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Nectar antimicrobial compounds and their potential effects on pollinators

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Nectar is a sugary, aqueous solution that plants offer as a reward to animal mutualists for visitation. Since nectars are so nutrient-rich, they often harbor significant microbial communities, which can be pathogenic, benign, or even sometimes beneficial to plant fitness. Through recent advances, it is now clear that these microbes alter nectar chemistry, which in turn influences mutualist behavior (e.g. pollinator visitation). To counteract unwanted microbial growth, nectars often contain antimicrobial compounds, especially in the form of proteins, specialized (secondary) metabolites, and metals. This review covers our current understanding of nectar antimicrobials, as well as their interplay with both microbes and insect visitors.

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Introduction

Plants make two types of nectar. Floral nectar (FN) is the major nutritional reward for pollinators, whereas extrafloral nectar (EFN) attracts pugnacious insects, like ants and wasps, for indirect defense against herbivores [1]. Both FN and EFN are secreted by specialized glands called nectaries, with their location on the plant (flowers versus vegetative tissues) being the main discriminator between nectary type. Please note, throughout this manuscript we refer to FN and EFN within the context of nectar only, not the specialized gland known as nectaries. Beyond sugars, nectars often contain biologically relevant concentrations of amino acids, lipids, specialized (secondary) metabolites, metals, and proteins (reviewed in Ref. [1]). Some of these compounds have demonstrable impacts on pollinator visitation [1]; however, since nectar is a nutrient-rich resource, it is perhaps unsurprising that it can also be an outstanding medium for microbial growth. Nectar microbes can range from being pathogenic to the plant to having a potentially benign impact on the nectar chemistry. For instance, *Erwinia* spp. are the causal agents of fire blight and can infect the plant vasculature through growth in nectar followed by invasion of the nectary in a variety of plant species (e.g. apple and pear) [2–5]. In addition to *Erwinia* spp., there are a variety of other pathogens that can infect plants through the nectary, including documented cases in cotton, bean, squash, apple, pear, aucuba, banana, pineapple, hawthorn and gourds [5–8].

In addition to pathogenic microbes, nectars often harbor nectarivorous yeasts [9–11] and bacteria at relatively high densities [12,13]. Nectar microbial communities are generally species-poor, containing either yeast or bacteria [14] and are often vectored by visiting insects [15,16]. It may be that in some, or even most, cases that nectarivorous microbes are neutral and have little to no impact on nectar, but nectar-adapted microorganisms can potentially alter nectar chemistry in ways that influence pollinator preference. Both nectar yeasts and bacteria can influence nectar pH [17,18], volatiles [19••,20••], temperature [21], sugar content [22–24,25•], amino acid content [25•,26–28,29•] and nectar volume [25•]. Some nectar microbiota may even help prevent the growth of microbes that may be deleterious to the plant [30]. There is also very interesting research beginning to look at how nectar microbes, both bacteria and yeasts, interact with one another and how that may influence their overall effects on pollinator attraction [reviewed in Ref. [31]]. Exactly how these alterations in nectar chemistry impact pollinator visitation and plant fecundity is a growing area of research. Here we provide an overview of how nectar composition might impact microbial communities in such ways that influence pollinator visitation.

It should be noted that not all floral visitors are pollinators, and that nectars may contain toxic or distasteful chemicals to deter 'nectar robbers' [62•]. Nectar robbers are classified as floral visitors that consume nectar but provide no pollen transfer or pollination services to the plant. This review will deal exclusively with pollinators in an effort to maintain focus, but we readily acknowledge the importance of nectar chemistry and its impact on the ecology of floral visitors outside of pollinators. As such, the focus of this review is on compounds in nectar that have demonstrated antimicrobial activity and how this activity, by

Table 1

Partial list of nectar antimicrobials and their impacts on nectar quality

Antimicrobial component of nectar	Nectar type	Species	Evidence for antimicrobial activity	Ref.
Nectarin I protein	Floral	Tobacco	NEC1 is a manganese superoxide dismutase that produces high amounts of hydrogen peroxide in floral nectar, which is antimicrobial in nature	[49,50]
Nectarin IV protein	Floral	Tobacco	Purified NEC4 protein displayed xyloendoglucanase inhibitory activity when incubated with xyloglucan-specific endoglucanase (XEG) from *Aspergillus aculeatus*. XEGs are hemicellulose degrading enzymes utilized by many pathogenic fungi to colonize plant tissues	[48]
Nectarin V protein	Floral	Tobacco	Berberine bridge enzyme (BBE)-like protein that displays glucose oxidase activity, generating hydrogen peroxide which then contributes to the nectar's antimicrobial nature	[51]
Pathogenesis-related (PR) proteins	Extrafloral	*Acacia cornigera, A. hindsii, A. collinsii*	Nectar inhibited the growth of *Phytophthora parasitica, Fusarium oxysporum, Verticillium dahliae* and *Alternaria alternata*. In-gel assays revealed glucanase and chitinase activity of various proteins from the nectar	[55,60]
BrLTP2.1	Floral	*Brassica rapa*	Non-specific lipid transfer protein (nsLTP) with direct antimicrobial activity against certain plant pathogens such as *Alternaria solani*	[61•]
GDSL lipase	Floral	*Jacaranda mimosifolia*	Raw nectar and the protein JNP1 display lipase/esterase activity. JNP1 is hypothesized to produce free fatty acids that act as antimicrobials	[59]
Acidic chitinase III protein	Floral	Apple	Machi3-1 protein inhibits growth and biofilm formation of *Erwinia amylovora*	[58]
Floral nectar (component unknown)	Floral	*Cucurbita pepo*	Nectar inhibited the growth of *Escherichia coli* and *Erwinia tracheiphila* when compared to glucose controls. Increased transmission of *E. tracheiphila* via flowers when nectar was artificially removed	[7]
Anabasine	Floral	Tobacco	The alkaloid anabasine in the nectar of *Nicotiana* spp., reduced pathogen load and transmission of the parasitic *Crithidia bombi* in bumblebees	[69,71]
Gelsemine	Floral	*Gelsemium sempervirens*	Gelsemine consumption reduced pathogen load of *Crithidia bombi* in bumblebees	[70]
Callunene	Floral	*Culluna vulgaris*	Callunene consumption causes *Crithidia bombi* inhibition by inducing flagellum loss	[83••]
Methylglyoxal/dihydroxyacetone	Floral	*Leptospermum* sp.	High levels of methylglyoxal in honey derived from dihydroxyacetone in *Leptospermum* sp. nectar shows strong positive relationship with non-peroxide antibacterial activity (against *Staphylococcus aureus* ATCC 25923)	[81,82]

influencing the growth of microorganisms, may impact nectar chemistry in such a way that attracts or deter pollinators.

Antimicrobial compounds in nectar

Nectar has been shown to limit the growth of certain microbes [32,33], with some of the known antimicrobial compounds being summarized in Table 1. Here we discuss these different nectar compounds, the mechanisms through which they limit microbial growth, and potential impacts on pollinator visitation.

Sugars

Sugars are the primary nectar solutes. Sugar concentration and composition (primarily sucrose, glucose and fructose) are important factors in mutualist attraction [34]. Some rarer sugars, such as arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose, and stachyose have been found in FN and, interestingly, can be toxic to some pollinators [35–37], but are not known to have direct antimicrobial properties. However, nectars can contain up to ~70% (w/w) sugar. This high osmolarity in itself could clearly limit microbial growth and serve as a filter as to what kinds of microorganisms can inhabit nectar, as has been seen in honey [38], but this hypothesis does not appear to have been tested *in vivo* and warrants further investigation. For instance, the nectars from flowers visited by insects are usually more concentrated than ones pollinated by vertebrates [39–43], but it is unclear if vertebrate-pollinated flowers generally contain more microbes than insect-pollinated ones. For a more detailed examination of how sugar-rich environments might influence microbiological dynamics, Lievens *et al.* is an excellent review [44].

Proteins

Nectars consistently contain arrays of a few major proteins, often referred to as nectarins, along with a larger number of minor ones. The specific proteins vary from species to species, but nectar proteins are usually involved in plant defense and constitutively secreted (uninduced by any biotic or abiotic stress). For instance, a recent report identified 144 proteins in the nectars of 23 *Nicotiana* species, with the majority having known or predicted roles in defense or stress responses [45•]. Some of these proteins included lipid transfer proteins, 'nectar redox cycle' proteins, and protease inhibitors [45•], which are detailed further below.

Proteins secreted into nectar may either directly or indirectly influence the growth of nectar-inhabiting microorganisms. Perhaps the most well characterized example of indirect inhibition, meaning the protein does not directly bind or interact with the inhibited microorganism, of microbial growth is the nectar redox cycle in the FN of ornamental tobacco (*Nicotiana* spp.). The nectar of ornamental tobacco produces high levels of hydrogen peroxide (accumulating up to ~4 mM) that can inhibit microbial growth [46,47]. There are five nectarins (NEC1-NEC5) that accumulate to concentrations of ~250 μg/mL [47,48]. NEC1, a superoxide dismutase [49,50], and NEC5, a glucose oxidase [51], both contribute to generating hydrogen peroxide in nectar, which has direct activity against known plant pathogens. We suggest hydrogen peroxide in nectar is most likely a defense mechanism against a broad spectrum of microbes, both bacterial and fungal. Interestingly, *Nicotiana alata* plants silenced for a trypsin proteinase inhibitor had higher levels of both nectarins and hydrogen peroxide, but were less visited by pollinators [52]. These results suggest that hydrogen peroxide homeostasis is important to maintain a balance between nectar defense and its palatability to pollinators.

Other nectarins may interact directly with microorganisms to reduce their growth. Pathogenesis-related (PR) proteins have been found in both EFN and FN [1,53,54]. An interesting study examined the proteins and antimicrobial activity in the EFN of myrmecophytes (plants that associate with mutualistic ants and constitutively secrete EFN) against non-myrmecophytes (these plants secrete EFN upon herbivore damage in order to attract ants as a defense mechanism) [55]. Since myrmecophyte EFN is constitutively produced, and may remain on the plant for long periods, it was hypothesized that this type of nectar would require enhanced protection from exploitation by microbes relative to non-myrmecophytes. Perhaps unsurprisingly, the study found that myrmecophyte nectar had more protein content and antimicrobial activity because their long-exposed nectar is potentially more vulnerable to microbial infestation. The myrmecophyte-nectar contained chitinases and glucanases that were effective at inhibiting the growth of yeasts [55,56]. Chitinases have also been found in FN [57,58]. Specifically, a recent report found that an apple cultivar with enhanced resistance to *Erwinia amylovora*, the causal agent of fireblight, contained a nectar chitinase that was absent in a susceptible cultivar [58]. It is worth noting that the source of chitinases (and other antimicrobials) in the prior examples were of plant origin. While yet to be demonstrated, it is possible that some nectar antimicrobials might be produced by other nectar-inhabiting microbes.

Another example that illustrates potentially conserved antimicrobial mechanisms between EFN and FN is the presence of GDSL-lipases. The lipid-rich nectar of the tropical tree *Jacaranda mimosifolia* contains a GDSL-lipase, which appeared to limit microbial growth [59]. Interestingly, the EFN of *Populus trichocarpa* (poplar) displays antimicrobial activity and also contains GDSL-lipases and other PR proteins [60]. Of course, this is a small sample size and warrants more studies that examine the protein content of both FN and EFN to see if there are conserved mechanisms by which these nectars limit microbial growth.

Non-specific lipid transfer proteins (nsLTPs) are members of a large protein family that has been found in a variety of nectars from various species [24,35,36], unpublished data]. A common feature of nsLTPs is that they generally have antimicrobial activity, particularly against fungi [62]. A recent study identified the major protein secreted into the nectar of *Brassica rapa* as a nsLTP (BrLTP2.1) [61•]. BrLTP2.1 is extremely heat stable, has a binding affinity for free fatty acids found in nectar, and is a potent antifungal agent against a series of plant pathogens [61•]. The mode of action for BrLTP2.1 as an antimicrobial agent is still unknown.

Preliminary evidence suggests that these nectar nsLTPs are effective at inhibiting the growth of pathogenic filamentous fungi but are not very active against some common nectarivorous yeasts and bacteria. These results suggest that, if the primary function of these nectar nsLTPs is to limit microbial growth, their activity might be selective. While the exact mechanisms of how nsLTPs inhibit fungal growth is still unknown, there is some data to suggest that they directly target the membrane integrity of certain fungi [63]. If the selectivity of nectar nsLTPs is confirmed, its activity may be interpreted to have evolved to target microbes that could be deleterious to nectar chemistry, plant health, and reproduction while enabling proliferation of microbial communities that are beneficial.

Taken together, the current body of literature suggests that plants secrete antimicrobial peptides into nectar in order to prevent it from being exploited by potentially deleterious microbial communities. These unwanted microorganisms could be directly pathogenic to the plant, as is the case with *Erwinia* spp., or they could simply alter nectar in such a way that is not as appealing to pollinators.

Interestingly, to date, no studies have evaluated the impacts of plant-derived nectar proteins on the growth of commonly occurring nectarivorous microbes.

Specialized (secondary) metabolites

Nectar contains a variety of specialized (secondary) metabolites that may also provide antimicrobial functions. Specialized metabolites have been suggested to have an assortment of functions in nectar (see reviews Refs. [1,64••]) and may also help mitigate the spread of pathogens among pollinators. There is an established body of literature that has demonstrated that specialized metabolites may add a level of toxicity or bitterness to nectar as a way to mediate floral visitors outside of its potential ability to limit microbial growth [65–67]. For example, the variance of nicotine concentrations in the flowers of *Nicotiana attenunata* lead to increased outcrossing [68]. This suggests that specialized metabolites by themselves, irrespective of their potential antimicrobial activity, can attract or deter pollinator visitation.

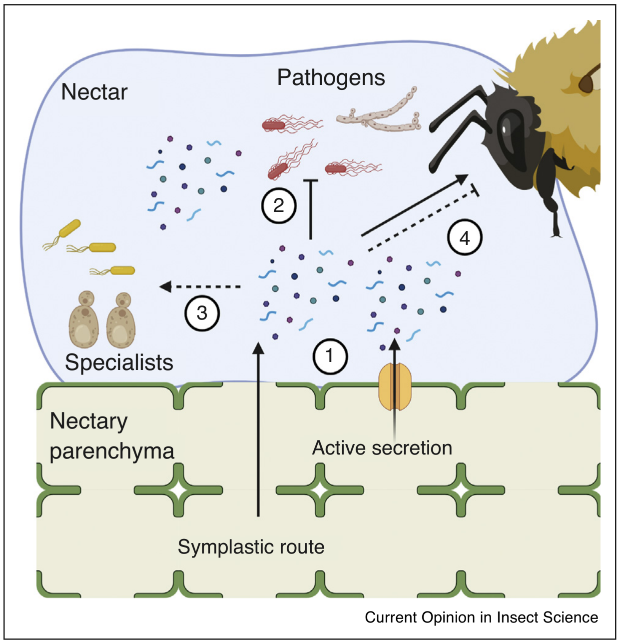
Recent studies suggest that pollinators consume nectar specialized metabolites as a way to help reduce pathogen load and pathogen spread among other members of pollinator colonies [69–73]. For example, the alkaloid anabasine in the nectar of *Nicotiana* spp., reduced pathogen load and transmission of the parasitic, unicellular eukaryote *Crithidia bombi* in *Bombus terrestris* [69,71]. Parasitized *Bombus* spp. are also more likely to visit flowers of *Chelone glabra* that contain higher concentrations of the iridoid glycosides aucubin and catalpol [74••]. In turn, this higher visitation leads to an increase in pollen donation to conspecific stigmas from those *C. glabra* with nectar containing higher concentrations of iridoid glycosides [74••]. It should be noted that the consumption of nectar containing gelsemine has also been shown to reduce the pathogen load of the microbial parasite *C. bombi* in some bee pollinators [70]. Although speculative, these results suggest that the variation in antimicrobial specialized metabolites in nectar may be a driver in the evolution of some plant-pollinator interactions.

Whether specialized metabolites reduce microbial growth in nectar is an area that requires further investigation. Interestingly, several alkaloids found in nectars — caffeine, nicotine and anabasine — were tested against nectar bacterial isolates, but there were no detectable impacts even at concentrations beyond those found naturally in nectar [12]. These experiments were conducted *in vitro*, so it could be possible that synergistic effects found among nectars' full suite of chemical compounds went undetected in this study. Yet, in another report, the composition of nectar bacterial species greatly changed in a nicotine-deficient *Nicotiana attenuata* mutant [75]. Nicotine in itself can inhibit microbial growth in a dose-dependent manner with detectable impacts as low as 1 μ g/mL for some microbial species [76]. Nicotine in the nectar *of N. attenuata* has an average concentration of 27.6 μg/mL [75] while *Nicotiana glauca*, used as an outlier in the study due to its low levels of nicotine in its nectar, has an average concentration of nectar nicotine which is 55 times lower. Surprisingly, the bacterial communities from *N. glauca* nectar were more comparable to those cultured from wild-type *N. attenuata* plants than in the nicotine-deficient mutant nectar. This suggests that nicotine, even at low concentrations, may influence bacterial assemblages in nectar. *N. glauca* also contains higher concentrations of anabasine (5.4 μg/mL on average [77]), which also has antimicrobial activity [78,79], suggesting that anabasine may have similar effects as nicotine at higher concentrations in nectar. An additional interesting finding was that a novel species of yeast, *Candida gelsemii*, was isolated from Carolina Jessamine (*Gelsemium sempervirens*), whose nectar is considered to be relatively poisonous as it contains the toxic alkaloid gelsemine [80]. These results suggest that some nectar specialized metabolites may be a factor in filtering the type of microbes that inhabit nectar, but more research is needed to clarify this role. There also needs to be a push to better understand the potential synergistic effects that specialized metabolites may have on the growth of nectar microorganisms in conjunction with other nectar solutes.

Another example of the antimicrobial role of nectar metabolites comes from studies with medical grade mānuka honey. Mānuka honey is unique for its known strong antimicrobial properties [81]. This therapeutic honey is derived from the nectar of *Leptospermum scoparium* (manuka) plants that are found in New Zealand and Australia. The causal agent of this antimicrobial activity has been purported to be methylglyoxal, which is produced from dihydroxyacetone [82]. Recent reports suggest that there is a positive correlation between methylglyoxal levels and the honey's antimicrobial activity [82].

There are still many avenues to be studied on the impacts of specialized metabolites on microorganisms that inhabit nectar, as well as animal mutualists. For example, very little is known about mechanisms of how specialized metabolites may reduce microbial growth, either *in planta* or in pollinators. An interesting report recently showed that callunene from the nectar of *Calluna vulgaris* can inhibit *C. bombi* growth through the removal of its flagellum, which is required for anchoring in its host bees [83••]. Another avenue worth pursuing might be to elucidate how domestication of crops that are reliant on pollinators may have their nectar chemistry altered in such a way that can be deleterious to the survival of their pollinators. For example, domesticated blueberries have a significant reduction in caffeic acid esters. These caffeic esters were found in higher concentrations in non-domesticated plants and may be important for mitigating pathogen load in visiting pollinators [84••]. Furthermore, while there has been some exploration of antimicrobial

Figure 1



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The secretion of antimicrobials into nectars and their influence on biotic interactions: (1) Arrival of actively transported or passively secreted antimicrobial molecules in nectar drops (2) inhibit harmful plant/insect pathogens, while (3) either stimulating or not affecting nectar specialist growth. (4) Microbial-derived metabolites can stimulate or negatively impact pollinator feeding preference. Created with BioRender.com.

peptides in EFN, at this time there appear to be very few studies on specialized metabolites in EFN [85]. An interesting avenue of study would be to explore what types of specialized metabolites are found in EFN and what influence they might have on its biological function. Lastly, it is important to understand how some microbial species interact with nectar specialized metabolites. Vannette and Fukami found that nectar microbes can reduce the levels of the nectar specialized metabolites aucubin, catalpol, caffeine, nicotine and ouabain, and that none of these compounds could reduce microbial growth enough to prevent changes to nectar sugars [29•]. It might be reasonable to suggest that nectar-adapted microbes have evolved the ability to metabolize antimicrobial specialized metabolites in nectar.

Metals

Metals in nectar could also be important for influencing and shaping both the nectar microbiome and pollinator attraction. Plants obtain metals from the soil. Numerous studies have shown that soil supplemented metals do translocate to floral organs and rewards such as nectar [86–88]. This has implications in reproductive success too since metals accumulated in floral rewards sometimes negatively affect foraging behavior of pollinators [87,89] and could be toxic to them [90]. We speculate that the presence of a very specific inorganic ion composition in nectar of various plants would dictate a specific microbiome. The nectars of metal hyperaccumulating plants are of special interest. Hyperaccumulation of metals has been suggested to be a defense mechanism against both herbivores and pathogens [91]. A field survey of selenium hyperaccumulators revealed lesser arthropod load in these plants [92]. Another study reported nickel hyperaccumulation in floral nectar and fewer flower visitations by pollinators when plants were grown on nickel-supplemented soils [89]. Nickel toxicity in microorganisms is not completely understood [93] and it would be important to study how higher nickel levels influence the microbiome of nectar in nickel hyperaccumulating plants.

Conclusions and future directions

Nectar chemistry is complex, with multiple layers of biotic interactions. The primary function of floral nectar is to attract and manipulate pollinators to maximize plant fecundity. Non-mutualistic visitors are known to exploit the sweet carbohydrate-rich resource that is nectar. Consequently, nectar must maintain some toxicity to inhibit the growth of some microorganisms that could be deleterious to plant reproductive success while still limiting its distastefulness or toxicity to pollinators.

In Figure 1 we illustrate a proposed model by which antimicrobial compounds function in nectar. Nectar antimicrobial compounds are secreted actively or via a passive symplastic route, although the mechanisms by which these solutes are secreted into the nectar is still relatively unknown and should be an intense focus of research going forward. These antimicrobial compounds then limit microbial growth, which left unchecked, could be deleterious to the plant or mutualist insects. The current literature suggests pathogenesis-related proteins, such as chitinases and lipid transfer proteins, may be the primary mechanism by which plants limit growth of deleterious microorganisms, particularly fungi, in their nectar while still harboring microbial communities that may be beneficial or neutral. The mechanisms by which these proteins act upon specific microorganisms is still an active area of research. Additionally, many specialized metabolites in nectars have antimicrobial activity (either *in vivo* or *in vitro*), although their experimentally demonstrated effects appears to be more of a resource for limiting pathogen growth and transmission among pollinators. Metals are also another potential source of toxicity in nectar, but how these metals directly impact pollinator preference and microorganisms growth is yet another area of research that warrants attention. Interestingly, a recent report showed that some nectar metals and hydrogen peroxide may be harmful to pollinators by disrupting bumblebee microbiomes [94].

In conclusion, a concerted effort is needed to understand the diversity and mode of action of antimicrobial nectar components, as well as their downstream impacts on microbial communities and pollinator visitation. Studies at the intersection of plant, microbial, and pollinator ecology, coupled with genomic and molecular approaches, will lead to key advances in this field, but will likely require strong collaborative and interdisciplinary efforts.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

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Floral and nectar volatiles are known to be important for attracting floral visitors. This study examined the volatile profiles of several microbial inhabitants of floral nectar and their potential effect on the generalist pollinator *Apis mellifera*. Interestingly, yeast nectar specialist *Metschnikowia reukaufii* emitted the most attractive volatile profile.

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Some pollinators use chemosensory cues to identify and evaluate nutritional resources. This report made some key findings that bumblebees (*Bombus impatiens*) have the capacity to respond to some microbial volatile compounds and, perhaps surprisingly, were more attracted to nectar solutions inoculated with the bacterium *Asaia astilbes* over *M. reukaufii*. Although, bumblebees consumed significantly more nectar that had been inoculated with *M. reukaufii*. This study highlights the potential effects that microbial metabolites may have on pollinator foraging and nectar consumption.

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