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Contrasting effects of yeasts and bacteria on floral nectar traits

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• **Background and Aims** Flowers can be highly variable in nectar volume and chemical composition, even within the same plant, but the causes of this variation are not fully understood. One potential cause is nectar-colonizing bacteria and yeasts, but experimental tests isolating their effects on wildflowers are largely lacking. This study examines the effects of dominant species of yeasts and bacteria on the hummingbird-pollinated shrub, *Mimulus aurantiacus*, in California.
• **Methods** Wildflowers were inoculated with field-relevant titres of either the yeast *Metschnikowia reukaufii* or the bacterium *Neokomagataea* sp. (formerly *Gluconobacter* sp.), both isolated from *M. aurantiacus* nectar. Newly opened flowers were bagged, inoculated, harvested after 3 d and analysed for microbial abundance, nectar volume, and sugar and amino acid concentration and composition.
• **Key Results** Yeast inoculation reduced amino acid concentration and altered amino acid composition, but had no significant effect on nectar volume or sugar composition. In contrast, bacterial inoculation increased amino acid concentration, enhanced the proportion of nectar sugars comprised by monosaccharides, and reduced nectar volume.
• **Conclusions** The results presented suggest that microbial inhabitants of floral nectar can make nectar characteristics variable among flowers through divergent effects of yeasts and bacteria on nectar chemistry and availability, probably modifying plant–pollinator interactions.

Keywords: Flower longevity, flower microbiome, host–microbe, *Mimulus aurantiacus*, nectar chemistry, plant defence, plant-pollinator.

INTRODUCTION

It is widely recognized that flowers vary greatly in nectar volume and chemical composition (Mitchell, 2004, and references therein), which can affect pollinator foraging and plant fitness (Cnaani *et al.*, 2006). Some of this variation reflects genetic differences among plants (Mitchell, 2004), but nectar characteristics can be highly variable even within individual plants (Biernaskie and Cartar, 2004; Herrera *et al.*, 2006). One factor that is gaining increased recognition as a major source of this variation is nectar-colonizing micro-organisms. Recent studies have linked microbial abundance to a variety of nectar characteristics, including temperature (Herrera *et al.*, 2010), pH (Vannette *et al.*, 2013; Tucker and Fukami, 2014), volatile profiles (Rering *et al.*, 2018), and the concentration and composition of sugars (Herrera *et al.*, 2008; Canto and Herrera, 2012; de Vega and Herrera, 2012; Canto *et al.*, 2015; Vannette and Fukami, 2017), secondary metabolites (Vannette *et al.*, 2013) and amino acids (Herrera *et al.*, 2008; Herrera and Pozo, 2010; Peay *et al.*, 2012; Vannette *et al.*, 2013), as well as pollinator foraging (Good *et al.*, 2014; Junker *et al.*, 2014; Schaeffer and Irwin, 2014; Vannette and Fukami, 2016) and plant reproduction (Herrera *et al.*, 2013; Vannette *et al.*, 2013). In addition, some studies have reported taxon-specific effects, particularly between bacteria and yeasts, both of which are frequently found in floral nectar (Vannette *et al.*, 2013; Good *et al.*, 2014; Tucker and Fukami, 2014; Rering *et al.*, 2018).

Most of these studies have used field observations examining correlational data, laboratory experiments using nectar extracted from plants (e.g. Peay *et al.*, 2012; Vannette *et al.* 2013, 2016), or field experiments where entire microbial communities are added to the nectar of real flowers (Canto *et al.*, 2007, 2008, 2011; de Vega and Herrera, 2013). It remains unknown whether bacteria and yeasts differentially influence nectar characteristics in real flowers. This knowledge is needed to understand fully microbial effects on nectar traits as microbial species composition can vary among flowers due to differences in microbial dispersal history and strong competitive interactions within flowers (Tucker and Fukami, 2014; Vannette and Fukami, 2017; Toju *et al.*, 2018). Given that nectar microbial communities might be often dominated by either yeasts or bacteria (Tucker and Fukami, 2014) and that yeasts and bacteria may have contrasting effects on nectar chemistry and pollination visits (Vannette *et al.*, 2013; Good *et al.*, 2014; Rering *et al.*, 2018), their separate effects in wildflowers are of particular interest. In this study, we inoculated flowers of wild *Mimulus auranticus* plants, a hummingbird-pollinated shrub in California, with a species of the dominant nectar yeast, *Metschnikowia reukaufii*, or that of the dominant nectar bacteria, *Neokomagatea* sp. (previously called *Gluconobacter* sp.), to quantify their effects on nectar characteristics.

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MATERIALS AND METHODS

Study organisms

We conducted this study using micro-organisms isolated from the nectar of flowering plants at Jasper Ridge Biological Preserve (JRBP) in the foothills of the Santa Cruz mountains near Stanford, California. Yeasts and bacteria were isolated from the nectar of the flowering shrubs *Mimulus aurantiacus* (sticky monkeyflower) and *Eriodictyon californicum* (yerba santa). Both plant species are visited by hummingbirds, and bees including *Bombus vosnesenskii*, *Ceratina acantha* and *Xylocopa micans*, although *M. aurantiacus* is primarily hummingbird pollinated, whereas *E. californicum* is mainly insect pollinated. The butterfly *Euphydryas chalcedona* also visits *E. californicum.*

The nectar microbes of *M. aurantiacus* have been studied previously (Belisle *et al.*, 2012; Dhami *et al.*, 2016; Tsuji *et al.*, 2016; Vannette and Fukami, 2017; Dhami *et al.*, 2018), and the dominant micro-organisms are readily cultured and inoculated into live flowers (e.g. Vannette *et al.*, 2013). In *M. aurantiacus*, both bacteria and yeasts appear to modify floral nectar traits including pH, sugar composition, H_2O_2 concentration, and amino acid composition, although this evidence comes from laboratory experiments using extracted nectar, not field experiments with nectar in real flowers (Vannette *et al.*, 2013). In a field correlational study, microbial abundance explained 35 % of the variation in sugar composition of *M. aurantiacus* (Vannette and Fukami, 2017), suggesting that micro-organisms influence nectar traits under field conditions. Microbial diversity within flowers is relatively limited. Often, single or a few species of micro-organism dominate a nectar sample (Belisle *et al.*, 2012; Vannette and Fukami, 2017). As a result, examining the effect of single microbial species is ecologically relevant for *M. aurantiacus* (Belisle *et al.*, 2012) and other plant species (Herrera *et al.*, 2009).

We chose microbial taxa that were common and dominant representatives of floral microbial communities in our study plants. The fungal species most frequently isolated from both plant species was the yeast *Metschnikowia reukaufii*. Given the considerable phenotypic diversity within this yeast species (Herrera *et al.*, 2014; Dhami *et al.* 2016, 2018), we used multiple strains: one isolated from *M. aurantiacus* and two from *E. californicum*. No significant differences in growth or effects on nectar parameters were detected between the strains ($P > 0.05$), so strains are pooled for all analyses below. We also used a bacterial strain isolated from *M. aurantiacus* (NCBI JX437138.1), a bacterium in the Acetobacteraceae and a close BLAST match to the genus *Neokomagatea* sp. (previously called *Gluconobacter* sp.) identified using a region of the 16S rRNA gene. The Gram-negative *Neokomagataea*, an acetic acid bacterium, is a frequent colonist of floral nectar in many species of California plant species (R. L. Vannette, unpubl. res.) and some other biogeographic regions (Samuni-Blank *et al.*, 2014; Reis and Teixeira, 2015). We used a single strain from the genus *Neokomagataea*. Yeasts were maintained on yeast malt agar (YMA) and *Neokomagataea* on Reasoner's 2A (R2A) medium supplemented with 20 % sucrose (Oxoid formula).

Experimental design

In the same area of JRBP as used by Belisle *et al.* (2012), we chose 29 *M. aurantiacus* plants, which spanned a gradient of sun exposure and water availability. Five of the 29 plants received experimental water addition, mimicking 2-fold the annual rainfall. For this purpose, from January to May of 2014, 10 L of tap water was delivered once a week, using a 10 L container placed at the base of each plant. The containers had a spigot, so that the water was delivered slowly. No significant effect of watering was detected on any measured variables ($P > 0.10$), so we do not include this factor in the statistical models below. During May and June of 2014, unopened buds on each plant were marked using a small jewelry tag that had a unique identification number and then bagged using a small organza bag, which prevented hummingbirds and other floral visitors from accessing the flower, inhibiting microbial dispersal to flowers via the flower-visiting animals (Belisle *et al.*, 2012; Vannette and Fukami, 2017). More flowers were marked and bagged than were used to ensure that experimental cohorts of flowers had opened on the same day. Bagged flowers were checked daily.

Microbial inoculation

Microbial suspensions were prepared using a sterile 20 % sucrose solution. On the morning of inoculation, suspensions of each yeast and bacterial strain were prepared at 1000 cells μL^{-1} from a colony formed on 3- or 4-day-old plates, which probably included both viable and non-viable cells. Into each newly opened flower, we pipetted 1 µL of suspension (1000 cells) of a single species or a sterile control solution comprised only of the 20 % sucrose solution. Flowers from all plants received each treatment, depending on flower availability. Most plants hosted between one and three replicates of each treatment (control, yeasts, and bacteria), which was limited by the number of flowers opening each day. Upon anthesis, *M. aurantiacus* flowers typically contain about 2–5 µL of nectar. Feeding by a hummingbird in artificial nectar solutions after banding (Vannette and Fukami, 2017) typically results in the deposition of, on average, 155 (range = 10–1180) colony-forming units (CFUs) on a YMA plate or, on average, 2000 (range = 10–10 000) CFUs on a R2A plate (unpubl. res.), so our experimentally introduced microbial titres were within the range of those probably deposited in nectar of *M. aurantiacus*.

Álvarez-Pérez and Herrera (2013) showed that bacteria and yeasts appeared to co-occur frequently in the flowers that they studied in Spain. However, this study analysed the presence and absence of bacteria and yeasts, not their abundance. Even if bacteria and yeasts co-occur frequently in terms of their presence/absence, it is possible that they rarely co-dominate, in the sense that bacteria are rarely abundant if yeasts are abundant, and vice versa. In fact, laboratory experiments that used bacterial and yeast strains isolated from *M. aurantiacus* flowers (Tucker and Fukami, 2014) indicated that bacteria and yeasts, in general, and *Neokomagataea* sp. (formerly *Gluconobacter* sp.) and *M. reukaufii*, in particular, would engage in strongly antagonistic interactions in nectar and, as a consequence, rarely co-dominate. This study suggests that whichever arrives in a

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flower earlier than the other would dominate in that flower, suppressing the other (Tucker and Fukami, 2014). Furthermore, a field experiment using wild *M. aurantiacus* flowers (Toju *et al.*, 2018) yielded results that are consistent with those of Tucker and Fukami (2014). Given that bacteria and yeasts are likely to affect nectar traits most extensively when they are abundant, these two earlier studies suggesting that bacteria and yeasts rarely co-dominate provide a biological justification for the lack of co-inoculation of bacteria and yeasts in our experimental design. We note, however, that it may be worthwhile in future research to try co-inoculation to better understand the effect of bacteria and yeasts on nectar traits.

Immediately after the inoculation, flowers were re-bagged. Three days following inoculation, flowers were clipped at the pedicel and stored in a cooler until nectar could be extracted. Individual flowers are typically open between 4 and 7 (Peay *et al.*, 2012). We chose to harvest after 3 d to maximize recovery of inoculated flowers. Storage in the cooler lasted no longer than 3 h. In total, 222 flowers were inoculated over 7 d, with the experiment spanning 2 weeks during peak flowering during 2014, from 22 May to 6 June. We did not observe stigma closure on any bagged flower, indicating that pollen was not deposited on the stigma (Fetscher and Kohn, 1999), and thus flowers were probably not visited by pollinators.

Laboratory analyses

In the laboratory, the corolla tube was separated from the calyx, and nectar collected from each flower using a 20-µL microcapillary tube. The volume of each sample was quantified and the nectar diluted in 15 µL of sterile water. Flower status, including corolla condition (turgid or wilted/senescent), was noted during nectar extraction. Serial dilutions of nectar samples were plated (0.1 and 0.01 µL of original sample plated) to determine CFU density in each flower. Yeast-inoculated samples were plated on YMA without the antibacterial chloramphenicol, and bacterial-inoculated samples were plated on R2A without the antifungal cycloheximide, to assess whether additional micro-organisms were present. Sucrose-inoculated flowers were plated on both media types to ensure that no detectable microbes were present. The number of CFUs on each plate was counted after 5 d. We analysed 123 flowers inoculated with *M. reukaufii*, 33 flowers inoculated with *Neokomagataea* sp., and 40 flowers inoculated with sucrose solution.

Sugars and amino acids were also quantified from the same nectar samples. For sugars, 1 µL of remaining nectar was diluted in 49 µL of 50:50 acetonitrile:water containing 0.5 µg/µL^{-1} maltose as an internal standard. For each sample, 1 µL of the above dilution was injected and separated using ultra performance liquid chromatography (UPLC). Sugars were separated on a Luna amide column (50 × 2 mm, 3 µm, Phenomenex, Torrance, CA, USA) and quantitated using evaporative light scattering detection (Waters, Milford, MA, USA). Mono- and disaccharides detected in each sample (with elution times matching fructose, glucose, and sucrose) were quantified using a series of external standards. The remaining nectar was used to quantify amino acid content using Waters AccQ-Tag derivatization and standards, separated on an AccQTag Ultra Column (2.1 × 100 mm, 1.7 µm, Waters) and analysed using UV detection. However, only a sub-set of total samples could be used for all analyses due to microbial effects on nectar presence and volume. Amino acid identity was based on the retention time of a series of external standards and concentration calculated from standard curves.

Statistical analyses

We examined whether microbial CFUs and nectar characteristics within individual flowers varied among inoculation treatments using a linear mixed model, where plant individual and inoculation date were included as random effects, using the R package nlme v3.1 (Pinheiro *et al.*, 2012). In each model, microbial inoculation treatment (yeasts, bacteria, or control) was used as a predictor. Separate models examined inoculation effects on $\log_{10}$-transformed total CFUs per flower, nectar volume, the concentrations of sucrose, glucose, and fructose, the proportion of monosaccharides [i.e. (glucose + fructose)/(glucose + fructose + sucrose)], and the total concentration of amino acids. We also analysed raw CFU counts using a negative binomial generalized linear mixed model (GLMM) using the package lme4 (Bates *et al.*, 2015) followed by a likelihood ratio test to assess the significance of inoculation treatment. To assess whether inoculation treatment influenced the probability that a flower contained nectar following inoculation (yes or no), we used logistic regression with inoculation treatment as a predictor. We examined sources of variation in the amino acid composition in nectar using a multivariate analysis of variance (MANOVA) including microbe inoculation treatment and plant identity as predictors.

All analyses were performed in the R statistical environment (v.3.3.1, R Core Team, 2013).

RESULTS

Treatments were effective, with focal microbes detected in 90 % of inoculated flowers, while <10 % of the uninoculated flowers had detectable microbial growth. When present, both yeasts and bacteria grew to high CFU densities in nectar (linear model Fig. 1A; $F_{2,141} = 90.16$, $P < 0.0001$; glmm $\chi^2 = 21.5$, $P < 0.001$) and influenced nectar characteristics (Fig. 1B–D), but the effects depended on the microbial species identity. Microbial inoculation influenced nectar volume (Fig. 1B; $F_{2,156} = 3.8$, $P = 0.024$). Specifically, flowers inoculated with *Neokomagataea* had, on average, 25 and 30 % lower nectar volume than flowers inoculated with yeast and sucrose control flowers, respectively, whereas yeasts did not significantly influence nectar volume (Fig. 1B). Microbial inoculation treatments did not significantly influence the concentration of sucrose ($F_{2,87} = 1.62$, $P = 0.20$), glucose ($F_{2,87} = 1.80$, $P = 0.17$) or fructose ($F_{2,87} = 1.9$, $P = 0.16$) in floral nectar. However, microbial inoculation influenced the ratio of nectar sugars (Fig. 1C; $F_{2,91} = 12.5$, $P < 0.001$). *Neokomagataea* doubled the proportion of monosaccharides in nectar compared with the control, whereas yeast inoculation did not significantly affect the proportion of monosaccharides in nectar (Fig. 1C). Microbial inoculation also influenced amino acid concentration ($F_{2,42} = 4.95$, $P = 0.01$). *Neokomagataea*-inoculated flowers increased the

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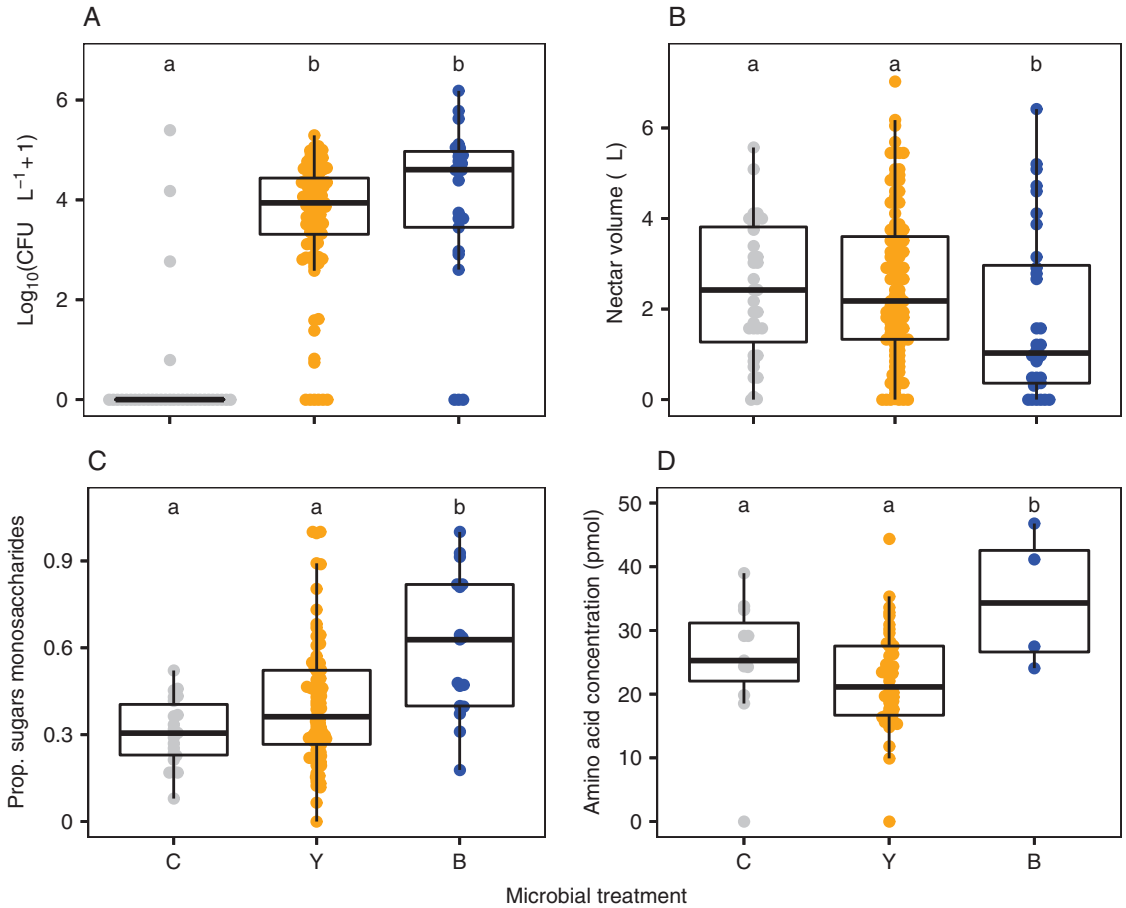

FIG. 1. Effects of microbial inoculation treatment on nectar characteristics 3 d after inoculation with yeast (Y), bacteria (B) or a sucrose control (C). Measured characteristics included (A) $\log_{10}$ (microbial CFUs + 1) in nectar assessed on YM or R2A plates; (B) total nectar volume (µL) per flower; (C) the proportion of monosaccharides in nectar (fructose + glucose)/(fructose + glucose + sucrose); and (D) the total amino acid concentration measured in nectar (pmol). The midline in the box represents the median, and edges represent Q1 and Q3. Letters in each panel represent treatments that differed ($P < 0.05$ based on a Tukey HSD test).

concentration of amino acids in nectar compared with sucrose- or yeast-inoculated flowers (Fig. 1D). Finally, inoculation with *Neokomagataea* increased the probability that flowers contained no nectar [Fig. 2; χ^2 = (2) = 6.26, P=0.044]. Flowers without nectar, particularly in the bacterial inoculation treatment, were also typically wilted and browning.

Inoculation treatment and plant identity were the main sources of variation in amino acid composition within floral nectar (Fig. 3; MANOVA Taxa Pillai's $\text{trace}_{2,42}$ = 1.16, P = 0.001; Plant ID Pillai's $\text{trace}_{15,42}$ = 5.38, P = 0.00028). The MANOVA indicated that proline, lysine, methionine, leucine, phenylalanine and isoleucine were reduced with yeast inoculation. *Neokomagataea* tended to increase the concentration of some amino acids in nectar, but the amino acid composition in *Neokomagataea*-inoculated flowers was not statistically distinguishable from that in sucrose-inoculated flowers, which may in part reflect the small sample size for *Neokomagataea*-inoculated flowers.

DISCUSSION

Our results indicate that the dominant species of yeasts and bacteria in our nectar system cause contrasting changes in floral characteristics, thereby contributing to increased variation in nectar chemistry among flowers. For example, bacterial inoculation more strongly affected sugar composition and the

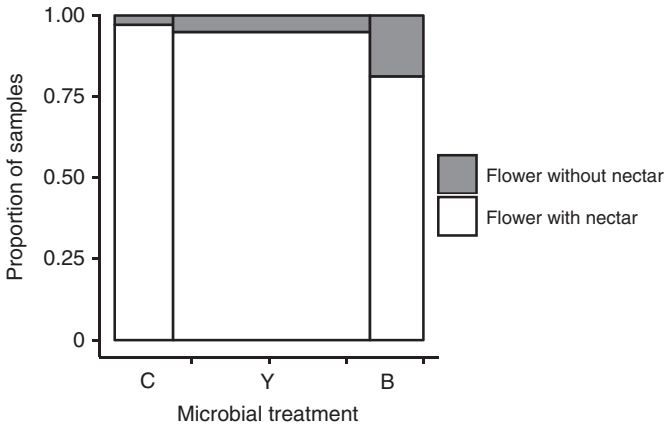

FIG. 2. Mosaic plot indicating the frequency of measurable nectar (yes >0 µL of nectar per flower) in flowers that were inoculated with sucrose control (C), yeast (Y) or bacteria (B) 3 d previously. Flowers without nectar were also wilted and browned. n = 185 recovered flowers (some were lost due to wind or florivory). Treatments differed at P = 0.015. Bar width indicates the relative number of replicates within each treatment group.

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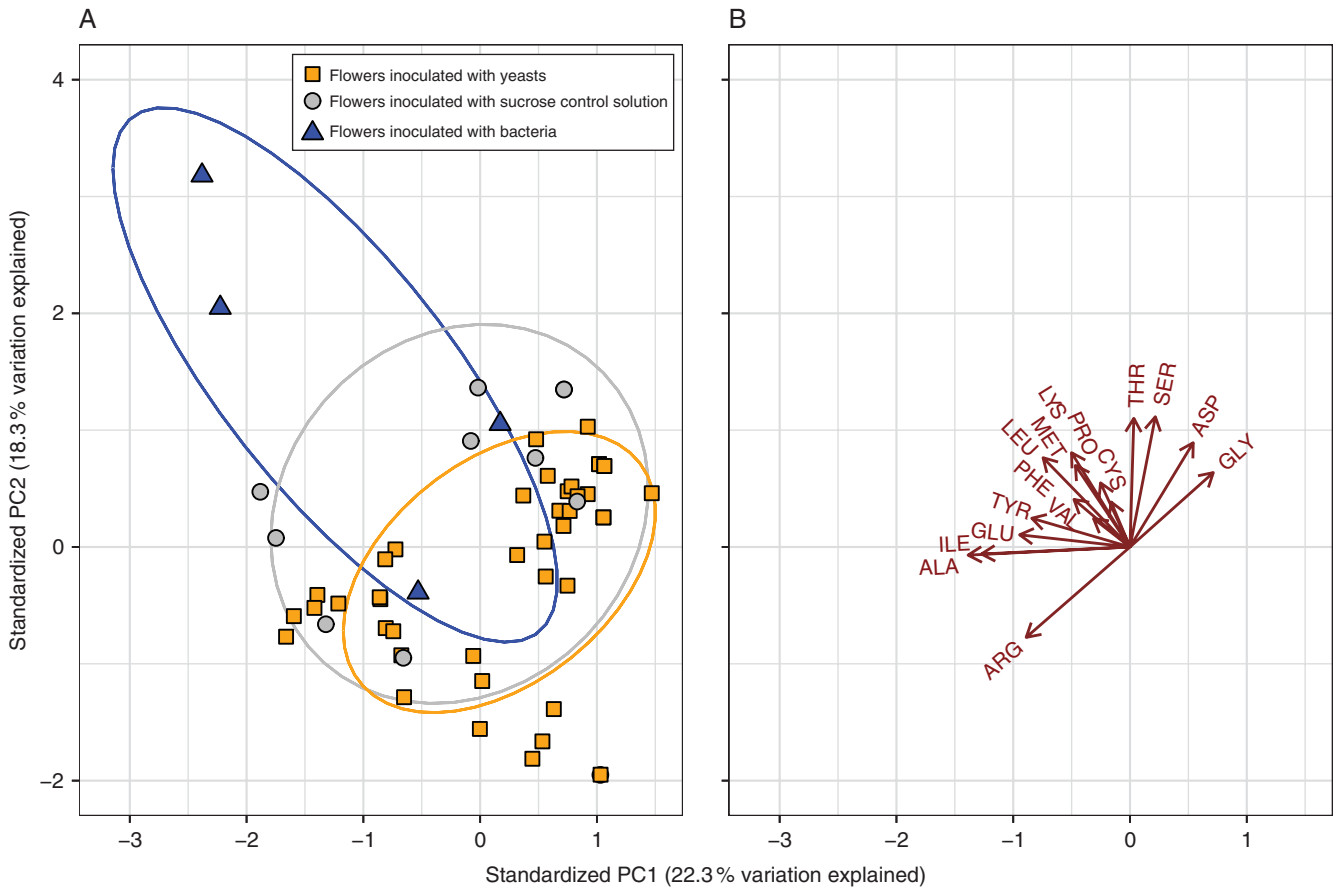

FIG. 3. Effect of microbial inoculation on the composition of amino acids in nectar. (A) Ovals indicate the 95 % confidence interval for taxonomic groups (yeasts, bacteria, or sucrose control). MANOVA treatment $P < 0.0001$. (B) Letters indicate amino acid vectors, with arrows pointing towards samples with a higher abundance of amino acids.

presence of floral nectar than did yeast inoculation, similar to *ex situ* studies using *M. aurantiacus* nectar (Vannette *et al.*, 2013). On the other hand, yeasts decreased the concentration of specific amino acids, while bacteria tended to increase amino acid concentration overall. Those amino acids that were reduced by yeasts were largely overlapping with those previously documented (Peay *et al.*, 2012; Vannette and Fukami, 2014, Tucker and Fukami, 2014). Microbe-induced changes in nectar characteristics may result from microbial metabolism or plant response to microbial presence. For example, bacterial-induced increases in proline, among other amino acids, in floral nectar (Figs. 1D and 3) might suggest plant resorption of floral nectar and the concentration of solutes or, alternatively, plant stress response (Fabro *et al.*, 2004). The observation of reduced nectar volume and increased floral senescence following bacterial inoculation (Fig. 2) may also suggest plant stress and subsequent floral senescence. This observation suggests that plants may respond to bacterial growth in nectar, and that at least some bacterial species can directly inflict biotic stress and damage on plant tissues, as has been previously documented in *Malus*–pathogen interactions (Venisse *et al.*, 2002). Further work is required to examine whether other species of floral bacteria or yeasts elicit similar plant responses, and whether co-inoculation with two or more microbial species attenuates or increases these effects.

Microbe-driven divergence in nectar traits may introduce variation in floral attractiveness to particular floral visitors. For example, bumble-bees are attracted to yeast-colonized flowers (Herrera *et al.*, 2013; Schaeffer and Irwin, 2014), while bacterial growth can reduce visitation by diverse floral visitors (Vannette *et al.*, 2013; Good *et al.*, 2014). Specifically, variation in amino acid composition (Inouye and Waller, 1984; Carter *et al.*, 2006) and nectar presence and volume (or indicators of these properties) influence pollinator choice, even among individual flowers (von Arx *et al.*, 2012). Because floral visitor species vary in attraction to particular nectar characteristics, visitor communities to flowers colonized by different microbial communities may diverge, even within the same plant species. The resulting heterogeneity in microbial communities and nectar characteristics among flowers of the same species may in turn increase the species diversity of floral visitors. These scenarios remain speculative, but suggest that it might be difficult to explain the abundance and distribution of plants, pollinators, or nectar microbes if interactions among the three groups were not considered simultaneously.

CONCLUSIONS

We found that micro-organisms could influence nectar characteristics and increase variability among individual flowers in the wild. Our results suggest that microbial communities that vary

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in species composition can also vary in their effects on plant traits. The ecological consequences of this variation on plants, pollinators, and micro-organisms remain to be fully explored.

DATA ACCESSIBILITY

Accession numbers for sequence data are included in the article.

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