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Microbial signatures of vertebrate visitation in floral nectar: a case study with two endemic Aotearoa New Zealand plant species

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ABSTRACT

Microbes found in nectar and dispersed by animal visitors can mediate pollination and host fitness. While previous studies have characterised floral nectar microbiomes and their effects on invertebrate pollinators, fewer studies have focused on vertebrate pollinators, especially outside of the Northern Hemisphere. In Aotearoa New Zealand, vertebrates, such as birds and bats, are critical pollinators for many native plant species. Here, we present nectar microbiome profiles for two endemic, vertebrate-pollinated plant species in Aotearoa New Zealand, wharariki, mountain flax (*Phormium cookianum*), and pua o te Rēinga, wood rose (*Dactylanthus taylorii*). We used vertebrate exclusion treatments and camera traps to monitor visitation. Camera trap footage revealed silvereye (*Zosterops lateralis*), ship rat (*Rattus rattus*), and possum (*Trichosurus vulpecula)* visitation. We detected shifts in microbial species turnover in wood rose and varying relative abundances of fungal and bacterial taxa across the vertebrate exclusion treatments for both mountain flax and wood rose. However, we did not detect strong effects on floral nectar microbiome community composition or richness. Future work should move beyond profiling the microbial communities and identify fitness consequences and pollination outcomes, which could affect conservation and management decisions.

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Introduction

Animals provide pollination services crucial for sustaining biodiversity, ecosystem functioning, and human wellbeing (Potts et al. 2016). However, this interaction between plants and animals does not occur in isolation, as microbes are ubiquitous. Increasing interest in plant-animal-microbe interactions has led to numerous studies characterising floral nectar microbiomes and their effects on plants and animals. Studies have identified

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diverse communities of bacteria, fungi, and viruses from floral nectar (Morris et al. 2020; reviewed in Vannette 2020), and identified animals as important vectors of microbes to flowers (Ushio et al. 2015; Vannette and Fukami 2017; Zemenick et al. 2018, 2021; Russell and Ashman 2019; Morris et al. 2020; de Vega et al. 2021). Some of these microbes have been shown to impact plant-pollinator interactions by changing nectar composition, with consequences on animal foraging behaviour and plant and animal fitness (Herrera et al. 2013; Vannette et al. 2013; Good et al. 2014; Junker et al. 2014; Schaeffer and Irwin 2014; Schaeffer et al. 2017, 2019; Russell and Ashman 2019; Pozo et al. 2020, 2021). However, most experimental work remains focused on invertebrate pollinators, especially bumblebees (reviewed in Cullen et al. 2021) despite the importance of vertebrate pollinators, such as mammals, reptiles, and birds for certain plant species (reviewed in Ratto et al. 2018).

Characterisation of the microbes hosted by vertebrate animals and the plants they visit are limited to a few floral and faunal species and remain understudied, especially outside of the Northern Hemisphere (Vannette et al. 2013; Belisle et al. 2014; Lee et al. 2019; de Vega et al. 2021; Donald et al. 2022). In Aotearoa New Zealand, vertebrate pollination is critical for native flora – including iconic species such as flax (*Phormium* spp.), kowhai (*Sophora* spp.), pōhutukawa (*Metrosideros excelsa*), and pua o te Rēinga wood rose (*Dactylanthus taylorii)* (Ecroyd 1996; Anderson 2003; Pattemore and Wilcove 2012; Bylsma et al. 2014). Despite this, to our knowledge, only one study has characterised microbial communities found in nectar and vectored by nectar-feeding birds in Aotearoa New Zealand (Donald et al. 2022). Thus, we emphasise the need to first characterise these nectar microbial communities and then determine the role of microbes in mediating these plant-pollinator interactions and their effects on plant and pollinator fitness. Here, we contribute to the understanding of Aotearoa New Zealand nectar microbiomes by identifying nectar bacteria and fungi found in two vertebrate-pollinated, endemic plant species: wharariki, mountain flax (*Phormium cookianum*) and pua o te Rēinga wood rose (*Dactylanthus taylorii)*, and document vertebrate visitation to these plants. We also compare these data with the published literature, pose key research questions, and call for the integration of microbial data in plant-pollinator studies and conservation efforts.

Natural history and data collection: Both mountain flax and wood rose are endemic to Aotearoa New Zealand, rely on vertebrate pollination for reproduction, and produce a substantial amount of nectar (supplemental data S1; Ecroyd 1996; Donald and Dhami 2022). Mountain flax is widespread throughout the country and is visited by various bird species (Gibb 2000; Webber et al. 2012). In contrast, wood rose is parasitic on a number of plant hosts and is geographically range restricted. Both wood rose and its primary pollinator, the short-tailed bat (*Mystacina tuberculata*), are considered Nationally Vulnerable (de Lange et al. 2018; O'Donnell 2021).

Both plant species are also visited by introduced mammals and insects. Mountain flax flowers have been observed to be visited by rats (*Rattus rattus*) and introduced honeybees (*Apis mellifera*) (Donald and Dhami 2022). Similarly, wood rose is known to be visited by rats (*Rattus rattus* and *R. norvegicus*), mice (*Mus musculus*), possums (*Trichosurus vulpecula*), invasive wasps (*Vespula* spp.), and other insects (Ecroyd 1996).

We present nectar microbiome profiles for mountain flax and wood rose. Samples were collected from flowers that were either open to all visitors or covered to exclude vertebrate pollinators. The characterisation and comparison of microbial taxa from these two treatment groups combined with observed vertebrate visitation permits identification of microbial taxa likely dispersing on vertebrate visitors.

Nectar collection and microbial characterisation: We sampled nectar from mountain flax on 21–24 January 2019 and wood rose on 11–12 March 2020. Site characteristics for mountain flax are provided in Donald and Dhami (2022). To investigate the influence of vertebrate visitors on nectar microbiomes, we covered some mountain flax inflorescences with fine mesh bags and left others uncovered. Unopened flowers in both treatments were tagged and nectar was sampled following bloom. For wood rose, we collected nectar from eight flowers under long-term vertebrate exclusion cages (pers. comm. David Mudge and Thomas Emmett, Department of Conservation). These flowers were at varying stages of anthesis and included both male and female flowers. Following the nectar collection, the cages were removed to permit vertebrate visitation. We returned the next day to collect nectar from eight flowers in the same patch and from an additional 48 uncaged flowers in the surrounding areas (5 m to ca. 80 m from the focal patch).

For all sampling, we used sterile pipette tips to extract the nectar from individual flowers and followed the protocol for DNA extraction, PCR, and bioinformatic processing described in Donald et al. (2022). Sequences were filtered for contaminants, samples with fewer than 200 and 1000 reads for fungi and bacteria respectively were excluded, and OTU counts were transformed to relative abundance to visualise the proportion of each fungal and bacterial taxa within the overall community.

Vertebrate visitation characterisation: We used camera traps to monitor vertebrate visitation to mountain flax and wood rose. In both cases, we used Reconyx XR6 cameras programmed to be motion activated and able to record both diurnal and nocturnal visitation. Following initial nectar collection from wood rose, the cages were removed, and the two cameras were installed. Visitors to wood rose flowers were monitored by camera for a 24-hr period with motion-activated photos captured. There was a 30-second delay between consecutive trigger events. Additional details for mountain flax monitoring are provided in Donald and Dhami (2022). In both cases, all photos and videos were manually reviewed.

Statistical analysis: First, we visually assessed fungal and bacterial community composition based on relative abundance for wood rose and mountain flax. Then to determine differences between the covered and uncovered samples, we compared community composition, species richness and species turnover for each plant species.

For wood rose, we used PERMANOVA in adonis from the vegan R package (Oksanen et al. 2019) to assess differences in community composition with the Bray–Curtis dissimilarity index. We also used linear models with a Gaussian distribution and either species richness (Shannon Index calculated within Phyloseq; McMurdie and Holmes 2013) or species turnover (distance to centroid calculated with beta disper within vegan; Oksanen et al. 2019) as the response variable and covered/uncovered as the predictor variable.

For mountain flax, we also used PERMANOVA in adonis with the Bray–Curtis dissimilarity index to assess differences in community composition. Because multiple flowers were sampled from individual mountain flax inflorescences, we incorporated inflorescence ID as a blocking variable within PERMANOVA. We used linear mixed effect models with a Gaussian distribution and either species richness (Shannon Index calculated within Phyloseq; McMurdie and Holmes 2013) or species turnover (distance to centroid calculated with beta disper within vegan; Oksanen et al. 2019) as the response variable and covered/uncovered as the predictor variable. These models were fit within the lme4 R-package (Bates et al. 2015), and included inflorescence ID as a random effect. We used Phyloseq (McMurdie and Holmes 2013) to work with the microbiome data. All analyses were done in R v.3.6.3 (R Core Team 2020).

Results

In comparing the microbial composition of covered and uncovered nectar microbiomes, we identified several taxa that had differing relative abundances corresponding with vertebrate pollinator exclusion (Figures 1 and 2). Wood rose nectar from open flowers hosted greater relative abundance of bacterial genera *Rahnella* and *Novosphingobium*, while covered flowers hosted *Leuconostoc, Candidatus Carsonella, Acinetobacter*, and *Stenotrophomonas*. These general patterns held when considering the additional samples as well (Figures S1 and S2). Within vertebrate-excluded mountain flax nectar, bacterial genera included *Arthrobacter, Pseudomonas,* and fungal genera *Metschnikowia, Pleospora,* and *Sporidiobolus*. Nectar from open mountain flax nectar samples had higher relative abundance of bacterial *Mesoacidotoga* and fungal *Botryotinia, Davidiella,* and *Mycosphaerella* genera.

For wood rose community composition for both bacterial and fungal taxa, we did not find a significant difference between treatment groups (Figure 3, bacterial: df = 1, R2 = 0.06, F = 0.60, $p = 0.91$; fungal: df = 1, R2 = 0.24102, F = 3.1755, $p = 0.103$). We also did not find a significant difference for species richness between treatment groups (bacterial: f = 1, F = 3.74, $p = 0.09$; fungal: df = 1, F = 0.14, $p = 0.72$) for wood rose. However, in accordance with our findings of varying relative abundance of taxa within wood rose nectar, we found statistically significant differences in species turnover between the uncovered and covered wood rose nectars (bacterial: df = 1, F = 14.12, $p = 0.001$;

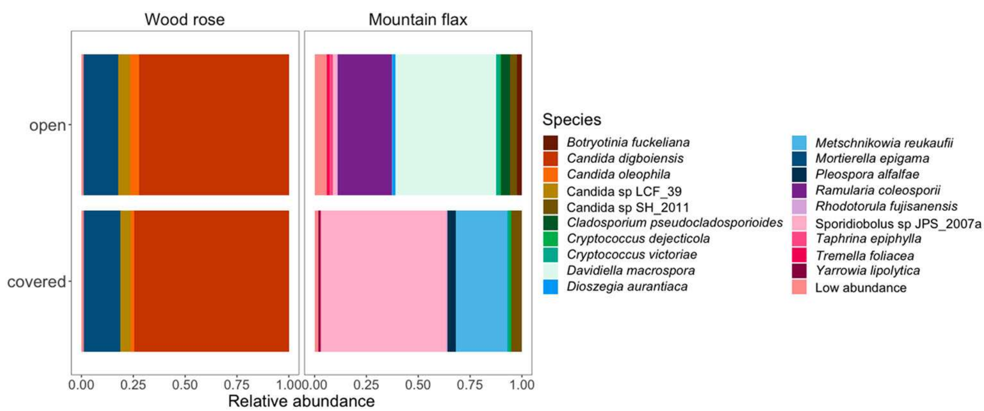

Figure 1. Relative abundance of fungal taxa that were greater than 1% of the total abundance found in open and vertebrate excluded wood rose (*Dactylanthus taylorii)* and mountain flax (*Phormium cookianum)*.

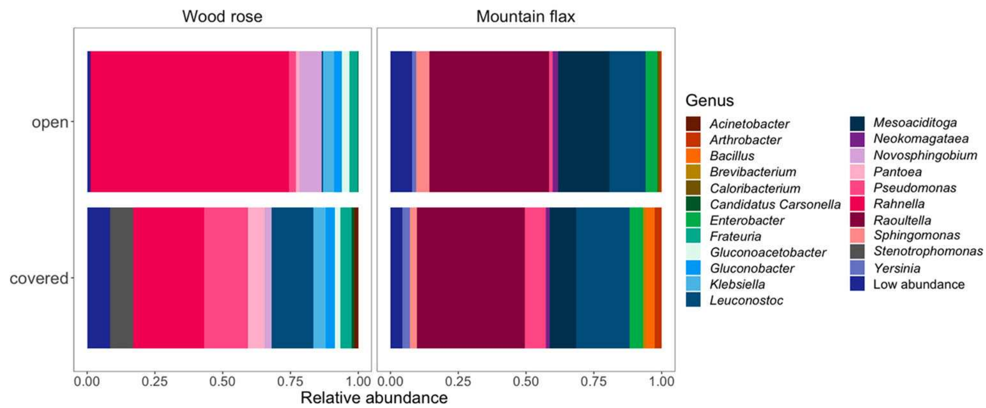

Figure 2. Relative abundance of bacterial taxa that were greater than 1% of the total abundance found in open and vertebrate excluded wood rose (*Dactylanthus taylorii)*, and mountain flax (*Phormium cookianum*).

fungal: df = 1, F = 4.79, $p = 0.03$). These differences were domain specific, as uncovered flowers had lower species turnover for bacterial taxa but higher turnover for fungal taxa. However,

Despite observing varying relative abundance for bacterial and fungal taxa across the treatments in mountain flax nectar, we did not detect statistically significant differences

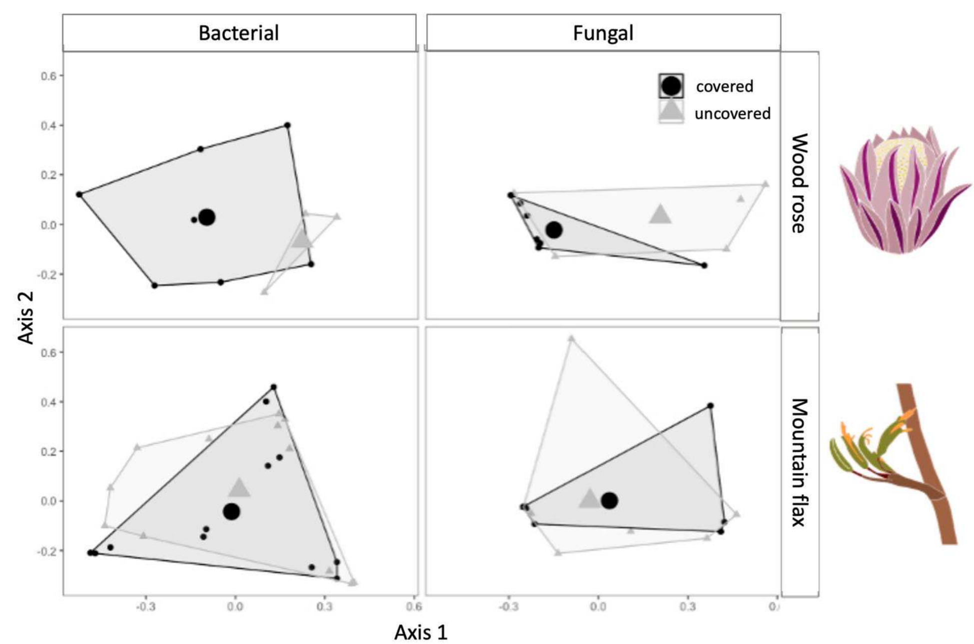

Figure 3. PCoA ordination of bacterial and fungal nectar microbial communities from wood rose (*Dactylanthus taylorii)* (top panels) and mountain flax (*Phormium cookianum*) (bottom panels). Nectar microbial communities from vertebrate excluded flowers are in black circles and those from open flowers are in grey triangles. The large points represent the centroids of each group.

for the three community metrics considered. Specifically, we did not find significant differences between the treatment groups for community composition (bacterial: df = 1, R2 = 0.03, F = 0.47, p = 0.75; fungal: df = 1, R2 = 0.05, F = 0.70, p = 0.91). Richness was also not significantly different between treatment groups (bacterial richness: df = 1, Chisq = 0.56, p = 0.45; fungal richness: df = 1, Chisq = 0.64, p = 0.42), and neither was turnover (bacterial: df = 1, F = 1.66, p = 0.21; fungal: df = 1, F = 1.19, p = 0.30, p = 0.21).

From the camera trap footage (Figure 4), we identified vertebrate visitation from possum (*Trichosurus vulpecula*) to wood rose. This visit was identified from multiple sequential photos. Additionally, a blackbird (*Turdus merula*) triggered the camera but did not appear to visit any of the wood rose flowers in the captured photos. Ship rat (*Rattus rattus*) and silvereye (*Zosterops lateralis*) visitation to mountain flax were previously identified by Donald and Dhami (2022).

Discussion and future directions

In this study, we characterised the floral nectar microbiome of two endemic plant species (wharariki, mountain flax and pua o te Rēinga, wood rose) identified visitation by a native bird and invasive mammals, and assessed the impact of vertebrate exclusion on nectar microbiomes. We found strong similarities between the nectar microbiomes of vertebrate excluded and open flowers, suggesting that the vertebrate visitation did not significantly influence nectar microbiome composition. Our results contribute to the limited knowledge on nectar microbiomes in Aotearoa New Zealand and highlight the need for more research on the role of microbes in plant-pollinator interactions. This is important as understanding plant-pollinator-microbe interactions may prove crucial for the conservation and management of vulnerable plant and animal species in Aotearoa New Zealand and globally.

Figure 4. Visitation by possum (*Trichosurus vulpecula*) to wood rose (*Dactylanthus taylorii)* (A and B) and ship rat (*Rattus rattus*) to mountain flax (*Phormium cookianum*) (C and D). Bottom panel images are modified from Donald and Dhami (2022).

Nectar microbes as indicators of vertebrate visitation: Recent studies have shown that vertebrate visitors can vector specific microbial taxa to the floral nectar microbiome (Vannette and Fukami 2017; Morris et al. 2020; de Vega et al. 2021), and other work from Aotearoa New Zealand has shown that silvereyes and tūī, key avian pollinators, share 74%–84% of bacterial taxa with nectar sources (Donald et al. 2022). In our study, we found that certain microbial taxa were differentially abundant across our vertebrate exclusion treatments and that species turnover varied for fungal and bacterial communities in wood rose nectar. However, the overall floral microbial community structure and taxonomic richness were not significantly influenced by the vertebrate exclusion treatments. Our observations of vertebrate animals visiting the flowers and consuming nectar were limited to silvereyes and rat visitation to mountain flax (Donald and Dhami 2022) and possum visitation to wood rose. The lack of clear community-level effects may also be due to our small sample sizes and limited observation periods. Additionally, due to the sampling design for wood rose it is likely that some flowers were resampled following the cage removal and possum visitation. Therefore, it is not unexpected that the community profiles between vertebrate exclusion and open flowers are similar. Despite observing possum visitation to the flowers and effort to sample these specific flowers, vertebrate vectored microbes may not be able to successfully establish within these nectar communities due to priority effects (Fukami 2015; Toju et al. 2017). While vertebrate exclusion studies can be useful, future work should concurrently sample microbes from nectar and animal visitors and extend sampling periods both temporally and spatially. Coupling microbiome profiles from floral nectar and their pollinators with data confirming visitation is essential for determining the role of vertebrates in dispersing nectar microbes.

Shared and exclusive nectar microbes: Wood rose and mountain flax nectars shared multiple fungal and bacterial taxa with nectar from other Aotearoa New Zealand plant species. Compared with nectar microbiome data from *Phormium tenax*, *Callistemon* sp., *Hymenosporum flavum,* and *Metrosideros excelsa* (Donald et al. 2022), we detected the following shared genera: *Cryptococcus*, *Davidiella*, *Rhodotorula*, *Taphrina*, *Tremella*, *Caloribacterium*, *Mesoaciditoga*, *Neokomagataea*, *Pantoea*, *Pseudomonas*, *Raoultella*, *Sphingomonas*, *Stenotrophomonas*, and *Yersinia*. This taxonomic overlap from nectar collected over 700 km apart suggests a generality and potentially a set of microbial genera associated with Aotearoa New Zealand nectar microbiomes and raises questions about the roles stochastic and deterministic processes in structuring the nectar microbiome. Future work, determining the importance of the environment, host, and animal visitors will help resolve the drivers of nectar microbial community composition. *Metschnikowia* was detected from mountain flax nectar but not in nectar from wood rose nor was it abundant in nectar from the other plant species in Aotearoa New Zealand (Donald et al. 2022). *Acinetobacter* is similar in this regard, as we detected it in low abundance in wood rose but not from mountain flax nectar. Yet, these two genera, *Acinetobacter* and *Metschnikowia*, are commonly described nectar inhabitants from the Northern Hemisphere (Belisle et al. 2012; Fridman et al. 2012; Álvarez-Pérez et al. 2012; Jacquemyn et al. 2013; Álvarez-Pérez and Herrera 2013; Vannette and Fukami 2017).

Nectar microbes and plant-pollinator conservation: Microbes in nectar can affect plants, pollinators, and plant-pollinator interactions (reviewed in Vannette 2020) and, therefore, may play an underappreciated role in conservation outcomes. While our study did not quantify the magnitude or direction of the relationship with microbes, we did detect putative pathogens as well as microbes known to beneficial. For instance, *Yersinia* and *Pantoea* are genera known to infect animals and plants (Niskanen et al. 2003; Völksch et al. 2009). *Bacillus,* and *Klebsiella* have been identified as promoting plant growth and defence against pathogens in plants (Radhakrishnan et al. 2017; Liu et al. 2018; Andrić et al. 2023), while *Metschnikowia* and other yeast species have been shown to promote bumblebee colony growth and reduce gut pathogens in bumblebees (Pozo et al. 2020). Further, several genera that we detected (*Rhodotorula*, *Yarrowia*, *Candida*, and *Metschnikowia)* have the potential for fermentation and previous studies have identified tolerance for alcoholic nectar across many species of bats (Orbach et al. 2010), and frequent and preferential consumption of alcoholic nectar by species of tree shrews, aye-ayes, and slow lorises (Wiens et al. 2008; Gochman et al. 2016). In Aotearoa New Zealand, wood pigeons kererū (*Hemiphaga novaeseelandiae*) and tūī (*Prosthemadera novaeseelandiae*) have been anecdotally reported to consume fermented berries and nectar (Hare 2008; Mills and Bayer 2013). While nectar microbes and their by-products may have key impacts on animal health (reviewed in Martin et al. 2022), this remains understudied in Aotearoa New Zealand and elsewhere.

Conclusion

Vertebrate pollination is essential for several key plant species in Aotearoa New Zealand, and from studies worldwide, it is evident that microbes can play important roles in animal preferences, fitness and pollination success. Recent work from Aotearoa New Zealand has characterised microbiome profiles for taxa found in nectar and on avian visitors (Donald et al. 2022). We have expanded on this by providing nectar microbiome profiles for two vertebrate-pollinated, endemic plant species and document vertebrate visitation. We recommend that future research not only characterise the microbial profiles found in nectar and on animal visitors but also experimentally determine the effects of these microbes on plant and animal fitness. This could be done by experimentally inoculating flowers with specific microbial taxa and assessing the effects on animal visitation, pollination success, and animal health outcomes. The incorporation of these microbial players into vertebrate fitness consequences and pollination outcomes could have implications for conservation and management and advance microbial ecology and zoology.

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