Two *Metschnikowia* nectar yeast species have similar volatile profiles, but elicit differential foraging in bee pollinators

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Acknowledgements: The authors would like to thank Mckaela Whilden and the NSF REU BeeMORE program at NC State University for assistance in the field components of this study. We would also like to thank Kristin Conrad and Em Trentham for their assistance in bee management and rearing, and Em Trentham and Eleanor Griggs for transcribing observational field data.

Author Contributions: **M. Elizabeth Moore:** Conceptualization (equal), data curation (lead), formal analyses (lead), funding acquisition (lead), investigation (lead), methodology (supporting), project administration (lead), visualization (lead), writing – original draft (equal), writing – review and editing (equal). **Lindsey Wilson:** Data curation (supporting), investigation

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Funding statement: This project was funded by the National Science Foundation's Postdoctoral Research Fellowship in Biology, Award No. 2109616.

Data Availability: Data will be made publicly available on Dryad after publication. ITS gene sequences have been deposited in GenBank (accessions PP756580-PP756623).

Conflict of Interest: The authors have no conflicts of interest to declare.

Two *Metschnikowia* nectar yeast species have similar volatile profiles, but elicit differential foraging in bee pollinators

Abstract

1. Nectar yeasts are a highly specialized group of fungi that may play key roles in pollination ecology. Nectar yeasts lack an independent dispersal mechanism to access new habitats with fresh resources. Yeasts, bumble bee pollinators, and flowering plants likely take part in a series of diffuse mutualisms, wherein yeast attract bees that provide phoretic travel between flowers. This interaction is thought to provide bees with improved foraging efficiency and plants with increased pollinator visitation and associated pollination services. However, the underlying mechanisms driving bee pollinator preferences for nectar with yeast and differences among yeast species in eliciting pollinator behavior are relatively unexplored.
2. We used an integrative approach to elucidate the underpinnings of bee pollinator preference for nectars that contain yeasts. We conducted a survey of local flower nectar for presence and species diversity of yeast. Using two prominent, local nectar yeast species (*Metschnikowia reukaufii* and *Metschnikowia koreensis*), we conducted observational field trials to ascertain the effects of the presence and identity of nectar yeast on bee visitation rates. We also analyzed the volatile profiles of both yeast species to explore if olfactory cues were associated with differential foraging behavior.
3. We found that *M. reukaufii* was the most common nectar yeast in our study area in the Southeastern USA, as did previously published global surveys. Intriguingly, we found co-occurrence of multiple yeast species in 22% of nectar samples, all of which contained *M. reukaufii* and another yeast typically from the *Metschnikowia* genus, such as *M. koreensis*. In a field trial we found that bee pollinators had higher visitation to flowers supplemented with *M. koreensis* over sterile flowers, while no difference in bee foraging behavior was evident in response to *M. reukaufii*. Despite this behavioral difference, the volatile profiles of both yeast species were not significantly different from one another.
4. The ecology and species interactions of wild yeasts are poorly understood, yet may play vital roles in many ecosystems. Our research highlights the importance of studying facultative mutualisms, and the necessity of testing their underlying assumptions. Elucidating the mechanisms behind insect-microbe symbioses will open new horizons in pollination ecology and conservation.

Keywords: insect-microbe symbioses, facultative mutualisms, pollination ecology, yeast, olfaction, volatile organic compounds

Introduction

Floral nectar is an important energy source and nutrients for many insects and some vertebrates, and contributes to both plant and animal fitness (Baker & Baker, 1973). More recently, nectar has been recognized as an important habitat for archaea, protists, viruses, bacteria, and yeast, and these microbial communities further mediate plant-insect interactions (Vannette, 2020). Insects and yeast, in particular, have an ancient and diverse co-evolutionary history, with yeast volatiles often playing a vital role in insect attraction for symbiotic relationships (Blackwell, 2017; Madden *et al.*, 2018; Stefanini, 2018). Despite recent advances in the ecological study of nectar yeasts, open questions remain on the biogeographical distribution of nectar yeasts, the degree to which they attract or repel insect pollinators at flowers, and how flower-insect-yeast interactions are mediated (Klaps *et al.*, 2020).

While flower nectar is a hostile environment for microbes due to osmotic stress associated with high sugar, low nitrogen availability, and competitive exclusion (Jacquemyn *et al.*, 2020; Vannette, 2020), specialized yeast (fungi) and bacteria are able to reach high densities in nectar: up to 105 for fungi and 107 for bacteria cells/µl (Herrera *et al.*, 2009b; Fridman *et al.*, 2012). With regard to fungi, field surveys show that a single yeast species often dominates the nectar community, and single yeast species often dominate individual flowers, likely due to strong competitive and priority effects (Peay *et al.*, 2011; Tucker & Fukami, 2014; Vannette & Fukami, 2014), dispersal limitation (Herrera *et al.*, 2009b; Ushio *et al.*, 2015), vector associations (Morris *et al.*, 2020; de Vega *et al.*, 2021), and environmental filtering caused by the nectar environment (Herrera *et al.*, 2009a; Vannette & Fukami, 2016). The most frequently identified yeast species in nectar include the nectar specialists *Metschnikowia reukaufii* and *Metschnikowia gruessi*, and the generalists *Aureobasidium pullulans* and *Cryptococcus* and *Candida* species (Brysch-Herzberg, 2004; Belisle *et al.*, 2012; Pozo *et al.*, 2012; Schaeffer *et al.*, 2015). Based on studies to date, *M. reukaufii* is the most ubiquitous nectar yeast, at least in the temperate regions where nectar has been most studied (Dhami *et al.*, 2018; Álvarez-Pérez *et al.*, 2021).

The roles of microbes in ecological interactions are poorly understood, but the recognition of their impact and importance is increasing across systems (Rering *et al.*, 2018a; Martin *et al.*, 2022; Mueller *et al.*, 2023; Deng *et al.*, 2024). Lab and field-based experiments have often found that pollinators, specifically bees, preferentially feed on nectar inoculated with *M. reukaufii* over nectar hosting bacteria (Rering *et al.*, 2018a; Sobhy *et al.*, 2018). In addition, a number of lab and field experiments have documented that bumble bees consume significantly more *M. rekaufii*-inoculated nectar than control “nectar” (e.g., sterile sugar water) (Rering *et al.*, 2018a; Sobhy *et al.*, 2018; Schaeffer *et al.*, 2019). *Metschnikowia* species are also found in and on pollinators (Stefanini, 2018; Madden *et al.*, 2022), suggesting that those pollinators also disperse yeasts (Belisle *et al.*, 2012; Pozo *et al.*, 2012; Schaeffer *et al.*, 2015; Vannette & Fukami, 2016), as has been hypothesized (e.g., Madden et al. 2022). The majority of studies investigating the effects of yeast on insect pollinator foraging behavior have focused on the yeast *M. reukaufii*. The degree to which results from *M. reukaufii* can be generalized to other nectar yeast taxa requires further investigation.

The ability of yeast to alter insect foraging behavior appears to be an ancient and evolutionarily conserved trait (Blackwell, 2017). Yeasts consume sugar from floral nectar and convert it into ethanol. The metabolic products of this conversion, particularly the volatile organic compounds (VOCs), have been hypothesized to provide an honest signal to insect pollinators of the presence of sugar sources (Madden *et al.*, 2018). There is a growing body of literature documenting the VOCs emitted from nectar inoculated with yeast and their effects on insect behavior (Martin *et al.*, 2022). *M. reukaufii* produces sweet-smelling esters/acetates (Rering *et al.*, 2018a, 2018b; Schaeffer *et al.*, 2019; Sobhy *et al.*, 2019). Electroantennographic assays that gauge the response of antennae to *M. reukaufii* volatiles differ between *Apis mellifera* and *Bombus impatiens*, but both bees respond to 2-ethyl-1-hexanol, 2-phenylethanol, and 3-methylbutyl acetate (Rering *et al.*, 2018a; Schaeffer *et al.*, 2019). Of particular interest is 3-methylbutyl acetate, also known as isoamyl acetate, which has a strong odor (banana, pear), and is also an important attractant for *Drosophila melanogaster* via *Saccharomyces cerevisiae* (Christiaens *et al.*, 2014). Work remains to document VOC profiles from yeast metabolic products beyond *M. reukaufii* and their effects on insect behavior.

Here we identified the abundance and diversity of nectar yeast in local flower populations in the subtropical southeastern USA, tested whether bee pollinators are differentially attracted to flowers containing different nectar yeast in the field, and characterized the volatile profiles of two nectar yeast species. First, we sampled the nectar of local funnelform flowers that have high rates of bee visitation. In line with other studies, *M. reukaufii* was the most abundant species identified. We also documented the co-occurrence of *M. reukaufii* with relatives *M. gruessi* and *M. koreensis*. *M. koreensis* has been previously observed in floral nectar, but has not been well characterized (Hong *et al.*, 2001). Second, from this screening of local nectar microbiota, we selected strains of *M. reukaufii* and *M. koreensis* and conducted observations of pollinator visits to flowers with and without augmented yeast. We predicted that free-foraging bees would prefer flowers with either yeast species over control flowers. We found that bee species (carpenter bees, bumble bees, honey bees and a few solitary bee species) showed a strong preference for *M. koreensis* relative to sterile, control nectar, but surprisingly showed no preference for *M. reukaufii* relative to control nectar. Third, we characterized the volatiles of *M. reukaufii* and *M. koreensis* using gas chromatography and mass spectrometry (GC-MS). Given the differences we observed in bee pollinator visitation, we predicted that the VOCs emitted from the two yeasts would differ in compounds known to affect bee visitation. However, we found the VOC profiles of *M. koreensis* and *M. reukaufii* to be largely similar, suggesting that other factors beyond olfaction may be driving the behavioral differences observed in the field. Taken together, these results corroborate previous findings that *Metschnikowia* species predominate nectar yeast communities, but that the most abundant species (*M. reukaufii*) may not be the most important microbial symbiont in influencing bee pollinator behavior.

MATERIALS AND METHODS

Nectar Yeast Survey

Nectar Sampling: We sampled 103 funnel-form flowers of various species in Raleigh, NC and Chapel Hill, NC, USA over a period of three seasons: September 2021 (fall), April 2022 (spring), and June 2022 (summer) (Table S1). We bagged open flowers using mesh bags to prevent pollinator access and allow for nectar accumulation. We collected nectar from bagged flowers approximately 24 hours later. We collected nectar by removing the flower from the calyx and gently squeezing the tapered end, collecting nectar with sterile 5 µl glass microcapillary tubes. If at least 2.5 µl of nectar could not be collected from a single flower, nectar from multiple flowers on the same plant were combined in a sample. Microcapillary tubes were stored in individual sterile 1.5 mL centrifuge tubes and maintained in a cooler until returned to the lab.

Nectar samples were expressed from the microcapillary tubes into 100 µl sterile water, vortexed, then plated on yeast peptone dextrose (YPD) media (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and cultured for 48-72 hours at room temperature. We sampled individual yeast colonies that differed in color, size, and texture from each plate. The diversity of growth on the plates was preserved by conducting total plate washes with YPD media that were stored at -80°C in 15% glycerol. We inoculated individual unique colonies in 2 mL YPD media and let the samples grow for 24-48 hours on a spinner at room temperature. Each sample was then archived in a cryotube at -80°C in 15% glycerol.

Yeast Isolation and Identification: We screened colonies for yeast species using polymerase chain reaction (PCR) with primers Pn3 (5' CCGTTGGTGAACCAGCGGAGGGATC 3') and Pn34 (5' TTGCCGCTTCACTCGCCGTT 3') that target the internal transcribed spacer (ITS) region, a commonly used locus for species identification in fungi. Cells were inoculated in 10 µL 0.2 M NaOH, incubated for 20 minutes, frozen at -80°C for 15 minutes, and spun down in 90 µL nuclease-free water for 1 minute. PCR was performed at a total volume of 20 uL using 10 µL Taq 2X master mix (New England Biolabs), 7 µL nuclease-free water, 1 µL of each primer, and 2 µL of the colony sample. We used 1% gel electrophoresis to confirm the success of the PCR and identify those that were "positive" for yeast. Each sample was screened at least 2 times. Positive samples were Sanger sequenced using forward (Pn3) and reverse (Pn34) primers. We analyzed the resulting sequences using NCBI BLAST to determine the genus and species of each sample (percent identity ≥ 97%). Samples with less than 97% identity or more than one species greater than 97% identity were reevaluated using D1/D2 primers (ITS1 - TCCGTAGGTGAACCTGCGG; NL4 - GGTCCGTGTTTCAAGACGG) (Spurley *et al.*, 2022). Finalized sequences were uploaded to GenBank (Table S2).

Data summary: We calculated numbers and proportions of nectar samples that contained yeast, the distribution of yeast species across plant families, and the number of instances of co-occurrence of yeast

species within the same flower sample. Calculations were conducted in the statistical program R (v. 4.4.1) via RStudio (v. 2024.04.2+764) (RStudio Team, 2020; R Core Team, 2021).

Effects of Nectar Yeasts on Insect Pollinator Behavior

Yeast cultures: We selected clones of the two most abundant yeast species, *M. reukaufii* (s2_1) and *M. koreensis* (s3_1) (Table S2), from the flower nectar survey to assess effects on pollinator behavior. Yeast were initially cultured on YPD agar for 48 hours, then inoculated into 5 mL of artificial nectar media (21.25% sucrose, 1.875% fructose, 1.875% glucose, 0.1mM amino acid mixture of alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, serine), modified from (Rering *et al.*, 2018a), and placed in a culture tube rotator at 30°C. After 24-72 hours, the optical density of the yeast cultures was measured using a spectrophotometer (Biowave Cell Density Meter CO8000). Yeast cultures were then diluted with sterile artificial nectar to 1x10^4 cells/µL, using a reference optical density determined by counting cells at a known optical density on a hemocytometer. This was done separately for each strain to account for differential relationships between cell concentration and optical density. This cell density was chosen to align with reported yeast cell concentration in sampled flower nectar ranging from 103 to 105 cells/µL (Herrera *et al.*, 2009c, 2011, 2014; Vannette *et al.*, 2013; Schaeffer & Irwin, 2014; Schaeffer *et al.*, 2014, 2015; Vannette & Fukami, 2016, 2018). Diluted yeast cultures were kept at 4°C until 12 hours before use in the field, at which point they were returned to room temperature. Diluted yeast cultures were used within 5 days of dilution (kept at 4°C) or discarded and new diluted cultures established.

Plants and field plot: We conducted the field behavioral assay in July 2022. We used the plant *Pentas lanceolata* (var. Glitterati Red Star and var. Graffiti Mix) (Rubiaceae) which had consistent flower presence that were highly attractive to bees. Plants were potted into 1 gallon (3.78L) plastic pots (Seed Kingdom, FL, US) with standard mix commercial potting soil and fertilized with Espoma Organic Flower-Tone (Espoma Organic, NJ, US) following manufacturer instructions. Plants were kept in a 3.05m x 3.05m x 2.13m mesh shade tent (CAMPMORE, Amazon, US) when not being used for experimental trials to prevent heat stress and herbivory. Plants were watered daily or as necessary, and senesced flower heads removed regularly to promote continual flowering. We assigned plants to one of two nectar treatments: sterile nectar or yeast-inoculated nectar. Nectar treatment assignments remained consistent across trials. For each trial, plants were arranged in an interdigitated array of 4 rows with 5 plants each, with plants spaced 1 m apart. The location of plants within the array was randomly assigned, and this assignment was changed between yeast species.

Behavioral assays: Prior to each behavioral assay, we counted and recorded the number of flowers on each plant; plants with <10 flowers open were replaced with spare plants, and plants with >100 flowers had mesh bags placed over some flower clusters to prevent pollinator access and reduce effective flower number.

Using a Fisherbrand repeater pipette, 4µL of either sterile artificial nectar or yeast-inoculated artificial nectar was placed into each flower based on treatment assignment. Because we did not remove nectar from flowers, our treatments represent dilution or augmentation of yeast that were present in flowers, respectively. After flowers were counted and treated, plants were placed into the interdigitated field array and trial observations began. Two researchers were present at each trial; one recorded pollinator observations, and one refilled flowers with artificial nectar to prevent pollinators associating one treatment as “no reward." The researchers and their roles were the same across all trials. Pollinators were observed individually from the time they entered the plot, to when they left the plot or were lost. Nectaring was defined as the insertion of the proboscis fully into the flower. For each nectaring event, we recorded the plant ID, the number of flowers visited, and the duration of nectaring on each flower using a hand-held voice recorder (EVISTR 64GB Digital Voice Recorder). Flowers were refilled with 4 µL of the appropriate nectar treatment as needed, and trials were ended daily when replacement nectar was exhausted (approx. 2 hrs).

Pollinator observation data were transcribed from the audio recordings, and each pollinator was assigned a unique ID. Pollinators were identified to genus or species on the wing for carpenter, bumble and honey bees, or given a descriptive class for solitary bee species (see Fig. S2). The transcribed data included plant ID, plant location within the plot, plant nectar treatment, yeast species, pollinator species, number of flowers visited per plant, and nectaring duration for each flower. We conducted 4 days of observation for each yeast species, ranging from July 7-11, 2022 (*M. reukaufii*) and July 18-22, 2022 (*M. koreensis*) from approx. 9:30-11:30 in the mornings.

Statistical Analyses: Four metrics of bee pollinator visitation were calculated and analyzed by nectar treatment on a per visitor basis: the number of plants visited, proportion of flowers visited per plant, visitation rate (number of plant visits times the proportion of flowers visited), and visit duration per flower (in seconds). The effects of sterile or yeast-inoculated artificial nectar on these metrics of bee pollinator visitation were analyzed with linear mixed effects models using the function 'lme' from the 'nlme' package using maximum likelihood. Plant nectar treatment was included as a fixed effect (factorial), and the date of each observational trial was included as a random intercept. For the analysis of time spent per flower, we also included plant ID as a random effect. Because *M. reukaufii* and *M. koreensis* were manipulated in separate trials, their effects on bee pollinator visitation relative to sterile nectar were analyzed separately. All data analyses, here and below, were conducted in the statistical program R (v. 4.4.1) via RStudio (v. 2024.04.2+764).

Volatile Organic Compound Profiles

Volatile collection and analysis: The volatiles for the strains of *M. reukaufii* and *M. koreensis* collected from the nectar survey and used in the pollinator behavioral assays were collected via solid phase microextraction

(SPME) and analyzed using gas chromatography and mass spectrometry (GC-MS). Yeast cultures were grown and diluted following the methods described in *Yeast cultures*, with the modification that cultures were diluted in sterile artificial nectar to a total volume of 10 mL with a concentration of 1x104 cells/µl to increase volatile production for SPME. Diluted cultures were stored at 4°C until use. Before volatile collection, cultures were transferred to sterile glass collection vials and incubated at 30°C for 12 hours in glass beads on a hot plate. Volatile collections were replicated 5 times for each nectar yeast species, and the cultures of both species were diluted on the same day. Replicates of each yeast species were run on the same day using the same SPME fiber.

Yeast volatiles were collected using a DVB/CAR/PDMS 50/30µm SPME fiber, conditioned at 270°C per manufacturer instructions before each collection. The fiber was exposed to volatiles for 90 minutes at 37°C. Collected volatiles were analyzed on a GC-MS (6890 GC and 5975 MS, Agilent Technologies, Palo Alto, CA, USA) which was equipped with a DB-WAXetr column (30 m × 0.25 mm, df = 0.25 µm, Agilent Technologies) and helium was used as the carrier gas at an average velocity of 32 cm/s. Oven program was set to 31°C for 2 min, increased at 5°C/min to 50°C, 10°C/min to 90°C, 5°C/min to 150°C, 20°C/min to 250°C and held for 2 min. The injector was set to splitless mode (4 psi) at 250°C, transfer line was also at 250°C, MS source was set to 230°C and the quadrupole was set to 150°C. Compounds were identified based on Kovats indices and electron ionization mass spectra.

Statistical Analyses: We excluded 11 compounds that were found in only one replicate, which were likely contamination from an unknown source, or were below the 50% confidence threshold (Table S3), leaving 18 compounds. The composition of volatile compounds collected from *M. reukaufii* and *M. koreensis* were visualized using Principal Component Analysis using Bray-Curtis dissimilarities using the PCA function from the FactoMineR package. Differences in the VOC profiles of the two yeast species were examined using perMANOVA using the adonis2 function from the vegan package. Our samples did not include an internal standard, so peaks were normalized relative to isoamyl alcohol, which was the largest and most consistent peak across all samples for both species, leaving 17 compounds for statistical analysis. All standardized peaks were square-root transformed for the perMANOVA analysis.

RESULTS

Nectar Yeast Survey

Out of 103 unique flower samples, 33.98% (35/103) of nectar samples contained yeast in Raleigh and Chapel Hill, NC USA (Table S1). We found that *Metschnikowia* yeast dominated local nectar communities surveyed, with 90.7% of all identified yeasts in our survey being in the *Metschnikowia* genus. Of these, we identified the nectar specialist *Metschnikowia reukaufii* as the most commonly occurring yeast

species present (68.57% of all yeast-positive samples, Fig 1). *M. koreensis*, *M. gruessi*, and *M. rancensis*, however, were also common (37.14% of all yeast-positive samples across all 3 species). One isolate (1/103) was only able to be identified to the genus *Metschnikowia*, and the species identification remains uncertain. Generalist and plant-associated fungi *Aureobasidium pullulans*, *Meira argovae*, *Papiliotrema flavescens*, and *Vishniacozyma melezitolytica* were each identified in one sample. While most nectar samples contained only a single distinct lineage, we identified 8 cases (22.9% of samples) of co-occurrence between yeasts, typically between *M. reukaufii* and another *Metschnikowia* species (Fig 2). The most common co-occurrence was *M. reukaufii* and *M. gruessi*, followed by *M. reukaufii* and *M. koreensis*.

Effects of Nectar Yeasts on Insect Pollinator Behavior

Bee pollinators exhibited similar numbers of plant visits (LMM, $F_{1,102}=0.93$, $p=0.3383$), flowers probed (LMM, $F_{1,73}=1.67$, $p=0.2006$), and visitation rates (LMM, $F_{1,75}=2.69$, $p=0.1052$; Table 1, Fig. 3, Fig. S2) when presented with plants treated with *M. reukaufii* or sterile nectar. In contrast, bee pollinators increased their visitation rates to flowers and plants supplemented with *M. koreensis*-inoculated nectar over those treated with sterile nectar (LMM, $F_{1,73}=15.15$, $p=0.0002$; Table 1A, Fig. 3). Bees visited 1.3 times more plants with *M. koreensis* treated nectar than sterile (LMM, $F_{1,73}=15.15$, $p=0.0002$; Table 1A, Fig. 3), and foraged on 2.64-times more flowers on yeast treated plants. Treatment with *M. koreensis* resulted in bees repeatedly foraging on flowers, with 128% of flowers visited (indicating repeat visits to the same flowers) versus only 54% flowers probed with sterile nectar (LMM, $F_{1,73}=14.69$, $p=0.0003$; Table 1C, Fig. 3). Nectar inoculation with either yeast species had no effect on the duration of flower visits over sterile nectar (LMM, *M. koreensis:* $F_{1,19}=0.97$, $p=0.3381$; *M. reukaufii*: $F_{1,19}=0.95$, $p=0.3427$; Table 1D, Fig. S2). During the observation days for *M. koreensis*, the majority of visitors to experimental flowers were carpenter bees (71.3%), with additional visits by bumble bees (23.8%) and solitary bees (5.0%) (Fig S1). During observation of flowers inoculated with *M. reukaufii*, the make up of bee visitors was more diverse, consisting of carpenter bees (40.0%), bumble bees (40.0%), solitary bees (7.1%), honey bees (4.3%), and other bees (8.6%).

Volatile Organic Compound Chemical Profiles

Despite the differences in observed pollinator behavior, the volatile profiles of *M. reukaufii* and *M. koreensis* were largely overlapping (Fig. S3) and not statistically different based on perMANOVA ($F_{1,9} = 1.874$, $P = 0.1559$). Of the 18 volatile compounds produced across *M. reukaufii* and *M. koreensis*, 16 were shared by both species and only two compounds (phenethyl acetate (2-phenylethyl acetate) and phenylethyl butyrate (2-phenylethyl butanoate)) were produced by a single species (*M. koreensis*; Table 2). For the two

compounds unique to *M. koreensis*, neither was a dominant component of the odor bouquet; phenethyl acetate was only detected in three of the five replicates, and phenylethyl butyrate was only in two of five replicates (Table 2). Both yeast species had 12 identified peaks that were found in all five replicates. The majority of volatiles were primary alcohols (8 compounds), followed by esters (5 compounds), acids (3 compounds), methyl ketones (1 compound), and secondary alcohol (1 compound) (Table 2).

Discussion

Our research aimed to connect several levels of biological organization to further our understanding of which yeasts are present in local flower nectar and how and whether they affect pollinator foraging decisions. Our results provided some of the first information on nectar yeast presence and species composition in the southeastern US. Our results are consistent with previous studies in other regions: *M. reukaufii* is often the predominant yeast found in nectar (Lachance *et al.*, 2001; Herrera *et al.*, 2009c; Pozo *et al.*, 2011; Schaeffer *et al.*, 2015). However, we observed frequent co-occurrences of multiple yeast species within flowers. The most common co-occurrence was that of *M. reukauffii* with *M. gruessi*, which, intriguingly, is reflective of previous findings in nectar sampled in Europe (Pozo *et al.*, 2011, 2016; Álvarez-Pérez *et al.*, 2016). It is unclear whether the shared yeast composition of European and North American flowers reflects large, natural geographic ranges of floral yeasts, or if invasion of floral yeasts has occurred. Overall, our results are consistent with other studies suggesting that the nectar microbiome is species poor, and add to the growing body of work from across North America, South America, and Europe demonstrating that *M. reukaufii* is the dominant nectar yeast with a widespread distribution.

One can hypothesize a scenario in which the most common yeast in flowers is also the most attractive to pollinators, with its commonness resulting in part from its ability to attract pollinators and, hence, to disperse phoretically. However, in our study, *M. reukaufii*, the most common yeast, was no more attractive to pollinators than sterile nectar. Instead, a less prevalent species, *M. koreensis*, showed much stronger pollinator attraction when compared to sterile nectar (Herrera *et al.*, 2013; Rering *et al.*, 2018a; Schaeffer *et al.*, 2019). If pollinators are the main method of yeast dispersal (as indicated by previous research), our results bring up interesting questions as to the method of *M. reukaufii*'s community dominance (Brysch-Herzberg, 2004; Good *et al.*, 2014). *M. reukaufii* might have adaptations that allow it to outcompete other yeasts in nectar, allowing it to dominate a nectar source even if co-introduced with other yeast species. It is also possible that *M. reukaufii* is better able to tolerate the conditions in nectar (e.g., environmental filtering), such as the particularities of sugar and amino acid composition, secondary defensive chemicals, and pH levels (Herrera *et al.*, 2006; Petanidou *et al.*, 2006; de Vega *et al.*, 2009; Tucker & Fukami, 2014; Lievens *et al.*, 2015). *M. reukaufii* growth in extreme sugar environments is mediated by methylation differences in response to sugar content and composition (Herrera *et al.*, 2012). This plastic response, in combination with strong host plant-mediated diversity of *M. reukaufii* genotypes,

may be a mechanistic explanation of its broad ecological niche (for a nectar yeast) and general ubiquitousness in flower nectar (Herrera, 2014). If *M. reukaufii* is a more competent colonizer of nectar, but has less potent pollinator attraction than other yeast species, it calls into question our assumptions of the role nectar yeast play in pollinator foraging choices, yeast transmission, and yeast community dynamics.

We had expected that both yeast species would be more attractive to bee visitors than sterile nectar, but this was not the case. While a growing body of evidence has documented bee (especially bumble bee) preference for flowers inoculated with yeast over sterile nectar (Herrera *et al.*, 2013; Schaeffer *et al.*, 2017; Deng *et al.*, 2024), this pattern is not universal (Good *et al.*, 2014; Rering *et al.*, 2018a; Schaeffer *et al.*, 2019; Colda *et al.*, 2021). Indeed, the existing literature indicates that while pollinators show no preference for *M. reukaufii* over sterile controls, the nectar yeast is not aversive, unlike other microbes (usually bacteria) that reduce visitation and nectar consumption, which is consistent with our results (but see (Rering *et al.*, 2021). There are not enough studies to draw general conclusions for why or under what conditions floral visitors prefer yeast-inoculated flowers or not. However, the species identities of the flower, visitor, and yeast may have an effect, along with the ecological background in which the experiments are conducted. For example, because we observed the effects of the two yeast species relative to sterile nectar at different time periods, the proportions of pollinator species or groups who visited the arrays differed. Preference studies for each bee species in how they respond to each yeast species relative to sterile nectar and relative to each other could yield important insights.

The mechanism of pollinator choice also remains elusive. Bee pollinators consistently fed more frequently on flowers supplemented with *M. koreensis* over sterile nectar, suggesting that olfactory cues associated with yeast might have guided bees to the inoculated nectar. However, there were no differences in foraging on *M. reukaufii*-supplemented nectar vs. sterile nectar, which is unexpected, given that *M. reukaufii* releases volatiles that can be detected by bumble bees and have been assumed to be attractive (Rering *et al.*, 2018a; Schaeffer *et al.*, 2019). Surprisingly, the volatile profiles of these two *Metschnikowia* species were virtually indistinguishable. There are several potential explanations for these results. First, the small differences we observed in volatile profiles may be sufficient to alter pollinator foraging choices. Related to this, it is possible that certain volatiles not trapped by SPME are key to guiding the differential responses of pollinators. Further investigations using alternate headspace trapping and chemical analytical techniques could illuminate differences we were not able to detect – such as dynamic headspace collection and thermal desorption, coupled with bee electroantennal responses to yeast volatiles. Second, yeast-associated behavior might be guided by gustation rather than olfaction (or, more plainly, taste rather than smell). In previous research, bumble bees showed preference for *M. reukaufii* nectar over bacteria inoculated nectar, but only after tasting the nectar (Schaeffer *et al.*, 2019).How and why pollinators are making foraging choices in response to microbial symbionts remains unresolved, but could provide important insights into insect-yeast interactions. Third, we measured volatiles produced by the two yeast species but not in the floral background in the field. Surprisingly, few studies of nectar yeast have considered the floral background. We cannot rule out the possibility that the floral background and other

environmental factors that may have differed between the two trials of observation modified VOC profiles or pollinator perceptions of those profiles.

Insect-fungal symbioses are an ancient and abundant network of ecological interactions, ranging from purely facultative to completely obligate. There must be strong evolutionary pressures on both insects and yeasts to maintain these symbioses. Indeed, the production of insect-attracting chemicals is a conserved, and often necessary, trait of many yeasts (Christiaens *et al.*, 2014; Becher *et al.*, 2018). One intriguing class of such chemicals is the acetate esters, which are produced by alcohol acetyltransferases (*ATF1* in *S. cerevisiae*). *Metschnikowia* species have 8-9 putative alcohol acetyltransferases, and characterization in *Saccharomyces* species and in *Saccharomycopsis fibuligera* suggests an increased number of alcohol acetyltransferases in non-*Saccharomyces* species, and evidence that orthologues produce different odor profiles (Stribny *et al.*, 2016; Moon *et al.*, 2021). These genes are intriguing targets for molecular mechanisms underlying differences in odors, and possibly taste, in yeast-insect interactions. Future work to elucidate the genetic underpinnings of nectar yeast - bee pollinator interactions, such as chemical signalling, nectar metabolism, and pathogen interference, will lead to new revelations of the mechanisms and the evolution of insect-yeast symbioses (Schiestl *et al.*, 2006; Christiaens *et al.*, 2014; Bogo *et al.*, 2021; Rering *et al.*, 2023).

Citations:

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Table 1. Linear mixed effects models of the effects of plant treatment (addition of sterile nectar or nectar inoculated with yeast) on metrics of bee pollinator visitation. A. The number of plants visited by each observed bee pollinator each trial day. B. The proportion of the total available flowers visited by bee pollinators each trial day. C. The visitation rate (number of plants visited * the proportion of flowers visited) of bee pollinators to each plant treatment. D. The duration of each flower visitation (in seconds). Plant treatment was included in models as a fixed effect, trial day was included as a random intercept, and models were fit using maximum likelihood.

A.	Number of Plants Visited							
	Metschnikowia reukaufii				*Metschnikowia koreensis*			
	nDF	dDF	F-value	p-value	nDF	dDF	F-value	p-value
Plant treatment	1	102	0.92545	0.3383	1	122	4.32158	**0.0397**
B.	Proportion of Flowers Visited							
	Metschnikowia reukaufii				*Metschnikowia koreensis*			
	nDF	dDF	F-value	p-value	nDF	dDF	F-value	p-value
Plant treatment	1	73	1.66775	0.2006	1	73	14.6866	**0.0003**
C.	Visitation Rate							
	Metschnikowia reukaufii				*Metschnikowia koreensis*			
	nDF	dDF	F-value	p-value	nDF	dDF	F-value	p-value
Plant treatment	1	75	2.68979	0.1052	1	73	15.1512	**0.0002**
D.	Visit Duration							
	Metschnikowia reukaufii				*Metschnikowia koreensis*			
	nDF	dDF	F-value	p-value	nDF	dDF	F-value	p-value
Plant Treatment	1	19	0.94718	0.3427	1	19	0.96581	0.3381

Table 2: Volatiles identified by GCMS of SPME collected volatiles of two nectar yeast species, *M. reukaufii* (**A**) and *M. koreensis* (**B**). Five replicate cultures were analyzed per yeast species, and compounds were identified based on Kovats indices and electron ionization mass spectra. Compounds included in this table were used for all analyses; compounds that had a confidence score of less than 50% and were not present in 2 or more replicates across both species..

		M. reukaufii				*M. koreensis*			
Primary Alcohols		N	Log Area	Ret. Time	Qual	N	Log Area	Ret. Time	Qual
	Isoamyl alcohol	5	19.06± 0.16	9.48± 0.01	90.00± 0.00	5	19.18± 0.25	9.47±0.01	90.40± 0.55
	Ethanol	5	18.50± 0.18	5.01± 0.02	78.0± 0.00	5	18.59± 0.29	4.99±0.01	78.00± 0.00
	Isobutyl alcohol	5	16.83± 0.42	7.88± 0.05	90.80± 0.44	5	17.18± 0.42	7.86± 0.05	91.00± 0.00
	Furfuryl alcohol	5	12.23± 0.28	18.01 ±0.01	73.8± 21.86	5	12.53± 0.33	18.01± 0.00	86.20± 14.14
	Phenylethyl alcohol	5	14.47± 0.29	22.15 ±0.00	96.6± 0.89	5	15.19± 0.27	22.15± 0.00	96.60±0.89
	2-ethyl-1-hexanol	5	14.37± 0.15	14.40 ±0.01	87.6± 2.19	5	14.42± 0.17	14.39± 0.00	86.00±0.00
	3-Buten-1-ol, 3-methyl-	5	13.86± 0.18	10.13 ±0.01	71.4± 18.85	5	13.87± 0.19	10.12± 0.01	86.20± 10.25
	4-Penten-1-ol	2	13.41± 0.03	10.95 ±0.01	57.00± 9.90	3	13.09± 0.36	10.93± 0.01	63.00± 5.39
Sec. alcohols									
	2-Hexanol, 3,4-dimethyl-	2	12.97± 0.07	12.60 ±0.00	51.5± 2.12	5	12.80± 0.15	12.59± 0.00	45.20± 6.57
Esters									
	Isoamyl acetate	5	16.45± 0.61	8.34± 0.03	90.00± 0.00	5	16.37± 0.49	8.32± 0.03	80.40± 5.37

	Isobutyl acetate	2	15.40± 0.18	6.59± 0.03	54.00± 25.46	3	15.28± 0.09	6.57± 0.01	74.00± 3.46
	Furfuryl acetate	5	12.36± 0.41	15.48 ±0.01	66.40± 17.34	1	12.94	15.47	95.00
	Phenethyl acetate	–	–	–	–	3	13.02± 0.13	21.04± 0.00	56.67± 6.35
	Phenylethyl butyrate	–	–	–	–	2	12.80	21.83	56.00
Acids									
	Acetic acid	5	14.63± 0.10	13.77 ±0.01	89.6± 1.52	5	15.18± 0.37	13.75± 0.00	89.20± 1.79
	2-Methyl-butanoic acid	5	14.71± 0.45	18.22 ±0.01	80.8± 5.76	5	14.61± 0.63	18.21± 0.00	79.60± 4.98
	Isobutyric acid	5	15.90	16.08	79.8	5	15.79	16.07	85.6
Methyl Ketones									
	Acetoin	5	14.06	10.82	82.4	5	14.00	10.80	72.00

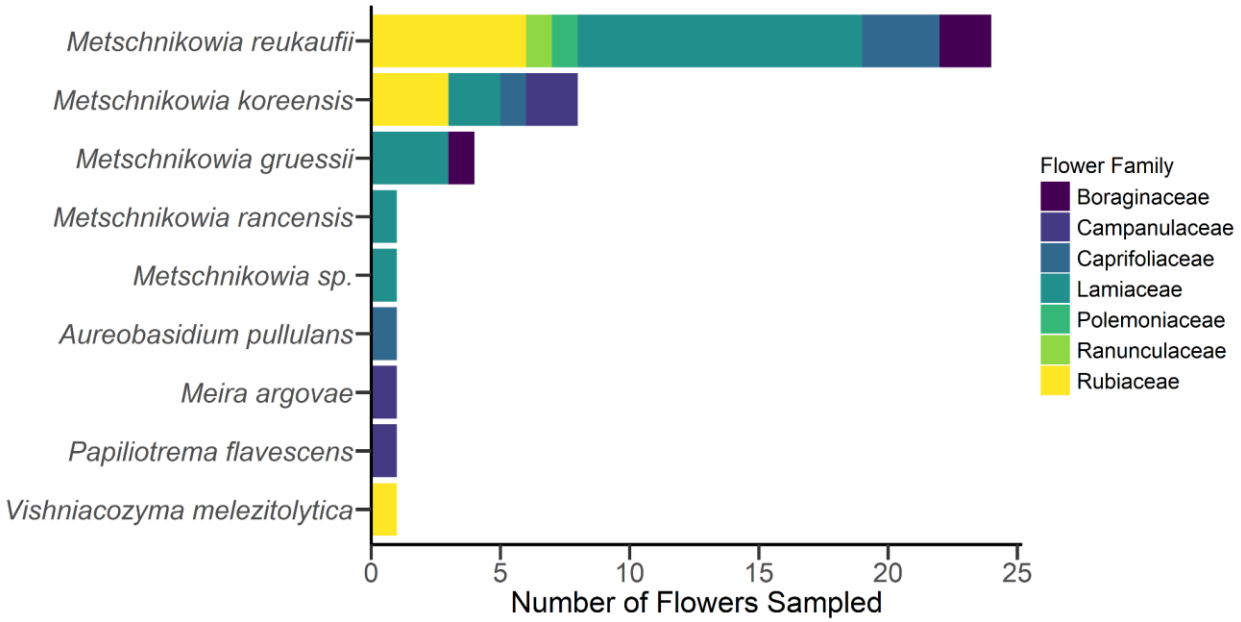

Figure 1. The distribution of yeast species across flower families sampled. Plants were selected based on flower structure; funnel-form flowers allowed for nectar collection without contamination from other plant tissues. Nectar samples were plated on rich media, and colonies that presented yeast-like morphology were sequenced and identified to genus or species.

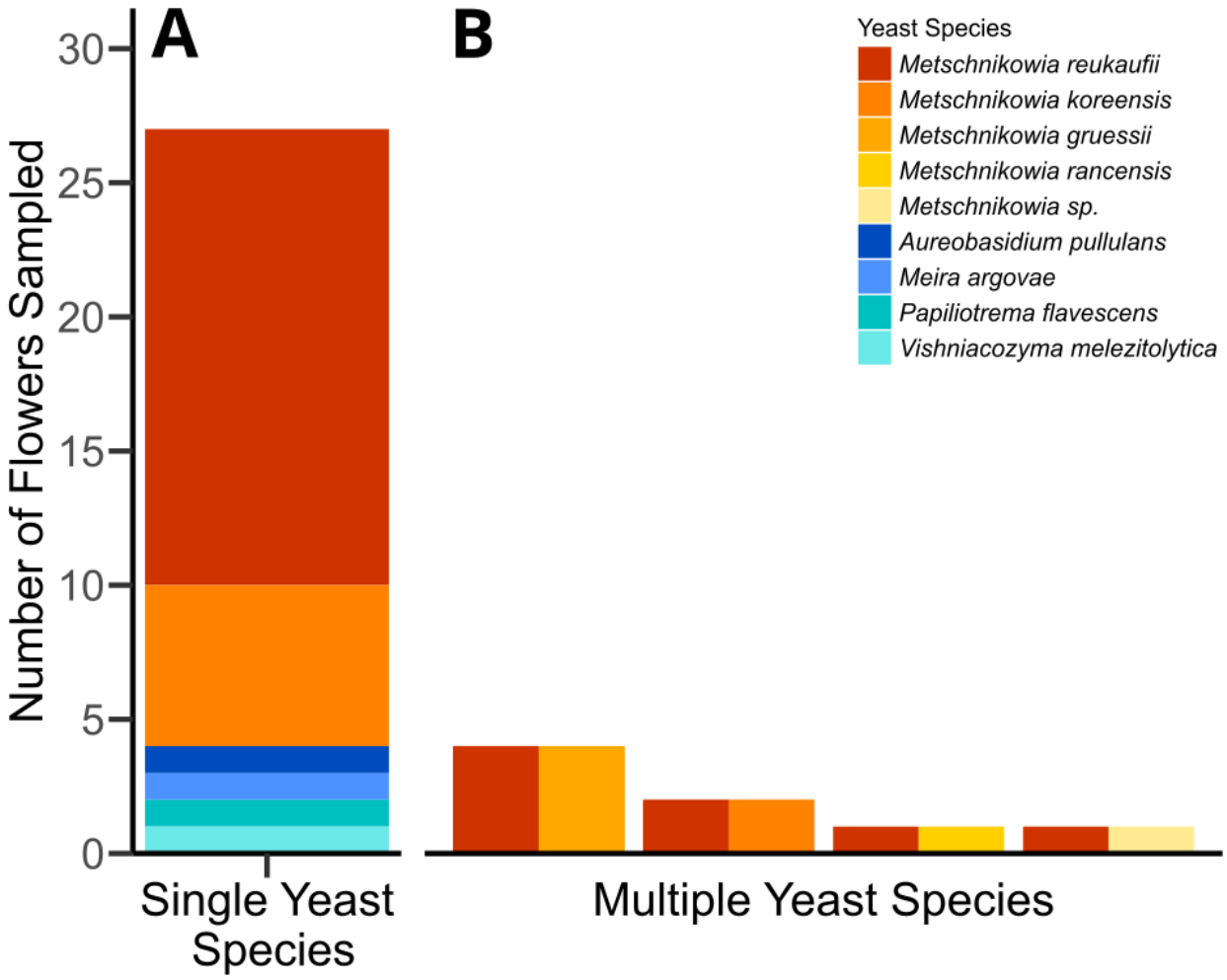

Figure 2. Most nectar samples contained only one species of yeast (A), which is congruent with the majority of published studies on nectar microbes. A small portion of the nectar samples contained multiple yeast species (B), with *M. reukaufii* being present in all samples.

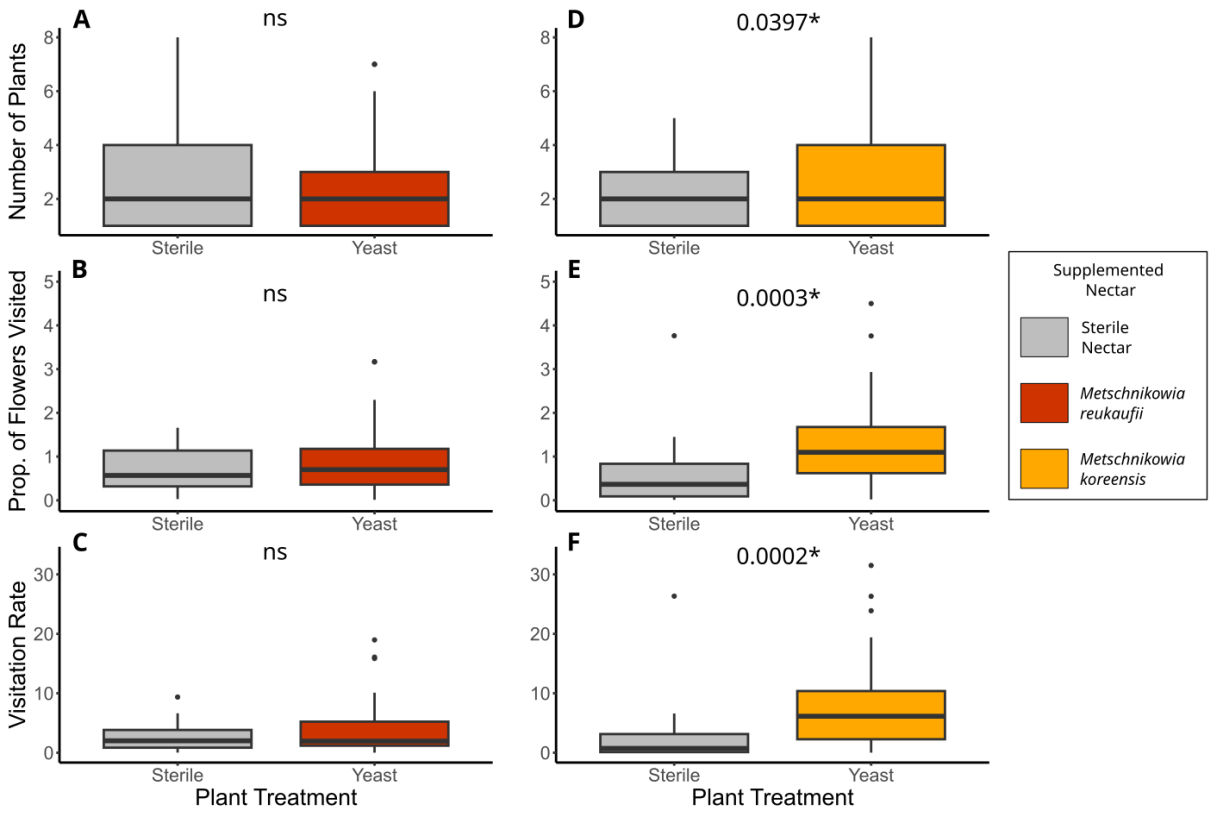

Figure 3. The effects of plant treatment (addition of sterile nectar or nectar inoculated with yeast) on metrics of bee pollinator visitation. A. The number of plants visited by each observed bee pollinator each trial day. B. The proportion of the total available flowers visited by bee pollinators each trial day. C. The visitation rate (number of plants visited * the proportion of flowers visited) of bee pollinators to each plant treatment.