Exploring the mānuka phyllosphere microbiome

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Thesis Abstract

The surface of plant leaves, termed *phyllosphere*, is a ubiquitous microbial habitat that harbours diverse communities of microorganisms. Although a growing body of experimental evidence demonstrates that these microorganisms can have an influential role in host physiology, the ecological processes that drive the assembly of natural phyllosphere communities remain poorly understood.

Leptospermum scoparium (mānuka) is an indigenous New Zealand tea tree widely known for the non-peroxide antibacterial properties of its honey. However, the host physiological traits associated with these properties exhibit variation that remain unexplained despite decades of research. Considering a preliminary study that identified spatially persistent host association in the mānuka phyllosphere microbiome with patterns congruent with those of a microbial community under strong host selection, the primary objective of this research was to generate a holistic understanding of the ecological processes underpinning community assembly in the natural mānuka phyllosphere.

Since the host specificity of the mānuka phyllosphere microbiome was unquantified, this PhD thesis research began with a multi-species, spatially hierarchical survey of a native forest to understand the relative influence of host species identity versus distance on the phyllosphere microbiome of mānuka and ecologically similar, adjacent native plant species. The results revealed that the relative influence of host species identity on the phyllosphere microbiome was quantitatively stronger in mānuka compared to other plant species, and mānuka species-specificity was not associated with leaf morphological traits.

Using a pair of morphologically indistinguishable and naturally co-occurring plant species (mānuka and *Kunzea ericoides* [kānuka]), I then explored the relative influence of host species identity and leaf morphology on inter-host dispersal. Specifically, I addressed a longstanding yet under-examined hypothesis that the relative strength of the phyllosphere microbiome as a source of dispersing microorganisms (i.e., *source-strength*) is contingent on leaf morphology. The results revealed considerable spatial heterogeneity among morphologically indistinguishable leaves at small spatial scales and suggested that mānuka may act as a stronger source of phyllosphere microorganisms than kānuka. These findings suggest that source-

strength is determined by the quantitative difference in the relative strength of host selection among plant species.

To contextualise this apparent spatial stability and host species-specificity of the mānuka phyllosphere microbiome, temporal variation was investigated by replicate sampling in three different seasons. My results revealed that the relative influence of individual host trees was larger than the season, and specific phyllosphere taxa persisted across time. Additionally, my results revealed an increased core microbiome during summer flowering, suggesting an association between host selection strength and host phenology.

Lastly, I explored the relationship between the mānuka phyllosphere microbiome and mānuka honey quality. I sampled trees and honey from three adjacent mānuka populations known to exhibit visually discrete phenological traits (i.e., flowering time). My results revealed correlations between phyllosphere community composition and chemical properties of mānuka honey, including the primary constituent of mānuka's non-peroxide antibacterial properties (i.e., methylglyoxal).

Through the incorporation of spatial and temporal sampling designs, as well as a multi-disciplinary case study, this thesis provides a holistic understanding of the relative influence of host selection (abiotic and biotic), dispersal (short- and long-distance), and climate, on the assembly of the mānuka phyllosphere microbiome. These results also provide new perspectives on prevailing controversies (e.g., host selection vs. dispersal), address unverified hypotheses (e.g., source-strength), and illustrate a path forward that will allow the emergence of a coherent and generalisable understanding of phyllosphere microbial ecology.

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Table of Contents

List of figures

Chapter II: Host selection is not a universal driver of phyllosphere community assembly among ecologically similar native New Zealand plant species

Chapter III: Phyllosphere microbial dispersal exhibits a source-sink relationship among ecologically and morphologically similar plant species

Chapter IV: The effect of season on the evergreen phyllosphere microbiome of mānuka (*Leptospermum scoparium*)

Chapter V:Investigating the relationship between the mānuka phyllosphere microbiome and mānuka honey quality: a case study

List of Tables

Chapter III: Phyllosphere microbial dispersal exhibits a source-sink relationship among ecologically and morphologically similar plant species

Chapter IV: The effect of season on the evergreen phyllosphere microbiome of mānuka (*Leptospermum scoparium*)

Chapter V:Investigating the relationship between the mānuka phyllosphere microbiome and mānuka honey quality: a case study

Chapter I

Introduction and Literature Review

1.1 Introduction

Plants and animals live in close association with diverse communities of microorganisms that colonise the discrete microhabitats provided by their various anatomical structures (McFall-Ngai *et al.* 2013; Trivedi *et al.* 2020). From the perspective of the eukaryotic host, association with the individual members of each of these microbial communities, or *microbiomes*, can be neutral, sometimes deleterious, but also beneficial (Delaux & Schornack 2021). In recent years, there has been an explosion of research across a wide range of plant and animal species aimed at identifying beneficial host-microorganism associations and elucidating the ecological processes that drive the assembly of these relationships (Hammer *et al.* 2019; Fitzpatrick *et al.* 2020). Understanding the intricate relationships between host microbiome composition and function, host biology, and the broader environment has far-reaching consequences for our natural and managed ecosystems around the world (Trivedi *et al.* 2020; Delaux & Schornack 2021). Moreover, generating such an understanding is essential in order to develop the ability to leverage microbiomes to protect and promote desirable compositions for agricultural sustainability, economic growth, and environmental conservation (Gilbert *et al.* 2014; Perreault & Laforest-Lapointe 2022; Beattie *et al.* 2024).

The surface of plant leaves, termed *the phyllosphere*, represents one of the largest biological surfaces on Earth and is colonised by a diverse microbiome, including bacteria, archaea, fungi, algae, protists, and viruses (Ruinen 1956; Lindow & Brandl 2003; Leveau 2019). Of these phyllosphere microorganisms, bacteria are the most abundant, and a global population as large as 10^{26} cells has been estimated (Morris & Kinkel 2002). In accordance with these estimates, phyllosphere bacteria are presumed to have a significant influence on many important global processes, as well as the physiology of their individual host plants (Lindow *et al.* 1978; Lindow & Brandl 2003; Innerebner *et al.* 2011; Vacher *et al.* 2016; Laforest-Lapointe *et al.* 2017; Rosado *et al.* 2018; Delaux & Schornack 2021; Li *et al.* 2022b). However, despite the size and potential significance of bacterial communities on leaf surfaces, relatively little research

has historically been carried out on the phyllosphere microbiome compared to other plant-associated microbial communities (Ruinen 1961; Yang *et al.* 2001; Lindow & Brandl 2003). Specifically, the microbial ecology of the natural phyllosphere microbiome has received surprisingly little attention and thus the ecological processes that drive the assembly of natural phyllosphere communities remain poorly understood (Ruinen 1961; Lindow *et al.* 1978; Hirano 1985; Hirano & Upper 2000; Finkel *et al.* 2012; Stone & Jackson 2016).

Recently, a spatially persistent host association was identified in the phyllosphere microbiome of *Leptospermum scoparium* (mānuka), a culturally and economically significant indigenous New Zealand tea tree species (Noble *et al.* 2020). Given that such a persistent tissue- and species-specific host association has seldom been previously observed in the phyllosphere microbiome (Rastogi *et al.* 2012; de Souza *et al.* 2016; Grady *et al.* 2019), mānuka therefore represents a unique and attractive model plant species in which to further investigate processes of phyllosphere community assembly.

In the remainder of this chapter, a review of available and relevant literature is presented and broadly divided into five sections. First, general features that characterise the leaf surface and phyllosphere microorganisms are described. Second, the conception and history of the field of phyllosphere research is synthesised with emphasis on *Pseudomonas syringae*. Although a historical recount is not necessary to understand the current state of a research field, in this context, it provides relevant information on why it is where it is, the challenges it has overcome, and where it needs to go. Third, the current understanding of phyllosphere microbial ecology is summarised. Here, several important conceptual and methodological controversies that are arguably limiting the progression of research in this field are also addressed. Fourth, an introduction to mānuka and the properties that make this plant species an attractive model system in which to study processes of phyllosphere community assembly are explained. To conclude, the primary goals and outline of this PhD thesis are presented.

1.2 The phyllosphere: characteristics

The surface of leaves is a harsh environment that imposes a unique combination of abiotic stressors on epiphytic microorganisms (Lindow & Brandl 2003). As the primary photosynthetic organs of plants, leaves are subject to prolonged sunlight exposure and possess multiple adaptations that prevent water and nutrient loss from internal tissues (Tukey 1966). An example

of one such adaptation includes the hydrophobic waxy cuticle, which not only prevents diffusion but also the adherence of water particles to the leaf surface (Knoll & Schreiber 2000). Consequently, phyllosphere microorganisms are generally thought to experience an extremely desiccated and nutrient-poor environment. Although the severity of these physicochemical conditions is unlikely to exceed those experienced by microorganisms in other extreme environments, such as hydrothermal vents or the Antarctic dry valley soils (Zierenberg *et al.* 2000; Cary *et al.* 2010), the extremity of the leaf surface as a microbial habitat is acknowledged due to the rapid and extreme physicochemical fluctuations that arise as a result of the diurnal cycle and stochastic weather events. From this perspective, the phyllosphere may represent one of the most prevalent yet extreme microbial habitats on earth (Hirano & Upper 2000; Lindow & Brandl 2003).

Despite these unifying characteristics, the leaf surface is also an extremely heterogeneous microbial habitat. At the micron scale, the bulges and troughs of epidermal cells, veins, stomata, and the presence of surface appendages (i.e., trichomes) creates a complex, mountainous topography that directly affects microhabitat quality and availability for colonisation by phyllosphere microorganisms (Mechaber *et al.* 1996). Epiphytic microorganisms are most commonly found in the 'protected sites' of the leaf surface, such as the troughs between epidermal cells and the base of trichomes, where the concentration of carbon-containing compounds and water availability tends to be the highest (Roos & Hattingh 1983; Leben 1988; Mercier & Lindow 2000; Miller *et al.* 2001). At the macroscale, differences in general leaf traits among plant species (i.e., cuticle composition) (Jetter *et al.* 2006), leaf longevity (i.e., deciduous versus evergreen) (Bao *et al.* 2020), regional environmental variation (Wang *et al.* 2023), and spatial location of individual leaves and trees (i.e., branch aspect, slope aspect) (Stone & Jackson 2019), is also thought to generate substantial heterogeneity. However, the relative influence of each of these factors remains to be quantified.

Due to the challenging conditions that characterise the leaf surface, it is perhaps unsurprising that phyllosphere bacteria differ significantly from bacteria found in other plant-associated microbiomes, such as the rhizosphere and endosphere, and possess numerous adaptations that presumably enhance their epiphytic fitness (Knief *et al.* 2012; Yao *et al.* 2020). For example, pigmentation is a common characteristic of many phyllosphere bacteria, which seems likely to confer a selective advantage in a habitat that is exposed daily to solar radiation (Sundin & Jacobs 1999). Many bacteria are also methylotrophic, which facilitates the metabolism of

reduced one-carbon compounds (i.e., methanol or methane) that are leached by plants (Tukey 1966; Corpe & Rheem 1989; Knief *et al.* 2012). A large number of bacteria that successfully establish in the phyllosphere also demonstrate the ability to create more favourable conditions for microbial growth by altering leaf surface conditions (Lindow & Brandl 2003). Such alterations include aggregate formation via extracellular polysaccharide production (Yu *et al.* 1999), increasing the wettability of leaves by producing compounds with surfactant properties (Knoll & Schreiber 2000), and increasing the release of carbon sources from the plant cell walls by the production of plant growth regulators, such as indole-3-acetic acid (Brandl & Lindow 1997; Brandl & Lindow 1998). Furthermore, beyond conferring increased physical protection, evidence also suggests that the aggregates in which epiphytic bacteria are commonly found further facilitates epiphytic survival via quorum-sensing (Dumenyo *et al.* 1998; Bassler 1999) and high rates of plasmid transfer (Lilley & Bailey 1997; Björklöf *et al.* 2000).

1.3 The phyllosphere: a brief history of the leaf

Although the presence of a specialised, non-pathogenic microbial community living near plant roots (i.e., the rhizosphere) was well recognised by the early 1950s (Hartmann *et al.* 2008), a comparable non-pathogenic microbial community on leaf surfaces was largely overlooked. Up until this time, research on leaf microorganisms was primarily focused on identifying pathogenic bacterial epiphytes that were responsible for foliar diseases and plant pathologists were achieving this by isolating pathogenic strains from diseased leaves (Hirano & Upper 2000). Given that most isolated strains of leaf epiphytic bacteria were, by virtue of the methodology used, pathogenic, the early perception of these microorganisms was largely biased towards the belief that pathogenicity was their primary role on the leaf surface.

This early dogma was first challenged by the pioneering work of Jakoba Ruinen who identified oligonitrophilic and nitrogen-fixing bacteria (e.g., *Beijerinckia*) in the phyllosphere of tropical plant leaves in Indonesia (Ruinen 1956; Ruinen 1961). At the time, these authors were studying the rhizosphere of a fern epiphyte and noticed that areas of the leaf surface that were not covered by the epiphyte were instead colonised by abundant bacteria. Subsequent surveys also revealed that *Beijerinckia* was prevalent across the leaf surface of different plant species and islands. Ruinen (1956) hypothesised that a beneficial relationship between the plants and *Beijerinckia* might exist, whereby leaf surface metabolites may provide a nutrient source for *Beijerinckia* and *Beijerinckia* might provide a source of fixed nitrogen for the plant. Thus, in

analogy with the rhizosphere, Ruinen (1956) defined this microbial habitat, *the phyllosphere*. Although the significant economic impact of foliar diseases continued (and continues) to motivate more pathologically-inclined researchers, the work by Ruinen (1956) signifies the emergence of interest in leaves as habitats for microorganisms, not merely the causal agents of disease (Ruinen 1961).

1.3.1 *Pseudomonas syringae*: a phyllosphere celebrity

Of all the microorganisms that colonise the phyllosphere, *Pseudomonas syringae* has undoubtedly attracted the most attention. *P. syringae* was first isolated from a diseased lilac (*Syringa vulgaris*) in 1899, and its role as a plant pathogen became firmly established (Young 1991). Indeed, pathogenicity was even one of the early criterions for *P. syringae* classification (Hirano & Upper 2000). Since its first isolation, the majority of research on *P. syringae* has focused primarily on the diseases it causes and more than 60 pathovars of *P. syringae* have now been identified (Xin *et al.* 2018). One significant observation from these research efforts was the considerable specialization with respect to the plants that some individual strains are able to interact. For example, some *P. syringae* strains only initiate disease in one or a few cultivars of a given plant species (Baltrus *et al.* 2012; Morris *et al.* 2019). The identification of these specific plant-strain interactions provided specific model systems, which were (and are still being) used to describe detailed mechanisms (Crosse 1959; Sarkar *et al.* 2006; Arnold *et al.* 2011; Xin *et al.* 2018). However, during these early studies, one observation started to challenge the dominant narrative of *P. syringae* as a pathogen: for some strains, no plants could be found on which they would cause disease (Hirano & Upper 1983).

A pioneering moment in the history of *P. syringae* research was the discovery of its role as an active ice nucleus (Maki *et al.* 1974; Arny *et al.* 1976). The implications of this finding were twofold. First, this discovery revolutionised strategies to control crop frost injury (Lindow *et al.* 1989). Of particular significance, an Ice^- strain of *P. syringae* was the first genetically modified microorganism to be released into the environment (Lindow 1987). Second, this discovery supported the findings of Ruinen (1961) and reinforced the idea that non-pathogenic phyllosphere microorganisms can have a ubiquitous distribution and presumably an important role in nature (Lindow *et al.* 1978; Hirano 1985).

1.3.2 Phyllosphere microbial ecology from the perspective of *P. syringae*

Following the discovery of ice nucleation activity in *P. syringae*, many questions regarding the ecology of natural phyllosphere microorganisms were raised. For example, 'How do these bacteria disperse across small (i.e., leaf-to-leaf) and large (i.e., regional) spatial scales?' Or, 'How do these natural populations persist year after year, even on leaves or plants that do not overwinter?' (Hirano 1985). Central to answering these questions was the need to sample and study natural phyllosphere communities. Culture-dependent studies sought to answer these questions in natural conditions using either individual *P. syringae* strains or the culturable portion of the phyllosphere community. Together, these studies demonstrated that epiphytic population sizes (individual *P. syringae*-strains or total culturable) exhibit significant spatial and temporal variation (Hirano & Upper 1986; Upper & Hirano 1994).

Among the studies aimed at quantifying spatial variation in epiphytic population size, it was observed that the amount of variation among leaves was both strain- and plant species-specific (O'brien & Lindow 1989). For example, 15 different strains of *P. syringae* were found to vary up to 10-fold on a single plant species. Furthermore, the total epiphytic population size of different plant species was found to vary up to 17-fold. Differences in carrying capacity among plant species tended to correlate with general leaf traits. For example, plant species with wettable leaf surfaces generally had larger bacterial populations (i.e., carrying capacities) than those with waxy hydrophobic leaves (Lindow *et al.* 1978).

Short-term temporal studies on annual crop species had already revealed that *P. syringae* population sizes could exhibit rapid (i.e., in less than 24 hours) and sporadic fluctuations of more than 100-fold several times throughout a growing season (Hirano & Upper 1989; Hirano *et al.* 1996). However, one particular study on the evergreen phyllosphere of olive was unique in that it was able to examine the individual effect of both leaf age and season without the confounding effect of plant development (Ercolani 1991). These authors reported that phyllosphere diversity decreased with leaf age and identified significant differences among leaves of different ages and at different times of the year.

However, of particular significance was the insight generated on the role of microbial dispersal, an ecological process that had remained unquantified in experimental research. Not only did these studies demonstrate that plant leaf surfaces constitute a major source of airborne bacteria which can thus disperse to and colonise the phyllosphere microbiome of adjacent or distant

host plants (Lindemann *et al.* 1982), but they also revealed emigration and immigration was a heterogeneous process. For example, rates of bacterial emigration from leaf surfaces varied at different times of the day, during different environmental conditions, and across host species (Lindemann *et al.* 1982; Lindemann & Upper 1985; Lindow & Andersen 1996). Moreover, immigration rates were found to vary among leaf canopy positions as well as with the presence of and distance to microbial sources (i.e., neighbouring plants) (Andrews *et al.* 1980; Jacques *et al.* 1995).

Although culture-based methods were unable to capture the full diversity of microorganisms now known to comprise the phyllosphere microbiome (Staley & Konopka 1985; Yang *et al.* 2001), these early investigations were undeniably pivotal in founding our early understanding of microbial ecology in the phyllosphere and generated many interesting hypotheses (Upper & Hirano 1994). One example is the 'hypothesis of accessibility' generated by Andrews *et al.* (1980), which states that variation in the accessibility of leaves to airborne bacteria is a primary determinant of variation in epiphytic population size. Another example is the 'hypothesis of source-strength' generated by Lindow and Andersen (1996), which states that the relative strength of a host plant's phyllosphere microbiome as a source of local airborne communities is correlated with the carrying capacity and thus leaf morphological traits of the plant species. Although some of these hypotheses have subsequently been tested and verified with culture-independent methods, many observations and hypotheses generated by these early studies remain to be tested with cultivation-independent methods.

1.4 Modern microbial ecology needs to be studied in natural systems

Research within the field of microbial ecology generally adheres to three basic objectives: 1) identify community patterns (i.e., the diversity, abundance, and distribution of microorganisms through space and time), 2) understand the mechanisms that generate these patterns (i.e., processes of community assembly), and 3) determine the effect that these community compositions have on ecosystems (i.e., function/ecological interactions) (Hirano & Upper 2000). Although the ability to achieve all three of these objectives does require experimental research (i.e., because mechanisms can be challenging to discern with only 16S rRNA gene amplicon sequencing/metagenomic characterisations of natural communities), the ecological relevance and generality of mechanisms remain unknown with only experimentation (Hirano 1985; Fitzpatrick *et al.* 2020; Nie & Wu 2021). For example, a phyllosphere microorganism

that demonstrates success under greenhouse conditions may not be successful in the field where it must compete with a large diversity of environmental microorganisms and withstand varying environmental conditions. Similarly, techniques that successfully manipulate plant microbiomes under experimental conditions have been unsuccessful when applied under field conditions (Weinhold *et al.* 2018). Thus, research approaches that begin with ecological characterisation of widespread and natural phenomena are generally more successful than those that start with experiments (Fitzpatrick *et al.* 2020).

One strategy that epitomises this approach is the search for a core microbiome to identify ecologically significant host-microbiome associations. The prevailing core microbiome concept is built on the premise that the prevalence of a microorganism across the spatial and temporal boundaries of its ecological niche can be used to infer its functional importance to the host microbiome in which it is found (Shade & Handelsman 2012; Shade & Stopnisek 2019). This framework thus permits the detection of putative microbial targets within diverse and complex host microbiome datasets that can then be subject to further ecological and experimental investigation. This approach has successfully revealed numerous significant host –microbiome interactions in a number of different hosts, such as humans (Turnbaugh *et al.* 2008), coral (Hernandez-Agreda *et al.* 2017), honeybees (Raymann & Moran 2018), and plant roots (Lundberg *et al.* 2012).

1.5 Microbial ecology of the *total* and *natural* phyllosphere microbiome

The transition to cultivation-independent methodology facilitated detailed characterisation of total phyllosphere bacterial communities (Yang *et al.* 2001). These communities have revealed that the leaf surface is commonly colonised by bacteria that are members of Proteobacteria, Actinobacteria, Bacteroidetes, and Acidobacteria (Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016a). Furthermore, the development of cheaper and faster sequencing methods has facilitated wider exploration of phyllosphere communities across an increasing number of plant species and environments (Li *et al.* 2022a). These biogeographical studies have corroborated a number of the observations made by culture-based studies. In particular, large spatiotemporal variation in total phyllosphere community structure has also been reported by cultivation-independent studies (Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016a). Furthermore, these studies have also revealed significant associations between phyllosphere community structure and factors such as: host species identity (Redford *et al.* 2010; Kembel *et al.* 2014; Laforest-Lapointe *et*

al. 2016a), geographic location (Finkel *et al.* 2011; Stone & Jackson 2016), climatic factors (Stone & Jackson 2021), season (Bao *et al.* 2020; Al Ashhab *et al.* 2021), and canopy position (Stone & Jackson 2019).

However, despite a steadily growing body of cultivation-independent data on the phyllosphere microbiome, the relative influence of these factors on phyllosphere community structure remains controversial. One controversy that prevails throughout the literature is whether the phyllosphere microbiome is more strongly influenced by the host or microbial dispersal. For example, some studies have identified dominant patterns of host species identity, which suggests the host plant may have a primary influence on phyllosphere assembly (Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016a; Smets *et al.* 2023). However, other studies have identified dominant biogeographic patterns and distance decay relationships, which suggests microbial dispersal may play a primary role instead (Finkel *et al.* 2011; Finkel *et al.* 2012). Similar controversies are also observed for other factors, including but not limited to: season (Laforest-Lapointe *et al.* 2016a; Al Ashhab *et al.* 2021), rain (Jackson *et al.* 2006; Stone & Jackson 2021), and canopy position (Stone & Jackson 2019; Al Ashhab *et al.* 2021). Clarifying these unresolved controversies and making generalisable progress towards a unified understanding of *total* and *natural* phyllosphere microbiome assembly thus needs to be made a priority of modern phyllosphere research.

1.6 Progress towards a unified understanding of phyllosphere microbiome assembly

A number of conceptual and methodological limitations critically hinder progress in the field of phyllosphere microbial ecology. These limitations include: 1) inconsistent used of the term ‘phyllosphere’, 2) lack of systematic focus on the individuality of plant species, and 3) preference of controlled systems over natural conditions.

1.6.1 Inconsistent used of the term ‘phyllosphere’

Given the inherent complexity of natural ecosystems, defining and sampling microbial habitats at microbiologically relevant scales is essential in order to study specific ecological processes that are relevant to a single community of microorganisms. Despite the explicit definition outlined by Ruinen (1956), over the last 70 years the term ‘phyllosphere’ has, however, been used broadly and inconsistently throughout the available literature. In the broadest sense, ‘phyllosphere’ has been used to describe the epiphytic microbial communities and endophytic

microbial communities that can be extracted from all aerial plant structures, including leaves, stems, bark, flowers, and fruit (Whipps *et al.* 2008; Müller & Ruppel 2014). In many other cases, 'phyllosphere' has been either been used to describe the epiphytic communities that can be extracted from all aerial plant structures (Morella *et al.* 2020) or the epiphytic and endophytic communities that associate with leaves (Knief *et al.* 2010; Copeland *et al.* 2015; Wagner *et al.* 2016). Given that these different plant structures harbour indisputably distinct microbial habitats, coarse sampling approaches (i.e. pooling functionally explicit communities into a single sample) encouraged by these less stringent definitions of 'phyllosphere' are likely to have hindered detection of microbiome-specific ecological processes (Fleishman *et al.* 2022). Furthermore, the use of such inexact terminology also promotes methodological inconsistencies that critically hinders generalisability across studies. For example, the inclusion of endophytic microorganisms requires plant tissue maceration and thus causes substantial host plastid contamination. This reduces the sequencing depth of many studies or chloroplast-excluding 16S rRNA gene PCR primers are used (Copeland *et al.* 2015). In comparison, studies that exclusively target epiphytes do not require tissue maceration and thus are able to use universal primers without sacrificing sequencing depth or introducing taxonomic bias in their PCR amplicons (Noble *et al.* 2020; Smets *et al.* 2023). Although these controversies are not a direct result of inexplicit sampling (i.e., conflicting results are still observed among studies that explicitly sample leaf epiphytes) (Redford *et al.* 2010; Finkel *et al.* 2011), improving our terminology can be expected to benefit progress by increasing the number of generalisable results.

1.6.2 Lack of systematic focus on individual plant species

Plant species exhibit enormous ecological, morphological, and physiological variation such that microorganisms most likely experience different physical (abiotic) conditions in the phyllosphere of different plant species. For example, the waxy cuticle of different plant species exhibits vastly different three-dimensional crystalline structures, creating species-specific differences in nutrient availability, leaf hydrophobicity, and thus leaf carrying capacities (Tukey 1966; Beattie & Lindow 1999). Furthermore, leaf surface properties are also significantly correlated with host plant ecological traits, including those linked to a plant species' position on the leaf economic spectrum (Zhao *et al*. 2016). The leaf economic spectrum is a conceptual framework in plant ecology that describes a continuum of leaf functional traits associated with resource use strategies. Specifically, it captures a trade-off

between a resource-acquisitive strategy (e.g., high nutrient uptake rates and fast growth) and a resource-retentive strategy (e.g., low resource uptake rates and slow growth). Plants with an "acquisitive" resource strategy tend to have thin, short-lived leaves with high nutrient concentrations. In contrast, plants with a "retentive" resource strategy typically have long-lived leaves with thicker cuticles and low nutrient concentrations. Moreover, interspecies variation in phyllosphere community composition has been associated with many such traits (Kembel *et al.* 2014; Li *et al.* 2022a). Given that the quantification of many ecological processes, such as spatially driven distance decays, requires careful control of environmental variables (Nekola & White 1999), the environmental heterogeneity that exists among leaves of different plant species therefore requires greater consideration. For example, many phyllosphere studies have searched for distance-decay relationships across heterospecific phyllosphere samples and have reported no such relationship (Smets *et al.* 2023). However, sampling heterospecific (i.e., ecologically inequivalent communities) may have potentially compromised the robustness of their findings. A similar argument can be made for the application of the core microbiome concept, which arguably requires sampling from tissue- and species-specific host microbiomes (Shade & Handelsman 2012; Berg *et al.* 2020; Noble *et al.* 2020). Nevertheless, many phyllosphere studies define 'core taxa' as those that persist across different plant species (Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016a; Laforest-Lapointe *et al.* 2016b; Li *et al.* 2022a; Smets *et al.* 2023), or even plant tissues (Hamonts *et al.* 2018; Cernava *et al.* 2019). As such, the 'core taxa' these studies define are more likely to represent over-dispersed phyllosphere generalists, which precludes the detection of specific host-microorganism associations.

1.6.3 Preference of controlled systems over natural conditions

Given the complexity and unpredictability of the natural environment, controlled experiments offer an attractive means by which to minimise the effect of confounding variables, such as environmental heterogeneity. Given the necessity of controlling environmental heterogeneity when investigating the processes of community assembly, many studies have thus sought to use controlled systems to address ecological questions (Maignien *et al.* 2014; Meyer *et al.* 2022; Meyer *et al.* 2023). Given the lack of generalisability of these systems to natural environments, the results of such studies demonstrate what *could* happen in nature rather than what *does* happen in nature. For example, as demonstrated through the pioneering culture-based work of Prof. Stephen Lindow, microbial dispersal has an important role in phyllosphere

assembly but is not reproducible in a greenhouse (Lindow *et al.* 1978; Hirano 1985). An additional limitation regarding the popularity of experimental systems is that a lot of research is thus being carried out on fast growing model plant species, such as *Arabidopsis thaliana*, which have vastly different ecologies to long-lived perennial tree species.

1.7 *Leptospermum scoparium* (mānuka)

Leptospermum scoparium J.R. et G. Forst. (mānuka; Myrtaceae family), a woody perennial shrub species indigenous to Aotearoa (New Zealand) (Stephens *et al.* 2005), has become widely known in recent decades for the unique non-peroxide antibacterial properties found in its honey (McDonald *et al.* 2018). These unique non-peroxide properties have been principally linked to the accumulation of a three-carbon sugar in mānuka nectar called dihydroxyacetone (DHA), which undergoes an irreversible dehydration reaction in mature honey to an antibacterial constituent called methylglyoxal (MGO) (Adams *et al.* 2009; Williams *et al.* 2014; Grainger *et al.* 2016). However, the concentration of DHA that accumulates in mānuka nectar is notoriously variable and thus the antimicrobial efficacy of mānuka honey and its commercial value also vary across space and time (Adams *et al.* 2009; Hamilton *et al.* 2013; Williams *et al.* 2014). Despite an expanding mānuka honey industry and decades of research, the mechanism by which nectar DHA is produced and accumulates in mānuka, as well as the functional and adaptive significance (if any), remains to be determined (Clearwater *et al.* 2021).

Beyond the apicultural significance of mānuka, mānuka is also a highly aromatic plant species with unique properties that have been treasured and used by Māori, the indigenous people of Aotearoa, as a source of rongoā rākau (traditional herbal medicine) since their arrival in New Zealand approximately 800 years ago. Mānuka leaf infusions were used to alleviate fevers and gastrointestinal conditions, the tree's gum served as a remedy for coughs, and mixtures derived from the bark were used as a sedative and provided relief from scalds and burns (Durie 1985).

1.7.1 Mānuka: biology and ecology

In total, 87 species are members of the genus *Leptospermum* and are distributed throughout south-east Australia, Southeast Asia, New Guinea, Rarotonga, and New Zealand (Thompson 1989). Nevertheless, mānuka is the only indigenous member of *Leptospermum* in New Zealand (Stephens *et al.* 2005). Although natural populations of *L. scoparium* are also found in Australia,

recent studies have revealed that the New Zealand and Australian *L. scoparium* populations diverged c. 9–12 million yr ago and are thus genetically distinct (Koot *et al.* 2022).

Ecologically, mānuka is fast growing and widespread throughout New Zealand. Mānuka can tolerate a range of environmental conditions and is often found as a dominant species where environmental conditions are adverse (i.e., low fertility soils, peat swamps, volcanic soils, geothermal areas, exposed coastal, sub alpine), or as a pioneering species in disturbed habitats (i.e., following deforestation or fire) (Stephens *et al.* 2005; Derraik 2008). As such, mānuka is considered an ecologically important species for erosion control, carbon sequestration, and vegetation restoration.

Mānuka exhibits significant phenotypic variation that is thought to be attributable, in part, to the different environmental conditions and habitats in which it is found (Stephens *et al.* 2005). For example, depending on exposure, mānuka can be found as a small (1 – 2 m) shrub or tall (10 – 15 m) trees. Mānuka leaves are xeromorphic and generally small and narrow with a sharp point (Johnson 1980). Nevertheless, variation in size is also common among regions, with leaves ranging in length from 4 – 12 mm long. Mānuka phenolic compounds also exhibit significant variation, and different chemotypes have been identified in different geographic regions (Douglas *et al.* 2004). However, significant phenolic variation has also been identified among mānuka trees and clones grown in the same environment, demonstrating that such physiological variation can exist independently of both abiotic factors and host genetics (Porter *et al.* 1998; Effah *et al.* 2022).

1.7.2 Variation in the nectar-DHA trait

Mānuka nectar is dominated by two major sugars, fructose and glucose, and also contains smaller and variable amounts of sucrose (< 2%) and DHA (< 2%). Significant intraspecies variation in mānuka nectar composition, such as total sugar per flower, is observed (Williams *et al.* 2014; Nickless *et al.* 2017). Interestingly, however, DHA production in mānuka nectar does not appear to be stoichiometrically linked to the production of the major sugars (i.e., fructose, glucose, and sucrose), and DHA content (quantified as the ratio of DHA to total sugars, DHA:Tsugar) varies significantly (Clearwater *et al.* 2018; Smallfield *et al.* 2018). Surveys of both wild and cultivated mānuka have reported significant site-to-site and plant-to-plant DHA variation. For example, a previous study by Noe *et al.* (2019) sampled 10 mānuka trees each from five geographically distant and environmentally diverse regions in the North Island of

New Zealand and found that the DHA:Tsugar ratio varied the most between sites (R^2 = 0.446). However, a significant and almost equivalent proportion of variation (R^2 = 0.438) was also observed within sites between individual plants. This contrasted with sucrose concentrations, which did not vary significantly across sites nor plants. Correlations between DHA:Tsugar and various abiotic and biotic factors have also been investigated by various empirical and experimental studies (Hamilton *et al.* 2013; Williams *et al.* 2014; Nickless *et al.* 2017; Clearwater *et al.* 2018; Smallfield *et al.* 2018; Grierson *et al.* 2024), but no definitive conclusion has been drawn. Similar to the phenolic variation observed in mānuka, DHA variation between different sites and co-occurring plants demonstrates that such variation can exist independently of both abiotic factors and host genetics.

1.7.3 The mānuka phyllosphere microbiome: a potential model system

The first study to investigate the mānuka phyllosphere microbiome characterised the composition and structure of epiphytic bacterial communities on leaf surfaces collected from five geographically distant and distinct native mānuka populations in the North Island of New Zealand (Noble *et al.* 2020). Using 16S rRNA gene amplicon sequencing, this study revealed that bacterial communities in the mānuka phyllosphere were distinct from those observed in surrounding surface soil and dominated by Alphaproteobacteria, Acidobacteria, Bacteriodetes, Firmicutes, and Verrucomicrobia. The findings of this study also revealed a dominant and phyllosphere-specific core microbiome comprising 10 OTUs that were present in all phyllosphere samples yet were either rare or entirely absent in surface soil. This finding was significant given the geographical separation and environmental heterogeneity of the sampled populations. For example, sites ranged from low-lying wetlands to subalpine environments and were separated by distances up to 330 km. Moreover, the detection of a tissue- and species-specific core phyllosphere microbiome had not been previously observed at such a fine taxonomic resolution in natural communities at such a spatial scale (Rastogi *et al.* 2012; de Souza *et al.* 2016; Grady *et al.* 2019). In accordance with the prevailing core microbiome concept (Turnbaugh *et al.* 2008; Shade & Handelsman 2012; Shade & Stopnisek 2019), and the notion that microbiomes under strong host selection are more likely to confer beneficial functions to the host compared to communities that assemble stochastically (Hammer *et al.* 2019; Jackrel *et al.* 2021), this persistent spatial association suggests that some taxa in the mānuka phyllosphere may have an influential role in mānuka physiology.

1.8 Research chapters and objectives

The primary objective of this PhD thesis is to investigate explicit processes of community assembly in the natural mānuka phyllosphere. Specifically, the overarching hypothesis of this work is that the assembly of the mānuka phyllosphere microbiome is primarily driven by strong host selection. The anticipated outcome of this research is twofold. First, by performing a hypothesis-driven series of investigations of explicit processes of community assembly, I anticipate this work will generate generalisable results that can be compared with future studies of other plant species. Second, through efforts to generate a fundamental understanding of mānuka phyllosphere microbial ecology, this work will provide new knowledge on a previously unexplored driving factor of mānuka physiological heterogeneity. The chapters of this thesis are outlined below:

Chapter II used a multi-species, spatially hierarchical survey of a native forest to quantify and compare the relative influence of host selection versus microbial dispersal on phyllosphere microbiome assembly in mānuka and three ecologically similar, neighbouring indigenous New Zealand plant species. The hypothesis of this study was that the relative influence of host species identity is stronger than the effect of stochastic dispersal in the assembly of the mānuka phyllosphere. Our results demonstrated that host selection is the primary driver of microbiome assembly in the mānuka phyllosphere and suggest a direct, chemically-mediated mechanism of host selection. Our results also reveal several crucial considerations for future microbial ecology research in the phyllosphere such as: 1) the relative influence of host species identity was quantitatively host dependent, and 2) some phyllosphere microbiomes may serve as stronger sources of dispersing microorganisms than others. This chapter has been submitted to the journal Microbiome for publication and is currently in review.

Chapter III used a pair of morphologically indistinguishable and naturally co-occurring plant species (mānuka and *Kunzea ericoides* [kānuka]) to address a longstanding yet untested hypothesis of inter-host dispersal established by early culture-based work of Lindow and Andersen (1996). The hypothesis states that the relative strength of a phyllosphere microbiome as a source of microorganisms to local airborne and phyllosphere microbial communities is correlated to the leaf morphological traits of the host plant species. However, in contrast to the tested hypothesis, we demonstrated that mānuka may act as a stronger source of phyllosphere microorganisms than kānuka, despite their morphologically similar leaves. Our results also

revealed that the dispersal and colonisation of total phyllosphere communities is heterogeneous even among the phyllosphere of similar trees at small spatial scales and implicates a potential role of priority effects. This chapter demonstrates the importance of small-scale spatial heterogeneity, which is often overlooked by modern sampling designs, and using current methodologies to test longstanding hypotheses that were established with culture-based methods.

Chapter IV utilised a replicate sampling design in three different seasons to investigate the temporal variation (i.e., due to seasonal abiotic factors) in the mānuka phyllosphere microbiome. In accordance with our overarching hypothesis that host selection plays a primary role in mānuka phyllosphere assembly, we thus hypothesised that the composition of the mānuka phyllosphere microbiome would exhibit no significant seasonal variation. In accordance with this hypothesis, our results demonstrated that the identity of the host had a stronger influence on phyllosphere community composition than season. Further, we identified a significant increase in the number and relative abundance of core microorganisms during the summer flowering season, suggesting an increase in the relative strength of host selection. This chapter also demonstrates the attractiveness of the evergreen phyllosphere as a model system for investigating long-term temporal changes independently from the confounding effects of leaf age, host development, and community succession.

Chapter V explored the relationship between the mānuka phyllosphere microbiome and mānuka honey quality by sampling trees and honey from three adjacent mānuka populations known to exhibit different phenological characteristics (i.e., flowering time). We identified correlations between phyllosphere community structure and the concentration of both mānuka chemical constituents and elements. These results demonstrate that interactions between mānuka and the phyllosphere microbiome represent a possible driver of mānuka honey quality.

Chapter VI highlights general conclusions. considerations and offers future directions from this work.

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Chapter II

Host selection is not a universal driver of phyllosphere community assembly among ecologically similar native New Zealand plant species

Anya S. Noble, Jaber Abbaszadeh, Charles K. Lee

(*In review)*

2.1 Abstract

A growing body of evidence demonstrates that host-associated microbial communities of plant leaf surfaces (i.e., the phyllosphere) can influence host functional traits. However, it remains unclear whether host selection is a universal driver of phyllosphere community assembly. We targeted mānuka (*Leptospermum scoparium*) and three neighbouring non-mānuka plant species along an 1,800-m transect in a New Zealand native bush to conduct a hypothesis-driven investigation of the relative influence of host species identity and stochastic dispersal on the composition of natural phyllosphere bacterial communities.

We detected significant correlations between host species identity and mānuka phyllosphere communities that are consistent with a dominant role of host selection in the assembly of the mānuka phyllosphere microbiome. In contrast, the phyllosphere community compositions of neighbouring, ecologically similar native plants were highly variable, suggesting that the phyllosphere microbiomes of those non-mānuka plants were more strongly driven by stochastic processes such as dispersal. Furthermore, the distribution of phyllosphere taxa among plant species was congruent with a scenario in which microorganisms had dispersed from mānuka to non-mānuka phyllosphere microbiomes.

We conclude that host selection of phyllosphere communities is not and should not be presumed to be a universal trait across plant species. The specificity of the mānuka phyllosphere microbiome suggests the presence of functionally significant bacteria that are under direct, possibly chemically-mediated, selection by the host. Furthermore, we propose that phyllosphere microbiomes under strong host selection, such as that of mānuka, may act as a source of microorganisms for the phyllosphere microbiomes of neighbouring plants.

2.2 Introduction

Plant leaf surfaces are colonised by diverse communities of epiphytic microorganisms, defined as the phyllosphere microbiome (Last 1955; Ruinen 1956). Despite having received limited attention from microbial ecologists until recent years, a growing body of evidence from laboratory model plant species has demonstrated that reciprocal interactions with epiphytic bacterial communities can have a significant influence on host plant functional traits (Innerebner et al. 2011; Devarajan et al. 2021; Gupta et al. 2022; Li et al. 2022b). Such microbiome-mediated host functional traits include increased disease resistance (Innerebner et al. 2011; Gupta et al. 2022), nutrient acquisition (Fürnkranz et al. 2008; Abadi et al. 2021), and stress tolerance (Devarajan et al. 2021). Gaining a fundamental and generalisable understanding of the processes that shape the assembly of epiphytic microbial communities is required to identify and promote beneficial compositions, which is especially desirable for economically and environmentally significant plant species.

Although there is a steadily growing body of empirical data on the 'phyllosphere microbiome', our understanding of epiphytic community assembly is severely limited by the broad and inconsistent use of the term 'phyllosphere'. In the broadest sense, 'phyllosphere' has been used to refer to the epiphytic and endophytic microbial communities associated with all aerial plant structures, including leaves, stems, bark, flowers, and fruit (Whipps *et al.* 2008; Müller & Ruppel 2014). In many other cases, 'phyllosphere' has been used to describe only the epiphytic communities associated with all aerial plant structures (Morella *et al.* 2020) or the epiphytic and endophytic microorganisms extracted from only leaves (Knief *et al.* 2010; Copeland *et al.* 2015; Wagner *et al.* 2016). Given that different plant structures represent ecologically distinct habitats and harbour ecologically distinct microbiomes (Leveau & Tech 2010; de Souza *et al.* 2016; Yao *et al.* 2020; Al Ashhab *et al.* 2021), coarse sampling approaches (i.e. pooling functionally explicit communities into a single sample) encouraged by these less stringent definitions of 'phyllosphere' are likely to obscure the detection of microbiome-specific ecological processes (Fleishman *et al.* 2022). In addition, inexact terminology promotes methodological inconsistencies that further hinders generalisability across studies. For example, studies that include endophytic microorganisms often use chloroplast-excluding 16S rRNA gene PCR primers due to the presence of large quantities of host plastids released during the maceration of plant tissue (Copeland *et al.* 2015). In comparison, studies that exclusively target epiphytes are more likely to use universal primers as plant tissue is not macerated during

sample processing (Smets *et al.* 2023). As a result, the body of 'phyllosphere microbiome' literature is growing haphazardly on a foundation of conceptual and empirical ambiguity. Defining and sampling habitats at microbiologically relevant scales remains essential in order to study specific ecological processes and host-microbiome associations. Herein, we define the phyllosphere *sensu stricto* as the epiphytic microbial communities present on the leaf surface and only refer to studies that adhered with this original definition.

The phyllosphere microbiome is continuously exposed to bacteria that disperse in the near-surface atmosphere from distant and local sources, such as soil and vegetation (Bowers *et al.* 2011). Nevertheless, the communities that establish on the leaf surface are generally distinct from those in the surrounding environment (Vokou *et al.* 2012; Vorholt 2012). In particular, host species- and even genotype-specific patterns of community composition have been widely observed, from which a dominant role of host selection in phyllosphere community assembly has been inferred (Redford *et al.* 2010; Kim *et al.* 2012; Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2017). However, the relative strength of host selection in structuring phyllosphere community composition remains enigmatic. For example, some studies have found that geographic region has a larger influence on the phyllosphere microbiome than host species identity, implying that phyllosphere communities may also be predominantly structured by stochastic processes, such as dispersal limitations, or regional environmental conditions (Finkel *et al.* 2011; Finkel *et al.* 2012). Although methodological differences (including spatial scale) may account for a significant proportion of this inconsistency, it is also probable that the drivers of phyllosphere microbiome assembly are not universal across plant species (Hammer *et al.* 2019). Some studies have presumed significant host selection across all plant species in their experimental design or data interpretation, which potentially undermined the robustness and generalisability of their findings (Li *et al.* 2021; Meyer *et al.* 2023). Evaluating the universality of host selection in phyllosphere microbiome assembly requires careful control of environmental variables, yet it is also critically important that such studies focus exclusively on stable, ideally natural, specimens since priority effects may have an overwhelming influence on simplified laboratory experimental systems that have not yet reached a steady state (Maignien *et al.* 2014; Fukami 2015).

The decline in community similarity with increasing geographic distance, or distance-decay relationship, is a widely recognised ecological pattern that represents a useful tool to test ecological theories, such as the relative role of selection versus dispersal in microbial

community assembly (Nekola & White 1999; Condit *et al.* 2002; Wang *et al.* 2017). For example, in a scenario in which community assembly is strongly influenced by dispersal limitation (relative to local selection), ecologically equivalent communities may become increasingly dissimilar in their composition as the physical distance between them increases (Evans *et al.* 2017). Meanwhile, ecologically discrete communities may become more similar in their composition as the physical distance between them decreases (Burns *et al.* 2017). In contrast, a relatively weaker distance-decay relationship is more likely to be observed in a scenario in which community assembly is strongly influenced by local selection (relative to dispersal) (Evans *et al.* 2017). Although a role of dispersal limitation in phyllosphere community assembly has been indirectly inferred from observations of large-scale biogeographic patterns in community composition (Finkel *et al.* 2011), only two studies have systematically investigated the effect of spatial distance in the phyllosphere (i.e. by employing sampling strategies to minimise confounding environmental heterogeneity). One study sampled the *Tamarix aphylla* phyllosphere microbiome along a 500- km transect in the Sonoran Desert and reported a significant relationship between distance and *Betaproteobacteria* (Finkel *et al.* 2012). Another study sampled the *Magnolia grandiflora* phyllosphere microbiome that was separated by distances of up to 450 m in a 20-ha Mississippi forest plot and identified a significant relationship between distance and total phyllosphere community dissimilarity (Stone & Jackson 2016). More generally, the role of stochastic processes in phyllosphere community assembly has also been examined in controlled experiments using annual model plants or crops (Maignien *et al.* 2014; Meyer *et al.* 2022). Although the effect of confounding environmental variables is generally reduced in an experimental setting, these results are not necessarily generalisable to natural communities nor perennial plant species (Maignien *et al.* 2014). Sampling natural phyllosphere communities at exponentially increasing distances will be necessary to test whether the stochastic dispersal of phyllosphere microorganisms conforms to the exponential model of distance-decay (Nekola & White 1999; Condit *et al.* 2002). Furthermore, deliberately targeting conspecific (i.e., ecologically equivalent) and heterospecific (i.e., ecologically discrete) phyllosphere samples will be important to shed light on the relative contribution of dispersal versus host selection among different plant species.

Leptospermum scoparium J. R. Forst et G. Forst, commonly known as "mānuka", is a culturally and economically significant flowering tea tree species, indigenous to Aotearoa New Zealand (Stephens *et al.* 2005). Honey derived from the nectar of the mānuka tree contains unique non-peroxide antibacterial properties and has become a highly valuable commodity (Stephens *et al.*

2005; McDonald *et al.* 2018). The unique antibacterial properties originate in the nectar of the mānuka flower due to the accumulation of a three-carbon sugar called dihydroxyacetone (DHA), which undergoes a chemical conversion to the main antibacterial constituent, called methylglyoxal (MGO), in mature honey (Mavric *et al.* 2008; Adams *et al.* 2009). However, the quantity of DHA that accumulates in the nectar of the mānuka flower is notoriously variable, consequently causing large regional and annual fluctuations in the antimicrobial efficacy of mānuka honeys (Williams *et al.* 2014; Noe *et al.* 2019). Despite extensive research efforts, no reliable correlation has been identified between DHA production and climate (Noe *et al.* 2019), soil properties (Nickless *et al.* 2017), host genetics (Clearwater *et al.* 2018), fungi (McKenzie *et al.* 2006), or endophytes (Johnston 1998; Wicaksono *et al.* 2016). In a previous study, we characterised the phyllosphere bacterial community structure of geographically distant and environmentally diverse populations of mānuka and identified a dominant and ubiquitous core phyllosphere microbiome (Noble *et al.* 2020). Such specific host association patterns have seldom been observed in the phyllosphere of other host species and are congruent with those of a microbial community under strong host selection (Shade & Handelsman 2012; Shade & Stopnisek 2019). However, the host specificity of the mānuka phyllosphere microbiome remains to be determined, particularly in relation to phyllosphere microbiomes of physiologically and/or ecologically similar plants native to New Zealand.

In the present study, we used a multi-species, spatially hierarchical sampling design to systematically investigate whether the mānuka phyllosphere microbiome is primarily influenced by host species identity or dispersal in the absence of confounding environmental gradients. Specifically, we sampled focal mānuka and a neighborhood of three non-mānuka plant species at replicate sites separated by quasi-exponentially increasing distances (i.e., 4, 16, 80, 400, and 1800 m) along an 1800-m transect of native vegetation. Given the close host-microbiome association previously identified in the mānuka phyllosphere, we hypothesised that the assembly of the mānuka phyllosphere microbiome is overwhelmingly driven by host selection relative to the effects of local stochastic processes. To evaluate this, we addressed the following questions:

1. Do phyllosphere taxa disperse stochastically among mānuka and neighbouring phyllosphere microbiomes (i.e., does the distribution of phyllosphere taxa within each site reflect a random probability distribution)?

2. Is the mānuka phyllosphere microbiome colonised by specific bacterial communities that are distinct from ecologically similar plant neighbours?

3. Do patterns of host species identity or distance-decay prevail in the phyllosphere bacterial communities of these four plant species?

4. Are phyllosphere bacterial communities distinct from the surrounding environment (i.e., surface soil)?

2.3 Materials and Methods

2.3.1 Study site

This study was carried out on lands administered by the Lake Rotoaira Forest Trust adjacent to the Dual World Heritage Tongariro National Park in the Central North Island of New Zealand at an altitude of 800 m ASL (39°10 S; 175°46 E). The total land area of this site comprises 660.9 ha of native vegetation, of which 31.7% is forest and 68.1% is dense shrubland dominated by mānuka (*Leptospermum scoparium*) and kānuka (*Kunzea ericoides*) (Manaaki Whenua — Landcare Research). The mean annual temperature at the nearest weather station at Turangi (39.0 S, 175.8 E, 366 m ASL, 17 km from site) is approximately 12°C and mean annual rainfall is approximately 1,600 mm. Within this site, a single 1,800 m northeast transect was established across an area of mānuka-dominated scrub that exhibited visually homogenous topography and vegetation.

2.3.2 Sample collection

Within a single day in the summer of 2021, branch and surface soil samples were collected from a focal mānuka and three surrounding non-mānuka plant neighbours. Neighbouring plant species were kānuka (*Kunzea ericoides*), tawiniwini (*Gaultheria antipoda*), and toatoa (*Phyllocladus alpinus*), and one of each of these species was sampled approximately 4 m from the focal mānuka (Fig. 1). We selected these neighbouring plant species so that our samples would include leaf morphologies that range in similarity to mānuka. Data on leaf and plant characteristics for each species was compiled from the literature. This sampling design was replicated at six sites along the 1,800 m transect, separated by quasi-exponentially increasing distances ranging from 4 m to 1,400 m (Fig. 1). These distances (to the point of origin) were chosen because distance-decay curves are best fit by negative exponential functions. Three

branches per focal mānuka and one branch per neighbouring non-mānuka plant species were cut with clippers sterilised on site with 70% *v/v* ethanol/water, placed in individual sterile zip lock bags, and immediately placed on dry ice. Sampled branches were seemingly healthy with no obvious signs of herbivory or disease, and similarly sized. Surface soil (1–2 mm) from around the base of each tree was collected into sterile 50 mL Falcon tubes using a spatula sterilised on site using 70% *v/v* ethanol/water and immediately placed on dry ice. Upon return to the Thermophile Research Unit at the University of Waikato, branch and soil samples were frozen at −20°C until further analysis. Several attributes of each sample tree were measured during sampling. These included tree height, tree basal diameter, the distance of the focal mānuka to each of the three non-mānuka plant neighbours, as well as the height and aspect of each branch collected. GPS coordinates (WGS84 (G1762) in decimal minutes) were determined for each tree at the time of sampling.

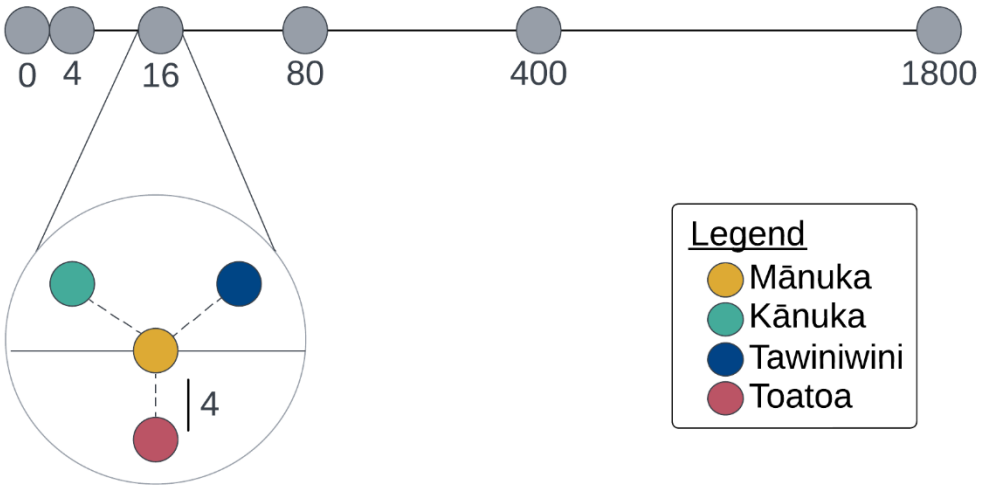

Fig. 1: Schematic representation of transect sampling design. Numbers represent quasi-exponentially increasing distances (m) between transect sample sites and individual sample trees at each site (inset). Colour represents host species identity of sample trees.

2.3.3 DNA extraction, amplification, and sequencing

Per branch, one gram of healthy, undamaged green leaves was carefully and aseptically excised and pooled. Epiphytes were recovered from the surface of excised leaves using a sonication protocol that has been previously described (Noble *et al.* 2020). Briefly, leaves were sonicated in 10 mL of phosphate buffer wash solution (100mM NaH2PO4, 1% tween 20) for 10 minutes using an ultrasonic cleaning bath (60 Hz). After sonication, the wash solution was decanted and the sonicated leaves were submerged and rinsed with another 10 mL of PBS. This rinse

step was repeated twice. The total wash solution (30 mL) was syringe filtered (90 µm) to remove fine plant debris and centrifuged (3,200 x g) for 30 minutes. The pellet was resuspended in 270 µl of PBS, transferred to a 2.0 mL bead tube containing 0.5 g each of 0.1 mm and 2.5 mm silica-zirconia beads, and frozen at −80°C until DNA extraction. Total epiphytic DNA was extracted from sonicated resuspensions using a modified cetyl trimethylammonium bromide (CTAB) bead-beating protocol, which has been proven to be highly effective for low biomass samples (Lee *et al*. 2011). For each surface soil sample, total DNA was extracted from 0.5 g of soil using the Power Soil DNA Extraction kit (Qiagen). DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, United States) and stored at −20°C until further analysis. The V4-V5 hypervariable region of the 16S rRNA gene was amplified with 515FY/926R fusion primers (5'GTGYCAGCMGCCGCGGTAA/5'CCGYCAATTY MTTTRAGTTT) and previously described PCR conditions (Noble *et al.* 2020). Briefly, 20 µl PCR reactions each contained: 5 ng of total DNA, 240 µM dNTPS, 1.2 x PCR buffer, 6 mM MgCl2, 0.016 mg/ml BSA, 0.2 mM of each primer, and 0.024 U Taq polymerase (Thermo Fisher Scientific, Massachusetts, United States). The following PCR conditions were used: 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR reactions were run in triplicate for each sample and subsequently pooled. A no template control (i.e., PCR blank) was also included in each PCR run. PCR products were cleaned and standardised with SequelPrepTM (Thermo Fisher Scientific). Standardised samples were pooled at an equimolar concentration into two libraries. An Illumina MiSeq 300PE sequencing run was performed on each library at Massey Genome Service (Palmerston North, New Zealand).

2.3.4 Sequence quality control and taxonomic assignment

The raw 16S rRNA gene amplicon sequences were processed using the DADA2 pipeline (Callahan *et al.* 2016). Forward and reverse reads were truncated at 237 and 232 bp, respectively, and quality filtered using the 'filterAndTrim' function with the following settings: maxN = 0, maxEE = c(3, 3), and truncQ = 2. Error rates were determined with the 'learnErrors' function and used to remove sequencing errors from forward and reverse reads, which were then assigned to amplicon sequence variants (ASVs) using the 'dada' function. Paired reads were then merged, converted into an ASV table, and chimeras removed with the removeBimeraDenovo function using the method 'consensus'. Taxonomy was assigned using the 'assignTaxonomy' and 'addSpecies' functions using the native implementation of the naive

Bayesian classifier and the SILVA database version 138.1 (Quast *et al.* 2012). Chloroplast and mitochondrial sequences were filtered out by removing all ASVs with a taxonomic assignment of 'Chloroplast' at the Order level and 'Mitochondria' at the Family level, respectively. Lastly, we applied the 'isContaminant' function (method = prevalence) from the package 'decontam' to our samples using our blank DNA extractions and PCR reactions to identify and remove putative contaminants introduced during processing (Davis *et al.* 2018).

After 16S rRNA gene sequence reads were quality filtered, one sample (Rp_03.1) stood out as an outlier. This sample was first identified due to its uniquely large number of sequencing reads (43,184) relative to all other samples (<27,000) (Supplementary Fig. 2A). This sample was dominated by the genus *Sodalis*, which comprised 32 ASVs and represented 62.4% of the reads of this sample (Supplementary Fig. 3). In comparison, *Sodalis* comprised only 0.25% and 0.57% of the reads in two other samples originating from the same tree and was entirely absent in all other samples. The genus *Sodalis* contains several insect endosymbionts and is thus very likely not a part of the native microbial phyllosphere population (Tláskal *et al.* 2021). Therefore, sample Rp_03.1 was removed from further analyses and 17,315 ± 4,128 quality-assured reads per sample remained.

2.3.5 Data analysis

All statistical analyses were conducted in R (R Core Team, 2022). Alpha diversity analyses were conducted using the 'vegan' package (Oksanen *et al.* 2007). Each sample was subsampled 100 times to an even sequencing depth (6,192 reads) and an average richness (observed number of ASVs) and Shannon-Wiener index was calculated for each sample. The Kruskal–Wallis test was used to evaluate significant differences across host species and sample type. Pairwise Bray-Curtis and Jaccard community dissimilarities were calculated using the 'vegdist' function on ASV relative abundance and presence/absence transformed data, respectively. Differences in community structure among host species were assessed using both an ANOSIM and PERMANOVA on community dissimilarities using the 'adonis' and 'anosim' functions in the 'vegan' package, respectively. Species-specific core microbiomes were determined using a 100% prevalence method with the underlying assumption that the spatial persistence of core ASVs across ecologically equivalent communities implies an important role of these ASVs in host plant fitness (Shade & Handelsman 2012; Shade & Stopnisek 2019). Indicator taxa analysis was performed using the 'multipatt' function in the 'indicspecies' package (De Cáceres *et al.* 2012). The normalized stochasticity ratio (NST) was used in the 'NST' package with the 'EF'

null model to estimate ecological stochasticity of community assembly; 50% was taken as the boundary point between more deterministic (< 50%) and more stochastic (> 50%) assemblies (Ning *et al.* 2019). Mantel tests were used to test the correlation between community dissimilarity and spatial distance. Benjamini-Hochberg corrected Kruskal-Wallis tests were used to evaluate significant differences in taxon relative abundance (i.e. core/indicator) and host species identity.

Occurrence probabilities provide a quantitative description of the likelihood of an organism being present at a location as well as a mechanism for testing hypotheses related to factors that influence occurrence (Royle *et al.* 2012). We used occurrence probabilities of taxa across the spatially structured phyllosphere microbiomes in our replicate transect sites to test hypotheses related to local dispersal. Assuming complete neutrality in the assembly of the phyllosphere microbiome (and assuming each tree at each site is exposed to a single homogenous pool of microorganisms), each ASV has the potential to exhibit one of 63 unique combinations of occurrence per site (corresponding to the unique combinations of presence/absence across each of the six microbiomes). The number of unique combinations of presence/absence were used to calculate zero-truncated probabilities that describe the likelihood of an ASV being present in any number of phyllosphere microbiomes (i.e. 1 – 6, herein termed *microbiome occurrence*) (See Fig. 2A-B for schematic explanation of how occurrence probabilities were generated, see Supplementary Table 2 for occurrence probabilities). Chi-square goodness-of-fit tests were used to determine whether the empirical presence/absence data (observed proportion of ASVs at each microbiome occurrence) differs from the distribution of occurrence probabilities, aka the predicted distribution. We next categorised the phyllosphere microbiomes as either "mānuka" or "non-mānuka". Organising the microbiomes as such provided two host groups of an equal number of spatially structured microbiomes per site (mānuka = 3, non-mānuka =3). Using the same assumptions of neutrality, we then used the same approach as above to calculate zero-truncated probabilities that describe the likelihood of an ASV being present at each microbiome occurrence (i.e. 1 – 3) within each host group (See Fig. 2C-D for schematic explanation of how occurrence probabilities were generated for individual host groups, see Supplementary Table 5 for occurrence probabilities). Chi-square goodness-of-fit tests were used to determine whether the empirical presence/absence data differs from the distribution of occurrence probabilities. Jennsen-Shannon divergence was used to calculate the distance between the empirical and predicted distributions for each host group. Lastly, we determined 'preferential host occupancy' for each ASV, defined as the host group in which any given ASV

had the largest occupancy. For example, if an ASV was present in all three mānuka microbiomes and one non-mānuka microbiomes, mānuka was considered the preferential host. The number of ASVs with ‘mānuka’, ‘non-mānuka’, or ‘no’ preferential host occupancy was determined for each microbiome occurrence and compared to the ratios generated for the predicted distribution.

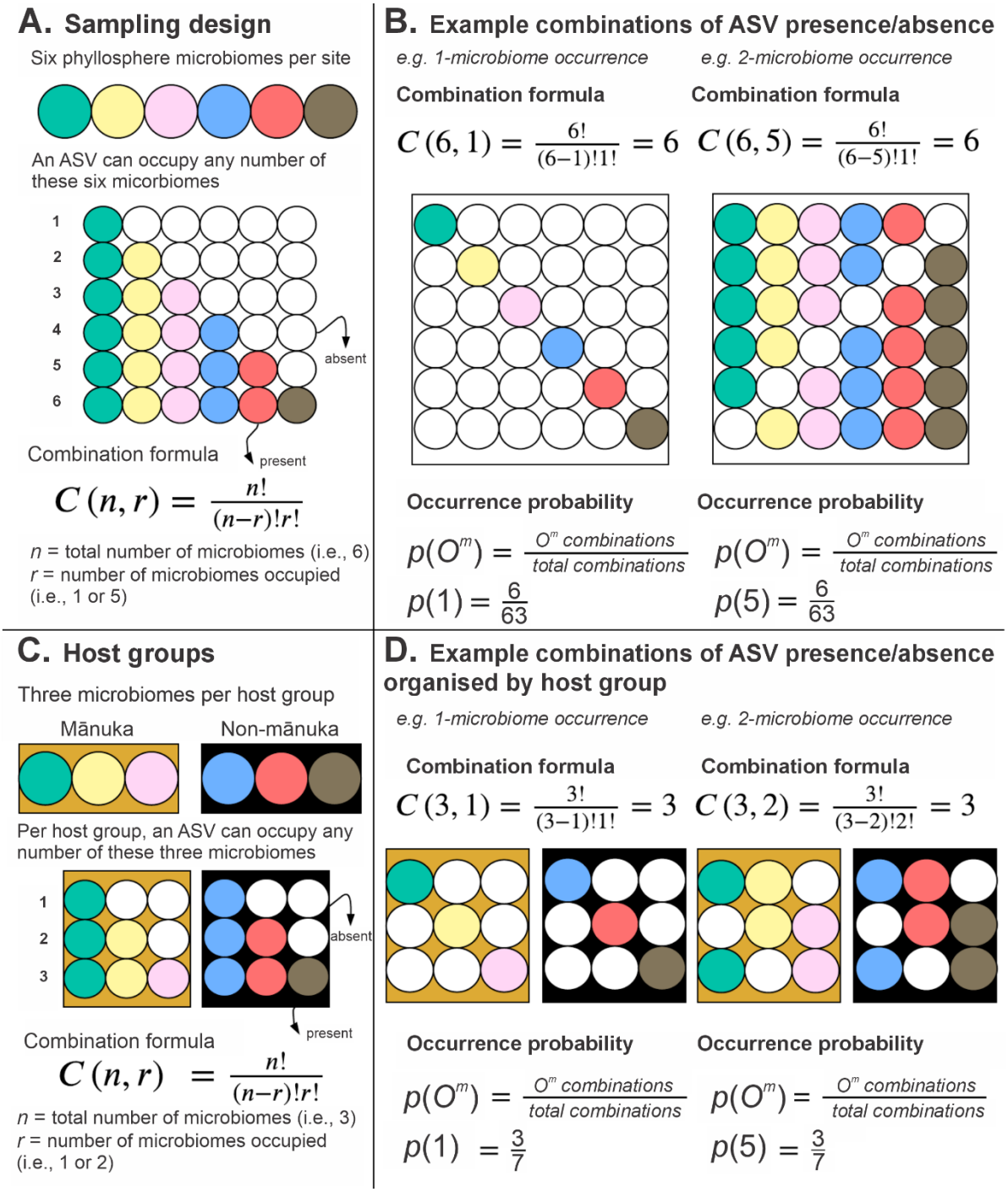

Fig. 2. Combinatorial schematic and occurrence probability approach. Each ASV has the potential to colonise any number of the six spatially structured phyllosphere microbiomes per site (termed microbiome occurrence, Om). For each microbiome occurrence, the number of unique combinations of ASV presence/absence is calculated using the combination formula. The total number of possible combinations for all microbiome occurrences (1 – 6) is 63. The probability than an ASV is present at a microbiome occurrence of N is calculated by dividing the number of unique combinations for N microbiome occurrence by the total number of combinations. **B)** For example, ASVs that are present in either one or five microbiomes can each exhibit six unique combinations of presence/absence. Therefore, the corresponding occurrence probability for each of these microbiome occurrences is 6/63. **C)** The

abstraction of two host groups, 'mānuka' and 'non-mānuka', were used to further organise the six phyllosphere microbiomes within each site. In this scenario, each ASV colonises any number of the three phyllosphere microbiomes per host group. For each microbiome occurrence, the number of unique combinations of ASV presence/absence is calculated using the combination formula. The total number of possible combinations for all microbiome occurrences per host group (1-3) is seven. The probability that an ASV is present in a host group at a microbiome occurrence of N is calculated by dividing the number of combinations of N microbiome occurrence by the total number of combinations. **D)** For example, ASVs that are present in either one or two microbiomes per host group can each exhibit three unique combinations of presence/absence. Therefore, the corresponding occurrence probability for each of these microbiome occurrences is 3/7.

2.4 Results

Phyllosphere samples were collected from focal mānuka (*L. scoparium*) and three neighbouring plant species, kānuka, tawiniwini, and toatoa, at six replicate sites separated by quasi-exponentially increasing distances along an 1800-m transect of native bush (Fig. 1). Neighbouring plant species were endemic to New Zealand and ranged in ecological and morphological similarity to mānuka (Supplementary Fig. 1, see Supplementary Table 1 for comparisons). Briefly, mānuka (family Myrtaceae) is a bushy evergreen shrub or tree that ranges in height up to 10 m but found at heights of 2 – 5 m in our study region (Whitehead *et al.* 2004; Stephens *et al.* 2005). Mānuka trees that were selected for sampling ranged in height from 2.6 – 3.6 m. Small white flowers emerge in our study region from late December to mid-January. Mānuka leaves are small (4-12 mm long) and narrow with a low leaf area index and a sharp point (Supplementary Fig. 1A). In addition, mānuka leaves are commonly described as xeromorphic, featuring adaptations such as a thick waxy cuticle (>10 μm) (Johnson 1980). Tawiniwini (family Ericaceae) is a bushy evergreen shrub generally 1 – 2 m in height (Bush *et al.* 2009). The tawiniwini plants selected for sampling ranged in height from 0.9 – 1.8 m. White or pink, solitary, bell-shaped flowers emerge in the sample region during November. Leaves on the tawiniwini plant are rounded (5 – 15 mm long), leathery, and toothed (Supplementary Fig. 1C). Toatoa (family Podocarpaceae) is a strongly aromatic gymnosperm shrub or tree that can reach heights up to 9 m (Wardle 1969). Toatoa trees that were selected for sampling ranged in height from 2 – 6 m. Members of the *Phyllocladus* genus, including toatoa, are distinguished by the presence of phylloclades, flattened stem structures that simulate the form and function of a foliage leaf (Supplementary Fig. 1D). Phylloclades are considered xeromorphic and are the only specialised photosynthetic organs in mature *Phyllocladus* plants (Keng 1974; Dörken *et al.* 2021). Given their morphological and functional equivalence to leaves, the phylloclades

on toatoa were sampled and processed synonymously with the leaf samples of the other plant species. Kānuka (family Myrtaceae) is a bushy, evergreen shrub or tree known to reach heights of up to 20 m but generally found at heights of 2 – 8 m in our study region (Burrows 1973; Whitehead *et al.* 2004). Kānuka trees selected for sampling ranged in height from 2.6 – 6.4 m. In our study region, clusters of small white flowers emerge from late December to mid-January. Kānuka leaves are small and narrow with a low leaf area index, similar to mānuka leaves, with a reduced point (Supplementary Fig. 1D). Kānuka exhibits many morphological and ecological similarities to mānuka and was formally known as *Leptospermum ericoides* until 1983 (Allen *et al.* 1992). Despite these superficial similarities, kānuka is in fact phylogenetically distinct from mānuka (Thompson 1983). Sequencing of bacterial 16S rRNA gene PCR amplicons from the phyllosphere of these plant species yielded 17,315 ± 4,128 quality-assured reads per sample. In total, 4,765 ASVs were identified at an average of 619 ± 120 ASVs per phyllosphere sample.

2.4.1 Patterns of local dispersal differ between mānuka and non-mānuka neighbouring plant species

Experimental phyllosphere studies have suggested that phyllosphere community composition can be directly influenced by the dispersal of microorganisms among plant neighbours, indicating phyllosphere microbiomes have the potential to act as both 'source' and 'sink' communities (Meyer *et al.* 2022). We therefore used occurrence probabilities (see Materials and Methods) and the observed distribution of taxon presence/absence to test a series of hypotheses aimed at tentatively inferring local patterns of dispersal within our six replicate transect sites.

> H1: microorganisms do not disperse between phyllosphere microbiomes (i.e. all six communities are sinks).

In a scenario of no inter-host dispersal, we would expect an entirely stochastic dispersal of microorganisms from the surrounding environment to each of our six spatially structured phyllosphere microbiomes (i.e. ASV presence/absence to be well described by a random probability distribution). We tested for this scenario by comparing the empirical distribution of ASVs (i.e. the proportion of ASVs that were observed at each microbiome occurrence) with their corresponding occurrence probabilities (Supplementary Table 2-3). We found that the empirical and predicted distribution of ASVs were significantly different (Chi-square goodness of fit $p < 0.001$, Fig. 3A, see Supplementary Table 4 for X^2 values). Compared to the predicted stochastic distribution, a larger proportion of ASVs were observed in a single microbiome. We

also observed a larger proportion of ASVs present in all six microbiomes than what was predicted for a completely stochastic dispersal. These observations suggest that chance alone is not sufficient to describe the distribution of taxa, indicating the possibility of dispersal from neighbouring plants.

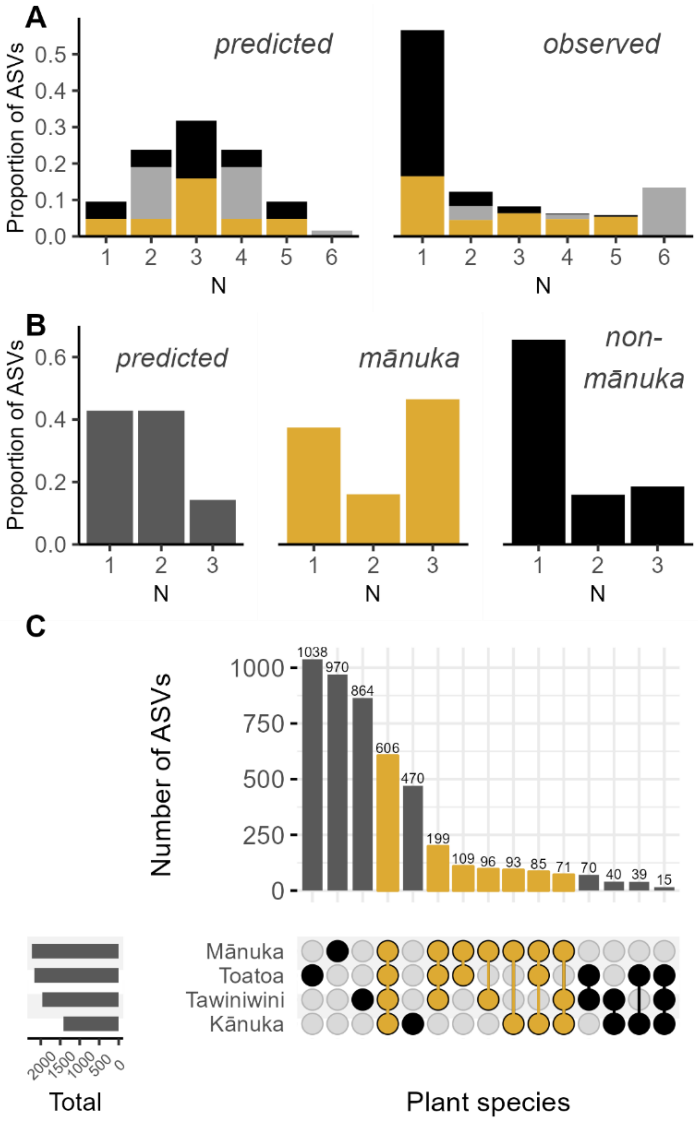

Fig. 3. The predicted versus observed distribution of ASVs across the spatially structured phyllosphere microbiomes within each site. **A)** Bar plots showing the proportion of ASVs predicted (left) versus the proportion of ASVs that were observed (right) for each microbiome occurrence. Within these bar plots, colour indicates the predicted (left) versus observed (right) proportion of preferential host occupancy: mānuka (yellow), non-mānuka (black), and no preference (grey). **B)** Bar plots showing the proportion of ASVs predicted (left) versus the proportion of ASVs that were observed for each microbiome occurrence within mānuka (middle) and non-mānuka (right) microbiome host groups. All proportions are averaged per site. **C)** Upset plot demonstrating the total number of shared and unique ASVs across each possible host species microbiome combination. Yellow represents host species combinations that include mānuka.

H2: microorganisms do not disperse between mānuka or non-mānuka phyllosphere microbiomes (i.e. mānuka and non-mānuka communities are sinks).

We next looked for differences between groups of host species by testing for a stochastic distribution of taxa within 'mānuka' and 'non-mānuka' phyllosphere microbiomes. As for H1, we tested this by comparing the empirical distribution of ASVs with their corresponding occurrence probabilities (Supplementary Table 5-6). We found that the observed distribution of ASVs in each of the mānuka and non-mānuka microbiome groups was significantly different to the predicted stochastic distribution (Chi-square goodness of fit $p < 0.001$, Fig. 3B, see Supplementary Table 7 for X^2 values). Compared to the predicted stochastic distribution, the non-mānuka phyllosphere had a larger proportion of ASVs that were present in only a single microbiome and a reduced proportion of ASVs that were present in two microbiomes (Fig. 3B). Meanwhile, the mānuka phyllosphere had a larger proportion of ASVs that were present in three microbiomes, and a reduced proportion of ASVs that were present in two microbiomes (Fig. 3B). Interestingly, the distance between the observed distribution of non-mānuka taxa and the prediction was less than the distance between the observed distribution of mānuka taxa and the prediction (One-sided Wilcoxon test on Jensen-Shannon divergence $p = 0.004$, see Supplementary Table 7 for Jensen-Shannon divergence values). Again, these observations suggest that factors other than chance, such as dispersal from neighbouring plants, influence the distribution of taxa within the microbiomes of each host group, and that the effect of such inter-host dispersal may be more prominent between mānuka phyllosphere microbiomes than between non-mānuka phyllosphere microbiomes.

H3: mānuka and non-mānuka phyllosphere microbiomes are equivalent sources of microorganisms.

To test our third hypothesis, we investigated whether there was an equal ratio of preferential host occupancy in taxa that were shared between mānuka and non-mānuka phyllosphere microbiomes (see Materials and Methods for definition of preferential host occupancy). Overall, we found that the majority of ASVs that are present in at least three out of six microbiomes had a preferential occupancy in mānuka (i.e. partially dispersed taxa were more likely to be absent from non-mānuka phyllosphere microbiomes) (Fig. 3A). Finally, looking at the total number of unique and shared ASVs across different combinations of host species, we noticed that a substantially smaller number of taxa are shared by any combination of non-

mānuka host species, compared to any combination of mānuka and non-mānuka host species (Fig. 3C). In other words, the unequal distribution of microorganisms across the phyllosphere microbiomes of mānuka and non-mānuka suggests that a larger number of microorganisms have dispersed from mānuka to neighbouring non-mānuka plants than from neighbouring non-mānuka plants to mānuka.

2.4.2 Mānuka phyllosphere communities are dominated by core microorganisms

In our previous study, we identified a widely distributed core microbiome in the mānuka phyllosphere. We sought to verify this finding by searching for a core microbiome within our regional and spatially structured study design. Given the importance of defining ecologically relevant core microbiomes within tissue- and species-specific host microbiomes, we used a stringent criterion of 100% prevalence within each individual host species. Using this criterion, we identified 280 core ASVs in the mānuka phyllosphere microbiome (Fig. 4A). These taxa comprised two phyla (Acidobacteria and Proteobacteria) and four genera (*Granulicella* and *Terriglobus*, as well as *1174-901-12* (Rhizobiales) and *Methylocella*, respectively) (Supplementary Fig. 4A). We also applied this core criterion to each neighbouring plant species. Across all kānuka samples, 212 core ASVs were identified. These taxa comprised two phyla (Acidobacteria and Proteobacteria) and four genera (*Granulicella*, as well as *1174-901-12* and *Methylocella*, respectively) (Supplementary Fig. 4B). Across all tawiniwini samples, 209 ASVs were identified. These taxa comprised two phyla (Acidobacteria and Proteobacteria) and three genera (*Granulicella*, as well as *1174-901-12* and *Methylocella*, respectively) (Supplementary Fig. 4C). Lastly, 92 core ASVs were identified across all toatoa samples. These taxa comprised a single phylum (Proteobacteria) and two genera (*1174-901-12* and *Methylocella*) (Supplementary Fig. 4D). The relative abundance of core taxa was significantly different for each host species; the core taxa in mānuka and kānuka had a greater relative abundance compared to core taxa in tawininwini and toatoa (Benjamini-Hochberg corrected Kruskal-Wallis $p < 0.05$, Fig. 4B, Supplementary Fig. 5A-C).

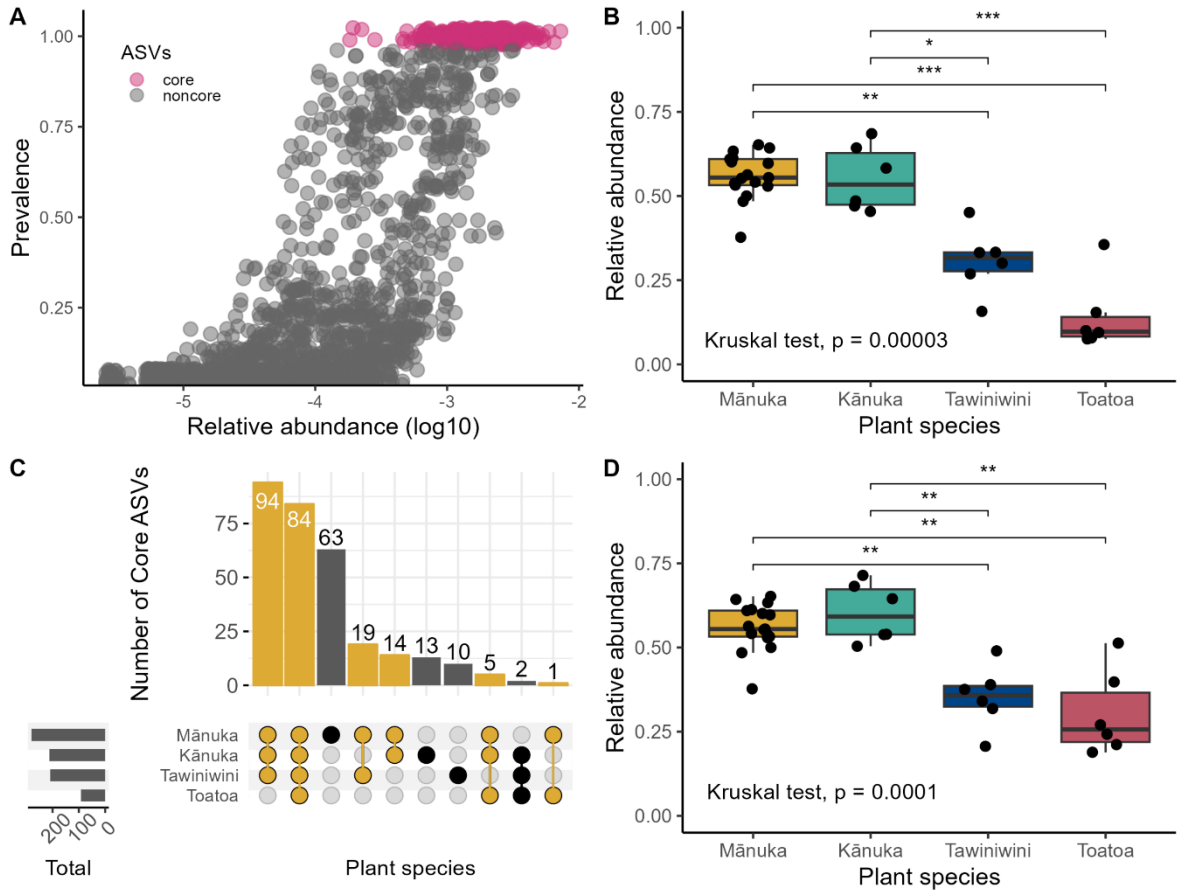

Fig. 4: The relative abundance and distribution of host species-specific core microbiomes. **A)** Relative abundance (log10) versus prevalence of ASVs in the mānuka phyllosphere microbiome. Pink represents 280 ASVs with 100% prevalence across all mānuka samples that were defined as members of the mānuka core microbiome. **B)** The cumulative relative abundance of species-specific core ASVs identified in each individual host species. **C)** Upset plot showing the distribution of core ASVs among host plant species. Yellow bars represent host species combinations that include mānuka. **D)** The cumulative relative abundance of mānuka core ASVs in each host species. Asterisks in **B)** and **C)** indicate significance level of Kruskal-Wallis test.

We next examined the overlap and relative abundance of core taxa across host species (Fig. 4C). In total, 63 ASVs in the mānuka core microbiome were unique to mānuka (i.e. not identified in the core microbiome of any other surrounding plant species). In contrast, very few ASVs in the core microbiome of neighbouring host plants were unique to these species as the majority of ASVs identified in the kānuka, tawiniwini, and toatoa core microbiomes were also identified as members of the mānuka core microbiome. The largest proportion of shared core taxa included 94 ASVs that were common to mānuka, kānuka, and tawiniwini. This was followed by 84 ASVs that were identified in the core microbiome of all four host species. Notably, the relative abundance of mānuka core taxa was significantly higher in mānuka and

kānuka compared to tawiniwini and toatoa (Benjamini-Hochberg corrected Kruskal-Wallis $p < 0.05$, Fig. 4D).

2.4.3 Phyllosphere community composition varies across host species

To investigate the role of host selection in shaping the phyllosphere microbiome, we examined the effect of host species identity on community structure. Across all phyllosphere samples, 16 phyla were detected of which Proteobacteria was the most represented phylum (average relative abundance 61.4%), followed by Acidobacteriota (23.7%), Verrucomicrobiota (5.5%), and Bateroidota (4.4%) (Supplementary Table 8). Host species-associated differences in composition were detectable at low levels of taxonomic resolution, with six out of the total 16 phyla exhibiting significant differences in relative abundance (Benjamini-Hochberg corrected Kruskal-Wallis $p < 0.05$, Supplementary Table 9-10). At the ASV level, microbial community composition was significantly different across host species (Fig. 5A-B) (ANOSIM based on Bray-Curtis and Jaccard; R = 0.75, $p = 0.001$ and R = 0.77, $p = 0.001$, respectively). Host species identity explained 26% of variation in overall phyllosphere community structure (PERMANOVA on Bray-Curtis and Jaccard dissimilarities, $p = 0.001$). Notably, no significant difference in phyllosphere richness or diversity were identified across host species ($p > 0.05$) (Supplementary Fig. 6A-B, see Supplementary Table 11-12 for raw and averaged alpha diversity values).

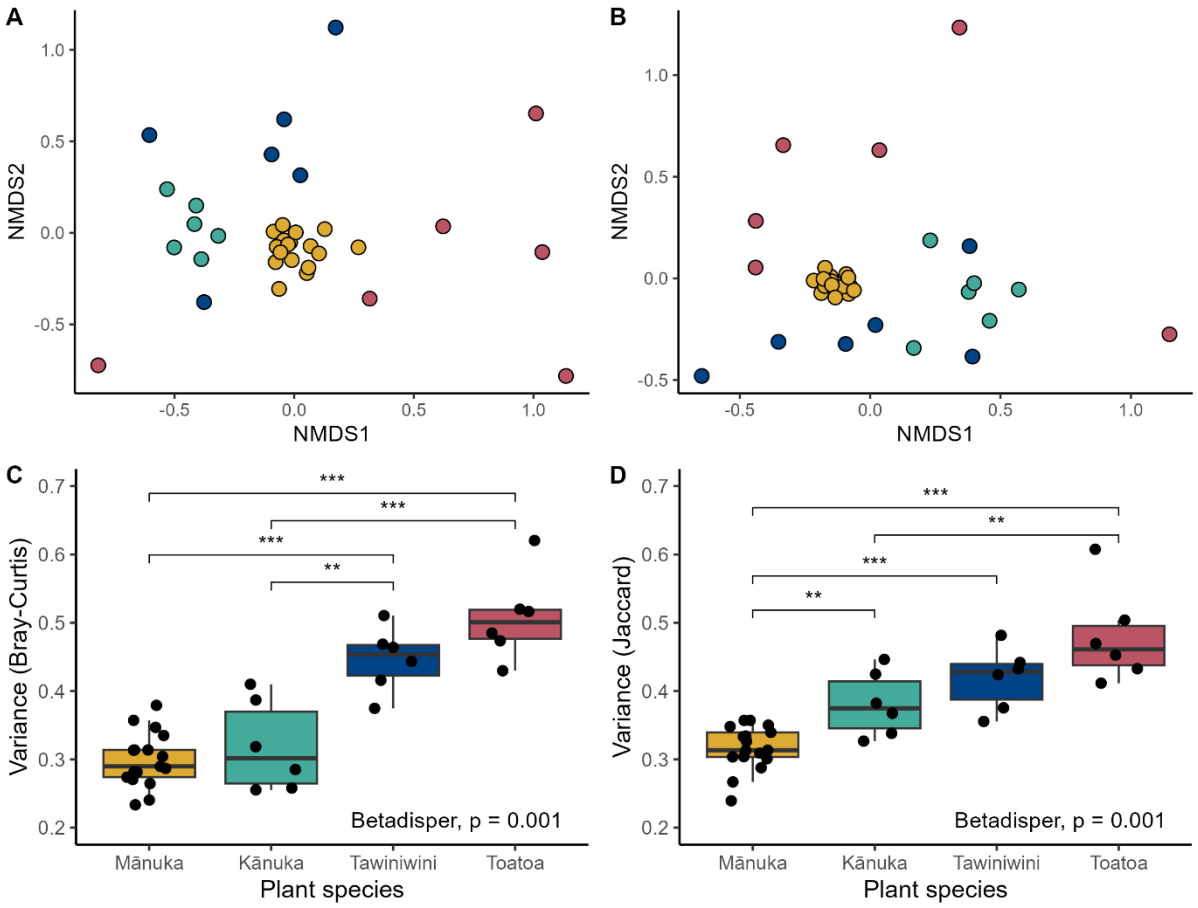

Fig. 5: Phyllosphere microbiome community structure shows significant differences across host species. NMDS ordination of A) Bray-Curtis (stress = 0.15) and B) Jaccard (stress = 0.13) community dissimilarities (PERMANOVA, $R^2 = 0.26$, $p = 0.001$). Box plot showing the corresponding intraspecies variance in Bray-Curtis **C)** and Jaccard **D)** phyllosphere community dissimilarity. NMDS colours in **A)** and **B)** depict host species identity, as in **C)** and **D)**. Asterisks denote significance values of pairwise Betadisper.

To test for significant associations between individual ASVs and each host species, an indicator species analysis was performed and 485 taxa were identified. In total, 217 ASVs were significantly associated with mānuka ($p < 0.05$) and comprised 31.8 ± 5% of the total community in the mānuka phyllosphere. A total of 129 ASVs were significantly associated with kānuka ($p < 0.05$) and comprised 38.9 ± 8.6% of the total community in the kānuka phyllosphere. Only 65 and 74 ASVs were significantly associated with tawiniwini and toatoa ($p < 0.05$), representing 8.5 ± 2.2% and 6.3 ± 3.2% of each host species' total community, respectively. Indicator taxa were distinctive at the genus level across each of the four host species (Fig. 6A-D). Indicator taxa in the mānuka phyllosphere largely comprised taxa belonging to the genera: *Bryocella*, *Granulicellam*, *LD29*, *Methylocella*, *Terriglobus*, and

1174-901-12. Meanwhile, indicator taxa in the kānuka phyllosphere largely belong to the genera: *Methylocella*, *Sphingomonas*, and *1174-901-12*. In contrast, indicator taxa in the tawiniwini and toatoa phyllosphere comprised small relative abundances of many of the genera identified in mānuka and kānuka, as well as *Aurantisolimonas* and *Edaphobacter* and *PMMR1*, respectively. Taxonomic differences also persisted at the phylum level (Kruskal-Wallis $p < 0.05$, Supplementary Table 13-14). Notably, mānuka indicator taxa were present at low relative abundances in neighbouring host species (Fig. 6A). A similar pattern was also observed for kānuka indicator taxa (Fig. 6B). However, indicator taxa identified for toatoa and tawiniwini were essentially absent from the mānuka and kānuka phyllosphere microbiomes (Fig. 6C-D).

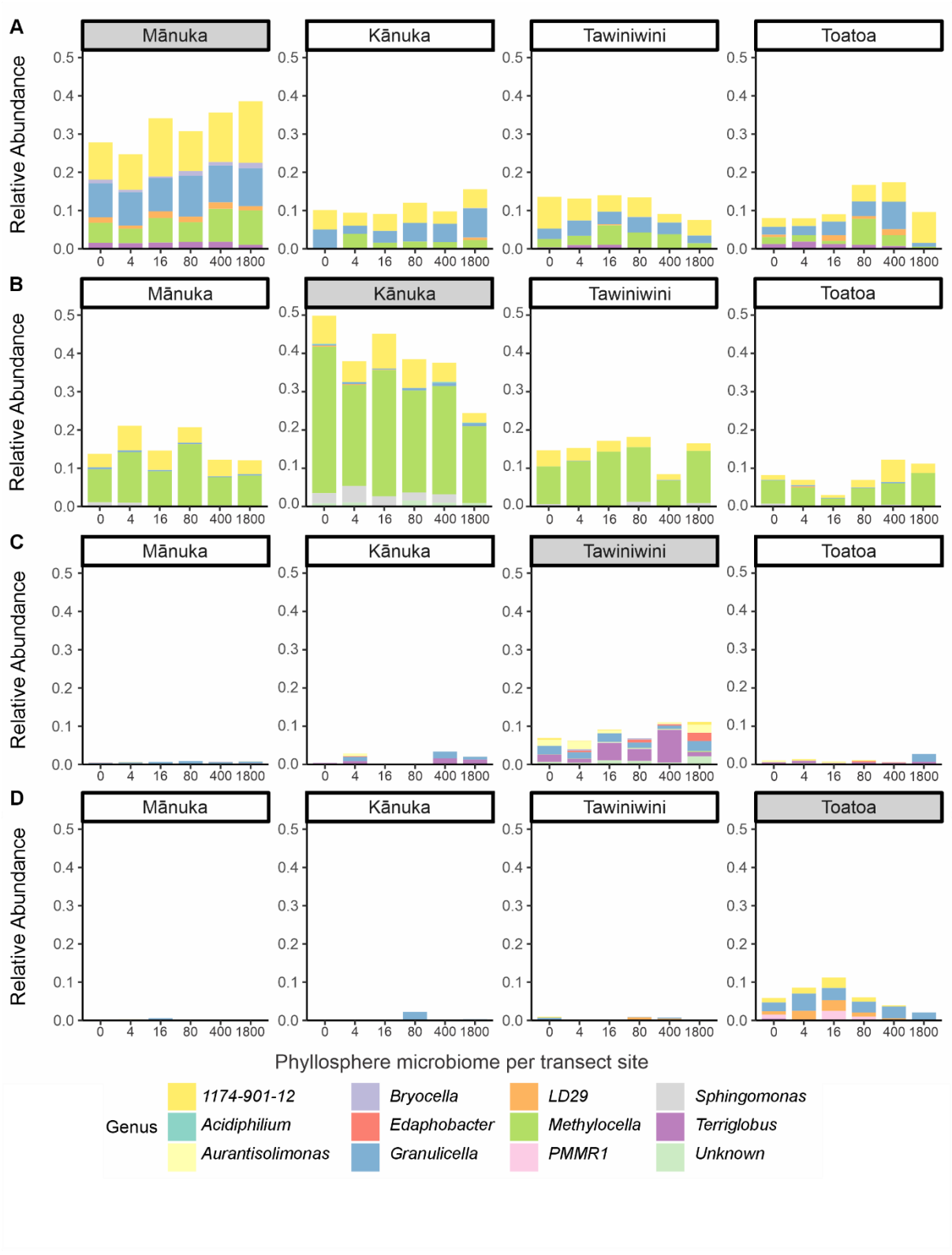

Fig. 6: The relative abundance of indicator taxa that are significantly associated with individual host species. Bar plots show the relative abundance of **A)** mānuka, **B)** kānuka, **C)** tawiniwini, and **D)** toatoa indicator taxa across all species. Colours represent taxonomic classification at the genus level.

2.4.4 Relative contributions of phyllosphere community assembly mechanisms differ across host species

We used intraspecies variation of phyllosphere community dissimilarity as an indicator of the relative strength of host selection on community structure. Notably, intraspecies variation differed significantly across each of the four host species. Mānuka and kānuka both exhibited significantly less relative abundance-based variation compared to tawiniwini and toatoa (Betadisper $p < 0.001$, Fig. 5C, Supplementary Table 15). Furthermore, mānuka also exhibited significantly less presence/absence-based variation compared to all other neighbouring plant species (Betadisper $p < 0.001$, Fig 5D, Supplementary Table 15). To complement these results, we also applied a null modelling approach using the NST index to quantify stochasticity in taxonomic community structure (Ning *et al.* 2019). On average, phyllosphere communities belonging to all host species had NST values less than 0.5, indicating the phyllosphere microbiome generally assembles in a deterministic manner (Supplementary Table 16). However, the NST values of mānuka and kānuka phyllosphere communities were significantly lower than the NST values of tawiniwini and toatoa phyllosphere communities, indicating a difference in the magnitude of stochasticity between host species (Supplementary Table 17).

2.4.5 Distance-decay in the phyllosphere microbiome

To better understand the role of dispersal limitation in the phyllosphere microbiome, we next used mantel tests to investigate the relationship between community dissimilarity and distance. No overall relationship was observed between phyllosphere community dissimilarity and distance (i.e. across heterospecific samples), highlighting the overarching presence of host species identity (and thus significant inter-species variation) irrespective of spatial distance (Figure 7a, Supplementary Table 18). We then tested the relationship between community dissimilarity and distance separately for each host species and significant host-specific distance-decay relationships were identified. Community dissimilarity in the mānuka phyllosphere exhibited a significant relationship with distance (Mantel R = 0.32, $p = 0.009$). This relationship was observed in low relative abundance community members (Mantel R = 0.45, $p = 0.001$), high relative abundance members (Mantel R = 0.39, $p = 0.001$), and taxon presence-absence (Mantel R = 0.41, $p = 0.001$) (Fig. 7B, Supplementary Table 19). A significant relationship was also present in the high abundance members of the Toatoa and Tawiniwini phyllosphere (Mantel R = 0.80, $p = 0.02$; Mantel R = 0.68, $p = 0.009$, respectively) (Fig. 7C-D, Supplementary Table 19). Similar trends were observed in the kānuka phyllosphere, however, none of these were statistically significant (Supplementary Table 19). Further, no

relationship was observed between taxon presence-absence, low abundance community members, and distance in the phyllosphere microbiome of toatoa and tawiniwini.

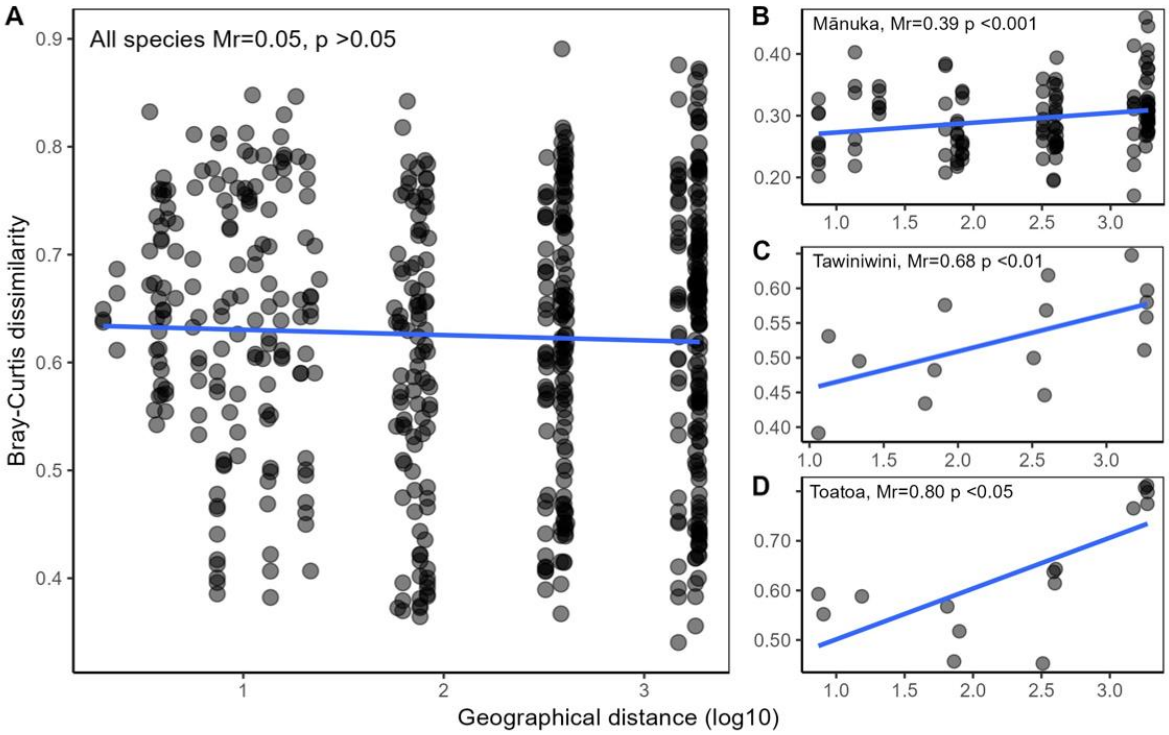

Fig. 7: The relationship between phyllosphere community dissimilarity (Bray-Curtis) and geographical distance (log10). **A)** No overall relationship was observed across all host species. Significant (p values < 0.05) species-specific distance-decay relationships were observed in the high abundance members of the **B)** mānuka, **C)** tawiniwini, and **D)** toatoa phyllosphere microbiomes.

2.4.6 The surface soil microbiome is distinct from the phyllosphere microbiome

Previous studies have suggested surface soil is a source of microorganisms for the phyllosphere microbiome (de Souza *et al.* 2016). We collected surface soil to evaluate potential contributions of surface soil microorganisms to the phyllosphere of different host species. In total, 24 surface soil samples were collected from the base of each sample tree and yielded 15,927 ± 3,241 quality-assured reads per sample. In total, 8,781 ASVs were identified, with an average of 550 ± 119 ASVs per sample. A total of 25 phyla were detected across all samples (Supplementary Table 20). Proteobacteria were the most represented phyla (average relative abundance 34.2%), followed by Bacteroidota (17.9%), Verrucomicrobiota (17.8%), Acidobacteriota (15.2%), and Planctomycetota (6.0%) (Supplementary Fig. 7). We examined whether host species identity of the tree from which each surface soil sample was collected had any influence on community

structure; however, no effect was observed (PERMANOVA on Bray Curtis and Jaccard dissimilarities $p > 0.05$) (Supplementary Fig. 8). Mantel tests were used to test for a decay of community similarity with distance, however, no significant relationship was observed (Mantel $p > 0.05$) (Supplementary Table 21). Notably, in contrast to the phyllosphere microbiome, no ASVs were 100% prevalent across surface soil samples (Supplementary Fig. 5D).

Overall, the community structure of surface soil was distinct from the phyllosphere (Supplementary Fig. 9). A significant difference in community richness ($p = 0.04$) but not diversity ($p > 0.05$) was identified between surface soil and phyllosphere microbiomes (Supplementary Fig. 6C-D, see Supplementary Table 22 for raw values). Notably, only 38 ASVs were shared between all phyllosphere microbiomes and surface soil samples (Supplementary Fig. 10A). The number of taxa shared between the phyllosphere microbiomes and surface soil samples was relatively consistent across host species, ranging from 22 – 29 ASVs (Supplementary Fig. 10B-E).

2.5 Discussion

Stochastic processes, such as dispersal, operate in conjunction with host selection to shape the composition of host-associated microbiomes. However, the relative importance of each of these processes in the assembly of the phyllosphere microbiome remains unclear. In our previous study, we identified a core microbiome in the *Leptospermum scoparium* (mānuka) phyllosphere that was persistent across five environmentally diverse and geographically distinct populations (Noble *et al.* 2020), making the mānuka phyllosphere an ideal model system for interrogating processes of community assembly. In this study, we used a multi-species, spatially explicit sampling design to examine the relative influence of host species identity and spatial distance in the assembly of the mānuka phyllosphere microbiome. Overall, host species identity had a stronger influence on phyllosphere community composition than distance. However, the relative influence of host species identity was different for each host species. Compared to ecologically and morphologically similar plant neighbours, a consistent and distinct community composition was observed in the mānuka phyllosphere microbiome, providing strong evidence in support of our hypothesis that host selection is the main driver of community assembly on the mānuka leaf surface. Conversely, the phyllosphere microbiome of kānuka (*Kunzea ericoides*), tawiniwini (*Gaultheria antipoda*) and toatoa (*Phyllocladus alpinus*) exhibited a higher degree of compositional stochasticity. Accordingly, the phyllosphere

microbiome of non-mānuka native plants exhibited stronger distance-decay relationships than the phyllosphere microbiome of mānuka. Furthermore, the distribution of taxa within each site deviated significantly from random probability distributions, suggesting that the local movement of microorganisms among phyllosphere microbiomes of adjacent plant species is not purely random.

Intraspecies variation in phyllosphere community composition was significantly different across the four host species in our study, suggesting that the capacity to select for a consistent phyllosphere microbiome, and thus the relative strength of host selection, is variable across plant species. Previous studies have reported contradictory results regarding the relative importance of host selection versus microbial dispersal in the assembly of the phyllosphere microbiome. For example, Redford *et al.* (2010) found that the intraspecies variation of phyllosphere communities on *Pinus ponderosa* trees separated by distances of up to 14,000 km was significantly less than the interspecies variation between *P. ponderosa* and sympatric *Pinus* species. In contrast, Finkel *et al.* (2011) reported that the interspecies variation between co-occurring *Tamarix* species was less than the intraspecies variation of allopatric *Tamarix* species. However, in light of our findings, it is plausible that plant species vary strongly in the degree to which they associate and interact with their phyllosphere microorganisms. Hence, we propose that host selection of phyllosphere communities is an essential hypothesis that requires empirical testing on a species-by-species basis before embarking on experimental studies. Given that microbiomes subject to strong host selection are more likely to perform functions beneficial to the host compared to communities that assemble stochastically (Hammer *et al.* 2019; Jackrel *et al.* 2021), identifying plant species that exert strong selection on their phyllosphere microbiome (i.e., mānuka in the current study) will be paramount for generating targeted and falsifiable hypotheses designed to enhance our understanding of specific plant-microbial relationships. These hypotheses can then be tested using rational experimental designs, which have historically founded our understanding of plant-pathogen interactions in the phyllosphere (Lindow & Brandl 2003).

Despite the growing number of empirical-based studies that demonstrate patterns of host species identity in phyllosphere community composition (Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016; Laforest-Lapointe *et al.* 2017; Yao *et al.* 2020; Li *et al.* 2022a; Smets *et al.* 2023; Duan *et al.* 2024), the mechanism(s) of host selection in the phyllosphere microbiome are still mostly elusive. Our unique comparison of phyllosphere communities

belonging to plant species that exhibit ecological and morphological similarity to one another permits mechanistic interpretation. We noticed that several general characteristics of the kānuka phyllosphere microbiome, such as intraspecies variation (Fig. 5C-D) and the relative abundance of core (Fig. 4B and Fig. 4D) and indicator (Fig. 6) taxa, were more similar to mānuka than either of the other two plant species. Considering that mānuka and kānuka are morphologically similar species, this may be a result of similar selection pressures exhibited by the shared characteristics of their leaf surfaces. For example, mānuka and kānuka trees in our study region have similar leaf nitrogen, stomatal conductance, specific leaf area, and wood density (Whitehead *et al.* 2004; Marden *et al.* 2021), traits that have been previously associated with interspecies variation in both bacterial and fungal phyllosphere community composition (Kembel & Mueller 2014; Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016). However, despite these similarities, the composition of the mānuka phyllosphere microbiome remained distinct from that of kānuka, suggesting that the mechanism of selection in the mānuka phyllosphere microbiome is independent of these general leaf traits. This observation, together with our previous study that identified a remarkably persistent host association (Noble *et al.* 2020), is parsimonious with mānuka having a direct influence on the structure and composition of its phyllosphere bacterial communities. Interestingly, mānuka is a highly aromatic plant species and mānuka leaves exhibit distinct chemical profiles compared to kānuka leaves. For example, the volatile organic compounds (VOCs) in mānuka leaves are comprised of monoterpene hydrocarbons (5%), sesquiterpene hydrocarbons (60-70%), and triketones (20%) (Perry *et al.* 1997a). In contrast, the VOCs in kānuka leaves are largely comprised of monoterpenes (75%) (Perry *et al.* 1997b). Therefore, it is conceivable that a chemically-mediated mechanism of host selection may be taking place in the mānuka phyllosphere microbiome. The direct recruitment of microorganisms via the secretion of specific chemical molecules, including plant-derived VOCs, has been well documented in the rhizosphere and endosphere (Junker & Tholl 2013; Pang *et al.* 2021). Furthermore, direct recruitment of a disease-suppressive microbiome via specific chemical-signalling has been recently demonstrated in the phyllosphere of tomato (Gupta *et al.* 2022).

Compared to mānuka, the community structure of the toatoa and tawiniwini phyllosphere microbiome appeared highly variable (Fig. 5A-B), suggesting that stochastic processes play a larger role than the identity of the host in shaping the phyllosphere microbiome of these plant neighbours. In accordance with this observation, theoretical studies have shown that stochastic processes are more likely to contribute to microbiome community structure when selective

forces by the host are considerably reduced (Evans *et al.* 2017). Further, abundant taxa in the toatoa and tawiniwini phyllosphere microbiome exhibited a strong and significant decay in community similarity with distance (Fig. 7C-D), highlighting a role of dispersal limitation in structuring the abundant members of their communities. A significant, albeit weaker, distance-decay relationship was also identified in mānuka phyllosphere communities (Fig. 7B), which also demonstrates the underlying role of stochastic processes in shaping the structure of the phyllosphere communities even in the presence of strong host selection. This finding is in-line with a previous greenhouse study that found spatial variation in the relative abundance of phyllosphere taxa on *Arabidopsis thaliana*, despite a strong convergence in communities over time (Maignien *et al.* 2014). It is notable that we only observed distance-decay relationships within individual host species, rather than across different host species (Fig. 7). This shows us that even though the assembly of the tawiniwini and toatoa phyllosphere microbiome was more stochastic than mānuka, the effect of dispersal in the presented study was not strong enough to obscure the overall effect of host species identity (i.e., intraspecies variation was on average smaller than interspecies variation). The identification of species-specific distance-decay relationships supports two previous studies that observed significant relationships within the phyllosphere of a single host species (Finkel *et al.* 2012; Stone & Jackson 2016), and may also explain why distance-decay relationships have generally not been observed across heterospecific phyllosphere samples (Smets *et al.* 2023). Moreover, these findings emphasise the importance of choosing appropriate sampling scales when testing for specific ecological patterns, such as using exponentially increasing distances for testing exponential models of distance-decay (Nekola & White 1999).

While mānuka's phyllosphere community was distinct, we did observe an overlap of abundant taxa across host species. Within our sampling design, focal mānuka and neighbouring plant species were separated by close and consistent distances (~ 4 m) to equalise dispersal opportunities among host species. However, the distribution of microorganisms within each site was not congruent with a scenario in which taxa were dispersed with equal probability across mānuka and neighbouring phyllosphere microbiomes (Fig. 3A-B). Instead, a large proportion of taxa was shared between mānuka and non-mānuka host species while few taxa were shared among non-mānuka host species (Fig. 3C). Further, taxa that were shared among multiple microbiomes typically had a higher occurrence in mānuka than neighbouring host species (Fig. 3A). While speculative, a logical interpretation of these results is that inter-host dispersal may be occurring in such a way that mānuka is acting as a source of specific

phyllosphere microorganisms for neighbouring plant species. Other evidence in support of this idea includes the presence of key members of the mānuka microbiome (i.e. core ASVs and indicator taxa) in neighbouring species and a correspondent lack of neighbouring plant species' key taxa in mānuka. Furthermore, this interpretation is in agreement with three previous lines of evidence: 1) plants can influence the composition of local airborne communities (Lymperopoulou *et al.* 2016), 2) the presence of plant neighbours is linked to variation in phyllosphere community size and composition (i.e., neighbour effect) (Lindow & Andersen 1996; Meyer *et al.* 2022), and 3) neighbour effect varies with crop plant species identity (Lindow & Andersen 1996; Meyer *et al.* 2022). Our study builds on these results by suggesting a source-sink dynamic may establish between phyllosphere microbiomes that experience different relative strengths of host selection. An alternative explanation could be that shared taxa between mānuka and neighbouring plant species are cosmopolitan within our sampling region. However, this interpretation is less congruent with the specificity of the mānuka phyllosphere. In addition, phyllosphere taxa were essentially absent in surface soil, confirming the distinctiveness of the phyllosphere microbiome from the surrounding environment. Together, our results raise the question as to whether conventional sampling designs used to infer the neutral role of dispersal (Nekola & White 1999; Condit *et al.* 2002) are sufficient to capture the complex reciprocal nature of dispersal in the phyllosphere microbiome. Incredibly strategic sampling designs will be required to shed further light on these complicated and intricately linked processes.

Our work demonstrates the potential of mānuka as an intriguing model plant species for developing our mechanistic understanding of plant-microbiome interactions in the phyllosphere of woody perennials. Now that we have established that a strong, species-specific association (and thus host-microbiome interaction) likely exists between mānuka and its associated phyllosphere microorganisms, identifying patterns in community structure or particular microorganisms (i.e. core taxa) that associate with differences in host functional traits will be useful to identify specific microbial targets for future functional analyses. A combination of shotgun metagenomic sequencing, cultivation approaches, manipulative experiments, and microscopy will be essential to elucidate the functional potential of target microorganisms and the influence they have on the functional traits of mānuka, including economically important traits such as nectar DHA. Finally, continued investigation utilising the unique comparison provided by mānuka and kānuka will be beneficial to confirm a role of chemical signalling in host selection of mānuka phyllosphere communities.

2.6 Conclusions

An ongoing debate exists in the literature regarding the relative importance of host selection versus microbial dispersal in the assembly of natural phyllosphere communities. Nevertheless, the design and data interpretation of a growing number of experimental studies using model plant species is based on the unverified assumption that host selection plays a significant and universal role. Using a systematic sample design in a native New Zealand bush, we demonstrate the first attempt to quantify and compare the contribution of host species identity and dispersal in the phyllosphere microbiome of different plant species. Moreover, our results reveal that the relative influence of each of these processes are not universal across plant species. The mānuka (*L. scoparium*) phyllosphere microbiome appeared to be more strongly influenced by host selection, whereas the phyllosphere microbiome of neighbouring native plant species appeared to be more strongly influenced by microbial dispersal. The mānuka phyllosphere microbiome was distinct from an ecologically and morphologically similar neighbouring plant species, suggesting that the specificity of the mānuka phyllosphere is not merely a result of general leaf characteristics. Instead, we propose a direct, chemically-mediated recruitment of microorganisms is taking place on the mānuka leaf surface. Furthermore, the distribution of phyllosphere taxa within each site reflects a scenario in which microorganisms disperse between neighbouring host plants (i.e. inter-host dispersal). However, as an extension to this previously established concept, we provide new evidence that suggests that the relative strength of host selection influences inter-host dispersal such that phyllosphere microbiomes that are strongly influenced by host selection may act as source of microorganisms to phyllosphere microbiomes that are only weakly influenced by host selection. Overall, the evidence presented in this study emphasises the importance of using explicit terminology, carefully structured sampling designs, and falsifiable hypotheses to investigate the complex ecological processes that drive the assembly of phyllosphere communities in natural environments. Several new perspectives are provided for future investigations focused on advancing our mechanistic understanding of community assembly and plant-microorganism relationships in the phyllosphere.

2.7 Data Availability

Raw sequencing data generated for this study is available on the NCBI Sequence Read Archive (SRA) under the accession number PRJNA1108893 and SRA accessions SRX24489499-SRX24489558. The R scripts used for main analyses and figures can be found on the GitHub page https://github.com/asn6/NZ-phyllosphere-transect.

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Chapter III

Phyllosphere microbial dispersal exhibits a source-sink relationship among ecologically and morphologically similar plant species

3.1 Abstract

Since early cultivation-based studies, the phyllosphere microbiome has long been recognised to act as both a source and a sink of dispersing airborne microorganisms. Among these early studies, work by Lindow and Andersen (1996) demonstrated that plants may vary in the extent to which they act as microbial sources and hypothesised that the relative strength of a host plant's phyllosphere microbiome as a source (herein termed *source-strength*) is correlated with leaf morphological traits. Under this hypothesis, no source-sink relationship is to be expected between plant species with similar leaf morphologies. Despite the potential significance of such inter-host dispersal for our understanding of phyllosphere microbial ecology, the hypothesis by Lindow and Andersen (1996) has not been systematically interrogated. Here, we use an ecologically and morphologically indistinguishable pair of plant species, *Leptospermum scoparium* (mānuka) and *Kunzea ericoides* (kānuka), to examine this untested conjecture. We demonstrate that the distribution of taxa among closely located phyllosphere microbiomes exhibits a high degree of spatial heterogeneity. Furthermore, the patterns of taxon presence/absence revealed by our study are consistent with a scenario in which mānuka may act as a stronger source of microorganisms for kānuka. Extending on the observations made by Lindow and Andersen (1996), these results suggest that factors other than leaf morphological traits may also determine phyllosphere source-strength. This work highlights the importance of investigating processes of microbial dispersal at small and relevant spatial scales and emphasises the relevance of both recognising and verifying the hypotheses established by pioneering cultivation-based studies on entire microbial communities with cultivation-independent techniques.

3.2 Introduction

A long-standing hypothesis by Lindow and Andersen (1996) states that the relative strength of a host plant's phyllosphere microbiome as a source of local airborne communities, and thus the phyllosphere microbiome of surrounding plant neighbours, is correlated with the leaf morphological traits of the plant species. The origin of this hypothesis was founded on several key observations derived from cultivation-based studies. First, plants constitute a major source of airborne bacteria, yielding greater numbers of emigrating bacteria than other terrestrial sources such as soil (Lindow *et al.* 1978; Lindemann *et al.* 1982). Second, plants with leaf traits conducive to increased phyllosphere nutrient availability (e.g., thin waxy cuticles and decreased hydrophobicity) exhibit larger epiphytic populations (i.e., leaf surface carrying capacities) and also larger numbers of emigrating phyllosphere bacteria (Tukey 1966; O'brien & Lindow 1989; Butterworth & McCartney 1991; Lindow & Andersen 1996). Third, the presence of plants with high leaf surface carrying capacities increases the epiphytic population size and induces microbially-mediated physiological changes in plants with low leaf surface carrying capacities (Lindow & Andersen 1996). However, these phenomena, as well as the hypothesis itself, have not been examined, much less verified, for the total phyllosphere microbiome using modern sequencing-based cultivation-independent techniques. Given that microbial ecologists generally agree that cultivation-independent techniques yield much more generalisable and consistent information on natural microbial communities, one can argue that the role of leaf morphological traits in the establishment of a source-sink relationship between the phyllosphere microbiomes of neighbouring plant species remains essentially unknown.

Implicit in the hypothesis by Lindow and Andersen (1996) is the premise that microbial dispersal and thus phyllosphere colonisation is heterogeneous among closely co-located trees. Indeed, studies prior to the formation of this hypothesis had already demonstrated that the numbers of viable airborne bacteria were different upwind and downwind of different crop species, at different times of the day, and during different environmental conditions (Lindemann & Upper 1985). Moreover, a hypothesis generated by Andrews *et al.* (1980), which was later supported by Jacques *et al.* (1995), stated that variation in the accessibility of leaves to dispersing microorganisms can also cause heterogeneous colonisation. In addition, other studies that exclusively focused on the transmission of pathogenic strains, such as *Pseudomonas syringae*, frequently identified non-random spatial patterns of disease among identical rows of crops (Hudelson *et al.* 1989). Nevertheless, the sample design and data

interpretation of many cultivation-independent empirical studies have generally assumed homogenous dispersal within individual study sites (Redford *et al.* 2010; Laforest-Lapointe *et al.* 2016). As such, whether the spatial position of the phyllosphere microbiome relative to local sources (e.g., plant neighbours) influences total phyllosphere community composition remains to be systematically investigated.

Mānuka (*Leptospermum scoparium*) and kānuka (*Kunzea ericoides*) are ecologically and morphologically indistinguishable, yet phylogenetically distinct, native shrub species that frequently co-occur throughout New Zealand (Allen *et al.* 1992; Whitehead *et al.* 2004). Despite their similarities, however, our results in Chapter II revealed mānuka and kānuka harbour distinct phyllosphere microbiomes. This unique host species pair therefore offers an exemplary model system in which to investigate explicit mechanisms of phyllosphere community assembly while controlling for abiotic factors that arise from variation in species-specific traits, which frequently confound most interspecies comparisons (Kembel *et al.* 2014). The aim of the current study was to use mānuka and kānuka to test the unproven conjecture that the relative strength of a host plant's phyllosphere microbiome as a source (herein termed *source-strength*) is determined by the leaf morphological traits of the plant species. Under the hypothesis of Lindow and Andersen (1996), we would expect to observe no source-sink relationship since mānuka and kānuka should be equal sources of phyllosphere bacteria. To test this hypothesis, we sampled one kānuka tree and three mānuka trees separated by relatively equal, straight-line distances (< 10 m) along each of three transects in a naturally regenerating, mānuka-dominated scrub. Specifically, this sampling design was established to test for the null hypothesis that kānuka acts as a stronger source of phyllosphere microorganisms than mānuka. To minimise the confounding influence of alternative vegetation sources, we ensured sample trees were not surrounded by other host trees (i.e., the transects covered fragmented vegetation). We used this sampling design to address the following questions:

1. Do phyllosphere taxa disperse homogenously between closely located kānuka and mānuka phyllosphere microbiomes (i.e., does the distribution of phyllosphere taxa within each transect reflect a random probability distribution)?

2. Do mānuka and kānuka provide equivalent sources of phyllosphere microorganisms?

3.3 Materials and Methods

3.3.1 Site description

This study was carried out on a single study site located in the Whanganui region of the North Island of New Zealand (39°51 S; 175°21 E) (Fig. 1A). In total, the study site comprises approximately 1500 hectares of grazed pasture, regenerating native scrub, and maize. The native scrub was dominated by mānuka and broadly divisible among three adjacent valleys that exhibit a range of different topographical descriptors, such as elevation, landforms, and slope aspect (Fig. 1B). Mānuka flowering typically lasts for six weeks, from which mānuka honey is harvested annually. Mid flow of mānuka nectar is approximately 10-15th December.

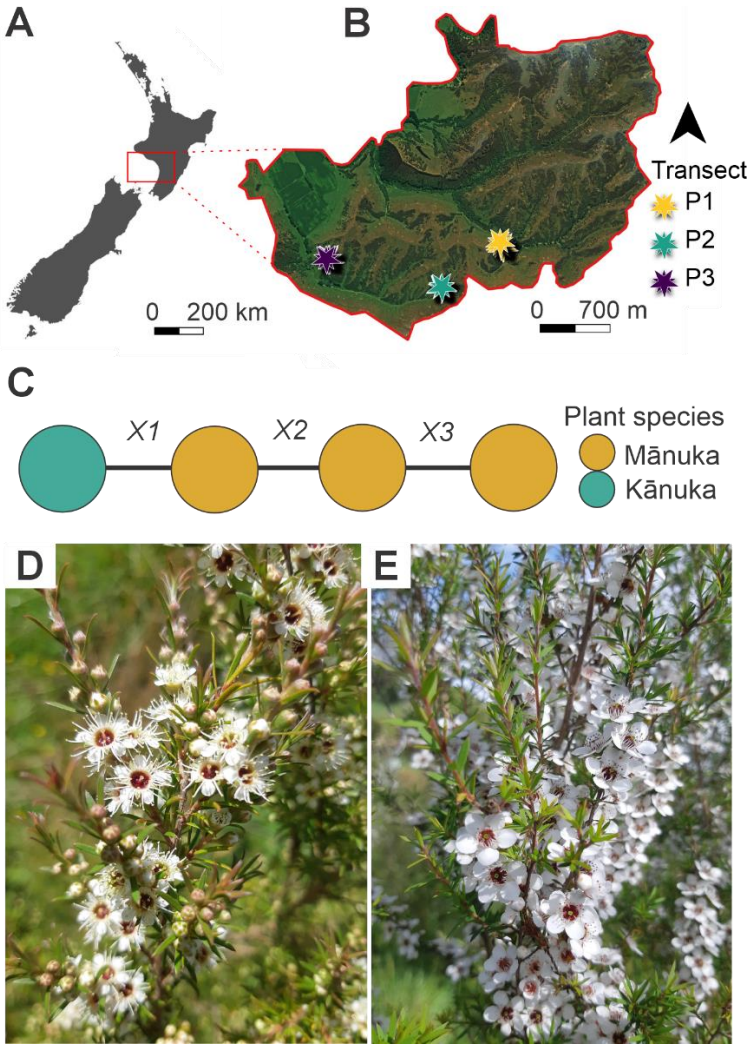

Fig. 1: Study site location and transect design. **A)** Study site was located in the Whanganui region of the North Island of New Zealand. **B)** Three transects were established in distinct regions of regenerating, mānuka-dominated native bush. **C)** Schematic of transect sampling design. Photos demonstrating the ecological and morphological similarity of **D)** kānuka and **E)** mānuka.

3.3.2 Sample collection

To permit detection and interpretation of complex spatial patterns among natural microbial communities, strategic and careful sampling methods are required. To date, empirical phyllosphere studies have most commonly used random sampling designs (e.g., quadrat sampling). However, such approaches are generally unable to detect, much less quantify, non-random spatial patterns (Clayton & Hudelson 1991). We therefore used a systematic, distance-based approach and precise measurements of inter-host distances to permit detection of non-random spatial patterns. Our null hypothesis, that kānuka is a source of phyllosphere microorganisms for mānuka, was primarily developed with specific consideration for the mānuka-dominated characteristic of the sample site. To test this hypothesis, we established three short-distance transects (P1-P3) (Fig. 1B). Per transect, one kānuka tree and three mānuka trees, separated by relatively equal (< 10 m) straight-line distances, were chosen for sampling (Fig. 1C). Selected trees were uniform in size and visually healthy. Each transect was determined such that the sample trees were not contiguous with the main areas of regenerating bush and did not neighbour any other tree species (i.e., the distance between sample trees and other plants exceeded the total length of the transect). Given the specific spatial characteristics of each transect and the infrequent presence of kānuka, our three transects were opportunistically established in distinct regions within two adjacent valleys. Each transect was 20-30 m length in total and distances between individual host trees ranged from 2.6 – 10.4 m (Table 1). The bearing of each transect varied from 51-308°. The slope aspect of each transect varied from 45-290°.

Table 1: Spatial descriptors of the three mānuka-kānuka transects.

Transect ID	Valley ID	Transect bearing	Slope aspect	Total distance (m)	Individual distances (m)		
					X1	X2	X3
P1	A	NW, 308	NE, 45	20	2.6	10.4	7
P2	B	NE, 51	SE, 115	20	5	5	10
P3	B	SW, 215	SW, 290	28	10	10	8

Samples were collected over the duration of two days in the summer of December 2020. Three branches per tree, at approximately the same height (1 – 2 m above the ground surface), with approximately 100-200 seemingly healthy leaves, were cut with clippers sterilised on site with 70% *v/v* ethanol/water, placed in individual sterile zip lock bags, and immediately placed on

dry ice. One surface (1 – 2 mm) soil sample from around the base of one tree was also collected into sterile 50 mL Falcon tubes using a spatula sterilised on site using 70% *v/v* ethanol/water and immediately placed on dry ice. Upon return to the Thermophile Research Unit at the University of Waikato, branch and soil samples were frozen at −20°C until further analysis

3.3.3 DNA extraction, amplification, and sequencing

Per branch, one gram of healthy, undamaged green leaves were carefully and aseptically excised and pooled. Total phyllosphere DNA was extracted from the surface of excised leaves using a previously described sonication and modified cetyl trimethylammonium bromide (CTAB) bead-beating protocol (Noble *et al.* 2020). For each surface soil sample, total DNA was extracted from 0.5 g of soil using the Power Soil DNA Extraction kit (Qiagen). DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, United States) and stored at −20°C until further analysis. The V4-V5 hypervariable region of the 16S rRNA gene was amplified with 515FY/926R fusion primers (5'GTGYCAGCMGCCGCGGTAA /5'CCGYCAATTYMTTTRAGTTT) and previously described PCR conditions (Noble *et al.* 2020). PCR products were cleaned and standardised with SequelPrepTM (Thermo Fisher Scientific). Standardised samples were pooled at an equimolar concentration into two libraries. An Illumina MiSeq 300PE sequencing run was performed on each library at Massey Genome Service (Palmerston North, New Zealand).

3.3.4 Sequence quality control and taxonomic assignment

The raw 16S rRNA gene amplicon sequences were processed using the DADA2 pipeline (Callahan *et al.* 2016). Forward and reverse reads were truncated at 237 and 232 bp, respectively, and quality filtered using the 'filterAndTrim' function with the following settings: maxN = 0, maxEE = c(3, 3), and truncQ = 2. Error rates were determined with the 'learnErrors' function and used to remove sequencing errors from forward and reverse reads, which were then assigned to amplicon sequence variants (ASVs) using the 'dada' function. Paired reads were then merged, converted into an ASV table, and chimeras removed with the removeBimeraDenovo function using the method 'consensus'. Taxonomy was assigned using the 'assignTaxonomy' and 'addSpecies' functions using the native implementation of the naive Bayesian classifier and the SILVA database version 138.1 (Quast *et al.* 2012). Chloroplast and mitochondrial sequences were filtered out by removing all ASVs with a taxonomic assignment of 'Chloroplast' at the Order level and 'Mitochondria' at the Family level, respectively. Lastly, we applied the 'isContaminant' function (method = prevalence) from the package 'decontam'

to our samples using our blank DNA extractions and PCR reactions to identify and remove putative contaminants introduced during processing (Davis *et al.* 2018).

After 16S rRNA gene sequence reads were quality filtered, two samples (P3_01.3 and P2_01.2) stood out as outliers. Although these two samples did not exhibit significantly different numbers of sequencing reads compared to other samples (Supplementary Fig. 1), these two samples appeared to exhibit unusual compositions relative to all other samples such that preliminary NMDS plots appeared highly skewed. Therefore, samples P3_01.3 and P2_01.2 were removed from further analyses and 17,332 ± 7,355 quality reads per sample remained.

3.3.5 Data analysis

All statistical analyses were conducted in R (R Core Team, 2023). Slope and branch aspect was converted into northing ($\cos \times \frac{\text{pi} \times \text{degrees}}{180}$) and easting ($\sin \times \frac{\text{pi} \times \text{degrees}}{180}$). Alpha diversity analyses were conducted using the 'vegan' package (Oksanen *et al.* 2007). Each phyllosphere and surface soil sample was first subsampled 100 times to an even sequencing depth (1,985 reads) and the average richness (observed number of ASVs) and Shannon-Wiener index was calculated for each sample. The Kruskal–Wallis test was used to evaluate significant differences across host species and sample type. Alpha diversity analyses were repeated for a phyllosphere-only subset of the data, whereby phyllosphere samples were subsampled a further 100 times to an even sequencing depth (6,491 reads) and the average richness and Shannon-Wiener index was calculated for each sample. These phyllosphere-only alpha diversity values were used to test for significant differences among plant species and transects using the Student's t-test and an ANOVA/Kruskal-Wallis test, respectively. Pearson's product-moment correlations were calculated between alpha diversity indices and continuous spatial descriptors. Pairwise Bray-Curtis and Jaccard community dissimilarities were calculated using the 'vegdist' function on ASV relative abundance and presence/absence transformed data, respectively. Differences in community structure among host species and transects were assessed using a PERMANOVA on community dissimilarities using the 'adonis' function in the 'vegan' package.

We used occurrence probabilities of phyllosphere taxa within each of our spatially structured mānuka-kānuka transects to test hypotheses related to dispersal (Royle *et al.* 2012). Assuming complete spatial randomness in the distribution of taxa in the surrounding environment, each ASV has the potential to exhibit one of 4,095 unique combinations of occurrence per transect.

The number of unique combinations of presence/absence were used to calculate zero-truncated probabilities that describe the likelihood of an ASV being present in any *N* number of phyllosphere microbiomes (i.e., 1 – 12, termed *microbiome occurrence*) (see Supplementary Table 4 for occurrence probabilities and corresponding combination formula). Chi-square goodness-of-fit tests were used to determine whether the observed taxon presence/absence of taxa differs from the predicted distribution of occurrence probabilities.

3.4 Results

3.4.1 Mānuka and kānuka phyllosphere community diversity

In total, 3,895 ASVs were detected across all mānuka and kānuka phyllosphere communities. No significant differences in alpha-diversity (richness or Shannon) were detected between mānuka and kānuka phyllosphere communities (Supplementary Fig. 3A-B, $p > 0.05$) nor between the three transects (Supplementary Fig. 3C-D, $p > 0.05$). However, significant correlations were observed between phyllosphere community diversity and phyllosphere microbiome spatial descriptors (Table 2). Interestingly, these correlations were plant species-specific. For example, phyllosphere community richness in mānuka exhibited a negative relationship with slope northing ($r = -0.43$, $p = 0.03$), whereby trees that were sampled on more northward facing slopes tended to have less species richness than trees on more southward facing slopes. Phyllosphere community richness in mānuka also exhibited a positive relationship with tree height ($r = 0.40$, $p = 0.04$), whereby communities sampled from taller trees tended to have greater species richness relative to shorter trees. Meanwhile, richness and Shannon in the kānuka phyllosphere exhibited a negative relationship with branch height ($r = -0.94$, $p = 0.002$, $r = -0.79$, $p = 0.03$, respectively), whereby communities sampled from lower branches tended to have greater species richness and evenness relative to branches sampled from greater heights. Shannon diversity in kānuka phyllosphere communities also exhibited a positive relationship with slope easting ($r = 0.80$, $p = 0.03$), whereby trees that were sampled on more eastward facing slopes tended to exhibit greater species evenness than trees on more westward facing slopes.

Table 2: Pearson's product-moment correlations between phyllosphere community diversity (Shannon and richness) and phyllosphere microbiome spatial descriptors.

Spatial descriptor	Plant species	Richness		Shannon	
		r	*p value*	*r*	*p value*
Branch easting	Mānuka	0.08	0.71	-0.14	0.48
	Kānuka	0.22	0.64	-0.08	0.87
Branch northing	Mānuka	-0.25	0.21	-0.21	0.29
	Kānuka	0.56	0.19	0.45	0.31
Branch height	Mānuka	-0.05	0.81	-0.02	0.91
	Kānuka	**-0.94**	**0.002**	**-0.79**	**0.03**
Tree height	Mānuka	**0.40**	**0.04**	0.25	0.20
	Kānuka	0.44	0.33	0.52	0.23
Slope easting	Mānuka	0.18	0.37	0.04	0.83
	Kānuka	0.71	0.07	**0.80**	**0.03**
Slope northing	Mānuka	**-0.43**	**0.03**	-0.09	0.65
	Kānuka	0.23	0.62	0.19	0.68
Elevation	Mānuka	-0.02	0.93	0.16	0.44
	Kānuka	0.37	0.42	0.34	0.45

3.4.2 Community composition of the mānuka and kānuka phyllosphere microbiomes

In total, phyllosphere communities were represented by 16 phyla (Supplementary Table 1). Proteobacteria were the dominant members of these communities (relative abundance 76.7 ± 7%), followed by Acidobacteriota (10.1 ± 3.6), Bacteroidota (5.73 ± 3.2), Bdellovibrionota (3.72 ± 2), and Myxococcota (1.27 ± 1.4). No significant differences in the relative abundance of phyla were detected between mānuka and kānuka phyllosphere communities (Benjamini-Hochberg corrected Wilcox test $p > 0.05$; Supplementary Fig. 4, Supplementary Table 2).

3.4.3 Overall community structure of the mānuka and kānuka phyllosphere microbiomes

At the ASV level, host species identity explained a small proportion of variation in overall phyllosphere community structure (PERMANOVA on Bray-Curtis dissimilarities, $R^2 = 0.06$, $p = 0.001$; PERMANOVA on Jaccard dissimilarities, $R^2 = 0.06$, $p = 0.003$). Meanwhile, 15% and 13% of variation in Bray-Curtis and Jaccard community dissimilarity, respectively, was attributable to the transect from which phyllosphere microbiomes were sampled (Fig. 2, Supplementary Table 3). For mānuka phyllosphere communities, transect explained 22% and 17% of variation in Bray-Curtis and Jaccard community dissimilarity, respectively

(Supplementary Table 3). Meanwhile, the individual host tree from which mānuka phyllosphere microbiomes were sampled explained 26% and 29% of variation in Bray-Curtis and Jaccard community dissimilarity, respectively (Supplementary Table 3). In addition, variance in mānuka phyllosphere community dissimilarity was significantly different between each transect. Specifically, mānuka phyllosphere microbiomes from transect P2 exhibited a significantly smaller amount of variance compared to transects P1 and P3 (Betadisper, $p < 0.05$). For kānuka phyllosphere communities, host tree and transect explained 39% and 43% of variation in Bray-Curtis and Jaccard community dissimilarity, respectively (Supplementary Table 3). How much of this variation is individually explained by host tree and transect was indeterminable as only one kānuka tree was sampled per transect.

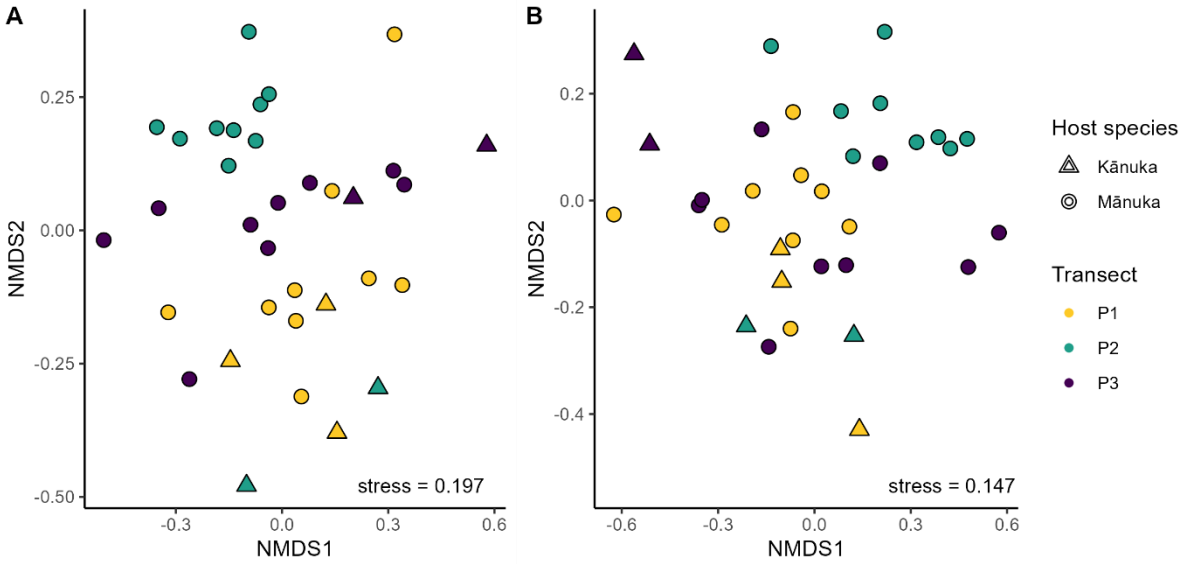

Fig. 2: Overall community dissimilarity of phyllosphere communities. Community dissimilarity was based on **A)** Bray-Curtis (relative abundance-based distance) and **B)** Jaccard (presence-absence-based distance). Colour represents transect, shape represents plant species.

Within each transect, community dissimilarity was calculated between kānuka and mānuka phyllosphere communities and the relationship with physical distance of separation was explored. A significant distance-decay relationship was observed in Jaccard-based community dissimilarity in transect P1 ($r = 0.47$, $p = 0.004$) and P3 ($r = 0.7$, $p = 0.002$) (Supplementary Fig. 5). In contrast, a significant distance-decay relationship was observed in Bray-Curtis community dissimilarity in transect P2 ($r = 0.55$, $p = 0.011$) (Supplementary Fig. 5).

3.4.4 Distribution of phyllosphere taxa along kānuka-mānuka transects

In a scenario in which closely located phyllosphere microbiomes are exposed to and colonised by one homogenous source pool of dispersing microorganisms (i.e., either no inter-host dispersal occurs *or* the effect of inter-host dispersal is negligible), the distribution of phyllosphere taxa is expected to reflect a random probability distribution. Therefore, we first tested for complete spatial randomness in the distribution of taxa among the 12 phyllosphere microbiomes in each replicate mānuka-kānuka transect by comparing the observed distribution of taxon presence/absence with occurrence probabilities (see Materials and Methods). We found that the observed distribution of taxa across each of the 12 microbiomes in each transect did not resemble the predicted stochastic distribution (Fig. 3A, Supplementary Table 4, Supplementary Table 5, Chi-square goodness of fit $p < 0.001$). Compared to the predicted stochastic distribution, a relatively larger proportion of taxa was observed in one to three microbiomes (Fig. 3A). In addition, a relatively larger proportion was also observed in ten to 12 microbiomes. In contrast, a relatively smaller proportion of taxa was observed in four to eight microbiomes. These observations suggest that stochasticity alone is not sufficient to describe the distribution of taxa among spatially organised phyllosphere microbiomes, and that dispersal may not be homogenous.

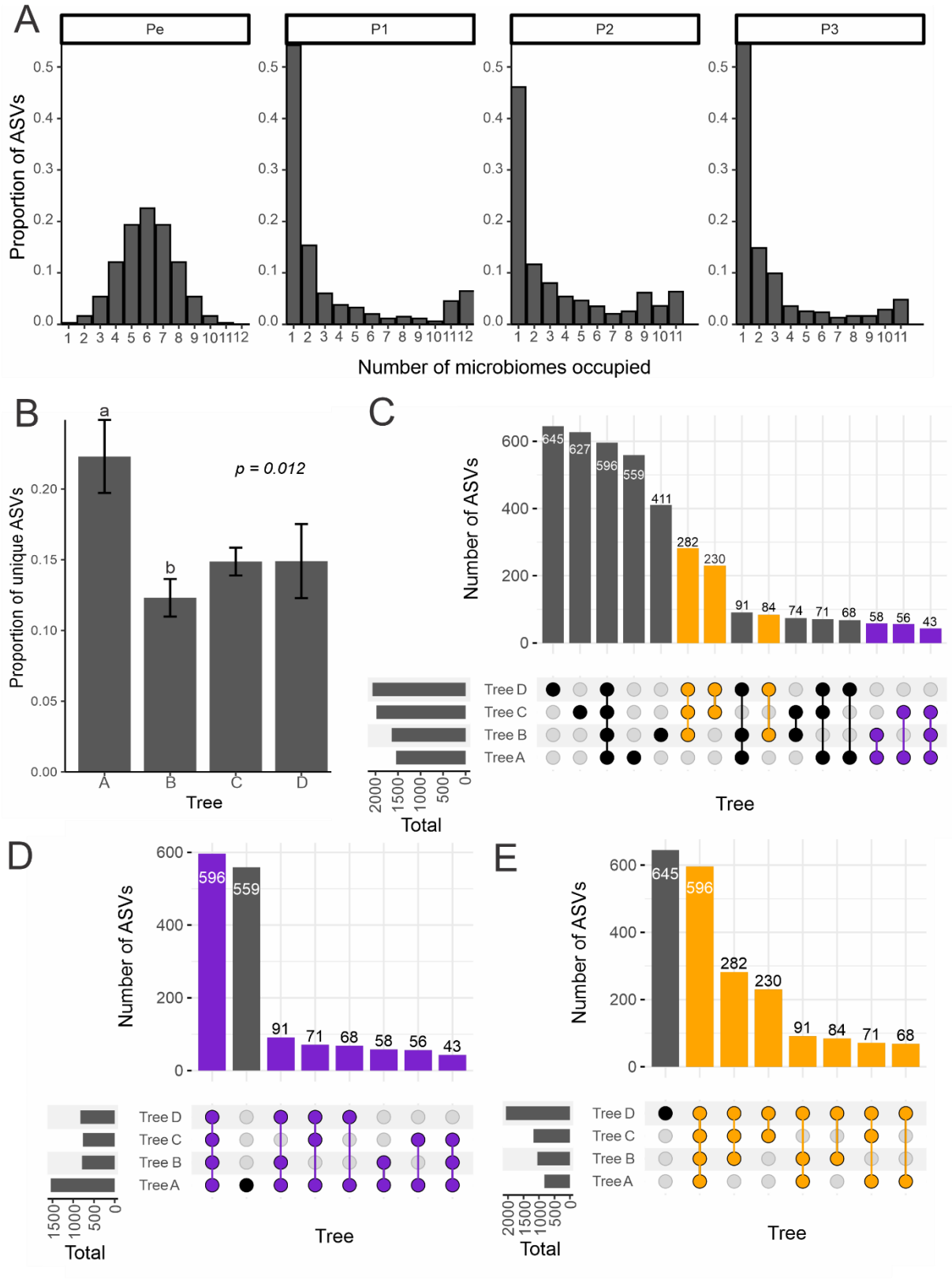

Fig. 3: The distribution of phyllosphere taxa across replicate mānuka-kānuka transects. **A)** Predicted (Pe) vs observed distribution of taxa across the 12 phyllosphere microbiomes within each transect P1-P3. **B)** The average proportion of unique taxa per sample within each transect tree. Letters denote

statistical significance. **C)** Upset plot displaying the distribution of phyllosphere taxa across all possible transect tree combinations (cumulative across all transects). Purple represents taxa shared between tree A (kānuka) and trees B-C (mānuka). Yellow represents taxa shared between tree D (mānuka) ad tree B-C (mānuka). **D)** Upset plot displaying the distribution of tree A taxa across all possible transect tree combinations. **E)** Upset plot displaying the distribution of tree D taxa across all possible transect tree combinations.

To further examine whether the observed distribution of phyllosphere taxa reflects a scenario in which taxa have dispersed stochastically from the surrounding environment to each host tree, the average proportion of unique taxa per phyllosphere sample was calculated for each tree (A-D) across all transects. In this scenario, the occurrence probability for one microbiome ($N = 1$) is equal among all host trees ($\frac{12}{4095}$ = ~0.0029, Supplementary Table 4) and thus the average number of unique taxa per phyllosphere sample is also expected to be equal. Instead, we found that kānuka (tree A) exhibited a significantly larger proportion of unique taxa per microbiome compared to neighbouring mānuka (tree B) (Fig. 3B, p = 0.012). Although tree B exhibited a slightly lower number of unique taxa on average than tree C or tree D, these differences were not statistically significant.

To shed further light on whether mānuka and kānuka phyllosphere microbiomes are equivalent sources of microorganisms, we compared the distribution of taxa across spatially equivalent pairs of host trees. In the scenario in which mānuka and kānuka phyllosphere microbiomes are equivalent sources of microorganisms, the number of shared taxa between tree A (kānuka) and tree B-C (mānuka) would be expected to be equivalent to the number of shared taxa between tree D (mānuka) and tree B-C (mānuka) (Fig. 1C). However, we observed that the number of taxa shared between tree A and tree B-C was smaller than the number of taxa shared between tree D and tree B-C (Fig. 3C).

Lastly, if either mānuka or kānuka acts as a significant source of phyllosphere bacteria that can disperse to other trees, we would expect that there would be an observable gradient of presence/absence away from the source of inoculum. Therefore, we investigated whether there was a gradient of phyllosphere taxa presence/absence along the length of the transect away from tree A and tree D. In total, 1,542 taxa were observed in tree A phyllosphere communities (Fig. 3D). Moreover, a relatively consistent number of these tree A taxa (766 – 826 ASVs) were also present in the phyllosphere communities belonging to trees B – D and taxon presence did not demonstrate a relationship with distance or order of separation away from tree A (Fig. 3D). A large number of tree A taxa were either identified in all four trees (596 ASVs) or only

within tree A (559 ASVs). All other possible combinations of trees in which taxa could be distributed within the transect shared relatively equal numbers of taxa (Fig. 3D). In total, 2,067 taxa were identified in tree D phyllosphere communities (Fig. 3E). Further, the number of taxa decreased in each subsequent tree along the transect away from tree D; decreasing from 1,179 ASVs in tree C, 1,053 ASVs in tree B, and 826 ASVs in tree A (Fig. 3E). A large number of taxa was either only found in tree D (645 ASVs) or all four transect trees (596 ASVs) (Fig. 3E). The next highest number of shared tree D taxa was observed between trees D – B (282 ASVs), followed by tree D and tree C (230 ASVs) (Fig. 3E). Other possible combinations of trees in which taxa could be distributed within the transect exhibited relatively equal numbers of taxa. Notably, the smallest number of taxa was shared between tree D and tree A (68 ASVs) (Fig. 3E).

3.4.5 Surface soil microbial communities

In total, 12 surface soil samples were collected from the base of each sample tree and yielded 17,332±7,355 quality-assured reads per sample. In total, 4,026 ASVs were identified, with an average of 568±217 per sample. No significant differences in microbial diversity (richness and Shannon) were observed between surface soils collected from different transects ($p > 0.05$, Supplementary Fig. 6A-B). Further, no significant correlations were observed between microbial diversity and spatial descriptors (Supplementary Table 6). In addition, no significant differences in diversity were observed between surface soil and phyllosphere microbial communities ($p > 0.05$, Supplementary Fig. 6C-D).

Surface soil microbial communities were structurally distinct from the phyllosphere microbiome (Supplementary Fig. 7). In total, surface soil microbial communities were represented by 24 phyla (Supplementary Fig. 8). On average, Proteobacteria were the dominant members of these communities (relative abundance 27.4 ± 7.4%), followed by Verrucomicrobia (25.3 ± 12), Bacteroidota (18.1 ± 7.7), Acidobacteriota (8.54 ± 3.3), Actinobacteriota (6.45 ± 3.6), and Planctomycetota (5.26 ± 2.1) (Supplementary Table 7). No significant difference in the relative abundance of taxa from the phylum to genus level was observed across transects (Benjamini-Hochberg corrected Kruskal-Wallis $p > 0.05$).

At the ASV level, variation in community structure was significantly associated with site (PERMANOVA on Bray-Curtis dissimilarities, $R^2 = 0.20$, $p = 0.02$; PERMANOVA on Jaccard dissimilarities, $R^2 = 0.19$, $p = 0.02$; Supplementary Table 3). Furthermore, the effect of host

species identity from which the surface soil sample was collected beneath did not explain any variation in community structure (PERMANOVA on Bray-Curtis and Jaccard dissimilarities, $p > 0.05$, Supplementary Table 3).

3.5 Discussion

The consensus that prevails from early cultivation-based studies is that surrounding vegetation is a significant source of phyllosphere microorganisms (Lindow & Brandl 2003). However, many empirical, cultivation-independent studies implicitly presume homogenous dispersal within individual study sites and thus spatial variability in the dispersal and colonisation of phyllosphere microbiomes among co-occurring plants has received little attention. In this study, we addressed this assumption as well as the longstanding hypothesis by Lindow and Andersen (1996), that the relative source-strength of the phyllosphere microbiome is correlated with leaf morphological traits. By characterising phyllosphere community composition and taxon distribution across linear transects comprising pairs of ecologically and morphologically indistinguishable plant species (i.e., mānuka and kānuka), we were able to test the null hypothesis that kānuka acts as a stronger source of phyllosphere microorganisms for mānuka.

3.5.1 Colonisation of the phyllosphere microbiome is heterogeneous within and between host trees

Within each of our replicate transects, the distribution of taxa across phyllosphere communities was significantly different from being perfectly stochastic (i.e., seeded by a single homogenous pool of microorganisms in the local surrounding environment). Notably, large proportions of taxa were identified in a single microbiome and host tree (Fig. 3A), demonstrating that dispersal and thus the colonisation of natural and total phyllosphere communities is spatially heterogeneous within and across ecologically and morphologically similar plants, even when separated by small distances (<1 – 30 m). This finding is in line with early cultivation-based studies that frequently reported large variability in epiphytic population size among individual leaves and trees (Andrews *et al.* 1980; O'brien & Lindow 1989; Kinkel *et al.* 1995; Kinkel *et al.* 2000). A small number of cultivation-independent studies have previously described intra- and inter- host variation in the phyllosphere microbiome of other plant species (Redford *et al.* 2010; Laforest-Lapointe *et al.* 2016). However, several aspects of their sample design limited the robustness of their results. First, pooling leaves from multiple branches around each host tree precluded the detection of spatial heterogeneity among distinct anatomical plant structures.

Second, by collecting samples from trees randomly positioned across large study sites (i.e., a 35-ha university campus and two geographically distinct Canadian forests) the inter-host variation reported in these studies was likely confounded by dispersal limitations and an unquantified level of environmental heterogeneity. Lastly, neither of these previous studies describe, much less control, the identity or proximity of microbial sources (i.e., plant neighbours). In contrast, the careful sample design of the current study revealed large spatial heterogeneity and minimised the confounding influence of large distance dispersal limitations, environmental heterogeneity, and plant neighbours.

Differences in the order of arriving microbial taxa (i.e., priority effects) has been shown to significantly influence the composition of other plant microbiomes and their multi-trophic functional interactions (Chappell *et al.* 2022). Although priority effects have not yet been investigated in total, much less natural, phyllosphere communities, evidence from early cultivation-dependent field studies does suggest that variability in the indigenous phyllosphere communities among leaves (i.e., initial colonisers) may contribute to differences in the subsequent survival of immigrating bacteria. For example, high levels of variability in bacterial population size was still evident among replicate leaf samples after uniform inoculation with individual bacterial strains (Kinkel *et al.* 2000). Moreover, previous experimental work also demonstrated that the presence of *Pantoea agglomerans* 299R on bean leaf surfaces was shown to significantly increase the survival probability of subsequent immigration by *P. agglomerans* 299R and *Pseudomonas fluorescens* A506, but not *Pseudomonas syringae* B728a (Monier & Lindow 2005). Although priority effects may not have a significant influence on the overall composition of the dominant members of the mānuka phyllosphere (i.e., because in our previous study and Chapter II the mānuka phyllosphere exhibits patterns of community structure that are indicative of a primary role of host selection) (Noble *et al.* 2020), priority effects may explain why we do not observe a stochastic distribution of low abundant taxa. Moreover, we cannot exclude the possibility that even a relatively small role of priority effects in mānuka phyllosphere assembly has significant functional consequences (Jousset *et al.* 2017). Interestingly, mānuka exhibits large intra-host physiological heterogeneity that remains unexplained (Stephens *et al.* 2005). For example, the concentration of nectar dihydroxyacetone has been shown to vary up to 4-fold between co-occurring mānuka trees (Noe *et al.* 2019). Driven by the motivation to understand such heterogeneity, the involvement of microbiomes, specifically the phyllosphere microbiome, has been recently hypothesised (Noble *et al.* 2020). However, in a scenario in which this hypothesis holds true, a growing recognition for the role

of priority effects in the assembly of such host microbiomes may also paradoxically mean that we have to accept that there will always be a proportion of community composition and function, and thus presumably host physiology, that cannot be explained through either deterministic (i.e., abiotic or biotic) or neutral (i.e., stochastic) processes.

3.5.2 Mānuka is a stronger source of phyllosphere microorganisms than kānuka despite similar leaf morphological traits

Overall, the distribution of taxa across the mānuka-kānuka transects was not consistent with a scenario in which trees were equivalent sources of phyllosphere microorganisms. Specifically, we observed a large number of single-microbiome taxa in tree A (kānuka) (Fig. 3B) and fewer taxa were shared between trees A-C than trees B-D (Fig. 3C). Given that these two plant species did not exhibit a significant difference in microbial diversity, these findings suggest that a relatively smaller proportion of kānuka phyllosphere taxa is distributed among plant neighbours. Furthermore, we identified a gradient of taxon presence/absence away from tree D (Fig. 3E) but not tree A (Fig. 3E). Contrary to our stated null hypothesis, these results are consistent with a scenario in which mānuka is a relatively stronger strength source of phyllosphere microorganisms for kānuka. In Chapter II, we identified a possible source-sink relationship between focal mānuka and neighbouring 'non-mānuka' plant species. However, owing to the abstraction of 'non-mānuka' to define plant species surrounding mānuka, this work was unable to examine the influence of leaf morphology. The current study builds on these results by demonstrating this dominant direction of inter-host dispersal is also apparent between pairs of species that have very similar leaf surface characteristics. Although our results do not definitively disprove the hypothesis posited by Lindow and Andersen (1996), they demonstrate that the factors that mediate processes of inter-host dispersal are largely unknown. Given that in Chapter II the assembly of the mānuka phyllosphere appears to be strongly influenced by host selection, a parsimonious theory is that phyllosphere microbiome source-strength is directly proportional to the strength of host selection. That is, phyllosphere microbiomes that comprise specific taxa provide a more consistent source of emigrating microorganisms into the proximal surrounding environment compared to phyllosphere microbiomes that exhibit greater compositional stochasticity. As a further point of discussion, recent studies have demonstrated that plants exhibit different plant-plant chemical signalling when grown next to conspecific versus heterospecific plant neighbours (Kigathi *et al.* 2019). Interestingly, this has been demonstrated for mānuka, which has been found to release different quantities and compositions of volatile organic compounds (VOCs) corresponding to the

identity of its plant neighbour (Effah *et al.* 2020; Effah *et al.* 2022). Further investigation of phyllosphere inter-host dispersal complemented with measures of host physiology therefore represents an interesting avenue for future research.

3.5.3 Mānuka and kānuka phyllosphere communities are distinct at a fine taxonomic resolution

The phyllosphere communities of mānuka and kānuka were compositionally and structurally similar. Specifically, no significant difference in microbial diversity nor the relative abundance of taxa down to the genus level was observed. This finding confirms the results of Chapter II, which observed a greater similarity between mānuka and kānuka phyllosphere communities compared to other native tree species, and further supports the idea that physically similar microhabitats on the leaf surface select for similar communities of microorganisms at a broad taxonomic resolution (Kembel *et al.* 2014). This also supports previous studies that have reported an association between plant functional traits and interspecies variation in phyllosphere community composition (Kembel *et al.* 2014). However, despite these general similarities, host species identity did still explain a small proportion (5 – 6 %) of variation in phyllosphere community structure at the ASV level. Furthermore, the microbial diversity in mānuka and kānuka phyllosphere communities exhibited significant relationships with spatial descriptors that likely reflect species-specific differences in community assembly processes. For example, a significant relationship was detected between species richness and mānuka tree height. In contrast, microbial diversity in the kānuka phyllosphere exhibited a significant relationship with branch height. The distinction of the mānuka phyllosphere microbiome at a fine taxonomic resolution continues to support our previously established hypothesis that the mānuka phyllosphere is driven by a direct, potentially chemically-mediated, mechanism of host selection that is distinct from ecological and morphological characteristics of the leaf surface.

3.5.4 The mānuka phyllosphere microbiome exhibits variation that could be attributable to slope aspect

Phyllosphere microbiomes within the same transect tended to be more similar than those sampled in different transects (Fig. 2). Although all three transects were geographically confined within adjacent valleys and thus experienced general similarities in regional environmental conditions (i.e. rainfall), each transect had a unique local topography. One obvious topographical difference between each transect was slope aspect (45°, 115°, 290°) (Table 1). Interestingly, mānuka trees that were sampled on more northward facing slopes

tended to harbour phyllosphere communities that were less species rich than trees on more southward facing slopes (Table 2). Slope aspect is an important topographical factor as it influences the intensity and duration of solar radiation and thus contributes to substantial heterogeneity of species diversity at local scales (Carter & Ciolkosz 1991; Bennie *et al.* 2008). Moreover, slope aspect has previously been found to have a larger influence than elevation on the bacterial community structure in other environmental microbial habitats, such as soil (Wu *et al.* 2016). As north facing slopes in New Zealand receive more solar radiation compared to south-facing slopes, a parsimonious interpretation of these results is that differential exposure to solar radiation could drive subtle differences in the mānuka phyllosphere microbiome between transects. In accordance with this observation, biogeographical patterns in phyllosphere community structure have also been previously observed across environmentally diverse populations of mānuka (Noble *et al.* 2020). Furthermore, this idea is also supported by a previous study that reported slope aspect might have explained some variation in the magnolia phyllosphere microbiome (Stone & Jackson 2016).

There are several mechanisms by which solar radiation could influence phyllosphere communities. First, the amount of solar radiation may have a direct impact on the abiotic conditions experienced by microorganisms on the leaf surface. Increased solar radiation increases UV stress, leaf temperature, and decreases leaf moisture (Williams *et al.* 2022). As microorganisms differ in their tolerance of temperature and moisture (Potts 1994), this could lead to differences in abiotic selection across slopes. Indeed, increased leaf surface moisture has been linked to an increase in general survivability of phyllosphere microorganisms (Monier & Lindow 2004). A previous study also found that water stress reduced leaf bacterial richness and homogenized bacterial community composition among plants (Debray *et al.* 2022). The intensity and duration of solar radiation may also indirectly influence phyllosphere communities by eliciting changes in plant physiology, growth, and development (Li *et al.* 2021; Williams *et al.* 2022). For example, plants on sun-exposed slopes commonly experience decreased water availability, which can reduce photosynthetic activity due to phytohormone-mediated stomatal closure (Salvi *et al.* 2021). Interestingly, previous studies have also reported that temperature can affect the emission of several biogenic VOCs in mānuka leaves, including a green leaf volatile, terpenoids, and other esters (Effah *et al.* 2020). Although speculative, this could have important consequences on the composition of the mānuka phyllosphere microbiome, which exhibits characteristics congruent with a microbiome under direct, chemically-mediated host selection (i.e., Chapter II). Moreover, as a further point of discussion,

light has also been shown to have a significant effect on the sugars that accumulate in the nectar of the mānuka flower, including mean total sugar per flower and DHA/total sugar ratio (Clearwater *et al.* 2021). Investigating the interaction between phyllosphere communities and the direct and indirect changes in host tree physiology in response to differential abiotic conditions represents a necessary avenue for future work aimed at developing our understanding of the mechanisms of selection on phyllosphere communities as well as their influence on host tree physiology.

3.6 Conclusions and significance

In summary, the results of this chapter are consistent with a scenario in which the mānuka phyllosphere microbiome provides a source of microorganisms to an ecologically and morphologically indistinguishable plant neighbour, suggesting that the ability of a plant species to act as a source is not only determined by leaf traits, disputing the longstanding hypothesis by Lindow and Andersen (1996). Furthermore, our results also reveal considerable spatial heterogeneity in the distribution of phyllosphere taxa between branches and closely located host trees, suggesting that colonisation of the phyllosphere is neither a homogenous nor a stochastic process. Greater consideration for a larger number of explanatory variables, such as the identity of plant neighbours, may not only increase the proportion of explainable variation in phyllosphere empirical studies but also shed light on the proportion of unexplainable variation that exists in natural communities due to undeterminable processes, such as priority effects. Furthermore, acknowledging that plants do not exist in isolation but are continuously exposed to a heterogeneous community of dispersing microorganisms from both distant and local sources will also help to improve our understanding of microbial dispersal, which has far-reaching consequences for all terrestrial microbial habitats. From an applied point of view, understanding the role of inter-host dispersal and thus the presence of neighbouring plant species on the composition of the phyllosphere microbiome will likely have significant consequences for future agricultural, horticultural, and environmental conservation strategies designed to maintain and promote beneficial phyllosphere compositions. Finally, this study demonstrates the importance of using modern technologies to verify longstanding hypotheses and assumptions that were established with traditional methodologies. Such an approach will become increasingly necessary as our volume of scientific literature and rate of technological innovation continues to increase.

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Chapter IV

The effect of season on the evergreen phyllosphere microbiome of mānuka (*Leptospermum scoparium*)

4.1 Abstract

A prevailing consensus from early culture-based studies is that the phyllosphere microbiome exhibits large temporal variation. Although these observations have been supported by cultivation-independent studies that have investigated how the total phyllosphere community of short-lived plant species changes over the duration of a single growing season, much less is understood for the evergreen phyllosphere microbiome. Nevertheless, the evergreen phyllosphere microbiome represents an attractive system to study temporal variation, since the effect of leaf age, plant phenology, and season can be decoupled. Previously, we have demonstrated that the *Leptospermum scoparium* (mānuka) phyllosphere microbiome exhibits strong host associations that are maintained over large spatial scales. However, the temporal variation in the phyllosphere microbiome of mānuka, an evergreen plant, remains uncharacterised. Given the dominant role of host selection observed by previous studies, we hypothesised that season does not have a significant effect on the mānuka phyllosphere microbiome relative to the influence of the host. We tested this hypothesis using a temporal sampling design to investigate the influence of season on the mānuka phyllosphere microbiome. In accordance with our hypothesis, our results demonstrated that the mānuka phyllosphere microbiome does not exhibit significant seasonal variation. Nevertheless, a notable increase in the mānuka core phyllosphere microbiome is observed during summer, which, together with the results from our previous spatial analyses, may suggest an increase in the relative strength of host selection. This work highlights the importance of conducting temporal analyses, particularly in evergreen plant species, to contextualise and complement spatial analyses and further our understanding of the abiotic and biotic factors that drive phyllosphere community assembly.

4.2 Introduction

A central question in early culture-based phyllosphere research was, 'How does the phyllosphere microbiome change over time?' Addressing this question was necessary in order to characterise pathogenesis in the phyllosphere of agriculturally significant crop species and many of these studies reported large changes in epiphytic population sizes within a single growing season (Upper & Hirano 1994). These changes were generally attributed to the rapid emigration of bacteria following specific weather events (e.g., wind gusts or rainfall) (Lindemann & Upper 1985), periods of exponential bacterial growth (e.g., after emigration events) (Hirano *et al.* 1996), and the ephemerality of aerial plant structures in these annual or herbaceous plant species (Thompson *et al*. 1993). Although culture-based methods limited the ability of these early studies to capture the full microbial diversity of these phyllosphere communities, many cultivation-independent studies have since verified that the phyllosphere microbiome does undergo predictable and repeatable successional changes in total community composition from emergence to senescence on short-lived leaves, such as those harboured by annual (Williams *et al*. 2013), herbaceous (Grady *et al*. 2019; Stone & Jackson 2021), and deciduous plant species (Redford & Fierer 2009). However, in contrast to the ephemerality of these phyllosphere microbiomes, evergreens maintain their leaves for multiple years and thus the evergreen phyllosphere microbiome offers a unique microbial habitat in which to study long-term (i.e., seasonal) temporal variation independently from the successional changes that arise as a result of annual leaf emergence.

Ercolani (1991) was the first to examine temporal variation in the evergreen phyllosphere. Specifically, this culture-based study identified differences in the bacterial communities of differently aged olive leaves irrespective of season, but also different communities in different seasons irrespective of leaf age. Since the progression to cultivation-independent methodology, few studies have investigated temporal variation in the evergreen phyllosphere microbiome. One study used DGGE to investigate annual and seasonal phyllosphere community composition in a single southern magnolia (*Magnolia grandiflora*) tree and observed large temporal changes (Jackson & Denney 2011). Another study compared the summer and winter phyllosphere communities of one deciduous and two evergreen plant species and found that season had a larger influence than host species identity (Bao *et al.* 2020). Furthermore, another study characterised the monthly phyllosphere communities in two pairs of two Acacia tree species over a year and also observed season had a larger influence on community composition

than host species identity (Al Ashhab *et al.* 2021). However, given that these studies collectively comprise a very small number of plant species, it remains unclear if seasonal variation is a universal trait of all plant species. Moreover, as our understanding of plant-microbiome associations in natural (i.e., long-lived) phyllosphere communities continues to grow, temporal analyses will be necessary to understand how these beneficial associations are formed and/or maintained by the host at different times of the year.

The assembly of the evergreen mānuka (*Leptospermum scoparium*) phyllosphere microbiome appears to be primarily influenced by a direct mechanism of host selection, such as chemical signalling via leaf exudates or volatile organic compounds (VOCs). This evidence is derived from a previous study, which identified a strong and consistent host association in the mānuka phyllosphere across large spatial scales (Noble *et al.* 2020), and Chapter II, which used spatially explicit sampling to demonstrate the mānuka phyllosphere microbiome exhibits host species-specificity even when compared to ecologically and morphologically indistinguishable plant species. Given the importance of controlling for different phases of host phenology, however, our previous work has explicitly sampled mānuka during the New Zealand summer season (December – February), which naturally coincides with mānuka flowering. Therefore, an outstanding question remains to be addressed: how does the mānuka phyllosphere microbiome change over time?

In the present study, we used a temporal sampling design to investigate whether the mānuka phyllosphere microbiome exhibits seasonal variation. Specifically, we performed replicate sampling of individual mānuka trees from a naturally regenerating, mānuka-dominated scrub at three different times of the year: September (spring), January (summer), and May (autumn). Given the dominant role of host selection suggested in Chapter II, we hypothesised that season does not have a significant effect on the mānuka phyllosphere microbiome relative to the influence of the host (null hypothesis: season has a significant influence on phyllosphere). To examine this, we addressed the following questions:

> Does alpha diversity in the mānuka phyllosphere exhibit significant differences across seasons?
>
> Is the mānuka phyllosphere microbiome colonised by distinct bacterial taxa at different seasons?

Is community structure in the mānuka phyllosphere more strongly influenced by season or individual host tree?

Are patterns of strong host association (i.e., a core microbiome) observed in the mānuka phyllosphere within and across different seasons?

4.3 Materials and Methods

4.3.1 Site description

This study was carried out on a single study site located in the Western King Country (Waikato) region of the North Island of New Zealand (38°51 S; 174°45 E) (Fig. 1A). In total, the study site comprises approximately 420 hectares of regenerating, mānuka-dominated native scrub with some grazed pasture (Fig. 1B). The study site lies within the Herangi range and is surrounded by the Whareorino Conservation Area, which is one of the largest forested areas in the Western King Country (Fig. 1B). Whareorino is known for its special range of undisturbed, indigenous fauna and flora. The top of the Herangi Range is 600–800 m ASL and its proximity to the west coast creates a generally cold and moist climate, with an annual rainfall of 1.6 – 2.5 m (McEwan 1987).

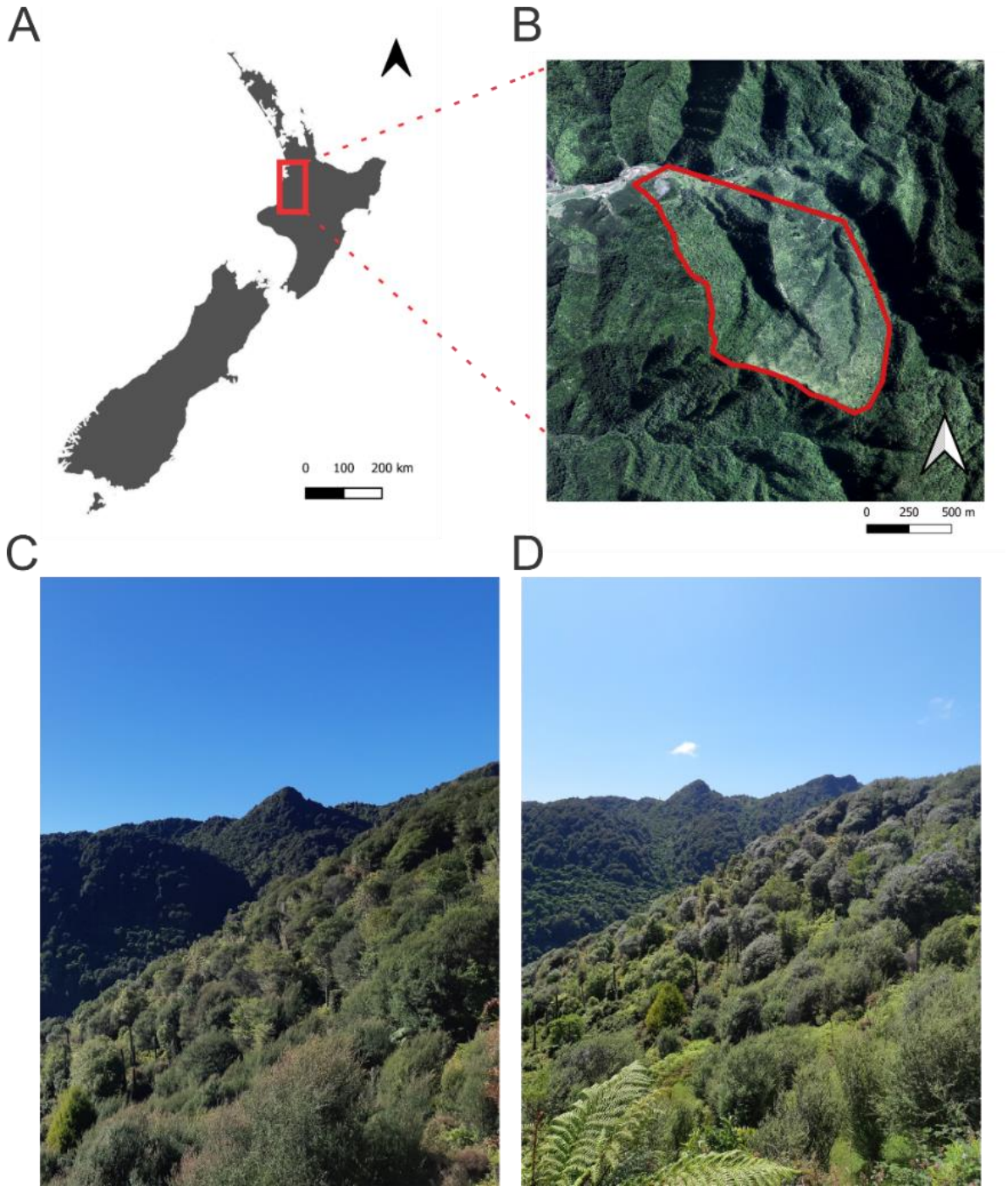

Fig. 1: Study site location and vegetation. **A)** Study site was located in the Western King Country (Waikato) region of the North Island of New Zealand. The site comprised approximately 420 hectares of regenerating, mānuka-dominated native scrub, surrounded by the native forest of the Whareorino Conservation Area. Photos illustrating the native vegetation of the study site at **C)** T1 (September/spring) and **D)** T2 (January/summer). **D)** White flowering is visible on mānuka.

4.3.2 Sample collection

Three days at three different times of the year during 2020 – 2021 were chosen for sampling. The times at which sampling took place (herein referred to as *timepoints*) were chosen so that they represented different seasons. To maintain consistency with our previous studies, the time of mānuka flowering (T2) was first identified as a target timepoint (Table 1). Two timepoints pre- and post- flowering were then determined. In order to minimise the confounding effect of short-term weather events that occur at different frequencies throughout the year (i.e., high winds and rainfall), we ensured that sampling was carried out after a minimum of four consecutive days of fine weather (i.e., low winds and zero precipitation). Although specific sampling dates were therefore weather-dependent, care was made to ensure timepoints were separated by approximately the same length of time (i.e., 4 months).

Table 1: Three sampling timepoints and the corresponding season and host phenology.

Timepoint	Month	Season	Host phenology
T1	September	Spring	Vegetative growth
T2	January	Summer	Flowering
T3	May	Autumn	Vegetative growth

In total, 9 – 10 trees throughout the study site were selected for sampling. Sample trees were visually healthy with no obvious signs of physical damage or disease. Furthermore, we ensured trees were a minimum of 10 m from the nearest access track in order to minimise the confounding influence of agricultural activities (i.e., mechanical damage by grazing animals or moving vehicles). In order to enable repeated sampling of trees at each timepoint, each tree was tagged with a removable wire tie and GPS coordinates (WGS84 (G1762); degree minutes) were recorded.

At each timepoint, sampling was conducted over the duration of a single day. Per tree, one branch with approximately 100 – 200 seemingly healthy leaves, was cut with clippers sterilised on site with 70% *v/v* ethanol/water, placed in individual sterile zip lock bags, and stored in an opaque carry container away from direct sunlight. In order to minimise differences in vegetation age, care was taken to avoid sampling zones of new growth. One surface (1 – 2 mm) soil sample from around the base of each tree was also collected into a sterile 50 mL Falcon tube using a spatula sterilised on site using 70% *v/v* ethanol/water and also stored in an opaque

carry container away from direct sunlight. Spatial descriptors recorded for each sample included: branch height, branch aspect, basal diameter of the tree base at 10 cm off the ground, tree height, slope aspect, and elevation. Upon return to the access vehicle (0.5 – 3 hours after collection), samples were immediately placed on dry ice. Upon return to the Thermophile Research Unit at the University of Waikato, branch and soil samples were frozen at -20°C until further analysis.

4.3.3 DNA extraction, amplification, and sequencing

Per branch, one gram of healthy, undamaged green leaves were carefully and aseptically excised and pooled. Given that each sample represented a pooled composite leaf sample, the effect of different individual leaf ages among trees and timepoints was minimised. Total phyllosphere DNA was extracted from the surface of excised leaves using a previously described sonication and modified cetyl trimethylammonium bromide (CTAB) bead-beating protocol (Noble *et al.* 2020). For each surface soil sample, total DNA was extracted from 0.5 g of soil using the Power Soil DNA Extraction kit (Qiagen). DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, United States) and stored at -20°C until further analysis. The V4-V5 hypervariable region of the 16S rRNA gene was amplified with 515FY/926R fusion primers (5'GTGYCAGCMGCCGCGGTAA/5'CCGYCAATTYMTTTR AGTTT) and previously described PCR conditions (Noble *et al.* 2020). PCR products were cleaned and standardised with SequelPrepTM (Thermo Fisher Scientific). Standardised samples were pooled at an equimolar concentration into two libraries. An Illumina MiSeq 300PE sequencing run was performed on each library at Massey Genome Service (Palmerston North, New Zealand).

4.3.4 Sequence quality control and taxonomic assignment

The raw 16S rRNA gene amplicon sequences were processed using the DADA2 pipeline (Callahan *et al.* 2016). Forward and reverse reads were truncated at 237 and 232 bp, respectively, and quality filtered using the 'filterAndTrim' function with the following settings: maxN = 0, maxEE = c(3, 3), and truncQ = 2. Error rates were determined with the 'learnErrors' function and used to remove sequencing errors from forward and reverse reads, which were then assigned to amplicon sequence variants (ASVs) using the 'dada' function. Paired reads were then merged, converted into an ASV table, and chimeras removed with the removeBimeraDenovo function using the method 'consensus'. Taxonomy was assigned using the 'assignTaxonomy' and 'addSpecies' functions using the native implementation of the naive

Bayesian classifier and the SILVA database version 138.1 (Quast *et al.* 2012). Chloroplast and mitochondrial sequences were filtered out by removing all ASVs with a taxonomic assignment of 'Chloroplast' at the Order level and 'Mitochondria' at the Family level, respectively. Lastly, we applied the 'isContaminant' function (method = prevalence) from the package 'decontam' to our samples using our blank DNA extractions and PCR reactions to identify and remove putative contaminants introduced during processing (Davis *et al.* 2018). After 16S rRNA gene sequence reads were quality filtered, the relationship between the number of ASVs versus number of sequencing reads was examined and preliminary NMDs ordinations were observed. However, no samples stood out as obvious outliers and therefore all 57 samples were retained for analysis. In total, 14,970 ± 5,067 quality-assured reads per sample remained.

4.3.5 Data analysis

All statistical analyses were conducted in R (R Core Team, 2023). Slope and branch aspect was converted into northing ($\cos \times \frac{\text{pi} \times \text{degrees}}{180}$) and easting ($\sin \times \frac{\text{pi} \times \text{degrees}}{180}$). Alpha diversity analyses were conducted using the 'vegan' package (Oksanen *et al.* 2007). Each phyllosphere and surface soil sample was first subsampled 100 times to an even sequencing depth (4,964 reads) and the average richness (observed number of ASVs) and Shannon-Wiener index was calculated for each sample. The Student's t-test and the Wilcox test was used to evaluate the significance of differences between the phyllosphere and surface soil microbiome for Shannon and Richness, respectively. Alpha diversity analyses were repeated for a phyllosphere-only subset of the data, whereby phyllosphere samples were subsampled a further 100 times to an even sequencing depth (4,964 reads) and the average richness and Shannon-Wiener index was calculated for each sample. These phyllosphere-only alpha diversity values were used to test for significant differences among timepoints with an ANOVA. Pearson's product-moment correlations were calculated between alpha diversity indices and continuous spatial descriptors. Pairwise Bray-Curtis and Jaccard community dissimilarities were calculated using the 'vegdist' function on ASV relative abundance and presence/absence transformed data, respectively. Differences in community structure among timepoints were assessed using a PERMANOVA on community dissimilarities using the 'adonis' function in the 'vegan' package.

4.4 Results

4.4.1 Phyllosphere microbial diversity

In total, 3,406 ASVs were detected across all phyllosphere samples at an average of 440 ± 146 ASVs per sample. No significant differences in alpha diversity (observed richness or Shannon) were observed across different timepoints ($p > 0.05$, Supplementary Fig. 2). Despite no significant difference in alpha diversity across timepoints, phyllosphere community diversity appeared more variable in samples collected at T1. Furthermore, some correlations were observed between phyllosphere microbial diversity and environmental variables (Table 2). Specifically, both richness and Shannon exhibited a negative relationship with elevation ($r = -0.47$, $p = 0.01$; $r = -0.37$, $p = 0.05$, respectively). Richness exhibited a negative relationship with slope easting ($r = -0.44$, $p = 0.02$). Furthermore, Shannon exhibited a positive relationship with tree height ($r = 0.38$, $p = 0.05$) (Table 2).

Table 2: Pearson's product-moment correlation between phyllosphere and surface soil alpha-diversity (Shannon and richness) and spatial descriptors. Bold indicates significant values ($p < 0.05$).

Spatial descriptor	Plant species	Richness		Shannon	
		r	*p value*	*r*	*p value*
Branch easting	Leaf	0.14	0.48	0.16	0.43
	Soil	*na*	*na*	*na*	*na*
Branch northing	Leaf	-0.08	0.69	-0.09	0.67
	Soil	*na*	*na*	*na*	*na*
Branch height	Leaf	0.25	0.21	0.15	0.46
	Soil	*na*	*na*	*na*	*na*
Tree height	Leaf	0.36	0.06	**0.38**	**0.05**
	Soil	0.01	0.96	0.02	0.92
Slope easting	Leaf	**-0.44**	**0.02**	-0.37	0.06
	Soil	-0.13	0.49	-0.10	0.60
Slope northing	Leaf	-0.11	0.57	-0.20	0.31
	Soil	0.10	0.60	0.15	0.45
Elevation	Leaf	**-0.47**	**0.01**	**-0.37**	**0.05**
	Soil	-0.07	0.71	-0.07	0.73

4.4.2 Phyllosphere community composition

In total, phyllosphere communities across all three timepoints were represented by 13 phyla (Supplementary Fig. 3, Supplementary Table 1). Proteobacteria were the most relatively abundant phylum (72.9 ± 8.9), followed by Acidobacteriota (9.79 ± 6.3), Bacteroidota (5.97 ± 7.4), Myxococcota (2.73 ± 4.3), and Deinococcota (2.31 ± 2.7) (Supplementary Table 1). No significant differences in the relative abundance of phyllosphere taxa classified at levels ranging from phylum to genus were detected across timepoints (Benjamini-Hochberg corrected Kruskal-Wallis $p > 0.05$, Supplementary Table 2). In total, 4 ASVs were assigned to genera known to be common members of the honey bee gut microbiome (Supplementary Table 3). Although these ASVs were mostly only identified at T2, these ASVs were identified in few samples and at low abundances.

4.4.3 Phyllosphere community structure

At the ASV level, sampling time did not explain any variation in either Bray-Curtis nor Jaccard community dissimilarity ($p > 0.05$, Fig. 2A-B, Supplementary Table 4). Instead, the individual host tree from which samples were taken explained approximately 50% of the variation in phyllosphere community structure (PERMANOVA on Bray-Curtis dissimilarities, $R^2 = 0.52$, $p = 0.001$; PERMANOVA on Jaccard dissimilarities, $R^2 = 0.47$, $p = 0.001$; Supplementary Table 4). Interestingly, inter-tree variation in phyllosphere community structure was significantly among timepoints (Fig. 2C-D). Specifically, T2 exhibited significantly less variance in Bray-Curtis and Jaccard community dissimilarity compared to T1 and T3 exhibited significantly less variance in Jaccard community dissimilarity compared to T1 (Betadisper < 0.05, Fig. 2C-D).

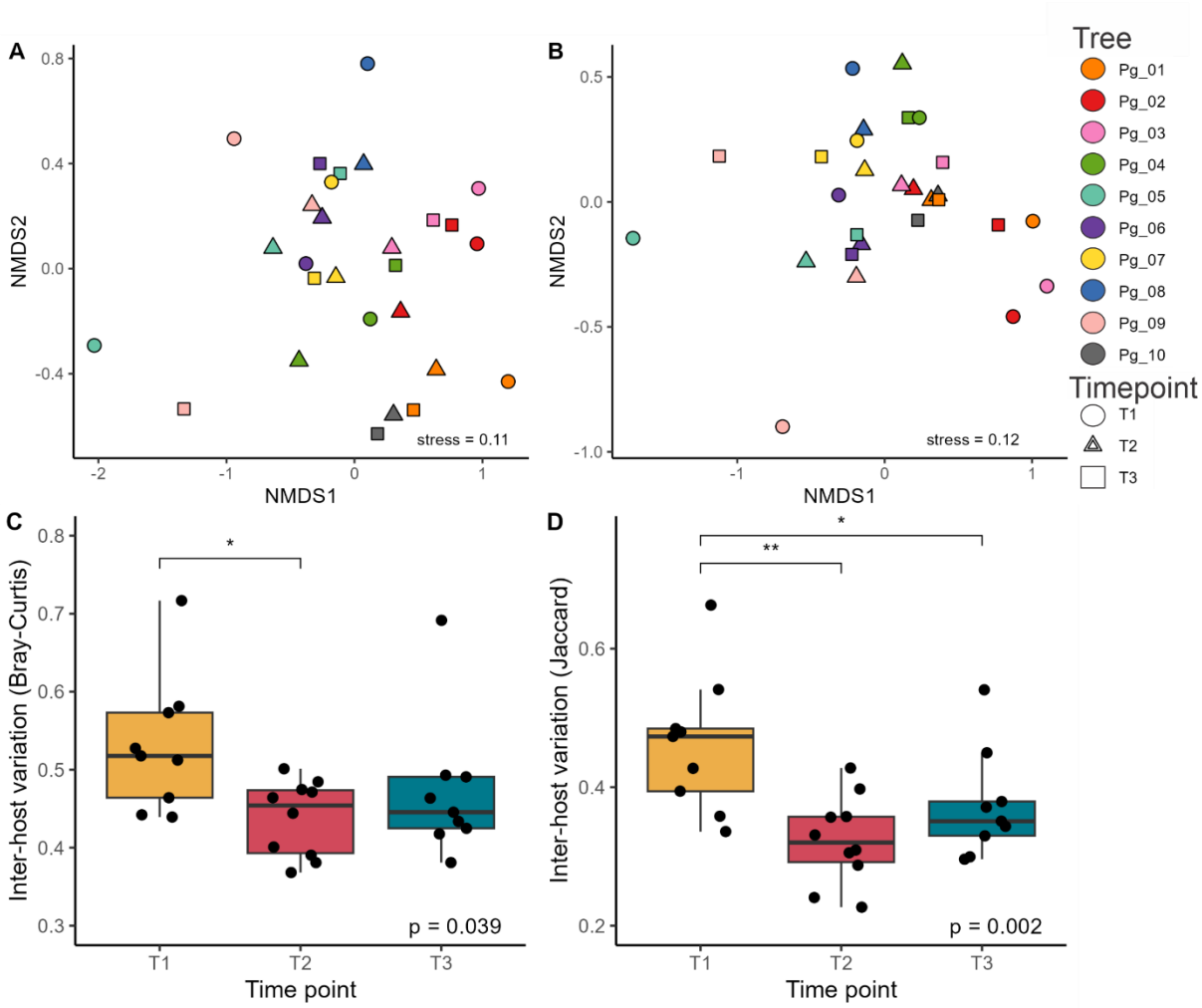

Fig. 2: Community structure of the mānuka phyllosphere microbiome across timepoints. NMDS ordination of **A)** Bray-Curtis (relative-abundance-based distance) and **B)** Jaccard (presence-absence-based distance). Colour represents host tree, shape represents timepoint. Box plot showing the corresponding inter-host variation in **C)** Bray-Curtis and **D)** Jaccard community dissimilarity at different timepoints. P value represents betadisper test and asterisks denote pairwise significance.

4.4.4 Core microbiomes

In order to assess the stability of the mānuka phyllosphere microbiome over time, we first searched for a core microbiome in all phyllosphere communities (i.e., phyllosphere samples collected across all timepoints). Notably, 16 ASVs were identified at 100% prevalence. These 16 ASVs were identified at an average relative abundance of 8.0 ± 4.4% and were members of the *Methylocella* and *1174-901-12* genera (Supplementary Fig. 4A).

In order to examine the influence of season on patterns of host association in the mānuka phyllosphere microbiome, we searched for a core microbiome across phyllosphere samples

collected from each individual timepoint. At T1, 16 ASVs were identified at 100% prevalence within the mānuka phyllosphere; these were the 16 ASVs that were previously identified across all timepoints (Fig. 3A). At T2, 117 ASVs were identified at 100% prevalence within mānuka phyllosphere (Fig. 3B). Lastly, 52 ASVs were identified at 100% prevalence within the phyllosphere communities at T3 (Fig. 3C). Furthermore, the cumulative relative abundance of these core taxa differed significantly across timepoints (Fig. 3D). The relative abundance was highest in phyllosphere communities from T2 at 30.16 ± 5.5%, followed by the phyllosphere communities from T3 at 23.5 ± 13.0%, and lowest in phyllosphere communities from T1 at 7.3 ± 3.8% (Benjamini-Hochberg corrected Dunn test $p < 0.05$, Fig. 3D). Similar to the 16 ASVs detected at 100% prevalence across all timepoints, core taxa belonging to each individual timepoint were members of the *Methylocella* and *1174-901-12* genera.

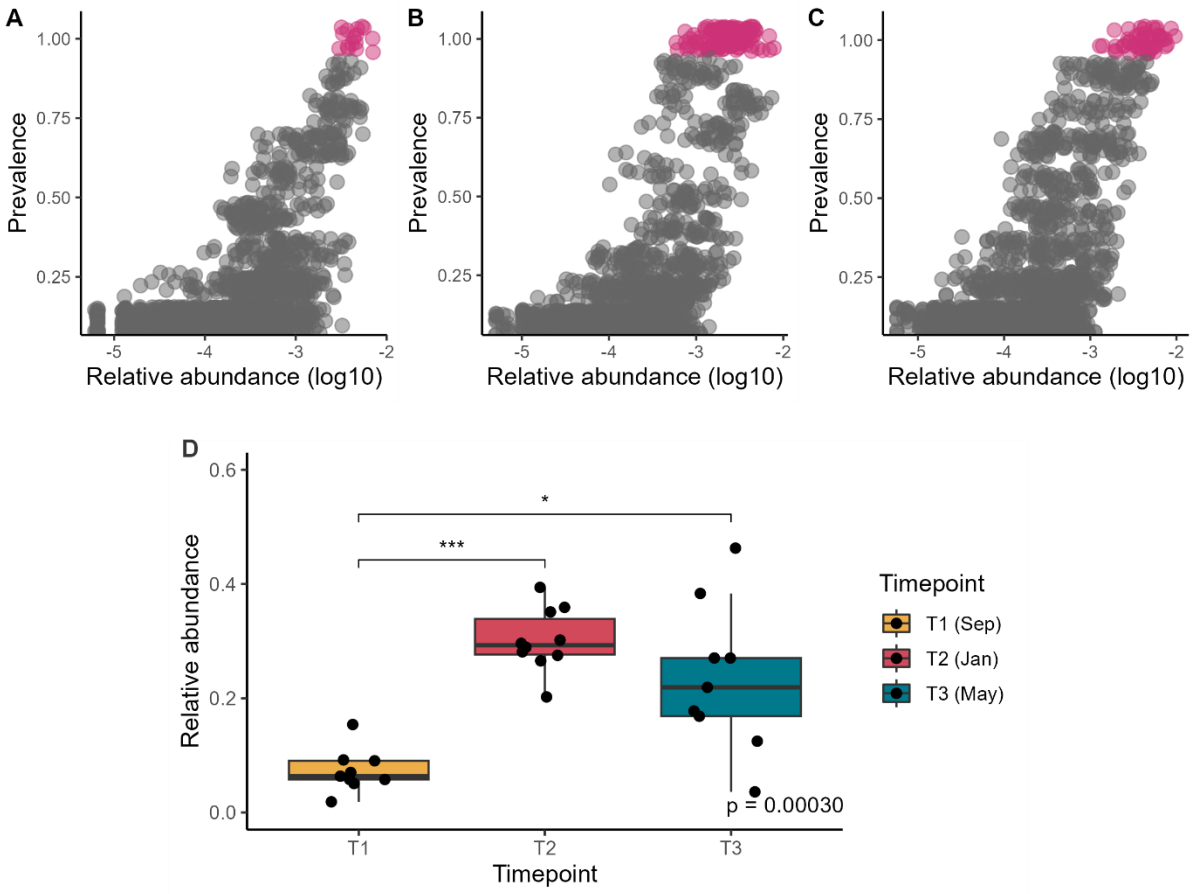

Fig. 3: The relative abundance (log10) of core taxa in the mānuka phyllosphere microbiome at each timepoint **A)** T1, **B)** T2, and **C)** T3. Pink represents core ASVs. Cumulative relative abundance of core taxa were significantly different at each timepoint **D)**. P value represents significance of Kruskal-Wallis test, asterisks denote significance of pairwise Dunn's test.

Intrigued by the large number of core ASVs identified at T2, we investigated the relative abundance and prevalence of these taxa in the mānuka phyllosphere at the other two timepoints. All 117 ASVs were identified in the phyllosphere microbiome in at 30% of the phyllosphere microbiomes at T1 and 50% of the phyllosphere microbiomes at T3 (Fig. 4A-C).

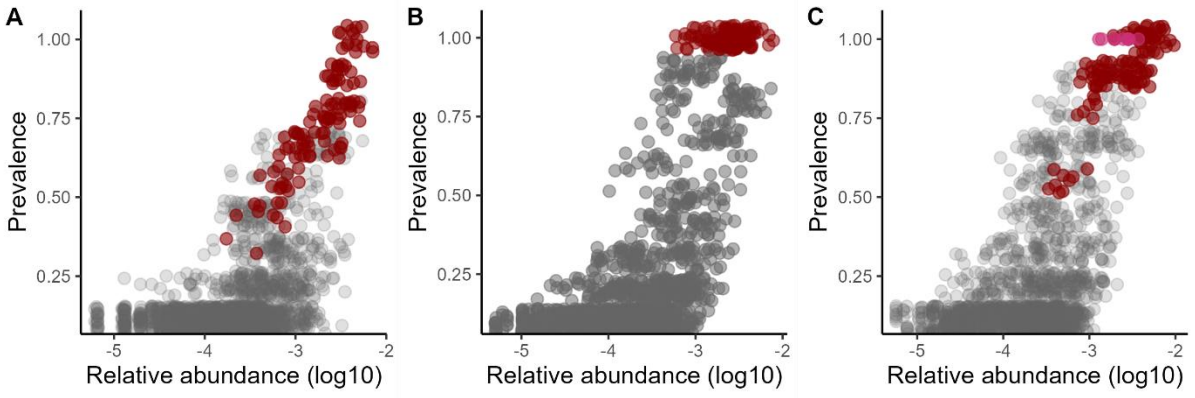

Fig. 4: The relative abundance (log10) versus prevalence of T2 core taxa in the mānuka phyllosphere microbiome at each timepoint **A)** T1, **B)** T2, and **C)** T3. Red represents the 117 ASVs identified at 100% prevalence at T2. Pink represents timepoint-specific core taxa that are not identified as T2 core taxa.

4.4.5 Surface soil microbial communities

In total, 29 surface soil samples were collected from the base of each sample tree and yielded 16,612 ± 5,067 quality-assured reads per sample. In total, 10,316 ASVs were detected, with an average of 532 ± 165 ASVs per sample. No significant differences in alpha-diversity (observed richness or Shannon) were observed across different timepoints (Kruskal-Wallis and ANOVA, $p > 0.05$). Further, no correlations were observed between community diversity and spatial descriptors (Table 2). Interestingly, community diversity appeared more variable in surface soil samples collected from T2. Moreover, significant differences in alpha-diversity (both observed richness and Shannon) were observed between phyllosphere microbial communities and surface soil.

Soil communities were represented by 24 phyla (Supplementary Table 5). On average, Proteobacteria were the phyla with the highest relative abundance (36.2 ± 8.7), followed by Verrucomicrobiota (17.5 ± 4.5), Bacteroidota (15.3 ± 5.9), Acidobacteroita (9.89 ± 3.4), and Planctomycetota (6.29 ± 2.2) (Supplementary Table 5). The relative abundance of taxa

classified at levels ranging from phylum to genus did not exhibit any significant difference across timepoints (Supplementary Table 6).

At the ASV level, time of sampling explained the largest proportion of variation in community dissimilarity (PERMANOVA on Bray-Curtis dissimilarities, R^2 = 0.35, p = 0.001; PERMANOVA on Jaccard dissimilarities, R^2 = 0.34, p = 0.001; Supplementary Table 4). The individual host tree from which samples were taken beneath also explained a significant, albeit small, proportion of variation in community structure (PERMANOVA on Bray-Curtis dissimilarities, R^2 = 0.08, p = 0.001; PERMANOVA on Jaccard dissimilarities, R^2 = 0.08, p = 0.002; Supplementary Table 4).

Notably, the structure and composition of surface soil microbial communities was significant different from the mānuka phyllosphere microbiome (Supplementary Fig. 5, Supplementary Fig. 6). This was observed across all levels of taxonomic resolution from the ASV level up to and including the phylum level. In total, 19 phyla exhibited significantly different relative abundances between phyllosphere and surface soil communities (Supplementary Table 7). Lastly, no core taxa (defined at 100% prevalence) were detected in surface soil communities collected across all timepoints (Supplementary Fig. 4B).

4.5 Discussion

A small number of studies have demonstrated that evergreen phyllosphere microbiomes can exhibit temporal variation in community structure (Jackson & Denney 2011; Bao *et al.* 2020; Al Ashhab *et al.* 2021). However, it remains to be determined if phyllosphere temporal variation is a universal characteristic of all plant species. With our quantitative spatial analyses in Chapter II and Chapter III, we have demonstrated the potential of the evergreen mānuka phyllosphere microbiome as a natural and long-lived perennial model plant species for investigating processes of community assembly. Specifically, our work thus far has revealed evidence consistent with a dominant role of host selection. In this study, we addressed a fundamental yet outstanding question: does the mānuka phyllosphere microbiome change over time? By performing replicate sampling of mānuka trees at three different times of the year, we investigated the relative influence of season on the community composition of the mānuka phyllosphere microbiome.

4.5.1 Mānuka phyllosphere microbial diversity is consistent across seasons

Notably, we did not observe a significant difference in phyllosphere alpha diversity across seasons (Supplementary Fig. 2A-B). This result is in line with the findings of Bao *et al.* (2020), who also did not observe a difference in alpha diversity in the evergreen phyllosphere microbiome of *Pinus bungeana* and *Sabina chinensis* during summer and winter. Interestingly, however, these authors did observe a significant difference in alpha diversity in the deciduous phyllosphere microbiome of *Ginko biloba* between seasons (Bao *et al.* 2020). Evergreen and deciduous phyllosphere microbiomes have also shown significantly different microbial diversities in a previous study (Laforest-Lapointe *et al.* 2016). Although many plant species-specific factors (i.e., traits that determine leaf carrying capacity) likely drive differences in phyllosphere alpha diversity among plants, the large ecological differences between deciduous and evergreen phyllosphere microbiomes (i.e., community residence time, stage and rate of leaf development, and leaf longevity) presumably play a major role. Our results also contrast with previous investigations of temporal variation in the total and culturable phyllosphere microbiome of annual and herbaceous plant species, which commonly report reductions in numbers of taxa over a growing season (Ercolani 1991; Thompson *et al.* 1993; Stone & Jackson 2021; Meyer *et al.* 2022). Such studies have suggested that this is due to 1) initial colonization by a large number of microorganisms that are subsequently outcompeted by preferentially adapted taxa (Meyer *et al.* 2022), and 2) reduced nutrient availability in senescing plant leaves (Stone & Jackson 2021). Thus, our results are consistent with a scenario in which microbial communities in the mānuka phyllosphere consist of taxa that are generally established (i.e., non-transient) members and that communities are in a stable steady state.

4.5.2 The relative influence of the mānuka host tree is stronger than season

On average, we found that phyllosphere communities sampled from the same host tree on three different days throughout the year were more similar than phyllosphere communities sampled on the same day from different host trees (Fig. 2A-B). The lack of a strong seasonal pattern is in agreement with our hypothesis and suggests that changes in climatic factors (e.g., solar radiation, average temperature, and relative humidity) as well as the seasonal frequency of disturbance effects (e.g., rainfall) do not have a strong relative influence on community structure in the mānuka phyllosphere. Instead, we identified a total core microbiome comprising 16 ASVs that were persistent across space (i.e., all trees) and time (i.e., all sampling dates). This finding is significant, considering that colonisation of the phyllosphere was

demonstrated in Chapter III to be a spatially heterogeneous process even among adjacent trees. However, this result is consistent with our work in Chapter II that suggests host selection is a dominant driver of community assembly in the mānuka phyllosphere (Noble *et al.* 2020). Moreover, the current study builds on these results by demonstrating that the relative influence of host selection persists despite seasonal changes in the surrounding environment and host tree phenology. Notably, the absence of pronounced seasonal variation in the mānuka phyllosphere contrasts with previous cultivation-independent studies that reported seasonality was a major determinant of evergreen phyllosphere community composition (Jackson & Denney 2011; Bao *et al.* 2020; Al Ashhab *et al.* 2021). However, in light of Chapter II, which also suggests the relative influence of host selection on phyllosphere assembly is quantitatively host dependent, it is conceivable that the degree to which the phyllosphere microbiome exhibits seasonal variation also varies among plant species. Given that significant temporal variation was previously observed in evergreen plant species that did not exhibit patterns of host species identity in phyllosphere community structure (Bao *et al.* 2020; Al Ashhab *et al.* 2021), whether the relative influence of season is inversely proportional to the relative strength of host selection represents an interesting hypothesis for future investigations. Moreover, disentangling whether seasonal variation is a feature of abiotic or biotic processes will require investigation on a species-by-species basis. Together with Chapter II, these observations further emphasise the need to consider plant species as ecologically discrete habitats when quantifying ecological processes of community assembly.

4.5.3 Patterns of host association in the mānuka phyllosphere increase during summer flowering

Although season did not appear to have a significant influence on overall community structure, a significant increase in the membership and relative abundance of the core microbiome was observed in the mānuka phyllosphere during summer flowering (i.e., timepoint T2) (Fig. 3A-D, Fig. 4A-C). Two previous studies have also reported a temporal convergence in phyllosphere community composition (i.e., changes that were not driven by alpha diversity) (Maignien *et al.* 2014; Grady *et al.* 2019), which both authors attributed to a strong selective force by the host. However, the use of short-lived plant species (i.e., *Arabidopsis thaliana* and herbaceous crops), limited the ability of these studies to disentangle whether this convergence was primarily driven by the host or community succession (i.e., leaf age related changes). Given that long-lived evergreen plant species produce and maintain mature leaves for multiple years, and that our pooled branch sampling design essentially controls for differences in

individual leaf age, we argue that the summer convergence observed in the mānuka phyllosphere microbiome is more likely to reflect seasonal changes in the host. Furthermore, given that core microbiomes are generally used to infer functionally significant host associations (Shade & Handelsman 2012; Shade & Stopnisek 2019), we postulate that this convergence is driven by a host selection that increases specifically during summer flowering. This interpretation is in line with previous studies have identified seasonal variation in the core microbiome of other hosts, such as the honeybee gut microbiome (Kešnerová *et al.* 2020). Repeated sampling over multiple years will help to shed light on whether this process has a cyclical nature and functional analyses will be necessary to infer whether a larger core microbiome confers a functional benefit to the host.

4.5.4 Why would host selection increase during summer flowering?

The correlation between the mānuka phyllosphere core microbiome and the summer flowering season raises many interesting speculations regarding the factors that could increase the relative influence of host selection on phyllosphere assembly.

First, it is possible that host selection is increased as a result of seasonal changes in host physiology (i.e., biotic drivers of community assembly). Interestingly, emissions of volatile organic compounds (VOCs) from mānuka leaves – specifically types of monoterpenoids, sesquiterpenoids, and green leaf volatiles – have been found to increase significantly in summer compared to other seasons (Effah *et al.* 2020). Terpenoids (or isoprenoids) are synthesised in the methylerythritol phosphate pathway of the chloroplast and represent a diverse class of plant secondary metabolites that play important roles in plant growth, stress protection (i.e., thermal protection to leaf photosynthesis), and interspecies signalling (i.e., herbivory protection) (Sharkey *et al.* 2008; McGenity *et al.* 2018; Zuo *et al.* 2019; Boncan *et al.* 2020). One hypothesis states that increased VOC production by mānuka leaves during summer reflects a heightened defence response against herbivorous insects, such as the endemic mānuka beetle, *Pyronota festiva*, which exhibits an adult stage of development from late November to mid-January (Stewart 1987; Effah *et al.* 2020; Effah *et al.* 2022). However, terpene metabolism is also a prevalent bacterial trait (Yamada *et al.* 2015). Bacterial isoprene degraders have been previously identified in the phyllosphere of a high isoprene emitting tree species (*Populus alba*) (Crombie *et al.* 2018). Moreover, a recent analysis also identified a surprising seasonal enrichment of pathways and genes involved in the metabolism of volatile terpenes (i.e., isoprene biosynthesis) in the (herbaceous) epiphytic phyllosphere microbiome of switchgrass

and miscanthus (Howe *et al.* 2023), two plant species in which a seasonal convergence has been previously reported (Grady *et al.* 2019). Given that a growing body of evidence demonstrates that plant-microbiome interactions are facilitated by VOCs and a growing number of studies document the metabolism of isoprene-related molecules in the phyllosphere, we speculate that core members of the mānuka phyllosphere microbiome are being preferentially enriched due to an increased production of organic compounds by the mānuka host. However, an alternative speculation could be that some of the VOCs emitted from mānuka leaves are of microbial origin, and the seasonal increase in VOC emissions is driven by a seasonal enrichment of core terpene-producing phyllosphere taxa. These speculations are particularly relevant since the results of Chapter II support a role for chemical signalling in mānuka phyllosphere assembly, a concept also suggested by previous studies (Lambais *et al.* 2014). Future work to understand this potential association between VOC emissions and convergence of the mānuka phyllosphere microbiome may shed light on a VOC-mediated host-bacteria interaction.

Other physiological changes also coincide with the transition between vegetative growth and flowering in evergreen woody perennials. One such change includes the internal transport of photosynthates as carbohydrates are preferentially mobilised from leaves to flowers (Kozlowski 1992). Phyllosphere microorganisms utilise a range of different nutrients that are leaked, leached, or exuded from internal leaf tissues to the leaf surface (Tukey 1966). Although epiphytic bacteria compete for many of the carbon sources in the phyllosphere, some carbon sources appear to be utilised only by specific genera. Moreover, it has been experimentally demonstrated that under carbon-limited conditions, the differential ability to catabolize a specific substrate can increase the population size of the catabolizing strain relative to other competing strains (Wilson *et al.* 1995). Therefore, a phenology-mediated decrease in available nutrients during summer could also preferentially increase selection for specific genera in the mānuka phyllosphere microbiome (Wilson *et al.* 1995; Leveau & Lindow 2001).

4.5.5 Other considerations

Beyond the physiology of the individual host plant, it is also necessary to consider other environmental or ecosystem-level changes that could confound the perceived relative strength of host selection during summer.

The first obvious factor that warrants consideration is climatic (i.e., abiotic) factors, such as temperature and water availability. New Zealand summers are generally characterised by prolonged periods of no rainfall. Water stress experienced by mānuka trees during summer, driven both by limited rainfall and increased transpiration via flowers, could therefore directly increase the abiotic stress experienced by phyllosphere microorganisms, which could drive the differential survival of taxa (Mohan Ram & Rao 1984; Potts 1994). Such a response has been experimentally demonstrated on the bean leaf surface, whereby leaves subjected to desiccation stress favoured the growth of *Pseudomonas syringae* aggregates over solitary cells (Monier & Lindow 2003). Furthermore, abiotic factors such as water availability can also have an indirect effect on the structure of plant-associated microbial communities via environmentally induced physiological changes in the host plant. Leaf-specific changes that could influence the phyllosphere include phytohormone-mediated stomatal closure and reduced photosynthesis (Salvi *et al.* 2021). Reduced physical disturbance is another means by which reduced rainfall could affect phyllosphere composition. Rain has been shown to remove a large number of microorganisms from leaf surfaces, which is then followed by short periods of exponential bacterial growth (Upper & Hirano 1994). Although previous culture-based studies have demonstrated large fluctuations in the abundance of individual phyllosphere microorganisms following rain, such as *Pseudomonas* spp. (Hirano *et al.* 1996), culture-independent studies have yielded conflicting results. For example, a previous study that investigated the short-term effect of rain in the evergreen magnolia phyllosphere did not report a strong influence on total phyllosphere community composition (Stone & Jackson 2019). Thus, the likelihood that an increased core membership was driven by a reduced frequency of rainfall-mediated disturbance events remains unclear.

At the broader ecosystem level, the summer flowering season is also characterised by increased visitation by pollinators and herbivorous insects (Stewart 1987). Therefore, it is possible that increased insect-mediated dispersal during summer could increase the prevalence of phyllosphere taxa among mānuka trees. Indeed, we did identify a small number of ASVs that were members of genera commonly identified in the honeybee gut microbiome (Kešnerová *et al.* 2020), which does suggest some microbial dispersal between host organisms. However, these were identified at a very low prevalence (i.e., 1 – 3 samples per timepoint) and abundance (i.e., < 0.03%). Moreover, given the heterogeneous distribution and activity of pollinators (i.e., foraging honeybees) (Beekman & Ratnieks 2000), an insect-mediated vector of dispersal does

seem like a less parsimonious explanation for such a systematic convergence in the mānuka phyllosphere microbiome.

4.6 Conclusions and significance

In summary, this chapter represents the first temporal analysis of the mānuka phyllosphere microbiome. The results demonstrate that bacterial community structure in the mānuka phyllosphere does not undergo drastic changes at times of the year that coincide with different seasons or stages of host phenology. Nevertheless, a notable increase in host association is observed during summer, which we postulate is due to a seasonal increase in the relative strength of host selection. Although multiple interpretations can be generated for our results, we argue that the informative power of this observational study is strengthened by our previous quantitative spatial analyses of community assembly in the mānuka phyllosphere microbiome. We demonstrate that complementing spatial analyses with temporal analyses can generate a great number of hypotheses for future investigation, which will not only broaden our knowledge of the ecological and mechanistic processes taking place within the phyllosphere microbiome of specific plant species but also contribute generalisable progress towards a unified understanding of phyllosphere community assembly. Furthermore, as the body of temporal research on the evergreen phyllosphere microbiome is poised to grow significantly, this study highlights a number of important considerations for future investigators. First, regarding plant species as ecologically discrete habitats will hopefully facilitate the development of a unified understanding of seasonal variation. Second, this study emphasises the importance of multi-disciplinary collaboration, as developing a mechanistic understanding of the drivers of temporal variation in the evergreen phyllosphere microbiome will require detailed knowledge of complex and species-specific plant physiological processes that influence the leaf surface at scales relevant to microorganisms.

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Chapter V

Investigating the relationship between the mānuka phyllosphere microbiome and mānuka honey quality: a case study

5.1 Abstract

Decades of research have been invested towards understanding the unique physiologies of the *Leptospermum scoparium* (mānuka) tree and the factors that are responsible for the unique non-peroxide antibacterial properties found in mānuka honey. Nonetheless, variation in the DHA-nectar trait of mānuka, and thus quality of mānuka honey, remain largely unexplained. Here, we use three adjacent populations of mānuka with discrete phenology (i.e., flowering time) to carry out a first exploration of the relationship between the mānuka phyllosphere microbiome and the physicochemical properties of mānuka honey. Our results reveal correlations between phyllosphere microbiome structure and several chemical constituents of mānuka honey, such as MGO, phenolic compounds, and trace elements. Together these findings are consistent with a relationship between the surrounding environment, mānuka trees, the mānuka phyllosphere microbiome, and mānuka honey quality. By describing a connection between mānuka and its wider environment via phyllosphere microorganisms, this work emphasises the importance of recognising the complex interconnected nature of our terrestrial ecosystems, the hidden role of multi-trophic interactions, and expanding our multidisciplinary approaches to address unresolved ecological questions. Finally, this work raises new ideas for future research, which will not only improve our understanding of mānuka and mānuka honey but could also be used to improve and protect the value of other NZ honey sources.

5.2 Introduction

It is widely acknowledged that honey composition is strongly influenced by its botanical and geographical origin (Anklam 1998). Specifically, the properties of honey strongly depend on the biochemical profile of the source nectar, which is qualitatively and quantitatively influenced by plant genetics and physiology. However, interactions between the nectar source and all aspects of the wider surrounding environment (i.e., climate, edaphic properties, plant and animal diversity, and pollinator species) also contribute unique physicochemical properties (Tomczyk *et al.* 2019). As such, identical nectar sources are capable of producing honeys with very different characteristics across locations and time (Anklam 1998; Tomczyk *et al.* 2019).

Understanding the unexplained variation in mānuka honey composition remains one of the major challenges faced by the New Zealand (NZ) mānuka honey industry (Molan 1992; Hamilton *et al.* 2013; Clearwater *et al.* 2021). Since Prof Peter Molan's pioneering discovery in 1981, it is now understood that the unique non-peroxide properties of mānuka honey originate in the nectar of the mānuka flower (Molan & Russell 1988; Adams *et al.* 2009; Williams *et al.* 2014). Specifically, a three-carbon sugar called dihydroxyacetone (DHA) accumulates in mānuka nectar and undergoes an irreversible enzymatic reaction in mature honey to methylglyoxal (MGO), the main (non-peroxide) antibacterial constituent (Grainger *et al.* 2016). However, the concentration of nectar DHA and thus honey MGO exhibits variation that remains largely unexplained despite decades of research on mānuka genetics (Dawson 1997; Clearwater *et al.* 2018; Sheridan 2019), physiology (Clearwater *et al.* 2021), climate (Noe *et al.* 2019), and soil (Nickless *et al.* 2017).

The challenges faced by the NZ mānuka honey industry are, however, not necessarily unique to apiculture. For example, the viticulture industry has also experienced similar challenges. Similar to honey, wine exhibits regionally distinct characteristics, known as "*terroir*", which is also influenced by grapevine genetics and physiology, as well as climate and soil (Gilbert *et al.* 2014; Liu *et al.* 2019). As such, the same clonal varieties of grapevine can produce different varieties of wine in different vineyards and therefore large scientific investments have been devoted towards elucidating the driving factors of the regionalised organoleptic properties of wine.

Progress in phyllosphere research over the last two decades has demonstrated a role of leaf epiphytic microorganisms in plant physiology (Lindow & Brandl 2003; Gupta *et al.* 2022).

Moreover, the application of this knowledge to industry has already proven hugely successful. For example, studies have revealed that the biogeographic patterns in grapevine phyllosphere communities represent another important driver of *terroir* (Gilbert *et al.* 2014; Gupta *et al.* 2022; Johnston-Monje *et al.* 2023). Throughout my previous studies and thesis chapters, I have demonstrated that the mānuka phyllosphere microbiome exhibits a spatiotemporally persistent and plant species-specific association with mānuka that increases during summer flowering (Noble *et al.* 2020). Given that such patterns of microbial community assembly are congruent with a microbiome that provides functional benefits to the host, we thus raise the question: does the mānuka phyllosphere microbiome influence mānuka honey physiology and thus quality?

At the study site for this chapter, mānuka honey is harvested annually from hives within three adjacent valleys of a privately owned site in the Whanganui region of New Zealand. Interestingly, the mānuka in each of these three valleys has been reported to exhibit different flowering phenology (i.e., flowering time), honey characteristics, and some hive sites tend to perform better than others (M. Walford, personal communication, November 2020). Differences in mānuka flowering phenology are common among distant mānuka populations and have been attributed to latitudinal gradients in environmental characteristics, such as day length and temperature, as well as host genetics (Dawson 1997). This site therefore provides an attractive system in which to carry out a first exploration of the relationship between the mānuka phyllosphere microbiome and mānuka honey characteristics. Specifically, this system allows us to investigate variation in phyllosphere communities and honey characteristics within discrete yet adjacent mānuka populations while controlling for the large-scale environmental heterogeneity (i.e., macroclimate factors such as rainfall and day length, plant and animal diversity, land-use classification, beekeeper, and pollinator population) that usually confounds inter-population empirical studies of mānuka (Meister et al. 2021). Here, we sampled mānuka trees, surface soil, and mānuka honey from each of these three valleys and explored correlational patterns between honey chemistry, elemental composition, and the phyllosphere microbiome.

5.3 Materials and Methods

5.3.1 Site description

This study was carried out on a single study site located in the Whanganui region of the North Island of New Zealand (39°51 S; 175°21 E) (Fig. 1A). In total, the study site comprises approximately 1500 hectares of grazed pasture, regenerating native scrub, and maize. The native scrub was dominated by mānuka and broadly divisible among three adjacent valleys that exhibit a range of different topographical descriptors, such as elevation, landforms, and slope aspect (Fig. 1B). Mānuka flowering typically lasts for six weeks, from which mānuka honey is harvested annually. Midflow of mānuka nectar is approximately 10-15th December.

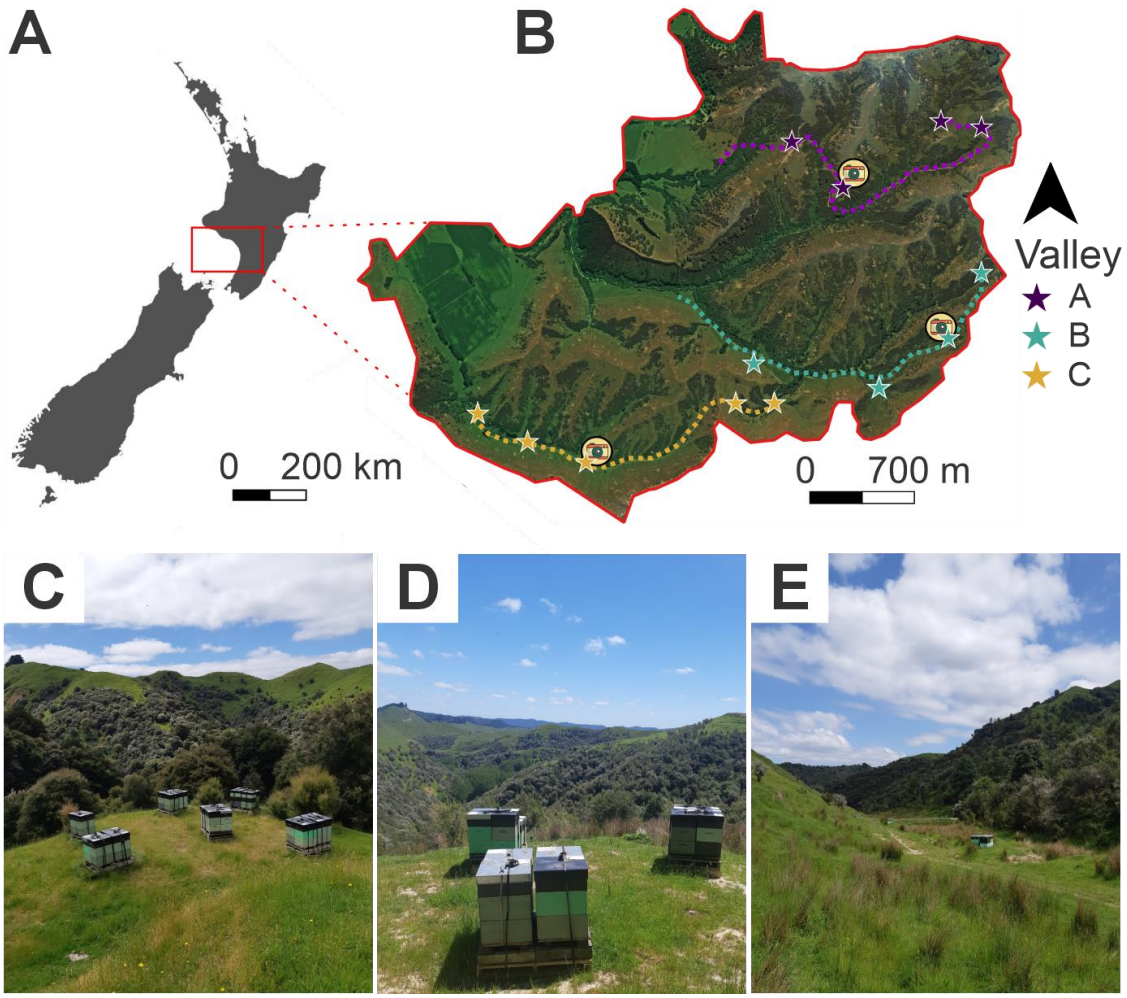

Fig. 1: Study site location and description. **A)** Study site was located in the Whanganui region of the North Island of New Zealand. **B)** Three distinct valley systems within the study site each contained hive sites (represented by stars) for mānuka honey production. Photos demonstrate the vegetative and topographical characteristics of **C)** valley A, **D)** valley B, and **E)** valley C. Camera symbols in site map **B)** represent the location of the corresponding photos **C-E**.

5.3.2 Phyllosphere and surface soil sample collection

Within the study site, each valley contained 4-5 hive sites for mānuka honey production (Fig. 1B). At each hive site, three mānuka trees were randomly chosen for sampling. Selected trees were between 1.9 – 5 m tall (i.e., of flowering age), visually healthy (i.e., no damage or disease), and generally within a small distance from their corresponding hives (i.e., 6 – 30 m) to ensure honeybee visitation. One exception was hive site Tk_13, whereby sampled trees were 78 – 96 m away due to the hives being positioned in the centre of grazed pasture. However, given the large foraging distance bees routinely cover (i.e., previous studies have reported mean foraging distances of 500 – 1200 m), our slightly larger hive – tree distances at Tk_13 are unlikely to affect pollinator visitation (Beekman & Ratnieks 2000).

Samples were collected over the duration of two days in the summer of December 2020. One branch per mānuka tree, with approximately 100-200 seemingly healthy leaves, was cut with clippers sterilised on site with 70% *v/v* ethanol/water, placed in individual sterile zip lock bags, and immediately placed on dry ice. One surface soil sample (1–2 mm) from around the base of one tree at each hive site was also collected into a sterile 50 mL Falcon tube with a spatula sterilised on site using 70% *v/v* ethanol/water and immediately placed on dry ice. Upon return to the Thermophile Research Unit at the University of Waikato, branch and soil samples were frozen at −20°C until further analysis.

5.3.3 DNA extraction, amplification, and sequencing

Per branch, 1 g of healthy, undamaged green leaves was carefully and aseptically excised and pooled. Total phyllosphere DNA was extracted from the surface of excised leaves using sonication and a modified cetyl trimethylammonium bromide (CTAB) bead-beating protocol that has been described previously (Noble *et al.* 2020). Total DNA was also extracted from 0.5 g of each surface soil sample using the Power Soil DNA Extraction kit (Qiagen). DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, United States) and stored at −20°C until further analysis. The V4-V5 hypervariable region of the 16S rRNA gene was amplified with 515FY/926R fusion primers (5'GTGYCAGCMGCCGCGGTAA/ 5'CCGYCAATTYMTTTRAGTTT) and previously described PCR conditions (Noble *et al.* 2020). PCR reactions were run in triplicate for each sample and pooled. PCR products were cleaned and each sample concentration was standardised with SequelPrepTM (Thermo Fisher Scientific). Standardised samples were pooled at an equimolar concentration into two libraries.

An Illumina MiSeq 300PE sequencing run was performed on each library at Massey Genome Service (Palmerston North, New Zealand).

5.3.4 Honey sample collection and preparation

Three honey samples (i.e., one sample per valley) were collected during the annual mānuka honey harvest at the end of the study site's 2020 flowering season. Each honey sample (i.e., 50 mL) represented a pooled blend from all hives within each valley (Fig. 1) and was used to measure the chemical constituents related to mānuka honey, elemental fingerprinting (ICP-MS), and visual pollen analysis. Plant and soil elemental chemistry was not included, given the large heterogeneity known to exist at small scales (Jarrell & Beverly 1981; Nicolson *et al.* 2007; Conn & Gilliham 2010; Clearwater *et al.* 2018; Noe *et al.* 2019).

Chemical characteristics of mānuka honey

Briefly, mānuka honey is primarily comprised of sugar (i.e., fructose and glucose) (65 – 80 %) and water (15 – 21 %), with small quantities of proteins, amino acids, and vitamins (Cianciosi *et al.* 2018). Honeys also contain polyphenols, products of secondary plant metabolism, which are specific to the nectar source. Relative to other honeys, mānuka honey has a high flavonoid content. Moreover, other polyphenols including phenyllactic acid and a group of methoxylated benzoic acids are also commonly described as the principal phenolic components in mānuka honey (Wilkins *et al.* 1993; Stephens *et al.* 2010; Wang *et al.* 2023). Within the current study, the mānuka-specific chemical constituents tested included: moisture, DHA, 5-hydroxymethyl-2-furfural (HMF), MGO, 2-methoxybenzoic acid, 4-hydroxyphenyllactic acid, 3-phenyllactic acid, and methylsyringate (for a brief description of each chemical see Table 1).

Table 1: Definition and description of the physicochemical properties measured in mānuka honey samples.

Physicochemical or chemical property	Definition
Moisture	One of the most important parameters of honey quality. The amount of water determines stability against fermentation and granulation (Singh & Singh 2018). Lower moisture (e.g., 19%) prevents fermentation.

Dihydroxyacetone (DHA)	The precursor to MGO. Accumulates in the nectar of the mānuka flower and undergoes an irreversible dehydration reaction to MGO in mature honey (Atrott *et al.* 2012; Grainger *et al.* 2016).
5-hydroxymethyl-2-furfural (HMF)	HMF is an intermediate product of the acid-catalysed dehydration reaction of hexose (i.e., fructose and glucose) during storage. Used as an indicator of thermal damage and/or ageing (Fallico *et al.* 2008). Limit is 40 mg kg^{-1} for export (CODEX STAN 2001; Bogdanov 2009).
Methylglyoxal (MGO)	Compound linked to the unique non-peroxide antibacterial properties found in mānuka honey (Adams *et al.* 2008). The higher the MGO concentration, the higher the antibacterial efficacy, and consequently the higher the price.
2-methoxybenzoic acid	Polyphenol commonly found in mānuka honey. Minimum concentration of 5 mg kg^{-1} is required for monofloral mānuka honey classification (Ministry for Primary Industries 2017).
4-hydroxyphenyllactic acid	Polyphenol commonly found in mānuka honey. Minimum concentration of 1 mg kg^{-1} is required for monofloral mānuka honey classification (Ministry for Primary Industries 2017).
3-phenyllactic acid	Marker polyphenol for mānuka honey. Minimum concentration of 400 mg kg^{-1} is required for monofloral mānuka honey classification (Ministry for Primary Industries 2017). Has a synergistic effect on MGO (Thierig *et al.* 2023).
Methylsyringate	Polyphenol found in high abundance in mānuka honey. Functionally related to syringic acid. Proposed as a phytochemical fingerprint of mānuka honey in the 1990's. Has been identified as a superoxide scavenger (Inoue *et al.* 2005).

Elemental fingerprinting (ICP-MS)

Small concentrations of elements are also present in mānuka honey (< 1 g 100 g^{-1}), which are largely influenced by geographical location and botanical source. Among the most represented are potassium, calcium, magnesium, sodium, manganese, copper, iron, and zinc (Cianciosi *et al.* 2018; Grainger *et al.* 2021). Therefore, elemental fingerprinting was also carried out on the honey samples. Samples were prepared for analysis using a briefly described method (Grainger *et al.* 2020). Briefly, honey samples were mixed and weighed (200 ± 20 mg). Weighed samples were combined with 0.2 mL of nitric acid and heated to 80°C for 60 min. Samples were cooled for 30 min, 0.1 mL hydrogen peroxide was added, and then samples were heated to 80°C for another 60 min. Ultrapure water was added to cooled samples to a final volume of approximately 6 mL. Each specific volume was recorded for each sample. Prior to ICP-MS analysis, all samples were centrifuged (10 min, 3000 rpm). Elemental analysis was carried out using a previously described protocol and an Agilent 8900 Triple Quadrupole Inductively Coupled Plasma Mass Spectrometer (Q-ICP-MS; Agilent Technologies, Santa Clara, California, USA) (Grainger *et al.* 2021).

5.3.5 Sequencing quality control and taxonomic assignment

The raw 16S rRNA gene amplicon sequences were processed using the DADA2 pipeline (Callahan *et al.* 2016). Forward and reverse reads were truncated at 237 and 232 bp, respectively, and quality filtered using the 'filterAndTrim' function with the following settings: maxN = 0, maxEE = c(3, 3), and truncQ = 2. Error rates were determined with the 'learnErrors' function and used to remove sequencing errors from forward and reverse reads, which were then assigned to amplicon sequence variants (ASVs) using the 'dada' function. Paired reads were then merged, converted into an ASV table, and chimeras removed with the removeBimeraDenovo function using the method 'consensus'. Taxonomy was assigned using the 'assignTaxonomy' and 'addSpecies' functions using the native implementation of the naive Bayesian classifier and the SILVA database version 138.1 (Quast *et al.* 2012). Chloroplast and mitochondrial sequences were filtered out by removing all ASVs with a taxonomic assignment of 'Chloroplast' at the Order level and 'Mitochondria' at the Family level, respectively. Lastly, we applied the 'isContaminant' function (method = prevalence) from the package 'decontam' to our samples using our blank DNA extractions and PCR reactions to identify and remove putative contaminants introduced during processing (Davis *et al.* 2018).

After 16S rRNA gene sequence reads were quality filtered, one sample (Tk_02.3) stood out as an outlier. This sample did exhibit a considerably smaller number of sequencing reads compared to other samples (Supplementary Fig. 1). Moreover, this sample also appeared to exhibit unusual compositions relative to all other samples such that preliminary NMDS plots appeared highly skewed (Supplementary Fig. 2). Therefore, sample Tk_02.3 was removed from further analyses and 13,718 ± 3,733 quality reads per sample remained.

5.3.6 Data analysis

All statistical analyses were conducted in R (R Core Team, 2023). Slope and branch aspect was converted into northing ($\cos \times \frac{\text{pi} \times \text{degrees}}{180}$) and easting ($\sin \times \frac{\text{pi} \times \text{degrees}}{180}$). Alpha diversity analyses were conducted using the 'vegan' package (Oksanen *et al.* 2007). Each phyllosphere and surface soil sample was first subsampled 100 times to an even sequencing depth (4,451 reads) and the average richness (observed number of ASVs) and Shannon-Wiener index was calculated for each sample. The Shapiro-Wilk test was used to test normality and the Student's t-test and Wilcoxon test was used to evaluate significant differences in richness and Shannon across sample type, respectively. The Kruskal-Wallis and ANOVA tests were used to evaluate significant differences in phyllosphere richness and Shannon across valleys, respectively. Pearson's product-moment correlations were calculated between alpha diversity indices and continuous spatial descriptors. Pairwise Bray-Curtis and Jaccard community dissimilarities were calculated using the 'vegdist' function on ASV relative abundance and presence/absence transformed data, respectively. Differences in community structure among valleys and sample type were assessed using a PERMANOVA on community dissimilarities with a crossed design using the 'adonis' function in the 'vegan' package. We identified relationships between bacterial community structure and honey chemistry/elements by calculating correlations between log-transformed honey data and the scores of samples on the axes of the bacterial community ordination using the 'envfit' function in the 'vegan' package.

5.4 Results

5.4.1 Phyllosphere microbial diversity

In total, 3,645 ASVs were detected across all mānuka phyllosphere communities. No significant differences in alpha-diversity (richness or Shannon) were detected between valleys ($p > 0.05$) (Supplementary Fig. 3A-B). However, a significant correlation was observed

between both indices of alpha diversity and tree height (r = 0.37, p = 0.02, Supplementary Table 1). No other significant correlations were observed between phyllosphere community diversity and spatial descriptors (Supplementary Table 1). Neither richness nor Shannon diversity index were significantly different between phyllosphere and surface soil microbial communities (p > 0.05) (Supplementary Fig. 3C-D).

5.4.2 Phyllosphere community composition

In total, mānuka phyllosphere communities were represented by 13 phyla (Supplementary Fig. 4). Proteobacteria were the dominant members of these communities (average relative abundance 77.9 ± 9.6%), followed by Acidobacteriota (8.78 ± 4.4), Bacteroidota (5.76 ± 6), Bdellovibrionota (3.45 ± 3), and Verrucomicrobiota (1.5 ± 1.6) (Supplementary Table 2). Community composition at the phylum level was relatively consistent across all mānuka samples and no significant differences in phyla relative abundance were detected across valleys (Benjamini-Hochberg corrected Wilcox test $p > 0.05$, Supplementary Table 3). At the genus level, mānuka phyllosphere communities were represented by 130 genera, of which 11 were detected at an average relative abundance greater than 1%. *1174-901-12* was the most abundant genus (43.1 ± 12%), followed by *Methylocella* (19.1 ± 10%), *Spingomonas* (7.23 ± 3.7%), *Granulicella* (5.95 ± 3.4%), and *Bdellovibrio* (3.44 ± 3%). Although some variation was observed in low-abundance genera, they were not statistically significant (Fig. 2).

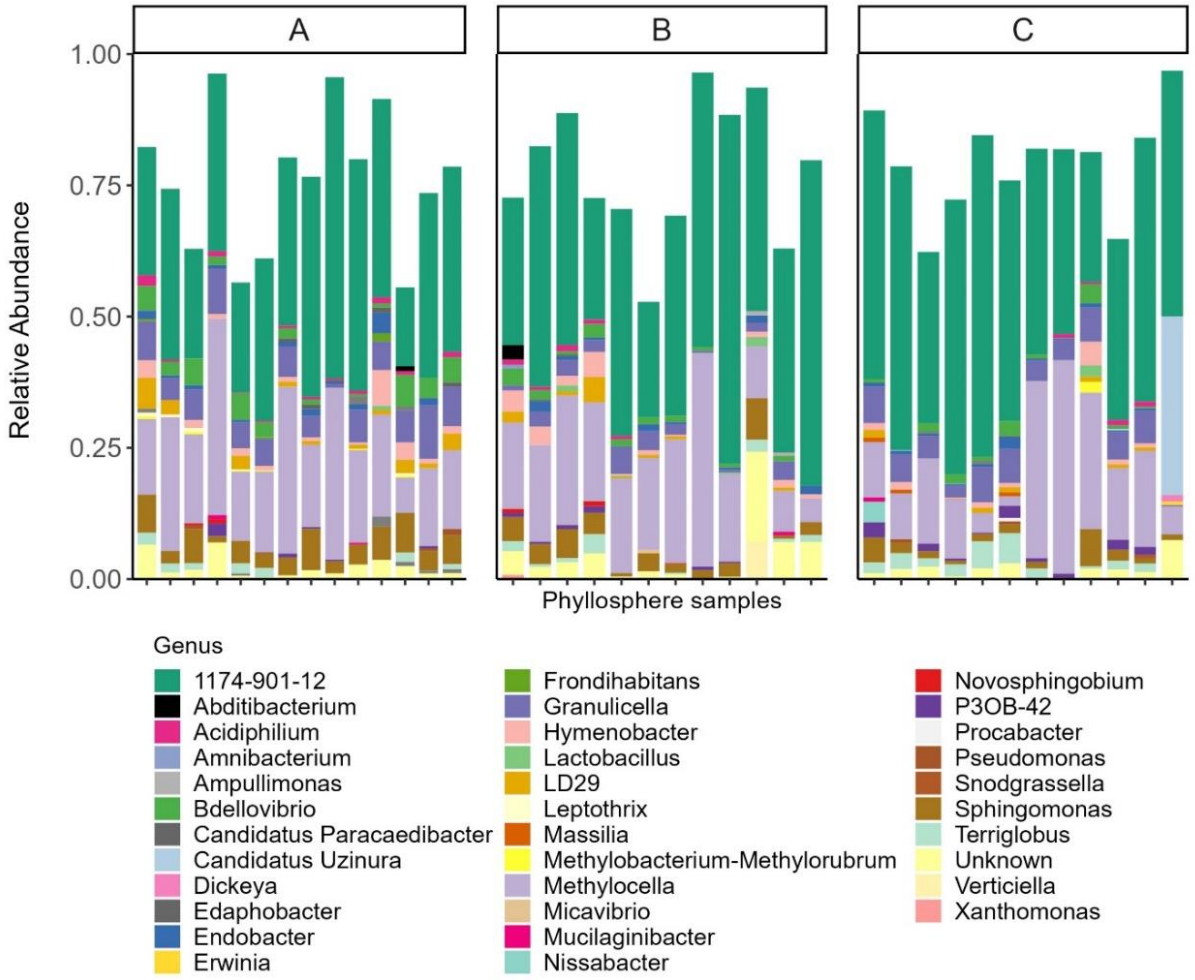

Fig. 2: Relative abundance of taxa in phyllosphere microbial communities across valleys. Colour represents taxonomic classification at the genus level.

5.4.3 Phyllosphere community structure

At the ASV level, the hive site from which each tree was sampled explained a significant proportion of variation in phyllosphere community dissimilarity (PERMANOVA on Bray-Curtis dissimilarities, $R^2 = 0.25$, $p = 0.001$; PERMANOVA on Jaccard dissimilarities, $R^2 = 0.21$, $p = 0.001$; Fig. 3; Table 2). Moreover, a small amount of variation was also explained by the valley in the trees were located (PERMANOVA on Bray-Curtis dissimilarities, $R^2 = 0.045$, $p = 0.002$; PERMANOVA on Jaccard dissimilarities, $R^2 = 0.041$, $p = 0.011$; Table 2).

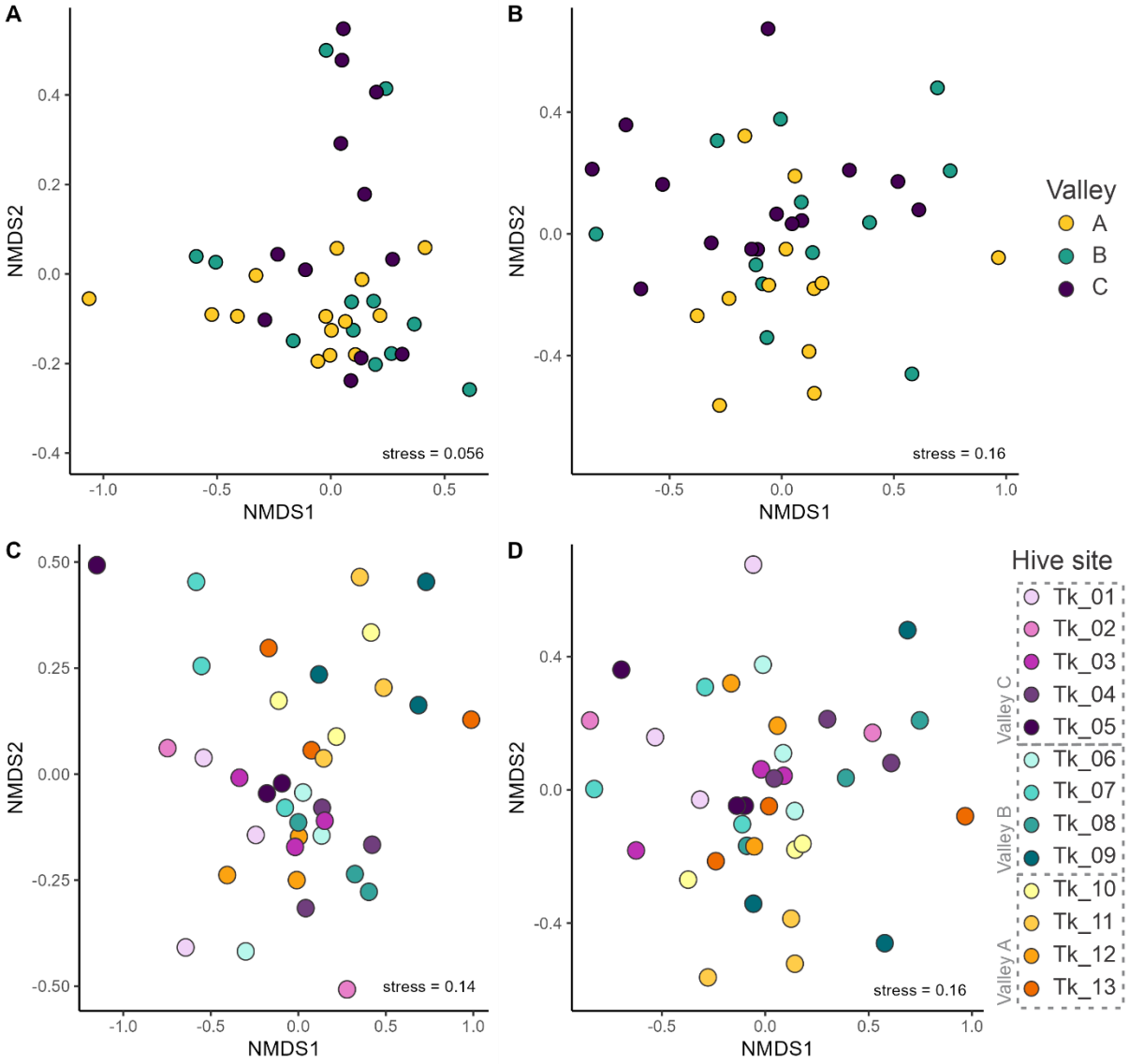

Fig. 3: Community dissimilarity of mānuka phyllosphere communities across valleys. Community dissimilarity is based on **A/C)** Bray-Curtis and **B/D)** Jaccard. **A-B)** Colour represents valley from which each phyllosphere sample was collected. **C-D)** Colour represents hive site from which each phyllosphere sample was collected.

Table 2: PERMANOVA on community dissimilarities. Bold values indicate significant values ($p < 0.05$).

Sample type	**Variable**	**Relative abundance**		**Presence/absence**	
		R^2	P value	R^2	P value
Phyllosphere	Valley	**0.045**	**0.002**	**0.041**	**0.011**
	Hive site	**0.25**	**0.001**	**0.21**	**0.001**
Soil	Valley	0.18	0.187	**0.18**	**0.024**

5.4.4 Core phyllosphere microbiomes

Given the identification of a core microbiome in all previous studies of the mānuka phyllosphere, we next searched for the presence of a core microbiome in the current study (i.e., phyllosphere samples collected from all valleys). Overall, 54 ASVS were identified at 100% prevalence. These 54 ASVs had a collective average relative abundance of 27.6 ± 9.4% and were members of Proteobacteria within the *1174-901-12* genus (Supplementary Fig. 5).

5.4.5 Surface soil microbial communities

In total, 13 surface soil samples were collected across all hive sites and yielded 14,648 ± 2,757 quality-assured reads per sample. In total, 4,774 ASVs were identified, with an average of 443 ± 124 ASVs per sample. No significant correlations were observed between surface soil alpha-diversity (richness and Shannon) and microbiome spatial descriptors (Supplementary Table 1). At the ASV level, a small proportion of community dissimilarity based on the presence/absence of taxa was explained by valley (PERMANOVA on Jaccard dissimilarities, $R^2 = 0.18$, $p = 0.024$; Table 2).

Surface soil microbial communities were represented by 30 phyla. Proteobacteria were the dominant members of these communities (average relative abundance 27.9 ± 3.8%), followed by Verrucomicrobiota (22.9 ± 6.1), Bacteroidota (16.2 ± 5.1), Acidobacteriota (8.89 ± 2.1), and Planctomycetota (4.93 ± 1.9). No significant differences in the relative abundance of phyla were detected across valleys (Benjamini-Hochberg corrected Wilcox test $p > 0.05$). At the genus level, surface soil microbial communities were represented by 313 genera, of which 17 were detected at an average relative abundances greater than 1%. *Candidatus Udaeobacter* was the most abundant genus (11.6 ± 6%), followed by ADurb.Bin063-1 (3.2 ± 1.3%), *Bradyrhizobium* (2.49 ± 0.74%), *Bryobacter* (2.49 ± 0.74%), and *Puia* (2.07 ± 1.4%). No significant differences in the relative abundance of genera were detected across valleys (Benjamini-Hochberg corrected Wilcox test $p > 0.05$).

Significant differences in composition were observed when comparing surface soil and phyllosphere bacterial communities. These differences were observed at all levels of taxonomic resolution ranging from the level of phyla to ASVs (Supplementary Fig. 7, Supplementary Fig. 7).

5.4.6 Honey chemistry

The physicochemical constituents outlined in Table 1 were measured in each honey sample (for raw values, see Supplementary Table 4). First, we found that the concentration of the three phenolic compounds (2-methoxybenzoic acid, 4-hydroxyphenyllactic acid, 3-phenyllactic acid) were above the minimum threshold required for identification of monofloral mānuka honey. In total, three constituents exhibited significant correlations with total variation in phyllosphere community structure (ordination sample scores). These included moisture (R^2 = 0.16, p = 0.007), HMF (R^2 = 0.20, p = 0.003), and 2-methoxybenzoic acid (R^2 = 0.14, p = 0.012). The same three constituents plus MGO and methylsyringate exhibited significant correlations with variation in the non-core phyllosphere community (Supplementary Table 5). These five constituents plus DHA and 3-phenyllactic acid also exhibited significant correlations with variation in the core phyllosphere community. Although the correlations between the core community and both DHA and 3-phenyllactic acid were small, it is particularly interesting that these correlations were only observed with the core members of the mānuka phyllosphere community (54 ASVs).

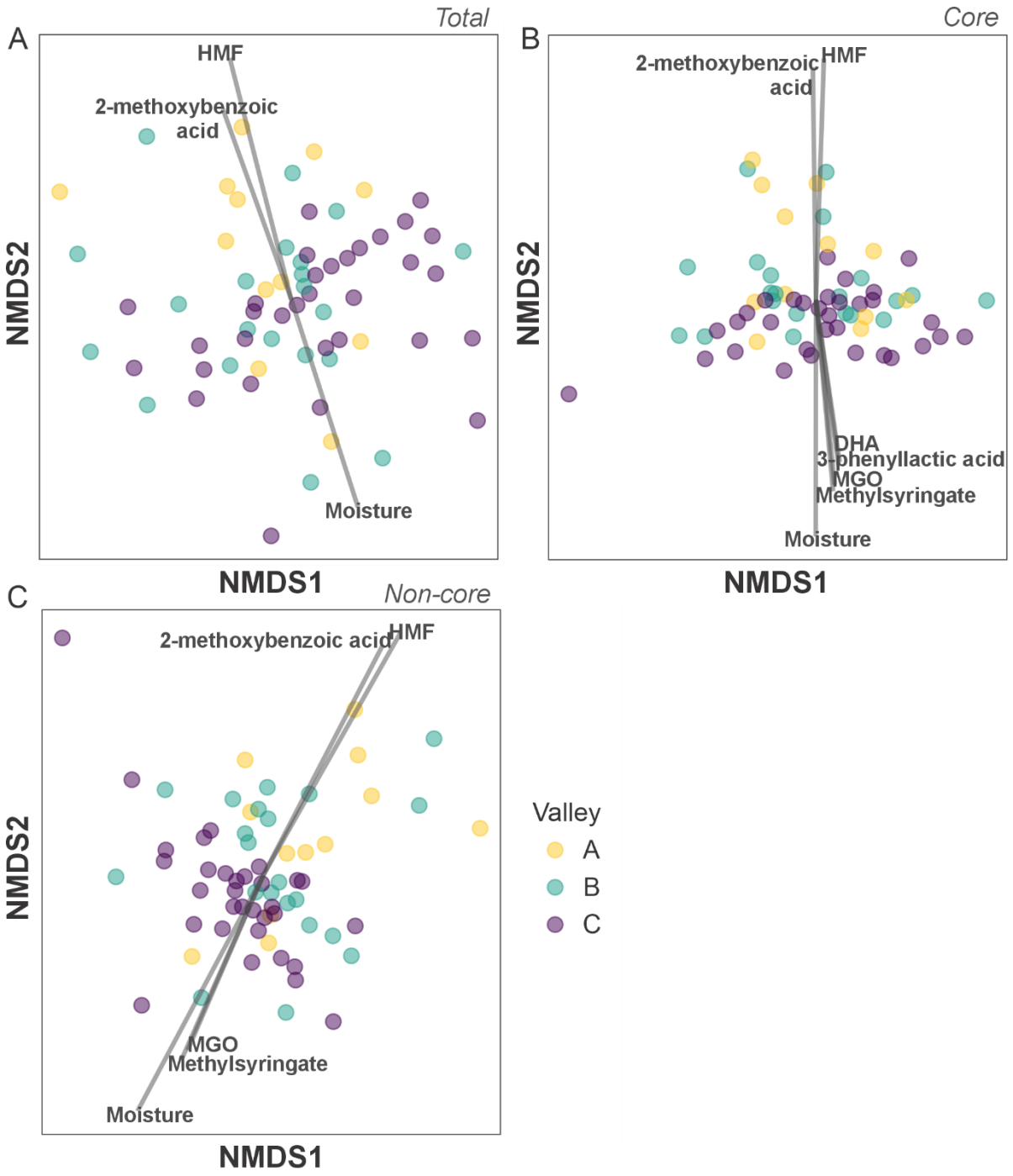

Fig. 4: Community dissimilarity of phyllosphere communities and significant correlations with mānuka honey chemistry. NMDS ordination of **A)** total, **B)** core, and **C)** non-core mānuka phyllosphere communities. Colour represents valley. Vectors represent chemistry components with significant ($p < 0.05$) correlations with sample scores on ordination axes.

5.4.7 Honey elemental analysis

Honey samples were analysed for 20 major, minor, and trace elements. In total, the concentrations of these elements in each honey sample were within the normal range of values that have been previously observed in New Zealand honey (Grainger *et al.* 2021; Grainger *et al.* 2024) (Supplementary Table 6). On average, the most abundant element was potassium (on average 2,310 ± 213 mg kg^{-1}), followed by Ca (92.7 ± 7.74 mg kg^{-1}), Na (76.0 ± 32.2 mg kg^{-1}), Mg (29.2 ± 3.21 mg kg^{-1}), and B (9.32 ± 1.03 mg kg^{-1}). These elements have also been reported as the most abundant elements in other NZ honey samples (Grainger *et al.* 2024). The next most abundant elements were Rb, Mn, Zn, Al, and Fe (concentrations ranging from 880 – 2,000 µg/kg). This was followed by smaller concentrations of Sr, Ba, Cu, Cr and Ni (30 – 270 µg kg^{-1}) (Supplementary Table 6). The elements with the smallest concentration were Tl, Co, Pb, Cd, and Cs (0.25 – 9 µg kg^{-1}) (Supplementary Table 6).

In total, 14 elements exhibited significant correlations with total variation in phyllosphere community structure (ordination sample scores) (Supplementary Table 7). Furthermore, 15 elements exhibited significant correlations with variation in the phyllosphere core community structure (Supplementary Table 7). These elements were the same as those that exhibited significant correlations with the total phyllosphere community, with the addition of Zn (R^2 = 0.12, p = 0.033). In total, 17 elements exhibited significant correlations with variation in the non-core phyllosphere community. The two elements that exhibited correlations that were only detected in the non-core phyllosphere community were Ni (R^2 = 0.11, p = 0.027) and Sr (R^2 = 0.11, p = 0.024).

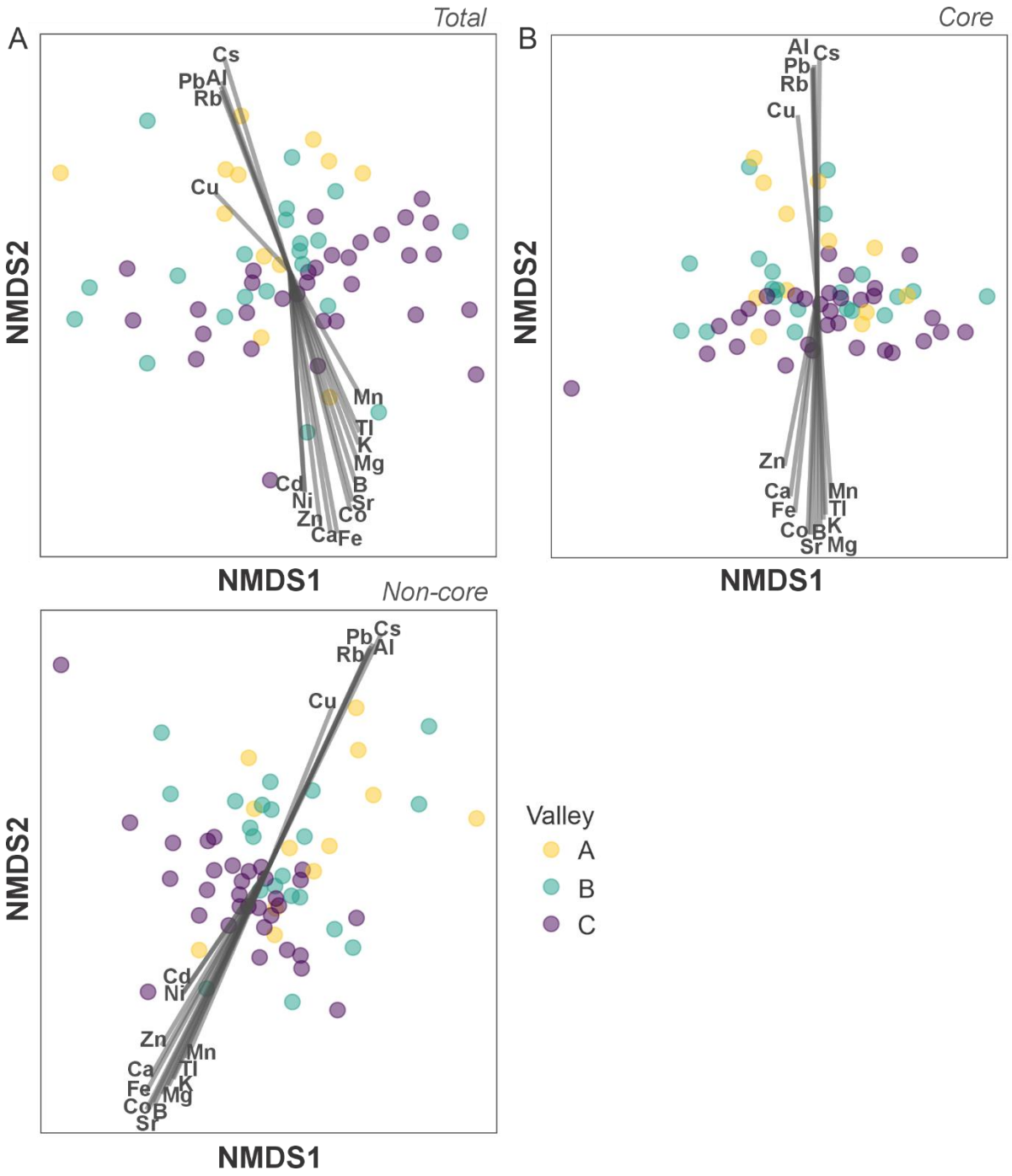

Fig. 5: Community dissimilarity of phyllosphere communities and significant correlations with honey elements. NMDS ordination of **A)** total, **B)** core, and **C)** non-core mānuka phyllosphere communities. Colour represents valley. Vector represent honey elements with significant ($p < 0.05$) correlations with sample scores on ordination axes.

5.4.8 Visual pollen analysis

A visual pollen analysis is a reliable and widely used method for identifying the plant origin of a honey and thus was carried out on the three honey samples in the current study in order to confirm a mānuka origin (Moar 1985). The concentration of pollen per 10 g of each honey sample was 174,739 (valley A), 212,169 (valley B), and 240,580 (valley C). The most prominent source of nectar pollen was mānuka/kānuka, comprising 45 – 58 % of counts (Fig. 6). This was followed by trefoil (17 – 31 %), clover (4 – 6 %), titoki (2 – 9 %), catsear and dandelion type (1 – 4 %), and buttercup (0.3 – 2 %) (see Supplementary Table 8 for raw pollen counts).

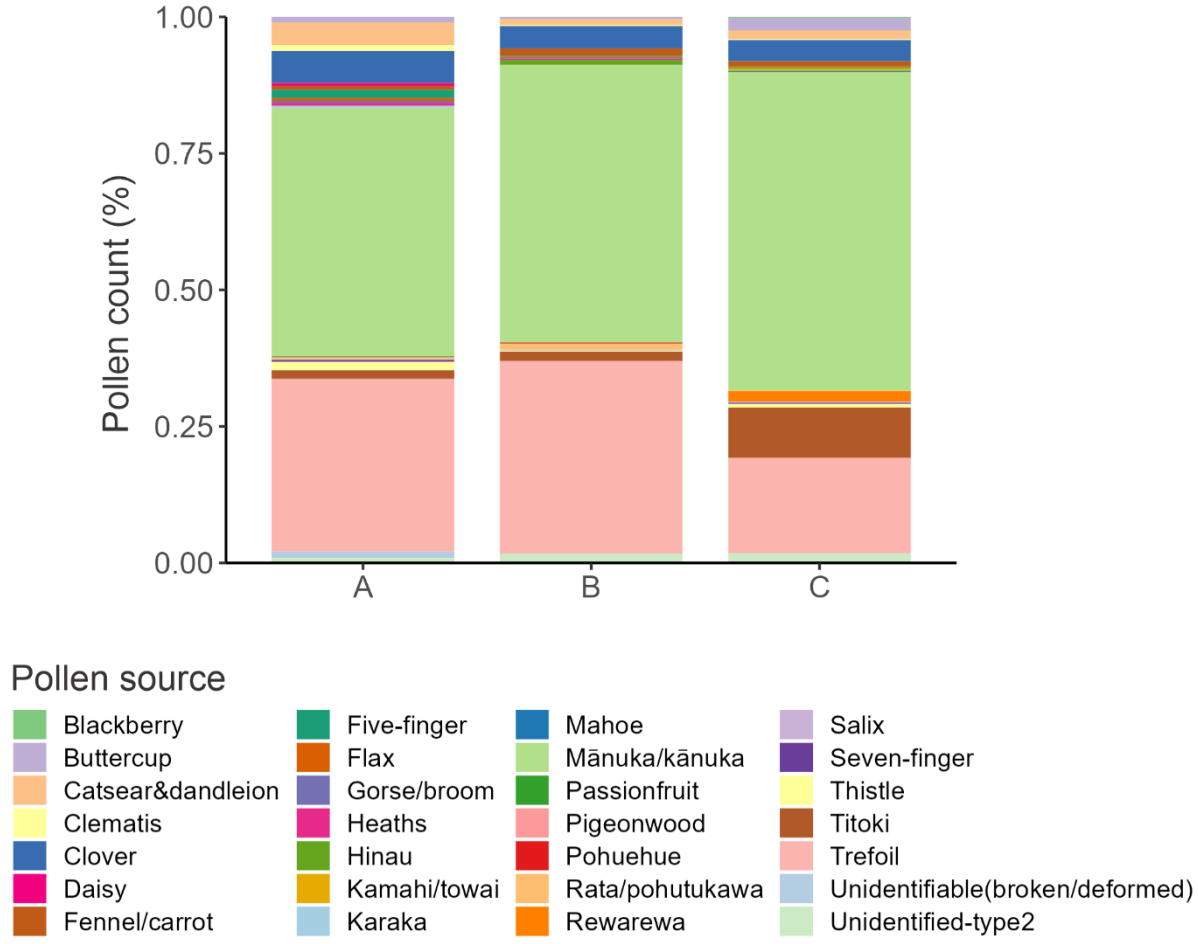

Fig. 6: Honey pollen composition (%) determined by visual pollen analysis. Colour represents plant source.

5.5 Discussion

Despite decades of research, the factors that drive variation in the physicochemical attributes of mānuka honey, such as MGO, remain unclear and thus new ideas require exploration. A series of quantitative and qualitative spatiotemporal analyses (i.e., Chapters II – IV of the here presented thesis) have demonstrated that the mānuka phyllosphere microbiome exhibits patterns of community assembly that are congruent with a microbiome that may influence host physiology. In this case study, we used three adjacent, yet geographically defined, populations of mānuka, which naturally exhibit distinct physiologies and honey characteristics, to explore the relationship between the phyllosphere microbiome and mānuka honey quality.

5.5.1 Variation in phyllosphere microbiome community structure correlates with mānuka honey MGO

Correlations were identified between the concentration of key chemical constituents in mānuka honey and variation in phyllosphere community structure across valleys (Fig. 4A-C). One notable correlation observed with both the core and non-core members of the phyllosphere microbiome was honey MGO. This observation is significant, given that the concentration of MGO largely determines the unique non-peroxide antibacterial properties and thus commercial value of mānuka honey (Fig. 4B-C). Moreover, given that MGO is produced via a non-enzymatic reaction of nectar DHA (Grainger *et al.* 2016), these results thus suggest a potential link between the composition of the phyllosphere microbiome and the concentration of nectar DHA.

Currently, the most parsimonious hypothesis for nectar DHA production posits that DHA, a derivative of triose-phosphate (DHAP), is produced by chloroplasts in the green nectary cells of the mānuka flower (Clearwater *et al.* 2021). In support of this hypothesis, a recent study also identified a candidate phosphatase gene, *LsSgpp2*, which was differentially expressed in the nectary tissue of mānuka trees that produced high and low nectar DHA and may thus play a role in facilitating the extracellular release of DHA from nectary cellular pools of DHAP (Grierson *et al.* 2024). In light of our results, however, we argue that an influence of the phyllosphere microbiome on mānuka physiology is compatible with this hypothesis. Although anatomically distinct, flowers and leaves are not functionally disconnected. For example, leaf-derived sucrose signalling is widely acknowledged to initiate flowering in other plant species (Kozlowski 1992; Cho *et al.* 2018). Furthermore, phyllosphere bacteria, such as *Pseudomonas*

syringae, produce phytohormones, such as IAA, which have key roles in plant development, growth, and flowering (Lindow *et al.* 1998; Lindow & Brandl 2003).

5.5.2 Variation in phyllosphere microbiome community structure correlates with honeydew-derived phenolic compounds

Beyond MGO, other notable correlations included those with phenolic compounds. Specifically, correlations were observed between the core phyllosphere members and 3-phenyllactic acid, all phyllosphere members and 2-methoxybenzoic acid, and both the core and noncore members and methylsyringate (Fig. 4A-C, Supplementary Table 5). Synringic acid and phenyllatic acid comprise some of the most abundant aromatic acid derivatives found in mānuka honey (Russell *et al.* 1990). Although phenolic compounds are strongly correlated with honey botanical origin, previous studies have demonstrated that these components are not present in mānuka flowers (Becerril-Sánchez *et al.* 2021). Instead, they are more likely to be collected by honeybees from elsewhere on the mānuka tree, such as honeydew deposits on the leaves or trunk from the scale insect *Acanthococcus orariensis*, *Ac. Leptospermi*, or *Ac. campbelli* (Tan *et al.* 1988; Weston *et al.* 1999; Stephens *et al.* 2005; McKenzie *et al.* 2006; Bohórquez *et al.* 2019). Given that herbivorous insects are generally considered to exhibit widespread interactions with many epiphytic bacteria (Silva-Sanzana *et al.* 2023), these observations therefore raise several interesting speculations regarding a possible quadripartite interaction between mānuka, mānuka-associated insects, the mānuka phyllosphere microorganisms, and foraging honeybees. Previous studies have demonstrated direct interactions between phloem-feeding insects and phyllosphere microorganisms. For example, some epiphytic bacteria (i.e., *Pseudomonas syringae*) are pathogenic to herbivorous insects (Smee *et al.* 2021). Furthermore, some phloem-feeding insects can detect and thus avoid plants with specific epiphytic *P. syringae* strains (Hendry *et al.* 2018). In light of these previous studies, it is possible that the mānuka phyllosphere community composition could alter the concentration of honeydew-derived phenolic compounds in mānuka honey by increasing or decreasing the visitation of *Acanthococcus* species. Previous studies have also demonstrated indirect interactions between phloem-feeding insects and phyllosphere microorganisms. For example, honeydew-derived carbon has been found to significantly change the phyllosphere community structure on European beech leaves (Potthast *et al.* 2022). Furthermore, honeydew deposits have also been linked to the growth of the sooty mould *Capnodium walteri* Sacc. (Fungi: Capnodiaceae) on the mānuka leaf surface, which reduces photosynthesis, induces plant defence responses, and is responsible for the mānuka blight disease (Mulcock 1954).

Unsurprisingly, sooty moulds have also been shown to strongly effect leaf surface bacterial communities (Lv *et al.* 2023). Thus, the concentration of honeydew-derived phenolic compounds in mānuka honey could also be indirectly linked to variation in phyllosphere community composition due to herbivory-mediated changes in resource availability, overall plant health, and microbial competition.

5.5.3 Mānuka honey elemental fingerprint exhibits correlations with the phyllosphere microbiome

Correlations were also observed between variation in phyllosphere community structure across valleys and the concentration of several elements in our honey samples (Fig. 5A-C). Specifically, these included major elements (i.e., K, Mg, Mn, Ca), minor elements (i.e., B, Al, Fe, Cu, Rb, Sr, Cs), and hazardous elements associated with anthropogenic activities (i.e., Co, Tl, Pb) (Supplementary Table 7). The elemental composition of honey is generally considered to reflect the geographic origin of the nectar source (i.e., elements are introduced into the nectar via root uptake from soil). This consensus has been largely confirmed by the ability to use honey elemental fingerprinting to predict the geographic region (with 76% accuracy) and even the land type category (96% accuracy) from which the honey was derived (Grainger *et al.* 2021). By identifying correlations between the phyllosphere microbiome and honey trace elements, our results describe another link between honey composition and other attributes of the local environment.

The relationship between the elemental composition of honey, other plant tissues, the environment, and plant-associated microbiomes at smaller spatial scales remains unclear. Given the differential translocation and storage of elements throughout the plant, it is likely that the existence of such relationships would be complex and possibly element-specific (Conn & Gilliham 2010; Che *et al.* 2018). For example, a previous study has found that potassium in plants and honey exhibits a positive correlation, which is likely attributable to the fact that potassium is highly mobile in the plant phloem (Meister et al. 2021). However, this would not be expected for calcium, which is thought to be phloem immobile (Conn & Gilliham 2010). In addition, it is possible that a relationship exists between nectar elemental composition and other key physicochemical properties in mānuka nectar (i.e., DHA). For example, the hypothesised production of DHA via nectary photosynthesis would enzymatically require Mg, Mn, Zn, or Co (Liang *et al.* 1993). Furthermore, manganese superoxide dismutase has been shown to generate high concentrations of hydrogen peroxide in the nectar of other plant species (Carter

& Thornburg 2004). Thus, although hydrogen peroxide, which can react with DHA to form glycolate, is usually only present at low levels in mānuka nectar, shifts in enzymatic reactions as a result of differing elemental concentrations may have the capacity to affect nectar DHA concentrations (Brudzynski *et al.* 2011). Lastly, another relevant consideration is the impact of elemental concentrations on pollinators, as high concentrations of certain elements in nectar have been shown to negatively affect the foraging behaviour of honeybees (Sivakoff & Gardiner 2017; Xun *et al.* 2018).

5.5.4 Case study considerations

Given the challenges associated with carrying out such work in a natural ecological system, we acknowledge a couple of uncertainties that could impact the interpretation of our results.

Given that mānuka honey is rarely collected from 100% mānuka nectar, we cannot exclude the possibility that other plant sources had an influence on the composition and characteristics of our sample honeys. Nevertheless, four observations give us confidence that our honey samples were predominantly of mānuka-origin. First, bees will generally visit all available nectar sources near their hive and the vegetation in the study site was mānuka-dominated (Fig. 1C-D). Second, mānuka/kānuka was the dominant pollen visualised in honey. Third, MGO and DHA concentrations were within the same range as those reported in previous studies (Adams *et al.* 2009; Stephens *et al.* 2010; Atrott *et al.* 2012). Finally, the concentration of our mānuka phenolic markers satisfied the criteria for mānuka monofloral honey classification.

Another consideration of our study was that the inability to confirm the contribution of our sampled trees as a nectar source to each honey sample. Source tracking is carried out in various scientific and environmental sectors for identifying and monitoring the origin and movement of various entities, such as microorganisms or pollutants (Scott *et al.* 2002; Simpson *et al.* 2002). Moreover, the ability to trace honey back to individual nectar sources would be highly desirable to improve our understanding of the processes that influence honey quality as well as a means of ensuring honey origin. Nevertheless, a method for accurately tracking nectar source to honey does not currently exist. However, given that bees are highly likely to visit nectar sources within close proximity of their hives (Beekman & Ratnieks 2000), we maximised the likelihood that the nectar of our sample trees contributed to the sample honeys by minimising this tree-hive distance.

A final consideration is that compared to our honey chemistry data, which most likely reflects the properties of the broader mānuka population and valley environment, our microbiome sampling was spatially patchy (i.e., three trees per hive site). This was largely attributed to the remote nature of this site, which limited the ability to include larger sample numbers and geographic coverage. However, our previous work gives us confidence that the patterns in phyllosphere community structure we observed (i.e., the variation between valleys) was not severely influenced by our sampling design. Specifically, many patterns in community structure we observed were consistent with observations in our previous work. For example, the positive relationship between alpha diversity and tree height has also been observed in Chapter 3 and Chapter 4. Furthermore, a core microbiome comprising the same genus-level taxonomic classification has previously been identified in all of our studies to date (i.e., Chapter II - IV) (Noble *et al.* 2020).

5.6 Conclusions and significance

In summary, our results are consistent with the overarching hypothesis established throughout our previous studies that host-microbiome interactions in the phyllosphere may influence the physiological properties of the mānuka tree. Furthermore, the results of this chapter illustrate a relationship between the surrounding environment, mānuka trees, the mānuka phyllosphere microbiome, and mānuka honey quality. Given the preliminary nature of this case study, we are unable to generate mechanistic interpretation. Nevertheless, the significance of this study lies in the unique speculations that are raised in our results, which will hopefully inspire new questions to motivate future research efforts. Furthermore, the correlations identified between the phyllosphere microbiome and mānuka honey characteristics emphasises the complex interconnected nature of our terrestrial ecosystems and highlights the necessity of implementing multidisciplinary approaches in order to address unresolved ecological questions.

5.6.1 The unique properties of mānuka honey: more than a sum of its parts

Protecting the authenticity of premium products is a worldwide issue (Zhou *et al.* 2018), and this has particularly been the case for the NZ mānuka honey industry (McDonald *et al.* 2018), which lost its bid to trademark the term 'Mānuka Honey' in 2023 (Murphy 2023). Although mānuka is a word sourced from te reo Māori and was used to brand the honey product as uniquely from Aotearoa NZ, *L. scoparium* is also present in Australia and Australian

beekeepers have won the right to also label their honey as ‘mānuka honey’. Five attributes are currently used to define mānuka honey: four chemicals (3-pheenyllactic acid, 2-methoxyacetophenone, 2-methoxybenzoic acid, and 4-hydroxyphenyllactic acid) and one mānuka pollen DNA marker (Ministry for Primary Industries 2017). However, the botanical nature of these markers fails to demarcate the value of *provenance*, which is concerned with multiple dimensions of a product’s history: space (i.e., place of origin), social (i.e., method of production), and cultural (i.e., quality and reputation) (Reid & Rout 2016). The concept of Australian mānuka honey is therefore challenging the integration of the indigenous worldview of Māori in Aotearoa NZ and western science. At its core, the Māori experience of the world is one of connectivity; there is a realisation that ecosystem functionality and sustainability is dependent on the existence of a diversity of life forms, including everything visible or invisible (Harmsworth 2004; Harmsworth & Awatere 2013). From this perspective, New Zealand requires a definition of mānuka honey that is more than its scientifically delineated composition. By describing a connection between mānuka and its wider environment via phyllosphere microorganisms, this work also aims to inspire ongoing convergence and collaboration between scientific principles and indigenous worldviews.

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Chapter VI

Summary, conclusions, and future work

6.1 Thesis summary

The phyllosphere microbiome is a ubiquitous microbial habitat in nature that harbours a diverse community of microorganisms (Lindow & Brandl 2003; Leveau 2019). Although pathogenesis was once assumed to be the primary role of microorganisms on the leaf surface (Hirano & Upper 2000), a growing body of experimental evidence now demonstrates that these microorganisms can engage in beneficial associations and interactions with their host (Gupta *et al.* 2022; Li *et al.* 2022b). The ability to identify beneficial microbial associations in the phyllosphere is thus becoming a goal of an increasing number of studies. However, the ecological processes that drive the assembly of natural phyllosphere communities remain poorly understood.

Cultivation-independent studies on natural phyllosphere communities are proficient at characterising biogeographic patterns of community composition across large numbers of host species and/or spatial scales (Redford *et al.* 2010; Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016a; Laforest-Lapointe *et al.* 2016b; Li *et al.* 2022a). However, the lack of quantitative information on the role of different processes in forming these spatiotemporal patterns presents a fundamental constraint on our understanding of phyllosphere microbial ecology.

The work presented in this thesis uses a top-down approach with natural communities in the mānuka phyllosphere to carry out a hypothesis-driven series of investigations on specific ecological processes of phyllosphere community assembly. The results of this thesis shed new light on controversial and under-explored processes of community assembly and illustrate a path forward that will allow the emergence of a coherent and generalisable understanding of phyllosphere microbial ecology. Further, by generating knowledge of microbial ecology in mānuka, the source of New Zealand's famed mānuka honey, the results of this thesis also generate new consideration for the potential role of phyllosphere microorganisms in driving the enigmatic heterogeneity observed in mānuka's economically desirable physiological traits.

This final chapter summarises the following: our findings from the perspective of the overarching hypothesis, notable observations made throughout these studies, general considerations for the field of phyllosphere microbial ecology, specific future directions using mānuka as a model plant species, and some closing words.

6.2 Host selection is the primary driver of community assembly in the mānuka phyllosphere

The overarching hypothesis throughout this thesis was that community assembly of the mānuka phyllosphere microbiome is primarily driven by host selection. This hypothesis was founded on our first biogeographical study of the mānuka phyllosphere, which revealed a geographically persistent and environmentally diverse core microbiome (Noble *et al.* 2020). Although the identification of such spatially persistent core taxa was highly suggestive of a dominant role of host selection (Shade & Handelsman 2012), an alternative explanation that we were unable to exclude was the possibility that these taxa represented over-dispersed, ubiquitous microorganisms and were not in fact specific to the host.

Together, the series of spatial and temporal investigations in this thesis have unequivocally demonstrated that the assembly of the mānuka phyllosphere is primarily driven by host selection. Compared to ecologically similar and naturally co-occurring plant species nearby, our results revealed that the mānuka phyllosphere microbiome is species-specific and stable over both space and time. The relative influence of the mānuka host was larger than the effect of dispersal limitations (within a spatial scale of 2 km), priority effects, and seasonal abiotic factors. Furthermore, the specificity of the mānuka phyllosphere microbiome was not associated with leaf surface abiotic conditions (i.e., leaf morphology), suggesting that biotic host factors may instead be the primary mechanism (i.e., chemical signalling).

The detection of a core phyllosphere microbiome in our first biogeographical study was also supported by the identification of a species-specific core microbiome in each of our five chapters. Core taxa were consistently identified as members of Beijerinckiaceae (Proteobacteria) within the genus *Methylocella* and *1174-901-12*, an uncultured genus within Rhizobiales. Importantly, the repeated identification of these taxa across time and space throughout this thesis gives us confidence that this finding is robust and not an artefact of contamination. Furthermore, given that plant leaf surfaces leach and emit various C1 compounds and the Beijerinckiaceae family demonstrates broad C1 metabolic diversity, the taxonomic classification of the core microbiome is consistent with the adaptations known to permit microbial survival on the leaf surface. Specifically, *Methylocella* spp. are acidophilic, facultative methanotrophs (Dedysh *et al.* 2000); they assimilate methane via the serine pathway and can utilise other multicarbon compounds. *Methylocella* spp. also fix atmospheric nitrogen,

and they have been identified in the phyllosphere of other plant species, such as the evergreen southern Magnolia (Jackson & Denney 2011). These core taxa represent ecologically relevant bacterial targets for future targeted cultivation and genome sequencing, which will be desirable to explore strain-level variation and describe strain-specific physiological characteristics.

6.3 Processes of community assembly are not universal across plant species

A prominent message of this thesis is that the relative influence of community assembly processes is not and should not be presumed to be universal across plant species. This was particularly emphasised by our work in Chapter II, which, to the best of our knowledge, is the first study to empirically demonstrate that plant species vary in the degree to which they select for phyllosphere microorganisms. This idea provides a logical resolution to the prevailing dichotomy in the literature, whereby some studies have reported community patterns indicative of a primary role of host selection while others reported patterns indicative of a primary role of dispersal (Redford *et al.* 2010; Finkel *et al.* 2011; Smets *et al.* 2023). This also likely explains why seasonal variation, which has been observed in other evergreen tree species (Al Ashhab *et al.* 2021; Stone & Jackson 2021), was not observed in the mānuka phyllosphere. Given that microbiomes under strong host selection are more likely to confer beneficial functions to the host than those that are acquired stochastically (Shade & Handelsman 2012; Jackrel *et al.* 2021), this paradigm (i.e., that not all plants select for a consistent microbiome) thus has significant implications for studies aimed at identifying functionally important plant-microorganism associations on the leaf surface. This is especially the case for experimental research, since the design and interpretation of many of these studies thus far are based on the unverified assumption that the host has a strong influence, when the influence of the host, in fact, remains unquantified (Li *et al.* 2021; Meyer *et al.* 2023). Future investigations seeking to quantify the relative influence of the host across a larger number of plant species will be necessary and valuable to verify this assumption.

6.4 Phyllosphere microbial ecology: we find what we look for

Despite the overwhelming evidence in support of a primary role of host selection on mānuka phyllosphere assembly, we still detected a significant distance-decay relationship (Chapter II) and a heterogeneous distribution of taxa at small spatial scales (Chapter III). Our spatially

hierarchical sample designs and control of confounding environmental variables permit the attribution of these patterns to dispersal limitations, inter-host dispersal, and priority effects. Thus, although host selection plays a substantial role in determining the spatiotemporal stability of mānuka phyllosphere communities, the influence of other processes is not, and should not be assumed to be, negligible.

Microbial dispersal, which includes the immigration and emigration of taxa to and from a given microbiome and the surrounding environment, has been widely recognised as a fundamental process in the assembly of natural microbial communities, such as the phyllosphere. This was particularly recognised by researchers during the 1970s-1990s who conducted pioneering culture-based work on the immigration/emigration of bacteria among natural leaf surfaces (Clayton & Hudelson 1991; Lindow 1996; Kinkel 1997), and others who quantified the relative influence of different routes, rates, and mechanisms of dispersal (i.e., aerosols, raindrops). Nevertheless, the design and data interpretation of many modern studies of phyllosphere microbial ecology implicitly assume a homogenous microbial distribution within their study sites (Kembel *et al.* 2014; Laforest-Lapointe *et al.* 2016a). Moreover, many long-standing theories and hypotheses (e.g., the source-strength hypothesis that we addressed in Chapter III) have not been verified with modern molecular techniques. It seems paradoxical that, for a field intrinsically motivated to understand natural variation, recognition for the importance of these fundamental processes on community composition has been largely disregarded by the cultivation-independent era of phyllosphere microbial ecology. Why?

For many, there seems to be an appeal (conscious or subconscious) in studying processes that are easily controllable and measurable. This reason has been ascribed to the historic popularity of laboratory or greenhouse studies over the investigation of natural systems (Lindow 1996). This has also been recently ascribed to the emerging popularity of synthetic communities, which can only be used to test hypotheses that are relevant for synthetic communities (Nie & Wu 2021). Another emerging problem in our modern era of microbial ecology is that the use of ‘meta-omic’ technologies and the desire to generate large quantities of data is being prioritised at the expense of careful study designs, thoughtful scientific questions, and hypothesis-driven research (Nie & Wu 2021).

In this context, this thesis epitomises the importance of using accessible natural systems and the simplest appropriate methodology to interrogate critical and falsifiable hypotheses. Doing

so requires careful consideration for each of the four basic ecological processes (i.e., immigration, emigration, growth, and death) and controlling (or at least acknowledging and measuring) variables at their basic constituents.

An example addressed by this thesis is the abstraction of host species identity to infer host selection. Although searching for patterns of host species identity represents a practical starting point for distinguishing between microbiomes that assemble more deterministically compared to those that assembly stochastically, this approach is generally unable to shed light on whether these patterns are driven indirectly by species-specific morphological traits (i.e., abiotic) or directly by host-microbiome interactions (i.e., biotic) (Yadav *et al.* 2005; Kembel *et al.* 2014; Lambais *et al.* 2014). However, by identifying and using a unique system that allowed us to essentially decouple leaf morphology from host species identity (i.e., mānuka and kānuka), we were able to generate unique mechanistic predictions in Chapter II and Chapter III that had not been previously achieved.

Another example addressed in Chapter IV is temporal variation. Throughout the literature, temporal variation has been correlated with plant development (Grady *et al.* 2019), leaf age (Ercolani 1991), and climate (Stone & Jackson 2021). However, from these correlations alone we are unable to extract information regarding the individual processes that drive these patterns. For example, correlations with leaf age and phyllosphere community composition could be a result of microbial immigration, annual climate cycle, host physiological development, senescing plant tissues, and microbially-mediated interactions such as priority effects (Jacques *et al.* 1995; Kinkel *et al.* 2000; Knoll & Schreiber 2000; Maignien *et al.* 2014). However, by investigating seasonal variation in the evergreen phyllosphere of mānuka, we were able to decouple the effect of leaf age from season/host phenology. Future investigations in the evergreen phyllosphere aimed at separating the effect of season (abiotic) and host phenology (biotic) represents a valuable avenue of future work. Furthermore, another interesting question raised as a result of both Chapter III and Chapter IV, is whether continuous intra-host dispersal among a permanent leaf canopy increases the rate of community succession (i.e., time taken to reach a steady-state) in the phyllosphere of emerging evergreen leaves relative to the deciduous phyllosphere. Lastly, Chapters II-IV also raise questions about whether intra-host dispersal influences the ability of a plant species to form and maintain stable host-microbiome associations on the leaf surface.

We anticipate a stricter consideration for the basic ecological processes that structure microbial communities will facilitate the identification and use of unique natural model systems by a larger number of research groups.

6.5 Future work

By generating a fundamental understanding of the ecological processes in the mānuka phyllosphere microbiome, this work presents mānuka as a model plant species for developing a mechanistic understanding of host selection and plant-microbiome interactions in the natural phyllosphere. Several hypotheses generated throughout this thesis provide an immediate next step and foundation for future research. These hypotheses and possible future directions are outlined in more detail below.

Hypothesis: functionally significant interactions exist between mānuka and phyllosphere microorganisms

Establishing causality with regard to the effect of the phyllosphere microbiome on mānuka will be a long process. Now that we have established that an ecologically significant, species-specific association (and thus host-microbiome interaction) likely exists between mānuka and its associated phyllosphere microorganisms, the natural progression of this work is to demonstrate functionality and causality. This would be best achieved by combining cultivation-independent and culture-based approaches. Comparative shotgun metagenomic sequencing would immediately permit a broad characterisation of community functional potential. Different sampling strategies would be needed to first generate information on the patterns of community function across space and time. A useful starting point could include a broad geographical survey, similar in nature to our first 16S rRNA investigation, in order to gauge whether the taxonomic stability of the mānuka is also observed at the level of functional potential. This would shed light on gene categories that are ‘core’ versus those that are variable among populations, as has been done for the human gut microbiome (Turnbaugh *et al.* 2008). Given the distinctiveness observed between the mānuka and kānuka phyllosphere microbiome at the ASV level, comparative metagenomic analysis would be useful to shed light on whether these microbiomes are also distinct at the gene level. This would shed light on genes that may confer a competitive advantage to phyllosphere microorganisms in the physical environment common to both mānuka and kānuka leaf surfaces, as well as predicted functions that are

mānuka-specific. Once a baseline understanding of the mānuka phyllosphere at the gene level has been achieved, comparative metagenomic sequencing of the mānuka phyllosphere among trees that exhibit distinct phenotypes, such as our case study site in Chapter V, could be useful to investigate whether any genes or microbial strains are associated with phenotypic variation. Beyond bacteria, metagenomic sequencing will also shed new light on the non-bacterial members of the microbiome, such as fungi, viruses, and micro-eukaryotes (Lindow & Brandl 2003).

The core taxa identified across our four chapters provide useful targets for cultivation. Given the biases and challenges associated with cultivation (Stewart Eric 2012), the complex physicochemical characteristics of the phyllosphere (Lindow & Brandl 2003), and the specificity of the core microbiome to mānuka (observed throughout this thesis), time would need to be invested towards the development of cell culture substrates that sufficiently replicate the conditions on the mānuka leaf surface. Information on the physicochemical properties of mānuka leaves is already readily available and thus could be combined with documented cultivation approaches (i.e., replica leaf surfaces) to create a tailored, mānuka-specific workflow (Johnson 1980; Perry *et al.* 1997; Van Vuuren *et al.* 2014; Effah *et al.* 2022; Monteiro *et al.* 2023). These approaches would further benefit from acquiring and using phenotypic information of the closest cultured sequences to core *Methylocella* taxa (Marín & Arahal 2014). The ability to isolate and culture members of the mānuka core phyllosphere microbiome would permit a stronger understanding of their specific physiologies and their role in the mānuka phyllosphere. This would be facilitated through metagenomics and whole bacterial genome sequencing, which would provide a greater sequencing depth and coverage compared to environmental metagenomes.

Hypothesis: assembly of the mānuka phyllosphere microbiome is driven by a chemically-mediated mechanism of host selection

Similar to the previous hypothesis, working towards the identification of a specific mechanism of host selection in the mānuka phyllosphere will depend on gathering a ‘chain of evidence’, which will start with the continuation of association studies (Chaudhari *et al.* 2021). An example of such an approach has been recently demonstrated in the phyllosphere microbiome of *Arabidopsis thaliana* (Unger et al. 2024). Mānuka has been reported to exhibit distinct volatile chemical compounds in their leaves among geographic populations, cultivars, and

clones (Porter *et al.* 1998; Douglas *et al.* 2004). Mānuka has also been reported to emit different volatile compositions regionally and across time (Effah *et al.* 2020; Effah *et al.* 2022). Thus, an attractive first step could use different mānuka chemotypes, determined with gas chromatography-mass spectrometry (GC-MS), to investigate whether an association exists between the mānuka phyllosphere microbiome and leaf chemical profiles and/or volatile emissions. To minimise the confounding influence of environmental heterogeneity and dispersal within such a study, it would be desirable for mānuka chemotypes to be located in the same region. For this reason, common gardens do represent an attractive system. However, baseline work would need to be done to ensure phyllosphere communities had reached a steady state. Furthermore, results would require verification with natural mānuka. The incorporation of kānuka and other *Leptospermum* spp. would also be useful to further quantify the species-specificity of the mānuka phyllosphere microbiome as well as the potential chemical targets.

6.6 Final words

In summary, this thesis epitomises the importance of using natural systems to address simple yet fundamental ecological questions. As summarised by Prosser (2020), "The real limitation to our understanding of microbial ecology lies, not in a lack of techniques, but in a lack of motivation, enthusiasm, desire and courage to identify and ask significant scientific questions in advance of experimental work, and a lack of testable hypotheses and theory."

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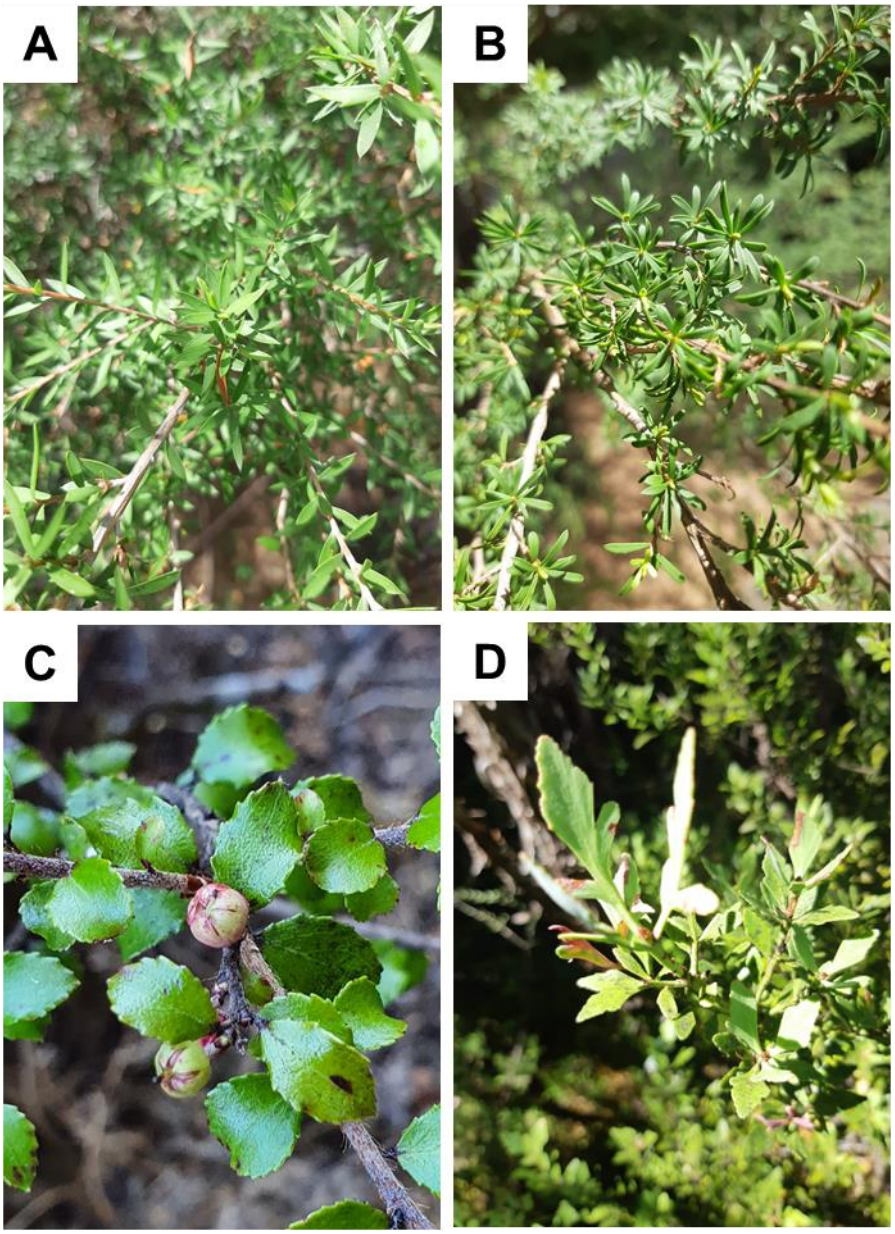
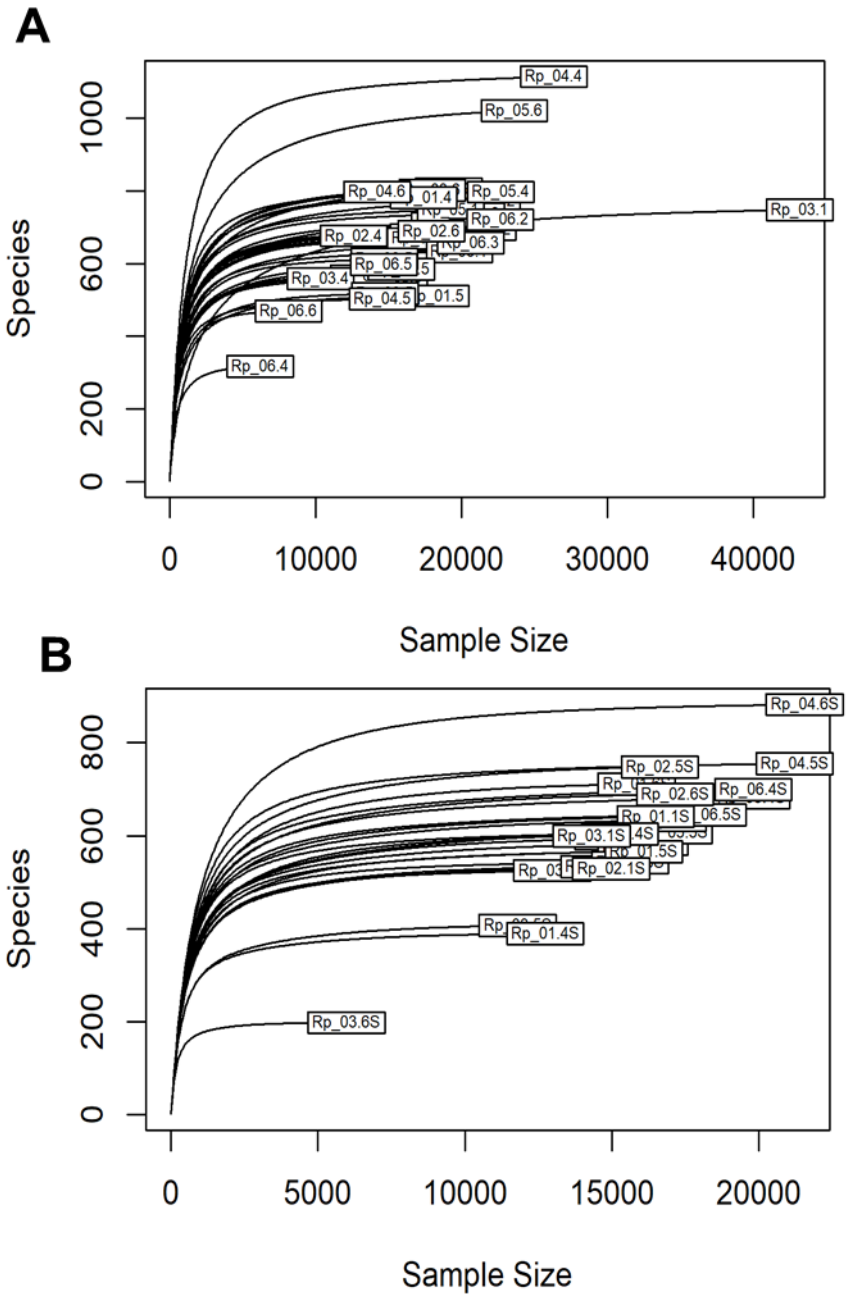
Appendix A

Supplementary Material for Chapter II

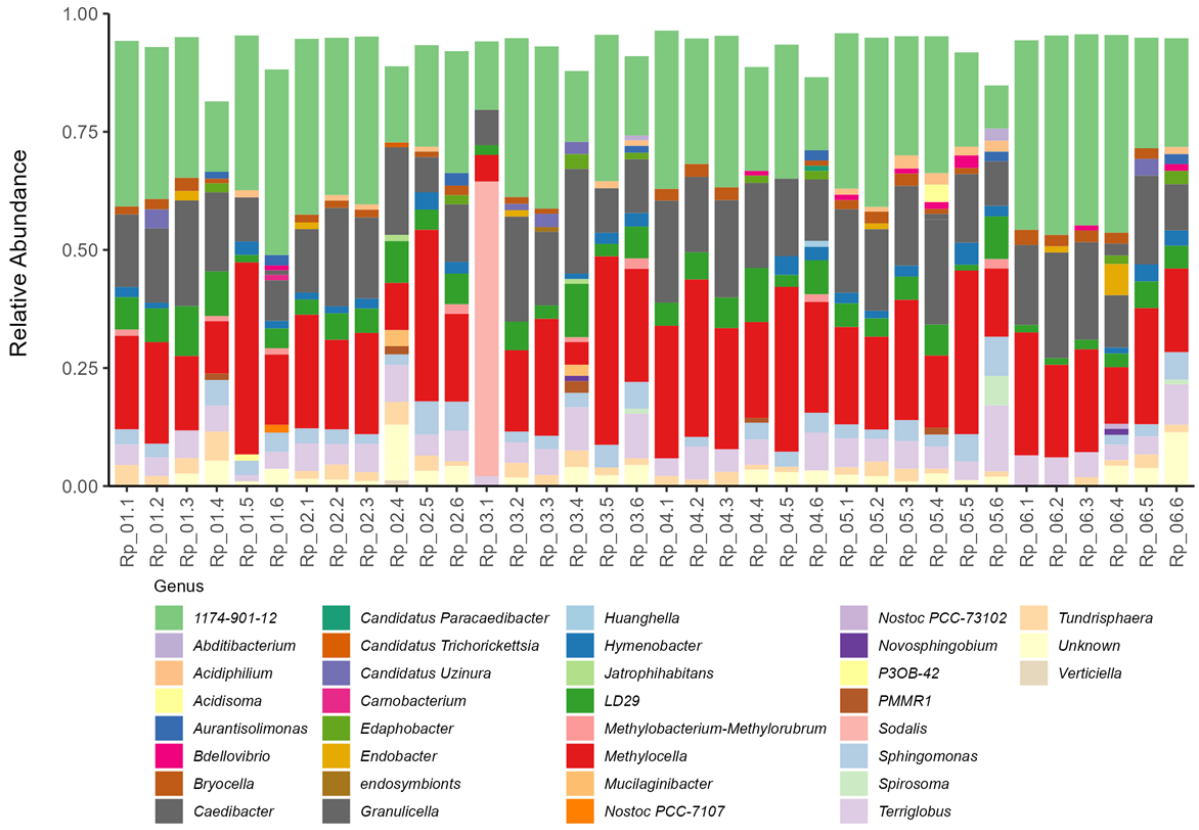
Supplementary Figures and Tables



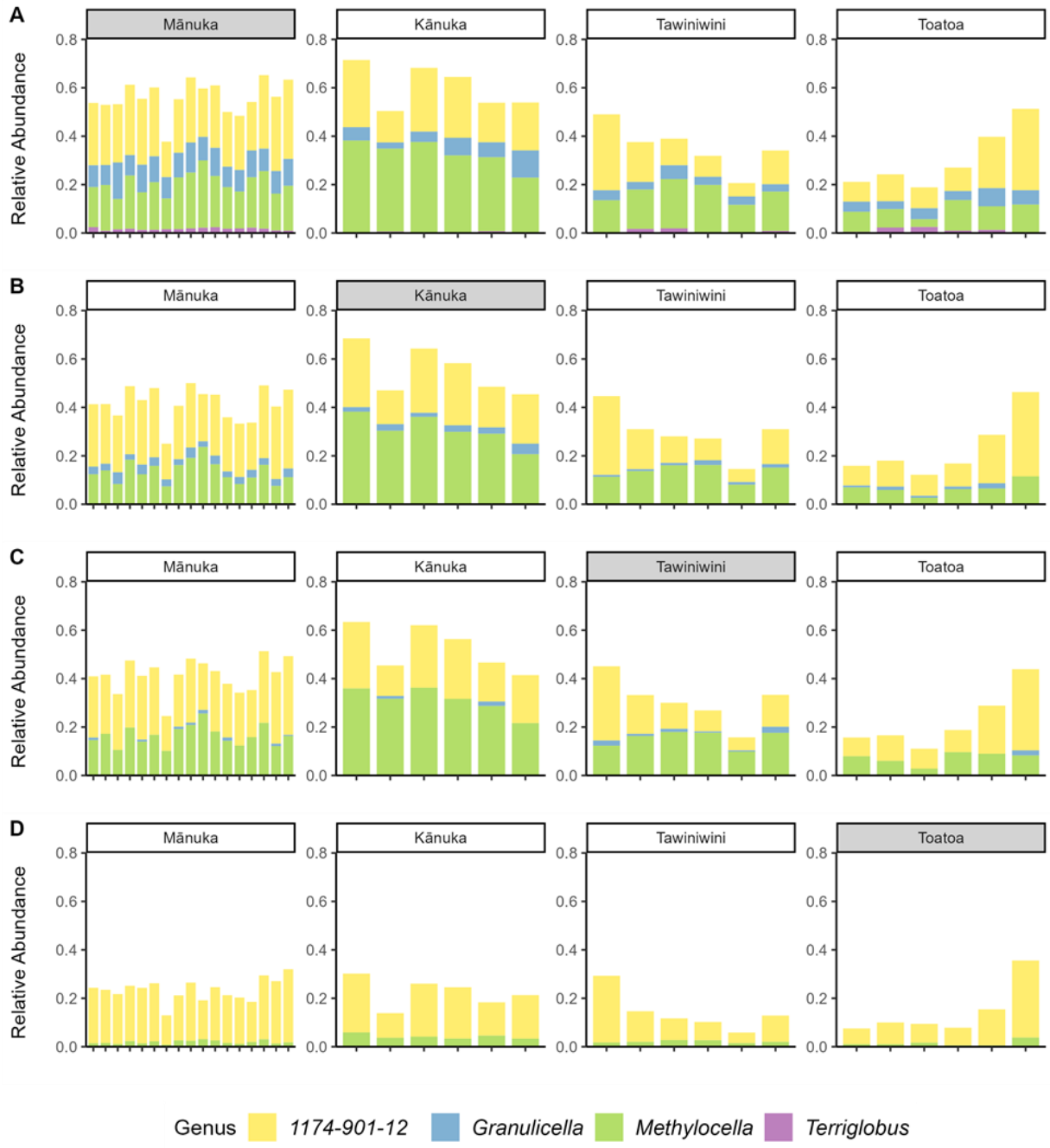
Supplementary Fig. 1: Leaf morphologies of sampled **A)** mānuka (*Leptospermum scoparium*) and neighbouring plant species **B)** kānuka (*Kunzea ericoides*), **C)** tawiniwini (*Gaultheria antipoda*), and **D)** toatoa (*Phyllocladus alpinus*).



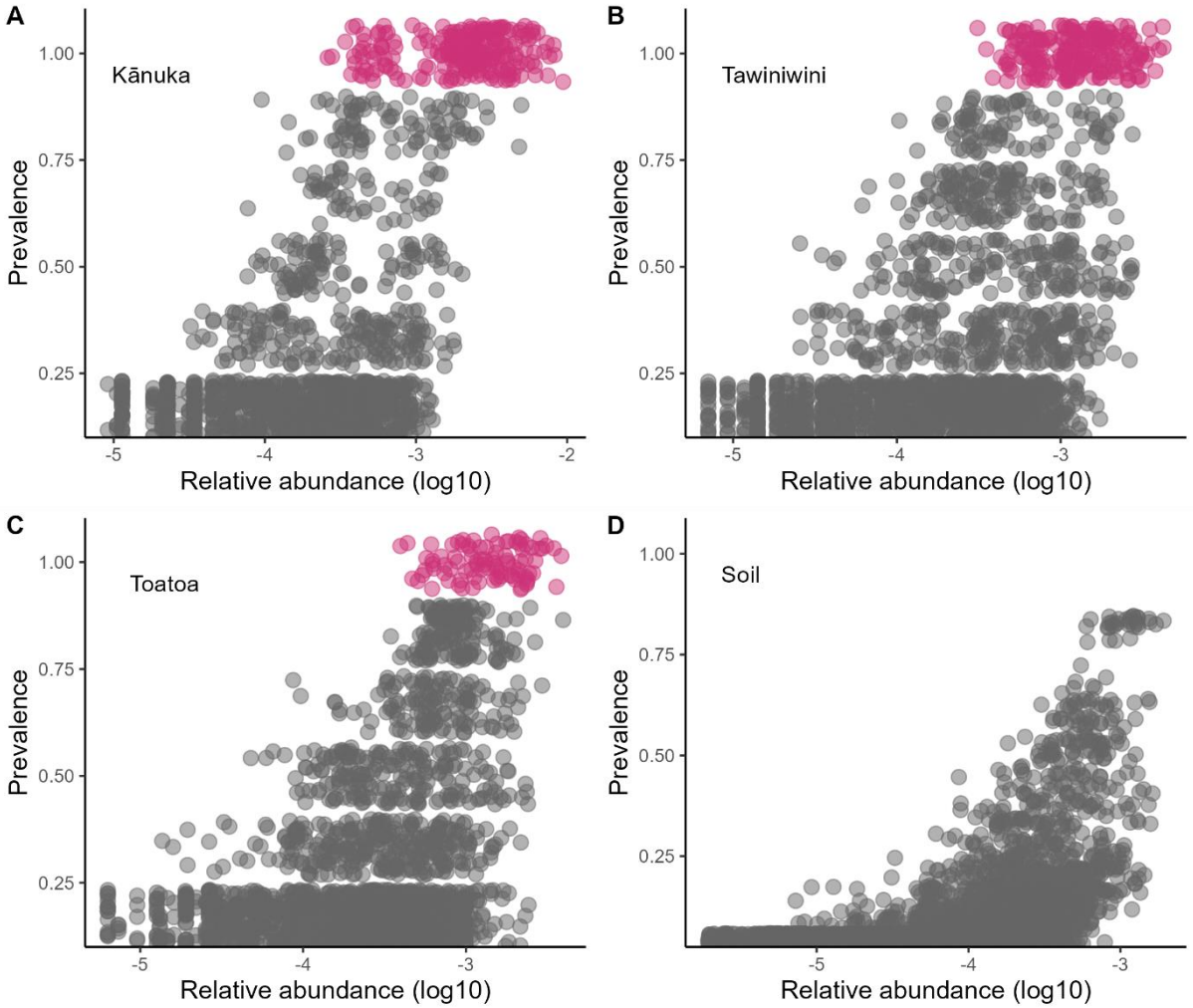
Supplementary Fig. 2: Rarefaction curves showing the relationship between the number of ASVs detected and the sample size (i.e. number of sequencing reads) for the phyllosphere **A)** and surface soil **B)**.



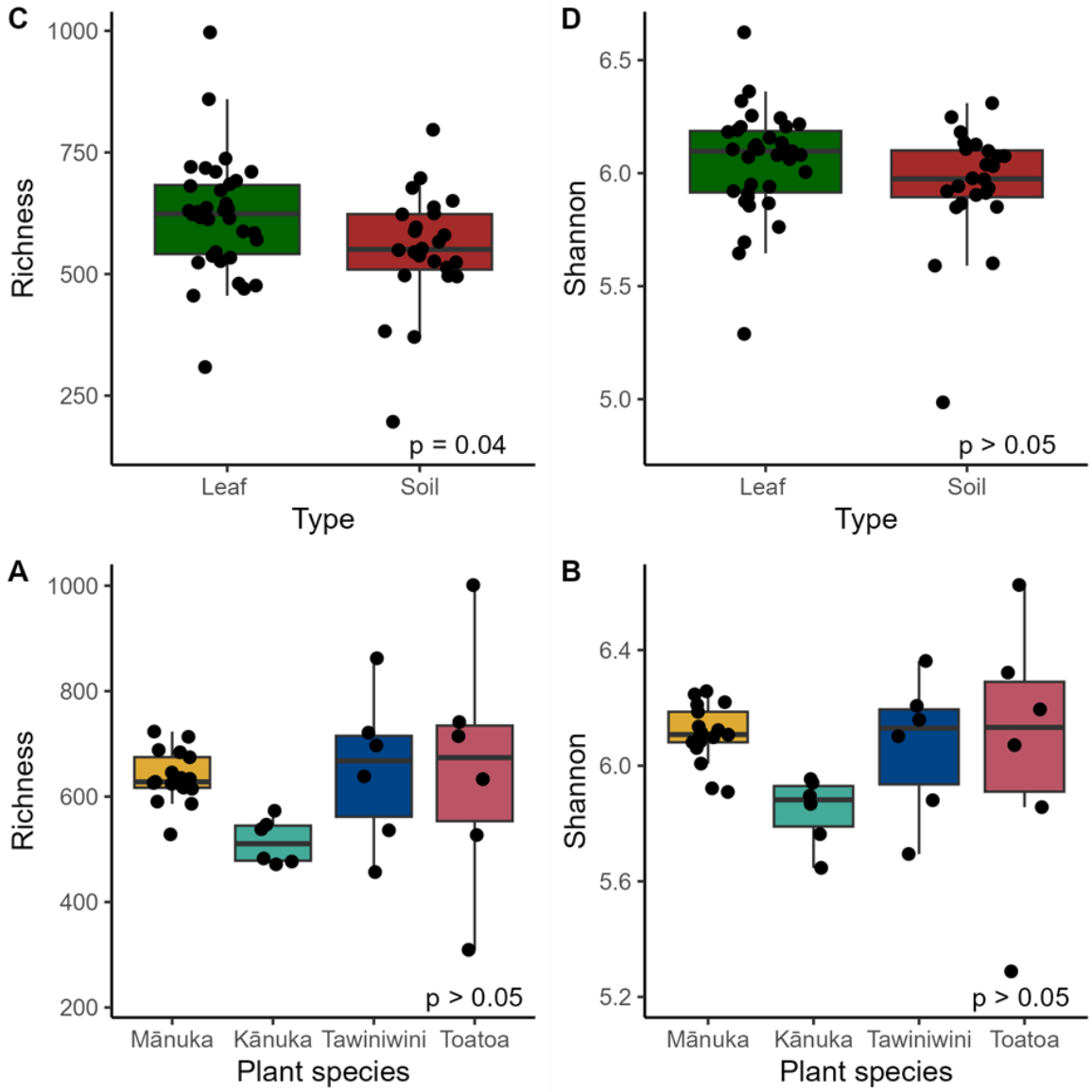
Supplementary Fig. 3: Relative abundance of genera (>0.01) in the phyllosphere. Rp_03.1 is dominated by 32 ASVs belonging to the genera *Sodalis*, which comprise 62.4% of reads.



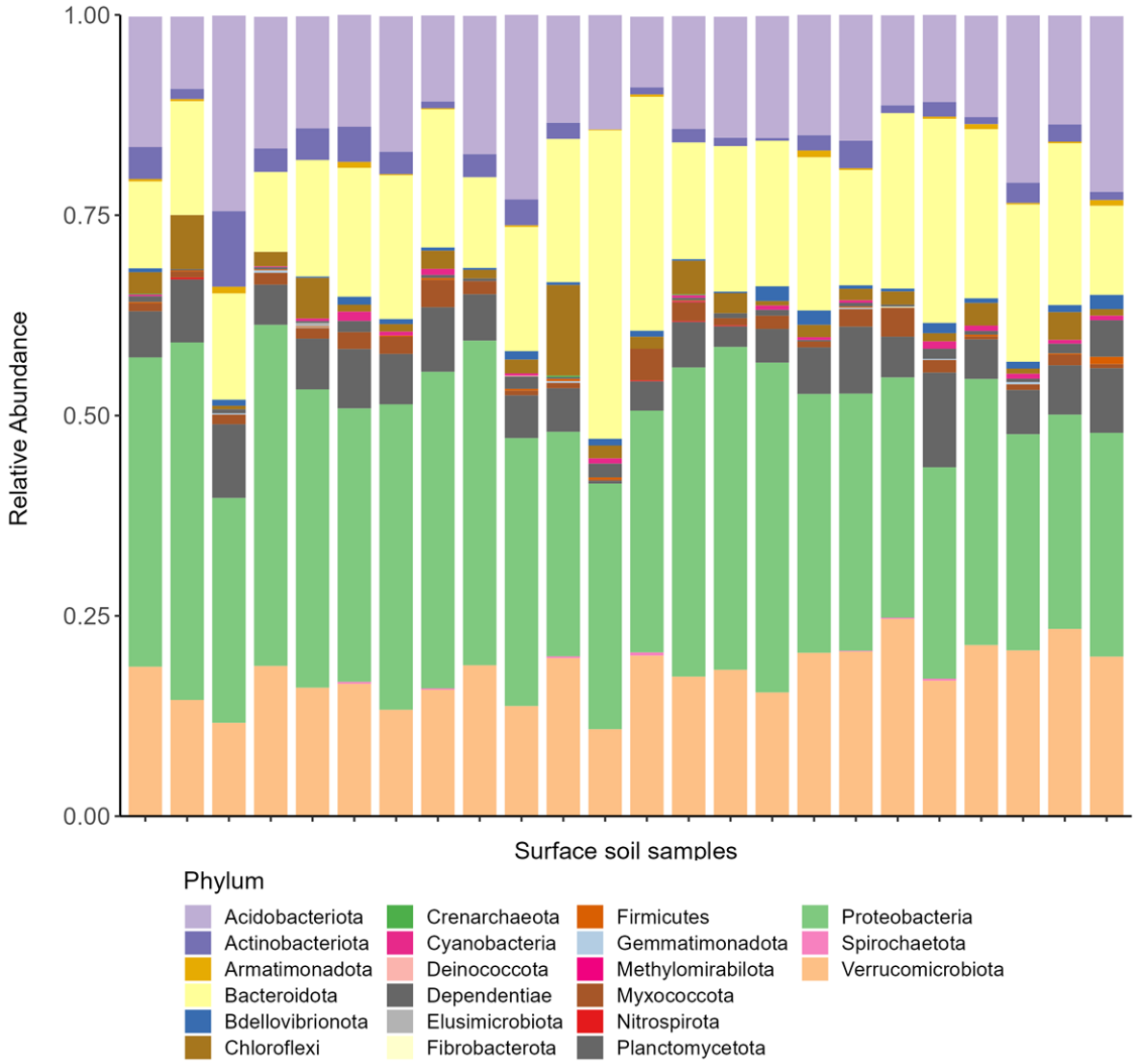
Supplementary Fig. 4: The relative abundance of core ASVs belonging to each host species: **A)** mānuka (280 ASVs), **B)** kānuka (212 ASVs), **C)** Tawininwini (209 ASVs), and **D)** Toatoa (92 ASVs), across the phyllosphere of each and every host species. Colour represents taxonomic assignment at the genus level.



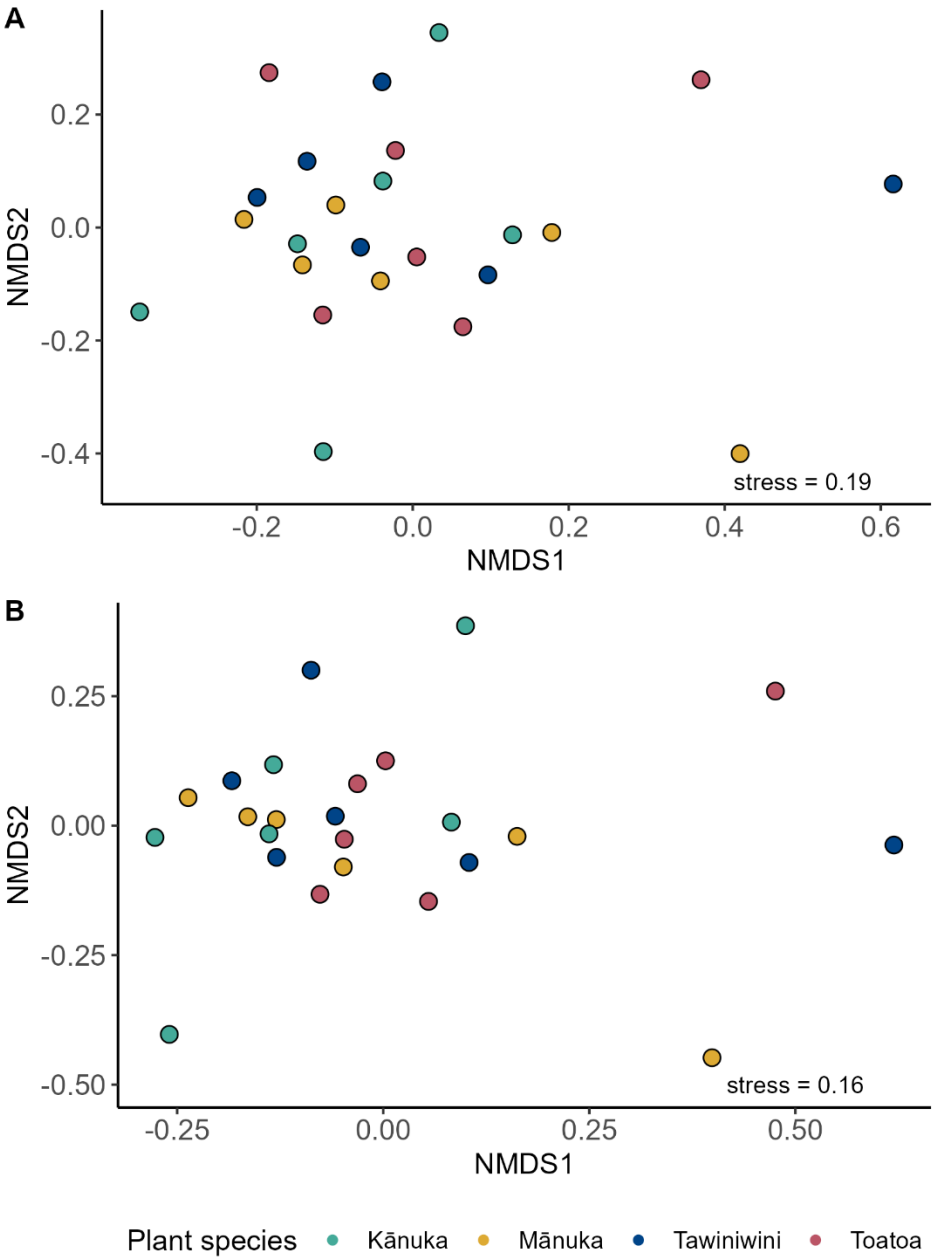
Supplementary Fig. 5: Relative abundance (log10) vs. prevalence of taxa in the phyllosphere microbiome of neighbouring host species **A)** kānuka, **B)** tawiniwini, **C)** toatoa, and **D)** surface soil. Colour indicates core taxa with 100% prevalence in each of these host species.



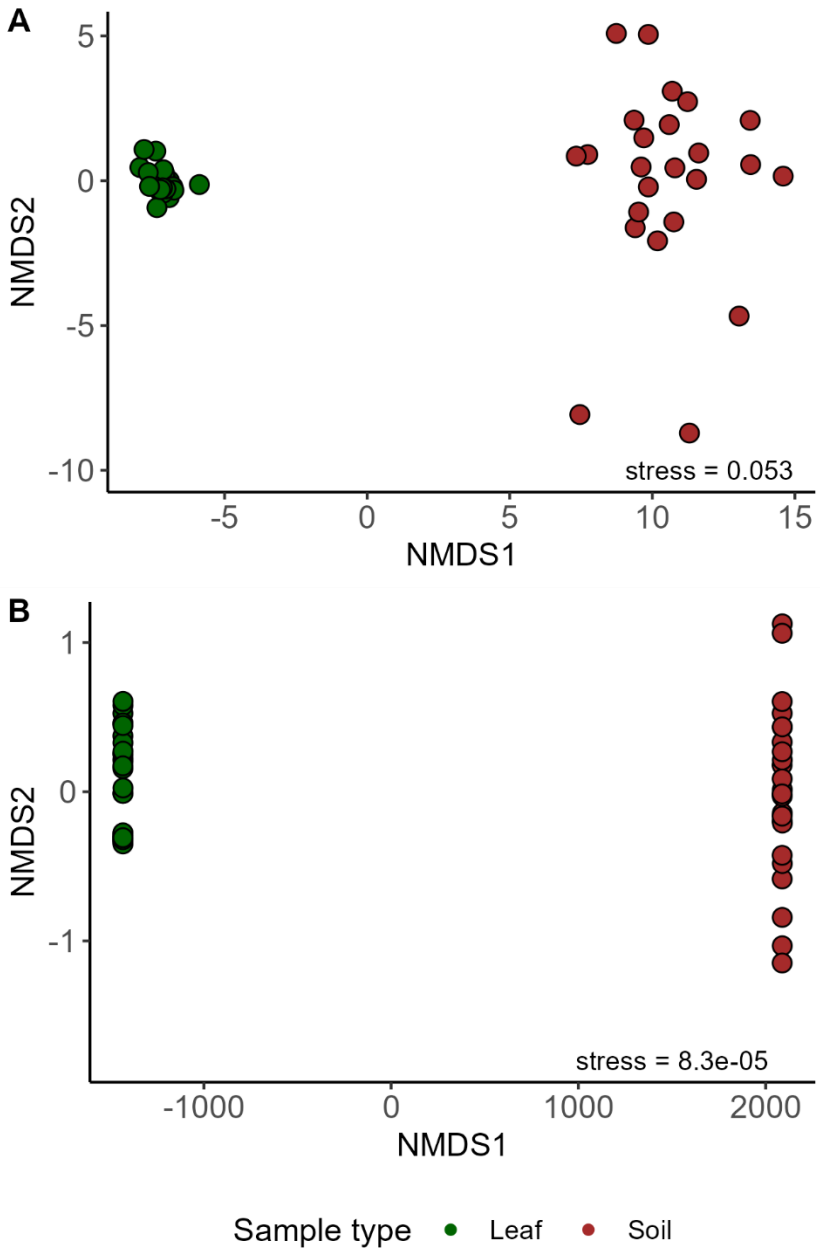
Supplementary Fig. 6: Alpha diversity (Shannon and richness) of the phyllosphere microbiome across different plant species **A-B)** and sample types **C-D)**. Each sample was subsampled (100x) to an even sequencing depth. Boxes indicate the interquartile range and the thick bar represent the median. Vertical segments extend to the fifth and 95th percentiles of the distribution of values.



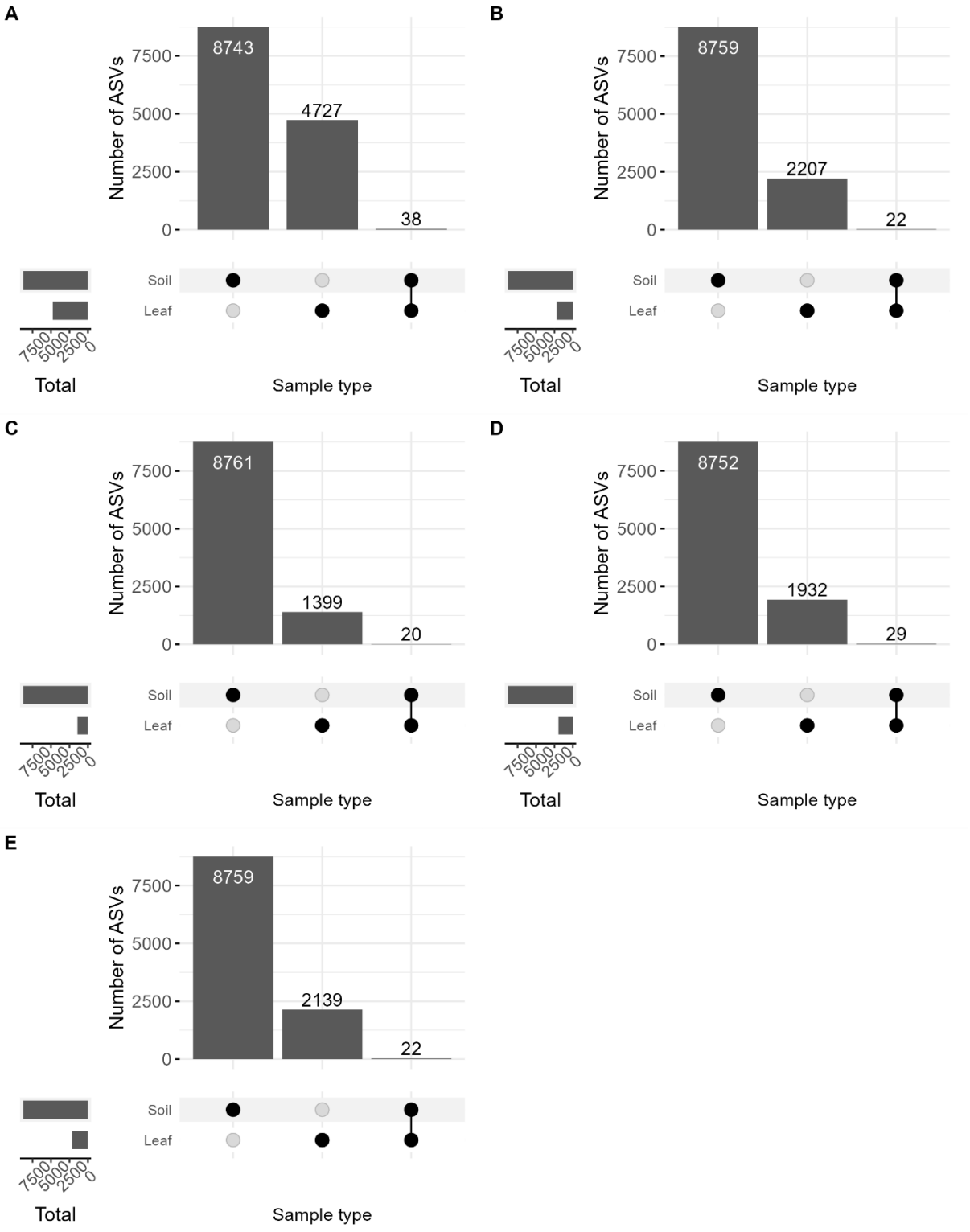
Supplementary Fig. 7: Relative abundance of phyla (>0.001) in surface soil. Colour depicts taxonomic assignment at the phylum level.



Supplementary Fig. 8: NMDS ordination of surface soil community dissimilarity based on **A)** Bray Curtis (based on relative abundance) and **B)** Jaccard (presence/absence).



Supplementary Fig. 9: NMDS ordination of surface soil and phyllosphere community dissimilarity with **A)** Bray Curtis (relative abundance) and **B)** Jaccard (presence/absence)



Supplementary Fig. 10: The total number of shared and unique ASVs across surface soil and phyllosphere samples for all **A)** and independently for mānuka **B)**, kānuka **C)**, tawiniwini **D)**, and toatoa **E)** host species.

Supplementary Table 1: Descriptions of mānuka (*Leptospermum scoparium*), Kānuka (*Kunzea ericoides*), tawiniwini (*Gaultheria antipoda*), and toatoa (*Phyllocladus alpinus*) species in New Zealand.

		Leptospermum scoparium[1]	*Kunzea ericoides*[2]	*Gaultheria antipoda*[3]	*Phyllocladus. alpinus*[4]
General information	Common name	Mānuka	Kānuka	Tawiniwini	Mountian toatoa
	Family	Myrtaceae	Myrtaceae	Ericaceae	Podocarpaceae
	Structural class	Dicotyledon	Dicotyledon	Dicotyledon	Dicotyledon
	Angiosperm/ gymnosperm	Angiosperm	Angiosperm	Angiosperm	Gymnosperm
	Distribution	Indigenous to NZ and Australia. Coastal to low alpine throughout NZ. Pioneer species. Commonly found with Kānuka [5]	Endemic to NZ Coastal to low alpine throughout North and northern South Islands, NZ. Pioneer species[6] Commonly found with mānuka.	Endemic to NZ Lowland to subalpine scrub in the South and central North Islands, NZ	Endemic to NZ Scrub forests to subalpine throughout NZ, particularly colder climates
	Chromosome number	Normally diploid (22)	Diploid (22)	Diploid (22)	Diploid (18)
	Mature plant height	2-10 m	Up to 20 m	1-2 m	Up to 9 m
Leaf characteristics	Foliage	Evergreen	Evergreen	Evergreen	Evergreen
	Leaf size	4-12 mm long	4-12 mm long	5-15 mm long	Phylloclades 5-25 mm long, 3-12 mm wide.

[1] Stephens, J., *et al.* 2005, *A review of Leptospermum scoparium (Myrtaceae) in New Zealand*,
[2] de Lange, P. J. 2014, 'A revision of the New Zealand Kunzea ericoides (Myrtaceae) complex', *PhytoKeys*, vol. no. 40, pp. 1.
[3] Bush, C. M., *et al.* 2009, 'The phylogeny, biogeography and morphological evolution of Gaultheria (Ericaceae) from Australia and New Zealand', *Australian Systematic Botany*, vol. 22, no. 4, pp. 229-242.
[4] Wardle, P. 1969, 'Biological flora of New Zealand', *New Zealand Journal of Botany*, vol. 7, no. 1, pp. 76-95.
[5] Burrows, C. 1973, 'The ecological niches of Leptospermum scoparium and L. ericoides (Angiospermae: Myrtaceae)', vol. no. pp.
[6] Allen, R., *et al.* 1992, 'Ecology of Kunzea ericoides (A. Rich.) J. Thompson (Kānuka) in east Otago, New Zealand', *New Zealand journal of botany*, vol. 30, no. 2, pp. 135-149.

	Specific leaf area	7.26±0.25 m^2kg^{-1} [7]	7.92±0.39 m^2kg^{-1}		
	Leaf arrangement	Single or in groups	Single or in groups	Singly along stem	Singly and distichously along stem
	General leaf description	Undivided, linear to ovate, sharply pointed[8] Anomocytic stomata Thick, striated cuticle (more than 10 µm) Non-glandular, unicellular, appressed hairs on their young leaves normally shed during leaf development No papillae	Undivided, narrow-linear, pointed tips	Undivided, round-oblong, leathery, shiny, small serrations	Phylloclades pinnately leaf-like, undivided, of variable shape Seedlings have needle leaves, but these become ephemeral and non-photosynthetic as plants develop
Flower characteristics	Flower size	8-25 mm diam	4-8 mm diam	3-4 mm diam	Pollen cone 4-6 mm long
	General flower description	Solitary, white or pink, 5 petals	Solitary but occur in clusters, white or prink, 5 petals	Solitary, white, 5 petals	-
	Regional flowering time	February	February	November	-
	Fruit	Dry	Dry	Dry	-
Seed mass/size		5-6 mm	2-3 mm		
Wood density		400-600 kg m^3 [9]	400-600 kg m^3 [10]		
	Breeding system	Andromonoecious	Hermaphrodite	Hermaphrodite	Hermaphrodite

[7] Whitehead, D., *et al.* 2004, 'Characteristics of photosynthesis and stomatal conductance in the shrubland species mānuka (Leptospermum scoparium) and kānuka (Kunzea ericoides) for the estimation of annual canopy carbon uptake', *Tree Physiology*, vol. 24, no. 7, pp. 795-804.
[8] Johnson, C. T. 1980, 'leaf anatomy of Leptospermum Forst. (Myrtaceae)', *Australian journal of botany*, vol. 28, no. 1, pp. 77-104.
[9] Marden, M., *et al.* 2021, 'Species-specific basic stem-wood densities for twelve indigenous forest and shrubland species of known age, New Zealand', *New Zealand Journal of Forestry Science*, vol. 51, no. pp.
[10] Marden, M., *et al.* 2021, 'Species-specific basic stem-wood densities for twelve indigenous forest and shrubland species of known age, New Zealand', *New Zealand Journal of Forestry Science*, vol. 51, no. pp.

Supplementary Table 2: ASV occurrence probabilities predicted for each microbiome occurrence

Microbiome occurrence*	Probability an ASV is found at an occurrence, with zero truncation+
0	NA
1	6/63 = ~0.095
2	15/63 = ~0.238
3	20/63 = ~0.317
4	15/63 = ~0.238
5	6/63 = ~0.095
6	1/63 = ~0.016

* Microbiome occurrence represents the number of phyllosphere microbiomes in which an ASV is present within each site out of a maximum of six.

+ Probabilities are calculated by dividing the number of unique microbiome combinations an ASV could occupy per microbiome occurrence, by the total number of possible combinations.

The number of unique combinations

$$C(n,r) = \frac{\mathrm{n!}}{(\mathrm{n-r})!\mathrm{r!}}$$

Probabilities were zero truncated due to the inability to measure undetected microbial taxa.

Supplementary Table 3: The observed number of ASVs found at each microbiome occurrence per site

Sites	**Microbiome occurrence**						
	1	**2**	**3**	**4**	**5**	**6**	**Total**
Site 1	1002	229	150	139	56	211	1787
Site 2	971	187	149	90	108	236	1741
Site 4	952	222	147	106	119	267	1813
Site 5	1181	209	108	91	95	274	1958
Site 6	634	196	142	110	129	143	1354

Supplementary Table 4: Chi-square goodness of fit test results of the occurrence probabilities (Supplementary Table 2) vs the observed number of ASVs (Supplementary Table 3).

Site	X2	Df	P value
Site 1	5908.6	5	< 2.2x10-6
Site 2	6175.1	5	< 2.2x10-6
Site 4	6172.8	5	< 2.2x10-6
Site 5	8115.8	5	< 2.2x10-6
Site 6	3047.2	5	< 2.2x10-6

Supplementary Table 5: ASV occurrence probabilities predicted for each microbiome occurrence per host group/site

Microbiome occurrence*	Probability a taxon is found at an occurrence+ with zero truncation (mānuka)	Probability a taxon is found at an occurrence+ with zero truncation (non-mānuka)
0	NA	NA
1	3/7 = ~0.429	3/7 = ~0.429
2	3/7 = ~0.429	3/7 = ~0.429
3	1/7 = ~0.143	1/7 = ~0.143

* Microbiome occurrence represents the number of phyllosphere microbiomes in which an ASV is present, out of a maximum of three per each host group (i.e. mānuka or non-mānuka).

+ Probabilities are calculated by dividing the number of unique microbiome combinations an ASV could occupy per microbiome occurrence, by the total number of possible combinations.

The number of unique combinations

$$C(n,r) = \frac{n!}{(n-r)!r!}$$

Probabilities were zero truncated due to the inability to measure undetected microbial taxa.

Supplementary Table 6: The observed number of ASVs found at each microbiome occurrence within mānuka and non-mānuka microbiomes per site

Site	Microbiome occurrence							
	Mānuka				Non-mānuka			
	1	2	3	Total	1	2	3	Total
Site 1	461	173	449	1083	865	162	223	1250
Site 2	460	166	446	1072	815	226	237	1278
Site 4	268	174	476	918	959	288	293	1540
Site 5	374	130	457	961	1111	189	304	1604
Site 6	312	153	464	929	564	166	163	893

Supplementary Table 7: Chi-square goodness of fit test results of the occurrence probabilities (Supplementary Table 5) vs the observed number of ASVs (Supplementary Table 6).

Site	Mānuka				Non-mānuka			
	X^2	Df	P value	JSD^+	X^2	Df	P value	JSD^+
Site 1	742.41	2	$< 2.2x10^{-6}$	0.0959	474.16	2	$< 2.2x10^{-6}$	0.0849
Site 2	747.44	2	$< 2.2x10^{-6}$	0.0985	335.63	2	$< 2.2x10^{-6}$	0.0558
Site 4	1069.2	2	$< 2.2x10^{-6}$	0.1254	369.35	2	$< 2.2x10^{-6}$	0.0506
Site 5	940.93	2	$< 2.2x10^{-6}$	0.1266	646.84	2	$< 2.2x10^{-6}$	0.0927
Site 6	996.54	2	$< 2.2x10^{-6}$	0.1235	218.43	2	$< 2.2x10^{-6}$	0.0514

Supplementary Table 8: Minimum, maximum, and average relative abundance (%) of phyla in all phyllosphere microbiome samples.

Phylum	minimum	maximum	Average
Abditibacteriota	0	2.5014	0.1839
Acidobacteriota	10.1446	34.8733	23.7166
Actinobacteriota	0	2.1631	0.3749
Armatimonadota	0	2.7995	0.7518
Bacteroidota	0.11	15.7523	4.385
Bdellovibrionota	0	2.7665	0.8514
Chloroflexi	0	0.0614	0.0018
Cyanobacteria	0	2.1603	0.1869
Deinococcota	0	0.2735	0.0342
Desulfobacterota	0	0.0338	0.001
Firmicutes	0	1.136	0.0648
Myxococcota	0	1.3368	0.2839
Nitrospirota	0	0.0272	0.0008
Planctomycetota	0.4568	6.7188	2.2585
Proteobacteria	35.9113	80.0674	61.4325
Verrucomicrobiota	1.198	11.6899	5.472

Supplementary Table 9: Minimum, maximum, and average relative abundance (%) of phyla in the phyllosphere microbiome of mānuka, kānuka, tawiniwini, and toatoa.

Phylum	Host_species	minimum	maximum	Average
Abditibacteriota	Kānuka	0	0.1915	0.0341
	Mānuka	0	0.2579	0.0243
	Tawiniwini	0.1749	2.2209	0.7115
	Toatoa	0	0.5474	0.111
Acidobacteriota	Kānuka	9.7824	20.6749	14.814
	Mānuka	19.2458	28.8484	22.934
	Tawiniwini	9.1709	21.9948	17.9094
	Toatoa	11.3846	26.7284	21.5378
Actinobacteriota	Kānuka	0.0196	0.8684	0.4348
	Mānuka	0	0.1948	0.0435
	Tawiniwini	0.2199	1.1704	0.601
	Toatoa	0.1366	1.6712	0.6697
Armatimonadota	Kānuka	0.0958	0.4972	0.329
	Mānuka	0	0.7552	0.2778
	Tawiniwini	0.4732	1.752	1.1389
	Toatoa	0.1489	2.1937	1.4581
Bacteroidota	Kānuka	3.3189	7.6052	5.1251
	Mānuka	0.1027	4.9919	1.7096
	Tawiniwini	4.7012	12.5753	8.6379
	Toatoa	0.2727	5.7385	3.1931
Bdellovibrionota	Kānuka	0.2936	2.5594	0.8666
	Mānuka	0	1.2142	0.6962
	Tawiniwini	0.3162	1.3083	0.7612
	Toatoa	0.1092	1.343	0.7363
Chloroflexi	Kānuka	0	0	0
	Mānuka	0	0	0
	Tawiniwini	0	0.0545	0.0091
	Toatoa	0	0	0
Cyanobacteria	Kānuka	0	0	0
	Mānuka	0	0	0
	Tawiniwini	0.0187	1.1419	0.3915
	Toatoa	0.0207	1.34	0.3931
Deinococcota	Kānuka	0	0.1657	0.0391
	Mānuka	0	0.2466	0.0354
	Tawiniwini	0	0.2399	0.04
	Toatoa	0	0	0
Desulfobacterota	Kānuka	0	0.0313	0.0052
	Mānuka	0	0	0
	Tawiniwini	0	0	0

	Toatoa	0	0	0
Firmicutes	Kānuka	0	0.0637	0.0106
	Mānuka	0	0.0831	0.0049
	Tawiniwini	0	0.7818	0.1747
	Toatoa	0	0.3163	0.0818
Myxococcota	Kānuka	0	1.2062	0.4629
	Mānuka	0	0.5156	0.1918
	Tawiniwini	0	0.3962	0.1875
	Toatoa	0	0.5169	0.243
Nitrospirota	Kānuka	0	0.0245	0.0041
	Mānuka	0	0	0
	Tawiniwini	0	0	0
	Toatoa	0	0	0
Planctomycetota	Kānuka	0.6399	3.3701	1.6742
	Mānuka	0.4182	4.1757	2.0288
	Tawiniwini	0.7767	1.5779	1.2041
	Toatoa	0.7444	5.265	2.5685
Proteobacteria	Kānuka	63.5682	82.0143	73.5
	Mānuka	63.6813	72.4008	67.8198
	Tawiniwini	48.9659	78.531	62.6956
	Toatoa	53.8798	82.6104	61.9965
Verrucomicrobiota	Kānuka	1.1083	4.6758	2.7003
	Mānuka	1.3102	8.237	4.234
	Tawiniwini	2.8855	8.9382	5.5376
	Toatoa	1.7866	10.851	7.011

Supplementary Table 10: Kruskal-wallis rank sum test on phylum relative abundance across host species. Benjamini-Hochberg corrected p value. Significant values are in bold.

Phylum	Statistic	P value	P adjusted
Abditibacteriota	18.5538	0.0003	**0.0018**
Acidobacteriota	15.9628	0.0012	**0.0037**
Actinobacteriota	16.1661	0.001	**0.0037**
Armatimonadota	10.205	0.0169	**0.03**
Bacteroidota	20.7632	0.0001	**0.0009**
Bdellovibrionota	0.7171	0.8692	0.8819
Chloroflexi	4.8333	0.1844	0.2459
Cyanobacteria	28.869	0	**0**
Deinococcota	2.0669	0.5586	0.6384
Desulfobacterota	4.8333	0.1844	0.2459
Firmicutes	15.1192	0.0017	**0.0046**
Myxococcota	0.6627	0.8819	0.8819
Nitrospirota	4.8333	0.1844	0.2459
Planctomycetota	4.3673	0.2244	0.2762
Proteobacteria	11.8036	0.0081	**0.0162**
Verrucomicrobiota	11.9406	0.0076	**0.0162**

Supplementary Table 11: Summary of alpha diversity in the phyllosphere microbiome of different host species. Values represent the mean and standard deviation of 100 subsamples to the minimum sequencing depth (6192 reads).

Sample_ID	Richness		Shannon		Host species
	mean	sd	mean	sd	
Rp_01.1	625.53	5.9501	6.0809	0.0114	Mānuka
Rp_01.2	723.38	7.2122	6.2471	0.0107	
Rp_01.3	624.42	4.9425	6.1138	0.0087	
Rp_01.4	714.93	5.5108	6.1945	0.0104	Toatoa
Rp_01.5	471.46	5.2309	5.646	0.0111	Kānuka
Rp_01.6	536.25	4.4481	5.8803	0.0104	Tawiniwini
Rp_02.1	628	5.3314	6.1232	0.0089	Mānuka
Rp_02.2	688.31	6.8721	6.2201	0.01	
Rp_02.3	614.6	5.8205	6.1077	0.0103	
Rp_02.4	633.02	5.1345	6.071	0.0099	Toatoa
Rp_02.5	573.12	4.9304	5.954	0.0123	Kānuka
Rp_02.6	638.61	5.253	6.1015	0.0098	Tawiniwini
Rp_03.2	713.31	5.6295	6.2576	0.0088	Mānuka
Rp_03.3	674.83	6.5691	6.1865	0.0112	
Rp_03.4	526.93	4.5644	5.8565	0.0107	Toatoa
Rp_03.5	482.7	5.1669	5.7634	0.0109	Kānuka
Rp_03.6	697.1	6.7293	6.1585	0.0103	Tawiniwini
Rp_04.1	645.77	6.6239	6.0616	0.0117	Mānuka
Rp_04.2	616.8	5.2724	6.0982	0.0093	
Rp_04.3	618.34	5.5126	6.0832	0.0108	
Rp_04.4	1001.44	7.1807	6.6253	0.0094	Toatoa
Rp_04.5	477.01	3.2737	5.8682	0.0086	Kānuka
Rp_04.6	721.31	6.743	6.2067	0.0121	Tawiniwini
Rp_05.1	684.2	6.5056	6.2109	0.0102	Mānuka
Rp_05.2	633.76	4.928	6.1355	0.0094	
Rp_05.3	528.01	4.2247	5.9088	0.0111	
Rp_05.4	741.22	6.3319	6.3221	0.0111	Toatoa
Rp_05.5	538.29	6.1698	5.8956	0.012	Kānuka
Rp_05.6	862.04	9.243	6.3622	0.011	Tawiniwini
Rp_06.1	586.26	5.0063	6.0068	0.0101	Mānuka
Rp_06.2	635.38	6.2098	6.1065	0.0088	
Rp_06.3	590.69	6.7265	5.9222	0.0137	
Rp_06.4	309.33	2.4579	5.2887	0.01	Toatoa
Rp_06.5	547.07	5.8556	5.9409	0.0104	Kānuka
Rp_06.6	456.59	3.062	5.6941	0.0125	Tawiniwini

Supplementary Table 12: ANOVA on phyllosphere microbiome alpha diversity across host species.

Alpha diversity index	Variable	Df	Sum Sq	Mean Sq	F value	P value
Shannon	Host species	3	0.3164	0.1055	2.092	0.122
	Residuals	31	1.5633	0.05043		
Richness	Host species	3	84392	28131	2.089	0.122
	Residuals	31	417358	13463		

Supplementary Table 13: Indicator species phyla relative abundance, kruskal-wallis rank sum test Benjamini-Hochberg corrected p value. Significant values are in bold.

Phylum	P adjusted
Acidobacteriota	**0.0002**
Bacteroidota	**0.0004**
Proteobacteria	**0.0004**
Verrucomicrobiota	**0.0005**

Supplementary Table 14: Indicator species genera relative abundance, kruskal-wallis rank sum test Benjamini-Hochberg corrected p value. Significant values are in bold.

Genus	P adjusted
1174-901-12	**0.0002**
Acidiphilium	**0.0011**
Aurantisolimonas	**0.0005**
Bryocella	**0.0001**
Edaphobacter	**0.0253**
Granulicella	**0.0003**
LD29	**0.0009**
Methylocella	**0.0002**
PMMR1	**0.0277**
Sphingomonas	**0.0253**
Terriglobus	**0.007**
Unknown	**0.0009**

Supplementary Table 15: Pairwise comparisons of betadisper test. Significant values are in bold.

Host species comparison	Bray-Curtis (p value)	Jaccard (p value)
Mānuka-Kānuka	0.399	**0.001**
Mānuka-Tawiniwini	**0.001**	**0.001**
Mānuka-Toatoa	**0.001**	**0.001**
Kānuka-Tawiniwini	**0.008**	0.189
Kānuka-Toatoa	**0.001**	**0.02**
Tawiniwini-Toatoa	0.077	0.109

Supplementary Table 16: Normalised stochasticity ratios (NST) of microbial community assembly based on taxonomy.

Host species	NST
Mānuka	0.13
Kānuka	0.13
Tawiniwini	0.34
Toatoa	0.48

Supplementary Table 17: Permanova on normalised stochasticity ratios (NST) of microbial community assembly based on taxonomy.

Host species comparison	P value
Mānuka-Kānuka	0.860
Mānuka-Tawiniwini	**0.001**
Mānuka-Toatoa	**0.001**
Kānuka-Tawiniwini	**0.001**
Kānuka-Toatoa	**0.005**
Tawiniwini-Toatoa	0.074

Supplementary Table 18: Mantel analyses for different partitions of the phyllosphere microbiome of all host species. Mantel tests performed on community dissimilarity (Bray Curtis relative abundance) and Euclidean distance. Significant values are in bold.

Community partition	**All phyllosphere samples**	
	Mantel r	**P value**
High abundance (>0.001)	0.079	0.192
Entire community	0.05	0.296
Low abundance (<0.001)	0.1527	0.059
Presence/absence	0.09	0.179

Supplementary Table 19: Mantel analyses for different partitions of the mānuka and non-mānuka phyllosphere microbiome. Mantel tests performed on community dissimilarity (Bray-Curtis/relative abundance or Jaccard/presence-absence) and Euclidean distance. Significant values are in bold.

Community partition	Mānuka		Kānuka		Toatoa		Tawiniwini	
	Mantel r	P value	Mantel r	P value	Mantel r	P value	Mantel r	P value
High abundance (>0.001)	**0.388**	**0.001**	0.4714	0.1514	**0.7964**	**0.02361**	**0.6786**	**0.0097**
Entire community	**0.3174**	**0.009**	0.5393	0.1181	**0.7786**	**0.0333**	0.4679	0.0750
Low abundance (<0.001)	**0.4457**	**0.001**	0.725	0.0597	0.6714	0.0680	0.3536	0.1167
Presence/absence	**0.4092**	**0.001**	0.3821	0.1763	0.6429	0.0930	0.4214	0.1014

Supplementary Table 20: Minimum, maximum, and average relative abundance (%) of phyla in the surface soil microbiome.

Phylum	Minimum	Maximum	Average
Acidobacteriota	8.8107	24.3939	15.1887
Actinobacteriota	0.0335	9.443	2.3567
Armatimonadota	0	0.844	0.2658
Bacteroidota	9.9583	38.4848	17.9208
Bdellovibrionota	0.0864	1.827	0.7169
Chloroflexi	0.4414	11.2985	2.4383
Crenarchaeota	0	0.1949	**0.0213**
Cyanobacteria	0	1.1586	0.3799
Deinococcota	0	0.1079	**0.0045**
Dependentiae	0.0721	4.5571	0.756
Desulfobacterota	0	0.0656	**0.0027**
Elusimicrobiota	0	0.4206	**0.0175**
Entotheonellaeota	0	0.0797	**0.0056**
Fibrobacterota	0	0.1402	**0.0072**
Firmicutes	0	0.9025	0.1441
Gemmatimonadota	0	0.3155	0.0838
Methylomirabilota	0	0.1129	**0.0078**
Micrarchaeota	0	0.011	**0.0005**
Myxococcota	0.1844	3.9418	1.5172
Nanoarchaeota	0	0.0117	**0.0005**
Nitrospirota	0	0.2435	**0.0381**
Planctomycetota	0.3352	11.819	6.0289
Proteobacteria	26.3768	44.612	34.2122
Spirochaetota	0	0.3991	0.0789
Verrucomicrobiota	10.8616	24.6129	17.806

Supplementary Table 21: Mantel analyses for different partitions of microbial communities in surface soil. Mantel tests performed on community dissimilarity (Bray-Curtis/relative abundance or Jaccard/presence-absence) and Euclidean distance.

Community partition	All samples	
	Mantel r	P value
High abundance (>0.001)	-0.0420	0.572
Entire community	-0.0377	0.600
Low abundance (<0.001)	0.0019	0.471
Presence/absence	0.0047	0.465

Supplementary Table 22: Summary of alpha diversity across phyllosphere and surface soil samples. Values represent the mean and standard deviation of 100 subsamples to the minimum sequencing depth (5966 reads).

SampleID	Richness		Shannon		Type	
	mean	sd	mean	sd		Host species
Rp_01.1	623.85	4.8812	6.0805	0.0108	Leaf	Mānuka
Rp_01.1S	588.29	5.6377	6.0312	0.011	Soil	
Rp_01.2	720.36	6.0244	6.2436	0.01	Leaf	
Rp_01.3	622.48	5.3	6.1152	0.0102	Leaf	
Rp_01.4	710.26	6.7981	6.1904	0.0107	Leaf	Toatoa
Rp_01.4S	370.63	3.6671	5.6009	0.0097	Soil	
Rp_01.5	469.8	4.4969	5.6452	0.0115	Leaf	Kānuka
Rp_01.5S	524.18	4.7893	5.9033	0.009	Soil	
Rp_01.6	533.7	4.8814	5.8754	0.0107	Leaf	Tawiniwini
Rp_01.6S	650.47	6.4939	6.1265	0.0097	Soil	
Rp_02.1	628.01	5.9365	6.1245	0.0096	Leaf	Mānuka
Rp_02.1S	495.11	4.5945	5.8648	0.0077	Soil	
Rp_02.2	684.86	6.6606	6.2169	0.0098	Leaf	
Rp_02.3	612.56	6.4375	6.1054	0.0102	Leaf	
Rp_02.4	629.98	5.545	6.0713	0.0101	Leaf	Toatoa
Rp_02.4S	566.55	4.6174	6.038	0.01	Soil	
Rp_02.5	570.64	5.0503	5.9492	0.0111	Leaf	Kānuka
Rp_02.5S	677.12	6.5185	6.1814	0.0088	Soil	
Rp_02.6	636.28	5.5761	6.1013	0.0107	Leaf	Tawiniwini
Rp_02.6S	624.61	6.3864	6.0983	0.0104	Soil	
Rp_03.1S	552.19	5.3252	5.92	0.0104	Soil	Mānuka
Rp_03.2	710.22	6.6265	6.2544	0.0108	Leaf	
Rp_03.3	672.08	6.6434	6.1826	0.0102	Leaf	
Rp_03.4	523.74	4.0741	5.8558	0.0109	Leaf	Toatoa
Rp_03.4S	496.4	4.1317	5.8506	0.0094	Soil	
Rp_03.5	480.39	4.8636	5.7616	0.0097	Leaf	Kānuka
Rp_03.5S	382.44	4.1593	5.5905	0.0088	Soil	
Rp_03.6	691.88	8.3995	6.1568	0.0118	Leaf	Tawiniwini
Rp_03.6S	196.33	1.1015	4.9855	0.0086	Soil	
Rp_04.1	644.47	6.2884	6.0621	0.0115	Leaf	Mānuka
Rp_04.1S	537.97	5.016	5.9771	0.0087	Soil	
Rp_04.2	614.96	5.3709	6.0971	0.0103	Leaf	
Rp_04.3	616.62	6.1262	6.08	0.0103	Leaf	
Rp_04.4	996.91	7.3417	6.623	0.0086	Leaf	Toatoa
Rp_04.4S	545.06	5.1006	5.942	0.01	Soil	
Rp_04.5	476.4	4.3647	5.867	0.0081	Leaf	Kānuka
Rp_04.5S	697.11	5.944	6.2477	0.0097	Soil	
Rp_04.6	718.02	7.0324	6.2045	0.0098	Leaf	Tawiniwini

Rp_04.6S	796.67	7.4523	6.3098	0.0113	Soil	
Rp_05.1	681.02	6.818	6.206	0.0109	Leaf	Mānuka
Rp_05.1S	497.1	4.7001	5.8491	0.0102	Soil	
Rp_05.2	631.03	5.5221	6.1334	0.01	Leaf	
Rp_05.3	526.65	4.3073	5.9091	0.0097	Leaf	
Rp_05.4	737.33	5.4663	6.319	0.0086	Leaf	Toatoa
Rp_05.4S	622.79	5.7547	6.1071	0.0102	Soil	
Rp_05.5	537.35	6.1814	5.8934	0.011	Leaf	Kānuka
Rp_05.5S	549.28	5.6641	5.9723	0.0103	Soil	
Rp_05.6	859.47	10.4374	6.3616	0.0124	Leaf	Tawiniwini
Rp_05.6S	579.92	5.8148	6.0754	0.0095	Soil	
Rp_06.1	584.28	5.1111	6.0046	0.0098	Leaf	Mānuka
Rp_06.1S	526.12	4.961	5.9332	0.0108	Soil	
Rp_06.2	633.92	6.4771	6.1048	0.0094	Leaf	
Rp_06.3	587.7	5.6667	5.9203	0.0109	Leaf	
Rp_06.4	308.77	2.5736	5.2885	0.01	Leaf	Toatoa
Rp_06.4S	637.33	6.189	6.1358	0.0097	Soil	
Rp_06.5	545.12	4.9712	5.9401	0.0106	Leaf	Kānuka
Rp_06.5S	595.87	5.5005	6.073	0.0096	Soil	
Rp_06.6	455.55	3.3886	5.6944	0.0107	Leaf	Tawiniwini
Rp_06.6S	513.18	4.6196	5.9125	0.01	Soil	

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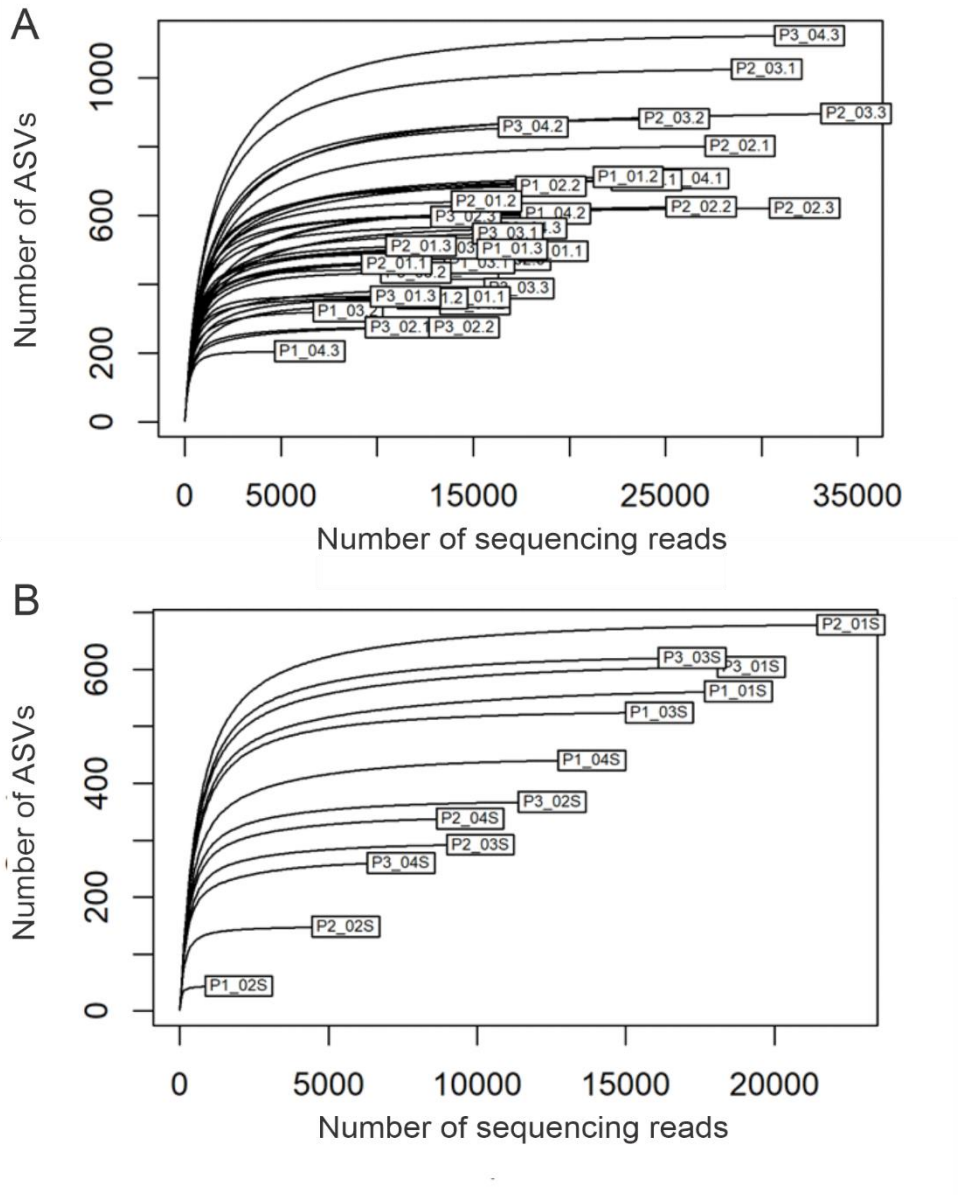
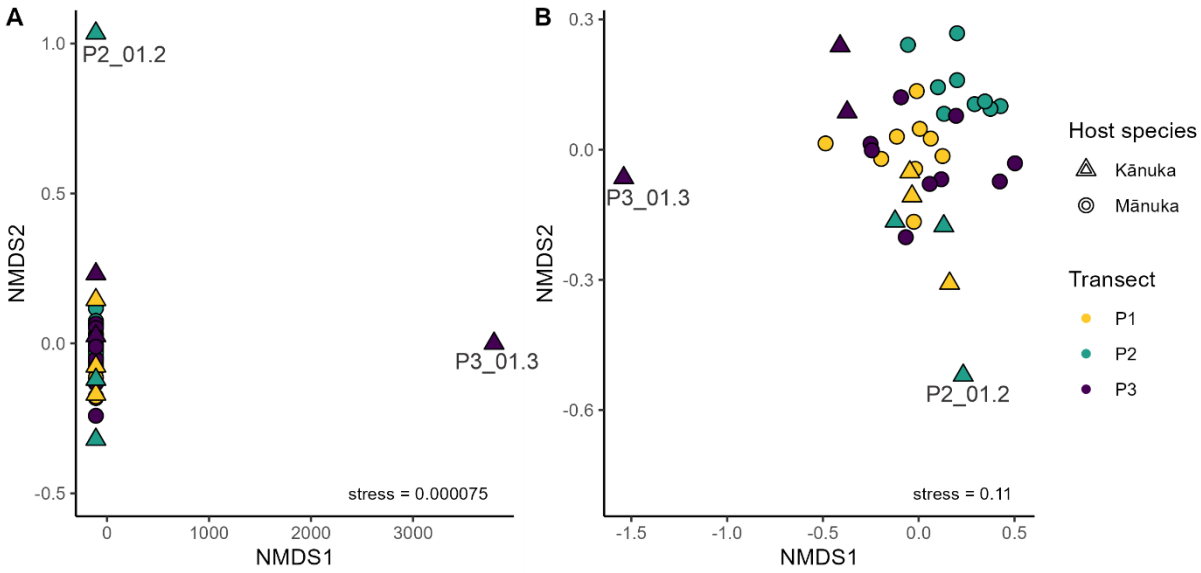
Appendix B

Supplementary Material for Chapter III

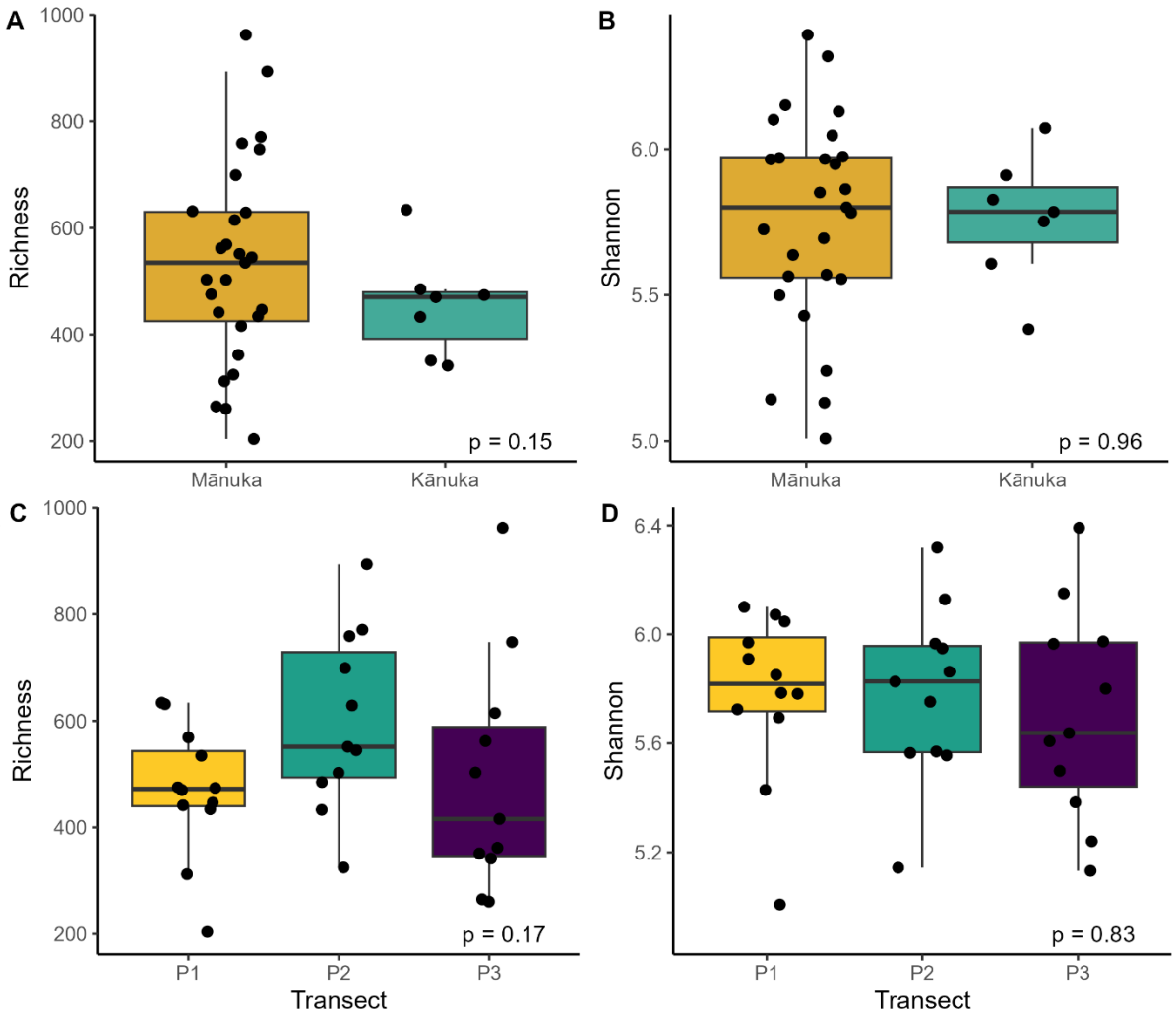
Supplementary Figures and Tables



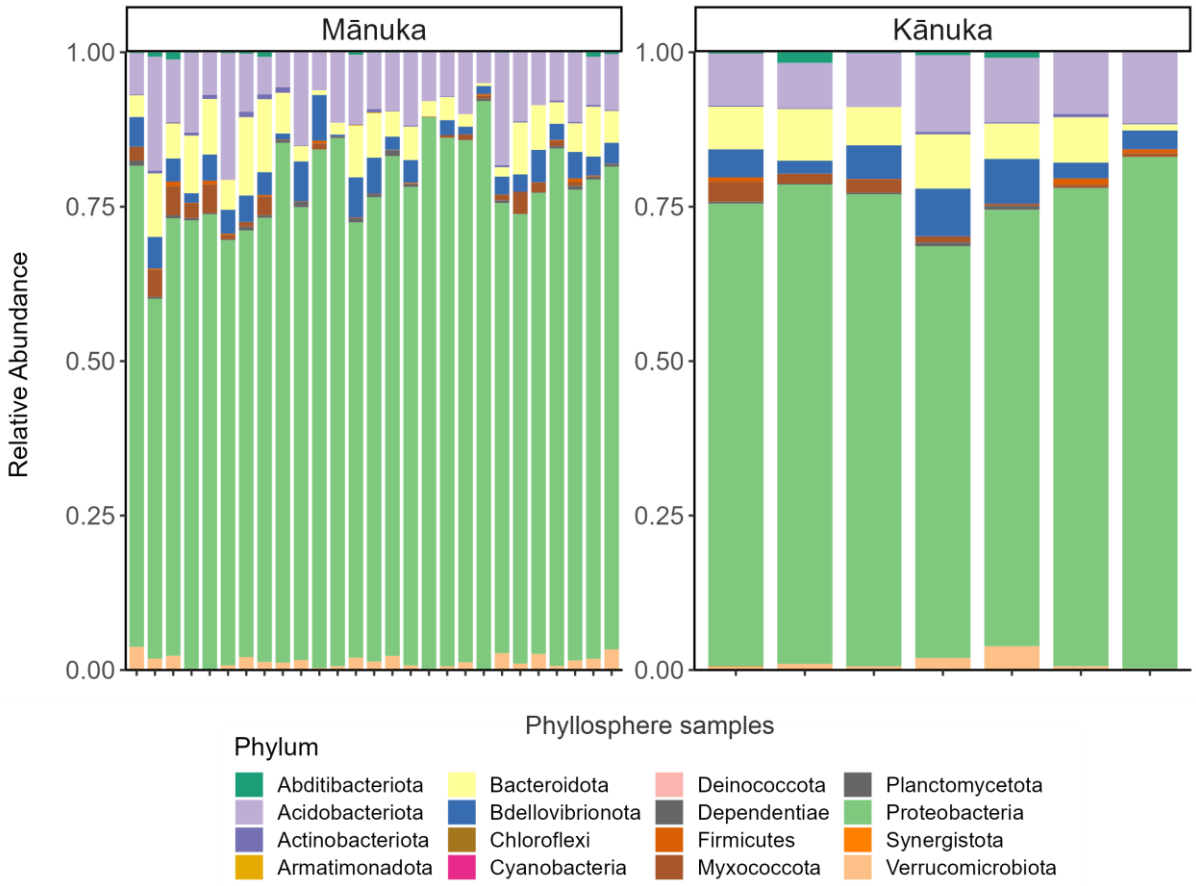
Supplementary Fig. 1: Rarefaction curves showing the relationship between the number of ASVs detected and the number of sequencing reads for **A)** phyllosphere and **B)** surface soil samples.



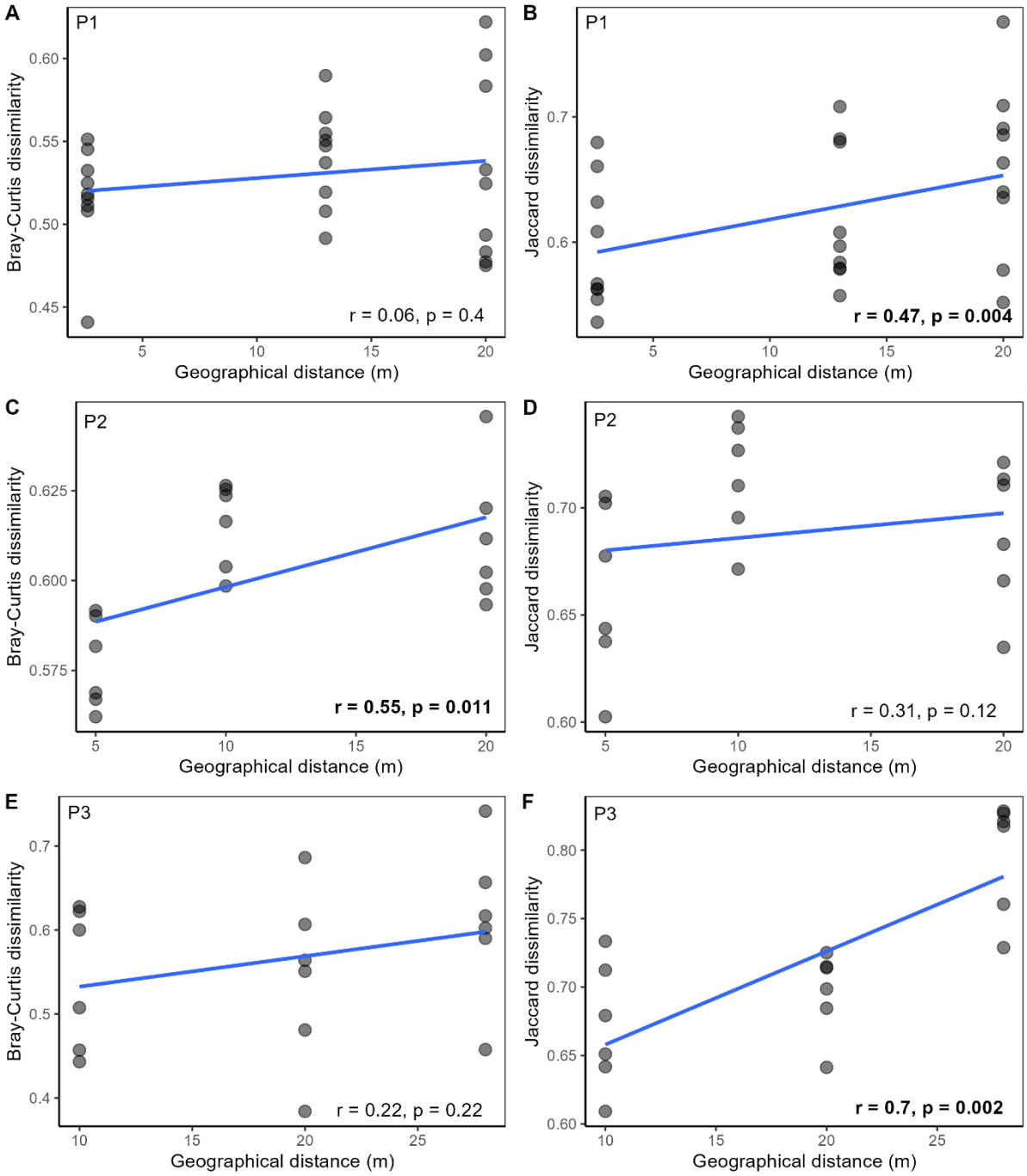
Supplementary Fig. 2: NMDS ordination of mānuka and kānuka phyllosphere community dissimilarities before the removal of outliers P2_01.2 and P3_01.3. Community dissimilarity is based on **A)** Bray-Curtis (relative abundance) and **B)** Jaccard (presence/absence).



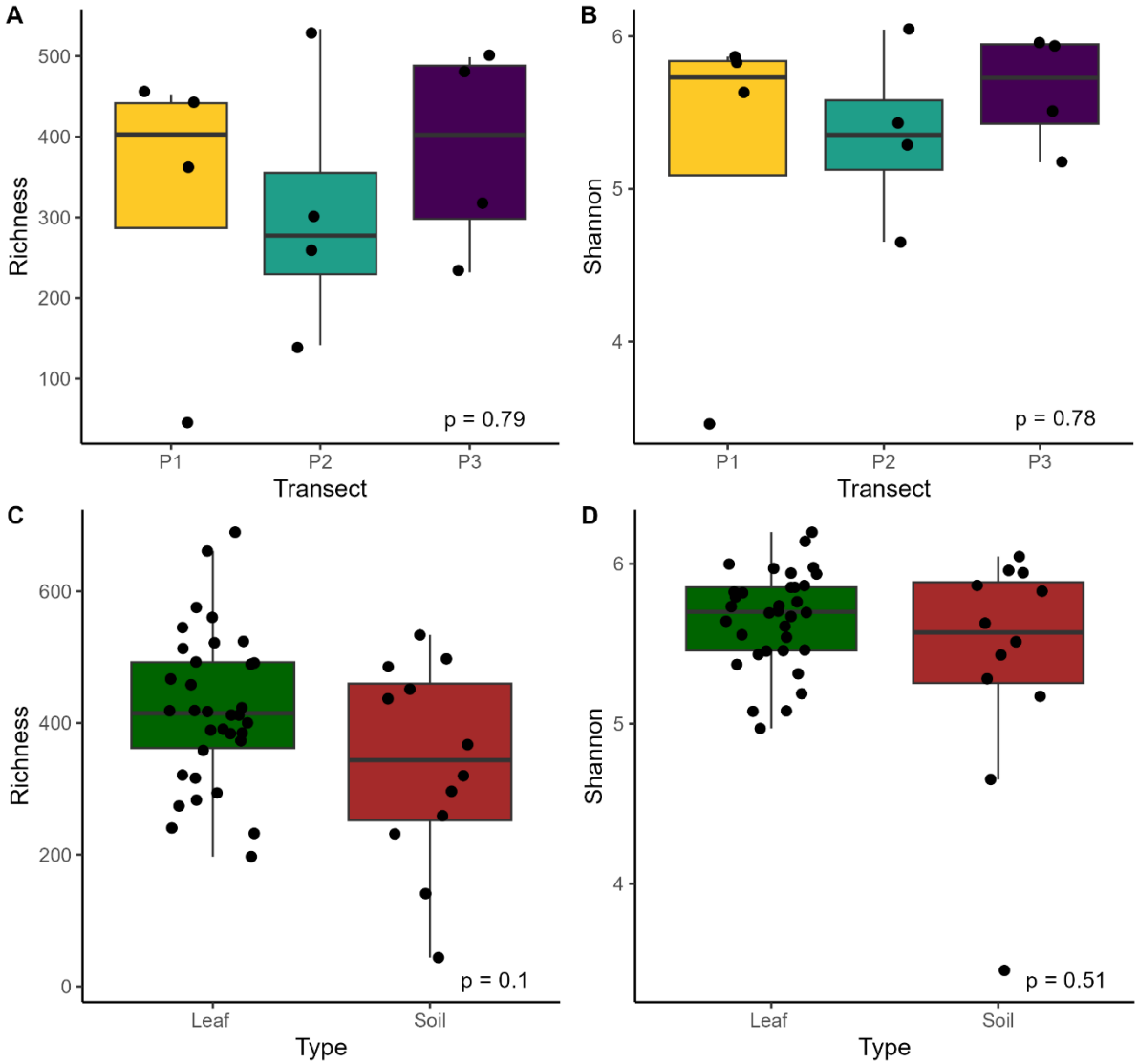
Supplementary Fig. 3: Alpha diversity (Shannon and richness) of the phyllosphere microbiome across **A-B)** different plant species and **C-D)** transects. Each sample was subsampled (100x) to an even sequencing depth (6,491 reads). Boxes indicate the interquartile range and the thick bar represents the median. Vertical segments extend to the fifth and 95th percentiles of the distribution of values.



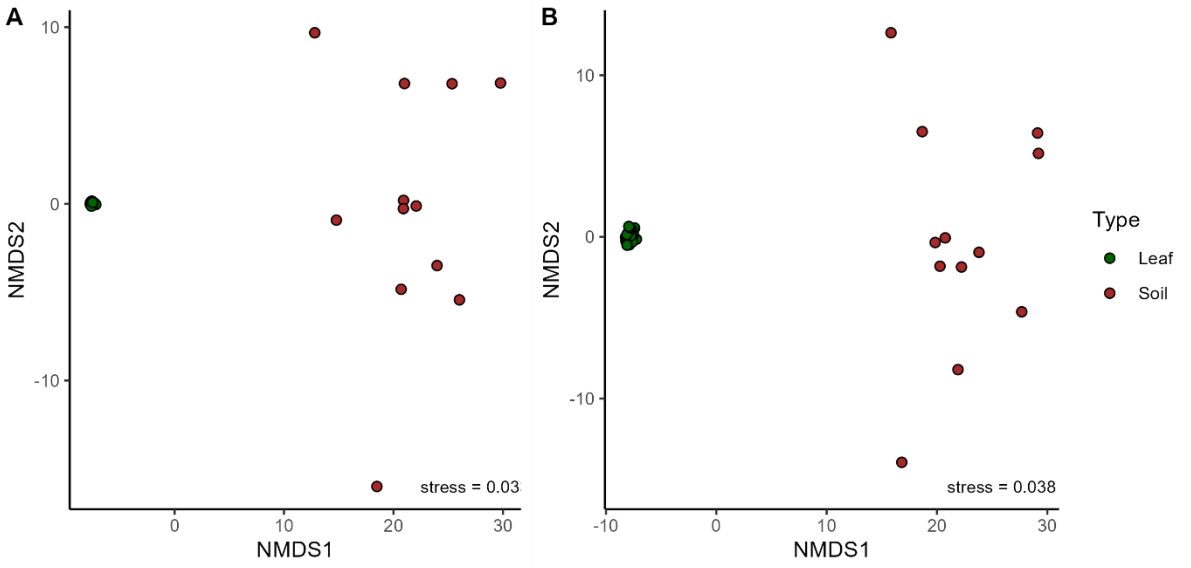
Supplementary Fig. 4: Relative abundance of taxa in the mānuka and kānuka phyllosphere microbiomes. Colour represents taxonomic classification at the phylum level.



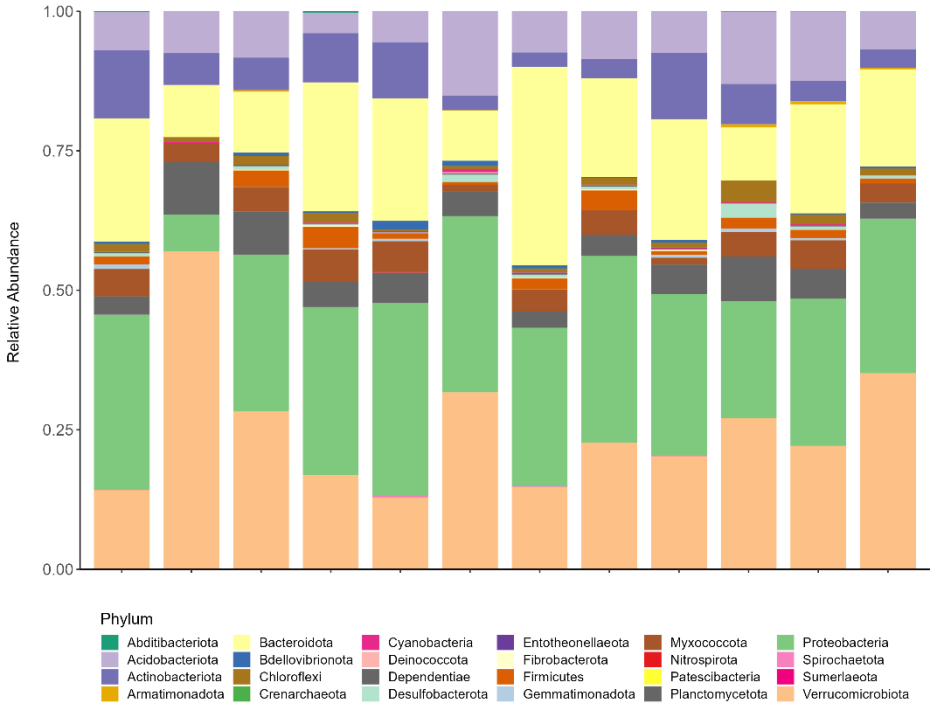
Supplementary Fig. 5: Mānuka-kānuka community dissimilarity versus distance within each transect. **A-B)** P1, **C-D)** P2, and **E-F)** P3. Inset r values represent Pearson's product-moment correlation and corresponding p value. Bold values represent significant relationships.



Supplementary Fig. 6: Alpha diversity (Shannon and richness) of surface soil microbial communities **A-B)** across transect sites and **C-D)** compared to phyllosphere microbial communities. Each sample was subsampled (100x) to an even sequencing depth (1,985 reads). Boxes indicate the interquartile range and the thick bar represents the median. Vertical segments extend to the fifth and 95th percentiles of the distribution of values.



Supplementary Fig. 7: Overall community dissimilarity of phyllosphere and surface soil microbial communities using **A)** Bray-Curtis (relative abundance based distance) and **B)** Jaccard (presence/absence based distance). Colour represents sample type.



Supplementary Fig. 8: Relative abundance of taxa in surface soil microbial communities. Colour represents taxonomic classification at the phylum level.

Supplementary Table 1: Minimum, maximum, and average relative abundance of phyla in mānuka and kānuka phyllosphere communities.

Phylum	Plant species	Minimum	Maximum	Average ± SD
Abditibacteriota	Kānuka	0	1.73	0.494 ± 0.63
	Mānuka	0	1.18	0.188 ± 0.31
	Combined	0	1.73	0.251 ± 0.4
Acidobacteriota	Kānuka	7.32	12.4	9.82 ± 1.8
	Mānuka	5	20.5	10.2 ± 4
	Combined	5	20.5	10.1 ± 3.6
Actinobacteriota	Kānuka	0	0.536	0.244 ± 0.18
	Mānuka	0	0.924	0.281 ± 0.29
	Combined	0	0.924	0.273 ± 0.27
Armatimonadota	Kānuka	0	0.0132	0.00189 ± 0.005
	Mānuka	0	0.161	0.0133 ± 0.04
	Combined	0	0.161	0.011 ± 0.036
Bacteroidota	Kānuka	0.993	8.75	6.32 ± 2.6
	Mānuka	0.461	12.7	5.58 ± 3.3
	Combined	0.461	12.7	5.73 ± 3.2
Bdellovibrionota	Kānuka	2.09	7.74	4.67 ± 2.3
	Mānuka	0	7.38	3.48 ± 1.9
	Combined	0	7.74	3.72 ± 2
Chloroflexi	Kānuka	0	0.00868	0.00124 ± 0.0033
	Mānuka	0	0	0 ± 0
	Combined	0	0.00868	0.000255 ± 0.0015
Cyanobacteria	Kānuka	0	0.0182	0.0026 ± 0.0069
	Mānuka	0	0	0 ± 0
	Combined	0	0.0182	0.000534 ± 0.0031
Deinococcota	Kānuka	0	0	0 ± 0
	Mānuka	0	0.00621	0.00023 ± 0.0012
	Combined	0	0.00621	0.000183 ± 0.0011
Dependentiae	Kānuka	0	0	0 ± 0
	Mānuka	0	0.0277	0.00103 ± 0.0053
	Combined	0	0.0277	0.000815 ± 0.0048
Firmicutes	Kānuka	0	1.03	0.366 ± 0.44
	Mānuka	0	0.768	0.153 ± 0.22
	Combined	0	1.03	0.197 ± 0.28
Myxococcota	Kānuka	0.35	3.17	1.32 ± 1
	Mānuka	0	4.68	1.25 ± 1.5
	Combined	0	4.68	1.27 ± 1.4
Planctomycetota	Kānuka	0	0.66	0.305 ± 0.25
	Mānuka	0	0.991	0.399 ± 0.27

	Combined	0	0.991	0.38 ± 0.27
Proteobacteria	Kānuka	66.6	82.8	75.2 ± 5.2
	Mānuka	58.3	92.1	77.1 ± 7.4
	Combined	58.3	92.1	76.7 ± 7
Synergistota	Kānuka	0	0.12	0.0172 ± 0.045
	Mānuka	0	0	0 ± 0
	Combined	0	0.12	0.00353 ± 0.021
Verrucomicrobiota	Kānuka	0.248	3.86	1.25 ± 1.3
	Mānuka	0	3.76	1.42 ± 1
	Combined	0	3.86	1.38 ± 1

Supplementary Table 2: Wilcoxon test on phylum relative abundance across plant species. Benjamini-Hochberg corrected p value.

Phylum	p.adjust
Abditibacteriota	0.631
Acidobacteriota	0.838
Actinobacteriota	0.886
Armatimonadota	0.939
Bacteroidota	0.788
Bdellovibrionota	0.788
Chloroflexi	0.313
Cyanobacteria	0.313
Deinococcota	0.815
Dependentiae	0.815
Firmicutes	0.801
Myxococcota	0.788
Planctomycetota	0.788
Proteobacteria	0.801
Synergistota	0.313
Verrucomicrobiota	0.788

Supplementary Table 3: PERMANOVA on community dissimilarities. Significant values are in bold. The residuals are presented for each independent PERMANOVA test.

Host species	Variable	Relative abundance		Presence/absence	
		R2	P value	R2	P value
Mānuka	Transect	**0.22**	**0.001**	**0.17**	**0.001**
	TreeID	**0.26**	**0.002**	**0.29**	**0.002**
	Residual	0.52		0.54	
Kānuka	TreeID + Transect	**0.39**	**0.05**	**0.43**	**0.05**
	Residual	0.61		0.57	
Total phyllosphere	Host species	**0.06**	**0.001**	**0.06**	**0.003**
	Transect	**0.15**	**0.001**	**0.13**	**0.001**
	Residual	0.78		0.82	
Soil	Transect	**0.20**	**0.02**	**0.19**	**0.008**
	Host species	0.10	0.2	0.09	0.623
	Residual	0.70		0.71	

Supplementary Table 4: ASV occurrence probabilities predicted for each microbiome occurrence.

Microbiome occurrence*	Probability an ASV is found at an occurrence, with zero truncation+
0	NA
1	12/4095 = ~0.0029
2	66/4095 = ~0.016
3	220/4095 = ~0.054
4	495/4095 = ~0.12
5	792/4095 = ~0.19
6	924/4095 = ~0.23
7	792/409 = ~0.19
8	495/4095 = ~0.12
9	220/4095 = ~0.054
10	66/4095 = ~0.016
11	12/4095 = ~0.0029
12	1/4095 = ~0.0002

* Microbiome occurrence represents the number of phyllosphere microbiomes in which an ASV is present within each transect out of a maximum of 12.

+ Probabilities are calculated by dividing the number of unique microbiome combinations an ASV could occupy per microbiome occurrence, by the total number of possible combinations.

The number of unique combinations

$$C(n,r) = \frac{\mathrm{n!}}{\mathrm{(n-r)!r!}}$$

Probabilities were zero truncated due to the inability to measure undetected microbial taxa.

Supplementary Table 5: Chi-square goodness of fit test results of the occurrence probabilities versus the observed number of ASVs

Transect	X2	Df	P value
Transect 1	118.78	11	$< 2.2x10^{-6}$
Transect 2	74.081	11	2.033e-11
Transect 3	103.19	11	$< 2.2x10^{-6}$

Supplementary Table 6: Pearson's product-moment correlations between surface soil community diversity and spatial descriptors.

Spatial descriptor	**Richness**		**Shannon**	
	cor	**P value**	**cor**	**P value**
Elevation	-0.029	0.929	0.011	0.972
Slope easting	-0.303	0.339	-0.316	0.316
Slope northing	0.121	0.709	-0.002	0.995

Supplementary Table 7: Minimum, maximum, and average relative abundance of phyla in surface soil microbial communities.

Phylum	**Transect**	**minimum**	**maximum**	**Average ± SD**
Abditibacteriota	P1	0	0.303	0.112 ± 0.14
	P2	0	0	0 ± 0
	P3	0	0.0559	0.0241 ± 0.029
	Combined	0	0.303	0.0453 ± 0.092
Acidobacteriota	P1	3.64	8.32	6.56 ± 2
	P2	5.52	15.1	9.15 ± 4.2
	P3	6.84	13	9.91 ± 3.2
	Combined	3.64	15.1	8.54 ± 3.3
Actinobacteriota	P1	5.69	12.3	8.14 ± 3.1
	P2	2.58	10.1	4.68 ± 3.6
	P3	3.27	11.9	6.52 ± 4
	Combined	2.58	12.3	6.45 ± 3.6
Armatimonadota	P1	0	0.304	0.076 ± 0.15
	P2	0	0.0538	0.0135 ± 0.027
	P3	0.026	0.567	0.357 ± 0.25
	Combined	0	0.567	0.149 ± 0.22
Bacteroidota	P1	9.37	23.1	16.4 ± 7.2
	P2	9.02	35.5	21 ± 11

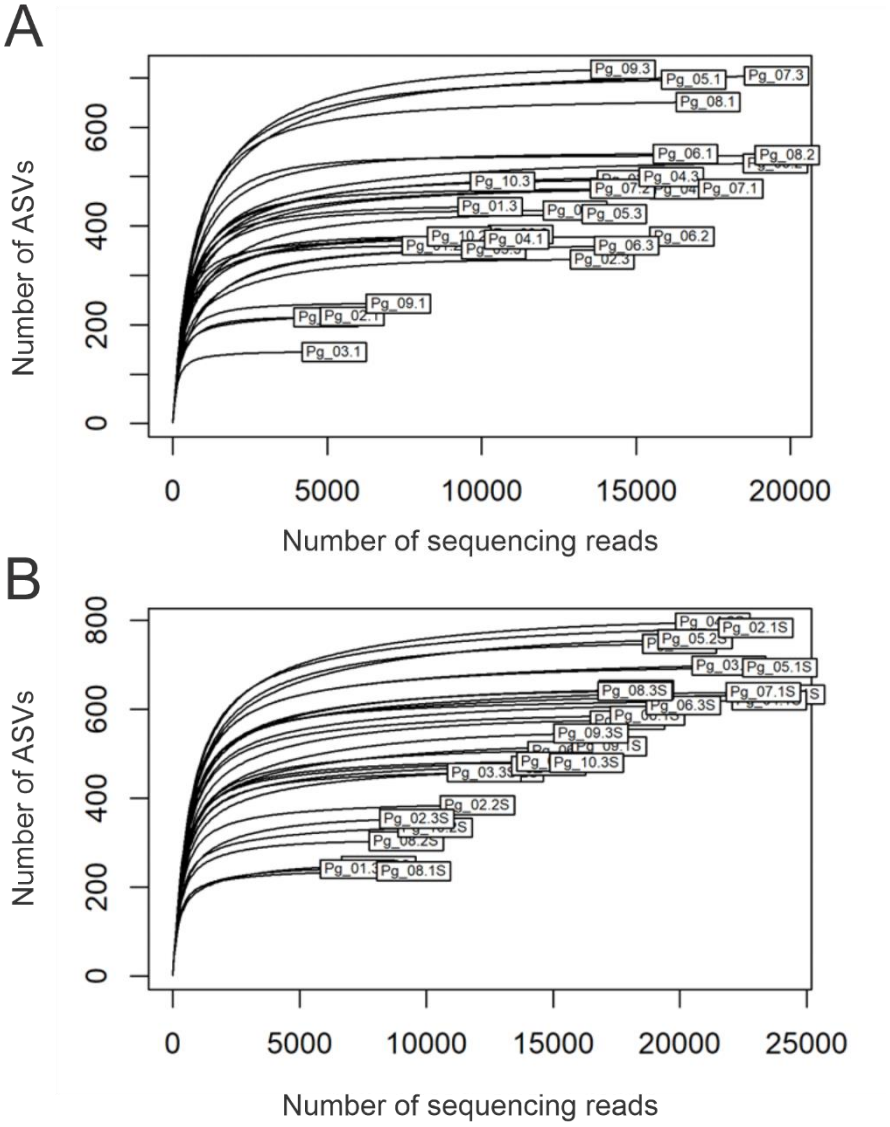
	P3	9.51	21.7	17 ± 5.3
	Combined	9.02	35.5	18.1 ± 7.7
Bdellovibrionota	P1	0	0.614	0.337 ± 0.26
	P2	0.164	1.52	0.801 ± 0.57
	P3	0	0.406	0.242 ± 0.19
	Combined	0	1.52	0.46 ± 0.43
Chloroflexi	P1	0.756	1.75	1.35 ± 0.43
	P2	0.35	1.24	0.709 ± 0.38
	P3	0.823	3.92	1.93 ± 1.4
	Combined	0.35	3.92	1.33 ± 0.94
Crenarchaeota	P1	0	0	0 ± 0
	P2	0	0	0 ± 0
	P3	0	0.177	0.0443 ± 0.089
	Combined	0	0.177	0.0148 ± 0.051
Cyanobacteria	P1	0	0.302	0.124 ± 0.15
	P2	0	0.556	0.179 ± 0.25
	P3	0	0.224	0.135 ± 0.097
	Combined	0	0.556	0.146 ± 0.16
Deinococcota	P1	0	0	0 ± 0
	P2	0	0.161	0.0404 ± 0.081
	P3	0	0	0 ± 0
	Combined	0	0.161	0.0135 ± 0.047
Dependentiae	P1	0	0.292	0.165 ± 0.15
	P2	0.102	0.356	0.241 ± 0.11
	P3	0	0.197	0.0994 ± 0.082
	Combined	0	0.356	0.168 ± 0.12
Desulfobacterota	P1	0	0.639	0.356 ± 0.31
	P2	0	1.31	0.609 ± 0.54
	P3	0	2.52	0.887 ± 1.1
	Combined	0	2.52	0.618 ± 0.7
Entotheonellaeota	P1	0	0	0 ± 0
	P2	0	0.102	0.0255 ± 0.051
	P3	0	0	0 ± 0
	Combined	0	0.102	0.00849 ± 0.029
Fibrobacterota	P1	0	0.253	0.0957 ± 0.12
	P2	0	0.143	0.0943 ± 0.064
	P3	0	0.286	0.0904 ± 0.14
	Combined	0	0.286	0.0935 ± 0.1
Firmicutes	P1	0	3.85	2.03 ± 1.7
	P2	0.502	3.52	1.75 ± 1.3
	P3	0.682	1.91	1.22 ± 0.57
	Combined	0	3.85	1.67 ± 1.2
Gemmatimonadota	P1	0	0.841	0.29 ± 0.38
	P2	0	0.425	0.116 ± 0.21

	P3	0.0269	0.639	0.401 ± 0.27
	Combined	0	0.841	0.269 ± 0.29
Myxococcota	P1	3.43	5.77	4.61 ± 0.99
	P2	1.18	5.55	3.74 ± 1.8
	P3	1.18	5.12	3.55 ± 1.7
	Combined	1.18	5.77	3.97 ± 1.5
Nitrospirota	P1	0	0	0 ± 0
	P2	0	0.106	0.0445 ± 0.053
	P3	0	0	0 ± 0
	Combined	0	0.106	0.0148 ± 0.035
Patescibacteria	P1	0	0.0124	0.0031 ± 0.0062
	P2	0	0	0 ± 0
	P3	0	0.0573	0.0183 ± 0.027
	Combined	0	0.0573	0.00714 ± 0.017
Planctomycetota	P1	3.26	9.37	6.23 ± 2.8
	P2	2.95	5.45	4.16 ± 1.1
	P3	2.86	8.01	5.38 ± 2.1
	Combined	2.86	9.37	5.26 ± 2.1
Proteobacteria	P1	6.6	31.4	24.1 ± 12
	P2	28.4	34.6	32 ± 2.7
	P3	21	29	26 ± 3.5
	Combined	6.6	34.6	27.4 ± 7.4
Spirochaetota	P1	0	0	0 ± 0
	P2	0	0.164	0.0682 ± 0.082
	P3	0	0.0469	0.0117 ± 0.023
	Combined	0	0.164	0.0266 ± 0.054
Sumerlaeota	P1	0	0.0426	0.0107 ± 0.021
	P2	0	0.0399	0.00997 ± 0.02
	P3	0	0.0156	0.0039 ± 0.0078
	Combined	0	0.0426	0.00818 ± 0.016
Verrucomicrobiota	P1	14.2	57	29.1 ± 20
	P2	12.9	31.7	20.5 ± 8.6
	P3	20.3	35.1	26.1 ± 6.7
	Combined	12.9	57	25.3 ± 12

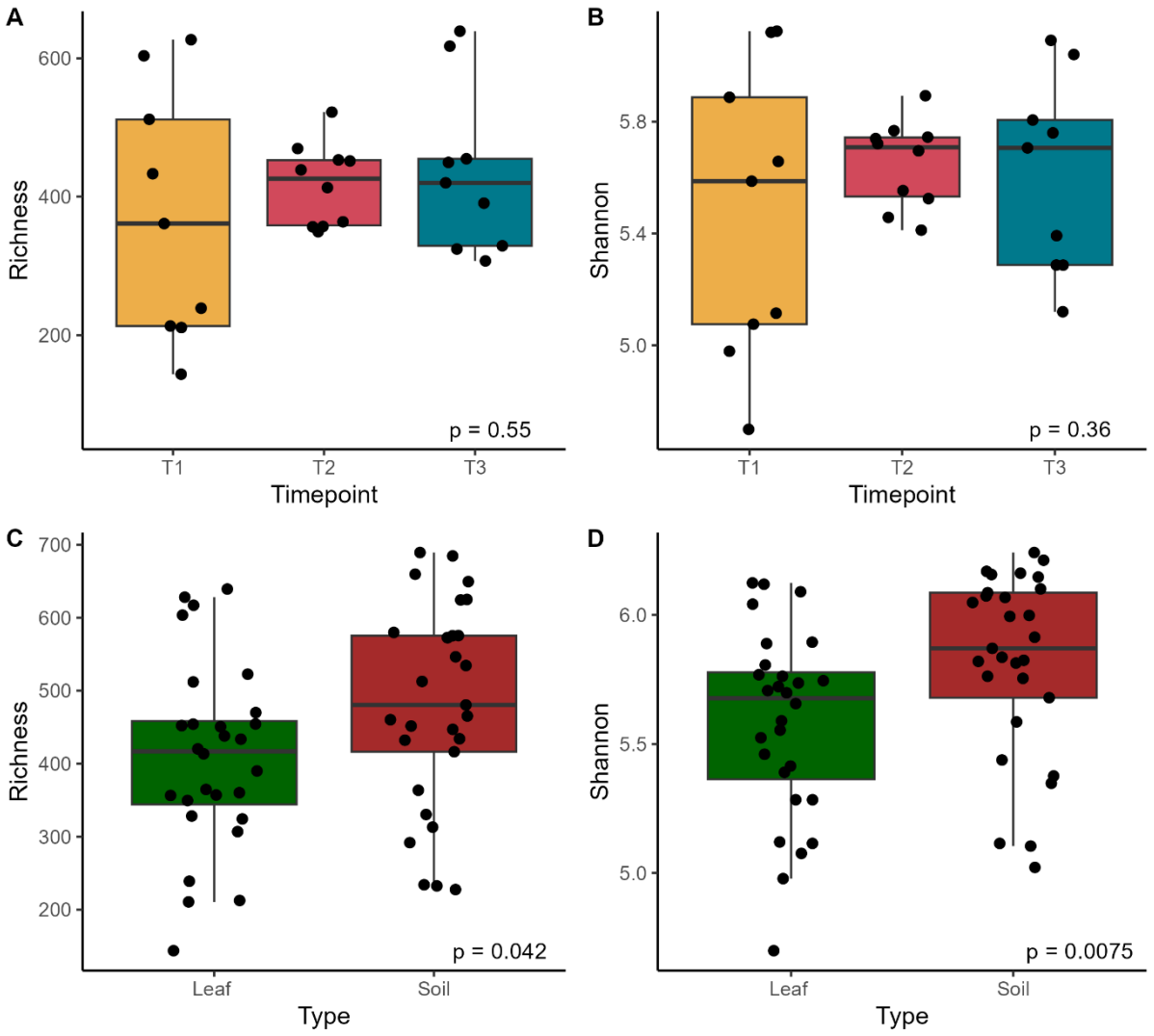
Appendix C

Supplementary Material for Chapter IV

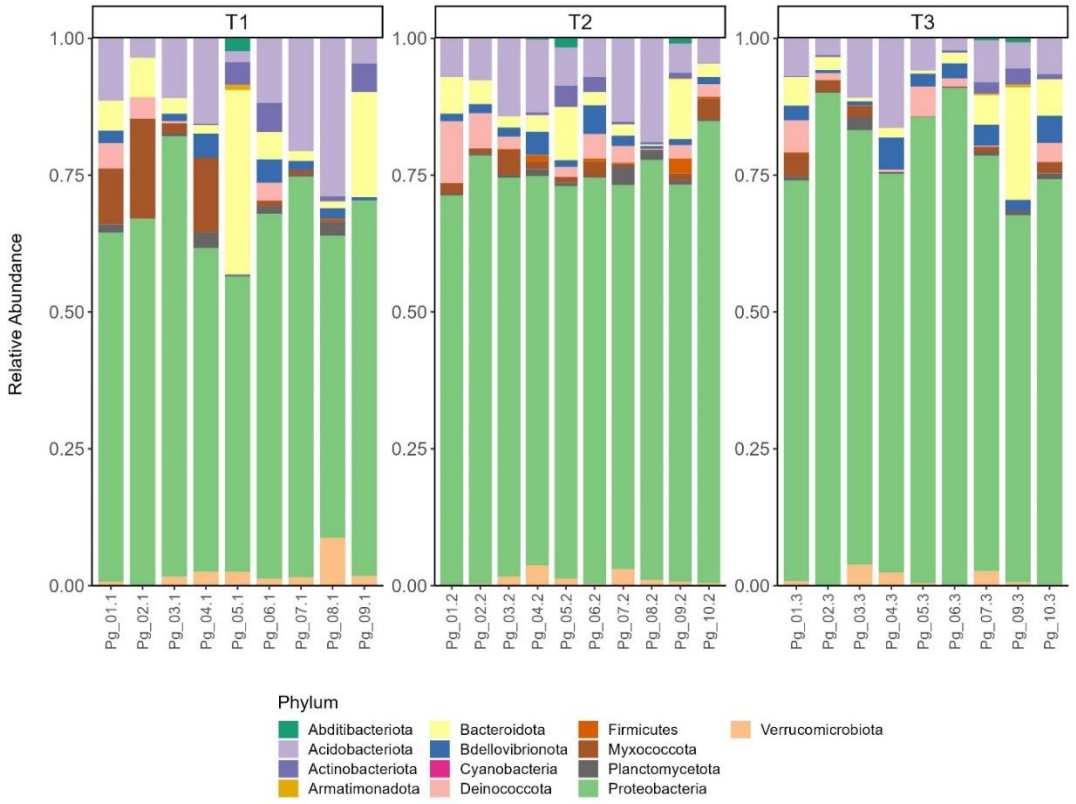
Supplementary Figures and Tables



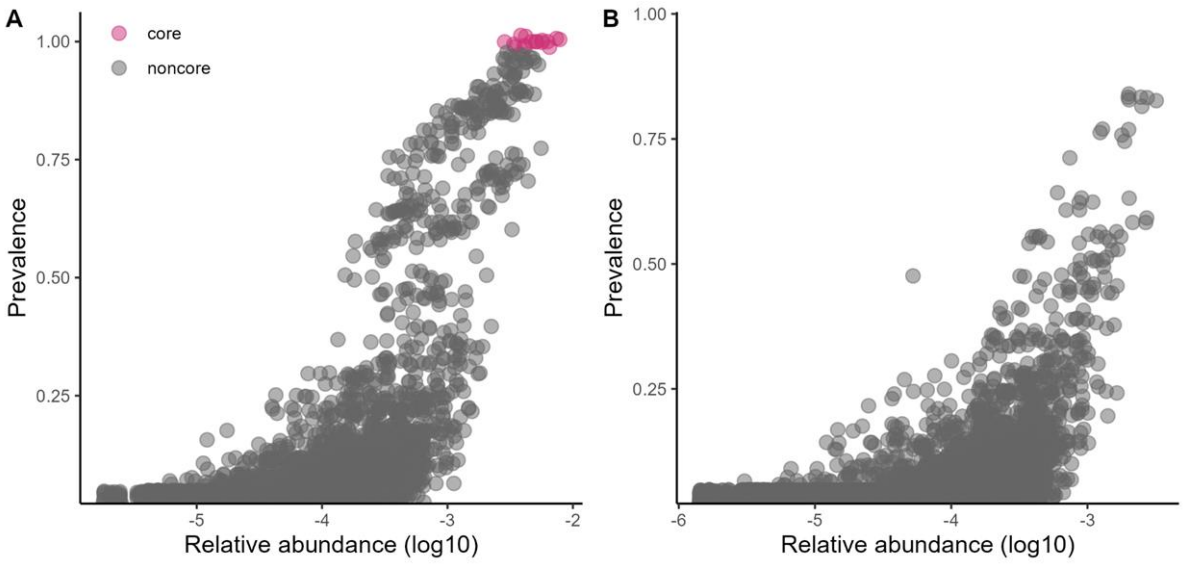
Supplementary Fig. 1: Rarefaction curves showing the relationship between the number of ASVs detected and the number of sequencing reads for **A)** phyllosphere and **B)** surface soil samples.



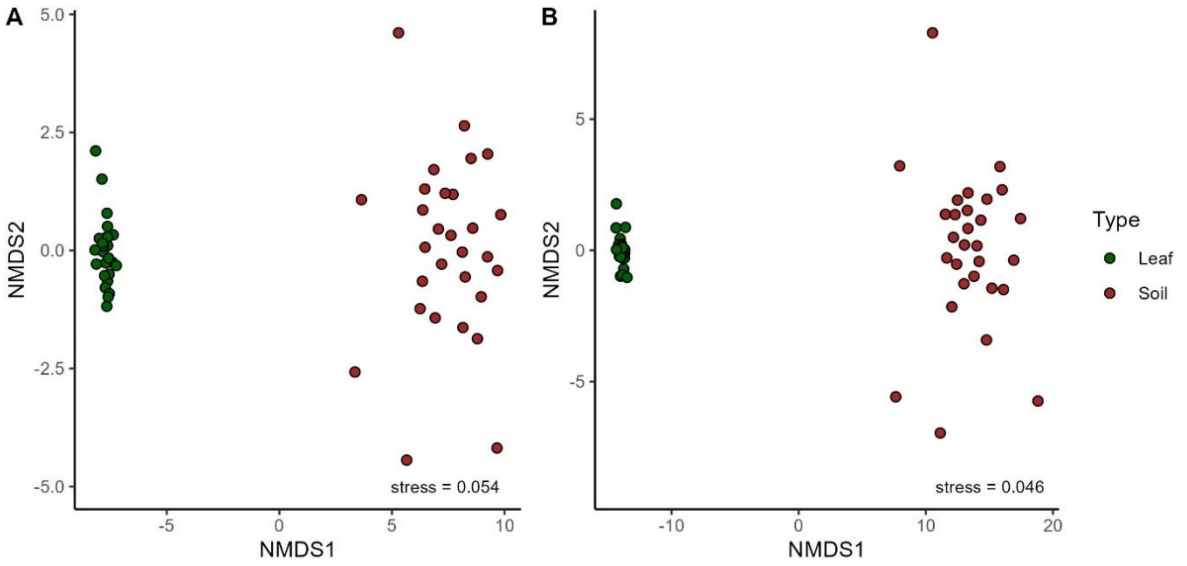
Supplementary Fig. 2: Alpha diversity (Shannon and richness) of **A-B)** the phyllosphere microbiome across timepoints and **C-D)** the phyllosphere versus surface soil microbiome. Each sample was subsampled (100x) to an even sequencing depth. Boxes indicate the interquartile range and the thick bar represents the median. Vertical segments extend to the fifth and 95th percentiles of the distribution of values.



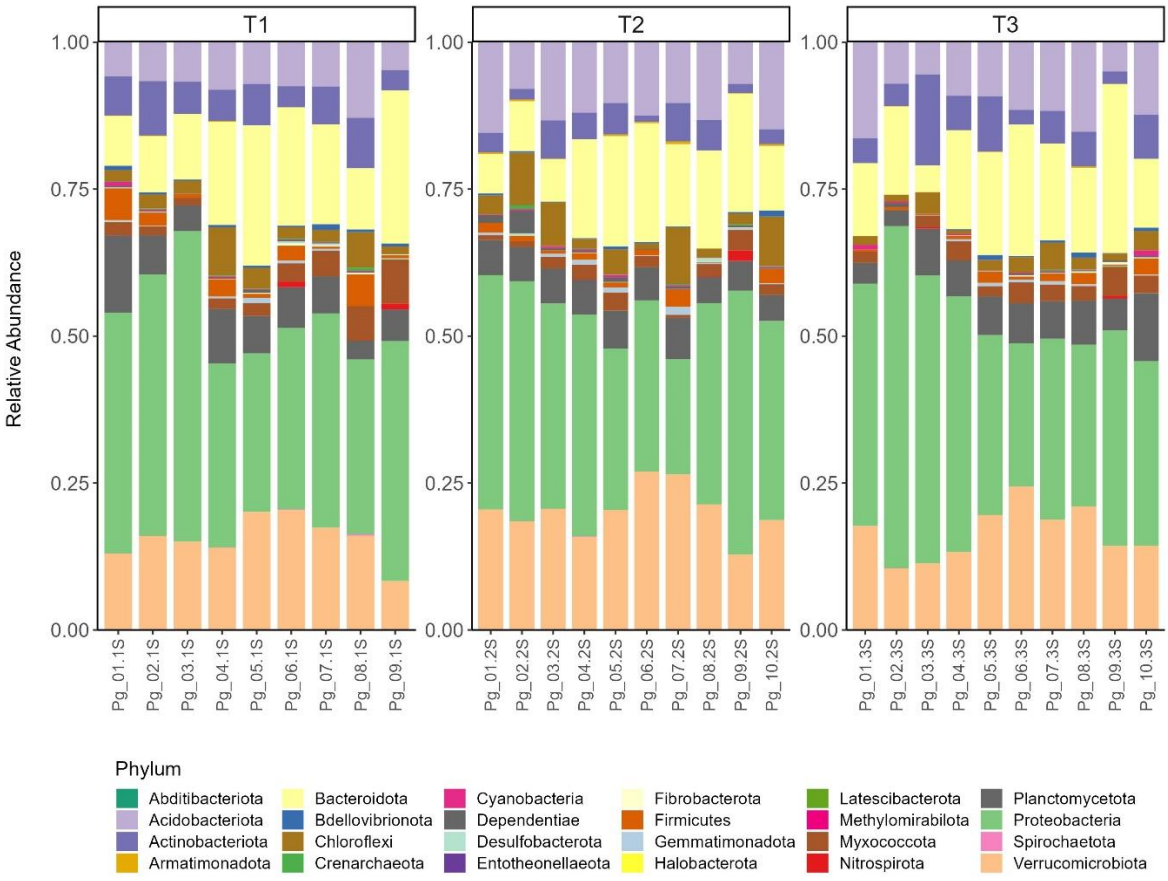
Supplementary Fig. 3 Relative abundance of phyla in the mānuka phyllosphere microbiome across timepoints. Colour represents taxonomic classification at the phylum level.



Supplementary Fig. 4: The relative abundance of taxa identified at 100% prevalence in **A)** mānuka phyllosphere communities and **B)** surface soil communities across all timepoints (T1-T3). Relative abundance is log10. Pink represents ASVs identified at 100% prevalence.



Supplementary Fig. 5: Overall community dissimilarity of phyllosphere and surface soil microbial communities using **A)** Bray-Curtis (relative abundance based distance) and **B)** Jaccard (presence/absence based distance). Colour represents sample type.



Supplementary Fig. 6: Relative abundance of taxa in surface soil microbial communities across timepoints (T1 – T3). Colour represents taxonomic classification at the phylum level.

Supplementary Table 1: Minimum, maximum, and average relative abundance of phyla in mānuka phyllosphere communities within and across timepoints.

Phylum	Plant species	Minimum	Maximum	Average ± SD
Abditibacteriota	T1	0	2.31	0.257 ± 0.77
	T2	0	1.67	0.287 ± 0.57
	T3	0	0.734	0.122 ± 0.26
	Combined	0	2.31	0.224 ± 0.56
Acidobacteriota	T1	2.06	28.9	12.2 ± 8.6
	T2	4.67	19	10.1 ± 4.9
	T3	2.18	16.3	7.12 ± 4.3
	Combined	2.06	28.9	9.79 ± 6.3
Actinobacteriota	T1	0	5.35	1.76 ± 2.4
	T2	0	3.88	0.906 ± 1.3
	T3	0	2.96	0.778 ± 1.1
	Combined	0	5.35	1.14 ± 1.7
Armatimonadota	T1	0	0.972	0.108 ± 0.32
	T2	0	0.13	0.013 ± 0.041
	T3	0	0.466	0.0802 ± 0.17
	Combined	0	0.972	0.0652 ± 0.2
Bacteroidota	T1	1.25	33.7	8.66 ± 11
	T2	0.291	11	4.39 ± 3.6
	T3	0.56	20.6	5.02 ± 6.2
	Combined	0.291	33.7	5.97 ± 7.4
Bdellovibrionota	T1	0	4.39	1.85 ± 1.6
	T2	0.548	5.27	2.03 ± 1.5
	T3	0.554	5.9	2.85 ± 1.8
	Combined	0	5.9	2.23 ± 1.6
Cyanobacteria	T1	0	0	0 ± 0
	T2	0	0	0 ± 0
	T3	0	0.0216	0.00448 ± 0.0089
	Combined	0	0.0216	0.00144 ± 0.0053
Deinococcota	T1	0	4.61	1.36 ± 2
	T2	0.0364	11.3	3.43 ± 3.3
	T3	0	5.85	2.02 ± 2.4
	Combined	0	11.3	2.31 ± 2.7
Firmicutes	T1	0	0.271	0.0301 ± 0.09
	T2	0	2.73	0.509 ± 0.86
	T3	0	0.185	0.0342 ± 0.064
	Combined	0	2.73	0.202 ± 0.55
Myxococcota	T1	0	18.3	5.11 ± 7
	T2	0.226	4.61	1.86 ± 1.4
	T3	0	4.25	1.31 ± 1.4

	Combined	0	18.3	2.73 ± 4.3
Planctomycetota	T1	0	2.86	1.06 ± 1.1
	T2	0.17	3.37	0.923 ± 0.98
	T3	0	2.35	0.696 ± 0.72
	Combined	0	3.37	0.893 ± 0.92
Proteobacteria	T1	54	80.5	65.4 ± 8.5
	T2	70.2	84.4	74.3 ± 4.4
	T3	67.1	90.9	78.7 ± 8.3
	Combined	54	90.9	72.9 ± 8.9
Verrucomicrobiota	T1	0	8.73	2.29 ± 2.5
	T2	0	3.67	1.25 ± 1.2
	T3	0	3.84	1.23 ± 1.4
	Combined	0	8.73	1.58 ± 1.8

Supplementary Table 2: Wilcoxon test on phylum relative abundance in the mānuka phyllosphere microbiome across timepoints. Benjamini-Hochberg corrected p value.

Phylum	p.adjust
Abditibacteriota	0.876
Acidobacteriota	0.652
Actinobacteriota	0.958
Armatimonadota	0.876
Bacteroidota	0.876
Bdellovibrionota	0.652
Cyanobacteria	0.485
Deinococcota	0.508
Firmicutes	0.336
Myxococcota	0.876
Planctomycetota	0.896
Proteobacteria	0.0656
Verrucomicrobiota	0.774

Supplementary Table 3: Relative abundance and occurrence of ASVs belonging to common genera of the honeybee gut microbiome

Timepoint	Sample	Abundance	Phylum	Genus	OTU
T1	Pg_08.1	0.0006	Firmicutes	*Lactobacillus*	ASV_3828
T2	Pg_04.2	0.0047	Gammaproteobacteria	*Snodgrassella*	ASV_2783
		0.011	Firmicutes	*Lactobacillus*	ASV_3828
	Pg_06.2	0.0067	Gammaproteobacteria	*Snodgrassella*	ASV_2783
		0.0058	Firmicutes	*Lactobacillus*	ASV_3828
	Pg_09.2	0.0107	Gammaproteobacteria	*Frischella*	ASV_10261
		0.016	Gammaproteobacteria	*Snodgrassella*	ASV_2783
		0.0273	Firmicutes	*Lactobacillus*	ASV_3828
		0.0064	Gammaproteobacteria	*Gilliamella*	ASV_4123
T3	Pg_07.3	0.0087	Gammaproteobacteria	*Snodgrassella*	ASV_2783
		0.0012	Gammaproteobacteria	*Gilliamella*	ASV_4123

Supplementary Table 4: PERMANOVA on community dissimilarities. Significant values are in bold.

Sample type	Variable	Relative abundance		Presence/absence	
		R^2	P value	R^2	P value
Phyllosphere	Time	0.068	0.602	0.079	0.312
	TreeID	**0.524**	**0.001**	**0.467**	**0.001**
Soil	Time	**0.354**	**0.001**	**0.344**	**0.001**
	TreeID	**0.081**	**0.003**	**0.078**	**0.002**

Supplementary Table 5: Minimum, maximum, and average relative abundance of phyla in surface soil microbial communities within and across all timepoints.

Phylum	Plant species	Minimum	Maximum	Average ± SD
Abditibacteriota	T1	0	0	0 ± 0
	T2	0	0.03	0.01 ± 0.01
	T3	0	0	0 ± 0
	Combined	0	0.03	0.01 ± 0.01
Acidobacteriota	T1	4.74	12.90	7.44 ± 2.30
	T2	7.07	15.40	11.70 ± 2.80
	T3	4.97	16.40	10.30 ± 3.80
	Combined	4.74	16.40	9.89 ± 3.40
Actinobacteriota	T1	3.45	9.26	6.20 ± 2.00
	T2	1.09	6.55	3.85 ± 2.10
	T3	2.12	15.50	6.24 ± 3.90
	Combined	1.09	15.50	5.40 ± 3.00
Armatimonadota	T1	0	0.15	0.04 ± 0.06
	T2	0	0.45	0.20 ± 0.14
	T3	0	0.21	0.04 ± 0.07
	Combined	0	0.45	0.09 ± 0.12
Bacteroidota	T1	8.49	26.10	16.00 ± 6.50
	T2	6.73	20.20	14.00 ± 5.30
	T3	4.56	28.80	16.00 ± 6.40
	Combined	4.56	28.80	15.30 ± 5.90
Bdellovibrionota	T1	0.21	0.96	0.47 ± 0.24
	T2	0	1.02	0.30 ± 0.29
	T3	0	0.88	0.313 ± 0.34
	Combined	0	1.02	0.36 ± 0.29
Chloroflexi	T1	1.31	8.20	3.27 ± 2.30
	T2	0.89	9.61	4.78 ± 3.50
	T3	0.63	4.61	2.25 ± 1.30
	Combined	0.63	9.61	3.44 ± 2.70
Crenarchaeota	T1	0	0.57	0.16 ± 0.17
	T2	0	0.67	0.14 ± 0.20
	T3	0	0.16	0.02 ± 0.05
	Combined	0	0.67	0.11 ± 0.16
Cyanobacteria	T1	0	0.73	0.19 ± 0.22
	T2	0	0.40	0.17 ± 0.13
	T3	0	0.96	0.26 ± 0.33
	Combined	0	0.96	0.20 ± 0.24
Dependentiae	T1	0.05	0.47	0.17 ± 0.13
	T2	0	3.85	0.80 ± 1.10
	T3	0.03	0.70	0.21 ± 0.20

	Combined	0	3.85	0.40 ± 0.72
Desulfobacterota	T1	0	0.35	0.10 ± 0.14
	T2	0	0.66	0.11 ± 0.23
	T3	0	0.20	0.03 ± 0.074
	Combined	0	0.66	0.08 ± 0.16
Entotheonellaeota	T1	0	0	0 ± 0
	T2	0	0.07	0.01 ± 0.02
	T3	0	0	0 ± 0
	Combined	0	0.07	0.01 ± 0.01
Fibrobacterota	T1	0	0.40	0.15 ± 0.14
	T2	0	0.10	0.03 ± 0.04
	T3	0	0.41	0.10 ± 0.13
	Combined	0	0.41	0.09 ± 0.12
Firmicutes	T1	0.37	5.41	2.30 ± 2
	T2	0	2.99	1.18 ± 0.96
	T3	0.17	2.78	1.02 ± 0.90
	Combined	0	5.41	1.47 ± 1.40
Gemmatimonadota	T1	0	0.93	0.29 ± 0.29
	T2	0	1.37	0.48 ± 0.42
	T3	0	0.57	0.26 ± 0.23
	Combined	0	1.37	0.35 ± 0.33
Halobacterota	T1	0	0.03	0.01 ± 0.011
	T2	0	0	0 ± 0
	T3	0	0	0 ± 0
	Combined	0	0.03	0.01 ± 0.01
Latescibacterota	T1	0	0.09	0.01 ± 0.03
	T2	0	0	0 ± 0
	T3	0	0.11	0.01 ± 0.04
	Combined	0	0.11	0.01 ± 0.03
Methylomirabilota	T1	0	0.01	0.001 ± 0.01
	T2	0	0	0 ± 0
	T3	0	0	0 ± 0
	Combined	0	0.013	0.01 ± 0.01
Myxococcota	T1	1.32	7.43	3.31 ± 2.10
	T2	0.49	3.50	1.93 ± 0.97
	T3	0.09	4.94	2.58 ± 1.30
	Combined	0.09	7.43	2.58 ± 1.60
Nitrospirota	T1	0	1.03	0.23 ± 0.44
	T2	0	1.76	0.19 ± 0.55
	T3	0	0.49	0.07 ± 0.16
	Combined	0	1.76	0.16 ± 0.41
Planctomycetota	T1	3.17	13.10	6.83 ± 2.90
	T2	4.41	7.05	5.66 ± 0.83
	T3	2.66	11.60	6.45 ± 2.40

	Combined	2.66	13.10	6.29 ± 2.20
Proteobacteria	T1	27.00	52.80	37.20 ± 8.4
	T2	19.60	44.90	34.30 ± 7.40
	T3	24.40	58.20	37.30 ± 11.00
	Combined	19.60	58.20	36.20 ± 8.70
Spirochaetota	T1	0	0.24	0.04 ± 0.08
	T2	0	0.12	0.01 ± 0.04
	T3	0	0.031	0.01 ± 0.011
	Combined	0	0.24	0.02 ± 0.05
Verrucomicrobiota	T1	8.31	20.40	15.60 ± 3.70
	T2	12.80	26.90	20.20 ± 4.30
	T3	10.50	24.40	16.50 ± 4.50
	Combined	8.31	26.90	17.50 ± 4.50

Supplementary Table 6: Kruskal-Wallis test on the relative abundance of surface soil phyla across timepoints. Benjamini-Hochberg corrected p value.

Phylum	p.adjust
Abditibacteriota	0.27
Acidobacteriota	0.22
Actinobacteriota	0.27
Armatimonadota	0.16
Bacteroidota	0.87
Bdellovibrionota	0.50
Chloroflexi	0.56
Crenarchaeota	0.22
Cyanobacteria	0.95
Dependentiae	0.27
Desulfobacterota	0.62
Entotheonellaeota	0.57
Fibrobacterota	0.56
Firmicutes	0.56
Gemmatimonadota	0.62
Halobacterota	0.56
Latescibacterota	0.56
Methylomirabilota	0.56
Myxococcota	0.57
Nitrospirota	0.95
Planctomycetota	0.57
Proteobacteria	0.91
Spirochaetota	0.57
Verrucomicrobiota	0.27

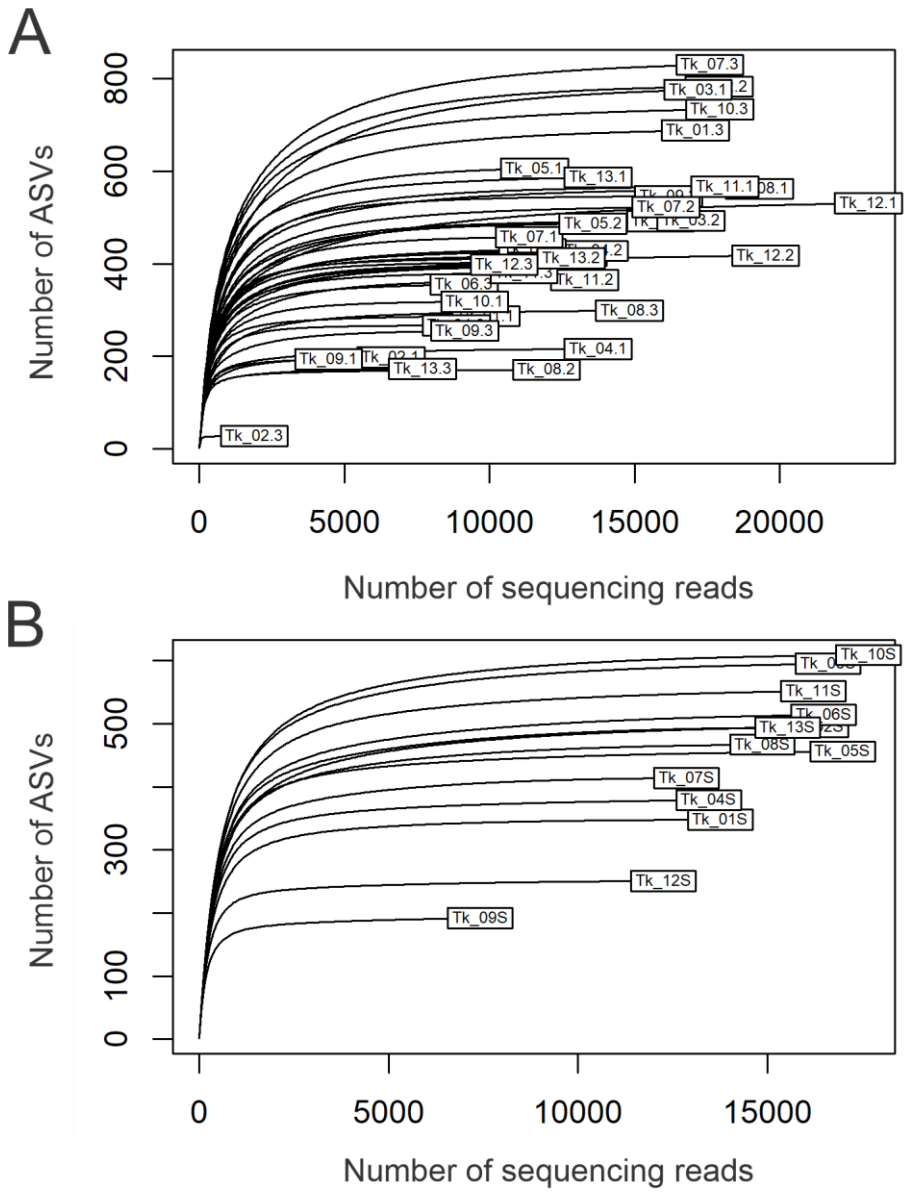
Supplementary Table 7: Kruskal-Wallis test on the relative abundance of phyla in phyllosphere and surface soil communities. Benjamini-Hochberg corrected p value.

Phylum	p.adjust
Actinobacteriota	1.09E-07
Armatimonadota	0.00317
Bacteroidota	1.76E-06
Bdellovibrionota	2.10E-07
Chloroflexi	1.36E-10
Crenarchaeota	2.82E-05
Cyanobacteria	9.83E-08
Deinococcota	7.22E-08
Dependentiae	2.50E-10
Desulfobacterota	0.00472
Fibrobacterota	5.36E-06
Firmicutes	2.10E-07
Gemmatimonadota	1.02E-08
Myxococcota	0.049
Nitrospirota	0.00267
Planctomycetota	6.58E-10
Proteobacteria	6.58E-10
Spirochaetota	0.0171
Verrucomicrobiota	6.58E-10

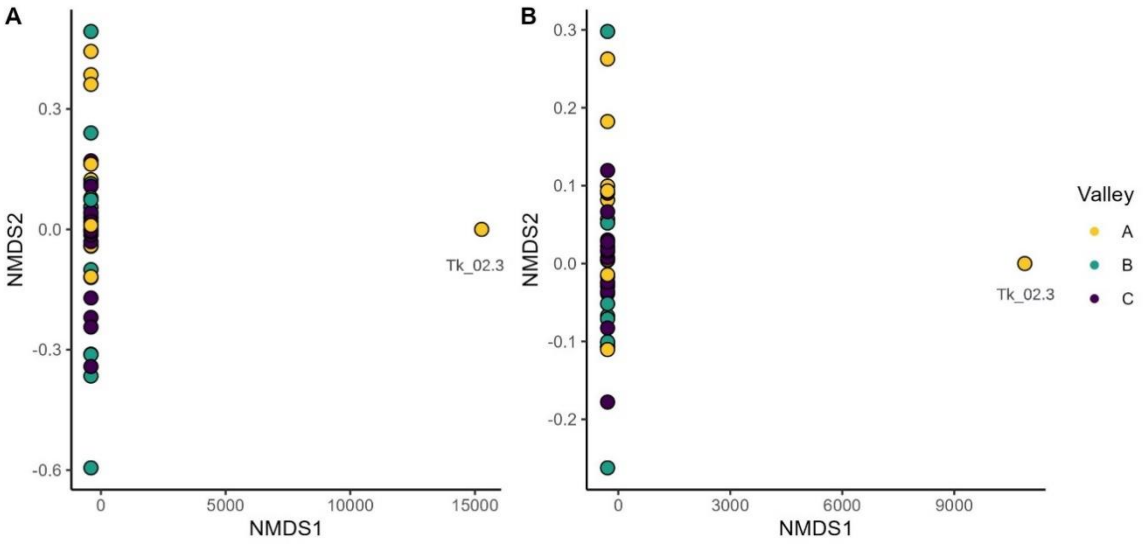
Appendix D

Supplementary Material for Chapter V

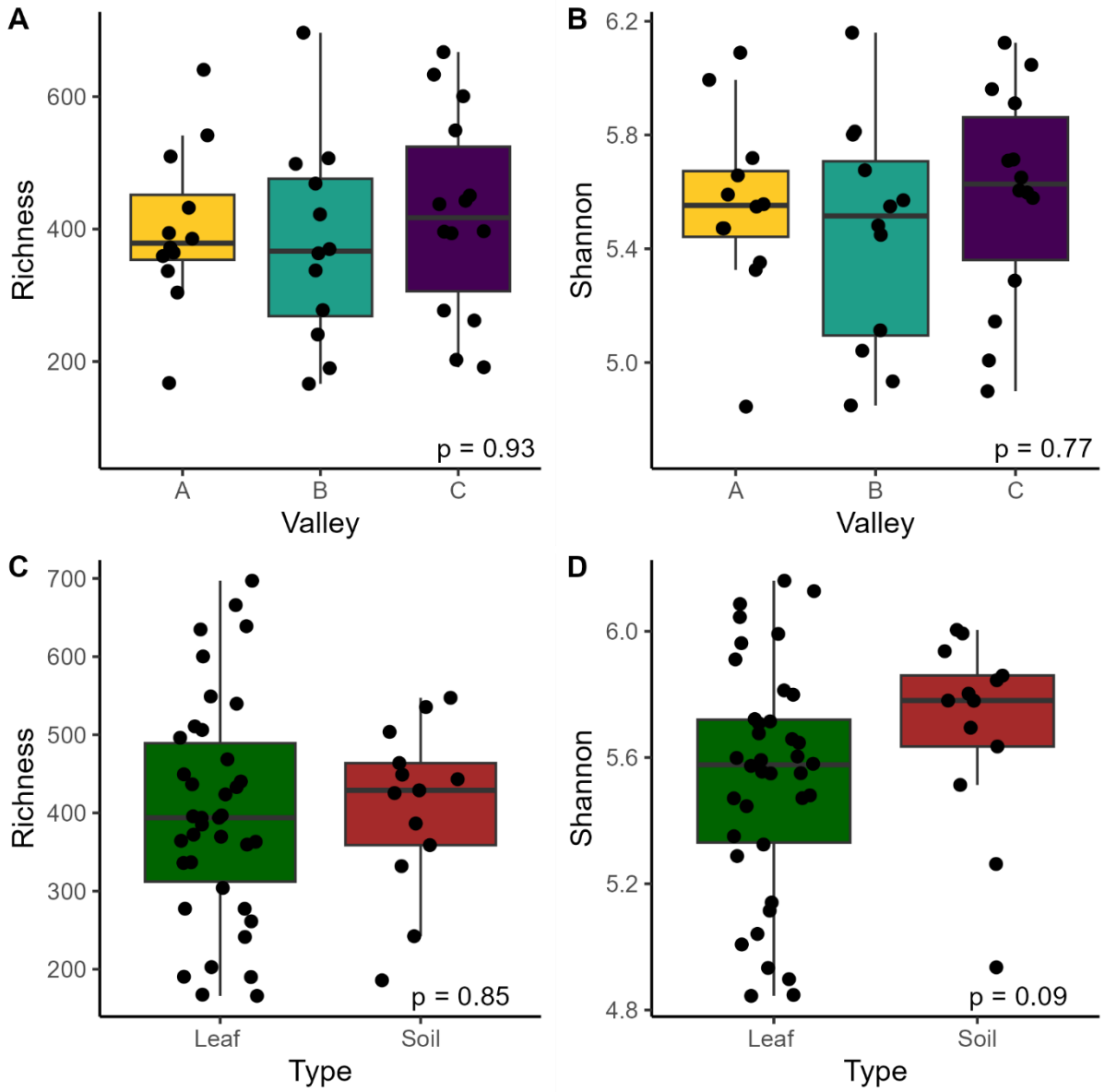
Supplementary Figures and Tables



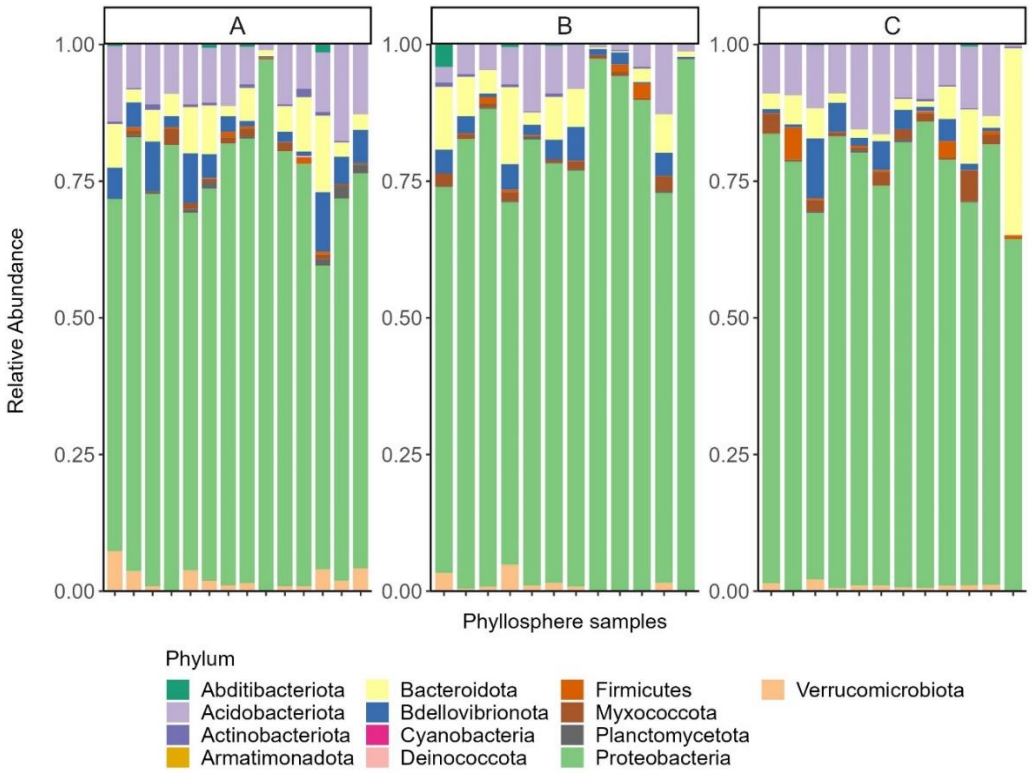
Supplementary Fig. 1: Rarefaction curves showing the relationship between the number of ASVs detected and the number of sequencing reads for **A)** phyllosphere and **B)** surface soil samples. A disproportionally reduced number of reads is visible in sample Tk_02.3.



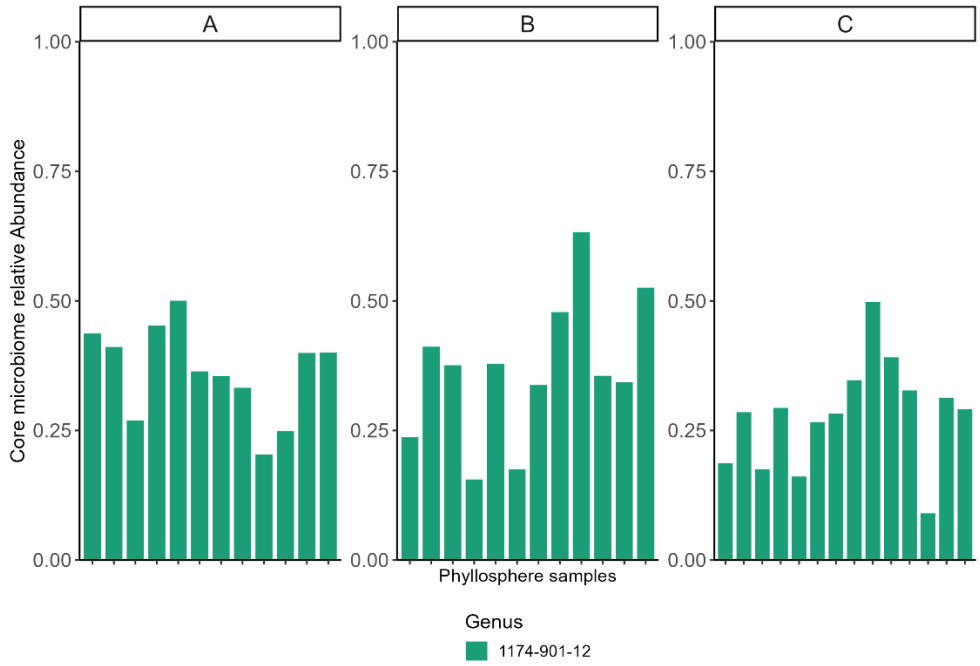
Supplementary Fig. 2: Phyllosphere community dissimilarity demonstrating outlier Tk_02.3 with **A)** Bray-Curtis and **B)** Jaccard. Colour represents valley from which each phyllosphere sample was collected.



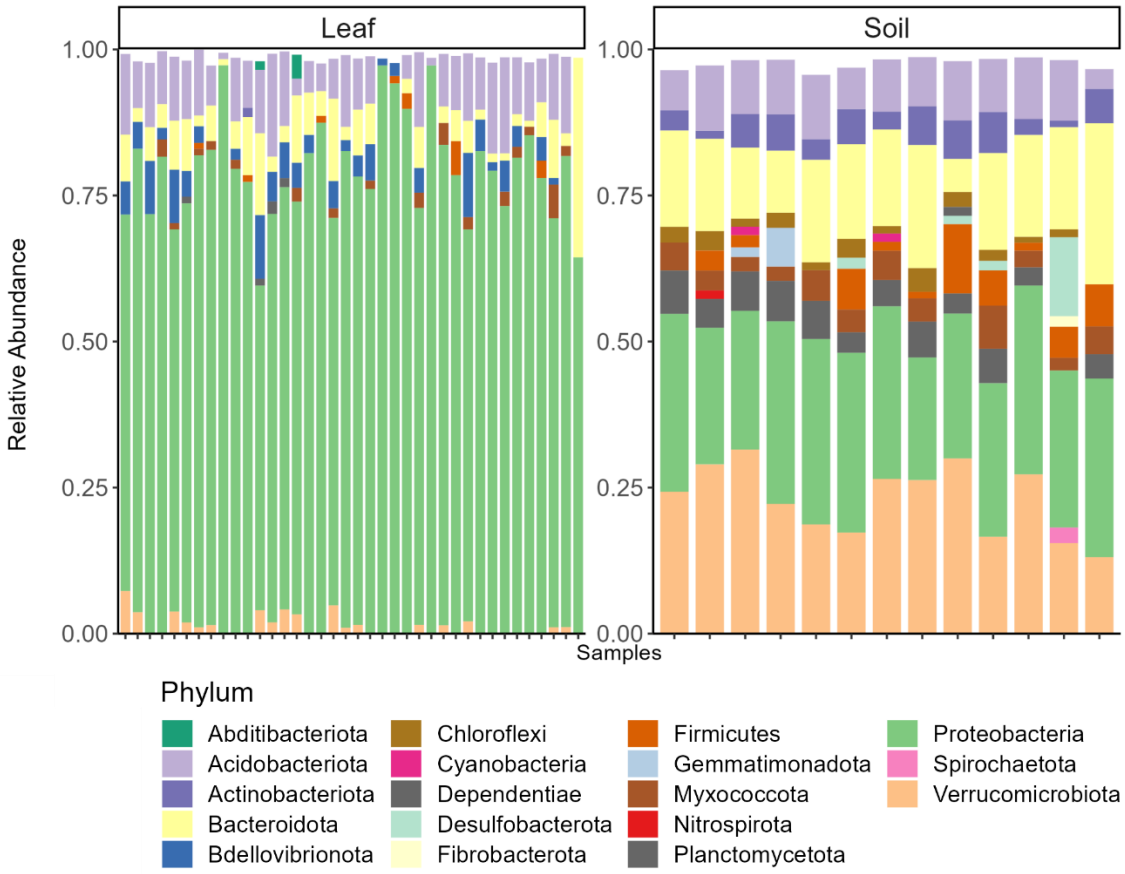
Supplementary Fig. 3: Alpha diversity (Shannon and richness) of **A-B)** the phyllosphere microbiome across valleys and **C-D)** the phyllosphere versus surface soil microbiome. Each sample was subsampled (100x) to an even sequencing depth. Boxes indicate the interquartile range and the thick bar represents the median. Vertical segments extend to the fifth and 95th percentiles of the distribution of values.



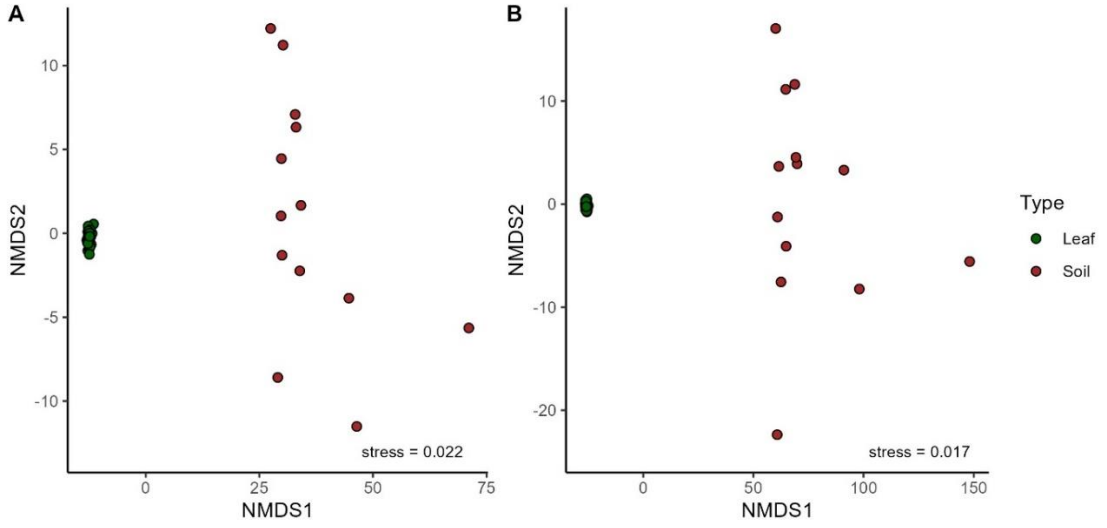
Supplementary Fig. 4: Relative abundance of taxa in phyllosphere microbial communities across valleys. Colour represents taxonomic classification at the phylum level.



Supplementary Fig. 5: The relative abundance of the mānuka phyllosphere core microbiome across valleys. Colour presents taxonomic assignment of core taxa (54 ASVs) at the genus level.



Supplementary Fig. 6: Relative abundance of taxa (> 0.01) in phyllosphere and surface soil microbial communities across valleys. Colour represents taxonomic classification at the phylum level.



Supplementary Fig. 7: Community dissimilarity of phyllosphere and surface soil bacterial communities. Colour represents sample type.

Supplementary Table 1: Pearson's product-moment correlations between community diversity (Shannon and richness) and phyllosphere and surface soil microbiome spatial descriptors. Bold indicates significant values ($p < 0.05$).

Spatial descriptor	Sample type	Richness		Shannon	
		corr	P value	corr	P value
Elevation	Leaf	-0.12	0.47	-0.10	0.54
	Soil	0.10	0.75	-0.01	0.97
Slope easting	Leaf	0.03	0.86	0.01	0.93
	Soil	0.30	0.32	0.35	0.24
Slope northing	Leaf	0.17	0.30	0.20	0.22
	Soil	-0.13	0.67	-0.10	0.76
Branch easting	Leaf	0.20	0.24	0.17	0.31
	Soil				
Branch northing	Leaf	0.13	0.45	0.12	0.46
	Soil				
Tree height	Leaf	**0.37**	**0.02**	**0.37**	**0.02**
	Soil	0.26	0.38	0.31	0.30
Branch height	Leaf	0.14	0.39	0.15	0.36
	Soil				
Tree diameter	Leaf	0.22	0.19	0.27	0.10
	Soil	0.10	0.76	0.13	0.67

Supplementary Table 2: Minimum, maximum, and average relative abundance of phyla in mānuka phyllosphere microbial communities within and across all valleys.

Phylum	Valley	minimum	maximum	Average_SD
Abditibacteriota	A	0	1.47	0.214 ± 0.41
	B	0	4.1	0.401 ± 1.2
	C	0	0.386	0.0414 ± 0.11
	Combined	0	4.1	0.218 ± 0.7
Acidobacteriota	A	1.08	17.6	10.2 ± 3.7
	B	0.401	12.8	5.71 ± 4.2
	C	0.403	16.4	10.2 ± 4.1
	Combined	0.401	17.6	8.78 ± 4.4
Actinobacteriota	A	0	1.56	0.436 ± 0.44
	B	0	0.758	0.268 ± 0.27
	C	0	0.379	0.111 ± 0.14
	Combined	0	1.56	0.28 ± 0.34
Armatimonadota	A	0	0.0726	0.00519 ± 0.019
	B	0	0	0 ± 0
	C	0	0	0 ± 0
	Combined	0	0.0726	0.00191 ± 0.012
Bacteroidota	A	1.07	14	5.75 ± 3.7
	B	0.209	14.1	5.42 ± 4.5
	C	1.02	34.2	6.11 ± 9.2
	Combined	0.209	34.2	5.76 ± 6
Bdellovibrionota	A	0.218	10.9	4.55 ± 3.4
	B	0.247	6.2	2.73 ± 1.9
	C	0	11	2.88 ± 3.2
	Combined	0	11	3.45 ± 3
Cyanobacteria	A	0	0.0147	0.00105 ± 0.0039
	B	0	0.0472	0.00394 ± 0.014
	C	0	0.065	0.00542 ± 0.019
	Combined	0	0.065	0.00334 ± 0.013
Deinococcota	A	0	0.282	0.0202 ± 0.075
	B	0	0	0 ± 0
	C	0	0.0615	0.00513 ± 0.018
	Combined	0	0.282	0.00905 ± 0.047
Firmicutes	A	0	1.17	0.312 ± 0.38
	B	0	2.67	0.513 ± 0.81
	C	0	5.82	0.978 ± 1.7
	Combined	0	5.82	0.586 ± 1.1
Myxococcota	A	0	2.95	0.832 ± 0.8
	B	0	2.61	1.02 ± 0.83
	C	0	5.74	1.74 ± 1.7
	Combined	0	5.74	1.18 ± 1.2
Planctomycetota	A	0	2.15	0.553 ± 0.7

	B	0	0.498	0.158 ± 0.16
	C	0	0.494	0.133 ± 0.17
	Combined	0	2.15	0.296 ± 0.48
Proteobacteria	A	55.6	97.3	74.9 ± 10
	B	66.3	97.3	82.6 ± 11
	C	64.4	85.3	76.9 ± 6.7
	Combined	55.6	97.3	77.9 ± 9.6
Verrucomicrobiota	A	0	7.31	2.29 ± 2
	B	0	4.85	1.2 ± 1.5
	C	0	2.1	0.884 ± 0.57
	Combined	0	7.31	1.5 ± 1.6

Supplementary Table 3: Kruskal-Wallis test on the relative abundance of phyllosphere phyla across valleys. Benjamini-Hochberg corrected p value.

Phylum	p.adjust
Abditibacteriota	0.44
Acidobacteriota	0.24
Actinobacteriota	0.44
Armatimonadota	0.61
Bacteroidota	0.64
Bdellovibrionota	0.44
Cyanobacteria	0.98
Deinococcota	0.67
Firmicutes	0.65
Myxococcota	0.54
Planctomycetota	0.44
Proteobacteria	0.44
Verrucomicrobiota	0.44

Supplementary Table 4: Concentration of mānuka honey chemical constituents in each honey sample.

Chemical component	Valley A	Valley B	Valley C
Moisture (%)	20.2	18.9	20.3
DHA[11] (mg/kg)	2715.8	2598.3	3851.0
HMF[12] (mg/kg)	1.7	2.3	1.8
MGO[13] (mg/kg)	412.3	343.8	625.0
2-methoxybenzoic acid[14] (mg/kg)	6.3	7.3	6.1
4-hydroxyphenyllactic acid[15] (mg/kg)	8.1	8.3	9.5
3-phenyllactic acid[16] (mg/kg)	1375.7	1318.3	1753.8
Methylsyringate[17] (mg/kg)	83.5	76.0	98.8

Supplementary Table 5: Correlations between the chemical constituents of mānuka honey and ordination samples scores of mānuka phyllosphere microbial communities (total, core, and non-core partitions). Bold represents significant values ($p > 0.05$). Blue represents correlations that are unique to the core microbiome.

Chemical component	Total community		Core community		Non-core community	
	R^2	P value	R^2	P value	R^2	P value
Moisture (%)	**0.157**	**0.007**	**0.193**	**0.001**	**0.238**	**0.001**
DHA[1] (mg/kg)	0.005	0.255	**0.094**	**0.042**	0.075	0.079
HMF[2] (mg/kg)	**0.196**	**0.003**	**0.200**	**0.001**	**0.263**	**0.001**
MGO[3] (mg/kg)	0.061	0.148	**0.120**	**0.022**	**0.112**	**0.024**
2-methoxybenzoic acid[4] (mg/kg)	**0.139**	**0.012**	**0.186**	**0.002**	**0.222**	**0.001**
4-hydroxyphenyllactic acid[5] (mg/kg)	0.036	0.331	0.063	0.138	0.036	0.317
3-phenyllactic acid[6] (mg/kg)	0.047	0.231	**0.099**	**0.037**	0.082	0.068
Methylsyringate[7] (mg/kg)	0.067	0.126	**0.128**	**0.020**	**0.124**	**0.017**

[11] **Dihydroxyacetone:** 3-carbon sugar that accumulates in mānuka nectar and is converted to MGO in mature honey.
[12] **5-(hydroxymethyl)furfural:** intermediate product of the acid-catalyzed dehydration reaction of hexose (i.e., fructose and glucose) during storage. Used as an indicator of thermal damage and/or ageing.
[13] **Methylglyoxal:** responsible for the nonperoxide antibacterial properties in mānuka honey
[14] **2-methoxybenzoic acid:** phenolic compound commonly found in mānuka honey. Minimum concentration of 5 mg kg^{-1} required for monofloral classification.
[15] **4-hydroxyphenyllactic acid:** polyphenol commonly found in mānuka honey. Minimum concentration of 1 mg kg^{-1} required for monofloral classification.
[16] **3-phenyllactic acid:** marker polyphenol for mānuka honey. Minimum concentration of 400 mg kg^{-1} required for monofloral classification.
[17] **Methylsyringate:** phenolic compound found in high abundance in mānuka honey.

Supplementary Table 6: Concentration (ug/kg) of 20 major, minor, and trace elements in each honey sample. Colour indicators represent the elements broadly defined in honey as major (green), minor (orange), and hazardous (red).

Element	unit	Honey Sample			Average
		Valley A	Valley B	Valley C	
B	mg/kg	10	7.85	10.1	9.32 ± 1.03
Na		121	59.9	47.1	76.0 ± 32.2
Mg		30.7	24.7	32.1	29.2 ± 3.21
K		2370	2020	2530	2310 ± 213
Ca		103	84.3	90.9	92.7 ± 7.74
Al	µg/kg	937	1080	909	975 ± 74.9
Cr		61.4	52.3	48.7	54.1 ± 5.34
Mn		1740	1340	2320	1800 ± 402
Fe		1030	756	876	887 ± 112
Co		6.46	5.23	6.17	5.95 ± 0.525
Ni		45.2	27	26.9	33.0 ± 8.60
Cu		233	250	191	225 ± 24.8
Zn		2030	727	909	1220 ± 576
Rb		1760	2700	1540	2000 ± 503
Sr		274	262	273	270 ± 5.44
Cd		1.68	< 1.2	< 1.2	0.560 ± 0.792
Cs		< 0.60	0.756	< 0.60	0.252 ± 0.357
Ba		194	238	243	225 ± 22.0
Tl		9.69	7.56	11	9.42 ± 1.42
Pb		4.84	7.85	4.22	5.64 ± 1.59

Supplementary Table 7: Correlations between the concentration of 20 major, minor, and trace honey elements and ordination samples scores of mānuka phyllosphere microbial communities (total, core, and non-core partitions). Bold represents significant values ($p > 0.05$). Colour indicators represent the elements broadly defined in honey as major (green), minor (orange), and hazardous (red). Pink represents correlations that are unique to the non-core microbiome.

Element		Total community		Core community		Non-core community	
		R^2	P value	R^2	P value	R^2	P value
	B	**0.174**	**0.004**	**0.196**	**0.002**	**0.2870**	**0.001**
	Na	0.002	0.958	0.029	0.396	0.0337	0.373
	Mg	**0.169**	**0.002**	**0.186**	**0.003**	**0.2598**	**0.001**
	Al	**0.169**	**0.002**	**0.186**	**0.003**	**0.2592**	**0.001**
	K	**0.163**	**0.002**	**0.176**	**0.003**	**0.2368**	**0.001**
	Ca	**0.100**	**0.042**	**0.145**	**0.009**	**0.2583**	**0.001**
	Cr	0.001	0.972	0.026	0.439	0.0217	0.544
	Mn	**0.138**	**0.009**	**0.145**	**0.005**	**0.1736**	**0.005**
	Fe	**0.125**	**0.023**	**0.168**	**0.006**	**0.2900**	**0.001**
	Co	**0.169**	**0.006**	**0.200**	**0.002**	**0.3139**	**0.001**
	Ni	0.022	0.512	0.061	0.149	**0.1096**	**0.027**
	Cu	**0.112**	**0.027**	**0.115**	**0.020**	**0.1199**	**0.021**
	Zn	0.063	0.142	**0.108**	**0.033**	**0.1976**	**0.002**
	Rb	**0.166**	**0.003**	**0.180**	**0.003**	**0.2461**	**0.001**
	Sr	**0.173**	**0.004**	**0.200**	**0.002**	**0.3073**	**0.001**
	Cd	0.023	0.493	0.062	0.145	**0.1119**	**0.024**
	Cs	**0.174**	**0.004**	**0.197**	**0.002**	**0.2899**	**0.001**
	Ba	0.012	0.693	0.048	0.227	0.0809	0.065
	Tl	**0.157**	**0.003**	**0.168**	**0.003**	**0.2200**	**0.001**
	Pb	**0.167**	**0.003**	**0.182**	**0.003**	**0.2517**	**0.001**

Supplementary Table 8: Raw pollen counts from visual pollen analysis

Pollen identification	Valley A	Valley B	Valley C
Nectar plants			
Banksia type			
Basil type (*Ocimum*)			
Blackberry (*Rubus fruticosus*)			1
Brassicas			
Bush lawyer (*Rubus australis*)			
Buttercup (*Ranunculus*)	6	2	13
Citrus			
Clematis	6	1	1
Clover (*Trifolium*)	34	22	22
Convolvulus			
Cress type (*Apium*)			
Daisy type (*Asteraceae*)	3		
Catsear & Dandleion type (*Crepsis/Taraxacum*)	24	6	9
Eucalyptus or other Myrtaceae			
Fabaceae undifferentiated			
Fennel/carrot family (*Apiaceae*)	4	7	5
Five-finger (*Pseudopanax*)	9	1	
Flax (*Phormium*)	3	1	1
Forget-me-not (*Myosotis*)			
Gorse/broom type (*Ulex/Cytisus*)	2	1	
Hangehange (*Geniostoma*)			
Heaths (*Ericaceae*)	3	1	
Hinau (*Elaeocarpus*)		5	2
Honeysuckle (*Leycesteria/Lonicera*)			
Ironweed (*Vernonia*)			
Jasmine type			
Kamahi/towai (*Weinmannia*)			2
Kapuka (*Griselinia*)			
Karaka (*Corynocarpus laevigatus*)	2		
Kiekie (*Freycinetia banksii*)			
Lacebark (*Hoheria*)			
Lily family (*Liliaceae*)			
Mahoe (*Melicytus*)			1
Mānuka/kānuka (*Leptospermum/Kunzea*)	263	272	330
Matagouri (*Discaria toumatou*)			
Mint family (*Lamiaceae*)			
Nasturtium (*Tropaeoleum*)			
Native bidibid (*Acaena*)			
Olive family (*Oleaeceae*)			
Palm type (*Arecaceae*)			
Passionfruit (*Passiflora*)	1		

Perching lily (*Collospermum*)			
Tansy (*Phaecelia*)			
Pigeonwood (*Hedycarya*)	1		
Pohuehue (*Muelhenbeckia*)		1	
Rata/pohutukawa type (*Metrosideros*)	2	6	1
Rewarewa (*Knightia excelsa*)			11
Rose family (Undifferentiated)			
Salix (*Willow*)		1	1
Seven finger (*Schefflera*)	2		1
Tawari (*Ixerba brexiodes*)			
Tawheowheo (*Quintinia*)			
Thistle (*Carduus/ Cirsium*)	9	1	4
Thyme (*Thymus*)			
Titoki (*Alectryon excelsus*)	9	9	52
Trefoil (*Lotus*)	183	189	99
Viper's bugloss (*Echium vulgare*)			
Wattle (*Acacia*)			
Unidentified types			
Unidentifiable (broken and deformed)	7		
Unidentified type 1			
Unidentified type 2	5	9	10
TOTAL NECTAR POLLEN	**578**	**535**	**566**
Fungal material	**5**	**1**	**10**
Non-nectar plants			
Chestnut type			
Coprosma			
Docs, sorrels (*Rumex*)			
Fathen			
Fern spore			
Grass (*Poaceae*)	8	16	5
Kawakawa (*Piper excelsum*)	1		
Native beech (*Fuscospora*)			
Other wind	1		1
Pine type (*Pinus*)			
Plantain (*Plantago*)	18	8	32
Podocarpaceae			
Poplar (*Populus*)			
Pukatea (*Laurelia*)			
Sedge type (*Cyperaceae*)			
Tutu (*Coriaria*)			
Total non-nectar plant pollen	28	24	38

Appendix E

Co-authorship Forms

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Co-Authorship Form

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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.	Thesis chapter II: Host selection is not a universal driver of phyllosphere community assembly among ecologically similar native New Zealand plant species
Nature of contribution by PhD candidate	Conceived study design, sample collection, data analysis, writing and editing of the manuscript
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Jaber Abbaszadeh	Assisted with sample collection
Charles Lee	Conceived study design, provided input during data analysis and manuscript editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Jaber Abbaszadeh		2024-07-29
Charles Lee		2024-07-26

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Thesis chapter III: Phyllosphere microbial dispersal exhibits a source-sink relationship among ecologically and morphologically similar plant species

Nature of contribution by PhD candidate	Conceived study design, sample collection, data analysis, writing and editing of the chapter
Extent of contribution by PhD candidate (%)	95

CO-AUTHORS

Name	Nature of Contribution
Charles Lee	Study design conception, established relationship with landowners, provided input during chapter editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Charles Lee		2024-07-26

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Thesis chapter IV: The effect of season on the evergreen phyllosphere microbiome of mānuka (Leptospermum scoparium)

Nature of contribution by PhD candidate	Conceived study design, sample collection, data analysis, writing and editing of the chapter
Extent of contribution by PhD candidate (%)	95

CO-AUTHORS

Name	Nature of Contribution
Charles Lee	Helped during study conception, provided input during chapter editing

Certification by Co-Authors

The undersigned hereby certify that:

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Name	Signature	Date
Charles Lee		2024-07-26

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Thesis chapter V: Investigating the relationship between the mānuka phyllosphere microbiome and manuka honey quality: a case study

Nature of contribution by PhD candidate	Worked alongside supervisors to conceive study design, sample collection, microbial data collection, data analysis, writing and editing of the chapter
Extent of contribution by PhD candidate (%)	90

CO-AUTHORS

Name	Nature of Contribution
Megan Grainger	Helped with study conception, honey and pollen analyses, and chapter editing
Charles Lee	Study conception, established relationship with landowner, provided input during chapter editing

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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Megan Grainger	Megan Grainger	29/07/2024
Charles Lee		2024-07-26

July 2015