Journal of Chemical Ecology
https://doi.org/10.1007/s10886-020-01169-3

Microbial Co-Occurrence in Floral Nectar Affects Metabolites and Attractiveness to a Generalist Pollinator

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Received: 1 November 2019 / Revised: 27 January 2020 / Accepted: 6 March 2020

Abstract

Microbial metabolism can shape cues important for animal attraction in service-resource mutualisms. Resources are frequently colonized by microbial communities, but experimental assessment of animal-microbial interactions often focus on microbial monocultures. Such an approach likely fails to predict effects of microbial assemblages, as microbe-microbe interactions may affect in a non-additive manner microbial metabolism and resulting chemosensory cues. Here, we compared effects of microbial mono- and cocultures on growth of constituent microbes, volatile metabolite production, sugar catabolism, and effects on pollinator foraging across two nectar environments that differed in sugar concentration. Growth in co-culture decreased the abundance of the yeast *Metschnikowia reukaufii*, but not the bacterium *Asaia astilbes*. Volatile emissions differed significantly between microbial treatments and with nectar concentration, while sugar concentration was relatively similar among mono- and cocultures. Coculture volatile emission closely resembled an additive combination of monoculture volatiles. Despite differences in microbial growth and chemosensory cues, honey bee feeding did not differ between microbial monocultures and assemblages. Taken together, our results suggest that in some cases, chemical and ecological effects of microbial assemblages are largely predictable from those of component species, but caution that more work is necessary to predict under what circumstances non-additive effects are important.

Keywords *Apis mellifera* · Olfaction · Gustation · Nectar microbes · MVOC · Pollination · Volatile organic compounds · Phytobiome

Introduction

Service-resource mutualisms involving plants and animals are often mediated by nutrient-rich food provisions (Bronstein 2015). Mutualists seek out hosts offering resources such as fruits or floral nectar, among other rewards, using visual, olfactory, and gustatory cues (Bascompte and Jordano 2007; Vander Wall 2001). Microbial colonization of food is common and can result in significant alteration of the nutritional value, taste, and associated cues animals use to assess food quality (Buchholz and Levey 1990; Davis et al. 2013; Peris et al. 2017; Ruxton et al. 2014). Despite such effects, and recognition that these nutrient-rich resources can harbor diverse microbial assemblages (Allard et al. 2018; Herrera et al. 2009a, b; Wassermann et al. 2019), most studies to date have focused on both the metabolic and resulting tritrophic impact of a single microorganism's colonization of a reward (Rering et al. 2018). However, communities containing more than one microbial species are likely to influence the metabolome differently than do monocultures (Fischer et al. 2017; Verginer et al. 2010).

Interactions among microbial community members can affect the associated metabolome in a number of ways (Ponomarova and Patil 2015). For example, syntrophy or cross-feeding, where one community member consumes the by-product of another, can facilitate emergent community-level biochemical pathways not observed in monocultures (Morris et al. 2013; Seth and Taga 2014). Antagonistic metabolic interactions among microorganisms are also common.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-020-01169-3) contains supplementary material, which is available to authorized users.

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Published online: 03 April 2020

For example, microorganisms can detect the presence of nearby cells and excrete antimicrobial metabolites in response (reviewed in Bauer et al. 2018; Granato et al. 2019). Or, in response to resource competition, microorganims may metabolize non-preffered nutrients (Beisel and Afroz 2016). Finally, in antagonistic interactions where release of an antimicrobial metabolite results in death of another, a corresponding release of nutrients into the environment can potentially improve growth of the predatory microorganism (Tang et al. 2020).

Floral nectar often hosts yeasts and bacteria that can attain high abundance (Herrera et al. 2009a, b; Vannette and Fukami 2017; Álvarez-Pérez et al. 2012), and alter nectar chemistry, volatile emission, and resulting pollinator visitation (Herrera et al. 2013; Rering et al. 2018). Despite low species diversity typically observed in flowers (Pozo et al. 2011), multiple species are often isolated from individual flowers. Moreover, microbial species are likely to interact through metabolites within nectar. Priority effects, where early colonizing species suppress the establishment of late-arrivers, have been reported in floral nectar (Mittelbach et al. 2016; Peay et al. 2012; Toju et al. 2018; Tucker and Fukami 2014) and are hypothesized to be mediated by competition for nitrogen (Dhami et al. 2016; Vannette and Fukami 2018). However, certain microorganisms co-occur in nectar more frequently than would be expected by chance (Álvarez-Pérez and Herrera 2013), suggesting facilitation may also occur. Thus, interactions among nectar-inhabiting microbes are predicted but the nature of these interactions are unclear (Álvarez-Pérez et al. 2019).

Microbial growth can change nectar chemistry (Pozo et al. 2015), altering both sugar and amino acid content and composition (Herrera et al. 2008; Lenaerts et al. 2016; Vannette and Fukami 2018), in addition to producing volatile and non-volatile metabolites. These metabolic products can be perceived by pollinators (Rering et al. 2018; Schaeffer et al. 2019) and can influence pollinator resource use (Junker et al. 2014; Rering et al. 2018; Schaeffer et al. 2019). Pollinator response to microbial cues can differ among microbial species (Pozo et al. 2019; Schaeffer et al. 2019; Vannette et al. 2013), likely due to species-specific growth patterns and metabolite production. To date, our knowledge of nectar microbe metabolism and subsequent impacts on pollinator acceptance have been evaluated for monocultures alone (Rering et al. 2018; Schaeffer et al. 2017), despite the observation that multiple microbial species can often be isolated from nectar of the same flower (Álvarez-Pérez and Herrera 2013).

In this study, we prepared a coculture of yeast and bacterium and respective monocultures. We examined microbial volatile and non-volatile metabolism in laboratory-based experiments with two nectars of differing sugar concentrations, one approximating a rain-diluted nectar and the other a typical floral nectar, to evaluate potential microbial-induced shifts in nectar chemistry. Finally, we examined honey bee foraging preferences when presented with mono- and cocultured synthetic nectars in a field study. We hypothesized that nectar microorganisms would suppress one another's growth in cocultures relative to monocultures, that cocultures and monocultures would produce unique volatile metabolite blends, and that bees would discern between the differing cultures. Our results reveal that coculture reduced the abundance of *Metschnikowia reukaufii* but not *Asaia astilbes* and volatile metabolites distinguish mono- and cocultures. In preference assays, honey bees consumed similar amounts of all microorganism-inoculated nectars and preferred the sterile nectar over any microorganism-inoculated nectars.

Methods and Materials

Study System

Two microorganisms commonly associated with floral nectar and pollinators were selected for study: *Metschnikowia reukaufii*, a cosmopolitan, nectar-specialist yeast (Lachance et al. 2001; Pozo et al. 2011) and *Asaia astilbes*, a plant- and insect-associated bacterium (Crotti et al. 2010; Fridman et al. 2012; Good et al. 2014). Both are documented to occur in floral nectar (Suzuki et al. 2010). Strains of *M. reukaufii* (GenBank ID: MF319536) and *A. astilbes* (GenBank ID: KC677740) were isolated from *Epilobium canum* (Onagraceae) and *Mimulus aurantiacus* (Phrymaceae) respectively (Rering et al. 2018). Previous work by our group has revealed that these species have divergent effects on nectar chemistry when grown in monoculture (Rering et al. 2018).

Two synthetic sterile nectars were prepared, "high" sugar concentration (60 g/L each fructose and glucose, 30 g/L sucrose, 15% *w/v* sugar) and "low" (6 g/L each fructose and glucose, 3 g/L sucrose, 1.5% *w/v* sugar) with identical amino acid supplementation (0.1 mM each: glycine, alanine, asparagine, aspartic acid, glutamic acid, proline, serine). The "high" sugar nectar reflects the chemistry of a more typical floral nectar, which can range anywhere between 6.3–85% sugar (Pamminger et al. 2019), while the "low" nectar approximates the carbohydrate content of a rain-diluted nectar, which occurs in many upward-facing flowers with unprotected nectar crops (Lawson and Rands 2019). A dilute (i.e., "low") nectar was selected to evaluate the impact of sugar content on microbial growth kinetics alone and in co-culture, as the high osmotic pressure of nectar often precludes or reduces the growth of less osmo-tolerant generalist microorganisms (such as *Asaia astilbes*) within nectar (Herrera et al. 2009a, b; Álvarez-Pérez et al. 2012). Samples ($n = 04$–5 per treatment, one replicate was lost) were prepared by suspending cells from 3 d old cultures (YMA and R2A media plates for yeast and bacteria, respectively) in 20 mL of each nectar solution. Monocultures contained approx. 10^3 cells/μL (0.4 A OD_{600} for bacteria) and cocultures contained twice the total number of cells (yeast +

bacteria) in the same 20 mL initial volume as monocultures in an additive design. Initial cell densities fell below those typically observed in nature (Herrera et al. 2009a, b; Tsuji and Fukami 2018). Sterile controls containing either nectar but no microorganisms were also prepared, and all experiments were performed in sterilized mason jars with lids modified as described in Rering et al. (2018). Microbial growth was assessed non-destructively at 0, 24, and 48 h post-inoculation from sample solutions by removing 1 μL nectar, diluting in sterile water, and plating to yeast- or bacteria-selective media to quantify colony forming units/μL (CFU/μL). Data collection and sample preparation was staggered over two consecutive weeks due to limited sample throughput, with mono- and coculture samples prepared in each nectar evenly distributed throughout the collection period.

Volatile Analysis

Microbial volatiles were sampled in a non-targeted approach with solid-phase microextraction fibers (SPME; Supelco, Bellafonte, p.a.; 50/30 μm, 2 cm, divinylbenzene/ carboxen/ polydimethylsiloxane) at intervals of 0, 24 and 48 h and analyzed by gas chromatography coupled to mass spectrometry (GC-MS) similar to as described in Rering et al. (2018). Method details are described in the supporting information (Supplementary S1). Briefly, a DB-Wax column (60 m x 320 μm x 0.25 μm, J &W Scientific, Folsom, ca.) was used to separate microbial volatiles and compare emission between samples and the MS electron ionization source was set to positive mode. Compound identities were confirmed via comparison of fragmentation pattern and retention indices on a polar and non-polar column with commercial standards when possible. Identification results, retention indices, and fragmentation patterns of microbial volatiles are described in Table S1. Compounds with similar abundance in sterile media were removed from the dataset. Those which were present in sterile media at reduced abundance however were retained, but sample peak areas were blank-subtracted (see Supplementary S1 for additional details).

Theoretical Additive Coculture Sample Set

To evaluate whether coculture headspace deviated from an additive combination of yeast and bacteria monoculture headspace, and if so, what compounds were more or less abundant than would be expected based on monocultures, we calculated a theoretical coculture sample set (hereafter described as "theoretical coculture"). The theoretical coculture samples were calculated by summing the analyte peak areas for yeast and bacteria monocultures, binned by sampling interval and nectar composition. This iterative summation results in a volatile emission dataset with equal replication as the experimental mono- and cocultures (4–5 theoretical coculture replicates for each nectar; 0, 24 and 48 h sampling intervals for each replicate). The experimental and theoretical coculture samples were then compared as described in the statistical analysis section below.

Sugar analysis

Nectar sugars were analyzed by liquid chromatography (LC) coupled with evaporative light scattering detection (ELSD). Samples were collected (0, 24, 48 h), filter-sterilized (0.22 μm, 13 mm, PVDF, Foxx Life Sciences, Salem, NH), and quantified with external calibration curves. Method details are provided in the supporting information (Supplementary S2).

Pollinator Preference Assay

To examine if a generalist pollinator (*Apis mellifera*) could distinguish among microbial mono- and cocultures, we performed a pollinator preference assay. An artificial foraging array was established at the Bee Biology Research Facility at the University of California Davis. Artificial flowers were constructed from white cardstock and 1.5 mL centrifuge tubes to mimic flowers. The artificial array consisted of 10 green-painted bamboo sticks (~ 1 m tall), with flowers fixed to each stick using florist's wire and green florist's tape. Each stick held 4 flowers, with each flower assigned to one of 4 nectar treatments (control, *M. reukaufii, A. astilbes, and M. reukaufii* + *A. astilbes*; incubated for 2 d prior to the bioassays). The array was placed ~ 1–2 m away from hives at the apiary. Prior to the start of the experiment, flowers were filled with 1 mL of control nectar. Nectar was replenished daily for a period of three days to allow honey bee foragers to identify and recruit to the array prior to the beginning of the experiment. During each replicate of the experiment, flowers were filled with 200 μL of treated nectar solution, with each stake assigned a treatment in a randomized order, including bagged controls to account for evaporation. Bees were allowed to forage for 2 h in the afternoon, then vials were collected, and remaining nectar quantified. The assay was repeated twice during fall 2018.

Statistical Analyses

To compare if growth in coculture vs. monoculture influenced microbial cell density, we analyzed CFU data with a linear mixed effect model with interactive effects of time, microbe treatment (*A. astilbes*, *M. reukaufii* and the coculture) and nectar composition with a random effect of experimental week. Sugar catabolism was compared among microbe-inoculated treatments with linear mixed effect models with interactive effects of time, microbe treatment and nectar composition, using molar concentration units and proportion of monomers/total sugars as dependent variables. First, sugar

catabolism was compared using all samples in the dataset including both 15 and 1.5% nectar. A second analysis compared among samples within a given nectar concentration, to more closely examine the influence of time and microbial treatment on resulting sugar content. Pollinator consumption of nectars was evaluated with a linear model with the effect of microbial inoculation with bioassay replicate date as a covariate. Tukey honest significance differences (HSD) were used to assess differences among microbial treatment groups. Linear mixed models were estimated using the nlme package (Pinheiro et al. 2019).

Volatile composition among microbe-inoculated treatments was visualized with a principle coordinate analysis (PCoA) based on Bray-Curtis dissimilarities. Volatile composition was then further investigated with a permutational analyses of variance (perMANOVA) using time, microbe treatment, nectar composition and their interaction as effects with the adonis function in the vegan package and homogeneity of variance was assessed using the betadisper function in vegan (Oksanen et al. 2018). To further evaluate differences between pre-defined groups, including which compounds (if any) distinguished theoretical and experimental coculture samples, differential analyses based on the negative binomial distribution were performed using DESeq2 (Love et al. 2014) with nectar composition and microbe treatment as effects and significance evaluated with the Wald test ($\alpha = 0.01$). Volatile data collected at 0 h was excluded from the differential analysis due to its high variability. All statistical analyses were carried out in R (version 3.5.1; R Core Team 2013).

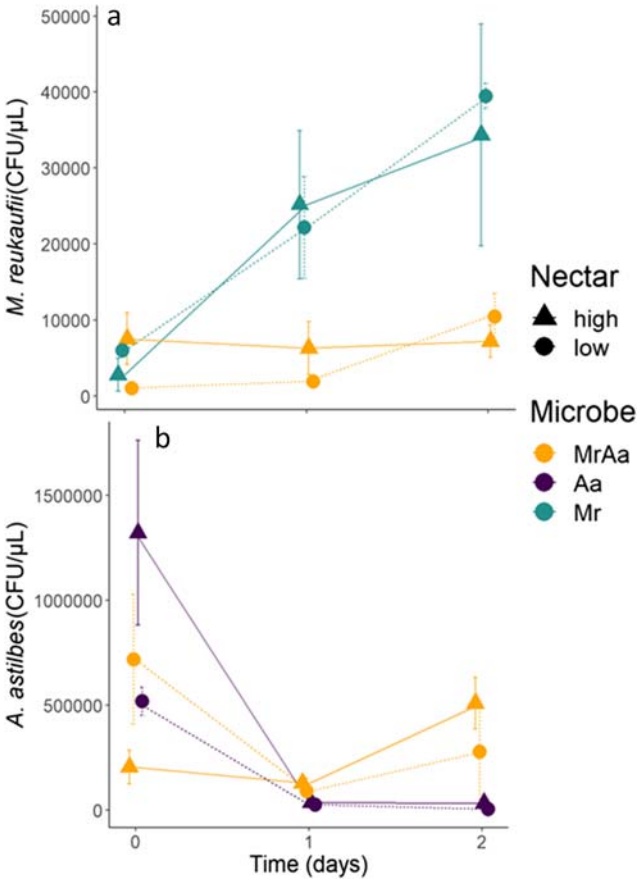

Fig. 1 Colony forming unit (CFU) density of (**a**) *Metschnikowia reukaufii* (Mr) and (**b**) *Asaia astilbes* (Aa) in mono- and cocultures (MrAa) prepared in 15% (square icon) and 1.5% (point icon) nectar ($n = 04–5$ per treatment). Points and error bars represent mean and standard error, respectively

Results

Microbial Growth in Mono- and Cocultures

Co-inoculation differentially affected yeast and bacteria cell densities. The cell density of *M. reukaufii* was significantly lower in cocultures compared to monocultures (> 70% at 48 h, Fig. 1, microbe treatment: $\chi^2 = 22.5$, df = 1, $p < 0.001$). In contrast, *A. astilbes* cell densities were not distinguishable between mono- and coculture treatments (microbe treatment: $\chi^2 = 0.0004$, df = 1, $p > 0.9$). Microbial density changed over time, but differently depending on taxa; yeast cell densities tended to increase with time (time: $\chi^2 = 18.5$, df = 2, $p < 0.001$) while bacteria densities decreased in both mono- and coculture for the first day, but increased on day 2 in coculture (Fig. 1; time: $\chi^2 = 30.7$, df = 2, $p < 0.001$). Nectar composition did not significantly influence cell densities for yeast (nectar composition: $\chi^2 = 0.0162$, df = 1, $p = 0.9$), but *A. astilbes* cell density differed with the interaction between nectar and microbial treatment (microbe treatment*nectar composition: $\chi^2 = 4.14$, df = 1, $p = 0.04$) and with the 3-way interaction between nectar, microbial treatment and time (microbe treatment*nectar composition*time: $\chi^2 = 14.6$, df = 2, $p < 0.001$; see Table S2 for full model results).

Microbial Volatile Emission in Mono- and Cocultures

Cocultures and yeast monocultures emitted a greater number and total abundance of volatiles than bacterial monocultures (day 2 VOC abundance: Tukey HSD $p < 0.001$; Fig. 2). Total volatile emission of mono- and cocultures differed with microbial treatment, time, and nectar composition, as well as their interactive effects ($p \leq 0.001$; Table S3 for full model results). Strikingly, total volatile emission was much higher in 15% nectars than in 1.5% nectar: volatile emission of monocultures was five-fold greater in 15% than 1.5% sugar nectars, and coculture volatile emission was three-fold greater.

The composition of microbial volatiles further distinguished mono- and cocultures (microbe treatment: $F_{2,57} = 41.1$, $p = 0.001$), nectar compositions ($F_{1,57} = 33.6$, $p = 0.001$), and timepoints ($F_{2,63} = 35.7$, $p = 0.001$), with significant interactions effects for all factors in the perMANOVA

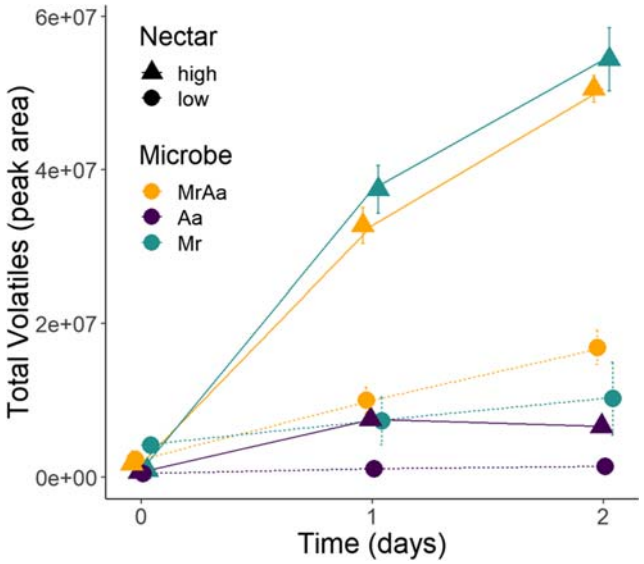

Fig. 2 Total volatile emission among mono- (Mr: *Metschnikowia reukaufii*; Aa: *Asaia astilbes*) and cocultures (MrAa) in low (point icon) and high (square icon) concentration nectars. Points and error bars represent mean summed peak areas for all microbial volatiles and standard error, respectively ($n = 04–5$ per treatment). Volatile emission from inoculated samples was background-subtracted with corresponding sterile controls

model (Table S4). Ordination based on Bray-Curtis dissimilarity matrices (Fig. 3), revealed *A. astilbes* monocultures diverged from the yeast and coculture samples, while cocultures and *M. reukaufii* monocultures largely overlap after 24 and 48 h growth. This pattern suggests that the yeast aroma dominated the volatile emission of the cocultured nectars. However, significant differences between mixed and *M. reukaufii* monocultures were observed, including enhanced emission of isoprene and acetoin in cocultures and greater emission of 1-hexanol and 4-penten-1-ol in single cultures of *M. reukaufii* ($p < 0.001$ each; Figure S2).

No previously undetected volatiles were found when the microbes were co-inoculated, suggesting the volatile profiles of these microbes are largely additive when introduced and allowed to grow in a shared nectar media. Only the emission of 4-penten-1-ol significantly differentiated theoretical and experimental cocultures, indicating a slight deviation from purely additive effects on volatile metabolite formation. In both nectars, emission of 4-penten-1-ol was lower in experimental cocultures than the calculated theoretical coculture samples would suggest (Figure S3). Unlike many volatiles, which were common to the headspace of both yeast and bacteria monocultures, this short-chain unsaturated alcohol was never detected in bacteria monocultures.

Sugar Catabolism in Mono- and Cocultures

When microbial effects on sugar concentration was assessed, total sugar content (M) only weakly decreased with time ($\chi^2 = 3.4$, df = 1, $p = 0.06$) and did not differ between mono- and cocultures (microbe treatment: $\chi^2 = 1.5$, df = 2, $p = 0.5$) despite the higher cell density in coculture (Fig. 4). Low and high nectar differed in sugar reduction (nectar composition: $\chi^2 = 5714$, df = 1, $p < 0.001$), where sugar content was reduced to a greater extent in 1.5% sugar nectar compared to 15% nectar.

Among component sugars, sucrose and glucose concentration declined with time (time: sucrose $\chi2 = 8.0$, df = 1, $p = 0.005$; glucose $\chi2 = 6.3$, df = 1, $p = 0.01$). However, fructose content did not change over time (time: fructose $\chi2 = 1.1$, df = 1, $p = 0.3$). Fructose, glucose and sucrose concentration were similar among mono- and cocultures (microbe treatment: $p > 0.2$; see Table S4 for all model results). However, microbe treatment significantly affected the ratio of monosaccharides to total sugars (([fructose] + [glucose])/([fructose] + [glucose] + [sucrose]); *mol/mol*; $\chi^2 = 10.6$, df = 2, $p = 0.005$), with significant decrease in monomer sugars in mixed inoculations compared to *M. reukaufii* monocultures (Tukey HSD $p = 0.005$).

Because graphical analysis suggested distinct patterns in sugar catabolism depending on nectar concentration, we performed separate analyses of each nectar type. Within 1.5%

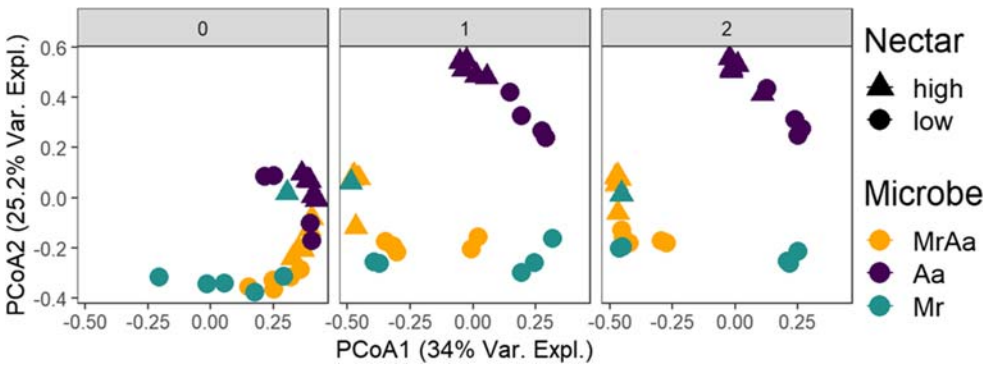

Fig. 3 Differences in volatile emissions between mono- (Mr: *Metschnikowia reukaufii*; Aa: *Asaia astilbes*) and cocultures (MrAa) prepared in low (point icon) and high (square icon) concentration nectars, visualized by principle coordinate ordination (PCoA) using Bray-Curtis dissimilarity ($n = 04–5$ per treatment). Each panel represents a sampling interval (day 0, 1, 2). Volatile emission from inoculated samples was background-subtracted with corresponding sterile controls

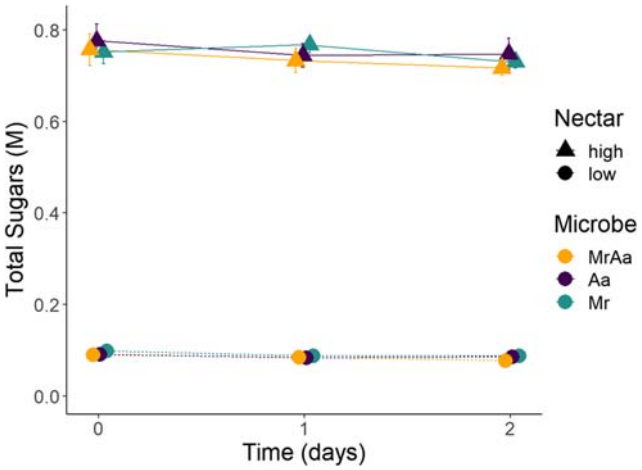

Fig. 4 Total sugars remaining (M) in mono- (Mr: *Metschnikowia reukaufii*; Aa: *Asaia astilbes*) and cocultures (MrAa), prepared in low (point icon) and high (square icon) concentration nectars. Points and error bars represent mean and standard error, respectively ($n = 04–5$ per treatment)

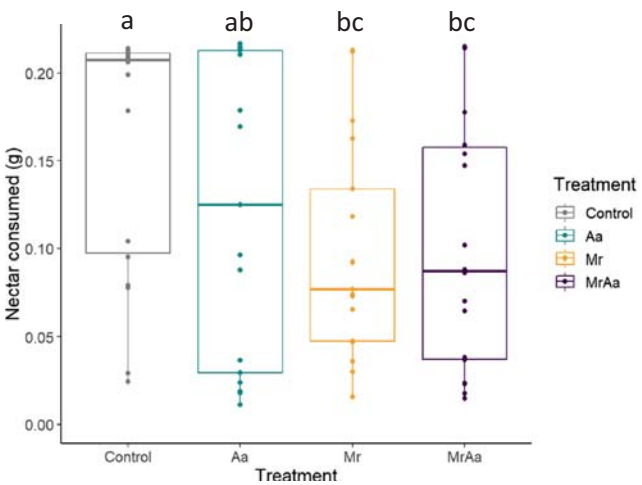

Fig. 5 Removal of mono- (Mr: *Metschnikowia reukaufii*; Aa: *Asaia astilbes*) and coculture (MrAa) nectar, prepared initially at a 15% sugar concentration, by honey bees ($n = 20$ per treatment)

nectars, fructose, glucose, and total sugar content declined with time (time: $p < 0.001$ in all cases, see Table S6 for full model results), however fructose remained unchanged throughout the incubation period (time: $\chi^2 = 0.758$, df = 1, $p = 0.3$). Cocultures contained significantly less total sugar content compared to yeast monocultures (microbe treatment: $\chi^2 = 8.73$, df = 2, $p = 0.01$, Tukey HSD, $p = 0.02$) and yeast monocultures maintained higher levels of glucose than bacteria mono- or cocultures (microbe treatment: $\chi^2 = 42.5$, df = 2, Tukey HSD, $p > 0.001$). In contrast, no detectable reduction in sugar concentration or in sugar composition over time or with microbial treatment was detected in inoculated samples prepared in 15% nectar ($p > 0.05$ in all cases; see Table S7 for full model results).

Pollinator Preference Between Mono- and Cocultures

Bees consumed nearly 60% more sterile nectar than microbial-inoculated nectars (ANOVA, $F_{3,58} = 3.35$, $p = 0.03$; Table S8, Tukey HSD, $p < 0.05$; Fig. 5). Though mono- and cocultures were not statistically distinguishable, honey bees removed on average 20% more *A. astilbes*-inoculated nectar than *M. reukaufii* monocultures or cocultures. Nectar consumption was different between the two bioassay replicates, with greater nectar removal in the first trial than the second (ANOVA, $F_{1,57} = 61.8$, $p < 0.001$). However, the same pattern of preferences was observed in both trials (Figure S4).

Discussion

Resources provisioned by plants to attract animal mutualists are frequently colonized by diverse microbial assemblages. The ecological and evolutionary effects of microbial colonization and multitrophic consequences of their metabolism have largely been restricted to study of individual microbial taxa. Here, we demonstrate that volatile production in an additive co-culture may be relatively well approximated by that of the respective monocultures, though this finding is likely dependent on the microbial species present and the host substrate. Volatile metabolites distinguished mono- and cocultures, but total sugar content and honey bee consumption of mono- and cocultures were similar. These results underscore the importance of examining the metabolic interactions and mutualist responses of microbial communities in addition to pure cultures.

Coculture influenced microbial growth for both species. Specifically, yeast density in nectar was lower when bacteria were also present, despite the additive design (Fig. 1a), while bacterial abundance was greater in the presence of yeasts (Fig. 1b). Although we did not investigate the mechanisms that may underlie these differences, it could be that the production of some bacterial metabolites reduced yeast growth or that bacteria could use by-products of yeast metabolism or cell death (Tang et al. 2020). Microbial species also differed in their patterns of abundance over time. In mono- and co-cultures, the yeast *M. reukaufii* increased in density over time, whereas bacterial cell counts decreased, then increased over the two day incubation period, particularly in coculture. Yeast cell densities were not impacted by the differing sugar content in the two nectars, whereas some significant interactive effects of nectar concentration were found for bacteria cell density.

Despite the differences in microbial abundance between treatments, volatile emission of the coculture could be effectively modeled by summing volatile emission of the monocultures, except for the emission of one short-chain alcohol produced by *M. reukaufii* but not *A. astilbes*, 4-penten-1-ol. This volatile appears to be relatively unique among microbial

metabolites; it is yet unregistered in the mVOC 2.0 database (Lemfack et al. 2014) and only one report, published by some of the authors of this experiment, has previously reported its emission from microbial headspace samples (Rering et al. 2018). 4-penten-1-ol, which possesses a musty, unpleasant odor to humans, was less abundant in coculture headspace than would be predicted based on yeast monoculture headspace samples. This could be a consequence of the reduced *M. reukaufii* cell density observed in cocultures. However, it is also possible that *A. astilbes* metabolizes 4-penten-1-ol.

Significantly higher volatile emission was observed in the 15% sugar nectars than in the 1.5% sugar nectars, regardless of which focal microorganisms were inoculated. Increased volatilization of chemicals from aqueous solutions containing higher amounts of solutes is a well-understood abiotic process, driven by reduced volatile solubility in the more saturated solution (Schwarzenbach et al. 2003). We investigated abiotic partitioning as potential cause for this disparity by comparing emission of microbial volatiles in sterile 1.5% and 15% sugar nectars (described in supplementary S3) and found that differences in volatilization driven by differing sugar concentrations were insufficient to explain the difference in emission observed in microbial cultures, ranging from only 8–12% enhanced emission in high vs. low nectar (Figure S1). This increased emission was also not correlated with a greater cell density in the 15% nectar. It may be that differences in volatile emission between the nectars is due to different metabolic activities of the focal microorganisms under different conditions, although this hypothesis remains to be tested. If volatile production differs depending on nectar concentration this may be detectable by pollinators and potentially serve as an 'honest signal" (Raguso 2004) of nectar sugar availability or resource quantity.

Despite marked increases in volatile emission from both *M. reukaufii* and cocultured nectars, honey bees preferentially foraged on *A. astilbes*-inoculated and sterile nectars. On average, honey bees removed 40% more nectar from flowers assigned to these treatments over the assay period. These results contrast with honey bee responses reported in previous studies by our laboratories and others (Good et al. 2014; Rering et al. 2018), where honey bees preferred yeast-inoculated and sterile nectars over bacteria. Differences between the previous and current studies are not immediately apparent; however, we suggest that honey bees incorporate previous experiences when foraging for resources (Menzel 1993) and their foraging preferences can be manipulated by volatiles present in the hive (Jakobsen et al. 1995), so their behavior can be highly context-dependent. It is possible the honey bees in this study had limited experience with these or other nectar microorganisms and possessed no general innate preference for microorganism-laden nectar, which may explain their general preference for sterile nectar over any of the inoculated nectars reported here. Seasonality may also have impacted the results of the bioassay, as it was conducted in the fall, when limited floral resources were available. Honey bee hive microbiomes also exhibit seasonal shifts (Kešnerová et al. 2019) which may also impact forager preferences.

Bees exhibit strong preferences among nectars, preferring high sugar content (Seo et al. 2019) and sucrose-rich over monosaccharide-rich nectars (Mommaerts et al. 2013; Wykes 1952). Though sugars were depleted in the inoculated nectars, reductions were minor and didn't exceed 6% of total sugars in the high sugar nectar after 48 h. Individual nectar sugars and total sugar content were not significantly different among the microbial treatments, suggesting that the microbes' catabolism of carbohydrates yielded similar quality nectars in terms of caloric value. Additionally, minor shifts (below 1%) in nectar carbohydrate chemistry were observed between microbe treatments, including changes in the proportion of monomers to total sugars, as has been previously reported for various nectar microbes (Schaeffer et al. 2015; Vannette and Fukami 2018). The shifts towards a higher proportion of sugar monomers in coculture may have contributed to reduced bee preference.

The data presented here suggest that in some communities, overall metabolic output of multiple microbial species can be predicted by their monocultures. In contrast to other systems where large differences in composition result from growth in coculture (Wakefield et al. 2017; Martínez-Buitrago et al. 2019), our data suggest that in this nectar system interspecific interactions have only subtle effects on metabolite composition. These results suggest that scaling up from single microbial effects in more complex microbial communities may require a combination of methods, including additive and subtractive experimental designs, and assessing multiple ecologically relevant phenotypes. Nevertheless, our results suggest that the dynamics of multispecies microbial community likely matter for community metabolism and effects on resource quality and should be considered in future study of the effects of microbes on multitrophic interactions.

Acknowledgments We thank M. Anderson, M. Handy, and I. Maalouf for laboratory and field assistance. This research was supported by USDA-ARS Research Project 6036-22000-028 (JB and CR), and 2016 ARS Administrator Research Associate program (CR). RS acknowledges support from a USDA NIFA Education and Literacy Initiative Postdoctoral Fellowship (2017-67012-26104). RV was supported by the National Science Foundation (award numbers DEB1846266 and DEB1929516), USDA Multistate award NE1501, the Hellman Foundation and the University of California, Davis.

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