Microbes in bee-plant networks: Composition characterization and their ecological implications

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Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus-Liebig-University Gießen for the Safeguarding of Good Scientific Practice".

Gießen, 26.06.2025 Haoran Shi

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List of abbreviations

AAI	average amino acid identity
ABPV	Acute bee paralysis virus
AMP	antimicrobial peptide
ANI	average nucleotide identity
ASV	amplicon sequence variants
BQCV	Black queen cell virus
CBPV	Chronic bee paralysis virus
CCD	colony collapse disorder
CFU	colony forming unit
CLR	centered log-ratio
dDDH	digital DNA-DNA hybridization
DWV	Deformed wing virus
FAME	fatty acid methyl ester
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IMD/JNK	immunodeficiency and c-Jun N-terminal kinase
ITS	internal transcribed spacer
JAK/STAT	Janus kinase-signal transducers and activators of transcription
KBV	Kashmir bee virus
LAB	lactic acid bacteria
LPS	lipopolysaccharide
mdFDR	mixed directional false discover rate
ML	maximum-likelihood
MP	maximum-parsimony
NJ	neighbor-joining
OUT	operational taxonomic units
PCA	principal component analysis
PCoA	principal coordinate analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
PERMDISP	Permutational Multivariate Analysis of Dispersion
PNA	peptide nucleic acid
POCP	percentage of conserved proteins
qPCR	Quantitative PCR
RDA	redundancy analysis
RNAi	RNA interference
SBV	Sacbrood virus
SNP	single nucleotide polymorphisms
StARS	Stability Approach to Regularization Selection
TEF	transcription elongation factor

Summary

Honey bees are vital pollinators in ecosystems around the world, and microbes play key roles in connecting bees and plants. Collectively, microbes, bees and plants form intricate tripartite interactions networks. Through a co-evolution, many bee- and plant-associated microbes have developed functions that benefit their hosts, including promoting growth, enhancing pathogen resistance, and aiding digestion. Microbiome associated with different hosts tend to be specific, and within bee-microbe-plant networks, both beneficial and pathogenic microbes are dynamically transmitted among hosts. In the current study, honey bee corbicular samples were collected over a two-year period from beehives at Justus-Liebig-University Giessen. Corbicular pollen is able to reflect both microbes and plants encountered by honey bees during foraging activity. Plant and microbial communities in honey bee corbicular pollen were profiled using 16S rRNA gene and plant ITS2 metabarcoding. The results indicated that the corbicular pollen microbiome exhibited clear seasonal variations, and was affected by multiple environmental factors as well as choices of forage plants. Co-occurrence network analysis further revealed specific plant-microbe associations and identified several hub plant taxa that may serve as hotspots for microbial transmissions. Following this study, we characterized bacterial and fungal microbiome of flowers from a highly insect-visited hub plant, bramble (genus *Rubus*), using 16S rRNA gene and fungal ITS2 metabarcoding. The data showed that insect visitation increased microbial loads on flowers and enriched specific microbial groups including fermentative and pathogenic microbes, highlighting the role of bramble flowers as hotspots for microbial transmission. In addition, insect visitation altered floral microbiome structure, potentially through the introduction of several hub microbial taxa and by increasing the centralization of the microbial interaction networks.

Honey bees were collected from beehives, and common bacterial, fungal and viral honey bee pathogens were screened. The expression levels of several immunity-related genes (*defensin-1*, *lysozyme-like*, *vitellogenin*, *glucose oxidase*) and the composition of the bee microbiome were examined to assess honey bee health status. Black queen cell virus (BQCV) was detected in almost all individuals, while *Vairimorpha* pathogens were only partially detected. *Paenibacillus larvae*, *Melissococcus plutonius*, Kashmir bee virus (KBV), Acute Bee Paralysis Virus (ABPV), Chronic bee paralysis virus (CBPV), Deformed Wing Virus (DWV), and Sacbrood bee virus (SBV) were not detected in the samples. The data indicated that BQCV and *Vairimorpha* infections had no significant impact on the expression of immunity-related gene.

Since the microbiome composition was assessed at the hive level and BQCV was present in every hive, its potential influence on the microbiome remains to be further clarified.

In addition, a bacterial isolate from birch pollen was phenotypically, genotypically, and chemotaxonomically characterized using a polyphasic approach. Based on 16S rRNA gene phylogeny and comparative genomic analysis, the isolate was identified as a novel species of the genus *Robbsia*. The bacterium was rod-shaped, non-motile, facultative anaerobic and grew optimally at 28 °C and pH 6–7. Unlike its closest relative *Robbsia andropogonis*, which is a known phytopathogen, the isolate exhibited no phytopathogenic traits such as flagellum formation, rhizobitoxine production, or induction of plant hypersensitive response. The proposed and accepted name of the isolate is *Robbsia betulipollinis* Bb-Pol-6^{T}.

Zusammenfassung

Weltweit sind Honigbienen unverzichtbare Bestäuber in Ökosystemen und Mikroorganismen haben eine zentrale Rolle in der Verknüpfung zwischen Bienen und Pflanzen. Gemeinsam bilden Mikroben, Bienen und Pflanzen komplexe dreigliedrige Interaktionsnetzwerke. Durch eine Koevolution haben viele mikrobiellen Begleiter von Bienen und Pflanzen Funktionen entwickelt, die ihren Wirten zugutekommen, darunter Wachstumsförderung, verbesserte Resistenz gegen Krankheitserreger und Unterstützung bei der Verdauung. Die mit verschiedenen Wirten assoziierten Mikrobiome sind meist spezifisch, und innerhalb von Bienen-Mikroben-Pflanzen-Netzwerke werden sowohl nützliche als auch pathogene Mikroben dynamisch zwischen den Wirten übertragen.

In der vorliegenden Studie wurden über einen Zeitraum von zwei Jahren Corbiculaproben von Honigbienen aus Bienestöcken der Justus-Liebig-Universität Gießen gesammelt. Corbiculapollen spiegeln sowohl die Mikroben- als auch die Pflanzenwelt wider, mit denen Honigbienen während ihrer Sammelflüge in Kontakt kommen. Die pflanzlichen und mikrobiellen Gemeinschaften in den Pollenhöschen wurden mithilfe von 16S rRNA Gen- und pflanzlicher ITS2-Metabarcoding analysiert. Die Ergebnisse zeigten, dass das Mikrobiom der Corbiculapollen deutliche saisonale Schwankungen aufwies und sowohl von verschiedenen Umweltfaktoren als auch von der Wahl der Trachtpflanzen beeinflusst wurde. Eine Kookkurrenznetzwerkanalyse offenbarte spezifische Pflanze-Mikroben-Assoziationen und identifizierte mehrere zentrale Pflanzentaxa, die als Hotspots für mikrobielle Übertragungen fungieren könnten. Außerdem wurde das bakterielle und fungale Mikrobiom von Blüten einer besonders häufig von Insekten besuchten zentralen Pflanze – der Brombeere (Gattung *Rubus*) – untersucht, ebenfalls mithilfe von 16S rRNA Gen- und ITS2-Metabarcoding. Die Daten zeigten, dass Insektenbesuche die mikrobielle Besiedlung der Blüten erhöhten und spezifische mikrobielle Gruppen, darunter fermentative und pathogene Mikroben, anreicherten, was die Rolle von Brombeerblüten als Hotspots für mikrobielle Übertragungen unterstreicht. Darüber hinaus veränderte der Insektenbesuch die Struktur des Blütenmikrobioms, möglicherweise durch die Einführung zentraler mikrobieller Taxa und durch eine stärkere Zentralisierung der mikrobiellen Interaktionsnetzwerke.

Honigbienen wurden außerdem aus den Bienenstöcken entnommen und auf häufige bakterielle, fungale und virale Krankheitserreger untersucht. Zur Bewertung des Gesundheitszustands der Bienen wurden die Expressionsniveaus mehrerer immunitätsrelevanter Gene (*defensin-1*, *lysozym-like*, *vitellogenin*, *glucose oxidase*) sowie die Zusammensetzung des Mikrobioms

analysiert. Das Black queen cell virus (BQCV) wurde in nahezu allen Individuen nachgewiesen, während *Vairimorpha*-Pathogene nur manchmal gefunden wurden. *Paenibacillus larvae*, *Melissococcus plutonius*, Kashmir bee virus (KBV), Acute Bee Paralysis Virus (ABPV), Chronic bee paralysis virus (CBPV), Deformed Wing Virus (DWV), und Sacbrood bee virus (SBV) wurden in den Proben nicht nachgewiesen. Die Daten zeigten, dass Infektionen mit BQCV und *Vairimorpha* keinen signifikanten Einfluss auf die Induktion und Expression der untersuchten Immunitätsgene hatten. Da die Zusammensetzung des Mikrobioms auf Bienenstockebene untersucht wurde und BQCV in jedem Stock vorkam, muss sein möglicher Einfluss auf das Mikrobiom in zukünftigen Studien weiter geklärt werden.

Darüber hinaus wurde ein bakterielles Isolat aus Birkenpollen mittels eines polyphasischen Ansatzes phänotypisch, genotypisch und chemotaxonomisch charakterisiert. Basierend auf der 16S-rRNA Genphylogenie und vergleichender Genomanalyse wurde das Isolat als eine neue Art der Gattung *Robbsia* identifiziert. Das Bakterium war stäbchenförmig, nicht-motil, fakultativ anaerob und wuchs optimal bei 28 °C und einem pH-Wert von 6–7. Im Gegensatz zu seinem nächsten Verwandten *Robbsia andropogonis*, einem bekannten Phytopathogen, zeigte das Isolat keine phytopathogenen Merkmale wie Flagellenbildung, Produktion von Rhizobitoxin oder Auslösung einer hypersensitiven Reaktion in Pflanzen. Der vorgeschlagene und akzeptierte Name des Isolats lautet *Robbsia betulipollinis* Bb-Pol-6^{T}.

Chapter 1 Introduction

1.1 Honey bee: history, importance, and crisis

Honey bees (genus *Apis*) in the family *Apidae*, order Hymenoptera (Crane, 2009), are important insects for humans and share a long history with us. Supported by numerous rock arts found in Africa, Europe, Asia, and Australia, wild honey gathering can be traced back to the Stone Age (Crane, 2005). The identification of bee wax residues in archaeological sites indicates that the domestication of honey bees started at least 9000 years ago (Roffet-Salque et al., 2015), and the most ancient artificial beehives and massive apiary date back to the 10th-9th centuries B.C.E, suggesting beekeeping activity was already an important and well-developed agricultural practice at that time (Bloch et al., 2010). The question of where honey bees originated is complicated. The Middle East and Africa have been proposed as the origin of honey bee expansions according to mitochondrial DNA marker or single nucleotide polymorphisms (SNP) analysis (Arias and Sheppard, 1996; Garnery et al., 1992; Whitfield et al., 2006). A recent study showed that all extant honey bee groups resulted from a more ancient split, and intragroup divergence of subspecies occurred more recently (Wallberg et al., 2014). Despite these contradictory hypotheses, it is undoubted that honey bees are spread extensively around the world by human activity and are constantly bred for desirable traits such as honey production, gentleness, and parasite resistance (Büchler et al., 2010; Lodesani and Costa, 2003). Currently, honey bees can be classified into five evolutionary lineages involving 33 subspecies, and Europe is inhabited by 13 subspecies from the lineages M, C and O (for review: Ilyasov et al., 2020). Commercially, honey bee subspecies *Apis mellifera ligustica* and *Apis mellifera carnica* are now the most favored breeds (Meixner, 2010), while the importation of the breeds could cause introgression to local bee subspecies such as *Apis mellifera mellifera* in north-western Europe (Ellis et al., 2018). As the indigenous subspecies are more adapted to local environment, such introgression may influence the overall survivorship and vulnerability of local bee colonies (Büchler et al., 2014, Schaumann et al., 2024).

The benefits generated by honey bees on a global scale are substantial and can be revealed both ecologically and economically. One major benefit of honey bees is their pollination service in different ecosystems. Hung et al. (2018) have performed a systematic and quantitative study based on 80 published plant-pollinator interaction networks worldwide to understand the importance of honey bees as pollinators in natural habitats. The pollination efficiency of honey bees is moderate among the insect pollinators, and even poor for some plant taxa with specialist pollinators (Aslan et al., 2016), however, they remain the most frequent floral visitors and important generalist pollinators in natural ecosystems, contributing to maintaining biodiversity.

The importance of honey bees in pollination also extends to agriculture. A study in 2007 reported that 35% of global food crop production was dependent upon animal pollination (Klein et al., 2007), and this dependency has exhibited an increasing trend since the 1960s, especially in developing regions (Aizen et al., 2009). While the importance of wild pollinators is increasingly recognized and there is a trend to bring them back to the crop field to sustain agriculture, this does not conflict with honey bee managing activity as agricultural fields provide excessive floral resources, beyond what can be utilized by insect pollinators (Garibaldi et al., 2011; Garibaldi et al., 2013; Rader et al., 2016; Winfree et al., 2008). In some cases, the development of flowers into fruits is even better with the presence of both honeybees and wild insects (Chagnon et al., 1993). Honey bee pollination can increase the yield, quality, and market value of a variety of crops (Ji et al., 2024; Sáez et al., 2020; Stein et al., 2017; Stoner, 2020) and honey bee management has been developed into a mature and convenient agricultural practice in many countries to ensure global food security (Phiri et al., 2022; vanEngelsdorp and Meixner, 2010; Zheng et al., 2011). In addition to agricultural services, honey bees also provide bee products with nutritional properties such as honey, propolis, and royal jelly. These consumable bee products exhibit various functions (antioxidant, anti-bacterial, anti-inflammation, etc.) and have been used as part of traditional medicine in many cultures (Viuda-Martos et al., 2008). A study in 2009 estimated that the economic value of insect pollination was around 150 billion EUR, and this value remained high (over 200 billion USD) in a more recent study in 2020 (Gallai et al., 2009; Porto et al., 2020). The proportion contributed by honey bees is also considerable, for example, honey bee pollination contributed to 12 billion USD in the USA alone in 2009 (Calderone, 2012).

Despite their vital importance to human societies and ecosystems, honey bees are also confronting threats from the environment. A typical framework for discussing honey bee threats is “4P”, which stands for pests (Rosenkranz et al., 2010), pathogens (Genersch, 2010), pesticides (Henry et al., 2012), and poor nutrition (Naug, 2009). In addition to these major threats, honey bees are also impacted by a range of other factors that can contribute to colony losses. These include agricultural intensification, land use changes, migratory management practices, seasonal variations, and other environmental factors (Durant and Otto, 2019; Kremen et al., 2002; Simone-Finstrom et al., 2022). The biotic and abiotic stressors mentioned above are associated with colony loss globally. Regional declines of honey bee colonies have been reported in the USA and Europe (Potts et al., 2010; vanEngelsdorp et al., 2009). Worldwide, although the number of honey bee colonies has shown an increasing trend since the 1960s, the growth rate of honey bee colonies tends to be insufficient to meet the increasing demand for

pollination services (Aizen and Harder, 2009). These situations are increasingly prompting research interest into honey bee-related studies to understand and mitigate the factors contributing to their decline.

1.2 Honey bee and its microbial partners

Given that microbes are ubiquitously present on Earth and tightly associated with eukaryotic lives in their evolution, development, and various biological processes (for review: McFall-Ngai et al., 2013), it is not surprising that honey bee-microbe interaction is also a crucial and indispensable aspect in the field of honey bee study. Honey bees are extensively exposed to various microbes during their lifetime. Some of these microbes are beneficial, for example, facilitating the digestive process in the honey bee gut (Engel et al., 2012), while some are detrimental, leading to abnormal development of infected individuals and even colony collapse (Genersch, 2010; Lanzi et al., 2006). The intensive foraging activity of honey bees also shapes them as vectors, dynamically exchanging microbes with the environment (Ushio et al., 2015).

1.2.1 Gut microbes of honey bee

Worker honey bees comprise the dominant population in the bee colony, and adult worker bees harbor a simple, consistent, and well-characterized community of microbes in their gastrointestinal tract. Honey bee larvae emerge germ-free and acquire an erratic microbial community through the feeding activities of worker bees (Hroncova et al., 2015; Martinson et al., 2012; Vojvodic et al., 2013). The microbial community is depleted due to gut lining shedding in the metamorphosis process and the new adult worker bees again have no or few gut microbes. Newly emerged worker bees gradually acquire the characteristic gut microbial communities through interactions with hive materials and other worker bees. The social interactions include fecal-oral transmission and oral trophallaxis. The bacterial load can reach $10^8 – 10^9$ bacteria per gut, which constitute the majority of bacteria in worker bees (Martinson et al., 2012; Powell et al., 2014). The gut microbes are not evenly distributed in the gastrointestinal tract of adult worker bees but are mainly concentrated in the posterior hindgut, while the bacterial load in the bee crop (also called honey stomach) and midgut is lower (Engel et al., 2015; Martinson et al., 2012). The gut microbiome of honey bee queens and drones is less studied, but in brief, drones have a more similar gut microbiome to worker bees, while the gut microbiome of the queen is more distinct potentially attributed to its unique physiology and diet (Hroncova et al., 2015; Kapheim et al., 2015).

Five bacterial lineages are identified in the gut of almost all worker honey bees worldwide and are known as the honey bee core gut microbes, namely *Bifidobacterium*, *Gilliamella*, *Snodgrassella*, *Bombilactobacillus*, and *Lactobacillus* (Bottacini et al., 2012; Kwong and Moran, 2013; Moran et al., 2012; Zheng et al., 2020). Other non-core gut microbes frequently identified in the honey bee gastrointestinal tract include *Bartonella*, *Bombella*, *Frischella*, and *Commensalibacter* (Engel et al., 2013; Kešnerová et al., 2016; Siozios et al., 2019; Yun et al., 2017). Some of these gut microbes have been referred to using synonyms or phylotypes in early studies, which could result in confusion with the up-to-date valid names. To standardize the use of bacterial names, the valid names of honey bee gut microbes and their synonyms/phylotypes are summarized in Table 1, and only valid names will be used in this dissertation.

Table 1. Valid names of honey bee gut microbes and their synonyms/phylotypes

Valid name	Synonym/Phylotype
Bartonella (Kešnerová et al., 2016)	phylotype Alpha-1 (Cox-Foster et al., 2007)
Bombella (Yun et al., 2017)	phylotype Alpha-2.2 (Martinson et al., 2011) *Parasaccharibacter* (Corby-Harris et al., 2014b)
Bombilactobacillus (Zheng et al., 2020)	phylotype Firm-4 (Martinson et al., 2011)
Commensalibacter (Siozios et al., 2019)	phylotype Alpha-2.1 (Martinson et al., 2011)
Frischella (Engel et al., 2013)	phylotype Gamma-2 (Cox-Foster et al., 2007)
Gilliamella (Kwong and Moran, 2013)	phylotype Gamma-1 (Cox-Foster et al., 2007)
Lactobacillus (Zheng et al., 2020)	phylotype Firm-5 (Martinson et al., 2011)
Snodgrassella (Kwong and Moran, 2013)	phylotype Beta or Beta-1 (Cox-Foster et al., 2007)

Although these microbes are often identified in the honey bee gut, they show differential localization in the gastrointestinal tract. The adult worker bee crop, despite its low bacterial load, is dominated by *Bombella* and *Apilactobacillus kunkeei* (Corby-Harris et al., 2014a; Corby-Harris et al., 2014b). The bacterial load in the honey bee midgut is slightly higher than that in the bee crop. Due to the presence of a continuously replaced peritrophic matrix, most parts of the midgut are not ideal for bacterial colonization and the majority of bacteria are located at the end of the midgut, dominated by *Gilliamella* (Martinson et al., 2012). The microbiome composition of the midgut is mainly shaped by diet (Ludvigsen et al., 2015). The midgut and hindgut are connected by a short region called the pylorus, and this region is mainly colonized by *Frischella perrara* (Engel et al., 2015). The hindgut is the primary site of bacterial colonization within the honey bee gastrointestinal tract and is divided into the ileum and the

rectum. The ileum gut wall is colonized by a layer of *Snodgrassella* with *Gilliamella* on top of it, while the rectum is dominated by fermentative bacteria including *Lactobacillus*, *Bombilactobacillus*, and *Bifidobacterium* (Kešnerová et al., 2020; Martinson et al., 2012). The co-localization of *Snodgrassella* and *Gilliamella* could be explained by their metabolic interdependence, i.e., the carbohydrate fermentation product of *Gilliamella* is utilized by *Snodgrassella*, while *Snodgrassella* creates microaerophilic zones to support *Gilliamella* survival (Kwong et al., 2014; Zheng et al., 2017). Overall, different sections in the honey bee gastrointestinal tract exhibit distinct microbial structures. The abundance of these gut microbes can be influenced by diet, seasons, honey bee age, and physiological conditions (Castelli et al., 2022; Copeland et al., 2022; Kešnerová et al., 2020; Ricigliano et al., 2017).

1.2.2 Environmental microbes encountered by honey bee

Beyond the gut microbiome, honey bees are exposed to diverse microbial communities present in their surrounding environment, both within the hive (in-hive) and outside (extra-hive). The majority of in-hive microbes are found in stored food such as honey and bee bread (fermented bee pollen with bee saliva and flower nectar). Early research on the microbial composition of honey bee food stores indicated that the food stores harbor some fermentative lactic acid bacteria (LAB) that facilitate food preservation, including *Apilactobacillus*, *Bifidobacterium*, *Bombella*, and *Lactobacillus* (Anderson et al., 2013; Vásquez and Olofsson, 2009). The bee bread in the hive is distributed vertically in the honeycomb. The bee bread in the lower layer shows decreased bacterial diversity compared to that in the higher layer, which could be caused by selective enrichment of specific microbes during the fermentation process (Wang et al., 2023).

Donkersley et al. (2018) identified the most common in-hive bacterial genera as *Pseudomonas*, *Arsenophonus*, *Lactobacillus*, *Erwinia*, and *Acinetobacter*, while Wang et al. (2023) found that the dominant bacterial genera are *Pseudomonas*, *Lactobacillus*, and *Serratia*. The universal presence of *Pseudomonas* and *Lactobacillus* within the hive is not surprising, as *Pseudomonas* is a large genus with extensive environmental distributions and genetic diversity, and *Lactobacillus* is commonly identified in the hive environment as fermentative bacteria (Girard et al., 2021; Vásquez and Olofsson, 2009). Besides these two genera, it is attempting to reach an agreement on a broader collection of in-hive core bacteria. The in-hive microbiome structure displays internal variations in different boxes of the same hive, and external variations driven by period of a year and hive location (Donkersley et al., 2018; Wang et al., 2023). Such variations were consistently revealed in a study investigating the hive microbiome under

different apicultural practices, confirming that the bee hive microbiome is affected by multiple factors and might be used as a unique fingerprint of each hive to reflect the hive condition (Santorelli et al., 2023).

Honey bees forage extensively in the field, searching for nectars and pollens as their food source. It is estimated that a honey bee can visit on average 9.2 flowers per minute during its foraging trip, and a honey bee hive requires 10 to 30 kilograms of pollen per year (Couvillon et al., 2015; Keller et al., 2005). Analysis of bee pollen and honey identity using the traditional microscopic approach or molecular metabarcoding approach both revealed a diverse forage plant inventory (Bareke et al., 2024; Lemessa, 2009; Milla et al., 2021; Richardson et al., 2021). Some example microscopic images of pollen grains are shown in Figure 1. The identity of foraging plants is dependent on the type of landscape. For example, a large proportion of corbicular pollen is from economic crops such as cabbage (*Brassica*) and soybean (*Glycine*) in the apiaries near agricultural fields, while apiaries in urban regions collect more pollens from ornamental plants such as *Hedera* or trees such *Ulmus* (Richardson et al., 2021). Overall, the intensive foraging activity of honey bees renders the foraging plants as hotspots for the acquisition of extra-hive microbes.

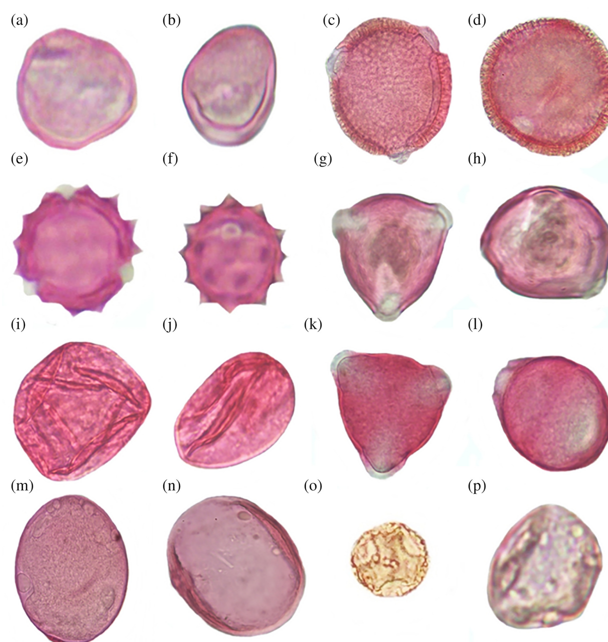

Figure 1. Light microscopy (LM) pollen microphotographs (polar view and equatorial view) taken at (40×) of (a, b) *Phoenix dactylifera*, (c, d) *Pelargonium inquinans*, (e, f) *Pelargonium inquinans*, (g, h) *Senna obtusifolia*, (i, j) *Sorghum bicolor*, (k, l) *Verbena tenuisecta*, (m, n) *Zea mays*, and (o, p*) Ziziphus mauritiana* (Attique et al., 2022).

Numerous studies have been performed to investigate the floral microbiome of different plants using cultivation or sequencing approaches (Álvarez-Pérez and Herrera, 2013; Ambika

Manirajan et al., 2016; Fridman et al., 2012; Junker et al., 2011; Ottesen et al., 2013; Ramakrishnan et al., 2024; Russell and McFrederick, 2022). Although some bacterial taxa were consistently identified across various plants and a wide geographical range, the floral microbiome structure still exhibits patterns associated with specific plant taxa or types. For example, Fridman et al. (2012) investigated the bacterial communities in the nectar of three antimicrobial agent-producing plant species, *Amygdalus communis*, *Citrus paradisi*, and *Nicotiana glauca*, and the microbiome structure is clustered according to these plant species. Ambika Manirajan et al. (2016) reported that the microbiome structure of pollen is significantly different between wind-pollinated and insect-pollinated plants, and the pollen of wind-pollinated plants has higher species richness than insect-pollinated plants.

1.2.3 Interactions between honey bees, microbes and plants

Honey bees, plants, and microbes form a tripartite symbiotic relationship and benefit from each other (Figure 2). In this system, plants serve as the primary energy source and honey bees provide pollination services in return. The stability and functionality of the system are supported by microbes as third partners (for review: Steffan et al., 2024). The tripartite relationship can also be exploited by pathogenic microbes, which can spread throughout the system once introduced at specific nodes (for review: Proesmans et al., 2021).

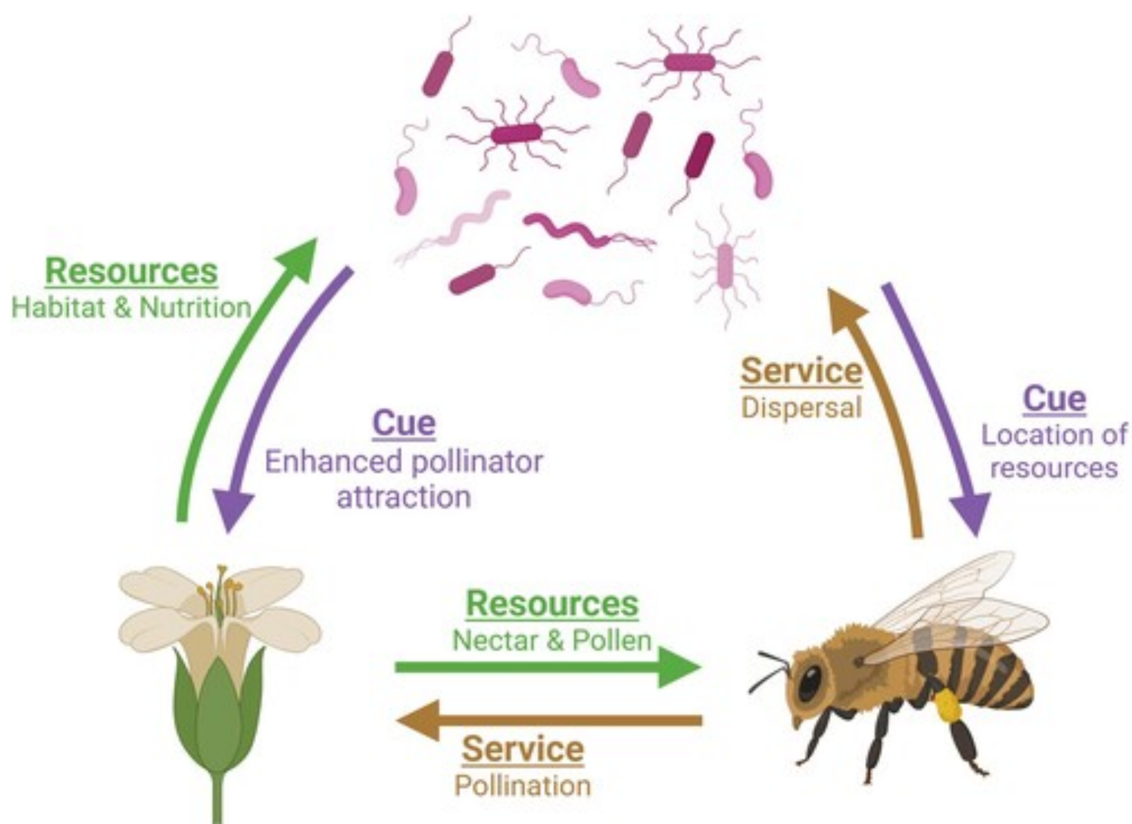

Figure 2. The relationship between honey bees, plants, and microbes. The three communities engage in a widespread and stable tripartite symbiosis, with each community benefiting from the others. Some specific relationships are shown in the figure (Lignon et al., 2025).

Honey bee core gut microbes have co-evolved with their host since the origin of the eusociality of corbiculate bees (Kwong et al., 2017), and perform essential functions supporting various physiological activities of honey bee. These microbes play an important role in the digestion and detoxification process in the honey bee gut. For example, *Bifidobacterium* and *Gilliamella* have significant enrichment in glycoside hydrolase and polysaccharide lyase genes, respectively, and thus function as the major degraders of pollen-derived hemicellulose and pectin (Zheng et al., 2019). *Gilliamella* can also metabolize an array of monosaccharides that are toxic to honey bees, including mannose, xylose, and arabinose (Zheng et al., 2016). In addition, the honey bee gut microbiome appears to influence the metabolism of amino acids and glycerophospholipids in the hemolymph and is associated with the expression of several developmental and behavioral genes. Through the circulatory system, the hemolymph metabolome associated with the gut microbiome could pose impacts on the nervous system, affecting the development, olfactory learning, and social interaction of honey bees (Kešnerová et al., 2017; Zhang et al., 2022a; Zhang et al., 2022b; Zheng et al., 2017). In contrast, gut microbe-deprived honey bees could have issues of reduced weight gain, reduced taste sensitivity, and impaired learning ability. Some honey bee gut microbes are also important protectors against invading pathogens. *Apilactobacillus kunkeei* and *Gilliamella* exhibit an inhibitory effect against the honey bee pathogen *Melissococcus plutonius* through the synthesis of antimicrobial peptides (Lang et al., 2023; Zendo et al., 2020). The established gut biofilm could also act as a physical barrier against invading pathogens (Steele et al., 2021).

Diet, season, and landscape are influencing factors of honey bee gut microbes and in-hive microbes (Castelli et al., 2022; Donkersley et al., 2018; Ricigliano et al., 2017). These factors indirectly reflect the influence of floral resources on honey bee-related microbes. Moreover, the bacteria in food stores can be identified in the bee gut and floral nectar in the surrounding pollination environment, implying frequent horizontal transmission between these ecological niches (Anderson et al., 2013).

Honey bees transfer their microbial community to flowers of their forage plants, and they simultaneously acquire flower-associated microbes. Multiple studies reported that natural flowers or flower-associated materials (nectar and pollen) visited by honey bees showed increased microbial diversity and increased abundance of honey bee-associated microbes, such

as *Bombella*, *Frischella*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella* (Aizenberg-Gershtein et al., 2013; Hietaranta et al., 2023; Prado et al., 2022; Wei et al., 2021). The extensive microbial dispersal of honey bees also to some extent homogenizes the microbial structure of their visited flowers (Wei et al., 2021). By performing laboratory contact experiments and field experiments investigating the microbial transfer from pollinator insects to flowers, Ushio et al (2015) found that flowers are inhabited by species-specific insect microbes after insect visitation, and these microbes on flowers could be used as a fingerprint of the corresponding insect (Ushio et al., 2015). As microbial transmission is bilateral, flowers visited by various insect pollinators could act as important hotspots for inter-species pathogen transmission (Adler et al., 2021). Graystock et al (2015) showed that bee pathogens could be effectively transmitted between honey bees and bumblebees via shared forage flowers. The floral microbial community is also associated with plant phytochemical composition including volatile organic compounds, surface sugars, and metabolic fingerprints (Gaube et al., 2023; Rering et al., 2018). These phytochemicals could function as attractants and influence the pollinator foraging preference. Zemenick et al (2021) reported that plants in the central position of a plant-pollinator network might not be equally important for microbial dispersion in the plant-microbe network, potentially due to the microbial filtration process by indigenous plant properties. This adds additional complexity to the bee-microbe-plant interaction network.

In addition to the "inoculation effect" of insect pollinator visitations, microbial community on the plant is influenced by various other factors. One major factor is the indigenous properties of host plants including immunity response, production of antimicrobial and volatile compounds, and nutrient content. These properties, together with abiotic conditions, provide specialized growth conditions for microbial colonization (Filipiak et al., 2022; González-Teuber et al., 2009; Harper et al., 2010; Huang et al., 2012). The microbial interactions, either positive or negative, on different flower niches also pose an impact on the colonization and succession of floral microbes (Álvarez-Pérez et al., 2012). The plant-associated microbes provide benefits to host plants in various ways. Nectar-associated bacteria *Acinetobacter* can stimulate pollen germination and bursting (Christensen et al., 2021). Some plant symbiont microbes can defend against invading pathogens by activating the host immune response or reducing pathogen establishment (Shalev et al., 2022; Steffan et al., 2024).

1.2.4 Honey bee pathogens

The health of honey bees is threatened by a variety of bacterial, fungal, and viral pathogens. The most notorious honey bee bacterial pathogen is probably *Paenibacillus larvae*, the

causative agent of American Foulbrood (Genersch et al., 2006). The spores of *P. larvae* germinate and proliferate in the midgut of bee larvae, and secrete highly active extracellular proteases to invade tissues, eventually leading to larvae death (Yue et al., 2008). The spores of *P. larvae* can remain infectious for decades and tolerate extreme environmental conditions, thus infected colonies are always burned to completely eradicate the pathogen (Spivak and Reuter, 2001). Another widespread honey bee bacterial disease is European Foulbrood caused by *Melissococcus plutonius*, and the symptoms are similar to American Foulbrood (Bailey, 1983). The pathogenesis of *M. plutonius* is yet unclear due to its sophisticated growth requirement, absence of reproducible laboratory infection assays, and limited availability of genetic data until recently (for review: Fünfhaus et al., 2018). Some bacterial species such as *Enterococcus faecalis* and *Paenibacillus alvei* have been proposed as secondary invaders of European Foulbrood, however, their roles in disease progression remain contentious (Anderson et al., 2023; Giersch et al., 2010; Lewkowski and Erler, 2019). *Spiroplasma apis* and *Spiroplasma melliferum* infect adult honey bees, leading to nervous disorder and reduced longevity, but are less detrimental to the entire colony (Clark et al., 1985; Mouches et al., 1982). Some opportunistic honey bee pathogens include *Serratia marcescens* and *Hafnia alvei*, which can cause septicemia in adult bees and are more virulent for those with disturbed gut microbiomes (Lang et al., 2022; Raymann et al., 2018).

The most harmful fungal disease is vairimorphosis caused by *Vairimorpha apis* and *Vairimorpha ceranae* (Fries, 1993; Fries et al., 1996). *Vairimorpha* is previously known as *Nosema*, and its infection can affect all members of the colony including larvae, worker bees, drones, and queens. The consequences of infection at the individual level can be suppressed immune response, impaired ability to produce royal jelly, precocious foraging, and shortened lifespan (Eiri et al., 2015; Goblirsch et al., 2013; Li et al., 2018). Colony-wise, *Vairimorpha* infection results in depressed brood-rearing activity, decreased population size, and colony loss (Botías et al., 2013; Hassanein, 1953; Iorizzo et al., 2022). Some mild fungal diseases include chalkbrood disease and stonebrood disease caused by *Ascosphaera apis* and *Aspergillus* spp., respectively (Gilliam et al., 1988; Jensen et al., 2013). These two fungal diseases only affect bee larvae and are always resisted by a healthy and populous colony.

The most common honey bee viral pathogens include Deformed wing virus (DWV), Sacbrood virus (SBV), Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), Chronic bee paralysis virus (CBPV), and Black queen cell virus (BQCV). DWV is the most prevalent honey bee virus worldwide, causing morphological deformities in the wings of infected adult bees, as

indicated by its name. The spread of DWV is primarily facilitated by the parasitic mite *Varroa destructor* (Gisder et al., 2009; Martin et al., 2012; Shen et al., 2005). Jointly, DWV and the *Varroa* mite are associated with colony weakening, reduced lifespan of winter bees, and increased susceptibility to insecticides (Barroso-Arévalo et al., 2019a; Dainat et al., 2012; Zhu et al., 2022). The *Varroa* mite could also act as a vector for the transmission of other viruses including SBV, ABPV, and KBV (Shen et al., 2005; Tentcheva et al., 2004). SBV-infected larvae appear as sacs filled with SBV-rich fluid, while SBV-infected adult bees exhibit no clear symptoms (Bailey, 1969). KBV usually persists as an inapparent viral pathogen without obvious symptoms in honey bees, while its virulence can be boosted by mite infestation (Hung et al., 1995). Both ABPV and CBPV can cause paralysis symptoms (trembling and flying disability) in bees, but ABPV kills diseased bees more rapidly than CBPV (Bailey et al., 1963). BQCV infection mainly influences queen larvae and pupae, characterized by the dark color on the wall of the queen cell. BQCV infection is always associated with the *Vairimorpha* infection, and they interact synergistically to decrease the honey bee survival rate (Bailey et al., 1981; Doublet et al., 2015; Gajda et al., 2021). As the oral uptake of BQCV has a minor effect on honey bee health, *Vairimorpha* might breach the midgut epithelial cells to provide a new infection route (Al Naggar and Paxton, 2020).

The transmission of honey bee pathogens can be both horizontal and vertical. The horizontal transmission can be achieved by an array of social interactions (fecal-oral transmission, trophallaxis, body contact) (Amiri et al., 2019; Coulon et al., 2018; Figueroa et al., 2019), vector-mediated transmission (Martin et al., 2012), robbing activity between colonies (Lindström et al., 2008), and air transmission (Sulborska et al., 2019). The vertical transmission route is relatively straightforward compared to horizontal transmission. Pathogens can be transmitted from queens to descendants via egg-laying activity and from mother to daughter colonies via swarming (Amiri et al., 2018; Fries et al., 2006). The horizontal transmission of honey bee pathogens via foraging activity in natural ecosystems has been poorly studied.

1.3 The immune system of honey bee

Insects do not have antibody-mediated adaptive immunity and primarily rely on innate immunity (for review: Ali Mohammadie Kojour et al., 2020). Although honey bees have lower immune gene diversity compared to the model insects *Drosophila melanogaster* and *Anopheles gambiae*, they still harbor a complete set of canonical innate immune pathways (Barribeau et al., 2015; Evans et al., 2006). The canonical innate immunity is composed of four major signaling pathways, Janus kinase-signal transducers and activators of transcription

(JAK/STAT), immunodeficiency and c-Jun N-terminal kinase (IMD/JNK), RNA interference (RNAi), and Toll pathways, and these pathways are activated simultaneously or specifically in response to different external stress (Figure 3).

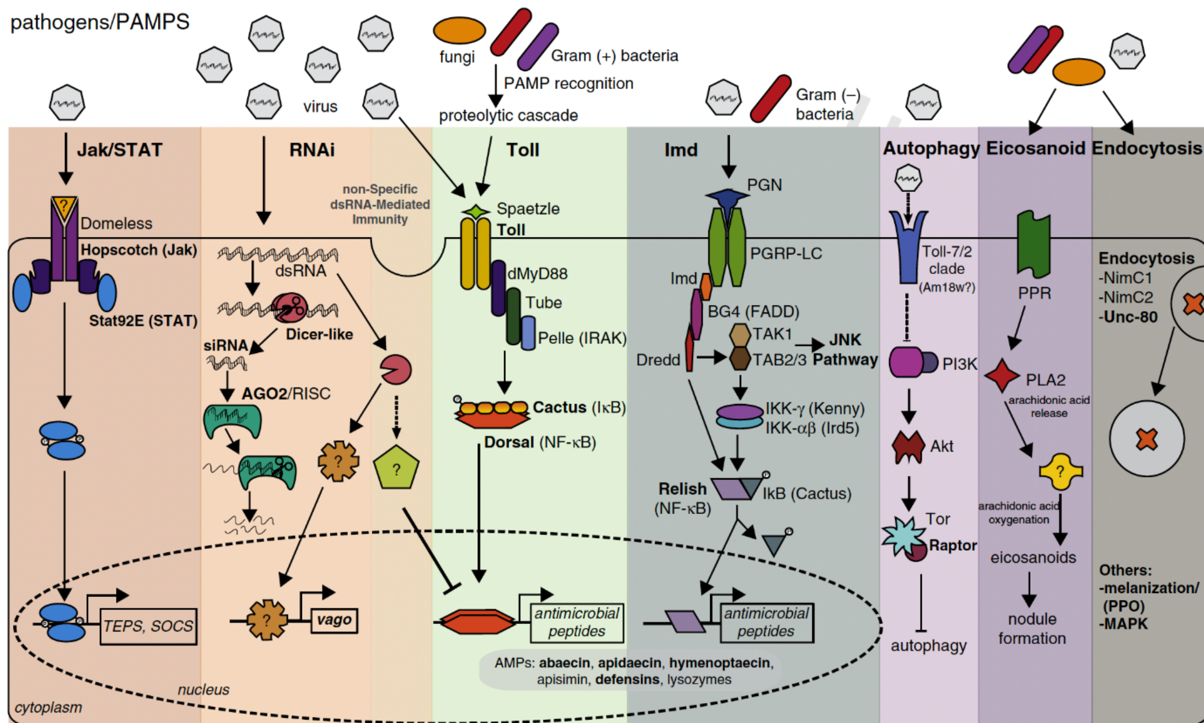

Figure 3. Honey bee canonical innate immunity pathways. The major innate immunity signaling pathways as well as pathways related to autophagy, eicosanoid, and endocytosis are shown. The figure is adapted from Brutscher et al. (2015) with minor modifications.

All four major pathways can be activated by viral infection, while the RNAi pathway tends to be the primary mediator (for review: Brutscher et al., 2015). RNAi pathway is highly conserved across multiple phyla, and its antiviral role has been revealed in plants and invertebrates (for review: Ding, 2010; Hammond et al., 2001). The cellular machinery in the RNAi pathway recognizes, processes and recruits virus-derived RNA sequences to target and cleave complementary viral sequences (for review: Wilson and Doudna, 2013).

Activation of the Jak/STAT pathway leads to the expression of thioester-containing protein. This humoral effector protein has been shown to bind to microbial surfaces via covalent interactions, eventually leading to phagocytosis of the pathogen in mosquitos and fruit flies (Fraiture et al., 2009; Stroschein-Stevenson et al., 2005). The Jak/STAT pathway in honey bees can be activated by the infection of DWV and IAPV (Galbraith et al., 2015; Quintana et al., 2019), however, its activation via bacterial and fungal pathogens was barely reported.

It has been shown that the IMD/JNK pathway can be triggered by Gram-negative bacteria, and the Toll pathway can be triggered by Gram-positive bacteria and fungi in fruit fly models (for review: Royet et al., 2005). In honey bees, in addition to viral activation (Barroso-Arévalo et al., 2019b; Nazzi et al., 2012), these two pathways can be activated by the common honey bee pathogens *P. larvae*, *M. plutonius*, and *Vairimorpha* pathogens (Doublet et al., 2017; Evans et al., 2006; Lewkowski et al., 2022). The downstream signaling of IMD/JNK and Toll pathways leads to the activation of Relish and Dorsal. These two proteins are both homologs of the transcription factor NF-κB, which is a crucial player in the insect innate immunity (Silverman and Maniatis, 2001). Through Relish and Dorsal, IMD/JNK and Toll pathways regulate the expression of antimicrobial peptides (AMPs) (De Gregorio et al., 2002). Four families of AMPs are present in honey bee hemolymph, namely apidaecins, abaecin, hymenoptaecin, and defensins. The general antimicrobial mechanism of AMPs is pore formation on the cell membrane, leading to leakage of cellular content, while AMPs also exhibit preferential targeting of specific types of microbes over others (Casteels-Josson et al., 1993; Klaudiny et al., 2005). AMPs and NF-κB homologs in honey bees can be used as indicators of pathogen infection and honey bee health conditions due to their central role in the immune response (Barroso-Arévalo et al., 2019b; Nazzi et al., 2012).

To reflect the honey bee health condition, lysozyme-like, vitellogenin, and glucose oxidase are also reasonable indicators. Honey bee lysozyme-like protein is a homolog of lysozyme, which targets bacterial peptidoglycan cell walls for degradation (Masschalck and Michiels, 2003). The level of lysozyme-like is upregulated in *P. larvae*-infected honey bee larvae and bee colonies with low level of hygiene (Al-Ghamdi et al., 2021; Lazarov et al., 2016). Vitellogenin is a storage protein for metabolic use and is correlated with longer lifespans due to its protective role against oxidative stress (Amdam and Omholt, 2002; Seehuus et al., 2006). It has also been shown as an immunomodulator, acting as a pathogen pattern recognition receptor and promoting phagocytosis (Li et al., 2008). Glucose oxidase is mainly expressed in the honey bee hypopharyngeal gland, and it exhibits antiseptic properties via catalyzing glucose to produce hydrogen peroxide (Ohashi et al., 1999). It is used to preserve food in honey bee colonies and is regarded as part of honey bee social immunity (Bucekova et al., 2014). The activity of glucose oxidase can be boosted by a polyfloral diet, and reduced when nutritional stress is present (Alaux et al., 2010; Castelli et al., 2020). The expression levels of lysozyme-like, vitellogenin, and glucose oxidase also decrease in response to nutritional stress (Castelli et al., 2020).

1.4 Metabarcoding: a method to uncover the details

Metabarcoding is defined as a high-throughput sequencing approach of DNA phylogenetic markers to identify a collection of organisms from a bulk sample (Ji et al., 2013). It has become a powerful tool to uncover the hidden biodiversity of various communities in the environment, including insects (Yu et al., 2012), plants (Parducci et al., 2017), microbes (Burki et al., 2021; Joos et al., 2020), and even fish communities (Hatzenbuhler et al., 2017). The DNA region used as a barcode should fulfill some criteria, including ubiquitous presence in the community with stable mutation rate, appropriate length for sufficient phylogenetic information, and flanking regions with low variation for primer targeting.

The prokaryotic 16S rRNA gene has been widely used as the phylogenetic marker for bacterial taxonomic classification. The full-length 16S rRNA gene is approximately 1500 bp and contains 9 hypervariable regions. As some mature sequencing technologies (Illumina and Ion Torrent Sequencing) are incapable of covering the entire 16S rRNA gene, the selection of the most informative region becomes crucial. The choice of region may vary depending on the sample type and bacterial composition (Chakravorty et al., 2007; Fadeev et al., 2021; Johnson et al., 2019; Lin and Ju, 2023; López-Aladid et al., 2023). Hypervariable regions V4-V6 were shown to be representative of the full-length sequence (Yang et al., 2016). The V4-V5 region was chosen for this study based on a previous microbiome study on flower pollen (Ambika Manirajan et al., 2016).

Multiple DNA regions can be candidates for plant barcodes, including *trnH*-*psbA*, *rbcL*, *matK*, and internal transcribed spacer (ITS) (Kress et al., 2005; Kress and Erickson, 2007; Lahaye et al., 2008). Notably, *trnH*-*psbA*, *rbcL,* and *matK* are maternally-inherited plastid DNA, limiting their application in pollen DNA barcoding, while the ITS region is a part of nuclear ribosomal DNA (Matsushima et al., 2011). In addition, it has been shown that the ITS2 region exhibits reasonable PCR efficiency, pronounced interspecific divergence, and high identification resolution compared to other regions (Chen et al., 2010). Compared to classical palynology methods, ITS2 metabarcoding shows improved taxonomic resolution and provides comparable quantitative information (Bänsch et al., 2020; Keller et al., 2015). ITS2 can also serve as a fungal DNA barcode with primers designed for selective fungal sequence amplification (Toju et al., 2012).

Multiple sequence databases are present for the 16S rRNA gene and ITS2, including Greengenes (DeSantis et al., 2006), Silva (Quast et al., 2013), Unite (Abarenkov et al., 2023), BOLD (Ratnasingham and Hebert, 2007), ITS2 Database (Ankenbrand et al., 2015), and EUKARYOME (Tedersoo et al., 2024).

1.5 Analysis of high-throughput metabarcoding data

Numerous pipelines have been designed for the analysis of high-throughput metabarcoding data, and despite variations in specific methodologies, the overall frameworks tend to be quite similar (Caporaso et al., 2010; Schloss et al., 2009; Truong et al., 2017). Some typical analyses include but are not restricted to alpha and beta diversity analysis, differential abundance analysis, correlation, and network analysis (for review: Liu et al., 2021). Some methodologies involved are adopted from traditional macro-ecology and show limitations when applied in the metabarcoding analysis.

Alpha diversity is a measure of the richness and evenness of an ecological community. Some widely used alpha diversity indices are species richness, Shannon, and Simpson. Species richness is simply the number of species or selected taxonomic units in a community and is highly sensitive to sampling efforts and strongly influenced by rare species (Lande et al., 2000). Compared to species richness, Shannon and Simpson indices provide more robust measurements as they additionally include relative abundance in the calculation. However, these traditional indices use different equations and units, complicating the justification of their selection. Moreover, when considerable changes in species richness occur, the corresponding changes in Shannon and Simpson indices are minimal, leading to challenges in interpreting their non-intuitive scaling (for review: Roswell et al., 2021). The problems with these three alpha diversity indices can be solved by Hill diversity, which uses a general equation with an adjustable rarity scale to cover the three indices (for review: Jost, 2006). When using an arithmetic scale, Hill diversity corresponds to Hill-richness, which gives high leverage to rare species. In contrast, Hill-Simpson diversity employs a reciprocal scale and emphasizes common species. Hill-Shannon diversity uses a logarithmic scale, serving as an intermediate, eclectic index. The advantages of Hill diversity include a unified equation and unit, proportional responsiveness to community change, and consistency across different sample standardization approaches. Notably, as Hill-richness is exactly the same as species richness, its high sensitivity to rare species makes it widely discouraged unless the community is fully characterized or enough information is available for richness estimation (Chase and Knight, 2013; Guillera-Arroita et al., 2019).

High-throughput metabarcoding data, particularly microbiome data, are typically characterized by high dimensionality, sparsity, and most importantly, compositionality. The compositionality of metabarcoding data is a critical feature that can easily lead to misinterpretation of the data. Compositional data represents parts of a whole, where the components are constrained to be

non-negative and sum to a constant value (Aitchison, 1982). As a consequence, the values in compositional data can only reflect relative but not absolute information, and these values are interdependent (for review: Gloor et al., 2017; Greenacre, 2021). In metabarcoding data, the compositional nature arises from the fixed sequencing capacity of the instrument. The most intuitive issue caused by data compositionality is the false-positive interpretation of correlations. When the count of a specific taxonomic unit increases or decreases within a sample, constrained by the constant sum, the count of other taxonomic units inevitably shifts in the opposite direction, resulting in spurious correlations (Gloor et al., 2017; Morton et al., 2019). In addition, compositional data often violate linearity, deviate from a normal distribution, and reside in a simplex, rendering standard statistical methods such as ANOVA, t-test, Pearson correlation, and linear regression analysis inapplicable for the data analysis (Aitchison, 1982; Mandal et al., 2015).

The first step in compositional analysis is always a ratio transformation of the data, as the ratios between the components in a dataset remain constant (Morton et al., 2019). Applying the logarithm of these ratios further makes the data into a symmetric and linearly related form. The log-ratio-transformed data eventually occupy a real vector space, making them suitable for standard statistical methods (Aitchison, 1982). The most widely used transformation approach is centered log-ratio (clr) transformation, which uses the geometric mean of all components in the dataset as the denominator (Aitchison, 1982).

Many following analyses are based on clr-transformed data, such as Aitchison distance-based beta diversity, ALDEx2 differential abundance analysis, and SpiecEasi co-occurrence analysis. Aitchison dissimilarity matrix is based on the Euclidean distance between components in the clr-transformed spaces (Aitchison, 1982; Aitchison and Greenacre, 2002). Compared to other distance measures (e.g., Bray-Curtis), Aitchison distance resides in an Euclidean space and enables ordination with principal component analysis (PCA). PCA offers advantages over principal coordinate analysis (PCoA) in that it provides consistent results regardless of the subset of components analyzed, demonstrating robustness against excessive sparsity (Bian et al., 2017). In contrast, beta diversity ordination based on many common distance metrics exhibits horseshoe artifacts, resulting from the limitations of these metrics to discriminate samples without shared components (Morton et al., 2017).

ALDEx2 is a statistical tool designed to identify differentially abundant components within compositional data. It uses Dirichlet-multinomial sampling to account for data variability, followed by clr-transformation of the generated Monte Carlo instances (Fernandes et al., 2013).

ALDEx2 incorporates both statistical significance evaluation and effect-size estimates in the analysis, providing a comprehensive assessment of differences with respect to both confidence and magnitude. A recent tool for differential abundance analysis is ANCOM-BC2, developed based on ANCOM-BC. ANCOM-BC deals with sparsity using pseudo-count addition, normalizes the data based on natural log, and performs bias correction using a log-linear regression model (Lin and Peddada, 2020). ANCOM-BC2 further extends ANCOM-BC for multiple groups and repeated measurements. It incorporates the mixed directional false discover rate (mdFDR) method when performing multiple pairwise comparisons. Moreover, as the choice of pseudo-count can influence the results, leading to inflated false positive rates, ANCOM-BC2 includes sensitivity analysis in response to a series of pseudo-counts (Lin and Peddada, 2024).

Metabarcoding data typically produce large datasets with numerous underlying interactions among components that influence community structures and functions, and network analysis is a robust tool for uncovering these interactions from the population-level data (Banerjee et al., 2018). Network analysis based on correlation coefficient has been a popular approach, but it is limited by spurious correlations due to compositionality (Layeghifard et al., 2018). SpiecEasi addresses both compositionality using clr transformation and sparsity using two graphical model estimators, neighborhood selection and sparse inverse covariance selection (Kurtz et al., 2015). To evaluate the network, numerous measures are available. The number of nodes and edges can give the most fundamental information about a network. The connectivity of a network can be reflected by the average number of edges, average path length, and attack robustness (Tipton et al., 2018), while multiple node centrality analysis (e.g., degree centrality, closeness centrality, and betweenness centrality) can reveal how central a node is in a network (for review: Golbeck, 2013).

1.6 Characterization of a novel *Robbsia* species isolated from birch pollen

Flower pollen is a natural repository of various bacteria, and pollen from different plant species could harbor specified microbiome composition and structure (Ambika Manirajan et al., 2016). Identifying and characterizing the undiscovered bacterial species from the environment could help elucidate its environmental distribution and potential ecological role. Ambika Manirajan et al. (2016) have isolated a collection of strains from different pollens, and a strain isolated from birch (*Betula pendula*) is a potential candidate as a novel *Robbsia* species. Currently, genus *Robbsia* only involves one species, *Robbsia andropogonis*. This species was initially described by Smith (1911) as *Bacterium andropogoni* and is pathogenic to over 10

agriculturally important crops (Morales-Galván et al., 2022; Smith, 1911). *Robbsia andropogonis* strains are able to produce rhizobitoxine and induce hypersensitive responses in infected plants (Lopes-Santos et al., 2017; Mitchell, 1994). The strains are Gram-negative, non-spore-forming, rod-shaped and the G+C content varies from 59 to 61.3 mol% (Gillis et al., 1995).

For novel bacterial taxa classification, a series of tests are required to assess the genotypic, phenotypic, and chemotaxonomic characteristics of the strain. In the genotypic assessment, 16S rRNA gene-based phylogeny is a traditional approach and can serve as a starting point to indicate the potential position and cluster in the phylogenetic tree. A strain sharing less than 98.7% 16S rRNA gene similarity with its next-relative species is considered a potential novel species (Stackebrandt, 2006). Further genotypic assessment includes comparative genome analyses such as digital DNA-DNA hybridization (dDDH), average amino acid identity (AAI), average nucleotide identity (ANI), and percentage of conserved proteins (POCP). These values also have accepted boundaries for taxon delineation (species boundary: 95% for AAI and ANI, 70% for dDDH; genus boundary: 50% for POCP) (Chun et al., 2018; Qin et al., 2014; Thompson et al., 2013). Moreover, compared to phylogenetic trees based on 16S rRNA gene or multilocus sequence analysis, the core genome tree can indicate a more stable phylogenetic position of the strain (Yu et al., 2017). Phenotypic assessment involves a set of traditional microbiological assays that elucidate the bacterial morphology, gram-staining, growth condition, and some strain-specific characteristics (in this case production of rhizobitoxine and induction of plant hypersensitive response) (for review: Bochner, 2008). Chemotaxonomic assessment characterizes chemical components of the bacterial cell such as respiratory quinones, cellular fatty acids, and polar lipids (Tindall et al., 2010). However, it has also been argued that chemotaxonomy analysis should no longer be mandatory for novel species description as bacterial genome sequencing has become increasingly advanced and accessible (Vandamme and Sutcliffe, 2021).

1.7 Aim of the study

Honey bees are valuable insect pollinators and form complicated interaction networks with microbes and plants in natural ecosystems (for review: Steffan et al., 2024). To understand the interactions among bees, microbes, and plants, research typically focuses on either the bee side, characterizing microbiome composition in the bee gastrointestinal tract or hive materials, or, on the plant side, examining the microbiome of floral pollen (Ambika Manirajan et al., 2016; Corby-Harris et al., 2014a). Corbicular pollen serves as an intermediate entity in the bee

foraging process, linking bee hives and plants. It reflects both the forage plants utilized by honey bees and the environmental microbes to which they are exposed. However, the plant and microbial composition in corbicular pollen has never been studied simultaneously. In addition, while the transmission of bee pathogens has been widely studied within and between hives (Fries et al., 2006; Lindström et al., 2008), research on their transmission in the natural ecosystems remains limited. Corbicular pollen serves as an ideal object for studying microbial transmission, and the plant and microbes it carries might also influence the health of honey bees.

Therefore, we proposed the following aims for the current study: i) to characterize the microbiome and plant composition in honey bee corbicular pollen using 16S rRNA gene and ITS2 metabarcoding; ii) to investigate the role of environmental and floral factors in shaping the corbicular pollen microbiome; iii) to explore the potential of corbicular pollen in studying microbial transmission in natural ecosystems and validate the role of hub plant taxa in microbial transmission; iv) to examine the impact of BQCV and *Vairimorpha* on the immunity genes and the microbiome of forager honey bees under field conditions; v) to characterize a novel *Robbsia* species with potential phytopathogenicity.

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Chapter 2 Microbiome of honey bee corbicular pollen: Factors influencing its structure and potential for studying pathogen transmission

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Microbiome of honey bee corbicular pollen: Factors influencing its structure and potential for studying pathogen transmission

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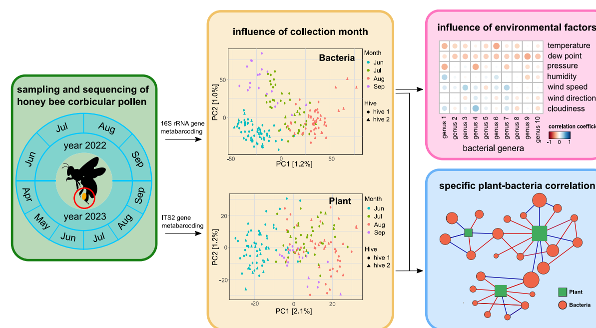
HIGHLIGHTS

- Microbiome of honey bee corbicular pollen changes over time.
- Environmental factors and forage plants influence corbicular pollen microbiome.
- *Rubus* is an important hub plant genus for microbe transmission.
- Corbicular pollen shows potential for studying pathogen transmission.

GRAPHICAL ABSTRACT



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ABSTRACT

Honey bees are exposed to a diverse variety of microbes in the environment. Many studies have been carried out on the microbiome of bee gut, beebread, and flower pollen. However, little is known regarding the microbiome of fresh corbicular pollen, which can directly reflect microbes acquired from the environment. Moreover, although evidences have suggested that floral resources in general can affect the bee-acquired microbes, whether specific forage plants affect the composition of these microbes is still unclear. Here, we characterized both the microbiome and plant composition of corbicular pollen in collection seasons over two years from six hives using 16S rRNA gene and ITS2 metabarcoding. The results reveal temporal changes in the microbiome and plant composition in corbicular pollen, which was influenced by environmental factors and the choice of forage plants. We identified several co-occurrences between plant and bacterial genera, indicating specific plant-microbe interactions. Many *Spiroplasma* species with various insect hosts, including a honey bee pathogen *Spiroplasma melliferum*, were shown to positively correlate with *Rubus*, suggesting this plant genus as an important node for microbial transmission. Overall, we demonstrated the potential of corbicular pollen for studying the transmission of microbes, especially pathogens. This framework can be applied in future research to explore the complicated pollinator-microbe-plant network in different ecosystems.

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1. Introduction

Honey bees (*Apis mellifera*) are agriculturally important insects that provide pollination services to increase crop yields and quality and contribute to significant economic values (Calderone, 2012; Geslin et al., 2017; Sáez et al., 2020). As industrialized agriculture magnifies, the negative impact has been continuously posed on the diversity and abundance of wild pollinators, and modern agriculture becomes more dependent on domesticated bee colonies (Aizen and Harder, 2009). However, honey bees are also vulnerable insects. Their health is affected by multiple interacting stressors, both biotic and abiotic, including pathogens (Evans and Schwarz, 2011), pesticides (Xiao et al., 2022), intensive agricultural practice (Kremen et al., 2002), land use (Durant and Otto, 2019), and other environmental factors (Le Conte and Navajas, 2008; Fisher et al., 2022; Simone-Finstrom et al., 2022). These external stressors are associated with widespread loss of bee colonies (Insolia et al., 2022), and consequently draw increasing concerns about honey bee health.

Among the factors that influence honey bee health, the bee-microbe interaction is a significant aspect. Honey bees exchange microbes extensively within the hive. Newly emerged honey bee adults are germ-free and gradually acquire gut microbiome through interaction with nestmates and hive components (Powell et al., 2014). The core gut microbiome of honey bees involves *Snodgrassella*, *Gilliamella*, *Bifidobacterium*, *Lactobacillus* and *Bombilactobacillus*, which have coevolved with the host since the emergence of eusocial bees (Kwong et al., 2017; Motta and Moran, 2024; Steffan et al., 2024). These microbes perform vital functions to facilitate digestion, detoxify hazardous chemicals, and protect against pathogens. For example, *Gilliamella* strains isolated from the honey bee gut showed a wide spectrum of carbohydrate utilization and degradation activity for pectin, a major component of the pollen cell wall (Engel et al., 2012; Zheng et al., 2016). *Lactobacillus* and *Bifidobacterium* isolates showed an antagonistic effect against *Paenibacillus larvae*, a devastating bee pathogen, both in vitro and in vivo (Forsgren et al., 2010). Honey bee hives also harbor hive-specific microbes that are involved in food preservation and fermentation (Anderson et al., 2013). Compared to the relatively stable and consistent gut microbiome composition, the microbiome in the hive environment can be quite distinctive, most likely affected by land use around apiaries and different choices of forage plants (Donkersley et al., 2018; Santorelli et al., 2023).

As the plant-pollinator interactions are ubiquitous in natural ecosystems, it is not surprising that honey bees are also exposed to a wide variety of microbes in the extra-hive environment during their foraging activity. One honey bee is estimated to visit hundreds of flowers per day, while flower pollen is inhabited by various microbes depending on pollination types (Corby-Harris et al., 2014; Ambika Manirajan et al., 2016). Plant metabarcoding results of corbicular pollen have revealed that honey bees forage diverse flora, and the foraging pattern also depends on the surrounding flora landscape (Milla et al., 2021; Richardson et al., 2021). Like other insect pollinators, honey bee visitation transfers the microbes from the insect to the flower, and thus the flower microbiome gets altered (Ushio et al., 2015; Hietaranta et al., 2023). As the interaction is bilateral, flower microbiome is also transferred to honey bees via pollen.

While many studies focus on bee bread and flower pollen, research on freshly collected corbicular pollen seems to be quite limited. Fresh corbicular pollen, as an intermediate state between flower pollen and bee bread, can be important from the following aspects. First, the plants are not equally visited by honey bees. It has been shown that the protein content of pollen is an important driver of foraging preference, for example, protein-rich pollen from *Trifolium repens* was collected more compared to pollen from *Oenothera biennis* with a lower protein content (Ghosh et al., 2020). Even different honey bee species (*A. mellifera* and *A. cerana*) at the same location exhibited distinctive foraging patterns with >70 % of the plant taxa in honey uniquely foraged by one of them (Namin et al., 2022). Therefore, corbicular pollen can indicate what plant honey bees are exactly foraging. Second, the microbiome of bee bread is a result of in-hive fermentation, which selectively enriches certain microbes, leading to a relatively stable microbiome structure (Wang et al., 2023). Thus, the bee bread microbiome cannot reflect the complete microbial profile to which honey bees are exposed in the extra-hive environment. Although the microbiome of freshly collected corbicular pollen has been briefly mentioned (Ghosh et al., 2022), a temporal description of the microbiome structure is lacking. Most importantly, many studies pointed out that the microbial composition of honey bee gut and hive is affected by season and landscape (Donkersley et al., 2018; Jones et al., 2018; Kešnerová et al., 2020), which strongly indicated an effect of floral resources. However, it is still unknown how forage plants affect the microbes that honey bees are acquiring from the environment. In this case, corbicular pollen is an optimal object to study both extra-hive microbes and plants related to honey bees and any potential interactions in between.

In this study, we collected fresh corbicular pollen from six honey bee hives over two years and profiled the bacterial microbiome and plant composition using 16S rRNA gene and ITS2 metabarcoding, respectively. The questions we want to answer include: (i) Does the microbiome structure of corbicular pollen change over time? (ii) Do environmental factors affect the microbiome structure? (iii) Is there any correlation between forage plants and microbiome composition? We hypothesized that the microbiome composition and structure of corbicular pollen may vary in different collection periods. This could be due to different flowering time of forage plants and environmental factors collectively. Moreover, forage plants may affect the corbicular pollen microbiome in two different approaches. First, an increase in forage plant diversity leads to an increase in microbiome diversity. Second, certain microbes in corbicular pollen are derived from specific forage plants.

2. Material and methods

2.1. Study sites and sample collection

Corbicular pollen samples were collected in the apiary of Justus-Liebig-University Giessen, Germany in 2022 and 2023. In 2022, samples were collected from two hives from June to September. For each hive, the collection was carried out three days per month and from sunrise to sunset per day. In 2023, samples were collected from four hives from April to September. In total, 230 samples were collected over two years. The detailed sampling procedure is as follow: a pollen trap was installed at the entrance of the bee hive before sunrise on each sampling day. The pollen trap contains a pollen screen that mechanically removes pollen from the corbicula of returning foragers, which then fall through a mesh screen into a removable tray underneath. Corbicular pollen in the tray was collected into a 15 ml centrifugation tube (Starlab International GmbH, Germany) at one-hour intervals, and stored immediately at −20 °C. The tray was surface disinfected with 70 % ethanol before and after each collection to remove potential residue microbes.

2.2. DNA extraction from corbicular pollen

Metagenomic DNA extraction from corbicular pollen was performed as described in Ambika Manirajan (Ambika Manirajan et al., 2016) with minor modifications. In brief, approximately 300 mg of each corbicular pollen sample was mixed with 1 ml extraction buffer (0.1 M NaCl (pH 8), 50 mM EDTA, 0.2 M sodium phosphate buffer (pH 8), 2.5 % w/v SDS) and 200 μl sterile zirconia beads in a 2 ml screw-cap tube. Cell disruption was performed using FastPrep-24 tissue and cell homogenizer (MP Biomedicals, USA) at 5.5 m s^{-1} for 45 s. Homogenized samples were centrifuged at 16,200 *g*, 4 °C for 5 min, and the supernatant was transferred into a new microcentrifuge tube, followed by 10 μl RNase A treatment (10 mg ml^{-1}) at 37 °C for 30 min. Samples were then

extracted twice with firstly 800 μl phenol/chloroform/isoamyl alcohol (25:24:1) and secondly 800 μl chloroform/isoamyl alcohol (24:1), and centrifuged after each extraction at 16,200 g, 4 °C for 5 min. The final aqueous phase was incubated with 1 ml precipitation buffer (20 % w/v PEG 6000 and 2.5 M NaCl) on ice for 30 min, and centrifuged at 16,200*g*, 4 °C for 30 min. The DNA pellet was washed with 800 μl ice-cold 75 % ethanol, dried out, and dissolved in nuclease-free water.

2.3. 16S rRNA and ITS2 ion torrent sequencing

The hypervariable regions (V4 & V5) of the bacterial 16S rRNA gene were amplified by PCR with primers 520F (5′-AYTGGGYDTAAAGNG-3′) (Claesson et al., 2009) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Engelbrektson et al., 2010). A 50-μl touchdown PCR (Korbie and Mattick, 2008) with peptide nucleic acid (PNA) blocking plastid (5′-GGCTCAACCCTGGACAG-3′) (Lundberg et al., 2013) and mitochondrial sequences (5′-AAACCAATTCACTTGAGT-3′) (Abdullaeva et al., 2021) was carried out to enhance the reaction specificity and yield of the target gene. The reaction contained 2 μl extracted DNA, 60 μM KAPA dNTP mix, 0.4 μM of each primer, 1 μM of each PNA, and 1 unit KAPAHiFi polymerase in 1× KAPAHiFi buffer (KAPABiosystems, Woburn, MA, United States). The PCR was run as follows: 95 °C for 3 min, 35 cycles of (98 °C, 20 s; 65 °C, 30 s for PNA annealing; 65 °C, 30 s for primer annealing with a decrement of 1 °C after each cycle until 55 °C; 70 °C, 30 s) and 70 °C for 5 min.

The plant ITS2 region was amplified with primers S2F (5′-ATGCGATACTTGGTGTGAAT-3′) (Chen et al., 2010) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). A 15-μl PCR reaction contained 1 μl extracted DNA, 60 μM KAPA dNTP mix, 0.4 μM of each primer, and 0.3 unit KAPAHiFi polymerase in 1× KAPAHiFi buffer. The PCR program was set as follows: 95 °C for 3 min, 35 cycles of (98 °C, 20 s; 58 °C, 30 s; 72 °C, 30 s) and 72 °C for 5 min.

Barcodes and adapter were added to 16S rRNA gene and ITS2 amplicons using a second PCR (50 μl) that contained 1 μl template, 60 μM KAPA dNTP mix, 0.1 μM of barcodes- and adapter-linked primers, and 0.5 unit KAPAHiFi polymerase in 1× KAPAHiFi buffer. The PCR was performed as follows: 95 °C for 3 min, 8 cycles of (98 °C, 20 s; 58 °C, 30 s; 72 °C, 30 s) and 72 °C for 5 min. PCR products were firstly purified from agarose gel to exclude primer dimers with NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), then cleaned with NucleoMag NGS clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and quantified using Qubit dsDNA HS assay kit by QubitVR 3.0 fluorometer (Life Technologies, Carlsbad, USA). PCR products of equal molar (300 pM) were pooled and the final concentration was adjusted to 100 pM. Ion Torrent sequencing of the pooled libraries was performed according to the protocol described by Ambika Manirajan et al. (2016). The sequencing data are available at the NCBI GenBank database under the BioProject PRJNA1080550 and PRJNA1080497.

Three negative controls consisting of empty tubes were included from the beginning of the DNA extraction step and followed the same procedure as real samples.

2.4. Sequence data analysis

Sequence data were processed in QIIME 2 2022.11 (Bolyen et al., 2019). Raw sequences were demultiplexed using cutadapt plugin with zero error rate for barcodes (Martin, 2011) followed by quality control, denoising, dereplication and chimera identification with dada2 plugin (Callahan et al., 2016). Specifically, reads with number of expected errors >2 were discarded, and trimmed at the 15th position. Bacterial 16S rRNA gene sequences were truncated at the 320th position and plant ITS2 sequences were truncated at the 350th position. Taxonomy affiliation of bacterial and plant amplicon sequence variants (ASV) was achieved by pre-fitted sklearn-based classifiers (Pedregosa et al., 2011; Bokulich et al., 2018) trained with SILVA 138.1 database (Quast et al., 2013) and UNITE 9.0 eukaryotes database (Koljalg et al., 2020), respectively. Eukaryotic, chloroplast, mitochondria and unassigned ASVs were additionally removed from the bacterial dataset. Fungal, metazoan and unassigned ASVs were removed from the plant dataset. Contaminant taxa in negative controls were checked by the R package microDecon (version 1.0.2) (McKnight et al., 2019).

The core bacterial and plant genera were calculated using the R package microbiome (version 1.20.0) (Lahti and Shetty, 2017) with detection threshold of 1 % and prevalence threshold of 50 %. Alpha diversity was examined with Hill-Shannon and Hill-Simpson indices using the R package MeanRarity (version 0.0.1.4) (Roswell and Dushoff, 2022). Rarefication of datasets were performed using the R package phyloseq (version 1.44.0) (McMurdie and Holmes, 2013) before the calculation of alpha diversity. Comparisons among collection years, months and hives were performed using Wilcoxon test with Benjamini-Hochberg correction (Wilcoxon, 1946). For beta diversity analysis, centered log-ratio (clr) transformation of datasets was first performed to address the compositional nature of sequencing data (Aitchison, 1982) using the R package ALDEx2 (version 1.30.0) (Fernandes et al., 2013). Aitchison distance-based dissimilarity matrix was calculated and ordinated with principal component analysis (PCA) for visualization. The differences among months and hives in each year were assessed using PERMANOVA analysis (Anderson, 2001) with 999 permutations using the R package vegan (version 2.6–4) (Oksanen et al., 2022).

Differential abundance analysis of bacterial genera was performed using the R package ANCOMBC (version 2.0.3) (Lin and Peddada, 2024). ANCOMBC applied a correction for bias introduced by the differential sampling fraction of each sample, and a pseudo-count of 0.5 was used to replace zeros. Two-sided Z-test using the test statistic (log fold change/standard error) was performed to calculate *p*-values with Benjamini-Hochberg correction. A *p*-value threshold of 0.05 was used to filter differentially abundant genera. As the choice of pseudo-count may lead to false positive result, we also included pseudo-count sensitivity analysis implemented in ANCOMBC to address this issue. Taxa with sensitivity score of 0 were not affected by pseudo-count choices, indicating consistent results.

2.5. Correlation and network analysis

The effect of environmental factors on bacterial community structure was evaluated using redundancy analysis (RDA) (Legendre et al., 2011). The bacterial dataset was clr-transformed, and the environmental factors were standardized using "decostand" function in vegan (version 2.6–4). The significance of RDA was evaluated for both global model and single variable using permutation test with 999 permutations. For the correlation analysis between bacterial genera and environmental factors, low abundance genera with total counts below 100 were firstly removed. Spearman's rank correlation coefficient was calculated using "aldex.corr" function in ALDEx2 (version 1.30.0) based on clr-transformed data, and *p*-values were corrected with Benjamini-Hochberg approach. Correlation matrix was visualized with the R package corrplot (version 0.92). Environmental factors with 1-hour step including temperature, dew point, pressure, humidity, wind speed, wind direction and cloudiness were downloaded from OpenWeather (https://openweathermap.org).

The correlation between bacterial and plant diversity was examined using linear mixed effects model implemented in the R package lme4 (version 1.1-35.1) (Bates et al., 2015). Hive and month were set as random effects, and likelihood ratio test was performed to assess the significance of the model by comparing the full model: *bacterial diversity* $\sim$ *plant diversity* + *(plant diversity|hive)* + *(plant diversity|month)* with the null model: *bacterial diversity* $\sim$ *(plant diversity|hive)* + *(plant diversity|month)*. The analysis of the single and cross-domain co-occurrence network at genus level was performed using the R package SpiecEasi (version 1.1.2) (Kurtz et al., 2015). Low abundance genera with total counts below 100 were removed. The function "spiec.easi" was run

using the neighborhood selection method, a lambda value of 100, lambda minimum ratio of 10^{-2}, the Stability Approach to Regularization Selection (StARS) for model selection, StARs subsamples of 99 and threshold of 0.05. The networks were further analyzed and visualized in Cytoscape 3.9.1 (Shannon et al., 2003). The significant difference in the average number of co-occurrences and shortest path length between the cross-domain and single domain networks was evaluated using Welch's *t*-test with unequal variances. Attack robustness analysis of the networks was performed as described in Tipton et al. (2018). Potential hub taxa were identified based on the node degree and betweenness centrality (Agler et al., 2016).

3. Results

3.1. Ion torrent sequencing analysis

For 16S rRNA gene, 233 samples were sequenced in three runs, yielding 14,717,665 raw reads. After quality control, denoising, and chimera filtering with dada2 plugin, 9,218,343 high-quality sequences remained, resulting in 8851 exact ASVs. After removal of eukaryotic, chloroplast, mitochondria and unassigned sequences, the bacterial dataset eventually contained 4,457,671 sequences (964 to 44,381 reads per sample) and 7434 ASVs. The same 233 samples were sequenced for ITS2 and 17,121,069 raw reads were produced, from which 1,630,126 high-quality reads remained after dada2 processing and 2073 ASVs were generated. After removal of fungal, metazoan and unassigned sequences, the plant dataset eventually contained 1,592,631 sequences (189 to 39,145 reads per sample) and 1720 ASVs. Three negative controls revealed 7 ASVs in bacterial dataset and 16 ASVs in plant dataset as potential contaminants. These ASVs were validated with microDecon R package, and the most likely contaminants were removed accordingly.

3.2. Corbicular pollen harbors diverse bacterial and plant composition

In this study, 45.5 % and 88.4 % of bacterial ASVs were classified to species and genus level, and 63.8 % and 95.3 % of plant ASVs were classified to species and genus level, respectively. In total, we identified 656 and 754 unique bacterial species and genera, 323 and 234 unique plant species and genera, respectively. Due to the limited length of phylogenetic markers used in IonTorrent sequencing, the resolution of phylogenetic assignment was not always clear at the species level. Thus, our description and discussion will mainly focus on the genus level, while species information will be mentioned when appropriate. *Pseudomonadota* and *Bacillota* were the dominant bacterial phyla identified in all samples with at least 0.8 % relative abundance. These two phyla comprised a large proportion in each sample (min: 62.2 %; max: 100 %, mean: 97 %). *Acetobacteraceae* and *Lactobacillaceae* were the most prevalent bacterial family present in all samples, followed by *Orbaceae*, *Oxalobacteraceae* and *Xanthomonadaceae* present in 99.1 %, 98.7 % and 97.4 % of the samples, respectively. We identified a core set of bacterial genera in each month using 1 % detection threshold and 50 % prevalence threshold (Fig. 1A). *Apilactobacillus* was the only core genus found in every month, while some core genera occurred more exclusively in certain months such as *Luteibacter*, *Spiroplasma* and an uncultured *Rickettsiales*. The five most prevalent genera included *Apilactobacillus*, *Gilliamella*, *Massilia*, *Bombella* and *Snodgrassella* which were identified in at least 93.9 % of the samples. Using the same detection and prevalence threshold, the core plant genera were also identified. As expected, plant genera were only core exclusively in certain time period due to their relatively fixed flowering phase (Fig. 1B). The five most prevalent genera were *Plantago*, *Hypochaeris*, *Rubus*, *Crepis* and *Cichorium* which were identified in at least 46.5 % of the samples. Overall, the limited number of core genera compared to the total number of identified genera indicated that the bacterial and plant composition in corbicular pollen were diverse and changed rapidly. The phylogenetic assignment to the species level of these core plant/bacterial genera can be found in

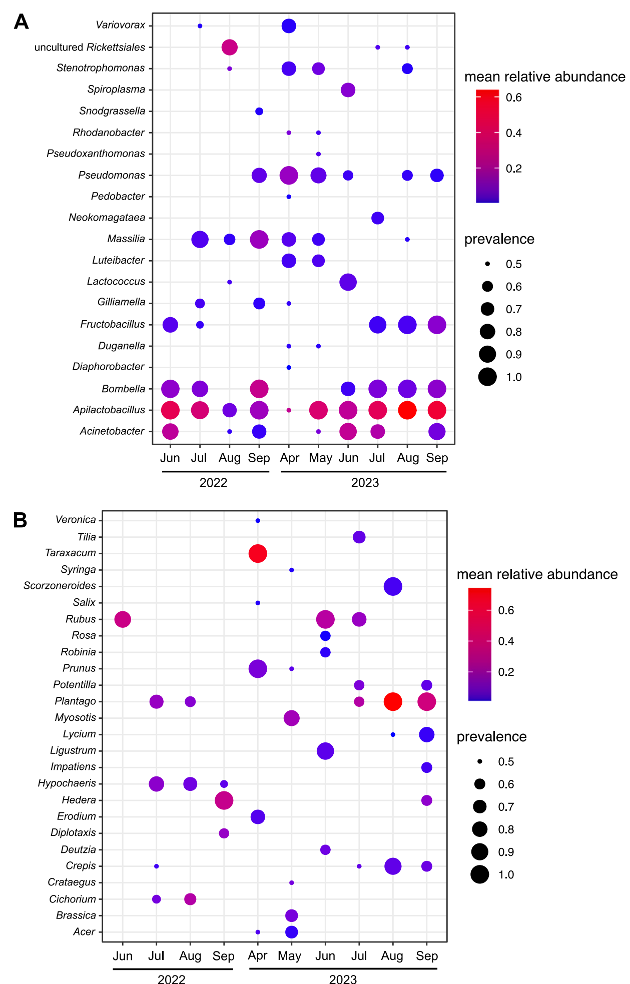

Fig. 1. Core genus analysis in each collection month. (A) Core bacterial genera in corbicular pollen. (B) Core plant genera in corbicular pollen. The detection threshold of core genera is 1 % of sequence number in each sample. The prevalence threshold of core genera is 50 % of sample number in each month.

Table S1.

3.3. The diversity of bacteria and plants in corbicular pollen changes over time

The alpha diversity of the samples were examined to see whether the richness and evenness of bacteria and plants in corbicular pollen were affected by collection period and hive. A significant difference was observed regarding plant alpha diversity between 2022 and 2023 (Hill-Shannon: $p = 9.7 \times 10^{-13}$; Hill-Simpson: $p = 2.1 \times 10^{-5}$). Despite the different plant alpha diversity, the bacterial alpha diversity remained stable with no significant difference between the two years (Hill-Shannon: $p = 0.82$; Hill-Simpson: $p = 0.78$). When comparing between hives in each year, we found that the alpha diversity of both bacteria and plant was independent of hive (Table S2). For month comparison of bacterial and plant alpha diversity, significant differences were observed between certain months, however, the differences and underlying trend were not consistent in the two years (Fig. 2A, Table S3). Bacterial alpha diversity in September was higher than the other months in 2022, while comparing the same months, they appeared to be similar in 2023. Plant alpha diversity in August was the highest in 2022, but tended to be the lowest in 2023. Thus, we concluded that although the alpha diversity of bacteria and plant in corbicular pollen exhibited some changes in each

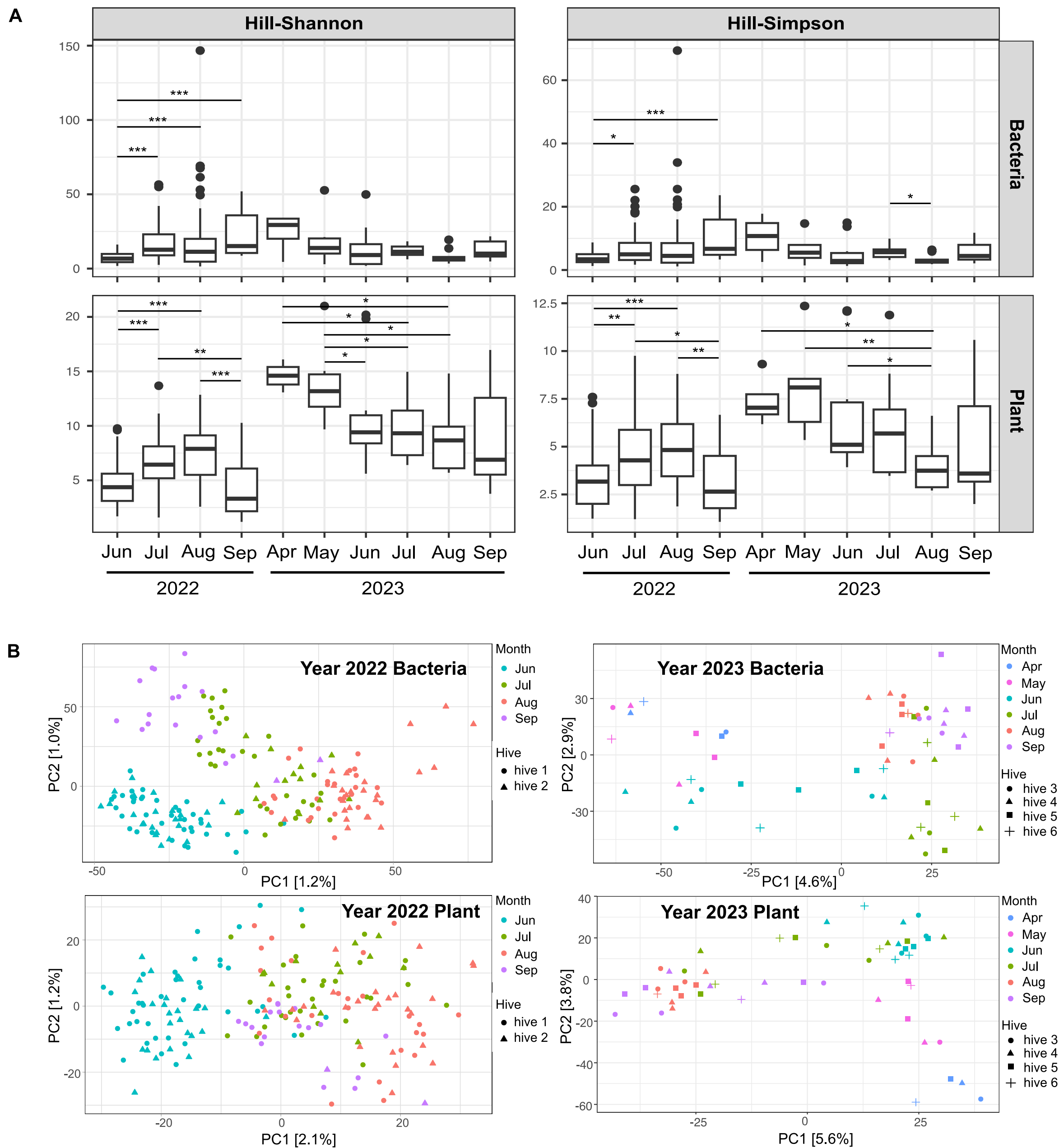

Fig. 2. Alpha diversity and beta diversity. (A) Hill-Shannon and Hill-Simpson indices of bacteria and plant in corbicular pollen in different collection months in 2022 and 2023. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (B) Principal component analysis based on Aitchison dissimilarity distance matrix for bacterial and plant composition in corbicular pollen samples in 2022 and 2023.

year, these changes were more sporadic and difficult to predict.

The beta diversity based on Aitchison dissimilarity matrix was further evaluated and a visualization using PCA ordination was performed (Fig. 2B). The results in 2022 revealed a clear clustering of bacterial communities according to collection month ($R^2 = 0.026$, F-statistic $= 1.537$, $p = 0.003$), while the separation of communities clustered by hive was suspicious due to a borderline *p*-value ($R^2 = 0.006$, F-statistic $= 1.104$, $p = 0.055$). This month-related but not hive-related microbiome structure was consistently revealed in 2023 with a significant *p*-value for month clustering ($R^2 = 0.129$, F-statistic $= 1.421$, $p = 0.001$) but not hive clustering ($R^2 = 0.055$, F-statistic $= 0.972$, $p = 0.268$). For plant composition in corbicular pollen, the clustering was significantly influenced by both collection month ($R^2 = 0.031$, F-statistic $= 1.845$, $p = 0.001$) and hive ($R^2 = 0.007$, F-statistic $= 1.256$, $p = 0.001$) in 2022. The plant composition was consistently driven by collection month in 2023 ($R^2 = 0.155$, F-statistic $= 1.761$, $p = 0.001$), but not influenced by hive ($R^2 = 0.054$, F-statistic $= 0.956$, $p = 0.701$). In general, collection month was the major driver of both bacterial and plant composition in corbicular pollen, while the influence of hive was minimal, if any.

We performed multi-group pairwise comparisons using ANCOMBC analysis to identify differentially abundant bacterial and plant genera in each month. The results revealed 34 bacterial and 9 plant genera differentially abundant in at least one of the comparisons in 2022 (Fig. 3A, Table S4), and 17 bacterial and 17 plant genera in 2023 (Fig. 3B, Table S5). In 2022, the bacterial genera that were uniquely and

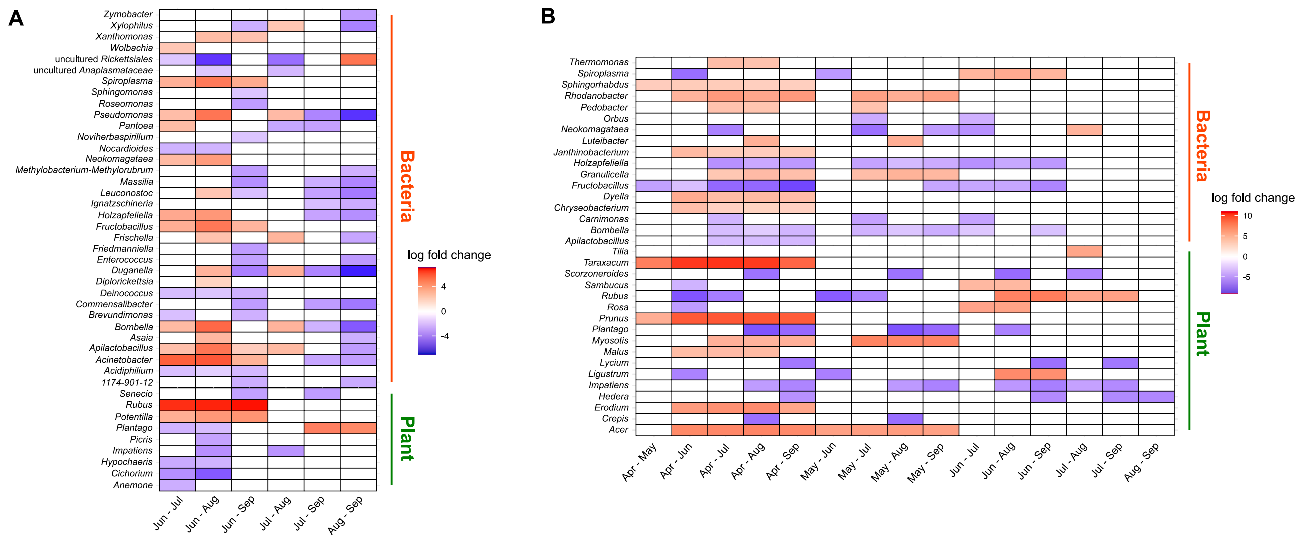

Fig. 3. ANCOMBC results for differential abundances of bacterial and plant ASVs collapsed to genus level of corbicular pollen samples. (A) Differentially abundant bacterial and plant genera in 2022. (B) Differentially abundant bacterial and plant genera in 2023. All differentially abundant genera have *p*-values below 0.05 and pseudo-count addition sensitivity scores of 0.

differentially abundant during specific months included *Acinetobacter*, *Apilactobacillus*, *Fructobacillus*, and *Spiroplasma* in June; uncultured *Rickettsiales* in August; and *Commensalibacter*, *Duganella*, *Leuconostoc*, and *Massilia* in September. The uniquely and differentially abundant plant genera were *Rubus* and *Potentilla* in June, and *Plantago* appeared to be differentially abundant during July and August. In 2023, the bacterial genera uniquely and differentially abundant during specific months included *Sphingorhabdus* in April; and *Spiroplasma* in June. *Holzapfeliella* was differentially abundant from July to September. The uniquely and differentially abundant plant genera were *Taraxacum* and *Prunus* in April; *Acer* in April and May; *Rubus* in June and July; and *Impatiens* in August and September. The relative abundance of these differentially abundant genera in each month is provided in Fig. S1 as a reference. Comparing the two years, although the differential abundance patterns were mostly different, some results were still quite consistent. The most prominent consistency was that *Spiroplasma* and *Rubus* were differentially abundant in June of both years, implying a potential relation between them.

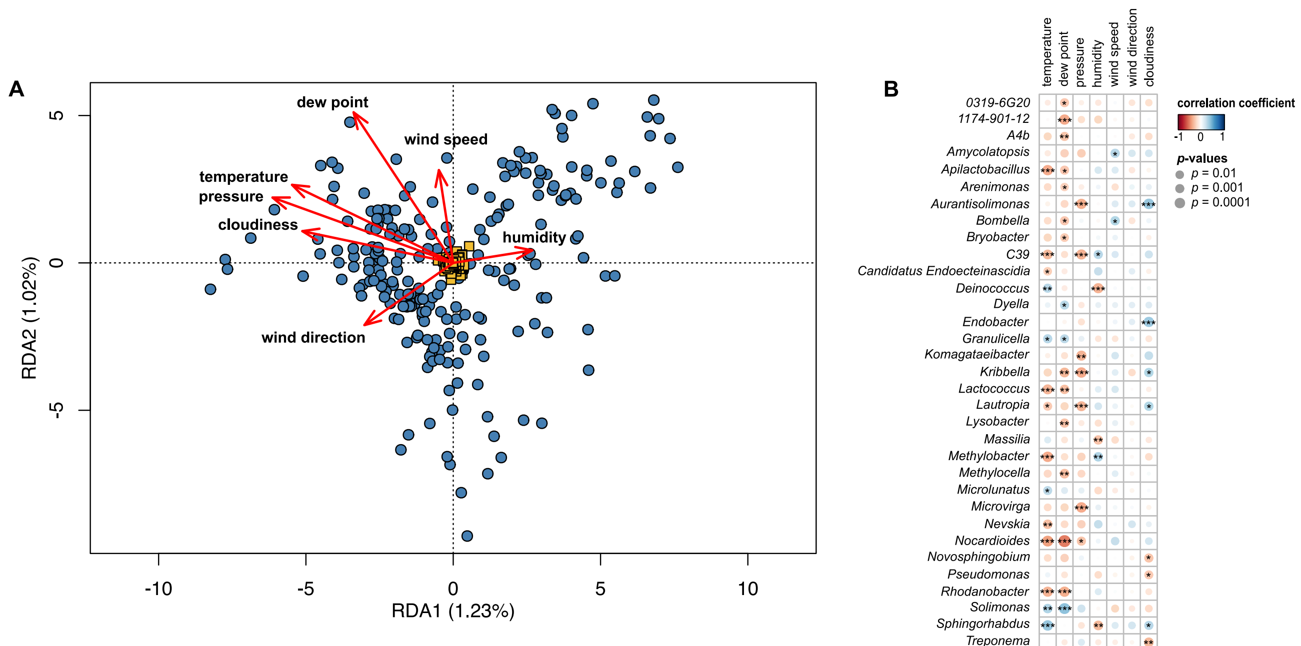

Fig. 4. Correlation of bacterial community of corbicular pollen samples with environmental factors. (A) Redundancy analysis of environmental factors on bacterial community structure based on clr-transformed bacterial dataset. Yellow dots indicate bacterial genera and blue dots indicate corbicular pollen samples. (B) Spearman correlation analysis of bacterial ASVs generated with ALDEx2 collapsed to genus level with environmental factors. * $p \leq 0.01$, ** $p \leq 0.001$, *** $p \leq 0.0001$.

3.4. Bacterial community in corbicular pollen is influenced by environmental factors

To explore the factors that drove the month-related microbiome differences, we modeled the effect of environmental factors on the bacterial community structure of corbicular pollen using RDA analysis. The global model revealed that the bacterial community structure was significantly influenced by these environmental factors ($R^2 = 0.051$, F-statistic 1.675, *p*-value 0.001) (Fig. 4A, Table S6). When considering the variables separately, five out of seven environmental factors (temperature, dew point, pressure, humidity, cloudiness) significantly influenced the bacterial community structure, while the effects of wind speed and wind direction were not significant (Fig. 4A, Table S6). Similarly, when we correlated bacterial genera with these environmental factors, the results revealed 33 genera that were significantly influenced by at least one of the environmental factors (Fig. 4B, Table S7). Among the seven environmental factors, dew point and temperature were the major influencing factors significantly correlating 16 and 14 bacterial genera, respectively. Cloudiness, pressure, and humidity significantly correlated with 8, 7, and 5 genera, respectively. Wind speed was the least influencing factor that significantly correlated with only two genera. No significant correlation was found for wind direction.

3.5. Co-occurrence analysis reveals specific microbe-plant correlation

In addition to environmental factors, another influencing factor of microbiome in corbicular pollen could be forage plants. We initially hypothesized that the forage plants might affect bacterial composition in corbicular pollen samples in two different manners, i.e., the forage plant diversity positively correlate with bacterial diversity, and the forage plants specifically co-occur with certain bacterial taxa. To test the hypothesis, we first fitted a linear mixed model using plant diversity as the fixed effect and hive and month as the random effects. In both years, small correlation coefficient values were observed for Hill-Shannon and Hill-Simpson alpha diversity indices, and the *p*-values were also non-significant (Table S8), indicating the bacterial diversity of corbicular pollen samples did not increase as the bees foraged a larger variety of plants.

The specific plant-bacteria correlation was then examined with co-occurrence analysis in each year. The cross-domain co-occurrence network of bacteria and plant genera had roughly the same number of nodes (188 nodes in 2022; 255 nodes in 2023) compared to the sum of nodes in single domain networks (171 nodes In 2022; 236 nodes in 2023), while the number of co-occurrences (599 co-occurrences in 2022; 911 co-occurrences in 2023) in cross-domain network was much greater than the sum of co-occurrences in single-domain networks (353 co-occurrences in 2022; 537 co-occurrences in 2023) (Fig. 5A, Table 1). This showed that with the presence of plant, the overall organization of the networks was reshaped. In both years, the cross-domain network showed a significantly higher average number of co-occurrences (*p*-value < 0.0001), and the average path lengths between any two bacterial nodes or plant nodes in cross-domain network were significantly shorter than their counterparts in single-domain networks (*p*-value $<$ 0.0001) (Table 1). We also examined the attack robustness of the networks in response to degree-based, betweenness-based, or random node removal, and the cross-domain network showed the largest area under the attack robustness curve under every condition (Fig. 5B, Table 1). These indicated that the cross-domain network is more connected and stable than single-domain networks, implying close relation between plant and bacterial composition in corbicular pollen. In the cross-domain network, there were 118 plant-bacteria co-occurrences representing 19.7 % of the total co-occurrences in 2022, and 174 plant-bacteria co-occurrences representing 19.1 % of the total co-occurrences in 2023. Despite the presence of unique plants in each year, 82 plant-bacteria co-occurrences were consistently revealed, and most of them (78 %) had a positive edge weight (Table S9). Many of these positive co-occurrences showed month-related patterns. For example, *Rubus* was predominantly abundant in June and July, and its positively co-occurring bacterial genera also displayed major abundances during the same period. Altogether, we concluded that bacterial composition in corbicular pollen is affected by specific forage plants, which could further explain the temporal change of microbiome in corbicular pollen in addition to environmental factors.

3.6. Corbicular pollen shows potential for studying pathogen transmission in natural ecosystems

We selected a subnetwork from the cross-domain network consisting of three plant genera with the highest node degree in each year (*Rubus*, *Hedera*, and *Cichorium* in 2022; *Rubus*, *Chenopodium*, and *Hedera* in 2023) along with their nearest bacterial neighbors (Fig. 6). Based on node degree and betweenness centrality, *Hedera* and *Chenopodium* were also identified as plant hub genera in 2023, while *Rubus* was a hub in both years (Fig. S2). These plants exhibited high connectivity with numerous bacterial genera and were potentially important nodes for the transmission of microbes, particularly pathogens, in natural ecosystems. In the current study, this was supported by the co-identification of plant hub genus *Rubus* with potential bee pathogen *Spiroplasma* in the core genus analysis, differential abundance analysis and co-occurrence analysis. As few ASVs of *Spiroplasma* were assigned to the species level, we identified the next relative species of these ASVs using EzBioCloud to improve the taxonomy resolution. Many ASVs were identified as the honey bee pathogen *Spiroplasma melliferum* with high similarity, and they accounted for a large proportion (91.6 %) of all *Spiroplasma* ASVs (Tables S1, S10). The other *Spiroplasma* species were identified as commensals of tick and various insects, and one *Spiroplasma* species was identified as a plant pathogen (Table S11). The diverse origins of these *Spiroplasma* species highlighted *Rubus* as an important plant genus for the plant-pollinator interactions and the transmission of microbes and pathogens.

4. Discussion

Pollen is the major protein source for honey bees, and a normal-sized bee hive is estimated to collect >10 kg of pollen per year (Crailsheim et al., 1992; Keller et al., 2005). As pollen harbors a wide variety of microbes (Ambika Manirajan et al., 2016), it is an important vector for the exchange of microbes between honey bees and the environment. Studies have been carried out to characterize either the microbiome or the plant composition of corbicular pollen (Corby-Harris et al., 2014; Richardson et al., 2019, 2021; Ghosh et al., 2022), while the plant-microbe correlation in corbicular pollen remains a gap area in this research field. Here, we characterized both the bacterial microbiome and plant composition of fresh corbicular pollen, and for the first time, illustrated how environmental factors and forage plants could affect the microbiome that honey bees are acquiring from the environment. We also proposed that our working scheme could be used to study the horizontal transmission of pathogens of insect pollinators in the environment.

Our results showed that the microbiome and plant composition of fresh corbicular pollen were quite diverse and changed over time, supported by significant month-related differences in beta diversity and differential abundance analysis, and a limited number of core bacteria or plant genera in each month. The diverse and temporally changing plant composition could reflect the foraging preference of honey bees or the different flowering times of local plants. Many core plant genera in corbicular pollen including *Taraxacum*, *Rubus*, *Hypochaeris*, *Plantago*, *Cichorium* and *Hedera* are known as important food sources for honey bees (Percival, 1950; Mayer and Lunden, 1991; Adamchuk et al., 2017; Wignall et al., 2020; Richardson et al., 2021), and the identification period of these core plant genera also matches the documented flowering time in a German floral database BiolFlor (www.biolflor.de). The

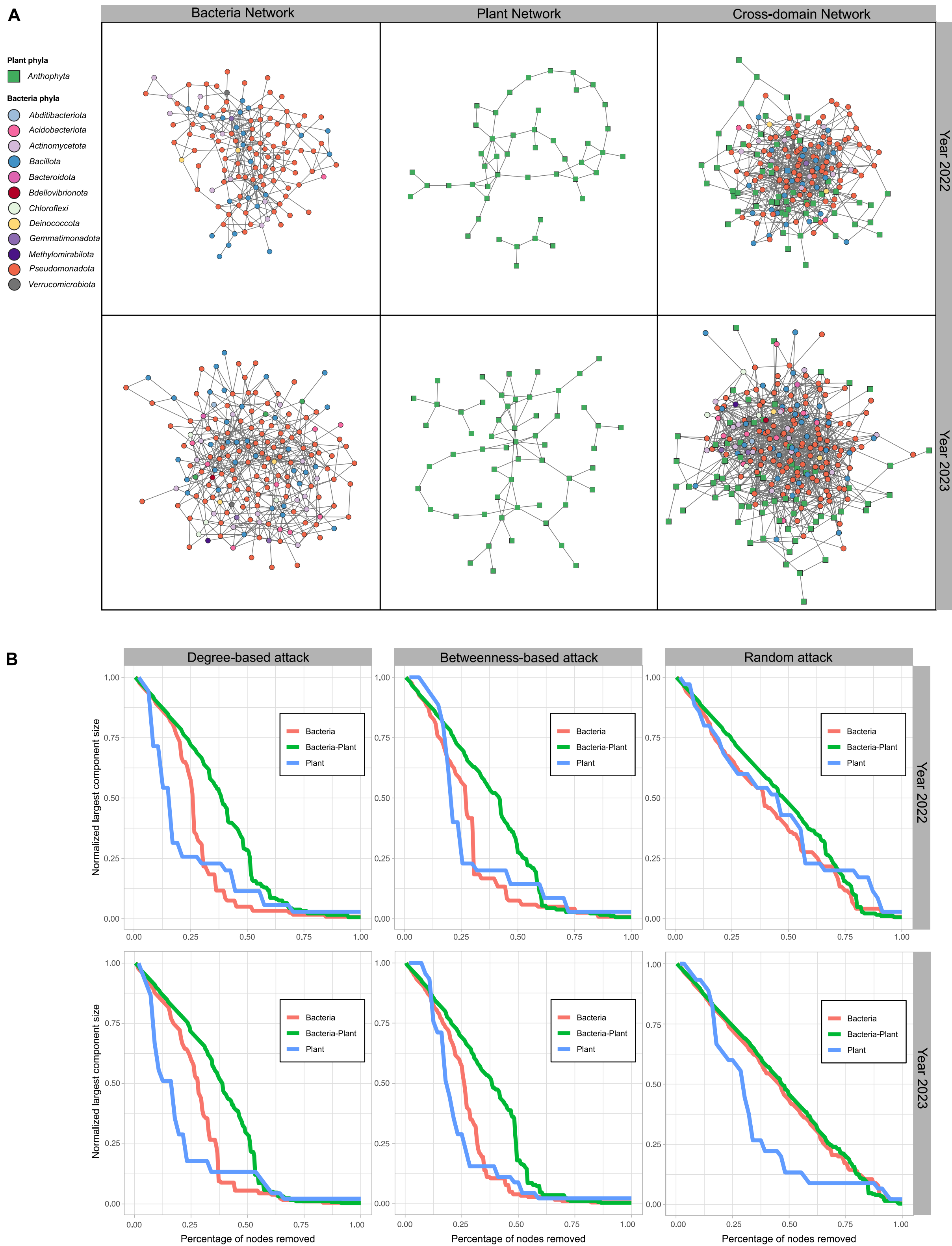

Fig. 5. Co-occurrence network analysis. (A) Network of bacteria and plant ASVs collapsed to genus level in corbicular pollen samples. Single domain and cross-domain network of bacteria and plant are shown. (B) Attack robustness curves for all networks under the situation of degree-based attack, betweenness-based attack and random attack. The highest value of largest component size is normalized to 1.

Table 1
Attributes of single-domain and cross-domain co-occurrence analysis from ASVs collapsed to genus level. The standard deviations were shown in brackets.

Co-occurrence network attributes		Bacterial network		Plant network		Cross-domain network	
		2022	2023	2022	2023	2022	2023
Number of nodes		124	180	47	56	188	255
Number of co-occurrences		263	467	60	70	599	911
Average number of co-occurrences		4.242 (3.194)	5.189 (3.254)	2.683 (1.396)	2.5 (1.640)	6.372 (4.001)	7.145 (4.045)
Average path length		3.858 (0.615)	3.551 (0.452)	4.496 (1.311)	4.291 (1.174)	Bacteria: 3.146 (0.367) Plant: 3.566 (0.521)	Bacteria: 3.137 (0.320) Plant: 3.556 (0.528)
Area under attack robustness curve	Degree-based	0.254	0.267	0.204	0.186	0.370	0.362
	Betweenness-based	0.262	0.252	0.263	0.216	0.373	0.353
	Random	0.402	0.451	0.415	0.312	0.459	0.464

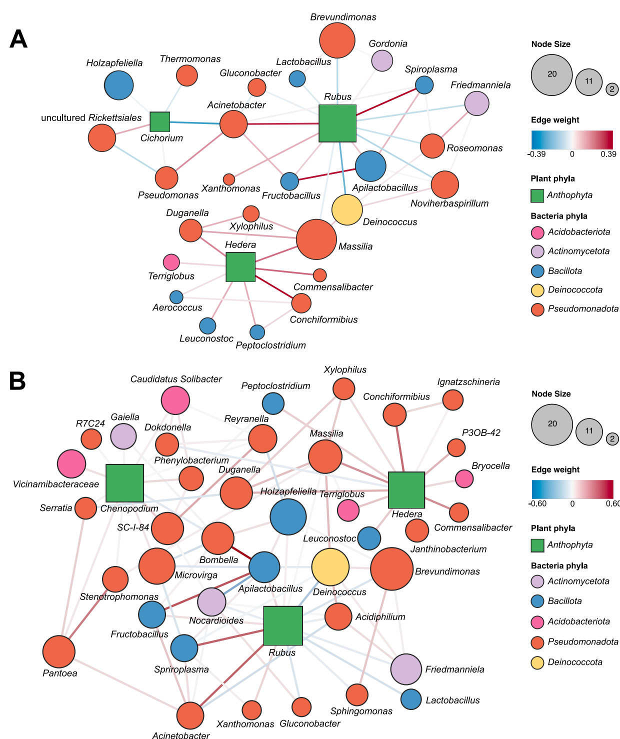

Fig. 6. Co-occurrence subnetwork of plant-bacteria cross-domain network in corbicular pollen samples with ASVs collapsed to genus level. (A) Subnetwork in 2022 with three plant genera with the highest node degrees and their nearest bacterial neighbors. (B) Subnetwork in 2023 with three plant genera with the highest node degrees and their nearest bacterial neighbors. Node size is based on the node degree of connectivity in the full cross-domain network.

microbiome of corbicular pollen samples consisted of diverse microbes derived from bees, other insects, plants, and other environmental sources. Bacterial genera with high prevalence were mostly originated from honey bees. For example, *Apilactobacillus* as a genus normally associated with flowers and insects (Zheng et al., 2020) was found in every corbicular pollen sample. Most ASVs under this genus were identified as *Apilactobacillus kunkeei* which are honey bee-related microbes typically isolated from the bee crop (also called honey stomach) (Corby-Harris et al., 2014; Olofsson et al., 2014), indicating they were introduced by honey bees during the foraging activity. A recent study exploring the effect of honey bees on the floral microbiome in a rural boreal ecosystem also showed that honey bees altered the floral microbiome and increased the relative abundance of bacterial order *Lactobacillales* in open flowers (Hietaranta et al., 2023). In addition to *Apilactobacillus*, we also identified other honey bee-related microbes with high prevalence such as *Bombella*, *Gilliamella*, and *Snodgrassella*. *Gilliamella* and *Snodgrassella* are dominant in the honey bee hindgut region (Martinson et al., 2012; Anderson et al., 2013), while their presence in the bee crop varies among worker bee types, i.e., they were identified in the forager crop with great abundance but absent from the crop of nurse bees and newly emerged bees (Anderson et al., 2013; Corby-Harris et al., 2014). As bee-related microbes in corbicular pollen are most likely derived from the bee crop due to pollen packing activity using crop liquid, our identification of these two genera with high prevalence in the corbicular pollen samples indirectly supports their presence in the forager crop. Interestingly, *Bifidobacterium* was the only core gut microbe completely absent from all samples. Like *Gilliamella* and *Snodgrassella*, *Bifidobacterium* mainly colonizes the hindgut region (Bottacini et al., 2012; Powell et al., 2014) and our finding suggest that its habitat in honey bee might be more fixed compared to other core gut microbes. Another highly prevalent genus was *Massilia*. Species under this genus are widely distributed in the environment with soil as the main habitat (Xu et al., 2023), thus its prevalence in corbicular pollen might reflect a more general interaction between honey bees and various environment sources.

The temporal change of corbicular pollen microbiome could be a result of environmental factors and choice of forage plants collectively. Most environmental factors used in this study affected the general microbiome structure and correlated with several bacterial genera. We speculated that flower pollen internally contained nutrition that supported bacterial growth and, together with external environmental conditions, acted as a mini-incubator favoring the growth of specific bacteria. The bacterial genera correlated with environmental factors did not show considerable overlap with core or differentially abundant genera, indicating the influencing factors of microbiome composition in corbicular pollen were multilayered and the influence of environmental factors alone was limited. The cross-domain network has been used as an efficient tool to study potential interactions between bacteria and fungi (Tipton et al., 2018), and here we expanded this method to study plant-microbe interaction. We found that plant composition reorganized the structure of the co-occurrence network, making it more connected and stable. Many positive plant-bacteria co-occurrences also aligned with the differential abundance results, suggesting the type of forage plants as a more dominant influencing factor of corbicular pollen microbiome. Previous studies have found that *Lactobacillus* spp. in honey bee gut showed seasonal changes in abundance and proposed that flowers could be a potential route of acquisition (Corby-Harris et al., 2014; Kešnerová et al., 2020; Castelli et al., 2022). The genus *Lactobacillus* was reclassified into *Lactobacillus*, *Paralactobacillus* and 23 novel genera in 2020, which include *Apilactobacillus* and *Holzapfelia* (Zheng et al., 2020). In our study, we found that *Apilactobacillus* consistently co-occurred with *Rubus* and *Ligustrum*, and *Holzapfelia* co-occurred with *Tilia*. All three plant genera exhibited a flowering phase around June in Germany. Although specific plant-microbe correlations were revealed, the

originality of these corbicular pollen microbes should be carefully interpreted. The plant-associated microbes could be harbored internally in the plant, for example, vertically transmitted from plant seed (Cope-Selby et al., 2017; Malinich and Bauer, 2018), or horizontally transmitted from plant-specific insect pollinators (Ushio et al., 2015).

Rubus was the most prominent plant hub taxon revealed by the high node degree and betweenness centrality in the cross-domain network. Its positive co-occurrence with the bee pathogen *Spiroplasma melliferum* further makes it an interesting object for discussion. *Spiroplasma* disease, also known as "May disease", occurs mainly between May and July (Mouches et al., 1982; Zheng and Chen, 2014), and this is in line with the flowering phase of *Rubus* and the period in which it was primarily identified in corbicular pollen. Thus, the occurrence of this honey bee disease could be due to intensive foraging from *Rubus*. The identified *Spiroplasma* species also exhibit a wide insect host range including honey bee, tick, horsefly, dragonfly, and beetle. This is consistent with a recent study showing diverse flower-visiting insects of bramble (*Rubus fruticosus* L. agg.) (Wignall et al., 2020). It has been reported that *Rubus* pollen is highly nutritious with considerable content of proteins, sugars and antioxidants, and bees fed with *Rubus* pollen are more tolerant against *Nosema* infection compared to bees fed with less nutritious pollen (Di Pasquale et al., 2013). We thus speculated that the high nutritional value makes *Rubus* a foraging hotspot for various insects in addition to honey bees, which further renders it a repository of diverse microbes and a potentially important node for pathogen transmission.

The positive co-occurrence of *Rubus* with *Spiroplasma* bee pathogen inspired us that analysis of corbicular pollen microbiome and plant composition could have great potential to reveal the horizontal transmission route of other bee pathogens. For example, *P. larvae* can be transmitted both vertically from mother to daughter colonies via swarming (Fries et al., 2006), and horizontally between colonies through robbing (Lindström et al., 2008). Little is known whether forage plants are also involved in the horizontal transmission of *P. larvae* and how they are involved. It has also been shown that interspecific transmission of fungal and viral bee pathogens is influenced by ecological and social factors, however, the influence of floral resources was not investigated in the same study (Tiritelli et al., 2024). Although a study by Graystock et al. (2015) highlighted flowers in general as key hotspots for the transmission of pollinator pathogens, a comprehensive framework to fully elucidate the complexities of this interaction network is still lacking. We propose that by analyzing corbicular pollen samples before and after infection by specific pathogen under natural conditions, and constructing cross-domain co-occurrence networks accordingly, the pathogen transmission route is likely to be uncovered. Moreover, pathogen transmission of other pollinator insects can also be studies, provided that a comparable sample to corbicular pollen is available, i.e., one that can reveal the complete set of forage plants and whose microbial composition has not been significantly altered, for example, by in-hive fermentation.

Compositionality of high throughput sequencing data has drawn increasing awareness in related research fields and emphasized the importance to use correct analytical approaches (Gloor et al., 2017). In this study, most analyses were based on clr-transformed sequencing data, which is a commonly used method to address compositional issue. However, we also presented data based on relative abundance without compositional adjustment such as core genus analysis. These data are incapable to reveal changes in absolute abundance. For example, we had no idea on the bacterial loads of honey bee-related microbes or plant-derived pathogens in corbicular pollen. This information could be uncovered in future studies using non-compositional methods such as qPCR.

In summary, our study revealed the temporal change of corbicular pollen microbiome and its influencing factors including environmental factors and type of forage plants. We demonstrated the potential of corbicular pollen to study the transmission of microbes between bees and forage plants. This framework could be applied in other regions with differential landscapes, climate or floral resources to further study the complicated pollinator-microbe-plant network or clarify the pathogen transmission route in different ecosystems.

CRediT authorship contribution statement

Haoran Shi: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Stefan Ratering:** Writing – review & editing, Validation, Supervision, Software, Methodology, Investigation. **Bellinda Schneider:** Investigation. **Sylvia Schnell:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.178107.

Data availability

The sequencing data are available at the NCBI GenBank database under the BioProject PRJNA1080550 and PRJNA1080497. Other data will be made available on request.

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Chapter 3 Microbiota and microbial transmission dynamics associated with bramble (*Rubus* spp.) flowers

Microbiota and microbial transmission dynamics associated with bramble (*Rubus* spp.) flowers

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Abstract

Microbes are integral players in plant-insect networks, and play crucial roles in host health and ecological interactions. However, the microbial transmission dynamics via flowers remain insufficiently understood, particularly in hub plants visited by diverse insect communities. Here, using 16S rRNA gene and ITS2 metabarcoding, we characterized the bacterial and fungal communities associated with open flowers, insect-excluded bagged flowers, and flower buds of bramble (*Rubus* spp.), a highly insect-visited hub plant. Our results showed that flowering and insect visitation significantly altered floral microbiota structure and microbial load. Insect visitation enriched fermentative and probably also pathogenic bacteria, including *Spiroplasma*, and introduced specific hub taxa that reshaped microbial interaction networks. While bagged flowers exhibited distinct microbiota structure and more internally connected microbial networks, open flowers displayed greater within-group variation and more centralized networks. Cross-domain network analysis revealed hub taxa unique to bacteria-fungi interactions, including *Cladosporium*, which was consistently detected across all flower groups. These findings highlight the dominant role of insect visitation in shaping floral microbiota and underscore the ecological significance of hub plants in microbial transmission across plant–pollinator networks.

Key words: floral microbiota, microbial transmission, bramble, hub plant, host-microbe interaction

Background

Microbes are functionally essential players for both plant and insect health. In plants, associated microbes can promote growth, facilitate nutrient uptake, and confer tolerance and resistance to environmental stressors and pathogens, and these microbe-mediated benefits are also observed in insects [1, 2]. As plants ubiquitously interact with insects in diverse ecological systems, microbes are inevitably transmitted between them, and one of the microbial transmission routes is via pollination. The microbes transmitted to flowers depend on the microbial pool carried by visiting insects [3, 4], as well as the assemblage of visiting insects filtered by various flower cues such as volatile organic compounds, floral morphology, floral color, and nutritional content [5-8]. The physical and chemical properties of plant flowers can further influence the survival and establishment of epiphytic microbes [6, 9, 10].

Pathogenic microbes can exploit plant-insect networks for their dispersal. For example, several bee-associated pathogens including *Vairimorpha* species (*V. apis* and *V. cerane*), *Spiroplasma* species (*S. apis* and *S. melliferum*), and *Ascosphaera* have been shown to utilize the floral transmission routes [11-14]. These pathogens can cause various diseases and pose threats to both insect individuals and entire communities [15-18]. This mode of pathogen transmission can occur not only within a single insect species but also between different insect species [19]. Plant-insect networks can also aid the spread of plant-associated pathogens. *Erwinia amylovora* is a bacterial phytopathogen that causes the destructive disease fire blight in some commercially important pome fruit trees such as apple and pear [20]. This pathogen is commonly spread by honeybees from diseased to healthy flowers [21]. In addition, plant fungal pathogens such as *Microbotryum violaceum* and *Monilinia vaccinii-corymbosi* can also be vectored by visiting insects [22, 23].

Insect-pollinated plants are not equally visited by the available insects within the local pollinator community, resulting in distinct pollinator profiles. Generalist plant species with easily accessible floral morphologies and rich nutritional reward tend to attract a broad array of pollinators [24, 25]. In contrast, other plants attract only specialized insects due to co-evolved physical or chemical traits [26, 27]. Plants that host diverse pollinators are considered hubs within plant-insect networks. These highly visited plants can also serve as key nodes for microbial transmission, contributing significantly to floral-microbe associations [28, 29]. Their co-occurrences with numerous microbes additionally render them potential hotspots for the transmission of pathogens [14]. Therefore, characterizing microbial composition and

transmission dynamics associated with hub plants holds significance for ecological epidemiology.

Bramble (*Rubus* spp.) belongs to the family Rosaceae and comprises hundreds of species and hybrids [30]. Economically important bramble crops include raspberry and blackberry, both of which have substantial global production [31, 32]. Bramble fruits are highly nutritious, rich in proteins and sugars, and exhibit strong antioxidant capacity, thus being considered as "superfoods" for human consumption [33, 34]. In addition, bramble is a valuable foraging resource, visited by insects from various orders including Diptera, Lepidoptera, Hymenoptera, Coleoptera [35]. A recent study analyzing plant and microbial composition in honeybee-collected pollen also identified bramble as a hub plant in plant-microbe networks and suggested its potential role in pathogen transmission [14]. Previous research on bramble microbiota has mainly focused on seeds, leaves, roots, and rhizosphere soil [36-38]. However, the floral microbiota and microbial transmission dynamics associated with bramble remain poorly understood.

In this study, we aimed to characterize the bacterial and fungal microbiota of bramble flowers using bacterial 16S rRNA gene and fungal ITS2 metabarcoding. By comparing open flowers, bagged flowers, and flower buds, we investigated microbial transmission dynamics via this highly insect-visited hub plant. We examined microbial loads and diversity, functional traits, and microbial co-occurrence network to better understand the underlying transmission processes.

Methods

Sample collection and DNA extraction

Bramble (*Rubus* spp.) flowers were collected around the Giessen area in July 2024 and allocated into three flower groups: open flowers, bagged flowers, and flower buds. The microbiota composition of these flower groups is primarily influenced by insect visitation, airborne deposition, and the indigenous microbiota of bramble, respectively. Open flowers and flower buds were directly placed into 50-ml tubes upon collection. For the collection of bagged flowers, flower buds were enclosed in mesh bags to exclude insect visitation for up to two weeks and were collected at full bloom. Three to four flowers from a single branch were pooled to form one sample. In total, 27 samples were collected and stored at -20 °C until further processing. For DNA extraction, samples were frozen in liquid nitrogen and mechanically

homogenized into fine powder. Metagenomic DNA extraction was then performed using the NucleoSpin Plant II kit (Macherey-Nagel GmbH, Germany) according to the manufacturer's instruction with a prior zirconia bead-beating step using FastPrep-24 tissue and cell homogenizer (MP Biomedicals, USA) at 5.5 m s^{-1} for 45 s.

Quantitative PCR

Total bacterial and fungal loads in the floral metagenomic DNA were quantified by quantitative real-time PCR. For bacterial quantification, primers 520F (5′-AYTGGGYDTAAAGNG-3′) [39] and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [40] targeting the 16S rRNA gene were used, along with peptide nucleic acids (PNAs) to block the amplification of mitochondrial (5′-AAACCAATTCACTTGAGT-3′) [41] and plastid sequences (5′-GGCTCAACCCTGGACAG-3′) [42]. For fungal quantification, primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 ngs (5′-TTYRCKRCGTTCTTCATCG-3′) [43] targeting the ITS1 region were used in combination with a blocking primer containing a 3′ C3 spacer to block the amplification of *Rubus* ITS1 (5′-GATCATTGTCGAAACCTGCCCAGCAG-3′) [36]. To normalize bacterial and fungal loads, the plant transcription elongation factor (*tef*) gene was quantified using primers tef_f (5′-ACTGTGCAGTAGTACTTGGTG-3′) and tef_r (5′-AAGCTAGGAGGTATTGACAAG-3′) [44].

The volume of each reaction was 10 µl, consisting of 1× ABsolute qPCR SYBR Green Mix (Thermo Scientific, USA), 300 nM of each primer, 1 µM of each PNA or 10 µM of blocking primer if required, and 1 µl DNA template. Amplification was performed using a Rotor Gene Q (QIAGEN GmbH, Germany) with program settings described in Table S1. Following amplification, a melting curve analysis was performed by gradually increasing the temperature from 60 °C to 95 °C with fluorescence measurements taken at 1 °C intervals to confirm the specificity of the qPCR products. The copy numbers of standard DNA samples were calculated according to [45], and the calibration curves were constructed using 10-fold serial dilutions of DNA standards ranging from 10^1 to 10^6 copies in four replicates. The gene copy numbers in real samples were obtained in Q-Rex software (version 1.1.04). The differences in microbial loads across the flower groups were assessed using the Wilcoxon test with Bonferroni correction.

Bacterial and fungal metabarcoding

The 16S rRNA gene V4-V5 regions and the ITS2 region were used as barcodes to profile the bacterial and fungal microbiota, respectively. The 16S rRNA gene was amplified using the same primers as in the quantitative PCR. The ITS2 region was amplified using primers ITS3 KYO2 forward (5′-GATGAAGAACGYAGYRAA-3′) [46] and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) [43]. Amplification, purification, and library preparation for the 16S rRNA gene followed the protocols described by [14], while the procedures for the ITS2 followed [47]. A negative control was included to confirm the absence of contamination. Prepared libraries were sequenced using Ion Torrent S5, and the sequencing data were deposited in the NCBI Sequence Read Archive under the BioProject PRJNA1261409 and PRJNA1261414.

We used QIIME 2 amplicon distribution (released in 2024.10) for sequencing data processing. Demultiplexed sequences were imported into QIIME 2, and the ITS2 sequences were trimmed using the ITSxpress plugin [48]. The dada2 plugin was then used to denoise and dereplicate the sequences into amplicon sequence variants (ASVs) [49]. The 16S rRNA gene sequences were truncated at the 300th position, while the ITS2 sequences were not truncated due to their highly variable lengths [50]. Taxonomy assignment of ASVs was performed using sklearn-based classifiers [51, 52] pre-trained with SILVA database (version 138.1) [53] for the 16S rRNA gene sequences and EUKARYOME database (version 1.9.4) [54] for the ITS2 sequences. ASVs identified with non-bacterial or non-fungal origins were excluded from downstream analyses.

Bioinformatic analyses

The taxonomy information was refined by replacing the unknown or uninformative taxonomy with the first informative taxonomy from a higher taxonomic rank, and taxonomy barplot at genus level was generated using the R package microViz (version 0.12.6) [55]. The shared and unique genera were analyzed using the R package UpSetR (version 1.4.0) [56]. Functional annotation of bacterial and fungal genera was performed using FAPROTAX database (version 1.2.10) and FungalTraits database (version 1.2), respectively [57, 58]. Functional traits of bacteria were grouped into functional guilds chemotroph, phototroph, pathotroph, biogeochemical cycle, and fermentation, and functional traits of fungi were grouped into saprotroph, pathotroph, and symbiotroph [59, 60]. Alpha diversity analysis was performed with the Hill-Shannon index using the R package MeanRarity (version 0.0.1.4) [61]. Beta diversity analysis was performed based on robust Aitchison distances and visualized through principal component analysis (PCA) ordination [62]. Differences across the flower groups were assessed

by permutational multivariate analysis of variance (PERMANOVA) [63] and permutation test for homogeneity of multivariate dispersions (PERMDISP) [64] with 999 permutations using the R packages microViz and vegan (version 2.6-4) [65]. Pairwise differences were assessed using the Wilcoxon test with Bonferroni correction.

Differentially abundant bacterial and fungal genera were identified using the R package ANCOMCB (version 2.0.3) [66]. Benjamini-Hochberg correction was used to adjust *p*-values and control the mixed directional false discover rate. Pseudo-count sensitivity analysis was performed as recommended by the package developer. Taxa with *p*-values lower than 0.05 and unaffected by pseudo-count choice were designated as differentially abundant taxa.

Bacterial, fungal and cross-domain co-occurrence networks were constructed using the web-based application SpeSpeNet [67]. In detail, the issues of compositionality and sparsity in amplicon sequencing data were mitigated using SparCC algorithms [68] and imputation of random pseudo-counts [69]. Occurrence and abundance thresholds were set at 2 and 0.1%, respectively. Only correlations with edge weights above 0.2 were included in network construction. Resulting networks were imported into Cytoscape (version 3.9.1) for the analysis of network attributes, including number of nodes and edges, average number of neighbors, characteristic path length, network density, clustering coefficient, and network centralization [70]. In each network, taxa within the top 10% for both degree and betweenness centrality were assigned as hub taxa.

Results

Bacterial and fungal composition of bramble flowers

We analyzed 27 bramble flower samples for their bacterial and fungal microbiota using 16S rRNA gene and ITS2 metabarcoding. After sequence quality control and removal of non-target sequences, the bacterial dataset contained 336,673 sequences, and the fungal dataset contained 169,628 sequences. Six samples were removed for fungal analysis due to low sequencing depth. The remaining sequences were grouped into 687 bacterial ASVs and 667 fungal ASVs, which were further collapsed into 219 bacterial genera and 307 fungal genera. The most prevalent bacterial genera included *Acinetobacter*, *Cupriavidus*, and *Enhydrobacter*, each identified in at least 92.6% of the samples (Fig. 1a). The most prevalent fungal genera were *Cladosporium*, *Aureobasidium*, and *Filobasidium*, present in at least 95.2% of the samples (Fig. 1b). Open flowers, bagged flowers and flower buds harbored 142, 149, and 66 bacterial genera,

respectively, with 41 genera shared across all three groups. Open and bagged flowers contained a considerable number of unique bacterial genera, while flower buds harbored comparatively fewer unique genera (Fig. 1c). For fungal communities, open flowers, bagged flowers and flower buds harbored 168, 219, and 108 fungal genera, respectively, with 58 genera shared across all groups. Bagged flowers exhibited the highest number of unique genera, followed by open flowers, while flower buds contained the fewest unique genera (Fig. 1d).

Microbiota structure and microbial load across bramble flower groups

Hill-Shannon diversity analysis revealed no significant differences in bacterial and fungal alpha diversity across open flowers, bagged flowers and flower buds of brambles (Fig. 2a, Table S2). However, beta diversity analysis based on robust Aitchison distances showed significant differences for both bacterial (PERMANOVA: $F = 1.94$, p-value = 0.001) and fungal communities (PERMANOVA: $F = 1.70$, p-value = 0.013) (Fig. 2b and 2c). The differences were mainly caused by bagged flowers, while open flowers and flower buds tended to be similar (Table S3). We also checked the dispersion of microbial communities across the flower groups. For bacterial communities, within-group variation differed significantly (PERMDISP: $F = 5.46$, p-value = 0.007), with open flowers showing greater dispersion than flower buds (p-value = 0.00049) (Fig. 2d). No significant differences in within-group variation were observed for fungal communities (PERMDISP: $F = 2.40$, p-value = 0.119) (Fig. 2d, Table S3). The bacterial load in open flowers was significantly higher than that in bagged flowers (p-value = 0.00025) and flower buds (p-value = 0.00086), while the fungal load in flower buds was the lowest compared the open flowers (p-value = 0.0233) and bagged flowers (p-value = 0.0023) (Fig. 2e).

Functional annotation of bacterial and fungal genera

Identified bacterial and fungal genera were annotated with functional traits using FAPROTAX and FungalTraits database, respectively. In total, 117 bacterial genera and 226 fungal genera were assigned at least one functional trait (Table S4 and S5). Shared genera across the flower groups indicated their consistent associations with plants and exhibited broad functional traits, with some classified as plant pathogens such as *Ralstonia*, and *Cladosporium*. Some pathogenic microbes, such as *Spiroplasma* and members of the family *Anaplasmataceae*, were uniquely identified in open flowers, suggesting their insect-mediated transmission. Considering the compositionality of amplicon sequencing data, we compared the occurrences of different functional guilds across the flower groups instead of relative abundance (Fig. 3). The occurrence of bacterial genera with fermentation function was significantly higher in open

flowers than in flower buds (*p*-value = 0.0066), and pathogenic bacteria showed a trend toward higher occurrence in open flowers with a borderline *p*-value (*p*-value = 0.094) (Table S6). For fungal functional guilds, no significant differences were detected across the flower groups (Table S7).

Differentially abundant taxa across bramble flower groups

Differential abundance analysis on bacterial and fungal genera was performed using ANCOM-BC2. Surprisingly, although many unique genera were identified across the flower groups, the number of differentially abundant genera was minor (Table S8). For bacterial genera, only *Neokomagataea* was differentially abundant, showing enrichment in open flowers compared to both bagged flowers and flower buds. For fungal genera, *Metschnikowia* was enriched in open flowers, whereas *Cryptococcus* and *Hannaella* were differentially abundant in bagged flowers relative to the other groups. The limited amount of differentially abundant taxa might be attributed to high within-group variation, which reduced the consistency of taxa within each flower group.

Co-occurrence network and hub taxa

To explore microbial interactions in different flower groups, we constructed bacterial, fungal, and cross-domain networks using the SparCC algorithm (Fig. 4). The networks derived from flower buds consistently exhibited the lowest number of nodes and edges, as well as the lowest average number of neighbors, while all three attributes were the highest in the bagged flower networks (Table S9). This is somewhat expected, as flower buds harbored notably fewer bacterial and fungal genera compared to open and bagged flowers. In addition, the bagged flower networks had the lowest characteristic path length and the highest network density and clustering coefficient, suggesting that these networks were more tightly connected and that microbial taxa were more interactive in this environment. In contrast, the open flower networks were more centralized compared to the bagged flower networks and the flower bud networks, as reflected by higher network centralization values. The centralization of the open flower networks was particularly pronounced when cross-domain interactions were considered.

Across the different networks, we identified distinct sets of bacterial and fungal hub taxa, and the composition of these hub taxa was strongly influenced by the flower groups (Fig. 5a). Most hub taxa were specific to a single flower group, with only a few exceptions. In the bacterial networks, five hubs were present in open flowers, four in bagged flowers and one in flower

buds, with no bacterial hub taxa shared across the flower groups. In the fungal networks, five hubs were detected in open flowers, eight in bagged flowers, and four in flower buds, with *Didymellaceae* as the only shared fungal hub. In the cross-domain networks, six hubs were identified in open flowers, nine in bagged flowers and four in flower buds. *Didymellaceae* and *Filobasidium* were hubs shared between two flower groups, while *Cladosporium* was shared across all three groups. Some hub taxa were uniquely present in one network but absent from the others. For example, *Metschnikowia* was a fungal hub in the open flower network, however, it was completely absent from the other fungal networks (Fig. 5b). Moreover, within each flower group, the hub taxa showed distinct patterns between the single-domain networks and the cross-domain network. Many hub taxa in the bacterial networks were non-hub in the cross-domain networks, and several non-hub taxa in the fungal networks emerged as hubs in the cross-domain networks.

Discussion

Plant-microbe interactions are ubiquitous across ecosystems, and hub plants that are visited by a diverse array of insects can play an important role in microbial transmission [28, 71, 72]. In this study, we characterized the floral microbiota of a highly insect-visited hub plant, bramble (*Rubus* spp.). We sampled and analyzed open flowers, bagged flowers, and flower buds to examine the microbial communities before flowering and how they change after flowering, and to investigate the influence of insect visitation. Our results showed that bramble flower buds were associated with a relatively small microbial community, which expanded and underwent structural shift during flowering, particularly influenced by insect visitation.

Bramble flower buds contained the fewest microbial genera and also the fewest unique genera among the flower groups, suggesting that with limited external introduction, microbial diversity in flower buds remained low. Many genera identified in flower buds were also shared with open and bagged flowers, indicating that these shared microbes may adopt a lifestyle tightly associated with plants. Some plant-associated microbes are capable of persisting within plant tissues as endophytes and can be transmitted horizontally via insects or vertically through plant reproduction [73], and several typical plant endophytes were present among the shared genera. For example, the phytopathogen *Ralstonia* is able to form biofilms and colonize plant xylem vessels using divergent respiratory strategies [74], while *Cladosporium* is commonly identified as a plant-associated fungus with diverse biotic effects [75]. As we did not perform surface sterilization on collected flower buds, the unique genera detected may have been introduced sporadically by passerby insects or through environmental deposition.

Floral microbial communities can be acquired from both insects and the surrounding environment [3, 76]. However, the relative contribution of different microbial transmission routes to the transmission dynamics remains insufficiently understood. Moreover, previous related studies often showed limitations, such as the lack of microbial quantification, lack of community structure information, or the use of analytical methods that do not account for the compositionality of amplicon sequencing data [76-78]. Here, we found that microbial diversity expanded substantially in both open and bagged bramble flowers compared to flower buds, as reflected by more unique genera in these two groups. This suggests that floral opening creates new opportunities for microbial colonization, either through environmental exposure or insect visitation. Notably, while the microbial community in individual flowers was not necessarily more diverse than that in individual flower buds, structural shift was evident, as revealed by the alpha and beta diversity analyses. The structural shift of microbial communities was more prominent in bagged flowers compared to open flowers, while the latter exhibited higher within-group variation regarding bacterial communities, indicating distinct bacterial assemblages among individual open flowers. This suggests that insect visitation may function to stabilize the existing microbial community structure, despite its potential to introduce diverse bacteria to flowers. In addition, bacterial load was significantly higher in open flowers compared to bagged flowers and flower buds, suggesting that insects are likely the major source of floral bacteria, whereas floral fungi appeared to originate from both insect visitation and environmental deposition. Altogether, these results show that both biotic and abiotic factors contribute to bramble floral microbiota assembly during flowering, and highlight the dominant role of insect-mediated microbial transmission.

Functional annotation of floral microbes provided additional insights into the microbial transmission dynamics via bramble flowers. Although no significant differences in fungal functional guilds were identified across the flower groups, we found that insect visitation enriched bacteria with fermentation functions and even pathogenic bacteria. Considering the high bacterial load in open flowers, the abundance of these bacteria could be further magnified. Some insects are associated with fermentative bacteria in their digestive tract; thus, these bacteria could be transferred to flowers during insect foraging activity [76, 79, 80]. We also noted that not all bacterial genera could be annotated using the latest version of FAPROTAX database (version 1.2.10 released in 2024). For example, several honeybee-associated bacterial genera were missing such as *Bombella* and *Gilliamella*. These bacteria also possess fermentation capacity [81, 82], therefore the fermentation group in our analysis may still be underestimated. In our previous study, we constructed a plant-microbe network based on the

plant and bacterial profiles in honeybee-collected pollen, and proposed that bramble could serve as an important hotspot for pathogen transmission, including *Spiroplasma* [14]. The current study confirmed the presence of *Spiroplasma* in bramble flowers and indicated its insect origin rather than being an indigenous plant microbe. The role of bramble as a pathogen hotspot was also supported by the enrichment of pathogenic bacteria in open flowers.

Co-occurrence networks are important tools to explore microbial interactions. In plant-associated microbial networks, hub microbes often play dominant roles in interacting with other microbes, and disturbances to hubs may result in shift in the overall microbiota structure [83]. Our co-occurrence network analysis revealed that the flower bud networks were relatively simple compared to the open flower and bagged flower networks, while the latter two were also distinct from each other. The bagged flower networks were more connected, whereas the open flower networks were more centralized, where many nodes are peripheral, connected primarily to the hub nodes but not to each other. The open flower networks exhibited distinct microbial hubs. Some of these hubs were uniquely identified and even overlapped with the few differentially abundant taxa, indicating insect visitation may introduce crucial microbes that reshape the floral microbiota interactions.

Neokomagataea and *Metschnikowia* were unique bacterial hubs and the only differentially abundant taxa in the open flower networks. Our results are consistent with previous studies indicating that these two microbial genera inhabit nectar environment and are more abundant in insect-visited flowers [78]. It has been shown that *Metschnikowia* is able to increase the pollinator visitation rate of a bumblebee-pollinated vine *Clematis akebioides*, possibly through the production of volatiles [84]. To our best knowledge, the effect of *Neokomagataea* on plant-pollinator interactions remains unknown. However, its close relative *Gluconobacter* has been reported to reduce nectar foraging activity of birds [85, 86]. These specific effects of microbial hubs may consolidate the existing plant-pollinator interactions and further contribute to the stabilization of floral microbiota structure.

Our analysis revealed that some hub taxa in the single-domain networks were not retained as hubs in the cross-domain network. This suggests that their influence may be limited to intra-domain interactions and not extend to cross-domain dynamics. Conversely, hubs in the cross-domain network may represent taxa that bridge bacterial and fungal communities, playing roles in inter-domain ecological processes. *Cladosporium* was a hub fungus in the cross-domain networks but not in the fungal networks, and it was the only hub consistently identified across all flower groups. This fungal genus shows either endophytic or epiphytic lifestyle in plants

[87, 88], and is capable of producing a group of plant growth-promoting volatiles [89]. However, its interaction with bacteria in plant-associated niches is still unclear, and we suggest that future studies should investigate the potential cross-domain interactions involving *Cladosporium* to better understand its ecological roles in floral microbiota.

In conclusion, by characterizing and analyzing the microbial profiles of open flowers, bagged flowers and flower buds of a highly insect-visited hub plant bramble, we showed that the microbial diversity and structure are substantially influenced by flowering and insect visitation. Our results demonstrate that insect visitation is the dominator influencer of bramble floral microbiota, through altering microbiota structure and functional properties, increasing microbial load, and introducing hub microbes that reshape microbial interactions. Based on these findings, we recommend that hub plants receive more research attention, as they may play key roles in microbial transmission across different ecosystems. Future studies focusing on functional interactions among floral microbes will also be essential to fully understand the ecological consequences of plant–microbe–pollinator networks.

Declarations

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Author contributions

HS conceived the study, conducted the experiment, analyzed the data, and wrote the manuscript. BS conducted the experiment. SR, DH and SS reviewed and finalized the manuscript. All authors read and approved the final manuscript.

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Data availability

The sequencing data are available at the NCBI GenBank database under the BioProject PRJNA1261409 and PRJNA1261414. Additional information is available in the supplementary files.

Competing interests

The authors declare no competing interests.

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Fig. 1

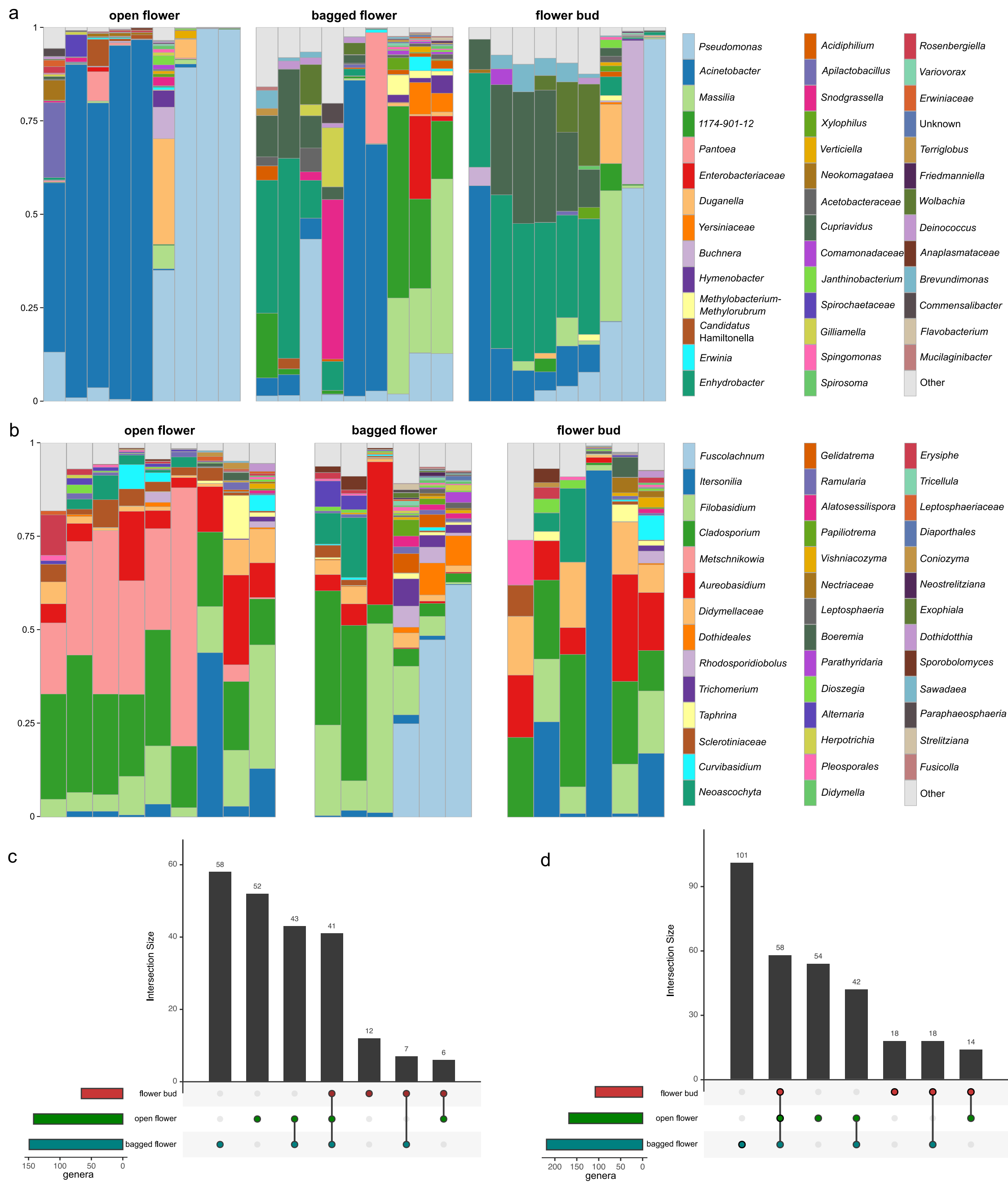

Figure 1. Bacterial and fungal composition in open flowers, bagged flowers, and flower buds of bramble. **a, b** Taxonomy barplots showing the relative abundance of bacteria (**a**) and fungi (**b**) across different flower groups. ASVs were collapsed to genus level and a higher taxonomic rank was used when genus was unavailable. Taxa with relative abundance lower than 0.5% were grouped into "Other". **c, d** UpSetR graphs showing the unique and shared bacterial (**c**) and fungal (**d**) genera across different flower groups.

Fig. 2

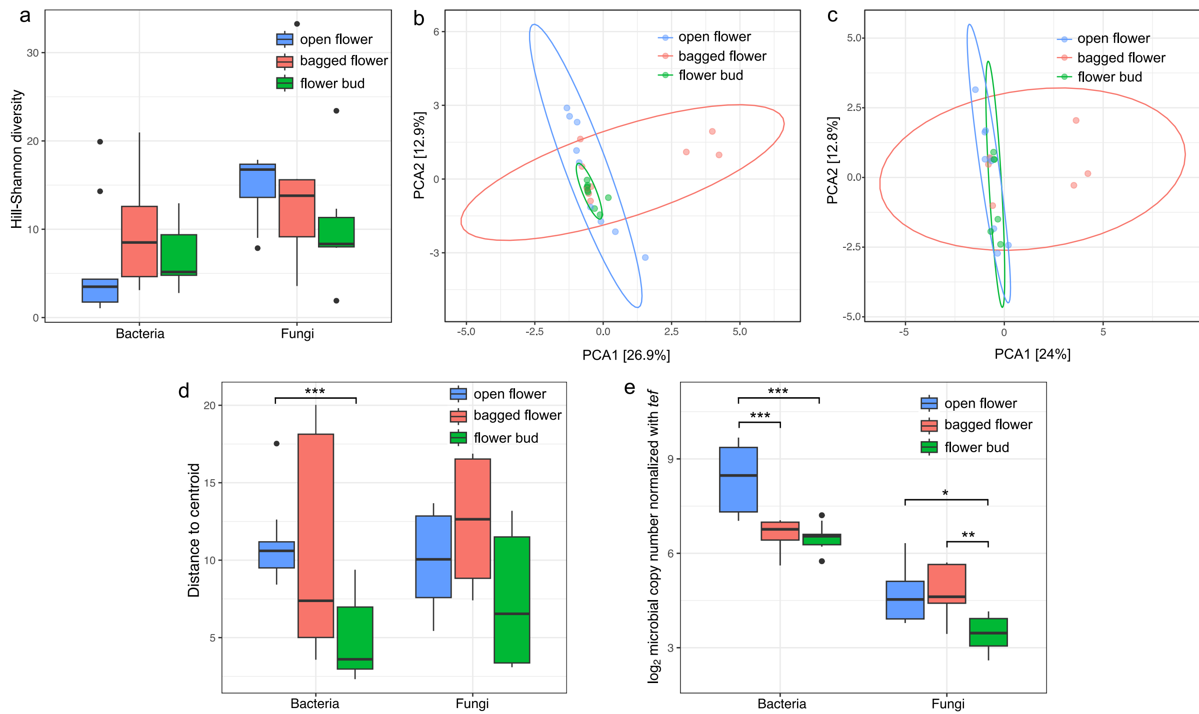

Figure 2. Microbial diversity and abundance in open flowers, bagged flowers, and flower buds of bramble. **a** Hill-Shannon diversity of bacterial and fungal communities across different flower groups. **b, c** Beta diversity analysis of bacterial (**b**) and fungal (**c**) communities across different flower groups based on robust Aitchison distances. The ellipses represent the 95% confidence intervals for each group. **d** Distances from each sample to the group centroid in the bacterial and fungal beta diversity plots. Larger distance indicates more pronounced within-group variation. **e** Microbial load of bacteria and fungi relative to plant *tef* gene quantified by quantitative real-time PCR across different flower groups. Wilcoxon test with Bonferroni correction was performed. *** $p \leq 0.001$.

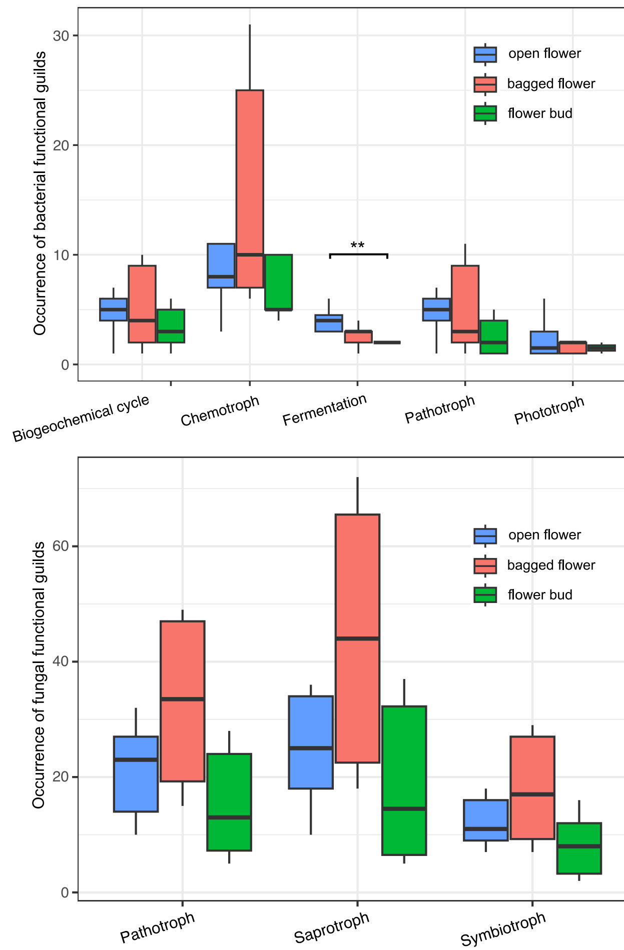

Figure 3. Occurrence of bacterial (upper graph) and fungal (lower graph) functional guilds in open flowers, bagged flowers, and flower buds of bramble. Wilcoxon test with Bonferroni correction was performed. ** $p \leq 0.01$.

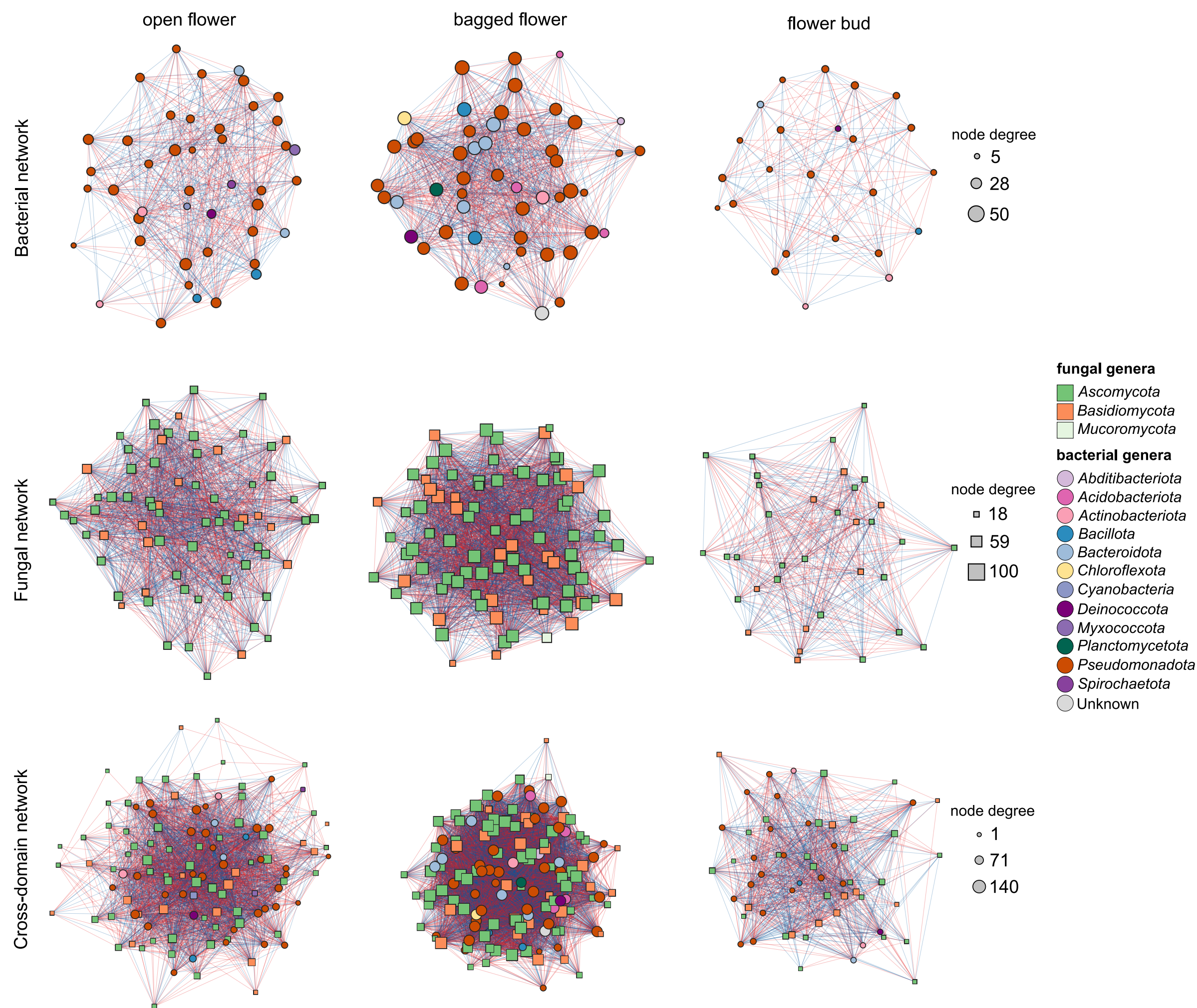

Figure 4. Single- and cross-domain networks from open flowers, bagged flowers, and flower buds of bramble. Red and blue lines indicate positive and negative co-occurrences, respectively. The node size represents node degree.

Fig. 5

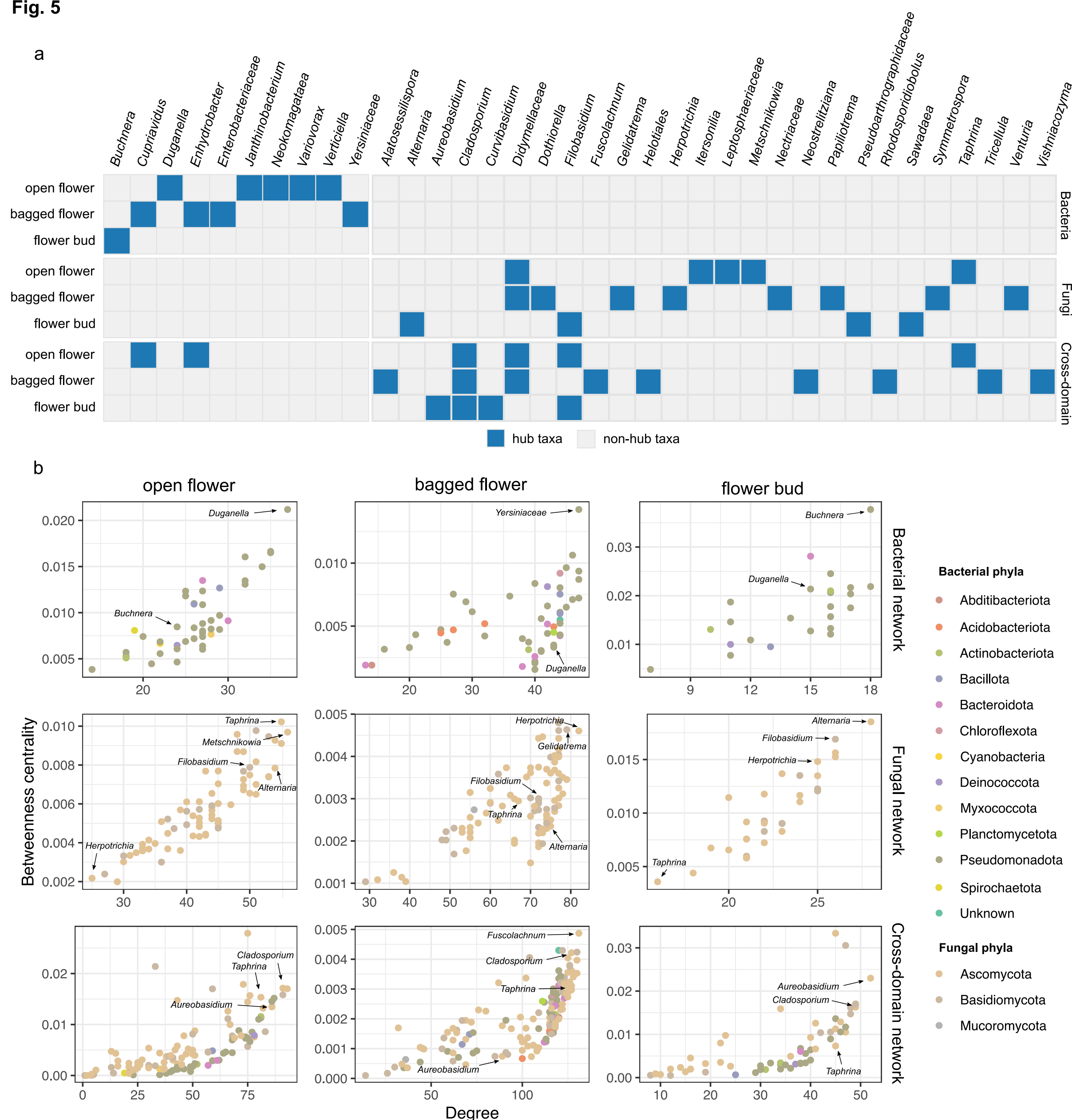

Figure 5. Hub taxa in the single- and cross-domain networks from open flowers, bagged flowers, and flower buds of bramble. **a** Heatmap of hub taxa identified in different networks based on degree and betweenness centrality. **b** Scatter plots of degree versus betweenness centrality for each node in the networks. Selected hub taxa and their positions as non-hubs in other networks are indicated with arrows.

Chapter 4 Impact of Black queen cell virus and *Vairimorpha* infection on the immunity genes and the microbiome of forager honeybees under field conditions

Impact of Black queen cell virus and *Vairimorpha* infection on the immunity genes and the microbiome of forager honeybees under field conditions

Running title: BQCV and *Vairimorpha* impact on forager honeybees

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Abstract

Black queen cell virus (BQCV) and *Vairimorpha* are honeybee pathogens that frequently co-occur and threaten honeybee health. While the impact of *Vairimorpha* alone on honeybees has been studied, few studies have examined the combined effect of BQCV and *Vairimorpha*. Here, we examined the immunity gene expression and the microbiome of honeybees naturally infected with BQCV and *Vairimorpha* pathogens by qPCR and metabarcoding of the 16S rRNA genes. The results confirmed the correlation between BQCV and *Vairimorpha* loads. We found for the first time that BQCV-*Vairimorpha* co-infection in the field did not influence several representative honeybee immunity genes including *defensin-1*, *lysozyme-like*, *vitellogenin*, and *glucose oxidase*. Our results supported the constitutive expression of *glucose oxidase* in honeybees, but also highlighted differential honeybee immune responses to various pathogens. The microbiome structure of co-infected bee colonies showed no difference from colonies infected with BQCV alone. No colonies were free of BQCV infection, thus its influence on honeybee microbiome remains to be studied. As BQCV-*Vairimorpha* co-infection is more detrimental than single infections, further research on this co-infection complex remain to be expanded.

Key words: BQCV, *Vairimorpha*, honeybee immunity, microbiome

Introduction

Honeybees are vital pollinators in agriculture, providing essential pollination services for economically important crops. Over the past few decades, honeybee research has gained increasing attention, particularly after the emergence of colony collapse disorder (CCD), a phenomenon characterized by the rapid and unexpected loss of honeybees within a hive (Cox-Foster et al., 2007). Colony losses continue to be reported worldwide in recent years, and the direct economic impact are substantial (Popovska Stojanov et al., 2021, Bruckner et al., 2023, Gray et al., 2023). The health of honeybees is threatened by various bacterial, fungal and viral pathogens, which can negatively affect honeybees at both the individual and the colony levels (Genersch, 2010, Evans and Schwarz, 2011).

Black queen cell virus (BQCV) is a non-enveloped RNA virus belonging to the genus *Triatovirus* from the family *Dicistroviridae*, and is commonly identified in apiaries around the world (Tentcheva et al., 2004, Mondet et al., 2014). It was firstly isolated from dead honeybee (*Apis mellifera*) pupae and prepupae (Bailey and Woods, 1977). BQCV mainly affects honeybee queen larvae and pupae, causing mortality at high viral titers, and the queen cell is also stained black by the necrotic remains (Spurny et al., 2017). Adult honeybees are normally regarded as carriers and transmitters of BQCV with no obvious clinical symptoms, while a recent study showed that BQCV may also influence the orientation ability of adult honeybees (Retschnig et al., 2019). In BQCV-infected honeybees, *Vairimorpha* (formerly known as *Nosema*) pathogens are frequently identified (Bailey et al., 1983, Chagas et al., 2020). The microsporidia *Vairimorpha apis* and *Vairimorpha ceranae* are spore-forming intracellular pathogens infecting the midgut epithelial cells of adult bees and causing vairimorphosis (Chen et al., 2009), and this fungal disease hampers honeybee health and reduces colony strength (Botías et al., 2013, Goblirsch et al., 2013). The co-infection of BQCV and *Vairimorpha* pathogens are more detrimental to honeybees, as it leads to increased mortality rate and shortened life span compared to infection by single pathogen (Gajda et al., 2021), and such deleterious effect may be exacerbated in the presence of sublethal doses of pesticides (Doublet et al., 2015).

To defend against pathogens, honeybees utilize canonical innate immunity composed of four major pathways: Toll, JAK/STAT, IMD/JNK, and RNAi. Among these, Toll pathway tends to play a dominant role in response to a variety of pathogens (Brutscher et al., 2015). One important group of downstream products in the innate immunity is antimicrobial peptide (AMP) such as defensin and lysozyme. As key components in humoral immunity, AMPs exhibit broad-

spectrum activity against microbes and can be used as indicators of pathogen infection (Danihlík et al., 2015, Barroso-Arévalo et al., 2019). Vitellogenin is a key storage protein that is not regulated by canonical innate immunity but shows numerous immunological functions, including immune priming, protection from oxidative stress, and maintenance of immune cells (Amdam et al., 2004, Li et al., 2009, Salmela et al., 2016). Honeybee social immunity is also important to protect the bee colony from pathogens and infections, and glucose oxidase can act as a parameter of social immunity. Glucose oxidase is expressed in the hypopharyngeal glands of worker bees and catalyzes the oxidation of glucose, producing antiseptic hydrogen peroxide to inhibit microbial growth (Ohashi et al., 1999).

Up-regulation of several genes from Toll, IMD/JNK, and RNAi pathways has been reported as the influence of BQCV alone or BQCV-*Vairimorpha* coinfection on honeybee immunity (Doublet et al., 2016), while upon the infection of *Vairimorpha* alone, honeybee immunity is either activated or suppressed (Antúnez et al., 2009, Li et al., 2017, Sinpoo et al., 2018, Lourenço et al., 2021). In addition to immunity genes, the honeybee microbiome might be also reflective of pathogen infection. Several infection experiments have shown that *V. ceranae* infection increases the abundances of core honeybee gut bacteria, such as *Bifidobacterium*, *Snodgrassella*, and *Gilliamella* (Huang et al., 2018, Rubanov et al., 2019, Zhang et al., 2019). In contrast, the microbiome structure of honeybees naturally infected with *Vairimorpha* appears to be minimally affected (Hubert et al., 2017). To the best of our knowledge, studies on the impact of BQCV infection on the honeybee microbiome are currently lacking.

Most existing studies have primarily examined the influence of *Vairimorpha* alone on the immunity genes and the microbiome of honeybees. In this study, we aimed to examine the expression of immunity genes and the microbiome in forager honeybees naturally infected with BQCV and *Vairimorpha* pathogens. Given that BQCV and *Vairimorpha* are frequently co-identified, understanding their combined impact on honeybees in natural settings is of practical significance and is essential for a more comprehensive perspective on pathogen interactions.

Materials & Methods

Sample collection from managed bee hives

Honeybees (*A. mellifera carnica*) were collected from three hives in the apiary of Justus-Liebig-University Giessen (50°34'14.6"N 8°40'07.9"E) in July and September 2023. The apiary was located in the university campus in an urban area. The major forage plants for honeybees around

this area included *Tilia* and *Rubus* in July, *Lycium*, *Impatiens* and *Hedera* in September, and *Plantago*, *Potentilla* and *Crepis* in both months (Shi et al., 2025). In each month, eight forager bees were collected at the hive entrance from each hive. The bees were collected in a 2-ml microreaction tube, placed on ice, and stored at -80 °C after transported back to the laboratory.

Nucleic acid extraction and cDNA preparation

Surface sterilization of honeybees was performed according to Hammer et al. (2015) before nucleic acid extraction. Honeybees were rinsed in sterile water, soaked sequentially in 70% ethanol and 10% bleach for 60 s each and then rinsed again in sterile water. Sterilized honeybees were then crushed in liquid nitrogen, and subsequently mixed with 1.6 ml extraction buffer (0.1 M NaCl (pH 8), 50 mM EDTA, 0.2 M sodium phosphate buffer (pH 8), 2.5 % w/v SDS) and 200 µl sterile zirconia beads in a 2 ml screw-cap tube. The cells were then disrupted using FastPrep-24 tissue and cell homogenizer (MP Biomedicals, USA) at 5.5 m s^{-1} for 45 s, followed by centrifuging at 16,200 g, 4 °C for 5 min. The supernatant was split and transferred to two separate microcentrifuge tubes for DNA (40% supernatant) and RNA (60% supernatant) extraction, respectively. The tubes for DNA extraction were treated with 10 µl RNase (10 mg ml^{-1}) at 37 °C for 30 min. Samples in both tubes were extracted first with 800 µl phenol/chloroform/isoamyl alcohol (25:24:1), followed by extraction with 800 µl chloroform/isoamyl alcohol (24:1), and centrifuged. The upper aqueous phase was mixed with 1 ml precipitation buffer (20 % w/v PEG 6000 and 2.5 M NaCl), incubated on ice for 30 min, and centrifuged at 16,200 g, 4 °C for 30 min. The pellet was washed twice with 800 µl 75 % ethanol, dried near a Bunsen burner for 5 min, and resuspended in 50 µl nuclease-free water. The tubes for RNA extraction were further treated with RNase-Free DNase Set (QIAGEN GmbH, Germany) according to the manufacturer's instructions, and the absence of DNA was verified by PCR with primers targeting honeybee actin gene (primers used in this study were summarized in Table S1). RNA was then cleaned up using RNA Clean & Concentrator-5 (Zymo Research, USA) and was finally eluted in 15 µl nuclease-free water. For cDNA preparation, 3 µl RNA was used as template, and reverse transcription was performed with TaqMan Reverse Transcription Reagents (Applied Biosciences, USA) using random hexamers, yielding 40 µl cDNA for each sample.

Quantitative PCR

Target DNA fragments of pathogens were synthesized as gBlock fragments (Integrated DNA Technologies, USA) and target immunity genes were PCR-amplified from honeybee DNA. The

fragments were then inserted into pGEM-T vectors, standard DNA was generated through PCR amplification using M13 forward and reverse primers and copy numbers of the standard gene segments were calculated as described by Kampmann et al. (2012). Standard curves were constructed using 10-fold serial dilutions of DNA standards (ranging from 10^1 to 10^6 copies) in four replicates.

Common honeybee pathogens including *Vairimorpha apis*, *Vairimorpha ceranae*, *Paenibacillus larvae*, *Melissococcus plutonius*, Black queen cell virus (BQCV), Kashmir bee virus (KBV), Acute Bee Paralysis Virus (ABPV), Chronic bee paralysis virus (CBPV), Deformed Wing Virus (DWV), and Sacbrood bee virus (SBV) were detected using TaqMan qPCR based on developed protocols (Chantawannakul et al., 2006, Bourgeois et al., 2010, Dainat et al., 2018). Each 10 µl TaqMan qPCR reaction contained 1× SensiFAST Probe No-ROX Mix (Meridian Bioscience, USA), 400 nM of each primer (Table S1), 100 nM of each probe, and 1 µl DNA template. The amplifications were performed with a Rotor Gene Q (QIAGEN GmbH, Germany) using the following program: 2 min at 95 °C and 40 cycles of 10 s at 95 °C and 30 s at 60 °C.

The expression levels of honeybee immunity genes including *defensin-1*, *lysozyme-like*, *vitellogenin*, *glucose oxidase*, as well as a housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) were quantified using SYBR Green qPCR. Each reaction was set up in a 10 µl volume, containing 1× ABsolute qPCR SYBR Green Mix (Thermo Scientific, USA), 200 nM of each primer (Table S1), and 1 µl DNA template. The amplification program was set up as follow: 15 min at 94°C, 40 cycles of 45 s at 94°C, 45 s at 63°C, 60 s at 72°C, and 20 s at 84°C. Q-Rex software v1.1.04 was used for raw data analysis.

The calculation of pathogen load per bee was based on the volume used at each step of qPCR. The virus load per bee was calculated using the following equation: virus load/bee = (n virus load/µl×40 µl)×(15 µl/3 µl)/0.6 bee. The *Vairimorpha* load was calculated based on the equation from Bourgeois et al. (2010). The relative expression levels of immunity genes were calculated according to Lourenço et al. (2021).

16S rRNA gene metabarcoding

Honeybee DNA samples from the same hive were pooled together, and V4-V5 regions of the 16S rRNA gene were amplified with primers 520F (5'-AYTGGGYDTAAAGNG-3') (Claesson et al., 2009) and 926R (5'-CCGTCAATTCMTTTRAGTTT-3') (Engelbrektson et al., 2010)

using KAPAHiFi polymerase (KAPABiosystems, Woburn, MA, United States). The amplification was performed with the following PCR program: 95 °C for 3 min, 35 cycles of (98 °C, 20 s; 55 °C, 30 s; 72 °C, 30 s) and 72 °C for 5 min. Ion Torrent sequencing was prepared and performed according to Shi et al. (2025). The sequencing data were deposited under the BioProject PRJNA1227468 at NCBI.

Data analyses

All statistical analyses were performed in R software (version 4.2.2). The correlation between BQCV and *Vairimorpha* loads was examined using Kendall's Tau correlation coefficient. Group comparison was performed using Wilcoxon rank sum test with Benjamini-Hochberg correction. The influence of pathogen loads on the relative expression levels of honeybee immunity genes was examined using generalized linear model.

Processing of sequencing data including demultiplexing, quality control, denoising, dereplicating and chimera filtering were performed in QIIME 2 2022.11 (Bolyen et al., 2019) with cutadapt (Martin, 2011) and dada2 plugins (Callahan et al., 2016). Taxonomy classification was performed using q2-feature-classifier plugin (Bokulich et al., 2018) based on SILVA 138.1 database (Quast et al., 2012). Microbiome analyses were performed with R package microViz (version 0.12.6) (Barnett et al., 2021). Beta diversity was visualized with principal component analysis of Aitchison dissimilarity matrix, and significance was assessed using PERMANOVA with 999 permutations.

Results

In total, 48 forager honeybees were collected from three hives in the apiary of Justus-Liebig-University Giessen. BQCV infection was highly dispersed in these hives, with 40 samples (83.33%) tested BQCV-positive. *V. apis* and *V. ceranae* were detected in 10 (20.83%) and 15 (31.25%) samples, respectively (Table 1). The two hives with the lowest BQCV loads were free of *Vairimorpha* infection. We found that BQCV loads were weakly correlated with *Vairimorpha* loads ($R = 0.26$, $p = 0.023$) (Fig. 1A). Moreover, significant difference in BQCV loads was observed between *Vairimorpha*-infected and non-infected groups (r effect size = 0.339, $p = 0.02$) (Fig. 1B). Common honeybee pathogens including *P. larvae*, *M. plutonius*, DWV, KBV, CBPV, ABPV and SBV were also screened in the samples and were not detected.

The infection status of these honeybee samples can be categorized as non-infected (n = 9), infected by BQCV (n = 23), infected by BQCV and one of the *Vairimorpha* pathogens (n = 7),

or infected by BQCV and both *Vairimorpha* pathogens (n = 9). The expression levels of four immunity genes (*defensin-1*, *lysozyme-like*, *vitellogenin*, *glucose oxidase*) relative to *gapdh* were examined in all honeybee samples. The median relative expression levels of these genes in non-infected honeybees were lower than those in the other groups, while the differences were found non-significant (Fig. 2, Table S2). In addition, the results from generalized linear model indicated that no pathogens or interactions among pathogens exhibited significant influence on the relative expression level of immunity genes (Table S3).

Metabarcoding of 16S rRNA gene yielded 89,512 high-quality reads which were grouped into 80 ASVs. These ASVs were collapsed into species level, while unclassified species were replaced with genera, resulting in 34 unique taxa. The most dominant taxa with relative abundance > 10% were *Snodgrassella alvi*, *Gilliamella*, *Commensalibacter*, and *Bombilactobacillus*. Sixteen taxa were present in all hives, accounting for 96.6% of all reads (Fig. 3A). As BQCV were present in all hives, we could only group the hives based on *Vairimorpha* infection status. PERMANOVA result showed that the microbiome structures between BQCV-*Vairimorpha* co-infected and BQCV-infected hives were not significantly different (F-value = 0.836, $p = 0.533$) (Fig. 3B).

Discussion

In honeybees, BQCV and *Vairimorpha* pathogens are frequently co-identified, but how they collectively influence honeybees under field conditions remain poorly studied. In this study, we quantified the pathogen loads of BQCV and *Vairimorpha* in naturally infected forager honeybees in an apiary in Giessen. The impact of these two pathogens on honeybees was assessed from two perspectives: the expression levels of several immunity genes, and the composition of the microbiome.

We identified significant higher BQCV loads in *Vairimorpha*-infected bees than non-infected bees and a weak positive correlation between the loads of BQCV and *Vairimorpha*, confirming their frequently identified co-occurrence. The BQCV loads vary widely in naturally infected honeybees, with an average load of approximately 10^7 per animal (Šimenc et al., 2021). The *Vairimorpha* loads in honeybees can gradually reach over 10^7 post-infection and forager honeybees typically exhibit higher infection level than in-hive members (Forsgren and Fries, 2010, Li et al., 2017). It has been proposed that *Vairimorpha* infection may provide an additional infection route for BQCV by disrupting the integrity of midgut epithelial cells. Al Naggar et al. (2020) compared the BQCV loads in honeybees exposed to virus via feeding or

injection and found that oral acquisition of BQCV was less efficient than injection (Al Naggar and Paxton, 2020). Honeybees fed with 10^8 BQCV acquired fewer than 10^4 viral particles, while injection with the same viral dose resulted in over 10^9 viral loads. In our study, the two hives with the lowest BQCV loads ($< 10^4$) were free of *Vairimorpha* infection. Combined with the observed positive correlation between BQCV and *Vairimorpha*, our findings also support that BQCV infection in honeybees can be amplified in the presence of *Vairimorpha*.

Available studies on the impact of BQCV-*Vairimorpha* coinfection in honeybees are limited. Doublet et al. (2016) conducted a brain transcriptomic study on honeybees experimentally infected with BQCV and *V. ceranae*, reporting the up-regulation of several AMP-related genes. However, the major immune components in honeybees such as fat body and hemolymph were not included. Such laboratory-based experiments may also not reflect field conditions, as honeybees are social insects, and social immunity plays a crucial role in defending colonies against invading pathogens (Cremer et al., 2018). In our study, field honeybee samples were grouped based on pathogen infection status, and immunity gene expression levels were compared between groups. Moreover, a generalized linear model was applied to assess the correlation between pathogen loads and gene expression levels. In both approaches, BQCV and *Vairimorpha* infection showed no significant influence on the expression levels of the selected immunity genes. López-Uribe et al. (2017) found that the expression of *defensin-1* and *glucose oxidase* were not induced by *P. larvae* infection, and suggested that these two genes might be constitutively expressed. *Vairimorpha* alone has been shown to have no effect on the expression of *glucose oxidase* (Alaux et al., 2010), and our results further support the stability of glucose oxidase levels in response to pathogen infections. However, the expression of *defensin-1*, *lysozyme-like* and *vitellogenin* has been reported to change following *Vairimorpha* and viral infections (Antúnez et al., 2013, Doublet et al., 2017, Lourenço et al., 2021, Kunat-Budzyńska et al., 2025). The differences between our study and previous studies suggest either differential honeybee immune responses to various pathogens and pathogen combinations or the presence of missing regulatory layers that influence pathogen-induced gene expression dynamics.

Regarding microbiome analysis, we found no significant differences in the honeybee microbiome between BQCV-*Vairimorpha* co-infected colonies and BQCV-infected colonies. The results are consistent with previous studies which showed that although *Vairimorpha* could alter the abundance of core honeybee gut bacteria under laboratory conditions, this influence was not observed under field conditions (Hubert et al., 2017, Huang et al., 2018). Honeybees fed with sugar water under laboratory conditions are physiologically distinct from field

honeybees with a polyfloral food source, exhibiting reduced bacterial diversity and levels of beneficial microbes, decreased expression of development-related genes, and increased vulnerability to pathogen infections (Powell et al., 2023). Therefore, field honeybees might establish more stable microbiome which is resilient to further changes. Unfortunately, we did not have colonies with no BQCV infection, thus its influence on honeybee microbiome remains to be studied.

In summary, we reported that BQCV-*Vairimorpha* co-infection has no impact on major honeybee immunity genes of forager honeybees under field conditions. The silent immune response of honeybees to this co-infection complex contrasts with their active response to other pathogens, highlighting the need for further investigations to understand the differential immune responses of honeybee to various pathogens. Due to lack of similar studies on this co-infection complex and diverse field conditions, the results should be carefully evaluated in the future.

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Table 1. The loads of BQCV and two *Vairimorpha* pathogens in honeybees.

Hive	**BQCV**		***V. apis***		***V. ceranae***	
	Infection ratio	Average load per bee	Infection ratio	Average load per bee	Infection ratio	Average load per bee
B7-2	8/8	5.1×10^{3}	0/8	0	0/8	0
B7-3	8/8	2.6×10^{5}	3/8	6.3×10^{7}	3/8	5.3×10^{7}
B7-4	7/8	1.2×10^{4}	4/8	4.2×10^{7}	6/8	4.3×10^{7}
B9-2	6/8	5.2×10^{6}	1/8	1.4×10^{7}	4/8	2.0×10^{7}
B9-3	5/8	2.1×10^{5}	2/8	1.1×10^{8}	2/8	1.4×10^{7}
B9-4	6/8	4.8×10^{3}	0/8	0	0/8	0

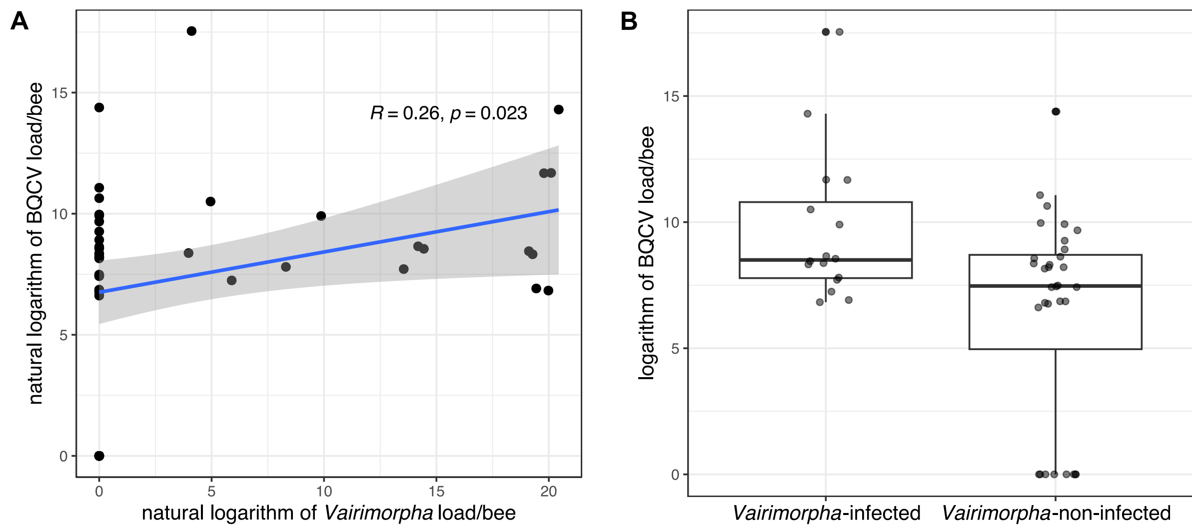

Figure 1. Correlation between BQCV and *Vairimorpha* in honeybees. (A) Kendall correlation between BQCV and *Vairimorpha* loads. (B) BQCV loads in *Vairimorpha*-infected and non-infected honeybees. The pathogen loads were transformed using natural logarithm.

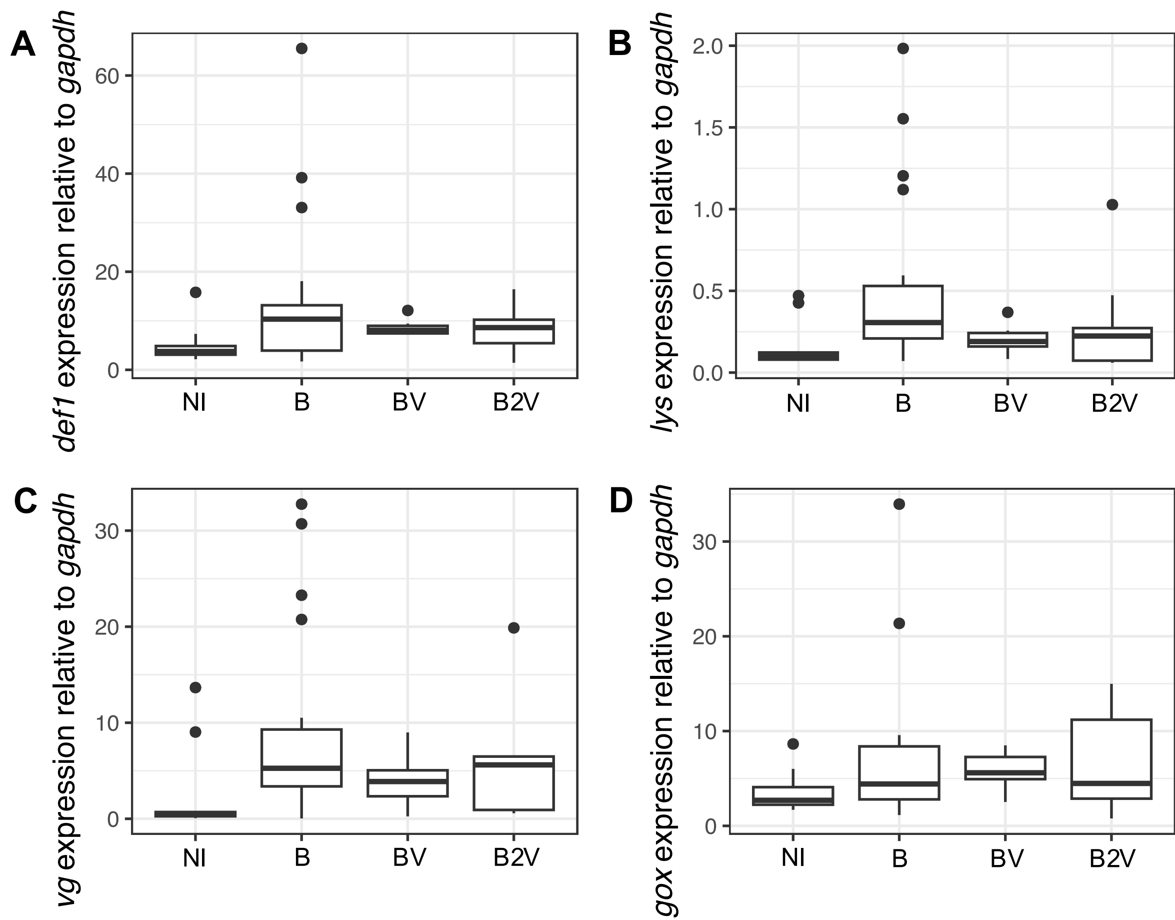

Figure 2. Relative expression levels of immunity genes in honeybees with different infection status. The examined immunity genes include (A) *defensin-1*, (B) *lysozyme-like*, (C) *vitellogenin*, (D) *glucose oxidase*. NI: non-infected; B: BQCV; BV: BQCV and one *Vairimorpha* pathogen; B2V: BQCV and two Vairimorpha pathogens.

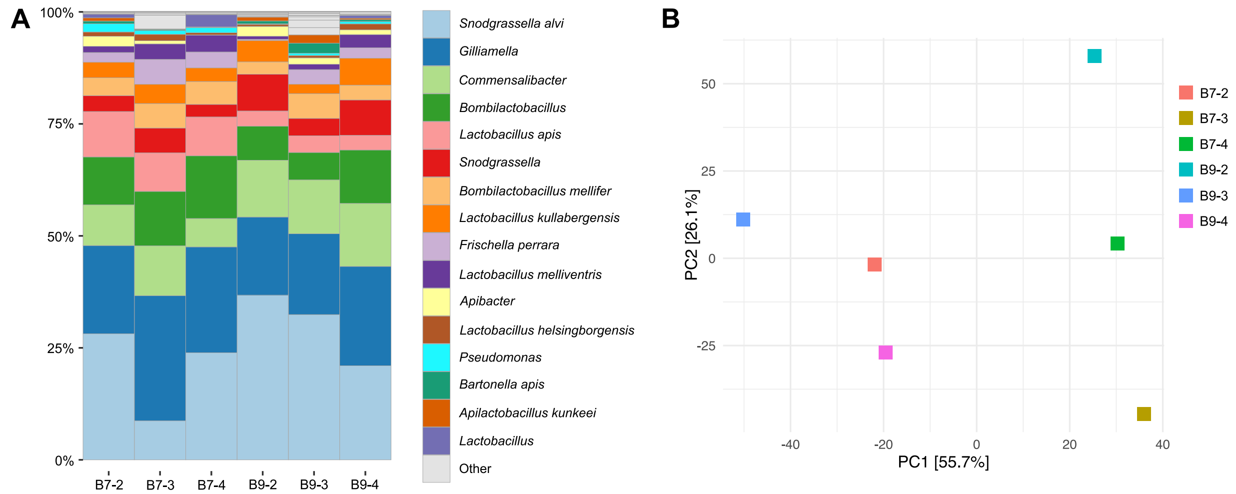

Figure 3. Honeybee microbiome composition and diversity. (A) Bacterial composition of honeybees from different hives based on ASVs collapsed to species level. Unclassified species were replaced with genera. Taxa with relative abundance lower than 0.5% were grouped into "Other". (B) Bacterial beta diversity analysis using PCA ordination of Aitchison dissimilarity matrix. Each dot represents a honeybee hive, pooled from eight honeybee samples.

Chapter 5 *Robbsia betulipollinis* sp. nov., isolated from pollen of birch (*Betula pendula*)

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Robbsia betulipollinis sp. nov., Isolated from Pollen of Birch (*Betula pendula*)

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Abstract
One gram-negative strain designated Bb-Pol-6 ^{T} was isolated from birch (*Betula pendula*) pollen at Giessen area, Germany. The analysis of 16S rRNA gene-based phylogenies indicated the next-relative genera were *Robbsia*, *Chitinasiproducens*, *Pararobbsia* and *Paraburkholderia* (96–95.6%). Further comparative genome analysis and phylogenetic tree-based methods revealed its phylogenetic position under the genus *Robbsia*. The genome of strain Bb-Pol-6 ^{T} was 5.04 Mbp with 4401 predicted coding sequences and a G + C content of 65.31 mol%. Average amino acid identity, average nucleotide identity, digital DNA–DNA hybridization and percentage of conserved proteins values to *Robbsia andropogonis* DSM 9511 ^{T} were 68.0, 72.5, 22.7 and 65.85%, respectively. Strain Bb-Pol-6 ^{T} was rod-shaped, non-motile, facultative anaerobic and grew optimally at 28 °C and pH 6–7. Ubiquinone 8 was the major respiratory quinone and the major cellular fatty acids were $C_{16:0}$, $C_{19:0\ cyclo}$ $\omega 7c$, $C_{17:0\ cyclo}$ $\omega 7c$ and $C_{17:1}$ $\omega 6c$. The dominant polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and an unidentified aminophospholipid. Based on the genomic physiological and phenotypic characteristics, strain Bb-Pol-6 ^{T} was considered a novel species under the genus *Robbsia*, for which the name *Robbsia betulipollinis* sp. nov. was proposed. The type strain is Bb-Pol-6 ^{T} (= LMG 32774 ^{T} = DSM 114812 ^{T}).

Introduction

The monotypic genus *Robbsia* belongs to the class *Betaproteobacteria* [1] and the family *Burkholderiaceae* [2], which harbors highly diverse environmental bacteria including saprophytic bacteria, and a variety of pathogens that infect plants, animals and humans [2]. The phylogenetically separated species and single species in the genus, *Robbsia andropogonis*, was isolated from sorghum with strip disease and first described by Smith as *Bacterium andropogoni* [3]. The DNA G + C content ranges from 59 to 61.3 mol% [4], and is 58.92 mol% for the type strain DSM9511^{T} [5]. After initial description, *R. andropogonis* has been reclassified several times into the genus *Pseudomonas* [6], *Burkholderia* [4], *Paraburkholderia* [7], and eventually *Robbsia* proposed by Lopes-Santos et al. [8]. It is a globally distributed phytopathogen that infects a wide range of hosts including some agriculturally important crops such as maize, sorghum, common bean and tomato [9–12]. Infected leaves can develop water-soaked lesions of pale yellow or greenish white that can enlarge and collapse to involve a larger area [10]. Distinct from most phytopathogens, *R. andropogonis* strains possess a single polar sheathed flagellum [13] and are capable to produce rhizobitoxine [14]. In this study, we isolated one bacterial strain from birch (*Betula pendula*) pollen designated as Bb-Pol-6 ^{T}. Based on genomic and phenotypic characterization, the strain is proposed to represent a novel species within the genus *Robbsia.*

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Methods and Materials

Strain Isolation

The pollen sample from a birch was collected from the Giessen area, Hesse state, Germany (50° 34′ 10.755″ N 8° 40′ 17.859″ E) in February 2016. The details of sample collection and strain isolation procedure were carried out according to Ambika Manirajan et al. [15]. In brief, pollen grains were collected from dissected anthers and stored

in sterile tubes. The pollen sample was then suspended in 0.05% (v/v) Tween 80 and 0.18% (w/v) $Na_4P_2O_7$, and serially diluted with 0.02% (v/v) Tween 80 and 0.085% (w/v) NaCl to 10^{-5}. Dilution was plated on 1:10 AC agar medium (Sigma Aldrich), and aerobically incubated at 25 °C for one week. Strain Bb-Pol-6 T was isolated by subculturing in the same medium and the purity was confirmed under light microscope. The pure culture was stored in 20% glycerol at −80 °C.

16S rRNA Phylogeny

Genomic DNA of strain Bb-Pol-6 T was extracted from a late-log phase axenic culture according to Pitcher et al. [16]. The 16S rRNA gene was amplified by PCR using the primers 9bfm from Mühling et al. [17] and 1512uR from Weisburg et al. [18], and sequenced in both directions by LGC genomics. A consensus sequence of 1408 bp was constructed with MEGA X [19]. The retrieved 16S rRNA gene was proved to be non-chimeric using software DECIPHER 2.20.0 [20]. Next relative species and conspecific species to Bb-Pol-6 T were identified by searching against the quality-controlled 16S rRNA sequence database in EzBioCloud [21] and Genbank (NCBI).

The analysis of 16S rRNA-based phylogenies was performed in ARB (version 7.0) [22] and the sequence of strain Bb-Pol-6 T was aligned with related taxa and merged with the pre-aligned 16S rRNA gene database LPT_12_2021 (February 2021) [23]. Phylogenetic trees based on 16S rRNA gene sequences were reconstructed using the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods integrated in the ARB program with 1000 replicates. The filters termini and Gap95_q0_to_q5 were applied in all tree calculations and the neighbor-joining tree was calculated with an additional Jukes-Cantor correction model and a termini filter between positions 101 and 1229 (*Escherichia coli* numbering) [24]. Maximum-parsimony and maximum-likelihood trees were calculated using the algorithms Phylip DNAPARS and RAxML 8, respectively.

Genome Sequencing and Analysis

Extracted genomic DNA was further sequenced with the Illumina Miseq V3 (2 × 300 bp) service from LGC Genomics. The draft genome assembly was performed using SPAdes 3.15.4 [25] and open reading frame determination and gene annotation were performed using the GenDB platform [26]. Signal peptides were predicted using SignalP 6.0 [27] and CheckM was used to estimate the genome completeness and contamination [28]. The copy number of 16S rRNA gene was estimated using acn.sh tool [29]. The digital DNA-DNA hybridization between strain Bb-Pol-6 T genome and the genome of the reference strain (GCA_902833845.1) was performed using formula 2 of the Genome-to-Genome Distance Calculator 3.0 with the recommended BLAST + alignment tool (https://ggdc.dsmz.de/ggdc.php) [30, 31]. Comparative genome analysis including percentage of conserved proteins (POCP), average amino acid identity (AAI), average nucleotide identity (ANI) and the calculation of genomic subsets was performed at the online platform EDGAR [32] with default settings. Secondary metabolite biosynthesis gene clusters were identified using antiSMASH 6.1.1 with strict detection strictness [33]. Resistome was predicted by CARD with default settings [34]. Plant interaction factors were detected using PIFAR-Pred on PLaBAse platform [35].

Morphological, Physiological and Biochemical Analyses

Physiological characteristics of strain Bb-Pol-6 T were determined together with the reference strain *R. andropogonis* DSM 9511 T. The Gram-staining was conducted as described in Gerhardt et al. [36]. Morphology of cells at late-log phase was assessed using Leica DM1000 light microscope under × 1000 magnification. Presence of flagellum was examined according to Heimbrook et al. [37] while cell motility was examined using the hanging-drop method. Catalase and oxidase activities were assessed by bubble production in 3% (v/v) H_2O_2 and oxidase test strips (Roth), respectively. Bacterial growth was tested on different media including R2A agar (Roth), Luria–Bertani agar (LB; Roth), trypticase soy agar (TSA; BD), nutrient agar (NA; BD), MacConkey agar (Merck) and AC 1:10 agar (Sigma). The optimal growth conditions were assessed by incubation in AC 1:10 broth at a range of temperatures (4, 15, 20, 28, 33, 37, 45 °C), pH (2.0–11.0, at intervals of 0.5) and salinity (0–5.0% w/v NaCl, at intervals of 0.5%). The broth pH-value was adjusted by 0.2 M Na_2HPO_4/0.4 M citrate for pH 2.0–8.0, and 0.2 M glycine/0.4 M NaOH for pH 8.5–11.0 with slight modification as described by Lin et al. [38]. Before the growth success was controlled, strain Bb-Pol-6 T and *R. andropogonis* DSM 9511 T were grown for five and three days respectively, except for inoculants at 4 °C, which were grown for two weeks. Strain Bb-Pol-6 T was incubated on AC 1:10 agar medium anaerobically using Anaerocult A system (Merck) to examine its oxygen requirement. Hypersensitive response was examined on tobacco (*Nicotiana tabacum* cv. Xanthi) and birch (*Betula pendula*) according to Klement and Goodman [39]. Rhizobitoxine production bioassay was performed based on Ruan and Peters [40]. Biochemical characteristics including enzyme activities and assimilation of carbon sources were determined with API ZYM, API 20 NE and API 50 CH strips (bioMérieux) according to the manufacturer's instructions. As strain Bb-Pol-6 T is a slow-grower, the incubation time of assimilation tests on API 20 NE and API 50 CH strips for strain

Bb-Pol-6 T was extended to five days for a clear result. The assimilation of acetate, succinate, malonate and propionate that are not involved in API system was also tested on API AUX medium.

Chemotaxonomic Characteristics

Cellular fatty acid, respiratory quinones and polar lipid composition of strain Bb-Pol-6 T and the reference strain were analyzed using corresponding DSMZ services. Late-log phase bacterial culture in AC 1:10 medium was harvested and freeze dried. Cellular fatty acids were converted into fatty acid methyl esters (FAMEs) using a modified method of Miller [41] and Kuykendall et al. [42], separated by gas chromatography and detected using Sherlock Microbial Identification System (MIDI, Microbial ID). Summed features are resolved and identities of fatty acids are confirmed by a GC–MS-based analysis. Respiratory quinones were extracted using hexane [43, 44], purified by a silica-based solid phase extraction, and analyzed by HPLC. Polar lipids were extracted based on Bligh and Dyer [45], separated by two-dimensional silica gel thin layer chromatography, and detected according to Tindall et al. [46].

Repositories

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Bb-Pol-6 T is KX450422. The whole-genome shotgun BioProject number is PRJNA875284 with the GenBank assembly accession number JAPMXC000000000. The strain has been deposited in the DSMZ under the number DSM 114812, and the BCCM/LMG under the number LMG 32774.

Results and Discussion

Isolation

From the same birch pollen sample where Bb-Pol-6 T was isolated, one gram of sample was found to contain $4.1 \pm 3.1 \times 10^5$ colony forming units (CFU) [15]. Culture-dependent method revealed 15 bacterial species from four phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. Culture-independent method revealed 441 operational taxonomic units (OTU), and the most dominant phyla were *Proteobacteria*, *Actinobacteria* and *Acidobacteria* [15]. Compared to insect-pollinated pollen species (autumn crocus and rape), birch pollen possesses significantly higher species richness and different communities [15]. Furthermore, it was shown that bacteria and pollen from wind pollinated plants like birch could have a higher allergic potential [47]. This possible higher allergic potential is caused by a high lipopolysaccharide concentration (LPS) concentration of the bacteria on the pollen. Interestingly, strain Bol-Pol-6 T has one of the highest measured LPS concentrations in this study with an LPS concentration of 76.6 ng l^{-1} [47] and five next relative uncultured bacteria (pairwise similarity values 98.72–99.70%) found in floor dust (FM872738) [48] and on human skin (HM270658, HM270467, JF167742, JF152894) [49] are also associated to allergic reaction in these studies.

16S rRNA Phylogeny

According to the highest pairwise gene similarity results in EzBioCloud (Ez) and ARB (Table S1), *R. andropogonis* DSM 9511 T (ARB 95.9, Ez 96.23), *Chitinasiproducens palmae* JS23T (ARB 96, Ez 95.94), *Pararobbsia silviterrae* DHC34T (ARB 95.7, Ez 95.58) *Paraburkholderia elongata* 5NT (ARB 95.6, Ez 95.51) were identified as the next-relative species with valid names to strain Bb-Pol-6 T. As the similarity is less than 98.7%, strain Bb-Pol-6 T can be considered a potential novel species [50]. Similarities with five conspecific species range from 98.72% to 99.70%. The ML tree (Fig. S1) showed that strain Bb-Pol-6 T and its conspecific species were clustered with *R. andropogonis* DSM 9511 T, forming a stable monophyletic group, which was consistently revealed in the NJ and MP trees (Figs. S2 and S3). This indicates the potential phylogenetic position of strain Bb-Pol-6 T under the genus *Robbsia*.

Genome Sequencing and Analysis

The draft genome assembly of strain Bb-Pol-6 T consisted of 46 contigs with a total size of 5.04 Mbp and the longest contig was 2.68 Mbp which was the N50 length. This genome contained 4401 predicted coding sequences among which 509 sequences were predicted to encode signal peptides, 57 tRNA genes and three rRNA genes, and had a G + C content of 65.31 mol%. Completeness and contamination of the genome was 98.44 and 0.05%, respectively. Although four copies of 16S rRNA gene were predicted only one 16S rRNA gene sequence from the draft genome was partially recovered with a length of 470 bp. *R. andropogonis* DSM 9511 T has the closest AAI and ANI values to strain Bb-Pol-6 T (72.42% and 72.53%) compared to the other next-relatives (Fig. S4). The dDDH and POCP values of strain Bb-Pol-6 T to *R. andropogonis* DSM 9511 T were 22.7 and 65.85%, respectively. These values were below the generally accepted species boundary values (AAI and ANI: 95%; dDDH: 70%) [51, 52], and above the proposed genus boundary value (POCP: 50%) [53], thus rendering strain Bb-Pol-6 T a promising novel species. The ML tree calculated from the genome of strain Bb-Pol-6 T and 19 closest-related species using 1307 core genes again revealed the monophyletic group formed by strain Bb-Pol-6 T and *R. andropogonis*

DSM 9511 ^{T}, confirming its phylogenetic position within the genus *Robbsia* (Fig. 1). Comparative genome analysis of strain Bb-Pol-6 ^{T} and *R. andropogonis* DSM 9511 ^{T} showed 7221 pan genes, 2720 core genes and 1186 strain Bb-Pol-6 ^{T} singleton genes. Identified secondary metabolite gene clusters by antiSMASH included non-ribosomal peptide synthetase (NRPS), redox-cofactor, phosphonate and terpene (Fig. S5). Two antibiotic resistance ontology terms with antibiotic efflux mechanism, qacG and adeF, were predicted using protein homolog model in CARD. The most abundant genes annotated by PIFAR-Pred were toxin-encoding genes (35%) (Fig. S6). Among these toxin-encoding genes, the majority encode syringomycin, a necrosis-inducing phytotoxin [54], and toxoflavin, a virulence factor of phytopathogens such as *Burkholderia glumae* in crop diseases [55, 56], indicating the phytopathogenic potential of strain Bb-Pol-6 ^{T}.

Morphological, Physiological and Biochemical Analyses

Cells of strain Bb-Pol-6 ^{T} were rod-shaped (0.4–0.7 µm in width and 0.9–1.6 µm in length) with no flagellum, Gram-stain-negative, facultative anaerobic, non-spore-forming and non-motile. Colonies of strain Bb-Pol-6 ^{T} were round, convex, viscous, creamy in color and has entire margin after grown on AC 1:10 agar for five days. The strain grew optimally on AC 1:10 agar and growth was also observed on R2A agar, but not on LB, TSA, NA and MacConkey agar. Strain Bb-Pol-6 ^{T} was catalase-positive and oxidase-negative. The temperature, salinity and pH value ranges for growth were 4–28 °C (optimum at 28 °C), 0–1% NaCl (optimum without NaCl) and 5–7.5 pH (optimum at 6–7). Unlike *R. andropogonis*, strain Bb-Pol-6 ^{T} cannot induce hypersensitive response on *Nicotiana tabacum* cv. Xanthi and *Betula pendula* (Fig. S7) and does not produce rhizobitoxine. The differential physiological and biochemical characteristics of strain Bb-Pol-6 ^{T} and its reference strain were summarized in Table 1.

Chemotaxonomic Characteristics

On AC 1:10 medium, ubiquinone 8 was the major respiratory quinone of both strain Bb-Pol-6 ^{T} (96.5%) and *R. andropogonis* DSM 9511 ^{T} (97.7%). The major cellular fatty acids of both strains were $C_{16:0}$, $C_{19:0\ cyclo}$ $\omega7c$, $C_{17:0\ cyclo}$ $\omega7c$ and

Table 1 Differential characteristics between strain Bb-Pol-6 ^{T} and its reference type strain

Characteristics	1	2
Motility	−	+
Hypersensitive response	−	+
Flagellum	−	+
Rhizobitoxine production	−	+
Growth at:		
Temperature range (°C) (optimum)	4–28 (28)	15–33 (28)
pH range (optimum)	5–7.5 (6–7)	4–10 (7–8)
NaCl range (optimum)	0–1 (0)	0–4 (0–1)
Enzyme activity		
α-Glucosidase	+	−
Alkaline phosphatase	−	+
Assimilation of:		
D-Adonitol, D-lyxose, D-sorbitol, inositol, D-arabinose, malonate, acetate	−	+
DNA G+C content (mol%)	65.31	58.92

Strains: 1, Bb-Pol-6 ^{T}; 2, *Robbsia andropogonis* DSM 9511 ^{T}. All data were obtained from this study (+, positive; −, negative), except the DNA G+C content of *R. andropogonis* DSM 9511 ^{T} [5]. All strains were Gram-stain-negative, oxidase-negative and catalase positive

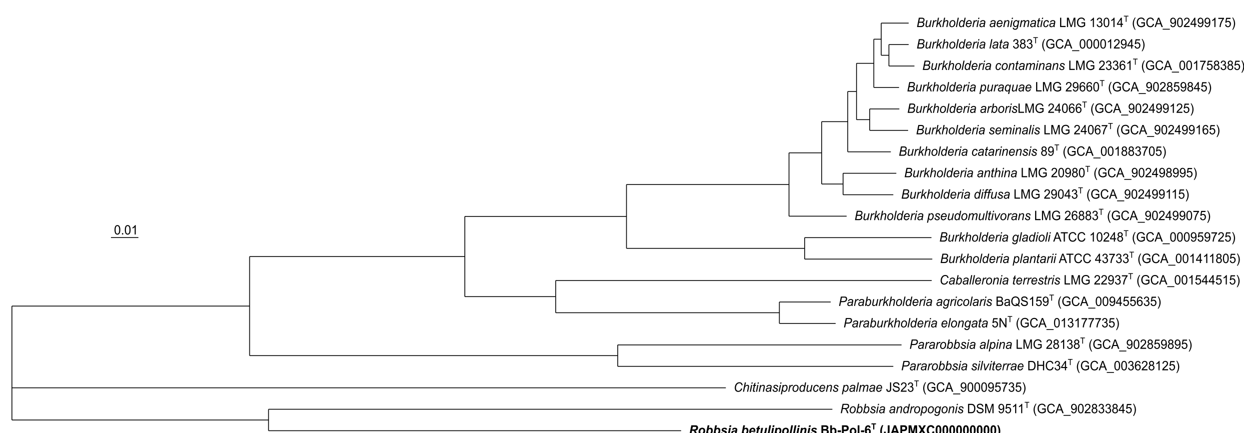

Fig. 1 Maximum-likelihood tree built out of a core of 1307 genes of the genomes showing the phylogenetic position of strain Bb-Pol-6 ^{T} in relation to 19 most closely related species. Bar, 0.01 substitutions per nucleotide position

$C_{17:1}$ $\omega 6c$ (Table S2). Compared to *R. andropogonis* DSM 9511 ^{T}, strain Bb-Pol-6 ^{T} contained a higher amount of $C_{12:0}$ (2.8%), $C_{18:1}$ $\omega 5c$ (2.9%) and $C_{16:1}$ $\omega 7c$ (8.8%), and a lower amount of $C_{14:0}$ (0.4%), $C_{17:0}$ (0.7%) and $C_{18:1}$ $\omega 7c$ (0.3%). Strain Bb-Pol-6 ^{T} contained trace amount of $C_{10:0}$, $C_{12:0}$ 3-OH and $C_{18:1}$ $\omega 7c$ 11-methyl, which were not detected in *R. andropogonis* DSM 9511 ^{T}, while $C_{15:0}$ 3-OH detected in *R. andropogonis* DSM 9511 ^{T} was not present in strain Bb-Pol-6 ^{T}. The major polar lipids in strain Bb-Pol-6 ^{T} were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, one unidentified aminolipid, one unidentified aminophospholipid, two unidentified phospholipids and six unidentified lipids (Fig. S8). Glycolipid was identified in *R. andropogonis* DSM 9511 ^{T} but not in strain Bb-Pol-6 ^{T}.

Conclusions

In summary, the phylogenetic analysis revealed that strain Bb-Pol-6 ^{T} belongs to the genus *Robbsia*. Further phenotypic and biochemical characterization distinguished strain Bb-Pol-6 ^{T} from its next-relative reference strain *R. andropogonis* DSM 9511 ^{T} suggesting it represents a novel species, for which the name *Robbsia betulipollinis* sp. nov. is proposed.

Description of *Robbsia betulipollinis* sp. nov

Robbsia betulipollinis (be.tu.li.pol′li.nis L. n. *betula* birch; L. n. *pollen* pollen; N.L. gen. n. *betulipollinis* of pollen from birch).

Cells are Gram-negative, facultative anaerobic, non-spore-forming, non-motile, rod-shaped (0.4–0.7 µm wide × 0.9–1.6 µm long) and do not have flagellum. Colonies were round, convex, viscous, creamy in color and has entire margin after five days of incubation on AC 1:10 agar. Grows between 4 and 28 °C (optimum at 28 °C), 0–1% NaCl (optimum without NaCl) and 5–7.5 pH (optimum at 6–7). Positive for catalase, esterase (C4), esterase (C8), acid phosphatase, β-galactosidase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α-Glucosidase, D-galactose, D-mannitol, D-mannose, D-ribose, L-arabinose, D-arabitol, D-glucose, D-fructose and succinate. Negative for oxidase, nitrate reduction, glucose fermentation, arginine dihydrolase, gelatin hydrolysis, esculin hydrolysis, indole production, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, N-acetyl-β-glucosaminidase, lipase (C 14), β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, urease, alkaline phosphatase, adipic acid, amygdalin, arbutin, capric acid, cellobiose, D-fucose, D-gentiobiose, lactose, maltose, melezitose, melibiose, raffinose, sucrose, D-tagatose, turanose, dulcitol, D-xylose, glycerol, inulin, L-arabitol, L-fucose, L-rhamnose, L-Xylose, malic acid, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, N-acetyl-D-glucosamine, phenylacetic acid, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, salicin, starch, xylitol, esculin ferric citrate, trehalose, glycogen, erythritol, methyl-ß-D-xylopyranoside, L-sorbose, trisodium citrate, propionate, acetate, D-adonitol, D-lyxose, D-sorbitol, inositol, D-arabinose, malonate, hypersensitive response and rhizobitoxine production. The major respiratory quinone is ubiquinone 8. The major cellular fatty acids are $C_{16:0}$, $C_{19:0\ cyclo}$ $\omega 7c$, $C_{17:0\ cyclo}$ $\omega 7c$ and $C_{17:1}$ $\omega 6c$. The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, one unidentified aminolipid, one unidentified aminophospholipid, two unidentified phospholipids and six unidentified lipids.

The type strain, Bb-Pol-6 ^{T} (= LMG 32774 ^{T} = DSM 114812 ^{T}), was isolated from pollen of birch in Giessen, Hesse, Germany (50° 34′ 10.755″ N 8° 40′ 17.859″ E). The genome of the type strain has a size of 5.04 Mbp with a G + C content of 65.31 mol%.

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Author Contributions HS designed the study, performed the phenotypic and biochemical experiments, analyzed the data, and wrote the original draft; BAM isolated the bacterium; RGP helped with the phenotypic and biochemical experiments; SR helped with the analysis of taxonomic data, designed and supervised the study, and edited the original draft; SS supervised the study, and edited the original draft.

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Data Availability The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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Chapter 6 General discussion

Plant-pollinator interactions are essential ecological processes that sustain biodiversity, provide ecosystem services and support food webs, while honey bees tend to be one of the most important pollinators across various ecosystems (Calderone, 2012; Hung et al., 2018; Simpson et al., 2022; Stanley et al., 2020). In bee-plant networks, the tight association of microbes with their hosts has been shaped through deep evolutionary time. These associated microbes confer a wide range of beneficial effects on their hosts, such as facilitating food storage and digestion in honey bees, enhancing floral attractiveness to pollinators, and providing protection against pathogens for both bees and plants (Dharampal et al., 2019; Dharampal et al., 2020; Forsgren et al., 2010; Schaeffer & Irwin, 2014; Tejerina et al., 2023; Yang et al., 2019). On the other hand, pathogenic microbes can also utilize bee-plant interactions as routes for their transmission (Figueroa et al., 2019). Thus, elucidating the microbial communities associated with bees and plants can help us decipher numerous key ecological processes within this network, such as microbial transmission dynamics and holobiont functioning, and inform strategies for better managing the health of bees and plants.

Numerous studies have been conducted on plant- or honey bee-associated materials, including flowers, pollen, nectar, bee bread, and hive environment (Aizenberg-Gershtein et al., 2013; Ambika Manirajan et al., 2016; Santorelli et al., 2023; Ushio et al., 2015; Wang et al., 2023). However, several components within this interaction network remain poorly understood, notably the microbiota of freshly collected honey bee corbicular pollen. Fresh corbicular pollen was specifically selected as the subject of this investigation because it uniquely captures both the plant species visited by honey bees and their associated microbial communities. A former study has compared the microbiota of bee bread and corbicular pollen from hives fed with oak or rapeseed pollen patties (Ghosh et al., 2022). The results showed that the microbiome composition of corbicular pollen was significantly different from that of bee bread. In our study, we further demonstrated that the microbiome composition of corbicular pollen underwent seasonal shift and was influenced by multiple environmental factors, particularly dew point and temperature, and identity of forage plants (Chapter 2). It is intuitive to conceptualize floral pollen as a micro-incubator for microbial communities, where the pollen provides a nutrient-rich substrate and environmental factors act as incubation conditions shaping microbial growth and composition.

Consistent with previous studies, many of the core plant taxa identified in corbicular pollen in our study are known to be frequently foraged by honey bees as important food sources, such as *Taraxacum*, *Rubus*, *Hypochaeris*, *Plantago*, *Cichorium* and *Hedera* (Adamchuk et al., 2017;

Mayer & Lunden, 1991; Percival, 1950; Richardson et al., 2021; Wignall et al., 2020). We found that some highly-prevalent microbes in corbicular pollen were commonly associated with honey bees such as *Apilactobacillus kunkeei*, *Bombella*, *Gilliamella*, and *Snodgrassella*. These microbes are likely introduced into corbicular pollen from the honey stomach of honey bees during the foraging activity (Anderson et al., 2013; Corby-Harris et al., 2014). Unlike stored pollen provisions, the microbiome of corbicular pollen was not homogenized through in-hive fermentation. Despite bee-originated microbes, corbicular pollen also harbored plant-specific microbes. The observed plant-microbe co-occurrence patterns suggested that certain plants might serve as natural ecological niches for specific microbes and functioned as microbial transmission hotspots within the bee-plant-microbe network (Chapter 2).

Our analysis of plant and microbial composition in corbicular pollen identified *Rubus* (brambles) as a crucial hub plant for the horizontal transmission of microbes. Across a two-year study period, *Rubus*, a highly nutritious and frequently insect-visited plant genus, was consistently identified as a core plant taxon in June, and was notably associated with the potential bee pathogen *Spiroplasma*. These findings highlighted the value of corbicular pollen as a model for investigating microbial transmission dynamics and offered new insights into the mechanisms underlying pathogen dissemination within ecological networks (Chapter 2). To validate the role of *Rubus* as a microbial transmission hotspot, we further analyzed the bacterial and fungal communities on bramble flowers, and compared flower buds, open flowers, and bagged flowers to assess the effect of flowering and insect visitation on the floral microbiome. We found that flower opening provided opportunities for microbial acquisition, and insect visitation significantly increased microbial loads on flowers. Functional annotation of the microbial taxa identified on bramble flowers further suggested that insect visitation might enrich specific groups of microbes, such as fermentative and potentially pathogenic microbes. Collectively, these findings confirmed that *Rubus* functioned as a critical hub for microbial transmission (Chapter 3). In addition, while previous studies have demonstrated that insect visitations could alter floral microbial communities, such effects have always been characterized as "gain-or-loss" of specific microbes (via differential abundance analysis) or as overall structural shifts (via beta diversity analysis) (Hietaranta et al., 2023; Ushio et al., 2015). However, few studies have explored the underlying mechanisms of these changes by examining how insect visitation reshapes the structure of microbial networks. Co-occurrence networks offer a range of topological metrics that can elucidate detailed interaction patterns and structural properties of microbiomes, including degree, betweenness centrality, average path length, robustness, centrality, etc. (for review: Kajihara & Hynson, 2024). Our results indicated that, in addition to

increased microbial loads and altered microbiome structure, insect visitation resulted in a more centralized microbial interaction network on flowers, most likely through the introduction of hub microbial taxa (Chapter 3).

Given the complexity of pollinator-microbe-plant network, investigating microbial transmission in natural ecosystems remains a considerable challenge. For example, *Paenibacillus larvae* is one of the most notorious honey bee pathogens, causing the disease American Foulbrood that is detrimental for individual larva and the whole colony (Yue et al., 2008). Existing studies have investigated its transmission within and between colonies through social interactions, such contaminated honey and infected adult bees (Stephan et al., 2020), as well as through robbing (Lindström et al., 2008) and swarming activities (Fries et al., 2006), while it is still unclear whether a floral transmission route exists for *P. larvae*. Many studies on transmission dynamics have been conducted under controlled or managed conditions, have focused on single microbial strains, or have drawn general conclusions without identifying specific plant–microbe associations (Durrer & Schmid-Hempel, 1994; Figueroa et al., 2020; Graystock et al., 2015; Hietaranta et al., 2023; Ogata et al., 2023). Our framework offered a high-throughput approach for systematically identifying plant–microbe associations (Chapter 2-3).

Co-occurrence network analysis based on high-throughput sequencing data has generated a substantial number of hub microbial taxa (as presented in Chapter 2 and 3), suggesting their potential importance within their respective ecological niches. However, the functional roles of many of these microbial hubs remain unclear, particularly in terms of their interactions with other microbes and their contributions to the stability and organization of the overall microbiome structure. A simple and commonly employed approach to answer such questions is the use of co-culture experiments. For example, a previous study has constructed fungi-bacteria co-occurrence networks based on skin microbiome data and validated a negative association between *Emericella nidulans* (also known as *Aspergillus nidulans,* when referring to its asexual form) and *Rothia dentocariosa* through co-culture assays (Tipton et al., 2018). Nonetheless, this approach becomes increasingly limited when multiple strains are involved, as positive and negative interactions may offset one another, thereby obscuring the true nature of higher-level interactions. As microbiota and their associated hosts together constitute a functional entity (also known as holobiont), studying the influence of overall microbiota on the hosts may provide additional information beyond those obtained from studies focusing on individual microbial strains. Previous studies have examined how microbial networks

influenced plant growth promotion, disease suppression, and facilitation (for review: Hassani et al., 2018). In the context of floral microbiomes, future research may focus on their roles in the production of volatile organic compounds, enhancement of blossom visits and fertilization as well as fruit quality, etc.

Along with the corbicular pollen collection in 2023, forager honey bee samples were also collected from the hive entrance. These samples were screened for the presence of common honey bee pathogens using quantitative PCR, including two bacterial pathogens (*Paenibacillus larvae* and *Melissococcus plutonius*), two fungal pathogens (*Vairimorpha apis* and *Vairimorpha ceranae*), and six viral pathogens (Black queen cell virus, Kashmir bee virus, Acute bee paralysis virus, Chronic bee paralysis virus, Deformed wing virus, and Sacbrood bee virus). Only *Vairimorpha* pathogens and BQCV were detected in these samples. *Vairimorpha* and BQCV are frequently identified as a co-infection complex, which is known to cause more severe effects on honey bees than single infections (Bailey et al., 1983; Chagas et al., 2020; Gajda et al., 2021). Our results revealed a positive correlation between BQCV and *Vairimorpha* in honeybees, supporting the commonly observed pattern of co-infection (Chapter 4). Most studies related to BQCV and *Vairimorpha* have examined their influence on honey bees independently, and only a limited number have been conducted under field conditions. In this study, the impact of BQCV-*Vairimorpha* co-infection under natural field settings was assessed by analyzing the expression levels of several immunity-related genes including *defensin-1*, *lysozyme-like*, *vitellogenin*, *glucose oxidase*, and the composition of the honey bee microbiome. Interestingly, no significant effects of the co-infection were observed in honey bees, indicating that field honey bees might establish more stable microbiome and are more resilient to BQCV-*Vairimorpha* co-infection. These findings were contradictory to several previous studies (Antúnez et al., 2013; Lourenço et al., 2021), thus future research will need to elucidate the potentially overlooked regulatory mechanisms that influence pathogen-induced gene expression dynamics. Such knowledge will be helpful to explain the differential immune responses of honey bees to various pathogens or pathogen combinations.

Microbes are ubiquitously present in bee–plant networks, which can therefore be regarded as microbial reservoirs harboring numerous yet unidentified microbial taxa. Ambika Manirajan et al. (2016) has characterized the bacterial composition of pollen from four plants species, rape, rye, birch, and autumn crocus, and the results showed that each plant harbored a distinct microbiome. Principle component analysis of bacterial metabarcoding data revealed significantly different clusters grouped by plant species. Furthermore, bacterial isolation

showed that each plant hosted specific microbial taxa, with only a limited number of species shared across them. Among the isolated bacterial strains, strain Bb-Pol-6 had a 96.23% 16S rRNA gene similarity to its next relative *Robbsia andropogonis*, and a polyphasic approach was performed for phenotypic, genotypic and chemotaxonomic characterization. The results indicated that strain Bb-Pol-6 was rod-shaped, non-motile, facultative anaerobic and grew optimally at 28 °C and pH 6–7. The draft genome of the strain has a size of 5.04 Mbp and a G + C content of 65.31 mol%. Comparative genomic analyses suggested a position of strain Bb-Pol-6 under the genus *Robbsia*, and the name *Robbsia betulipollinis* sp. nov. was proposed. Prior to the identification of *R. betulipollinis*, *R. andropogonis* was the only recognized species under the genus *Robbsia* and was known as a globally distributed plant pathogen. *R. andropogonis* can induce plant hypersensitive response, produce rhizobitoxine, and possesses a single polar sheathed flagellum (Borkar & Yumlembam, 2017; Fuerst & Hayward, 1969; Mitchell, 1994). In contrast, these traits are absent in *R. betulipollinis*, suggesting that the genus *Robbsia* may encompass a greater diversity of species than previously recognized (Chapter 5).

In our study, metabarcoding was the central technique for profiling microbial composition within bee-plant networks. Despite its high-throughput capacity, this approach confronts several limitations and challenges at various stages of analysis. These include the presence of PCR inhibitors from DNA extraction, biases introduced during DNA extraction and PCR amplification, and risks of cross-contamination due to inappropriate amplicon library preparation, etc. (Baer et al., 2024; González et al., 2023; Samarakoon et al., 2013). Among these challenges, two specific ones will be discussed in detail in the context of the current study.

For bacterial and fungal microbiome profiling, the 16S rRNA gene and ITS region are commonly used as molecular barcodes. However, in the context of plant-associated microbiome, primers targeting the 16S rRNA gene can also amplify non-target sequences from plant mitochondria and plastids. Although blocking primers such as peptide nucleic acids have been designed to specifically bind and inhibit the amplification of mitochondrial and plastid DNA (Abdullaeva et al., 2021; Lundberg et al., 2013), their performance is often inconsistent. In our study, for example, chloroplast and mitochondrial sequences still constituted the majority of reads in certain samples, resulting in a low yield of effective microbial sequences (Chapter 2). Similarly, primers designed to amplify fungal ITS2 region can unpredictably co-amplify plant ITS2 sequences. As a result, six bramble flower samples yielded an extremely low number of fungal reads and were removed from the subsequent analyses (Chapter 3). These undesired amplifications can significantly compromise downstream analyses and lead to unnecessary

sample loss, which is an especially critical issue when sample collection is difficult or impossible to replicate. This issue may be more effectively addressed by designing plant-specific blocking primers when investigating microbiomes associated with a particular plant species (Saati-Santamaría et al., 2023). Another challenge of microbial metabarcoding is the taxonomic resolution of sequence assignment. In the present study, metabarcoding was performed using IonTorrent sequencing and the length of barcodes was around 300 bp. Although over 90% of amplicon sequence variants (ASVs) could be classified at the genus level, fewer than 70% could be resolved to the species level. Consequently, most downstream analyses were conducted at the genus level. However, this limitation may obscure ecologically or functionally relevant differences, as members of the same genus such as *Pseudomonas* can exhibit substantial functional diversity. Such taxonomic ambiguity may result in the loss of important biological insights. Future studies are encouraged to use full-length 16S rRNA gene sequencing, as it is considered a fundamental requirement for accurate taxonomic classification when characterizing novel taxa. Recent research has also demonstrated that using the whole 16S rRNA gene as a barcode improves taxonomic resolution in microbiome samples, enabling more reliable species-level identification and reducing the ambiguity associated with short-read amplicons (Buetas et al., 2024).

In summary, this study investigated the microbial community associated with various components of bee-plant networks, including corbicular pollen, bramble flowers, and honey bees. The findings demonstrated that metabarcoding-based analysis of microbial and plant composition in corbicular pollen offered substantial potential for exploring plant–microbe interactions and microbial transmission dynamics across ecosystems. Using bramble flowers as a model system, the study further elucidated how flowering and insect visitation shaped the floral microbiome. Additionally, field-collected honey bees exhibited a resilient microbiome and stable immune gene expression in response to BQCV/*Vairimorpha* co-infections. Complementing these findings, a novel bacterial species, *Robbsia betulipollinis*, was isolated from birch pollen and formally characterized.

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春有百花秋有月，
夏有凉风冬有雪。
若无闲事挂心头，
便是人间好时节。