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RESEARCH ARTICLE

Disease management during bloom affects the floral microbiome but not pollination in a mass-flowering crop

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Abstract

1. Flowering crops are heavily managed during bloom to both promote pollination and prevent disease. Disease management practices can alter the floral microbiome, including pathogens and nontarget microbes. However, whether agrochemical presence or altered microbiome composition affect pollinator foraging and pollination services is unclear.
2. We assessed effects of orchard management tactics and landscape context on the flower microbiome in almond, *Prunus dulcis*. Fourteen orchards (five conventional, four organic and five conventional with habitat augmentation) were sampled at early and peak bloom to characterize bacterial and fungal communities associated with floral tissues. The surveys were complemented by an artificial flower experiment to assess effects of fungicides and microbes on honey bee foraging. Finally, a field trial was conducted to test effects of fungicides and microbes on pollination.
3. As bloom progressed, bacterial and fungal abundance and diversity increased across all floral tissue types and management strategies. The magnitude by which microbial abundance and diversity were affected varied, with proximity to apiaries and orchard management having notable effects on bacteria and fungi respectively.
4. Experiments revealed that fungicides reduced nectar removal by honey bees; however, neither fungicide nor microbe treatments affected pollination, as measured through pollen tube initiation and growth.
5. *Synthesis and applications*. Our results reveal that microbiota associated with flowers of a pollinator-dependent crop are temporally dynamic and sensitive to management practices. However, pollination services in almond may be resilient to both agrochemical disturbance and microbial augmentation of flowers, the latter of which may become more prominent as microbial solutions to disease management are embraced in agroecosystems.

KEYWORDS
agricultural landscapes, ecosystem services, flower microbiome, fungicide, pollination, *Prunus dulcis*, sustainable agriculture

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1 | INTRODUCTION

Crop tissues harbour distinct microbiomes that affect host health and yield. Despite such recognition, research on microbiome assembly and function is only recently beginning to move away from efforts on cataloguing diversity, to those that inform direct manipulations that more precisely improve crop performance, food safety, associated ecosystem services and sustainability (Allard & Micallef, 2019; Busby et al., 2017; French et al., 2021; Mueller & Sachs, 2015; Toju et al., 2018). Importantly, holistic assessments of crop microbiomes under diverse growing conditions, coupled with direct manipulation of microbial associates and the monitoring of their effects, are needed to advance progress on microbiome-informed management strategies for the crops of tomorrow (French et al., 2021).

Among plants tissues, the flower and its associated microbiome represents an important target for both study and management (Burgess & Schaeffer, 2022; Vannette, 2020). While flowers are not necessarily sterile upon anthesis (Vannette, 2020), it is after opening that flowers tend to acquire their microbiome, with microbes dispersing to floral tissues via air, water or insect visitation (Keller et al., 2021; Thomson & Gouk, 1992). Recent evidence also suggests a role for within-host emigration via the vasculature (Kim et al., 2019; Massoni et al., 2021). Given that some flower microbes are plant pathogens, with flowers serving as primary sites of infection for orchard crops, the bloom window is often a period of intense disease management, with fungicides, bactericides or other agents applied. Beyond flower-invading pathogens, however, flowers can also harbour a diversity of commensal and beneficial microbial taxa (Vannette, 2020). Evidence is accruing to suggest that factors such as host genotype, surrounding vegetation and management practices can shape the assembly and composition of the floral microbiome (Schaeffer et al., 2021; Wei et al., 2022; Wei & Ashman, 2018). For instance, application of fungicides can have nontarget effects on member constituents (Álvarez-Pérez et al., 2016; Bartlewicz et al., 2016; Schaeffer, Vannette, et al., 2017) and pollinators (Frazier et al., 2015; Johnson, 2015). Whether agrochemical effects on microbiome structure affect pollinator behaviour or pollination services remains an open question.

Approximately 35% of crops produced globally benefit from pollination by arthropods (Klein et al., 2007). With an economic value exceeding $300 billion world-wide (Lautenbach et al., 2012), there is strong incentive to manage pollination of flowering crops during bloom. Recently, this includes incorporation of flowering strips, hedgerows and nesting structures in the cropping landscape in effort to support native pollinator populations and the services they could potentially provide (Kremen et al., 2019; Scheper et al., 2015; Williams et al., 2015). Given that pollinators harbour distinct microbiomes and disperse microbes among flowers, practices that influence the abundance, diversity and activity of pollinators in a cropping system could translate to effects on floral microbiome assembly (Russell et al., 2019; Vannette & Fukami, 2017). Furthermore, addition of flowering strips, which can harbour source populations of flower microbes, could also affect floral microbiome assembly and composition (Lindow & Andersen, 1996; Lymperopoulou et al., 2016; Pathma et al., 2021).

Here, we assessed how agricultural management affected flower microbiome assembly and function in mass-flowering almond (*Prunus dulcis*). In California (CA, United States), approximately 470,000 ha are dedicated to almond production, yielding over 80% of the world's supply and $5.8B in farmer revenue (California Department of Food and Agriculture, 2019; Sumner et al., 2014). Despite these numbers, fruit set in almond orchards typically ranges from 20% to 40% (Pitts-Singer et al., 2018), partly due to limited pollinator availability and the quality of services they provide, in addition to disease and inclement weather during bloom. Almond orchards are heavily managed prophylactically during bloom for prevention of pathogens, namely *Monilinia laxa*, the causal agent of brown rot blossom blight. Synthetic fungicides are typically applied to preempt *M. laxa* establishment in conventional orchards (Adaskaveg et al., 2017). However, increasing demand for sustainably produced almonds has spurred adoption of alternative management tactics in many orchards (Brodt et al., 2009; Plattner & Perez, 2013), including use of copper and microbial biological control agents instead of synthetics for disease control (Crowley-Gall et al., 2021). Given that pollinators of almond, including honey bees and bumble bees, are sensitive to the chemical alterations that floral microbes induce through metabolism (Rering et al., 2018; Schaeffer et al., 2019; Schaeffer, Mei, et al., 2017), shifts in microbiome structure arising from different management schemes, including direct microbial augmentation, may affect pollination services (Herrera et al., 2013; Schaeffer & Irwin, 2014; Vannette et al., 2013).

To address these linkages, we first conducted a field survey of microbial diversity associated with floral tissues of almond in orchards with different management schemes. To do so, we investigated orchards with conventional or organic management practices, including a subset that have augmented floral resources to improve native pollinator populations. We then conducted two complementary experiments to examine how agrochemical residues and microbial augmentation of flowers could impact almond pollination, focusing on honey bee foraging and the services they provide. Since evidence has indicated that honey bee behaviour can be affected by floral microbes (Good et al., 2014; Rering et al., 2018), it follows that modification of the microbiome could affect pollination services and yield outcomes in this pollinator-dependent crop. Overall, our results provide evidence that variation in management of a mass-flowering crop can shape microbiome assembly and diversity; however, pollination services may be robust in the face of diverse management strategies employed during bloom.

2 | MATERIALS AND METHODS

2.1 | Orchard survey

We surveyed 14 orchards (four organic, five conventional and five with supplemental forb plantings) across the Sacramento Valley of CA (Figure 1; Table S1). Supplemental forb plantings included a mix

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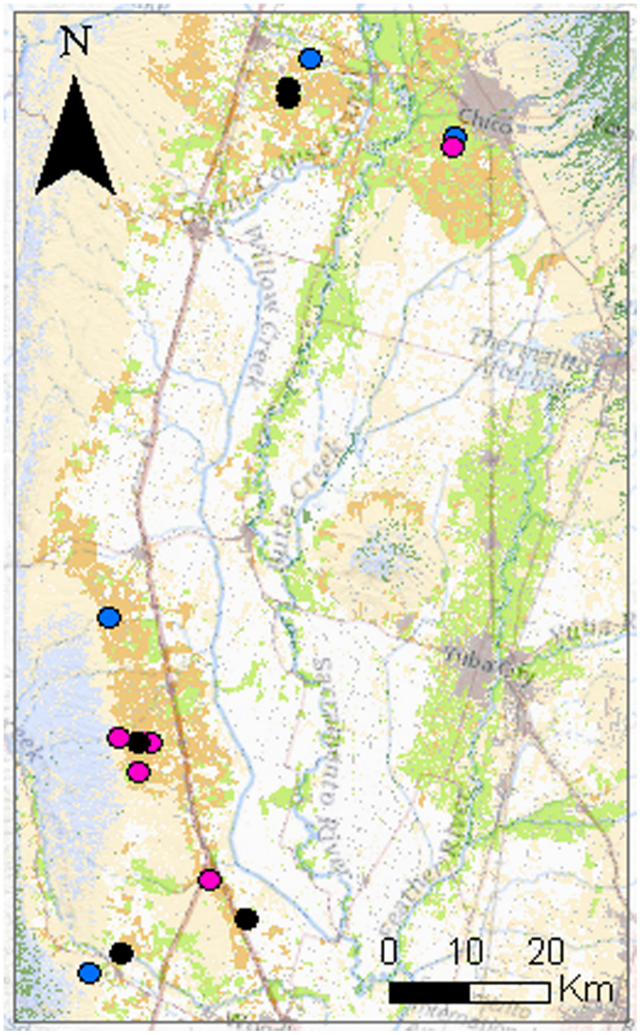

FIGURE 1 Map of the study area, showing heterogeneity in land-cover classes (Almond: Brown, Forest: Green; Shrubland: Blue; Grass/Pasture: Tan) across the Sacramento Valley of California, a major production area for almond in the United States. Points represent sampled orchards and are colour coded by management scheme (Conventional: Black; Forb-amended: Purple; Organic: Blue). Forb-amended orchards are conventionally managed, except have supplemental forb plantings on their border.

of annual species native to CA, including *Calandrinia ciliata*, *Collinsia heterophylla*, *Eschscholzia californica*, *Nemophila maculata*, *Nemophila menziesii*, *Phacelia campanularia* and *Phacelia ciliata*. Beyond the addition of forb plantings, forb-amended orchards follow conventional management practices, which typically employ the use of synthetics for disease and pest control, as well as intense orchard floor management given the nature in which almonds are harvested and associated food safety concerns (Table S2). Orchards were sampled twice between 15 and 24 February 2017, once at early bloom (~10% of flowers open) and then at peak bloom (>50% of flowers open). At each orchard and sampling event, six trees ('Nonpareil' variety) were sampled; three near the edge of the orchard, and adjacent to the forb planting if available. These trees were located in the second row in from the orchard edge ('edge'). The other three trees were sampled from the orchard interior (row 10; 'interior'). We chose this sampling scheme because semi-natural habitat in the surrounding landscape can increase visitation by native pollinators such as bees and flies (Klein et al., 2012). Pollinators can also be important dispersal agents for microbes (Aizenberg-Gershtein et al., 2013; Vannette & Fukami, 2017); thus, we may see greater microbial abundance or diversity in flowers that are in close proximity to these natural habitats. For each site (edge or interior) and sampling event, 30 open flowers ($N = 10$ per tree) were collected using aseptic technique and then pooled at the site level. Flowers with flat, fully reflexed petals that had been open for approximately 3 days were chosen for collection (Yi et al., 2006). This choice increased the probability that flowers had been visited by pollinators that disperse microbes. Once collected, flowers were placed in a cooler and transferred to the laboratory, then stored at 4°C until processing (within 24 h). In sum, four pooled flower samples were collected for each orchard: early (edge vs. interior) and peak (edge vs. interior) bloom.

To assess effects of pollinator foraging on the floral microbiome, we used two proxies for pollinator activity in orchards. First, during flower collection, we measured the average distance between trees sampled and the nearest set of honeybee hives in the orchard. Honey bees often forage near their hive (Gary et al., 1978); thus, we assumed that tree proximity to the hive would be a proxy for honey bee visitation frequency. Second, we measured the percent cover of semi-natural habitat within a 1 km buffer of each orchard, as prior work has shown that increased semi-natural habitat can increase the diversity and visitation rates of native pollinators in almond orchards (Klein et al., 2012; but see Lundin et al., 2017). We classified land cover from the Cropland Data Layer product (USDA, 2017) within a 1 km radius of each orchard edge using ArcGIS (ESRI, Redlands). Natural habitat near orchards in this study can primarily be classified as chaparral, oak woodland or valley and foothill riparian woodland (Barbour et al., 1977). We predicted that more semi-natural habitat would promote more diverse floral microbiomes as pollinator species harbour distinct microbiomes (Graystock et al., 2017; Koch et al., 2013) and disperse microbes to flowers (Russell et al., 2019; Vannette & Fukami, 2017).

2.2 | Sample processing

Whole flowers were dissected acropetally in sequence to minimize cross-contamination, as previous work has shown that floral microbial communities can display taxonomic structuring across tissue types (Junker & Keller, 2015; Pozo et al., 2012). Petals were removed first using sterile forceps. Petals from all 30 flowers in a sample were then pooled in a 50 ml Falcon tube (Corning), massed for fresh weight (g), then suspended in 20 ml of 1x-0.15% PBS-Tween solution ('Petal' sample). The androecium and gynoecium (hereafter collectively referred to as 'Anther' sample) were then removed from the base of each flower, pooled in a 15 ml Falcon tube, massed, then suspended in 5 ml of 1x-0.15% PBS-Tween solution. Given the low standing nectar crop of flowers harvested, to sample nectaries, we

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'washed' each hypanthia with 2 μl of 1x-0.15% PBS-Tween solution using a pipette, and pooled for each set of 30 flowers ('Nectary' sample). Each wash was diluted with 1 ml of 1x-0.15% PBS-Tween solution. Petal and Anther samples were then sonicated (Branson CPX5800H) for 10 min to dislodge epiphytic microbes. After sonication, debris was removed from sample tubes by pouring samples through autoclaved cheesecloth into a sterile Falcon tube. Tubes containing debris-filtered samples were then centrifuged at 704 g for 10 min at 4°C to pellet microbial cells. We then decanted the supernatant, re-suspended cell pellets in 1 ml of sterile PBS solution, vortexed tubes, then transferred the cell suspensions to new 1.7 ml microcentrifuge tubes.

2.3 | Microbial abundance

To estimate microbial abundance across tissue types, we used dilution plating to estimate the density of colony-forming units (CFUs) for each sample. Selective media for growth of fungi [yeast malt agar (YMA) + chloramphenicol (100 mg L^{-1})] and bacteria [R2A + cycloheximide (100 mg L^{-1})] were used. Although not all microbes are culturable, previous work suggests that most dominant species observed to be associated with flowers are culturable on these media types (Morris et al., 2020). Plates were incubated for 5 days at 25°C and colonies counted.

2.4 | DNA extraction and sequencing

Genomic DNA was extracted from samples using a ZymoBIOMICS® DNA Microprep kit (Zymo Research) at the University of California, Davis, following the manufacturer's protocol. Extracted DNA was then sent to the Centre for Comparative Genomics and Evolutionary Bioinformatics at Dalhousie University for library preparation and 16S/ITS amplicon sequencing. Amplicon sequence variants (ASVs) were assigned using DADA2 (Callahan et al., 2016). See *SI Materials and Methods* for information concerning amplification, sequencing and bioinformatic processing of data, including rarefaction (Figure S1).

2.5 | Pollination

2.5.1 | Pollination experiment one

To test effects of agrochemical residues and microbial augmentation on pollinator foraging behaviour we performed a field assay. Briefly, artificial flowers (Figure S2) designed to mimic features of those of almond were set out in an array near an apiary at the Harry H. Laidlaw Jr. Honey Bee Research Facility. Flowers were treated with 200 μl of artificial nectar in a fully crossed design, with three levels for each treatment. Artificial nectar was prepared by making a filter-sterilized 15% (w/v) glucose: fructose (1:1) solution, supplemented with 0.32 mM amino acids from digested casein (Vannette & Fukami, 2014), to mimic almond nectar (London-Shafir et al., 2003). For fungicides, treatment levels were: (1) no fungicide (control), (2) organic (copper, 7500 ppb) or (3) conventional (propiconazole, 7500 ppb), with concentration chosen based on residue analysis of almond flowers (Frazier et al., 2015). We chose propiconazole, a triazole, demethylation-inhibiting fungicide, as it is widely used in CA during almond bloom for control of brown rot blossom blight. Just prior to our study (2016), pesticide use records tracked by the Almond Board of California revealed that this fungicide had been applied on ~140,000 ha of orchard, which ranked it among the most widely used fungicides for disease control during bloom. Beyond that fact alone, we also chose this fungicide as it can be readily found in residue form in almond floral rewards (N. Williams, unpub data), as well as interact with other chemicals to affect pollinator health (Wade et al., 2019). With respect to nectar-inhabiting microbes, treatments were: (1) no bacterium or yeast (control), (2) *Neokomagataea thailandica* (bacterium) or (3) *M. reukaufii* (yeast). These species are frequently found on floral tissues and nectar, including almond (Aizenberg-Gershtein et al., 2013; Fridman et al., 2012; Schaeffer, Vannette, et al., 2017). Strains used were isolated from almond nectar or *Epilobium canum* (Onagraceae), a perennial herb native to the foothills of CA (Morris et al., 2020). Yeast and bacterial strains were cultured on YMA and R2A, respectively, and grown at 25°C. Individual colonies from 3-day-old cultures were then used to inoculate control or fungicide treated artificial nectars at a concentration of *c*. 400 cells μl^{-1}, after which nectars were incubated for an additional 3 days before use in a foraging assay. For each assay performed, a new set of treated nectars were prepared using the steps above.

Experimental arrays were set ~1–2 m from the hives at the apiary in the morning each day the experiment was performed. Two hours after the start of the experiment each day the remaining nectar from each flower's tube was capped, brought back to the laboratory, and weighed to estimate changes in volume. This assay was performed four times ($N = 9$ flowers per treatment combination per assay). See *SI Materials and Methods* for full experimental details.

2.5.2 | Pollination experiment two

We performed an in-vivo field assay at an orchard (Orchard 13) to test for consequences of agrochemical residues and microbial augmentation of nectar on the quality of pollination services, using measures of pollen tube initiation and growth as proxies. Briefly, fungicide/microbe treatments mirrored those used in the first pollination assay, with treatment identity randomized among nine unvisited flowers within an individual tree ($N = 20$ 'Nonpareil' variety trees, spaced across alternating rows, with five haphazardly selected in each row). Two microliters of treated nectar was applied to each flower, and after 2 days of exposure to pollinators, flowers were carefully removed along with the pedicel and placed in individual 1.5 ml microcentrifuge tubes containing 0.5 ml of water. This

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treatment volume approximates that of 24 h, per flower nectar production in Nonpareil (Abrol, 1995), as well as falls within the range of production volumes observed for related cultivars (Abrol, 1995; Karunakaran et al., 2021). Flowers were positioned such that the stigma did not touch the tube's surface and the pedicle was in water (Brittain et al., 2013). Once returned to the laboratory, flowers were stored in the dark at room temperature for 72 h to allow pollen tube growth. After this period, pistils were fixed (Farmer's fixative) and then stored at 4°C until further processing. Pollen tube growth was assessed using a staining and microscopy procedure, following a previously established protocol (Brittain et al., 2013). See *SI Materials and Methods* for full experimental details. An important caveat to note is that our study did not control for background levels of fungicide application in this conventional orchard, including use of cyprodinil (contact) and metconazole (locally systemic) during the bloom window (Table S2).

2.6 | Statistical analyses

All analyses were performed in R v.4.0.2 (R Core Team, 2013). We fit linear mixed-effect models with the LME4 package (Bates et al., 2014) to assess the impact of orchard management, and other measured variables on microbial abundance (log-10 transformed CFU counts) and diversity (ASV richness and Shannon diversity index). For each model and response variable examined, management, bloom stage (early/peak), site (edge/interior) and a three-way interaction among them, along with geographic zone (north/south), amount of semi-natural habitat surrounding orchards, and distance to the nearest apiary were included as predictors, with orchard identity as a random effect to account for repeated sampling. Geographic zone was included as a variable to account for microclimatic differences between the northern and southern orchards sampled (Figure 1), as temperature was found to differ significantly between the two sets of sites at both early ($t_{8.96} = 3.40$, $p = 0.008$) and peak ($t_{9.43} = -3.41$, $p = 0.007$) bloom (PRISM Climate Group, Oregon State University, http://prism.oregonstate.edu). Bacterial and fungal data were analysed separately for each floral tissue examined. Once fit, we used backward stepwise model selection in the LMERTEST package (Kuznetsova et al., 2017) to identify the best-fit model for each response variable examined. Log-likelihood ratio tests were used to determine which fixed model terms were retained in best-fit models, with significance of each calculated using *F* tests, based on the Satterthwaite approximation for denominator degrees of freedom (Kuznetsova et al., 2017). Finally, to determine if the relative abundance of individual ASVs responded to orchard variables of interest, we used *DESeq2* with Benjamini–Hochberg corrections for multiple testing (Love et al. 2014). Orchard management, bloom stage and apiary distance were treated as predictors in separate models.

Pairwise dissimilarities between fungal and bacteria communities were calculated using the Bray–Curtis dissimilarity metric. We then used permutational multivariate analysis of variance (PERMANOVA) to assess the contribution of management, bloom stage (early/peak), site (edge/interior) and all (two- and three-way) interactions among them, along with amount of semi-natural habitat surrounding orchards and distance to the nearest apiary on community composition. This analysis was performed using *vegan*, based on 1000 permutations (Oksanen et al., 2013).

For the behaviour assay (*Pollination Experiment One*), we fit a linear mixed-effects model with nectar remaining as the response, fungicide and microbe treatments as fixed factors, as well as their interaction. Floral stake identity was treated as a block and nested within trial number as a random effect. For the in-orchard pollination service assay (*Pollination Experiment Two*), we also fit linear mixed-effects models with pollen germination and number of tubes as response variables, fungicide and microbe treatments as fixed factors, as well as their interaction. Tree identity, nested within tree row, was included as a random effect.

3 | RESULTS

3.1 | Microbial abundance and diversity

Floral microbial abundance and diversity increased from early to peak bloom across all tissue types, and management strategies, for both bacteria and fungi (Tables S3–S5). The magnitude in which microbial abundance increased, however, varied considerably among these factors. Culturable bacterial CFU abundance from Petal, Anther and Nectary samples were 11- ($F_{1,54} = 647.71$, $p < 0.0001$), five- ($F_{1,41} = 489.86$, $p < 0.0001$) and eightfold ($F_{1,54} = 929.91$, $p < 0.0001$) higher at peak bloom, respectively than at the initial sampling (Figure 2; Table S3). Like bacteria, fungal CFU abundance (Table S3) also increased from early to peak bloom, increasing twofold for Petal ($F_{1,40} = 104.11$, $p < 0.0001$), twofold for Anther ($F_{1,52} = 70.97$, $p < 0.0001$) and threefold for Nectary tissues ($F_{1,41} = 42.33$, $p < 0.0001$).

Beyond bloom progression, additional orchard factors affected microbial abundance, but in disparate ways for bacteria and fungi across tissue types. Culturable fungi associated with Anther tissues were 27% more abundant in organic orchards than those that were conventional or forb-amended in management practice ($F_{2,52} = 4.61$, $p = 0.01$). Moreover, regardless of management scheme, fungi associated with Petals were 11% more abundant along the edges of orchards compared to the interior ($F_{1,40} = 4.36$, $p = 0.04$). In contrast, and regardless of tissue type, management scheme had no detectable effect on bacterial abundance, nor did sampling location within orchards or apiary distance. For bacteria associated with Anthers, however, significant effects of landscape cover (i.e. amount of semi-natural habitat) and geographic zone were detected (Table S3).

Bacterial diversity increased as bloom progressed for communities associated with Petal (richness: $F_{1,54} = 8.85$, $p < 0.01$; Shannon index: $F_{1,54} = 6.05$, $p = 0.02$), Anther (richness: $F_{1,35.60} = 25.99$, $p < 0.0001$; Shannon index: $F_{1,51} = 33.28$, $p < 0.0001$) and Nectary (richness: $F_{1,40.59} = 103.91$, $p < 0.0001$; Shannon index: $F_{1,40.63} = 25.73$, $p < 0.0001$; Figure 3; Tables S4 and S5) tissues. All

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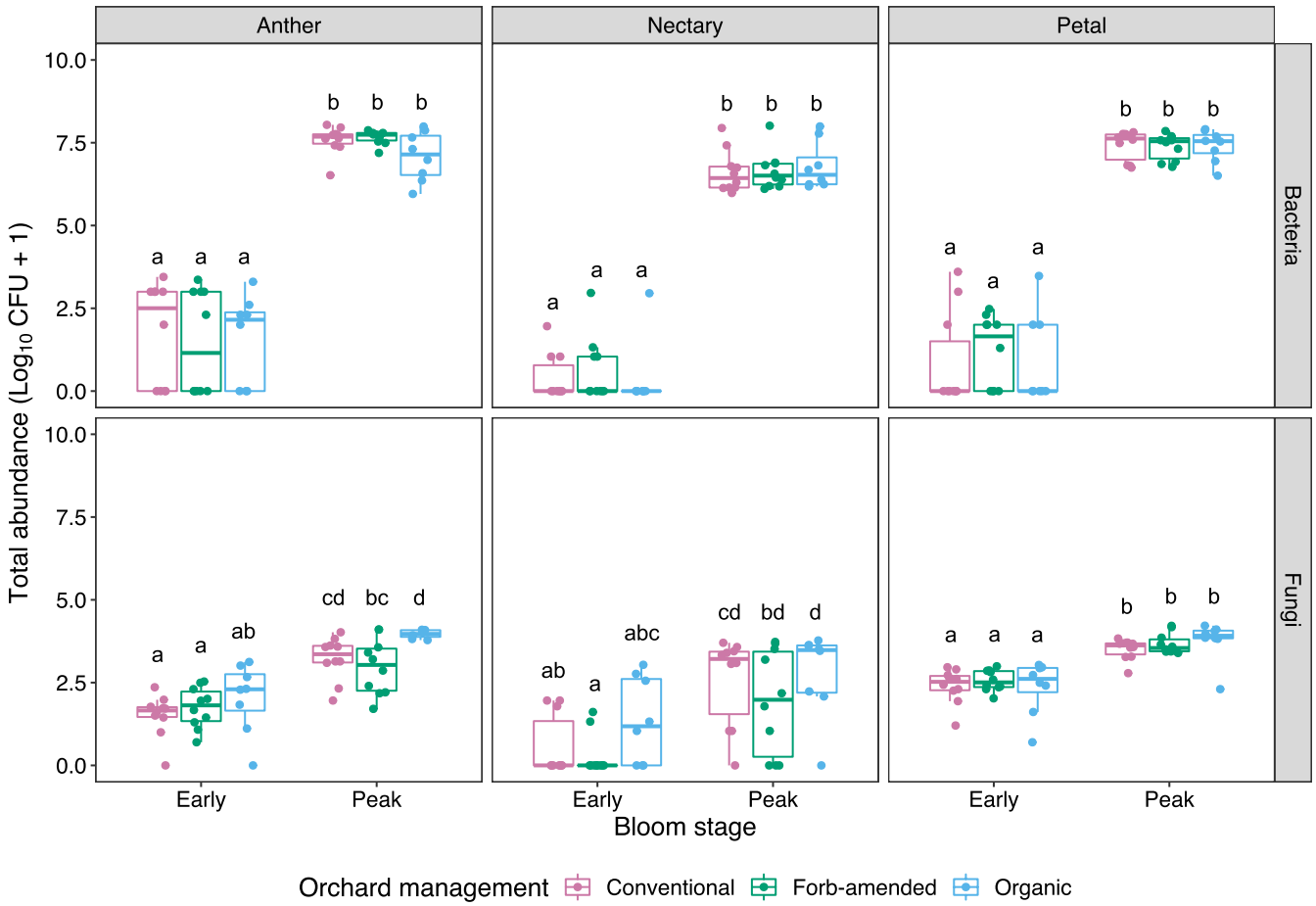

FIGURE 2 Abundance of colony-forming units (CFUs) on R2A media (bacteria) and YMA (fungi) associated with floral tissues (Anther, Nectary or Petal) of almond. Flowers were sampled at two stages of bloom (Early or Peak) from orchards that employed different management schemes (Conventional, Forb-amended or Organic). Forb-amended orchards are conventionally managed, except have supplemental forb plantings on their border. Lowercase letters denote differences between treatments.

tissues were dominated by Proteobacteria (Figure 4), particularly members of the Pseudomonadaceae, with two taxa being enriched from early to peak bloom overall (BactSeq2: *Pseudomonas* sp., log2-fold change = 2.50, $p_{adj} < 0.001$; BactSeq10: *Pseudomonas* sp., log2-fold change = 3.64, $p_{adj} < 0.01$). Tree proximity to apiaries within an orchard was found to be positively associated with bacterial diversity, although the association was weak (Figure S3A): Shannon diversity was higher on flowers closer to apiaries than those that were collected further away, although a significant effect was only detected for Anther ($F_{1,51} = 7.39$, $p < 0.0001$) samples in our models. Finally, Nectary samples collected from orchards in the northern zone were more diverse than those in the south (richness: $F_{1,11.16} = 8.86$, $p = 0.01$; Shannon index: $F_{1,11.10} = 12.79$, $p < 0.01$).

With the exception of organic orchards, fungal ASV richness generally increased over bloom (Figure 3). This trend was significant for Nectary (richness: $F_{1,52} = 7.51$, $p = 0.01$) and Petal (richness: $F_{1,41} = 5.32$, $p = 0.03$) tissues. Shannon diversity followed a similar pattern, with a significant increase observed for Nectary ($F_{1,52} = 11.29$, $p < 0.01$) and Petal samples; however, for the latter this effect depended on orchard management scheme (Management × Bloom stage: $F_{2,39} = 3.50$, $p = 0.04$). Specifically, fungal diversity (Shannon index) increased in both conventional and forb-amended orchards by 59% and 12% respectively, while decreasing in organic orchards by 26%. Other orchard-level variables examined, such as apiary distance and site sampled within orchards, generally had no effect on observed fungal ASV richness or Shannon diversity (Tables S4 and S5). However, as with nectary bacteria, we observed that fungal diversity was greater in northern sites compared to those in the south (richness: $F_{1,52} = 11.64$, $p < 0.01$; Shannon index: $F_{1,52} = 11.49$, $P < 0.01$). We also detected no correlation between apiary distance and either diversity metric (Figure S3b); however, DESeq2 analyses revealed *Vishniacozyma carnescens* (syn. *Cryptococcus carnescens*) ASVs that significantly declined in abundance with tree distance from apiaries (FunSeq13: log2-fold change = −0.02, $p_{adj} < 0.001$; FunSeq58: log2-fold change = −0.02, $p_{adj} < 0.01$).

Fungal communities associated with floral tissues were generally dominated by members of the Aureobasidiaceae and Bulleribasidiaceae (Figure 4), including *Aureobasidium pullulans*, *V. victoriae* (syn. *C. victoriae*) and *V. carnescens*. Over bloom, *A. pullulans* in particular was found to significantly increase in relative abundance (FunSeq1: log2-fold change = 2.96, $p_{adj} < 0.0001$), along with *Gelidatrema spencermartinsiae* (syn. *C. spencermartinsiae*, FunSeq16:

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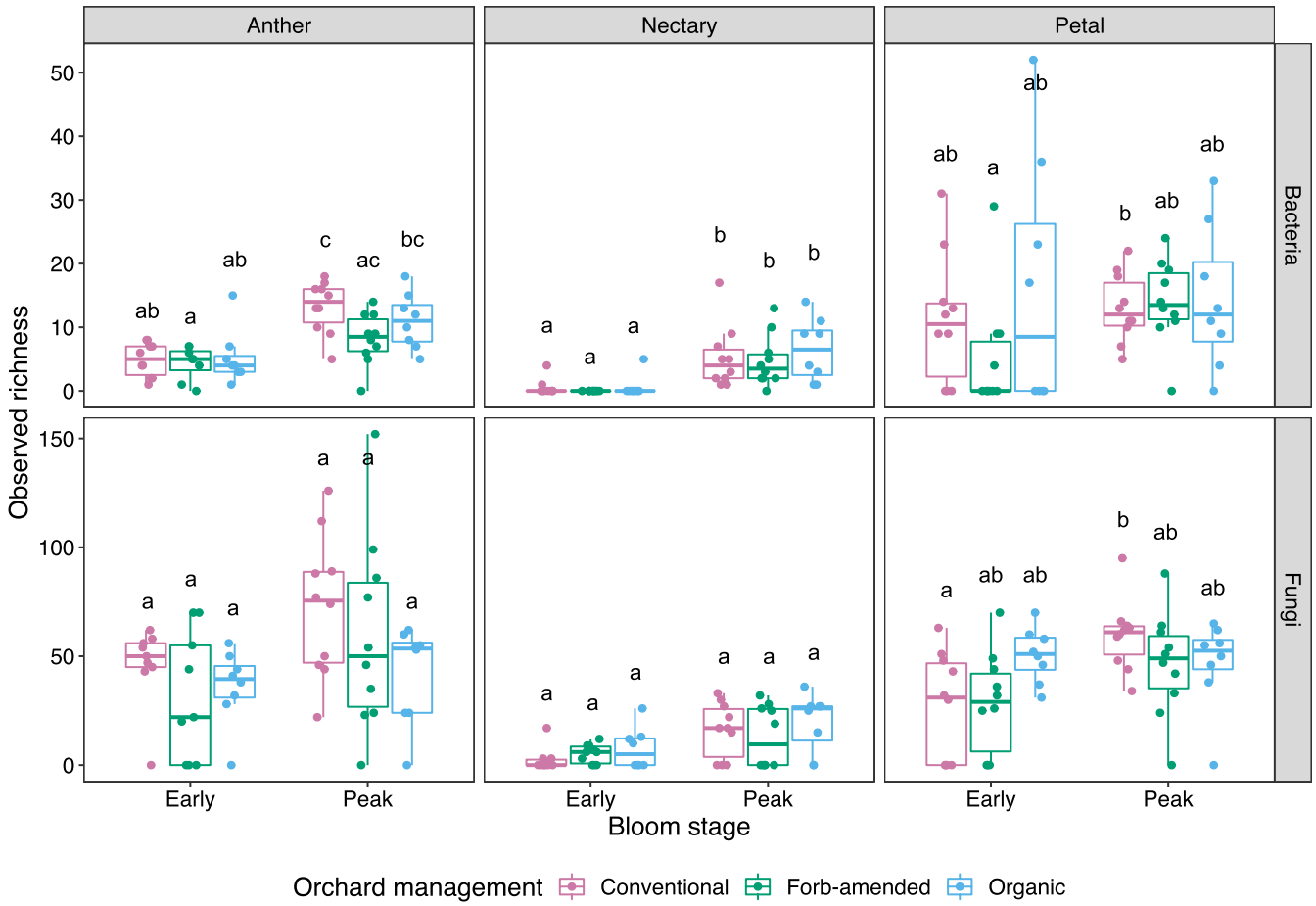

FIGURE 3 Observed sequence variant richness of bacteria and fungi associated with floral tissues (Anther, Nectary or Petal) of almond flowers. Flowers were sampled at two stages of bloom (Early or Peak) from orchards that employed different management schemes (Conventional, Forb-amended or Organic). Forb-amended orchards are conventionally managed, except have supplemental forb plantings on their border. Lowercase letters denote differences between treatments. Note differences in scale of *x* and *y* axes.

log2-fold change = 2.27, $p_{adj} < 0.01$), *Filobasidium wieringae* (syn. *C. wieringae*, FunSeq34: log2-fold change = 3.87, $p_{adj} < 0.0001$) and *Buckleyzyma aurantiaca* (syn. *Rhodotorula aurantiaca*, FunSeq26: log2-fold change = 1.14, $p_{adj} < 0.01$). Taxa observed to significantly decline in relative abundance included *Cladosporium delicatulum* (FunSeq3: log2-fold change = −1.52, $p_{adj} < 0.001$), a widely distributed saprobe species, and *Naganishia friedmannii* (syn. *C. friedmannii*; FunSeq27: log2-fold change = −3.28, $p_{adj} = 0.02$).

Bacterial and fungal species composition differed between sampling times (Table 1; Figure 5), with bloom stage explaining 3–21% of variation in composition, depending on flower tissue. Orchard-level predictors (e.g. semi-natural habitat) generally explained less variation in composition (Table 1). Orchard management was not associated with bacterial species composition, but did predict variation in fungal composition in Anther ($R^2 = 0.073$, $F_{2,32} = 2.22$, $p = 0.003$) and Petal ($R^2 = 0.078$, $F_{2,32} = 2.79$, $p = 0.001$) samples. The amount of semi-natural habitat in the surrounding landscape, apiary distance, as well as geographic zone were generally found to be associated with shifts in bacterial and fungal community composition, with a particularly notable effect of apiary distance on bacteria in Nectary samples ($R^2 = 0.176$, $F_{2,19} = 5.90$, $p = 0.002$).

3.2 | Microbial and fungicide effects on honey bee foraging and pollination

Fungicides reduced the amount of nectar removed by honey bee foragers (Figure 6; $F_{2,280} = 127.25$, $p < 0.0001$). In contrast, microbes were observed to have no effect on nectar removal ($F_{2,280} = 1.73$, $p = 0.18$), nor did we detect a significant interaction between microbial inoculation and fungicide treatment ($F_{4,280} = 1.87$, $p = 0.12$). In the orchard experiment, neither fungicide application nor nectar-inhabiting microbes affected pollen germination or pollen tube number (Table S6).

4 | DISCUSSION

The results presented here demonstrate that orchard management practices can mediate flower microbiome structure, although the magnitude of these effects can hinge on a variety of factors. Of those examined in this study, timing of bloom was the most consistent predictor: both culturable bacterial and fungal abundance, as well as sequence-based estimates of diversity, were higher at peak

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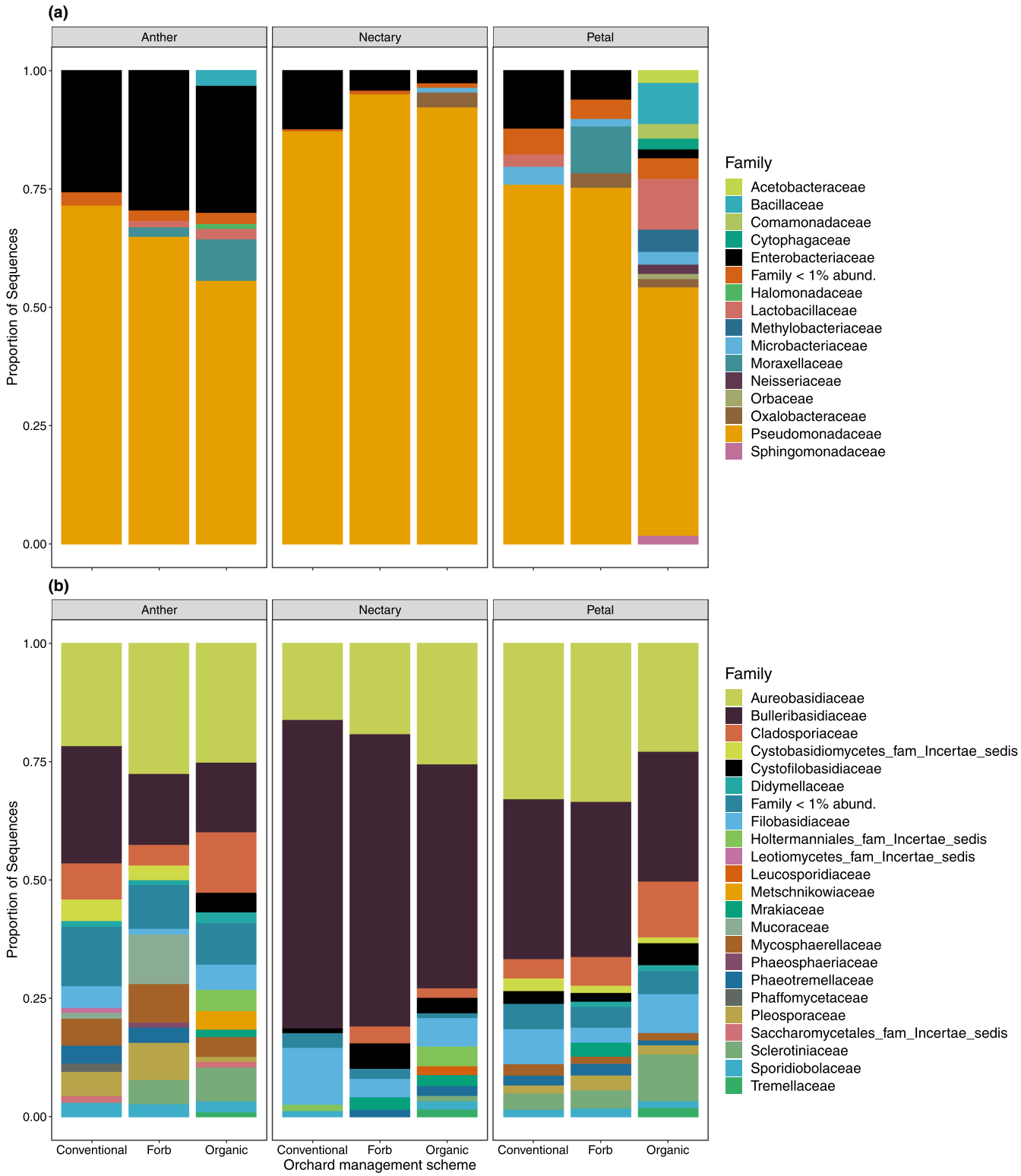

FIGURE 4 Average relative abundance (Proportion of Sequences) of (a) bacterial and (b) fungal families associated with floral tissues (Anther, Nectary or Petal) of almond. Flowers were collected from orchards that vary in management scheme (Conventional, Forb-amended or Organic). Forb-amended orchards are conventionally managed, except have supplemental forb plantings on their border.

bloom than at the start across all floral tissue types and management strategies. Sampling time with respect to bloom intensity has been documented previously (e.g. Smessaert et al., 2019), and may be driven by variation in a combination of interrelated variables, including temperature, pollinator activity and host plant metabolism (Belisle et al., 2012; Pozo et al., 2014). However, other orchard-level variables affected microbial abundance and diversity, with host proximity to apiaries and orchard management having significant

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TABLE 1 PERMANOVA results of Bray–Curtis dissimilarity between bacterial and fungal communities associated with almond floral tissues (Anther, Nectary and Petal) collected from orchards that vary in management scheme (conventional, forb-amended or organic).

Bacteria	Anther			Nectary			Petal		
Variable	R^2	F	p	R^2	F	p	R^2	F	p
Management	0.043	1.12	0.319	0.039	0.66	0.703	0.061	1.33	0.112
Bloom stage	0.033	1.74	0.060	0.075	2.53	0.062	0.069	3.03	0.001
Site	0.024	1.25	0.239	0.030	1.00	0.369	0.018	0.79	0.710
Management × Bloom stage	0.022	0.57	0.960	0.034	1.14	0.387	0.063	1.38	0.095
Management × Site	0.028	0.74	0.826	0.022	0.38	0.949	0.038	0.84	0.720
Bloom stage × Site	0.013	0.68	0.783	–	–	–	0.026	1.14	0.294
Management × Bloom stage × Site	0.044	1.15	0.287	–	–	–	0.028	0.62	0.963
Natural habitat	0.041	2.14	0.010	0.010	0.35	0.851	0.022	0.95	0.478
Apiary distance	0.017	0.92	0.519	0.176	5.90	0.002	0.031	1.40	0.134
Geographic zone	0.035	1.82	0.041	0.045	1.51	0.202	0.055	2.43	0.002
Fungi	**Anther**			**Nectary**			**Petal**		
Variable	R^2	F	p	R^2	F	p	R^2	F	p
Management	0.073	2.22	0.003	0.077	1.61	0.050	0.078	2.79	0.001
Bloom stage	0.137	8.32	0.001	0.145	6.05	0.001	0.210	15.08	0.001
Site	0.016	0.99	0.415	0.035	1.45	0.146	0.012	0.89	0.507
Management × Bloom stage	0.056	1.72	0.014	0.049	1.10	0.329	0.038	1.35	0.150
Management × Site	0.035	1.07	0.357	0.071	1.51	0.066	0.019	0.67	0.849
Bloom stage × Site	0.020	1.25	0.182	0.041	1.81	0.069	0.016	1.15	0.298
Management × Bloom stage × Site	0.033	1.00	0.440	0.046	1.03	0.416	0.021	0.77	0.755
Natural habitat	0.029	1.74	0.035	0.052	2.16	0.017	0.052	3.70	0.003
Apiary distance	0.032	1.93	0.010	0.029	1.20	0.290	0.056	4.01	0.002
Geographic zone	0.044	2.69	0.002	0.024	0.54	0.869	0.052	3.72	0.003

effects on both bacteria and fungi respectively. More specifically, organic practices tended to promote fungal abundance, likely due to the relaxed pressure imposed by fungicides or other agents.

The results described here support a role for pollinator foraging in the assembly of the almond flower microbiome. In our orchard survey we detected a signature of tree proximity to apiaries on epiphytic bacterial diversity of almond flower nectary and reproductive structures, as both bacterial richness and diversity decreased the further trees were from apiaries. Within orchards, honey bees generally forage more intensively near their apiary (Gary et al., 1978), which could perhaps explain the pattern observed, as honey bees disperse bacteria among more local flowers, generating spatial variation in microbial transmission and resulting flower microbial communities. Moreover, while foraging on flowers, individual honey bees can display consistent behaviours, including a focus on either pollen or nectar collection, which would affect the degree of physical contact with intrafloral tissues (Bosch & Blas, 1994; Thomson & Goodell, 2001). Finally, although contact with petals can occur as foragers land and side-work for nectar (Thomson & Goodell, 2001), these collective behaviours may explain the lack of a significant effect of apiary distance on bacterial diversity observed on petals versus nectar and reproductive structures of the flower; however, this was not tested directly in the current study and would make for a good future investigation. In sum, these patterns point to increased consideration of both apiary spacing, and pollinator foraging behaviour, as interest grows in leveraging pollinators as vectors of microbial biocontrol agents to combat disease (Kevan et al., 2008; Menzler-Hokkanen & Hokkanen, 2017).

Honey bee foraging was affected by fungicides, but not nectar-inhabiting microbes, in contrast to previous work (Colda et al., 2021; Good et al., 2014; Rering et al., 2018). This lack of a microbial effect can likely be attributed to fungicide effects on microbial establishment and growth, as observed in previous studies with *Metschnikowia* spp. (Álvarez-Pérez et al., 2016; Bartlewicz et al., 2016; Schaeffer, Vannette et al., 2017), although not directly measured here or in the second pollination experiment. Regardless of fungicide type, or the presence of nectar microbes, honey bees removed less nectar from artificial flowers with copper and propiconazole residues, demonstrating that fungicide application and residual contamination of floral rewards can affect forager decisions. Reduced foraging in the presence of fungicides has also been observed in cranberry (Jaffe et al., 2019), with honey bee foragers reducing pollen collection in response to fungicide application. This aversion to fungicides may not be universal, however, as honey bees

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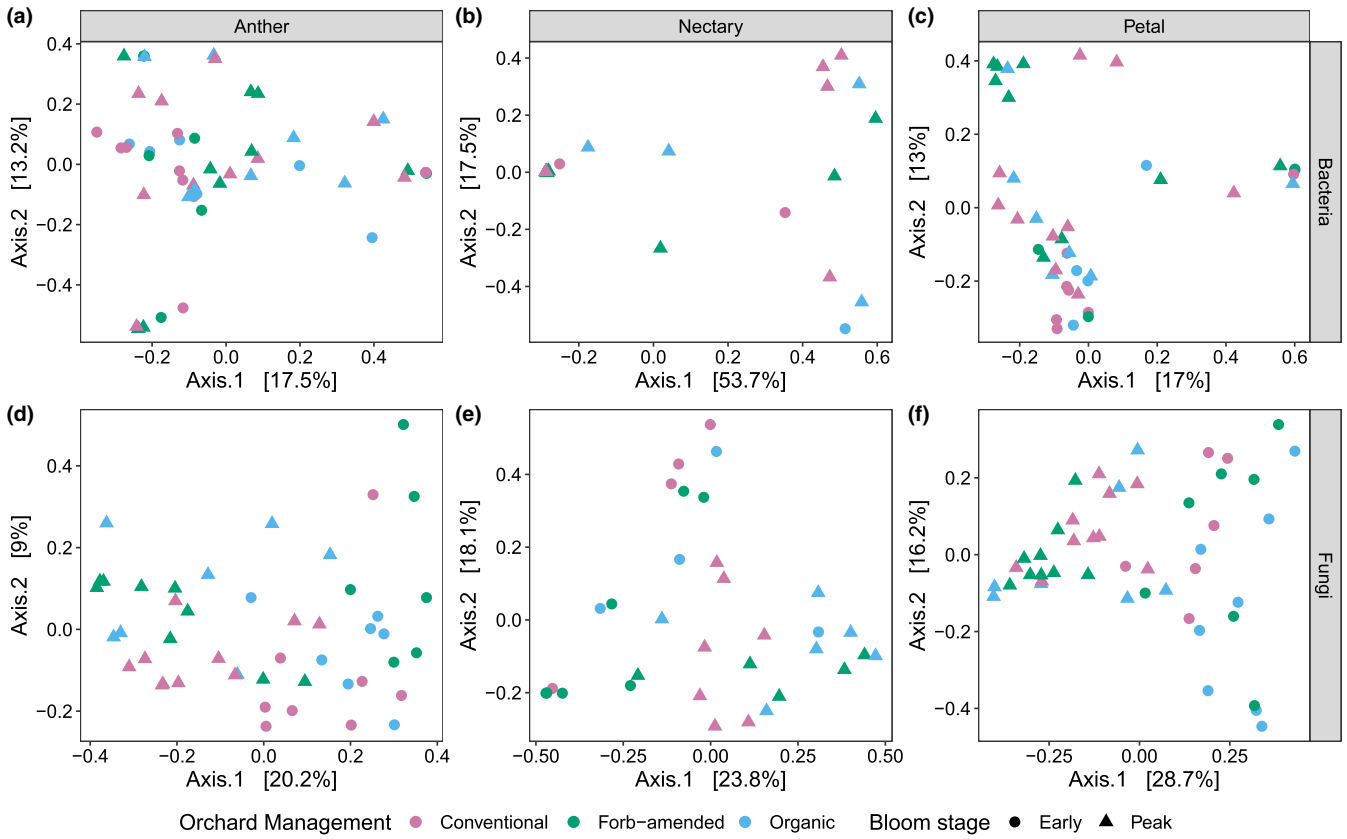

FIGURE 5 Principle coordinate analysis (PCoA) of Bray–Curtis distances for bacterial (a–c) and fungal (d–f) microbiomes associated with almond floral tissues (Anther, Nectary and Petal). Flowers were collected at early and peak bloom from orchards that varied in management scheme (Conventional, Forb-amended and Organic). Forb-amended orchards are conventionally managed, except have supplemental forb plantings on their border. Note differences in scale of *x* and *y* axes.

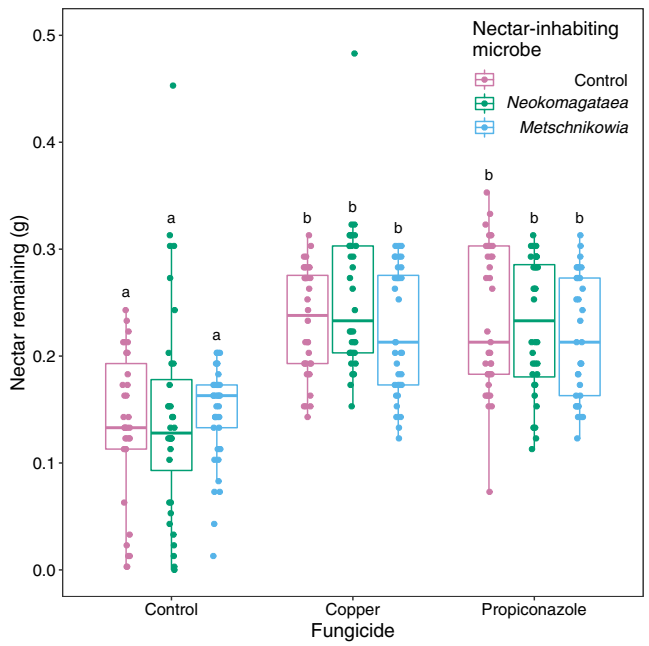

FIGURE 6 Nectar remaining (g) in artificial almond flowers treated with fungicides (Copper or Propiconazole) and nectar-inhabiting microbes (green = bacterium *Neokomagataea thailandica*, blue = yeast *Metschnikowia reukaufii*). Lowercase letters denote differences between treatments.

have also been shown to have an attraction to chlorothalonil (Liao et al., 2017), a fungicide widely used in almond orchards (Table S2). Exposure to fungicide-contaminated rewards can affect pollinator health and pollination in agroecosystems, including almond. Such consequences range from negative effects on larval development, pollinator cognition and even mortality (Johnson, 2015), as has been observed with honey bee workers directly exposed to a range of fungicides commonly employed for pathogen control in our focal crop (DesJardins et al., 2021; Fisher II et al., 2021). While almond growers have increasingly adopted the practice of not spraying fungicides during windows of active honey bee foraging, residues can persist on flowers and still have sublethal effects.

Although fungicide residues in nectar deterred honey bees, these shifts in foraging behaviour may not translate to noticeable effects on pollination services in almond. In our in-vivo pollination assay, we found no noticeable effect of fungicide residues, or nectar-inhabiting microbes, on pollen germination or tube number, effective proxies for reproductive success in this system (Brittain et al., 2013). In a related study involving nectar-inhabiting microbes, Colda et al. (2021) also observed a lack of an effect of nectar-inhabiting microbes on yield of pear (*Pyrus communis*), despite a positive response of both honey bees and hoverflies to flowers augmented with yeast and bacteria. With respect to almond, honey bee workers often alternate between foraging for

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nectar or pollen (Bosch & Blas, 1994). These nectar and pollen foragers differ markedly in the quality of services that they provide, with pollen foragers being on average five times more effective in affecting fruit set than those foraging for nectar (Bosch & Blas, 1994). Given that our flowers were exposed for a long enough duration to allow pollinators to forage for both resources, those that foraged for pollen alone likely conferred adequate pollination observed in our experiment. Moreover, our findings, as well as that of Colda et al. (2021), suggest that tree fruit yields may be more likely constrained by resource availability and balanced investment across the whole plant.

Our results highlight multiple orchard management practices that can shape the assembly of crop-associated microbiota during flowering and pollination. We documented temporal changes in microbial abundance and composition, but also detected effects of managed pollinators and natural areas, suggesting a role of immigration in determining species composition in many floral tissues. Combined with the potential for agrochemicals to differentially affect microbial growth and species interactions, we outline a few factors that likely contribute to flower microbiome assembly. Because flowers form the template for potential reproductive output that is translated through interactions with pollinators, understanding linkages between management, the assembly of the floral microbiome and its impact on pollination in crops may reveal how microbial interactions affect both crop yield and quality. As microbial solutions for disease and pollination management become increasingly embraced in agroecosystems, our results, as well as that of others (Crowley-Gall et al., 2021), suggest that pollination services may be resilient in the face of such changes.

AUTHOR CONTRIBUTIONS

Robert N. Schaeffer conceived the idea for the study and collaborated with David W. Crowder, John J. Beck, Tadashi Fukami, Neal M. Williams and Rachel L. Vannette in designing the survey and experiments performed. Robert N. Schaeffer carried out the survey, experiments, analyses and wrote the first draft of the manuscript. David W. Crowder and Javier Gutiérrez Illán contributed ArcGIS data and analysis. All authors contributed to revisions.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequences are available on the NCBI Short Read Archive (SRA) under BioProject PRJNA660037. Additional data on microbial abundance and pollination are archived in the Dryad Digital Repository https://doi.org/10.5061/dryad.tht76hf2s (Schaeffer et al., 2022).

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