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

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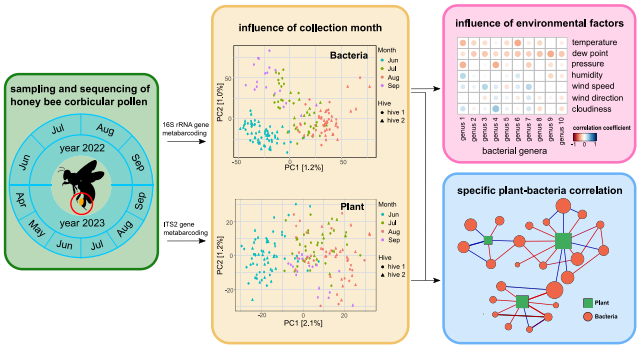
HIGHLIGHTS

- 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 16,200g, 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* ~ *plant diversity* + *(plant diversity|hive)* + *(plant diversity|month)* with the null model: *bacterial diversity* ~ *(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 Table S1.

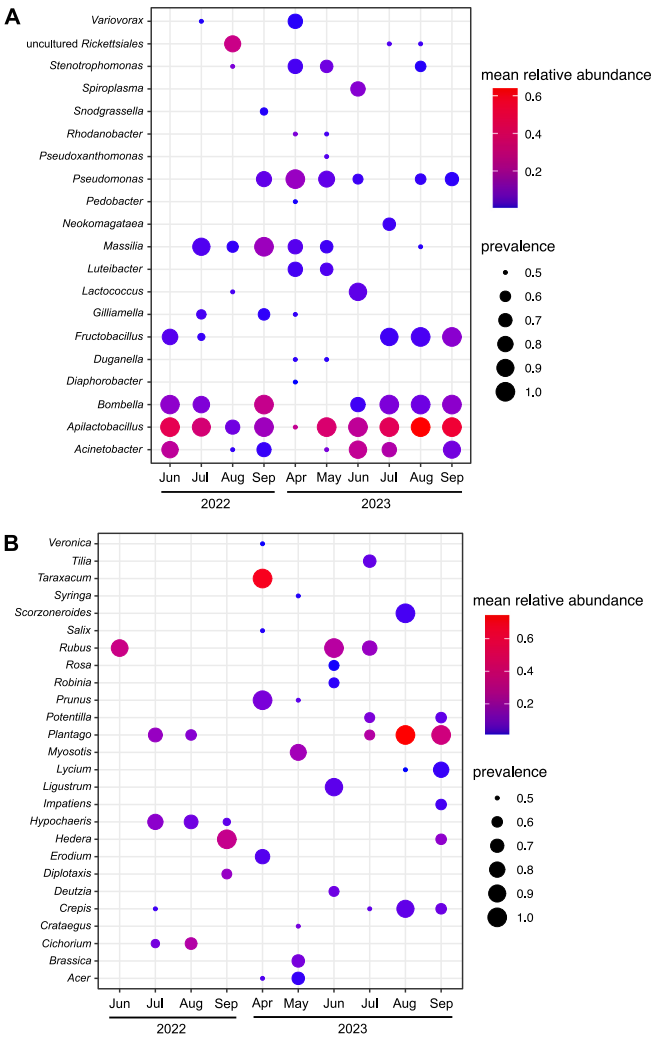

Fig. 1. Core genus analysis in each collection month. (A) Core bacterial genera in corbicular pollen. (B) Core plant genera in corbicular pollen. The detection threshold of core genera is 1 % of sequence number in each sample. The prevalence threshold of core genera is 50 % of sample number in each month.

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

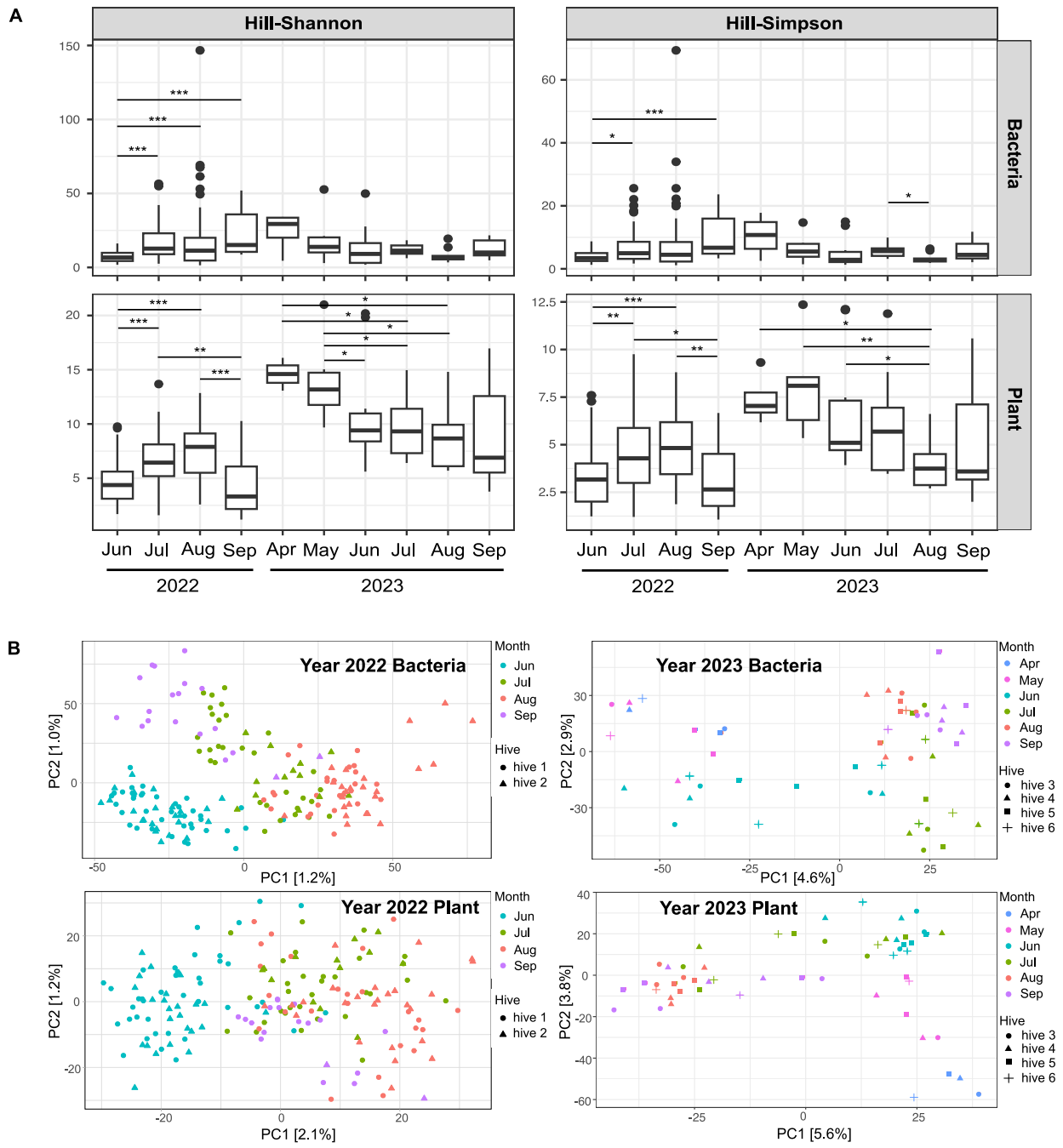

Fig. 2. Alpha diversity and beta diversity. (A) Hill-Shannon and Hill-Simpson indices of bacteria and plant in corbicular pollen in different collection months in 2022 and 2023. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (B) Principal component analysis based on Aitchison dissimilarity distance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

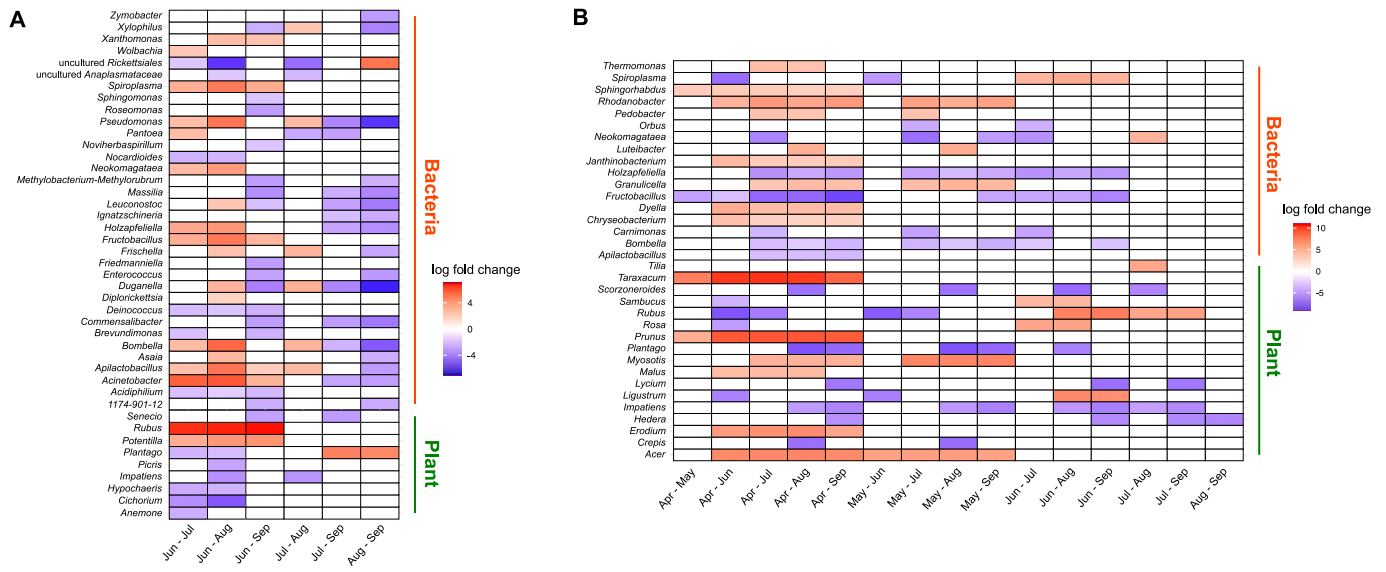

Fig. 3. ANCOMBC results for differential abundances of bacterial and plant ASVs collapsed to genus level of corbicular pollen samples. (A) Differentially abundant bacterial and plant genera in 2022. (B) Differentially abundant bacterial and plant genera in 2023. All differentially abundant genera have *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.

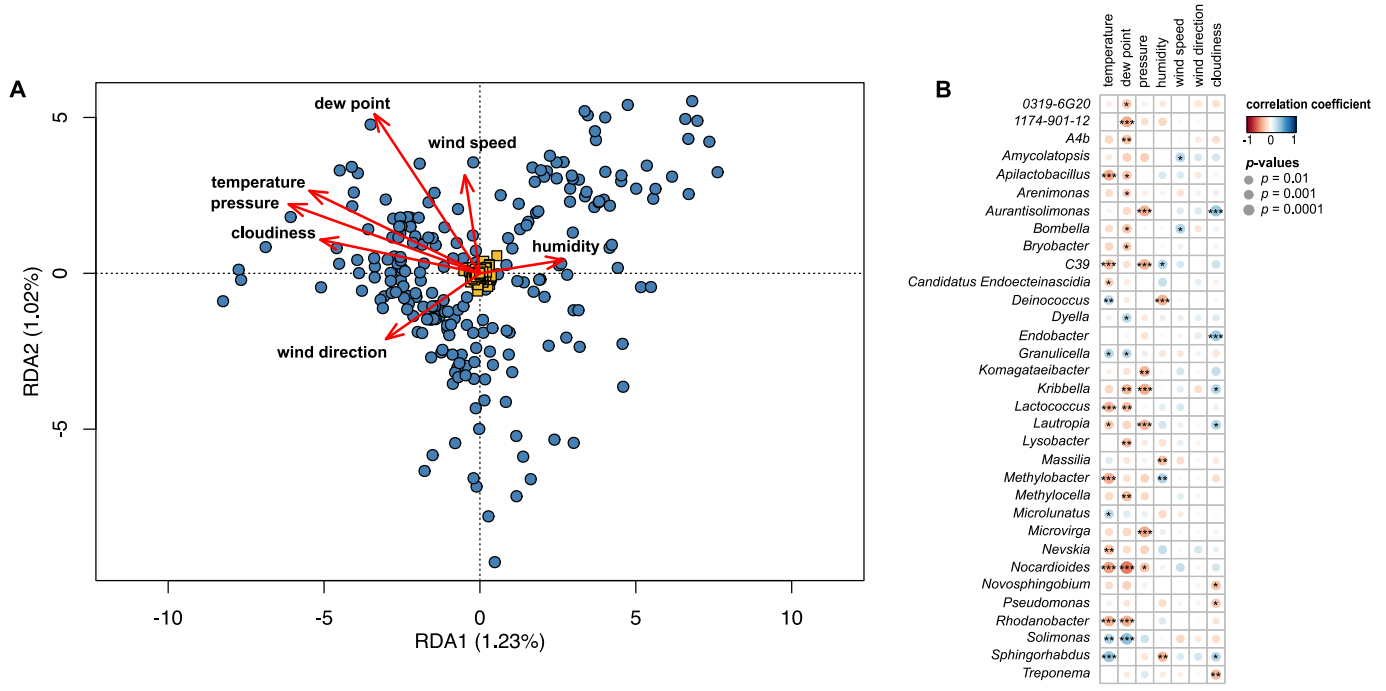

Fig. 4. Correlation of bacterial community of corbicular pollen samples with environmental factors. (A) Redundancy analysis of environmental factors on bacterial community structure based on clr-transformed bacterial dataset. Yellow dots indicate bacterial genera and blue dots indicate corbicular pollen 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

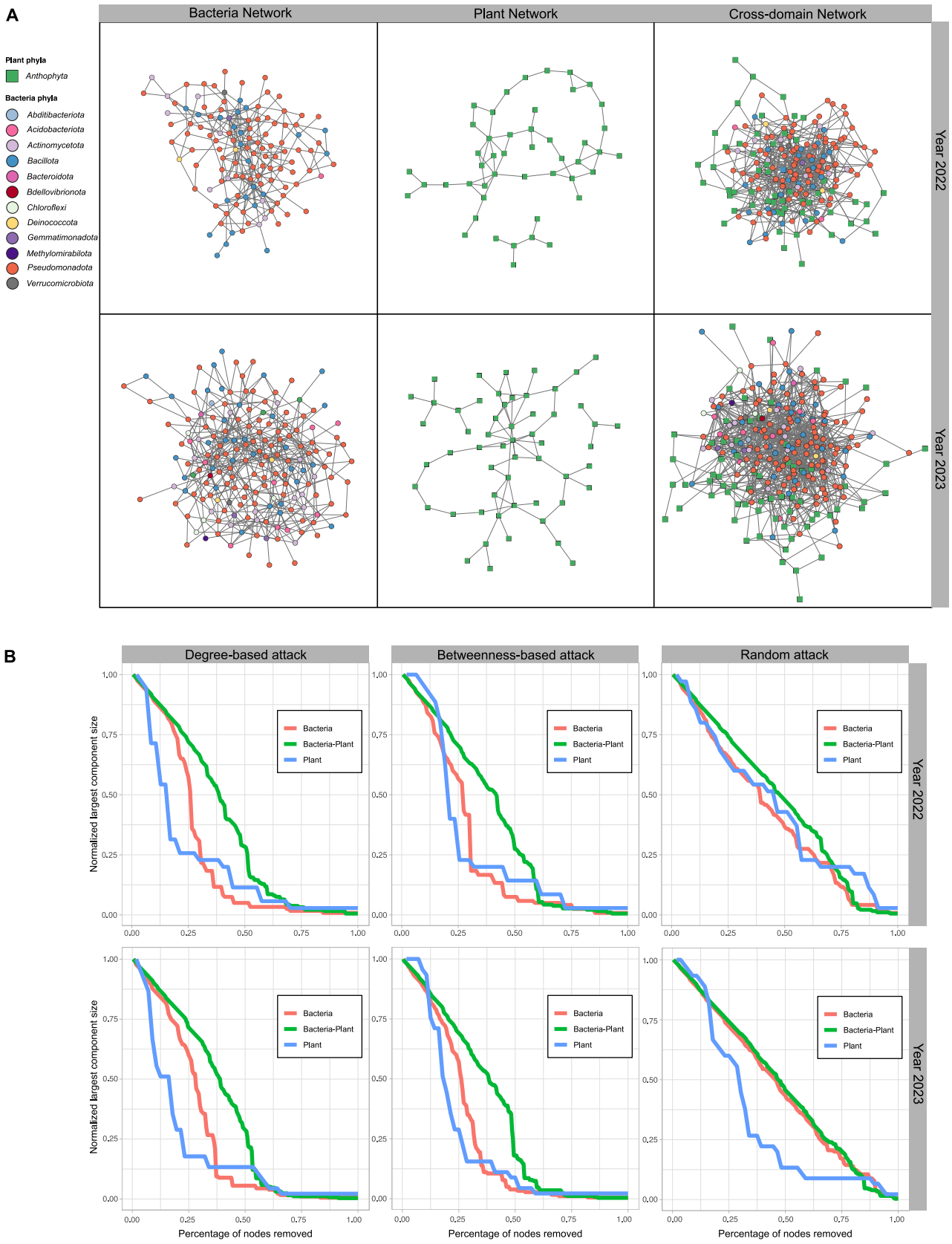

Fig. 5. Co-occurrence network analysis. (A) Network of bacteria and plant ASVs collapsed to genus level in corbicular pollen samples. Single domain and cross-domain network of bacteria and plant are shown. (B) Attack robustness curves for all networks under the situation of degree-based attack, betweenness-based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

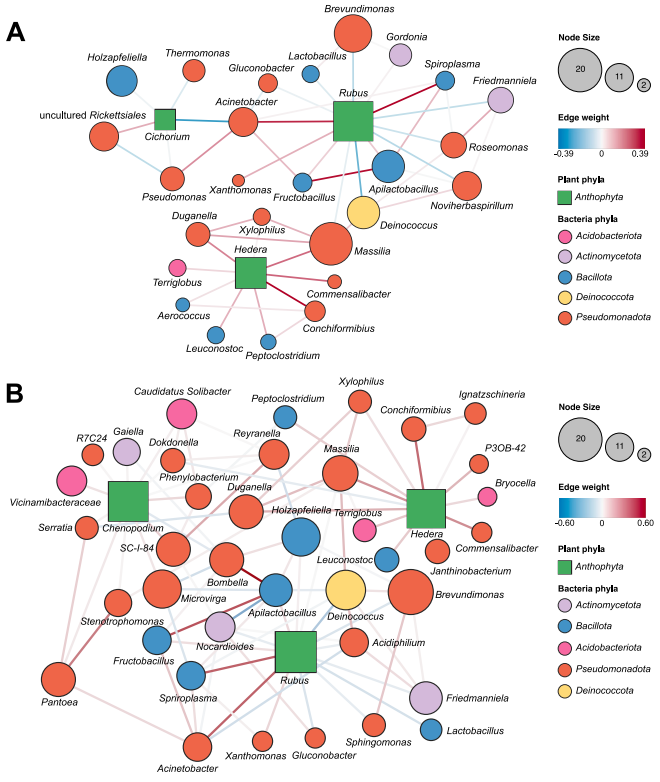

Fig. 6. Co-occurrence subnetwork of plant-bacteria cross-domain network in corbicular pollen samples with ASVs collapsed to genus level. (A) Subnetwork in 2022 with three plant genera with the highest node degrees and their nearest bacterial neighbors. (B) Subnetwork in 2023 with three plant genera with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