Microbiota interactions and dynamics in megachilid bee nests

Interaktionen und Dynamiken der Mikrobiota in Nestern der Megachilidae-Bienen

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Summary

Insect microbiota plays an essential role on the hosts' health and fitness, regulating their development, nutrition and immunity. The natural microbiota of bees, in particular, has been given much attention, largely because of the globally reported bee population declines. However, although the worker honey bee has been associated with distinctive and specialized microbiota, the microbiota of solitary bees has not been examined in detail, despite their enormous ecological importance. The main objectives of the present thesis were a) the bacterial community description for various solitary bee species, b) the association of the solitary bee microbiota with ecological factors such as landscape type, c) the relation of the bee foraging preferences with their nest bacterial microbiota, d) the examination of the nest building material contribution to the nest microbiota, e) the isolation of bacterial strains with beneficial or harmful properties for the solitary bee larvae and f) the pathological investigation of bacteria found in deceased solitary bee larvae.

The findings of the present study revealed a high bacterial biodiversity in the solitary bee nests. At the same time, the bacterial communities were different for each bee host species. Furthermore, it was shown that the pollen bacterial communities underwent compositional shifts reflecting a reduction in floral bacteria with progressing larval development, while a clear landscape effect was absent. The examination of the nest pollen provisions showed different foraging preferences for each included bee species. Both the pollen composition and the host species identity had a strong effect on the pollen bacteria, indicating that the pollen bacterial communities are the result of a combinatory process. The introduced environmental material also contributed to the nest natural microbiome. However, although the larval microbiota was significantly influenced by the pollen microbiota, it was not much associated with that of the nest material.

Two *Paenibacillus* strains isolated from *O. bicornis* nests showed strong antifungal activities, while several isolated strains were able to metabolize various oligosaccharides which are common in pollen and nectar. Screening for potential pathogenic bacteria in the nests of *O. bicornis* unveiled bacterial taxa, which dominated the bacterial community in deceased larvae, while at the same time they were undetectable in the healthy individuals.

Finally, larvae which were raised *in vitro* developed distinct bacterial microbiomes according to their diet, while their life span was affected.

The present thesis described aspects of the microbiota dynamics in the nests of seven megachilid solitary bee nests, by suggesting which transmission pathways shape the established bacterial communities and how these are altered with larval development. Furthermore, specific bacterial taxa were associated with possible services they might provide to the larvae, while others were related with possible harmful effects. Future studies should integrate microbiota examination of different bee generations and parallel investigation of the microbiota of the nests and their surrounding environment (plant community, soil) to elucidate the bacterial transmission paths which establish the nest microbiota of solitary bees. Functional assays will also allow future studies to characterize specific nest bacteria as beneficial or harmful and describe how they assist the development of healthy bees and the fitness of bee populations.

Zusammenfassung

Insektenmikrobiota spielt eine entscheidende Rolle für die Gesundheit und Fitness ihres Wirtes, indem sie dessen Entwicklung, Nahrung und Immunität reguliert. Dem natürlichen Mikrobiom der Honigbiene ist bereits viel Aufmerksamkeit gewidmet worden, was vor allem auf die Berichte des globalen Rückgangs der Bienenpopulationen zurückzuführen ist. Insbesondere sind die Arbeiterinnen der Honigbiene in Verbindung mit unverkennbaren und spezialisierten Bakterien gebracht worden, die hauptsächlich durch soziale Kontakte übertragen werden. Demgegenüber wurden die Mikrobiome der Solitärbienen, trotz ihrer enormen ökologischen Bedeutung, bisher noch nicht im Detail untersucht. Die Hauptziele der vorliegenden Doktorarbeit waren a) die Beschreibung der Bakteriengemeinschaften von unterschiedlichen Solitärbienenarten, b) die Assoziation von Mikrobiota der Solitärbienen mit ökologischen Faktoren wie dem Landschaftstyp, c) die Erforschung der Präferenzen der Nahrungssuche von Solitärbienen in Bezug auf die bakteriellen Gemeinschaften ihrer Nester, d) die Untersuchung des Beitrages des Nestbaumaterials zur gesamten Mikrobiota des Nestes, e) die Isolierung von Bakterienstämmen mit vorteilhaften oder schädlichen Eigenschaften auf die Entwicklung der Solitärbienenlarven und f) die Untersuchung von pathologischen Bakterien, die in verstorbenen Solitärbienenlarven gefunden wurden.

Die Ergebnisse der vorliegenden Studie zeigten eine hohe bakterielle Biodiversität in den Nestern der Solitärbienen. Gleichzeitig waren die bakteriellen Gemeinschaften bei jeder Wirtsbienenart unterschiedlich. Es wurde weiterhin gezeigt, dass die Bakteriengemeinschaften der Pollen, Verschiebungen in der Zusammensetzung unterlagen. Diese Verschiebung spiegelt eine Abnahme von Blütenbakterien mit fortschreitender Larvenentwicklung wider. Dabei wurde kein Landschaftseffekt festgestellt. Die Untersuchung des Pollenvorräte der Nester ergab unterschiedliche Präferenzen der Futtersuche für jede einbezogene Bienenspezies. Sowohl die Zusammensetzung des Pollens als auch die Identität der Wirtsspezies wirkten sich stark auf die Pollenbakterien aus, was darauf hindeutet, dass die Pollenbakteriengemeinschaften das Ergebnis eines kombinatorischen Prozesses sind. Das eingetragene Umweltmaterial trug auch zum natürlichen Mikrobiom des Nestes bei. Die Mikrobiota der Larven wurden

zudem signifikant durch die Pollenmikrobiota beeinflusst, jedoch nicht sehr stark durch das Nestmaterial.

Zwei *Paenibacillus*-Stämme, die aus Nestern von *O. bicornis* isoliert wurden, zeigten starke antimykotische Aktivitäten. Darüber hinaus konnten mehrere isolierte Stämme verschiedene Oligosaccharide metabolisieren, die in Pollen und Nektar üblich sind. Das Screening auf potenziell pathogene Bakterien in den Nestern von *O. bicornis* enthüllte bakterielle Taxa, welche die Bakteriengemeinschaft in verstorbenen Larven dominierten und nicht in den gesunden Individuen nachweisbar waren. Letztendlich entwickelten Larven, die *in vitro* gezüchtet wurden, ihrer Ernährung entsprechend, unterschiedliche bakterielle Mikrobiome. Außerdem wurde dadurch ihre Lebensdauer beeinträchtigt.

In der vorliegenden Arbeit wurden Aspekte der Mikrobiota-Dynamik in den Nestern von sieben Solitärbienen der Familie Megachilidae beschrieben, indem suggeriert wurde, welche Übertragungswege die etablierten Bakteriengemeinschaften prägen und wie diese mit der Entwicklung der Larven verändert werden. Darüber hinaus wurden bakterielle Taxa identifiziert, die für die Wirte mit einem möglichen funktionellen Nutzen verbunden sind, während andere mit möglichen schädlichen Wirkungen in Verbindung stehen. Zukünftige Studien sollten sowohl Mikrobiota-Untersuchungen verschiedener Bienengenerationen als auch die parallele Untersuchung der Mikrobiota der Nester und ihrer Umgebung (Pflanzengemeinschaft, Boden) einschließen, um die bakteriellen Übertragungswege umfassend aufzuklären, die die Nestmikrobiome von Soltärbienen begründen. Außerdem könnten funktionelle Assays in zukünftigen Untersuchungen dazu dienen, spezifische Nestbakterien als nützlich oder schädlich zu charakterisieren, und beschreiben, wie sie die Entwicklung gesunder Bienen und die Fitness der Bienenpopulationen unterstützen.

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1. Introduction

1.1. The importance of insect microbiota studies and the case of bees

The microbiota of organisms has profound effects on the hosts. Gut microbiota, in particular, has been investigated as a main factor affecting organismal health. In mammals, the implications of the gut flora are associated with energy and lipid metabolism and with metabolism disorders inducing disease development (Sarafian *et al.* 2016), with pathogen resistance (Lozupone *et al.* 2012) and with the regulation of the brain activity and the development of stress-related diseases (Heijtz *et al.* 2011, Holzer 2016, Sudo 2016, Ariefdjohan *et al.* 2017). Similarly, insects show dependence on gut bacteria for a wide range of basic functions (Engel and Moran 2013). Although most insects are associated with few intestinal microbial species when compared to mammalian guts, some are hosts of large and diverse intestinal microbial communities (Engel and Moran 2013). Also, even though some are treated as model organisms for microbiota studies in general because of their simplicity, there are several insect traits which make the study of their microbiome particularly intriguing.

Firstly, insects constitute the most diverse clade in the animal kingdom considering the number of different species (Stork 2018), while they form the most abundant terrestrial animal group globally in terms of biomass (Basset *et al.* 2012, Bar-On *et al.* 2018). Their vast abundance and diversity are linked to ample ecological habitats and incalculable possible relationships with environmental microbes. Furthermore, in holometabolous insects, metamorphosis causes drastic shift of the whole microbial community during development, presumably leading to newly emerged adults with sterile guts (Moll *et al.* 2001). Finally, the existence or the absence of a social structure in the insects' life cycle can determine the nature of the host-microbe transmission routes. In the cases where the only social behavior is mating, opportunities for direct transfer of microbes between individuals are limited, while in the cases where sociality ensures contact between and within generations, specialized host-microbe relationships are actively promoted (Hongoh *et al.* 2005, Martinson *et al.* 2012, Pasquaretta *et al.* 2018).

In general, insect microbiota have direct effects on the insects' health and fitness. There are microbes which can regulate insect development (Chouaia *et al.* 2012), the sex ratio of their offspring (Hosokawa *et al.* 2010), their nutrition (Douglas 2009, Engel *et al.* 2012) and also their immunity (Kaltenpoth *et al.* 2005, Brownlie and Johnson 2008, Hedges *et al.* 2008, Kaltenpoth 2009, Koch and Schmid-Hempel 2011, Vásquez *et al.* 2012, Ferguson *et al.* 2018). Additionally, the vast majority of terrestrial ecosystems depends on insects to a high degree and insect microbiota has proven to play an important role in many insect-environment interactions. For example, insect microbiota can affect biomass decomposition (Bignell *et al.* 1997; Fierer *et al.* 2009), assist herbivory (Hammer *et al.* 2015, Mason *et al.* 2018) and influence disease vectoring efficiency (McMeniman *et al.* 2009; Ricci *et al.* 2012). Pollination is another activity which is considered as an invaluable benefit provided foremost by insects and has been brought under the spotlight as a major ecological service (Winfree *et al.* 2011). Therefore, it has been suggested that the microbiota of insects who act as pollinators may affect their pollination competence (Anderson *et al.* 2013).

Ecologists have focused on bees, which are considered insects of major economic value, partly because of their importance as main pollinators in all ecosystems with flowering plants (Holzschuh *et al.* 2012, Nicolson and Wright 2017, Hung *et al.* 2018) and also because of the reported drastic declines of their populations (Ghazoul 2013). Research on bee microbiota has increased during the previous years, focusing on bee nutrition, health and offspring recruitment, as well as on factors which may disrupt the bees' natural microbiota. These studies have primarily dealt with social bees and foremost honeybees; integrating the taxonomic, genomic and functional dimensions of their gut symbionts. Bee species which form no structured society have been frequently neglected, despite their being the vast majority considering species numbers among Apiformes and despite their inestimable ecological value. An outlook on the current knowledge on both social and solitary bee microbiota will be presented in the following chapters and is summarized in **Figure 1**.

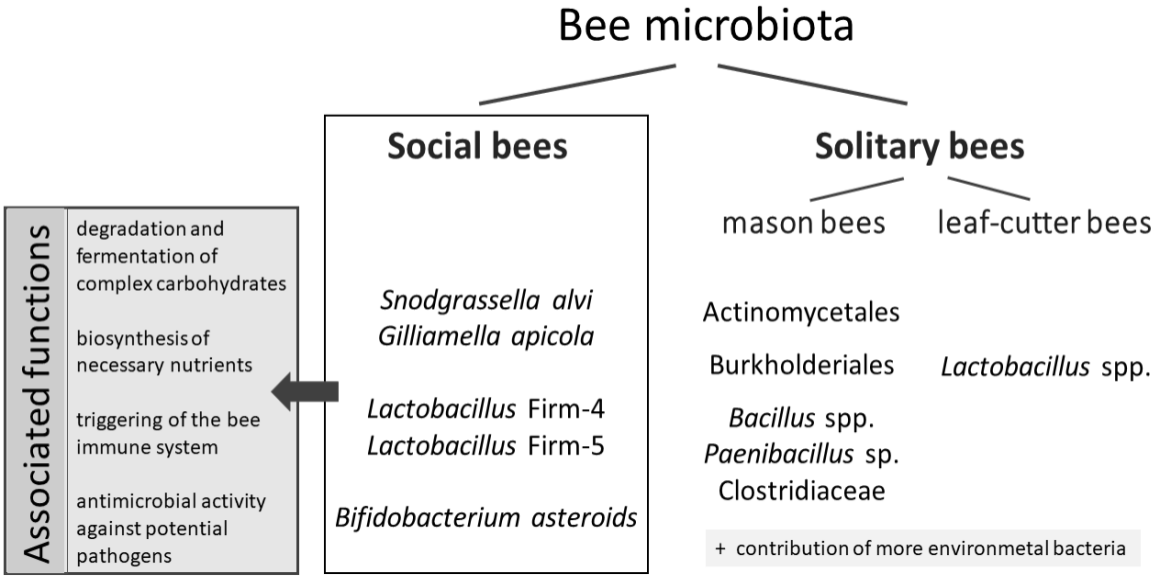

Figure 1. Current knowledge on the recurring bacteria of the bee microbiota. Social corbiculate bees have been claimed to share a basic core set of bacteria which has beneficial effects on bee health and fitness. On the contrary, solitary bees harbor bacteria of greater biodiversity.

1.1.1. The social bee core microbiota

Honey bees (*Apis* spp.), bumble bees and stingless bees all belong to the group of social bees (Michener 2007). They are the three main tribes among the subfamily of Apinae, or else corbiculate bees. Corbiculate bees are mostly social, although the degree of their sociality can vary. They are referred to as corbiculate because they acquire a pollen collecting apparatus, or corbicula, as part of the tibia of their hid legs and they are referred as social because they live in societies with division of labor which separates duties such as colony construction, colony defense, reproduction and active nursing of the offspring (Michener 2007). *Apis* spp. are common in East and South Asia, while the Western honey bee *A. melifera* is introduced worldwide (Ruttner 1988) and *A. cerana* is introduced in parts of Australia (Koetz 2013). Bumble bees are widely spread through Europe, America and a large part of Asia (Williams 1998). Lastly, stingless bees prefer tropical and subtropical regions (Vit *et al.* 2012). Honey bees are highly valued as crop pollinators which enhance the global food production (Calderone 2012). The decline of honey bee and bumble bee colonies has set off the alarm both for ecological and economic reasons (Cameron *et al.* 2011, Fürst *et al.* 2014).

The Western worker honey bee is reported to have a distinctive and recurring gut microbiome, harboring a set of nine main bacterial phylotypes (Moran *et al.* 2012, Corby-Harris *et al.* 2014, Kwong and Moran 2016). These bacteria are transmitted through social contact (Powel 2014) and are specialized for the hive environment and the bees (Martinson *et al.* 2011). Bumble bees harbor microbiota which are similar to those of the Western honey bee (Martinson *et al.* 2011, Koch and Schmid-Hempel 2011b, Koch *et al.* 2013). However, they are more likely to undergo microbial composition shifts due to different region of origin, colony, fitness and food availability (Kwong *et al.* 2017). In studies with stingless bees, microbiota varied between different colonies while at the same time it was much more heterogeneous and variable in composition and diversity when compared with honey bees and bumble bees (Leonhardt and Kaltenpoth, 2014, Kwong *et al.* 2017).

More specifically, the main bacterial species clusters which constitute the honey bee core gut microbiome are *Snodgrassella alvi* and *Gilliamella apicola* (Proteobacteria) (Kwong and Moran 2013), *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5 (Firmicutes) (Babendreier *et al.* 2007, Martinson *et al.* 2011) and *Bifidobacterium asteroids* (Actinobacteria) in lower abundance (Scardovi and Trovatelli 1969, Bottacini *et al.* 2012). Most of the members of the honey bee core microbiome have been found also in other *Apis* spp. (Yoshiyama and Kimura 2009, Saraithong *et al.* 2015). Four less frequent Proteobacteria complete the set of the Western honey bee microbiome. These are *Frischella perrara* (Engel *et al.* 2013), *Bartonella apis* (Kešnerová *et al.* 2016), *Parasaccharibacter apium* (Corby-Harris *et al.* 2014) and *Gluconobacter* (Martinson *et al.* 2011).

There are only a few bacterial taxa, such as Enterobacteriaceae, *Lactobacillus kunkeei* and *P. apium*, which have been found in the honey bee hive environment (Anderson *et al.* 2013). *L. kunkeei* thrive under acidic conditions in sugar rich substrates such as bee bread, honey crop, honey and pollen, and are also found in developing larvae (Anderson *et al.* 2013, Tamarit *et al.* 2015). *P. apium* is rare in the gut of the worker bee, however it is prevalent in stored food, royal jelly, as well as in larvae and queen guts (Corby-Harris *et al.* 2014b, Anderson *et al.* 2013, Tarpy *et al.* 2015).

Most members of the honey bee core gut microbiome have also been found in bumble bees (Koch and Schmid-Hempel 2011b, Martinson *et al.* 2011, Lim *et al.* 2015). *Apibacter*

adventoris (Bacteroidetes) (Moran and Kwong 2016) has been found in bumble bees and in lower abundances and more rarely in honey bees (Sabree *et al.* 2012). Furthermore, *Schmidhempelia bombi* (Proteobacteria) is a gut symbiont exclusively identified with bumble bees (Martinson *et al.* 2014) and *Bombiscardovia coagulans* (Bifidobacteriaceae) is mostly found in *Bombus* spp. (Killer *et al.* 2010). *Snodgrassella* is found both in *Apis* spp. and *Bombus* spp., as well as in some stingless bee species. Finally, *Gilliamella*, *Bifidobacterium*, *Lactobacillus* Firm-4, and *Lactobacillus* Firm-5 exist in all three honey, bumble and stingless bee groups (Leonhardt and Kaltenpoth 2014, Kwong *et al.* 2017).

1.1.2. Solitary bees' importance and microbiota

Solitary bees are often neglected in favour of social bees, despite their enormous diversity. As a matter of fact, only approximately one tenth of the world's 20,000 bee species are social, and only a small percentage of these construct hives (Michener 2007). Due to their high diversity, they vary in seasons of flight activity, preferred climate, geographic region, landscape type, nesting ecology, foraging preferences and compatible flower morphology (Brittain *et al.* 2013).

Moreover, solitary bees' efficiency in pollination has long been overlooked despite its enormous ecological importance (Ollerton *et al.* 2011, Christmann and Hasaan 2012, Garibaldi *et al.* 2013). The absence of corbicula in female solitary bees -a pollen collection apparatus common in most eusocial bees- leads to higher pollen loss at each flower visit and higher subsequent pollination efficiency (Michener 2007). Studies have shown that honey bees in Britain supply at most one third of the overall bee-mediated pollination (Breeze *et al.* 2011) and that wild bees enhance crop yields even where managed honey bee populations are present (Garibaldi *et al.* 2011), providing unsubstituted pollination services (Garibaldi *et al.* 2013). Additionally, solitary bees have been proven more effective than *Apis melifera* in pollinating apple trees (Vicens and Bosch 2000), cherry trees (Holzschuh *et al.* 2012) and rapeseed (Woodcock *et al.* 2013). Also, combination of honey bees and solitary bees has been proven beneficial for pollination in many cases (Greenleaf and Kremen 2006, Brittain *et al.* 2013b, Klein *et al.* 2003).

Despite the above, it was in the late 1990s when solitary bees were given some attention as alternative pollinators, when beekeepers started to report severe declines of bee

populations and high mortality of larvae in honey bee hives from all across the globe (Potts *et al.* 2010). Their populations have been reported as declining both because of anthropogenic factors (destruction, damaging or fragmentation of suitable habitats and agricultural inputs) and natural factors (infections, parasites, inclement weather) (Pfiffner and Müller 2016). Furthermore, wild bees have the highest ratio of endangered species among insects (Zurbuchen and Müller 2012).

Although there have been studies concentrating on the honey bee gut microbiome and on the hive's natural microbiota (see *Chapter 1.1.1.*), only few studies have dealt with the solitary bees' natural microbiota. Microbial profiling of solitary bees and of their nest environment is without a doubt a challenging task. The multiple aspects of their ecology and their diverse foraging preferences (Strickler 1979) form distinctive conditions that could establish different microbial transmission routes. Furthermore, lack of active nursing of the offspring in the nests suggests a greater environmental susceptibility, which could prevent devoted host-microbe associations and co-evolution. In general, solitary bees have been reported as adaptors of highly diverse bacterial communities, particularly when compared with social bees (Gilliam *et al.* 1990, Mohr and Tebbe 2006, Keller *et al.* 2013, Lozo *et al.* 2015, McFrederick and Rehan 2016, McFrederick *et al.* 2017).

Osmia bicornis larvae have been associated with large proportions of Burkholderiales (Mohr and Tebbe 2006, Keller *et al.* 2013), *Bacillus* spp., Clostridiaceae, Enterobacteriaceae and Acetobacteraceae (Keller *et al.* 2013). *Bacillus* spp. have been isolated also from the wild bee species *Crawfordapis luctuosa* (*Gilliam et al.* 1990) and *Osmia cornuta* (Lozo *et al.* 2015). Moreover, the pollen provision of *O. cornuta* nests has been associated with Firmicutes such as *L. kunkeei*, *Paenibacillus polymyxa* and *Clostridium baratii*, with Proteobacteria such as *Serratia marcescens* and *Pantoea agglomerans*, as well as with *Curtobacterium flaccumfaciens* (Actinobacteria) (Lozo *et al.* 2015). As already discussed, *L. kunkeei* is a frequent honey bee hive microbe (Anderson *et al.* 2013). In the case of solitary bees, its transmission through contact with floral microbiota and pollen transfer in the nest has been proven, as well (McFrederick *et al.* 2012).

Furthermore, the newly characterized *Lactobacillus micheneri, Lactobacillus timberlakei* and *Lactobacillus quenuiae* (McFrederick *et al.* 2018) have been found in pollen, bee bread,

larvae and adults of various wild bee species (*Cauplicana yarrowi*, *Diadasia opuntiae*, *Megachile* spp., *Osmia* spp., *Augochlorella pomoniella*, *Agapostemon* spp., *Dialictus* sp., *Halictus tripartitus* and *Halictus ligatus*) Especially in *Megachile* spp., they have been found in high abundances and they are phylogenetically closer with bee associated bacteria (McFrederick *et al.* 2017). More specifically, they are closely related to the honey-bee associated bacteria *L. kunkeei* (97% 16S rRNA gene sequence similarity) and *Lactobacillus apinorum* (97.0% 16S rRNA gene sequence similarity) (McFrederick *et al.* 2018).

1.1.3. Significant functions of the bee microbiome

The microbiome of an organism is considered a component of its health. Similarly, the honey bee microbiome has been proven to be an essential part of nutrition and pathogen defense in the colonies (Engel *et al.* 2012, Engel and Moran 2013b). Recently, a study showed that the bacterial composition of the honey bee surface and gut was different between thriving and non-thriving hives (Ribière *et al.* 2018). The thriving colonies were characterized by higher bacterial diversity and higher relative abundance of bacteria associated with healthy colonies (Anderson *et al.* 2013, Ribière *et al.* 2018).

Bioassays have demonstrated that a number of honey bee-associated bacteria, such as *Lactobacillu*s and *Bifidobacterium*, inhibit the causative agents of American and European foulbrood *in vitro* (Killer *et al.* 2014). Indirectly, the gut microbiota might also be able to activate the host's immune system (Schwarz and Huang 2015). Yet, the honey bee core microbiome should not be always presumed as entirely beneficial to honey bees. More specifically, sterile honey bees exposure to *F. perrara* but not to other bacterial members of the honey bee core microbiome has been observed to cause scab formation (Engel *et al.* 2015).

Other roles of the honey bee gut bacteria are the fermentation of complex carbohydrates, the digestion and the biosynthesis of necessary nutrients (Lee *et al.* 2014). For instance, bees cannot digest the complex pollen grains themselves since they do not biosynthesize pectate lyases; a task undertaken by the symbiont *Gilliamella apicola* (Engel *et al.* 2012). It had previously been suggested that microbes living in the stored pollen of the hive gradually degrade it, producing more nutritious and easier to digest components.

However, few bacteria have been found in stored pollen, suggesting that this process is conducted in the worker gut (Anderson *et al.* 2014).

In European bumble bees, deprivation of their gut microbiota led to high susceptibility to parasites (Koch and Schmid-Hempel 2011). Moreover, infection by the gut parasite *Crithidia* was negatively associated with the symbiont *Gilliamella* and positively associated with non-core bacteria in a wild bumble bee study (Cariveau *et al.* 2014). Furthermore, the general *Crithidia bombi* infection load in bumble bee colonies was proven to be associated with the microbiota, rather than with the individuals' genotypes (Koch and Schmid-Hempel 2012). Other bumble bee symbionts, like *Schmidhempelia bombi*, can ferment carbohydrates (Martinson *et al.* 2014) and others may form syntrophic interactions for partitioning of metabolic resources (Kwong *et al.* 2014).

In solitary bees, *Lactobacillus* strains have been isolated in multiple occasions (Lozo *et al.* 2015, McFrederick *et al.* 2018). In the solitary bee nest, where larvae have to feed on their own on the provided pollen, the presence of symbionts which could assist with pollen fermentation and digestion would be beneficial. Also, in the susceptible to the environment nest, solitary bee larvae might need microbial associates to resist disease caused by microbial pathogens. Indeed, there are bacterial strains with which have been isolated from solitary bee nests and have shown strong antifungal and antibacterial activity in designed bioassays (Keller *et al.* 2018). The origin of possible symbionts in solitary bee nests is yet to be characterized (Keller *et al.* 2013, McFrederick *et al.* 2017).

1.1.3.1. Lactobacillus spp.

Lactic acid bacteria, in general, have been suggested as valuable symbionts of the honey bee (Vásquez *et al.* 2012, Butler *et al.* 2013). Apart from inhibiting the lethal bacterial pathogens *Paenibacillus larvae* and *Melissococcus plutonius* (Forsgren 2010, Vásquez *et al.* 2012), bee-specific lactobacilli encode various genes for carbohydrate utilization (Ellegaard *et al.* 2015) and phosphotransferase systems involved in the uptake of sugars (Kwong *et al.* 2014). *Lactobacillus* Firm-5 also has groups of genes connected with the biosynthesis of trehalose, a disaccharide used for storing energy in insects (Ellegaard *et al.* 2015). Gene content, associated with carbohydrate use and exopolysaccharide biosynthesis, varies

between different *Lactobacillus* strains, which have been isolated from honey bees (Kwong *et al.* 2014b).

In solitary bees, the isolated novel *Lactobacillus* species described in McFrederick *et al.* 2018 possess a wide range of lytic enzymes. Operational taxonomic units corresponding to these species were found in a metagenomic study in high relative and absolute abundances in adult and larval bee guts, as well as in pollen provisions. However, the same bacterial types were found in flowers, supporting the hypothesis that flowers can share the same bacteria with bees or can act as transmission hubs for bees (McFrederick *et al.* 2017).

1.1.3.2. Paenibacillus spp.

Paenibacillus is a notorious genus in insect microbiota studies, mostly because of *Paenibacillis larvae*, which is the causative agent of American Foulbrood of honey bees (Genersch 2008, Genersch 2010). Furthermore, *Paenibacillus alvei* can establish in the larval remains of diseased honey bee colonies (Forsgren 2010). Examined genomes of *P. larvae* encode numerous virulence factors, such as toxins and collagenases (Djukic *et al.* 2014). Also, the ability to degrade the peritrophic matrix of the bee midgut epithelium, which is a key step in pathogenesis of *P. larvae* infections, has been attributed to specific chitin-degrading proteins (Garcia-Gonzalez *et al.* 2014). Yet not all *Paenibacillus* spp. should be considered as potentially harmful, since there are species which possess antifungal and antibacterial bioproperties, important in environmental biocontrol (Raza *et al.* 2008; Naing *et al.* 2014).

Paenibacillus bacteria have been reported from the nests of two solitary bee species, *O. bicornis* (Keller *et al.* 2013) and *O. cornuta* (Lozo *et al.* 2015), as part of their natural microbiome. Indeed, the larvae in the examined nests appeared healthy and normally developed. It has been suggested that their origin is environmentally dependent (Potts *et al.* 2005, Keller *et al.* 2013). The genome of a *Paenibacillus* strain isolated from the solitary bee species *Osmia caerulescens* was lacking genes encoding chitin-degrading proteins and was, in general, phylogenetically distinct from harmful members relevant to honey bee colony diseases. The bacterium was common in Megachilid bee nests, pollen, guts and surface and what is more, the isolated strain showed strong antimicrobial bioproperties (Keller *et al.* 2018). Furthermore, a *P. polymyxa* strain from the provisions of the stingless

bee *Melipona scutellaris* was active against entomopathogenic fungi and *P. larvae* (Menegatti *et al*. 2018).

1.2. Factors affecting the bee microbiome

The bee microbiota is likely to be influenced by both abiotic (e.g. climate, season, temperature) and biotic elements (e.g. pathogens, parasites). Furthermore, there are influential factors closely connected with anthropogenic activities (e.g. agricultural intensification, land use alterations). The main factors which have been studied as possibly affecting and/or shaping the bee microbiota are presented in the following paragraphs and summarized in **Figure 2**. These are the degree of sociality in the bee lifecycle, the developmental stage of the bee, the foraging preferences and plant availability, the overall environment and surrounding landscape type, the contact with potential pathogenic microbes or parasites and several anthropogenic activities.

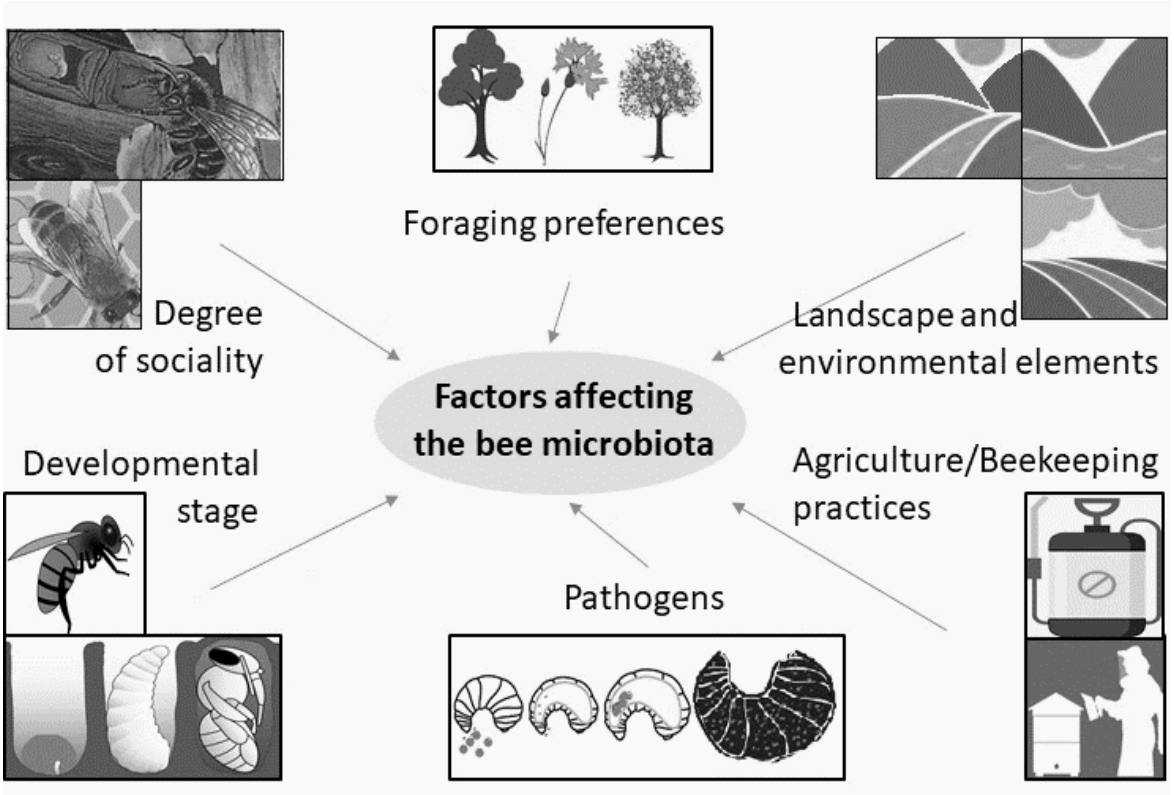

Figure 2. Main factors which influence the bee microbiota

1.2.1. Sociality

Sociality, interpreted as the sharing of the nesting habitat and the maintenance of division of labor and social interactions between and within generations, is central in the study of microbiota transmission. In general, it has been shown that the evolution of intimate associations between the host and the microbiome is favored in social hosts (Troyer 1984, Lombardo 2008). Stable close social contact of individuals is likely to aid transmission of beneficial symbionts both from parent to offspring and also between colony members, assisting the emergence and evolution of mutualistic interactions (Koch *et al.* 2013, Powell *et al.* 2014).

Honey bee adults are supposed to emerge with sterile guts from their protected cells where they are kept during pupating (Powell *et al.* 2014). It has been proposed, that the newly emerged bees get their first gut symbionts by chewing their way out of their capped cells and thus acquiring remnant gut bacteria (Kwong *et al.* 2014). The use of qPCR showed that if the young adults are taken from their cells and are kept under sterile conditions in the lab, they will never acquire substantial gut bacteria (Powell *et al.* 2014).

In addition, the bacterial species clusters which belong to the honey bee core gut microbiota have not been found in environments outside the bee hive or even out of the bee gut, showing devoted niche restriction. This dedication, which is most probably also driven by the fact that these bacteria are facultative anaerobes and cannot replicate successfully under aerobic conditions, shows that bacterial transmission between hosts depends greatly on social interactions (Engel *et al.* 2013b).

The main microbial transmission routes in the social honey bee hive include direct fecal – oral connectivity, oral trophallaxis and contact with the hive material (Martinson *et al.* 2012). Non-typical bacteria or bacteria which occur frequently but are not considered part of the core microbiota are established when workers are exposed to oral trophallaxis or hive components, such as honey comb, honey and bee bread (Powell *et al.* 2014). Furthermore, the transmission of *Frischella perrara*, *Gilliamella apicola*, *Snodgrassella alvi* were connected with the presence of nursing worker bees or with hindgut material, while at the same time Firmicutes were often transmitted through exposure to the hive (Powell *et al.* 2014).

On the other hand, the solitary insect lifestyle prevents transfer of bacterial symbionts between generations and significantly limits the connection between individuals of the same generation (Lombardo 2008, Engel and Moran 2013). Lack of active nursing of the offspring in the nests suggests that larvae are susceptible to a wide range of environmental threats, since active transfer of specialized bacteria through direct social interactions during their development is lacking. Higher environmental influence could also prevent devoted host-microbe associations and co-evolution.

In the case of solitary bees, the role of the mother bee on the microbiome of their offspring, as well as the connectivity between individuals of the same generation, has not been investigated. Mixing of the pollen provision with nectar and salivary gland secretions by the mother bee, prior to the egg laying, might inoculate the offspring's diet with beneficial bacteria. These might be essential for the developing larvae, assisting with the pollen digestion and the defense against environmental pathogens. Several insect species have been reported to have females able to inoculate their eggs with specific bacteria, preserving a stable host–microbe relationship (Hosokawa and Kikuchi 2007). Furthermore, the selection of the nesting site by the mother bee, with its accompanying microclimate, floral resource diversity and overall environment, might affect microbial communities.

Furthermore, apart from the connectivity between individuals, it is possible for non-social bees to be related to bacteria with conserved interactions through environmental transmission routes or food acquisition; their extent and importance, though, remain understudied. For instance, it is known that certain solitary bee species visit specific plant species (Westrich 2015). This high selectivity mediates stable bee -flower interactions, allowing flowers to play the role of hubs for bacterial transmission amongst bee hosts (McFrederick *et al.* 2017).

1.2.2. Developmental stage

It has been suggested that the newly eclosed honey bee larvae do not harbor any bacteria (Powell *et al.* 2014). The larvae are supposed to acquire bacterial symbionts after they start to receive nursing by the worker honey bees. During their first days, most of the bacteria which are transmitted to larvae are typical for the hive materials, whereas other bacteria are adult gut symbionts. Nevertheless, this initial bacterial composition is erratic and there

is no differentiation between different larval gut parts (Martinson *et al.* 2012, Ahn *et al.* 2012, Vojvodic *et al.* 2013, Hroncova *et al.* 2015).

The number of the bacteria in the honey bee larval gut raises drastically during the first days (Kwong and Moran 2016). In general, the most prevalent bacteria of the initial community are Lactobacilli and Acetobacteria (Anderson *et al.* 2016). Directly after metamorphosis and the gut reorganization, the bee guts have again few or no bacteria. Larval guts will be colonized during the first few days of adult life, in order for the adult bees to have the normal honey bee microbiota, before they are ready to leave the hive (Hroncova *et al.* 2015).

The formed gut communities will keep the same bacterial taxa even through transition of the worker bees through various states of labor division (Kapheim *et al.* 2015). Also, the gut microbiome of adult workers is more stable than the one harbored by male bees or queens (Kapheim *et al.* 2015). However, despite the stability of the bacterial taxa that are present, there are shifts in relative abundances that are observed with time and are associated with the age of the individuals (Hroncova *et al.* 2015) and with their behavioral task (Jones *et al.* 2018).

In the case of solitary bees, little is known on whether there are forces driving bacterial succession in the larvae and the adults in the nest environment. It has been suggested that the environment and foremost the available flora is a main factor affecting the nest microbiota (Keller *et al.* 2013, McFrederick *et al.* 2017). In a study with the halictid bees *Megalopta centralis* and *M. genalis*, environmentally acquired bacteria and not the existence of a social structure appeared to drive bacterial community shifts between different developmental stages of the larvae (McFrederick *et al.* 2014). More specifically, *Lactobacillus kunkeei* dominated the bacterial community of pollen and young larvae, but was less dominant in mature larvae and pupae. Foraging adults often reacquired the taxon, probably through foraging (McFrederick *et al.* 2014).

1.2.3. Foraging preferences

Although honey bee microbiota has been extensively investigated, the effect that foraging preferences and the imported pollen have on the characteristic core set of bacteria is

poorly studied. It has been observed that the nectar foragers of the colony can show preference for a food source over another (Weaver 1965, Mayer and Lunden 1991, Fohouo *et al*. 2008, Sushil *et al.* 2013) or for flowers from a specific plant height (Mattu *et al.* 2012). Also, the bee bread production is dependent on multiple plant species pollens (Camazine *et al.* 1998, Di Pasquale *et al.* 2013) and pollen and resin foragers prefer some pollen resources over others (Abou-Shaara 2014).

A recent study used honey bee colonies reared under identical conditions in order to investigate whether different landscapes along with their accompanying plant diversity and availability would influence the bee microbiota (Jones *et al.* 2018b). More, specifically, the colonies were placed in two landscape types; the one was situated in a region with established oilseed rape farmland, which is a beloved food source for honeybees (Danner *et al.* 2017), and the other in agricultural farmland away from oilseed rape fields. After six weeks, the characterized gut bacterial communities of adult bees from the colonies showed trivial differences (Jones *et al.* 2018b), however the overall results suggested that the broad environment can have some influence on the relative abundance of some honey bee microbiota members.

The bee bread in honeybee hives has been proven to contain a set of taxa (Anderson *et al.* 2013, Donkersley *et al.* 2018) also found in nectar and pollen of insect-pollinated plants (Junker and Keller, 2015, Ambika Manirajan *et al.* 2016; Lenaerts *et al.* 2016). Another recent study showed that the bee bread bacteria varied significantly with hive location and it was suggested that the reduced floral diversity in improved grasslands lead to reduction in bacterial diversity (Donkersley *et al.* 2018). However, it could not be determined whether bee gut microbiota or floral sources were more influential on bacterial composition (Donkersley *et al.* 2018).

The foraging preferences of different solitary bee species show great diversity. There are species described as oligolectic which prefer certain plant taxa(e.g. *Heriades truncorum* and Asteraceae) and others described as generalists or polylectic (e.g. *Osmia bicornis*) (Westrich 2015). In Central Europe the pollen sources of oligolectic bees are 24 plant families, while about one third of the nest building bee species are oligolectic (Westrich 1996). These foraging preferences have been studied as a candidate way of forming the

solitary bee microbiome, since the absence of a social structure has indicated that the establishment of steady host-microbe interactions could be the result of passive transmission through imported pollen in the nests (McFrederick and Rehan 2016; McFrederick *et al.* 2017, Rothman *et al.* 2018).

When foraging preferences are well conserved, they could establish conserved routes for bacterial colonization in the nest. Previous studies have identified the plant composition of pollen provisions (Sickel *et al.* 2015, Villanueva-Gutiérrez and Roubik 2016). Also, the examination of both pollen composition and pollen microbiota from the nests of a wild bee species has shown that they co-vary across different landscapes (McFrederick and Rehan 2018). Moreover, the association between the pollen microbiome and the pollen composition in the nests of a single bee species proposed possible plant mediated bee-microbe relationships (McFrederick and Rehan 2016). Finally, a recent study showed the ability of *Megachile rotundata* to deposit plant pathogens into its nests through pollen transfer (Rothman *et al.* 2018). Combination of this recent study with the reported negative responses of bumblebees towards several floral bacteria (Junker *et al*. 2014), indicates the complex interactions in the plant-microbe-pollinator triangle.

1.2.4. Nest material

Wild honey bees make hives in natural cavities, such as hollow trees or rock crevices, which are selected by scout bees as appropriate. The hives are then constructed with softened wax by bonding it into the honeycomb cells. The hive is generally used for food storage and for housing the offspring (Michener 2007). The nest-building and provisioning behavior of bees facilitates the maintenance of a consistent microbiome by securing the interactions between the members of the colony and the contact with the hive material (Anderson *et al.* 2013, Salem *et al.* 2015). In the interior of the hive, there are formed niches which host distinct bacterial clusters (Anderson *et al.* 2013) and they all contribute to the overall microbial community (Powell *et al.* 2014).

On the contrary, solitary bees use a wide range of different natural materials to construct the interior of their nests. Most of them are miners and nest in the ground, whereas others nest in natural cavities in dead wood or rock. Others use empty snail shells, vacated galls of the chloropid fly and stems (Westrich 1996). Also, there is a wide range of natural

materials that bees use to construct the interior of their nest and the different nest chambers for each egg. Mining bees usually line the nest's chambers with secretions. Others are selective for natural materials in their proximity. Different bee species have been documented to use clay (mason bees), leaves (leaf-cutters), masticated plant parts, cottony plant material, petals and resin (Westrich 1996, Westrich 2015).

The inclusion of natural materials in the nest highlights the significance of the nest's environment to the overall nest microbiota and the contact of the larvae with the nest material implies that the bee microbiome might be affected by nesting material. Microbiological studies with mason bees have indicated bacterial taxa which might have their origin in soil (Keller *et al.* 2013, Lozo *et al.* 2015). However, the extent to which the nest building material can influence the solitary bees remains unknown.

1.2.5. Microbial pathogens

Threats affecting bee health have causes which are associated with a number of different ecological pressures (Vanbergen *et al.* 2013). Most studies which examine possible perils against bees have focused on parasites, land use intensity and climate change (Hegland *et al.* 2009, Strohm 2011, González-Varo *et al.* 2013, Goulson *et al.* 2015, Woodcock *et al.* 2017, Goulson *et al.* 2018, Jones *et al.* 2018b, Schenk *et al.* 2018). Nevertheless, studies on honey bees have also revealed bacterial agents which can disrupt their natural microbiota and cause acute mortality in the hive (McKee *et al.* 2004, Genersch 2010, Fünfhaus *et al.* 2018).

More specifically, *Paenibacillus larvae* (Genersch 2010) and *Melissococcus plutonius* (McKee *et al.* 2004) are widely accepted as the main causative pathogens for American and European Foulbrood in honey bees, respectively. Furthermore, the honey bee pathogens *Spiroplasma apis* and *Spiroplasma melliferum* (Mouches *et al.* 1982, Mouches *et al.* 1983) have been proven to reduce adult bee longevity. Finally, the widespread dispersal of the honey bee microsporidian pathogen *Nosema* showed how anthropogenic introduction of pathogens between bee species can result in crucial situations (Klee *et al.* 2007).

Nevertheless, the precise impacts of some pathogens on honey bee health and fitness are unclear (Evans and Schwarz 2011). Two common microsporidian pathogens, for instance,

Nosema apis and *Nosema ceranae*, remain controversial, as there are reports which mention that they are harmful to honey bees (Martín-Hernández *et al.* 2007, Higes *et al.* 2008, Botías *et al.* 2013) and other which doubt the severity of their pathogenicity (Huang *et al.* 2015, Milbrath *et al.* 2015). Similarly, *P. apiarius* has also been considered as harmful to honey bees, although its effects have not received much attention (Nakamura 1996, Grady *et al*. 2016).

Apart from the described bacterial pathogens, there are other bacterial taxa which demonstrate an opportunistic behavior. The group of opportunistic environmental bacteria which affect the honey bee colonies includes Enterobacteriaceae and more specifically the genera such as *Enterobacter*, *Hafnia*, *Klebsiella*, *Pantoea* and *Serratia*, as well as several Gammaprotoebacteria (Corby-Harris *et al.* 2014). These opportunists are often involved with microbial shifts observed in individual worker honey bees. Bumble bees appear to be more susceptible than honey bees to such shifts (Cariveau *et al.* 2014).

Moreover, there have been a wide range of viruses which harm the honey bee and have been described so far (Chen and Siede 2007, McMenamin and Genersch 2015). Among the identified viruses, there are members associated with the deformed wing virus (Dainat *et al.* 2012) and the acute bee paralysis virus, which are lethal pathogens for entire colonies. However, there are also a lot of newly discovered viruses whose impact on honey bee health is unclear and others which can remain asymptomatical for a long period of time within bee colonies (McMenamin and Genersch 2015). The harmful effects caused by viruses can range from physiological changes to deformities, behavioral alterations and mortality. Also, the degree of pathology may differ significantly between different hosts (De Miranda *et al.* 2013).

It is supported that the combination of pathogens, as well as the combination of pathogens with pesticides, can cause negative effects on bee health synergistically (Evans and Schwarz 2011, Cornman *et al.* 2012, Dainat *et al.* 2012, Nazzi *et al.* 2012, Doublet *et al.* 2015). Also, honey bee viruses can often infect other hosts such as bumblebees, solitary bees and wasps with overlapping geographic ranges (Fürst *et al.* 2014, Mcmahon *et al.* 2015). It has been suggested that the shared pathogens between different pollinator species are possibly transmitted through the use of common floral sources (Singh *et al.* 2010, Fürst *et*

al. 2014, Graystock *et al.* 2015). Also, honey bee colony density may also affect the spore transmission of P. larvae (Lindström *et al*. 2008).

When it comes to solitary bees, during the recent years there have been studies concentrating on their nest bacterial microbiome (Keller *et al.* 2013; Lozo *et al.* 2015; McFrederick and Rehan 2016). Studies investigating potential microbial pathogens, however, have focused on viral/fungal infections, which are common between honey bees and solitary bees (Ravoet *et al.* 2014) and in one case on several *S. melliferum* strains, which were isolated from honey bees, bumble bees, digger bees and the mason bee *Osmia cornifrons* (Clark *et al.* 1985). Solitary bees have been proven to transfer plant pathogens into their nests (Rothman *et al.* 2018), implying that environment might play a main role also in the transmission of solitary bee nest pathogens. Studies screening for potential pathogens in solitary bees are currently lacking.

1.2.6. Human impact

Human caused global changes have long been put under the spotlight as the main force threatening the environment (Vitousek *et al.* 1997). Land use changes is the main threat against biodiversity (Murphy and Romanuk *et al.* 2014) and biodiversity losses raise concerns for the need to conserve valuable ecosystem functions (Ricketts *et al.* 2016). Pollination networks, in particular, have suffered both reduction of pollinator and plant populations and loss of co-occurrence between them (Burkle *et al.* 2013). The main anthropogenic activities which affect bee populations around the world are habitat fragmentation and agricultural intensification (Kremen *et al.* 2002, Steffan-Dewenter and Westphal 2008, Winfree *et al.* 2011, Kennedy *et al.* 2013).

A big part of intense agriculture has been the large scale introduction of chemicals in ecosystems which is considered as a main factor harming biodiversity (Mahmood *et al.* 2016). Although these chemicals are often directly lethal to bees, it is an issue whether their harmful effects are mediated or facilitated by disturbing their microbiome (Raymann *et al.* 2018). Also, correlations between honey bee related bacterial communities and land use have been significant in some cases, although they could not show a strong effect, like the one caused by application of antibiotics (Jones *et al.* 2018b).

Antibiotics used according to common beekeeping practices can affect the gut microbiome of honey bees by increasing bacterial resistance. One example is the use of antibiotics which was extensively increased to prevent *Paenibacillus larvae* infections (Tian *et al.* 2012). Moreover, the use of antibiotics can alter the immune response of bees, making them more susceptible to opportunistic infections (Di Prisco *et al.* 2013, Li *et al.* 2017, Motta *et al.* 2018).

In a recent study, exposure to glyphosate, which is one of the most used herbicides globally, increased the mortality of worker honey bees after subsequent exposure to the opportunistic pathogen *Serratia marcescens* (Motta *et al.* 2018). In another test of honey bee colony exposure to chlorothalonil, a common compound used against fungi in pollen and beehives, putative bacterial genes for oxidative phosphorylation increased, while sugar metabolism and peptidase potential declined (Kakumanu *et al.* 2016).

Although there are fewer studies on the effect of anthropogenic activities on the microbiome of solitary bees, it is widely accepted that human impact can be detrimental also in their case (Goulson *et al.* 2015). Apart from the use of pesticides, introduction of pathogens (Fürst *et al.* 2014) or of nonnative bee species (Hedtke *et al.* 2015) and the practices of global trade (Murray *et al.* 2013) may also have harmful effects.

1.3. Objective of the present study

The main aim of the present study is to identify and describe natural microbiota of several solitary bee species comprehensively. At the same time, the influence of several factors which may form, regulate or disrupt the natural bee nest microbiota will be addressed. Subsequently, the plan of the present study is to investigate possible relations of the bacterial communities and the factors which structure them with the health of the bees and their offspring. To achieve that, a number of solitary bee species with differences in various aspects of their ecology and occurrence in different landscapes will be included.

The main objectives of the project are the following:

- **Comparative bacterial community description and bacterial diversity assessment for various solitary bee species and different larval developmental stages.**

Hypothesis 1. Solitary bee nests harbor a high bacterial biodiversity; particularly in comparison with the one discovered in the nests of social bee species.

Hypothesis 2. The natural solitary bee microbiome nest mainly harbors bacteria of environmental origin.

Hypothesis 3. Bee host species is a discriminant factor separating the nest bacterial communities

Hypothesis 4. There are bacterial taxa showing consistent occurrence in the larvae and the pollen provision samples within the same bee species.

Hypothesis 5. The larval and the pollen bacterial microbiome undergoes bacterial succession benefiting bacteria contributing to the larval development.

- **Association of bacterial microbiota variation with ecological factors such as landscape diversity and land use intensity**

Hypothesis 6. The bacterial community in the solitary bee nest is influenced by the land use types of the sampling region.

Hypothesis 7. The bacterial community in the solitary bee nest is influenced by the diversity of the surrounding landscape.

Hypothesis 8. The bacterial community in the solitary bee nest is influenced by the geographic region it is located at.

- **Investigation of the foraging preferences of the sampled solitary bees and association of these preferences with the bacterial microbiome of their nests**

Hypothesis 9. Different solitary bee species are selective for specific plant species.

Hypothesis 10. The foraging preferences of solitary bees species influence the solitary bee nest microbiome by the direct introduction of environmental and foremost floral bacteria.

Hypothesis 11. Plant biodiversity of the pollen provisions inside the solitary bee nests affects the biodiversity of their bacterial microbiota.

Hypothesis 12. Taxonomic variations in the pollen inside solitary bee nests co-relate with variations in the bacterial composition within the same bee species and between different species.

Hypothesis 13. There are specific bacteria in the bees nests which co-occur with pollen from specific plant species.

- **Investigation of the nest building material contribution to the overall nest bacterial microbiome**

Hypothesis 14. The use of the nest building material introduces environmental bacteria which contribute to the solitary bee nest natural microbiota.

Hypothesis 15. The type of the nest building material influences the nest natural microbiome.

- **Isolation of bacterial strains from solitary bee larvae and their pollen provisions and investigation of possible bioproperties with beneficial effects for the larvae**

Hypothesis 16. There are nest bacteria with antimicrobial activities which benefit the bees by protecting the larvae from microbial pathogens.

Hypothesis 17. There are nest bacteria which help the larvae degrade and digest the complex pollen provision.

- **Comprehensive pathological investigation of microbial agents found in diseased and dead solitary bee larvae**

Hypothesis 18. The solitary bee nests are susceptible to a variety of opportunistic environmental pathogens.

Hypothesis 19. Bacterial infections of the larvae can entirely shift the natural larval and nest bacterial microbiome.

Hypothesis 20. There are bacterial pathogens which can cause acute mortality to the solitary bees' offspring in the nests.

2. Materials and methods

2.1. Sampling design

Sampling was conducted with the use of artificial trap-nests. Opportunistic solitary bee species like the mason bees and the leaf-cutter bees can nest in a wide variety of places and therefore these opportunists can easily be attracted by artificial nesting sites (Westrich 1996). Females start assembling pollen into the elongated narrow cavities a few days after mating (Strohm *et al.* 2002). When they form one sufficient pollen provision, they lay an egg on it and they construct a chamber using natural materials, which range from soil (*O. bicornis*) to leaves (*Megachile* spp.) and other plant parts (e.g. *O. caerulescens*, *O. leaiana*) or resin (*H. truncorum*) (Westrich 2015). A nest usually consists of two to ten nest chambers (Bosch and Vicens 2006) and when it is completed, the female seals the entrance and leaves. A few days after eggs are laid, they eclose and develop into larvae (Raw 1972). The bees hibernate as pupae or as larvae in cocoons, until their emergence (Bosch and Kemp 2004).

The artificial trap-nests were established on the 28th of March 2016. The sampling sites were chosen, as they had been previously used for sampling of solitary bees during projects conducted at the University of Würzburg. Sampling was planned to be conducted at a time covering the entire flight activity period of solitary bees in the region. Samples were collected in the time frame from 25/05/2016 until 30/09/2016 and from 06/06/2017 until 01/10/2017.

2.1.1. Artificial nests

Sampling was conducted with the use of artificial nests consisting of reed internodes (**Image 1**). Each trap nest consisted of two 20 cm long PVC tubes which were adjusted on the top of 1 meter high wooden sticks and contained 30 to 50 reeds. Each reed internode had a diameter of 4 to 10 mm. The artificial nests were constructed in early spring 2016, before the emergence of the solitary bees from hibernation and they were examined every 15 days during the bees‘ flight activity.

Image 1. Artificial nests used for sampling

2.1.2. Sampling region

They artificial nests were placed at 12 localities, spread through an area with a radius of 32 km at northern Bavaria, Germany (**Figure 3**). For each of the stations, two trap-nests were established in neighboring sites. Minimum distance between two sampling stations was 3.3 km and maximum distance between two sampling stations was 63.77 km. The localities were mainly occupied by agricultural land with adjacent land consisting of semi-natural vegetation. The two most abundant land use categories, which were also present in all sampling sites, were non-irrigated arable land and transitional woodland among all sampling sites.

Land cover for a radius of 1km around each sampling site was assessed with the use of *QGIS v2.18.16* software from the CORINE Land Cover (CLC) inventory (**Figure 4**). Land categorized as non-irrigated arable land, vineyards, fruit tree plantations and land with complex cultivation patterns were treated as agricultural land. Those categorized as transitional woodland, natural grassland, grasslands for pastures and forest were treated as land with semi-natural vegetation. Discontinuous urban fabric and relevant artificially surfaced areas were categorized as anthropogenic environment and were noted as such where present (**Table 1**) (Voulgari-Kokota *et al.* 2019).

Table 1. Geographic coordinates and land coverage information of the sampling sites for a radius of 1 km around them. Numbers under these three categories represent the percentage they hold in the conceivable area.

Site ID	Coordinates	Agricultural land	Land with semi-natural vegetation	Anthropogenic environment	Landscape diversity (Shannon)
A	49.54644, 10.06067	61.41	18.55	20.04	1.33
B	49.66317, 10.12187	59.05	34.35	6.59	1.24
C	49.77384, 10.00210	67.85	26.66	5.49	1.42
D	49.77842, 10.25783	71.99	28.01	0.00	0.69
E	49.79370, 9.797338	80.14	19.86	0.00	0.50
F	49.82359, 10.04256	64.25	35.75	0.00	0.65
G	49.84813, 9.837698	45.91	51.95	2.14	1.10
H	49.99919, 9.770108	44.66	49.84	5.50	1.15
I	50.03234, 9.906883	62.48	32.17	5.35	0.92
J	50.05504, 9.800053	51.35	42.39	6.27	0.88
K	50.09653, 9.921330	57.12	40.84	2.04	1.16
L	50.10711, 9.878093	32.54	67.46	0.00	1.22

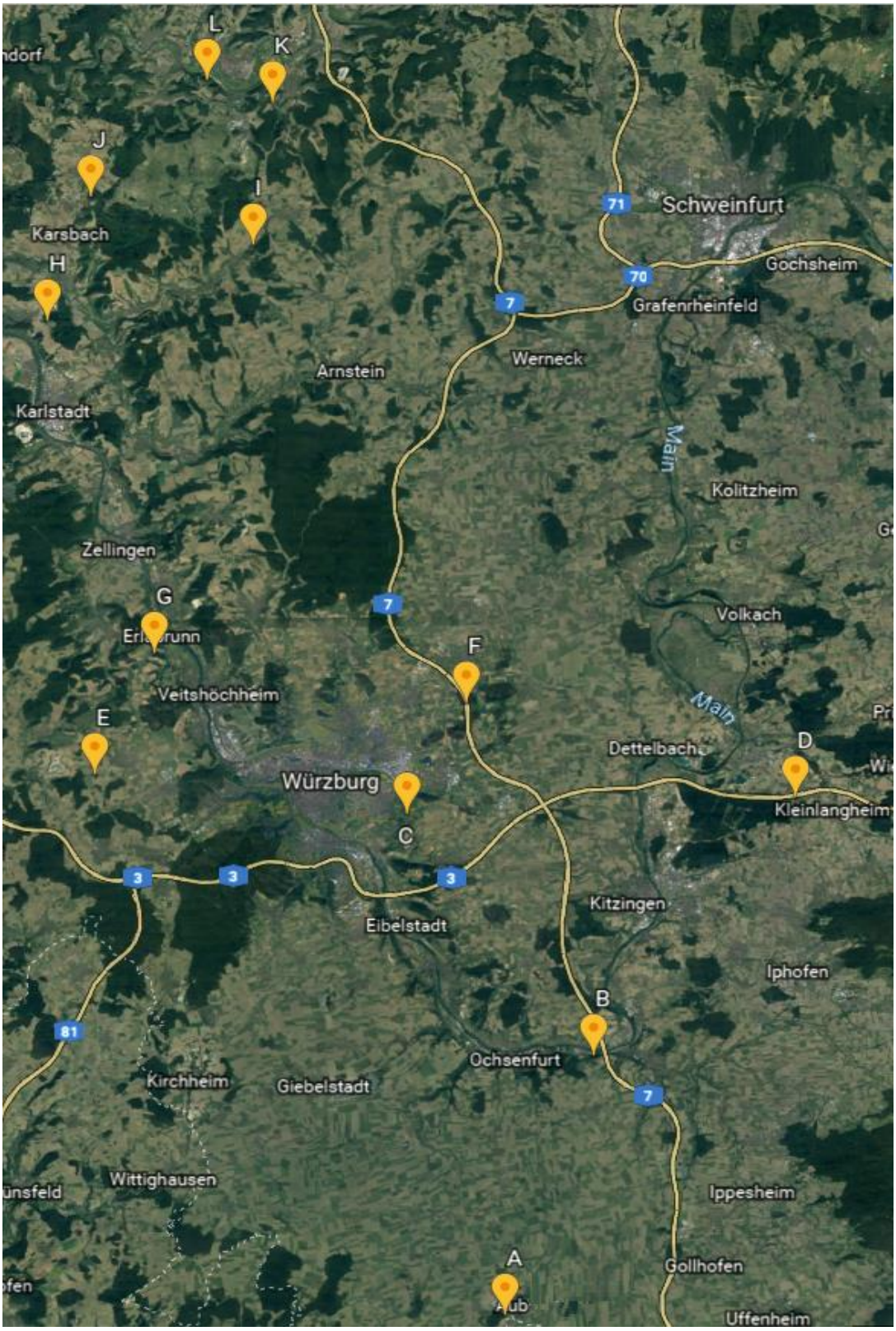

Figure 3. Geographic locations with established sampling sites

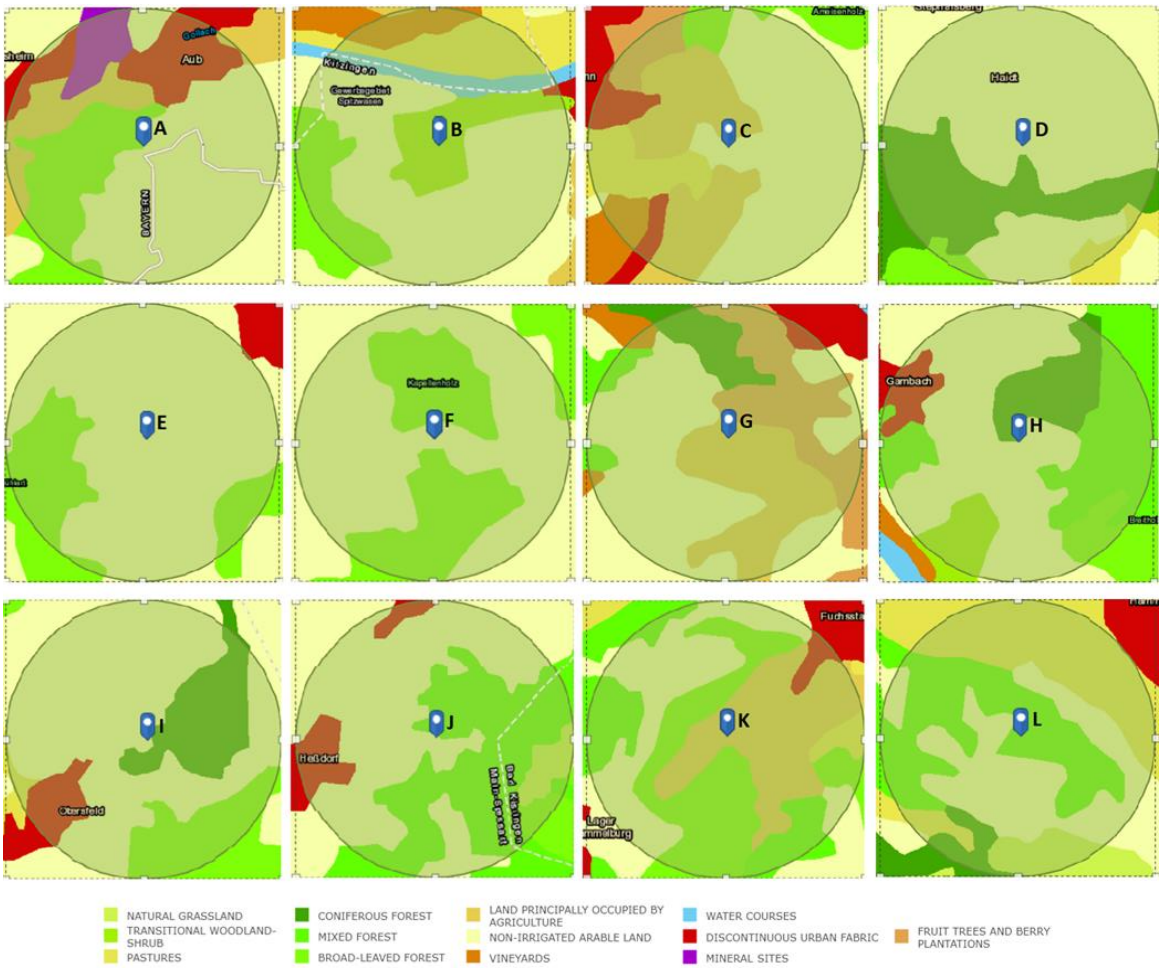

Figure 4. Land cover information for a radius of 1km around each sampling site

2.1.3. Laboratory entry and handling of the sampled material

Reed canes with clogged entrance were considered as occupied and were directly transferred into the lab. The date of sampling and place of origin of each nest was recorded, before they were opened in order to remove the larvae, pollen and material used for nest chamber construction. Nests were kept and opened horizontally in order not to disrupt the larvae. Nest chambers were marked as a) containing healthy larvae and not visibly affected by pathogens or as containing diseased and dead individuals and b) according to the solitary bee species they belonged to. Open nests belonging to different solitary bee species are shown in **Image 2**. Samples were taken for three main purposes: a) for bacterial cultures b) for *in vitro* rearing and experimental manipulation of larvae and c). for inclusion in DNA metabarcoding with next generation sequencing (NGS).

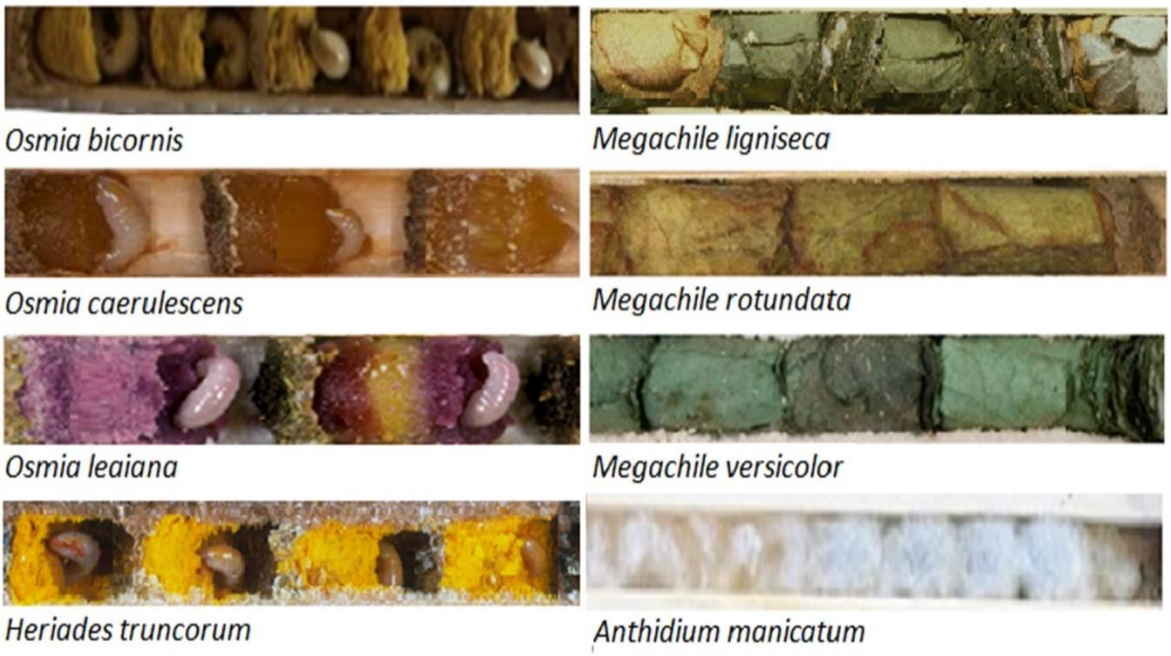

Image 2. Open solitary bee nests belonging to eight different species

2.1.3.1. Sample preparation for bacterial cultures

The mason bee *O. bicornis* and the leaf cutter bee *M. rotundata*, which were sampled in abundance in early summer 2016, were chosen for the selection of environmental samples which would be included in bacterial cultures. 99 samples taken from bee larvae, pollen provision and nest material were transferred with the use of sterile tweezers into sterile 1.5 ml eppendorf tubes. Glycerol was immediately added in the environmental material and the tubes were kept at -80°C. After samples were thawed, they were included in a series of microbial cultures with the aim of distinguishing morphologically different bacterial colonies and identifying the selected strains with 16S rDNA Sanger sequencing.

2.1.3.2. Sample preparation for in vitro rearing

O. bicornis was the most abundant bee species sampled in late Spring and early Summer of 2016. After measuring the length of all larvae, the 31 *O. bicornis* nests which included the smallest larvae in size were chosen. The size of the larvae was used as an indication to find the most recently constructed nests. The smallest female larvae (all <3 mm in size) from 31 chosen nests with healthy larvae were selected and transferred into sterile 48 well plates (Becker and Keller 2016).

2.1.3.3. Sample preparation for next generation sequencing

The contents of at least one nest chamber per sampled nest, containing a female bee larva, was chosen to be stored for NGS. After the opening of each nest, the pollen provision, the nest material and the larva of each chosen nest chamber were removed with the use of sterile tweezers and were transferred into sterile 1.5 ml eppendorf tubes. The tubes were then immediately frozen down at −25°C. Furthermore, after the *in vitro* manipulation of the 31 larvae (see *2.1.3.2.*), all larvae were stored into sterile 1.5 ml eppendorf tubes at -25°C, as well.

2.2. Laboratory workflow

All specimens from the sampled nests were included in three main experimental procedures. These procedures were: DNA metabarcoding, bacterial cultures and manipulation of living larvae. The species *Anthidium manicatum* was excluded because only one nest from this species was sampled and the larvae in its interior had already consumed the pollen provision. The numbers of nest chambers used for each laboratory procedure are presented in **Table 2**. From the 82 *O. bicornis* nest chambers which were included in 16S rDNA metabarcoding (**Table 2**), twelve contained deceased larvae which were not affected by fungi, parasitic insects or nematodes. Also, three out of the 24 *O. bicornis* larvae which were selected for the bacterial cultures were deceased at the time of the nest opening.

2.2.1. Bacterial cultures

Environmental samples were taken out of the deep freeze (-80°C) and were left to thaw in ice. Approximately 10 mg of each environmental specimen was added in 1ml of sterile physiological solution (0.9% NaCl) and was thoroughly but not vigorously mixed. Four different protocols were applied to achieve efficient bacterial growth and were implemented to isolate bacteria both selectively and non- selectively. Also, the selected culture growth conditions targeted both aerobic and facultatively anaerobic bacteria. All bacterial media were autoclaved prior to use and all bacterial cultures were prepared under sterile conditions. All the used protocols for the bacterial cultures are summarized in **Table 3**.

Table 2. Specimens included in all parts of the laboratory workflow.

Bee species	Material	16S rDNA metabarcoding	ITS2 rDNA metabarcoding	Bacterial cultures	*in vitro* rearing and manipulation of larvae
H. truncorum	larvae	43	-	-	-
	pollen	43	35	-	-
	nest material	21	-	-	-
M. ligniseca	larvae	8	-	-	-
	pollen	8	8	-	-
	nest material	8	-	-	-
M. rotundata	larvae	21	-	9	-
	pollen	20	20	9	-
	nest material	21	-	9	-
M. versicolor	larvae	4	-	-	-
	pollen	4	4	-	-
	nest material	4	-	-	-
O. bicornis	larvae	82	-	24	31
	pollen	82	21	24	-
	nest material	82	-	24	-
O. caerulescens	larvae	8	-	-	-
	pollen	8	8	-	-
	nest material	8	-	-	-
O. leaiana	larvae	4	-	-	-
	pollen	4	4	-	-
	nest material	4	-	-	-

Table 3. Overview of applied protocols for bacterial cultures

	Protocol A.	Protocol B.	Protocol C.	Protocol D.
Liquid culture	none	LB broth	none	MRS broth
		32°C		35°C
		72 hours		120 hours
		aerobic conditions		anaerobic conditions
Agar plate culture	Luria agar	Luria agar	MRS agar	MRS agar
	32°C	32°C	35°C	35°C
	96 hours	48 hours	168 hours	48 hours
	aerobic conditions	aerobic conditions	aerobic conditions	anaerobic conditions

Firstly, 100 µl of each resulting solution was directly plated on Luria agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) plates, without prior enrichment and was incubated at 32°C for 96 hours under aerobic conditions. Luria agar is recommended for non selectively isolating bacteria in low culture temperatures (Bertani 1951). The second protocol included microbial enrichment, by adding 100µl of each specimen solution in 100ml vials containing 10 ml of LB broth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The vials were closed with aluminum foil to avoid contamination and placed in a horizontal shaker set at speed of 120rpm at 32°C for 72 hours. Shaking of the vials is necessary for ensuring aerobic conditions of the culture. After this procedure, another 100µl from each liquid culture was then plated onto LB agar plates and incubated at 32°C for 48 hours.

Another approach was oriented towards selectively growing environmental lactic acid bacteria. 100µl of each selected specimen solution was applied on MRS agar plates (Otto Nordwald GmbH, Hamburg, Germany), which were then incubated under anaerobic conditions at 35°C for 7 days. MRS medium[i] was originally designed to favor the growth of *Lactobacilli* in extensive laboratory studies (De Man and Rogosa 1960). Anaerobic conditions were achieved by using the anaerobic sachets of the BD Gas-Pak System (BD,

Franklin Lakes, New Jersey, USA). Another version of the protocol included an extra step of bacterial enrichment in 10 ml of MRS broth (Sigma-Aldrich, St. Louis , USA). Anaerobic conditions in the vials with the MRS broth were achieved with the use of oxyrase for broth (Sigma-Aldrich, St. Louis, USA). After incubation at 35°C for 5 days, 100μl from each liquid culture was plated onto MRS agar plates and incubated at 35°C for 48 hours.

Different grown colonies from each specimen were carefully selected after incubation and were transferred with streaking onto plates of the same medium to grow for an additional time period of maximum 48 hours under the same conditions. Strains were isolated from all specimens. When necessary, colonies were picked and transferred again until pure colonies were obtained. Pure colonies were carefully examined so as to select those which most probably represented different bacterial taxa. A number of 15 colonies were selected and streaked again onto agar plates where they were left to grow under optimal conditions.

All selected aerobic bacterial strains were co-cultured with mold of the genus *Aspergillus*, which grew on several of the culture plates. Co-cultures were made to test the bacterial strains for potential antifungal properties. Bacterial strains showing possible antifungal activity were transferred in liquid cultures. Liquid cultures were filtered after three days with polycarbonate membranes (Thomas Scientific, Swedesboro, New Jersey, USA) and 10 μl of the remnant liquid was plated on petri dishes with grown fungi, to observe possible fungal inhibition.

Furthermore, all selected strains were tested on the Vitek 2 platform (BioMérieux, Marcy-l'Étoile, France), at the facilities situated at the environmental microbiology lab of the Institute of Biology of the University of Neuchâtel, to characterize substrate use and measure various metabolic activities such as acidification, alkalinization and enzyme hydrolysis. More specifically, the selected strains were tested for utilization of D-amygdalin, D-xylose, cyclodextrin, D-galactose, D-ribose, D-sorbitol, lactose, D-maltose, D-mannitol, D-mannose, D-raffinose, salicin, saccharose/sucrose, D-trehalose and for ability of growth in 6.5% NaCl. Also, they were tested for enzymatic activity for phosphatidylinositol phosphatase C, arginine dihydrolase 1, beta-galactosidase, alpha-glucosidase, Ala-Phe-Pro Arylamidase, L-aspartate arylamidase, beta galactopyranosidase,

alpha-mannosidase, phosphatase, leucine arylamidase, L-proline arylamidase, beta glucuronidase, alpha-galactosidase, L-pyrrolydonyl-arylamidase, beta-glucuronidase, alanine arylamidase, tyrosine arylamidase, urease, L-lactate alkalinization, N-acetyl-D-glucosamine and arginine dihydrolase 2.

Total DNA from the 15 selected colonies was extracted with the use of the Macherey Nagel NucleoSpin Microbial DNA commercial kit. A commonly used set of primers (16S_27f, 16S_1492r) (Frank *et al.* 2008) was used for the amplification of a fragment of the 16S rDNA. The PCR product was purified with the use of Macherey Nagel NucleoSpin Gel and PCR Clean-up commercial kit. The aforementioned forward primer was also used for the Sanger sequencing of the amplified area. Sanger sequencing was performed by Eurofins Genomics, Ebersberg, Germany. Taxonomic assignment of the received sequences was made by searching the best matched hits in the EzBioCloud 16SrDNA-based database (Yoon *et al.* 2017).

2.2.2. *In vitro* rearing and manipulation of larvae

The larvae which were transferred into sterile 48 well plates had a mean size of 2.4 mm at the time of the transfer. They were divided into three groups as summarized in **Table 4**. Eleven of the larvae were placed on pollen clumps retrieved from one *O. bicornis* nest without any further treatment (groupA). Ten larvae were placed on pollen clumps which had been treated with oxytetracycline (group B). Another ten were placed on pollen clumps which were first sterilized and then inoculated with a bacterial solution which contained a *Bacillus* sp. strain at 0.1 OD (group C). The *Bacillus* sp. strain had been previously isolated from one sampled deceased *O. bicornis* larva on Luria agar. The optical density of the used bacterial solution was measured with the use of the ELx808™ Absorbance Microplate Reader (Biotek, Vermont, USA).

After the larvae fed on the manipulated provisions for five days, they were transferred with the use of sterile tweezers into sterile 1.5 ml eppendorf tubes and stored at -25°C. Six out of the ten larvae of group B died on the fourth day of the experiment and were therefore removed and stored at -25°C one day earlier than the rest. All larvae along with part of the pollen, on which larvae of group A fed on, were later included in next generation sequencing for the 16S rDNA (see *section 2.2.3.*).

Table 4. *In vitro* rearing of *O. bicornis* larvae and treatment of their pollen provisions

Treatment	Number of larvae	Pollen provision
A	11	untreated pollen from an *O. bicornis* nest
B	10	sterile, treated with oxytetracycline
C	10	sterilized and inoculated with *Bacillus* strain (0.1 OD solution)

2.2.3. DNA metabarcoding

Laboratory workflow for DNA metabarcoding included genomic DNA isolation from mixed environmental samples, library preparation, indexing, quality control, normalization, pooling, quantification and sequencing.

2.2.3.1. DNA extraction

Genomic DNA from each of the specimens was isolated using the Macherey-Nagel Nucleospin (Düren, Germany) kits for Food and Soil (Burbach *et al.* 2016), following a protocol modified to better handle hard-to-lyse bacterial cell walls with an extra step of incubation with *proteinase K*. Original material was mechanically homogenized prior to the cell lysis step. After the whole genomic DNA extraction, the lab procedure continued to the PCR amplification of the *16S rRNA* gene for all selected larvae, pollen and nest material samples and to the PCR amplification of the *ITS2* gene for all pollen samples.

2.2.3.2. Library preparation for the 16S rDNA

After the acquisition of whole genomic DNA, the PCR amplification of the 16S ribosomal DNA was based on the Illumina platform (Illumina 2013; Illumina 2017) for 16S rDNA meta-barcoding (**Table 5**). The dual-indexing strategy suggested by Kozich *et al.* (2013) was followed to generate a pooled amplicon library based on the V4 variable region of the gene. The used dual-indexing strategy for multiplexing included the following primers to amplify the V4 region: AATGATACGGCGACCACCGAGATCTACAC [8bp-i5 index]

ATGGTAATTGTGTGCCAGCMGCCGCGGTAA and CAAGCAGAAGACGGCATACGAGAT [8bp-i7 index] AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT (Illumina 2016a).

To reduce random effects, PCR reactions were conducted in triplicates with 1 µl of template DNA in each reaction. New England Biolabs (UK) PCR Master Mix, along with the two indexed primers in a unique combination for each sample and an appropriate quantity of PCR grade dH2O were used for every reaction, along with two indexed primers in a unique combination for each sample and an appropriate quantity of PCR grade dH2O. PCR conditions were adjusted according to the primers guidelines (Kozich *et al.* 2013). Samples were initially denaturated at 95°C for 2 minutes, then amplified by using 30 cycles of 95°C for 20 seconds, 55°C for 15 seconds and 72°C for 5 minutes. A final extension (72°C) of 10 minutes ensured complete amplification. After the end of the reaction, triplicates of each reaction were combined and successful amplification was checked with the use of gel electrophoresis in a 1% agarose gel.

2.2.3.3. Library preparation for the ITS2 rDNA

The same dual-indexing strategy introduced by Kozich *et al.* (2013) was used in order to generate the pooled amplicon library for the ITS2 rDNA region used for pollen metabarcoding (**Table 5**). A combination of plant barcoding primers expanded for Illumina conformity were used, as described in Sickel *et al.* (2016). The primer sequences were: AATGATACGGCGACCACCGAGATCTACAC [8bp -i5 index] CCTGGTGCTGGTATGCGATACTTGGTGTGAAT and CAAGCAGAAGACGGCATACGAGAT [8bp -i7 index] AGTCAGTCAGCCTCCTCCGCTTATTGATATGC-3'. The primers amplify a total fragment of approximately 470–480 bp, including the complete ITS2 sequence, enabling safe plant identification up to species level.

PCR reactions were conducted in triplicates with 1 µl of template DNA in each reaction. New England Biolabs (UK) PCR Phusion Master Mix, along with the two indexed primers in a unique combination for each sample and an appropriate quantity of PCR grade dH2O were used for every reaction. PCR conditions were adjusted according to the primers guidelines. For the 16S rDNA, samples were initially denatured at 95 °C for two minutes, then amplified by using 30 cycles of 95 °C for 20 seconds, 55 °C for 15 seconds and 72 °C for 5 minutes. A final extension (72 °C) of 10 minutes ensured complete amplification. For

the ITS2 rDNA, samples were initially denatured at 95 °C for four minutes, then amplified with 37 cycles of 95 °C for 40 seconds, 49 °C for 40 seconds and 72 °C for 5 minutes. For final extension the program ended with a step of 72 °C for 10 minutes. After the end of the reaction, triplicates of every reaction were combined and PCR success was checked through gel electrophoresis in a 1% agarose gel.

Table 5. Protocols for the PCR amplifications of the 16S rDNA and the ITS2 rDNA for library preparation for amplicon sequencing on the Illumina Miseq platform.

Primers used for library preparation			
16S rDNA	primer 1	AATGATACGGCGACCACCGAGATCTACAC [8bp- i5 index] ATGGTAATTGTGTGCCAGCMGCCGCGGTAA	
	primer 2	CAAGCAGAAGACGGCATACGAGAT [8bp-i7 index] AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT	
ITS2 rDNA	primer 1	AATGATACGGCGACCACCGAGATCTACAC [8bp -i5 index] CCTGGTGCTGGTATGCGATACTTGGTGTGAAT	
	primer 2	CAAGCAGAAGACGGCATACGAGAT [8bp -i7 index] AGTCAGTCAGCCTCCTCCGCTTATTGATATGC-3'	
i5 Illumina indices		SA501-SA508 and SB501-SB508	
i7 Illumina indices		SA701-SA712 and SB701-SB712	
PCR conditions			
Initial denaturation		95°C	2 minutes
30 cycles		95°C	20 seconds
		55°C	15 seconds
		72°C	5 minutes
Final extension		72°C	10 minutes

2.2.3.4. DNA normalization and sequencing

The DNA amount was normalized between samples of each library using the Invitrogen SequalPrep Plate Normalization Kit (ThermoFisher Scientific, Life Technologies, Carlsbad, CA, USA). The BioAnalyzer 2200 (Agilent, Santa Clara, USA) with High Sensitivity DNA Chips was used for verification of fragment length distributions. The final pool was also quantified

using a Qubit II Flurometer and the dsDNA High-Sensitivity Assay Kit (ThermoFisher Scientific, Life Technologies, Carlsbad, CA, USA).

The final library pools were loaded into 500 cycle reagent Illumina Miseq cartridges (500 cycle, v2) along with the respective read 1 and read 2 sequencing primers. Since the MiSeq requires base diversity on every cycle, libraries were loaded with 5% PhiXv3, a control library for Illumina sequencing runs (Illumina 2016b). All samples were sequenced in-house on a Miseq platform in the Department of Human Genetics of the University of Würzburg, Germany.

2.3. Bioinformatic analysis

Next generation sequencing raw data were processed in order to produce two types of files for all downstream analyses. 16S rDNA data and ITS2 rDNA data were processed in order to construct two tables with community composition data for bacterial OTUs and plant species, respectively. After the acquisition of the community composition data, the composition tables were included along with the metadata for all the samples in further analysis. All the used bioinformatic tools are listed in **Table 6**.

2.3.1. Process of Illumina Miseq raw sequencing data

2.3.1.1. 16S rDNA data

After the data from the Illumina Miseq sequencing were acquired, *fastq-join* (Aronesty 2013) was used to join paired ends pairwise alignments of forward and reverse reads. Paired reads were accepted if longer than 250bp. *USEARCH* was used for length truncating, quality filtering and file conversion. Chimera filtering, operational taxonomic unit (OTU) clustering to a minimum identity of 97% and OTU table construction were performed with *USEARCH* (Edgar 2013; Edgar 2016). Data were restricted to high quality reads by filtering low quality reads after setting the maximum number of expected errors at *Emax*=1 (Edgar and Flyvbjerg 2015). Reads with ambiguous characters or singletons were excluded from the downstream analyses. Taxonomy was assigned for the *de novo* picked OTUs of the 16S rDNA library using the *RDP v16* reference database with an identity cut-off threshold of 97%.

Table 6. Tools used in bioinformatic analysis

Software		Description	Reference
fastq-join		joins two paired-end sequencing reads on the overlapping ends	Aronesty 2013
USEARCH		searches database for top global hits and combines sequence analysis software with processing features such as quality filtering and chimeric sequence filtering.	Edgar 2013; Edgar 2016
VSEARCH		alternative to USEARCH for greater accuracy and handling of large databases	Rognes *et al.* 2016
R 3.2.4.		software environment for statistical computing and graphics	R core 2017
R packages	ggplot2	graphics creation	Wickham 2009
	phyloseq	analysis of microbiome census data	McMurdie and Holmes 2013
	dplyr	data manipulation	Wickham *et al.* 2018
	bipartite	bipartite networks visualization and calculation of ecological indices	Dormann *et al.* 2009
	microbiome	analysis of microbiome profiling data	Lahti and Shetty 2017
	Hmisc	data analysis, sample size computation, missing values imputation and variable clustering	Harrell 2017
	GGally	pairwise plots, parallel coordinates plots and network plots construction	Emerson *et al.* 2013
	reshape2	data transformation	Wickham 2007
	vegan	ordination methods, diversity analysis and functions for community ecology	Oksanen *et al.* 2013
	cooccur	probabilistic species co-occurrence analysis	Griffith *et al.* 2016
	lme4	fit linear and generalized linear mixed-effects models computation	Bates *et al.* 2015

	lmerTest	conduction of tests in linear mixed effects models	Kuznetsova *et al.* 2016
	GMD	non-parametric distance measurement between two discrete frequency distributions	Zhao & Sandelin 2012
	varSelRF	variable selection using random forests	Diaz-Uriarte 2007
	randomForest	Breiman and Cutler's random forests for classification and regression	Liaw and Wiener 2002
	MASS	functions for distribution exploration, econometrics, environmetrics and multivariate numerical mathematics	Ripley 2011
	igraph	network analysis and visualization	Csardi and Nepusz 2006
SparCC		python module for computing correlations of compositional data in metagenomics	Friedman and Alm 2012

2.3.1.2. ITS2 rDNA data

In the case of the acquired ITS2 rDNA dataset, only the forward reads were kept for downstream analysis, as reverse reads showed less satisfying quality. The reads with high expected error rate or ambiguous characters were filtered following the same parameters as described above for the bacterial dataset, and low quality bases at the read ends were trimmed (<Q30). Reads were accepted if longer than 150 bases. The ITS2 rDNA reads were directly mapped against a Bavarian floral reference database for ITS2 (Keller et al. 2015) derived from the ITS2-database (Ankenbrand et al. 2015) with VSEARCH (Rognes et al. 2016) using an identity cut-off threshold of 97% and global alignments.

2.3.2. Description of the bacterial composition and bacterial biodiversity

After the acquisition of the bacterial OTU table , data were further analyzed in *R 3.2.4*. (R core 2017). Figures visualizing results were constructed with the package *ggplot2* (Wickham 2009). The OTU table with all samples was filtered to exclude OTUs annotated as chloroplasts or mitochondria with the package *phyloseq* (McMurdie and Holmes 2013)and final filtered samples were further used if they had more than 1000 remaining reads.

After filtering, rarefaction curves, bacterial OTU-richness (plain total number) and bacterial OTU Shannon diversity were computed for all samples (Shannon 1948; Whittaker 1972; Magurran 2004) with *phyloseq* (McMurdie and Holmes 2013). Analysis of variance (ANOVA) was performed for Shannon index values of all larvae and pollen samples setting the bee species as the source of variation, to detect differences in levels of biodiversity between bee host species. Wilcoxon and t-tests were conducted to compare the means of bacterial OTU richness for larvae, pollen and nest material samples for each species.

Data were relativized and differences in bacterial community composition were visualized for all specimens with Bray–Curtis based non-metric multidimensional scaling (NMDS) of OTU identities with *phyloseq* (McMurdie and Holmes 2013). Clusters were identified and were associated with bee species and sample type. To describe the bacterial communities related to each bee host species and sample type, the text refers to relative abundances of bacterial taxa, which represent the contribution of the 16S rDNA reads. Barplots describing taxonomic composition were constructed with *dplyr* (Wickham *et al.* 2018) and ggplot2 (Wickham 2009). Revealed bacterial communities for each bee species were also visualized as bipartite network after low abundance filtering (>1%) with the package *bipartite* (Dormann *et al.* 2009).

Furthermore, taxa with consistent occurrence in specimen subsets defined by the host species and the type of the specimen were retrieved (Salonen *et al.* 2012) with the package *microbiome* (Lahti and Shetty 2017). The analysis was conducted with setting the parameter of OTU prevalence at 95% of all samples.

2.3.3. Correlation of larval size with relative abundances of several bacterial taxa

Relative abundances for each prevalent bacterial family as well as the Shannon index for each sample of the same bee host species were correlated with the measured larval size at the time of the opening of each nest, using the packages *Hmisc* (Harrell 2017) and *GGally* (Emerson *et al.* 2013). Spearman's coefficient *Rho* denotes the statistical dependence between the ranking of each two variables, assessing the grade that the selected variables are related according to a monotonic function.

2.3.4. Description of the plant species composition found in pollen provisions

All samples from the acquired plant composition table were checked to confirm they have more than 1000 reads after quality filtering. OTU richness and Shannon diversity estimation (Shannon 1948) for plant communities was performed with *phyloseq* (McMurdie and Holmes 2013) and was based on the number of assigned plant species for each pollen sample. Rarefaction curves and diversity graphs were constructed with *ggplot2* (Wickham 2009) and *reshape2* (Wickham 2007).

Beta diversity was visualized with *ggplot2* (Wickham 2009) using nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis distance matrices of plant species. Descriptions of plant communities related to each host species were based on the relative abundances of identified plant species for each specimen, which represent the contribution of the ITS2 rDNA reads assigned to each plant species. Revealed plant communities for each bee species were visualized as bipartite networks after low abundance filtering (>1%) with the package *bipartite* (Dormann *et al.* 2009).

2.3.5. Association of bacterial communities and pollen composition

Spearman's correlations were conducted between alpha diversity of bacterial OTUs and plant species with *Hmisc* (Harrell 2017). Furthermore, pollen and bacterial OTU Bray-Curtis distance matrices were compared with Mantel tests based on Pearson's product correlation to explore the degree of association between them (Legendre and Legendre 1998) with *vegan* (Oksanen *et al.* 2013).

Furthermore, co-occurrence patterns between bacterial and plant taxa were investigated with the use of a probabilistic approach with the package *cooccur* (Griffith *et al.* 2016). This package defines the observed frequency of co-occurrence as positive, negative or random association (Veech 2012). The analysis was based on matrices of plant species and bacterial taxa agglomerated up to genus level, containing binary data to indicate the absence or existence of each taxon in each sample over a relative abundance threshold of 1%.

Furthermore, NMDS ordination coordinates of the datasets describing pollen composition, pollen bacterial community and larval bacterial community were included in mixed effect models to assess the causal variables, which shape the bacterial community structures in

pollen and larvae. Firstly, the NMDS coordinates for bacterial communities in larvae were set as dependent on the NMDS coordinates of pollen bacterial communities. Secondly, the NMDS coordinates for bacterial communities in pollen were tested as dependent on the NMDS coordinates of pollen composition. Finally, host species was included in all models as a categorical value and treated as a random variable. The statistical significance of the random effect was estimated with the method of single term deletion. The *lme4* package (Bates *et al.* 2015) was used for the construction of each model and the *lmerTest* package (Kuznetsova *et al.* 2016) was used for their evaluation.

Samples were grouped according a) to bee host species and b) to clusters depending on the pollen composition of the respective provision with the package *GMD* (Zhao & Sandelin 2012). Clustering of the plant species found in pollen provisions was conducted using k-means after cluster number selection according to the *Elbow* method (Kodinariya & Makwana 2013). Subsequently, random forest analysis was used to assign bacterial communities of pollen and larvae a) to host bee species and b) to pollen composition clusters and to estimate the significance of these factors for correct classification (Prasad *et al.* 2006; Junker *et al.* 2011; Junker and Keller 2015) with the packages *varSelRF* (Diaz-Uriarte 2007) and *randomForest* (Liaw and Wiener 2002). Confusion matrices demonstrate number of correctly assigned communities to either species or plant clusters as well as class error and total out-of-basket (OOB) error rate. To identify indicator bacterial OTUs per tested group, variable selection with the OOB error rate estimate set as a minimization criterion was used.

2.3.6. Association of bacterial communities and type of the nesting material

To investigate the effect of the nesting material type on the bacterial communities of the larvae, the computation of Bray–Curtis dissimilarities between samples was conducted with the package *vegan* (Oksanen *et al.* 2013). The permutational multivariate analysis of variance (PERMANOVA/Adonis) to test the homogeneity between group levels for larvae, by setting the type of the nesting material as discriminant factor was also conducted with *vegan*. A pre-requisite of PERMANOVA is that the multivariate spread among different groups is not statistically different. Therefore, the homogeneity of variances among groups was estimated with betadisper, a multivariate analogue of Levene's test (Levene 1960).

Random forest analysis was used in order to assign bacterial communities of nest material to the type of nesting material and to estimate the significance of the nest material type for correct group classification (Prasad *et al.* 2006; Junker *et al.* 2011; Junker and Keller 2015). The random forest analysis was implemented with the packages *varSelRF* (Diaz-Uriarte 2007) and *randomForest* (Liaw and Wiener 2002). To identify indicator bacterial OTUs per tested group, a variable selection was performed with the OOB error rate estimate set as a minimization criterion.

Furthermore, Spearman's correlations between alpha diversity of bacterial OTUs in the nest material and the larvae were conducted with the package *Hmisc* (Harrell 2017). Nest material and larval bacterial OTU Bray-Curtis distance matrices were compared with Mantel tests based on Pearson's value to explore if they are significantly corelated, with the package *vegan* (Oksanen *et al.* 2013).

2.3.7. Pathogen screening

Anova tests were conducted to compare the means of OTU richness and diversity for healthy and deceased *O. bicornis* larvae, estimated with *phyloseq* (McMurdie and Holmes 2013). Data were relativized and differences in community composition were visualized with Bray-Curtis based non-metric multidimensional scaling of OTU identities (NMDS) with *ggplot2* (Wickham 2009) and *phyloseq* (McMurdie and Holmes 2013). Differences in bacterial community composition between healthy and deceased larvae were also visualized with linear discriminant analysis (LDA) with the package *MASS* (Ripley 2011).

In an overview of the bacterial communities related to either healthy or deceased larvae, the mentioned relative abundances of bacterial taxa, represent the contribution of the 16S rDNA reads assigned to each mentioned taxon. Bacterial OTUs were agglomerated up to genus level (family level if not better classifiable) and the barplots describing taxonomic composition were constructed with *dplyr* (Wickham *et al.* 2018) and *ggplot2* (Wickham 2009).

To explore possible connections between the most abundant bacterial OTUs in healthy and also in deceased larvae, interaction networks were constructed with the program *SparCC*, which uses OTU abundances in samples of a dataset to identify correlations (Friedman and

Alm 2012) between them. 1000 bootstrap replicates were applied to calculate significance values and correlation coefficients were included in the network only if they were greater or less than 0.3 and -0.3, respectively with p-values less than 0.001. OTU networks in each dataset was visualized with the package *igraph* (Csardi 2008).

The influence of the pollen treatment on the larval bacterial microbiome was tested with linear discriminant analysis with treatment type set as the discriminative class. Linear discriminant analysis (LDA) was conducted with the package *MASS* (Ripley 2011) and estimated the effect of each implemented treatment on the bacterial community of all *in vitro* reared larvae.

2.3.8. Investigation of the landscape effect on the bacterial communities

To investigate the effect of landscape on the bacterial communities, two different metrics were used. First, all sampling sites were assigned to two landscape types. The first type included nine sites mainly occupied by agricultural activities and the second included three sites mainly occupied by semi-natural vegetation within a radius of 1 km around the established trap-nests (**Table 1**). Anthropogenic environment was ignored as non-interfering with the type of landscape, since it stands for artificially surfaced ground like wooden constructions for pasture, asphalted country roads and in one case, one recreational camping site (Site ID: A). Second, the procedure of Redlich, Martin and Steffan-Dewenter (2018) was followed in order to calculate Shannon's diversity of land use types, within a radius of 1 km around each established trap-nest. Land use categories as different main habitat types in the respective landscapes and calculated Shannon's diversity based on the relative cover of each land use. The minimum Shannon value was 0.50 with a maximum to 1.42. Sampling sites were then separated into two groups of landscape diversity. The first group included five localities with a landscape Shannon's diversity of less than one and the second included seven localities with a Shannon index value of more than one.

Bray–Curtis dissimilarities between samples and permutational multivariate analysis of variance (PERMANOVA/Adonis) were computed with the package *vegan* (Oksanen *et al.* 2013). PERMANOVA was used to test the homogeneity between group levels for larvae and

pollen separately, by setting landscape type and landscape diversity as independent factors. Homogeneity of variances among groups with betadisper, a multivariate analogue of Levene's test (Levene 1960).

The datasets coming from the three sampling sites where more than one bee species was sampled were included in testing host species specificity of bacterial communities. Bray–Curtis distances between samples were computed and PERMANOVA was applied to test the homogeneity between groups for larvae and pollen separately, using host species as an independent variable.

The effect of locality on the shaping of the microbiota of all samples was investigated through Mantel correlation tests with the package *vegan* (Oksanen *et al.* 2013) for larvae and pollen specimens. Correlation of matrices used Bray–Curtis dissimilarities for all samples and geographical distances between sampling stations.

To test if landscape has an effect on the microbiome of larvae and pollen of a particular developmental stage *O. bicornis* samples were sampled according to larval size and a PERMANOVA/Adonis test was conducted, using landscape and region as independent factors. Sample groups were set as follows: (i) eleven chambers with larval length of 4.0 to 4.9 cm from seven sampling sites, (ii) 31 chambers with larvae length of 5.0 to 5.9 cm from eight sampling sites, (iii) eleven chambers with larva length of 6.0 to 6.9 cm from six sampling sites, (iv) seven chambers with larva length of 7.0 to 7.9 cm from four sampling sites, (v) six chambers with larva length of 8.0 to 8.9 cm from three sampling sites and (vi) four chambers with larval size of 9.0 to 9.5 cm from three sampling sites.

3. Results

3.1. Sequencing results

All samples which were sequenced for the V4 region of the bacterial 16S rDNA on the Illumina MiSeq platform generated on average 7307.22 high-quality reads per sample after quality and control filtering. All samples with fewer than 1000 remaining reads were excluded from all downstream analysis. Sequencing depth is plotted against the absolute bacterial OTU number and the bacterial OTU based Shannon diversity for every sample in **Figure 5** and information on the sequencing depth per specimen type is given in **Table 7**.

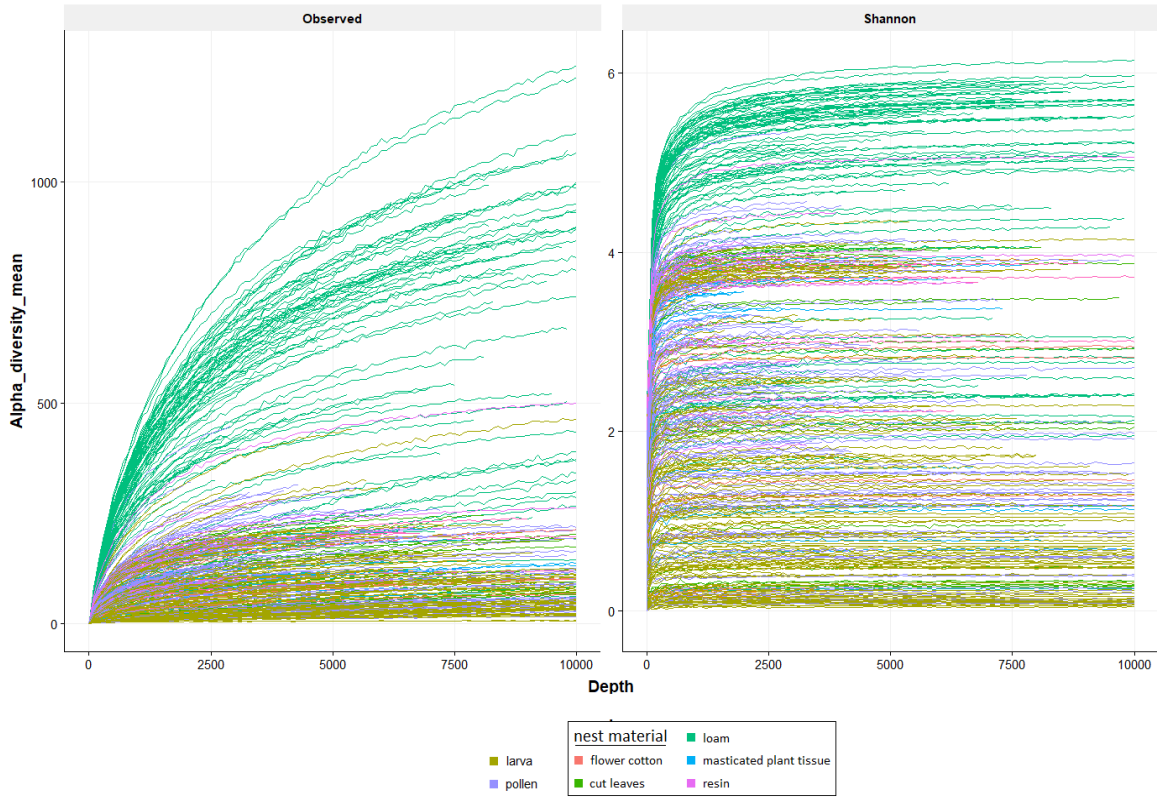

Figure 5. Rarefaction curves for 16S rDNA next generation sequencing based on absolute bacterial OTU richness and on bacterial OTU based Shannon diversity. The average number of OTUs and the respective Shannon diversity are plotted over the average number of sequence reads per specimen at a sampling pace of 10 reads. Sequencing depth is demonstrated for up to 10000 reads. The legend indicates the type of specimens for each sample.

Table 7. Information on the 16S rDNA sequencing depth per specimen type for all specimens after quality filtering.

Type of specimen		Number of specimens	Range of reads	Average of reads
larvae		188	1008-53729	7752
pollen		157	1006-29021	5317.8
nest material	cut leaves	24	1920-28558	11328
	flower cotton	4	14425-31486	22281
	loam	96	1726-16614	9306.4
	other plant tissue	19	1030-18505	4909.9
	resin	23	1386-11084	4087.7

All samples sequenced for the ITS2 rDNA on the Illumina MiSeq platform generated on average 13057 high-quality reads per sample after quality and control filtering (range: 2310 - 61846). Sequencing depth is plotted against the absolute plant species number and the plant species Shannon diversity for every sample in **Figure 6**.

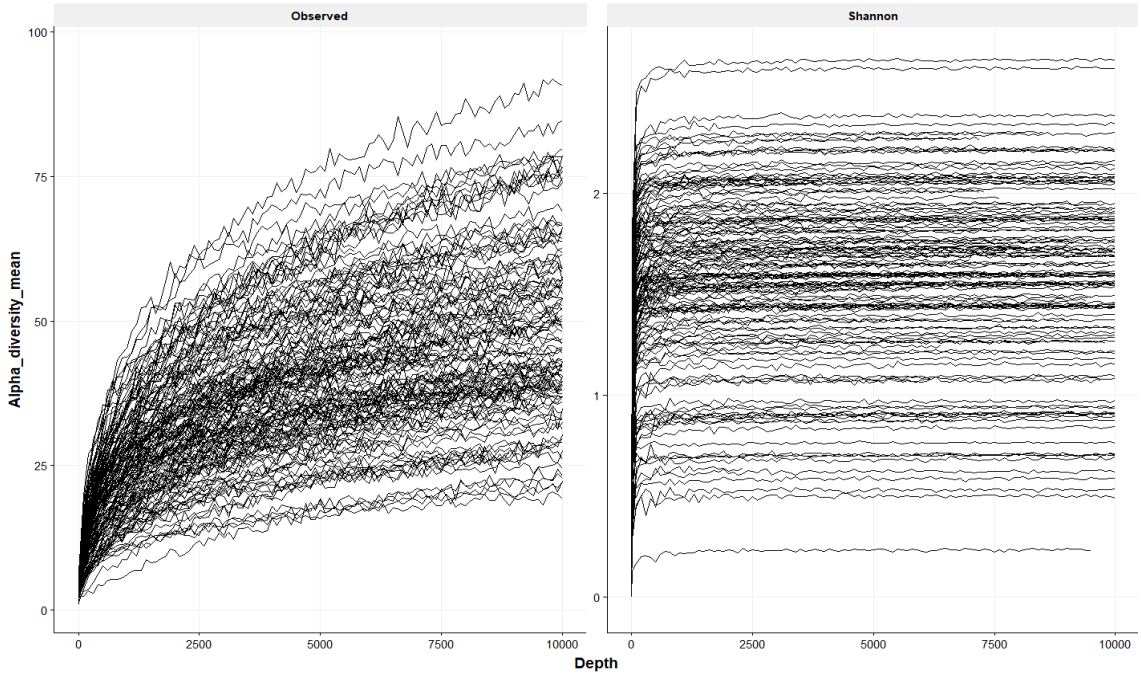

Figure 6. Rarefaction curves for ITS2 rDNA next generation sequencing based on absolute plant species richness and on plant species Shannon diversity. The average number of plant species and the respective Shannon diversity are plotted over the average number of sequence reads per specimen at a sampling pace of 10 reads. Sequencing depth is demonstrated for up to 10000 reads.

3.2. Comparative bacterial community description

Bee larvae, pollen provisioning and nest material specimens which derive from solitary bee nests with healthy larvae and were included in the 16S rDNA sequencing library (as shown in **Table 2**) returned 7267 bacterial OTUs. Nest material was loam for *O. bicornis*, cut leaves for all *Megachile* species, masticated plant tissue for *O. caerulescens* and *O. leaiana* and resin for *H. truncorum*. The NMDS ordination plot of all specimens is demonstrated in **Figure** 7.

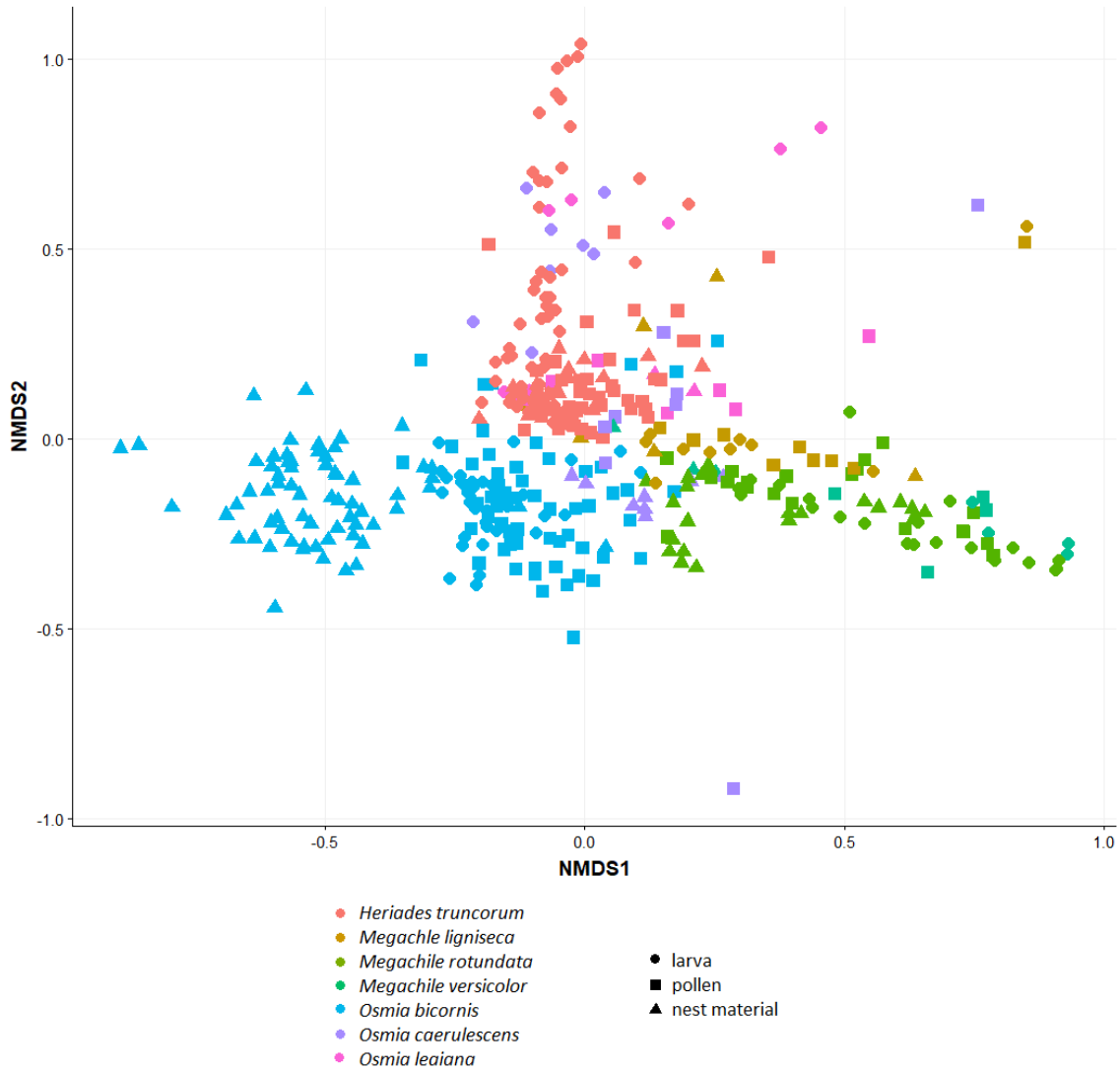

Figure 7. NMDS ordination of larvae, pollen and nest material specimens from seven solitary bee species nests. Sample points are coloured according to bee species and shaped according to type of specimen.

3.2.1. Bacterial diversity assessment

OTU richness and OTU based Shannon diversity values were higher for nesting material and particularly for loam (used by *O. bicornis*). Furthermore, the same diversity values were higher for pollen specimens than for the respective larvae. Wilcoxon test between all nest material and respective pollen samples returned statistically significant results both for absolute OTUs and Shannon values (*p<0.001****). In addition, the same tests returned a statistically significant difference in means (*p < 0.001****) for pollen and larvae (**Figure 8**). Spearman correlations for Shannon index values between bacterial communities of larvae and pollen were significant (Rho=0.38, *p<0.001****).

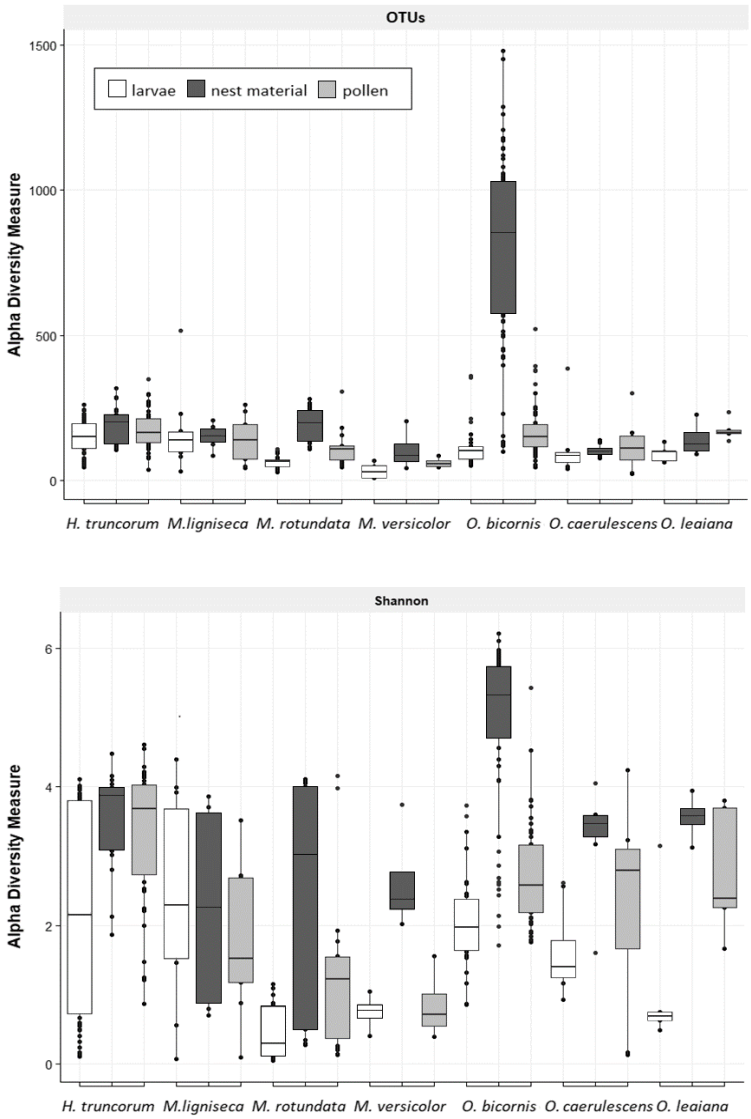

Figure 8. Up: OTU richness of bacterial communities in larvae, nest material and pollen samples for different solitary bee species. Down: OTU based Shannon diversity for the same specimens.

3.2.2. Bacterial community composition in the nests of different host bee species

The taxonomic composition of larval, pollen and nest material bacterial communities is summarized in **Figure 9**. Bacterial OTUs were agglomerated up to genus level. Taxonomy at family level is demonstrated when the respective OTU group was not better classifiable. Bacterial OTUs which were detected in all samples of a certain specimen category defined by the bee host species and the type of material are listed in **Table 8**.

Lactobacillus is the most abundant taxon in larvae and pollen of all three *Megachile* species included, while it occurs in the larvae and pollen of all species; however in lower relative abundances. *Fructobacillus* is also a genus containing lactic acid bacteria occurring in lower relative abundances in *Megachile* genera, while *Lactococcus* is the most abundant lactic acid bacterial genus in *O. leaiana* pollen provisions.

Bacillus and other aerobic Firmicutes is abundant in the nest material of *O. bicornis*, which is the only one consisting of loam and soil among the included bee species. A high bacterial biodiversity shown in **Figure 8**, interprets as many bacterial taxa contributing to the O. bicornis nest bacterial microbiome with low relative abundances (lower than 1%) and therefore, these taxa are not distinct in the respective barplot of **Figure 9**.

Gamma-proteobacteria consisted mostly of *Erwinia*, *Pseudomonas*, *Acinetobacter* and *Halomonas* and are represented in high relative abundance in all sample groups, while they are more prevalent in pollen than in larval bacterial communities. *Pseudomonas* occurs also in all types of nest material and particularly in those originating from plant tissues.

The genera *Rickettsia* and *Achromobacter* are highly abundant in *O. caerulescens* and *O. bicornis* larvae, respectively. The family of Acetobacteraceae, belonging to Alphaproteobacteria, is mostly found in pollen provisions of *H. truncorum*, *M. ligniseca* and *M. rotundata*. Enterobacteriaceae is a family highly abundant in the nesting material (cut leaves) of *M. ligniseca*. Finally, *Sphingomonas*, also belonging to Alphaproteobacteria, is abundant in the nest material of *M. rotundata* and *O. caerulescens*.

Sequences of the symbiotic/parasitic *Wolbachia* genus were highly abundant in *H. truncorum*, *O. caerulescens* and *O. leaiana* larvae. Finally, the endosymbiotic genus *Candidatus Portiera* was mostly found in *H. truncorum*.

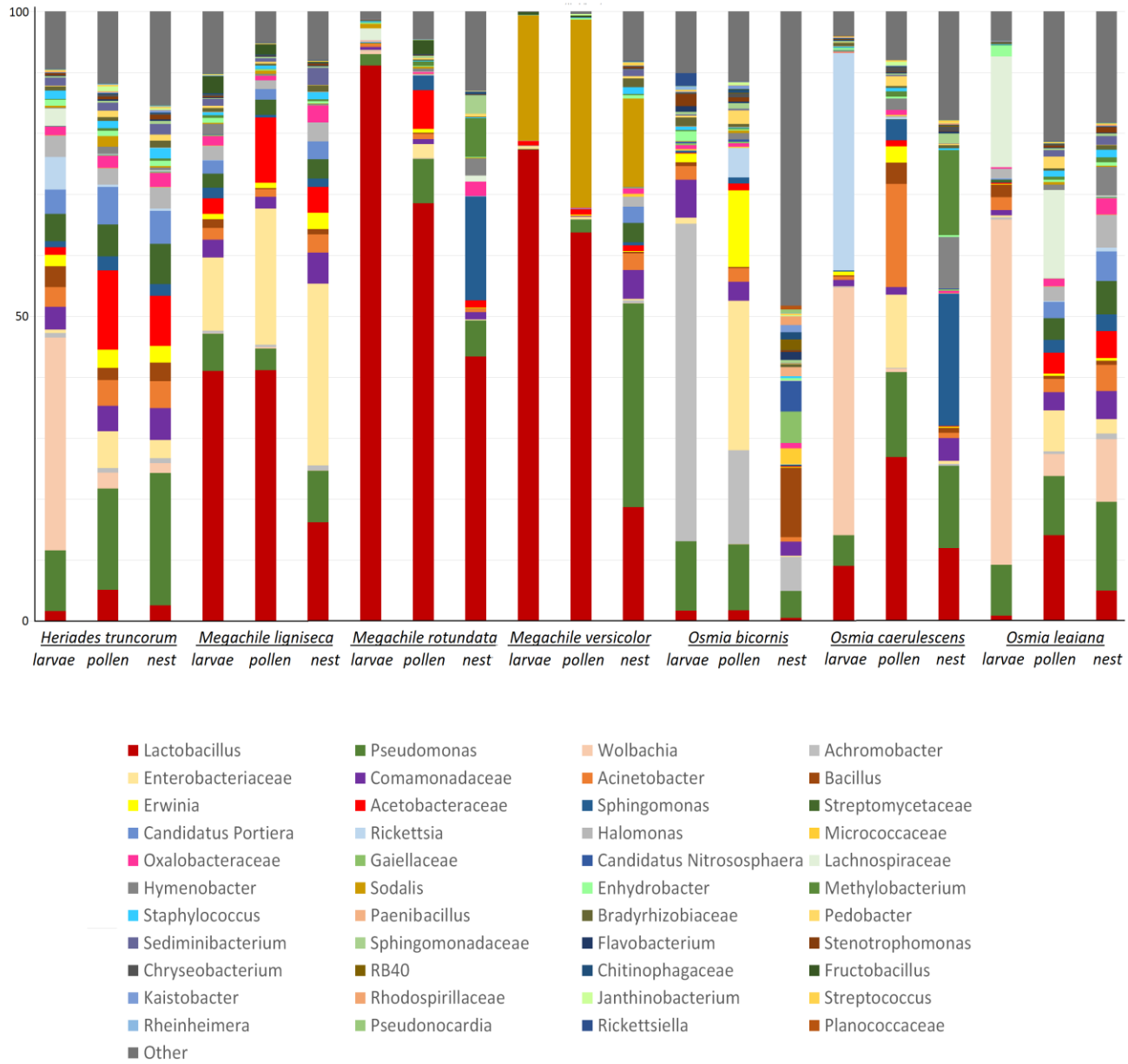

Figure 9. Bacterial profiles for seven solitary bee species nests. Relative abundances stand for the mean contribution in 16S rDNA sequence reads. Bacterial taxa up to genus level are included only if they were present with a ratio of >1% in the dataset. All the rest taxonomic identities are grouped under "other".

Table 8. Bacterial OTUs detected in all the samples of each specimen category defined by the host bee species and the material type.

	larvae	pollen	nest material
Heriades truncorum	OTU_2241_Comamonadaceae OTU_13_Pseudomonas OTU_58_Bradyrhizobiaceae OTU_24_Pseudomonas OTU_46_Acinetobacter	OTU_2241_Comamonadaceae OTU_31_Comamonadaceae OTU_13_Pseudomonas OTU_58_Bradyrhizobiaceae OTU_24_Pseudomonas OTU_46_Acinetobacter OTU_407_Erwinia OTU_7_Enterobacteriaceae OTU_26_Pseudomonas_viridiflava OTU_18_Sphingomonas OTU_45_Sphingomonas_echinoides OTU_52_Lactobacillus	OTU_10_Enterobacteriaceae OTU_441_Comamonadaceae OTU_14_Bacillus OTU_100_Caulobacteraceae OTU_72_Comamonadaceae OTU_384_Sphingomonadaceae OTU_48_Oxalobacteraceae OTU_448_Pseudomonas OTU_68_Xanthomonadaceae OTU_1790_Bradyrhizobiaceae OTU_34_Sediminibacterium OTU_1768_Bacillus_cereus OTU_73_Agrobacterium OTU_38_Acinetobacter OTU_130_Ochrobactrum OTU_496_Methylobacterium OTU_41_Sphingomonas OTU_28_Halomonas
Megachile ligniseca	OTU_5_Achromobacter OTU_2241_Comamonadaceae OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_48_Oxalobacteraceae OTU_7_Enterobacteriaceae OTU_2_Lactobacillus OTU_20_Acetobacteraceae	OTU_2241_Comamonadaceae OTU_13_Pseudomonas OTU_24_Pseudomonas OTU_46_Acinetobacter OTU_7_Enterobacteriaceae OTU_2_Lactobacillus OTU_38_Acinetobacter OTU_20_Acetobacteraceae OTU_28_Halomonas	OTU_5_Achromobacter OTU_31_Comamonadaceae OTU_138_Ralstonia OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_100_Caulobacteraceae OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_36_Staphylococcus OTU_407_Erwinia OTU_48_Oxalobacteraceae OTU_7_Enterobacteriaceae OTU_1790_Bradyrhizobiaceae OTU_1804_Comamonadaceae OTU_73_Agrobacterium OTU_41_Sphingomonas OTU_12_Sodalis OTU_5217_Oxalobacteraceae OTU_10707_Pseudomonas OTU_184_Phyllobacteriaceae OTU_46_Acinetobacter

Megachile rotundata	OTU_13_Pseudomonas OTU_169_Lactobacillus OTU_2_Lactobacillus OTU_12_Sodalis	OTU_5_Achromobacter OTU_13_Pseudomonas OTU_2_Lactobacillus OTU_26_Pseudomonas_viridiflava OTU_18_Sphingomonas	OTU_31_Comamonadaceae OTU_1558_Hymenobacter OTU_13_Pseudomonas OTU_58_Bradyrhizobiaceae OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_384_Sphingomonadaceae OTU_46_Acinetobacter OTU_407_Erwinia OTU_48_Oxalobacteraceae OTU_598_Hymenobacter OTU_93_Hymenobacter OTU_26_Pseudomonas_viridiflava OTU_128_Agromyces OTU_18_Sphingomonas OTU_38_Acinetobacter OTU_187_Methylocystaceae OTU_69_Methylobacterium OTU_496_Methylobacterium OTU_294_Methylosinus OTU_45_Sphingomonas_echinoides OTU_61_Sphingomonas OTU_41_Sphingomonas OTU_290_Sphingomonas OTU_107_Methylobacterium
Megachile versicolor	OTU_169_Lactobacillus OTU_2_Lactobacillus OTU_12_Sodalis	OTU_5_Achromobacter OTU_10_Enterobacteriaceae OTU_43_Enhydrobacter OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_169_Lactobacillus OTU_48_Oxalobacteraceae OTU_68_Xanthomonadaceae OTU_2_Lactobacillus OTU_271_Enterobacteriaceae	OTU_5_Achromobacter OTU_2241_Comamonadaceae OTU_31_Comamonadaceae OTU_43_Enhydrobacter OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_24_Pseudomonas OTU_36_Staphylococcus OTU_104_Lactobacillus OTU_46_Acinetobacter OTU_130_Ochrobactrum
Osmia bicornis	OTU_5_Achromobacter OTU_2241_Comamonadaceae OTU_31_Comamonadaceae OTU_37_Stenotrophomonas OTU_138_Ralstonia OTU_43_Enhydrobacter OTU_13_Pseudomonas OTU_82_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_46_Acinetobacter	OTU_10_Enterobacteriaceae OTU_31_Comamonadaceae OTU_13_Pseudomonas OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_384_Sphingomonadaceae OTU_407_Erwinia OTU_7_Enterobacteriaceae	OTU_31_Comamonadaceae OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_14_Bacillus OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_535_Paenisporosarcina OTU_16_Bacillus OTU_17_Micrococcaceae OTU_288_Intrasporangiaceae OTU_5_Achromobacter

Osmia caerulescens	OTU_5_Achromobacter OTU_31_Comamonadaceae OTU_138_Ralstonia OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_100_Caulobacteraceae OTU_24_Pseudomonas OTU_36_Staphylococcus OTU_46_Acinetobacter OTU_11_Rickettsia OTU_4_Wolbachia	OTU_13_Pseudomonas OTU_24_Pseudomonas OTU_2_Lactobacillus OTU_26_Pseudomonas_viridiflava	OTU_5_Achromobacter OTU_31_Comamonadaceae OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_839_Microbacteriaceae OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_384_Sphingomonadaceae OTU_407_Erwinia OTU_60_Methylobacterium_ad-haesivum OTU_2_Lactobacillus OTU_598_Hymenobacter OTU_93_Hymenobacter OTU_40_Hymenobacter OTU_400_Microbacteriaceae OTU_18_Sphingomonas OTU_69_Methylobacterium OTU_45_Sphingomonas_echinoides OTU_2457_Sphingomonas_wittichii OTU_191_Sphingomonas_wittichii OTU_155_Spirosoma OTU_61_Sphingomonas OTU_371_Sphingobacteriaceae OTU_41_Sphingomonas OTU_290_Sphingomonas OTU_825_Hymenobacter OTU_107_Methylobacterium OTU_116_Aurantimonadaceae OTU_173_Sphingomonas_wittichii
Osmia leaiana	OTU_5_Achromobacter OTU_2241_Comamonadaceae OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_58_Bradyrhizobiaceae OTU_100_Caulobacteraceae OTU_72_Comamonadaceae OTU_36_Staphylococcus OTU_46_Acinetobacter OTU_12071_Acinetobacter OTU_407_Erwinia OTU_68_Xanthomonadaceae OTU_1790_Bradyrhizobiaceae OTU_2_Lactobacillus OTU_1768_Bacillus_cereus OTU_28_Halomonas OTU_4_Wolbachia	OTU_5_Achromobacter OTU_10_Enterobacteriaceae OTU_2241_Comamonadaceae OTU_31_Comamonadaceae OTU_138_Ralstonia OTU_43_Enhydrobacter OTU_13_Pseudomonas OTU_441_Comamonadaceae OTU_14_Bacillus OTU_72_Comamonadaceae OTU_24_Pseudomonas OTU_36_Staphylococcus OTU_46_Acinetobacter OTU_12071_Acinetobacter OTU_407_Erwinia OTU_48_Oxalobacteraceae OTU_201_Rhodococcus OTU_7_Enterobacteriaceae OTU_93_Hymenobacter OTU_1197_Comamonadaceae OTU_73_Agrobacterium OTU_22_Streptomycetaceae OTU_18_Sphingomonas OTU_38_Acinetobacter OTU_20_Acetobacteraceae OTU_5217_Oxalobacteraceae OTU_10707_Pseudomonas OTU_64_Enterobacteriaceae	OTU_5_Achromobacter OTU_2241_Comamonadaceae OTU_43_Enhydrobacter OTU_13_Pseudomonas OTU_14_Bacillus OTU_58_Bradyrhizobiaceae OTU_36_Staphylococcus OTU_384_Sphingomonadaceae OTU_46_Acinetobacter OTU_7_Enterobacteriaceae OTU_1790_Bradyrhizobiaceae OTU_38_Acinetobacter OTU_496_Methylobacterium OTU_45_Sphingomonas_echinoides OTU_41_Sphingomonas OTU_5217_Oxalobacteraceae OTU_28_Halomonas OTU_5413_Methylocystaceae

3.2.3. Bacterial community composition in line with larval development

The most prevalent bacterial families from larvae and pollen specimens were correlated to the developmental stage of the larvae for the three most abundant bee species in the dataset (*O. bicornis*, *H. truncorum*, *M. rotundata*). The larval developmental stage is estimated from their measured length. Multiple bacterial families in the pollen provisions returned statistically significant correlations with the measured larval length (**Table 9**, **Figure 10**). The shifts of specific bacterial families abundances in the pollen provisions show an overall dynamic change with progressing larval development (Voulgari-Kokota *et al.* 2019).

Table 9. Spearman's coefficient for correlations between bacterial families in pollen and larval size. Families were included only if they were present with a ratio of >1% in the respective community and if they returned a statistical significant correlation (*:*p<0.05*, **:*p <0.01* and ***:*p < 0.001*).

	***O. bicornis* pollen**		***H. truncorum* pollen**		***M. rotundata* pollen**	
	p	**rho**	**p**	**rho**	**p**	**rho**
Lactobacillaceae					*	−0.54
Enterobacteriaceae	***	−0.47	***	-0.76		
Pseudomonadaceae			***	-0.70	*	-0.62
Sphingomonadaceae	***	0.46			**	0.73
Acetobacteraceae	***	0.39	***	0.83		
Sphingobacteriaceae	**	0.34				
Cytophagaceae	***	0.45				
Methylocystaceae	***	0.40			**	0.65
Oxalobacteraceae			*	-0.47		
Streptococcaceae			*	0.41		

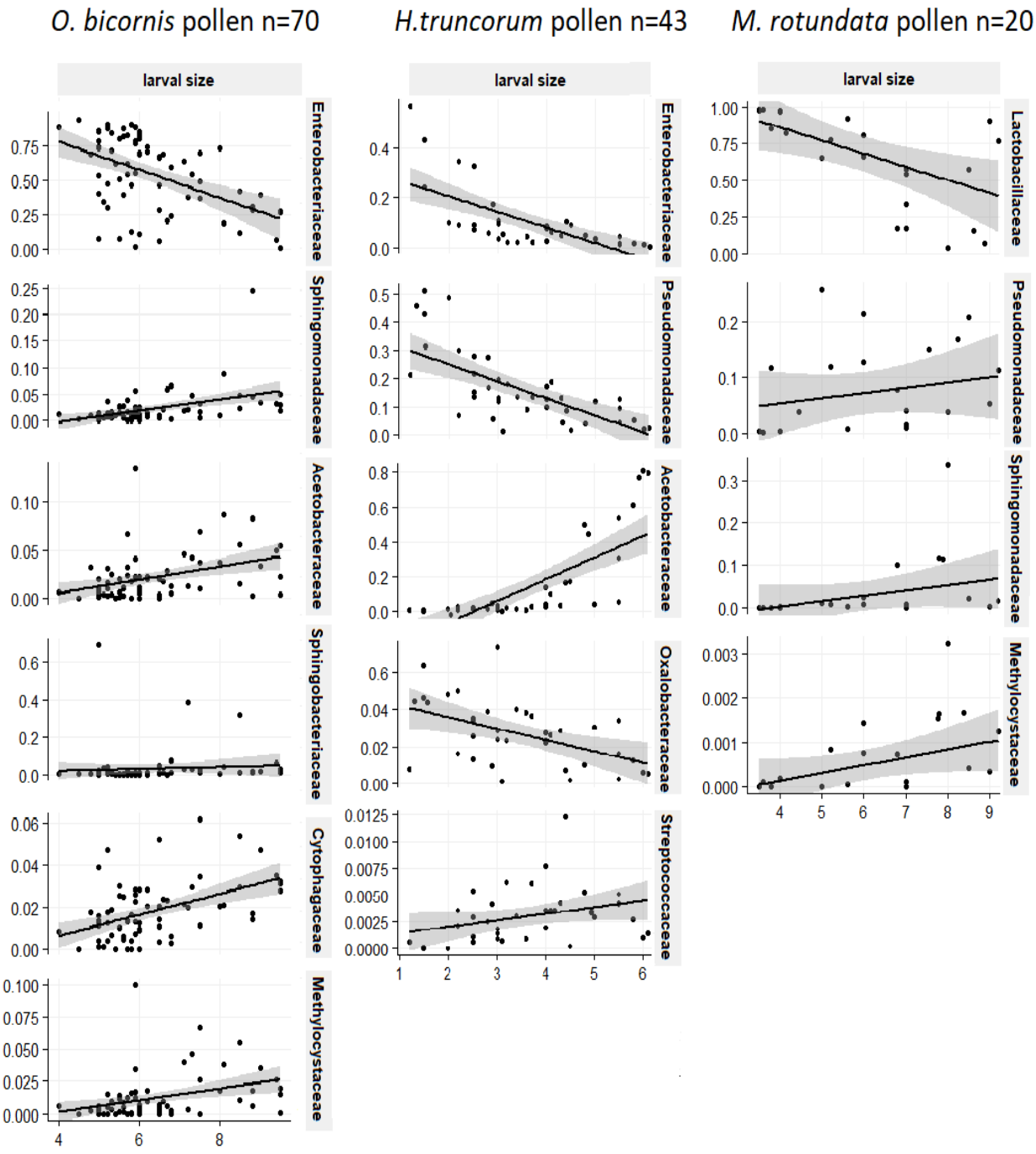

Figure 10. The relative abundances of certain bacterial families in the pollen provisions of three solitary bee species are plotted over the size of the respective larvae, when the correlations between them are statistically significant (*p<0.05*).

3.3. Association of the bacterial microbiota variation with the surrounding landscape

Sampling sites were divided into two categories: a) according to the landscape type they belonged to and b) according to their landscape diversity. Regions were characterized as agricultural land or as land principally occupied with semi natural vegetation (**Table 1**). Landscape diversity for each region is shown in **Table 1**. All larvae and pollen samples investigated for their bacterial communities (**Table 2**) were tested to explore the effect which the landscape type, the landscape diversity and the geographic region had on their microbiota.

The type of the landscape or the landscape diversity did not have a significant effect on the dataset. However, bacterial communities from the same bee host species but from different regions showed important differences. This was the case particularly with pollen bacterial communities.

3.3.1. Effect of landscape on the microbiota structure

3.3.1.1. Landscape effect on the interspecific level

For all larval specimens which were included in the 16S rDNA metabarcoding survey for bacterial metabarcoding, the landscape factor in the *Adonis* test could not explain the variation in the dataset (r^2=0.01, $p > 0.05$); with homogeneous multivariate dispersions among all groups (betadisper $p > 0.05$). For all pollen specimens, the result of *Adonis* was also non-significant (r^2 = 0.01, $p > 0.05$), with homogeneous multivariate dispersions among groups (betadisper $p > 0.05$).

In addition, landscape diversity was also statistically non-significant as a driving factor for the microbiome structure variance in the included datasets. Both for the larval specimens(r^2 = 0.01, $p > 0.05$) and the pollen specimens (r^2 = 0.01, $p > 0.05$) landscape diversity had no explanatory power for the structure of the datasets, while the multivariate dispersions among groups were also homogenous (betadisper $p > 0.31$ and betadisper $p > 0.05$ for larvae and pollen, respectively).

3.3.1.2. Landscape effect on the intraspecific level

O. bicornis and *H. truncorum* were sampled from both types of landscapes and their larvae and pollen datasets were therefore tested to investigate if their intraspecific variation can be explained by the landscape factor. Neither *O. bicornis* larvae and pollen, nor *H. truncorum* larvae and pollen showed statistical significant differences between groups (r^2 = 0.01, *p > 0.05*), with homogeneous multivariate dispersions among groups (betadisper *p > 0.05*) (**Table 9**).

Finally, the most abundant host bee species among the samples was used in order to test if landscape had an effect on any specific developmental stage of the larvae. *O. bicornis* larvae and pollen (n=70) were divided into six groups, according to the size of the larva in each respective nest chamber. Each of the six groups were tested to investigate any effect of landscape. No group showed statistically significant landscape effects either for larvae or for pollen (*p > 0.05*).

3.3.2. Correlation of geographic coordinates with the microbiota structure

To test the effect of the geographical location on the microbiota of samples, the Bray–Curtis correlation between a dissimilarity matrix for the included samples and the matrix of geographic distances between all sampling sites was examined. The datasets for the host bee species which were collected from more than two locations were included in the analysis. These were *O. bicornis* (sampled from 10 sampling sites), *H. truncorum* (sampled from five sampling sites) and *M. rotundata* (sampled from three sampling sites).

All datasets did not show statistically significant *Mantel* correlations with geographic distances. More specifically, *O. bicornis* larvae and pollen returned a *Mantel* statistic value equal to r = 0.07, *p > 0.05* and a *Mantel* statistic value equal to r = −0.00, *p > 0.05*, respectively. The values for *M. rotundata* larvae and pollen were r = −0.23, *p > 0.05* and r = −0.252, *p > 0.05*, respectively. Finally, the values for *H. truncorum* were r=0.02, *p<0.05* and r=0.07, *p>0.05* for larvae and pollen specimens, respectively.

3.3.3. Intraspecific microbiota variation according to geographic region

Intraspecific variation of microbiota structure according to the geographic region was tested for all three species which were sampled from more than two regions (**Table 10**). The region factor returned the following *Adonis* values: r=0.25 and *p<0.05** for *H. truncorum* larvae, r=0.21 and *p<0.05** for *H. truncorum* pollen; r=0.09 and *p>0.05* for *M. rotundata* larvae, r=0.49 and *p<0.01*** for *M. rotundata* pollen, r=0.30 and *p>0.05* for *O. bicornis* larvae and r=0.34 and *p>0.05* for *O. bicornis* pollen. Beta dispersity among all tested groups was homogeneous for all levels (*p>0.05*).

Finally, all *O. bicornis* sample groups which were divided according to larval size, were also tested for investigating the potential effect of the region. The *Adonis* tests returned a statistically significant result with high explanatory power (r^2=0.80, *p < 0.05**) only for pollen samples originating from *O. bicornis* nest chambers with the smallest larvae (group A. 4.0 to 4.9 cm), with homogenous multivariate dispersions among sample groups (betadisper *p > 0.05*).

Table 10. Effect of the landscape and of the sampling region on the microbiota structure of three bee species, estimated with PERMANOVA/Adonis.

		Landscape			Sampling region		
O. bicornis (n=70)	larvae	2 levels	r=0.01	*p>0.05*	10 levels	r=0.30	*p>0.05*
	pollen	2 levels	r=0.01	*p>0.05*	10 levels	r=0.34	*p>0.05*
H. truncorum (n=43)	larvae	2 levels	r=0.01	*p>0.05*	5 levels	r=0.25	*p<0.05**
	pollen	2 levels	r=0.01	*p>0.05*	5 levels	r=0.21	*p<0.05**
M. rotundata (n=21)	larvae	-			3 levels	r=0.09	*p>0.05*
	pollen	-			3 levels	r=0.49	*p<0.01***

3.4. Foraging preferences of different solitary bee species

The pollen samples included in the ITS2 rDNA sequencing library (as shown in **Table 2**) returned assignments for 415 plant species after quality filtering. The NMDS ordination plot of all specimens is demonstrated in **Figure 11**.

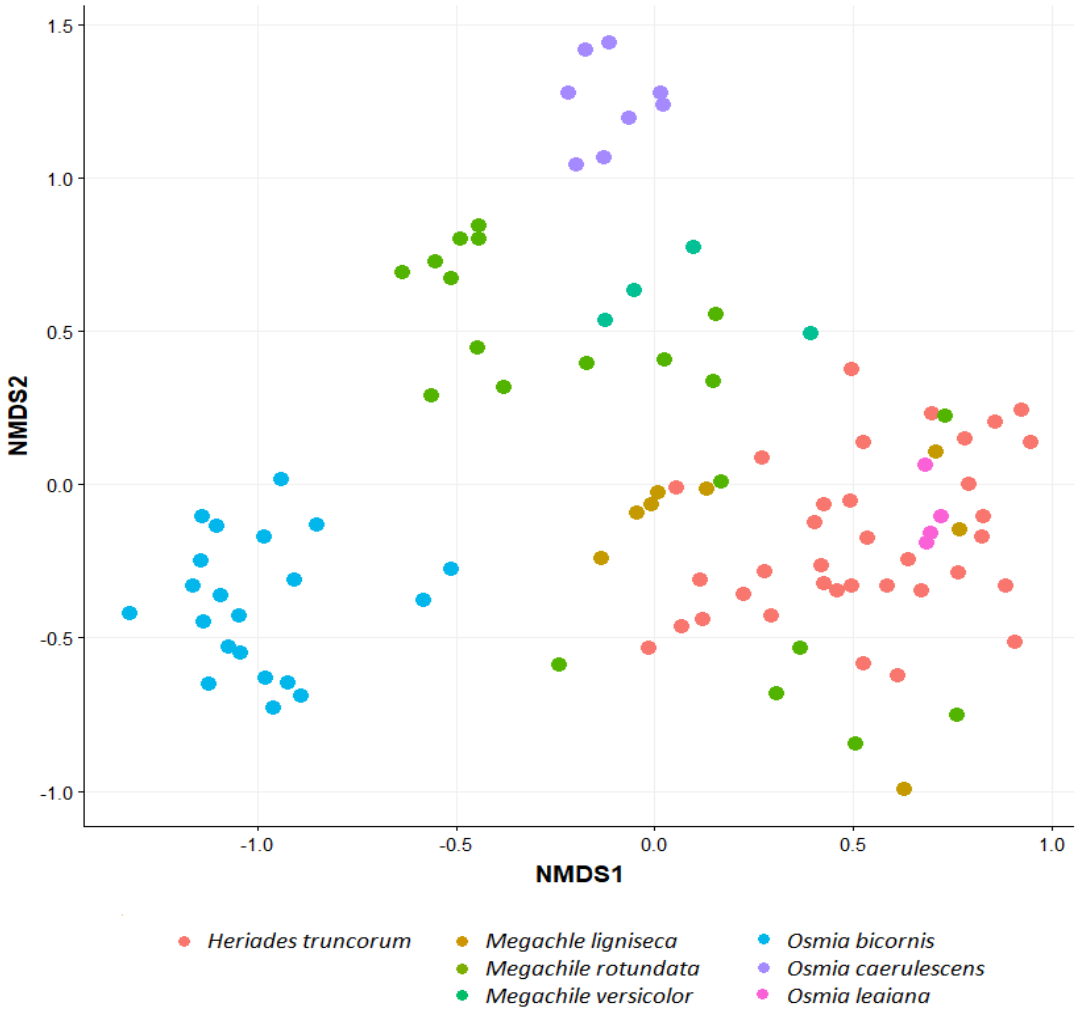

Figure 11. NMDS ordination for the plant species composition of pollen provisions from seven solitary bee species nests. Sample points are colored according to bee species.

3.4.1. Plant species biodiversity in the pollen provisions

The plant species absolute numbers and the plant species Shannon diversity values were higher for *Megachile versicolor* (**Figure 12**). However, the anova test for the plant species Shannon diversity values did not show significant differences in means between the seven bee species (F=2.20, *p>0.05*).

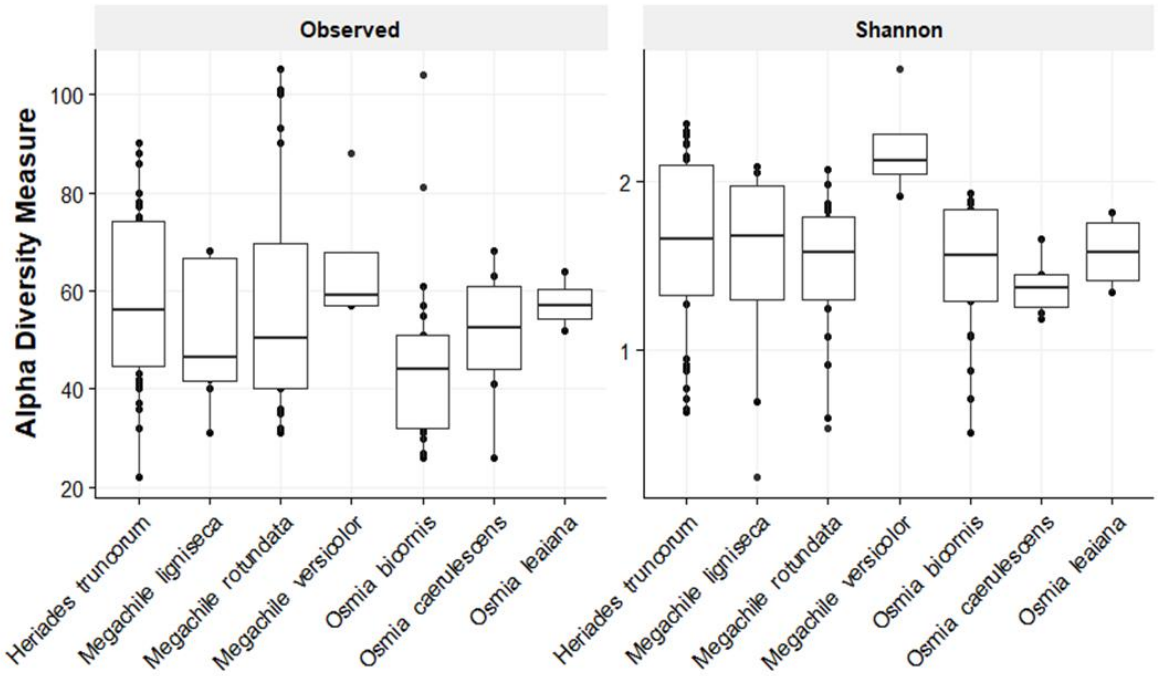

Figure 12. Plant species richness and plant species Shannon diversity in pollen provisions for seven different solitary bee species

3.4.2. Plant taxa composition in the pollen provisions

In the nests of three oligolectic bee species, pollen consisted mainly by one plant family. Asteraceae was dominant in *H. truncorum* (91.14%) and *O. leaiana* (98.27%), while pollen provisions from *O. caerulescens* nests were almost entirely composed by Fabaceae (97.24%) (**Figure 13**). The pollen provisions of the rest four bee species consisted of more than one plant family. *M. ligniseca* pollen provisions consisted mainly of Asteraceae (75.20%) and Araliaceae (8.33%). The pollen provisions in *M. rotundata* and *M. versicolor* nests consisted mainly of Asteraceae (42.48% and 50.17%), Rosaceae (22.50% and 3.50%), Fabaceae (4.35% and 22.27%) and Papaveraceae (13.61% and 20.70%). Finally, *O. bicornis* pollen consisted of many plant families with Sapindaceae (39.75%) and Brassicaceae (31.91%) being the most prevalent (**Figure 13**). Polylectic bee species show a higher intraspecific variation in the plant families they prefer to collect pollen from. Differences among nest chambers of the same bee species and also between different bee species are shown in the plant family heatmap which includes all samples (**Figure 14**).

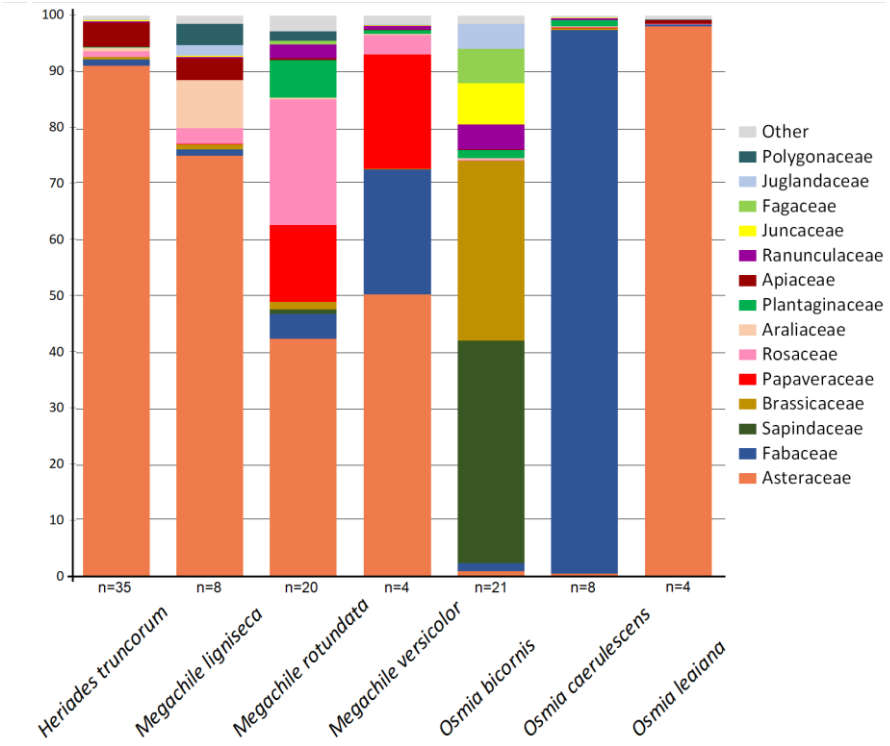

Figure 13. Plant family composition of the pollen provisions in the nests of seven solitary bee species. Relative abundances stand for the mean contribution in ITS2 rDNA sequence reads. Plant families are demonstrated if they were present with a ratio of >1% in the dataset of each host bee.

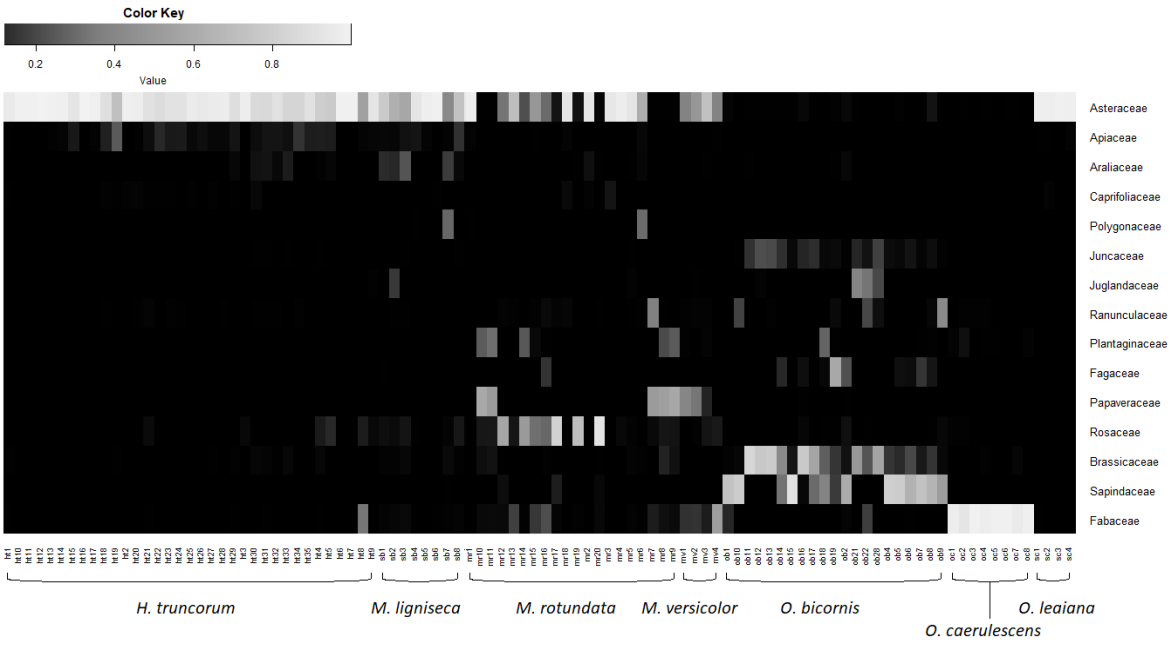

Figure 14. Plant family composition in the pollen provisions of 100 nest chambers belonging to seven solitary bee species.

3.5. Association of the bee bacterial microbiota with their foraging preferences

100 nest chambers from seven solitary bee species were used for pollen metabarcoding of their pollen provisions (**Table 2**) and inspected the association between the revealed plant species composition and the larval and pollen bacterial microbiota of the same nest cells. A tripartite network connecting bee species with plant species, pollen bacterial microbiota and, finally, larval bacterial microbiota is demonstrated in **Figure 15**.

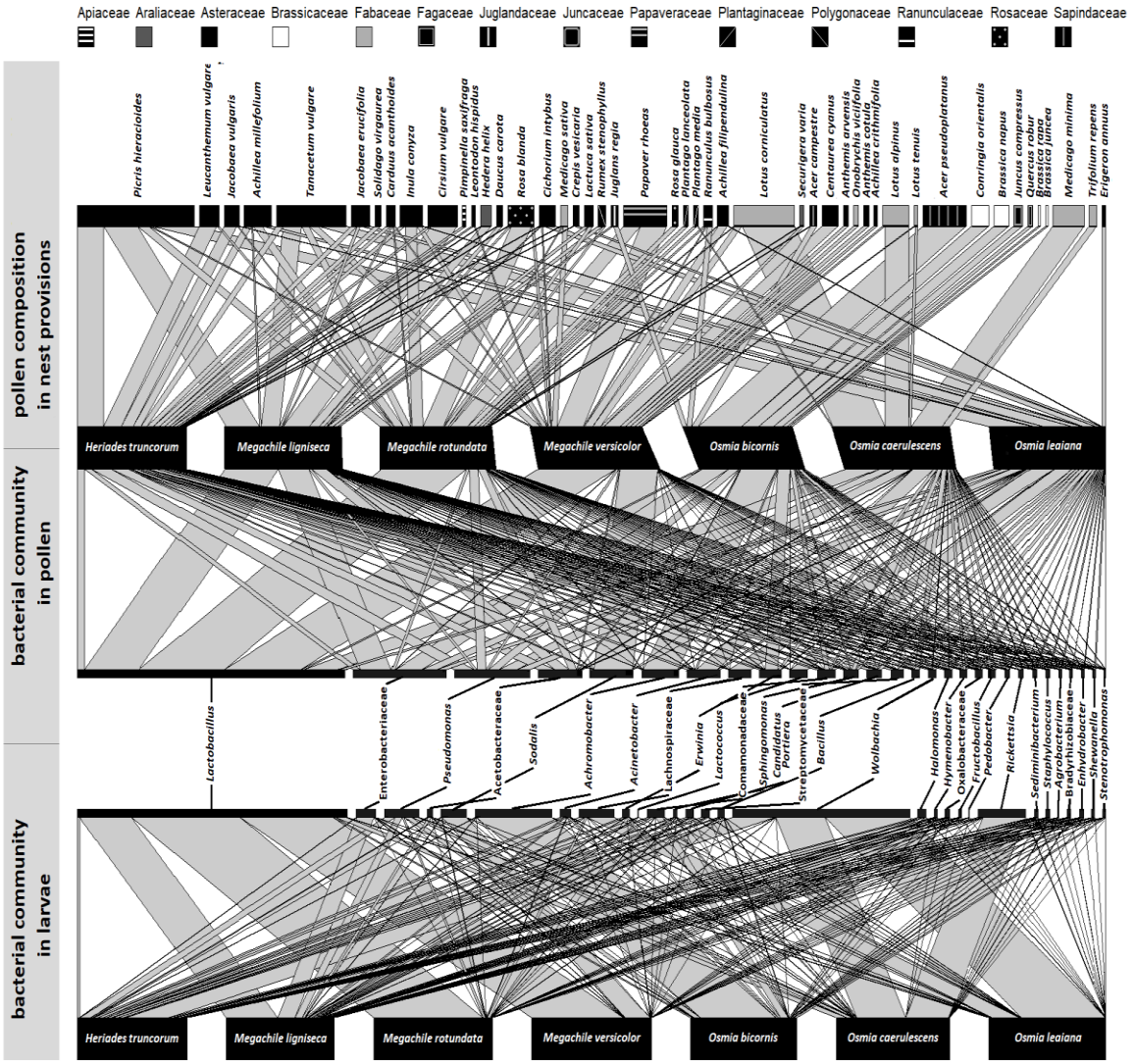

Figure 15. Tripartite network indicating interactions between seven solitary bee species and plant species and between the same bee species with bacterial taxa found in larvae and pollen. Plant species and bacterial taxa are included if they occur in relative abundance of at least 1% in the respective dataset.

3.5.1. Correlation of plant diversity with bacterial biodiversity

Pairwise Spearman rank correlations were conducted to investigate associations between bacterial diversity for larvae, bacterial diversity for pollen and pollen type diversity (**Figure 16**). Shannon values showed weak Spearman correlation between bacterial diversity in pollen and plant species diversity (Rho=0.21, *p<0.05**). Bacterial OTU Shannon diversity values in larvae and plant species diversity were not significantly correlated (Rho=0.07, *p>0.05*).

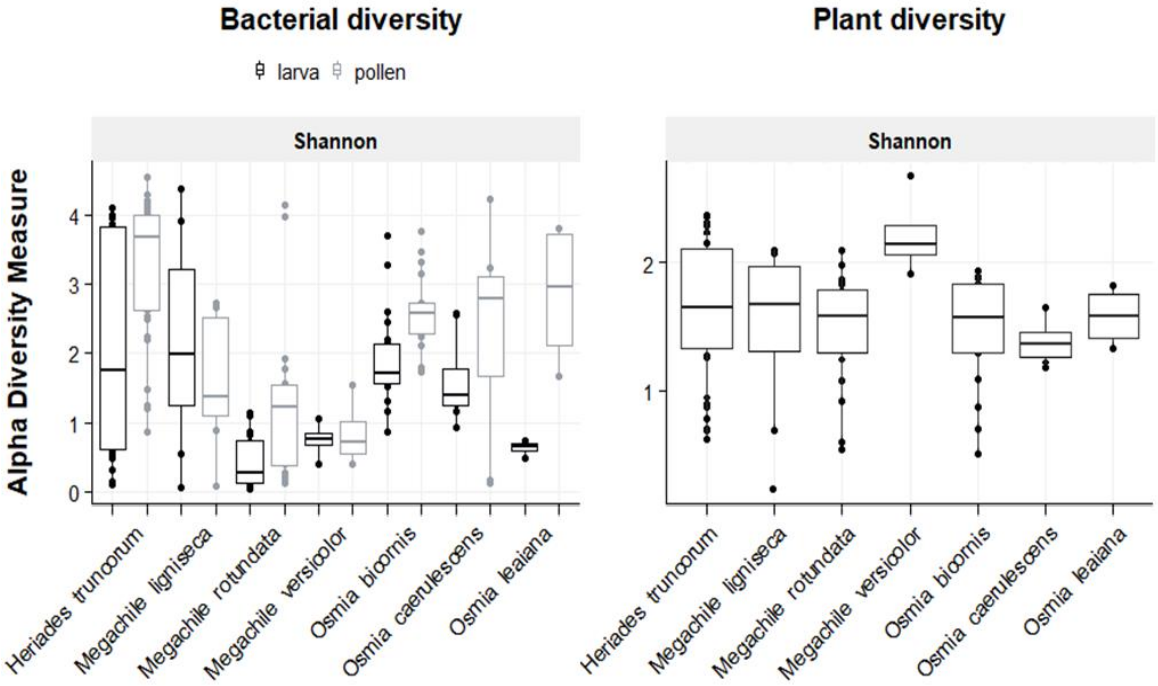

Figure 16. Left: Shannon diversity of bacterial communities in nest chambers for seven solitary bee species based on bacterial OTUs. Right: Shannon diversity of pollen species in pollen provisions of the same nest chambers.

3.5.2. Co-occurrence of plant species with bacteria in the pollen provisions and co-occurrence of bacteria in pollen and larvae

The probabilistic co-occurrence analysis between the bacterial community in the pollen provisions and the respective pollen composition for all samples showed possible connections between bacterial taxa and plant species. Positive, negative, and random

interactions are summarized in **Figure 17**. The analysis is based on absence - presence data with a relative abundance threshold for all taxa at 1% in the dataset.

Pollen and larval bacterial communities shared several bacterial OTUs which occurred in all samples (**Table 8**). More specifically, OTUs assigned as Comamonadaceae, Bradyrhizobiaceae, *Pseudomonas* and *Acinetobacter* were shared between all *H. truncorum* larvae and pollen samples. Respective shared OTUs were assigned as Comamonadaceae, Enterobacteriaceae, Acetobacteraceae, *Pseudomonas* and *Lactobacillus* for *M. ligniseca*, as *Pseudomonas* and *Lactobacillus* for *M. rotundata* and as *Lactobacillus* for *M. versicolor*. For *O. bicornis*, the respective taxa were *Pseudomonas* and Comamonadaceae, for *O. caerulescens* it was *Pseudomonas* and for *O. leaiana* the shared OTUs were assigned as *Achromobacter*, Comamonadaceae, *Pseudomonas*, *Acinetobacter* and *Erwinia*.

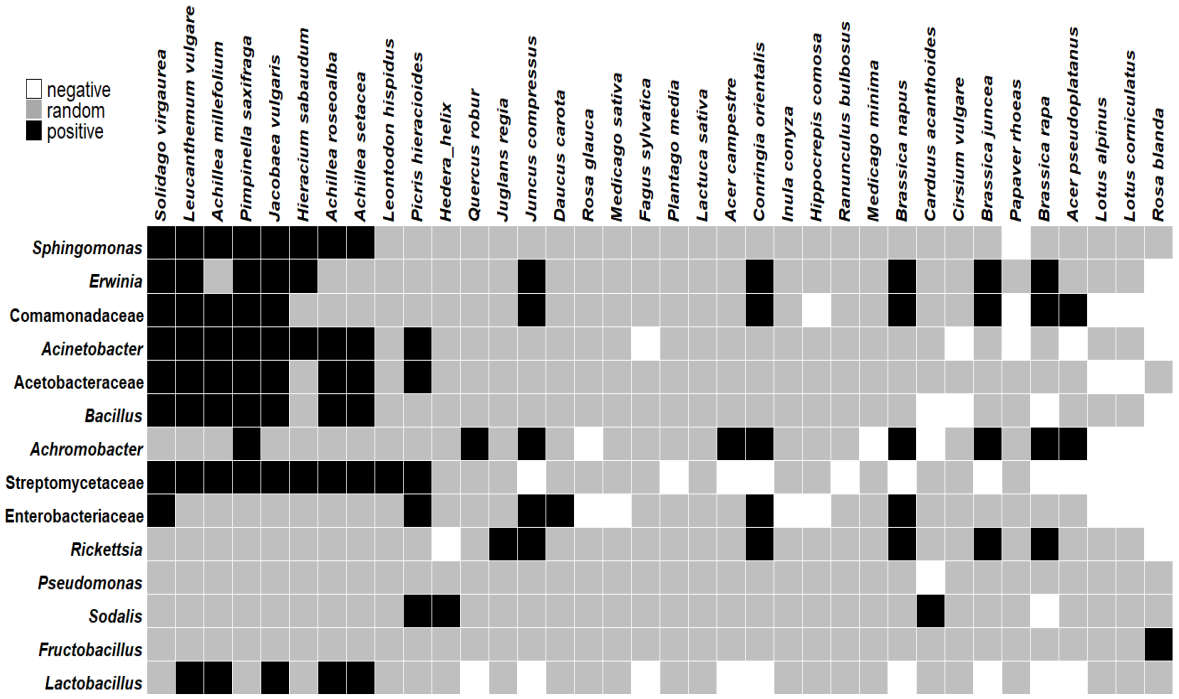

Figure 17. Probabilistic co-occurrence analysis results for the most abundant bacterial taxa found in pollen and the most abundant plant species consisting the pollen provisions. Bacterial taxa shown here are agglomerated up to genus level or up to family level if not better classifiable.

3.5.3. Correlation of the pollen provision composition with the bacterial community composition

Statistically significant *Mantel* correlations between pollen composition and pollen bacterial communities and also between pollen composition and larval bacterial communities were observed in the whole dataset (**Table 11**). When the same tests were conducted within each host bee species, no significant correlations between pollen plant species and pollen bacterial taxa were detected, except for *M. rotundata* (**Table 11**).

Table 11. *Mantel* correlations between *Bray-Curtis* distance matrices for plant species and bacterial OTUs in pollen, plant species and bacterial OTUs in larvae, as well as for bacteria OTUs in pollen and larvae.

	plant species x pollen bacteria	plant species x larval bacteria	pollen bacteria x larval bacteria
whole dataset (n=100)	**r=0.32, *p<0.001******	**r=0.31, *p<0.001******	**r=0.37, *p<0.001******
H. truncorum (n=35)	r=0.08, *p=0.095*	r=0.05, *p=0.15*	**r=0.18, *p<0.01****
M. ligniseca (n=8)	r=0.05, *p=0.37*	r=-0.23, *p=0.81*	r=0.07, *p=0.35*
M. rotundata (n=20)	**r=0.23, *p<0.05****	r=0.16, *p=0.06*	r= 0.17, *p=0.18*
M. versicolor (n=4)	r=0.32, *p=0.29*	r=0.31, *p=0.25*	r= 0.34, *p=0.29*
O. bicornis (n=21)	r= 0.07, *p=0.22*	r=-0.06, *p=0.68*	r= 0.19,*p=0.11*
O. caerulescens (n=8)	r= -0.03, *p=0.47*	r=-0.13, *p=0.69*	r= 0.36,*p=0.05*
O. leaiana (n=4)	r= 0.56, *p=0.21*	r=0.86, *p=0.25*	r=0.56, *p=0.17*

3.5.4. Bee species and foraging preferences as drivers of the nest bacterial microbiota

3.5.4.1. Mixed effect model

The information on host bee species and also the NMDS coordinates (**Figure 18**) of all samples were included in the construction of mixed effect models to investigate the relationships between all variables (**Figure 19**). Host species had a significant effect on all three NMDS coordinate pairs for pollen composition, pollen bacterial community and larval bacterial community. At the same time, the ordination of pollen composition had an effect on the pollen bacterial microbiome ordination and the pollen bacterial microbiome ordination had an effect on larval bacterial communities (**Figure 19**).

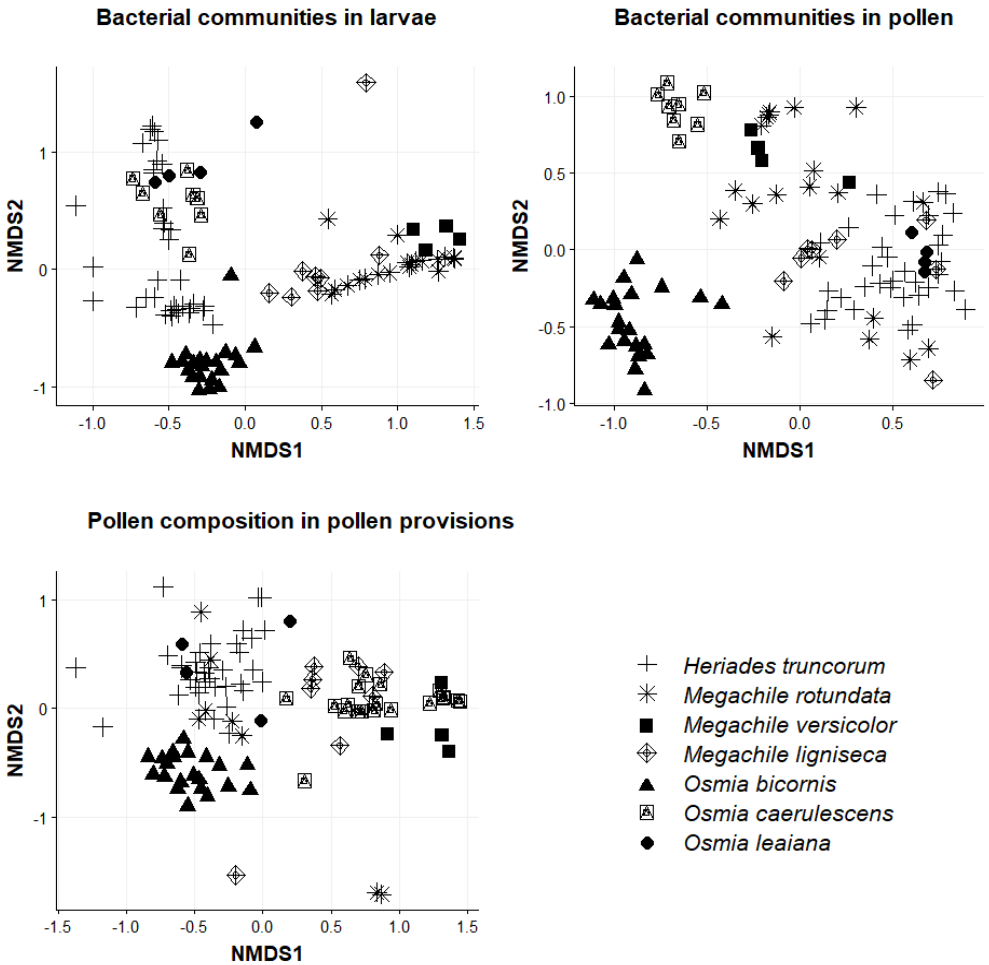

Figure 18. NMDS ordination of all samples sequenced both for 16S rDNA and ITS2 rDNA based on Bray-Curtis distances of bacterial OTUs and plant species. Sample points are shaped according to host bee species

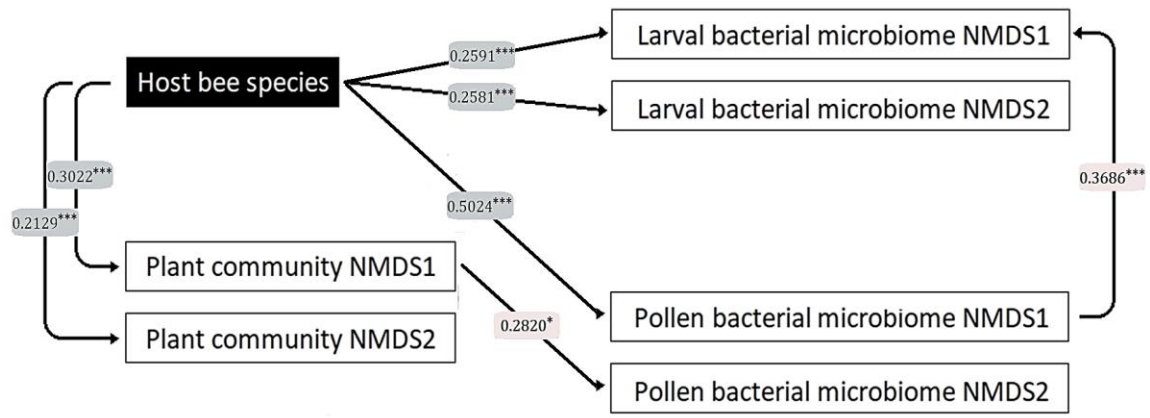

Figure 19. Results of mixed effect models, investigating the role that host species and foraging preferences play in the shaping of the bacterial communities in bee nests. Random and fixed independent variables are demonstrated only if they returned statistically significant effects (*p<0.05**, *p<0.01***, *p<0.001****).

3.5.4.2 Random forest analysis

Pollen provisions were divided into seven clusters according to their composition in plant species (**Figure 20**). Also, the indicator bacterial taxa for pollen and larvae of all bee species were defined with random forest analysis (**Figure 21**). The random forest regression analysis assigned 77% of bacterial pollen communities to host species and 70% to pollen cluster (as shown in **Figure 20**), correctly (**Table 12**). For bacterial communities in larvae the correct random forest regression assignments were 89% to host species and 58% to pollen composition cluster.

Assignment of bacterial pollen communities to pollen composition clusters within each bee species returned low error rates (from 0% to 14.29%, **Table 13**). More specifically, 31 out of 35 *H. truncorum* pollen bacterial communities were assigned correctly to two pollen composition clusters and 18 out of 21 pollen bacterial communities were assigned correctly to two pollen composition clusters, as well. The pollen bacterial communities from *M. ligniseca*, *M. versicolor* and *O. caerulescens* were all successfully assigned to two pollen composition clusters each. Finally, 18 out of 20 *M. rotundata* pollen bacterial communities were assigned correctly to three pollen composition clusters (**Table 13**).

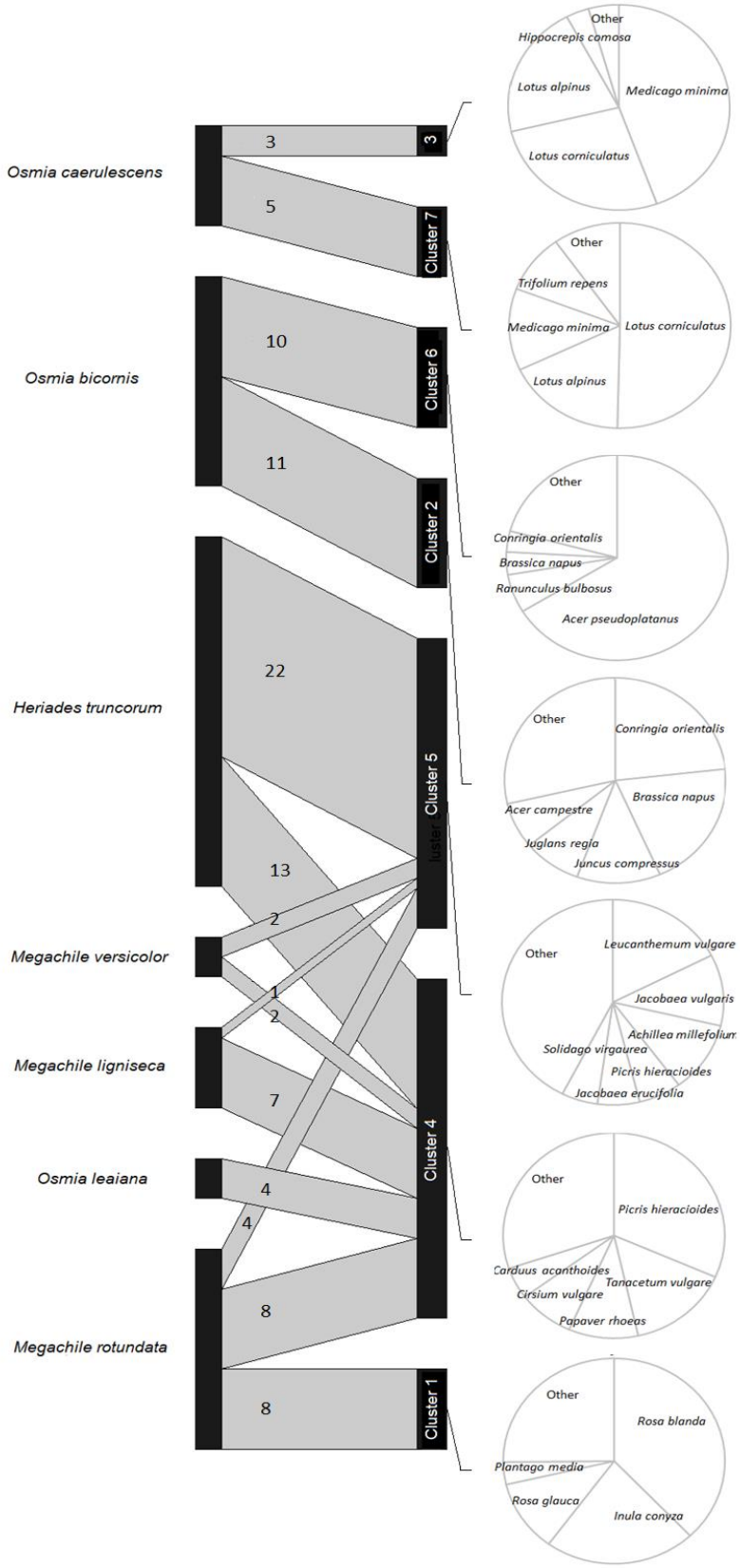

Figure 20. Samples were divided into seven clusters according to their composition in plant species. Pie charts demonstrate the mean relative abundance of plant species among all samples grouped under each cluster. Numbers in the grey areas stand for the respective number of samples from each bee species assigned to each cluster.

Table 12. Random forest analysis shows associations of all pollen bacterial communities associated with host species and pollen composition. Confusion matrices show the number of correctly assigned communities, the proportional class error for each category and the total OOB estimate of the error rate.

Assignment of pollen bacterial communities to bee host species								
OOB estimate of error rate: 23%								
Confusion matrix:								
	H. truncorum	*M. ligniseca*	*M. rotundata*	*M. versicolor*	*O. bicornis*	*O. caerulescens*	*O. leaiana*	class error
H. truncorum	34	0	0	0	0	1	0	0.03
M. ligniseca	1	2	4	0	0	1	0	0.75
M. rotundata	1	1	18	0	0	0	0	0.1
M. versicolor	0	0	4	0	0	0	0	1
O. bicornis	0	0	0	0	21	0	0	0
O. caerulescens	4	0	0	0	2	2	0	0.75
O. leaiana	4	0	0	0	0	0	0	1
Assignment of pollen bacterial communities to pollen composition cluster								
OOB estimate of error rate: 30%								
Confusion matrix:								
	1	2	3	4	5	6	7	class error
Cluster 1	8	0	0	0	0	0	0	0
Cluster 2	0	9	0	0	0	2	0	0.18
Cluster 3	0	0	0	2	1	0	0	1
Cluster 4	0	0	0	26	8	0	0	0.24
Cluster 5	1	0	0	8	20	0	0	0.31
Cluster 6	0	3	0	0	0	7	0	0.3
Cluster 7	0	1	0	1	2	1	0	1

Table 13. Random forest regression results of all pollen bacterial communities to the respective pollen composition clusters within each bee host species. Confusion matrices show the number of correctly assigned communities, the proportional class error for each category and the total OOB estimate of the error rate.

Assignment of pollen bacterial communities to pollen composition cluster for:									
H. truncorum						*M. ligniseca*			
OOB estimate of error rate: 11.43%						OOB estimate of error rate: 0%			
Confusion matrix:						Confusion matrix:			
	4	5	class error				4	5	class error
Cluster 4	10	3	0.23			Cluster 4	7	0	0
Cluster 5	1	21	0.05			Cluster 5	0	0	NaN
M. rotundata						*M.versicolor*			
OOB estimate of error rate: 10%						OOB estimate of error rate: 0%			
Confusion matrix:						Confusion matrix:			
	1	4	5	class error			4	5	class error
Cluster 1	8	0	0	0		Cluster 4	2	0	0
Cluster 4	0	8	0	0		Cluster 5	0	2	0
Cluster 5	1	1	2	0.5					
O. bicornis						*O. caerulescens*			
OOB estimate of error rate: 14.29%						OOB estimate of error rate: 0%			
Confusion matrix:						Confusion matrix:			
	2	6	class error				3	7	class error
Cluster 2	9	2	0.18			Cluster 3	3	0	0
Cluster 6	1	9	0.1			Cluster 7	0	5	0

3.6. Association of the bees bacterial microbiota with their nesting material

The nest material samples included in the 16S rDNA sequencing library (**Table 2**) returned assignments for 6693 bacterial OTUs after quality filtering, 6242 of which occur in all loam specimens derived from *O. bicornis* nests and 1580 of which occur in all the rest. The bacterial composition of the nest material and larvae samples for each bee species is summarized in **Figure 9**. NMDS ordination of all nest material samples shows separation of the specimens according to species and type of material (**Figure 21**).

3.6.1. Association of the bacterial biodiversity and composition of the nesting material with that of the bee larvae

Alpha biodiversity of larvae and nest material samples is demonstrated in **Figure 8**. The bacterial biodiversity of the nest material samples dataset was correlated with the bacterial biodiversity of the respective larvae. Spearman's correlation coefficient was statistically significant among the samples of all bee species (Rho=0.48, *p<0.001****). However, Spearman's correlation results between bacterial biodiversity of nest material and larvae samples were not statistically significant among the same species (*p>0.05*). Mantel correlation of the bacterial community structure between all nest material and larvae specimens was also statistically significant for all samples (r=0.54, *p<0.001****). Nevertheless, no correlation was observed within the samples of the same species (*p>0.05*).

Beta-dispersity among all larvae grouped according to the different nest material types was significantly unequal (betadisper *p<0.001****). Therefore, PERMAOVA results were not used to compare material type's effect on the groups . However, random forest analysis and subsequent regression of all larval bacterial communities with nest material set as a discriminant category (leaves, loam, plant tissue and resin) was successful (error rate: 8.79%, number of category levels:4). At the same time, the same analysis with bee species set as the discriminant category was also successful (error rate: 13.19%, number of category levels:7).

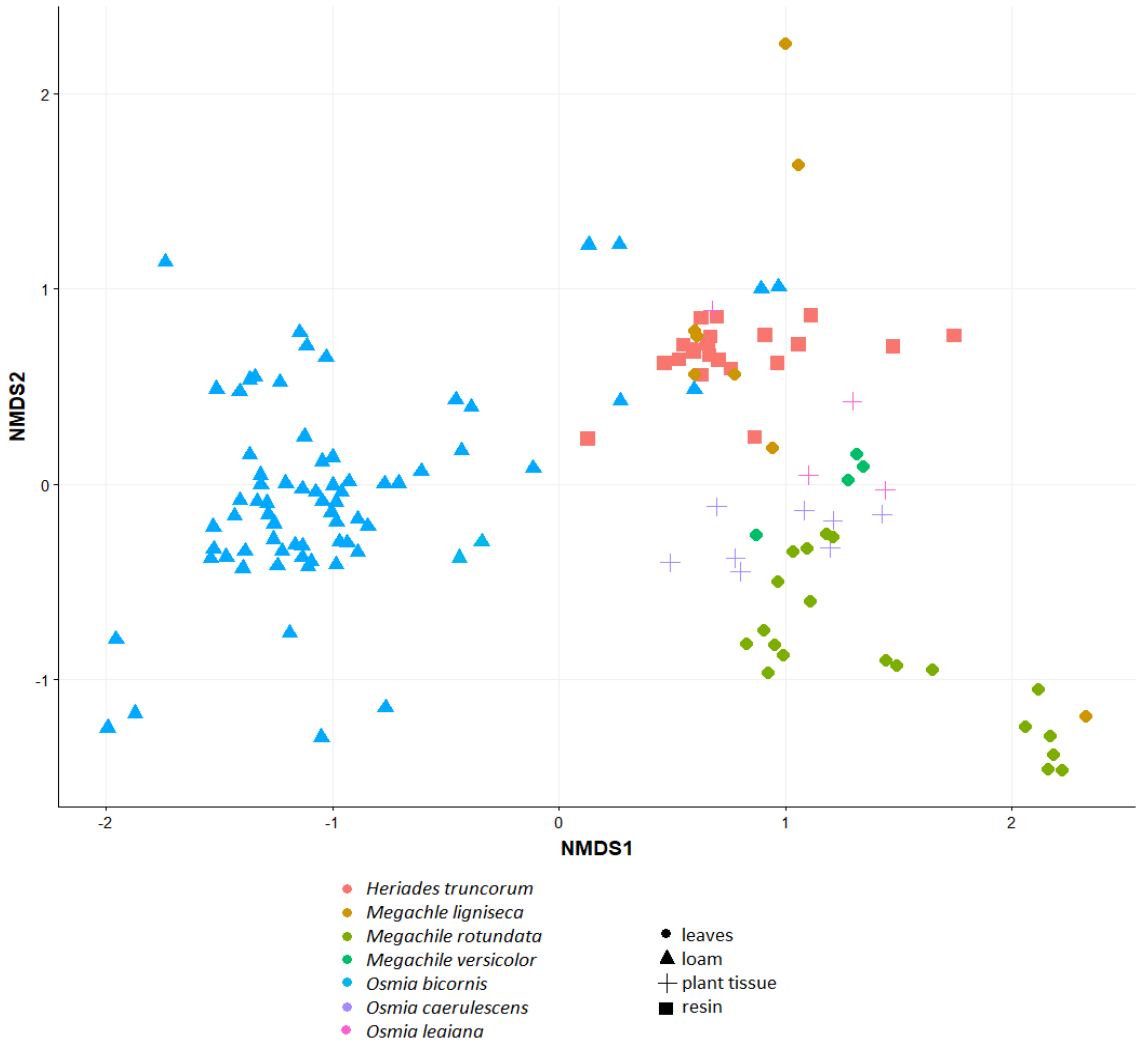

Figure 21. NMDS ordination of nest material specimens from seven solitary bee species nests. Sample points are colored according to bee species and shaped according to type of specimen.

In many cases, larvae and nest material share bacterial OTUs which occur in all their samples, however not in the respective pollen samples (**Table 8**). In the case of *H. truncorum*, *M. rotundata* and *M. versicolor*, the nest material and the larvae shared no such OTUs. *M. ligniseca* leaves and larvae shared OTUs assigned as Achromobacter, Comamonadaceae, Bradyrhizobiaceae and Oxalobacteraceae. *O. bicornis* and *O. caerulescens* nest material and larvae shared OTUs assigned as *Achromobacter* and Comamonadaceae and *O. leaiana* nest material and larvae shared OTUs assigned as Bradyrhizobiaceae.

3.6.2. Association of the type of the nest material with the nest microbiota

Bacterial OTUs were selected as variables from random forests as indicative taxa for the bacterial communities in each of the nest material types. Indicative bacterial taxa for each nest material type are summarized in **Figure 22**. Random forest analysis and subsequent regression of all nest material bacterial communities with material type set as a discriminant category (leaves, loam, plant tissue and resin) was successful (error rate: 7.69%, number of category levels:4). The same regression analysis for nest material bacterial communities with bee species set as the discriminant category was also successful (error rate: 8.79%, number of category levels:7).

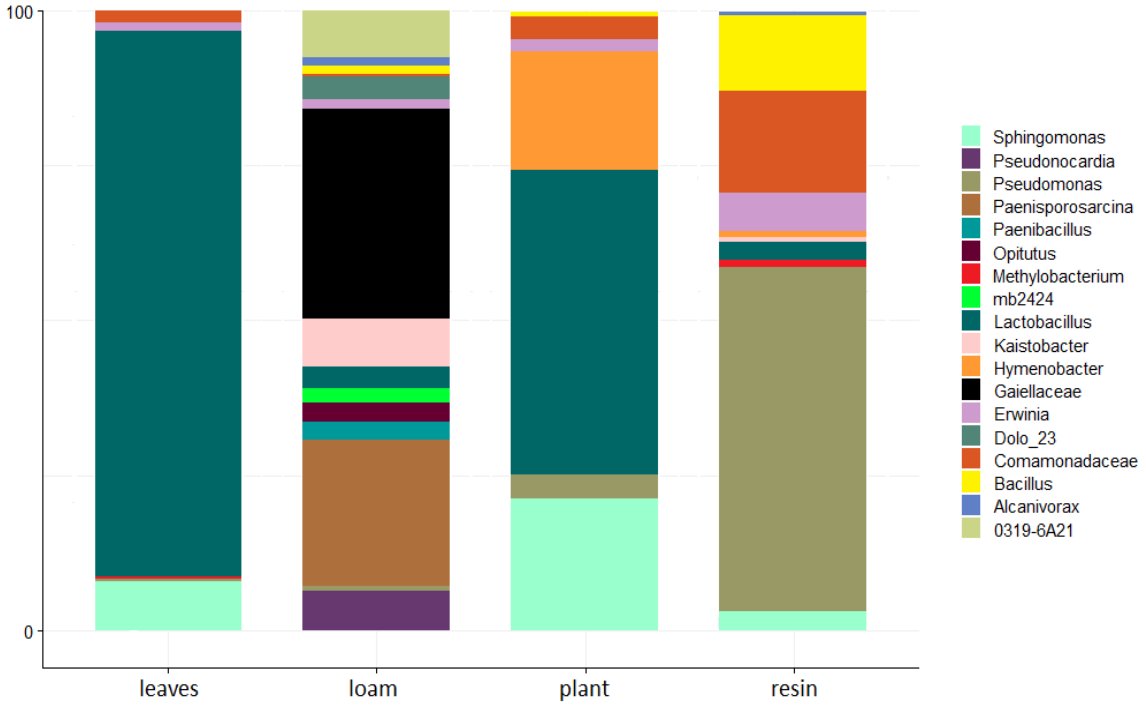

Figure 22. Indicative bacterial taxa for each nest material type after random forest variable selection. Relative abundances stand for the mean contribution in 16S rDNA sequence reads. Taxa are agglomerated up to genus level or up to family level if not better classifiable.

3.7. Isolated bacterial strains from bee nests

Bacillus strains were isolated from all types of specimens which were included in bacterial cultures. Furthermore, two *Paenibacillus* strains were isolated from *O. bicornis* larvae and soil. Other Firmicutes were isolated both from pollen and nest material from *O. bicornis* and *M. rotundata*. One *Lactobacillus* strain was isolated from *M. rotundata* pollen in

multiple occasions, while one *Acinetobacter* strain was isolated from *O. bicornis* pollen. All strains which were selected for further inclusion in bioassays are listed in **Table 14**.

Table 14. Isolated strains from *O. bicornis* and *M. rotundata* nests selected for biochemical tests.

	O. bicornis larvae	*O. bicornis* pollen	*O. bicornis* nest material	*M. rotundata* larvae	*M. rotundata* pollen	*M. rotundata* nest material	Taxonomic match of 16S rDNA sequence (>97%)
A11			x				*Bacillus subtilis*
A244						x	*Bacillus licheniformis*
A4		x					*Bacillus cereus*
A217					x		*Bacillus* sp.
A221			x				*Bacillus pseudomycoides*
A243						x	*Bacillus* sp.
A226	x						*Aneurinibacillus* sp.
A45	x						*Brevibacillus brevis*
A150				x			*Brevibacillus reuszeri*
A147			x				*Paenibacillus polymyxa*
A55	x						*Paenibacillus ehimensis*
Z52						x	*Micrococcus aloeverae*
A145				x			*Enterobacter cloacae*
A1A3					x		*Lactobacillus* sp.
A1J		x					*Acinetobacter sp.*

3.7.1. Carbon source uptake and enzymatic activity

Selected strains as listed in **Table 14** were tested for carbon source utilization and enzymatic activities. Results for each strain are shown in **Table 15**. Bacteria were tested for nutrient utilization and various enzymatic activities.

Table 15. Biochemical profiling including carbon source utilization tests for all selected bacterial strains. The symbol + stands for positive reaction, - for negative reaction and (+) indicates weak possible reaction.

	Bacterial strains														
	A11_*B. subtilis*	A244_*B. licheniformis*	A4_*B. cereus*	A217_*Bacillus sp.*	A221_*B. pseudomycoides*	A243_*Bacillus sp.*	A226_*Aneurinibacillus sp.*	A45_*B. brevis*	A150_*B. reuszeri*	A147_*P. polymyxa*	A55_*P. ehimensis*	Z52_*M. aloeverae*	A145_*E. cloacae*	A1A3_*Lactobacillus sp.*	A1J_*Acinetobacter sp.*
nutrient uptake															
D-amygdalin	-	+	-	-	+	-	-	-	-	+	+	-	-	-	-
D-xylose	-	+	-	-	-	-	-	-	-	+	+	-	+	-	-
cyclodextrin	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
D-sorbitol	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
D-galactose	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+
D-ribose	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-
lactose	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+
N-acetyl-D-glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
D-maltose	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
D-mannitol	+	-	-	-	-	-	-	-	-	+	+	-	+	+	+
D-mannose	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+
D-raffinose	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-

salicin	+	+	(+)	+	-	-	+	-	-	+	+	-	+	+	-
saccharose/sucrose	+	+	+	+	-	+	+	-	-	+	+	-	-	+	+
trehalose	+	+	+	+	+	+	(+)	-	-	+	-	-	+	-	-
growth in 6.5% NaCl	+	+	+	+	-	-	-	-	-	(+)	-	-	(+)	-	-
enzymatic activity-															
phosphatidylinositol phosphatase C	-	-	-	-	+	-	+	+	+	-	-	-	-	-	+
arginine dihydrolase 1	+	+	(+)	+	-	-	+	-	-	+	+	+	-	-	-
beta-galactosidase	(+)	+	-	-	-	-	-	-	-	-	-	-	+	+	-
alpha-glucosidase	-	-	-	-	-	-	-	+	+	+	+	+	-	+	-
Ala-Phe-Pro Arylamidase	-	-	-	-	+	-	+	-	-	-	+	+	-	-	-
L-aspartate arylamidase	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
beta galactopyranosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
alpha-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
phosphatase	-	-	-	-	-	-	+	+	+	-	+	-	-	+	-
leucine arylamidase	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-
L-proline arylamidase	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+
beta glucuronidase	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
alpha-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
L-pyrrolydonyl-arylamidase	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
beta-glucuronidase	-	+	-	-	-	(+)	-	-	-	-	-	-	-	-	-
alanine arylamidase	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
tyrosine arylamidase	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+
urease	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-lactate alkalinization	+	+	-	-	-	-	+	-	-	-	-	+	-	+	+
arginine dihydrolase 2	+	+	-	+	-	-	+	-	-	-	-	+	-	-	-

3.7.2. Antimicrobial properties

Two *Paenibacillus* strains isolated from *O. bicornis* soil and larvae, taxonomically assigned after 16S rDNA sequence match, showed strong antifungal activity both in co-cultures with fungi and after addition of liquid culture filtrate on petri dishes with fungal spores (**Image 3**).

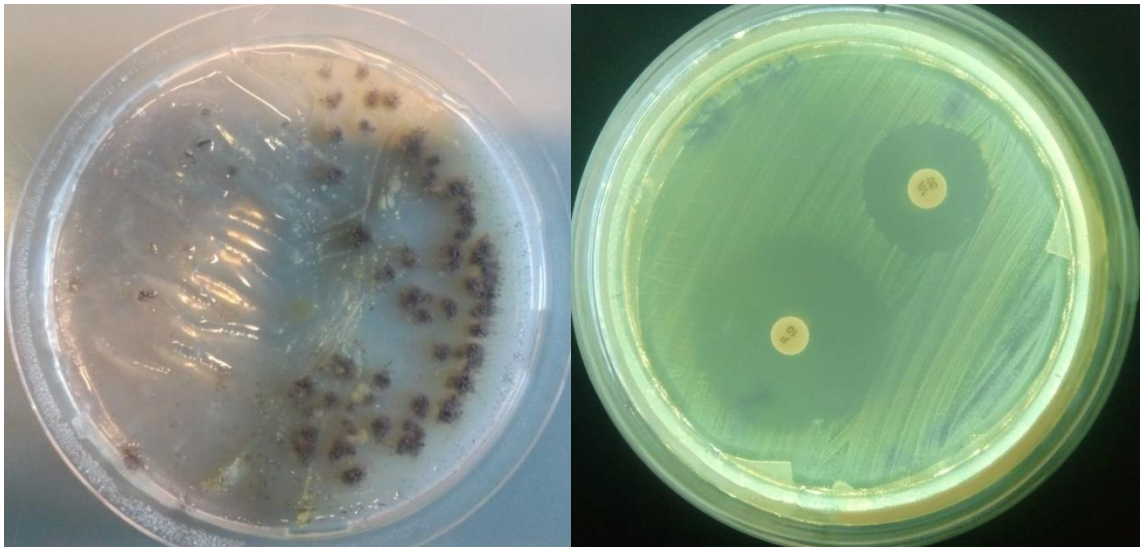

Image 3. Co-culture of *Aspergillus sp*. fungus and *Paenibacillus polymyxa* (left) and culture of *Aspergillus sp.* loan around inhibitory culture filtrates of *P. polymyxa* and *P. ehimensis* (right)

3.8. Screening of potentially pathogenic bacteria

The larvae, pollen provisions and soil from 12 *O. bicornis* nest chambers with deceased larvae along with 31 *O. bicornis* nest chambers with healthy larvae sampled from the same sampling sites were included in 16S rDNA metabarcoding. One pollen sample returned less than 1000 filtered reads and was excluded from any downstream analysis. For the rest of the samples, sequencing generated a range of 1008 to 15843 filtered reads (average: 4372.8 reads) for larvae, 1006 to 14664 reads (average: 3538.3) for pollen and 1726 to 29697 (average: 10748.3) for soil.

Furthermore, 31 larvae which were reared and manipulated *in vitro* were also included in 16S rDNA metabarcoding. Larvae which fed on untreated pollen returned 1017 to 12490 filtered reads (average: 5261.3) and larvae which fed on pollen which was previously inoculated with bacterial solution (*Bacillus* strain closest assigned to *B. pumilis* and to OTU 23) returned 1090 to 3736 filtered reads (average:2344.3). The larvae which fed on sterile

pollen returned a low count of filtered sequencing reads (40 to 726, averge:313) and were therefore excluded from any downstream analysis. The pollen sample which was used to feed the first group of larvae was also sequenced for its bacterial community (1643 filtered reads).

3.8.1. Bacterial biodiversity comparison between nests with healthy and deceased larvae

Mean Shannon alpha-diversity based on OTU richness was lower for deceased larvae, their nest material and their pollen provisions (**Figure 23**). The difference of means between healthy and deceased was not statistically significant for larvae (anova: F=0.19, *p>0.664*) or for soil (F= 3.31, *p>0.076*). However, the respective difference was significant for pollen samples (anova: F=11.14, *p<0.0018***).

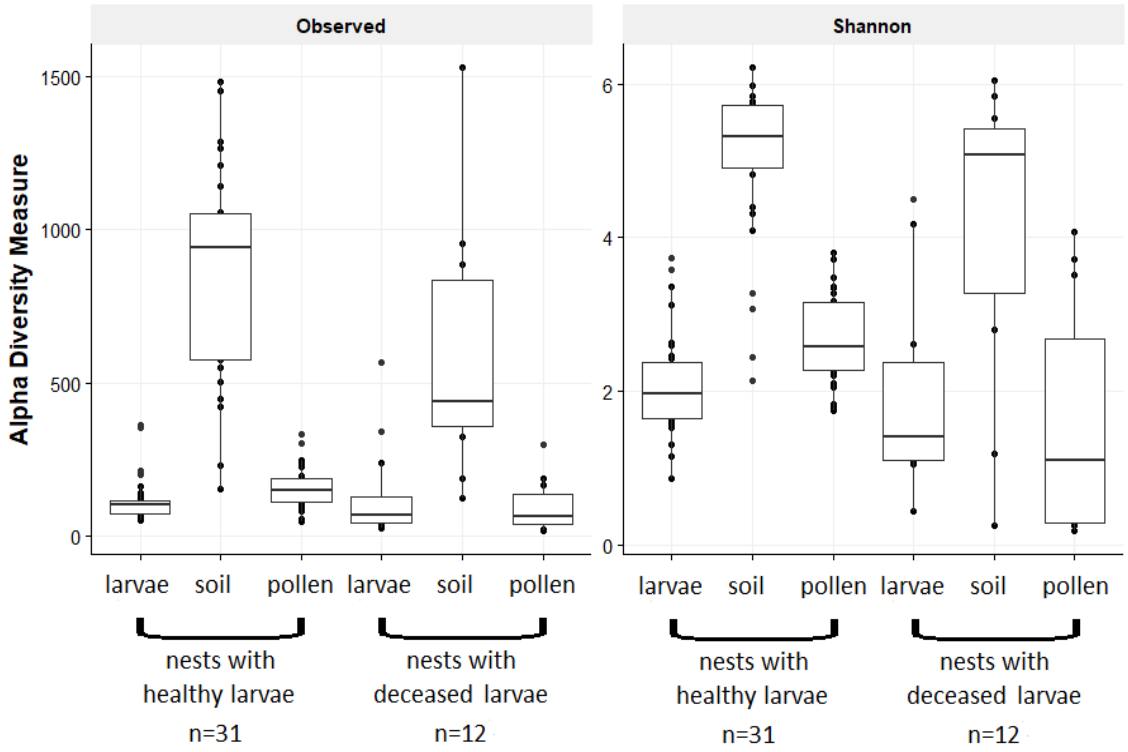

Figure 23. Shannon diversity based on the OTU identities found in larvae, pollen and soil from 31 nest chambers with healthy *O. bicornis* larvae and 12 nest chambers with deceased *O. bicornis* larvae.

3.8.2. Bacterial community comparison between nests with healthy and deceased larvae

NMDS ordination and linear discriminant analysis showed differences in the bacterial microbiome structure between the two groups (**Figures 24** and **25**). PERMANOVA results for bacterial microbiome differences between healthy and deceased larvae and their pollen provisions were statistically significant, yet weak (r^2=0.214, *p<0.001**** and r^2=0.167,*p<0.001****, respectively), while beta dispersity of both datasets was homogeneous for deceased and healthy larvae (*p>0.05*) and their respective pollen provisions (*p>0.05*). PERMANOVA results for differences in soil bacterial microbiome between the two groups were even weaker (r^2=0.04, *p<0.05**, betadisper *p>0.05*).

The bacterial communities in healthy larvae were more homogeneous between samples than the ones in deceased larvae (beta dispersity: 0.29 and 0.56, respectively). Comparison between healthy and deceased larvae, showed a rise of aerobic Firmicutes at expense of Proteobacteria in deceased larvae and their pollen provisions. The dominant phylum in the bacterial communities of healthy larvae was Proteobacteria (88.43%), while in the deceased larvae, Firmicutes constituted the 59.67% of the community (mostly due to OTUs assigned as *Bacillus*, *Paenibacillus*, *Sporosarcina*, **Figure 26**).

Bacterial OTUs from all larvae, both healthy and deceased, were tested for statistically significant correlations and negative and positive relationships were retrieved (**Figure 27**). Three OTUs which prevailed in the compositional data from the deceased larvae show negative relationships with four OTUs which occur in all healthy samples tested. The OTU 19 which was found in the deceased larvae was assigned to the *Paenibacillus pabuli/amylolyticus/xylanexedens* complex.

The bacterial communities in the respective pollen provisions were slightly more homogeneous between samples for healthy larvae (beta dispersity: 0.48) than for deceased larvae (beta dispersity: 0.57). Moreover, there was also a rise of Firmicutes (47.60% in provisions of deceased larvae) at the expense of Proteobacteria (86.58% in provisions of healthy larvae). However, this was mostly due to the abundance of OTUs assigned as Lactobacilli in the community (**Figure 26**). At the same time pollen of healthy larvae showed low or undetectable levels of Lactobacilli.

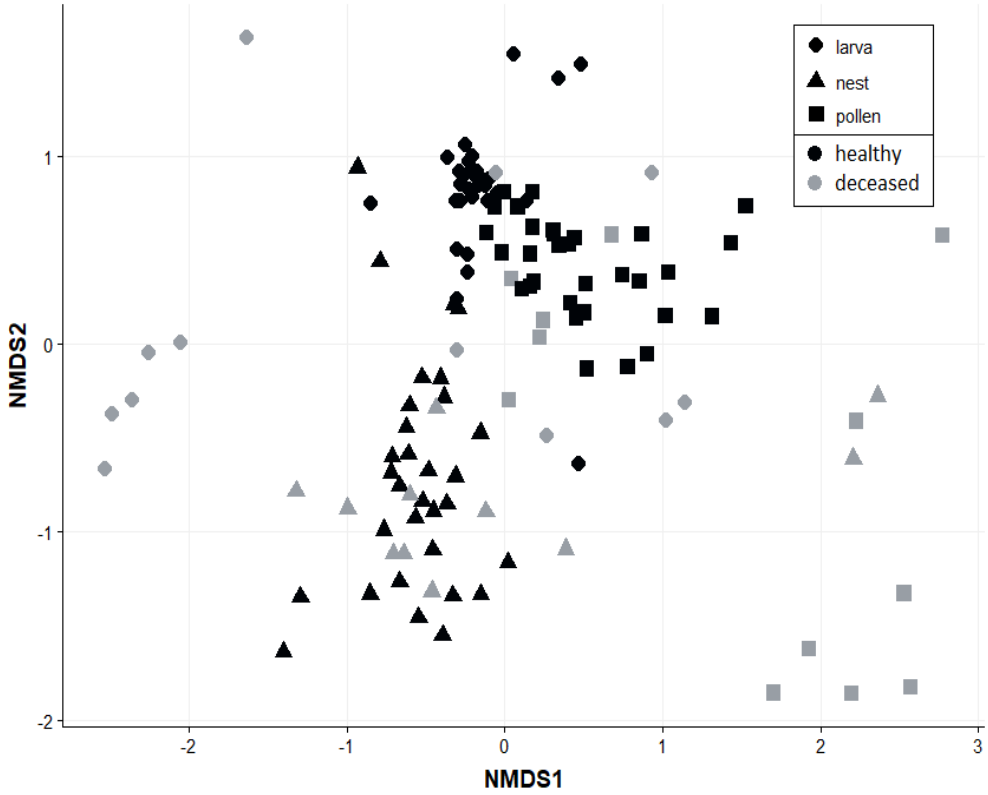

Figure 24. Bray Curtis based NMDS ordination of all samples (larvae, pollen and soil pellets) acquired from 43 *O. bicornis* nest chambers. Of the latter, 31 contained healthy larvae and 12 contained deceased larvae.

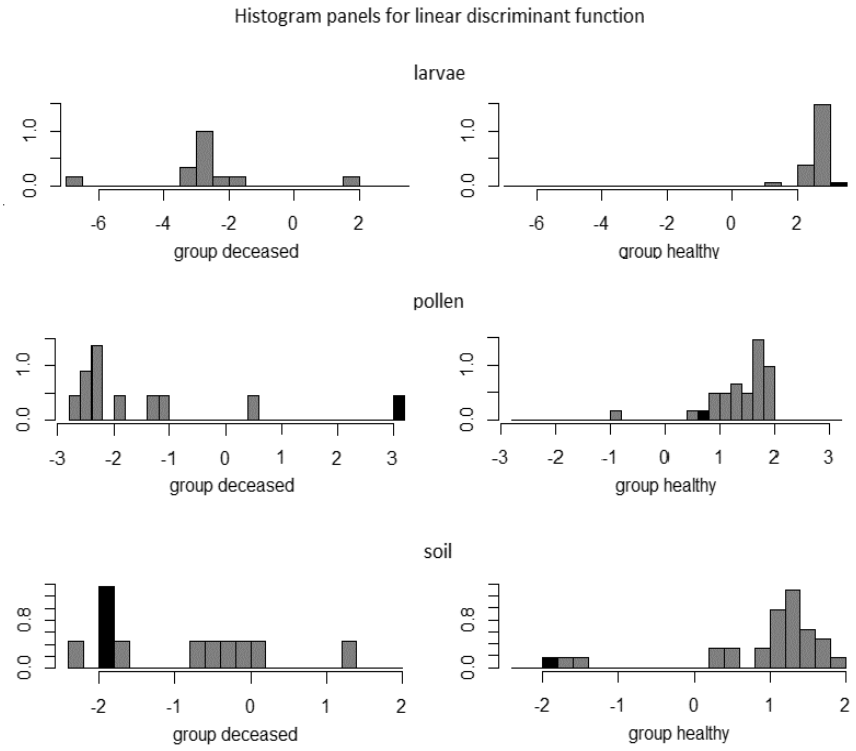

Figure 25. Histogram panels for linear discriminant function of all samples after setting the status of health (healthy/deceased) as discriminative class.

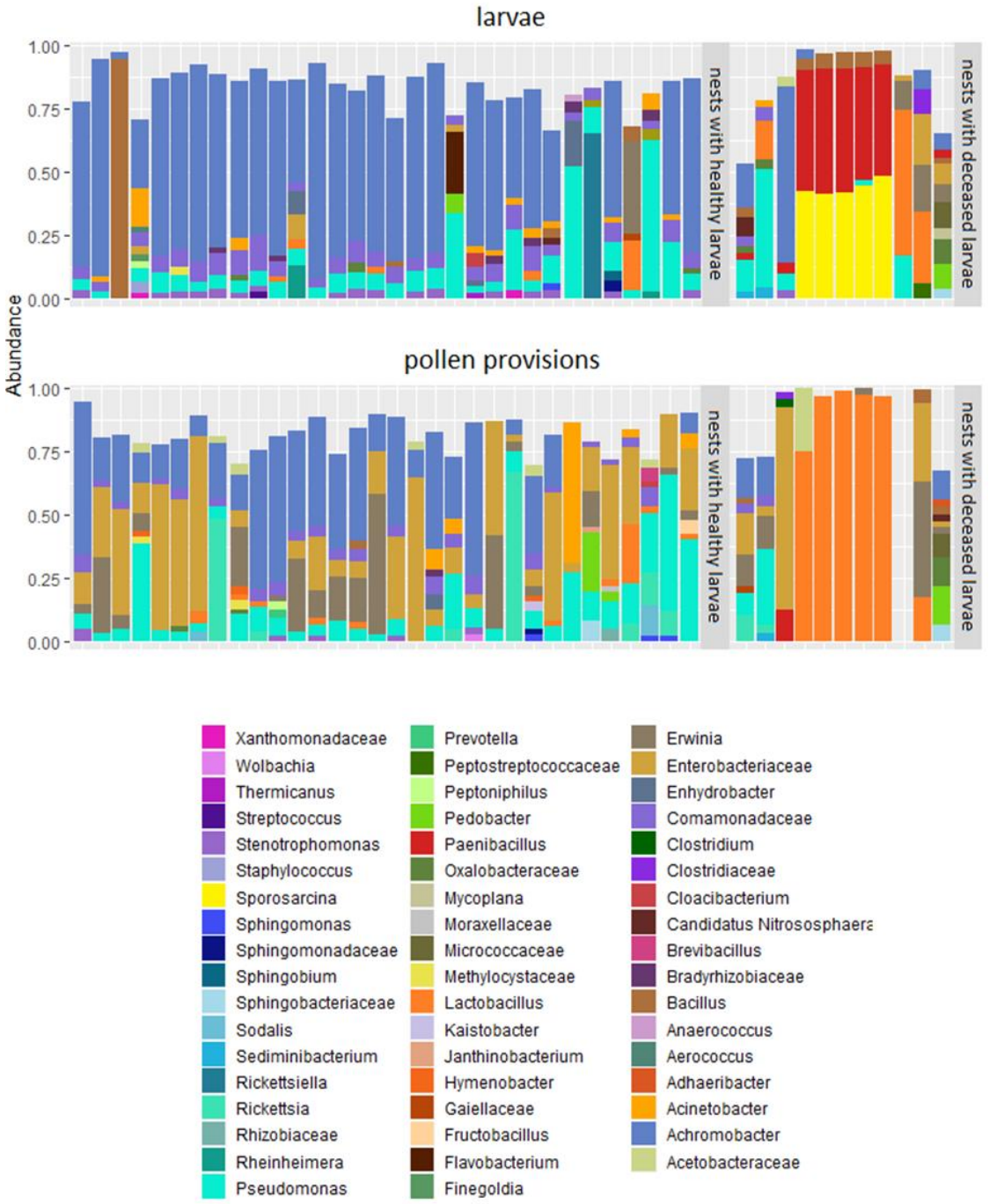

Figure 26. Bacterial composition in larvae and pollen samples acquired by 43 O. bicornis nest chambers belonging to different nests. 31 nest chambers contained healthy individuals and 12 contained deceased individuals. Bacterial OTUs are agglomerated up to genus level (family level if not better classifiable).

The bacterial composition datasets between larvae and their respective pollen provisions were correlated with Mantel tests and returned statistically significant associations. The matrix correlation value between the bacterial compositions of healthy larvae and their provisions was r=0.30 (*p<0.05**) and the respective value for deceased larvae and their provisions was r= 41 (*p<0.05**)

The bacterial communities in soil were much more diverse (**Figure 23**) and thus their composition was constituted by more taxa with lower relative abundances. The most prevalent taxa were the genus *Bacillus* (Firmicutes) (10.33% and 10.57% for nests with healthy and deceased larvae. respectively), the family of Gaiellaceae (Actinobacteria) (5.40% and 5.46% for nests with healthy and deceased larvae. respectively) and the genus *Achromobacter* (Proteobacteria) (5.95% and 2.30% for nests with healthy and deceased larvae, respectively).

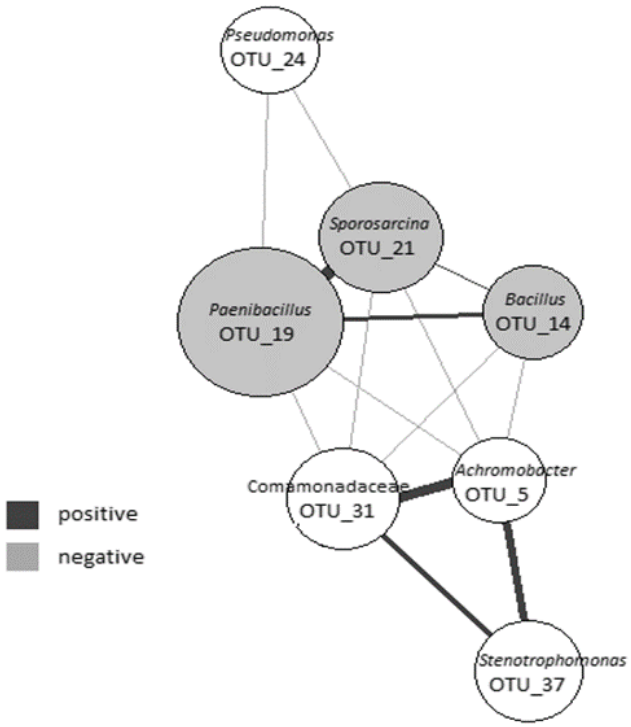

Figure 27. OTUs from the collective bacterial composition dataset from 31 healthy and 12 deceased larvae, showing positive or negative correlations (shown when correlation value is over0.35, and *p<0.001*). The width of the drawn lines in analogous to the correlation value.

3.8.3. Bacterial community of *in vitro* manipulated larvae

Alpha diversity of the two groups of larvae (larvae which fed on not manipulated pollen acquired from an *Osmia bicornis* nest and larvae which fed on pollen inoculated with the isolated *Bacillus* strain, which was closest assigned to *B. pumilis*) is shown on **Figure 28**. Multivariate dispersions within each group were heterogeneous (dispersity values: 0.30 and 0.07, respectively, permutation test for homogeneity *p<0.001****).

The type of the treatment was successful as a discriminant factor for the bacterial communities found in manipulated larvae in linear discriminant analysis (**Figure 29**). The bacterial composition of the second group of larvae were characterized by *Bacillus* spp (**Figure 30**).

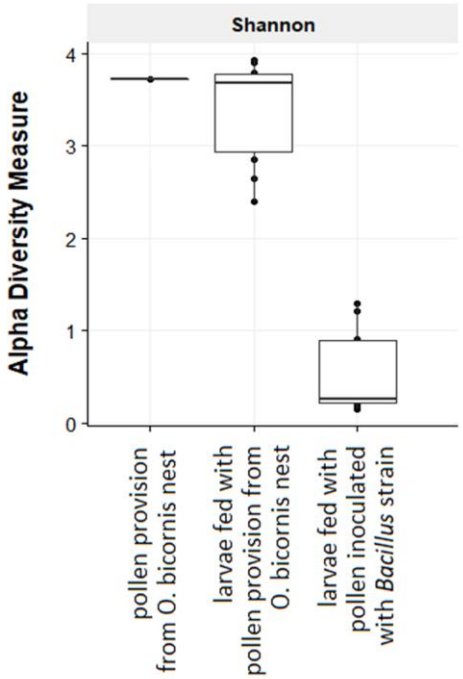

Figure 28. Shannon diversity of bacterial OTUs found in: a) one pollen sample on which 11 larvae fed on, b) 11 larvae which fed on the pollen sample and c) in larvae which fed on sterile pollen which was inoculated with one *Bacillus* sp. strain.

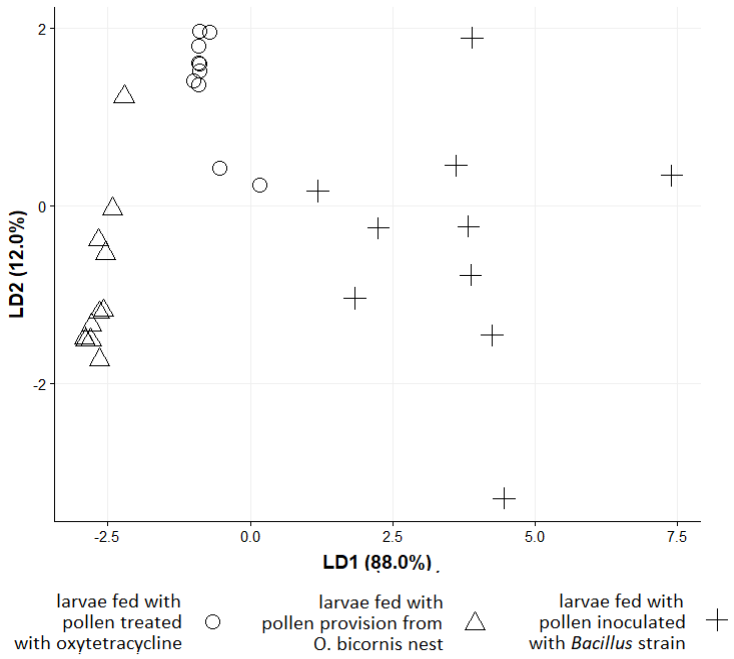

Figure 29. The two Linear Discriminants (LD1 and LD2) from Linear Discriminant Analysis of the bacterial OTU composition dataset from a) one pollen sample on which 11 larvae fed on, b) 11 larvae which fed on the aforementioned pollen sample and c) 10 larvae which fed on sterile pollen which was inoculated with one Bacillus sp. strain.

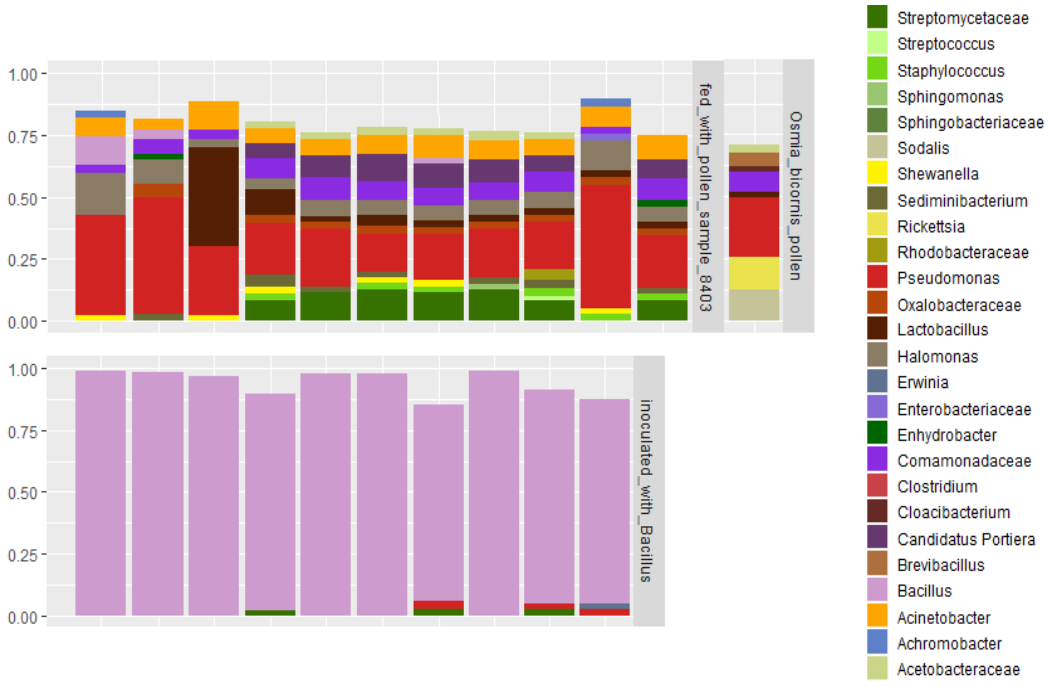

Figure 30. Bacterial composition in a) larvae which fed for five days on a pollen sample taken from one *O. bicornis* nest and b) from larvae which fed for up to five days on pollen inoculated with one *Bacillus* sp. strain. Bacterial OTUs are agglomerated up to genus level (family level if not better classifiable).

4. Discussion

4.1. Nest bacterial microbiome of seven solitary bee species

The environmentally susceptible conditions in the interior of the solitary bee nests, where larvae receive no active nursing and grow unattended, are connected with complex and diverse bacterial microbiota (Keller *et al.* 2013, McFrederick and Rehan 2016, Voulgari-Kokota *et al.* 2019). This complexity makes the bacterial community description for bee nests with no social structure a quite challenging task, since it can mask existing patterns and conserved paths of bee-microbe relationships. Indeed, the results revealed high bacterial biodiversity (**Figure 8**) and bacterial taxa showing consistent occurrence in the nests (**Table 8**), at the same time.

4.1.1. Nest bacterial diversity assessment

The bacterial biodiversity was significantly higher for the nest material specimens than for the pollen specimens and the bacterial biodiversity for pollen was higher than that of the larvae in the case of all seven examined solitary bee species (**Figure 8**). Furthermore, bacterial biodiversity values were significantly correlated between larvae and pollen. Since the larvae are firmly attached to the pollen clump and in contact with the nest building material during their transition to pupae, the difference of bacterial diversity suggests an ability of the larvae to actively or passively filter environmentally introduced microbes. Also, this finding suggests that the environment of the nest is beneficial for the growth of several bacteria.

Solitary bee nests harbor a high bacterial biodiversity; particularly in comparison with the one discovered in the nests of social bee species, where the core bacterial microbiome of the adults is constituted by less than ten bacterial taxa (Kwong and Moran 2016) and that of the larvae consists mainly by Acetobacteraceae and Lactobacillaceae (Anderson *et al.* 2016). Environmental susceptibility of the solitary bee nests is also indicated by the fact that the nest building material is a major path for bacterial transmission into the nests. Particularly in the case of *O. bicornis* nests, loam is characterized by an immense bacterial

biodiversity (**Figure 8**) as it is typical for soil specimens (Bardgett and Van der Putten 2014) and it maintains this high bacterial biodiversity after its inclusion in the nest.

4.1.2. Bacterial community description

The bacterial community structure in the nests showed differences between the seven bee host species (**Figure 7**). In the case of the *Osmia* spp. and *H. truncorum* nests, a high Proteobacteria to Firmicutes ratio in the larvae and pollen provisions was observed. The families of Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae and Acetobacteraceae were the most prevalent among Proteobacteria. *Lactobacillus* was the most prevalent genus in *Megachile* larvae and pollen. Proteobacteria was the dominant phylum in the nest building material of all bee species which use plant material for the construction of their nests. In contrast, *O. bicornis* nest material showed a different and highly diverse bacterial composition typical for soil with contribution of several bacterial phyla (**Figure 9**).

Enterobacteriaceae and especially *Erwinia* spp. are bacterial taxa closely associated with flowers (Gnanamanickam 2006, Junker *et al.* 2011, Junker and Keller 2015) and also reported from other wild bee microbiota studies (McFrederick and Rehan 2016). Also, Pseudomonadaceae occurred particularly in pollen and nest material specimens. The family is diverse and typically associated with plant tissues, while it has been characterized as beneficial for plants and soil (Roberson and Firestone 1992, Chang *et al.* 2007).

Moraxellaceae and particularly *Acinetobacter*, a genus typically associated with insect pollinated plants (Alvarez Perez *et al.* 2013), was detected in all sample groups but mostly from *Osmia* and *Heriades* pollen data. Furthermore, Acetobacteraceae was detected in all sample subgroups and in *H. truncorum* pollen in particular. The family has been reported as important in food uptake and subsequent insect survival (Crotti *et al.* 2010).

Firmicutes are represented mostly by Bacilli in all host bee species. *Lactobacillus* spp. occurs is all sample types from all examined hosts. However, its community ratio is quite low in *H. truncorum* and *Osmia* spp., while it is closely connected with the pollen provisions as well as with the larvae of *Megachile* bees. The most abundant *Lactobacillus* phylotypes identified from *Megachile* spp. were closest assigned to phylotypes from a wild megachilid bee microbiota study (McFrederick *et al.* 2017).

Further examination of functional traits for these Lactobacilli could relate these bacteria with the Lactobacilli isolated from social bee colonies, where they are considered to contribute to pollen fermentation and bee defence against microbial pathogens (Vasquez *et al.* 2012), possibly by antimicrobial substance secretion (Killer *et al.* 2014). The consistent occurrence of *Lactobacillus* spp. in the pollen provisions of the solitary bee species (**Table 8**) could suggest a consistent relationship of the bees with bacterial agents of possible beneficial bioproperties.

Furthermore, *Bacillus* spp. occurred in all *H. truncorum* and *Osmia* spp.. Members of the genus have been reported as beneficial for honey bee guts (Gilliam *et al.* 1990, Sabaté *et al.* 2009). At the same time, there are species under the genus known as toxin producing and harmful for insect larvae and pupae (Jurat-Fuentes and Jackson 2012). Moreover, *Paenibacillus* is a notorious genus in bee microbiota studies, as *Peanibacillus larvae* is the cause of American Foulbrood of honey bees (AFB) (Genersch 2008, Genersch 2010). However, many members of the genus are important in environmental biocontrol, since they possess beneficial antifungal and antibacterial bioproperties (Raza *et al.* 2008, Naing *et al.* 2014).

Finally, the endosymbiotic genus *Wolbachia* was highly abundant in *H. truncorum*, *O. caerulescens* and *O. leaiana* larvae*,* while the genus *Sodalis*, recently reported as symbiotic in the eusocial form of several Halictidae (Rubin *et al.* 2018), occurred in *M. versicolor* larvae. All in all, the occurrence of endosymbiotic bacteria and the different levels of typical floral bacteria in pollen and larvae indicate that there is a barrier for passive bacterial transmission to the larvae, even though the natural solitary bee microbiome nest harbors many bacteria of environmental origin.

4.1.3. Bacterial succession in line with larval development

In social bee colonies, processed pollen and nectar offers the larvae a protein-rich nutritional mixture (Ellis and Hayes 2009). The quantity and quality of this mixture affects egg production and larval growth (Schmickl and Crailsheim 2002). The pollen grains are initially broken by the worker bees, while digestion relies on enzymes and low PH (Velthuis 1992). Actively inoculated lactic acid bacteria contribute to the bee bread production (Vásquez and Olofsson 2009).

Accumulated pollen inside the solitary bee nests is the source of nutrition that the larvae feed on during their development. This process takes place without any help from social interactions, therefore the digestion of the provided pollen is a major issue. In the solitary bee nest, eggs are laid directly onto a raw pollen clump and it is not clear how larvae can handle the complex pollen grains. The microbiome of the pollen mixtures could be the key of this process. Possible community transitions or selection from typically floral bacteria towards bacteria assisting in pollen pre-digestion should be at the center of this investigation.

The relationships between larval size and relative abundance of all bacterial families were examined for *H. truncorum*, *O. bicornis* and *M. rotundata*, which were the bee species represented with the most samples in the dataset. Larval development was accompanied with a fall of Enterobacteriaceae for *O. bicornis*, a fall of Enterobacteriaceae and Pseudomonadaceae for *H. truncorum* and a fall of Lactobacillaceae and Pseudomonadaceae for *M. rotundata* (**Table 9**, **Figure 10**). In general, flower specific bacterial taxa of the initial community (Enterobacteriaceae and Pseudomonadaceae; Junker *et al.* 2011, Junker and Keller 2015) are replaced by others, probably better adapted to grow on the accumulated pollen provision.

Further investigation should include biochemical testing of the early and late stage bacterial community in the pollen provisions. Thus, the ability of late stage bacteria to ferment pollen would be assessed as sufficient or insufficient to provide pre-digestion of the complex pollen mixtures. In such a case, the pollen bacterial microbiome succession would be proven as a major aid to the larval development.

4.2. Association of solitary bee nest bacterial microbiota with the surrounding environment

Solitary bees demonstrate a variety of different preferences, when it comes to the proper construction of their nests and the foraging of pollen and nectar. The inclusion of environmental materials in the nest construction, as well as availability and preferences for different pollen sources, could significantly influence the microbiome of the resulting nest.

The composition of nest construction materials and pollen may change between landscapes and biogeographical regions even within the same bee species. Indeed, plant community variability between regions has shown an effect on bee forage (Steffan-Dewenter and Kuhn 2003, Danner *et al.* 2017, Persson *et al.* 2018). The information regarding the sampling sites was used to investigate the impact that landscape and region may have on the discovered bee nest bacterial communities.

4.2.1. Association with landscape type and landscape diversity

Landscape type and landscape diversity had no direct effect on the shaping of the microbiome structure of the larvae and pollen samples in this study, both at an interspecific and at an intraspecific level (**Table 10**). As the present study was restricted to regions with similar land use composition, further studies with populations from more contrasting landscapes might show significant environmental effects on solitary bee microbiomes.

4.2.2. Association with geographic region

Despite the absence of a clear landscape effect, the pollen bacterial communities from early stage *O. bicornis* larvae, *H. truncorum* larvae and pollen and *M. rotundata* pollen were dependent on the sampling site they originated from. Although region had an effect on the pollen bacterial microbiota (**Table 10**), larvae were not significantly influenced by region with the exception of *H. truncorum* larvae. This finding adds to the conclusion that larvae are able to filter environmentally introduced bacteria.

4.3. Association of solitary bee nest bacterial microbiota with their foraging preferences

Pollen metabarcoding allowed the discovery of the plant species, which consisted the pollen provisions in the sampled nests without the need of palynological observations (Keller *et al.* 2015, Sickel *et al.* 2015, Bell *et al.* 2016). At the same time, next generation sequencing of the 16S rDNA gene allowed the characterization of bacterial diversity and composition of the same specimens. Thus, the testing of possible relationships between pollen and bacterial species community became possible.

4.3.1. Foraging preferences of the sampled solitary bees

The examination of the pollen composition showed different foraging preferences for the included bee species (**Figure 11**). The dataset included both oligolectic bee species which showed preference for one specific plant family (*H. truncorum*, *O. caerulescens*, *O. leaiana*) and also polylectic generalists **(Figures 13**, **14**). Nevertheless, plant species diversity was not lower for oligolectic bee species (**Figure 12**).

From the plant side, the revealed plant-pollinator associations showed several plant species, pollen of which mainly exists only in the nests of one bee species (**Figure 15**). This indicates the important of bee diversity for the efficient pollination of a wide variety of plants. In fact, out of the most abundant plant species in the plant dataset, five are specifically associated with *O. bicornis*. These are *Acer campestre*, *Acer pseudoplatanus*, *Conringia orientalis*, *Juncus compressus* and *Brassica napus*. Furthermore, *Jacobaea vulgaris*, *Solidago virgaurea* and *Pimpinella saxifraga* are specially associated with *H. truncorum* and *Medicago minima* with *O. caerulescens*.

Additionally, if plants act as reservoirs or transfer hubs for bacteria (McFrederick *et al.* 2017), specialized bee-plant interactions in a landscape could secure specialized bee-bacteria relationships. Thus, plant availability and specialized interactions with plants would be significant not only for the nutrition of the bees, but also for the maintenance of their necessary nest microbiome; and consequently for the health of the larvae.

4.3.2. Association of plant biodiversity with the bacterial biodiversity

Introduced pollen in solitary bee nests could be a major bridge for bacterial colonization inside the nest chambers. One way the pollen provision could acquire its bacterial community would be through active inoculation by the mother bee, while diversity in plant sources could also contribute to its bacterial diversity. The results showed a weak correlation between bacterial and pollen alpha-diversity (**Figure 16**), showing that introduced pollen can bring a wide diversity of microbes into the nests (McFrederick and Rehan 2016).

4.3.3. Co-occurrence of plant species with bacteria

Co-occurrence analysis enabled the investigation of the bacterial taxa from pollen, which may be associated with specific plants. On one hand, it was expected to find bacterial taxa which are commonly associated with plants (Gnanamanickam 2006, Junker *et al.* 2011, Álvarez-Pérez 2013, Junker and Keller 2015), to be part of co-occurrence relationships with various plant species. The genus *Pseudomonas*, for instance, was randomly correlated with most of the plant species, while the genus *Erwinia* co-occured with plants from different families such as Asteraceae, Apiaceae, Juncaceae and Brassicaceae (**Figure 17**).

On the other hand, co-occurrence analysis can help to focus on bacterial taxa which are likely to adopt key functions for the larval health. More specifically, Lactobacilli could be acquired from several Asteraceae plants, with which they have a positive co-occurrence relationship (**Figure 17**). *Achillea millefolium*, in particular, which is associated with *Lactobacillus* spp. is visited by all three *M. rotundata*, *H. truncorum* and *O. leaiana*. *O bicornis* bees, on the other hand, do not feed on Asteraceae and the existence of Lactobacilli in their provisions and larvae are very low.

4.3.4. Association of pollen composition with the bacterial community

Pollen composition was significantly correlated with the bacterial community in pollen, as well as with the bacterial community in larvae through the whole dataset (**Table 11**). Causal analysis suggested an effect of the plant community structure on the pollen bacterial microbiome, which then significantly affected the larval bacterial microbiome. Host bee species had a strong effect on all steps of the path, indicating that bacterial communities are the result of a combinatory process (**Figure 19**). These findings are furthered supported by the fact that pollen bacterial communities were successfully assigned to both host species and pollen composition with regression analysis (**Tables 12** and **13**), while larval bacterial communities were less successfully assigned to pollen composition.

Correlations of pollen composition with bacterial communities within each host bee species did not return significant results (**Table 11**). It has been previously proposed that the influence of the pollen composition on the brood provision microbiome can be masked when examined at a small scale (for instance, when interactions within a bee species are

investigated), while the same influence can be made apparent when different bee species with distinctive foraging preferences are compared (McFrederick *et al.* 2017). However, successful assignment of pollen bacterial communities to pollen composition within each host species (**Table 13**) showed that the nest microbiome of a species depends on the pollen provided to the larvae. The foraging preferences of solitary bees species influence the solitary bee nest microbiome by the direct introduction of environmental and foremost floral bacteria.

4.4. Contribution of the nest building material to the overall nest bacterial microbiome

In the social bee hives, the microbiome of the nest is actively controlled by the worker bees, while formed niches in its interior host distinct bacterial communities (Anderson *et al.* 2013). In the solitary bee nests, the inclusion of the nest building material introduces a wide biodiversity of environmental bacteria which contribute to the nest natural microbiota.

4.4.1. Effect of the nest material to the larval bacterial microbiome

In the examined solitary bee nests, larvae, pollen and nest material often shared bacterial taxa. In some cases, these bacterial taxa were shared between larvae and nest material, but they did not occur in the respective pollen provisions from the same nest chambers (**Table 8**), indicating that larval bacterial microbiome might be affected by the nest chamber walls. Indeed, the larval bacterial communities structure and diversity were associated with the type of the nesting material.

Furthermore, the bacterial biodiversity of the nest material samples from seven solitary bee species was assessed and correlated with the respective larval bacterial biodiversity, adding to the conclusion that the introduced environmental material contributes to the nest natural microbiome. Bacterial biodiversity was not significantly different for the nest materials of the different bee species, with the exception of *O bicornis* nests. Loam from *O. bicornis* nests hosted a rather high, soil-typical bacterial biodiversity in comparison with the plant derived nest materials(**Figure 8**).

Although the bacterial community structure and diversity of the nest material were associated with that of the larvae, the number of the bacterial OTUs which were consistently shared between the larvae and nest material was restricted. For *H. truncorum*, *M. rotundata* and *M. versicolor*, in particular, there was no such shared OTUs. The combination of these facts propose that to a certain degree the nest environment contribution to the larval microbiome might be erratic.

4.4.2. Each nest material type introduces different kind of bacteria in the nest

Indicative bacterial taxa for each nest material type were different for loam, resin and plant derived materials, showing that the type of the nest building material influences the nest natural microbiome (**Figure 22**). The bacterial communities from all nest material specimens were successfully assigned to each material type.

Leaves for *Megachile* spp. and plant tissue for *O. caerulescens* and *O. leaiana* were characterized by Comamonadaceae and the genera *Lactobacillus*, *Sphingomonas*, *Erwinia* and *Pseudomonas*, which are typical epiphytic taxa and phyllosphere specific (Wilson and Lindow 1993, Innerebner *et al.* 2011, Williams and Marco 2014, Pontonio *et al.* 2018). Plant tissue was also linked with *Hymenobacter*, studies for which from apple tree orchards and grapevines have shown that members of the genus are common for the phyllosphere (Ottesen *et al.* 2009, Leveau and Tech 2011).

The nest material in *H. truncorum* consists of small pebbles and other environmental materials stabilized with the use of resin. Resin plant extracts are well known for its antimicrobial properties (Shuaib *et al.* 2013), therefore the use of resin might restrict the bacterial transmission between nest chambers. Examination of the *H. truncorum* nest material showed that it is characterized by Comamonadaceae, *Erwinia*, *Bacillus* and mostly by the genus *Pseudomonas*, a taxon reported as beneficial for plants and soil (Roberson and Firestone 1992, Chang *et al.* 2007).

Finally, *O. bicornis* nest material hosted bacterial microbiota of great biodiversity. The indicative taxa for loam in the nests include highly bioactive Actinobacteria, such as Pseudonocardia and Gaiellaceae (Hermans *et al.* 2017, Wang *et al.* 2017) and aerobic Firmicutes such as *Bacillus* spp. and *Paenisporosarcina* spp..

4.5. Beneficial properties of bacteria in the nests

The honey bee microbiota has been proven to be a component of the bees' health, playing a significant role in the nutrition of the bees and the defense of the colony against a variety of pathogens (Engel *et al.* 2012, Engel and Moran 2013). In the solitary bee nests, the presence of symbiotic bacteria could play a similar part, particularly since solitary bee larvae do not benefit from social interactions and active nursing.

4.5.1. Antimicrobial activities of nest bacteria

Two *Paenibacillus* strains (A147, A55), assigned as *P. polymyxa* and *P. ehimensis* and isolated from *O. bicornis* nest material (loam) and an *O. bicornis* larva, respectively, showed strong antifungal activities against a fungus which had grown in different *O. bicornis* nests (**Image 3**). The two species often associate with biocontrol services (Raza *et al.* 2008, Naing *et al.* 2014) as they protect many plant species from plant pathogenic fungi and other microorganisms (Beatty and Jensen 2002, Haggag and Timmusk 2008, Son *et al.* 2009).

Even though the genus includes severe bee pathogens (Genersch 2010, Forsgren 2010, Grady *et al*. 2016), *Paenibacillus* bacteria have been reported from nests of mason solitary bee species as part of their natural microbiota (Keller *et al*. 2013, Lozo et al. 2015). In general, the humidity, the richness in available nutrients and the overall conditions in the mason bee nests can benefit fungal growth which can harm the larvae. Therefore, the presence of bacteria such as the isolated *Paenibacilli* can be proven very important for the larval health (Keller *et al.* 2018).

4.5.2. Enzymatic activity of nest bacteria

A number of bacteria inhabiting the social bee gut have been attributed roles in the bees' digestion process, as they are proven to ferment complex carbohydrates and biosynthesize nutrients in favor of their hosts (Engel *et al.* 2012, Lee *et al.* 2014). Although the same has been suggested for the bacteria which live in the stored pollen of the social bee hive, their number is low and it has been suggested that all major digestion processes are mediated by the worker bees' gut (Anderson *et al.* 2014). For the solitary larvae which grow unattended, nevertheless, the presence of bacteria in the nest has been suggested as a

necessary substitute for the socially aided pollen fermentation and nutrient uptake process (Keller *et al.* 2013, McFrederick *et al.* 2017, Voulgari-Kokota *et al.* 2019). Yet, no study has undertaken the biochemical profiling of solitary bee nest bacteria so far.

Since bees forage on pollen and nectar, it is necessary to consider how they metabolize the plant derived nutrients and how their microbiota helps in this process. Nectar is predominantly composed of sucrose and its component monosaccharides, fructose and glucose (Doner 1977, Nicolson and Thornburg 2007). Other sugars that are most likely to exist in nectar are the monosaccharides mannose, arabinose, xylose, the disaccharide maltose, the oligosaccharide raffinose and the sugar alcohol sorbitol (Nicolson and Thornburg 2007). As for pollen, honey bees are not able to survive only on raw pollen grains, because they have hard cell walls and are therefore difficult to digest (Haydak 1970). Pollen maturation has proven to be assisted by bacteria in the honey bee gut (Lee *et al*. 2014). Bacteria with glycosidase and peptidase activity can participate in the degradation of plant polysaccharides, oligopeptides and complex plant material, in general. Shorter saccharides, peptides and amino acids produced by this digestive process could be taken up by other bacterial members of the bee microbiome (Lee *et al*. 2014).

In the present study, a number of biochemical tests was conducted in order to test whether there are nest bacteria which could potentially help the larvae degrade and digest the complex pollen provision. Some isolated strains were typical endophytic bacteria, providing no apparent service to the larvae. For example, *M. aloeverae*, a member of Actinobacteria, which was isolated from *M. rotundata* nest material (**Table 14**), is a typical endophytic bacterium with beneficial biochemical properties for the plant (Prakash *et al*. 2014). However, others, such as *E. cloacae*, isolated from a *M. rotundata* larva (A145), were bacteria which could potentially be a part of digestive processes. *E. cloacae*, in particular, occurs as commensals in the intestinal tract of animals including insects (Grimont and Grimont 2006).

The bacterial strains which were isolated and tested included several *Bacillus* spp.. The genus has a wide variety of species with diverse bioproperties which have been reported both as beneficial and as harmful for insects and bees in particular (Gilliam *et al.* 1990, Sabaté *et al.* 2009, Jurat-Fuentes and Jackson 2012). Among the isolated *Bacillus* strains, the strain

which was assigned as *B. licheniformis* (A244) was the sole genus member showing strong beta galactosidase activity, implying that it can use several substrates which result from fermentation chains, such as lactosylceramides, lactose and various glycoproteins (Trân *et al.* 1998). Also, all *Bacillus* strains were able to take up trehalose as their sole carbon source, a disaccharide which is used for energy storing in various insects (Ellegaard *et al.* 2015).

Aneurinibacillus and *Brevibacillus* strains could also play a part in the metabolism of smaller saccharides. *Aneurinibacillus* has been described as beneficial for plants metabolism of smaller saccharides such as lactose (Chauhan *et al.* 2017). Also, *Brevibacillus* is one of the most widespread genera of Gram-positive bacteria (Panda *et al.* 2014). Although it shows inability to use the majority of common saccharides (**Table 15**), it uses the phosphotransferase system for fructose as well as kinases for fructose and glycerol (Asatani and Kurahashi 1977) to take up fructose as a carbon source (Panda *et al.* 2014), becoming a good candidate for inhabiting the fructose-rich nectar.

Strains assigned as *P. polymyxa* and *P. ehimensis* (A147, A55) possess glucosidases, a trait typical for these species (Aktuganov *et al.* 2008, Lal and Tabacchioni 2009). Some glucosidases, in particular, are highly specific against cellobiose and show ability to degrade molecules of high polymerization degree. The species are also known for producing a vast amount of extracellular proteases taking part in natural biocontrol (Raza *et al.* 2008, Naing *et al.* 2014); a trait which could prove beneficial for the bee larvae in the solitary bee nest (Keller *et al*. 2018, Menegatti *et al*. 2018), as discussed in the subchapter *4.5.1.*.

One *Lactobacillus* strain (A1A3), which was isolated from *M. rotundata* pollen, showed a positive results for metabolizing a variety of oligosaccharides. Oligosaccharide metabolism has been discussed in detail for the genus and particularly for its role in fermentation of pollen in social bee hives (Anderson *et al.* 2014, Lee *et al.* 2014). Fructo-oligosaccharides, galacto-oligosaccharides and oligosaccharides of the raffinose-family are all carbon sources which are fermented by the members of the genus (Gänzle and Follador 2012), are intermediate products of the pollen fermentation in the bee nests (Killer *et al.* 2014) and are utilized as carbon sources from the isolated *Lactobacillus* strain in the present study (**Table 15**).

Finally, the *Acinetobacter* strain (A1J), which was isolated from *O. bicornis* pollen, could utilize sucrose and D-mannose, resembling the floral *A. nectaris*, in contrast with other strains of the genus (Álvarez-Pérez *et al.* 2013). The strains which have been assigned to *A. nectaris* were isolated from nectar of several plant species and it is considered a floral bacterium (Álvarez-Pérez *et al.* 2013). The presence of this strain in the pollen provision of *O. bicornis* shows that introduced floral bacteria in the nests can participate in sugar uptake.

4.6. Candidate pathogenic bacteria for solitary bee larvae

Solitary bees are subject to pressures, which may cause severe decline on their populations. 16S rDNA metabarcoding allowed the screening for potential bacterial pathogens in *O. bicornis* nests. Moreover, the larval bacterial microbiome of treated individuals was compared to test the effect of the bacterial microbiome of the pollen provision to their health.

4.6.1. Bacterial community in deceased larvae

The bacterial alpha diversity was in general lower for the larvae, pollen and nest material from nests with deceased individuals (**Figure 23**). Nevertheless, this difference was significant only in the case of the pollen. The bacterial composition, however, was significantly distinct between the two groups (**Figures 24** and **25**) and mostly for larvae and their respective pollen provisions, showing that the health state of the individuals in a nest is connected with their microbiome.

As previously shown (Keller *et al.* 2013, Voulgari-Kokota *et al.* 2019), the natural nest microbiome of *O. bicornis* is composed of a community of high bacterial diversity with Proteobacteria being the most prevalent phylum (**Figure 26**). Also, the bacterial alpha diversity in healthy larvae is lower than the respective diversity in the nest materials (pollen and soil, **Figure 23**). Moreover, the bacterial communities were fairly homogeneous between healthy larvae, in contrast with the ones found in deceased individuals. This finding suggests that either the bacteria which harm the larvae might be able to disrupt their natural microbiome structure or that the environment which harms the larvae supports a different and/or erratic bacterial community.

Screening for potential pathogenic bacteria in the nests of the widely spread *O. bicornis* unveiled several bacterial taxa, which dominated the bacterial community in deceased larvae, while at the same time they were undetectable in the healthy individuals. Pathogen screening for the deceased larvae revealed three bacterial OTUs with dominant presence assigned to the genera *Paenibacillus*, *Sporosarcina* and *Bacillus*. The microbiome network for all larvae (**Figure 27**) shows that the three candidate pathogenic taxa are positively correlated with each other (especially *Paenibacillus* spp. and *Sporosarcina* spp.), while at the same time they are negatively correlated with OTUs primarily found in the microbiome of healthy larvae. Similar networks have been proposed to indicate candidate taxa for a number of desirable or undesirable outcomes like the presence or absence of specific infections in plants (Poudel *et al.* 2016).

Paenibacillus is a notorious genus in insect microbiota studies. More specifically, *P. larvae* is considered to be the cause of acute mortal intestinal larval infections and colony collapse in honey bees (Genersch 2010) and *P. alvei* is a saprophytic, aerobic bacterium which does not grow in healthy bee larvae, but can establish in diseased honey bee colonies in larval remains (Forsgren 2010). At the same time, *P. polymyxa* has been described as a beneficial member of the bee microbiota (Keller *et al*. 2018, Menegatti *et al*. 2018). The discovered *Paenibacillus* OTU in the present study was closest assigned to the *P. pabuli/amylolyticus/xylanexedens* complex.

Sporosarcina, on the other hand, is less characterized as insect related, although strains of the genus have been isolated from *Galleria mellonella* larvae which were infected with nematodes (Georgieva *et al.* 2005). *Bacillus* spp. form a wide and diverse bacterial group with members known as toxin exerting and lethal for insect larvae and pupae (Jurat-Fuentes and Jackson 2012). Functional assays should be oriented into examining whether the discovered taxa are opportunistic secondary invaders or main causes for the larval mortality.

Lactobacillus spp. was prevalent in several pollen samples from the nests of deceased larvae, in contrast with the pollen provisions of healthy larvae, where the ratio of the genus was very low. Although Lactobacilli were found in the pollen provisions of the *Megachile* solitary bees and the species *O. caerulescens* (McFrederick *et al.* 2017, Voulgari-Kokota *et*

al. 2019), they were absent from healthy *O. bicornis* nests (Keller *et al.* 2013, Voulgari-Kokota *et al.* 2019). The discovered Lactobacilli in the pollen provisions of the deceased larvae and in some cases in the larvae themselves (**Figure 26**) might be thriving because of the death of the individuals, fermenting the accumulated organic material in the nest.

The soil used as nest material showed the highest bacterial diversity in the nests (**Figure 23**), as expected. Furthermore, the most dominant bacterial taxa were the same between soil pellets from nests with healthy and from nests with deceased larvae. The similarity of the soil bacterial microbiome between the two groups indicates that soil can sustain its natural microbiome in the bee nest. Therefore, apart from protection against intruders, its use as a nest construction element could also provide protection against environmentally transmitted bacteria.

Screening of potential bacterial pathogens in more bee populations would enhance current knowledge on candidate pathogens. Also, inclusion of a variety of landscapes would enable the discovery of pathogens from a broad geographic scale in the nests of specific species, in case they do not occur opportunistically in limited areas.

4.6.2. Pollen provisions as a source of introducing harmful bacteria in the nest

Larvae which were raised *in vitro* developed distinct bacterial microbiomes according to their diet (**Figure 29**) and their health was affected. The *in vitro* manipulation of *O. bicornis* larvae showed that the pollen provision was able to drastically affect the bacterial microbiome of the larvae. Bacterial diversity for larvae which fed on untreated pollen was comparable to that of the actual pollen provision they fed on, while bacterial diversity found in larvae which fed on *Bacillus* inoculated pollen was low with their microbiome composed almost entirely by the introduced potential pathogen (**Figures 28** and **30**). Also, the health of the life span of the second group of larvae was reduced.

Unfortunately, it was not possible to look into the composition of larvae which fed on sterile pollen. However, if the newly eclosed larvae in the nests are sterile, as it has been proposed for honey bees (Kwong and Moran 2016), this would explain the high dependence of the larval microbiome to the that of the pollen provision and their susceptibility, since microbial antagonists are lacking.

Indeed, the association of larval and pollen bacterial microbiome was also significantly positive for the individuals sampled from actual nests, showing that the larval bacterial microbiome depends on the provided pollen. When combined with the results from the *in vitro* treatment experiment, this indicates that larvae can also acquire pathogens from their food. It has been recently shown that solitary bee larvae can also acquire plant pathogens from their food (Rothman *et al.* 2018); the effect on bee health however is yet unclear.

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