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Research

Intraspecific variation in realized dispersal probability and host quality shape nectar microbiomes

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Summary

- Epiphytic microbes frequently affect plant phenotype and fitness, but their effects depend on microbe abundance and community composition. Filtering by plant traits and deterministic dispersal-mediated processes can affect microbiome assembly, yet their relative contribution to predictable variation in microbiome is poorly understood.
- We compared the effects of host-plant filtering and dispersal on nectar microbiome presence, abundance, and composition. We inoculated representative bacteria and yeast into 30 plants across four phenotypically distinct cultivars of *Epilobium canum*. We compared the growth of inoculated communities to openly visited flowers from a subset of the same plants.
- There was clear evidence of host selection when we inoculated flowers with synthetic communities. However, plants with the highest microbial densities when inoculated did not have the highest microbial densities when openly visited. Instead, plants predictably varied in the presence of bacteria, which was correlated with pollen receipt and floral traits, suggesting a role for deterministic dispersal.
- These findings suggest that host filtering could drive plant microbiome assembly in tissues where species pools are large and dispersal is high. However, deterministic differences in microbial dispersal to hosts may be equally or more important when microbes rely on an animal vector, dispersal is low, or arrival order is important.

Introduction

Phyllosphere microbes frequently influence plants' expressed phenotype and ecological interactions. Plants benefit when microbes mitigate the effects of stress, enhance plant growth, or reduce the growth of antagonists (Stone *et al.*, 2018). Yet, other microbes are plant pathogens, deplete critical nutrients, or support the growth of other antagonists (Liu *et al.*, 2020). Given the diverse and important effects of microbial communities on plant traits and fitness, understanding the processes driving plant microbial community assembly is a key goal. A predictive framework of plant microbiome assembly holds promise for both agricultural application (Busby *et al.*, 2017; Toju *et al.*, 2018) and deepening our understanding of ecological interactions in natural plant communities (Fitzpatrick *et al.*, 2020).

Plant microbiome assembly is shaped by selection, dispersal, drift, and speciation (Dini-Andreote & Raaijmakers, 2018). Stochastic or neutral processes impact communities without regard to microbial species identity or host traits, do not result in predictable community trajectories (Vellend *et al.*, 2014), and generate nondeterministic variability in microbiome communities (e.g. in *Arabidopsis thaliana* Maignien *et al.*, 2014; Vega & Gore, 2017; and *D. melanogaster* Zapién-Campos *et al.*, 2020). Alternatively, deterministic community assembly processes are driven by trait variation among individual plants, lines, or species (Peiffer *et al.*, 2013; Wagner *et al.*, 2016; Leopold & Busby, 2020) that predictably affect microbial establishment or interactions with other microbiome members (Fukami, 2015; Leopold & Busby, 2020; Mueller *et al.*, 2023). Plant traits may also influence species interactions that affect microbial dispersal or growth (e.g. with herbivores Humphrey & Whiteman, 2020; other plants Meyer *et al.*, 2022; or the environment Pusey & Curry, 2004; Gaube *et al.*, 2021). Interspecific variation among host plants can explain significant variation in microbiome composition, ranging from 15% (Wagner, 2021) to 41% (Yang *et al.*, 2023) while intraspecific variation also explains *c.* 40% of variation in microbiome composition in the leaves and roots of cotton (Wei *et al.*, 2019). Often, microbial dispersal is considered a solely stochastic process, yet there is growing theoretical and empirical support that some of its components may be predicable (Evans *et al.*, 2017) and interact with host selection to result in deterministic plant microbiome assembly (Martini *et al.*, 2015; Dini-Andreote & Raaijmakers, 2018; Rasmussen *et al.*, 2019; Custer *et al.*, 2022). Because isolating deterministic and stochastic factors shaping plant microbiome assembly is challenging (but see Edwards *et al.*, 2018; rev. in Fitzpatrick *et al.*, 2020) and is often inferred from surveys, the relative strength of these factors in shaping plant microbiomes is still unclear (Dini-Andreote & Raaijmakers, 2018; Cordovez *et al.*, 2019).

The floral microbiome specifically has a central, unique, and typically brief role in shaping plant fitness and ecology. Some

pathogens use flowers to access plant vasculature, sterilizing flowers, or causing tissue death (e.g. Anther-smuts and *Erwinia* Elmqvist *et al.*, 1993; Sasu *et al.*, 2010). Alternatively, nonpathogenic floral microbes are common (Vannette, 2020). In floral nectar, bacteria or yeasts may affect plant fitness via changes to floral phenotype that change pollinator visitation (Vannette *et al.*, 2012; Schaeffer *et al.*, 2017; Vannette & Fukami, 2018), shift pollinators' on-flower behavior (Herrera *et al.*, 2013; de Vega *et al.*, 2022), or by competing with or facilitating other beneficial, commensal, and pathogenic microbes (Crowley-Gall *et al.*, 2022; Mueller *et al.*, 2023). Compared with the microbiomes of leaves or roots, floral microbiomes are highly variable among flowers on a plant, among individual plants, and among plant species (Rebolleda-Gómez *et al.*, 2019; Vannette, 2020). Both host selection and dispersal have been hypothesized to explain this variation, but their relatively influence has not been experimentally compared.

In flowers, host selection is likely an important deterministic process in floral microbiome assembly because floral microbiomes are a phylogenetically and phenotypically restricted subset of environmental microbes (Herrera *et al.*, 2010; Rebolleda Gómez & Ashman, 2019; Rebolleda-Gómez *et al.*, 2019). Plants can vary in their resistance to floral pathogens, for example, apple cultivars differ in resistance to the florally transmitted pathogen *Erwinia amylovora* (Emeriewen *et al.*, 2019). But less is known about host filtering of commensal or beneficial nectar microbes and the mechanisms driving it. Floral traits, such as nectar volume and chemistry, that could affect microbial survival and growth vary among individuals and species, and are likely to impact microbiome assembly because flowers are short-lived communities (Ashman & Schoen, 1994).

Dispersal is also a central process in floral microbiome assembly. In most floral communities, less than half of flowers contain culturable yeasts or bacteria (Herrera *et al.*, 2009; Vannette *et al.*, 2021), which is generally attributed to dispersal limitation. Most nectar-inhabiting microbes depend on zoophilic dispersal, and clear vertical transmission of the nectar microbiome has not been documented (rev. in Vannette *et al.*, 2021; except for some pathogens; e.g. Alexander, 1989). Floral microbe dispersal could be deterministic if plant traits caused predictable visitation by pollinators or other dispersers. There is some circumstantial evidence for this: Floral visitor networks predict the bacterial microbiomes of co-flowering plant species (Zemenick *et al.*, 2021), and broad pollination guild can predict floral microbiome (de Vega *et al.*, 2021). Nevertheless, plant–pollinator interactions are characterized by consistently low pollination driven by inadequate and partially stochastic pollinator visitation (Knight *et al.*, 2005; Richards *et al.*, 2009), making it likely that stochasticity may be particularly important in the assembly of nectar microbiomes compared with other plant tissues. Furthermore, low dispersal probability can increase the relative importance of stochastic processes in microbiome assembly (Evans *et al.*, 2017) especially in short lived ecosystems like flowers (Zapién-Campos *et al.*, 2020). Together, the short lifespan of flowers and variation in the probability of pollinator visitation raise the possibility that stochastic dispersal-mediated impacts on microbiome assembly might swamp out deterministic portions of dispersal or host selection.

Here, we tested the relative strength of host-plant selection on microbial growth vs: deterministic dispersal and establishment of microbes among hosts; and stochastic processes in shaping intraspecific variation in the nectar microbiome of *Epilobium canum*. We inoculated microbes into flowers, removing any dispersal differences among hosts, and monitored their growth to test for differences in host suitability among individual plants and cultivars (Fig. 1; study 1). We then compared standing microbial communities in openly visited flowers on the same plants to test for differences in the presence or absence of microbes – a measure of dispersal and establishment (Fig. 2; study 2). Finally, we compared nonzero microbial densities between inoculated and natural communities in flowers on the same plants to test whether plants that are most suitable for yeast and bacterial growth when inoculated with synthetic communities also have the highest microbial densities when under natural animal visitation (Fig. 1; studies 1 and 2 combined).

If host filtering is the primary driver of differences in nectar microbiome, we have three predictions. First, individuals and/or cultivars will differ in bacterial or yeast abundance when inoculated (study 1). Second, plants or cultivars will differ in nonzero (i.e. postdispersal and establishment) microbe abundances in study 2. Third, plants that were the best hosts in study 1 (i.e. high yeast or bacterial growth) also will have the highest microbial densities in study 2 (Fig. 1c). Alternatively, if deterministic dispersal and establishment are more important, then individual plants or cultivars should vary predictably the presence or absence of microbes in their flowers (Fig. 1b; study 2), but the microbiome of open flowers will not reflect the microbiome of inoculated flowers (Fig. 1c; study 3). Finally, if stochastic processes are very strong compared with deterministic processes, we should not detect individual or cultivar differences in study 1 and/or 2 and no correlation between the two studies.

Materials and Methods

Common garden design

All experimental plants were grown in a common garden on the campus of the University of California, Davis (38°53′71″N, 121°77′28″W) embedded in a matrix of agricultural land to control for environmental influence on the nectar microbiome, variation in regional microbial species pools, or differences in the pollinator landscape. The garden consisted of 15 plots (7.6 m × 4.6 m), each planted with a community of co-flowering plants a year before the experiment (see Supporting Information Table S1 for a full species list by plot). At planting, every bed contained five individuals of four morphologically distinct cultivars of the California endemic *Epilobium canum* (Greene, Onagraceae) including the wild accession *E. canum* ssp. canum, (Canum); two horticultural cultivars: *E. canum* cv Chaparral Silver (Silver, *E. canum*) and cv Everett's Choice (Everett's); and the regional ecotype *E. canum* cv Calistoga (Calistoga). We confirmed that cultivars have distinct floral morphology and nectar

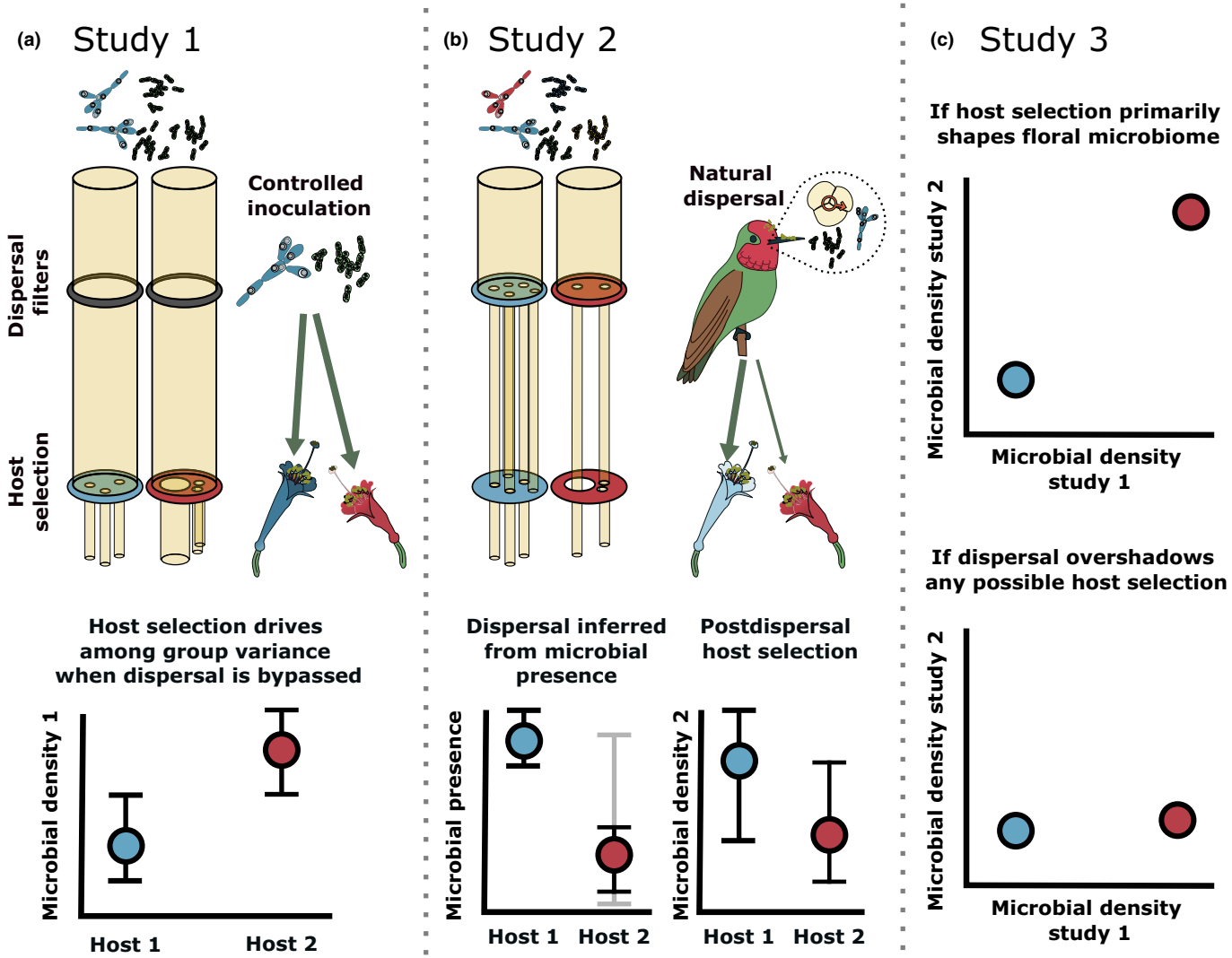

Fig. 1 Hypothesized filtering processes affecting nectar microbiome assembly and predictions for studies 1 (left), 2 (middle), and 3 (right). Microbial species pools are represented by yellow columns that are filtered by stochastic and deterministic processes including dispersal (upper disk) or plant host (lower disk). The bottom of each column displays predicted outcomes, where points represent group means and error bars are a measure of within-group variation. Left panel (a) – study 1: we removed dispersal filters by hand inoculating a synthetic community of a yeast and bacteria (represented by gray open disks) to isolate host selection differences among groups. If groups differ in host selection on nectar microbiome, we predicted that among group variation would be higher than within group (i.e. statistically significant differences among groups). Center panel (b) – study 2: we allowed natural animal visitation to the same plants. The resultant nectar microbe communities are the product of both dispersal (upper disks) and host selection (lower disks) filters. The probability of flowers having microbes is a measure of dispersal limitation (left plot). Deterministic differences in microbial dispersal would contribute to among plant variation (gray dotted line), while stochastic variation would increase residual variation (e.g. stochasticity would be higher in the gray vs black error bars). Significant differences in the mean of the nonzero densities of microbes among groups (right plot) would indicate postdispersal host filtering. Right panel (c) – study 3: we compared the microbial densities in study 1 to microbial densities in study 2. If the same host-plant quality is consistent between studies, plants or cultivars that had the highest microbial growth in study 1 should have the highest microbial growth in study 2 (upper plot). Alternatively, if differences in (1) dispersal dynamics or (2) in host selection between synthetic vs real communities were strong, we predicted that microbe density in study 1 would not predict microbe density in study 2 (lower plot).

phenotypes by collecting 172 flowers where we restricted animal visitation (control flowers from study 1 described below). *Epilobium* cultivars differ in standing nectar volume ($P < 0.01$; Fig. S1), sugar concentration ($P < 0.01$; Fig. S1), corolla width at widest point ($P < 0.05$; Fig. S1), and corolla length ($P < 0.00001$; Fig. S1). Additionally, these four cultivars are qualitatively phenotypically distinct, varying in growth form (prostrate to upright), leaf coloration, floral color, etc. While there are notable phenotypic differences among these cultivars, we do not have any information on genetic variability within or among these groups. Fieldwork was conducted from 24 September 2020 to 26 October 2020.

Study 1: Experimental inoculation

Inoculation protocol To test for differences among individual plants and cultivars in host filtering, we inoculated bagged flowers with the yeast *Metschnikowia koreensis* and bacteria

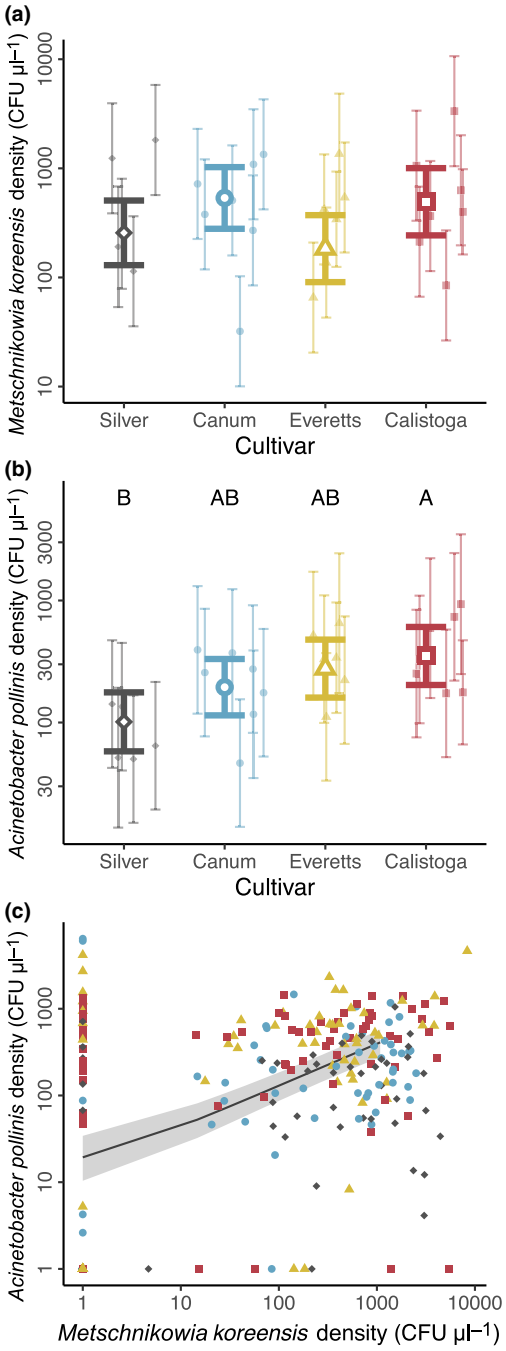

Fig. 2 Density of (a) *Metschnikowia koreensis* and (b) *Acinetobacter pollinis* in inoculated *Epilobium canum* flowers from study 1 in colony forming units (CFUs μl^{-1}). Small, closed points are the modeled mean ± 95% confidence intervals density for each plant (grouped, color, and shape by cultivar). Large open points are the cultivar-level modeled mean and 95% confidence intervals. There was significant variation among individual plants in both *M. koreensis* and *A. pollinis* (log-linear model). Cultivars significantly differed from each other in *A. pollinis* densities (log-linear mixed effects model, *post hoc* significance indicated by letters). (c) Flower level correlation between *M. koreensis* and *A. pollinis* with modeled relationship (black line) and 95% confidence intervals (gray fill, log–log-linear model). Points color and shape corresponds to cultivar. All densities shown on log scale (gray diamonds, Silver; blue circles, Canum; yellow triangles, Everett's; red squares, Calistoga).

Acinetobacter pollinis, both isolated from *E. canum* in our common garden. These genera commonly co-occur (Álvarez-Pérez & Herrera, 2013; Tsuji & Fukami, 2018), are the most common genera in *Epilobium* at our site (Morris *et al.*, 2019), and were qualitatively the most common morphospecies in our open samples (study 2). These strains were identified using MALDI-TOF using a custom-built library of Sanger Sequenced microbial accessions (Morris *et al.*, 2019 Bruker UltraFlextreme MALDI-TOF/TOF). We created freezer suspensions of this artificial microbial community made up of 5000 cells μl^{-1} of each species in 15% sucrose, 15% glycerol, and 70% sterile ultrapure H_2O. Cells were quantified via hemocytometer. We created a single freezer stock at the beginning of the experiment, stored it at −80°C, and used aliquots across all inoculations to ensure that every flower was inoculated with the identical initial microbial community.

At least 48 h before inoculation, we removed all the male-phase flowers from a section of a plant and enclosed that section of the plant in large pollinator exclusion bags (1 and/or 5-gallon paint filter bags, 200 µm, Cascade tools). *Epilobium canum* is protandrous and takes *c.* 2 d to proceed from male to female phase (Morris *et al.*, 2019), so any male-phase flowers in our bags opened while visitors were excluded. We bagged flowers from 30 individual plants in study 1. Bags were effective at excluding large visitors to *Epilobium* (e.g. hummingbirds and bees), but less so for smaller animals (e.g. thrips and ants).

On the morning of each inoculation bout, we resuspended freezer stock in sterile 15% sucrose at 9 : 1 ratio, giving us an inoculum with 500 cells μl^{-1} of each microbe. After thawing, the solution was vortexed for 30 s and stored for a maximum of 2 h before inoculating flowers. A control inoculum contained the same sterile 15% sucrose and glycerol mixture used to create freezer stocks. We randomly selected 14 male-phase flowers from within a bag on each plant for inoculation with microbial suspensions or control solutions (210 flowers of each treatment across the study). Using sterile 10 µl microcapillary tubes (VWR, Radnor, PA, USA), we added 4 µl of experimental solution to each flower (2000 cells each of *M. koreensis* and *A. pollinis*) or 4 µl of control solution, and flowers were marked using numbered jeweler's tags. Unmanipulated bagged flowers contained on average 13.2 µl of nectar. We inoculated flowers between 9:30 and 11:00 h across 3 d. During inoculation, we excluded any flowers that we observed being visited by animals while the bag was removed for experimental manipulation. After inoculation, all bags were replaced on the plants. After 72 h, we excised inoculated and control flowers (then female phase), transported flowers to the laboratory in coolers, and extracted nectar in a sterile condition as described below. Most flowers we inoculated persisted on the plant for 72 h ($N_{control} = 172$ and $N_{inoculated} = 173$, *c.* 81% of inoculated flowers in each treatment persisted, did not differ among cultivars Poisson GLM; $P = 0.51$). To estimate background microbial dispersal and contamination in bagged flowers, we sampled flowers inoculated with sterile control solutions (Table S2).

Quantifying microbial establishment and growth We used culture-based methods to quantify microbial presence and abundance. We collected nectar using 10 µl microcapillary tubes and

measured nectar volume to the nearest 0.05 µl. If flowers contained > 2 µl of recoverable nectar, we destructively measured the sugar concentration on 1 µl of the sample (to the nearest 0.5% brix) using a handheld refractometer. The remaining nectar was diluted in 20 µl of sterile ultrapure water and diluted 10× with phosphate-buffered saline (PBS) and an aliquot plated on yeast media agar (containing 0.1 mg ml^{-1} chloramphenicol to reduce bacterial growth), and 100× in PBS plated on fructose-supplemented tryptic soy agar plates (containing 0.1 mg ml^{-1} cycloheximide to reduce fungal growth). Plates were incubated at 26°C for 48 h and colony-forming units (CFUs) counted. Roughly 4% of plates generated uncountable colonies where microbes grew in a continuous mat or did not form discrete small units. For these, a single researcher classified plates as high, medium, or low coverage. We assigned the upper quartile, median, or lower quartile CFU counts of all plates of that type (e.g. Tryptic Soy Agar or Yeast Media Agar) to high medium and low coverage respectively. Finally, we accounted for dilution to calculate CFU μl^{-1} for each nectar sample. Comparison between culture-free shotgun metagenomes and culture libraries suggests that nectar microbes of *E. canum* are culturable on the media types used here, and all analyses here are referring only to culturable microbes (Morris *et al.*, 2019).

Measuring floral traits We assessed Brix of all nectar samples that contained > 2 µl using a handheld refractometer. When measured Brix exceeded 50% sucrose (13 samples), samples were diluted with 20 µl of DI water and re-measured. We also measured flower length (from the distal tip of the ovule to the furthest distal petal tip) and width (the widest point between petal tips) of each flower.

Studies 2 and 3: Open flower sampling

To assess microbial presence and abundance when microbial dispersal was not controlled, we sampled flowers from seven to eight individuals of each of the four cultivars used in study 1. For each plant, 10 female-phase flowers per plant were sampled. Female flowers were chosen to reflect the age of flowers in study 1. One individual Everett's was sampled twice, 8 d apart (seven plants of this cultivar, one with 20 flowers). We collected flowers and extracted then plated nectar to estimate microbial presence and abundance, as described above. We quantified the abundance of CFUs on TSA plates as bacteria and those on YMA as fungi, which are supported by previous validation (Morris *et al.*, 2019) and is a coarse, but conservative, measure of variation in microbiomes compared with species-level measures. Floral traits were also quantified as described above. In addition, we also removed stigmas of openly visited flowers immediately after removing flowers from plants in the field and stored them in 70% ethanol to quantify pollen receipt.

Measuring pollen receipt We assessed whether flowers received conspecific and heterospecific pollen to infer animal visitation using stigmas of openly visited flowers (Engel & Irwin, 2003; Price *et al.*, 2005). Stigmas were collected with cleaned tweezers and placed in 70% ethanol in the field. To quantify pollen, stigmas were mounted in phenol-free fuchsin gel (Kearns & Inouye, 1993) and melted at 80°C. To quantify pollen that had been dislodged from stigmas, the stigma-storage solution was pelleted (1.5 min at 16 000 ***g***), resuspended in 70% ethanol, and pollen mounted in fuchsin gel. Total pollen receipt was the sum of the on-stigma and pollen rinse counts.

Conspecific and heterospecific pollen were quantified visually under ×100–400 magnification as two indicators of pollinator visitation. *Epilobium* pollen is morphologically distinct from the pollen of co-flowering species, allowing determination of conspecific and heterospecific pollen receipt (Fig. S2). *Epilobium* flowers bear both male and female reproductive parts and produce copious amounts of pollen. Flowers display spatial and temporal herkogamy (separation of anthers and stigma) which may reduce self-pollination but we could not determine whether conspecific pollen was self or outcrossed in this study. Previous work in this species suggests that animal visitation to *E. canum* increases conspecific pollen deposition on stigmas and seed set (Snow, 1986), so we anticipated that conspecific pollen receipt would be an imperfect, noisy, proxy for animal visitation. Alternatively, heterospecific pollen on stigmas in this study is more likely to have been brought by animal visitors. We did not find any clearly wind-dispersed pollen grains in our samples (e.g. large *Pinus* pollen), and the majority of co-flowering plants are animal pollinated, so we consider heterospecific pollen a clearer proxy for animal visitation than conspecific pollen in this system.

Statistical analyses

All statistical analyses were completed in R 4.1.2 (R Core Team, 2021). Broadly, we used log-linear models (base R) and log-linear mixed effects models implemented in LME4 (Bates *et al.*, 2015) to assess differences in microbial density and abundance among plants and cultivars. We included a fixed effect of date to account for unmeasured time-varying factors. We also ran separate models testing for differences in microbial abundance among plots. If plot was a significant predictor, we included it in models testing for effects of interest, and if the final model included date, we nested plot in date. Additionally, we ran models with plot as a random intercept for plant-level models and plants nested within plot as a random intercept for cultivar-level models. The results for studies one and two were qualitatively similar, but we report the results of the linear models for plant-level differences because R^2 is better defined for these models (Nakagawa & Schielzeth, 2013). When testing for differences among cultivars, we accounted for repeated measures on individuals using a random intercept for plant. We used likelihood ratio tests and *F*-tests to test for significance in mixed effects and linear models, respectively. For all plant-level mean values, we used estimated marginal means to get predicted values after accounting for significant variation in covariates such as plot or sampling date. Broadly, we began with fully specified models but dropped nonsignificant terms for reported statistical values.

Study 1: Experimental inoculation

To test for host filtering, we built models testing whether individual or cultivar predicted microbial growth (model details Table 1). To quantify the relative effect of plant host on microbial abundance, we calculated partial r^2 values for models with individual plants as a predictor. Additionally, to assess whether some flowers or plants were better hosts for both yeasts and bacteria, we tested correlations among their densities at the flower and plant level. To test whether plant-level nectar sugar concentration predicted microbial growth, we regressed mean Brix from sterile control flowers (120 of 172 flowers) against mean bacterial and yeast densities. For all plant-level analyses, we excluded plants that had < 5 flowers sampled ($n = 5$).

Study 2: Open flower sampling

To detect deterministic differences in dispersal and host filtering in open flowers, we used two-stage hurdle models using microbial presence and abundance as response variables (*c.* 45% of open flowers did not contain microbes). We first tested whether plants or cultivars differed in the probability of containing bacteria or fungi (binomial GLMM). Because 95% of inoculated flowers contained microbes in study 1, we assume that *Acinetobacter* spp. and *Metschnikowia* spp. can establish in nectar, so variation in microbial presence is likely due to the presence or absence of dispersal. However, because microbial absence could be due to a lack of either dispersal or establishment, we refer to microbial presence as realized dispersal (defined in Custer *et al.*, 2022). We then tested whether plants or cultivars differed in nonzero fungal and bacterial densities using a log-linear GLMM (model details in Table 2). We calculated separate r^2 values for each stage of the hurdle model, for fungi and bacteria separately to test the relative strength of deterministic dispersal and host selection at the plant level. To determine whether plants and cultivars differed in pollen receipt, we built similar two-stage hurdle models.

If pollinators are the main source of microbes, the presence of pollen and microbes should be correlated. We tested for correlations between microbial and pollen presence at the floral level using chi-squared tests comparing the presence of conspecific, heterospecific, or any pollen deposition with the presence of

Table 1 Model results for study 1 assessing the effects of plant individual, date, plot, and cultivar on density of inoculated bacteria *Acinetobacter pollinis* and yeast *Metschnikowia koreensis* in the nectar of *Epilobium canum*.

Response	Fixed effects	Mixed effects	ΔAIC	*P*
Individual plants				
Log (*Acinetobacter pollinis* density + 1)	**Individual**	**na**	**−2.63**	**0.017**
	Date	na	−1.26	0.43
Log (*Metschnikowia koreensis* density + 1)	**Individual**	**na**	**−1.89**	**0.047**
	Date	na	0.09	0.19
Log (*Acinetobacter pollinis* total + 1)	**Individual**	**na**	**−5.68**	**0.033**
	Date	na	−0.98	0.36
Log (*Metschnikowia koreensis* total + 1)	**Individual**	**na**	**−4.35**	**0.081**
	Date	na	−1.30	0.45
Cultivar				
Log (*Acinetobacter pollinis* density + 1)	**Cultivar**	**1|Plant**	**−4.05**	**0.0085**
	Date	1|Plant	5.78	0.22
Log (*Metschnikowia koreensis* density + 1)	**Cultivar**	**1|Plant**	**3.17**	**0.027**
	Date	**1|Plant**	**2.99**	**0.023**
Log (*Acinetobacter pollinis* total + 1)	**Cultivar**	**1|Plant**	**−3.98**	**0.024**
	Date	1|Plant	1.59	0.511
Log (*Metschnikowia koreensis* total + 1)	**Cultivar**	**1|Plant**	**−6.76**	**0.0052**
	Date	1|Plant	3.34	0.25
Plot				
Log (*Acinetobacter pollinis* density + 1)	Plot	1|Plant	1.23	0.22
Log (*Metschnikowia koreensis* density + 1)	**Plot**	**1|Plant**	**−12.52**	**0.00038**

Bold lines indicate significant fixed effects, ΔAIC (Akaike information criterion) are single term deletions for fixed effects accounting for other covariates as described in the Materials and Methods section. na, not applicable.

Table 2 Models results for study 2 assessing the effects of plant individual, date, plot, and cultivar on the presence and abundance of bacteria and yeast in the nectar of open flowers of *Epilobium canum*.

Response	Fixed effects	Mixed effects	ΔAIC	*P*
Individual plants				
Proportion of flowers containing bacteria	**Individual**	**na**	**−59.47**	**1.3E-12**
Binomial error	Date	na	−1.45	0.063
Proportion of flowers containing yeasts	**Individual**	**na**	**−9.99**	**0.000049**
Binomial error	Date	na	−0.96	0.33
Log (bacteria density + 1)	**Individual**	**na**	**−11.25**	**0.00038**
	Date	na	1.92	0.8
Log (yeast density + 1)	**Individual**	**na**	**−20.62**	**0.00015**
	Date	**na**	**−16.90**	**0.00020**
Cultivar				
Proportion of flowers containing bacteria	**Cultivar**	**1|Plant**	**−8.71**	**0.0021**
Binomial error	**Date**	**1|Plant**	**−4.14**	**0.017**
Proportion of flowers containing yeasts	**Cultivar**	**1|Plant**	**−8.37**	**0.00240**
Binomial error	Date	1|Plant	−1.60	0.055
Log (bacteria density + 1)	Cultivar	1|Plant	1.09	0.17
	Date	1|Plant	0.82	0.16
Log (yeast density + 1)	Cultivar	1|Plant	5.12	0.83
	Date	1|Plant	−0.53	0.089
Plot				
Proportion of flowers containing bacteria	Plot	1|Plant	7.63	0.50
Proportion of flowers containing yeasts	Plot	1|Plant	7.35	0.47
Log (bacteria density + 1)	Plot	1|Plant	7.31	0.46
Log (yeast density + 1)	Plot	1|Plant	1.99	0.10

Bold lines indicate significant fixed effects, ΔAIC (Akaike information criterion) are single term deletions for fixed effects accounting for other covariates as described in the Materials and Methods section. na, not applicable.

culturable yeasts, bacteria, or any microbes (nine comparisons) and corrected for multiple comparisons using a false discovery rate. We used the presence or absence of both pollen and microbes because many factors can impact the magnitude of pollen transferred (e.g. nectar volume; Thomson, 1986), and the total abundance of microbes (e.g. time since dispersal; Vannette, 2020). At the plant level, we regressed the model-estimated proportion of flowers that received conspecific or heterospecific pollen on a plant against the mean number of flowers containing yeasts or bacteria.

To test whether plant traits impacted pollinator visitation, we used beta regressions to examine the relationship between plant mean nectar sugar concentration (Brix) or volume, flower width, or flower length and the probability of con- or heterospecific pollen receipt (Grun *et al.*, 2012). We built separate models for each plant trait. Furthermore, we tested whether mean plant traits impacted microbial dispersal by building similar beta regressions predicting the proportion of flowers on a plant that contained fungi or bacteria.

Study 3: Does variation in inoculated microbial communities predict microbial density in open flowers?

To test whether plants that had the highest microbial densities after inoculation (study 1) also had the highest microbial densities when openly visited (study 2), we constructed two linear models comparing mean microbial densities in inoculated and open flowers at the plant level. We used *z*-transformed and centered modeled densities *c.* 0 to account for differences in total microbial densities in studies 1 and 2. Twenty-one plants were represented in both the inoculated and open flower data sets, and we only included flowers that contained microbes when estimating density for flowers in study 2.

Results

Study 1: Do plants or cultivars predictably differ in microbial growth (independent of dispersal)?

We observed host selection by plant individual and cultivar when we controlled dispersal. There was a 30-fold range among plants in the mean density of the yeast *M. koreensis* (Figs 2a, 3; Table 1) and a 13-fold difference in the *A. pollinis* densities among plants (Figs 2b, 3; Table 1). Plant individual explained about a quarter of the variation in *M. koreensis* ($r^2 = 0.23$) and *A. pollinis* ($r^2 = 0.25$) densities. The results were qualitatively similar for total microbial cells per flower (Table 1). Cultivars also differed in *M. koreensis* and *A. pollinis* growth (Fig. 2a; Table 1), but pairwise differences among cultivars were not strong (all *post hoc* pairwise comparisons $P > 0.05$). Plots differed from each other in the density of *M. koreensis* but not *A. pollinis* (Table 1).

We also examined whether bacterial or fungal growth covaried. Indeed, yeast and bacteria densities were positively correlated in individual flowers (log–log-linear mixed effects model, cultivar effect $\chi^2 = 11.03$, $P < 0.001$; correlation $\chi^2 = 42.47$, $P < 0.0001$). At the plant level, *M. koreensis* and *A. pollinis* growth were positively but not significantly correlated (Fig. S2; log–log-linear model, $P = 0.069$), suggesting mechanisms occurring more strongly at the local flower scale.

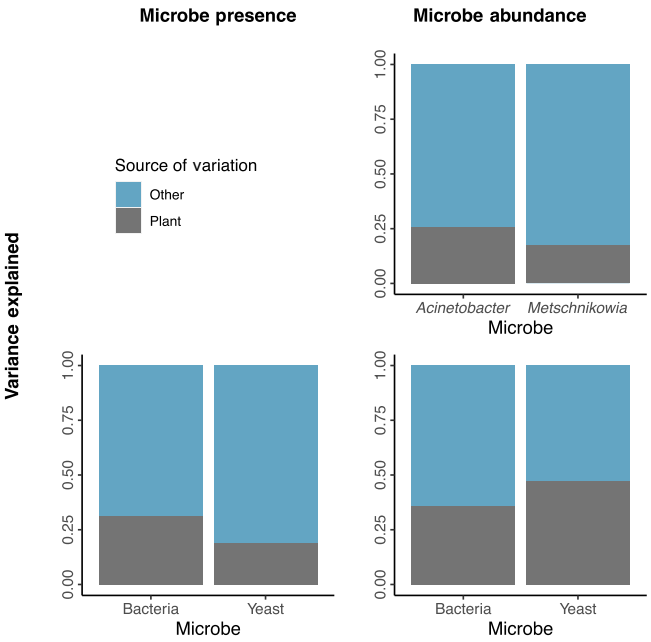

Fig. 3 Variance explained by individual *Epilobium canum* plant identity in microbial abundance in study 1 (upper right), abundance in study 2 (lower right), and microbial presence (lower left). Lower portion of the bars (gray area) represents variation explained by individual plant identity, upper portion of the bars (blue area) represents residual variation or variation explained by covariates (e.g. plot or date) in the final models indicated in Tables 1 and 2.

There were significant differences among individual plants in their nectar sugar concentrations (Brix) in sterile control flowers (linear model, $F = 4.11$, $P < 0.001$; Fig. 4a). However, plant-level mean sugar concentration did not predict *M. koreensis* or *A. pollinis* densities at the plant level (log-linear models, *M. koreensis* $t = 0.57$, $F = 0.32$, $P = 0.58$; Fig. 4c; *A. pollinis* $t = -1.06$, $F = 1.12$, $P = 0.28$; Fig. 4b).

Nearly all inoculated flowers had microbial growth; 94.8% contained fungi (mean = 363 CFU μl^{-1}) and 94.7% contained bacteria (mean = 1727 CFU μl^{-1}; Table S2). Control flowers had low microbial incidence suggesting inoculation was the main source of microbes; 10.0% of control flowers contained fungi (mean 29 CFU μl^{-1}) and 25.9% contained bacteria (mean 712 CFU μl^{-1}), which we suspect may have been due to thrips visitation (Vannette *et al.*, 2021).

Study 2: Do openly visited flowers predictably differ in microbial presence or growth?

In open flowers, plant individuals and cultivars differed in the presence of yeasts and bacteria, suggesting that deterministic differences in realized dispersal are important under natural conditions (individual level yeast $r^2 = 0.18$; bacteria, $r^2 = 0.31$; Fig. 5a, b; Table 2; individual level yeast $r^2 = 0.18$; bacteria, $r^2 = 0.31$; Fig. 5a,b; Table 2). In addition, individual plants, but not

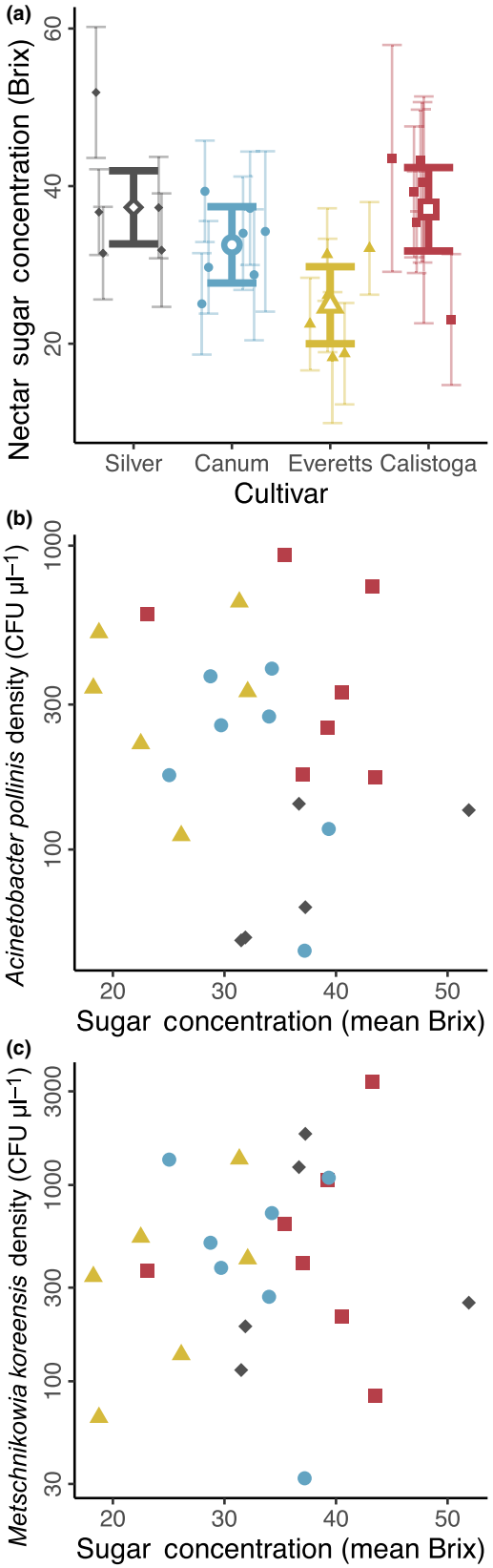

Fig. 4 (a) Nectar concentration (Brix) in control flowers varied among *Epilobium canum* plants (small points, linear model) and cultivars (open points, linear mixed effects model). Error bars represent 95% confidence intervals estimated from linear mixed effects model accounting for repeated measures on plants. There was no correlation between modeled mean *Acinetobacter pollinis* (b) or *Metschnikowia koreensis* (c) densities and modeled mean nectar concentrations at the plant level (gray diamonds, Silver; blue circles, Canum; yellow triangles, Everett's; red squares, Calistoga).

cultivars, differed in microbial abundance, suggesting postdispersal host filtering (Fig. 5b,c; Table 2). Plant individual also explained a substantial portion of variation in microbial abundance ($r^2 = 0.36$ bacteria, $r^2 = 0.42$ yeast; Fig. 3).

Plant individuals and cultivars differed significantly in pollen receipt (Table S3). If pollinators are the main source of pollen and microbes, pollen and microbial presence should be correlated. Indeed, plants with greater bacterial incidence in flowers were also more likely to receive heterospecific pollen (Table 3), suggesting shared pollinator movement of heterospecific pollen and bacteria. However, this correlation did not hold for conspecific pollen, as we suspect significant self-pollen deposition via wind and physical movement. By contrast, the plant-level mean amount of pollen receipt (conspecific or heterospecific) did not predict the proportion of flowers that contained yeasts (Table 3). At the flower level, individual flowers that received at least one conspecific pollen grain were more likely to contain bacteria, or any microbe at all (although all FDR > 0.05; Table 3).

Nectar and physical traits were associated with microbial incidence at the plant level. Plants with long and wide flowers were much more likely to contain yeasts (beta-reg, $z = 5.00$, $P < 0.001$ and $z = 5.72$, $P < 0.001$), while plants with higher nectar volumes were more likely to contain bacteria (beta-reg, $z = 2.07$, $P = 0.038$). Plants with longer flowers were more likely to receive conspecific pollen (beta-reg, $z = 5.00$, $P = 0.01$; Table S4), but neither nectar concentration, volume, nor flower width was associated with pollen presence.

Study 3: Comparing between study 1 and study 2: Does microbial growth in inoculated flowers predict microbial abundance in open flowers?

Microbial densities in inoculated and openly visited flowers on the same plant were not correlated. Plant-level mean *M. koreensis* and *A. pollinis* growth did not predict nonzero densities of yeasts or bacteria sampled from open flowers (linear model, yeasts, $t = -1.15$, $P = 0.27$; bacteria, $t = -0.86$, $P = 0.40$; Fig. 6), suggesting different processes driving microbial abundance between studies.

Discussion

We detected signatures of both deterministic plant selection and realized dispersal in community assembly of the nectar

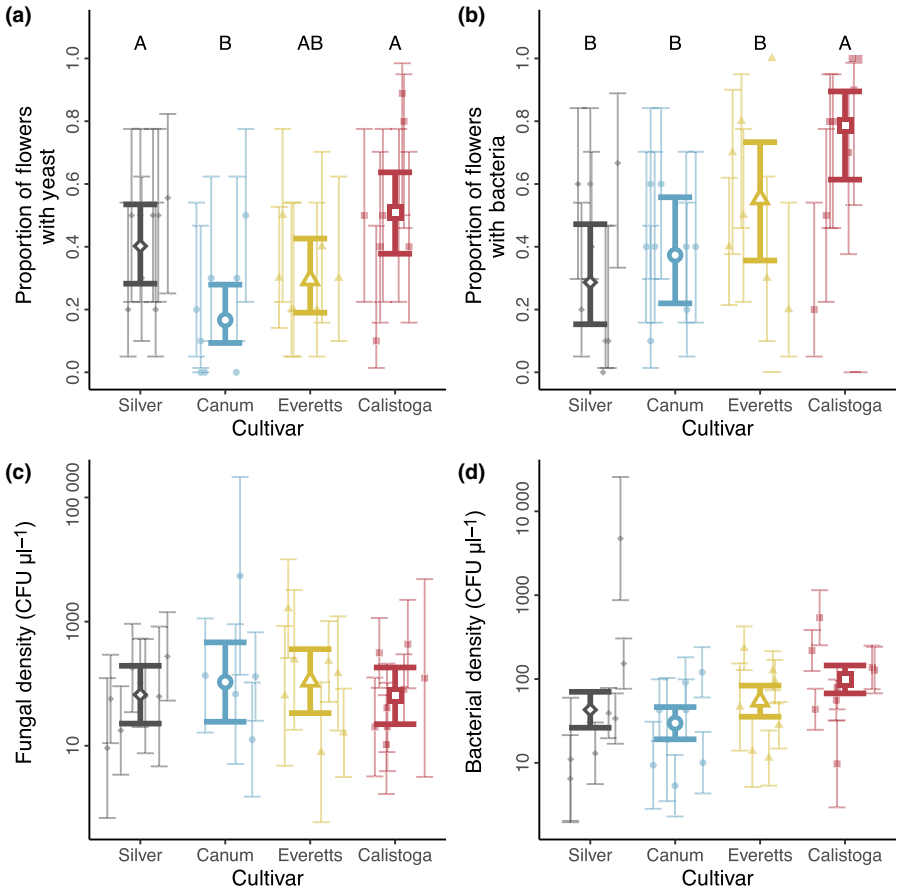

Fig. 5 Modeled mean proportion of *Epilobium canum* flowers that contain culturable yeasts (a) and bacteria (b) by cultivar (large open points) and individual (small closed points, binomial generalized linear model) colored and grouped by cultivar (binomial generalized linear mixed effects model). Letters indicate significant differences among cultivars. Mean density of culturable yeasts (c) and bacteria (d) in nectar samples that contained at least one colony forming unit (CFU) of each respectively by cultivar (large open points), and individuals (small, closed points). Error bars represent modeled 95% confidence intervals, note that density is log-scaled (Silver: gray diamonds, Canum: blue circles, Everett's: yellow triangles, Calistoga: red squares).

Table 3 Chi-squared tests of correlation between the presence of pollen and the presence of microbes at the individual flower level in *Epilobium canum*, and linear models at the plant level of probability of pollen receipt vs probability of microbe presence.

Pollen type	Microbe type	*T*-value	*P*
Plant level			
Conspecific presence	Bacteria present	0.3	0.74
Conspecific presence	Yeast present	0.76	0.38
Heterospecific presence	**Bacteria present**	**2.82**	**0.0037**
Heterospecific presence	Yeast present	−0.95	0.28

Pollen type	Microbe type	*P*	Adjusted *P*
Flower level			
Conspecific presence	Microbes present	**0.0076**	0.069
Conspecific presence	Bacteria present	**0.039**	0.17
Conspecific presence	Yeast present	0.11	0.35
Heterospecific presence	Microbes present	0.44	0.54
Heterospecific presence	Bacteria present	0.48	0.41
Heterospecific presence	Yeast present	0.2	0.54

Bold text indicates significant effects after correction for false discovery rate ($P < 0.05$).

microbiome of *Epilobium canum*. Multiple lines of evidence suggest that predictable differences in realized dispersal (dispersal and establishment) had an equal or stronger effect than differences in postestablishment microbial growth under realistic pollinator visitation. First, variation among cultivars in microbial presence (and inferred realized dispersal limitation) overshadowed variation in host selection among cultivars (Fig. 5). Furthermore, the plants that had the highest microbial growth when inoculated did not have the highest microbial densities when openly visited, suggesting that other factors, including dispersal and establishment are the primary factors affecting differences in the presence and abundance of *Epilobium* nectar microbiomes among cultivars (Figs 5, 6).

Host filtering of plant microbiomes is well documented in many plant tissues (e.g. in leaves Bálint *et al.*, 2013; and roots Xiong *et al.*, 2021; rev. in Fitzpatrick *et al.*, 2020) and was also evident in this experiment. In study 1, plant individual explained nearly 25% of variation in microbial abundance, despite previous evidence that host filtering of floral microbiomes may be less detectable than in other tissues (Wei & Ashman, 2018). While we found a signature of host selection, the specific plant traits driving these differences are not clear. Plant-level nectar sugar concentrations (Brix) in uninoculated flowers did not predict microbial growth among plants for either microbe (Fig. 4a,b). Yet, nectar is a complex mixture of mono- and disaccharides, free amino acids, secondary compounds, and proteins (Nicolson & Thornburg, 2007). Many of these can affect microbial growth (Schmitt *et al.*, 2018; Mueller *et al.*, 2023) and vary predictably among plants (e.g. nectar secretion rate; Mitchell, 2004); sugar concentration and composition (Herrera & Pozo, 2010;

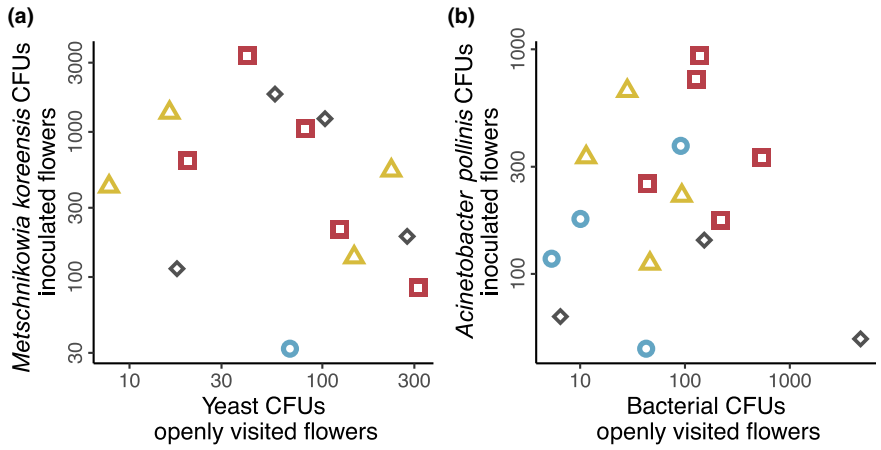

Fig. 6 Scatterplots showing modeled mean microbial densities at the plant level from studies 1 (vertical axes) and 2 (horizontal axes), (a) There was no correlation between mean yeast colony forming unit (CFU) density in open *Epilobium canum* flowers that had yeast cells present and *Metschnikowia koreensis* growth in inoculated flowers on the same plant (P = 0.20). (b) Similarly, mean bacterial CFUs in flowers that contained bacteria did not predict *Acinetobacter pollinis* growth on the same plant (P = 0.36; Silver: gray diamonds, Canum: blue circles, Everett's: yellow triangles, Calistoga: red squares).

Parachnowitsch *et al.*, 2019); amino acid concentration (Ryniewicz *et al.*, 2020); and the presence of secondary compounds, reactive oxygen species, or antimicrobial peptides (Adler, 2000; Carter & Thornburg, 2004; Palmer-Young *et al.*, 2019; Christensen *et al.*, 2021; Schmitt *et al.*, 2021). Future work should better link plant-level differences in nectar chemistry or other floral traits to nectar microbiome assembly and functional outcomes for plants, microbes, and pollinators.

Although we were unable to identify specific traits driving variation in microbial growth, yeasts and bacteria responded similarly among flowers when co-inoculated (Fig. 2c), suggesting shared microbial responses to variation in floral conditions or traits. We did not detect a signature of competition; instead, yeast and bacterial growth were positively correlated within individual flowers. Similar patterns of covariation have been previously detected, but are not universal (Tsuji & Fukami, 2018; Álvarez-Pérez *et al.*, 2019). A few hypotheses may explain this pattern. First, individual flowers on a plant may vary in quality, due to variation in light, temperature, nectar traits, or possibly even epigenetic mosaicism affecting floral traits (Herrera *et al.*, 2021). Second, positive correlations in natural flowers may be due to co-dispersal. However, the pattern reported here was in flowers where we controlled for dispersal via inoculation. Finally, we hypothesize that these correlations could be caused by microbial facilitation within a flower via the release of limiting nutrients (Christensen *et al.*, 2021) or the detoxification of shared environments as has been demonstrated between *Metschnikowia* and other bacteria (Mueller *et al.*, 2023).

In addition to host filtering, multiple lines of evidence suggest that deterministic differences in dispersal may be one of the main drivers of microbiome assembly in this system. First, differences among cultivars and individuals in microbial presence were nonrandom, and individual explained nearly as much variation in incidence as abundance. Individual differences accounted for 19–31% of the variation in microbial presence or absence, which is similar to variation explained by plant individual identity in microbial abundance in this study and in other plant tissues (< 15% in other tissues; Wagner, 2021). Between 38% and 73% of open flowers on a plant contained bacteria and 17–50% contained fungi despite near universal establishment in study 1 (*c.* 95%; Fig. 4). The incidence rates observed here reflect previous work in the Mediterranean (yeasts 32–44%; Herrera *et al.*, 2009), and California Coast Range (Yeasts 20%, Bacteria 49%; Vannette *et al.*, 2021) surprisingly closely. This study adds to those by demonstrating that intraspecific differences in microbial incidence are predictable and can be as high or higher than differences among co-flowering plant species. Because the microbial propagule density used in the current experiment may overestimate establishment (Hausmann *et al.*, 2017), work that compares establishment across inoculation densities would further separate the effects of dispersal in the strict sense from realized dispersal. Nevertheless, supporting the importance of dispersal, cultivars that differed in microbial abundance when we inoculated flowers showed no measurable differences in microbial abundance when openly visited but did show strong predictable differences in microbial presence. This is consistent with the hypothesis that dispersal is a major driver of differences in microbial community assembly at the cultivar level, overwhelming differences in microbial growth we detected in study 1.

The second line of evidence for a primary role of dispersal is that after removing open flowers with no dispersal or establishment, plants that had the highest growth in study 1 did not have the highest nonzero densities of microbes in study 2 (Fig. 6). This could be due to several factors. One possibility is that microbial communities in open flowers are more diverse than the inoculated communities. Previous work supports the overwhelming dominance of the two microbial genera inoculated in this study in many flowering systems world-wide (e.g. Álvarez-Pérez & Herrera, 2013; Tsuji & Fukami, 2018; Chappell *et al.*, 2022). However, this line of evidence is contingent on the assumption that *Metchnikowia* and *Acinetobacter* are representative of other members of natural communities (Morris *et al.*, 2019) and some habitats may host more diverse nectar microbial communities (e.g. Canto *et al.*, 2017; de Vega *et al.*, 2021). Another possibility is that in our open flowers, the timing and frequency of microbe inoculation likely varied. Variability in dispersal time could impact microbe–microbe interactions such as competition, facilitation, and priority effects (Fukami, 2015; Álvarez-Pérez *et al.*, 2019), which may have been more pronounced in study 2. In our open flowers, we are unable to know how long microbial

communities had been growing in flowers, adding substantial noise to any abundance measures in study 2. With these caveats, if host suitability for microbial growth broadly had a very strong impact on microbial density, we predicted that densities in studies 1 and 2 would be correlated, but they were not.

A third line of evidence for the importance of dispersal is the correlation between heterospecific pollen receipt and bacterial presence. Although some traits predict both pollen and microbial presence (Table 3), evidence for pollinators as microbial dispersers was not as universal as we predicted. We did not find relationships between conspecific pollen receipt and bacteria or any pollen receipt variables and yeasts. We hypothesize that temporal lags between microbial dispersal and growth to detectable levels, visitation by animals that move microbes but do not pollinate, or substantial self-pollination in *Epilobium* might partially explain the lack of correlations between conspecific pollen receipt and microbe presence, especially at the floral level. Previous work has documented different dispersal dynamics between yeasts and bacteria (Vannette *et al.*, 2021). This result does not exclude the possibility that microbes co-disperse to flowers but possibly suggests reliance on different dispersal vectors among yeasts and bacteria, differential establishment, or possibly competitive exclusion under realistic dispersal. Previous work supports the hypothesis that animals vary in their vectoring of microbial taxa, and this may underlie such patterns (de Vega *et al.*, 2021; Vannette *et al.*, 2021; Zemenick *et al.*, 2021).

The finding that 25% of variation in microbial incidence was explained by host identity in this system, suggests that heritability of the 'microbial phenotype' may be low and might limit the impact of microbes on floral trait evolution. Furthermore, separating microbial-influenced selection on floral traits from other factors will require careful experiments. Detecting whether differences in nectar microbiome are the cause or effect of variation in pollinator visitation, given that these two variables are inconsistently correlated, will be difficult. However, the correlation between microbial dispersal and pollination shown here suggests a novel hypothesis: Plant species that require few animal visits for adequate pollination (e.g. plants with high Pollen : Ovule ratios or high pollen deposition), may be less impacted by microbial changes to floral phenotypes. For floral microbes to shape plant trait evolution, we posit that: microbial dispersal would have to be consistently high; and flowers would have to be long-lived, needing multiple visits by pollinators for adequate pollination. Alternatively, microbial colonization would have to be extremely costly/beneficial (as is the case with pathogenic microbes where the eco-evolutionary dynamic may be different; Alexander, 1989; Elmqvist *et al.*, 1993).

Taken together, our results suggest that floral microbiome assembly is contingent on interactions between differences in host-plant quality and deterministic dispersal limitation. In some cases, deterministic dispersal can be as strong as host selection in driving variation in microbiome. Because floral microbes are dispersed primarily by animals who make predictable decisions based on plant traits, nectar microbiomes may be unique from other plant tissues because host traits not only act on growth rates but also on dispersal probabilities. However, we suggest that the role of deterministic dispersal processes may be an underrecognized driver across other plant tissues. Recent evidence from phyllosphere microbes suggests that possible dispersal from co-occurring plants is an important factor in driving leaf microbiome assembly (Meyer *et al.*, 2022), and co-dispersal of seeds and rhizosphere fungi may be predictable (Correia *et al.*, 2019). Furthermore, new work suggests that at large biogeographic scales, plant-associated bacteria and fungi can be dispersal-limited (Zhang *et al.*, 2021), suggesting that the dynamics we characterize here may be more broadly applicable. Our work adds experimental evidence that deterministic dispersal can overwhelm host selection in some cases and rival it in others, so we suggest that future empirical studies of plant microbiome assembly should consider this possibility.

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Competing interests

None declared.

Author contributions

All authors contributed to the conceptualization, data collection, and preparation of the manuscript. JSF performed statistical analyses with feedback from TGM and RLV and prepared the original draft of the manuscript. All authors contributed to revisions.

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Data availability

All data are available at https://datadryad.org/stash/dataset/doi:10.25338/B84P8N.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Cultivars of *Epilobium canum* differ in corolla length corolla width, nectar volume, and nectar sugar concentration.

Fig. S2 Microscopic image of *Epilobium canum* stigma showing conspecific and heterospecific pollen grains.

Fig. S3 There is no correlation between average *Acinetobacter pollinis* and *Metschnikowia koreensis* density at the plant level.

Table S1 List of plant species planted in each plot in our common garden.

Table S2 Data regarding the presence of bacteria or fungi in our bagged control flowers broken down by cultivar in *Epilobium canum* in study 1.

Table S3 Model results for hurdle models of pollen receipt on flowers in study 2.

Table S4 Model results for beta regressions testing for correlations between plant traits, microbial presence, and pollen receipt.

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