naser et al. (2005a), respectively. The most closely related sequences observed on the basis of individual comparisons of hsp60 and pheS genes were those of *Vagococcus carniphilus* (80.8% sequence similarity; GenBank accession number JX576771) and *Vagococcus salmoninarum* (80.0% sequence similarity; GenBank accession number JQ363693), respectively. Individual comparisons of rpoA and atpA genes revealed closely related sequences from species of the genus *Enterococcus* with sequences similarities 82.3%. Nevertheless, the GenBank database does not contain sufficient data related to the sequences of these genes in members of the family *Enterococcaceae*. Strains VOSTP2^T, VOSTP5 and VOSTP6 had identical hsp60 and pheS gene sequences (not shown). The internal transcribed

spacer (ITS) was also sequenced according to the protocol of Dobson et al. (2002). It was revealed that the 16S–23S rRNA intergenic spacer of *Enterococcus saccharolyticus* KCTC 3643^T was the sequence most similar to the sequences of the ITS region amplified from bacterial strains from the digestive tract of wasps, with sequence similarity of 77.2%. Obtained results indicate that the novel bacterial strains represent a novel species belonging to the family *Enterococcaceae*. Phylogenetic analysis based on 16S rRNA and housekeeping gene sequences were performed in order to clarify the phylogenetic position of the investigated strains. Sequences of 16S rRNA genes and other housekeeping gene sequences were identical in all three isolates from wasps. For this reason, the type strain VOSTP2^T was the predominant strain characterized.

The clonal status of strains VOSTP2^T, VOSTP5 and VOSTP6 and differentiation from other representatives of species of the genus *Vagococcus* were characterized using $(GTG)_5$-PCR fingerprinting, automated ribotyping and whole-cell protein profiling and compared with the type strains representing all hitherto described species of vagococci in order to clarify their taxonomic position within the genus *Vagococcus*. Repetitive-sequence-based PCR fingerprinting with the $(GTG)_5$ primer was performed according to the protocol of Svec et al. (2008). Automated ribotyping with the *Eco*RI restriction enzyme was per-formed in the same strains using the RiboPrinter microbial characterization system (DuPont Qualicon) in accordance with the standard protocol provided by the manufacturer. Whole-cell protein analysis was performed according to the protocol described by Pot et al. (1994). The isolates were grown on BHI agar at optimal temperature for 24 h. Harvested cells were disrupted by sonication using a Labsonic M ultrasonic homogenizer (Sartorius). Protein extracts were separated using SDS-PAGE. Fingerprint profiles resulting from individual methods were digitized, processed and analysed using Bionumerics version 6.6 software (Applied-Maths). The dendrograms were calculated using Pearson's correlation coefficients with the unweighted pair group method using arithmetic averages (UPGMA). The resulting dendrograms calculated from $(GTG)_5$-PCR, automated ribotyping and whole-cell protein profiling results are shown in Figs. S1a, S1b and S1c (Supplementary materials are available online: http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.054940-0#tab5). Obtained fingerprints revealed from individual methods clearly separated strains VOSTP2^T, VOSTP5 and VOSTP6 from representatives of remaining species of the genus *Vagococcus* and demonstrate their close similarity. $(GTG)_5$-PCR fingerprints, ribotype patterns and whole-cell protein profiles revealed by strains VOSTP2^T, VOSTP5 and VOSTP6

were visually nearly identical and showed similarity values reaching 93.5%, 96.5% and 96%, respectively. These values were generated using the Bionumerics v. 6.6 software (Applied-Maths). On the basis of these results, it appears that two bacterial strains VOSTP5 and VOSTP6 could be clones. VOSTP2^T had slightly different fingerprinting profiles from the other two strains tested.

Phylogenetic trees were reconstructed using MEGA 5.05, Gblocks and MrBayes (Ronquist et al., 2012) programs as described previously (Killer et al., 2013). The phylogenetic tree based on 16S rRNA gene sequences of representatives of the family *Enterococcaceae* showed that members of the genera within the family *Enterococcaceae*; *Vagococcus*, *Enterococcus*, *Melissococcus*, *Catellicoccus*, *Tetragenococcus*, *Bavariicoccus* and *Pilibacter* were clustered into distinct phylogenetic groups. VOSTP2^T and VOSTP6 were situated on a separate phylogenetic branch within the group of vagococci (Fig. 1). Phylogenetic trees based on available hsp60 and pheS (Fig. S2 and Fig. S3) sequences of representatives of the family *Enterococcaceae* were reconstructed to confirm the assumption that strain VOSTP2^T belongs to the genus *Vagococcus*. Sequences of these genes are not available in the GenBank database for all species of vagococci. Therefore, fragments of the genes were amplified and sequenced in type strains of species of the genus *Vagococcus* species as described above. Also, the pheS phylogenetic tree confirmed the phylogenetic delineation of vagococci (except *V. salmoninarum*) and enterococci. As shown in the phylogenetic tree, the novel strain was grouped within a cluster of vagococci together with *V. acidifermentans* KCTC 13418^T (Fig. S3). Species of enterococci and vagococci were also separated into two phylogenetic groups based on partial hsp60 gene sequences. However, some species of vagococci formed separate phylogenetic branches. Within the phylogenetic tree, the novel strain was located on a separate branch between the phylogenetic groups of enterococci and vagococci (Fig. S2). Phylogenetic studies based on rpoA and atpA gene sequences and the ITS region have not been performed due to the lack of sufficient data on sequences of representatives of the family *Enterococcaceae*.

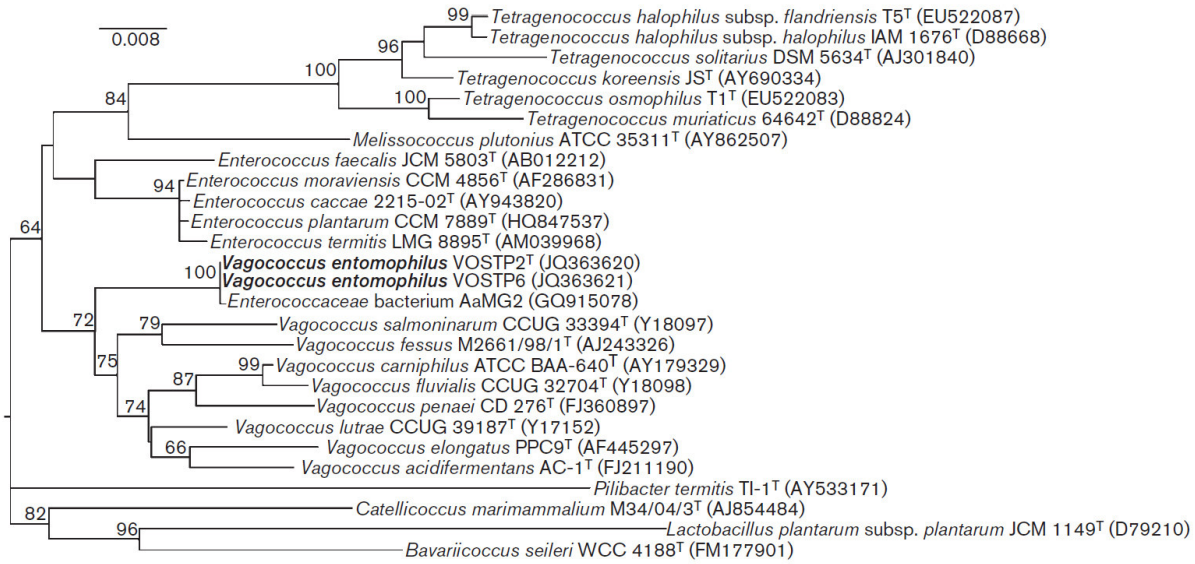

Fig. 1. Phylogenetic tree showing that the novel strains VOSTP2^T and VOSTP6 belong to the genus *Vagococcus*. It was reconstructed based on 16S rRNA (length of 1303 nt) gene sequences using the maximum likelihood algorithm within the MEGA version 5.05 software after removing hypervariable positions using the program Gblocks. Sequence data were aligned using the CLUSTALW algorithm. The Jukes–Cantor model was used for reconstruction of the phylogenetic tree. Bootstrap values, expressed as percentages of 1000 datasets, are given at nodes. Numbers in parentheses correspond to the GenBank accession numbers. The tree was reconstructed as unrooted. Bar 0.008 substitutions per nucleotide position.

A modified enzymatic degradation method (Killer et al., 2011) was used for determination of the DNA G+C contents in the VOSTP2^T strain and other type strains of known species of the genus *Vagococcus*. The DNA G+C mol% content was determined according to the method of Mesbah et al. (1989) with some minor modifications of the analytical set-up. The separation of four deoxynucleosides (Sigma–Aldrich) was achieved with an analytical HPLC system Dionex Summit (Dionex), consisting of a P680 quaternary gradient pump, diode array detector UVD340U,and a column thermostat, interfaced with the Waters 717 autosampler (Waters), using a Phenomenex Fusion C18 column (2506 4.6 mm internal diameter, 5 mm particle size, Phenomenex). The column temperature was 22 °C. Nucleosides were eluted under gradient conditions using A: 20 mM ammonium acetate (pH 4.5) and B: acetonitrile. The linear gradient started at 4% of A and changed linearly to 26% B. The linear gradient started at 4% of B and changed linearly to 26% B in 12 min. The flow rate was set to 0.8 ml

min^{-1} and the injection volume was 20 ml. The value of 39.7 mol% (mean of three experiments, SD50.07) was determined for strain VOSTP2^T. DNA G+C contents in the range from 32.6 to 45.2 mol% have been found in representatives of the genus *Vagococcus* (Table 1). Some of the values detected by the modified enzymic degradation method are lower compared with those that had been determined by authors who described some of the novel species of the genus *Vagococcus. V. fessus* and *V. elongatus* are examples of species for which lower values of C+G content were determined by thermal denaturation spectrophotometric methods (Hoyles et al., 2000; Lawson et al., 2007).

Table 1. Differences in DNA G+C contents, peptidoglycan structure, growth characteristics and results of enzymic characterization among the novel strains and species of the genus *Vagococcus* with validly published names.

Taxa: 1, *V. entomophilus* sp. nov. strains VOSTP2^T, VOSTP5 and VOSTP6; 2, *V. salmoninarum* CCM 4305^T; 3, *V. penaei* CCM 8416^T; 4, *V. fluvialis* CCM 4304^T; 5, *V. carniphilus* CCM 8414^T; 6, *V. lutrae* CCM 4937^T; 7, *V. fessus* CCM 8413^T; 8, *V. elongatus* CCM 8415^T; 9, *V. acidifermentans* CCM 8417^T. All strains produced arginine arylamidase, phenylalanine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. All species fail to produce β-galactosidase-6-phosphate, β-fucosidase, β-galactosidase, α-arabinosidase, β-glucuronidase, glutamic acid decarboxylase (except for strains of *V. entomophilus*), proline arylamidase, leucyl glycine arylamidase, glutamyl glutamic acid arylamidase, lipase (C14), valine arylamidase, trypsin, urease (variable reactions among strains of *V. fluvialis* and *V. fessus* according to the results of Teixeira et al., 1997 and Hoyles et al. 2000, respectively), catalase (tested with 3% hydrogen peroxide), oxidase (tested with 1% tetramethyl-p-phenylenediamine dihydrochloride), indole from L-tryptophan and protease (gelatin hydrolysis based on the method of Smith & Goodner, 1958). All strains were unable to reduce nitrates. Data are from this study, except where indicated. +, Positive reaction; 2, negative reaction; W, weakly positive reaction; V, variable reactions among strains; NA, no data available; meso-Dpm, meso-diaminopimelic acid.

Taxa/Characteristic	1	2	3	4	5	6	7	8	9
DNA G+C content (mol%)*	39.3–40.1	37.5	35.1	33.3	32.6	37.7	36.5	38.6	45.2
Peptidoglycan structure	A1γ; meso-Dpm	NA	A4 α ; L-Lys–D-Asp	NA	NA	NA	NA	NA	NA
Temperature range for growth on TYSE broth (°C)	10–40	5–30	10–40	10–40	10–45	20–40	10–40	20–40	15–40
RAPID ID32A, API ZYM:									
α-Chymotrypsin	W	+	V**	W	2	+	+	2	+
α -Galactosidase	2	2	2	2	2	+	2	+	2
α -Glucosidase	+	2	V**	W	2	+	2	2	+
β-Glucosidase	2	W	2	+	2	+	V**	W	2

RAPID ID32A, API ZYM:									
α -Mannosidase	2	2	2	W	2	+	2	2	2
Acid phosphatase	+	+	+	+	2	+	2	2	2
Alkaline phosphatase	W	W	2	2	W	W	V**	W	+
N-acetyl- β -glucosaminidase	2	2	2	V**	2	+	2	2	2
Cystine arylamidase	2	2	+	2	+	2	2	2	+
Arginine dihydrolase	2	V	V**	2	2	2	2	2	2
Esterase (C4)	+	W	+	W	2	W	W	W	+
Esterase lipase (C8)	+	W	+	+	2	+	W	W	+
Leucine arylamidase	+	+	V**	2	+	+	+	2	+
Naphthol-AS-BI-phosphohydrolase	+	2	+	+	2	W	W	2	+
Pyroglutamic acid arylamidase	2	+	+	W	+	+	+	2	2
Production of acetoin (Voges– Proskauer test)	2	2	+	2	2	+	2	2	2

*Values for DNA C+G content are mean of three experiments: SDS=0.02–0.07.
**Data from previous studies (Teixeira et al., 1997; Hoyles et al., 2000 and Jaffre`s et al., 2010).

Phenotype characterization using Rapid ID32A Strep and API ZYM systems (bioMerieux) has been proved to be a useful tool for distinguishing species of the genus *Vagococcus* (Collins, 2009). These test kits were used to differentiate strains VOSTP2T, VOSTP5 and VOSTP6 from the remaining type strains of all hitherto described species of vagococci. Production of 15 enzymes displayed differences among species of the genus *Vagococcus* (Table 1). Subsequently, the characteristics covered by the Biolog Identification System (GP2 MicroPlate) and API 50 CH test strips (bioMerieux) were determined in order to obtain extensive phenotypic data on the isolates VOSTP2^T, VOSTP5 and VOSTP6 and type strains of all hitherto described species of vagococci. Species of vagococci differ in the utilization of 35 substrates (Table S1). The novel strains were not able to utilize maltotriose or glycerol, unlike the type strains of species of the genus *Vagococcus* with validly published names. On the other hand, the novel strains are able to utilize inulin, unlike members of other species of the genus *Vagococcus*.

Trypticase soy yeast extract medium (TSYE; Sigma-Aldrich) suitable for cultivation of vagococci and enterococci was used to evaluate the ability to grow in aerobic, microaerophilic and anaerobic environments, at different temperatures and pH values by methods described previously (Killer et al., 2013). Best growth of the strains VOSTP2^T, VOSTP5 and VOSTP6 was observed on TSYE medium under aerobic conditions. However, poor growth was observed also under microaerophilic and anaerobic conditions. Growth occurred at temperatures between 10 and 40 °C. The temperature ranges for growth of the type strains of

other species of the genus *Vagococcus* in TSYE broth are shown in Table 1. The minimum initial pH for growth under aerobic conditions in TSYE broth was 5.0 and the maximum was 10.0. Motility was tested on stab-inoculated semi-solid medium according to the method of Svec et al. (2012) and showed that VOSTP2^T is non-motile. The detailed morphology of the cells was studied by scanning electron microscopy (Killer et al., 2009). The ovoid cell morphology of strain VOSTP2^T is shown in Fig. 2.

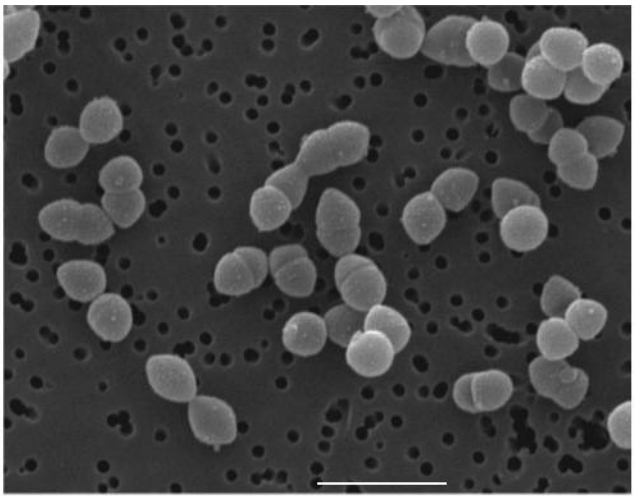

Fig. 2. Ovoid-shape cell morphology of *Vagococcus entomophilum* VOSTP2T shown by scanning electron microscopy. Bar, 2 μm.

Lactic, acetic and propionic acids at concentrations of 16.5 mmol l21 (71% of the total short-chain fatty acids produced), 5.6 mmol l21 (24%) and 1.1 mmol l21 (5%), respectively, were quantified using the izotachophoresis analytical method (Killer et al., 2011) after cultivation at 37 °C for 24 h. The aerobic TSYE broth supplemented with 5 g glucose l21was used for determination of end products of hexose catabolism in strain VOSTP2*T*. Obtained results showed that this strain is a representative of the homo-fermentative lactic acid bacteria.

Production of lactic acid optical isomers was tested by the D/L-lactic acid kit (Megazyme) according to the manufacturer's instructions. VOSTP2^T produced only L-lactic acid, which corresponded with results obtained for the other species of the genus *Vagococcus* (Collins, 2009).

For the quantitative analysis of cellular fatty acids, strain VOSTP2^T was grown at 30 °C for 72 h in the trypticase soy broth (Sigma–Aldrich) under aerobic conditions. Growth was stopped in the stationary phase of the growth curve. The profile of cellular fatty acids was

determined as described previously by Jaffres et al. (2010) with some modifications described by Killer et al. (2009). The major fatty acids detected in VOSTP2^T strain were 10-methyl C16:0, C14:0 and C16:0, respectively. Compared with other species of vagococci (Wang et al., 2011), the tested strain has a higher proportion of 10-methyl C16:0 and a lower proportion of C18:1ω9c fatty acids (Table S2).

VOSTP2^T was also tested for susceptibility to 33 different antibiotics and two chemotherapeutics using a disc diffusion method (Vlkova et al., 2006). Sensitivity to most tested cephalosporines, macrolides, penicillin-derived antibiotics, vancomycin, rifampicin, clindamycin, chloramphenicol, tetracycline and gentamicin was observed. On the other hand, the tested strain was resistant to three different cephalosporines, meropenem, aminoglycosides, fluoroquinolones, sulphonamides, mupirocine and metronidazole (Table S3). In contrast to these results, *V. fluvialis* and *V. salmoninarum* were resistant to clindamycin but susceptible to cefotaxime and trimethylprim–sulfamethox-azole based on results from Teixeira et al. (1997).

Structure and amino acid composition of the cell-wall peptidoglycan and the profile of polar lipids were evaluated by the Identification Service (DSMZ, Braunschweig, Germany) by the methods described previously (Killer et al., 2010). It was concluded that VOSTP2^T displayed peptidoglycan type Alγ mesodiaminopimelic acid; type A31 according to DSMZ www.dsmz.de/catalogues/catalogue-microorganisms/specific-catalogues/peptidoglycans.html. The cell-wall peptidoglycan has been determined only for *V. fluvialis* among the species of the genus Vagococcus. Collins et al. (1989) determined the peptidoglycan type to be L-lysine-D–aspartic acid in the species. Polar lipids detected in cells of VOSTP2^T strain consisted of phosphatidylglycerol, two phosphoglycolipids, six phospholipids, three unidentified aminolipids, six glyco-lipids and three unknown polar lipids, respectively (Fig. S4). Fischer & Arneth-Seifert (1998) found unusual membrane polar lipids such as cardiolipin [bis(phosphatidyl)glycerol], D-alanylcardiolipin, lysocardiolipin, α-D-glycinecardiolipin, D-alanylphosphatidylglycerol and D-alanylphosphatidylgly-cerol in *V. fluvialis*. However, the compositions of the polar lipids in other species of the genus *Vagococcus* have not been determined yet. Therefore, it was impossible to attempt the identification of distinctive polar lipids.

The results of a wide range of genotypic, phenotypic and phylogenetic analyses demonstrated that the bacterial strains VOSTP2^T, VOSTP5 and VOSTP6 represent a novel species for which the name *Vagococcus entomophilus* sp. nov. is proposed.

3.1 Description of *Vagococcus entomophilus* sp. nov.

Vagococcus entomophilus [(en.to.mo´phi.lus. Gr. n. entomon insect; N.L. adj. philus -a -um (from Gr. adj. philos –e´ -on), friend, loving; N.L. masc. adj. entomophilus insect loving)].

Cells growing in liquid aerobic TSYE medium are Gram-stain-positive, non-spore-forming, non-motile, ovoid-shaped cocci, measuring 0.7–0.9 x 0.5–1.1 mm. They are arranged mainly in pairs or singly (Fig. 2). Colonies on TSYE agar under aerobic conditions after 48 h incubation at 37 °C are generally triangular or disc-shaped with regular edges, white, smooth and reach 0.82–2.75 mm in diameter. Cells are facultative anaerobic, obligately homofermentative and produce L-lactic acid. Growth occurs at temperatures between 10 and 40 °C and at a pH range of 5–10. Has the ability to use the following carbon sources via respiration: inulin, N-acetyl-D-glucosamine, amygdalin, arbutin, cellobiose, D-fructose, gentiobiose, α-D-glucose, D-mannose, methyl-β-D-glucoside, salicin, sucrose, trehalose, adenosine and uridine. Negative for utilization of α-cyclodextrin, β-cyclodextrin, glycogen, man-nan, Tween 40, Tween 80, L-arabinose, D-arabitol, L-fucose, D-galactose, D-galacturonic acid, D-gluconic acid, myo-inositol, α-lactose, lactulose, maltose, maltotriose, D-mannitol, melezitose, melibiose, methyl-α-D-galactoside, methyl-β-D-galactoside, 3-methyl glucose, methyl-α-D-glucoside, methyl-α-D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, D-ribose, sedoheptulosan, D-sorbitol, stachyose, D-tagatose, turanose, xylitol, D-xylose, acetic acid, α -hydro-xybutyric acid, β-hydroxybutyric acid, c-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, β-ketogluta-ric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, pyruvatic acid methyl ester, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, 2´-deoxya-denosine, inosine, thymidine, adenosine-5´-monophosphate, thymidine-5´-monophosphate, uridine-5´-monophosphate, D-fructose-6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Variable results between strains for dextrin utilization; the type strain is not able to utilize dextrin. Produces α-chymotrypsin, α-glucosidase, acid phosphatase, alkaline phosphatase, esterase (C4), esterase

lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, arginine arylamidase, phenylala-nine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Does not produce α-galactosidase, β-galactosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, α-mannosidase, N-acetyl-β-glucosaminidase, cystine arylamidase, arginine dihydrolase, pyroglutamic acid arylamidase, β-galactosidase-6-phosphate, α-fucosidase, proline arylamidase, leucyl glycine arylamidase, glutamyl glutamic acid arylamidase, lipase (C14), valine arylamidase, trypsin, urease, catalase, oxidase, indole from L-tryptophan and protease (gelatin hydrolysis). Negative for acetoin production. Susceptible to some cephalosporines, macrolides and some penicillin-derived antibiotics. Resistant to aminoglycosides, fluoroquinolones, sulphonamides, mupirocine and metronidazole (Table S3). The major fatty acids detected were 10-methyl C16:0, C14:0 and C16:0, in that order (Table S2). The peptidoglycan type is A1γ meso-diaminopimelic acid. Principal polar lipids are phosphatidylglycerol, two phosphoglycolipids, five phospholipids, three unidentified aminolipids and six glycolipids.

The type strain, VOSTP2^{T} (=DSM 24756T=CCM 7946T) was isolated from the digestive tract of wasp (*Vespula vulgaris*) which had been caught in the locality of Modřany (Prague, Czech Republic) in 2010. The DNA G+C content for VOSTP2T is 39.7 mol%. Values of DNA G+C content of other strains are in the range from 39.3 to 40.1 mol%.

3.2 Acknowledgements

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4

Lactobacillus bombi sp. nov., from the digestive tract of laboratory-reared bumblebee queens (*Bombus terrestris*)

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Conceived and designed the experiments: J. Killer, A. Votavova, I. Valterova, V. Rada. Performed the experiments: J. Killer, E. Vlkova, V. Rada, **Z. Hroncova**. Analysed the data: J. Killer, A. Votavova, I. Valterova**, Z. Hroncova**. Contributed reagents/materials/analysis tools: A. Votavova, I. Valterova, V. Rada. Wrote the paper: J. Killer, A. Votavova, I. Valterova. Prepared the isolates: J. Killer, E. Vlkova, **Z. Hroncova**.

Abstract

Three bacterial strains belonging to the genus *Lactobacillus* were isolated from the digestive tract of laboratory-reared bumblebee queens (*Bombus terrestris*) using MRS agar under anaerobic conditions. The isolates were identified according to 16S rRNA gene sequences as yet undescribed *Lactobacillus* sp. with the highest 16S rRNA gene similarity (96.9%) to uncharacterized bacterial strain *Lactobacillus* sp. Mboho2r2 isolated from the stomach of a European honeybee (*Apis mellifera*). Another unclassified lactobacilli from the digestive tract of honeybees seemed to be related to new isolates. *Lactobacillus tucceti* was found to be the closest relative valid species with 92.9% 16S rRNA gene sequence similarity. However, phylogenetic analyses based on different markers revealed that the species is phylogenetically very distant from the new strains. The DNA G+C content of the proposed type strain BTLCH M1/2^T is 37.8 mol%. The fatty acids C19:1v6c and/or C19:0 cyclo ω10c/19ω6, C18:1ω9c and C16:0 were predominant in all strains. Diphosphatidylglycerol, phosphatidylglycerol, a phospholipid, seven glycolipids and two phosphoglycolipids were detected in the novel strains. Growth was observed at 47 °C. The peptidoglycan type A4α L-Lys–D-Asp was determined for strain BTLCH M1/2^T. Genotypic characteristics and phylogenetic analyses based on phylogenetic markers such as *hsp60, pheS, rpoA* and *tuf* genes, as well as results of phenotypic characteristics and chemotaxonomic analyses confirmed that new isolates belong to a new *Lactobacillus* species. The name *Lactobacillus bombi* sp. nov. was proposed for group of new isolates. The type strain is BTLCH M1/2^T (=DSM 26517^T = CCM 8440^T).

Keywords: bumblebee, *Lactobacillus* sp. nov., *Bombus terrestris*, digestive tract, MLSA (Multilocus Sequence Analysis)

Genus *Lactobacillus* belongs among the largest genera within the bacterial phylum Firmicutes. Lactobacilli are found primarily in environments that are rich source of carbohydrates and other organic substrates. Many species of lactobacilli are obligate bacterial symbionts of the digestive tract of mammals (Hammes & Hertel, 2009). Digestive tract of insects is an environment that is inhabited by a wide variety of bacterial symbionts, many of which are among the yet undescribed species (Engel et al., 2012; Colman et al., 2012). Some researchers believe that the bacterial symbionts of insects, especially lactic acid bacteria, can to some extent positively influence the immune system and host health (Evans & Armstrong, 2006; Forsgren et al., 2010; Koch & Schmid-Hempel, 2012; Mattila et al., 2012). Many studies have been recently published on the prevalence of lactobacilli and other lactic acid bacteria in the digestive tract of insects, especially in the digestive tract of important pollinators (Mohr & Tebbe, 2006; Killer et al., 2010b; Tajabadi et al., 2011; Martinson et al., 2012; Tang et al., 2012; Killer et al., 2014). Some authors have shown that in the stomach of honeybees appear new, probably host-specific species of lactobacilli (Olofsson & Vásquez, 2008; Forsgren et al., 2010). Bacteria inhabiting the digestive tract of bumblebees are not sufficiently explored (Killer et al., 2010b; Koch & Schmid-Hempel, 2011). Studies on the isolation and characterization of bacteria present in the digestive tract of bumblebees have so far focused only on the representatives of the family *Bifidobacteriaceae*. Three species of bifidobacteria and a new genus within the family *Bifidobacteriaceae* were recently discovered in the digestive tract of bumblebees (Killer et al., 2009; Killer et al., 2010a; Killer et al., 2011).

Isolation and detailed characterization of a new representative of the genus *Lactobacillus* occurring in the digestive tract of bumblebees is presented in this study. Based on results of genotypic, phylogenetic and phenotypic analyses, it was concluded that group of three bacterial strains represent a new *Lactobacillus* species.

Bumblebees of *Bombus terrestris* species were bred in the laboratory of the Agricultural Research, Ltd. (Troubsko, Czech Republic) in the spring of 2012. Bumblebees were kept in wooden hives at 26 °C and 50% RH (Relative Humidity). They were fed by fresh frozen honeybee pollen pellets (mix of pollen with dominance of Brassicacae, Rosaceae and *Papaver* from a local beekeeper) and sugar solution: saccharose (1000 g, white beet sugar, local producer) and fructose (460 g, Fructopur, Natura, Czech Republic) dissolved in water (1200g). Living bumblebees were transported to the Laboratory of anaerobic microbiology (Institute of Animal Physiology and Genetics in Prague, The Academy of Sciences of the

Czech Republic) and immediately killed by decapitation. Fresh digestive tracts of three queens originated from different nests were then placed in sealed tubes containing anaerobic MRS Broth (Oxoid, UK) and serially diluted in the same medium. Diluted samples were cultivated on MRS agar under anaerobic conditions (Anaerobic jars, Oxoid) at 37 °C for 48 hours. Genes encoding the 16S rRNA were amplified from bacterial isolates originated from the 10^{-6}-diluted samples using primers 616V and 630R under conditions as described by Ehrmann et al. (2003). Three bacterial isolates originating from the three bumblebee queens were then identified according to similarities (Killer et al., 2011) of almost complete 16S rRNA gene sequences (1490 bp).

The three new isolates, designated BTLCH M1/2^T, BTLCH M3/2 and M250 3MRA, were most closely related phylogenetically to unclassified bacterial strains *Lactobacillus* sp. Mboho2r2 (GenBank accession no. HM534813) and *Lactobacillus* sp. AcjLac3 (AB810024) isolated from the digestive tracts of European (*Apis mellifera*) and Japanese (*Apis cerana japonica*) honeybees, respectively. These new isolates were found to share 96.7–96.9% and 96.0–96.2% 16S rRNA gene sequence similarity with these unclassified isolates from the digestive tracts of honeybees. The jPHYDIT software (Jeon et al., 2005) was used for calculation of sequence similarities. *Lactobacillus tucceti* CECT 5920^T (GenBank accession no. NR_042194) was found to be the closest related member of a species with a validly published name, with 92.9% 16S rRNA gene sequence similarity. The three new bacterial isolates had high 16S rRNA gene sequence similarity to each other (99.7%). These results suggest that the new bacterial isolates belong to the same bacterial phylotype, probably representing a novel bacterial species of the genus *Lactobacillus* (Stackebrandt & Ebers, 2006). Multilocus sequence typing, phylogenetic, phenotypic and chemotaxonomic analysis was then used to test this assumption.

Genes encoding the 60 kDa heat-shock protein (*hsp60*), phenylalanyl tRNA synthase alpha subunit (*pheS*), RNA polymerase alpha subunit (*rpoA*) and translation elongation factor Tu (*tuf*) were sequenced from the three strains as additional phylogenetic markers. Detailed information about the primers and PCR parameters for amplification of these genes was published previously by Goh et al. (2000), Dobson et al. (2002), Naser et al. (2005, 2007) and Ventura et al. (2003). Amplified fragments were subsequently checked by electrophoresis on 1.5% PCR agarose gel (Top-Bio), purified using a PCR purification kit (Qiagen) and sequenced by using an ABI PRISM 3130xl automatic genetic analyser (Applied Biosystems). Defined sequences of phylogenetic markers were compared with sequences of

type strains of the closest related species based on the search results in gene databases through the BLAST program. Results of *hsp60, pheS, rpoA* and *tuf* gene sequence similarity testing revealed that the closest related sequences were those of *L. tucceti* DSM 20183^T (81.6–82.3%; GenBank accession no. KJ144259), *L. nagelii* LMG 21593T (78.7–79.2%; AM087708), *L. curvatus* LMG 9198T (73.3–73.6%; AM087783) and *L. crustorum* LMG 23699T (83.2–83.6%; FN395011), respectively. The authors who proposed the above phylogenetic markers reported much higher values of interspecies sequence similarity (Goh et al., 2000; Naser et al., 2007). Identical *hsp60* and *pheS* gene sequences were found in strains BTLCH M1/2^T and BTLCH M3/2. For this reason, only strains BTLCH M1/2T and M250 3MRA were included in further phylogenetic analyses based on these phylogenetic markers. Similarities of *hsp60, pheS, rpoA* and *tuf* gene sequences among the three strains were 99.0, 98.6, 99.4–99.7 and 99.5–100%, respectively. These results suggest that the novel strains represent a novel species within the genus *Lactobacillus*. Phylogenetic analyses subsequently confirmed this conclusion.

A procedure similar to that described recently (Killer et al., 2013) was used for reconstruction of phylogenetic trees based on partial 16S rRNA, *hsp60, pheS, rpoA* and *tuf* gene sequences. Phylogenetic trees were reconstructed based on sequences of type strains of species of the genus *Lactobacillus* by using the maximum-likelihood algorithm within the MEGA 5.05 program (Tamura et al., 2011) and the Jukes-Cantor model. Topology of trees was also checked by using the neighbour joining and maximum parsimony algorithms. Alignments generated by the CLUSTAL W algorithm were improved by removing hypervariable positions using the program Gblocks (Castresana, 2000). A phylogenetic tree reconstructed based on 16S rRNA gene sequences of species of the genus *Lactobacillus* present in the digestive tract of animals revealed that strains BTLCH M1/2^T, BTLCH M3/2 and M250 3MRA are situated together with uncharacterized strains of *Lactobacillus* from the digestive tract of honeybees in a separate phylogenetic cluster (Fig. 1). These lactobacilli from the digestive tract of pollinators represent a novel phylogenetic lineage within the genus *Lactobacillus*. Thus, the novel lactobacilli are phylogenetically distant from the *Lactobacillus* alimentarius cluster (Chenoll et al., 2006), which includes the species *L. tucceti*, the most closely related taxon with a validly published name based on 16S rRNA gene sequence similarity. Significant differences between this species and the isolates from the digestive tract of bumblebees were demonstrated by analysis of biochemical characteristics. For these reasons, the type strain of *L. tucceti* was not used for comparative analyses of other phenotypic characteristics.

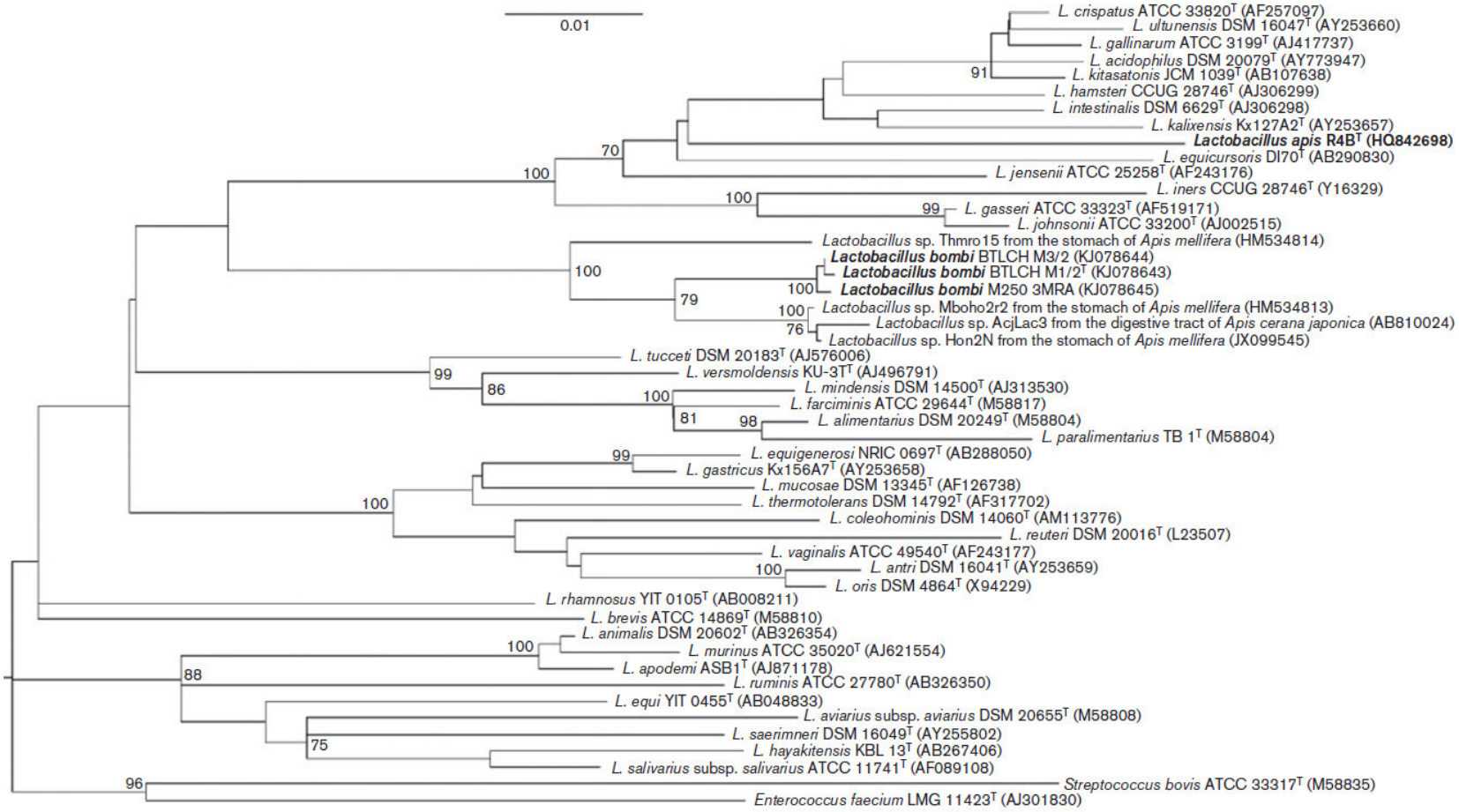

Fig. 1. Unrooted phylogenetic tree of species of the genus *Lactobacillus* that occur in the digestive tracts of humans and animals, showing the position of strains representing *Lactobacillus bombi* sp. nov. They occur in a separate cluster together with uncharacterized lactobacilli originating from the digestive tracts of honeybees. The tree was reconstructed by the maximum likelihood method based on 16S rRNA gene sequences (1336 nt) using MEGA version 5.05 software and the Jukes–Cantor model. Bootstrap values, expressed as percentages of 1000 datasets, are given at nodes. Numbers in parentheses correspond to GenBank accession numbers. Bar, 0.01 substitutions per nucleotide position.

Phylogenetic trees reconstructed using *hsp60, pheS, rpoA* and *tuf* gene sequences confirmed that the three studied strains can be assigned to a novel taxon of the genus *Lactobacillus*. They are positioned on separate phylogenetic branches among species of the genus *Lactobacillus* (Figs. S1–4 Supplementary materials are available online: http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.063602-0#tab5).

However, the topology of the trees did not match that obtained on the basis of 16S rRNA gene sequences, because of the use of shorter gene fragments and the absence of relevant gene sequences for unclassified, probably novel, taxa of lactobacilli from the digestive tracts of pollinators.

PCR–denaturing gradient gel electrophoresis was chosen as a tool to demonstrate the presence of the described novel species of the genus *Lactobacillus* in the digestive tracts of laboratory-reared bumblebee queens (*Bombus terrestris*) originating from four different localities in Moravia (Czech Republic). Total bacterial DNA from the digestive tracts of bumblebee queens was isolated and analysed exactly as described previously (Killer et al., 2014a). Amplified fragments (200 bp) of the 16S rRNA gene highly similar to that of strain BTLCH M1/2^T (99–100% sequence similarity) were observed in all samples (Fig. S5).

A modification of the enzymatic degradation method of Killer et al. (2011) was used to determine the DNA G+C contents of strains BTLCH M1/2^T, BTLCH M3/2 and M250 3MRA. The values obtained, 37.8 (mean of three experiments, SD50.4), 37.2 (SD50.1) and 38.0 (SD50.6) mol%, are in the range of values (32–55 mol%) reported for species of lactobacilli (Hammes & Hertel, 2009).

API 50 CHL, Rapid ID 32A and API ZYM commercial kits (all from bioMerieux) were applied to determine biochemical characteristics of the three tested strains and *L. tucceti* DSM 20183^T. Tests were performed according to the manufacturer's instructions, except that the API 50 CHL test strips were incubated under anaerobic conditions (anaerobic jars; Oxoid) at 37 °C for 48 h. Bacterial strains were also tested for oxidase activity (Liu & Jurtshuk, 1986) and hydrolysis of gelatin by the API 20E system (bioMerieux). Physiological properties such as the ability to grow at a range of different temperatures and pH and environments with varying oxygen tension were deter-mined by methods described previously (Killer et al., 2013). Production of D- and L-lactic acid was also tested by using a D-/L-lactic acid kit (Megazyme). Durham tubes in MRS broth were used to test for gas production from glucose. The tested strains differed in the utilization of 18 substrates and production of 10 enzymes (Table 1).

Table 1. Differences in biochemical characteristics among the novel strains and *L. tucceti* DSM 20183^T

Strains: 1, BTLCH M1/2^T; 2, BTLCH M3/2; 3, M250 3MRA; 4, *L. tucceti* DSM 20183^T. All strains utilized D-glucose, D-fructose and D-mannose. None produced acids from glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, lactose, inulin, melezitose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, D- or L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. All tested strains produced glutamic acid decarbox-ylase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, histidine arylamidase, valine arylamidase, serine arylamidase and naphthol-AS-BI-phosphohydro-lase. All were negative for production of β-galactosidase-6-phosphate, α-glucosidase, α-arabinosidase, β-glucuronidase, α-fucosidase, reduction of nitrates, indole from L-tryptophan, leucylglycine arylamidase, pyroglutamic acid arylamidase, glutamyl glutamic acid arylamidase, esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-mannosidase, gelatin hydrolysis, catalase and oxidase. +, Positive; W, weakly positive; 2, negative. Data are from this study.

Characteristic	1	2	3	4
Utilization of :				
L-Arabinose	+	+	-	-
D-Xylose	+	+	+	-
D-Galactose	-	+	+	-
L-Rhamnose	w	-	w	+
D-Mannitol	-	-	-	+
N-Acetylglucosamine	w	+	w	+
Cellobiose	+	-	-	-
Amygdalin	+	+	+	-
Arbutin	+	+	+	-
Esculin	+	+	+	-
Salicin	+	+	+	-
Maltose	-	-	-	+
Melibiose	+	+	+	-
Sucrose	+	+	+	-
Trehalose	w	+	+	-
Raffinose	+	+	+	-
Gentiobiose	+	+	+	-
L-Fucose	-	-	-	+
Production of :				
Urease	-	-	+	-
Arginine dihydrolase	-	+	+	-
α-Galactosidase	+	+	+	-

Production of :				
Urease	-	-	+	-
N-Acetyl-β-glucosaminidase	+	w	+	-
Alkaline phosphatase	-	+	-	-
Acid phosphatase	+	+	-	-
Tyrosine arylamidase	w	-	w	-
Alanine arylamidase	w	-	w	+
Glycine arylamidase	-	-	-	+

L. tucceti DSM 20183^T had very different substrate utilization and enzyme activity patterns in comparison with the three novel strains (Table 1), in agreement with the phylogenetic distance (Fig. 1). The novel strains differed among themselves in utilization of L-arabinose, D-galactose, L-rhamnose and cellobiose and production of urease, arginine dihydrolase, alkaline phosphatase, acid phosphatase, tyrosine arylamidase and alanine arylamidase. Substrate utilization and enzyme activity patterns tested by the API 50 CHL and Rapid ID 32A kits did not reveal any similarity to profiles of species of *Lactobacillus* deposited in the Apiweb database (https://apiweb.biomerieux.com/servlet/Authenticate). Both D- and L-lactic acids were produced by cells of all analysed bacterial strains. No gas production from glucose was found. Growth at 20–47 °C and pH 4.0–8.5 was observed in the new isolates. Growth at temperatures higher than 45°C has been detected in only a few species of *lactobacilli* (Pedersen et al., 2004; Hammes & Hertel, 2009). The best growth was found under strictly anaerobic conditions on TPY (Scardovi, 1986) and MRS agar. Poor growth was also observed under microaerophilic conditions.

Determination of the end products of hexose catabolism in strain BTLCH M1/2^T was performed using capillary isotachophoresis (Killer et al., 2011). Lactic, acetic and propionic acids were determined at concentrations of 85.4 mmol l^{-1} (65% of all short chain fatty acids produced), 32.7 mmol l^{-1} (25%) and 13.2 mmol l^{-1} (10%), respectively. These results, along with the ability to utilize some pentoses, suggest that strain BTLCH M1/2^T belongs to the facultatively heterofermentative lactobacilli (group B according to Hammes & Hertel, 2009).

Cellular fatty acid profiles of the three novel strains were determined using methods described by Kampfer & Kroppenstedt (1996) and Miller (1982). Summed C19:1ω6c and/or C19:0 cyclo ω10c/19ω6; C18:1ω9c and C16:0 were detected as the major fatty acids (Table 2). These fatty acids have been identified previously as the main components in lactobacilli (Gomez Zavaglia et al., 2000).

Table 2. Cellular fatty acid profiles of the novel strains

Relative concentrations (%; w/v) of fatty acids are shown. Data are from this study.

Bacterial strains / Fatty acid	***BTLCH M1/2^T***	***BTLCH M3/2***	***M250 3MRA***
Summed $C_{19:1\ \omega6c}$ / cyclo $C_{19:0\ \omega10c/19\omega6}$	37.12	29.86	27.83
$C_{18:1\ \omega9c}$	35.64	33.25	29.40
$C_{16:0}$	13.15	18.32	20.53
$C_{18:1\ \omega7c}$	4.34	3.77	5.45
$C_{14:0}$	4.11	5.80	6.24
Summed $C_{16:1\ \omega7c}$ / iso $C_{15\ 2OH}$	2.48	2.31	1.27
$C_{18:0}$	1.80	< 0.1	1.70
$C_{17:1\ \omega7c}$	0.58	1.47	0.86
$C_{15:0}$	0.50	< 0.1	< 0.1

The profile of cellular polar lipids and the structure of peptidoglycan were determined for strain BTLCH M1/2^T by the Identification Service of the DSMZ by using methods described previously (Killer et al., 2010a). Polar lipids detected in the strain were diphosphatidylglycerol, phosphatidylglycerol, a phospholipid, seven glycolipids and two phosphoglycolipids (Fig. 2).

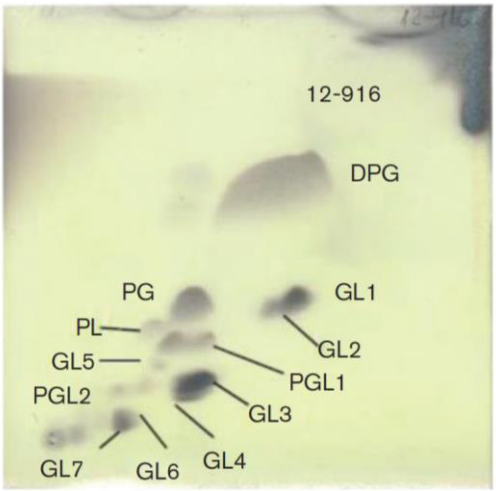

Fig. 2. Profile of cellular polar lipids detected in strain BTLCH M1/ 2^T. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; GL, unidentified glycolipid; PL, unidentified phospholipid; PGL, unidentified phosphoglycolipid.

Phosphatidylglycerol and unidentified phospholipids and glycolipids seem to be widely distributed among different species of the genus *Lactobacillus* (Arbogast & Henderson, 1975; Kim et al., 2011; Killer et al., 2014a, b). On the other hand, the occurrence of unidentified phosphoglycolipids and lipids and phosphatidylethanolamine can differ in cells of different taxa of lactobacilli (Kim et al., 2011; Liang et al., 2011; Killer et al., 2014a, b). Chemical analysis revealed peptidoglycan type A4α L-Lys–D-Asp (type A11.31 according to the DSMZ; http://www.dsmz.de/?id=449). The molar ratio of the amino acids in the peptidoglycan hydrolysate was as follows: 2.9 : 0.9 : 1.0 : 0.7 Ala : Asp : Glu : Lys. This peptidoglycan structure has been reported for most species of lactobacilli and other representatives of the order *Lactobacillales* (Schumann, 2011).

The results of this study allow us to assign the group of analysed bacterial strains to a novel species within the genus *Lactobacillus*, for which the name *Lactobacillus bombi* sp. nov. is proposed.

4.1 Description of *Lactobacillus bombi* sp. nov.

Lactobacillus bombi (L. n. bombus a boom, a deep hollow noise, buzzing, and also the zoological genus name of the bumblebee; N.L. gen. n. bombi of Bombus, of a bumblebee).

Cells growing on soft 0.5% MRS agar under anaerobic conditions are Gram-stain-positive, catalase- and oxidase-negative, regular, more or less curved, long rods with rounded ends, organized mostly singly and in pairs, 0.7–1.0 mm wide and 2.2–7.2 mm long. The best growth is observed in anaerobic TPY and MRS broth or agar; weaker growth is observed under microaerophilic conditions on the same media. Colonies on MRS agar under anaerobic conditions after 72 h are cream in colour with sharp edges. Colonies are disc-shaped in profile, but also triangular in approximately one-third of colonies. Colonies are 1.21– 2.82 mm in diameter. The optimum temperature for growth is 37 °C; growth occurs at 20–47 °C. Growth occurs at pH 4.0–8.5. Utilizes D-glucose, D-fructose, D-mannose, D-xylose, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, melibiose, sucrose, trehalose, raffinose and gentiobiose. Variable for utilization of L-arabinose, D-galactose, L-rhamnose and cellobiose. Negative for utilization of glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-mannitol, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α-D-manno-pyranoside, methyl α-D-glucopyranoside, lactose, maltose, inulin, melezitose, starch, glycogen, xylitol, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-

ketogluconate. Produces α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, glutamic acid decarboxylase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylami-dase, histidine arylamidase, valine arylamidase, serine arylamidase and naphthol-AS-BI-phosphohydrolase. Negative for β-galactosidase-6-phosphate, α-glucosidase, α-arabinosidase, β-glucuronidase, α-fucosidase, reduction of nitrates, production of indole from L-tryptophan, leucylglycine arylamidase, pyroglutamic acid arylamidase, glutamyl glutamic acid arylamidase, esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-mannosidase, gelatin hydrolysis, catalase and oxidase. Variable for production of urease, arginine dihydrolase, alkaline phosphatase, acid phosphatase, tyrosine arylamidase and alanine arylamidase. The determined peptidoglycan structure is type A4α L-Lys–D-Asp. Major fatty acids are summed C19:1ω6c and/or C19:0 cyclo ω10c/19w6; C18:1ω9c and C16:0.The polar lipid profile includes diphosphatidylglycerol, phosphatidylglycerol, a phospholipid, seven glycolipids and two phosphoglycolipids. The type strain, BTLCH M1/2^{T} (=DSM 26517^{T} =CCM 8440^{T}), was isolated from the digestive tract of a bumblebee queen (*Bombus terrestris*) reared in the laboratory at Agricultural Research, Ltd (Troubsko, Czech Republic), in 2012. Additional strains of the species are BTLCH M3/2 and M250 3MRA. The DNA G+C content of the type strain is 37.8 mol%.

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5

Variation in honey bee gut microbial diversity affected by ontogenetic stage, age and geographic location

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Abstract

Social honey bees, *Apis mellifera,* host a set of distinct microbiota, which is similar across the continents and various honey bee species. Some bacteria, such as lactobacilli, have been linked to immunity and defence against pathogens. Pathogen defence is crucial, particularly in larvae, as many pathogens affect the brood. However, information on larval microbiota is conflicting. Seven developmental stages and drones were sampled from 3 colonies at each of the 4 geographic locations of *A. mellifera carnica*, and the samples were maintained separately for analysis. We analysed the variation and abundance of important bacterial groups and taxa in the collected bees. Major bacterial groups were evaluated over the entire life of honey bee individuals, where digestive tracts of same aged bees were sampled in the course of time. The results showed that the microbial tract of 6-day-old 5th instar larvae were nearly equally rich in total microbial counts per total digestive tract weight as foraging bees, showing a high percentage of various lactobacilli (Firmicutes) and *Gilliamella apicola* (Gammaproteobacteria 1). However, during pupation, microbial counts were significantly reduced but recovered quickly by 6 days post-emergence. Between emergence and day 6, imago reached the highest counts of Firmicutes and Gammaproteobacteria, which then gradually declined with bee age. Redundancy analysis conducted using denaturing gradient gel electrophoresis identified bacterial species that were characteristic of each developmental stage. The results suggest that 3-day 4th instar larvae contain low microbial counts that increase 2-fold by day 6 and then decrease during pupation. Microbial succession of the imago begins soon after emergence. We found that bacterial counts do not show only yearly cycles within a colony, but vary on the individual level. Sampling and pooling adult bees or 6th day larvae may lead to high errors and variability, as both of these stages may be undergoing dynamic succession.

Keywords: Honey bee, Larvae, Foraging bees, Pupae, Gut microbial diversity, Gammaproteobacteria, Lactobacilli, *Gilliamella apicola*, DGGE

5.1 Introduction

Honey bees are a key species for agriculture, contributing significantly to the human food supply (Klein et al., 2007). Recent losses of *Apis mellifera* and the potential association of these declines with various infectious agents highlight the need for an increased understanding of innate bee immunity and mechanisms that help them adapt to environmental stress. One of these factors may be their gut microbiota; however, little is known regarding the role of beneficial microbes in honey bee health (Genersch, 2010, Forsgren et al., 2010).

Social insects provide unique resources for studying microbial symbionts because of the high density of individuals within colonies, sharing of food and other resources and the coexistence of colony members from multiple generations (Evans and Lopez, 2004). These bacterial communities vary immensely in total size, composition, location and functions within the individual parts of the gut (Martinson et al., 2012). The adult honey bee hosts up to 10^9 bacterial cells, 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). The maintenance of this stable and distinct microbial community depends on the nutrition and social lifestyle of these insects (Engel and Moran, 2013, Mattila et al., 2012), environment (Mattila et al., 2012, Mrazek et al., 2008) and ontogenetic stage (Martinson et al., 2012, Yoshiyama and Kimura, 2009, Mohr and Tebbe, 2006). This dynamic system has also been shown to follow seasonal trends (Corby-Harris et al., 2014a, Ludvigsen, 2013). Numerous studies have been conducted to characterize adult honey bee diversity using new cultivation-independent techniques, but fewer studies have examined the larvae. The microbiome of honey bee larvae can be highly variable, and particularly older, culture-based studies have not revealed any highly specific microbial patterns (Gilliam and Prest, 1987). However, later PCR-based methods in the larval and adult intestine and rectum revealed a few classes of Gammaproteobacteria, recently identified as *Gilliamella apicola* and *Frischella perrara* (Mohr and Tebbe, 2006, Kwong and Moran, 2013) as well as a presence of Betaproteobacteria (*Snodgrassella alvi*) (Martinson et al., 2012, Engel and Moran, 2013, Corby-Harris et al., 2014a, Kwong and Moran, 2013, Moran et al., 2012, Koch et al., 2013, Engel et al., 2014) and Acetobacteria (Corby-Harris et al., 2014b) species in the larval gut. The presence of members of the genus *Lactobacillus* appears to be rather random; however in larval stages were detected (Mohr and Tebbe, 2006, Ahn et al., 2012). Unlike many other insects, honey bee larvae defecate only shortly before pupation, making it difficult to control the microbial environment (Mohr and Tebbe, 2006). Specific knowledge regarding the dynamics and variation in the larval gut microbiome is very

important, as larvae are considered to be a focus for probiotic applications and in aiding defence against pathogens and colony health (Evans and Lopez, 2004). Probiotic bacteria are known to be promoters of host body defence by triggering a humoral immune response and creating an intestinal immunological barrier (Evans and Lopez, 2004, Olofsson and Vasquez, 2008, Patruica and Mot, 2012). As such, many studies examining bee microbiota were related to defence against the major pathogen *Paenibacillus larvae*, the causative agent of American foulbrood (Yoshiyama and Kimura, 2009).

Several bacterial strains or a mixture of strains are thought to be beneficial for honey bees and have been considered for probiotic supplementation, mainly of the *Lactobacillus* and *Bifidobacterium* spp.; however, the assumption that lactobacilli or bifidobacteria have beneficial effects in honey bees may be oversimplified as the physiological dynamics in the bee gut microbiome are not well-understood. Between 3 and 4 distinct classes of lactobacilli have been identified and were recently characterised: *Lactobacillus apinorum* (Olofsson et al., 2014), which is phylogenetically similar to *Lactobacillus kunkeei*; Firmicutes (Firm) 4 clade represented by *Lactobacillus mellifer* and *Lactobacillus mellis* and the Firm 5 clade represented by recently identified phylogenetically close species of Lactobacillus melliventris, *Lactobacillus kimbladii, Lactobacillus helsingborgensis, Lactobacillus kullabergensis* (Olofsson et al., 2014) and *Lactobacillus apis* (Killer et al., 2013). Bifidobacteria are present in relatively low abundance in honey bees. Higher variability of bifidobacteria was recently observed in closely related bumblebees (Killer et al., 2009, Killer et al., 2010, Killer et al., 2011). However, the role of Gamma (Gammaproteobacteria represented by the strains *G. apicola* or *F. perrara*) (Kwong and Moran, 2013, Engel et al., 2013) as a large and honey bee-specific microbial group remains unexplored, although they may also take part in pathogens defence (Engel et al., 2012).

Previous studies of bacterial populations as honey bee gut symbionts have not examined the large variation between geographical locations or individuals. Although differences in microbiota were seen between honey bees from different parts of the world (Yun et al., 2014) nothing is known about differences in bacterial populations of relatively close apiary sites with slightly different environments. Information on such interactions and inter-and intra-colony microbial variation is lacking, particularly for larval and pupal stages. The aim of this study was to compare the microbial populations in 7 developmental stages (+ drones) in honey bee colonies living within same location and within different apiary sites. We searched for patterns and variability in the honey bee microbiome, focusing on selected *Gilliamella* and

lactobacilli strains using 16S rRNA denaturing gradient gel electrophoresis (DGGE) and quantitative real-time (qRT)-PCR. We examined whether honey bee microbial populations are affected by location and thus food sources of microbial inoculation. Our goal also was to gain insight into microbial dynamics during honey bee development.

5.2 Materials and Methods

5.2.1 General experimental approach

Two experiments were performed. The experiment referred to as EXP1 involved sampling of various developmental stages from 4 different locations and was used for DGGE analysis and follow-up statistical evaluation. The other referred to as EXP2 was conducted in a single hive, tracking microbial populations of bees of similar oviposition time and was also referred to as a "single bee" model.

5.2.2 Honey bee samples

In EXP1, we compared microbiota in various developmental stages on 4 geographical locations and samples from total 12 colonies of *A. mellifera carnica* were collected from the following locations: Dol (50°12'23.9"N 14°21'58.8"E), Ustrasice (49°20'35.6"N 14°40'55.2"E), Postrizin (50°13'34.0"N 14°22'42.5"E) and Hostice (50°12'23.3"N 14°24'10.8"E) (Fig. S4; Supplementary materials are available online: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0118707#sec022). Hereby, we certify that the samples were collected on either private land (land of Bee Research Institute Dol) or land commercially rented, we solely hold responsibility for ethical approaches and can be contacted later for confirmation. The field studies did not involve endangered or protected species. EXP2 samples originated from one hive in the Dol location. Sampling was conducted on 31 July 2012 (EXP1), and continued for 60 days for the time-course experiment (EXP2).

For EXP1, 3 hives were randomly selected at each location and from each hive, samples of 1-, 3- and 6-day-old larvae, white and black pupae, young bees and foraging bees and drones were collected. One-day-old larval sample corresponded to the 1st instar larva shortly after breaking the egg chorion and forming the C-shaped position. The 6th-day-old larvae corresponded to the 5th instar, with the gut completely filled with a yellowish material and corresponding to the last feeding stage LF3 (Soares, 1993, Rembold, 1980) prior to sealing. White pupae were collected with red brown to dark brown eyes and no signs of body

pigmentation (Pr–Pd) (Rembold, 1980). Black pupae were acquired when they showed medium thorax pigmentation. Young bees and drones were bees randomly collected after emergence from the combs and nectar foraging bees were sampled from the landing board. Approximately 5–10 individuals were collected in disposable tubes and frozen immediately on dry ice. Bee management and samples represented traditional beekeeping practices in the Czech Republic.

For EXP2, to obtain controlled oviposition and larvae of the same age, the queen was secured to a broodless comb in a colony using an excluder cage for a 4-h period. Next, the brood was left to develop and was sampled on days 2, 5, 8, 12, 16 etc. Each newly emerged bee was labelled with paint and later, only marked bees were sampled. Each sample was a pooled sample of 3–10 individuals. A total of 16 samplings were conducted during this period.

Entire tube-like digestive tracts (crop, midgut, ileum and rectum) were removed from each honey bee stage, pooled and weighed. Exceptions included samples of 1-day larvae which were swabbed from 10 honeycomb cells by cotton swab, submerged in 200 μL sterilized water and centrifuged within 90 sec to 9000 rpm. Next, the pellet was used for isolation of total bacterial DNA. Three-day-old whole larvae were homogenised. For other samples, approximately 50–200 mg of pooled digestive tracts were used for isolation of total bacterial DNA using the ZR Faecal DNA MiniPrep kit (Zymo Research, Irvine, CA, USA).

5.2.3 Denaturing gradient gel electrophoresis and sequencing

Amplification of the total bacterial community was conducted by targeting 200 bp partial 16S rRNA gene sequences with the universal bacterial primers 338GC and RP534 under previously described conditions (Mrazek et al., 2008). PCR products were analysed on a DGGE gel (gradient from 35–65%) according to the method of Mrazek et al. (2008).

Appropriate standards containing a mixture of PCR products of 5 known microorganisms were loaded in the centre of gels defined to minimize gel variability and used for multi-gel comparison was conducted using BioNumerics 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). Lanes were manually aligned and band positions were identified from corrected intensity plots. Band matching was accomplished by using the following BioNumerics settings: 7% minimum profiling, 0% gray zone, 0% minimum area, and 0 shoulder sensitivity. Comparison between samples loaded on different DGGE gels (Fig. S1) was completed using normalized values derived from known standards (used as external

references). A matrix of the relative band intensity values of 17 major identified bands was prepared for all gel lanes (Table S1).

Two to three DNA bands with the same normalised Rf values were cut out of the polyacrylamide gel using a sterile scalpel blade to confirm their correct alignment (selected bands shown in Fig. S1). Bacterial species were detected using the primers FP341 and RP534. The bands of interest were sequenced on a 3100 Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA) in the Centre of DNA Sequencing (Institute of Microbiology of the ASCR, v. v. i.). The resulting sequences were compared with the GenBank database using the BLAST algorithm. Eukaryotic DNA bands were omitted for profiling. Some bands showed the presence of heterogeneous DNA, but were used for statistical analysis. The sequencing revealed that 2 of the single 16S rRNA sequences generally appeared as multiple bands at 2 Rf values, likely because of variation between the GC-clamp primers, which was previously described by Rettedal et al. (2010). For multivariate statistical analysis, these were considered as different bands but were pooled for heatmap construction. Some of the bands of low abundance or from the parts where matching may be of low precision were omitted.

Three of the lactobacilli, *G. apicola* and *F. perrara* strains used as standards for the DGGE profiling were isolated from adult honey bee digestive tracts. Their 16S rRNA gene sequences and those of honey bees related to Firm, or selected strains of the family Orbaceae obtained from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov), were aligned using ClustalW within the MEGA 5.05 software (Tamura et al., 2011). Alignments provided by the ClustalW algorithm were improved by removing hypervariable positions using the program Gblocks (Castresana, 2000). Evolutionary distance matrices were generated using the Cantor & Juke model. Two phylogenetic trees for both lactobacilli (see Fig. S2) and *G. apicola/F. perrara* were constructed (see Fig. S3).

5.2.4 Real-time PCR analysis

Quantification of bacterial DNA was conducted using mx3005P thermocycler (Stratagene, La Jolla, CA, USA) with Gammaproteobacteria (1080γF, γ1202R), Bacteroidetes (798cfbF, cfb967R), Firmicutes (928F-Firm, 1040FirmR) and Actinobacteria (Act920F3, Act 1200R) (De Gregoris et al., 2011).

5.2.5 Statistical analysis

To examine the relationship between colony location and honey bee development stage on the total distribution of major abundant bacterial strains, we performed redundancy analysis (RDA) on all data using the CANOCO 4.5 program (Microcomputer Power, Ithaca, NY, USA) (Braak and Šmilauer, 2002). RDA was preferred over principal component analysis because of the advantages of direct association of constrained canonical axes with groups of independent variables. CANOCO provides an advantage of separate centring and/or standardization within response variables (bacterial strain abundance) and within samples (each combination of ontogenetic stage, location and replicate). To test our hypothesis, 2 models were arranged where both included the abundance of bacterial strains as response variables and location-to-ontogenetic phase interaction as independent variables. When absolute values of strain abundance were investigated, only the centring (results in zero average) of each strain were used. If only the proportions of strain abundance was considered, the centring of each strain as well as the centring and standardizing (results in norm equal to one) of samples were used.

The statistical significance of first and all other constrained canonical axes was determined using the Monte Carlo permutation test (499 permutations). An ordination diagram was created in CanoDraw for graphical visualization of the results. Heatmaps were constructed in MS Excel (Microsoft Corporation, Redmond, WA, USA). For analysis and visualisation of qRT-PCR data, we used IBM SPSS Statistics ver. 20 (IBM, Armonk, NY, USA).

5.3 Results

5.3.1 DGGE fingerprinting patterns

Samples of honey bees in various ontogenetic stages were acquired from 4 apiary sites within 10–100 km distance of each other (Fig. S4), and 3 colonies from each apiary site were investigated to determine bacterial diversity. This experiment is referred to as EXP1. DGGE fingerprinting profiles of 200-base pair (bp) amplicons of the 16S rRNA V3 region showed between 20–30 bands. Of these, the 17 abundant or well aligning bands (Fig. S5) were matched manually through multi-gel alignment of all 96 samples. Selected major band intensities were displayed in a heatmap (Fig. 1). This visualisation revealed that all three *G. apicola* (Gamma1) strains occurred rather randomly and at very low intensities in 1st instar larvae (L1) and at even lower intensities in 4th larval instar (L3), but became abundant bands

in 5th instar larvae prior capping and defecation (L6). In later stages, they were mostly absent in both pupal stages (white pupa, PW, and black pupa, PB) and began occurring randomly in samples of young bees (BY) in some apiary sites. These strains were very abundant in foraging bees (BF) and drones (DR) with similar distribution profiles. At each apiary site, there were colonies with absent or highly abundant *Gilliamella sp.* (Gil) strains. Gil 1 (100% similarity to wkB1T) and Gil 2 was generally present as a more intense band than Gil 3. *Frischella perrara* (Fri) (Gamma-2) showed a very similar pattern to *Gilliamella* sp. strains (Fig. 1).

Betaproteobacterium *Snodgrassella alvi* (Sno), a typical and recently characterised honey bee host-specific microorganism, showed different distribution patterns from that of Gamma. Sno was nearly absent in L6 samples with an apparent exception: 1 colony from the Ustrasice site, where it also appeared in a higher abundance in both PW and PB. Interestingly, this colony in Ustrasice showed clinical signs of sacbrood virus infection, with larvae changing in colour and their mouth parts turning black. However, no general conclusion could be drawn from this case. *Snodgrassella alvi* appeared to occur with higher band intensities in PB samples than Gamma. In foraging bees, this bacterium was ubiquitous.

In the heatmap (Fig. 1), band intensities of the 4 most abundant and clear bands corresponding to *Lactobacillus* spp. strains are shown. All 4 selected lactobacilli strains clearly appeared to have higher band intensities in later ontogenetic stages. Lac 3 (100% similarity to *L. apis* R4BT (Killer et al., 2013) and 99% to Hma 11 (Olofsson et al., 2014)) was clearly more abundant than the others, followed by Lac 2 (100% similarity to *L. kimblandii* Hma2NT (Olofsson et al., 2014)). Lac 2 generally occurred randomly as a very intense band twice in different hives in the Dol apiary. In contrast to Lac 3, presence of Lac 2 was much rarer and was mostly absent in pupal or larval stages, with the exception of the Postrizin location where this strain appeared to be more abundant in pupae rather compared to Lac 3. The Lac 1 strain (99% similarity to *L. helsingborgensis* Bma5NT (Olofsson et al., 2014)) showed lower and rather patchy distribution in all stages.

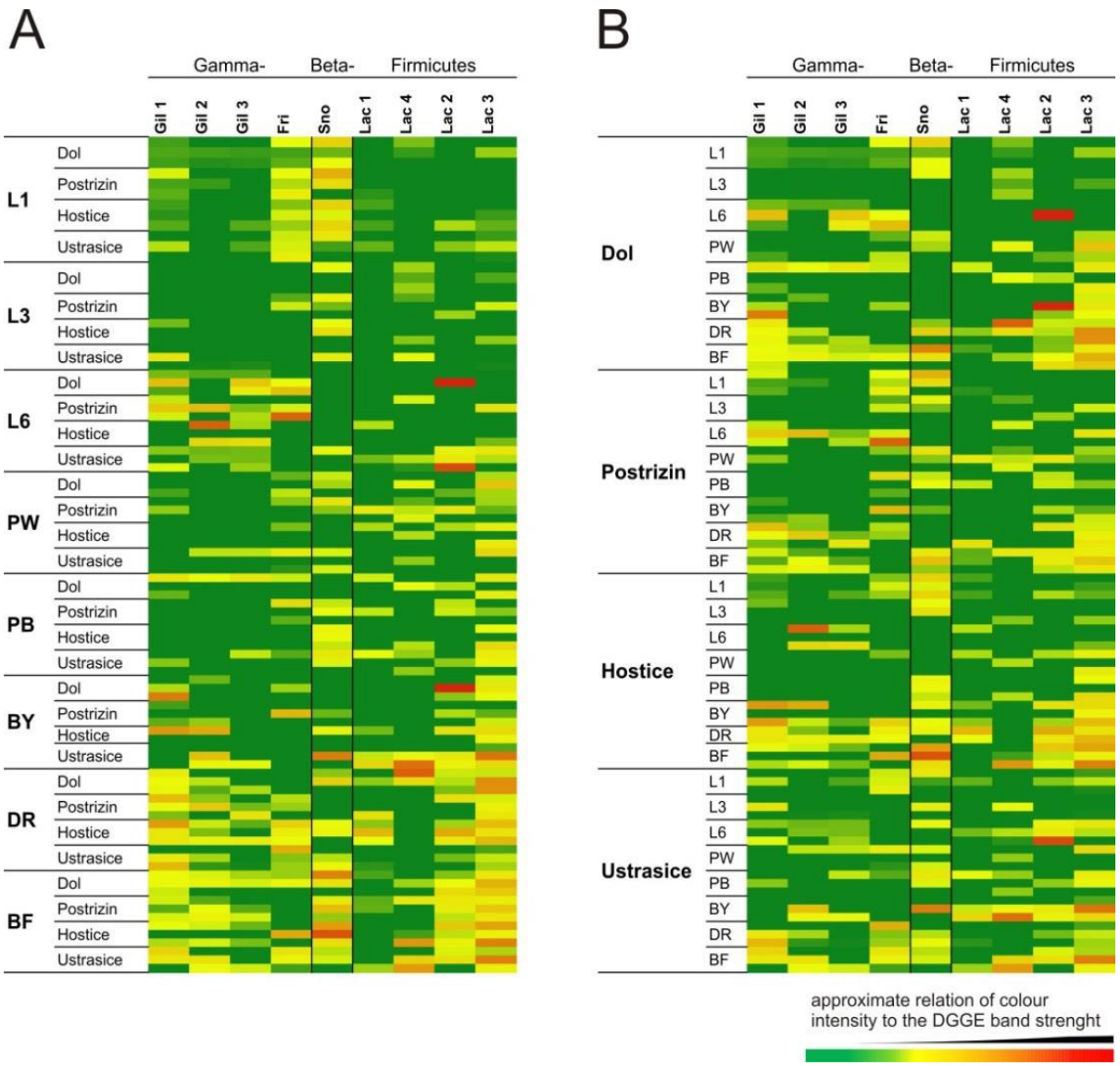

Fig. 1. Heatmap summarising the relative density of dominant denaturing electrophoresis bands of the 16S rRNA amplicon profiles of the total gastrointestinal tract contents of several honey bee ontogenetic stages. 1-, 3- and 6-day old larvae (L1, L3 and L6, respectively), white and black pupae (PW, PB), young bees, drones and flying bees (BY, DR and BF, respectively) collected in 4 different locations (Dol, Postrizin, Ustrasice, Hostice). Samples are sorted by ontogenetic stage (A) and location (B). The colours refer to relative band strength according to the colour key.

Some bands, such as Gil 2, Fri, Lac 2 and Lac 3, appeared to be more typical for adult stages, while Sno, Lac 3 and Lac 4 (98% similarity to *L. melliventris* Hma8NT (Olofsson et al., 2014)) appeared more frequently in pupae. In contrast to our expectations, no typical patterns of these selected strains were observed to be characteristic for one particular location.

5.3.2 Redundancy analysis of DGGE fingerprints

Microbial diversity based on DGGE profiles was statistically evaluated using redundancy analysis. A matrix of relative band intensities was used after grouping results from 3 colonies at each apiary site for simplification.

When the absolute abundance of strains was examined, the apiary site-to-ontogenetic stage interaction was significant (P = 0.002) and explained 43% of strain variability (all canonical axes), which was nearly 2-fold higher than when the 2 factors were evaluated separately (data not shown). Approximately half of the variability in total abundance of microbial strains may contribute to the difference among the 7 major ontogenetic categories and 4 apiary sites.

In Fig. 2A, the first canonical axis (horizontal) explained 15.3% (i.e. 35% of total explained) of strain variability and separated stages according to age with L1, L3, PW and PB on the left, L6 in the middle of the plot and adult stages mainly on the right side of the plot. The abundance of nearly all strains clearly increased with age (towards the right side). The highest variability according to locality was observed within drones and young bees, where the total strain abundance of these stages may be more similar to adults (e.g. drones in Dol) or larval stages (e.g. drones in Ustrasice). The data distribution for the second canonical axes (vertical) showed a trend in the differences within adult stages with drones in the lower plot, young bees in the middle and foraging bees in the upper part of the plot. *Lactobacillus* Lac 3, Lac 4, *Snodgrassella alvi* (Betaproteobacteria-Beta) and unknown *Rhizobiales bacterium* (Rhi) (Alphaproteobacteria-Alpha) appeared to be strains that were generally characteristic for foraging bees, while *Gilliamella* strains were characteristic for the microbiota of young bees, particularly drones. Although the samples were pooled, young bees at some locations appear to be less populated by distinct microbiota and are thus appear on the left side of the first canonical axis with larvae and pupae. This might be to the fact, that microbial successions in young bees may be faster at some sites or colonies compared to others.

The highest variability was observed within young bees and drones in the direction of first axis, in contrast to 6^{th}-day larvae and foraging bees in the direction of the second axis. This variation may have resulted from our experimental design, varying age of the individuals, time since the emergence and phase of microbial succession.

When the relative proportion of strain abundance was evaluated using redundancy analysis (RDA), the combination of location and ontogenetic stage was also significant (P = 0.002)

and explained 34% of strain variability (all canonical axes). In Fig. 2B, the first canonical axis explained 6.2% of variability and clearly distinguished 6th-day-old larvae in the left area from other larval or pupal stages on the right, showing higher proportion of *Gilliamella* strains on the left, compared to higher proportions of *Lactobacillus* Lac 4 and *Snodgrassella alvi* on the right. The second axis suggests a specific proportion of microbiota of L3 larvae (lower part) with a higher proportion of unknown *Rhizobiales bacterium* and *F. perrara* in contrast to most of the L6 larvae, drones and young bees with a higher proportion of *G. apicola* Gil 1–Gil 3 and Lac 3. Similarly to total abundance, proportions of strains were strongly affected by the apiary site. Variability was mainly in the direction of the second axis for drones, young bees and partially L6 larvae. A limitation of DGGE studies may be that some bands were not examined, as they occurred at rather low intensities and correct matching would be difficult.

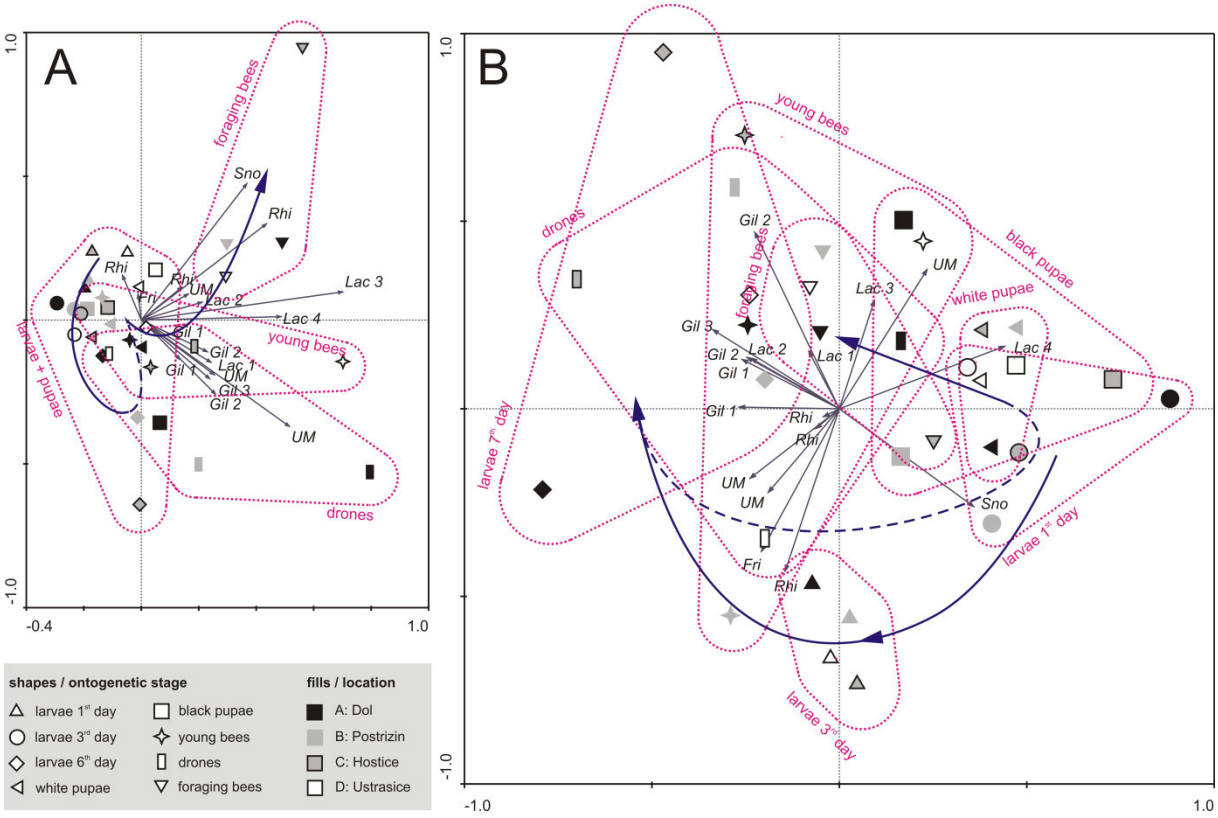

Fig. 2. Biplot from redundancy analysis (RDA) explaining the distribution of honey bee ontogenetic stages according to major bacterial strain abundance. Test of interactions between factors location and ontogenetic stage: A, crude data considering absolute DGGE band intensities; B, centred and standardized data considering relative band intensities. Abbreviations: Gil, *Gilliamella apicola*; Sno, *Snodgrassella alvi*; Lac, *Lactobacillus* sp.; Rhi, *Rhizobiales bacterium*; Fri, *Frischella perrara*, UM, unknown multiple - probable DNA heterodimer. For further strain descriptions, see Fig. S5. Dotted shapes surrounding each

ontogenetic stage were created as an aid in visualization. Eighteen bacterial strains occurring as major 16S rDNA DGGE bands were used for statistical analysis, while only selected strains are plotted as arrows. Same descriptions are for bands of the same sequence occurring at multiple locations of the line. Blue arrows show hypothetical developmental timeline. Its dotted part is ambiguous.

5.3.3 Quantitative PCR of microbial populations between apiaries

Real-time qPCR was conducted to quantify bacterial abundances using primers specific to major microbial populations, including Firmicutes, Gammaproteobacteria and minor groups of Actinobacteria and Bacteroidetes. Although bacterial counts varied highly among samples of the same stage, several patterns were recognized. During early development until day 3, in more than 90% of colonies, Gammaproteobacteria (Gamma = G) prevailed over Firmicutes (Firm = F) (G: 5.6×10^6 vs. F: 1.4×10^5; the means of gene copies per gram of digestive tract content), whereas when the 5th instar larva (L6) was prior to sealing, the ratio changed and Firm dominated over Gamma in 83% of hives (G: 1.9×10^7 vs. F: 2.5×10^8). After larval defecation and during pupation, bacterial counts decreased significantly; however, the dominating bacterial group was again Gamma in 80% of hives (G: 8.5×10^6 vs. F: 7.2×10^5) and was the major component in 60% of pigmented pupae (G: 6.8×10^7 vs. F: 1.5×10^6). Among newly emerged bees collected randomly from the comb, 55% contained higher counts of Firm (G: 1.7×10^7 vs. F: 2.3×10^8), and this ratio was further increased in drones and flying bees where Firm dominated in approximately 90% of samples (G: 1.3×10^7 vs. F: 3.1×10^8 and G: 5.1×10^7 vs. F: 5.4×10^8).

Moreover, the Bacteroidetes group and Actinobacteria were analysed using qRT-PCR. These groups were much less abundant; however, they appeared to follow a similar pattern. Variation between samples was high. In this study, we did not focus on 2 other microbial taxa present in the honey bee population: Alpha and Betaproteobacteria.

5.3.4 Changes in bacterial populations during the life span of a “single” honey bee

Another experiment (EXP2) was conducted to gain insight into the variation and dynamics of bacterial population changes during honey bee ontogenesis in one selected hive. The results showed that 4th and 5th larval instars were dominant in Firmicutes (Fig. 3); although the counts (gene copies per gram of digestive tract content) were generally low (2.9×10^7 in L6).

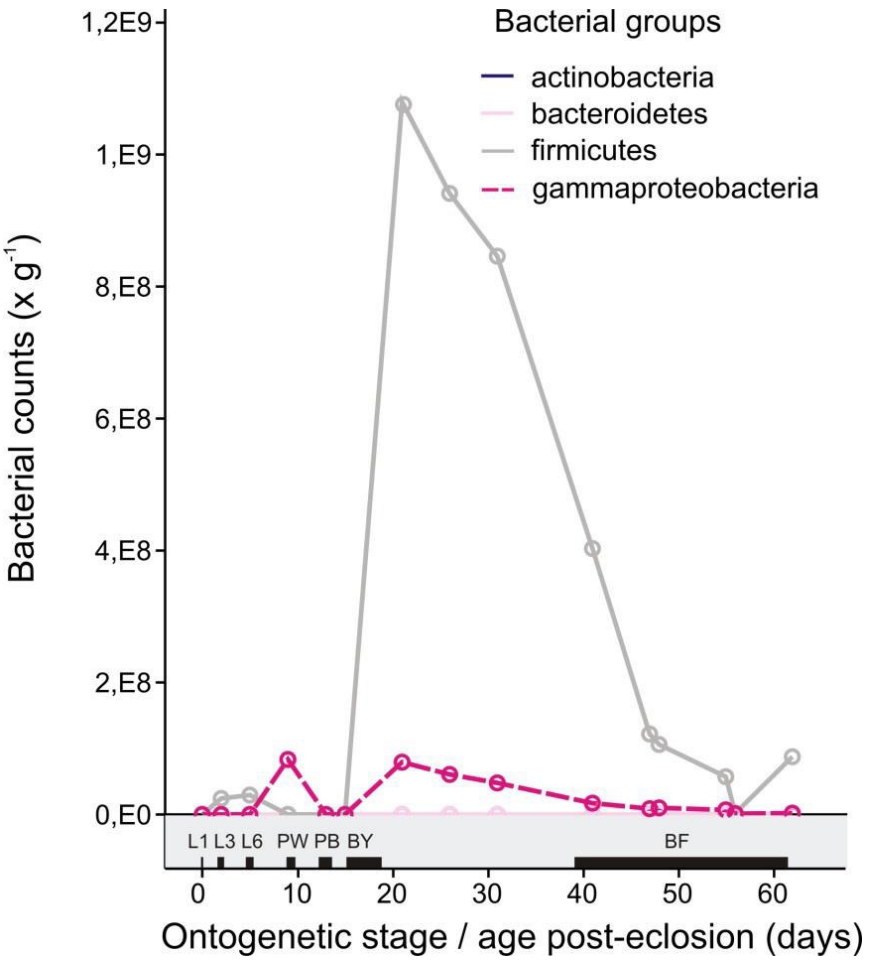

Fig. 3. Dynamics of selected bacterial groups in the total gastrointestinal tract during development and aging of a "single" honey bee. Data were obtained by collecting pooled samples of sister honey bees from the eggs of the same oviposition. Young bees were marked by paint shortly after emergence. Legend in the grey field provides a link to the first experiment EXP2 described here and shows at which approximate time points the samples for EXP1 were collected.

After defecation and capping, their counts decreased to (3×10^2). During pupation, an interesting rise in Gamma abundance was observed in white pupae samples (8.3×10^7), which was in this case an order of magnitude higher than the mean of the counts observed in EXP1 and rather similar to its maximum value. In black pupae, these bacteria were suppressed and newly emerged bees continued to show very low bacterial counts. The newly emerging bees appeared to be inoculated and bacterial counts of both Gamma and Firm groups within 6 days post-emergence quickly increased to the highest bacterial counts (G:7.9×10^7 vs. F: $1.8 \times$

10^9). Shortly after this rapid inoculation, both bacterial groups began decreasing in abundance, primarily in a linear manner as the honey bee aged (Fig. 4). Between 17–24 days after emergence, honey bees become foragers under normal circumstances (Huang and Robinson, 1996); both counts of Firm and Gamma were reduced by half compared to young bees at 6 days postemergence. At day 40, counts of Firm were as low as 4×10^8. It is clear that the sampling of young bees of unspecified age for microbiological analyses is linked with large variation error as the bees were in the process of inoculation and rapid succession.

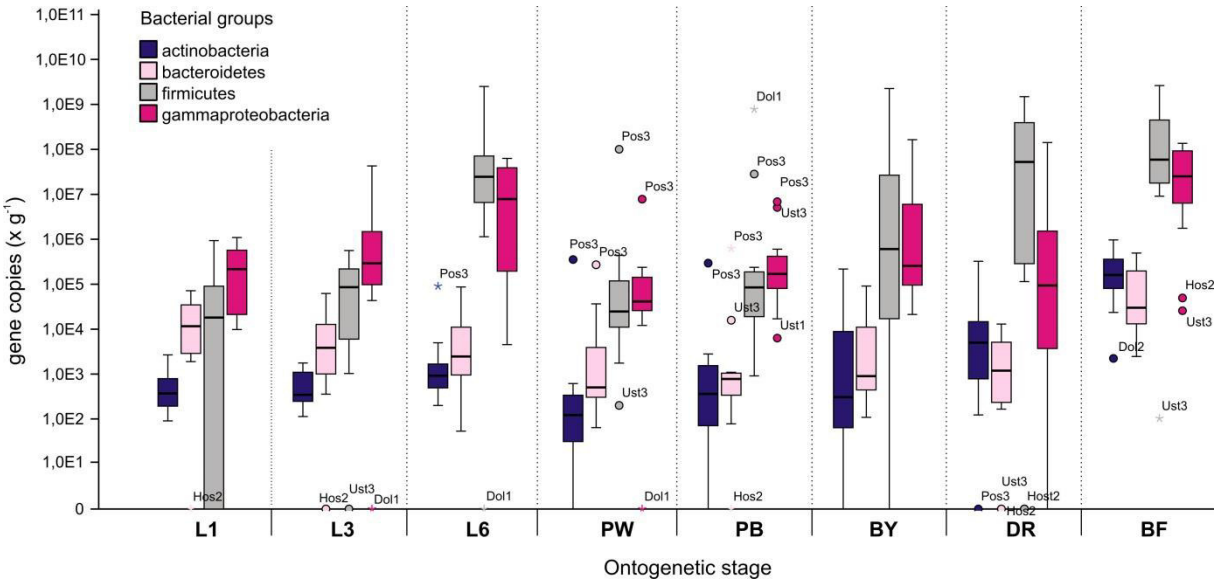

Fig. 4. Boxplot of quantitative real-time PCR (qRT-PCR) data of the abundance of selected bacterial groups in pooled samples of total gastrointestinal tract of each honey bee ontogenetic stage. The Y axis shows log-transformed copies of the 16S rRNA gene per gram of honey bee gastrointestinal tract. Boxes show pooled data from 4 locations and 3 hives at each location. 1-, 3- and 6-day old larvae (L1, L3 and L6, respectively), white and black pupae (PW, PB), young bees, drones and flying bees (BY, DR and BF, respectively). The codes of the outliers refer to the location (Pos: Postrizin, Hos: Hostice, Ust: Ustrasice) and colony number (1, 2, 3).

5.4 Discussion

5.4.1 Microbiota of 1st instar larvae

Our study revealed several interesting insights into the dynamics of honey bee microbial communities. First instar larvae generally showed very low bacterial counts, which is in agreement with previous studies (Vasquez et al., 2012, Vojvodic et al., 2013, Gilliam, 1971, Forsgren et al., 2010).

Whereas the Gamma group was slightly more abundant in this ontogenetic stage with 3.3×10^5 gene copies/g, the counts of Firm were 1.3×10^5 copies/g. This appears to be in contrast with the generally accepted notion that Gamma are only minimally present during in this early stage. However, Mohr and Tebbe (2006) previously found Gamma OTU in honey bee larvae and thus, their presence even in later larval instars cannot be ruled out. Our primers did not specifically distinguish between the core microbial set including *Gilliamella* and *Frischella* strains and other bee-non-specific Gamma. In this early ontogenetic stage, royal jelly proteins are thought to suppress microbial growth (Forsgren et al., 2010). Earlier studies have suggested that the presence of microbes in the larval stage digestive tract was due to unwanted contamination (Gilliam, 1971). Although our amplicon-based study revealed numerous copies of the 4 bacterial groups examined and the occurrence of bands characteristic of core honey bee microbiota in the DGGE profiles in our study suggest their presence in this developmental stage, the results might be biased by the fact that we used a cell content smear rather than an isolated larval tract.

5.4.2 Microbiota of 2–4th instar larvae

As the larva ages towards the 4th instar on day 3 of larval development, Alpha and Betaproteobacteria represented by an unknown *Rhizobiales* strain, *S. alvi* and, rarely in some of the samples, *F. perrara* remained among the frequently observed DGGE bands (Figs. 1 and 2B). Fingerprinting did not reveal any other typical bands, only the very random occurrence of the Firm and Gamma groups and low intensities of lactobacilli. The qRT-PCR method, however, showed that Gamma counts increased to 5.5×10^6 gene copies/g of the entire tract, whereas Firm remained at approximately the same level (1.5×10^5) (Fig. 3). This is clear even from evaluation of the DGGE patterns (Fig. 1). Box plot values in Fig. 3 may appear lower than the counts mentioned, as they do not show mean but median values after elimination of outliners.

5.4.3 Microbiota of 5th instar larvae

Major changes were observed in 5th instar larvae collected shortly after the last feeding. The Firm group averaged 2.5×10^8 and Gamma 1.9×10^7 gene copies per gram of digestive tract content. DGGE fingerprinting revealed a decrease in *S. alvi* and increase in the diversity of all Lac and Gil strains. RDA (Fig. 2B), showed very high variation among these L6 samples, particularly varying Gil strain abundance. Previously, *S. alvi* was found to be an important factor in biofilm formation, forming a bottom layer directly associated with the epithelial tissue, followed by the thick layer of Gil. Layered *Snodgrassella/Gilliamella* biofilm may function as a pathogen barrier as observed in *Bombus* spp. larval samples (Engel et al., 2012). It is possible that the reduction of *S. alvi* during ontogenesis is related to morphological changes, during which the gut intima is shed (Kikuchi et al., 2007); between locations, it may be affected by developmental speed in each colony.

The abundances of Firm and Gamma in larval and pupal instars were not fully supported by the EXP2 time-course experiment. The results of these experiments are shown in Fig. 3. The abundances of Gamma and Firm were lower during the larval development stages, which may be because of the natural variance between samples and because many of the strains occur at sporadic abundance; this is consistent with the hypothesis of a disease state. EXP1 and EXP2 results were difficult to compare because of their different designs.

5.4.4 Microbiome of young bees, drones and flyer bees

After pupation, the counts of both Firm and Gil decreased by nearly 2 orders in magnitude to means of 8.5×10^6 and 7.2×10^5 gene copies/g, after which growth continued slowly (Fig. 3). With the exception of one sample, RDA (Fig. 2B) distinguished black and white pupae from the L6 larvae based on the higher abundance of either *L. apis* or *L. melliventris*, whereas *L. apis* was more typical for black pupae and young bees. New bees are quickly inoculated within a maximum of 6 days post-emergence and showed mean values of 2.3×10^8 gene copies of Firm and 1.8×10^7 of Gamma, counts which were very similar to those observed in 5th instar larvae L6. This has recently been confirmed by Powel et al., who found that the maximum is reached depending on hive in 16 days (Powell et al., 2014).

The profile and bacterial counts of drones and young bees were very similar (Figs. 1 and 3) with drones showing only slightly lower counts of Gamma and higher counts of Firm. RDA revealed that adult groups were well-separated according to strain abundance along the

second axis in Fig. 2A, indicating some characteristic differences in strains distribution among stages.

Comparing to young bees, the number of gene copies of Firm and Gamma in pooled foraging bees, was significantly higher: 5.4×10^8 and 5.1×10^7, respectively.

5.4.5 Role of location in microbial composition

RDA revealed that despite clear drift in strain proportion from larval stages back to pupal stages and to adults, groups of bees and drones highly varied with location (Fig. 2B), showing some visible similarities of stages within a location (e.g. drones, young bees and L6 from Hostice in upper left side of the figure). The relative frequencies of core phylotypes are known to vary considerably among individuals in the same hive; in many cases, bacteria identified as core to the gut were not consistently found in foraging bees (Corby-Harris et al., 2014a), and the same may apply for L6 larvae. However, based on the heat map, no characteristic pattern was observed for any of the locations. At each location, all 3 hives appeared to be very different from each other and, rather than location, simple variability between hives was important.

5.4.6 Seasonal rhythms and ontogenetic changes in microbial profiles

Some studies have provided insight into seasonal time-course changes in honey bee microbiota; however these results are still very fragmented and studies have not examined complexity. A study by Ludvigsen (2013) clearly showed that while *G. apicola* follows seasonal changes and its counts continually decrease during the year, with the lowest values observed in October bees, while *S. alvi* counts rise during that period. Some periods of the year show higher variation among individuals in a single colony and colonies. Similarly, recent studies have shown a reduction in Firm-5 lactobacilli from spring to the fall by approximately 25% in foraging bees (Corby-Harris et al., 2014a). Studies on honey bee microbiota conducted at a particular time point may be affected by these seasonal rhythms. High variability may also be observed in different years. Mohr and Tebbe (2006) observed significant differences in microbial profiles from one year to another. This information is very important, as it may help in the design of proper probiotic supplementation strategies with respect to physiological conditions in the honey bee gut. We found that new bees are inoculated within a maximum of 6 days post-emergence; after that, bacterial counts decrease gradually over their lifetimes. Recent study of the Yale University team (Powell et al., 2014)

came to similar time frame of six to eight days post-emergence needed for the honey bee microbiome to reach maximum communities' richness and abundance, however, our study goes further by following the bees for their entire life.

5.4.7 Controversies in larval microbiota richness and composition

Colonisation of the larval gut is thought to start with strains of *L. kunkeei* (Vasquez et al., 2012) occupying the niche, dominating *Acetobacter* spp. (Alpha 2.2) abundance in early instars, and may be related to changes in the diet, which now contains pollen. Many pollen-associated bacteria species are considered to be contaminants, not belonging to the honey bee core-microbiota (Forsgren et al., 2010). Older culture-based studies generally found zero-to-low microbial abundance in larvae (Gilliam and Prest, 1987, Gilliam, 1971), but may be biased by the method and sometimes show contrasting conclusions, particularly for PCR-based methods. However, even with the widespread use of PCR-based methods, information regarding larval microbiota remains inconsistent, with many opposing theories. Few studies have examined this topic, however, they have not examined variability, which may have a large impact; these studies also sampled only a few individuals of *A. mellifera* or used pool samples from 1 colony (Martinson et al., 2012, Mohr and Tebbe, 2006, Ahn et al., 2012). Moreover, is not clear how management practices, breeding lines or colony location shape the microbial population. A study by Vojvodic et al. (2013) found large differences between managed *A. mellifera* and unmanaged Africanized bees patterns of larval microbial succession. Larval stages of Africanized bees were much more diverse in microbial species richness, in contrast with previous studies contained nearly 50% Firm-4 and Firm-5. Their conclusions were based on pooled samples from 3 colonies. In agreement with our results, a study of Vojvodic et al. (2013) confirmed that 4^{th} and 5^{th} instar larvae are already populated by Firm-5 and Firm-4 strains, although Gammaproteobacteria were not found. Gammaproteobacteria such as *Gilliamella* spp. have, however, been reported as part of the larval microbiota earlier (Mohr and Tebbe, 2006, Ahn et al., 2012).

An important limitation and source of discrepancies between recent studies focused on honey bee larvae microbiota may be that authors do not use a clear classification of honey bee preimaginal stages. As larval succession is very dynamic, similarly to physiological processes during morphogenesis, these studies require proper descriptive criteria. Thus, we propose using strict classification described by previous studies (Soares, 1993, Rembold, 1980) for *A. mellifera carnica* and Africanized bees, respectively.

In our study, larval samples of later instars in some colonies were essentially free of *Lactobacillus* strains with sporadic and quite low abundances, but nearly all samples showed bands of *F. perrara, S. alvi* and *G. apicola* Gil 1 (Fig. 1). The results obtained by DGGE profiling should be interpreted with caution because although all bands at the same Rf were matched with the highest thoroughness and each line was checked from 2–3 randomly sequenced bands, errors may occur and this data should be examined using statistical methods.

5.5 Conclusions

Active microbiota in honey bees differs in species richness and total abundances across the ontogenetic stage of honey bee and hive location. There were no clear patterns visible between different geographic locations; however, using DGGE and RDA, we identified several strains of *Lactobacillus* and *Gilliamella* spp. with characteristically higher but patchy abundance in various developmental stages of honey bees. We conclude that the digestive tract of larvae is not sterile or scarcely populated, as reported in earlier studies, but harbours 10^8 microorganisms primarily from the Firmicutes group. We also found that young bees are inoculated by Firm-5 during a maximum of 6 days post-pupation, and after this time, the counts of Gammaproteobacteria and Firmicutes decline as the honey bee ages.

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6

Repellence and attraction of *Apis mellifera* foragers by nectar alkaloids

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Abstract

Plant secondary metabolites present naturally in nectar, such as alkaloids, may change the behavioural responses of floral visitors and affect pollination. Some studies have shown that nectar containing low concentrations of these secondary metabolites is preferred by honey bee foragers over pure nectar. However, it remains unclear whether this is caused by dependence or addictive behaviour, a simple taste preference, or by other conditions such as self-medication. In our choice experiment, free-flying bees were presented with artificial flowers holding 20% sucrose containing 0.5–50 μg/mL of one of the naturally occurring nectar alkaloids caffeine, nicotine, senecionine, and gelsemine. Nectar uptake was determined by weighing each flower and comparing the weight to that of the control flower. Our experimental design minimised memorising and marking; despite this, caffeine was significantly preferred at concentrations 0.5–2 μg/mL over control nectar; this preference was not observed for other alkaloids. All of the compounds tested were repellent at concentrations above 5 μg/mL. We confirmed previous reports that bees exhibit a preference for caffeine, and hypothesise that this is not due only to addictive behaviour but is at least partially mediated by taste preference. We observed no significant preference for nicotine or any other alkaloid.

Keywords: Nectar preference, caffeine, nicotine, senecionine, gelsemine

6.1 Introduction

A characteristic feature of higher plants is their capacity to synthesise a variety of organic molecules known as secondary metabolites, which can protect them against a wide variety of pests (Wink, 1988). Several adaptive hypotheses have been proposed to explain the ecological and evolutionary roles of secondary metabolite alkaloids in nectar. They may deter nectar robbers (Johnson et al., 2006), prevent microbial degradation of nectar (Herrera et al., 2009), enhance cross-pollination by encouraging pollinators to move more quickly between flowers (Adler, 2000; Kessler and Baldwin, 2007), permit insect self-medication (Baracchi et al., 2015; Gherman et al., 2014), or even enhance connections between plants and certain insect species by eliciting addictive behaviour (Renwick, 2001). The effect of alkaloids on bee colony fitness and mortality has been tested in several studies (Cook et al., 2013; Gegear et al., 2007; Köhler et al., 2012; Manson et al., 2013; Reinhard et al., 2009; Singaravelan et al., 2005), which suggest that alkaloids provide benefits to weak colonies under certain circumstances. Some studies show that bees prefer nicotine and caffeine in choice experiments, perhaps because they develop dependence to these compounds (Thomson et al., 2015). Despite the possible evolutionary and ecological implications, the concurrent effects of floral attractiveness and bee preference on pollinator visitation have not been widely studied. These studies are important because diet has a significant effect on pathogen infections in animals and the consumption of secondary metabolites can either enhance or mitigate the severity of infections (Manson et al., 2010).

The present study investigates the influence of secondary metabolites in floral nectar on nectar preferences in pollinators by measuring the preference of *Apis mellifera carnica* for differing concentrations of secondary metabolites that are known to be present in nectar (caffeine, senecionine, nicotine and gelsemine) in artificial flowers.

6.2 Materials and methods

The design of the experiment followed that of Gegear et al. (2007), with several modifications. For the behavioural assay, nectar (20% sucrose solution) containing nicotine, caffeine, gelsemine, and senecionine as a free base (Sigma-Aldrich, St. Louis, USA) was used in artificial flowers.

The artificial flowers were constructed by attaching 2.5 cm wide yellow cardboard rims to the mouths of 1.5 mL microcentrifuge tubes. These flowers were weighed, filled with nectar

solutions and placed in a spiral formation on a 70 × 70 cm green Styrofoam board. Two independent overlapping concentration sets (0–0.5–1–2–5.5 and 0–0.5–2–5.5–17–50 µg/mL) were tested and later pooled for statistical evaluation. Each compound was used in triplicate per set and each set was tested in five or six independent experimental replicates, resulting in n = 15, 18, or 33 for each data point. The experimental concentration range was thus 0.5–50 µg/mL. Control flowers contained only a 20% sucrose solution.

Each flower held 1.2 mL of nectar. The green board was placed 1 m from the entrance of an outdoor hive housed in a bee-proof flight enclosure (3 × 4 × 2.5 m). The hive was housed in this enclosure for 1 week prior to the experiment, and the *Apis mellifera carnica* were supplied with pollen and honey frames during this time. No natural sources of nectar or pollen were available to the bees. At the beginning of the experiment, the bees were stimulated by dusting approximately 300 mg of pollen over the green board. The approximate volume of the solution in the control flowers was monitored over the course of the experiment, and the experiment was terminated when this volume dropped below 500 µL (which took approximately 60–90 min). The difference in the weights of the artificial flowers before and after the experiment was used to calculate the volume of nectar that was removed by the bees.

Experimental replicates were conducted twice a day, in the morning and in the afternoon, in July 2013. Between each replicate, flowers were re-filled and their positions were newly randomised. No further data filtering was applied.

Statistical analysis was done using General linear models followed by Dunnett's (2-sided) post-hoc multiple comparison test using the IBM SPSS Statistics ver. 20.0 (IBM, Armonk, NY, USA).

6.3 Results

In the present study, honey bees preferred caffeine concentrations between 0.5–2 µg/mL, with up to 22% higher uptake from the flowers containing 2 µg/mL of nectar (121.7% ± 7.0% S.E.M, n = 33, P = 0.045) than from those of the control flowers. The other alkaloids tested did not show this effect, and the attraction of all lower concentrations of the alkaloids to foraging bees was comparable to that of the control flowers (Fig. 1). In concentrations higher than 5.5 µg/mL, all compounds were repellent ($P < 0.05$). Caffeine and nicotine were slightly better tolerated than gelsemine and senecionine, which showed more significant repellence

at 17 µg/mL. In the highest concentration tested (50 µg/mL), nectar uptake was approximately zero for all compounds.

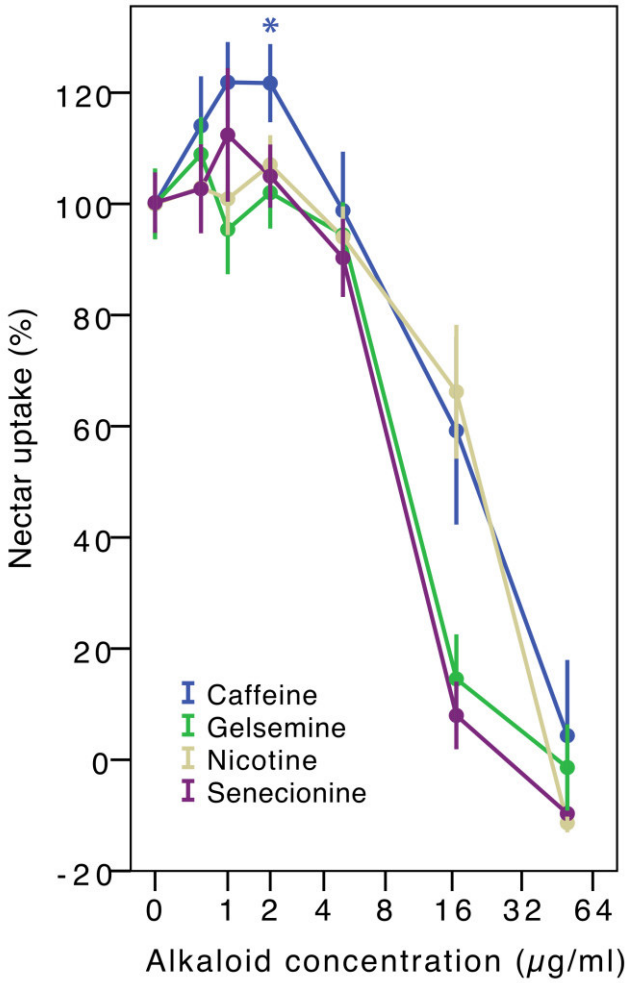

Fig. 1. Average uptake of artificial nectar containing alkaloids based on their concentration. Each point represents means of 15–33 independent replicates. Asterisk indicates statistically significant value from the control ($P < 0.05$).

6.4 Discussion

Singaravelan et al. (2005) found that bees preferred 25 ppm of caffeine in artificial nectar compared to sugar solution only, which reflects the amounts naturally present in nectar of citrus flowers (11.61–94.26 ppm). In the same study, the presence of nicotine in nectar (at concentrations of 0.5 and 1 ppm) also elicited a significant feeding preference. Bees have also

been shown to prefer nicotine derivatives, such as the neonicotinoids used for pest control (Kessler et al., 2015), which may have negatively affect their health.

We confirmed the preferential behaviour of bees towards caffeine but not towards nicotine. Moreover, neither of the two other alkaloids tested, senecionine and gelsemine, was preferred over the control. This shows that the preference of bees for caffeine (and for nicotine based on previous studies) is relatively specific for these alkaloids. This study differed from previous studies (such as Gegear et al., 2007) in the design and in the randomisation of the flowers; we also changed the flower rims after certain experimental sets to prevent the bees from forming associations between floral colour and position and nectar properties. This was done in order to reduce the number of addicted individuals, as addictive behaviour has been previously recognised in insects (Bainton et al., 2000; Schafer, 2004). Under our experimental conditions, preference was expressed not as an increase in the frequency of visits to a flower, but rather as an increase in feed intake per visit. Interestingly, in this experiment, the preference for caffeine observed at 10× lower concentrations than in the study by Singaravelan et al. (2005).

The results of this experiment support the theory that the long-term preference of caffeine and nicotine is based on addiction rather than unintentional self-medication (Baracchi et al., 2015; Gherman et al., 2014). Certain dietary elements appear to suppress the development of taste sensitivity to deterrents in insects, while the presence of specific stimulants in the diet may result in the development of dependence on these compounds (Renwick, 2001). Moreover, this suggests that taste preference depends on the presence of other compounds or concentrations simultaneously offered in nectars during experiments, or in surrounding forage. This is supported by studies in which simultaneous testing of different ranges of concentrations resulted in different preferential responses (Singaravelan et al., 2005).

To the best of our knowledge, this is the first time senecionine (as a free base) has been used in preference studies. According to our results, the presence of senecionine as a hazardous honey pollutant cannot be explained by the preferential behaviour of honey bees towards senecionine-containing flowers. Similarly, gelsemine solutions were neutral or repellent, in accordance with previous studies (Adler and Irwin, 2005; Gegear et al., 2007).

6.5 Conclusions

In conclusion, we tested preference for and repellence by four alkaloids in a nectar solution. We randomised the positions of flowers, which prevented the bees from memorising the position of the preferred nectar. Data suggest that honey bees prefer caffeine not only because it elicits addictive behaviour, but also because of a taste preference. In contrast with other studies, we did not observe a preference for nicotine-containing nectars.

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7

Plant alkaloid sanguinarine and novel potential probiotic strains *Lactobacillus apis*, *Lactobacillus melliventris* and *Gilliamella apicola* promotes 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, they are powerful tool to study insect immunity and used in biological control. Both mix of non-pathogenic bacteria and even nectar alkaloids were previously hypothesized to positively modulate honey bee health. We used EPNs for evaluation of the overall immune resistance of honey bee larvae treated with potentially immuno-modulating substances – a plant alkaloid sanguinarine and non-pathogenic strains of *Lactobacillus apis*, *L. melliventris* and *Gilliamella apicola*, native honey bee gut isolates. Honey bee L5 larvae 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 the 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 increased survivorship to 23.25±1.53 or 11.0±6.0 % for *S. feltiae* or *H. bacteriophora*. This is the first record that the nematobacterial infection was used for evaluation of immune status of beneficial insect. Both addition of low doses of sanguinarine and non-pathogenic strains of selected bacteria positively affected the honey bee resistance to pathogen. This method revealed to be a valuable tool for immunological tests in honey bees.

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

7.1 Introduction

Honey bees are used by human for several thousand years, but their immune system is still far from being fully understood. Moreover, we still don't have 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, 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 belong to social insect where 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 insect, e.g. well studied Lepidoptera or Diptera (Evans et al., 2006).

Honey bees can be naturally infected by 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. Entomopathogenic nematodes *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. So called infective juveniles (IJs; non-feeding parasitic larvae of EPNs) 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 (e.g. Ehlers, 2001). EPNs natural infection model is widely used in *Drosophila melanogaster* and *Galleria mellonella* research

to test their immunocompetence (Hallem et al., 2007, Hyrsl et al., 2010, Wang, 2010, Dobes et al., 2012, Arefin et al., 2013). In honey bees several studies showed their non-susceptibility to nematode infection under natural conditions (e.g. Kaya et al., 1982, Baur et al., 1995). In this study, our effort was to prove that also honey bee larvae and pupae under laboratory conditions can host nematobacterial complexes and exploit this interaction to 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, Audisio and Benitez-Ahrendts, 2011, Endo and Salminen, 2013, Forsgren et al., 2010, 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 increase in resistance, an effect referred 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 before (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,* and mix of three non-pathogenic species of probiotic bacteria previously isolated from honey bee gut.

7.2 Materials and methods

7.2.1 Honey bees and experimental design

Experimental beehives were arranged in apiary at Kyvalka near Brno, Czech Republic in two following 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 shaken off bees from 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, therefore experimental larvae were treated from eggs or early larval stage. Control group obtained sucrose syrup only. Brood combs with honey bee L5 – LS larvae and Pw pupae (partly sealed fifth instar in the age of 8^{th}-9^{th} day post eclosion and pupae white in accordance to Rembold et al., 1980) were collected and transferred vertically into laboratory at 25°C. 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). Whole experiment was repeated three times independently within two following years (control, sanguinarine and bacteria treated nuclei in first year and two another replicates of all three groups in second year); summary results are presented.

7.2.2 Sanguinarine and mix of non-pathogenic bacteria

Sanguinarine as powder extract of *Macleaya cordata* (Naturalin Bio-Resources Co., Ltd., China) was mixed with fructose syrup to obtain 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 30mM formic acid and acetonitril on a Phenomenex Gemini column (5 µm C18 110 Å, LC Column 250 x 4.6 mm), a slightly modified method previously published by Chen et al. 2009. Finally, concentrated stock solution was diluted to 1 g of sanguinarine per 1 liter of sucrose syrup. This dose was proved to be safe and effective for honey bees in 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 *Gilliamella apicola*, which was cultured in the same medium for 48 h. After that, 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 x 10^7 bacteria of *Lactobacillus melliventris* per mL, 2.2 x 10^8 *Lactobacillus apis* per mL and 1.4 x 10^6 *Gilliamella apicola* per mL.

7.2.3 Infection assay

Isolated honey bee larvae or pupae were collected on moist tissue paper. For each experimental nucleus the group of 20 individuals was collected and used for experimental infection. Nematodes *H. bacteriophora* (H222, isolated from Pouzdrany, Czech Republic) and *S. feltiae* (isolated from Prosenice, Czech Republic), were multiplied on *Galleria 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)

7.2.4 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.

7.3 Results

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

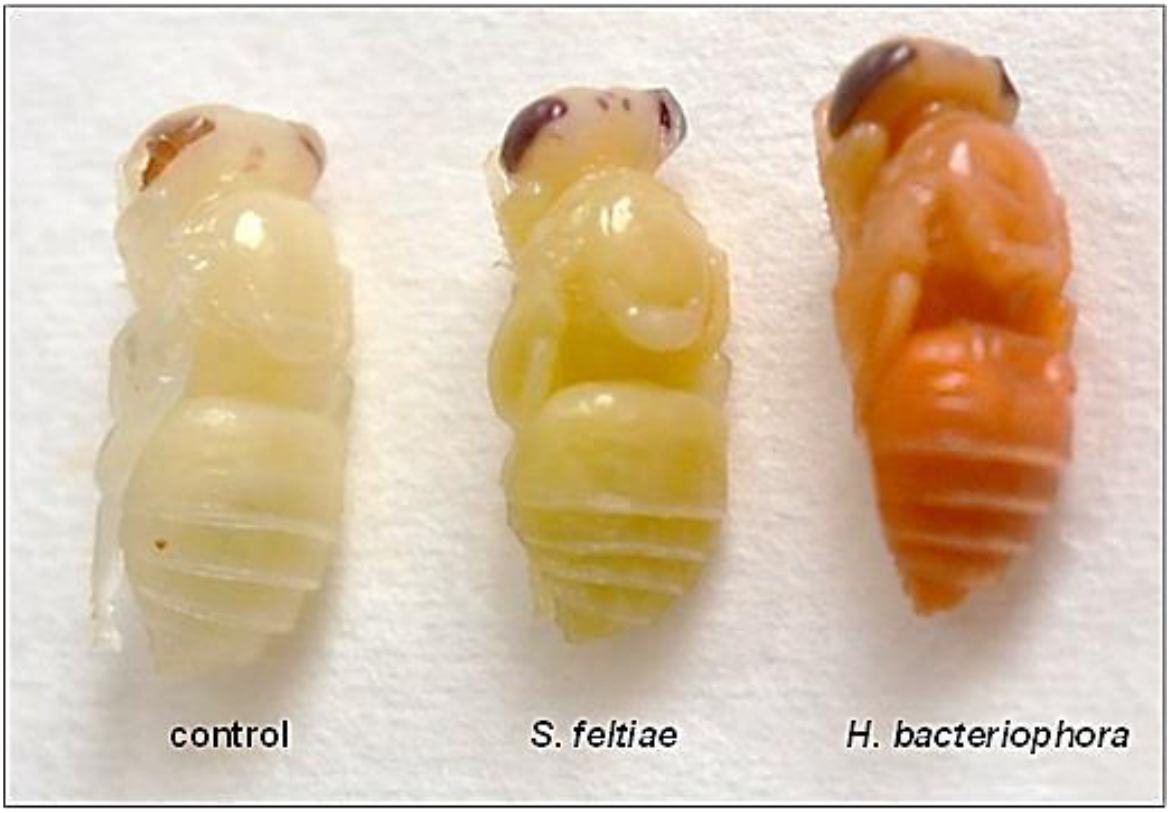

Fig. 1. Honey bee pupae 48 hours after infection by *Steinernema feltiae* (middle) or *Heterorhabditis bacteriophora* (right) with their typical coloration caused by their symbiotic bacteria *Xenorhabdus bovienii* or *Photorhabdus luminescens*. Typical coloration of honey bee pupae is visible at uninfected control pupa (left).

Fig. 2. Honey bee pupa infected by nematobacterial complex *Heterorhabditis–Photorhabdus*. New generation of IJs is released from cadaver after approx. 7 days.

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 (Fig. 3). To keep our model widely accessible and natural, we used wild-type *Heterorhabditis-Photorhabdus* complex in following experiments.

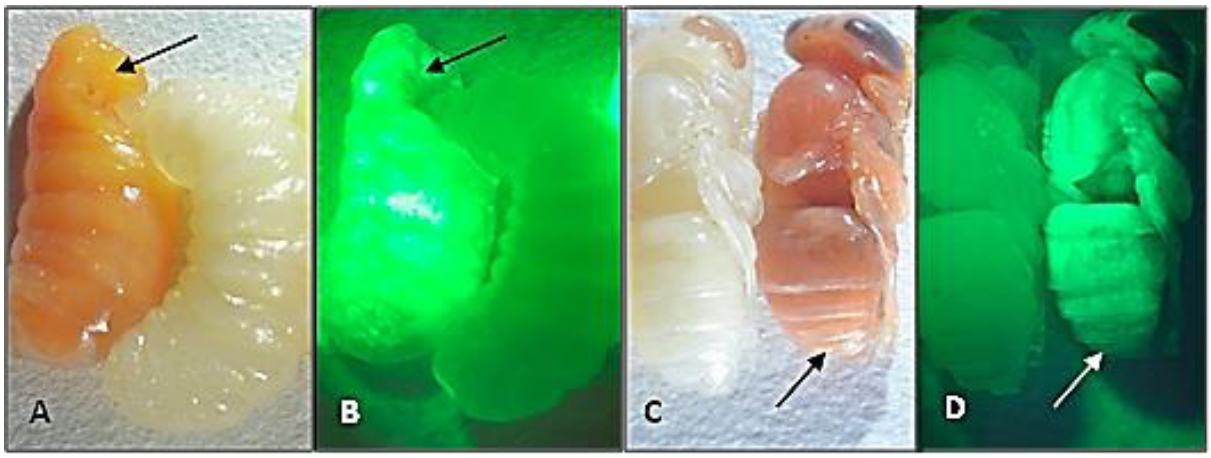

Fig. 3. To demonstrate the role of symbiotic bacteria of EPN, the natural symbiont *Photorhabdus luminescens* was replaced with GFP expressing strain. The bacteria are localized in the gut of IJs and cause septicemia after release into the insect hemocoel. Pictures shows uninfected and infected (arrows)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

Dose dependence of mortality on number of IJs per honey bee larva or pupa was clearly demonstrated as shown on Fig. 4 A, B. 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 following experiments.

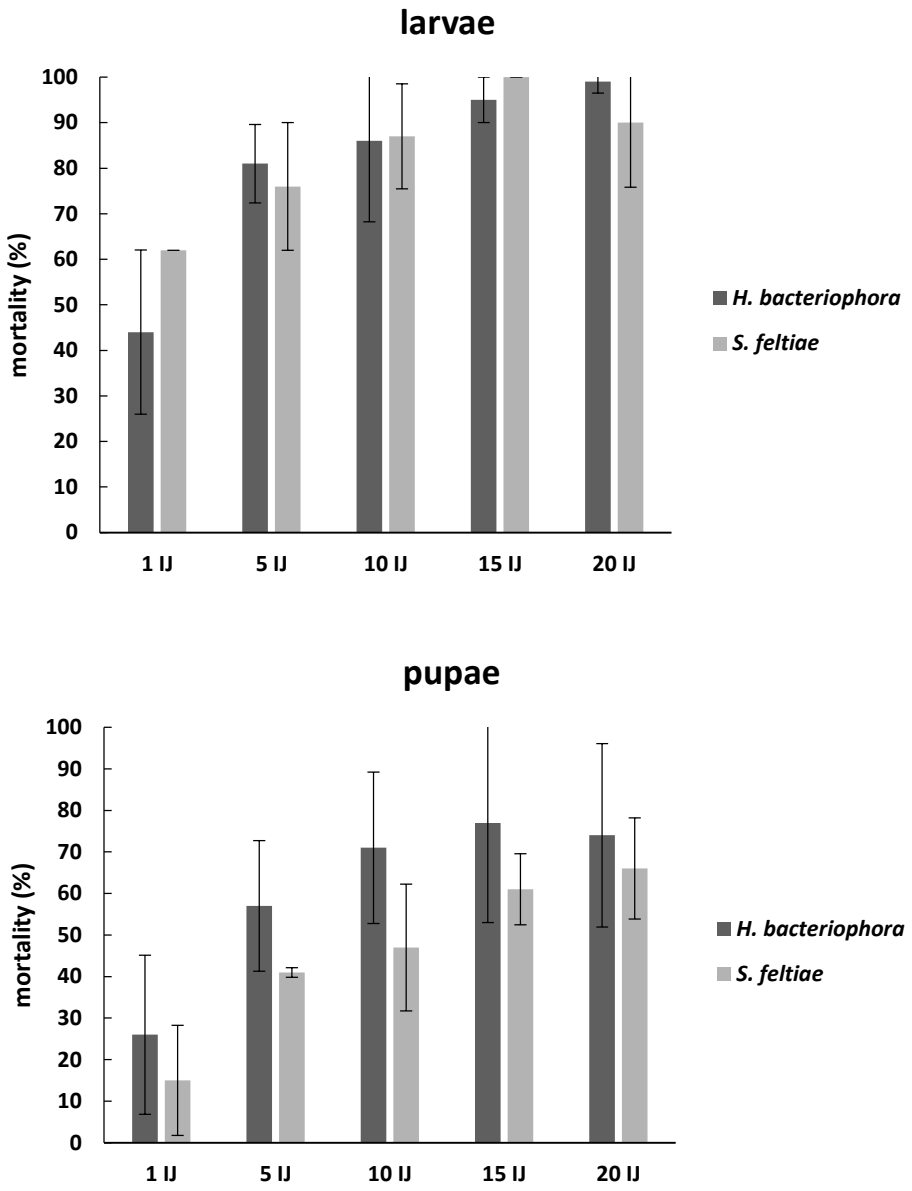

Fig. 4. Mortality of honey bee larvae (A) and pupae (B) 48 hours after infection is dependent on dose of IJs used for infection. Honey bees were infected with nematobacterial complex *Heterorhabditis–Photorhabdus* and *Steinernema-Xenorhabdus* (mean ± SD). Significant differences are indicated by different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

Application of selected non-pathogenic bacteria and sanguinarine led to the better survival of honey bee larvae after nematobacterial infection compared to control, Fig. 5. 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.

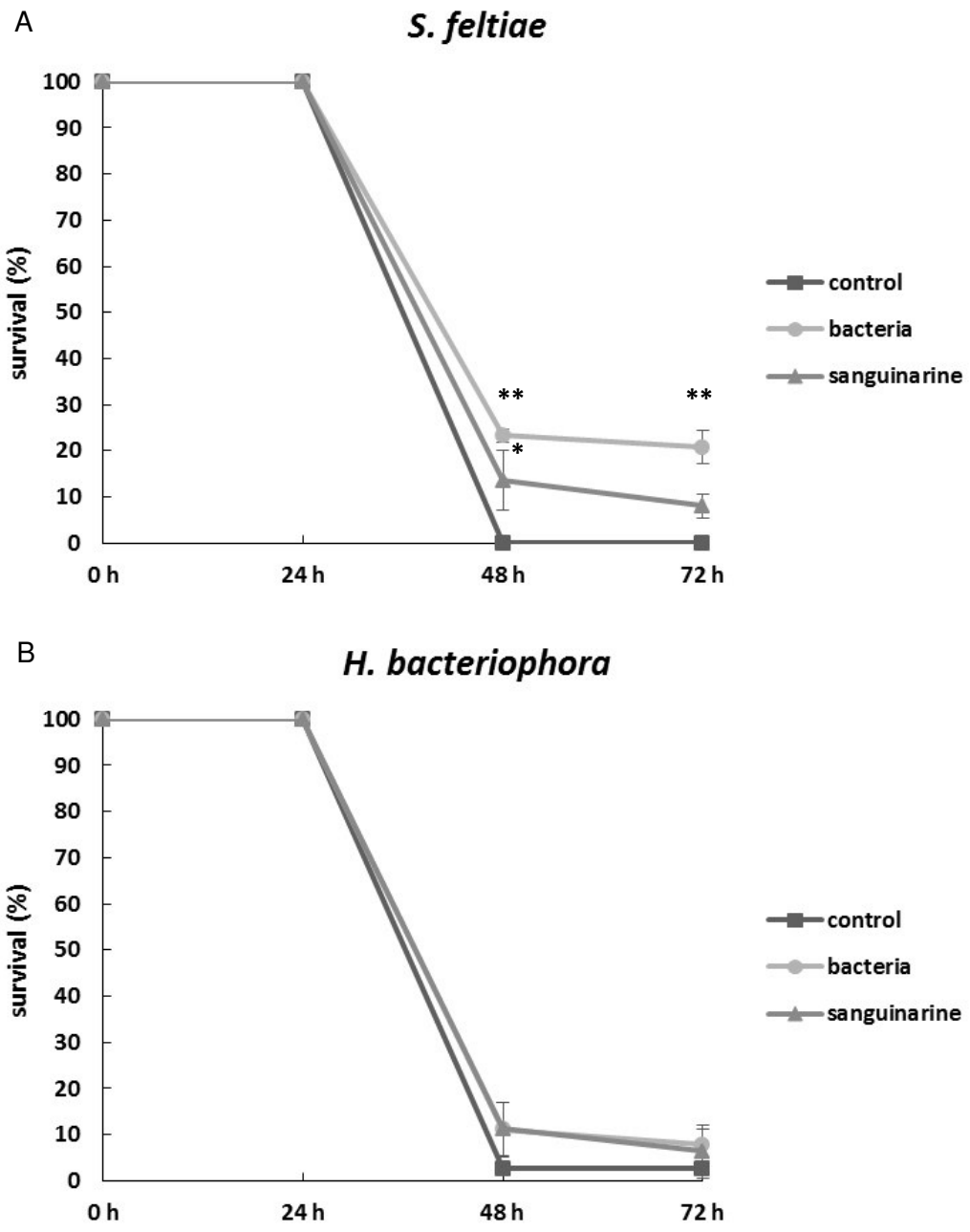

Fig. 5. Immuno-stimulating effect of plant alkaloid sanguinarine and mix of non-pathogenic bacteria (*Lactobacillus apis*, *Lactobacillus melliventris, Gilliamella apicola*) on honey bee larvae infected with 10 IJs of *S. feltiae* (A) and *H. bacteriophora* (B) per larva. Data are expressed as percentage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

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.

7.4 Discussion

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 basis for their immunocompetence is quality and diversity of nutrients available for honey bees (Alaux et al., 2010). However, 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 (e.g. Porrini et al., 2014, Pistorius et al., 2015). As social immunity is present in honey bee colonies, we have to think also about the effect of potential immunostimulant on social behaviour and chemical communication inside the hive (Richard et al., 2012).

Plant *Macleaya cordata* (Papaveraceae) is traditionally used in Chinese medicine. It contains several isoquinolone alkaloids and sanguinarine and chelerythrine are considered to be responsible for plant´s pharmacological effects (Zdarilova et al., 2008). 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 slight 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 as promising tool to improve honey bee health were reviewed by Crotti et al. (2013). One part of our aims was to test the role of naturally occurring non-pathogenic bacteria from digestive tract of honey bees in 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 *Paenibacillus larvae* and *Melissococcus plutonius* (Evans and Lopez, 2004, Audisio and Benitez-Ahrendts, 2011, Endo and Salminen, 2013). 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 *Paenibacillus 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, 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 increase 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 number of proteases and virulence factors affecting invaded insect (Hao et al., 2012). We used EPNs infection to study immunity of *Drosophila melanogaster* in our previous studies (Hyrsl et al., 2010, Dobes et al., 2012, Arefin et al., 2013) and here we show that also honey bee larvae and pupae *in vitro* conditions are suitable hosts for nematobacterial complex. We optimized the natural infection of honey bee larvae and pupae for EPNs species *Heterorhabditis bacteriophora* and *Steinernema feltiae*. There is only single research recorded for *in vitro* infections of honey bee larvae by *S. feltiae* and *S. affinis* (Zoltowska et al., 2003a, b). 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 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 *Galleria 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 entering side 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.

7.5 Conclusions

In summary, we observed supportive effect on survival rate after EPNs infection of honey bee larvae after 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.

7.6 Acknowledgements

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8

In-hive variation of microbial composition of honey bee larvae and pupae of the same oviposition time

Unpublished data

Researcher's Contributions

Conceived and designed the experiments: J. Havlik, **Z. Hroncova**, J. Killer. Performed the experiments: **Z. Hroncova**, J. Killer. Analysed the data: J. Havlik, **Z. Hroncova**, J. Killer. Contributed reagents/materials/analysis tools: Z. Hroncova, J. Killer. Wrote the paper: J. Havlik, **Z. Hroncova**, J. Killer. A statistical experiment has carried out under the supervision of: J. Havlik, **Z. Hroncova**.

Abstract

A detailed knowledge of bacterial colonization of honey bee gut, persistence, transmission and overall community function is needed if strategy of supplementation a microbial community with beneficial microbes (probiotics) is adopted. We have focused on the fifth instar because this stage is the target of many major pathogens and information about microbial variation is lacking, particularly for larval and pupal stages. The aim of this study was to raise awareness about the variations in microbiota composition among individual larvae and pupae; and compared microbiota of these two developmental stages within one hive using the denaturing gradient gel electrophoresis profiling and quantitative (real-time) PCR. Collected specimens were 6-days-old larvae (5^{th} instar) and black pupae, which have been super sisters (sharing both a queen mother and a drone father) or half-sisters (sharing only a mother). Both of used methods revealed effect of ontogenetic stage of the individual on gut microbial composition and indicate that all tested bees contained same bacteria but frequencies vary considerably. In the majority of individuals in 5^{th} instar Firmicutes were the predominant. After larval defecation and during pupation, Firmicutes decreased and being outweighed by Gammaproteobacteria. Also Bacteroidetes prevailed over Actinobacteria but with less inter-individual difference than in the guts of 5^{th} instar larvae. We can conclude presence of the same phylotypes in individual bees, and their presence in honey bees worldwide support the hypothesis that these bacteria have central functions in bees and variation in gut microbiota has been implicated in the health of humans and other animals.

Keywords: Honey bee, 5^{th} instar, Black pupae, Gut microbiota, Bacterial groups, Frequency

8.1 Introduction

Bees contribute significantly to agricultural productivity and profitability. Over the past several years, governments, beekeepers, and the general public in the United States and Europe have become concerned by increased losses of bee colonies, calling for more research on how to keep colonies healthy while still employing them extensively in agriculture (Rangberg et al., 2012). Nowadays research is focused on microbiota of digestive tract which is speculated to play a role in honey bee health (Wu et al., 2014, Killer et al., 2013, Koch and Schmid-Hempel, 2012, Mattila et al., 2012, Forsgren et al., 2010, Vasquez et al., 2012). Researchers are experimenting with many substances to promote honey bee microbial health, including applications of bacteria cocktails (Koch and Schmid-Hempel, 2011, Forsgren et al., 2010). This approach has merit in the sense that probiotics should be considered from the point of view of community ecology. It is typically the balanced nature of a microbial community that provides maximum benefits to the host. Bacterial spectrum of honey bees is affected by interactions between individuals that comprise the colony, the hive and the pollination environment, activities related to age, diet and developmental stage (Martinson et al., 2012). The hive itself acts as a microbial buffer from the external landscape as evidenced by the existence of a characteristic hive microbiota distinct from both the hindgut microbiota and microbes of the pollination environment (Anderson et al., 2013).

In social index, individuals are orally sharing of food (trophallaxis), which is generally perceived as a factor leading to a homogenisation of microbial profiles between individuals of one colony (Koch and Schmid-Hempel, 2011, Martinson et al., 2011, Koch et al., 2012, Martinson et al., 2012, Koch et al., 2013, Colman et al., 2012, Nixon and Ribbands, 1952). Minor variations of bacterial spectrum within the same colony could be a reflection of health status or short-term differences in the physiology or ontogenetic stage of individual bees (Moran et al., 2012). This homogeneity is often presumed as a fact when designing research studies and considering the numbers of individuals for sampling.

Microbial spectrum changes during the ontogenesis of the insects and honey bee and bumble bee larvae exhibit different bacterial profiles than adult bees (Mohr and Tebbe, 2006) because unlike the compartmentalised nature of the adult honey bee gut, developing larvae possess only a midgut, which connects with the hindgut at the pre-pupae stage (Winston, 1991). Larval midgut represents a unique niche for bacterial or fungal growth, and this stage of the honey bee life cycle is the target of many major pathogens including bacterial diseases

European and American foulbrood, and fungal diseases stonebrood and chalkbrood (Bailey and Ball, 1991, Seeley, 1995, Vojvodic et al., 2010, Rauch et al., 2009). Fifth instar larvae has up to 7 bacterial clades but there is not clear evidence of which group dominates and what the main drivers of this balance are. Recent research suggests Firmicutes are the most prevalent taxon in this group (Vojvodic et al., 2013), while a previous study (Cox-Foster et al., 2007) showed dominance of Gammaproteobacteria. It appears that after defecation and during pupation, bacterial counts decrease and low counts of Gammaproteobacteria persist in the gut, which might serve as a proxy for later bacterial colonisation after morphogenesis (Hroncova et al., 2015) but other studies suggest the gut is devoid of microbiota and are reinoculated after they emerge (Martinson et al., 2012).

Specific knowledge regarding the dynamics and variation in the larval gut microbiome is of importance from two main reasons: at first to build robust study protocols; and second, larvae are considered to be a focus for probiotic applications and in aiding defence against pathogens and colony health (Evans and Lopez, 2004, Yoshiyama and Kimura, 2009). Despite the recent literature on the microbiota of adult honey bees, there is very little and inconsistent information on the microbial communities in honey bee larvae and pupae. The aim of this study was to raise awareness about the variations in microbiota composition among individual larvae and pupae. In our study, we compared microbiota of two developmental stages (6-day-old larvae, black pupae) of *A. mellifera carnica* in individuals within one hive using the denaturing gel electrophoresis profiling (DGGE) and qRT-PCR.

8.2 Materials and Methods

8.2.1 Honey bee samples

Honey bees of two developmental stages (6-days-old larvae and black pupae) of *A. mellifera carnica* were sampled from one single hive at Dol, CZ (50°12'23.9"N 14°21'58.8"E) location. Genetic background of individuals was not reviewed, thus they might have been super sisters (sharing both a queen mother and a drone father) with coefficient of genetic relationship of 0.75; or half-sisters (sharing only a mother) and having a genetic relationship of 0.25 (Page et al., 1989, Page and Laidlaw, 1988). Sampling was conducted on 31 July 2012. Bee management and samples represented traditional beekeeping practices in the Czech Republic. Hereby, we certify that the samples were collected on either private land (land of Bee Research Institute Dol); we solely hold responsibility for ethical approaches and can be

contacted later for confirmation. The field studies did not involve endangered or protected species.

Collected specimens were 6-days-old larvae and black pupae. The 6-days-old larvae corresponded to the 5th instar, with the gut completely filled with a yellowish material and corresponding to the last feeding stage LF3 (Soares, 1993, Rembold, 1980) prior to sealing. Black pupae were acquired when they showed medium thorax pigmentation. Individuals were collected in disposable tubes and frozen immediately on dry ice. Entire tube-like digestive tracts (crop, midgut, ileum and rectum) were removed from each honey bee and weighed. Approximately 50 mg of digestive tract was used individually for isolation of total bacterial DNA using the ZR Faecal DNA MiniPrep kit (Zymo Research, Irvine, CA, USA).

8.2.2 Real-time PCR analysis

Quantification of bacterial DNA was conducted using the MX3005P thermocycler (Stratagene, La Jolla, CA, USA) with Gammaproteobacteria (1080γF, γ1202R), Bacteroidetes (798cfbF, cfb967R), Firmicutes (928F-Firm, 1040FirmR) and Actinobacteria (Act920F3, Act 1200R) (De Gregoris et al., 2011). For analysis and visualisation of qRT-PCR data, we used IBM SPSS Statistics ver. 20 (IBM, Armonk, NY, USA).

8.2.3 Denaturing gradient gel electrophoresis and sequencing

Amplification of the total bacterial community was conducted by targeting 200 bp partial 16S rRNA gene sequences with the universal bacterial primers 338GC and RP534 under previously described conditions (Mrazek et al., 2008). PCR products were analysed on a DGGE gel (gradient from 35–65%) according to the method of Mrazek et al. (2008).

Appropriate standards containing a mixture of PCR products of 5 known microorganisms were loaded in the centre of gels defined to minimize gel variability and used for multi-gel comparison was conducted using BioNumerics 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). Lanes were manually aligned and band positions were identified from corrected intensity plots. Band matching was accomplished by using the following BioNumerics settings: 7% minimum profiling, 0% gray zone, 0% minimum area, and 0 shoulder sensitivity.

Two to three DNA bands with the same normalised Rf values were cut out of the polyacrylamide gel using a sterile scalpel blade to confirm their correct alignment. Bacterial species were detected using the primers FP341 and RP534. The bands of interest were

sequenced on a 3100 Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA) in the Centre of DNA Sequencing (Institute of Microbiology of the ASCR, v. v. i.). The resulting sequences were compared with the GenBank database using the BLAST algorithm.

8.3 Results

Our results indicate that all tested bees contained same groups of bacteria and as noted, frequencies vary considerably. Total unseparated guts of individual bees in both morphogenetic stages of 5th larval instar (LF3) and black pupae (PB) were evaluated for the presence of including Firmicutes (Firm), Gammaproteobacteria (Gamma), Actinobacteria (Act) and Bacteroidetes (Bct). In the majority of individuals in 5th instar (70%) (Fig. 1.), Firmicutes were the predominant group while Gammaproteobacteria prevailed in gut of three out of ten larvae.

In samples 1, 3, 8 and 10, more than 98% of the bacterial population were of the Firm clade. Group of Actinobacteria was the most abundant in digestive tract of one sample (7). Individuals 2 and 9 hosted 17-20% of Bacteroidetes species, significantly more than others. After larval defecation and during pupation, Firmicutes decreased to 40% of the original bacterial counts, being outweighed by Gammaproteobacteria which corresponded to 60% of the population. Two black pupae 17 and 20 were predominantly colonized by Gamma (90%), while Firm clade was the major component in individuals 11, 14 and 19 (94-99%).

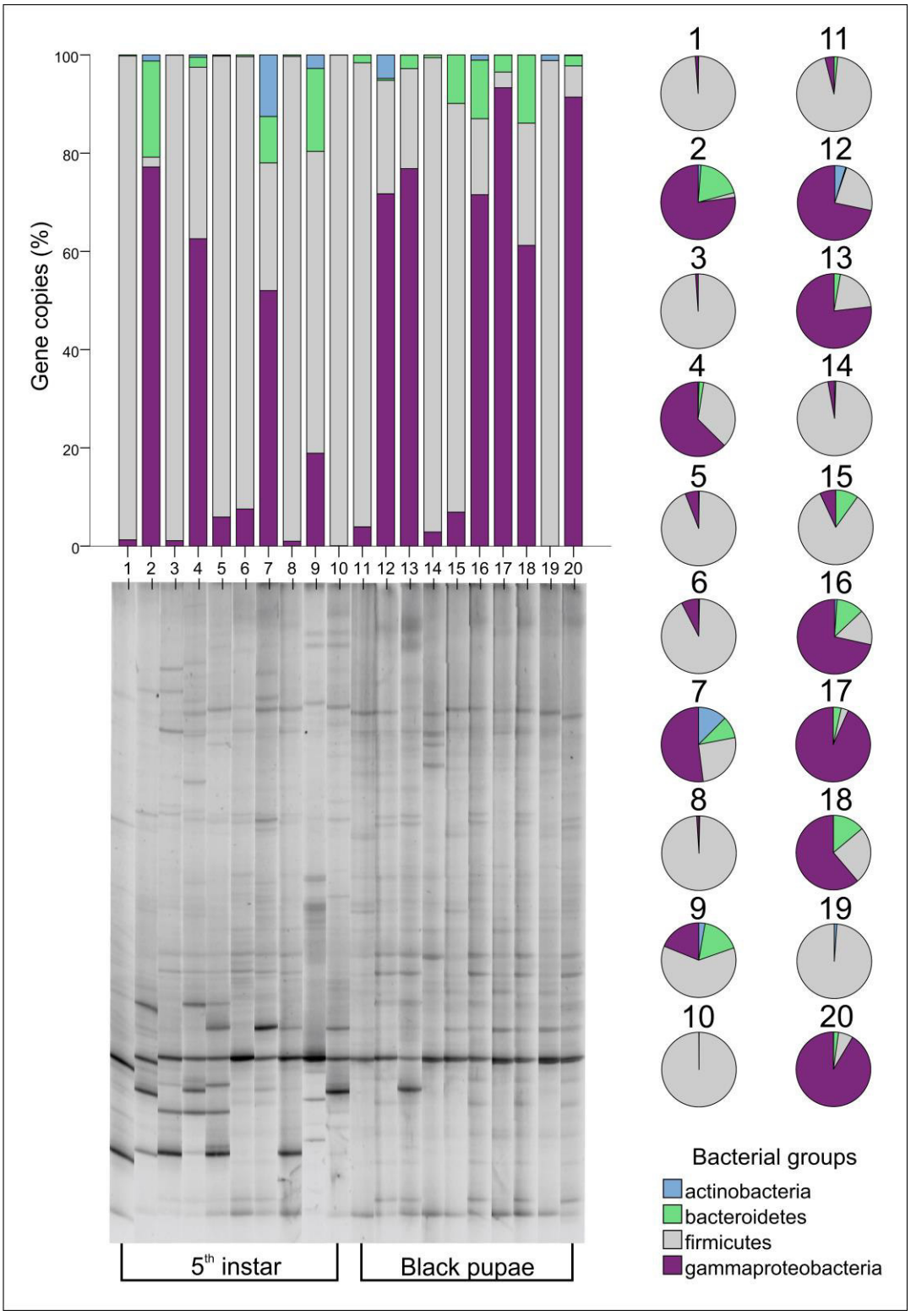

Fig. 1. Charts of quantitative real-time PCR (qRT-PCR) data of the abundance of selected bacterial groups in samples of total gastrointestinal tract of individual honey bee in different ontogenetic stages. The Y axis shows copies of the 16S rRNA gene per gram of honey bee gastrointestinal tract scaled to 100%. Graphs show data from individuals in different ontogenetic stages (5th instar and black pupae).

Our study revealed effect of individual's age or morphogenetic stage of the individual on gut microbial composition. In the digestive tract of 5th instar larvae Firm significantly dominated over Gamma in nearly 2 orders in magnitude (Firm: 1.8×10^6 vs. Gamma: 4.0×10^4; the means of gene copies per gram of digestive tract content) and Bacteroidetes prevailed over Actinobacteria (Bct: 3.3×10^3 vs. Act: 3.6×10^2) (Fig. 2).

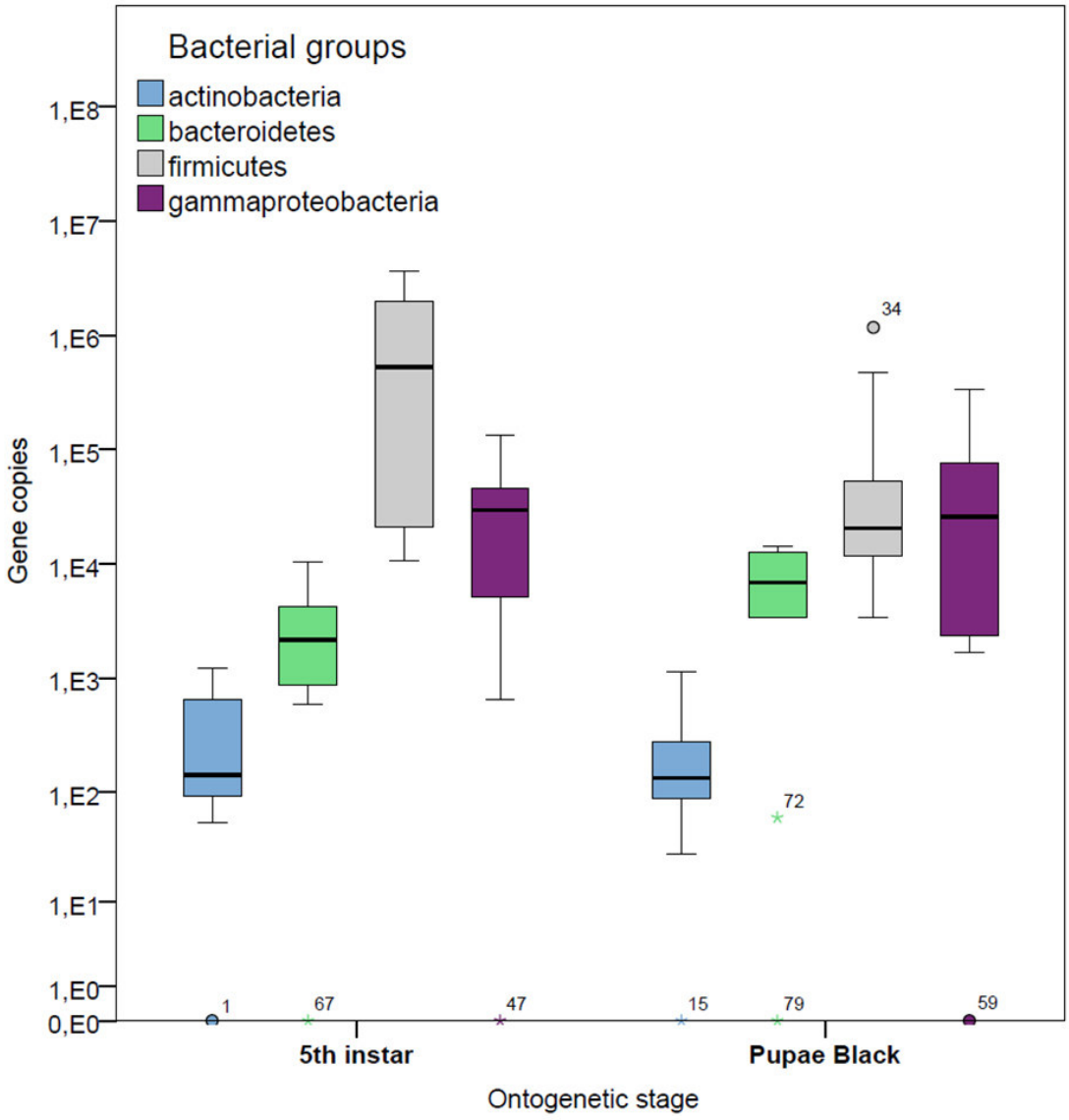

Fig. 2. Boxplot of quantitative real-time PCR (qRT-PCR) data of the abundance of selected bacterial groups averaged from samples of total gastrointestinal tract of individual honey bee in different ontogenetic stages. The Y axis shows log-transformed copies of the 16S rRNA gene per gram of honey bee gastrointestinal tract. Boxes show averaged data from individuals in different ontogenetic stages (5th instar and black pupae).

In black pupae, ratio of both groups was balanced (Firm: 1.8×10^5 vs. Gamma: 9.5×10^4; the means of gene copies per gram of digestive tract content) and Bacteroidetes prevailed over Actinobacteria (Bct: 6.9×10^3 vs. Act: 2.8×10^2) but with less inter-individual difference than in the guts of 5th instar larvae.

8.4 Discussion

A detailed knowledge of bacterial colonization, persistence, transmission and overall community function is needed if strategy of supplementation a microbial community with beneficial microbes (probiotics) is adopted. Our results indicate that ontogenetic stage of honey bee is important factor causing changes in gut microbiota. We have focused on the fifth instar because developing larvae have a discontinuous gut (the foregut is not connected to the hindgut) (Winston, 1991) and thus, this stage is the target of many major pathogens and infected larvae begin to die from 4th to 6th instar (Bailey and Ball, 1991, Rauch et al., 2009, Blanchard et al., 2014, Guo et al., 2015). In addition, the fifth larval instar harboured more diverse microbiota in A. mellifera than later stages (Mohr and Tebbe, 2006, Hroncova et al., 2015). Such differences may be associated with changes in nutrition and metabolism. Appropriate microbial composition in this instar might be crucial in later development; research suggests that well-established community within the gut may provide exclusionary effects against potential pathogens, either in terms of space-exclusion or nutrient competition (Martinson et al., 2012). Exclusionary effects have been previously documented, correlating the presence of Bifidobacteria and other LAB strains with the absence of the pathogens *Melissococcus plutonius* and *Paenibacillus larvae* respectively (Olofsson and Vasquez, 2008, Mattila et al., 2012). Also newly described *Parasaccharibacter apium* increased Nosema resistance in larvae (Corby-Harris et al., 2016). As suggested by Cremer et al. (2007) also adult-processed foods could be altered to inhibit microbial growth or enriched for a certain subset of nonpathogenic/probiotic microbes, insulating the young from opportunistic pathogens (Cremer et al., 2007). This may be especially important for honey bee, since many of its most destructive diseases attack brood (fungal, bacterial, arachnid, and protozoan (Schmid-Hempel, 1998, Shimanuki and Knox, 2000), but still leads to considerable variations between individuals because there are many fringe environments and interaction networks within the hive and colony that may support bacteria (Powell et al., 2014). However, the distribution of the roles within the hive such as foraging for food and nest material, nest defence or food storing (Wilson, 1971, Bonabeau et al., 1997) gave the grounds to the idea that honey bees share the same microbiota and microbial diversity is low (Moran et al., 2012,

Gilliam, 1997, Evans et al., 2006, Gilliam and Valentine, 1976, Gilliam and Morton, 1978, Gilliam et al., 1990, Piccini et al., 2004, Mohr and Tebbe, 2006, Evans and Armstrong, 2006). But this might be not the case. These interactions may lead to an accumulation of bacterial species from hive materials in their blind guts, as well as of some species that are usually found in the guts of adult bees; however, both the composition and abundance of this larval gut microbiota seems erratic (Martinson et al., 2012, Ahn et al., 2012, Hroncova et al., 2015, Mohr and Tebbe, 2006, Vojvodic et al., 2013). Nonetheless, even workers of the same age in a colony can harbour very different proportions of the core species of bacteria in the gut (Moran et al., 2012, Powell et al., 2014, Kapheim et al., 2015). Information on such interactions and inter-and intra-colony microbial variation is lacking, particularly for pupal stages. In agreement with our previous results (Hroncova et al., 2015), we confirmed that after larval defecation and during pupation, bacterial counts decreased significantly; however, the dominating bacterial group was Gammaproteobacteria. Also from these results is evident that colonies are also undergo age-related or seasonal shifts in the relative proportions of the core species of bacteria (Ludvigsen, 2013, Hroncova et al., 2015). The extent to which these shifts are specific to particular geographic regions or environmental conditions is unclear, partly because it is not possible to directly compare community profiles that are generated by different laboratories using different protocols. Due to honey bee gut microbiota which acts as forerunner for studying gut of higher animals design of experiments have be uniform. The most studies have relied on pooled samples from several bees and as note, digestive tract of individual bees harbours different frequency of bacterial groups. Also diversity of strain or rare phylotypes is expected to be missed by most studies to date, given the limited design of experiment or pooled samples. These differences in abundance and prevalence between bacterial species are probably due to their each having highly specialized localizations or metabolic niches in the gut, as is common in other animal microbiomes (Donaldson et al., 2016). It means every animal is unique and individual microbiome of every bee is important in creating a design experiment, mainly in sampling, where one pool sample would be from 10 individual bees. Bees provide an excellent case for studying the forces that influence the assembly and composition of the microbiome. As an emerging system with extensive similarities to human and other mammalian microbiomes, studies of the gut microbial community in bees are poised for rapid breakthroughs in the near future.

8.5 Conclusion

Recent studies show that social behaviours of honey bees create consistent associations of bacteria in digestive tract. However, our study revealed several interesting insights into the diversity of honey bee microbial groups. In agreement with our expectations was observed different frequencies of bacterial phylum in bee sisters' guts.

The presence of the same phylotypes in individual bees, and their presence in honey bees worldwide support the hypothesis that these bacteria have central functions in bees. If so, variation in gut bacteria, including possible functional differences among bacterial classes, may be an important factor in honey bee biology and colony health, just as variation in gut microbiota has been implicated in the health of humans and other animals.

8.6 Acknowledgements

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9

Summary

The research presented in this thesis concerns the complex mechanisms of bees and wasps immune system focused on microbiota as component of immunity. As shown in the introduction, highly social and managed species of bees like honey bees and bumble bees, play key roles in natural and agricultural ecosystems worldwide. Recent losses of bees have been attributed to pesticide exposure, poor nutrition, increased parasite loads and habitat degradation. Over the past several years, governments, beekeepers, and the general public worldwide have become concerned by increased losses of honey bee colonies, calling for more research on how to keep colonies healthy. Our main aim was to explore the complex mechanisms of bees and wasps' immunity and the possibility of increasing the immune response focusing on microbiota and dietary supplements.

The first aim of this thesis was to characterise the wasp and bumble bee gut microbiota using 16S RNA sequencing in a search for potentially novel bacterial species. Chapters 3 and 4 are focused on solve this goal. *Vespula germanica* has not characteristic gut bacterial profile what shows a great variety of bacterial profiles, and the fact that *V. germanica* is not dependent on a particular mutualistic microflora for its nutrition. A number of bacterial strains appeared widespread, but community composition varied between nests (Reeson et al., 2003). Furthermore, the comparison with other bees and wasps suggests that changes in social lifestyle may have had a stronger effect on the evolution of the gut microbiota than the dietary shift from predatory ancestors to pollen feeding (i.e. herbivorous) species (Kaltenpoth, 2011). In chapter 3 we describe three novel strains of bacteria isolated from a wasp (*Vespula vulgaris*) and representing a novel species of the genus *Vagococcus*; the strains were distinct from all other species of bacteria isolated from the digestive tracts of insects. The results of a wide range of genotypic, phenotypic and phylogenetic analyses demonstrated that the bacterial strains VOSTP2^{T}, VOSTP5 and VOSTP6 represent a novel species for which the name *Vagococcus entomophilus* sp. nov. is proposed.

Isolation and detailed characterisation of a new representative of the genus *Lactobacillus* occurring in the digestive tract of bumblebees is presented in chapter 4. Our tested bumble bees of *Bombus terrestris* species were bred in the laboratory of the Agricultural Research, Ltd. (Troubsko, Czech Republic) in 2012. Recent studies confirmed that gut microbiota of wild and indoor-reared *Bombus terrestris* contain a core set of bacteria, which consisted of Neisseriaceae (*Snodgrassella*), Orbaceae (*Gilliamella*), Lactobacillaceae (*Lactobacillus*), and Bifidobacteriaceae (*Bifidobacterium*). In wild *B. terrestris* were detected several non-core bacteria having a more variable prevalence (Meeus et al., 2015, Parmentier et al., 2016). Our

new described species (*Lactobacillus bombi*) is a representative of the genus *Lactobacillus* belonging to core set of bacteria. This pattern is similar to honey bees' microbiota, which characterisation was our second aim. Chapter 5 also deal effect of ontogenetic stage, age and geographic location of bees and their impact on the development of microbiota (third aim). Insects such as the honey bee possess a relatively simple digestive tract, suggesting a much less complex microbiota (Martinson et al., 2011) but the honey bee microbiota exists at two major levels; within the relatively simple alimentary tract, and throughout the extended organism of the hive that houses the developing young and food stores. Our research was focused on gut microbiota and we revealed several interesting insights into the dynamics of honey bee microbial communities. First instar larvae generally showed very low bacterial counts, which is in agreement with previous studies (Forsgren et al., 2010, Vasquez et al., 2012, Vojvodic et al., 2013, Gilliam, 1971). Whereas the Gammaproteobacteria (Gamma) group was slightly more abundant in this ontogenetic stage with 3.3×10^5 gene copies/g, the counts of Firmicutes (Firm) were 1.3×10^5 copies/g. Major changes were observed in 5^{th} instar larvae collected shortly after the last feeding. The Firm group averaged 2.5×10^8 and Gamma 1.9×10^7 gene copies per gram of digestive tract content. DGGE fingerprinting revealed a decrease in *Snodgrassella alvi* and increase in the diversity of all *Lactobacillus* sp. and *Gilliamella* strains. After pupation, the counts of both Firmicutes and *Gilliamella* spp. decreased by nearly 2 orders in magnitude to means of 8.5×10^6 and 7.2×10^5 gene copies/g, after which growth continued slowly. The profile and bacterial counts of drones and young bees were very similar. No characteristic pattern was observed for any of the locations. At each location, all 3 hives appeared to be very different from each other and, rather than location, simple variability between hives was important. Also other sequence based experiments examining the adult honey bee alimentary tract suggest a relatively simple and stable bacterial flora regardless of geography (Jeyaprakash et al., 2003, Mohr and Tebbe, 2006, Cox-Foster et al., 2007, Hroncova et al., 2015) but culture-based results revealed incredible microbial diversity in the extended hive environment (Gilliam, 1997). This microbial community from the gut of the adult honey bee is composed primarily of 8 bacterial groups from five major bacterial classes that account for over 95% of the 16S rRNA sequences (Martinson et al., 2011).

Last fourth aim of this thesis was to determined changes in honey bee microbiota under different management conditions and dietary supplements. Part of our research presented in chapter 7 used entomopathogenic nematodes (EPNs) for evaluation of the overall immune resistance of honey bee larvae treated with potentially immuno-modulating substances - a

plant alkaloid sanguinarine and non-pathogenic strains of *Lactobacillus apis, L. melliventris* and *Gilliamella apicola*, native honey bee gut isolates. We observed supportive effect on survival rate after EPNs infection of honey bee larvae after application of sanguinarine and non-pathogenic bacteria, which can act as a novel potential probiotic. We focused on larvae because this stage of the honey bee life cycle is the target of many major pathogens including European and American foulbrood, stonebrood and chalkbrood (Bailey and Ball, 1991, Seeley, 1995, Vojvodic et al., 2010, Rauch et al., 2009). Specific knowledge regarding the dynamics and variation in the larval gut microbiome is of importance from two main reasons: at first larvae are considered to be a focus for probiotic applications and in aiding defence against pathogens and colony health (chapter 7); and second to build robust study protocols (Evans and Lopez, 2004, Yoshiyama and Kimura, 2009), what we also focused on (chapter 8 - unpublished results).

8.8 Conclusion and future perspectives

In conclusion, the new results presented in this thesis have led to explore the complex mechanisms of bees and wasps immunity and the possibility of increasing the immune response. However, what protects bees against pathogens and chemicals in the natural environment? Our research is focused on gut microbiota as component of immune system of bees. Bees harbour well-defined bacterial communities in their guts. We have tried to explain how microbiome interacts with the host and showed that major members of these communities appear to benefit the host. The simple gut communities of social bees present ideal model systems to investigate the underlying evolutionary and genetic processes of such interactions. Information based on our results may help in the design of proper probiotic supplementation strategies with respect to physiological conditions in the honey bee gut.

Future studies will elucidate the precise relationship between bacteria and bees, determining the bacterial factors involved and the impact of the interaction on bee health and gut homeostasis. Also, gene functions linked to host interaction, biofilm formation, or secretion are good candidates for involvement in protective mechanisms. By modulating the immune system of the host, some of these functions probably have an indirect role in defence and hence, they will be object of examination in our future research.

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List of Abbreviations

ABPV	Acute Bee Paralysis Virus
Act	Actinobacteria
AFB	American Foulbrood
Alpha	Alphaproteobacteria
API ZYM	a Simple Rapid System for the Detection of Bacterial Enzymes
Bct	Bacteroidetes
Beta	Betaproteobacteria
BF	Forage Bees
BLAST	Basic Local Alignment Search Tool
bp	base pair
BQCV	Black Queen Cell Virus
BY	Young Bees
CBP	Chronic Bee Paralysis Virus
CCM	Czech Collection of Microorganisms
CIGA	Internal Grant Agency of the Czech University of Life Sciences Prague
CWV	Cloudy Wing Virus
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DR	Drones
DSMZ	German Collection of Microorganisms and Cell Cultures
DWV	Deformed Wing Virus
EcoRI	Restriction Endonuclease from *Escherichia coli*
EPNs	Entomopathogenic Nematodes
EXP	Experiment
Firm; F	Firmicutes
FP341	Forward primer 341
Fri	*Frischella perrara*
$(GTG)_5$-PCR	Repetitive sequence-based polymerase chain reaction using the $(GTG)_5$ primer

G+C	Guanine + Cytosine
Gamma; G	Gammaproteobacteria
GFP	Green Fluorescent Protein
Gil	*Gilliamella apicola*
GI-tract	Gastrointestinal Tract
HPLC	High-Performance Liquid Chromatography
ICPV	Israeli Acute Paralysis Virus
IJs	infective juveniles
ITS	The Internal Transcribed Spacer
KBV	Kashmir Bee Virus
L1	1st instar larvae
L3	4th larval instar
L6	5th instar larvae
LAB	Lactic Acid Bacteria
Lac	Lactobacilli
LF3	Last Feeding Stage
MeOH	Methanol
MLSA	Multilocus Sequence Analysis
MRS	De Man, Rogosa and Sharpe Broth
MTPY	Modified Tryptone–Phytone–Yeast Extract
NAZV	Czech National Agency for Agricultural Research
NCBI	National Center for Biotechnology Information
OP	Organophosphate Pesticides
OTC	Oxytetracycline Hydrochloride
OTU	Operational Taxonomic Unit
PB	Black Pupa
PCR	Polymerase Chain Reaction
ppm	parts per million
Pr–Pd	body pigmentation Red brown – Dark brown

PW	White Pupa
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RDA	Redundancy Analysis
rDNA	Ribosomal Deoxyribonucleic Acid
RH	Relative Humidity
Rhi	*Rhizobiales bacterium*
RNA	Ribonucleic Acid
RP534	Reverse Primer 534
rRNA	Ribosomal Ribonucleic Acid
RVO	the Institutional Research Concept
SBPV	Slow Bee Paralysis Virus
SBV	Sacbrood Virus
Sno	*Snodgrassella alvi*
spp.	Species
TPY	Tryptone–Phytone–Yeast Extract
TSYE	Trypticase Soy Yeast Extract Medium

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Chapter 3

Chapter 4

Chapter 5

List of Publication

KILLER, J., SVEC, P., SEDLACEK, I., CERNOHLAVKOVA, J., BENADA, O., **HRONCOVA, Z.**, HAVLIK, J., VLKOVA, E., RADA, V., KOPECNY, J. & KOFRONOVA, O. 2014. *Vagococcus entomophilus* sp nov., from the digestive tract of a wasp (*Vespula vulgaris*). *International Journal of Systematic and Evolutionary Microbiology,* 64**,** 731-737.

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