**Title: Nectar compounds impact bacterial and fungal growth and shift community dynamics in a nectar analog**

**Running head: NECTAR COMPOUNDS IMPACT GROWTH AND SHIFT COMMUNITY**

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**Originality-significance statement**

We examine the hypothesis that filtering due to inhospitable nectar chemistry is a main driver of the low diversity of microbes found in floral nectar. This study is the first to broadly compare if microbes, isolated from nectar and other habitats, vary in resistance, and therefore colonizing ability, to a range of nectar compounds and if these compounds impact microbe-microbe interactions.

**Summary**

Floral nectar is frequently colonized by microbes. However, nectar microbial communities are typically species-poor and dominated by few cosmopolitan genera. One hypothesis is that nectar constituents may act as environmental filters. We tested how five non-sugar nectar compounds as well as elevated sugar impacted the growth of 12 fungal and bacterial species isolated from nectar, pollinators, and the environment. We hypothesized that nectar isolated microbes would have the least growth suppression. Additionally, to test if nectar compounds could affect the outcome of competition between microbes, we grew a subset of microbes in co-culture across a subset of treatments.

We found that some compounds such as H2O2 suppressed microbial growth across many but not all microbes tested. Other compounds were more specialized in the microbes they impacted. As hypothesized, the nectar specialist yeast *Metschnikowia reukaufii* was unaffected by most nectar compounds assayed. However, many non-nectar specialist microbes remained unaffected by nectar compounds thought to reduce microbial growth. Our results show that nectar chemistry can influence microbial communities but that microbe-specific responses to nectar compounds are common. Nectar chemistry also affected the outcome of species interactions among microbial taxa, suggesting that non-sugar compounds can affect microbial community assembly in flowers.

**Introduction**

Most angiosperms produce floral nectar to attract pollinators. Floral nectar (hereafter simply nectar) is an aqueous solution often predominantly composed of sugars including sucrose, glucose, and fructose (Baker and Baker, 1983). However, nectar is much more than a simple sugar solution; approximately 10% of nectar’s dry weight is composed of non-sugar compounds including free amino acids, proteins, lipids, vitamins, and alkaloids among other compounds (Baker, 1977; Nicolson *et al.*, 2007; Roy *et al.*, 2017) and can differ substantially among and within species (Nicolson *et al.*, 2007; Ryniewicz *et al.*, 2020).

Nectar can be colonized by microbes, primarily yeasts and bacteria, which are deposited by floral visitors (Sandhu and Waraich, 1985; Russell *et al.*, 2019; Zemenick *et al.*, 2021). Surveys typically find 20 - 50% of flowers contain culturable microbes depending on plant species and environment (de Vega *et al.*, 2009; Pozo *et al.*, 2011; Álvarez-Pérez *et al.*, 2012; Jacquemyn *et al.*, 2013; Vannette *et al.*, 2021). The microbes found in nectar can range from plant and pollinator pathogens, to putatively mutualistic, to microbes that may be commensal or have no documented effects on plants or pollinators (Adler *et al.*, 2021). Once deposited, nectar microbes can reach high densities, growing to more than 105 cells/μL for yeasts and 107 cells/μL for bacteria (Álvarez-Pérez *et al.*, 2019). However, microbial communities often exhibit low alpha diversity within individual nectar samples, consisting of a few globally dominant genera, including fungi, such as *Metschnikowia* and *Aureobasidium* (de Vega *et al.*, 2009; Pozo *et al.*, 2011; Chappell and Fukami, 2018)*,* and bacteria, such as *Acinetobacter* (Fridman *et al.*, 2012; Álvarez-Pérez *et al.*, 2012; Alvarez-Pérez and Herrera, 2013; Tsuji and Fukami, 2018)*.* The microbes that establish in nectar are a subset of the microbes carried by pollinators and in the environment (Herrera *et al.*, 2010; Pozo *et al.*, 2012; Alvarez-Pérez and Herrera, 2013). While it is clear that many microbes deposited in floral nectar fail to establish (Herrera *et al.*, 2010; de Vega and Herrera, 2012; Pozo *et al.*, 2012), numerous processes may generate the low microbial diversity observed in nectar. Possible mechanisms include differential dispersal of microbes (Zemenick *et al.*, 2021); competitive exclusion that favors early arriving, faster growing, or inhibiting species (Fukami, 2015; Dhami *et al.*, 2016); or strong filtering by the chemistry of the nectar environment (Herrera *et al.*, 2010). These mechanisms are not mutually exclusive and likely vary in importance depending upon context. However, in some systems animal-flower visitation networks alone cannot explain nectar microbial communities suggesting that filtering may play a role (Zemenick *et al.*, 2021).

Some nectar traits are thought to provide antimicrobial activity (Herrera *et al.*, 2010; Schmitt *et al.*, 2021). The high sugar concentrations in nectar leads to extreme osmotic pressure and high C:N ratios both of which limit microbial growth (Brysch-Herzberg, 2004; Herrera *et al.*, 2010; Lievens *et al.*, 2015). Additionally, antimicrobial compounds are commonly produced in nectar (Schmitt *et al.*, 2018, 2021). In ornamental tobacco (*Nicotiana langsdorffii × Nicotiana sanderae*), hydrogen peroxide levels can reach 4mM (Carter and Thornburg, 2004), suppressing some but not all microbes’ growth (Carter *et al.*, 2007; Parra *et al.*, 2022). Other antimicrobial proteins are thought to have activity against specific groups of microbes (Schmitt *et al.*, 2021). In previous comparative studies, nectar compounds including hydrogen peroxide, the antimicrobial protein BrLTP2.1, and the floral volatile linalool showed species-specific effects, reducing microbial growth for some species but not others (Carter *et al.*, 2007; Burdon *et al.*, 2018; Schmitt *et al.*, 2018; Block *et al.*, 2019). However, few studies have broadly compared if microbes isolated from nectar and other habitats, vary in resistance to a range of nectar compounds (however, see Pozo *et al.*, 2012; Mittelbach *et al.*, 2016; Burdon *et al.*, 2018), and if these compounds impact microbe-microbe interactions.

Here, we use *in vitro* growth assays to test the degree to which nectar chemistry alone, or in combination with competitive dynamics, impacts microbial growth in a nectar analog. First, we tested the hypothesis that common nectar microbes can better tolerate a variety of nectar chemistries compared to microbes isolated from non-nectar habitats. If non-nectar specialists grow well in the presence of nectar compounds, it would indicate that filtering by these compounds is not a major driver of community assembly, and that other factors such as dispersal limitation or competition are more important. However, if only nectar specialists can maintain growth in the presence of common compounds found in nectar, it would suggest that environmental filtering may play a major role in nectar microbial community assembly. Second, we tested the hypothesis that the presence of nectar compounds affects the outcome of microbial competition in nectar.

**Experimental Procedure and Results**

*Microbial strains*

We tested the effects of nectar compounds on the growth of the fungi *Metschnikowia reukaufii, Aureobasidium pullulans, Starmerella bombi, Rhodotorula fujisanensis, Saccharomyces cerevisiae, Zygosaccharomyces bailii,* and the bacteria, *Acinetobacter nectaris, Rosenbergiella nectarea, Bacillus subtilis, Pantoea agglomerans, Pseudomonas mandelii, Pectobacterium carotovorum.* The species assayed include microbes commonly isolated from nectar, pollinators, and the environment (Table 1). We tested compounds detected in nectar that have been hypothesized or demonstrated to be antimicrobial and used concentrations in line with levels documented in nectar (Supplemental Table 1). We tested hydrogen peroxide (H2O2), a reactive oxygen species found in some nectars, at two concentrations (2mM and 4mM, (Carter and Thornburg, 2004)); deltaline, a norditerpene alkaloid found in the nectar of *Delphinium spp.* and a potent toxin for eukaryotes (22ug/ml, (Cook *et al.*, 2013)); BrLTP2.1, a lipid transfer protein isolated from *Brassica rapa* nectar, hereafter referred to as LTP (150μg/ml, (Schmitt *et al.*, 2018)); linalool, a common volatile found in nectar (100ng/ml, (Burdon *et al.*, 2018)); ethanol (EtOH), a common byproduct of fermentation in nectar (1%, (Wiens *et al.*, 2008)) and elevated sugar at 30%, along with a 15% base control nectar solution (which covers the low and moderate levels of natural sugar concentrations) (Nicolson *et al.*, 2007). These compounds were chosen because they represent a broad range of compounds found across floral nectars and were feasible to obtain. See Supplemental Methods 2 for the recipes and process of creating control and treatment “nectars”.

*Plate reader growth assay*

To test the effect of individual compounds on the growth of single microbe species, we used 96 well plate growth assays and synthetic nectars to observe the change in optical density (OD) as a proxy for microbial growth with OD measurements at 600 nm every 15 minutes for 72 hours. We used mathematical models to fit logarithmic curves to OD measurements and adjusted wells to account for plate effects (see Supplemental figure 1 plate mapping). To compare a treatment's relative impact on growth across microbes, we computed a scaled growth rate (𝛍) and maximum growth (𝚨) by adjusting each microbe’s growth in treatment relative to their growth in control nectar across all plates [*log ((scaled value = treatment* 𝛍 or 𝚨 */ mean control* 𝛍 or 𝚨*) + 1)]*.A scaled value over *log(2)* indicates a treatment 𝛍 or 𝚨 greater than that microbe’s control and scaled value below *log(2)* indicates a 𝛍 or 𝚨 lower than that microbe’s control. These transformations allow us to compare the effects of nectar compounds across many microbes that varied in absolute growth. See supplemental Methods 3 for all data analysis.

*Treatment impacts across all microbes*

Nectar compounds differed in their effect on maximum scaled OD (Figure 1); H2O2 strongly suppressed the growth of most microbes at 2mM (negative binomial model coefficients and standard error: -0.9 +/- 0.27, p < 0.001) and 4mM (-1.95 +/- 0.39, p < 0.001). 30% sucrose (-0.07 +/- 0.18, p = 0.7), LTP (-0.08 +/- 0.18, p = 0.64), linalool (-0.13 +/- 0.18, p = 0.49) and EtOH had no significant effect (0.06 +/- 0.17, p = 0.75). In contrast, the diterpene alkaloid deltaline increased maximum OD overall (0.34 +/- 0.15, p = .03). Scaled maximum OD was correlated with scaled maximum growth rate (r = 0.67, p < 0.001) and effects of treatments on both were congruent, although not identical (Supplemental Figure 2).

*Microbe-specific response to treatments*

Microbial species varied in their maximum OD and growth rate in control nectar and in response to treatment additions (Supplemental Figures 3-4, p < 0.05). All microbes were impacted by at least one treatment, but treatments differed in their effect on maximum OD (Figure 2) and growth rate (Figure 3) across microbial species. Species’ responses to nectar composition depended on the specific nectar compound tested: no microbe had significantly reduced maximum OD or growth rate across all treatments (Figure 2). When comparing across all treatments, the scaled maximum OD was not significantly different across degrees of nectar specialization (p > 0.05; Figure 4a), however, scaled growth rate was significantly different: microbes infrequently isolated from nectar had a lower scaled growth rate than both the highly and medium specialized group (p < 0.05; Figure 4b).

*Differences between yeast and bacteria*

Yeasts and bacteria differed significantly in the maximum OD attained, with yeasts (0.82 +/- 0.35, p = 0.04) having a higher max OD than bacteria (0.01 +/- 0.25, p = 0.96) (Supplemental Figure 5a). When assaying treatments’ scaled impact on max OD, yeast (-0.05 +/- 0.12, p = 0.67) were significantly less affected by treatments compared to bacteria (-0.7 +/- 0.25, p = 0.004) (Supplemental Figure 5b), suggesting that yeasts may be more resistant to the inhibitory effects of nectar chemicals than bacteria. However, there was no significant phylogenetic signal present that was driving the scaled max OD (λ = 0.59, p = 1; K = 0.2, p = 0.81) or growth rate (λ = 0.2, p = 1; K = 0.19, p = 0.91) indicating that while bacteria and yeasts as a whole may broadly differ, there is strong variation within each kingdom and relatedness does not drive the response to nectar chemistry (Supplemental Figure 6).

*Co-growth experiment*

To test if nectar composition could shift microbial interactions, we grew pairs of microbes across several treatment solutions: 1) *Starmerella bombi & Zygosaccharomyces bailii (*a facultative nectar yeast with a non-nectar yeast*),* 2) *Metschnikowia reukaufii & Rosenbergiella nectarea (*a nectar specialist yeast with a nectar specialist bacteria*),* and 3) *Saccharomyces cerevisiae & Rosenbergiella nectarea (*a non-nectar specialist yeast with a nectar specialist bacteria*)*. We also ran a pairing of *Metschnikowia reukaufii & Saccharomyces cerevisiae,* however, the vial lids burst open during incubation due to extremely rapid fermentation. These species pairings were chosen from many cogrowth combinations as they produced colonies that were easily distinguishable from one another during preliminary cogrowth tests.If the dominance of nectar specialists is driven by nectar chemicals shifting microbe-microbe competition we predict nectar specialists will increase in relative abundance in the presence of nectar compounds, while the relative performance of environmental microbes should be reduced compared to control co-growth trials. We chose a subset of treatments for co-growth assays, including 4mM H2O2, 22μg/ml deltaline, 100ng/ml linalool, and 1% EtOH. Treatments used the same recipes as the growth experiments described above. See supplemental Methods 3 for full experimental procedure.

The presence of competitors and nectar compounds together affected microbial abundance after 3 days for all species pairings (Figure 5). For example, in a co-culture of the food spoilage specialist *Z. bailii* and bee-associated *S. bombi*, *Z. bailii* never formed CFUs in the presence of a competitor, but did when grown alone, suggesting strong competitive exclusion*.* In contrast, *S. bombi* in the same pairing showed increased CFU formation in co-culture relative to its growth alone (p < 0.001), in control nectar, 22μg/ml deltaline, 1% EtOH, and 4mM H2O2 treatment nectars (Figure 5a). In the pairing of two nectar ‘specialists’, neither the bacteria *R. nectarea* nor the yeast *M. reukaufii* showed an altered CFU density in co-culture compared to growth in isolation (Figure 5b). When co-culturing *R. nectarea* and *S. cerevisiae,* we found that contrary to our original hypothesis, the non-nectar yeast *S. cerevisiae* did not show a significant reduction (p > 0.05) in growth compared with growth alone*.* Notably, however, the addition of H2O2 reduced *S. cerevisiae* and made *R. nectarea* growth undetectable (Figure 5c) *—* in contrast to the ability of *R. nectarea* to persist in the presence of *M. reukaufii* in H2O2-containing nectar.

**Discussion**

All nectar constituents tested had species-specific effects on microbial growth, significantly impacting certain microbes while showing no impact on others. Hydrogen peroxide showed strong antimicrobial properties across most microbes assayed, both nectar specialists and non-specialists. It is unknown how common H2O2 is in nectar, but it has been detected in several genera of plants including *Nicotiana* and *Cucurbita* (Carter *et al.*, 2007; Nocentini *et al.*, 2015)*.* Despite strong suppressive effects on most species (including those with documented catalase activity) (Álvarez-Pérez *et al.*, 2012), the antimicrobial effect of H2O2 was not universal. Notably the maximum OD of the yeast *M. reukaufii* and *Zygosaccharomyces bailli* were unaffectedby any concentration of H2O2 tested and *S. bombi* was only affected at 4mM. It should be noted, however, that H2O2 has a very short half-life and likely degraded over the course of each assay. In floral nectar, H2O2 can be continuously produced suggesting that our study may underestimate its antimicrobial properties. Other tested compounds were more selective in their growth suppression and impacted different microbes including those frequently and seldom isolated from nectar. We only tested 1 isolate per species here, but it is possible there could be strain specific adaptation or susceptibility to different compounds. This is an intriguing hypothesis for future work.

The observed differences in the selectivity of compounds suggest that nectar antimicrobial compounds (NACs) may fall into two broad classes with different functions: general antimicrobials and selective filters. General NACs (e.g., H2O2 here) may keep a flower from being colonized by most microbes and are possibly common in nature. In some ecosystems as many as 80% of plants have no culturable yeasts and some have very low incidence of culturable bacteria (Herrera *et al.*, 2009; Vannette *et al.*, 2021). We predict that general NACs, or other mechanisms to limit microbial growth, might be more common in ecosystems where plants have a high likelihood of colonization by antagonistic microbes but a low probability of colonization by beneficial microbes (or where the costs of antagonists consistently outweigh the benefits of mutualists). Conversely, we predict that selective filtering NACs might be more common in ecosystems where plants have equal likelihoods of being colonized by beneficial or antagonistic microbes. Direct effects of NACs on pollinator behavior and health, however, should not be discounted and likely also plays a role in the selection on NACs (Manson *et al.*, 2013). While we lack data on the plant traits that shape communities of antagonistic and beneficial microbes (Adler *et al.*, 2021), and there are likely other modes beyond NACs that work in conjunction such as floral morphology or other nectar constituents including enzymes, ions, lipids, among others, these data suggest that selective NACs may be one route by which plants shape their nectar microbiome. However, with the extreme diversity in floral nectar chemistry, many general and selective NACs have likely not yet been identified or may escape notice by being context dependent. Characterizing the relative abundance of general and selective NACs across different microbial landscapes might be particularly fruitful in disentangling how microbes shape selection on nectar traits.

Our findings suggest that NACs can also shift competitive dynamics and the trajectories of nectar microbial communities as previously suggested (Álvarez-Pérez *et al.*, 2019). While we found no relationship between degree of nectar specialization and treatment impacts on maximum growth, the growth rate of non-nectar specialists was more suppressed in the presence of nectar compounds, and bacteria were more negatively affected than yeasts, both of which could affect end community assembly. Our co-culture experiment further shows that treatments can impact communities not only by decreasing the growth of some microbes, but also increasing the growth of others in co-culture. Here, *Z. bailii* did not grow in co-cultures with *S. bombi*, however, *S. bombi* showed elevated growth in co-culture, even in the presence of H2O2. We hypothesize that the presence of *Z. bailii* may have facilitated the growth of *S. bombi* by potentially providing additional nutrition. Alternatively, it appears that some microbes may facilitate each other’s growth. For example, *R. nectarea* grewin H2O2-containing nectar in the presence of *M. reukaufii* but not *S. cerevisiae*, perhaps suggesting that *M. reukaufii*, which itself does not appear to be impacted by H2O2,may have methods for detoxifying H2O2 that extend to other inhabitants of the same nectar environment.

The impact of plant chemistry on ecological interactions can be difficult to predict and some presumptive NACs may even benefit certain microbes. We predicted that the norditerpene alkaloid deltaline would broadly suppress microbial growth, but our results generally suggest otherwise. Deltaline only decreased the growth of *M. reukaufii,* with most other microbes increasing in maximum OD relative to their control. This is surprising considering that other norditerpene alkaloids, extracted from flowering plants in the same family as *Delphinium*, have strong antimicrobial properties (Ahmad *et al.*, 2008). Prior work looking at the antimicrobial effects of norditerpenes, however, tested concentrations higher than those occurring in nectar (Ahmad *et al.*, 2008). For microbes that do not experience growth suppression, it is possible that deltaline is a source of otherwise limiting compound such as nitrogen (Vannette and Fukami, 2014), although our study had much higher levels of nitrogen compared to most floral nectar (Nicolson *et al.*, 2007). It is possible that compounds that might be otherwise anti-microbial in growth media or in other plant tissues may benefit microbes in nectar. These findings highlight that generalizing across plant tissues and among whole classes, or even subclasses, of compounds should be done with caution.

Although the impact of nectar secondary metabolites on microbes may be an understudied ecological role, other abiotic and biotic ecological drivers should also be considered. Nectar chemicals are widespread (Adler, 2000) but may be non-adaptive consequences of chemical defense in other plant tissues (Adler, 2000; Adler *et al.*, 2012) where they can effect florivores or pollinators and their behavior (Wright *et al.*, 2013). Additionally, nectar chemicals are often in low concentrations when compared to compounds in other plant tissues (Palmer‐Young *et al.*, 2019). Compounds in other plant tissues may also influence the nectar environment and shape microbial communities, for instance, when pollen gets deposited into floral nectar. Nectar is a complex and dynamic solution, changing with enzyme activity, host-mediated secretion and resorption, and via contact with floral tissues – ­all precluded by our use of synthetic nectar. It is possible that these complex interactions of chemicals may increase or decrease the effect of the specific compounds tested here. Whether the impacts of NACs observed here are stronger or weaker than these other factors (and thus are ecologically relevant) is an open question.

Taken together, our results suggest variable effects of nectar chemistry and that different microbes may be excluded from nectar for varying reasons. The findings that nectar compounds can shift microbial colonization and community dynamics raise more questions for further study. Given that nectar is chemically diverse (Palmer‐Young *et al.*, 2019), and microbes vary in dispersal limitation (Vannette *et al.*, 2021), what does the observed selectivity of NACs mean at a landscape scale? On one hand, it could lead to a diversity of microbial niches where different floral species have different selective NACs, and thus floral diversity would likely increase microbial diversity at the landscape scale. However, this is not found in nectar surveys, suggesting that other strong drivers, such as dispersal (Russell *et al.*, 2019; Vannette *et al.*, 2021), competitive ability (Fukami, 2015; Dhami *et al.*, 2016), or intraspecific variation in microbial sensitivity to NACs, also contribute to low species diversity in floral microbial communities(Herrera *et al.*, 2014; Dhami *et al.*, 2018). Finally, given our result that nectar secondary chemistry can affect microbial growth, and may affect yeasts to a lesser extent than bacteria, characterizing variation in antimicrobial potential among plant populations and species may allow a better understanding of how microbes, pollinators and other forces shape the ecology and evolution of nectar traits.

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**Statements and Declarations**

Author contributions:

TGM and RLV conceived of and designed the study with input provided by JSF. Data collection was performed by TGM with help from JSF. Data analysis was performed by TGM. The first draft of the manuscript was written by TGM with all authors contributing to the writing and editing process. All authors read and approved of the final manuscript.

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Competing interests:

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Data availability:

All datasets generated during the study as well as data analysis scripts and outputs can be found on GitHub at <https://github.com/tobiasgmueller/nectar_growth_assay>