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Pollinators mediate floral microbial diversity and microbial network under agrochemical disturbance

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Abstract

How pollinators mediate microbiome assembly in the anthosphere is a major unresolved question of theoretical and applied importance in the face of anthropogenic disturbance. We addressed this question by linking visitation of diverse pollinator functional groups (bees, wasps, flies, butterflies, beetles, true bugs and other taxa) to the key properties of the floral microbiome (microbial α- and β-diversity and microbial network) under agrochemical disturbance, using a field experiment of bactericide and fungicide treatments on cultivated strawberries that differ in flower abundance. Structural equation modelling was used to link agrochemical disturbance and flower abundance to pollinator visitation to floral microbiome properties. Our results revealed that (i) pollinator visitation influenced the α- and β-diversity and network centrality of the floral microbiome, with different pollinator functional groups affecting different microbiome properties; (ii) flower abundance influenced the floral microbiome both directly by governing the source pool of microbes and indirectly by enhancing pollinator visitation; and (iii) agrochemical disturbance affected the floral microbiome primarily directly by fungicide, and less so indirectly via pollinator visitation. These findings improve the mechanistic understanding of floral microbiome assembly, and may be generalizable to many other plants that are visited by diverse insect pollinators in natural and managed ecosystems.

KEYWORDS
bacterial and fungal communities, bactericide and fungicide, floral microbiome, microbial network, pollinator visitation, strawberry

1 | INTRODUCTION

Microbiomes can profoundly influence plant fitness in natural and agricultural settings (Berendsen et al., 2012; Bulgarelli et al., 2013; Vorholt, 2012). Relative to the rhizosphere (root) and phyllosphere (leaf), the anthosphere (floral) microbiome is least well studied but has the most direct impact on plant reproductive success (Aleklett et al., 2014; Rebolleda-Gómez et al., 2019; Wei & Ashman, 2018). Our understanding of what governs microbiome assembly in flowers—a highly dynamic niche—has advanced only recently (Aleklett et al., 2014; Vannette, 2020). The identified drivers include, for instance, microbial dispersal mediated by pollinators (Morris et al., 2020; Rebolleda-Gómez & Ashman, 2019; Vannette & Fukami, 2017), floral traits that can influence niche availability (e.g., flower size, nectar volume) (Vannette et al., 2020) and microbial source pool (e.g., flower abundance; this study), and disturbance imposed by bactericides and fungicides especially in crop plants (Bartlewicz et al., 2016; Schaeffer et al., 2017). Yet, as these different drivers have often been studied independently, we lack a clear view as to how they act together and thus their relative importance in shaping the floral microbiome.

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Pollinator-mediated microbial dispersal is an important determinant of the floral microbiome (Rebolleda-Gómez & Ashman, 2019; Vannette, 2020; Vannette & Fukami, 2017). Pollinators facilitate microbial colonization and transport (Herrera et al., 2013; Russell et al., 2019; Vannette et al., 2013), but their effect on the floral microbiome may differ among pollinator taxa (or flower visitors more generally). This is because pollinators may show different uses of flowers and preferences for floral resources (Armbruster, 2017; Kantsa et al., 2018; Wei et al., 2020) and microbes (e.g., bacteria vs. yeasts) (Good et al., 2014; Schaeffer et al., 2019). In addition, pollinators may differentially influence the floral microbiome due to different likelihoods of dispersing microbes based on the level of interaction with flowers or flower compartments (Russell et al., 2019) or by bringing different microbes to the flowers owing to their remarkably different lifestyles that could affect associated microbes (Engel et al., 2016; Kwong et al., 2017). While most plant species are pollinated by diverse groups of pollinator species (Kantsa et al., 2018; Wei et al., 2020), significant knowledge gaps exist as to how different pollinators govern floral microbiome assembly (Morris et al., 2020), and further how the effect of pollinators is modulated by other factors that can influence pollinators and the floral microbiome, such as floral traits (e.g., flower abundance) and external disturbance by bactericides and fungicides.

Floral traits can influence the floral microbiome directly by affecting niche availability and imposing habitat filtering for microbes (Hayes et al., 2021; Rebolleda-Gómez & Ashman, 2019; Vannette, 2020; Wei & Ashman, 2018) and indirectly by affecting pollinator visits (Armbruster, 2017; Kantsa et al., 2018; Wei et al., 2020) that can mediate microbial dispersal. In particular, flower abundance has rarely been studied in the floral microbiome but can potentially influence a given flower's microbial community by affecting the source pool of microbes hosted by neighbouring flowers (Toju et al., 2018). Such source pool of microbes is dynamic due to rapid habitat extinction (short flower longevity) and temporally shifted habitat availability (changes in flower abundance over time) (Aleklett et al., 2014). Thus, flower abundance may directly influence the abundance and richness of microbes that colonize individual flowers independently of pollinators (e.g., by wind or rain splash). In addition, flower abundance can indicate resource availability to pollinators (Benadi & Pauw, 2018; Wei et al., 2020), and thus large displays attract more visits and can potentially affect the microbiome indirectly by enhancing microbial dispersal to flowers within the display. Therefore, flower abundance and pollinators act together in shaping the floral microbiome, and their independent and interactive effects on the floral microbiome remain to be quantified.

Theory predicts that disturbance alters niche availability, the nature of species interactions (positive, facilitation or negative, competition), and the species composition of communities (Mouillot et al., 2013). For microbiomes in an agricultural setting, an apparent disturbance can come from bactericides and fungicides that broadly or selectively target microbial taxa (Matson et al., 1997; McManus et al., 2002). Chemically mediated disturbances are becoming more common in the Anthropocene as agrochemical use is increasing, and can reduce the abundance and richness of plant-associated microbes, shift microbial composition (Bartlewicz et al., 2016; Schaeffer et al., 2017), and potentially rewire microbe–microbe interactions via physical and metabolic mechanisms (e.g., habitat sharing via fungal hyphae and bacterial biofilm and by-product cross-feeding) (Deveau et al., 2018; Frey-Klett et al., 2011; Goldford et al., 2018; Smith et al., 2019). Agrochemical disturbance has also shown off-target effects on pollinators, including altering their visitation patterns (Fisher et al., 2017; Park et al., 2015; Stejskalová et al., 2018). Thus, agrochemical disturbance of bactericides and fungicides probably affects the flower microbiome (Bartlewicz et al., 2016; Schaeffer et al., 2017) both directly by reducing targeted microbial taxa and indirectly by influencing pollinator visitation. It remains, nevertheless, a major unresolved question whether the effect of agrochemical disturbance is generalizable across taxonomically diverse pollinators and between bacterial and fungal constituents of the floral microbiome.

Here we used a field experiment to quantify how different pollinators govern the key properties of the floral microbiome (i.e., microbial α- and β-diversity and microbial network) under agrochemical disturbance (bactericide and fungicide; Figure 1). We focused on the epiphytic floral microbiome of cultivated strawberry (*Fragaria* × *ananassa*). Strawberry crops are commercially important worldwide (FAOSTAT, 2020) and rely heavily on pesticide use for productivity (Environmental Working Group, 2020). While capable of autonomous self-pollination, the plant is visited by a wide array of insects, and insect visitation is essential for pollination success and marketable fruit production (Klatt et al., 2014). Thus, strawberry represents a model system to investigate how agrochemicals and pollinators affect the floral microbiome. Rather than manipulating pollinator access (Rebolleda-Gómez & Ashman, 2019; Vannette & Fukami, 2017), we linked the natural variation in visitation of different functional groups of pollinators (bees, wasps, flies, butterflies, beetles, true bugs and other taxa) to bacterial and fungal α- and β-diversity and microbial network in flowers. To minimize habitat filtering due to host variation, our experiment used clonal replicates of four genotypes of strawberry. The four genotypes differ dramatically in flower abundance but are similar in other floral traits. To test for the effect of agrochemical disturbance, we performed a replicated factorial design using a broad-spectrum bactericide and fungicide (Figure 1a). By doing so, we first characterized the communities of visiting pollinators and floral microbiota (bacteria and fungi), and examined separately how they responded to agrochemical disturbance and plant genotype. We further constructed microbial networks to infer intrakingdom (bacteria–bacteria and fungi–fungi) and interkingdom (fungi–bacteria) correlations among microbes and the network centrality of the floral microbiome. Lastly, to link pollinator communities to microbial communities, we used structural equation modelling to quantify how agrochemical disturbance and genotypic variation in

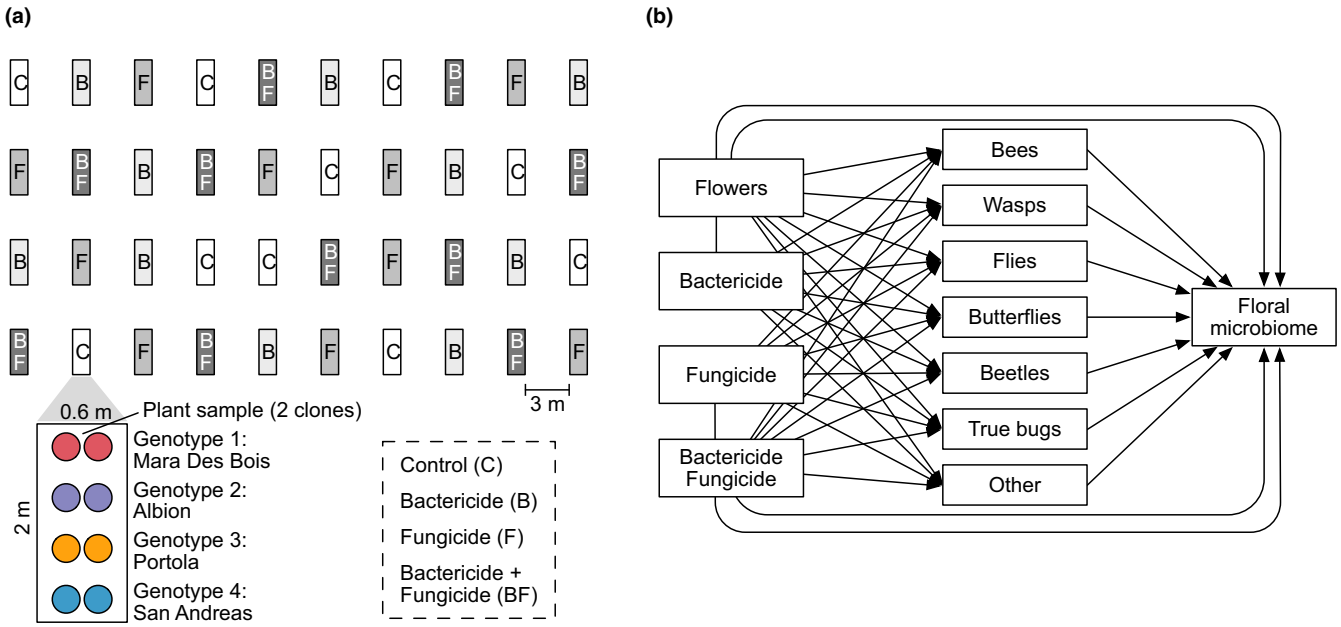

FIGURE 1 Summary of the experimental design and hypotheses in structural equation modelling. (a) A completely randomized, replicated factorial design was used to examine how pollinators influence the floral microbiome under agrochemical disturbance. Each treatment had 10 replicates ('blocks'). Each block consisted of four strawberry genotypes, with two clonal plants per genotype (referred to as one plant sample) to increase floral display for pollinators. Blocks were spaced to minimize potential treatment drift (Appendix S1). (b) Hypothesized effects of flower abundance and agrochemical disturbance on pollinator visitation and floral microbiome. Although the floral microbiome may also influence pollinator visitation, this effect was unlikely in this study (see main text) and thus one-way arrows were used to represent the effect of pollinator visitation on the floral microbiome. The floral microbiome was characterized using bacterial and fungal α- and β-diversity and microbial network centrality here

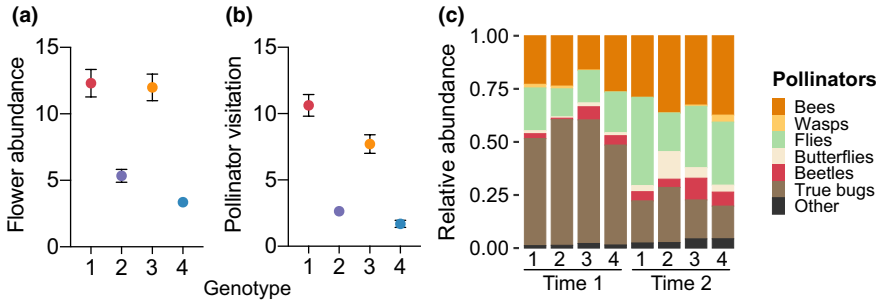

FIGURE 2 Plant genotypes differ in flower abundance and visting pollinators. Genotypic variation in (a) flower abundance matched (b) pollinator visitation (i.e., number of visiting pollinators per ~36 min) per plant sample during a 2-week time period. (a,b) The least-squares means are plotted with error bars (1 SE) after controlling for agrochemical treatment and time period (the first vs. last 2 weeks, Time 1 vs. Time 2) in generalized linear mixed models (Tables S1 and S2). (c) Pollinator community composition changed significantly over the two time periods and among genotypes (Table S2). The four strawberry genotypes were: Mara Des Bois (1), Albion (2), Portola (3) and San Andreas (4)

flower abundance affected the visitation of pollinator functional groups and thereby together affected microbiome properties (α- and β-diversity and network centrality; Figure 1b). These analyses revealed that (i) pollinator visitation influenced the floral microbiome with different pollinator functional groups affecting different properties of the floral microbiome; (ii) flower abundance strongly affected the floral microbiome both directly and indirectly via enhancing pollinator visitation; and (iii) agrochemical disturbance affected the floral microbiome primarily directly, and the effect was caused by the fungicide rather than bactericide.

2 | MATERIALS AND METHODS

2.1 | Experimental design

2.1.1 | Strawberry plant system and factorial experimental design

We used four day-neutral strawberry cultivars (Mara Des Bois, Albion, Portola, San Andreas; Nourse Farms) that show pronounced differences in flower production (Figure 2a). For each

genotype (cultivar), we planted 80 clonal 'bare root' plants in 2.4-L pots filled with Fafard 4 mix (Sun Gro Horticulture) on May 3, 2018. These plants were grown at 24°C day/18°C night temperatures 12-h days in a glasshouse at the University of Pittsburgh for 2 weeks until they were transported to a field at the Pymatuning Laboratory of Ecology, Linesville, Pennsylvania (41.57011°N, 80.45619°W). Potted plants grew and accumulated natural microbiota for 5 weeks prior to the start of a 4-week experiment of agrochemical treatment, pollinator observation, flower abundance survey and floral microbiome collection from June 24 to July 21, 2018. For agrochemical treatments, we used oxytetracycline and azoxystrobin, which are among the most widely used bactericides (McManus et al., 2002; Vidaver, 2002) and fungicides (Battaglin et al., 2010). The factorial design included water control ('C'), bactericide ('B,' oxytetracycline, 150 p.p.m.; Sigma-Aldrich; Appendix S1), fungicide ('F,' azoxystrobin, Heritage, 0.2 lb per acre; Syngenta), and bactericide and fungicide ('BF'). The four treatments were applied at the 'block' level (N = 40 with 10 blocks per treatment), with each block spaced 3 m apart to limit potential treatment drift (Figure 1a; Appendix S1). Each experimental block consisted of four pairs of clones (one pair of each genotype) and the location of pairs within block was randomized. Pairs were used to ensure floral display for pollinators and are referred to as one 'plant sample' hereafter (Figure 1a). Treatments were applied weekly using a backpack sprayer, with plant samples receiving an equal volume across treatments each week ('C': 25 ml water per plant sample; 'B': 12.5 ml water and 12.5 ml bactericide; 'F': 12.5 ml water and 12.5 ml fungicide; 'BF': 12.5 ml bactericide and 12.5 ml fungicide). Plants were protected from large herbivores using fencing with buffer distance (10 m) to the site.

During the 4-week experiment, due to temporal dynamics in flower production and pollinator visitation between the first 2 weeks and last 2 weeks (Figures S1 and S2), we divided the experiment into the corresponding two time periods (Time 1 and Time 2).

2.1.2 | Pollinator observation

Pollinator observations were conducted by the same observer 4 days each week between 9 AM and 5 PM. The observer spent 2 min per block for all 40 blocks in a single observation round. At each block, all flowers were observed simultaneously and the number of pollinators (bees, wasps, flies, butterflies, beetles, true bugs and other taxa) was recorded for each plant sample. Here, the 'other' category accounted for 2% of the visiting pollinators, including arthropod taxa not in any of the given categories (e.g., spiders) or unidentified in the field. Each day one to three rounds of observations were completed with the order of block visits systematically alternated each round. As a result, we spent 2874 min in total for pollinator observations in this experiment, and reported the visitation data as the number of visiting pollinators per plant sample per ~36 min during each 2-week time period.

2.1.3 | Flower abundance survey

We scored the number of flowers including both open flowers (typically lasting for 2 days in the field) and flower buds of each plant sample once per week, and reported flower abundance data as the sum of flowers per plant sample per 2-week time period. We also confirmed that the four genotypes differed significantly in flower abundance (Figure 2a; Appendix S1) and not other traits (e.g., flower size and pollen production, Figure S1; Appendix S1) that reflect attraction and resources for pollinators (Ashman, 2000).

2.1.4 | Floral microbiome collection and sequencing

Two flowers were collected from each plant sample each week 2 days after agrochemical application. Following Wei and Ashman (2018), flowers (without pedicel) were collected into a sterile 15-ml centrifuge tube using ethanol-rinsed forceps and were transferred to a −20°C freezer within 2 h. For microbial DNA extraction, we pooled flowers standardized by size (N = 1–4) per plant sample per 2-week time period and obtained 246 samples in total. Epiphytic microbes were collected by sonicating flowers in 3 ml phosphate-buffered saline at 40 kHz for 10 min and vortexing for 5 min, and were pelleted by centrifuging at 10,000 g for 5 min. Microbial DNA was extracted using Quick-DNA Fecal/Soil Microbe Kits (Zymo Research). Two negative controls without flower samples were included in the process of microbe isolation and DNA extraction. Samples and negative controls were sent to Argonne National Laboratory for bacterial (16S rRNA V5–V6 region, 799f–1115r primer pair) (Redford et al., 2010) and fungal (ITS1f–ITS2) (Smith & Peay, 2014) library preparation. Because the negative controls failed in polymerase chain reactions (PCRs) in library preparation, the 246 samples were sequenced on two lanes of a paired-end (PE) 250-bp Illumina MiSeq.

2.2 | Bioinformatic and statistical analyses

2.2.1 | Microbial sequence processing

Demultiplexed PE reads were used for detecting bacterial and fungal amplicon sequence variants (ASVs) using the package DADA2 version 1.12.1 (Callahan et al., 2016) in R version 3.6.0 (R Core Team, 2019) and QIIME 2 version 2019.4 (Bolyen et al., 2019). For bacterial ASV analysis in DADA2, PE reads were trimmed and filtered [truncLen = c(245, 245), trimLeft = c(10, 0), maxN = 0, truncQ = 2] after initial quality inspection. Then end-specific variants were identified after taking into account sequence errors, prior to joining the PE reads (minOverlap = 20, maxMismatch = 4) for ASV detection. Bacterial ASVs were further filtered against chimeras and assigned with taxonomic identification based on the SILVA reference database (132 release) implemented in DADA2. For fungal ASV analysis in DADA2, the PE reads were first screened to remove potential primer contaminations. Due to the low-quality end (~50 bp) of the fungal reads, we then truncated reads

at 200 bp during quality filtering [truncLen = c(200, 200), maxN = 0, truncQ = 2] to ensure the accuracy in ASV detection. By doing so, our method provided a conservative estimate of fungal ASVs, due to the potential loss of information based on ITS length variation (Callahan et al., 2016). After chimera removal, fungal taxonomic assignment was conducted based on the UNITE reference database (version 8.0 dynamic release) using QIIME 2.

Bacterial and fungal ASV tables were further filtered separately before conversion into microbial community matrices using package PHYLOSEQ (McMurdie & Holmes, 2013). First, we removed nonfocal ASVs (Archaea, chloroplasts and mitochondria). Second, we filtered out low-depth samples (<100 reads; N = 13 and eight samples for the bacterial and fungal data set, respectively). Third, we normalized per-sample reads to the same number (i.e., the median reads, 18,788 and 37,516 for the bacterial and fungal data set, respectively) following Wei and Ashman (2018). Lastly, we removed low-frequency ASVs (<0.001% of total observations). The final community matrices consisted of 1237 and 1165 ASVs for bacteria (N = 223) and fungi (N = 240), respectively, and are available on Dryad (Wei et al., 2021).

2.2.2 | Pollinator visitation and community composition

To evaluate how pollinator visitation responded to agrochemical treatment, genotype and time period, we examined pollinator fauna as a whole and individual functional groups using zero-inflated generalized linear mixed models (zGLMMs). In each zGLMM, the predictors included treatment (levels: C, B, F and BF), genotype (1: Mara Des Bois; 2: Albion; 3: Portola; 4: San Andreas) and time period (Time 1 vs. Time 2), with random effects being block and plant sample (due to repeated measurements over time) using the package GLMMTMB (Brooks et al., 2017). Negative binomial errors were used in zGLMMs and model fits were confirmed using the package DHARMA (Hartig, 2019). Statistical significance (type III sums of squares) and least-squares means (LS-means) of predictors were assessed using the packages CAR (Fox & Weisberg, 2011) and EMMEANS (Lenth, 2019).

To evaluate how the composition of the pollinator communities responded to agrochemical treatment, genotype and time period, we converted the pollinator functional group matrix into proportional data, and conducted permutational multivariate analysis of variance (PERMANOVA; predictors: treatment, genotype and time period; random effect: block) using the package VEGAN (Oksanen et al., 2019). Once significant predictors were detected, we further identified the specific functional groups underlying pollinator community variation caused by the predictors using random forest (RF) classification in the packages CARET (Kuhn, 2019) and RANDOMFOREST (Liaw & Wiener, 2002). The RF classification models were run for the full data with 1000 trees, and the number of randomly selected variables (i.e., functional groups) at each split of a decision tree was optimized using 10-fold cross validation in CARET. RF model performance was assessed using out-of-bag (OOB) error. The set of important variables (functional groups) was selected using backwards variable elimination with the package VARSELRF (Diaz-Uriarte, 2007), and their relative importance was evaluated using mean decrease in classification accuracy.

2.2.3 | Floral microbial α- and β-diversity

To evaluate how microbial α-diversity responded to agrochemical treatment, genotype and time period, we calculated Shannon diversity for bacterial and fungal communities separately using VEGAN, and performed general linear mixed models (LMMs) using the package LME4 (Bates et al., 2015). The predictors included treatment, genotype and time period as well as their two-way interactions, and the random effects included block and plant sample. The response variable was power transformed to improve normality, with the optimal power parameter determined using the Box–Cox method in the package CAR. The statistical significance of predictors in LMMs was evaluated using the package LMERTEST (Kuznetsova et al., 2017).

Microbial β-diversity (Bray–Curtis dissimilarity) was evaluated using PERMANOVA and constrained principal coordinates analysis (cPCoA) in VEGAN for bacterial and fungal communities separately. To assess the significance of the main effects, PERMANOVA and cPCoA included treatment, genotype and time period with a block random effect. To assess the significance of two-way interactions, PERMANOVA and cPCoA included both the main effects and their interaction terms. Once significant predictors were identified, RF classification was used to detect the set of important, nonredundant ASVs (in the presence of potentially correlated ASVs) that underlay bacterial or fungal community variation caused by the predictors, as described for pollinator community composition.

2.2.4 | Structural equation models (SEMs) linking pollinators to microbial diversity

To evaluate how pollinators influenced microbial diversity under agrochemical disturbance, we used SEMs to link flower abundance and agrochemical treatment to visitation by pollinators of specific functional groups to α- and β-diversity of bacteria or fungi in the floral microbiome (Figure 1b). The SEMs included flower abundance because genotypic variation was only significant in this trait (Figure 2a) and not the others (Figure S1). The SEMs were used on data collected across both time periods but did not include time period as an exogenous (independent) variable, because time-period variation was defined and reflected by variation in flower abundance and pollinator visitation (Tables S1 and S2). For exogenous categorical variables of treatments, we coded the control treatment as the reference level so that the effects of all other treatments were relative to the control. The endogenous (dependent) variable of the α-diversity metric (Shannon diversity) was power transformed as in the section 'Floral microbial α- and β-diversity' (power parameter = 1 and 2 for bacteria and fungi, respectively). The endogenous variable of β-diversity used the first axis of the cPCoA (see section 'Floral

microbial α- and β-diversity') that accounted for 25% and 60% of the total variation in bacterial and fungal communities, respectively. Model estimation used robust maximum likelihood with Satorra–Bentler scaled χ^2 that can accommodate non-normality using the package LAVAAN (Rosseel, 2012). Model fits were confirmed (comparative fit index, CFI > 0.9; root mean squared error of approximation, RMSEA, the lower bound of 90% confidence interval <0.05; standardized root mean squared residual, SRMR < 0.1) (Kline, 2015). We conducted the SEMs using the original and square-root-transformed pollinator visitation data separately, which yielded qualitatively similar results, but the former exhibited better model fits and thus were reported here. Standardized coefficients (*r*) were indicated for paths with notable effects ($p < .1$).

2.2.5 | Microbial networks and SEMs linking pollinators to network centrality

Microbial correlation networks were constructed to infer intra- and interkingdom correlations among microbes in flowers, and how pollinators influenced microbial network centrality under agrochemical disturbance. We focused on two centrality metrics (degree and eigenvector). In a network, degree centrality describes a microbe's importance based on the total number of direct connections (or links) with other microbes. Eigenvector centrality weighs each connection by giving higher weights to connections to well-connected (i.e., high-degree) microbes and considers both direct and indirect connections, and thus is a weighted sum of all connections of a microbe to other microbes (Bonacich, 2007). Therefore, degree and eigenvector centrality measure the network importance of microbes based on unweighted direct interactions and weighted network-wide interactions, respectively.

To build microbial networks, we merged the bacterial and fungal community matrices and normalized per-sample read numbers (to the median of the bacterial data set). This resulted in 2,346 ASVs (1232 bacteria and 1114 fungi) across 218 samples that had data for both bacteria and fungi. To infer positive and negative microbe–microbe correlations, we used SPARCC (Friedman & Alm, 2012), which is robust to spurious correlations among microbes due to microbiome compositionality (i.e., nonindependence among ASVs due to relative abundances) using the package SPIECEASI (Kurtz et al., 2015). To do this, we extracted the raw counts of these ASVs, and focused on the ASVs that were present in ≥10 samples. Networks were constructed and visualized for each treatment (based on correlation estimates ≥0.4 and ≤−0.4) using SPARCC and the package IGRAPH (Csardi & Nepusz, 2006).

To infer how pollinators influenced microbial network (degree and eigenvector centrality) under agrochemical disturbance using SEMs (Figure 1b), we targeted ASVs that were responsive to (i) visitation of pollinator functional groups and (ii) flower abundance based on maximal information coefficient (MIC ≥ 0.2) (Reshef et al., 2011) and (iii) agrochemical treatments using RF classification. MICs that can detect nonlinear associations were calculated using the package MINERVA (Albanese et al., 2013). RF classification was performed to detect the ASVs that differed between pairwise comparisons of treatments for bacteria and fungi separately. This resulted in 238 ASVs (146 bacteria and 92 fungi) in the 218 samples for SEMs. To do this, we performed a leave-one-out approach to measure network change caused by the removal of a sample. Specifically, we constructed the microbial network with and without a sample based on MIC (≥0.2) due to its nonlinear detectability. We then calculated node-level degree and eigenvector centrality for individual nodes (ASVs) using IGRAPH. Network change in node-level centralities was calculated as the distance between two vectors of ASV centralities (i.e., with and without a sample) using the package PDIST (Wong, 2013) for degree and eigenvector centrality separately. Such network change reflected an individual sample's importance to microbial network centrality and was used in the SEMs.

3 | RESULTS

3.1 | Pollinator visitation and community composition

The visitation of all pollinator fauna ($\chi^2 = 4.7$, $df = 3$, $p = .20$, zGLMM) and that of individual functional groups (Table S2) were similar among different agrochemical treatments, except butterflies and the 'other' category (Figure S2). Butterfly visitation tended to increase when both bactericide and fungicide were applied (BF vs. C; zGLMM, $t = 2.15$, $p = .030$, Figure S2b). The 'other' group of pollinators decreased under fungicide treatment relative to the control (F vs. C; $t = -2.01$, $p = .045$, Figure S2c). Different from the agrochemical treatment, plant genotype strongly influenced visitation of all pollinators combined ($\chi^2 = 178$, $df = 3$, $p < .001$, Figure 2b) and that of individual functional groups (all $p < .05$, Table S2). In addition, pollinator visitation declined over time period ($\chi^2 = 41.6$, $df = 1$, $p < .001$, Figure S2a), especially for true bugs (Table S2). Similar to visitation, pollinator community composition also varied significantly over time period (PERMANOVA, $F = 34.5$, $df = 1$, $p = .001$, 12% of variation, Figure 2c) and among plant genotypes ($F = 2.2$, $df = 3$, $p = .025$, 2%, Figure 2c; Figure S2d), which were driven by changes in the relative abundance of true bugs and flies (RF, OOB = 21.4%, Table S3; Figure 2c) and that of true bugs and beetles (OOB = 63.3%), respectively.

3.2 | Floral microbial α- and β-diversity and network

Agrochemical treatment significantly affected the α-diversity of fungal communities (LMM, $F = 3.59$, $df = 3$, $p = .022$, Table S4), after accounting for the effects of genotype ($F = 4.09$, $df = 3$, $p = .009$, Figure S3) and time period ($F = 2.04$, $df = 1$, $p = .16$). As expected, fungal Shannon diversity was reduced when fungicide was applied (F vs. C, $\chi^2 = 7.16$, $df = 1$, $p = .007$; BF vs. C, $\chi^2 = 7.74$, $df = 1$, $p = .005$,

Figure 3c). This reduction was especially strong at Time 1 (treatment × time period, $F = 4.20$, $df = 3$, $p = .007$), whereas at Time 2 all treatments remained low in fungal Shannon diversity including the control (Figure S3b), probably due to the combined effect of agrochemical use and reduced flower abundance (over time period), which had a strong effect on fungal diversity (described below in SEMs).

Unexpectedly, bacterial Shannon diversity was significantly reduced by fungicide (LMM, F vs. C, $\chi^2 = 5.71$, $df = 1$, $p = .017$; BF vs. C, $\chi^2 = 5.34$, $df = 1$, $p = .021$) rather than bactericide (B vs. C, $\chi^2 = 1.97$, $df = 1$, $p = .16$, Figures 3a and S3a). The weak effect of bactericide on bacterial diversity was consistent with the observations of microbial networks (Figure 3e). Bactericide increased positive bacteria–bacteria correlations (B, 522 out of 796 total intra- and interkingdom links) relative to the control (399 of 795), whereas fungicide reduced positive bacteria–bacteria (232 and 146 in F and BF vs. 399 in C) and positive fungi–bacteria correlations (12 and 14 in F and BF vs. 43 in C), as well as the total links (432 and 254 in F and BF vs. 795 in C).

Similar to α-diversity, fungal community composition was affected by agrochemical treatment, which explained the largest source of variation in fungal communities (PERMANOVA, 15%, $F = 15.2$, $df = 3$, $p = .001$, Table S4) relative to time period (5%, $F = 15.9$, $df = 1$, $p = .001$) and plant genotype (3%, $F = 2.7$, $df = 3$, $p = .001$). The cPCoA further revealed that fungicide caused the divergence of fungal communities of F and BF treatments from the control (along the first axis, cPCoA1, Figure 3d). This divergence was driven by a small set of nonredundant ASVs (F vs. C, $N = 40$, OOB = 11.5%; BF vs. C, $N = 27$, OOB = 9.2%), among which Basidiomycota and Ascomycota fungi exhibited the largest importance (Figure S4 and Table S5). In particular, *Rhodotorula graminis* (nectar yeast; Pozo et al., 2014) was significantly enriched, whereas *Sporobolomyces phaffii* and *Aureobasidium* sp. (nectar yeasts; Pozo et al., 2014) and *Cladosporium delicatulum* (with biocontrol activities; Venkateswarulu et al., 2018) were significantly depleted in F and BF treatments relative to the control (adjusted p for multiple comparisons <.001, Table S5).

Different from fungi, bacterial communities were primarily segregated over time period along cPCoA1 ($F = 6.96$, $df = 1$, $p = .001$, Figure 3b; PERMANOVA, $F = 7.62$, $p = .001$, Table S4). Albeit subtler than fungi (Figure 3b,d), bacterial communities varied among agrochemical treatments (cPCoA, $F = 1.38$, $df = 3$, $p = .017$; PERMANOVA, $F = 1.44$, $p = .015$, Table S4), with all other treatments (B, F and BF) deviating significantly from the control (PERMANOVA, pairwise comparisons, all $p < .05$, Table S4). Such deviation was driven by a small set of nonredundant ASVs ($N = 8$, 14 and 3 for B, F and BF, respectively, Table S5), among which *Hymenobacter* ASVs (common bacteria in strawberries; Sylla et al., 2013; Wei & Ashman, 2018) were most influential and significantly depleted in other treatments relative to the control (adjusted $p < .001$, Table S5; Figure S5).

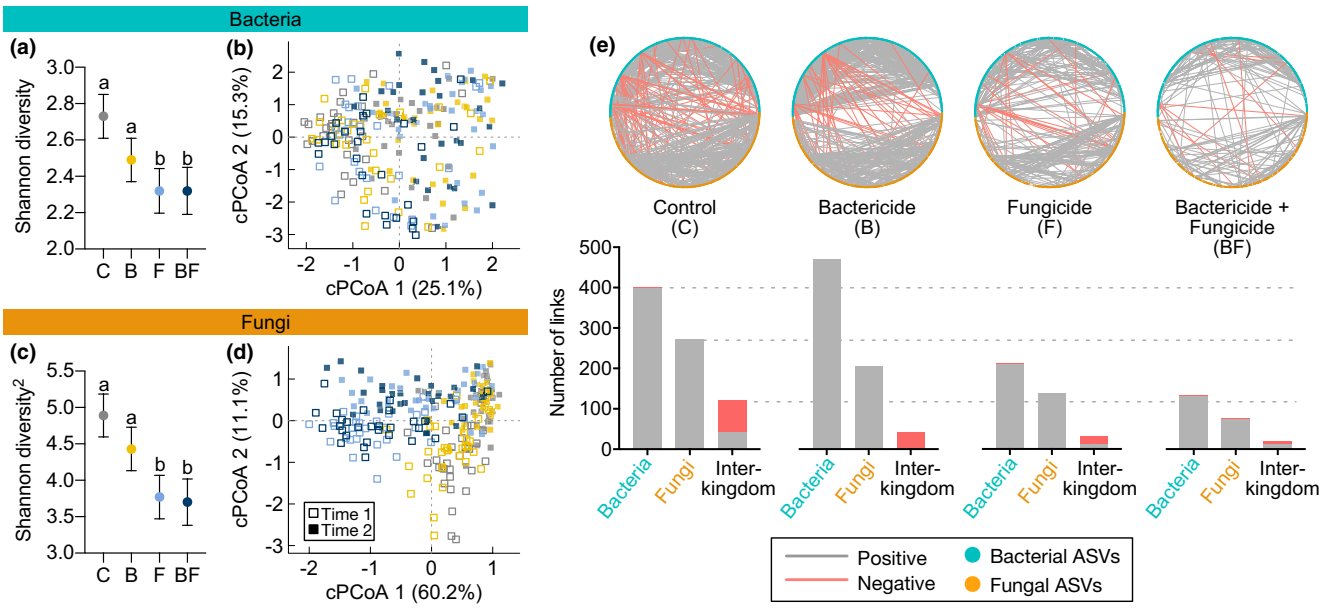

FIGURE 3 Microbial α- and β-diversity and network respond to agrochemical disturbance. The least-squares means of Shannon diversity for bacteria (a) and fungi (b; power-transformed, power parameter = 2) are plotted with error bars (1 SE) for each treatment (C, control; B, bactericide; F, fungicide; BF, bactericide and fungicide), after controlling for the effects of genotype and time period in general linear mixed models (Table S4). (b,d) Constrained principal coordinates analyses (cPCoAs) indicated microbial community separation by agrochemical treatment (colour) and time period (the first vs. last 2 weeks, Time 1 vs. Time 2) for bacteria (b, $N = 223$ samples) and fungi (d, $N = 240$). (e) Microbial correlation networks were based on SPARCC (correlation estimates ≥0.4 or ≤−0.4). The same set of bacterial ($N = 359$) and fungal ($N = 343$) amplicon sequence variants (ASVs) are represented as nodes in the networks across treatments (e, top panel). Positive (grey) and negative (red) intrakingdom (bacteria–bacteria and fungi–fungi) and interkingdom (fungi–bacteria) correlations are represented as links within the networks (e, top panel). The number of these microbial correlations (links) are summarized (e, bottom panel)

3.3 | Pollinators influenced the floral microbiome

SEMs revealed that pollinator functional groups varied in their influence on different properties of the floral microbiome (α- and β-diversity and network centrality), independent of the significant direct effects of flower abundance and agrochemical treatments (Figure 4). Bee visitation had a notable positive effect on bacterial Shannon diversity (r = .12, p = .070, Figure 4a) but a negative effect on fungal Shannon

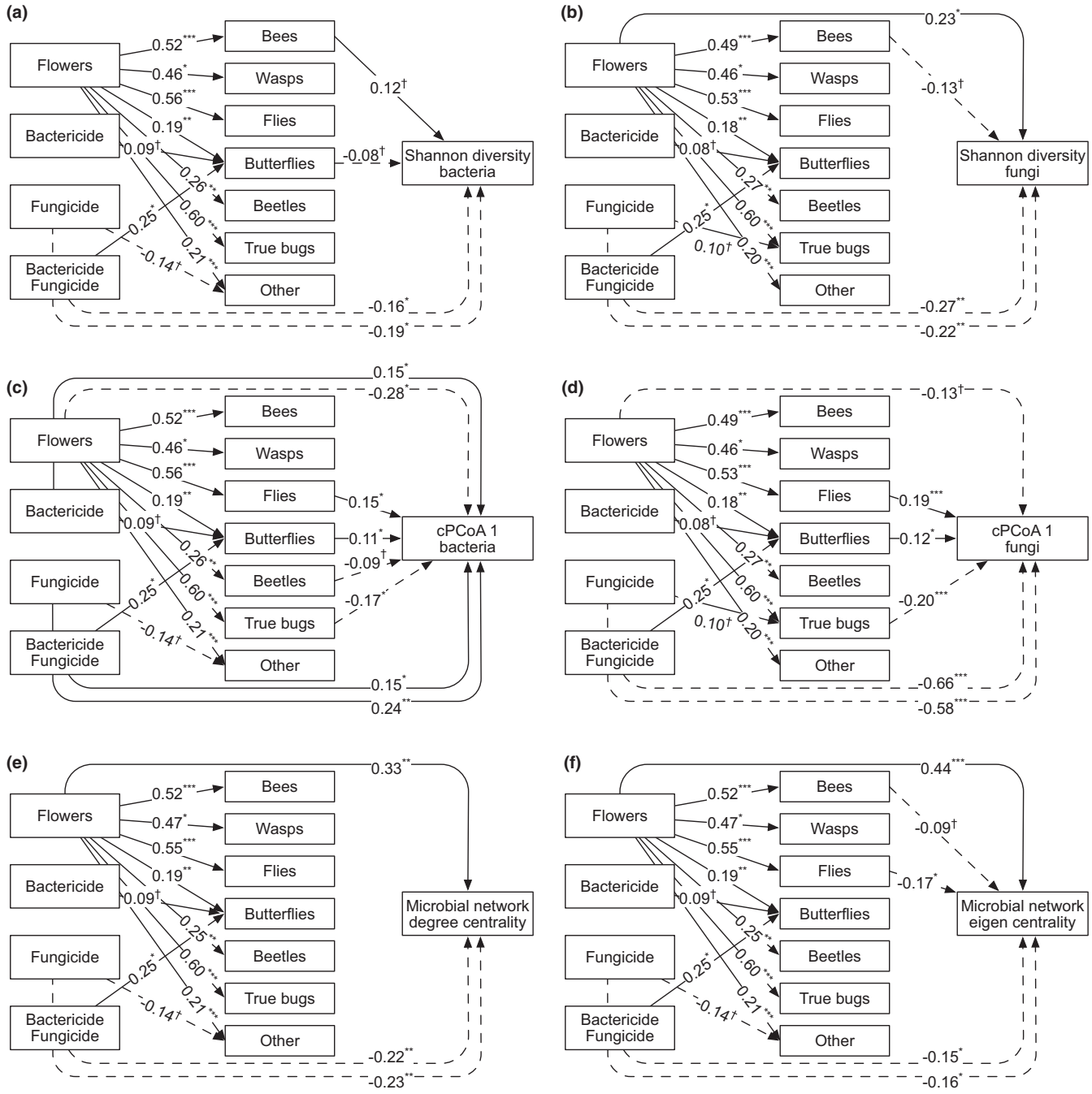

FIGURE 4 Structural equation models (SEMs) of flower abundance and agrochemical disturbance explaining pollinator visitation and floral microbiome. Arrows indicate notable positive (solid) and negative (dashed) relationships: †p < .10; *p < .05; **p < .01; ***p < .001. Numbers adjacent to arrows indicate standardized path coefficients. Agrochemical treatments were coded using the control treatment as the reference level in the SEMs. The α- and β-diversity of bacterial and fungal communities used Shannon diversity (a,b) and the first axis of constrained principal coordinates analysis (cPCoA1, c,d; see also Figure 3), respectively. The microbial network was built on a reduced set of amplicon sequence variants (ASVs; N = 238, 146 bacteria and 92 fungi) using maximal information coefficient (MIC ≥ 0.2; see Materials and Methods for details). (e) The degree centrality and (f) eigenvector centrality measure network importance of individual ASVs based on the number of direct interactions and the weighted sum of both direct and indirect interactions in a network, respectively. Details of SEM model fit and parameter estimation are given in Table S6

diversity (r = −.13, p = .068, Figure 4b), whereas butterfly visitation tended to reduce bacterial Shannon diversity (r = −.08, p = .065). In contrast to α-diversity, the β-diversity of microbial communities (cPCoA1) was influenced by a different set of pollinators (Figure 4c,d). Visitation of flies and butterflies showed contrasting effects relative to true bugs on bacterial (r = .15 and .11 vs. −.17, p = .015, .046 and .036, respectively, Table S6) and fungal community composition (r = .19 and .12 vs. −.20, p < .001, p = .011 and p < .001, respectively). Different from α- and β-diversity, pollinator visitation did not affect microbial network degree centrality (Figure 4e). However, visitation of bees and flies influenced eigenvector centrality (r = −.09 and −.17, p = .083 and .014, respectively, Figure 4f).

3.4 | Flower abundance affected the floral microbiome directly and indirectly

SEMs revealed that flower abundance affected the floral microbiome both directly and indirectly via influencing pollinator visitation (Figure 4). Flower abundance had a direct positive effect on fungal Shannon diversity (r = .23, p = .024, Figure 4b), and also directly affected microbial community composition (bacteria, r = −.28, p = .011, Figure 4c; fungi, r = −.13, p = .088, Figure 4d) and network centrality (degree, r = .33, p = .006, Figure 4e; eigenvector, r = .44, p < .001, Figure 4f). In addition, flower abundance affected microbiome indirectly by increasing pollinator visitation consistently across all functional groups (all p < .05, Figure 4). In particular, bacterial Shannon diversity was affected by flower abundance only indirectly via pollinators (bees, r = .06 [.52 × .12]; butterflies, r = −.02 [.19 × −.08], Figure 4a), whereas fungal Shannon diversity was affected by flower abundance both directly and indirectly (bees, r = −.06 [.49 × −.13], Figure 4b), similar to other properties of the microbiome (community composition and eigenvector centrality). The indirect effect of flower abundance was similar between bacterial community composition (flies, r = .08 [.56 × .15]; butterflies, r = .02 [.19 × .11]; beetles, r = −.02 [.26 × −.09]; true bugs, r = −.10 [.60 × −.17], Figure 4c) and fungal community composition (flies, r = .10 [.53 × .19]; butterflies, r = .02 [.18 × .12]; true bugs, r = −.12 [.60 × −.20], Figure 4d). For network eigenvector centrality (Figure 4f), the indirect effect of flower abundance was via bees (r = −.05 [.52 × −.09]) and flies (r = −.09 [.55 × −.17]).

3.5 | Agrochemical disturbance affected the floral microbiome primarily directly by fungicide

While the significant effect of agrochemical treatment on microbiome described above (LMMs, PERMANOVA/cPCoA and network links, Figure 3) reflected the overall effect of agrochemical treatment, SEMs dissected the overall effect into direct vs. indirect effects (Figure 4). The indirect effect of agrochemical treatment (B, F and BF) via pollinators was weak, because pollinator visitation was rarely affected by agrochemical treatment except for that of butterflies (r = .25, p < .05, Figure 4) and the 'other' group (r = −.14, p < .10) consistent with the zGLMMs results above, as well as true bugs (SEMs, r = .10, p < .10; zGLMM, t = 1.67, p = .097). The indirect effect of agrochemical treatment was only present via butterflies on bacterial Shannon diversity (B, r = −.01 [.09 × −.08]; BF, r = −.02 [.25 × −.08], Figure 4a) and community composition [B, r = .01 [.09 × .11]; BF, r = .03 [.25 × .11], Figure 4c), and on fungal community composition via butterflies (B, r = .01 [.08 × .12]; BF, r = .03 [.25 × .12], Figure 4d) and true bugs (F, r = −.02 [.10 × −.20]). In contrast to the weak indirect effect, the direct effect of agrochemical treatment was strong for all measured microbiome properties and for both bacteria and fungi (all p < .05, Figure 4). Similar to the overall effect revealed by other methods (LMMs, PERMANOVA/cPCoA and network links, Figure 3), the direct effect of agrochemical treatment on the microbiome was driven by fungicide (F and BF, Figure 4).

4 | DISCUSSION

Disentangling potentially interacting mechanisms in shaping microbiomes is critical for theoretical and applied advances in the principles of microbiome assembly (Hawkes & Connor, 2017). Our work has demonstrated the role of taxonomically diverse pollinators and their interactions with other drivers in shaping the properties of the floral microbiome. Pollinator functional groups showed distinct effects on microbial α- and β-diversity and network centrality. The effect of pollinator visitation was strongly influenced by flower abundance, but less so by agrochemical disturbance. Instead, agrochemical disturbance primarily from fungicide directly affected both bacterial and fungal communities. By linking these mechanisms previously studied in isolation (see reference citations in Introduction), our results provide an integrated understanding of the drivers and their respective importance in governing microbiome assembly in the anthosphere in the face of agrochemical disturbance.

Our results revealed that pollinator functional groups influenced bacterial and fungal communities, even after accounting for the strong direct effects of flower abundance and agrochemical disturbance. In particular, the most abundant functional groups that visited strawberry flowers (bees, flies and true bugs, Figure 2c) played the most important roles in governing microbial diversity relative to other pollinators. These abundant pollinators differed in respective effects on microbial α- vs. β-diversity (Figure 4), with bee visitation influencing α-diversity and true bugs and flies influencing β-diversity. These distinct effects may reflect different rates of microbial dispersal mediated by different functional groups. Microbial dispersal via bees, the most important pollinators of cultivated strawberry (Klatt et al., 2014), can be potentially extensive and thus increase α-diversity (e.g., in bacterial communities), but high rates of microbial dispersal can also homogenize microbial communities, thereby contributing little to community differentiation (along cPCoA1) relative to other functional groups (Figure 4c,d). We found that bacterial taxa associated with bee visitation (post-hoc tests, Appendix S1; Table S7) often belong to *Acinetobacter* (including

A. nectaris), *Methylobacterium*, *Rosenbergiella* and *Sphingomonas*, which are common in nectar (Pozo et al., 2014) and flowers (Hayes et al., 2021; Wei & Ashman, 2018). Relative to bees, the effect of visitation by true bugs and flies on microbial community differentiation may reflect microbial dispersal limitation mediated by these abundant functional groups. While overlapping in bacterial taxa with bees, true bugs and flies also associated with other taxa (Table S7): for instance, visitation by flies was associated with *Lactobacillus micheneri* and *Leuconostoc* that are common in insects (Pais et al., 2018; Vuong & McFrederick, 2019); visitation by true bugs was associated with many generalist bacteria that are present in flowers and other habitats (leaves and/or roots) such as *Xylophilus*, *Massilia* and *Deinococcus* (Wei & Ashman, 2018). Yet, whether microbial dispersal limitation is caused by shorter travel distance among flowers or fewer microbes being transported and delivered by true bugs and flies relative to bees needs further investigation. In addition to the distinct effects among pollinator functional groups, the same functional group can affect bacterial and fungal communities differently. For instance, bees tended to increase bacterial but decrease fungal α-diversity. While not affecting pollinator visitation (see Discussion below), resident microbes may affect whether and how much pollinators sample and consume nectar with fungi (Good et al., 2014; Vannette et al., 2013). Interestingly, different from bacteria, the fungal taxa associated with bee visitation are often not nectar-inhabiting (but see *Sporobolomyces phaffii*, Table S7). While nectar yeasts (Pozo et al., 2014) are common in strawberries here such as *Candida*, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Aureobasidium* and *Debaryomyces* (Dryad data, Wei et al., 2021), their weak associations with bee visitation may suggest dispersal may not compensate for the potential consumption of nectar with fungi. This may contribute to the negative net outcome of bee visitation on fungal diversity. Taken together, this suggests that diverse pollinators can influence the key properties of bacterial and fungal communities.

Pollinator-mediated dispersal did not act alone but interacted with other processes in driving the floral microbiome. Contrary to the expectation of off-target effects on pollinators of agrochemical use (Park et al., 2015; Stejskalová et al., 2018), visitation of most functional groups did not respond to bactericide and/or fungicide. Rather, pollinator visitation was strongly influenced by flower abundance that indicates resource availability, in agreement with the observations across a broad range of plant lineages in natural ecosystems (Benadi & Pauw, 2018; Wei et al., 2020). Here, flower abundance not only influenced pollinator-mediated microbial dispersal, but also had direct effects on microbiome properties, suggesting it influenced the source pool of microbes. In particular, microbes that are known to be adapted to the floral environment were positively associated with flower abundance, including nectar taxa (e.g., bacteria: *Rosenbergiella*, *Methylobacterium*, *Acinetobacter*, *Sphingomonas*, *Pseudomonas*, *Curtobacterium*; fungi: *Sporobolomyces*, *Rhodotorula*; Table S7) and fungal pathogens in strawberries (e.g., *Cladosporium*), whereas other microbes (Table S7) may be opportunistic or proliferate on flowers via mechanisms yet to be explored. Overall, the direct and indirect effects of flower abundance underlined the importance of considering this driver in the principles of microbiome assembly in flowers, in addition to other floral traits (e.g., volatiles, ultraviolet patterns; Hayes et al., 2021; Schaeffer et al., 2019).

Although floral microbes have been implicated in mediating plant–pollinator interactions (reviewed in Vannette, 2020), in our field study flowers with significantly altered microbiota did not seem less or more attractive to pollinators. This discrepancy probably has multiple reasons and also highlights some important gaps in our knowledge of plant–microbiome–pollinator interactions. First, previous manipulative experiments of nectar microbes have demonstrated their importance in mediating pollinator visitation and/or nectar consumption (Herrera et al., 2013; Schaeffer & Irwin, 2014; Vannette et al., 2013; Yang et al., 2019). Although nectar microbes are common in the floral microbiome here, the overall limited effect on pollinator visitation underlined the complexity in inferring the relative importance of microbes and floral traits (including flower abundance) in mediating plant–pollinator interactions in nature. Second, effects on only a few pollinator taxa have been specifically tested to date (e.g., honeybees, bumblebees and hummingbirds; Good et al., 2014; Herrera et al., 2013; Vannette et al., 2013), so it remains an open question how taxonomically diverse pollinators respond to floral microbes and how consistent the responses are given the dynamic nature of pollinator preference for microbial taxa (Good et al., 2014; Schaeffer et al., 2019). Lastly, previous studies have focused on one or a few microbes in the relatively simple nectar microbiome (reviewed in Vannette, 2020), which differs from the complex microbe–microbe interactions observed here in the whole floral microbiome. Thus, whether and how the floral microbiome consisting of thousands or more taxa of bacteria and fungi in many flowering plants mediates plant–pollinator interactions merits further investigation.

Agrochemical disturbance from fungicide directly affected the floral microbiome, consistent with observations in other systems and/or plant organs (Bartlewicz et al., 2016; Debode et al., 2013; Gu et al., 2010; Schaeffer et al., 2017). Although oxytetracycline is among the most widely used bactericides (McManus et al., 2002; Vidaver, 2002), it caused nonsignificant reductions in bacterial α-diversity and mild changes in composition such as *Hymenobacter*, the common bacteria in strawberry flowers (Wei & Ashman, 2018). Yet, we could not rule out that it may reduce the absolute abundance of bacteria, given that sequencing-based characterization of microbiomes considered only relative abundances. In contrast to the bactericide, azoxystrobin, one of the most popular fungicides that target eukaryotes (Battaglin et al., 2010), affected both fungal and bacterial communities. It is perhaps not surprising that fungicide affected fungal communities (Bartlewicz et al., 2016; Debode et al., 2013; Gu et al., 2010; Schaeffer et al., 2017). However, the strong effect of fungicide on bacterial communities underlined important interkingdom interactions. Fungi–bacteria interactions have been found predominantly to be negative in the rhizosphere (Duran et al., 2018), but these interactions can be context-dependent and shift in directions from negative to more positive in resource-limited environments (Velez et al., 2018), such as the anthosphere here (Figure 3e). The

observed positive fungi–bacteria correlations probably had physical (e.g., habitat sharing via fungal hyphae and bacterial biofilm) and/or metabolic reasons (e.g., by-product cross-feeding) (Deveau et al., 2018; Frey-Klett et al., 2011). More importantly, microbe–microbe interactions were beyond pairwise but formed a complex network, leading to cascading effects of agrochemical disturbance via network links. Therefore, reducing fungi affected not only positive fungi–bacteria but also positive bacteria–bacteria correlations (Figure 3e). Overall, our results indicated complex species interactions underlying how the floral microbiome responded to agrochemical disturbance.

In conclusion, the functional links between pollinator-mediated dispersal, flower abundance and agrochemical disturbance improve our mechanistic understanding of microbiome assembly in flowers. These findings may be generalizable to many other plants in natural and managed ecosystems, as we considered not only a plant species visited by diverse pollinators, but also the common agrochemical disturbance (Battaglin et al., 2010; Vidaver, 2002) that plants may encounter. In light of agricultural intensification (Matson et al., 1997; McManus et al., 2002) and pollinator loss worldwide (Potts et al., 2010), it is becoming more urgent than ever to understand how these anthropogenic changes in plant–pollinator interactions and disturbance alter microbiomes of on- and off-target plant species.

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AUTHOR CONTRIBUTIONS

N.W., A.L.R. and T.L.A. conceived the study. N.W. analysed the data and wrote the manuscript. A.L.R. and T.L.A. contributed to manuscript revision. N.W., A.L.R. and A.R.J. collected the data.

DATA AVAILABILITY STATEMENT

Sequence data are available from NCBI SRA (PRJNA713377). Experimental data are available from Dryad (https://doi.org/10.5061/dryad.qnk98sfg4).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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