**The paper reformatted into the sections INTRODUCTION, RESULTS, DISCUSSION. then at the end experimental procedures, or more ideally put those into supplemental methods section**

**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.

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 the highest concentrations previously 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 X 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 (see Supplemental methods X for assay methods and plate mapping). We used mathematical models to fit logarithmic curves to OD measurements.growth rate ()maximum growth ()

nectar additions

, however there was no significant phylogenetic signal present that was driving the scaled max OD indicating that

*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 vials 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.

*Co-growth assay*

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 this experiment. 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. While here we only tested 1 isolate per species it is possible that there could be strain specific adaptation or susceptibility and more work could be done to see if there is intraspecific variation in susceptibility.

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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**New SUPPLEMENTAL METHODS**

Creating Microbial suspensions

*Preparing synthetic nectars*

To prepare the synthetic nectars (treatment and control solutions), we weighed dry reagents on a microbalance to a precision of 0.0025 grams before washing them into a volumetric flask and dissolving the reagents in DI water. Liquid reagents were added and then the entire solution was diluted with DI water to the proper concentration before being vortexed and sterilized using a syringe filter (0.2 μm cellulose acetate membrane, Corning, Corning NY, product number 431219). Base nectar consisted of 15% sugar (50:25:25 sucrose:glucose:fructose) w/v, 1% peptone w/v, 3% yeast extract w/v, 50% 100x non-essential amino acids v/v. This high nitrogen nectar analog was chosen so that all tested microbes could grow in the base solution to detectable levels, allowing us to test the impacts of nectar compounds alone on growth separately from the effects of nutrient limitation, however it is possible that nutrient limitation and growth suppressive compounds may work in conjunction in floral nectar.

*Co-growth experiment*

We performed the co-growth experiment in 200μL 8-strip PCR tubes with 6 tubes per treatment–microbe combination. Each tube consisted of 190μL of synthetic nectar and 5μL (10,000 cells) of each microbial freezer stock in that pairing. We vortexed the tubes for 5 seconds and incubated them at 25°C for 72 hours. To assess the effect of co-growth, we also grew each microbe in isolation, following the same methods, with tubes consisting of 190μL of synthetic nectar and 5μL of microbial suspension (N=4).

After 72 hours of incubation, we serially diluted the microbial suspensions and plated 100μL of diluted microbial suspension onto TSA and YMA plates. We diluted TSA plates 2x (plating 50μL of original suspension), YMA plates from the *Starmerella bombi* and *Zygosaccharomyces* pairing both 20x (5μL plated) and 200x (0.5μL plated), and YMA plates with *Metschnikowia* or *Saccharomyces* 200x (0.5μL plated). We chose these dilutions as they created countable CFUs. We then incubated plates at 25°C for 72 hours to allow microbial colonies to form, after which we counted the number of colonies per plate. *Rosenbergiella* did not form single colonies and instead the percent of the plate covered by growth was estimated and adjusted relative to the maximum CFU count of its yeast pairing (100% coverage = maximum CFU count).

**Data Analysis**

All analyses were performed in RStudio using R version 4.0.1 (RStudio Team, 2020).

*Curve fitting and data curation*

We used the Grofit package (Kahm *et al.*, 2010) to fit logarithmic curves to the optical density (OD) timeseries. The initial OD value for each well was deducted from all readings to account for starting solution OD. Best-fit growth curve models were selected using AIC and each fitted curve was visually inspected after which growth rate (𝛍) and maximum OD (𝚨) were extracted from the fitted curves.

To ensure data quality, we performed the following checks on all growth curves before curve fitting: if a well did not change OD over 72 hours, the 𝚨 and 𝛍 were set to zero. If the OD increased and then returned to the starting value within 72 hours, we considered the well having no growth and set both parameters to zero. This was likely driven by a period of growth followed by cell death and in turn decreased OD. While it is possible that clumping of cells could lead to a slight decrease of OD, plates showing this growth pattern were visually inspected under a microscope after 72 hours and no evidence of clumping in wells was found. If only 1 treatment well out of the 6 did not grow, we considered this to be due to an error (possibly no microbe addition) and removed the well from the analysis. If no mathematical fit could be plotted to the OD readings, we removed the well from the analysis (less than 5% of growth curves). Many of these unfittable wells showed flatline curves with a single value change during the 72 hours likely not caused by microbial growth. One well showed an 𝚨 100 times greater than all other wells in that treatment and was removed. For a single plate (deltaline), condensation caused a temporary drop in the OD for the first 45 minutes. For these wells we set the starting OD as the lowest OD reading from the first hour.

*Treatment impacts across all microbes*

To compare the effects of treatment across all microbes we fit a negative binomial model (Venables *et al.*, 2002) with scaled maximum OD as a function of treatment. To test if the scaled maximum OD and scaled growth rate were correlated we calculated the Pearson’s correlation coefficient.

*Microbe-specific response to treatments*

We compared each microbe’s growth in different nectar chemistries to their growth in control nectar using a Kruskal-Wallis test followed by a Dunnett’s test (Signorell, 2021), with separate models for maximum OD and maximum growth rate. To test if treatment impacts were related to the frequency microbes occur in nectar (hereafter referred to as nectar specialization) we ranked microbes as “high”, “medium”, or “low” according to their relative incidence and abundance in nectar (Table 1). We ran a Kruskal-Wallis test comparing scaled maximum OD and scaled growth rate to the level of specialization. Significant results were followed by a Dunn’s test with a Holm-Bonferroni correction to compute pairwise differences (Signorell, 2021).

*Differences between yeast and bacteria*

To compare if nectar chemistry differentially affected yeasts compared to bacteria, we used a linear random effects model (for maximum OD) and negative binomial model (for scaled maximum OD) comparing microbial growth between kingdoms, with microbe and treatment as random intercept terms.

*Co-growth assay*

To compare how nectar chemistry can change community dynamics, we used a Kruskal-Wallis test followed by a Dunn’s test comparing each microbe's growth in co-culture across different nectar chemistries and alone in control nectar.

*Phylogenetic signal*

To determine if there was an effect of relatedness on the growth or inhibition of microbes we calculated both Pagles lambda as well as Blomberg’s K using the phytools package in R. Phylogenetic trees with divergence times were constructed using TreeTime of Life (Kumar *et al.*, 2022). For Rhodotorula fujinensis, no NCBI data was available so it was replaced with Rhodotorula graminis and Auerobasidium pallulans was replaced with the co-family member Sydowia polyspora, however, neither substitution will impact divergence time in relation to other members of the tree.

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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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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>