

An emerging actor in pollinator attractiveness: the nectar microbiome

Abstract

Ecosystems are threatened by extreme weather and global warming caused by global climate change. Angiosperms are key players in ecosystems and support various animals, plants and fungi. In addition, most angiosperms rely on animal-pollinators to disperse their pollen for fertilization. However, the interaction between pollinators and angiosperms is influenced by nectar microbes. Nectar microbes, mostly *Proteobacteria* or fungal yeasts, inhabit floral nectars in the flowers. Nectar may be a harsh environment with high osmotic pressure and antimicrobial compounds, but the microbes possess tolerance traits and have high cell densities in older flowers. Due to the relatively short lifespan of flowers, nectar microbes need pollinators to disperse to other habitats. Also, nectar microbes alter nectar chemistry, such as pH, sugar and amino acids concentrations, and increased nectary temperatures. The antimicrobial secondary metabolites were reduced by nectar microbes as well. The volatile organic compound compositions were altered by the nectar microbial species, but the overall effect on pollinator attractiveness was species-specific. Other components were influenced by nectar microbes as well: nectar bacteria decreased stigma closure after pollen reception and microbes affected pollinator fitness. Overall, nectar microbes had a wide range of effects on pollinator attractiveness. It remains unclear what the combined effect of the nectar microbiome is on pollinator attractiveness and fitness of angiosperms. Currently, we have little knowledge on the impacts of nectar microbiome on pollinator attractiveness. With future global warming it is most important to understand these interactions to protect our ecosystems.

Layman

Global climate change can have various consequences for our surroundings (ecosystems). The increase in greenhouse gases can lead to more extreme weather and temperature increases. Our ecosystems and its residents, such as plants, animals and fungi, are vulnerable to these changes. Moreover, many residents rely on each other for food or shelter, so these interactions are endangered as well. For instance, flowering plants support many other organisms, like pollinator bees and birds. Flowering plants also rely on pollinators to reproduce and attract pollinators with a food source, a sugary liquid called nectar. To prevent the loss of these interactions by global climate change, we need to be able to protect them. To do so, more knowledge on ecosystems and the interactions between the residents is needed. One player in the interaction between flowering plants and pollinators is yet overlooked: the bacteria and fungi (microbes) in nectars. Current studies show that nectar microbes can have contrasting effects on the pollinator attraction. Whereas one microbe can increase pollinator attraction to the flowers, others can repel the pollinators or have no effect. As well, nectar microbes affect different aspects of flower nectar, including nectar foods and scents. By understanding the importance of nectar microbes in the interaction between flowering plants and pollinators, we can protect these interactions and limit the effects of global climate change.

Chapter 1: Introduction

Ecosystems are threatened by climate change (Abbass et al, 2022). Global climate change is predominantly caused by fossil fuel emission and deforestation (United Nations, 2023). One of the consequences is an increase in atmospheric greenhouse gases concentration, such as carbon dioxide and methane gases. This leads to weather extremes and global warming (Rummukainen, 2012), that can threaten our current ecosystems and cause declining species populations (McCarty, 2001). Therefore, there is an urgency to better understand ecosystems' interactions to predict the effects of climate change.

1.1 Angiosperms are important in ecosystems

Land angiosperms are important in ecosystems due to their various interactions with diverse groups of species (Benton et al, 2022). Notably, the habitat of around 78% of insect species are dependent on angiosperms; and species belonging to epiphytes, fungi, birds, or mammals are supported by angiosperms as well. Currently, angiosperm species make up around 90% of the vascular plant diversity. The high diversity in angiosperms is partly due to flower adaptation for cross-pollination. Different methods of pollination exist: 8% of land angiosperms are wind pollinated, while most are pollinated by insects, bats, or birds. The evolution of the flower enabled angiosperms to interact with their animal pollinators.

1.2 Flower fertilization

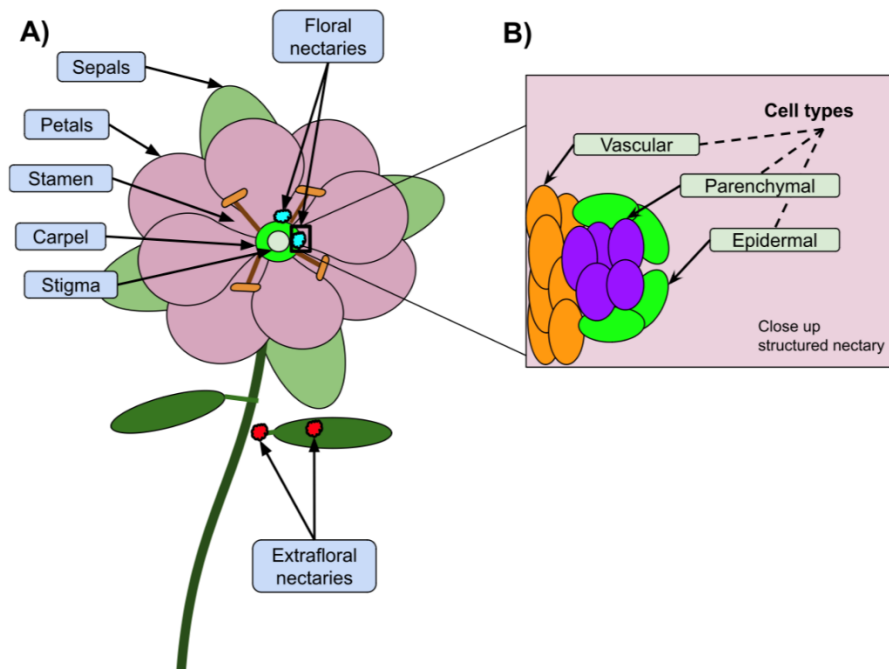


Figure 1. Schematic of flower and nectary anatomy. A) Sepals (green) and petals (pink) encase the reproductive organs: the male organ is a stamen (brown) and the female organ is a carpel (light green + white) with a stigma (white). Angiosperms can have floral nectaries (light blue) to attract pollinators and extrafloral nectaries (red) as indirect defense against herbivores. B) Close up of cell tissues in a structured nectary. The nectaries are involved in nectar production and secretion for attracting pollinators. The vascular cell layer (orange) supplies the pre-nectar to parenchymal cells (purple) where the pre-nectar is modified. After, the nectar is secreted by the epidermal cells (light green).

The flowers of angiosperms encase the plant reproductive organs (Melzer et al, 2010). The typical flower of angiosperms has four different organs and is structured in a whorl as seen in Figure 1A. From the margin to the center, the flower is structured as followed: sepals, petals, male and female reproductive organs. The male organs are structured in stamens and female organs in carpels with a receptive tip called the stigma. For seed production, the pollen grains produced by the stamens need to be deposited on the stigma of a flower from the same species (Chaudhury et al, 1998). The transport of pollen to the stigma is called pollination (Torezan-Silingardi et al, 2021). When compatible pollen grains are deposited on the stigma, the pollen grains germinate and fertilize the egg cells in the carpels (Chaudhury et al, 1998). In double fertilization, an embryo is formed and after diversification, a zygote and an endosperm are produced. Eventually, the zygote becomes a seed.

1.3 Pollinator attraction and effectiveness

Around 87% of angiosperm species are animal-pollinated (Roy et al, 2017), so the reproductive success of many angiosperm species is dependent on the interaction with their pollinators. Pollinators need to transfer pollen to compatible stigmas and therefore flowers need to attract specific pollinator species. Pollinator effectiveness is the degree to which a pollinator maximizes the fitness of the male and female organs (Moreira-Hernández et al, 2019). In other words, the effectiveness of the pollinator to disperse pollen of angiosperms to stigmas from flowers of the same species. Pollinator effectiveness is divided into quantity (the number of visits from the pollinators) and quality (the amount of pollen transferred per pollinator visit), that are partially dependent on effectiveness of pollinator attraction. Pollen misplacement and pollen loss due to the pollinator are negatively affecting the male fitness and can indirectly affect female fitness. In addition, heterospecific pollen transfer (transfer to other species) leads to a decrease in female fitness. Thus, pollinator effectiveness is essential to the fitness of angiosperms as well as attracting the right pollinator species.

The flower has an important role in pollinator attractiveness. The flower shape, such as flower size and organ morphology, and flower texture are suited to specific pollinator species (Whitney et al, 2007). Moreover, flower color and scent are important in attracting specific pollinator species. There are multiple rewards for the pollinator to increase visits to the flowers of angiosperms, such as pollen grains, nectar, and floral tissue (Torezan-Silingardi et al, 2021).

1.3.1 Nectar is a reward to pollinators

Above all other flower rewards, nectar is an important nutrient source for insects, birds, bats and mammals (Roy et al, 2017) as it is an organic liquid composed of sugars and other molecules, such as amino acids, proteins and ions (Pyke et al, 2023). Nectar production and secretion are significant in the reproductive success of angiosperms since nectar is an important attractant to pollinators. There are two types of nectar present in angiosperms (Figure 1A; Roy et al, 2017): floral nectar (FN) acts as a reward for pollinators, whilst extrafloral nectar (EFN) acts as part of an indirect defense against herbivores, because EFN is an energy source for predatory insects, like ants. Nectar is synthesized in and secreted from nectaries glands. EFN is produced in nectaries in vegetative tissue near the floral tissues, e.g., in the petiole or leaf blade. In contrast, FN is produced in nectaries located in various floral tissues, such as the sepals,

petals, stamen, or carpels. Most nectaries are differentiated from the surrounding tissue, resulting in structured nectaries as seen in Figure 1B. There are three cell types found in nectaries: vascular, parenchymal and epidermal. First, metabolites required for pre-nectar are supplied by vasculature tissue. Pre-nectar metabolites are then modified by the parenchymal cell layer, resulting in nectar. Finally, the nectar is secreted by epidermal tissue. The sugar concentrations in the nectars vary between species and are determined by the vasculature tissue type. When phloem is mostly present in vascular tissue, nectar can have a sugar content up to 80%. In contrast, when xylem is mostly present, the nectar sugar content can be much lower, around 8%. Overall, nectar contains mainly sugars, specifically sucrose, glucose, and fructose. The sucrose/hexose ratio is found to be crucial in the attraction of specific pollinators. Whereas hummingbirds prefer nectar that is high in sucrose, flies prefer nectar with a higher hexose content. Moreover, amino acids are found in all nectars and are important nitrogen sources for pollinators. Like sugar preference, pollinators have preferences for certain essential amino acids. Besides, nectarins (nectar proteins) in FN and EFN are found to attract pollinators or predatory insects but to reduce microbial growth

via the production of antimicrobial compounds, like hydrogen peroxide in the redox cycle (Park et al, 2009). However, despite the presence of nectarins and other antimicrobial compounds, certain microbes grow successfully in the nectar.

1.4 Microbes can interact with and impact different parts of the plant

Numerous studies have been performed to understand plant-microbe interactions as all plant organs harbor microbes (Berg et al, 2016). Microbes are found in the rhizosphere (soil close to the roots), phyllosphere (aerial surface of plants) and endosphere (within plant tissues; Turner et al, 2013). The microbial community includes bacteria that are beneficial or non-beneficial to the host plant. In the rhizosphere, beneficial bacterial strains can antagonize pathogenic bacteria or fungi. In contrast, phyllosphere bacteria *Pseudomonas syringae* can for example interfere with hormone signaling causing stomatal opening with the result of aiding pathogens in entering plant tissues. Besides, endophytic bacteria can be latent pathogens and cause disease when circumstances change. In short, microbes in the plant microbiome can have a wide range of effects on the plant host. Likewise, the nectar microbiome is not necessarily beneficial to the host plant as the nectar microbiome's effects on nectar and pollination attractiveness are not yet well understood.

This review summarizes and discusses findings on the nectar microbiome and its influence on pollinator attractiveness and plant fitness. First, the habitat and formation of the nectar microbiome will be explored. Next, the nectar microbiome effects on the flower rewards and pollinator attraction will be investigated. The review will end with a general discussion followed by a focus on the effects of elevated temperature (due to climate change) on the nectar microbiome.

Chapter 2: Nectar microbiome composition, habitat and formation

2.1 Nectar microbial species and traits

2.1.1 Nectar microbiome general composition

To explore the influence of nectar microbiomes on pollinator attractiveness, it is important to first describe the composition and member species (Table 1). Overall, nectar microbiomes are dominated by either bacterial or fungal species (De Vega et al, 2021; Vannette et al, 2020; Tucker et al, 2014). Bacteria belonging to phylum *Proteobacteria* are common nectar microbiome members since they were found in the nectar microbiomes of varying plant species (Alvarez-Perez et al, 2012; Lee et al 2019; Von Arx et al, 2019). For instance, *Proteobacteria* were frequently (77.4%) part of the nectar microbiome composition in 21 species of 13 families in KwaZulu-Natal province of South Africa (Alvarez-Perez et al, 2012), whereas other bacterial phyla were found less frequently (15,1% *Actinobacteria* and 7.5% *Firmicutes*). Likewise at another location, *Proteobacteria* belonging to genera *Pantoea/Erwinia* and *Rosenbergiella* were found in nectar microbiomes of *Datura wrightii* (Solanaceae) and *Agave palmeri* (Agavaceae) in the Sonoran Desert in Spain (Von Arx et al, 2019). The nectars of *D. wrightii* and *A. palmeri* were dominated by bacteria (>70%) compared to fungi. However, in many other species, the nectar microbiome consists mostly of fungi. Specifically, yeasts were common in nectars of varying plant species (De Vega et al, 2009; Herrera et al, 2008; Herrera et al, 2009), like in 40 species of 19 families from KwaZulu-Natal, where flowers contained about 43.2% yeast (De Vega et al, 2009). Besides, in three separate locations in Southeast Spain, yeast dominated the floral nectars of perennial herbs (*Helleborus foetidus*, *Aquilegia vulgaris* and *A. pyrenaica cazorlensis*; Herrera et al, 2008). Yeast was found in 90.0% of nectar microbiomes of *H. foetidus*, 60.0% of *A. vulgaris*, 62.9% of *A. p. cazorlensis*. In addition, yeast was found frequently (30-55%) in nectars of 130

species in Doñana and Cazorla in Spain and Yucatán in Mexico (Herrera et al, 2009). The diversity of yeast species between all locations (beta diversity) in Spain and Mexico was high, but the species diversity in each location (alpha diversity) was similar. In short, nectar microbiomes were bacteria- or fungi-dominated. Predominantly, *Proteobacteria* and yeasts species were found in nectar microbiomes of many angiosperm species.

2.1.2 Nectar bacteria have tolerance traits to nectar, but fungal traits are mostly unexplored

Since nectar contains antimicrobial compounds, such as hydrogen peroxide, and exerts osmotic pressure due to fairly high sugar concentrations, it is expected that nectar microbes possess traits to survive under these specific conditions. Functional traits give even more insight into the characteristics of nectar microbes (Table 1). Tolerance traits to different nectar components were well described for nectar bacteria, but for yeasts merely tolerance to glucose was tested. For instance, tolerance traits of 53 bacterial strains isolated from floral nectar of 21 plant species from KwaZulu-Natal in South Africa were characterized (Alvarez-Perez et al, 2012). Three traits were used in the characterization: catalase activity (tolerance to hydrogen peroxide), sugar tolerance (tolerance to sucrose) and microaerobiosis (tolerance to low oxygen). All 53 bacterial strains grew under microaerobiosis and showed tolerance to hydrogen peroxide. The sucrose concentrations tested for sugar tolerance were estimated from the sugar concentrations in floral nectars from the South African plant species and their nectars generally had a low sugar concentration (6-27%). Most bacterial strains tolerated such sucrose ranges (10-30%), but certain *Proteobacteria* (genera *Burkholderia*, *Methylobacterium* and families *Sphingomonadaceae*, *Xanthomonadaceae*) did not tolerate the highest concentrations. Thus, tolerance to hydrogen peroxide, sucrose and low oxygen levels were essential traits for these bacteria to survive in nectar. Furthermore, yeast species isolated from nectars of tropical plants in India were tested on osmotolerance (tolerance to glucose) and 14 from the 24 yeast species grew at 40% glucose concentration (Tiwari et al, 2022). Yeast *M. gruessi* isolated from *Digitalis obscura* and *Atropa baetica* even tolerated 50% glucose concentration (Pozo et al, 2012). Thus, nectar yeast species differed in osmotolerance, with only a few species tolerating the highest glucose concentrations. In short, nectar bacteria possess traits to endure microaerobic and antimicrobial compounds present in nectar. Sucrose was not completely tolerated by certain bacteria and glucose by yeasts, so the expected tolerance to osmotic pressure associated with sugar concentrations in nectar was not observed in all of tested nectar microbial species.

2.2 Nectar microbiome habitat

The habitat of the nectar microbiome gives insight into the requirements to spread and grow in the nectar. Nectar provides a different microhabitat compared to air and plant phyllosphere (Poza et al, 2012), since in comparison to the other plant tissues, the nectar and pollen had the lowest microbial abundance.

2.2.1 Nectar bacteria prefer organic nitrogen as nutrient source and yeast's sources are not extensively researched

Nectar provides microbes with various nutrient sources, such as sugars and amino acids (Table 1). Even though yeasts are found in many nectars, their nutrient source in nectar is not well described, contrary to nectar bacteria. For instance, the nutrient sources of bacterial strains of two *Acinetobacteri* and six *Rosenbergiella* species isolated from bees or flowers were tested by growing them in 12 types of artificial nectar with high or low sugar and organic nitrogen (i.e. amino acids) concentrations, and varying sugar compositions (sucrose and/or hexose; Morales-Poole et al, 2023). The bacteria were isolated from flowers in various locations (Europe, USA, South Africa, and Japan), giving a general overview on preferred nutrients of diverse nectar bacteria. All strains grew in artificial nectars with high nitrogen concentration. Also, the strains performed the best in nectars with low sugar content compared to high content and grew the most when hexose was present (either solely or in combination with sucrose). Thus, nectar with high nitrogen and low sugar content consisting of hexose is needed for optimal growth of *Acinetobacteri* and *Rosenbergiella* species. In addition, nectar microbes had pollen as a nutrient source, since nectar bacterium *Acinetobacter pollinis* and yeast *M. reukaufii* did not grow without pollen in the flowers (Christensen et al, 2021). The growth of nectar yeasts *Starmerella orientalis* and *Torulaspora delbueckii* increased in a pollen-dependent way, but the degree of growth was pollen species-specific. Thus, nectar yeasts use pollen as their nutrient sources, but growth in other sources is not yet tested. In short, nectar bacteria prefer organic nitrogen as their nutrient source and nectar microbes, especially yeasts, also consume pollen. Also, high sugar concentrations limited bacterial growth.

2.2.2 Nectar microbiome thrives in nectar of aged flowers

The flower type plays a role in whether bacteria or fungi are predominately present in the nectar, with both bacteria and fungi mostly found in flowers with exposed nectaries (Table 1; Vannette et al, 2020). Bacteria were found in flowers with exposed nectar, whereas fungi were found in flowers with long petals that (partially) enclose the nectar. So, nectar yeast and bacteria prefer a different habitat and could cause either one to be overrepresented in the nectar of specific flower types. Further on, as the flower

environment changes with flower age, the microbiome composition is also expected to change. The microbial density was the highest in older flowers. For example, nectar microbiomes in hermaphrodite flowers had the highest yeast density in latest stage, independent of the maturation order of male and female organs (Herrera et al, 2008; Schaeffer et al, 2014). Likewise, post anthesis (opening of the flower bud), the floral nectars of *A. palmeri* and *D. wrightii* contained the most microbes (Von Arx et al, 2019). On top of that, prior to anthesis, almost no microbes were seen in nectars of *A. palmeri*. Nectars of *D. wrightii* did contain microbes prior to anthesis, but thrips were found on the plant and could have increased the microbial density prior to anthesis. Generally, nectar microbes are almost not present in flowers before they open. In short, bacteria preferred exposed nectars in contrast to fungi and the microbial densities had a positive relation with flower age.

2.3 Animal pollinators: vectors for microbes that are then specifically selected in nectar

The flowers cannot provide the nectar microbiome with a continuous habitat, because their lifespan is relatively short compared to the rest of the plant (Pozo et al, 2012). In other words, dispersal is essential for the nectar microbiome to establish in a new habitat.

2.3.1 Animal pollinators are essential in microbial dispersal

To understand the formation of the nectar microbiome, it was explored how these microbes were dispersed to the flower (Table 1). The nectar microbiome dispersal depends on wind currents and biotic vectors (Morris et al, 2020). Biotic vectors were particularly important as without pollinator visits, nectars had a different microbial composition compared to visited nectars (Aizenberg-Gershtein et al, 2013; Morris et al, 2020). Visited nectars had an overall higher microbial abundance (Morris et al, 2020; Von Arx et al, 2019) and a higher yeasts abundance (Herrera et al, 2010a) compared to unvisited nectars. In addition, the nectar microbiome of visited flowers was composed of only a few bacterial and fungi genera (Morris et al, 2020; Alvarez-Perez et al, 2013). Because angiosperm species are pollinated by specific pollinator species, the main pollinator guild (pollinator groups) causes a different nectar microbiome formation. The nectar microbial communities differed with each pollinator guild in 48 angiosperm species from ten diverse ecological locations (De Vega et al, 2021). Plants pollinated by Coleoptera (beetles) had the most microbes in their nectar (De Vega et al, 2021) and bird-pollinated flowers had a high yeast density in nectar (De Vega et al, 2009). Not only are nectars of angiosperms visited by pollinators, but also by

parasitic organisms, such as nectar robbers (Morris et al, 2020). The visitor's identity impacted the microbial community as flowers visited by pollinators had a more diverse microbial community compared to flowers visited by robbers. Moreover, the pollinators had identical microbial species in their microbiota as the nectar microbiome of the plant species they pollinated (Aizenberg-Gershtein et al, 2013; Vannette et al, 2017; Herrera et al, 2010a; Anderson et al, 2013) and specific yeast species were found for each pollinator guild (De Vega et al, 2021). In short, the nectar microbes were dependent on animal pollinators guilds for dispersal to flowers and these species were found in the microbiota of the pollinators.

2.3.2 Specific microbes are selected by nectar environment

Even though the nectar microbiome formation is dependent on pollinator guild and identity, there was a notable species diversity in nectar microbiomes between plant species. Even when the same pollinator species pollinated two different plant species, like in *Citrus paradisi* and *Amygdalus communis*, the nectar microbiome differed between the two species (Aizenberg-Gershtein et al, 2013). However, angiosperms belonging to the same family had similar nectar bacteria species, independent of pollinator guild (De Vega et al, 2021), so the nectar microbiome formation and composition may be dependent on the plant phylogeny as well as pollinator guild. Overall, the species richness of the microbiome in the nectar was limited compared to other plant organs, but the nectars contained specific microbial species. This selection in microbial composition may be dependent on the plant's phylogeny, with flower shapes and nectar traits being critical factors.

Table 1. Overview of the nectar microbiome characteristics

Nectar microbes	Bacteria	Fungi
General composition	Mostly <i>Proteobacteria</i>	Mostly yeasts
Traits	Tolerance <ul style="list-style-type: none"> • Hydrogen peroxide tolerance • Microaerobiosis tolerance • Partial sucrose tolerance 	Tolerance <ul style="list-style-type: none"> • Partial glucose tolerance
Habitat	Preferred nutrient sources: <ul style="list-style-type: none"> • High nitrogen • Hexose • Pollen Exposed nectaries Exposed nectars Aged flowers	Preferred nutrient sources: <ul style="list-style-type: none"> • Pollen Exposed nectaries Unexposed nectars Aged flowers
Means of dispersal	Animal pollinators	Animal pollinators <ul style="list-style-type: none"> • Birds

Chapter 3: Nectar microbiome consequences to pollinator attraction and fitness

3.1 Nectar microbiome impacts nectar composition and temperature

The impact of the nectar microbiome on nectar composition was explored to understand whether the microbiome changed its environment or not. Nectars have specific properties, like sugar composition, pH, and antimicrobial compounds that can be impacted by the presence of microbes as results of their metabolism activities (Table 2).

3.1.1 Nectar microbes decrease sucrose and glucose but increase fructose concentrations

Sugars in nectar are a nutrient source to the pollinators but can be a nutrient source for the nectar microbes as well. As previously described, the nectar bacteria *Rosenbergiella* and *Acinetobacter* used mainly organic nitrogen for growth, but other nectar bacteria could have sugars as their main resource as nectar microbes were found to decrease total sugar concentrations in nectar (Table 2; De Vega et al, 2009; Herrera et al, 2008; Vannette et al, 2013). Besides, sugars can increase osmolarity in the nectar and can be a limiting factor in growth, so decreasing the sugar concentrations can be beneficial. Indeed, in the floral nectar of angiosperms, there was a negative relation between yeast density and nectar total sugar concentration (De Vega et al, 2009; Herrera et al, 2008). Both bacteria *Gluconobacter* sp. and fungi *M. reukaufii* reduced the sugar concentration in sterilized nectar from *Mimumulus aurantiacus* (Vannette et al, 2013), but the reduction by *Gluconobacter* (35%) was around two times stronger compared to *M. reukaufii* (17%). Sugar compounds were differently influenced by nectar bacteria and yeasts but overall, an increase in fructose was observed. Nectar yeast increased fructose concentrations and decreased sucrose with higher cell densities (Herrera et al, 2008). *Gluconobacter* decreased the glucose concentration (64%) in *M. aurantiacus* nectar and increased fructose by 42% while *M. reukaufii* barely decreased fructose concentration (Vannette et al, 2013). The increase in fructose was also seen in artificial nectar with bacterium *A. platycodi*, where *A. platycodi* decreased sucrose but increased glucose and fructose (Lenaerts et al, 2017). Thus, nectar microbes can increase the fructose concentration whilst reducing the total sugar concentration. The increase in fructose could be explained by the conversion of glucose and sucrose to fructose via glycolysis and hydrolysis, respectively. Most likely, bacteria used glucose and sucrose as nutrient source and yeast sucrose resulting in lowered sugar concentrations with higher microbial densities. Also, lowered sugar concentrations cause less osmotic stress for the microbes.

3.1.2 Nectar microbes impact nectar amino acids composition

Nectar bacteria changed the amino acids concentrations in nectars (Table 2). The nectar of *M. aurantiacus* inoculated with *Gluconobacter* spp. had increased amino acid concentrations, but when inoculated with *M. reukaufii* the concentration did not change (Vannette et al, 2018). In contrast, nectar bacteria *R. nectarea* and *A. platycodi* decreased total amino acids in artificial nectar (Lenaerts et al, 2017). The observed increase in amino acids content by *Gluconobacter* spp. could be the plants response to the bacterial presence (Fabro et al, 2004) and overall nectar bacteria used amino acids (organic nitrogen) as their nutrient source.

3.1.3 Nectar microbes lower pH in nectars

Because of microbial metabolism, the pH was also influenced by the nectar microbes and most decreased the pH in nectars (Table 2). The nectar of *M. aurantiacus* became more acidic in the presence of yeast and bacteria: *Gluconobacter* decreased pH by 5 units compared to a decrease of 2 units by *M. reukaufii* (Vannette et al, 2013). Other nectar bacteria (*A. platycodi*, *L. garvieae*, *R. nectarea*, *A. astilbes*, *E. tasmaniensis* and *L. kunkeii*) decreased the pH as well in artificial nectars (Lenaerts et al, 2017; Good et al, 2014), whereas yeast *M. reukaufii* only slightly decreased pH (Good et al, 2014). Overall, mostly nectar bacteria lowered the nectars' pH.

3.1.4 Nectar microbiome impact on sugars, pH and amino acids results in less pollinator consumption

Aside from altered nectar compositions, regarding altered sugar, amino acids and pH, pollinator, by nectar microbes, changed pollinator attraction and consumption was seen (Table 2). However, the direct links between changed compound abundance and pollinator attraction were not researched. Most nectar bacteria decreased pollinator attractiveness. For instance, bacterium *Gluconobacter* sp. that reduced sugars and pH in nectars decreased nectar removal by various pollinators in synthetic flowers by 27% (Vannette et al, 2013), independent of nectar origin. Also, fake sunflowers with artificial nectars with bacterial species that decreased pH (*A. astilbes*, *E. tasmaniensis* and *L. kunkeii*) were consumed less (3-31%) by *A. mellifera* (Good et al, 2014), while *A. mellifera* consumed nectars with yeast *M. reukaufii* (that changed pH the least) the same as control (Good et al, 2014). The decreased consumption of nectars with *A. astilbes* was related to nectar chemistry because bees still removed less nectar post filtering, but other nectar aspects, like volatiles, could be altered as well. In contrast, pollinator *A. ervi* did not consume synthetic nectar differently when either nectar bacteria *A. platycodi*, *L. garvieae* or *R. nectarea* were present, even though these bacteria reduced pH, sugars and amino acids in nectars (Lenaerts et al, 2017).

The total nectar volume of *M. aurantiacus* decreased with *Gluconobacter spp.* in nectars and flowers with depleted nectar were mostly wilted and brown (Vannette et al, 2018). Since the plant seemed to respond to *Gluconobacter spp.* presence, the flower wilting may be part of their defense. Thus, the change in composition and loss of nectar volume provoked by bacteria had a negative impact on flowers and could decrease pollinator attractiveness and fitness by not being as rewarding to pollinators. In short, the changed nectar compositions, especially by nectar bacteria, had a general negative effect on pollinator attractiveness and nectar consumption.

3.1.5 Microbes are susceptible to nectar secondary metabolites, but change their composition

Plants produce and secrete secondary metabolites in their flowers as part of their defense. Most secondary compounds have an antimicrobial function and are a limiting factor to nectar microbial growth. Overall, microbes (bacteria and yeast) from floral nectar and hummingbird's beak did not reach optimal growth densities in presence of five secondary metabolites at concentrations similar to concentrations found in floral nectar (Vannette et al, 2016). Nicotine, caffeine, catalpol and aucubin decreased cell densities of most nectar microbes, but nectar bacterium *Gluconobacter spp.* and yeast *M. reukaufii* maintained unchanged cell densities under catalpol. In addition, nicotine had a positive effect on bacterium *Erwinia sp.* cell densities. So overall, the tested secondary metabolites had limiting effect on growth of nectar bacteria and yeast species. The secondary compound concentrations were negatively affected by the nectar microbes (Table 2), possibly to make their environment more suitable for growth. Bacteria *Erwinia sp.* and *Gluconobacter spp.* decreased nicotine concentrations (Vannette et al, 2016) and the concentrations of ouabain were decreased by yeasts *S. bombicola* and *C. rancensis*. Also, aucubin was reduced in nectars by bacteria *A. astilbes*, *Gluconobacter spp.* and yeast *M. reukaufii* with *Gluconobacter spp.*, decreasing its concentrations by more than 50%. Both *Gluconobacter spp.* and *M. reukaufii* decreased the hydrogen peroxide concentration in the floral nectar of *M. aurantiacus* by 80% (Vannette et al, 2013). However, nectar yeast *M. reukaufii* and bacteria (*Asaia astilbes*, *Erwinia tasmaniensis*, *Lactobaccillus kunkeei*) in artificial nectars did not influence hydrogen peroxide concentrations (Good et al, 2014). The altered secondary compounds did not affect pollinator attractiveness. In short, the antimicrobial compounds of nectar are reduced by most nectar bacteria and yeasts. The reduction could increase the susceptibility of the nectar to more microbial species and possibly lead to more successful pathogenic invasions which decreases the fitness of the flower.

3.1.6 Bacteria impact nectar temperature

Other aspects of nectar such as temperature are also modified by nectar microbes (Table 2). The nectary temperatures of *H. foetidus* with *M. reukaufii* yeasts were higher (Herrera et al, 2010b), independent of location (Las Navillas and Puerto Llano). Moreover, higher *M. reukaufii* cell densities lead to even more nectary temperature increases. Floral warming can act as a reward for pollinators and can increase overall reproductive success.

3.2 Volatiles are changed by the presence of yeast

Volatile Organic Compounds (VOCs) are released by angiosperms to attract pollinators, but nectar microbes can emit volatiles as well. The nectar microbes changed the volatile profiles emitted by nectars, but the effect differed for nectar bacterial and fungal species (Rering et al, 2018). The VOC composition of nectars with only fungi consisted mostly of esters and ketones and in bacterial VOCs, volatile 2,5-dimethylfuran was always present. Overall, certain nectar bacteria provoked a positive reaction to nectars in pollinators (Table 2). Nectar bacteria of different phyla, (*Firmicutes T. saccharophilus* and *S. epidermidis*, *Actinobacteria Curtobacterium sp.* and *Proteobacteria Pantoea*) had a positive effect on pollinator attraction, since female pollinator *Trissolcus basalis* always preferred VOCs of artificial nectars with bacteria over sterile nectar (Cusumano et al, 2023). However, VOCs of nine other *Firmicutes*, *Actinobacteria* and *Proteobacteria* species had no effect on the females. Also, the VOC composition of artificial nectar with *T. Saccharophilus* was similar to control, so pollinator attraction was not caused by volatiles. Mainly nectar yeast specialist species impacted VOC compositions and attracted pollinators. Nectar yeast specialist *M. gruessi* and *M. reukaufii* and generalist *A. pullulans* changed the VOC composition of synthetic nectar (Sobhy et al, 2018) and, especially *M. reukaufii*, attracted pollinator *A. ervi*. Two other generalist yeast species did not change VOC compositions and generalist *S. roseus* provoked a negative response to the nectar. So, the VOC composition of certain bacteria species and nectar yeast specialists is beneficial in pollinator attraction. Pollinators, like bees, can detect volatiles with their antennae and the antennal response to specific VOCs is used as a measure of attractiveness. Honeybees *Apis mellifera* detected 14 of the 20 microbial VOCs with their antennae. The volatile compound alcohol 2-butanol produced by yeast *M. reukaufii* triggered the most antennal responses in *A. mellifera* and the honeybees were able to detect species-specific microbial VOCs, like 2-nonanone produced by fungus *A. pullulans*. The behavior (acceptance of nectar) of honeybee *A. mellifera* was influenced by VOCs emissions as confirmed by a choice experiment. 70% of artificial nectars with control

or *M. reukaufii* VOCs were accepted, so *M. reukaufii* did not influence pollinator attraction. The acceptance rate of nectars with bacterium *A. astilbes* was 48% and with fungus *A. pullulans* was 36%, so these microbes decreased pollinator attraction. *Gluconobacter* species VOCs had the lowest acceptance (17%) and made nectars the least attractive. Yet, natural nectar contained more VOCs than what was found in the artificial nectars, so the effects may differ under natural circumstances. In addition, under natural circumstances, nectar bacteria and fungi are both present in the nectar and will impact the nectar compositions together. The VOCs composition of a co-culture with yeast *M. reukaufii* and bacterium *A. astilbes* were dominated by VOCs from *M. reukaufii* (Rering et al, 2020) and the same volatile compounds were in *M. reukaufii*'s monocultures. However, *A. mellifera* preferred nectars with solely *A. astilbes* over nectars with *M. reukaufii*, so the domination of *M. reukaufii* VOCs in nectar is negative to *A. mellifera* attraction. In contrast, bumblebee *B. impatiens* consumed more (50%) nectar when *M. reukaufii* VOCs were present (Schaeffer et al, 2019). In short, mainly yeast changed VOC compositions and sometimes increased pollinator attraction with their volatile emissions.

3.3 Pollen germination and bursting is stimulated by nectar bacteria, but consequences are negative in fertilization

Pollen is used by nectar microbes as a nutrient source as it is high in proteins. However, pollen germination and pollen tube forming are important steps in fertilization and when impacted can lead to reduced fertilization and seed production (Table 2). Certain nectar bacterial species increase pollen germination and bursting to use pollen as nutrient source. Indeed, nectar bacteria *A. boissieri* and *A. pollinis*, and honeybee gut bacterium *A. apis* increased *Eschscholzia californica* pollen germination and bursting, but nectar yeast *M. reukaufii* and nectar bacterium *A. nectaris* did not (Christensen et al, 2021). Nectar bacteria *A. pollinis* increased pollen burst and germination in artificial nectar, in a density-related manner. Also, twice as many proteins were released from germinable pollen in presence of *A. pollinis* compared to uninoculated. The presence of germinable pollen also led to higher densities of *A. pollinis*. However, even though pollen bursting and germination were increased by *A. pollinis*, the pollen tubes were shorter after germination. So, nectar bacteria *A. pollinis* stimulated pollen bursting and germination while benefiting from the pollen proteins as nutrient source, and the increased protein release could have caused the shorter pollen tubes. Thus, the presence of nectar bacteria may lead to increased pollen germination, but this is not positive for successful fertilization.

3.4 Stigma closure is negatively changed by bacteria

Since nectaries are found in multiple parts of the flower, the nectar microbes could also affect the reproductive organs. For example, stigma plays an essential role in the reception of pollen and the behavior of the organ can be negatively impacted by nectar bacteria (Table 2). For instance, shrub *M. aurantiacus* closes their stigmas after pollinator visitation (Vannette et al, 2013). When the amount of received pollen is sufficient for fertilization, the stigmas stay closed. Otherwise, the stigma opens. Without pollinator visitations, the stigma closure was decreased by 28-60%. When flowers were inoculated with bacteria *Gluconobacter*, 23% less stigma closed compared to uninoculated and the presence of *Gluconobacter* decreased seed production by 18%. In contrast, yeast *M. reukaufii* had no effect on stigma closure and seed set. Thus, only bacteria had a negative effect on stigma closure and seed set and decreased plant fitness.

3.5 Nectar microbes generally did not impact pollinators' fitness

Besides the influence on the flowers, the nectar microbiome impacts pollinator fitness as well (Table 2). The fitness of the pollinator species is important for the angiosperms' reproduction, because with smaller pollinator population less pollination can take place. Not all nectar microbes affected insect fitness. Whereas nectar bacteria *A. platycodi* and *L. garvieae* affected the longevity of adult *A. ervi*, bacterium *R. nectarea* did not (Lenaerts et al, 2017). Further on, the observed effect was species-specific: *L. garvieae* increased insect longevity, while *A. platycodi* reduced longevity. Three generalist yeast species also decreased the longevity of *A. ervi*, whereas nectar yeast specialists did not influence insect longevity (Sobhy et al, 2018). Specialist *M. reukaufii* presence did not affect *B. impatiens*' egg laying, number, and weight of eggs/larvae (Schaeffer et al, 2017). In short, mainly generalist yeasts decrease pollinator fitness and nectar yeast specialists had no effect on pollinator fitness. In nectar bacteria, the impacts were species-specific, and most bacteria affected insect fitness positively. Overall, nectar microbes did not decrease significantly insect fitness and pollinators population sizes were not changed.

Table 2. Overview of nectar microbiome effects on pollinator attractiveness and fitness

(--) decrease; (+) increase; (0) no impact; (x): no data

Nectar aspects	Nectar microbe	Impact on aspect	Impact on pollinator attractiveness	Impact on angiosperm fitness
Sugars, amino acids, pH	Bacteria	-- / +	0/--	--
	Fungi	-- / +	+	0
Secondary compounds	Bacteria	--	0	--
	Fungi	--	0	--
Nectar temperature	Bacteria	x	x	x
	Fungi	+	+	+
Volatiles	Bacteria	+	+/0/--	+/0
	Fungi	+	+/--	+/0/--
Other flower/pollinator aspects				
Pollen germination and bursting	Bacteria	+		--
	Fungi	0		0
Stigma closure	Bacteria	--		--
	Fungi	0		0
Pollinator fitness	Bacteria	+/0/--		+/0/--
	Fungi	0/--		0/--

Chapter 4: Discussion

4.1 Nectar fungal traits are mostly unexplored

Nectar is a harsh environment to microbes mostly due to the presence of antimicrobial compounds and high osmotic pressure (Roy et al, 2017; Park et al, 2009). Tolerance traits in nectar bacteria are characterized and shown to have tolerance to pH, hydrogen peroxide and partially to sugars. However, nectar fungi are not well characterized for these traits and only osmotolerance to glucose was tested (Tiwari et al, 2022; Pozo et al, 2012). To understand how fungi survive in the nectar and especially why yeasts are common, similar experiments as described by Alvarez-Perez et al (2012) can be performed with isolated nectar yeasts *M. reukaufii*, *M. gruessii* and fungi *A. pullulans* for example. In the experiments, the yeast species could be tested on catalase activity (by growing in solutions with 3% hydrogen peroxide), sugar tolerance (by growing under various sucrose concentrations) and microaerobiosis (by growing in depleted oxygen). With these experiments, the tolerance traits of nectar fungi and yeasts will be characterized.

4.2 Nutrient sources of nectar yeast and bacteria need to be confirmed

Nutrient sources are important factors in habitats, but the nutrient sources for nectar fungi are not well described. The decrease in sugar concentrations in nectar and increase in nectar fungi abundance implies that these fungi may use sugar as a nutrient source (De Vega et al, 2009; Herrera et al, 2008). By testing, as is done by Morales-Poole et al (2023) for nectar bacteria, fungal species growth response to artificial nectars with different nitrogen and sugar levels, their most favorable nutrient could be confirmed, and nectar fungi can be characterized better. Morales-Poole et al (2023) only performed their experiments on two genera of nectar bacteria. Therefore, it is possible that sugars are a nutrient source to other nectar bacteria, because nectars with bacteria still decreased sucrose and glucose concentrations and increased fructose (Vannette et al, 2013; Lenaerts et al, 2017), indicating that bacteria indeed convert sugars. Bacteria possibly used the sugars in glycolysis or hydrolysis as nutrient source, but the decrease in sugars lowered the osmotic pressure in the nectar as well. It remains to be investigated whether nectar bacteria use sugars as a nutrient source, to lower osmotic pressure, or both as it would help to better understand how these bacteria survive in the nectar.

4.3 Responses from plants to the nectar microbes are unknown

As previously described, nectar microbes decreased pH and antimicrobial compounds, such as hydrogen peroxide (Vannette et al, 2016; Vannette et al, 2013; Good et al, 2014). By reducing the antimicrobial compounds, microbes are able to optimize their environment for growth. Nectar production is costly to angiosperms and the decreases in antimicrobial compounds can increase the plant's susceptibility to pathogens. In addition, nectar microbes modified other aspects of the nectar, like sugar, amino acid, and pH. Since there is a negative relation between nectar composition and microbial densities, it is likely that high microbial densities were possible in aged flowers due to altered nectar. Studies are missing on the pre-nectar metabolism to understand whether the plant perceives and responds to the changed nectar compositions. Indeed, it is possible that the plant compensates for decreased compound concentrations which is even more costly to the plant. RNA-seq on nectary tissues in flowers with sterile nectar or nectar with microbes can give insights into the plant response to nectar microbes in general. In addition, nectar microbial densities increased over time and thrived more in old than young flowers (Herrera et al, 2008; Schaeffer et al, 2014; Von Arx et al, 2019). Comparing the expression data at different flower ages (pre-/post-anthesis) will give insight on responses of the plant to different microbial densities and nectar composition change as well as the costs for the plant to produce nectars.

4.4 Nectar microbes can negatively and positively impact pollinator attractiveness

Nectar microbes indeed often altered pollinator attractiveness of nectars, but the effect was not always positive or negative. The nectar composition was changed by nectar microbes, specifically the sugar compositions and volatiles, resulting in changed pollinator attraction. However, the changed attraction was species specific and dependent on the microbial species as well as the pollinator species. Since pollinators have preferences for sugars and amino acids, the nectar microbes could have influenced pollinator attractiveness by changing their overall concentrations (Herrera et al, 2008; Lenaerts et al, 2017; Vannette et al, 2013). However, other nectar components, such as pH, secondary compounds concentrations and temperature, were also changed by the nectar microbes (Vannette et al, 2018; Lenaerts et al, 2017; Good et al, 2014; Vannette et al, 2013; Vannette et al, 2016; Herrera et al, 2010b). The overall response to altered nectars was less consumption by pollinators, but direct links to the components causing reduced pollinator attractiveness were not studied in detail. To test the link between

changed nectar compositions and pollinator attractiveness, the nectar consummation experiment by Sobhy et al (2018) can be repeated in fake sunflowers with filtered floral nectars obtained directly or a few days post anthesis. If the changed nectar compositions are confirmed to impact pollinator consumption, then each aspect, like amino acids concentrations and pH, could be modified separately to determine its role in pollinator attraction.

In addition, nectar yeast impacted volatiles the most and nectar yeast specialist had positive relation to pollinator attraction in contrary to nectar bacteria volatiles. However, the acceptance of nectars differed per pollinator species, as *A. mellifera* reacted negatively to *M. reukaufii* volatiles (Rering et al, 2020), whilst *B. impatiens* reacted positively (Schaeffer et al, 2019). Since the microbial effect on volatile profile is tested in artificial nectars without flowers, the combined effect of volatiles emitted by flower tissues and microbes on pollinator attraction is not studied.

Nectar microbes impact pollen germination and stigmas in flowers as well. The overall impacts by nectar bacteria were negative to fertilization. The mechanisms on how the microbes impact fertilization processes in flowers are yet unexplored. Most likely the metabolites produced by bacteria are perceived by the pollen and stigma counteracting the signals given by the plant or pollen. Not only did nectar bacteria impact the flower fitness, but the pollinator fitness was also sometimes impacted. Since different pollinators guilds visit the flowers, the nectar is a fitting place for pathogens exchanges. As the effects of microbes on pollinators were species-specific, the combined effect of the microbiome could impact pollinator fitness differently. Since nectar microbes are dependent on pollinators for dispersal, the effect on pollinator attraction is expected to be neutral or positive.

The main shortcoming in current studies is that the different impacts of the nectar microbiome on pollinator attractiveness and fitness are tested one species at a time. The nectar microbiome contains multiple bacterial and fungal species, even though it has a relatively low species richness. Specific species seem to be selected by the nectar environment, but their effect on the nectar and pollinator behavior is not the same for every species. In natural circumstances, the microbes also interact with each other, which for example influences the nutrient availability (due to cross-feeding or competition; Seth et al, 2014; Goyal et al, 2018) and the abiotic factors (due to changing pH or antimicrobial compounds; Herschend et al, 2018; Vannette et al, 2016). The nectar composition and volatiles can therefore differ because of the communal metabolism. Since a variety of nectar microbial species are isolated by labs, their combined effect can be explored. Investigating the impact of a synthetic community of microbes, and not just single

species, on pollinator attraction, would greatly help to understand what occurs in natural and agricultural conditions.

4.5 Future remarks

There is an urgency to understand the role of the nectar microbiome on pollinator attraction, since there is no consensus on its impacts on angiosperm fitness and could be critical to their reproduction. Currently, global climate change is leading to rising temperatures which can affect the nectar microbiome compositions and metabolisms (Wahid et al, 2020).

Indeed, while temperature rise is not always affecting the nectar microbial community, like in *Brassica rapa* (Russell et al, 2022a) and *Penstemon heterophyllus* (Russell et al, 2022b), the warming can impact pollinator attraction as bumble bee *B. impatiens* significantly consumed more nectar with microbes at current temperatures than warmed flowers (Russell et al, 2022a). So far, no positive effects of the temperature rise on pollinator attractiveness have been reported. Since in our current climate the effects of nectar microbiome are not well understood, a rise in temperature can therefore lead to unexpected outcomes regarding nectar microbiome impacts on pollinator attraction and this unpredictability is a threat to ecosystems.

Angiosperms are important in ecosystems since they provide various habitats and resources to other organisms. To understand and grasp the consequences of global warming on the ecosystems, predicting the future consequences on nectar microbiomes and associated consequences to angiosperms' fitness is necessary. A first step is to expand our global knowledge on the influence of the nectar microbiome on pollinators and angiosperms, and ultimately, knowledge could be applied to protect our ecosystems and global climate change effects may be limited.

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