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Research Article

RESEARCH ARTICLE

Microbial abundance, composition, and function in nectar are shaped by flower visitor identity

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One sentence summary: This study uses a floral nectar model system to show that vector identity, in this case legitimate floral pollinators versus nectar robbers, determines microbial assembly and function.

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ABSTRACT

Microbial dispersal is essential for establishment in new habitats, but the role of vector identity is poorly understood in community assembly and function. Here, we compared microbial assembly and function in floral nectar visited by legitimate pollinators (hummingbirds) and nectar robbers (carpenter bees). We assessed effects of visitation on the abundance and composition of culturable bacteria and fungi and their taxonomy and function using shotgun metagenomics and nectar chemistry. We also compared metagenome-assembled genomes (MAGs) of *Acinetobacter*, a common and highly abundant nectar bacterium, among visitor treatments. Visitation increased microbial abundance, but robbing resulted in 10× higher microbial abundance than pollination. Microbial communities differed among visitor treatments: robbed flowers were characterized by predominant nectar specialists within Acetobacteraceae and Metschnikowiaceae, with a concurrent loss of rare taxa, and these resulting communities harbored genes relating to osmotic stress, saccharide metabolism and specialized transporters. Gene differences were mirrored in function: robbed nectar contained a higher percentage of monosaccharides. Draft genomes of *Acinetobacter* revealed distinct amino acid and saccharide utilization pathways in strains isolated from robbed versus pollinated flowers. Our results suggest an unrecognized cost of nectar robbing for pollination and distinct effects of visitor type on interactions between plants and pollinators. Overall, these results suggest vector identity is an underappreciated factor structuring microbial community assembly and function.

Keywords: community assembly; nectar yeast; microbiome; microbial ecology; dispersal; *Xylocopa*; hummingbird

INTRODUCTION

Many microbes can be dispersal limited and rely on a vector, such as wind (Smith *et al.* 2013), currents (Barton *et al.* 2010; Müller *et al.* 2014) or hitchhiking on other organisms (Lussenhop 1992; Whitaker, Grogan and Taylor 2003; Belisle, Peay and Fukami 2012) for spread among habitats. Although it is well recognized that pathogenic microorganisms rely on dispersal by specific vectors for transport among hosts (Mauck *et al.* 2012), the degree to which environmental microorganisms rely on phoresis or dispersal by specialized vectors, and effects of such dispersal patterns on community patterns and function remain unclear (Lindström and Langenheder 2012; Burns *et al.* 2016). In flowers, like many other discrete habitats, microbes can be abundant (Fridman *et al.* 2012; Vorholt 2012) and their metabolism

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can influence both floral traits (Herrera, García and Pérez 2008; de Vega and Herrera 2012) and pollinators (Vannette and Fukami 2016). Like many other microbes (Whitaker, Grogan and Taylor 2003), floral nectar microbes are often dispersal limited (Belisle, Peay and Fukami 2012) and animals may be important vectors of both bacteria and fungi (Gilbert 1980; Brysch-Herzberg 2004; Herrera *et al.* 2010; Canto and Herrera 2012; Vannette and Fukami 2017). Floral visitation by birds, bees or other insects often increases microbial incidence, abundance in nectar, and changes species composition (Belisle, Peay and Fukami 2012). Subsequent environmental filtering and species interactions generate non-random communities of microorganisms (Herrera *et al.* 2010; Álvarez-Pérez, Herrera and de Vega 2012).

Microbes that disperse and successfully colonize nectar can substantially alter nectar conditions, including sugars, amino acids, pH and volatile emission (Canto and Herrera 2012; Vannette and Fukami 2018; Rering *et al.* 2019), with consequences for pollinator visitation (Vannette, Gauthier and Fukami 2013; Schaeffer *et al.* 2014). For example, the common nectar yeast *Metschnikowia reukaufii* can increase visitation to flowers by bumble bees (Schaeffer et al. 2014; Yang *et al.* 2019) while colonization by the bacterium *Neokomagataea* (Gluconobacter) can reduce pollinator visitation (Vannette, Gauthier and Fukami 2013; Good *et al.* 2014), likely due to its distinct effects on floral attractiveness. While these studies have linked microbial identity in floral nectar with measured effects on the nectar environment, and visitation frequency to divergence in microbial composition (Vannette and Fukami 2017), it remains difficult to predict what conditions, including variation in dispersal (e.g. Germain, Jones and Grainger 2019), will lead to specific microbial community structure or function.

Plant–pollinator relationships are regarded as an iconic example of mutualisms in nature. However, variation in partner quality is a common feature of mutualisms, and coevolved mutualisms are often at risk for interference by antagonists (Irwin, Adler and Brody 2004). Flowering plants are visited by pollinators, visitors that consume floral rewards and transfer pollen among flowers, but can also be visited by robbers, which consume nectar rewards without conferring pollination benefit (Irwin *et al.* 2010). Nectar robbing can decrease floral longevity, attractiveness to legitimate pollinators and reproductive success (Castro, Silveira and Navarro 2008), with consequences for the long-term stability of plant–pollinator mutualisms (Bronstein 2001). The indirect effects of robbers on nectar chemistry and microbiome structure (Zemenick, Rosenheim and Vannette 2018) and function are poorly understood, but may mediate plant reproduction and pollinator behavior and foraging (Biere and Bennett 2013; Vannette, Gauthier and Fukami 2013; Rering *et al.* 2019) and represent unrecognized mechanisms by which nectar robbers influence pollination.

Here, we examine how distinct dispersal vectors—pollinators and nectar robbers—influence floral nectar and microbial abundance and composition within nectar across flower development. We hypothesized that the two floral visitor types deposit distinct microbial communities (Ushio *et al.* 2015), because floral visitors differ in numerous aspects of biology including foraging habits (Hixon, Carpenter and Paton 1983; Gathmann and Tscharntke 2002) and therefore carry and deposit unique communities of bacteria and microfungi (Jacquemyn *et al.* 2013; Zemenick, Rosenheim and Vannette 2018), which vary in both structure and function (Robinson, Bohannan and Young 2010; Raes, Letunic and Yamada 2011). Previous work relies on culture work and amplicon studies, which may be subject to primer bias and could limit our description of nectar-inhabiting microbial communities or their functions. To overcome these potential biases, we integrate a whole shotgun metagenomics approach (Dinsdale *et al.* 2008; Gilbert and Dupont 2011) to avoid primer bias and link microbial taxa to function with metagenome-assembled genomes (MAGs; Delmont *et al.* 2015; Wilkins *et al.* 2019)—two methods that identify variation in gene content and functional potential. Our results suggest that disperser identity influences both the community structure and function of microbes in floral nectar.

MATERIALS AND METHODS

Epilobium canum (Onagraceae) is a perennial flowering shrub native to California. Red-orange flowers are produced from early June to late November, each lasting 5–8 days post-anthesis. Flowers are protandrous, presenting a male stage first, followed by hermaphroditic and then female, with each stage lasting between 2 and 3 days (Raven 1976; Snow 1986). *Epilobium canum* is primarily pollinated by hummingbirds (*Calypte anna*) but also receives frequent visits from nectar-exploiting carpenter bees (*Xylocopa californica*) that chew a slit in the corolla, facilitating secondary robbing by honey bees (M. Morris, pers. obs.). Flowers display evidence of each visitation type: hummingbird pollinators deposit pollen on stigmas, while carpenter bee robbing leaves a characteristic slit or hole in the corolla (Fig. 1). Our observations suggest that carpenter bees are unable to access nectar in the narrow tubular flower of *E. canum* without a hole in the corolla and do not deposit pollen on floral stigmas.

Floral nectar composition survey

To examine the effects of dispersal and flower age on microbial communities and nectar chemistry, we surveyed floral nectar samples from three *E. canum* populations in Davis, CA, USA, in October–November 2015. Two sites were within the UC Davis Arboretum (38°32′3″ N, 121°45′8″ W; 38°32′8″ N, 121°44′56″ W), and one semi-natural site in Davis, CA (38°33′11.9′N 121°44′27.3′W). Flowers were tagged individually before anthesis and manipulated to control dispersal by covering with nylon mesh bags to prevent visitor access to nectar, or leaving flowers exposed to allow visitation by pollinators and/or carpenter bee robbers, with at least 50 flowers per treatment (100 total) tagged at each of the three sites. Flowers were monitored daily for anthesis and progression through the floral stages, and bags preventing visitor access were draped loosely over floral parts so as not to inhibit floral development. Flowers were collected post-anthesis and classified into one of five developmental stages based on presentation of floral parts—Petal (reproductive parts not yet elongated/emerged/receptive), Male (anthers emerged and dehisced; stigma emerged but not receptive), Hermaphroditic (anthers emerged and dehisced; stigma emerged and receptive), Female (anthers emerged but not dehisced; stigma emerged and receptive) and Senescing (petals beginning to close; wilting of floral parts). At each site, 10–20 samples of each developmental stage were collected and classified into floral visitation treatments based on physical evidence—Bagged (covered with mesh bag to prevent visitor access), No Visitation (left exposed but no evidence of visitation), Pollinated (pollen deposited on stigma), Robbed (hole in corolla) and Pollinated + Robbed (pollen on stigma; hole in corolla; Fig. 1; Supplementary material S1, Supporting Information), for 202 samples collected in total. Collected flowers were kept on ice

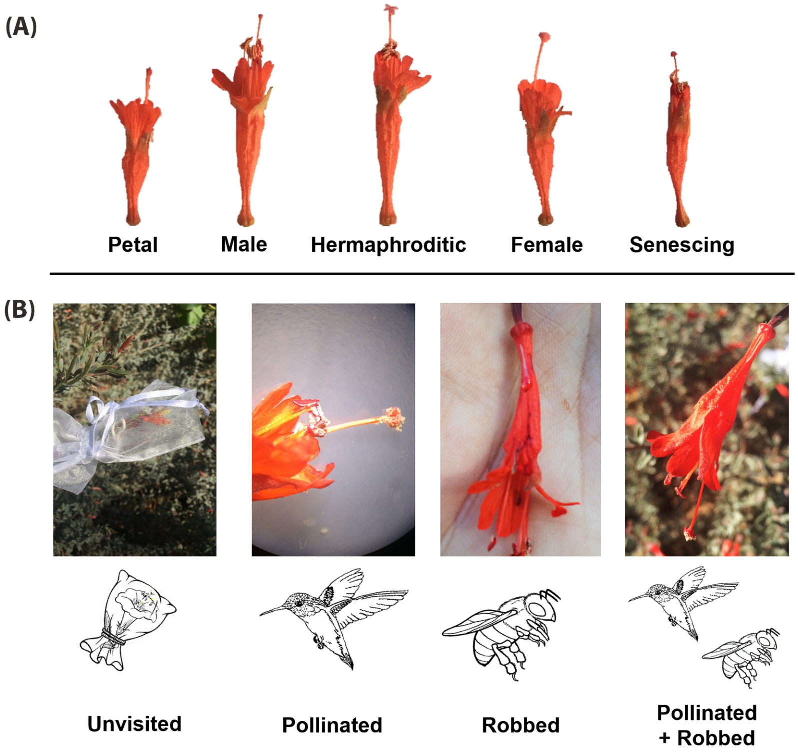

Figure 1. *Epilobium canum* nectar samples were categorized into one of five floral stages and one of four visitation types by morphological characteristics and visual cues. In **(A)**, *E. canum* flowers progress from post-anthesis (petal) to the senescing floral stage. In **(B)**, we classified visitation type based on evidence of visitation activity, where pollen present on the stigma indicated that pollination had occurred, and the presence of a slit at the base of the corolla indicated that robbing had occurred. To prevent visitation, we covered flowers with nylon mesh bags (Unvisited).

until nectar extraction in the laboratory, no more than 1 h following collection. Nectar was extracted from individual flowers using sterile glass calibrated microcapillary tubes and the nectar volume was recorded. Extracted nectar from each flower was serially diluted in MilliQ H_2O as per methods developed and described in Peay, Belisle and Fukami (2011) and Vannette, Gauthier and Fukami (2013), to both maintain microbial cell viability for enumeration and retain nectar sample integrity for downstream chemistry analysis.

Microbial enumeration

To estimate bacterial abundance in floral nectar, 50 μl of nectar was diluted 1:100 and plated onto Luria–Bertani agar (LBA) (Difco, Sparks, MD), Reasoner's 2A agar (R2A; Difco, Sparks, MD) supplemented with 20% sucrose, with cycloheximide (100 mg l^{-1} c.f.) to inhibit fungal growth. To estimate fungal abundance in floral nectar, 50 μl of nectar was diluted 1:10 and plated onto yeast media agar (YMA; Difco, Sparks, MD) with chloramphenicol (100 mg l^{-1} c.f.) to inhibit bacterial growth. Plates were incubated at 25°C for 7 days and colony forming units (CFUs) were recorded per microliter of plated nectar and $\log_{10}(x+1)$ transformed for normalization.

To non-exhaustively characterize the culturable microbes in nectar, bacterial and fungal colonies were classified by morphotype (shape, margin, elevation, texture and color) and multiple representatives of each morphotype were sequenced, depending upon availability (Supplementary material S2, Supporting Information). Growth on plates was typically a monoculture of one morphotype (personal observation), so one morphotype was selected from any one given plate for isolation by subculture.

Taxonomic assignment of microbial isolates

A region of taxonomy-inferring genes from isolated colonies was amplified using PCR primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) for bacterial strains (Lane et al. 1985), NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) for yeast strains (Kurtzman and Robnett 1998), and ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for non-yeast fungal strains (Mitchell et al. 1994) (Supplementary methods S1, Supporting Information). Amplicons were Sanger sequenced by GenScript (Piscataway, NJ).

Trimmed sequences were annotated by MegaBLAST search against the NCBI database using minimum cutoff values of 95% identity. Taxonomic assignments were corroborated by the RDP Classifier (Wang et al. 2007). Phylogenetic placement of query and reference sequences was conducted in Geneious v. 8.1.8 (Biomatters, Auckland, NZ) and analyzed in RStudio v. 0.99.893 (R Core Team 2015) using packages 'ape' (Paradis, Claude and Strimmer 2004) and 'phyloseq' (McMurdie and Holmes 2013).

Nectar chemistry analysis

Sugars in each diluted nectar sample were separated on an Amide HILIC column (Thermo Fisher Scientific, Waltham, MA; 50 mm x 2.1 mm x 2.6 μm) using a 5 min gradient separation starting at 80% acetonitrile in water with 0.2% triethylamine at 0.17 ml/min, ramping linearly to 50% acetonitrile, followed by a re-equilibration at initial conditions on a Thermo Fisher HPLC (Thermo Fisher Scientific). Each sample included maltose as an internal standard, as this sugar was not detected in nectar during our preliminary analysis. Detection was achieved using a

Charged Aerosol Detector (Thermo Fisher Scientific). The concentration of monosaccharide (fructose, sucrose) and disaccharide (sucrose, maltose) sugars in each sample was quantified using a series of external standards (Sigma-Aldrich, St Louis, MO).

Statistical analysis of survey data

We analyzed the effects of floral stage and dispersal categories on nectar traits and microbial abundance [$\log_{10}(x+1)$ transformed CFU counts] using linear regression models with floral stage and visitation as predictor variables in RStudio. To analyze the differences in measured variables among floral stages and visitation groups, we used analysis of variance (ANOVA) with Tukey HSD post-hoc analyses, using adjusted *P*-values for multiple comparisons. We found no statistical difference between bagged flowers and unbagged flowers that showed no evidence of visitation, suggesting that visitation is detectable based on physical evidence (Fig. 1); however, because we cannot confirm that there was no visitation to unbagged samples, we present only the results from our bagged treatment in the main text and refer to this group as 'Unvisited' (Supplementary material S3, Supporting Information).

Visitation-driven microbial dispersal experiment

Metagenomic sequencing of nectar-associated microbial communities

To investigate the effects of dispersal on microbial community structure and function in floral nectar without biases introduced by the culture methods or primers selected, we used shotgun metagenomics. We conducted an additional experiment to manipulate dispersal on *E. canum* flowers at one site used previously for the floral nectar composition survey, where pollinators and carpenter bees were particularly abundant (38°32′8″ N, 121°44′56″ W; June to July 2016). We established four visitation treatments similar to those described above: Unvisited (bagged to prevent visitation), Pollinated (corolla protected by a segment of a red plastic straw to prevent robbing), Robbed and Pollinated + Robbed. As above, flowers were categorized based upon physical evidence of visitation activity (pollen on stigma and/or slit at base of the corolla). Flowers were bagged and tagged at the bud stage (pre-anthesis), and floral nectar was collected once flowers reached the hermaphroditic stage. Nectar was extracted using methods described previously, and nectar from 10 flowers within the same visitation treatment was pooled to obtain sufficient microbial DNA content for downstream sequencing. Replicate samples (10 flowers each) were collected from treated plants across multiple dates with 4–5 replicate samples per treatment.

DNA was extracted from samples and fragmented to a target length of 500 bp, libraries were prepared using the Accel-NGS 2S Plus DNA library preparation kit and samples were sequenced using Illumina MiSeq (Supplementary methods S2, Supporting Information), including negative controls to check for contamination. Resulting forward reads (R1) were analyzed only, as long library fragments prohibited successful pairing (<65%). Trimmed fastq files were uploaded to the MG-RAST server (Meyer *et al.* 2008) for taxonomic annotation (Pruitt, Tatusova and Maglott 2007) against the RefSeq database, and functional annotation (Overbeek *et al.* 2005) using the SEED Subsystems database that classifies functional groups in a hierarchical manner analogous to taxonomic classifications, with four levels of resolution from broad functional subsystems (level 1) down to individual gene functions (level 4).

Analyses of community and functional composition

For microbial taxa and function separately, we assessed adequate sequencing depth of microbial communities by visualizing rarefaction curves of metagenomes. All metagenome rarefaction curves plateaued; thus, annotated reads within each metagenome were normalized by converting number of hits to relative proportions within each sample (Dinsdale *et al.* 2013; McMurdie and Holmes 2014; Supplementary material S4, Supporting Information), and downstream multivariate analyses were performed in RStudio with packages 'phyloseq' (McMurdie and Holmes 2013), 'vegan' (Dixon 2003) and 'randomForest' (Liaw and Wiener 2002). We examined if samples from dispersal treatments differed in relative taxonomic composition or functional annotations visualized using nonmetric multidimensional scaling (NMDS) and compared statistically using permutational ANOVA using the 'adonis' function in R (Dixon 2003) based on Bray-Curtis dissimilarities, and which OTUs or functional groups (variables) were responsible for differentiation among treatment groups using random forest analyses (Liaw and Wiener 2002). Variable (OTU or functional group) importance was assessed through Mean Decrease in Gini, and the performance of the random forest classifier model was determined by out-of-bag (OOB) error metric, which indicates the proportion of improperly classified samples, based on a bootstrap of the original dataset. To decrease the OOB error, we combined 'Robbed' and 'Pollinated + Robbed' for this analysis. We further compared which functional annotations were differentially abundant between robbed and unvisited flowers using DESeq2 (Love Huber and Anders 2014). For taxonomic profiles, analyses were conducted at the genus level for bacteria, the family level for fungi (some groups were poorly annotated) and functional profiles were examined at the level 3 subsystem, due to poor consensus and high variability at the lower taxonomic and functional ranks.

Metagenome-assembled genomes

To link structure and function of floral visitor-dispersed microbial communities, draft metagenome-assembled genomes (MAGs) were curated from metagenomic sequences as follows, using a similar pipeline as described in Papudeshi *et al.* (2017). First, raw sequenced microbial metagenomes were combined into a single file for each respective floral visitation treatment from which they were collected (Unvisited; Pollinated; Robbed; Pollinated + Robbed). Next, raw reads were trimmed for quality and short reads were removed using Trimmomatic (Bolger, Lohse and Usadel 2014). Trimmed reads longer than 25 bp were assembled into contigs using MEGAHIT (Li *et al.* 2015) and aligned to assembled contigs using Bowtie2 (Langmead and Salzberg 2012) and SAMtools (Li *et al.* 2009). Contigs were binned based on oligonucleotide profiles using MetaBAT2 (Kang *et al.* 2015) and bins were assessed for completeness and coverage using CheckM (Parks *et al.* 2015). Bins > 50% complete were uploaded to the RAST server (Aziz *et al.* 2008) for closest neighbor taxonomic assignment and annotation of functional profiles against the SEED Subsystems and KEGG databases. We focus on MAGs annotated as *Acinetobacter*, as these draft genome bins were most complete, had high coverage and represent all three of the treatments, which included a floral visitor (Pollinated; Robbed; Pollinated + Robbed). Subsequent comparative analyses of *Acinetobacter* functional profiles were performed in RStudio (R Core Team 2015).

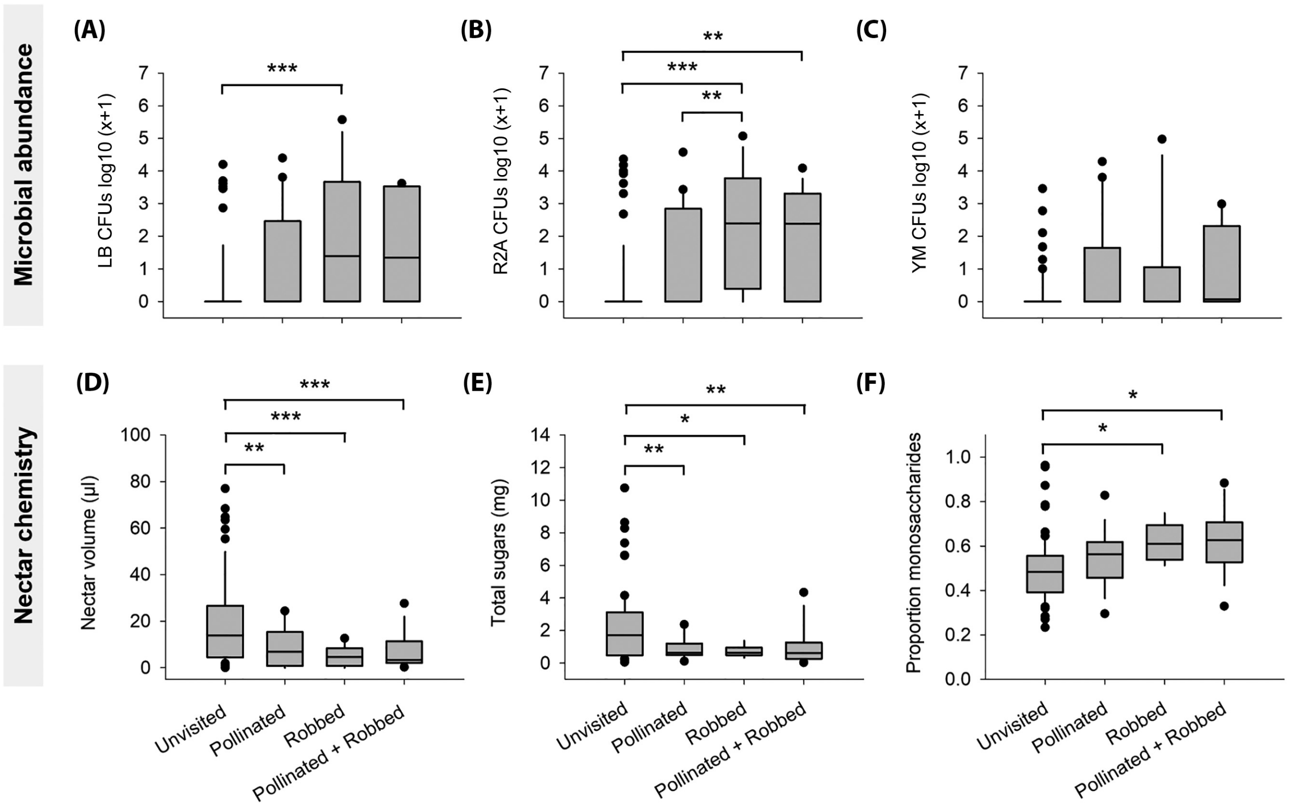

Figure 2. Our study describes the composition of *E. canum* floral nectar across floral stages and under different types of floral visitation. Here, flowers at the various stages (Petal, Male, Hermaphroditic, Female, Senescing) are collapsed within the four visitation treatment groups—Unvisited (Bagged) ($n = 77$), Pollinated ($n = 20$), Robbed ($n = 12$) and Pollinated + Robbed ($n = 14$). We present the culturable microbial abundance as measured on **(A)** LBA, **(B)** R2A and **(C)** YMA media, and (D) volume of nectar (µl), sugar content in **(E)** total sugars (mg) and **(F)** proportion of monosaccharides as measured by HPLC, and. Boxplots indicate the median and 95% CI (whiskers). Asterisks indicate significant differences between groups as measured by Tukey HSD post-hoc tests from the linear model including both stage, treatment and their interaction ($<0.05^{*}$, $<0.01^{**}$, $<0.001^{***}$).

RESULTS

Floral nectar composition survey

Microbial incidence and abundance in E. canum floral nectar

While nectar of unvisited flowers had the lowest incidence of culturable bacteria (LBA: 83.12%; R2A: 67.53% of flowers microbe free) and fungi (YMA: 79.22% microbe free), flowers displaying evidence of any floral visitation were the most likely to contain bacteria ($\chi^2 = 32.304$, $P < 0.001$) and fungi ($\chi^2 = 17.758$, $P < 0.001$), and hosted the greatest density of bacteria and fungi (Supplementary material S5, Supporting Information; Fig. 2A–C). Bacterial and fungal abundance in floral nectar increased with flower age in all visitation treatments but to the greatest extent in visited flowers (Fig 2; Supplementary material S6, Supporting Information). As floral age progressed from the petal stage to senescing, the mean abundance of bacteria increased from nearly undetectable to 1.007×10^{4} (LBA: 2803% increase) or 4.813×10^{3} (R2A: 1591% increase) CFUs μl^{-1}, while fungi increased from undetectable to 3.009×10^{3} CFUs μl^{-1} (YMA: 22.272% increase; Supplementary material S6, Supporting Information).

Taxonomic composition of culturable nectar-associated microbes

We isolated 145 microbial colonies representing 51 morphotypes, and 118 (64 bacteria, 54 fungi) were successfully sequenced and annotated (Supplementary material S2, Supporting Information). Thirteen bacterial genera were represented, with *Acinetobacter* ($n = 21$), *Micrococcus* ($n = 17$) and *Rosenbergiella* ($n = 6$) as the most common identified genera (Fig. 3A). From the 54 successful fungal isolates, 11 fungal genera were identified and were dominated by *Metschnikowia* ($n = 29$; Fig. 3B) followed by *Penicillium* ($n = 7$).

While we found nectar from unvisited flowers to exhibit the lowest microbial abundance, it contained the greatest count of unique culturable microbial taxa of the four visitation treatments, with 10 bacterial and 9 fungal genera identified, comprised mainly of bacterial genera *Micrococcus* and *Enterobacter* and the fungal species *Aureobasidium pullulans* and *Cryptococcus* spp. In contrast, robbed or pollinated flowers hosted fewer genera of bacteria (six) and fungi (five), and robbed flowers contained the lowest richness of any visitation treatment, with just two bacterial and one fungal genus represented. The genera *Acinetobacter* and *Metschnikowia* were only isolated from visited nectar (Fig. 3) and we did not detect colonies with *Acinetobacter* nor *Metschnikowia*-like morphological characteristics from cultures of unvisited floral nectar. Notably, microbial strains showed evidence of visitor specificity: strains of *Acinetobacter boissieri* (BLAST identity 99%) were isolated only from robbed flowers. In addition, clades of *Metschnikowia koreensis* were separated by visitation type, with separate clades for robbed and pollinated flowers (Fig. 3).

Nectar chemistry

Mean floral nectar volume varied among floral age categories, with visitation, and their interaction ($F_{11,176} = 2.874$, $P = 0.002$;

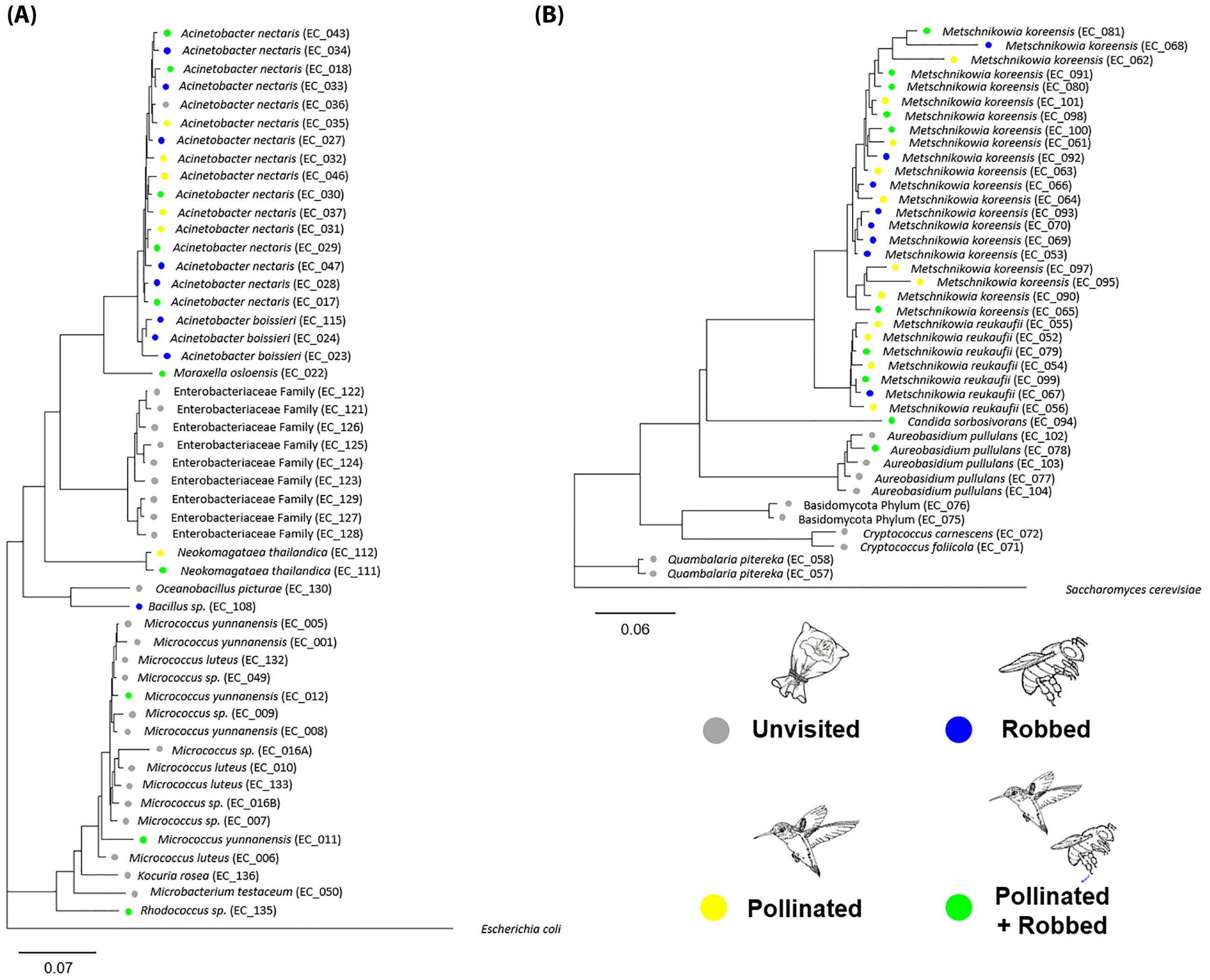

Figure 3. Neighbor-joining trees were generated from taxonomy-inferring DNA regions of microbial isolates cultured from *E. canum* floral nectar. Here, we show **(A)** bacterial 16S and **(B)** yeast LSU. Colors correspond to the visitation treatment (Unvisited = grey, Pollinated = yellow, Robbed = blue, Pollinated + Robbed = green). Selected outgroup species were not isolated in the current study, but are closely related to microbial isolates and yet fall outside clades of interest. Outgroup species in each tree are shown without identifying sample number and treatment color.

Supplementary material S7, Supporting Information). Without floral visitation, nectar volume increased between anthesis and the hermaphroditic stage, and then decreased as flowers reached senescence ($F_{4,72} = 9.053$, $P < 0.001$; Supplementary material S7, Supporting Information). Visitation activity by either pollinators (P adj. $= 0.001$), robbers (P adj. < 0.001) or both (P adj. < 0.001) reduced nectar volume in intermediate stages by nearly 75% (Supplementary material S7, Supporting Information). Nectar volume did not differ significantly among visited flowers (robbed, pollinated or both; Fig. 2D; Supplementary material S8, Supporting Information).

Unvisited floral nectar was comprised mainly of sucrose (~55%), followed by relatively equal proportions of the monosaccharides glucose (~22%) and fructose (~23%). Saccharide composition in nectar, including both concentration of individual sugars and proportion of monosaccharides to disaccharides, varied by visitation treatment, but was not affected by floral age (prop mono: $P = 0.106$) nor the interaction between visitation and age (prop mono: $P = 0.200$; prop mono full model: $F_{17,140} = 2.487$, $P = 0.002$) (Supplementary material S9, Supporting Information). Both mean fructose and glucose concentrations in floral nectar (mg/ml) were increased in nectar of visited flowers (fru: $P < 0.001$; glu: $P < 0.001$), with highest concentrations observed in robbed flowers. As a consequence, the proportion of monosaccharides was greatest when flowers were robbed (visitation $F_{4,140} = 5.503$, $P < 0.001$; Fig. 2F). Inclusive of all treatment groups, microbial abundance was positively correlated with proportion of monosaccharides in floral nectar (LBA: $t_{1,156} = 1.837$, $P = 0.068$; R2A: $t_{1,156} = 2.172$, $P = 0.031$; YMA: $t_{1,156} = 3.032$, $P = 0.003$) (Supplementary material S10, Supporting Information).

Visitation-driven microbial dispersal experiment

Metagenomes from 18 samples ($n = 5$ Unvisited, 4 Pollinated, 4 Robbed, 5 Pollinated + Robbed) were recovered (Supplementary materials S11 and S12a–c, Supporting Information). After quality control, the metagenomes contained between 88 218 and 944 553 reads each, with an average of 517 851 reads per metagenome. Here, we present analyses of bacterial and fungal diversity, and

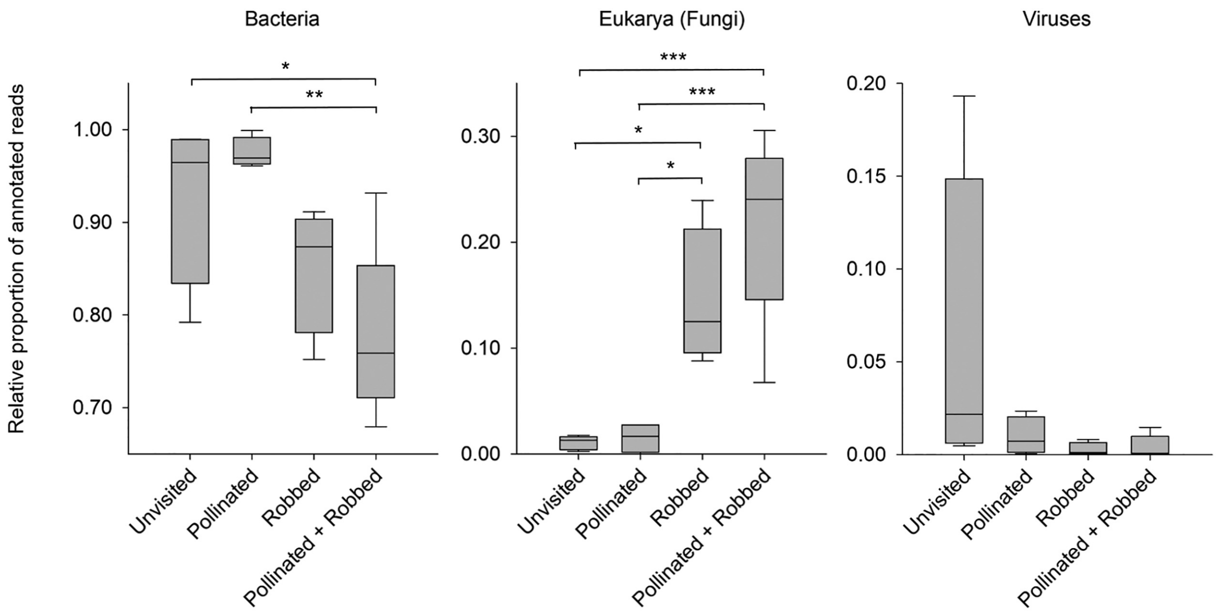

Figure 4. Relative abundance of microbes within the domains of Bacteria, Eukarya (fungal phyla) and viruses represented in metagenomes collected from *E. canum* floral nectar under four distinct visitation treatments—Unvisited ($n = 5$), Pollinated ($n = 4$), Robbed ($n = 4$) and Pollinated + Robbed ($n = 5$). The proportions of reads annotated to Archaea were <0.05% for all metagenomes and are not shown. The number of read annotations (hits) was normalized to proportion relative to the total number of annotated hits across all domains within each respective metagenome. Asterisks indicate significance detected between groups from Tukey HSD post-hoc tests ($<0.05^{*}$, $<0.01^{**}$, $<0.001^{***}$).

taxonomic composition and bacterial functional potential. Limited recovery of fungal sequences precluded functional analysis of fungal function (Supplementary material S12d, Supporting Information).

Microbial community composition and diversity

In total, 1678 unique OTUs were identified in at least 1 of the 18 metagenomes, including archaea (59 OTUs), bacteria (1397 OTUs), fungi (112 OTUs) and viruses (110 OTUs). The majority of hits within fungi were annotated to the Phylum Ascomycota (91 OTUs), followed by Basidomycota (11 OTUs), Blastocladiomycota (1 OTU), Chytridiomycota (6 OTUs) and Microsporidia (3 OTUs).

Floral visitation altered the relative abundance of bacterial and fungal reads and diversity within each group. In unvisited or pollinated flowers, bacteria comprised over 95% of annotated reads, while visitation by a nectar robber reduced bacterial dominance to as low as 67% (14% decrease in mean relative abundance) and increased fungal abundance to as high as 25% of annotated reads (18% increase in mean relative abundance; Fig. 4). Visitation by pollinators or nectar robbers did not affect microbial richness ($P = 0.262$), but tended to reduce evenness (Shannon's entropy $F_{3,14} = 3.320$, $P = 0.051$; Simpson's measure $F_{3,14} = 3.111$, $P = 0.060$; Supplementary material S13a, Supporting Information). Pollinated + Robbed samples hosted the least diverse communities (Shannon's entropy $P = 0.042$; Simpson's index $P = 0.044$; Supplementary material S13a, Supporting Information), with a 22% decrease in diversity compared to nectar of unvisited flowers. Visitation also influenced community composition for bacteria (PerMANOVA $F_{3,14} = 3.519$, $R^2 = 0.430$, $P = 0.004$) and fungi ($F_{3,14} = 5.831$, $R^2 = 0.555$, $P = 0.008$; Fig. 5). Visitation by robbers, but not pollinators, tended to homogenize microbial communities (Betadisper bacteria $P = 0.078$; fungi $P = 0.031$).

Of those bacterial taxa represented, 65% of annotated sequences were represented by just 18 bacterial genera. Within the 18 most abundant bacterial genera, the relative abundance of *Acinetobacter, Gluconobacter* (*Neokomagataea*), *Gluconacetobacter* and *Acetobacter* was greater in robbed flowers compared to unvisited or pollinated flowers (Fig. 5; Supplementary material S4, Supporting Information). Using a random forest model, we found that both highly abundant genera, such as *Acinetobacter* (0.201 mean decrease in Gini) and less abundant genera of *Leifsonia* (0.186), *Moraxella* (0.167), *Granulibacter* (0.161) and *Geobacter* (0.140; 28.57% OOB error rate) were influential in distinguishing between visitation groups. With the exception of *Leifsonia*, each of these genera were more abundant in robbed flowers (Robbed and Pollinated + Robbed) compared to pollinated flowers and unvisited flowers (*Moraxella*: 215%; *Granulibacter*: 694%; *Geobacter*: 241% increase in mean relative abundance). In contrast, the relative abundance of the *Leifsonia* was greater in the nectar of unvisited and pollinated flowers compared to robbed nectar (99.54% decrease in mean relative abundance).

Relative to bacteria, fungi were less abundant in the overall community profile, with 54 families containing a combined average of 3.33% of hits across all metagenomes. Visitation by robbers increased relative abundance of Debaryomycetaceae ($P < 0.001$), Metschnikowiaceae ($P < 0.001$), Saccharomycetaceae ($P = 0.002$) and unclassified Saccharomycetales ($P < 0.001$; Fig. 5; Supplementary material S14, Supporting Information). Random forest analysis indicated that treatments were best distinguished by the relative abundance of Metschnikowiaceae (0.554 mean decrease in Gini) and Debaryomycetaceae (0.526), and less abundant fungal families including Trichocomaceae (0.486), Phaeosphaeriaceae (0.482), Pleosporaceae (0.472) and Ajellomycetaceae (0.465; 14.29% OOB error rate). While Metschnikowiaceae and Debaryomycetaceae were more abundant in the nectar of robbed flowers (either Robbed or Pollinated + Robbed) compared to the nectar of pollinated or unvisited flowers, Trichocomaceae, Phaeosphaeriaceae and Pleosporaceae were present at higher relative abundances in nectar of unvisited flowers.

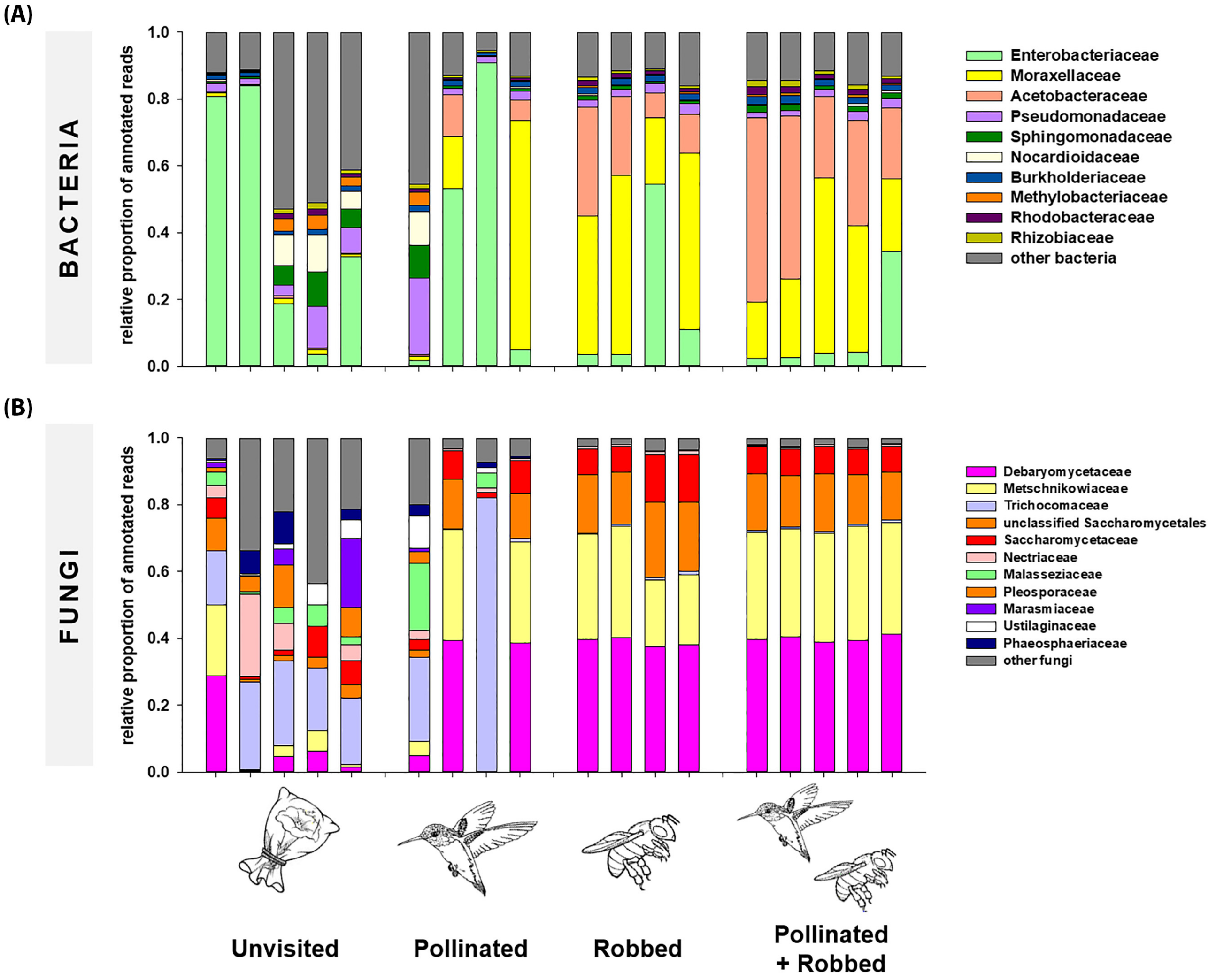

Figure 5. Here, we assess nectar-associated microbial community structure under different types of floral visitation, including legitimate pollination and nectar robbing. Bars represent the relative proportional abundance of the most abundant **(A)** bacterial and **(B)** fungal families within each respective taxonomic group, annotated within each microbial metagenome. Microbial community samples were curated from the nectar of 10 pooled flowers of each visitation type, and replicate pooled samples were collected and sequenced (Unvisited $n = 5$, Pollinated $n = 4$, Robbed $n = 4$, Pollinated + Robbed $n = 5$).

Community functional potential

In total, 3941 protein-encoding functions within 877 level 3 subsystems, 150 level 2 subsystems and 28 level 1 broad subsystems were annotated in at least 1 of the 18 metagenomes (Fig. 6). Visitation did not influence functional diversity (Supplementary material S13, Supporting Information), but altered the composition of bacterial functions at all subsystem levels (PerMANOVA level 1: $P = 0.002$; level 2: $P = 0.005$; level 3: $P = 0.004$; level 4: $P = 0.004$) (Fig. 7B). Flowers visited by a robber exhibited convergent functional composition (Betadisper level 1: $P = 0.009$; level 2: $P = 0.018$; level 3: $P = 0.036$; level 4: $P = 0.049$).

On the broadest functional level, bacterial community functional profiles varied by visitation treatment in 11 of the 28 categories—amino acids and derivatives; carbohydrates; clustering-based subsystems; cofactors; vitamins; prosthetic groups; pigments; dormancy and sporulation; fatty acids; lipids and isoprenoids; iron acquisition and metabolism; membrane transport; miscellaneous; photosynthesis; and respiration (Supplementary material S15, Supporting Information). Visitation also influenced relative abundance of 30 of the 150 level 2 subsystems; microbial communities within robbed flowers exhibited high abundance of genes related to plasmid-related functions, osmotic stress, transporters and secretion, anaerobic degradation of aromatic compounds and sugar hydrolysis, but showed a reduced abundance of sugar phosphotransferase systems and carotenoid biosynthesis (Fig. 6).

The bacterial functions (level 2) that best distinguished robbed from unvisited flowers (Fig. 7A) included protein and nucleoprotein secretion system, type IV (level 1 membrane transport; 0.413 mean decrease in Gini), electron donating reactions (level 1 respiration; 0.343), selenoproteins (level 1 protein metabolism; 0.303), ABC transporters (level 1 membrane transport; 0.277) and di- and oligosaccharides (level 1 carbohydrates; 0.242; OOB error estimate 21.43%), differentiating samples correctly 78.57% of the time.

Within fungal functional annotations, 583 functional genes were categorized within 251 level 3, 69 level 2 and 23 level 1 subsystems. The majority of functional groups annotated were

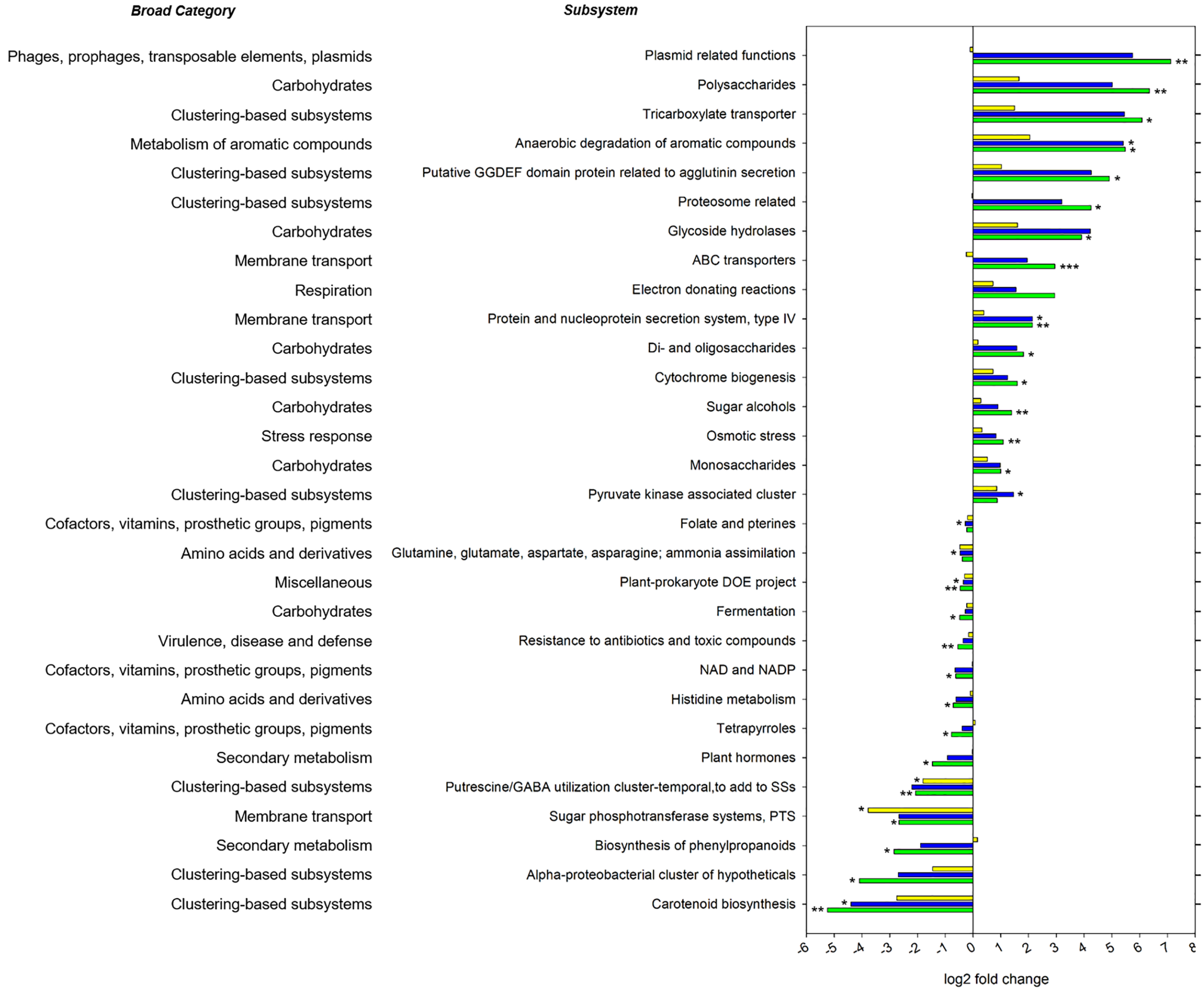

Figure 6. Microbial metagenomes were annotated for function on MG-RAST against the SEED Subsystems database, providing the number of annotation hits to functions classified within the database. Here, we show differentially abundant (log2 fold) genes annotated at the level 2 subsystem. Bars indicate average log2 fold change between Pollinated (yellow), Robbed (blue) and Pollinated + Robbed (green), each compared to the Unvisited treatment (mean set at zero, not shown). The Wald test in DESeq2 identified 30 level 2 subsystems, classified within 12 level 1 broad categories). Asterisks indicate significance detected by the Wald test comparing the respective visitation group to the Unvisited group (P. adj. $<0.05^{*}$, $<0.01^{**}$, $<0.001^{***}$).

classified within respiration ($\bar{x}_{R} = 42.43\%$, $\bar{x}_{P+R} = 46.02\%$) and protein metabolism ($\bar{x}_{R} = 17.35\%$, $\bar{x}_{P+R} = 18.67\%$), followed by carbohydrates ($\bar{x}_{R} = 7.51\%$, $\bar{x}_{P+R} = 5.88\%$; Supplementary material S16, Supporting Information).

Linking microbial structure and function using MAGs

Eighteen bacterial genome bins in total were recovered from metagenomes of visitor-dispersed microbial communities in floral nectar ($n =$ by each treatment). Of these, 11 draft MAGs were >50% complete and represent five genera, including *Acinetobacter* ($n = 3$), *Cronobacter* ($n = 2$), *Erwinia* ($n = 1$), *Gluconobacter* ($n = 3$) and *Pantoea* ($n = 2$) (Supplementary material S17, Supporting Information). For subsequent comparative analysis, we focus on the three *Acinetobacter* MAGs, as these were most complete (94.29–99.28% complete), had high coverage (34 901–228 892 unassembled metagenomic reads aligned per bin) and represent three of the floral visitor treatments (Pollinated; Robbed; Pollinated + Robbed). We compare the presence and absence of genes within metabolic pathways (Supplementary materials S17 and S18, Supporting Information).

The functional profile of the robber-dispersed *Acinetobacter* MAG was distinct from the Pollinated and Pollinated + Robbed *Acinetobacter* MAGs, whereas the latter two were nearly identical. The primary functional groups that differentiated the visitor-associated *Acinetobacter* MAGs were the amino acids and derivatives and the carbohydrate subsystems containing related protein encoding genes (Supplementary material S19, Supporting Information). Within the amino acids and derivatives functional category, the robber-associated *Acinetobacter* MAG was deficient in protein-encoding genes related to assimilation, metabolism and utilization of organic and amino acids, including genes within the arginine pathway, polyamines, and aromatic amino acids and derivatives (Rob: $n = 6$; Poll: $n = 30$; Poll+Rob: $n = 32$ genes). In addition, the robber-associated *Acinetobacter* MAG was deficient in genes for assimilation of glutamine, glutamate, aspartate, asparagine, and ammonia (Rob: $n = 9$; Poll: $n = 18$; Poll+Rob: $n = 18$ genes) and proline metabolism (Rob: $n = 3$;

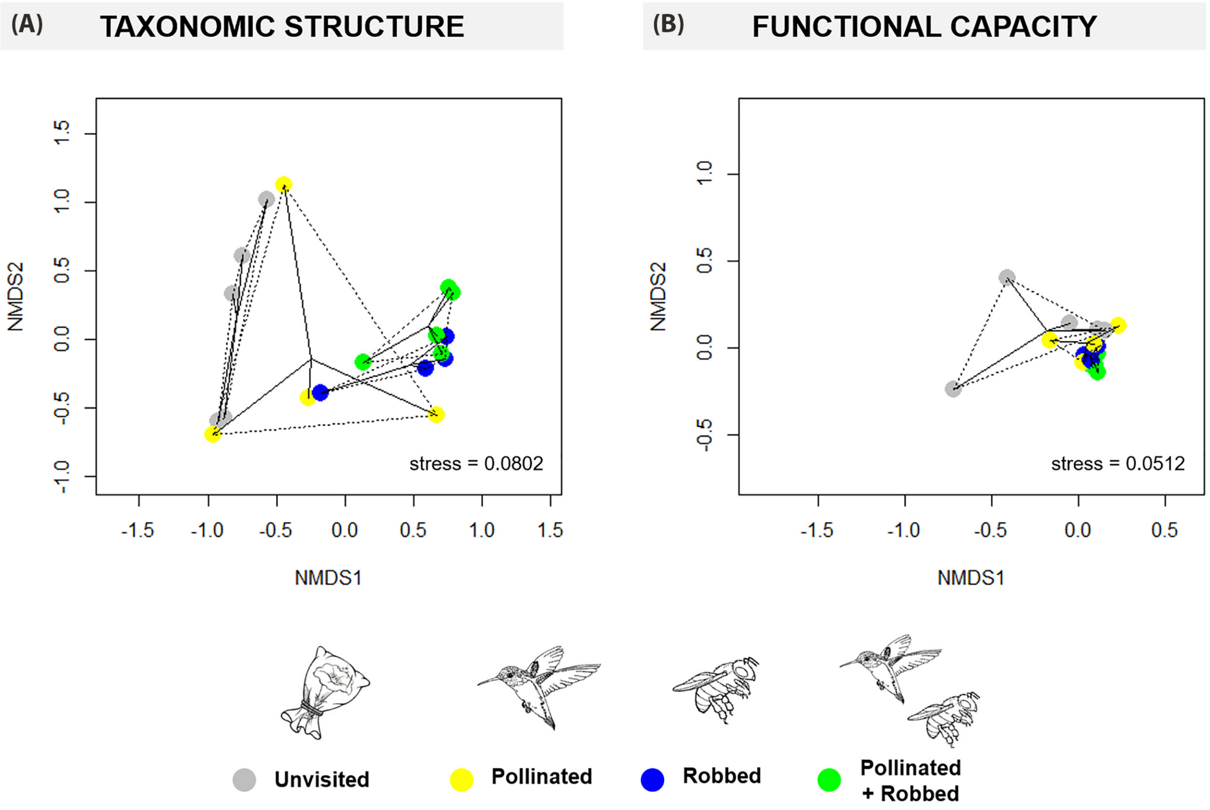

Figure 7. NMDS based on Bray–Curtis similarity of the relative proportion of features within each microbial metagenome. Visitation influenced both taxonomic structure (bacterial genera: PerMANOVA $F_{3,14} = 3.519$, $R^2 = 0.430$, $P = 0.001$; fungal families: $F_{3,14} = 5.831$, $R^2 = 0.555$, $P = 0.008$) and functional capacity (level 3 subsystem: $F_{3,14} = 2.035$, $R^2 = 0.304$, $P = 0.004$) of nectar-associated microbial communities at multiple levels tested. Here, we show the relatedness of microbial metagenomes for **(A)** composition of microbial genera inclusive of all domains, and **(B)** bacterial functional potential (SEED level 3 subsystem). Individual points compressed into 2D space represent replicate metagenomes from nectar-associated microbial communities of visited flowers (Unvisited $n = 5$, Pollinated $n = 4$, Robbed $n = 4$, Pollinated + Robbed $n = 5$).

Poll: $n = 12$; Poll+Rob: $n = 12$ genes), and lacked genes for histidine metabolism (Rob: $n = 0$; Poll: $n = 8$; Poll+Rob: $n = 8$ genes), compared to the *Acinetobacter* associated with visitation groups including a pollinator (Pollinated; Pollinated + Robbed; Fig. 8; Supplementary material S20a, Supporting Information).

Carbohydrate functional profiles were also distinct between the same groups. Specifically, the robber-dispersed *Acinetobacter* lacked protein-encoding genes within carbohydrate-related metabolic pathways, including the pentose phosphate pathway (Rob: $n = 0$; Poll: $n = 5$; Poll+Rob: $n = 5$ genes) and fermentation pathway (Rob: $n = 0$; Poll: $n = 9$; Poll+Rob: $n = 9$ genes) compared to the MAGs assembled from the other two visitation groups. The robber-dispersed *Acinetobacter* was distinguished by genes within functional groups for metabolism and utilization of saccharides, including an enrichment of genes for aminosugars (Rob: $n = 2$; Poll: $n = 0$; Poll+Rob: $n = 0$ genes), monosaccharides (Rob: $n = 10$; Poll: $n = 5$; Poll+Rob: $n = 5$ genes), di- and oligosaccharides (Rob: $n = 1$; Poll: $n = 0$; Poll+Rob: $n = 0$ genes), D-gluconate and ketogluconates, and xylose utilization, yet lacked genes for D-ribose utilization and mannose metabolism (Fig. 8) (Supplementary material S20b, Supporting Information).

DISCUSSION

Combining culture-dependent and independent approaches with shotgun metagenomics revealed that dispersal agents (i.e. floral visitors) introduce specialized microorganisms to floral nectar, shaping microbial community composition, functional potential and resulting sugar chemistry.

Bacterial taxa and functions described by shotgun metagenomics suggest that the dominant microbial colonists in nectar are culturable, including bacteria from the genera *Acinetobacter* and *Gluconobacter* (annotated as *Neokomagataea* from 16S database), and other acetic acid Proteobacteria. The bacterial functions characterized here suggest specialization to the nectar microhabitat, as communities in visited flowers were highly enriched in genes relating to osmotic stress and electron-donating reactions (Papadimitriou *et al.* 2016), and carbohydrate metabolism, including poly-, oligo-, di- and monosaccharides, and sugar alcohols (Fig. 6). When bacteria were abundant, genes associated with membrane transport of plasmids, gene transfer agents, and secretion systems were more abundant, which may indicate that horizontal gene transfer among between bacterial cohorts could be one mechanism underlying habitat specialization. These functional genes may reflect both the specific adaptations required for survival in nectar and/or the particular biology of dominant microorganisms, specifically *Acinetobacter* species, which comprised on average 40% of all reads within visited samples (Fig. 5). *Acinetobacter nectaris* and *A. boissieri* have been described to exhibit high osmotolerance, catalase activity and microaerobiosis for bacterial survival in nectar (Álvarez-Pérez, Herrera and de Vega 2012). Curiously, amino acid assimilation-related and metabolism genes were less abundant in robbed nectar samples (Fig. 6). Notably, different visitor guilds were associated with distinct microbial

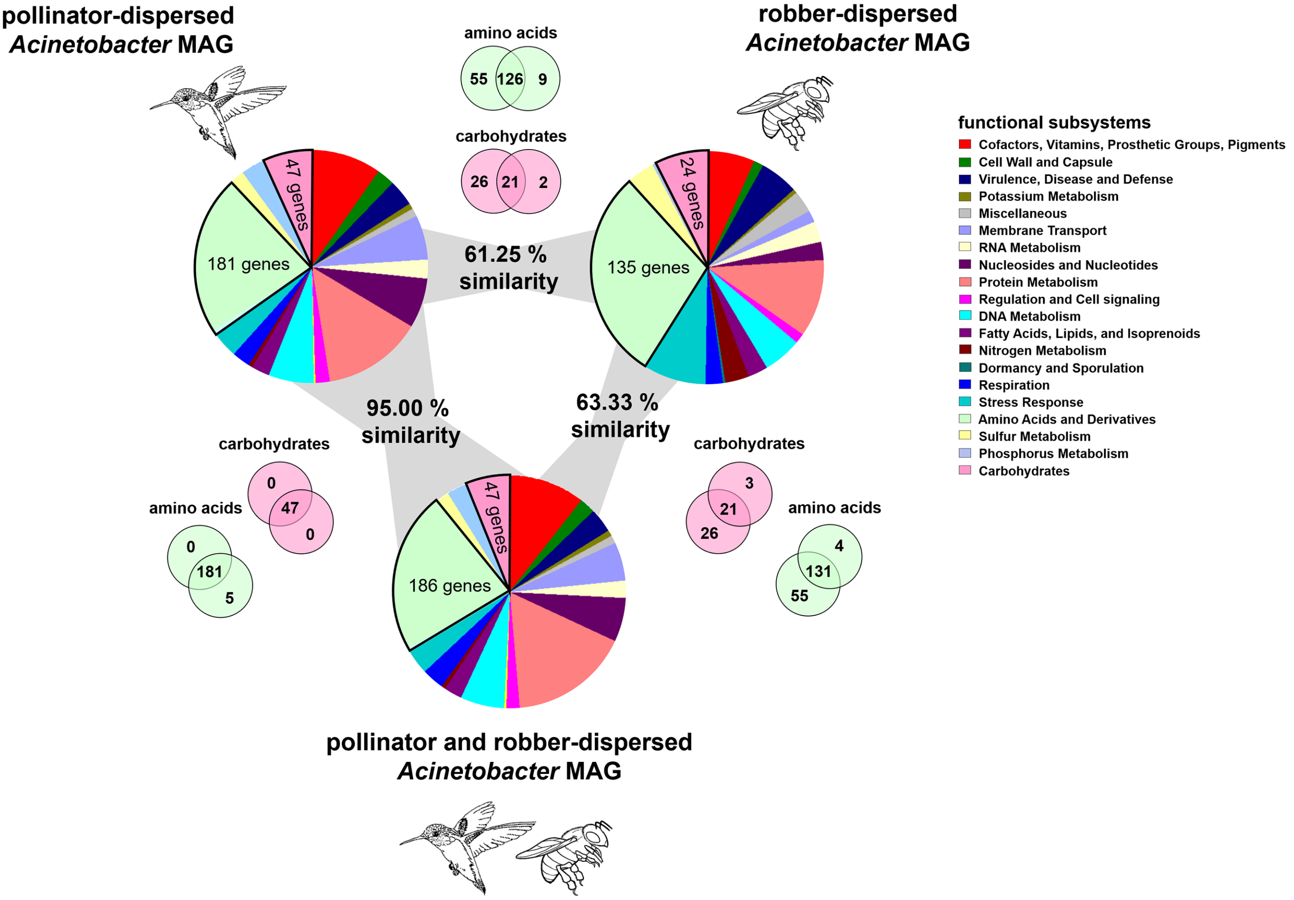

Figure 8. Comparative visualization of gene functions annotated in MAGs from floral visitor-dispersed *Acinetobacter*. Here, we show the composition of the 20 broad functional categories (SEED level 1) within each *Acinetobacter* MAG, highlighting the number of genes annotated for amino acids and derivatives and carbohydrates (others may differ but are not visualized here). The Jaccard index was used to calculate similarity percentages between each visitation treatment (Pollinated; Robbed; Pollinated + Robbed) for presence/absence of genes in amino acids and derivatives and carbohydrates. Venn diagrams compare the presence of amino acids and derivatives (teal) and carbohydrates (pink) genes, where numbers indicate the count of genes annotated within either or both visitor-dispersed *Acinetobacter* MAGs.

communities, including species within the dominant bacterial genus (*Acinetobacter*) and their function, specifically amino acid and carbohydrate pathways and effects on nectar chemistry. In particular, the robber-associated *Acinetobacter* MAG lacked 106 key genes within the amino acids pathway, which were identified in other MAGs, including arginine biosynthesis and aromatic amino acid synthesis. Amino acids including glutamine and proline are common in floral nectar (Nicolson and Thornburg 2007). In contrast, xylose metabolism genes were identified in the robber-associated MAGs. Xylose is uncommonly found in floral nectar, but is a constituent of wood (where carpenter bees nest), so xylose usage by robber-associated *Acinetobacter* strains suggests the potential to use wood substrates as a carbon source and further adaptation to association with carpenter bees.

Our analyses indicate that the differential functional capacity of visitor-dispersed *Acinetobacter* is not simply an artifact of lower coverage of the robber-associated MAG, as all *Acinetobacter* MAGs were >94% complete (Supplementary material S17, Supporting Information) and had a high number of assembled metagenomic reads aligned back to bins (Supplementary material S18, Supporting Information), but instead suggests that this robber-associated *Acinetobacter* strain differs in nutrient use from the pollinator-associated strain. Other nectar-dwelling microbes have a higher capability for amino acid utilization, in particular yeast *M. reukaufii*, which has undergone gene duplication and exhibits high expression of amino acid transporters associated with enhanced amino acid uptake and competitive ability (Dhami, Hartwig and Fukami 2016). The lack of these features in *Acinetobacter* suggests an alternative ecological strategy or distinct nutrient source. Moreover, distinct metabolic pathways in *Acinetobacter* species found in pollinator or robber-visited flowers suggest that despite resource use overlap between hummingbirds and carpenter bees (both visit flowers), these species may vector congeneric microbes that differ in genome content and effects on nectar.

For fungi, shotgun sequencing revealed both frequently cultured yeast and taxa not typically captured in culture-dependent studies of temperate nectar communities (Pozo, Herrera and Bazaga 2011; Álvarez-Pérez and Herrera 2013), particularly osmophilic fungi from the family Debaryomycetaceae including the genera *Debaryomyces*, *Lodderomyces*, *Meyerozyma*, *Millerozyma* and *Scheffersomyces*; other yeasts within the Saccharomycetales, including *Candida* spp., were also common in robbed flowers. Fungal genes for respiration dominated annotated sequence pools, followed by RNA metabolism, carbohydrate metabolism and amino acid-related functions (Supplementary material S16, Supporting Information), reflecting insights gained from whole-genome sequencing of dominant nectar yeast (Dhami, Hartwig and Fukami 2016). We postulate that this skewed annotation profile likely results from the low diversity of fungal taxa inhabiting the floral nectar, poor annotation of fungal genes and a lack of well-described genes curated across a wide array of metabolic pathways. While this study is not an exhaustive one, to our knowledge, this is the first study to apply whole-genome shotgun metagenomic sequencing in conjunction with empirical ecological experiments in a floral nectar model study system, the combination of which more sufficiently describes the microbial community potential.

Our study may have implications for the ecology of plant–pollinator interactions. In this system, visitation by nectar robbers was the strongest driver of differentiation in microbial community abundance, composition and specialized functions in floral nectar, with a subsequent increase in the concentration of monosaccharides in nectar, compared to visitation by legitimate pollinators (Fig. 2) or changes over time in non-visited flowers (Supplementary materials S7 and S6, Supporting Information). We cannot distinguish if effects of nectar robbers on microbial abundance are due to the deposition of microbes, floral damage itself or facilitate secondary visitors, all of which could affect nectar chemistry or antimicrobial defense (Carter and Thornburg 2004; Artico *et al.* 2014). Furthermore, although our study contrasts the microbial community structure and function from pollinator and robber-visited flowers, it is unclear to what extent other visitors differ in their effects on nectar microbial communities. Nevertheless, resulting microbial growth and effects on nectar quality and attractiveness to pollinators (Vannette, Gauthier and Fukami 2013; Rering *et al.* 2019) may contribute to negative effects of nectar robbing on pollinator visitation (Irwin and Brody 2000; González-Gómez and Valdivia 2005; Caballero *et al.* 2013) and plant reproduction (Adler, Leege and Irwin 2016; Richman *et al.* 2016). In addition, robbing was consistently associated with *Gluconobacter* and *Acetobacter*, and osmophilic yeasts. Even when similar microbial taxa were identified in floral nectar under robbing versus pollination, in particular, *Acinetobacter*, our comparative analysis of MAG profiles showed that robber- and pollinator-associated *Acinetobacter* had divergent functional capacities, notably in utilization and metabolism of amino acids and carbohydrates. As both amino acids and carbohydrates (saccharides) are integral to nectar quality and attracting subsequent pollinators , robber-mediated changes in nectar quality by both *Gluconobacter* and *Acinetobacter* may have implications in the maintenance of plant–pollinator mutualisms.

Overall, our study suggests that nectar-dwelling bacteria and yeasts rely on floral visitors to disperse among habitat patches, where they attain high abundance and influence nectar properties. The MAG analysis suggests that microbial strains within species (e.g. *Acinetobacter*) may differ among floral visitor vectors, with distinct genomic potential and metabolism of floral nectar following visitation by different vectors. However, further work on the dispersal of floral microorganisms and their attraction of animal vectors (Rering *et al.* 2019) will be necessary to elucidate vector specificity and ecological and evolutionary consequences for microbial diversity. Nevertheless, our results suggest that microbial dispersal is essential to understand and predict microbial community dynamics and functions in microenvironments. Given the relatively high habitat specificity of many microorganisms (e.g. Monard *et al.* 2016), specialized dispersal to suitable microhabitats may be an important but underappreciated factor influencing microbial colonization and function. Increased study of microbial dispersal traits, including those related to dormancy, dispersal mode and effective dispersal distances, will be key to understanding and predicting the role of dispersal and phoresis in microbial assembly and function.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

AUTHORS’ CONTRIBUTIONS

MMM and RLV conceived and designed the experiment, performed nectar chemistry analysis and analyzed sequencing results. MMM collected floral samples and observational data and isolated floral microbes. MMM, NJF and EAD performed metagenomic sequencing and analysis. ACB curated MAGs and MMM performed comparative analyses. MMM wrote the first draft of the manuscript, and all others contributed substantially to revisions.

AVAILABILITY OF SEQUENCING DATA

Sequences are available on MG-RAST (https://www.mg-rast.org/linkin.cgi?project=mgp21610).

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Competing interests. The authors declare no conflict of interest.

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