THE ROLE OF MICROORGANISMS IN SHAPING THE NECTAR CHEMISTRY OF MĀNUKA PLANTS (*Leptospermum scoparium*).

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LIST OF ABBREVIATIONS

DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
HA	Hydroxyacetone
H_2	Hydrogen
NaOH	Sodium hydroxide
H_2O_2	Hydrogen Peroxide
HCN	Hydrogen cyanide
MGO	Methylglyoxal
pH	Power of Hydrogen
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
16S	16S component of the 30S subunit of the prokaryotic ribosome
V4	Hypervariable sequence region of the 16S component
ITS1-2	Internal transcribed spacer of the nuclear ribosomal
PERMANOVA	Permutational Multivariate Analysis of Variance
ANOSIM	Analysis of Similarities
OTU	Operational taxonomic unit
DADA2	Divisive Amplicon Denoising Algorithm 2
ASV	Amplicon Sequence Variants

edgeR	Differential analysis of sequence read count data
FDR	False discovery rate
DESeq2	Differential expression analysis
MENA	Microbial Ecology Network Analysis
RMT	Random matrix theory
SparrCC	Sparce correlations for compositional data
TMM	Trimmed mean of M-values normalization
UMF	Unique Mānuka Factor
SWEET9	Sucrose efflux pathway named
A	Adenine
G	Guanine
C	Cytosine
T	Thymine
PCR	Polymerase chain reaction
VSEARCH	Vectorized search
QUIIME2	Quantitative Insights Into Microbial Ecology
UCLUST	Centroid based medium to high-identitiy clustering
USEARCH	Ultra-fast sequence analysis
PFBHA	O-(2,3,4,5,6-Pentaflurobenzyl) hydroxylamine
MSYR	(methyl syringate 4-O-β-D-gentiobiose)
UVD	Ultraviolet radiation detector
dNTP	Dideoxy nucleotypes
$MgCl_2$	Magnesium cloride
Platinum Taq HiFi	High Fidelity Polymerase
HPLC	High-performance liquid chromatography
ACN	Acetonitrile
KW	Kruskal-Wallis
hdpA	Dihydroxyacetone phosphate desphosphorylase
HAD	Haloacid dehalogenase
PCoA	Principal component Analysis

GABA	GABA (γ-amino butyric acid)
NPAAs	Non protein aminoacids

ABSTRACT

Mānuka (*Leptospermum scoparium*) honey is one of the main export products from New Zealand. The distinctiveness of this honey comes from the floral nectar, which possesses dihydroxyacetone, a carbohydrate with UV protection capabilities, which converts to methylglyoxal, usually during the honey maturation process. Methylglyoxal gives mānuka honey its biological activity (antimicrobial and antioxidant properties) and is often used as a quality marker. While it is acknowledged that microorganisms play an important role in plant ecology, their abundance and impact on nectar chemistry remain understudied. Microorganisms can arrive in the nectar by pollinator activity or through the phloem and can change nectar chemical properties. This thesis aims to fill a gap in the knowledge of the microbial diversity of the nectar; the impact of pollinators in the microbial nectar community, and the effect of bacteria on dihydroxyacetone production and sugar content in mānuka nectar. To achieve this aim, a combination of metagenomics, bacterial cultivation, chemical analyses, and biological *in vivo* and *in vitro* assays was conducted. The results show that mānuka nectar bacterial communities were dominated by Pseudomonadales, followed by Rhizobiales, Sphingomonadales, Corynebacteriales, Baccillales, Enterobacteriales, Xanthomonadales, Clostridiales, and Lactobacillales, while nectar fungi communities were dominated by Microbotryomycetes, Dothideomycetes, Malasseziomycetes, Sordariomycetes, and Ustilaginomycetes. When comparing bagged (pollinator restricted) and unbagged (with pollinator access) flowers, a bioinformatic and statistical analysis showed the bacterial community did not differ in community composition. In contrast, the fungal community was affected by pollinator visitation, and by plant genotype. The inoculation of an important functional bacterial *Pantoea agglomerans*, that previously was identified as a biomarker for the bacterial community, determined by linear discriminant analysis and network analysis, showed a significant increase in DHA using natural nectar under the laboratory. However, *P. agglomerans in vivo* inoculation did not change the composition of the main nectar sugars, suggesting that bacterial inoculation can maximize important plant metabolites like DHA,

with minor disruption to nectar sugar content. The cultivable bacterial community differed little from the main groups found through metabarcoding, meaning the stability or structure of a core bacterial community in the nectar can be maintained through cultivation. This thesis fills an important gap in the knowledge of microbial ecology of mānuka plants and provides insights into how to manipulate key bacteria to increase DHA nectar content.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. INTRODUCTION

Pollination is vital for plant reproductive biology in most terrestrial ecosystems (Kevan & Viana, 2003). This process involves interactions between the plant and several community members e.g. insects, birds (Olesen & Valido, 2003), and microorganisms (Rering, Beck, Hall, McCartney, & Vannette, 2017). Commercial products resulting from these interactions include honey and other sub-products like beeswax, propolis, and pollen, which are widely consumed worldwide. The production of honey and related products is an important economic activity in New Zealand, with nearly 23,000 tonnes of honey produced in 2019, out of which over 9,000 were exported generating 355 million dollars in 2019 (Ministry for Primary Industries, 2020). Among the exports, mānuka (*Leptospermum scoparium*) honey is possibly the most prominent one, since it is native to New Zealand, and its honey has been reported to have exceptional antioxidant and antimicrobial properties (e.g. (Carter et al., 2016; Taormina, Niemira, & Beuchat, 2001; Weston, 2000).

The antimicrobial properties of mānuka honey have been linked to the presence of methylglyoxal (MGO) (N. G. Porter & Wilkins, 1999). Dihydroxyacetone (DHA) in the nectar is the precursor of MGO. The origin of the DHA remains unclear; it may be plant-produced, microorganism-derived or a combination of both. Evidence shows that different mānuka genotypes vary in their DHA production, suggesting plant effects (Clearwater et al., 2018, Noe et al., 2019). However, previous studies show that mānuka endophytes affect some morphological and chemical properties of the plant including DHA production. Recent

research also shows that some bacteria can produce DHA from glycerol (Lidia & Stanisław, 2012). Therefore, a closer look at the nectar microorganisms may aid in elucidating the origin of DHA.

Microorganisms are an essential part of all ecosystems and organisms, having an impact on different types of hosts and their life processes (McFall-Ngai *et al*., 2013). Plants evolve by changing their biochemistry and behaviour to cope with microbes, and these adaptations shape their microbial communities (Manriquez et al., 2021). Pollinators may change their preferences based on nectar composition, and transport microbes from one plant to the other, further shaping plant microbial communities. Therefore, an understanding of the interactions between pollinators, plants and microorganisms is important if we are to improve production systems based on apiculture. This thesis aimed to characterise and compare the microbial diversity in the nectar of different mānuka cultivars and clones, establish the role of nectar microorganisms on DHA production, and explore the influence of pollinator visitation on microbial communities and nectar composition.

1.2. RESEARCH OBJECTIVES AND THESIS OUTLINE

This thesis has the following objectives:

1. Characterize and compare the microbial communities from mānuka flowers in different cultivars and clones

A) Identify cultivable bacteria in the nectar and select key functional bacteria for mānuka chemistry

B) Describe and compare bacterial diversity and abundance (16S rRNA amplicon sequencing)

C) Describe and compare fungal diversity and abundance (ITS1/2 amplicon sequencing)

2. Establish the effect of bacterial inoculation on DHA and sugars by comparing DHA and sugar measures in control and inoculated flowers *in situ* and *in vitro* conditions.

3. Establish the effect of pollinator visitation on the microbial community composition and metabolites (nectar) by comparing the communities in the nectar of open flowers vs. those enclosed within restriction nets.

1.3. HYPOTHESES

Following the aims and objectives of this research, relevant hypotheses are as follows:

1) There will be a varied microorganism community in mānuka plants, and the diversity and abundance of bacteria and fungi will differ between clones and cultivars
2) Microorganisms present in the flowers or nectar will affect nectar chemistry (mainly DHA and sugar production)
3) Pollinator visitation will have an effect on the microbial and chemical composition of the nectar

1.4. LITERATURE REVIEW

1.4.1. Mānuka generalities and ecology

Leptospermum scoparium (mānuka) belongs to the family Myrtaceae. It is a shrub species that is highly adaptable, being able to survive in different environments across a range of climate and soil conditions (Harris, 2002) with extensive genotypic variation (Harris, 2002; Ronghua, Mark, & Wilson, 1984; Stephens, Molan, & Clarkson, 2005). A brief botanical description from Allan (1961) is as follows: "mānuka (*Leptospermum scoparium*) is a variable shrub or small tree, usually about 2 metres tall but can also reach 4 metres or more. Leaves are present along all the branches and vary in both size and shape, ranging from elliptical to lanceolate. Flowers for wild plants are white or rarely pink or red, and they are distributed axillary or occasionally terminal on branchlets, usually solitary and sessile. Flowering occurs in October-February (Stephens et al., 2005). During this time hypanthia are

usually globous with a distinct pedicel, sepals are deciduous, oblong to broadly deltoid, petals are suborbicular and slightly clawed, and stamens occur in bunches. The style is inset with a large stigma and is often reduced or absent Also, the ovary is penta locular, with each ovary containing about 100 ovules. Finally, fruits are woody persistent with penta valved capsules. *L scoparium* is an andromonoecious species" (Allan, 1961).

Flowering is initially activated by a long-day flowering cue, and bud development is restrained by cool temperatures throughout winter, leading to spring flowering when the temperature is lifted (Zieslin & Gottesman, 1986). The majority of New Zealand's insect-pollinated flora has weakly coloured flowers, which has been historically related to non-specific insect associations (Godley, 1979; Lloyd, 1985; Wardle, 2008). The small white flowers of *Leptospermum scoparium* are classified as open access with a dish/bowl shape and they are visited by a range of insect pollinators (Newstrom & Robertson, 2005). The type of pollinators in *L. scoparium* were described in the South Island by Primack (1978), where he described flies from the genus *Protohystricia*, solitary bees in the genera *Lasioglossum* (Halictidae), also *Leioproctus (Colletidae)* in a lesser frequency. In lower abundance were *syrphid* flies and *calliphorid* flies. The bees were more commonly recorded during mid-morning periods and bees and flies in the late afternoon. In settled weather, moths (families *Pyralidae, Geometridae,* and *Noctuidae*), and craneflies (*Tipulidae*) were observed. Also, nocturnal moth visitation has been reported (Newstrom & Robertson, 2005), and the introduced honeybee (*Apis mellifera*) also collects both pollen and nectar (Butz Huryn, 1995). These observations confirm that a wide variety of pollinators are associated with *L. scoparium.* There is variability among the wild populations of *L. scoparium,* as there are single white-, pink-, red-flowered specimens, and double white- or pink flowered plants (Dawson, 1997). The flowering times within a population, among adjacent and geographically widely separated populations, and between seasons are highly variable (Primack & Lloyd, 1980).

1.4.2. Mānuka honey and nectar

The production of honey and related products is an important economic activity in New Zealand, with nearly 27,000 tonnes of honey produced in 2015, out of which over 10,288

tonnes exported in 2015 (Ministry for Primary Industries, 2020). Among the exports, mānuka (*Leptospermum scoparium*) honey is possibly the most prominent one and certainly the most highly valued, since this plant is native to New Zealand, and its honey has been reported to have exceptional antioxidant and antimicrobial properties (D. A. Carter et al., 2016; Taormina et al., 2001; Weston, 2000).

The antimicrobial capabilities of mānuka honey have been linked to the presence of methylglyoxal (MGO) (N. G. Porter & Wilkins, 1999). Dihydroxyacetone (DHA) (a compound present in the nectar) is the precursor of methylglyoxal. DHA is an intermediate product of the glycolysis as DHA phosphate and used by aerobic organisms (prokaryotes and eukaryotes) to release stored energy as it can reversible transform into glyceraldehyde 3-phosphate (Mavric, Wittmann, Barth, & Henle, 2008). The conversion of DHA to MGO occurs naturally in the process of honey storage, where conditions relating to temperature and pressure are kinetic parameters suitable for this type of reaction (Mavric et al., 2008), the reaction is described as a non-enzymatic dehydration, and it involves an enediol intermediate (Fedoroňko et al. 1980). This reaction starts in the nectar and will continue in the honey, as a result DHA is an indicator of quality either in the nectar and in the honey. There are other antibacterial compounds in mānuka honey like peroxide and leptosperin; however, the most studied compound is MGO Dihydroxyacetone (Kato et al., 2014; Kato et al., 2012). In fact, the commercial value of mānuka honey comes predominantly from the level of MGO and is expressed in Unique Mānuka Factor (UMF) levels (Adams, Manley-Harris, & Molan, 2009).

The properties of honey are largely determined by nectar quality and quantity. The floral nectar of mānuka (*Leptospermum scoparium*) and some other *Leptospermum* species is dominated by fructose and glucose; and contains small and variable amounts (usually <2% each) of sucrose and DHA (Nickless, Holroyd, Hamilton, Gordon, & Wargent, 2016; Norton, McKenzie, Brooks, & Pappalardo, 2015).

Surveys of nectar composition from wild and cultivated mānuka have revealed significant variations in the nectar composition of sugars and DHA per New Zealand location, and sometimes between individual plants (Nickless, Anderson, Hamilton, Stephens, & Wargent, 2017; S. Williams et al., 2014). In a recent study examining the influence of genotypes, the flower stage and induced water stress on the nectar composition, researchers found an effect of the genotype, water stress and flowering stage on nectar sugar content (Clearwater, Revell, Noe, & Manley-Harris, 2018). However, only the genotype influenced DHA content, and

specific flower stages or water stress had no significant effect on DHA content. The sugars (hexoses) are known to be under strong environmental control, hence, Clearwater concluded that DHA production is somehow separated from the hexoses.

The exact origin of DHA remains unclear. It is possible that DHA originates from the nectar parenchyma and enters the nectar independently of the sucrose efflux pathway named SWEET9 (Lin et al., 2014). Alternatively, DHA can be produced by microbial enzymatic activity in the nectar or via the combined interaction between the plant and microorganisms.

1.4.3. Chemical properties of nectar and microbial establishment

Floral nectar is a sugar-rich habitat for microbes across different environments. Other sugar-rich habitats include plant phloem, plant exudates, non-floral nectar, honeydew and the haemolymph of insects (Bové & Garnier, 2003; J. P. Williams & Hallsworth, 2009). Despite being an energy-rich source, nectar presents multiple challenges to microbial establishment, (Bartlewicz, Lievens, Honnay, & Jacquemyn, 2016).

It is necessary for bacterial and fungal cells to maintain the turgor pressure of their membranes to retain the integrity and activity of their organelles (Brown, 1990). In addition, their macromolecular systems require water for tertiary and quaternary structure, both at the level of hydrophilic and hydrophobic interactions that maintain the cell functions (Cray et al., 2013). The physicochemical conditions of nectar are challenging to microorganisms as nectar has a high osmotic potential due to low water activity, and contains secondary metabolites from plants and bacteria, and alcohols such as ethanol and glycerol. These conditions cause the nectar media to be very stressful and with generally low richness of microbial communities (M. I. Pozo, Herrera, & Bazaga, 2011; Pozo, Lachance, & Herrera, 2012).

Secondary metabolites in nectar can have antimicrobial properties, presenting an additional challenge to microbial establishment, and plants can regulate secondary metabolite concentration in specific tissues (Stevenson, Nicolson, & Wright, 2017). For instance, broad-activity antibiotics such as streptomycin, ampicillin and kanamycin have been isolated from banana (*Musa paridasiaca*) and rubber (*Ficus elastica*) nectar (Solomon, Santhi, & Jayaraj, 2006). There are also antifungal compounds such as chitinases and glucanases that have been

identified in the extra-floral nectar of acacias (*Acacia* spp.) (González‐Teuber, Eilmus, Muck, Svatos, & Heil, 2009). These compounds defend plants and insects from pathogen attack (Manson, Otterstatter, & Thomson, 2010) but may make nectar a challenging environment for more generalised microorganisms.

In addition to the nectar's chemical properties, interaction with UV light might facilitate the presence of free radicals such as hydrogen peroxide (H_2O_2), which will prevent toxin build up and reduce the breakdown of sugars and membrane proteins by altering protein structure and redox cycle (C. Carter et al., 2007; C. Carter & Thornburg, 2004). Furthermore, proteins called nectarines have been found in the nectar of Tobacco plants with the capability of producing hydrogen peroxide via the nectar redox cycle (Park & Thornburg, 2009). Other proteins have been found in various plants with the same function (Hillwig et al., 2011) and non-protein amino acids could play a similar role (Nepi, 2014). Despite the challenges presented by the nectar environment it has been shown to contain a large diversity of microbial inhabitants (Vannette & Fukami, 2016; Vannette, Gauthier, & Fukami, 2013). However, the challenges and variations that the environment produces on the plants might have an impact on the nectar microbial communities, for example environmental changes related to urbanization may impact microbial communities (Bartlewicz et al., 2016).

1.4.4. Microbial diversity in nectar and its effects on nectar chemistry

The relationships between plant and microorganisms are complex and dynamic, and can vary from beneficial to pathogenic (Newton et al., 2010). Plants are colonized on their exterior with microbes (epiphytes) as well as their interior (endophytes). Both types are known to interact with the host plant in a way that complements metabolic or defence functions (Turner, James, & Poole, 2013). In reproductive organs of plants like flowers, microorganisms play important biological and ecological roles. For instance, they can change flower smell and nectar properties (Aleklett, Hart, & Shade, 2014). There are several bacteria and fungi that modify nectar metabolite compositions (Vannette & Fukami, 2016; Vannette et al., 2013). For example, the bacteria *Gluconobacter spp*. alters nectar's chemistry by reducing the hydrogen peroxide concentration by about 80%, making the nectar a more habitable

environment for other microorganisms. *Gluconobacter spp.* also had a significant impact on both pH (reduced by 5 units) and sucrose (reduced by 35%), whereas *M. reukaufii* had a smaller impact (2 pH units and 17% in sucrose reduction). There were also modifications in the concentrations of other sugars. For instance, *Gluconobacter spp.* increased glucose concentration and reduced sucrose concentration. In contrast, *M. reukaufii* did not show an effect on these sugars. Therefore, these differences in sugar composition in flowers containing *Gluconobacter spp.* and *M. reukaufii*, might be a driving force for pollinators to choose plants that are inoculated by these organisms. (Good, Gauthier, Vannette, & Fukami, 2014).

Gluconobacter spp. also has the metabolic capability of producing DHA from glycerol (Jani, Seely, Peabody, Jayaraman, & Manson, 2017; X.-j. Zheng, Jin, Zhang, Wang, & Liu, 2016; Z. Zheng, Luo, Yu, Wang, & Ji, 2012) but the production is dependent on nectar pH. Low pH (in the range of 4.0 to 5.5) favour its production (Hu, Zheng, & Shen, 2011). Therefore, *Gluconobacter spp.* is a candidate bacterium to analyse if the presence of relative high values of DHA in mānuka nectar is a consequence of the activity of this bacteria or other species e.g. yeast or *Acetobacter spp*. (Mamlouk & Gullo, 2013).

Interactions between products of bacteria metabolism are likely to happen in the nectar (Noutsos, Perera, Nikolau, Seaver, & Ware, 2015) since the products of the metabolic process of one bacterium can be reactive to the metabolic pathways of other bacteria in the nectar, and as a result, metabolic dependencies can be driving species co-occurrence in nectar microbial communities (Zelezniak et al., 2015). For this reason, it is important to identify the composition of the microorganisms present in the nectar to better understand their impacts on nectar properties.

Fungi are also common inhabitants of nectar, in particular, yeasts which have been shown to be very active in changing nectar sugar composition (A. Canto & Herrera, 2012; Schaeffer, Vannette, & Irwin, 2015). The yeasts found in different research studies in Europe and in tropical areas belong to two phyla: Ascomycota and Basidiomycota (A. Canto & Herrera, 2012; M. I. Pozo et al., 2011). Mutualism between bumblebees and plants in Germany was studied in successional years and species in the *Metschnikowia* clade, the *Starmarella* clade and two genera *Debaryomyces* and *Zygosaccharomyces* were identified, the attractiveness of the flowers to the bumblebees was increased in correlation with the yeast diversity, as a result of the interaction the insect provides pollinations services to the flowers while they have a nutrition source from the yeast (Brysch-Herzberg, 2004). Moreover, Brysch-Herzberg and

Seidel (2015) found that physical and chemical properties of nectar had a limited influence on the abundance of nectar yeasts; by contrast, flower-visiting insects appear to have a greater impact on the yeast composition in the nectar of different plant species.

Yeasts can attract pollinators by producing volatiles or other secondary metabolite products of fermentation (M. J. Pozo, de Vega, Canto, & Herrera, 2009). Moreover, it has been shown that because of their metabolic activity through fermentation processes, there is an increase in nectar temperature (Herrera & Pozo, 2010). The increase of nectar temperature can be a direct stimulus for honeybees' preference as shown in Nicolson, de Veer, Köhler, and Pirk (2013). This study shows that warm nectar is more attractive for honeybees because of its reduced viscosity, which seems to be a more important aspect than sugar concentration. This research evidence the importance of yeasts for nectar ecology and plant fitness.

1.4.5. Nectar properties and pollinator visitation

Nectar chemical components are a vital element shaping plant-pollinator interactions as they constitute a main source of nutrition and may influence pollinator health and behaviour (Prasifka et al., 2018). Amino acids are the most abundant nectar solutes after sugars, and all twenty protein amino acids have been found in nectar (Nicolson and Thornburg 2007). Plants harbour also other important secondary metabolites as non-protein amino acids (NPAAs) that affect plant-pollinator interactions through the direct effect on the nervous system, by regulating phagostimulant, and by increasing flight muscle activity (Nepi, 2014). Examples of NPAAs include molecules such as GABA (γ-amino butyric acid), β-alanine, ornithine, taurine (Vranova et al., 2011). For bumblebees, a positive effect on longevity was observed for *B. terrestris* workers that fed on GABA (γ-amino butyric acid), by contrast this was not found for *A. mellifera* showing that NPAAs can have species-specific effects (Bogo et al., 2018).

The memory for pollinators can be also enhanced by association of nutritional rewards with other compounds commonly present in the nectar of certain plant species e.g., caffeine in coffee plants (Wright et al., 2013). Plant secondary metabolites can strongly influence pollinator preference for or avoidance of certain plants. Compounds in the nectar that typically attract pollinators are hexoses, sucrose, and amino acids. Honeybees are attracted to different type of amino acids as they are key requirements for some metabolic functions, and

as a result, the choice behaviour of foraging bees is influenced, for instance by phenylalanine and glycine (Hendriksma et al., 2014). On the other hand, other compounds such as phenols and alkaloids can reduce attractiveness for pollinators, with the effect depending on concentration and amount consumed, as well as on pollinator sensitivity (Barachi et al., 2017; Llamsa et al., 2018). Other molecules, such as iridoid glycoside compounds, could influence pollinator preference due to their observed health benefits, e.g., by reducing parasite loads (Stevenson et al., 2017).

1.4.6. Pollinator visitation and microbial diversity

Aizenberg-Gershtein, Izhaki, and Halpern (2013) described different bacteria communities in nectar and found that the visitation of honeybees was a factor affecting bacterial diversity. Another study carried out in three different arid regions of Israel by Samuni-Blank et al. (2014) examined the treatments of bagged and open flowers, across three different sites and they found significant variation in OTUs among sites but no significant variation in OTUs among treatments (bagged, unbagged flowers)

In contrast, another study conducted in central Texas (U.S.A.) by McFrederick et al. (2017) showed that flowers work as hubs for bacteria and that, even in absence of aerial pollinators, all flowers shared similar types of bacteria whether in open or bagged situations. The presence of bacteria in pollinator-restricted flowers suggests that these bacteria may also be transmitted to flowers via plant surfaces, air or other minute vectors and supports the hypothesis that flowers may provide habitats for metapopulations of easily dispersed microbes, as has been found in other systems that contain free-living microbes (Finlay, 2004).

Nectar seems to be an interface between honeybees and bacteria with more frequent horizontal transmission of microbes than expected (Anderson et al., 2013). Different plants may also harbour different bacterial communities, as shown in a study conducted by Aizenberg-Gershtein et al. (2013) on *Amygdalus communis* (Almond) and *Citrus paradise* (grapefruit). They found different dominant bacteria in each type of plant pollinated by honeybees (*A. mellifera*) across an area of 20 km^2. In Belgium, a study with cultivable bacteria of the forest herb *Pulmonaria officinalis* found a large variation in community structure of microorganisms among plant populations. Their findings concluded that the

assembly of the nectar microbiota is context-dependant (Jacquemyn, Lenaerts, Tyteca, & Lievens, 2013). These results indicate that pollinators may introduce microorganisms into the nectar but that other sources such as the air can act as carriers too, and that plants may have their own microbiomes. Flowers have been found to increase their microbial communities after anthesis, both in flowers exposed and unexposed to pollinators for bacteria, while fungi increased in abundance with pollinator visitation (von Arx, Moore, Davidowitz, & Arnold, 2019). Therefore, exploring the impacts of pollinator visitation and plant genotype is critical to understand the factors shaping nectar microbial communities and their effects on nectar properties.

1.4.7. Metagenomics and its use to explore microbial communities

Microbial communities can be explored as to their bacterial and fungal components with amplification of molecular markers and high throughput sequencing (T. M. Porter & Hajibabaei, 2018). Metagenomics is the study of nucleic acids, mostly DNA, that are extracted from environmental samples. When the genetic material refers to amplification of a certain barcode region that is used to identify microorganisms, we use the term metabarcoding. Amplification of the 16S rRNA unit has been applied to identify bacteria, and more specifically, the hypervariable subregion V4 has been used to study nectar microbiota (Vannette et al., 2017) since it has the advantage of recovering archaea as well as eubacteria (Willis, Desai, & LaRoche, 2019). This technique is ideal for characterising the diversity of microbial communities, which are composed of active cultivable and non-cultivable cells, and of dormant cells. Dormancy is a common strategy that allows microbial species to contend with temporal variability in environmental conditions, and dormant cells represent up to a half of the total cells of a certain community (Lennon & Jones, 2011). The active microbial community has metabolic traits that determine different bacteria phenotypes, and niche theory suggests that similar microbial phenotypes should share habitat affinities. The perspective that microorganisms are competing for limited resources arises from the principle of competitive exclusion that considers communities as open, non-equilibrium assemblages of ecologically microbial similar species, whose abundance are governed by random speciation and extinction, dispersal, and ecological drift (Gilbert & Levine, 2017). Metabarcoding has given insights to test these theories and to understand bacterial population genetics (Rocha, 2018).

1.4.8. History of metabarcoding

The foundations of molecular phylogeny studies of bacteria began once a consensus was formed on using the 16S rRNA sequence as a prokaryotic marker (Woese & Fox, 1977; Woese et al., 1975), and posterior use in sequence analysis for establishing relationships among nucleotide sequences in the definition of bacterial species (Stackebrandt et al., 2002) and is useful for a first phylogenetic affiliation and posterior use of other techniques for an accurate classification of bacteria (Rosselló-Mora & Amann, 2001). This gene encodes the small ribosomal subunit that is present in all prokaryotic cells. The gene that codes for this protein has characteristics that make it suitable as a taxonomic marker: 1) the 16S rRNA gene is found in all bacteria and archaea (Roy, 2014), 2) the small size 1500 bp and degree of conservation amongst prokaryotes (Goodwin, McPherson, & McCombie, 2016), 3) the presence of variable regions within the 16S rRNA gene as a result of diverse rates of evolution among species, which can be used to distinguish different bacterial groups (Clarridge, 2004), and 4) the existence of highly conserved regions in the gene sequence, which can be used to design universal primers flanking different hypervariable regions defined from V1 to V9 (Gray, Sankoff, & Cedergren, 1984). However, despite these advantages, the use of 16S rRNA for bacterial identification has limitations. First, the number of copies varies in bacterial genomes from one to 15 in bacteria species to hundreds in eukaryotic species (Kembel, Wu, Eisen, & Green, 2012). Second, there is low taxonomic resolution at the species level for some bacterial groups (Poretsky, Rodriguez-R, Luo, Tsementzi, & Konstantinidis, 2014), and third, the choice of the sub variable region within the gene can produce different results (Bukin et al., 2019).

The typical metabarcoding sequencing strategy has evolved in order to get longer fragments with good depth of coverage (Pollock, Glendinning, Wisedchanwet, & Watson, 2018). The first methods, developed by Sanger introduced significantly enhanced the study of microbial ecology since it enabled the assessment of microbial biodiversity through the construction of clone libraries that provided better phylogenetic resolution due to the longer reads (Leigh,

Taylor, & Neufeld, 2010). However, it had the limitation of requiring cloning of fragments, which requires in a very time-consuming, expensive process with limitations due to variation in efficiency of the cloning of amplicon fragments (Pace, 1997). More recently, people have turned to next-generation, Illumina sequencing, which is now very widely used because of its efficiency and cost-effectiveness and depth in the sequencing. For bacterial work, the fragments used are mostly the regions V1-V2 or V3-V4 of 16S rRNA using paired-end libraries (Bukin et al., 2019) and libraries of about 300bp in length are finally sequenced on the Illumina platform, though this level of resolution is generally limited to genus taxonomic identification (Kono & Arakawa, 2019).

1.4.9. Bioinformatic processing of the sequences

Illumina sequencing produces a large amount of sequence data, which has required the development of new algorithms for analysis (Bartlewicz et al., 2016). Filtering of the data is mainly done by pairwise sequence alignments performed by a vectorized implementation of the Needleman-Wunsch algorithm with ends-free gapping. A heuristic k-mer distance screen is applied before alignment, with a threshold applied to the k-mer distance based on an error model on the transition probability between aligned nucleotides that depends on the original nucleotide, substituting nucleotide and associated quality score (Needleman & Wunsch, 1970). These concatenated stages are typically implemented with the open-source package DADA2 (Callahan et al., 2016). This implementation to the output of the Illumina data allows chimera elimination and the assembly of both forward- and reverse-sequences to better identify more real variants and results in fewer spurious sequences, giving more biological meaning to the obtained data, and consequently more accuracy to the analysis of ecological metrics (Callahan, McMurdie, & Holmes, 2017). There are other algorithms to generate OTUs like USEARCH or UCLUST, though, they produce high number of spurious sequences and as a consequences inflated diversity measure (Edgar, 2017). The package DADA2 was preferred here as first choice, because it uses a parametric model to infer true biological sequences from reads. The model relies on input read abundances and distances. As a result, it reflects with more accuracy the sequences, and provides highest possible biological resolution

(Prodan et al., 2019). Joining and assembly forward and reverse sequences helps to obtain a straightforward contrast with the databases for either bacteria or fungi sequences. The output in amplicon sequence variants is useful for future comparisons with other works, as it is the more accepted form to conduct upstream statistical analysis. To consider the compositional nature of metagenomic data, all the variations of comparative methods were used and are explained in the next sections.

1.4.10. Approaches to the analysis of the 16S rRNA gene sequences

Here, I provide a brief summary of the typical approaches used in the molecular studies of microbial ecology and in the current application to nectar microbiomes.

1.4.11. Diversity indexes

In microbiome studies, the term diversity principally refers to either alpha (within-sample) or beta (across-sample) diversity obtained by the analysis of the 16S rRNA sequences (Walters & Martiny, 2020).

Alpha diversity

The diversity of an individual microbiome can be approached by estimating the number of different organisms, termed richness, supplemented by how evenly distributed their abundances are, termed evenness. The most direct measure of alpha diversity is the number of unique OTUs (operational taxonomic units) observed in a sample (Shannon, 1948). More advanced approaches consider the evenness in the microbiomes using diversity indices, e.g. Shannon index (Shannon, 1948); as richness can also be studied by other diversity indices,

e.g. Chao1 (Chao & Jost, 2015), that quantify richness considering the proportion of rare organisms in the sample.

Beta diversity

Beta diversity in microbiome research refers to the overlap or similarity of two microbiomes in terms of their microbial complement. Various metrics can be used to assess the similarity (or dissimilarity) of two microbiomes. Most simply, beta diversity can be quantified by counting the number of shared and unique OTUs between samples (the Jaccard index) or can use more complex metrics that consider the relative abundances of each organism in each microbiome (Lozupone & Knight, 2005). Calculating beta diversity between all the samples in a study generates a distance matrix representing the compositional similarity between samples. These outputs can be used in analyses such as PERMANOVA, to identify associations between phenotypes and wide-scale microbiota composition, or principal co-ordinates analysis, which reduces the dimensionality of the OTU data enabling visualisation of the clustering of samples by microbiota composition (Goodrich et al., 2014) . Although these analysis are typically limited by using a rarefaction cut in the microbiome data to make the samples more comparable, and, different library sizes can result in apparent differences in the compositional data, as a consequence rarefying can end in high rate of false discovery, and has limitations to be applied to microbiome studies and analysis (Weiss et al., 2015).

There are several indices and measures for both alpha and beta diversity, however, most do not consider the taxonomic similarity between the OTUs. Phylogenetic diversity provides a measure of taxonomic alpha diversity (Faith & Baker, 2007) and the UniFrac distance was developed to incorporate phylogenetic distances between communities in order to quantify taxonomic similarity between microbiome profiles (Lozupone & Knight 2005).

1.4.12. Differential proportional analysis

Whether the bioinformatic pipeline uses amplicon sequence variants (ASVs) or operational taxonomic units (OTUs), the analysis is based on compositional data, therefore does not represent the number of occurrences in the sample but the number of times that OTU was

observed relative to all the other counts observed (McMurdie & Holmes, 2014). To analyse microbiome data taking into consideration its compositional nature, several normalization approaches have been proposed (McMurdie & Holmes, 2014; S. Weiss et al., 2017). Rarefying microbiomes at even depth by sub-sampling of the ASVs or OTUs without replacement ends in a loss of information, reducing discriminatory power in further steps of the analysis, and when library sizes are different, loss of information is inevitable, with counts proportions that vary more than expected. Several new approaches have been proposed for normalization and data comparison.

When analysing results from metagenome studies, thousands of hypothesis tests are conducted simultaneously. As a result, several numbers of tests are performed and a correction for multiple tests is included to avoid inflation of type I errors (Haynes, 2013). The correction is the False Discovery Rate implemented by (Benjamini & Hochberg, 1995). There are new methods that apply the control for the false discovery rate (FDR) as a way to identify as many significant features as possible correction, and in addition they implemented Bayesian approaches in part of the analysis. These new approaches are called EDGES methods and are now available in software packages such as edgeR, and DESeq2 (Tang, Sun, Shimizu, & Kadota, 2015). The edgeR method uses TMM normalization which stands for trimmed mean of M-values normalization (M. D. Robinson & Oshlack, 2010); edgeR estimates a common or trended dispersion for all features and then applies an empirical Bayes strategy for squeezing the tag-wise dispersions toward the common or trended dispersion (Mark D. Robinson & Smyth, 2007), and is now available in software packages.

Another method of proportional differential analysis is DESeq2, which uses a median of ratios to normalized counts, and similar to edgeR assumes non differential proportion to analyse the overall counts; this is useful to detect outliers and reduce their impact (Love, Huber, & Anders, 2014).

DESeq2 was developed for comparing differential gene expression but is equally applicable to analysis of metabarcoding data. DESeq2 provides a new statistical fitting routine to account for the variance heterogeneity often observed in sequence data; it uses the negative binomial distribution as an error distribution to compare abundance of each OTU between groups of samples in the framework of generalised linear modelling. The method also incorporates an empirical Bayesian approach.

1.4.13. Network Analysis

1.4.13.1. Microbial interactions

Inter-microbial interactions and host-microbe interactions shape the microbiome composition of a certain habitat. Several types of interaction between microbes can occur (Faust & Raes, 2012). A positive interaction for one microbe with a negative outcome for the other microbe may be the result of parasitism or predation. A neutral interaction for one microbe and negative interaction for the other is a result of an amensalism. An interaction that is negative for one microbe and negative for the other is a result of a competitive interaction. An interaction that is neutral for one microbe and positive for the other is a result of a commensalism. Finally, when the interaction is positive for both microbes the type of interaction is called mutualism.

Interspecies interactions can be used to predict the relative taxonomic composition of microbial communities (Friedman & Alm, 2012) and these interactions are important to maintain community structure and ultimately resilience against stressors (Mandakovic et al., 2018). Interactions within members of the microbial community can also lead to the stimulation of host metabolism. For instance, the import of host nutrients or exporting metabolic by-products can end up in competition for resources or cooperative interactions as metabolic cross-feeding (Sung et al., 2017).

1.4.14.2. Methods to evaluate co-occurrence networks

MENA (Microbial Ecology Network Analysis) uses classical Pearson correlation coefficients, with a threshold above which interactions are considered important. The important, threshold is determined using a random matrix theory (RMT) approach (Deng et al., 2012) and selected where the eigenvalues of the matrix transition from fitting a Gaussian distribution to a Poisson distribution (Luo et al. 2006).

SparCC analysis, is a novel quantification of co-occurrence and was designed specifically for compositional OTU data. It utilises the log-ratio transformation, that is used in compositional analyses. Data that is described as proportions or probabilities, or with a constant or irrelevant sum, is referred to as compositional data (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017).

It is important to note that SparCC, like any method that employs log transformations, requires some pre-processing to eliminate zero values (Aitchison, 1982). SparCC employs a variation of the well-known pseudo-count method, which assigns a small fraction to OTUs that were not detected in a sample. This approach implicitly assumes that all components are in fact present in the sample, and that all zero values result from finite detection resolution, and utilises the ratio variance between two OTUs to infer the coefficient of their association (Friedman & Alm, 2012).

The graphical output of these algorithms are networks, and microbial communities can be identified within networks, although clearly defined structures are not always found (Faust & Raes, 2012). When all units fall within one connected graph, it is still possible to identify natural partitions within a nodes structure. More densely connected areas interlinked by more sparse connections could represent communities (Newman, 2006). When nodes within a network can be assigned to different communities we can define a modularity on a certain network (Newman & Girvan, 2004). Community partitioning is the number of edges found between communities. The best partitions would be expected to minimise the number of edges between communities. Modularity provides a measure of how well community partitions fit a network structure, but also an indication that there is more community structure than would be expected by stochastic effects (Newman 2006).

Networks are also used in nectar microbiomes and the approach has given insights into the biological importance of structuring within nectar microbial communities (Zemenick, Vannette, & Rosenheim, 2019).

1.4.15 Thesis aims

The purpose of this thesis is to identify the microbial community in the nectar of different mānuka genotypes that differ in their flower phenotypes and dihydroxyacetone content and evaluate the effect of pollinator visitation on the nectar microbial community. To accomplish these objectives, DNA extraction and bioinformatic analyses will be applied to assess the diversity of the microbial community (fungi and bacteria) and identity key functional microorganisms that might interact to determine the DHA levels in mānuka plants. Classical and new approaches in statistics that combine Bayesian and network analysis will be used to identify the microbial community structure and differences between plant genotypes and treatments.

1.5. REFERENCES

Adams, Christopher J., Merilyn Manley-Harris, and Peter C. Molan. 2009. "The origin of methylglyoxal in New Zealand Mānuka (Leptospermum scoparium) honey." *Carbohydrate Research* 344 (8):1050-1053. doi: https://doi.org/10.1016/j.carres.2009.03.020.

Aitchison, J. 1982. "The Statistical Analysis of Compositional Data." *Journal of the Royal Statistical Society. Series B (Methodological)* 44 (2):139-177.

Aizenberg-Gershtein, Yana, Ido Izhaki, and Malka Halpern. 2013. "Do Honeybees Shape the Bacterial Community Composition in Floral Nectar?" *PLOS ONE* 8 (7):e67556. doi: 10.1371/journal.pone.0067556.

Aleklett, Kristin, Miranda Hart, and Ashley Shade. 2014. "The microbial ecology of flowers: an emerging frontier in phyllosphere research." *Botany* 92 (4):253-266. doi: 10.1139/cjb-2013-0166.

Allan, H. H. 1961. *Flora of New Zealand. Volume I*: Government Printer, Wellington, N.Z.

Anderson, K. E., T. H. Sheehan, B. M. Mott, P. Maes, L. Snyder, M. R. Schwan, A. Walton, B. M. Jones, and V. Corby-Harris. 2013. "Microbial Ecology of the Hive and Pollination Landscape: Bacterial Associates from Floral Nectar, the Alimentary Tract and Stored Food of Honey Bees (*Apis mellifera*)." *PLOS ONE* 8 (12).

Baracchi, D., Marples, A., Jenkins, A. J., Leitch, A. R., & Chittka, L. (2017). Nicotine in floral nectar pharmacologically influences bumblebee learning of floral features. *Scientific reports*, *7*(1), 1951. https://doi.org/10.1038/s41598-017-01980-1

Bartlewicz, Jacek, Bart Lievens, Olivier Honnay, and Hans Jacquemyn. 2016. "Microbial diversity in the floral nectar of *Linaria vulgaris* along an urbanization gradient." *BMC Ecology* 16 (1):18. doi: 10.1186/s12898-016-0072-1.

Benjamini, Yoav, and Yosef Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society. Series B (Methodological)* 57 (1):289-300.

Bové, Joseph, and Monique Garnier. 2003. *Phloem-and xylem-restricted plant pathogenic bacteria*. *Plant Science* 164: 423-438. 10.1016/S0168-9452(03)00033-5.

Bogo, G., Bortolotti, L., Sagona, S. *et al.* Effects of Non-Protein Amino Acids in Nectar on Bee Survival and Behavior. *J Chem Ecol* **45,** 278–285 (2019). https://doi.org/10.1007/s10886-018-01044-2

Brown, P. R. 1990. "Promiscuous plasmids of gram-negative bacteria." *FEBS Letters* 275 (1-2):239-240. doi: 10.1016/0014-5793(90)81481-3.

Brysch-Herzberg M. (2004). Ecology of yeasts in plant-bumblebee mutualism in Central Europe. *FEMS microbiology ecology*, *50*(2), 87–100. https://doi.org/10.1016/j.femsec.2004.06.003

Brysch-Herzberg, Michael, and Martin Seidel. 2015. "Yeast diversity on grapes in two German wine growing regions." *International Journal of Food Microbiology* 214:137-144. doi: https://doi.org/10.1016/j.ijfoodmicro.2015.07.034.

Bukin, Yu S., Yu P. Galachyants, I. V. Morozov, S. V. Bukin, A. S. Zakharenko, and T. I. Zemskaya. 2019. "The effect of 16S rRNA region choice on bacterial community metabarcoding results." *Scientific Data* 6 (1):190007. doi: 10.1038/sdata.2019.7.

Butz Huryn, Vivian M. 1995. "Use of native New Zealand plants by honey bees (*Apis mellifera L.*): A review." *New Zealand Journal of Botany* 33 (4):497-512. doi: 10.1080/0028825X.1995.10410621.

Callahan, Benjamin J., Paul J. McMurdie, and Susan P. Holmes. 2017. "Exact sequence variants should replace operational taxonomic units in marker-gene data analysis." *The ISME journal* 11 (12):2639-2643. doi: 10.1038/ismej.2017.119.

Callahan, Benjamin J., Paul J. McMurdie, Michael J. Rosen, Andrew W. Han, Amy Jo A. Johnson, and Susan P. Holmes. 2016. "DADA2: High-resolution sample inference from Illumina amplicon data." *Nature Methods* 13 (7):581-583. doi: 10.1038/nmeth.3869.

Canto, A., and C. M. Herrera. 2012. "Micro-organisms behind the pollination scenes: microbial imprint on floral nectar sugar variation in a tropical plant community." *Ann Bot* 110 (6):1173-83. doi: 10.1093/aob/mcs183.

Carter, Clay, Rosanne Healy, Nicole M. O'Tool, S. M. Saqlan Naqvi, Gang Ren, Sanggyu Park, Gwyn A. Beattie, Harry T. Horner, and Robert W. Thornburg. 2007. "Tobacco Nectaries Express a Novel NADPH Oxidase Implicated in the Defense of Floral Reproductive Tissues against Microorganisms." *Plant Physiology* 143 (1):389-399. doi: 10.1104/pp.106.089326.

Carter, Clay, and Robert W. Thornburg. 2004. "Is the nectar redox cycle a floral defense against microbial attack?" *Trends in Plant Science* 9 (7):320-324. doi: https://doi.org/10.1016/j.tplants.2004.05.008.

Carter, Dee A., Shona E. Blair, Nural N. Cokcetin, Daniel Bouzo, Peter Brooks, Ralf Schothauer, and Elizabeth J. Harry. 2016. "Therapeutic Mānuka Honey: No Longer So Alternative." *Frontiers in Microbiology* 7:569. doi: 10.3389/fmicb.2016.00569.

Chao, Anne, and Lou Jost. 2015. "Estimating diversity and entropy profiles via discovery rates of new species." *Methods in Ecology and Evolution* 6 (8):873-882. doi: 10.1111/2041-210X.12349.

Clarridge, Jill E. 2004. "Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases." *Clinical Microbiology Reviews* 17 (4):840-862. doi: 10.1128/cmr.17.4.840-862.2004.

Clearwater, Michael J., Maria Revell, Stevie Noe, and Merilyn Manley-Harris. 2018. "Influence of genotype, floral stage, and water stress on floral nectar yield and composition of mānuka (*Leptospermum scoparium*)." *Annals of Botany* 121 (3):501-512. doi: 10.1093/aob/mcx183.

Cray, Jonathan A., Andrew N. W. Bell, Prashanth Bhaganna, Allen Y. Mswaka, David J. Timson, and John E. Hallsworth. 2013. "The biology of habitat dominance; can

microbes behave as weeds?" *Microbial Biotechnology* 6 (5):453-492. doi: 10.1111/1751-7915.12027.

Dawson, M. 1997. "A history of *Leptospermum scoparium* in cultivation – discoveries from the wild." *The New Plantsman* 4:51-59.

Deng, Ye, Yi-Huei Jiang, Yunfeng Yang, Zhili He, Feng Luo, and Jizhong Zhou. 2012. "Molecular ecological network analyses." *BMC Bioinformatics* 13 (1):113. doi: 10.1186/1471-2105-13-113.

Edgar RC. Accuracy of microbial community diversity estimated by closed- and open-reference OTUs. PeerJ. 2017; 5:e3889. https://doi.org/10.7717/peerj.3889 PMID: 29018622

Faith, D. P., & Baker, A. M. (2007). Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evol Bioinform Online, 2*, 121-128.

Faust, Karoline, and Jeroen Raes. 2012. "Microbial interactions: from networks to models." *Nature Reviews Microbiology* 10 (8):538-550. doi: 10.1038/nrmicro2832.

Fedoroňko, Michal & Temkovic, Peter & Königstein, Josef & Kováčik, Vladimir & Tvaroška, Igor. (1980). Study of the kinetics and mechanism of the acid-base-catalyzed enolization of hydroxyacetaldehyde and methoxyacetaldehyde. Carbohydrate Research. 87. 35–50. 10.1016/S0008-6215(00)85189-7.

Finlay, Bland J. 2004. "Protist taxonomy: an ecological perspective." *Philosophical Transactions of the Royal Society B: Biological Sciences* 359 (1444):599-610.

Friedman, Jonathan, and Eric J. Alm. 2012. "Inferring Correlation Networks from Genomic Survey Data." *PLOS Computational Biology* 8 (9):e1002687. doi: 10.1371/journal.pcbi.1002687.

Gilbert, Benjamin, and Jonathan M. Levine. 2017. "Ecological drift and the distribution of species diversity." *Proceedings of the Royal Society B: Biological Sciences* 284 (1855):20170507. doi: doi:10.1098/rspb.2017.0507.

Gloor, Gregory B., Jean M. Macklaim, Vera Pawlowsky-Glahn, and Juan J. Egozcue. 2017. "Microbiome Datasets Are Compositional: And This Is Not Optional." *Frontiers in Microbiology* 8 (2224). doi: 10.3389/fmicb.2017.02224.

Godley, E. J. 1979. "Flower biology in New Zealand." *New Zealand Journal of Botany* 17 (4):441-466. doi: 10.1080/0028825x.1979.10432564.

González-Teuber, Marcia, Sascha Eilmus, Alexander Muck, Ales Svatos, and Martin Heil. 2009. "Pathogenesis related proteins protect extrafloral nectar from microbial

infestation." *The Plant Journal* 58 (3):464-473. doi: 10.1111/j.1365-313X.2009.03790.x.

Good, Ashley P., Marie-Pierre L. Gauthier, Rachel L. Vannette, and Tadashi Fukami. 2014. "Honey Bees Avoid Nectar Colonized by Three Bacterial Species, But Not by a Yeast Species, Isolated from the Bee Gut." *PLOS ONE* 9 (1):e86494. doi: 10.1371/journal.pone.0086494.

Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE. Human genetics shape the gut microbiome. Cell. 2014 Nov 6;159(4):789-99. doi: 10.1016/j.cell.2014.09.053. PMID: 25417156; PMCID: PMC4255478.

Goodwin, Sara, John D. McPherson, and W. Richard McCombie. 2016. "Coming of age: ten years of next-generation sequencing technologies." *Nature Reviews Genetics* 17 (6):333-351. doi: 10.1038/nrg.2016.49.

Gray, Michael W., David Sankoff, and Robert J. Cedergren. 1984. "On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA." *Nucleic Acids Research* 12 (14):5837-5852. doi: 10.1093/nar/12.14.5837.

Harris, Warwick. 2002. "Variation of inherent seed capsule splitting in populations of *Leptospermum scoparium* (Myrtaceae) in New Zealand." *New Zealand Journal of Botany* 40 (3):405-417. doi: 10.1080/0028825X.2002.9512802.

Haynes, Winston. 2013. "Benjamini–Hochberg Method." In *Encyclopedia of Systems Biology*, edited by Werner Dubitzky, Olaf Wolkenhauer, Kwang-Hyun Cho and Hiroki Yokota, 78-78. New York, NY: Springer New York.

Herrera, Carlos M., and María I. Pozo. 2010. "Nectar yeasts warm the flowers of a winter-blooming plant." *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2009.2252.

Hendriksma, H. P., Oxman, K. L., & Shafir, S. (2014). Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant–pollinator interactions. *Journal of Insect Physiology*, *69*, 56–64. https://doi.org/https://doi.org/10.1016/j.jinsphys.2014.05.025

Hillwig, Melissa S., Anthony L. Contento, Alexander Meyer, Danielle Ebany, Diane C. Bassham, and Gustavo C. MacIntosh. 2011. "RNS2, a conserved member of the RNase T2 family, is necessary for ribosomal RNA decay in plants." *Proceedings of*

the National Academy of Sciences of the United States of America 108 (3):1093-1098. doi: 10.1073/pnas.1009809108.

Hu, Z. C., Y. G. Zheng, and Y. C. Shen. 2011. "Use of glycerol for producing 1,3-dihydroxyacetone by Gluconobacter oxydans in an airlift bioreactor." *Bioresource Technology* 102 (14):7177-7182. doi: 10.1016/j.biortech.2011.04.078.

Jacquemyn, Hans, Marijke Lenaerts, Daniel Tyteca, and Bart Lievens. 2013. "Microbial diversity in the floral nectar of seven *Epipactis (Orchidaceae)* species." *MicrobiologyOpen* 2 (4):644-658. doi: 10.1002/mbo3.103.

Jani, S., A. L. Seely, V. Gl Peabody, A. Jayaraman, and M. D. Manson. 2017. "Chemotaxis to self-generated AI-2 promotes biofilm formation in Escherichia coli." *Microbiology*. doi: 10.1099/mic.0.000567.

Kato, Yoji, Rie Fujinaka, Akari Ishisaka, Yoko Nitta, Noritoshi Kitamoto, and Yosuke Takimoto. 2014. "Plausible Authentication of Mānuka Honey and Related Products by Measuring Leptosperin with Methyl Syringate." *Journal of Agricultural and Food Chemistry* 62 (27):6400-6407. doi: 10.1021/jf501475h.

Kato, Yoji, Natsuki Umeda, Asuna Maeda, Daiki Matsumoto, Noritoshi Kitamoto, and Hiroe Kikuzaki. 2012. "Identification of a Novel Glycoside, Leptosin, as a Chemical Marker of Mānuka Honey." *Journal of Agricultural and Food Chemistry* 60 (13):3418-3423. doi: 10.1021/jf300068w.

Kembel, Steven W., Martin Wu, Jonathan A. Eisen, and Jessica L. Green. 2012. "Incorporating 16S gene copy number information improves estimates of microbial diversity and abundance." *PLoS Computational Biology* 8 (10):e1002743-e1002743. doi: 10.1371/journal.pcbi.1002743.

Kevan, Peter G., and Blandina F. Viana. 2003. "The global decline of pollination services." *Biodiversity* 4 (4):3-8. doi: 10.1080/14888386.2003.9712703.

Kono, Nobuaki, and Kazuharu Arakawa. 2019. "Nanopore sequencing: Review of potential applications in functional genomics." Development, Growth & Differentiation 61 (5):316-326. doi: 10.1111/dgd.12608.

Leigh, M. B., L. Taylor, and J. D. Neufeld. 2010. "Clone Libraries of Ribosomal RNA Gene Sequences for Characterization of Bacterial and Fungal Communities." In *Handbook of Hydrocarbon and Lipid Microbiology*, edited by Kenneth N. Timmis, 3969-3993. Berlin, Heidelberg: Springer Berlin Heidelberg.

Lennon, Jay, and Stuart Jones. 2011. "Microbial seed banks: The ecological and evolutionary implications of dormancy." *Nature Reviews Microbiology* 9:119-30. doi: 10.1038/nrmicro2504.

Lidia, Stasiak-Różańska, and Błażejak Stanisław. 2012. "Production of dihydroxyacetone from an aqueous solution of glycerol in the reaction catalyzed by an immobilized cell preparation of acetic acid bacteria *Gluconobacter oxydans* ATCC 621." *European Food Research and Technology* 235 (6):1125-1132. doi: 10.1007/s00217-012-1846-0.

Lin, I. Winnie, Davide Sosso, Li-Qing Chen, Klaus Gase, Sang-Gyu Kim, Danny Kessler, Peter M. Klinkenberg, Molly K. Gorder, Bi-Huei Hou, Xiao-Qing Qu, Clay J. Carter, Ian T. Baldwin, and Wolf B. Frommer. 2014. "Nectar secretion requires sucrose phosphate synthases and the sugar transporter SWEET9." *Nature* 508:546. doi: 10.1038/nature13082https://www.nature.com/articles/nature13082#supplementary-information.

Lämsä J, Kuusela E, Tuomi J, Juntunen S, Watts PC. Low dose of neonicotinoid insecticide reduces foraging motivation of bumblebees. Proc Biol Sci. 2018 Jul 25;285(1883):20180506. doi: 10.1098/rspb.2018.0506. PMID: 30051863; PMCID: PMC6083263.

Lloyd, David G. 1985. "Progress in understanding the natural history of New Zealand plants." *New Zealand Journal of Botany* 23 (4):707-722. doi: 10.1080/0028825x.1985.10434239.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." *Genome Biology* 15 (12):550. doi: 10.1186/s13059-014-0550-8.

Lozupone, Catherine, and Rob Knight. 2005. "UniFrac: a New Phylogenetic Method for Comparing Microbial Communities." *Applied and Environmental Microbiology* 71 (12):8228-8235. doi: 10.1128/aem.71.12.8228-8235.2005.

Luo, F., Zhong, J., Yang, Y., Scheuermann, R. H., & Zhou, J. (2006). Application of random matrix theory to biological networks. *Physics Letters A*, *357*(6), 420-423.

Mamlouk, Dhouha, and Maria Gullo. 2013. "Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation." *Indian Journal of Microbiology* 53 (4):377-384. doi: 10.1007/s12088-013-0414-z.

Mandakovic, Dinka, Claudia Rojas, Jonathan Maldonado, Mauricio Latorre, Dante Travisany, Erwan Delage, Audrey Bihouée, Géraldine Jean, Francisca P. Díaz, Beatriz

Fernández-Gómez, Pablo Cabrera, Alexis Gaete, Claudio Latorre, Rodrigo A. Gutiérrez, Alejandro Maass, Verónica Cambiazo, Sergio A. Navarrete, Damien Eveillard, and Mauricio González. 2018. "Structure and co-occurrence patterns in microbial communities under acute environmental stress reveal ecological factors fostering resilience." *Scientific Reports* 8 (1):5875. doi: 10.1038/s41598-018-23931-0.

Manriquez, B., Muller, D., & Prigent-Combaret, C. (2021). Experimental Evolution in Plant-Microbe Systems: A Tool for Deciphering the Functioning and Evolution of Plant-Associated Microbial Communities. *Frontiers in microbiology*, *12*, 619122. https://doi.org/10.3389/fmicb.2021.619122

Manson, J. S., M. C. Otterstatter, and J. D. Thomson. 2010. "Consumption of a nectar alkaloid reduces pathogen load in bumble bees." *Oecologia* 162 (1):81-9. doi: 10.1007/s00442-009-1431-9.

Mavric, Elvira, Silvia Wittmann, Gerold Barth, and Thomas Henle. 2008. "Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Mānuka (*Leptospermum scoparium*) honeys from New Zealand." *Molecular Nutrition & Food Research* 52 (4):483-489. doi: 10.1002/mnfr.200700282.

Ministry for Primary Industries. (2020). Apiculture: Ministry for Primary Industries 2020 apiculture monitoring report.

McFrederick, Q. S., Thomas, J. M., Neff, J. L., Vuong, H. Q., Russell, K. A., Hale, A. R., & Mueller, U. G. (2017). Flowers and Wild Megachilid Bees Share Microbes. *Microbial ecology*, *73*(1), 188–200. https://doi.org/10.1007/s00248-016-0838-1

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., . . . Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences, 110*(9), 3229-3236. 10.1073/pnas.1218525110

McMurdie, Paul J., and Susan Holmes. 2014. "Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible." *PLOS Computational Biology* 10 (4):e1003531. doi: 10.1371/journal.pcbi.1003531.

Needleman, Saul B., and Christian D. Wunsch. 1970. "A general method applicable to the search for similarities in the amino acid sequence of two proteins." *Journal of Molecular Biology* 48 (3):443-453. doi: https://doi.org/10.1016/0022-2836(70)90057-4.

Nepi, Massimo. 2014. "Beyond nectar sweetness: the hidden ecological role of non-protein amino acids in nectar." *Journal of Ecology* 102 (1):108-115. doi: 10.1111/1365-2745.12170.

Newman, M. E. J. 2006. "Modularity and community structure in networks." *Proceedings of the National Academy of Sciences* 103 (23):8577-8582. doi: 10.1073/pnas.0601602103.

Newman, M. E. J., and M. Girvan. 2004. "Finding and evaluating community structure in networks." *Physical Review E* 69 (2):026113. doi: 10.1103/PhysRevE.69.026113.

Newstrom, Linda, and Alastair W. Robertson. 2005. "Progress in understanding pollination systems in New Zealand." *New Zealand Journal of Botany* 43 (1):1-59. doi: 10.1080/0028825X.2005.9512943.

Newton, R. J., L. E. Griffin, K. M. Bowles, C. Meile, S. Gifford, C. E. Givens, E. C. Howard, E. King, C. A. Oakley, C. R. Reisch, J. M. Rinta-Kanto, S. Sharma, S. Sun, V. Varaljay, M. Vila-Costa, J. R. Westrich, and M. A. Moran. 2010. "Genome characteristics of a generalist marine bacterial lineage." *ISME J* 4 (6):784-98. doi: 10.1038/ismej.2009.150.

Nickless, Elizabeth M., Christopher W. N. Anderson, Georgie Hamilton, Jonathan M. Stephens, and Jason Wargent. 2017. "Soil influences on plant growth, floral density and nectar yield in three cultivars of mānuka (*Leptospermum scoparium*)." *New Zealand Journal of Botany* 55 (2):100-117. doi: 10.1080/0028825X.2016.1247732.

Nickless, Elizabeth M., Stephen E. Holroyd, Georgie Hamilton, Keith C. Gordon, and Jason J. Wargent. 2016. "Analytical method development using FTIR-ATR and FT-Raman spectroscopy to assay fructose, sucrose, glucose and dihydroxyacetone, in *Leptospermum scoparium* nectar." *Vibrational Spectroscopy* 84:38-43. doi: https://doi.org/10.1016/j.vibspec.2016.02.011.

Nicolson, Susan W., Leo de Veer, Angela Köhler, and Christian W. W. Pirk. 2013. "Honeybees prefer warmer nectar and less viscous nectar, regardless of sugar concentration." *Proceedings of the Royal Society B: Biological Sciences* 280 (1767):20131597. doi: 10.1098/rspb.2013.1597.

Nicolson SW, Thornburg RW (2007) Nectar chemistry. In: Nicolson SW, Nepi M, Pacini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 215–264

Noe, Stevie Merilyn Manley-Harris & Michael J. Clearwater (2019) Floral nectar of wild mānuka (*Leptospermum scoparium*) varies more among plants than among sites, New

Zealand Journal of Crop and Horticultural Science, 47:4, 282-296, DOI: 10.1080/01140671.2019.1670681

Norton, Amanda M., Leah N. McKenzie, Peter R. Brooks, and Linda J. Pappalardo. 2015. "Quantitation of Dihydroxyacetone in Australian *Leptospermum* Nectar via High-Performance Liquid Chromatography." *Journal of Agricultural and Food Chemistry* 63 (29):6513-6517. doi: 10.1021/acs.jafc.5b01930.

Noutsos, Christos, Ann M. Perera, Basil J. Nikolau, Samuel M. D. Seaver, and Doreen H. Ware. 2015. "Metabolomic Profiling of the Nectars of *Aquilegia pubescens* and *A. Canadensis*." *PLOS ONE* 10 (5):e0124501. doi: 10.1371/journal.pone.0124501.

Olesen, Jens, and Alfredo Valido. 2003. *Lizards as pollinators and seed dispersers: An island phenomenon*. Vol. 18.

Pace, N. R. 1997. "A molecular view of microbial diversity and the biosphere." *Science* 276 (5313):734-740. doi: 10.1126/science.276.5313.734.

Park, Sanggyu, and Robert W. Thornburg. 2009. "Biochemistry of Nectar Proteins." *Journal of Plant Biology* 52 (1):27-34. doi: 10.1007/s12374-008-9007-5.

Pollock, Jolinda, Laura Glendinning, Trong Wisedchanwet, and Mick Watson. 2018. "The Madness of Microbiome: Attempting To Find Consensus “Best Practice” for 16S Microbiome Studies." *Applied and Environmental Microbiology* 84 (7):e02627-17. doi: 10.1128/AEM.02627-17.

Poretsky, Rachel, Luis M. Rodriguez-R, Chengwei Luo, Despina Tsementzi, and Konstantinos T. Konstantinidis. 2014. "Strengths and Limitations of 16S rRNA Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics." *PLOS ONE* 9 (4):e93827. doi: 10.1371/journal.pone.0093827.

Porter, Noel G., and Alistair L. Wilkins. 1999. "Chemical, physical and antimicrobial properties of essential oils of *Leptospermum scoparium* and *Kunzea ericoides*." *Phytochemistry* 50 (3):407-415. doi: https://doi.org/10.1016/S0031-9422(98)00548-2.

Porter, Teresita M., and Mehrdad Hajibabaei. 2018. "Scaling up: A guide to high-throughput genomic approaches for biodiversity analysis." *Molecular Ecology* 27 (2):313-338. doi: https://doi.org/10.1111/mec.14478.

Pozo, M. I., C. M. Herrera, and P. Bazaga. 2011. "Species richness of yeast communities in floral nectar of southern Spanish plants." *Microb Ecol* 61 (1):82-91. doi: 10.1007/s00248-010-9682-x.

Pozo, María I., Marc-André Lachance, and Carlos M. Herrera. 2012. "Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in

community assembly." *FEMS Microbiology Ecology* 80 (2):281-293. doi: 10.1111/j.1574-6941.2011.01286.x.

Pozo, Maria J., Clara de Vega, Azucena Canto, and Carlos M. Herrera. 2009. "Presence of yeasts in floral nectar is consistent with the hypothesis of microbial-mediated signaling in plant-pollinator interactions." *Plant Signaling & Behavior* 4 (11):1102-1104. doi: 10.4161/psb.4.11.9874.

Prasifka, Jarrad, Mallinger Rachel, Portlas Zoe, Hulke Brent, Fugate, Karen, Paradis Travis, Hampton Marshall , Carter, Clay. (2018). Using Nectar-Related Traits to Enhance Crop-Pollinator Interactions. Frontiers in Plant Science. 9. 812. 10.3389/fpls.2018.00812.

Primack, Richard B. 1978. "Evolutionary aspects of wind pollination in the genus plantago (Plantaginaceae)." *New Phytologist* 81 (2):449-458. doi: 10.1111/j.1469-8137.1978.tb02650.x.

Primack, Richard B., and David G. Lloyd. 1980. "Andromonoecy in the New Zealand montane shrub mānuka, *Leptospermum scoparium* (myrtaceae)." *American Journal of Botany* 67 (3):361-368. doi: doi:10.1002/j.1537-2197.1980.tb07661.x.

Prodan A, Tremaroli V, Brolin H, Zwinderman AH, Nieuwdorp M, Levin E. 2020. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *PLoS One* 15:e0227434. 10.1371/journal.pone.0227434. Pu

Rering, Caitlin C., John J. Beck, Griffin W. Hall, Mitchell M. McCartney, and Rachel L. Vannette. 2017. "Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator." *New Phytologist*. doi: 10.1111/nph.14809.

Robinson, M. D., and A. Oshlack. 2010. "A scaling normalization method for differential expression analysis of RNA-seq data." *Genome Biol* 11 (3):R25. doi: 10.1186/gb-2010-11-3-r25.

Robinson, Mark D., and Gordon K. Smyth. 2007. "Moderated statistical tests for assessing differences in tag abundance." *Bioinformatics* 23 (21):2881-2887. doi: 10.1093/bioinformatics/btm453.

Rocha, Eduardo P. C. 2018. "Neutral Theory, Microbial Practice: Challenges in Bacterial Population Genetics." *Molecular Biology and Evolution* 35 (6):1338-1347. doi: 10.1093/molbev/msy078.

Ronghua, Yin, A. F. Mark, and J. B. Wilson. 1984. "Aspects of the ecology of the indigenous shrub *Leptospermum scoparium* (Myrtaceae) in New Zealand." *New Zealand Journal of Botany* 22 (4):483-507. doi: 10.1080/0028825X.1984.10425282.

Rosselló-Mora, Ramon, and Rudolf Amann. 2001. "The species concept for prokaryotes." *FEMS Microbiology Reviews* 25 (1):39-67. doi: https://doi.org/10.1111/j.1574-6976.2001.tb00571.x.

Roy, Samit. 2014. "Molecular Markers in Phylogenetic Studies-A Review." *Journal of Phylogenetics & Evolutionary Biology* 02. doi: 10.4172/2329-9002.1000131.

Samuni-Blank, Michal, Ido Izhaki, Sivan Laviad, Avi Bar-Massada, Yoram Gerchman, and Malka Halpern. 2014. "The Role of Abiotic Environmental Conditions and Herbivory in Shaping Bacterial Community Composition in Floral Nectar." *PLOS ONE* 9 (6):e99107. doi: 10.1371/journal.pone.0099107.

Schaeffer, Robert N., Rachel L. Vannette, and Rebecca E. Irwin. 2015. "Nectar yeasts in *Delphinium nuttallianum* (Ranunculaceae) and their effects on nectar quality." *Fungal Ecology* 18:100-106. doi: https://doi.org/10.1016/j.funeco.2015.09.010.

Shannon, C. E. 1948. "A mathematical theory of communication." *The Bell System Technical Journal* 27 (3):379-423. doi: 10.1002/j.1538-7305.1948.tb01338.x.

Solomon, R. D. Jebakumar, V. Satheeja Santhi, and Vimalan Jayaraj. 2006. "Prevalence of Antibiotics in Nectar and Honey in South Tamilnadu, India." *Integrative Biosciences* 10 (3):163-167. doi: 10.1080/17386357.2006.9647297.

Stackebrandt, Erko, Wilhelm Frederiksen, George M Garrity, Patrick A D Grimont, Peter Kämpfer, Martin C J Maiden, Xavier Nesme, Ramon Rosselló-Mora, Jean Swings, Hans G Trüper, Luc Vauterin, Alan C Ward, and William B Whitman. 2002. "Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology." *International Journal of Systematic and Evolutionary Microbiology* 52 (3):1043-1047. doi: https://doi.org/10.1099/00207713-52-3-1043.

Stephens, J. M. C., P. C. Molan, and B. D. Clarkson. 2005. "A review of *Leptospermum scoparium* (Myrtaceae) in New Zealand." *New Zealand Journal of Botany* 43 (2):431-449. doi: 10.1080/0028825X.2005.9512966.

Stevenson, Philip C., Susan W. Nicolson, and Geraldine A. Wright. 2017. "Plant secondary metabolites in nectar: impacts on pollinators and ecological functions." *Functional Ecology* 31 (1):65-75. doi: https://doi.org/10.1111/1365-2435.12761.

Sung, J., Kim, S., Cabatbat, J. et al. Global metabolic interaction network of the human gut microbiota for context-specific community-scale analysis. Nat Commun 8, 15393 (2017). https://doi.org/10.1038/ncomms15393

Tang, Min, Jianqiang Sun, Kentaro Shimizu, and Koji Kadota. 2015. "Evaluation of methods for differential expression analysis on multi-group RNA-seq count data." *BMC Bioinformatics* 16 (1):360. doi: 10.1186/s12859-015-0794-7.

Taormina, Peter J., Brendan A. Niemira, and Larry R. Beuchat. 2001. "Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power." *International Journal of Food Microbiology* 69 (3):217-225. doi: https://doi.org/10.1016/S0168-1605(01)00505-0.

Turner, Thomas R., Euan K. James, and Philip S. Poole. 2013. "The plant microbiome." *Genome Biology* 14 (6):209. doi: 10.1186/gb-2013-14-6-209.

Vannette, Rachel L., Marie-Pierre L. Gauthier, and Tadashi Fukami. 2013. "Nectar bacteria, butnot yeast, weaken a plant–pollinator mutualism." *Proceedings of the Royal Society B: Biological Sciences* 280 (1752). doi: 10.1098/rspb.2012.2601.

Vannette, Rachel L., and Tadashi Fukami. 2016. "Nectar microbes can reduce secondary metabolites in nectar and alter effects on nectar consumption by pollinators." *Ecology* 97 (6):1410-1419. doi: 10.1890/15-0858.1.

Vannette, R. L., Bichier, P., & Philpott, S. M. (2017). The presence of aggressive ants is associated with fewer insect visits to and altered microbe communities in coffee flowers. *Basic and Applied Ecology*, *20*, 62-74.

von Arx, Martin, Autumn Moore, Goggy Davidowitz, and A. Elizabeth Arnold. 2019. "Diversity and distribution of microbial communities in floral nectar of two night-blooming plants of the Sonoran Desert." *PLOS ONE* 14 (12):e0225309. doi: 10.1371/journal.pone.0225309.

Vranova V, Rejsek K, Skene KR, Formanek P (2010) Non-protein amino acids: plant, soil and ecosystem interactions. Plant Soil 342: 31–48

Walters, Kendra E., and Jennifer B. H. Martiny. 2020. "Alpha-, beta-, and gamma-diversity of bacteria varies across habitats." *PloS One* 15 (9):e0233872-e0233872. doi: 10.1371/journal.pone.0233872.

Wardle, Peter. 2008. "New Zealand Forest to Alpine Transitions in Global Context." *Arctic, Antarctic, and Alpine Research* 40 (1):240-249. doi: 10.1657/1523-0430(06-066)[WARDLE]2.0.CO;2.

Weiss, Sophie J., Zhenjiang Xu, Amnon Amir, Shyamal Peddada, Kyle Bittinger, Antonio Gonzalez, Catherine Lozupone, Jesse R. Zaneveld, Yoshiki Vazquez-Baeza, Amanda Birmingham, and Rob Knight. 2015. "Effects of library size variance, sparsity, and compositionality on the analysis of microbiome data." *PeerJ PrePrints* 3:e1157v1. doi: 10.7287/peerj.preprints.1157v1.

Weiss, Sophie, Zhenjiang Zech Xu, Shyamal Peddada, Amnon Amir, Kyle Bittinger, Antonio Gonzalez, Catherine Lozupone, Jesse R. Zaneveld, Yoshiki Vázquez-Baeza, Amanda Birmingham, Embriette R. Hyde, and Rob Knight. 2017. "Normalization and microbial differential abundance strategies depend upon data characteristics." *Microbiome* 5 (1):27. doi: 10.1186/s40168-017-0237-y.

Weston, Roderick J. 2000. "The contribution of catalase and other natural products to the antibacterial activity of honey: a review." *Food Chemistry* 71 (2):235-239. doi: https://doi.org/10.1016/S0308-8146(00)00162-X.

Williams, Jim P., and John E. Hallsworth. 2009. "Limits of life in hostile environments: no barriers to biosphere function?" *Environmental Microbiology* 11 (12):3292-3308. doi: 10.1111/j.1462-2920.2009.02079.x.

Williams, S., J. King, M. Revell, M. Manley-Harris, M. Balks, F. Janusch, M. Kiefer, M. Clearwater, P. Brooks, and M. Dawson. 2014. "Regional, annual, and individual variations in the dihydroxyacetone content of the nectar of Mānuka (*Leptospermum scoparium*) in New Zealand." *J Agric Food Chem* 62 (42):10332-40. doi: 10.1021/jf5045958.

Willis, Ciara, Dhwani Desai, and Julie LaRoche. 2019. "Influence of 16S rRNA variable region on perceived diversity of marine microbial communities of the Northern North Atlantic." *FEMS microbiology letters* 366 (13):fnz152. doi: 10.1093/femsle/fnz152.

Woese, Carl R., and George E. Fox. 1977. "Phylogenetic structure of the prokaryotic domain: The primary kingdoms." *Proceedings of the National Academy of Sciences* 74 (11):5088-5090. doi: 10.1073/pnas.74.11.5088.

Woese, Carl R., George E. Fox, Lawrence Zablen, Tsuneko Uchida, Linda Bonen, Kenneth Pechman, Bobby J. Lewis, and David Stahl. 1975. "Conservation of primary structure in 16S ribosomal RNA." *Nature* 254 (5495):83-86. doi: 10.1038/254083a0.

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., . .Stevenson, P.C (2013). Caffeine in Floral Nectar Enhances a Pollinator's *Science, 339*(6124), 1202-1204. 10.1126/science.1228806

Zelezniak, Aleksej, Sergej Andrejev, Olga Ponomarova, Daniel R. Mende, Peer Bork, and Kiran Raosaheb Patil. 2015. "Metabolic dependencies drive species co-occurrence in diverse microbial communities." *Proceedings of the National Academy of Sciences* 112 (20):6449-6454. doi: 10.1073/pnas.1421834112.

Zemenick, Ash T., Rachel L. Vannette, and Jay A. Rosenheim. 2019. "Linked networks reveal dual roles of insect dispersal and species sorting for bacterial communities in flowers." *bioRxiv*:847376. doi: 10.1101/847376.

Zheng, Xiao-juan, Kui-qi Jin, Lei Zhang, Gang Wang, and Yu-Peng Liu. 2016. "Effects of oxygen transfer coefficient on dihydroxyacetone production from crude glycerol." *Brazilian Journal of Microbiology* 47 (1):129-135. doi: https://doi.org/10.1016/j.bjm.2015.11.020.

Zheng, Zhi, Min Luo, Jianer Yu, Jianli Wang, and Jianbing Ji. 2012. "Novel Process for 1,3-Dihydroxyacetone Production from Glycerol. 1. Technological Feasibility Study and Process Design." *Industrial & Engineering Chemistry Research* 51 (9):3715-3721. doi: 10.1021/ie201710h.

Zieslin, N., and Varda Gottesman. 1986. "Environmental factors involved in growth, flowering and post-harvest behaviour of flowers of *Leptospermum scoparium* J.R. & Forst." *Israel Journal of Botany* 35 (2):101-108. doi: 10.1080/0021213X.1986.10677045.

CHAPTER 2.

Microbial diversity analysis of mānuka (*Leptospermum scoparium*) nectar

ABSTRACT

Microorganisms in plants play important ecological roles. Bacteria and fungi in the nectar can modify its chemistry and the interactions with pollinators. Pollinators can also act as vectors, transporting microorganisms from one flower to another. To understand plant-pollinator-microorganism interactions in the nectar of mānuka plants (*Leptospermum scoparium*), I bagged branches, restricting the access of aerial pollinators to flowers from six different mānuka cultivated genotypes and one wild cultivar, and then compared the results to those of unbagged plants. I used a metabarcoding next generation sequencing approach to identify bacterial and fungal nectar communities and HPLC analysis to estimate sugar (glucose, fructose, and sucrose) and DHA (dihydroxyacetone) content in the nectar. Bacterial community composition of the nectar was not modified by restricting pollinators although there was differential abundance of taxa comparing unbagged (control) versus bagged flowers. Some bacteria were present in all the genotypes but in varying abundance. Unlike bacteria, fungal communities were correlated with aerial pollinators as changes in alpha and beta diversity in fungal communities were detected as a treatment effect. Differences in the DHA content was found among genotypes but not between bagged and unbagged flowers, suggesting that DHA production is not related to the microorganisms associated with aerial pollinators. These results highlight the importance of microbial communities in the physiology and ecology of mānuka plants.

2.1. INTRODUCTION

Microorganisms are an essential part of several systems, having an impact on their hosts and their life processes (McFall-Ngai et al., 2013). In ecological processes such as pollination, there are multiple interactions between plants, pollinators, and microorganisms. Notably, pollinators are able to transport bacteria and fungi between flowers (Ushio et al., 2015), this can affect the composition of nectar (Wright et al., 2013).

Bacteria and yeast can change the nectar sugar ratios (Herrera et al., 2008), which in turn can alter amino acid content and concentration (Kevan et al., 1988, Peay et al., 2012). Both of these traits can influence foraging decisions by pollinators (Alm et al., 1990; Dyer et al., 2006). Moreover, bacteria are known to affect floral smell and thus pollinator attraction (Del Giudice et al., 2008), and floral volatiles can also affect interactions between bacteria and fungi (Effmert, Kalderás, Warnke, & Piechulla, 2012).

The diversity of bacteria and fungi in flowers and nectar is intriguing due to their adaptations to the chemistry and physical environment of nectar. The nectar contains sugars and amino acids that are very suitable for microorganisms. However, the presence of antimicrobial proteins and reactive oxygen species, such as hydrogen peroxide (H_2O_2), can influence the type and amount of nectar microbiota (Carter et al. 2007; Heil, 2011).

Several scientific reports have addressed the diversity of fungi and bacteria in plants of Northern latitudes. For example, Pozo et al. (2011) described the diversity of yeasts in Mediterranean plants and Shaeffer et al. (2015) studied the effect of nectar yeasts on the quality of nectar in Colorado plants. Furthermore, a recent study by Canto, Herrera, and Rodriguez (2017) investigated the imprint of nectar yeast species in nectar sugar composition and concentration in tropical plants from Yucatan-Mexico. However, no reports exist for the Southern hemisphere.

The techniques for identification and characterization of floral bacterial communities have extensively improved in the last decade. For instance, Junker and Keller (2015) studied the bacterial communities on flowers and leaves of *Metrosideros polymorpha* (Myrtaceae) and

found differences in community composition within the microhabitats of the flowers and leaves. Their findings showed that these differences strongly exceeded the influence of environmental variables such as precipitation, altitude, and geographic distance. In other research, Zarraonaindia et al. (2015) examined vine-associated bacteria across different grapevine organs (leaves, flowers, grapes, and roots) and found that the soil serves as a key source of vine-associated bacteria.

Mānuka nectar is known and valued for its dihydroxyacetone (DHA) content, a precursor of methylglyoxal, one of the quality markers for mānuka honey (Adams et al., 2009). In New Zealand, different mānuka clones and cultivars have been developed that have different flower morphologies and levels of DHA in their nectar (Clearwater et al., 2017), suggesting that there is a plant genetic component to DHA production. However, microorganisms also have the capability to either produce DHA by themselves (Z. Zheng et al., 2012) or to dephosphorylate DHAP to DHA (Wang, Meng, Li, Li, & You, 2019) that can then be used as an intermediate metabolite in several plant organelles, especially in chloroplasts (Chen & Thelen, 2010). Mānuka flowers possess photosynthetically active nectaries. Therefore, the interface of nectar-nectaries is a potential place for microbial DHA production.

Therefore, based on the ecological and economical characteristics of mānuka plants, the first aim of this study is to investigate the microbial diversity (bacterial and fungal) in six cultivated mānuka genotypes and one wild cultivar and to analyse sugars (glucose, fructose, and sucrose) and the DHA content in the nectar. A second objective is to determine the impact of aerial pollinators on the microbial community, DHA, and sugar content in the nectar. I predicted that there would be a varied microbial community in the genotypes studied and that there would be differences in the microorganisms within the plant genotypes. I also hypothesized that pollinators would influence the fungal and bacterial communities and nectar chemistry in the plant genotypes studied.

2.2. MATERIALS AND METHODS

2.2.1. Mānuka plants

This study involves mānuka plants growing at Massey University's experimental plots in Palmerston North, where different clones and wild cultivars are grown in patches of 10 plants (Fig.SF1), replicated four times per clone/cultivar, with only clover plants surrounding them. These particular mānuka plants consist of hyperproductive DHA mānuka clones bred and crossed from different regions of New Zealand and maintained by cuttings, and wild cultivars from Northland and Waikato that were planted from seeds at the field. A honeybee (*Apis mellifera*) hive was located nearby the mānuka clones and cultivars to ensure pollinator visitation.

The mānuka cultivars were colour coded: B blue, MG mint green, G green, O orange, P pink, Y Yellow and Cv cultivar (Fig.S2.7.1, Fig.S2.7.2a, and Fig.S2.7.2b). These cultivars had different rates of DHA concentration according to a previous two-year study (2014 and 2015), Y had the lowest DHA concentration in both years, while O and P produce the highest DHA concentration in 2014 and MG the highest in 2015 (Bohórquez Rodríguez de Medina, 2018)

2.2.2. Nectar sampling

Two treatments were applied to 10 mānuka plants from six clones and one cultivar: one set of plants was exposed to pollinators (unbagged) and the other set was pollinator restricted (bagged). For the bagged treatment, a branch with flowers was bagged at the budding stage and flowers were allowed to develop inside a breathable mesh (17 x 22 cm) up to the adult stage with 6 days of flower development, when they were picked. Four sides of each mānuka plant were bagged and branches marked. flowers were collected and stored at -20 ºC. Additional flowers at the same stage of development were collected in a similar manner from unbagged plants.

For each plant, 20 flowers from naïve or exposed flowers were studied. Nectar was removed from each flower by rinsing the receptacle with 10 µl of sterile MilliQ water. A total volume of 200 µl diluted nectar was removed from each type of plant per treatment. From this volume, 150 µl of the nectar solution was used for DNA extraction of the total community and the remainder for DHA and sugar analyses.

2.2.3. DNA extraction and PCR amplification

The volume of nectar separated for total community studies (150 µl) was filtered through a nitrocellulose membrane of 0.2 µm and the membrane was used for DNA extraction with a power soil kit (Qiagen). The metacommunity DNA after extraction was stored at –20 ºC for amplification procedures.

PCR was carried out by amplifying the V4 region of the 16S rRNA gene using the primers 515F (5′-GTGCCAGCMGCCGCGGTA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011) (Degenerate primers R = A,G ; Y = C,T; M = A,C; W = A,T; H = A,C,T; V = A,C,G) or the ITS1 region of the 18S rRNA gene using the primers ITS1_KYO1 (5′-TAGAGGAAGTAAAAGTCGTAA-3′) and ITS2_KYO2 (5′-TTYRCTRCGTTCTTCATC-3′) (Toju, Tanabe, Yamamoto, & Sato, 2012) to assess for prokaryotic and fungal communities, respectively. Full primer constructs used also included attached Illumina adaptors at the 5′ ends of each primer for library preparation (forward primer adapter 5′-(TCGTCGGCAGCGTCAGATGTATAAGAGACAG); reverse primer adapter, 5′-(GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG).

Reactions were prepared to a total of 25 µL with nuclease-free water as follows: For 16S, reactions contained 200 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.8X Platinum Taq HiFi buffer, 4 µl of GC enrich buffer (Invitrogen, MA, USA), 1 U Platinum Taq HiFi polymerase (Invitrogen, MA, USA), and 10 ng of template. Thermal cycling was carried out using a Mastercycler (Roche, USA), using the following conditions: For 16S, an initial 95ºC for 2 min, followed by 26 cycles of 95ºC for 45 s, 52ºC for 30 sec and 72ºC for 1 min followed by a final extension at 72ºC for 10 min. For ITS, an initial 95ºC for 10 min, followed by 26 cycles of 95ºC for 30 seconds 47ºC for 30 seconds and 72ºC for 1 minutes, followed by a final extension of 72ºC for 10 minutes. All PCR reactions for each primer set per sample were carried out in duplicate for bacteria to minimize stochastic PCR effects, and in triplicate for the ITS.

2.2.4. Bioinformatic Analysis

Samples comprised of 100% single run on the Illumina Miseq Platform. The adapters and primer sequences were removed from raw reads using SolexaQA++DynamicTrim (Cox, Peterson, & Biggs, 2010) for quality trimming at $p<0.001$ (equivalent Phred=20).

Paired-end reads were imported into Qiime2 (Bolyen et al. 2019). For merging of the forward and reverse sequence reads, VSEARCH was used and DADA2 was used for denoising. Taxonomic classification using Bayesian classifier against the SILVA SSU non-redundant database version 119 as reference alignment (Yilmaz et al., 2013) was performed with QIIME2. Fungal trimmed sequences were denoised with DADA2 optimizing trunc and trim to improve sequence length for identification. Taxonomic classification was performed with Bayesian trained classifier format of the UNITE version 8-99 database (Nilsson et al., 2018).

2.2.5. Sugars and DHA Analyses

2.2.5.1.) HPLC quantification of DHA and normalization

To perform the separation of the sample and analysis, High-performance liquid chromatography (HPLC) system requires two phases. The phase A was a solution of nanopure water combined with acetonitrile (ACN) at ratio of 70:30 (v/v). The phase B was 100% ACN. Reaction solutions were hidroxyacetone (HA) (3.01 mg/ml) with a O-(2,3,4,5,6-Pentaflurobenzyl) hydroxylamine (PFBHA) as a derivatising reagent (19.8 mg/ml in citrate buffer of 0.1 M adjusted to pH 4 with sodium hydroxide (NaOH) 4M). Six DHA standard aliquots (100, 75, 60, 50, 25, 0 µl) were taken from a stock solution of DHA (3.88 mg/ml). To analyse samples, 20 µl of nectar or standard sample was pipetted and added to a tube (4 ml). 25 µl of HA were added to every sample and samples were then mixed and shaken on a rotator table for one hour. A derivatizing reagent (PFBHA) was added, and samples were set to rest one hour. Finally, 1.5 ml ACN and 0.5 µl deionised Milli-Q water was added to the samples and filtered (new filter of 0.22 µm) before being measured by HPLC with a UV Diode array detector (λ=263 nm, UVD340U) using a Synergy Fusion column (size 4.6x7.5 mm, 4 µm particle size). The column was kept at 30 °C to assure stable conditions.

The DHA was normalized with the levels of total sugars (Tsugars) per sample (DHA/Tsugars), results were then multiplied by the dilution factor to obtain the final volume (mg/mL).

2.2.5.2.) HPLC quantification of fructose, glucose, and sucrose in nectar

To analyse the sugars, sucrose, glucose, and fructose, were separated in a column Sugar-Pak I (Waters, size 6.5x300mm). This column was kept at 75 °C. The single mobile phase solution was prepared by dissolving ethylenediaminetetraacetic acid (50mg/L) in deionised Milli-Q water and run using an isocratic elution system and a constant flow rate of 0.6 ml per minute. Samples were run at a volume of 20 µl using a RI detector (Shodex RI-101).

For each sugar, a 1% stock solution was made, and from that, five standards were prepared at different concentrations from 0.0025% to 0.5%.

2.2.6. Statistical Analyses

Amplicon sequence variants (ASVs) for fungi were filtered in 10% abundance and variance (for any feature to be retained a minimum in counts and in variance was set as a cut-off), normalization total sum scaling (TTS) (divides the number of reads that cluster within the same OTU by the total number of reads in each sample), and the α-diversity for fungi was explored by computing Shannon and evenness indices using qiime2 plugin and MicrobiomeAnalyst, while β-diversity was compared with PERMANOVA, and ANOSIM tests for Bray-Curtis and weighted UniFrac distances (Lozupone & Knight, 2005). The α-diversity was calculated using the number of observed amplicon sequence variants. The Kruskal-Wallis (pairwise) test was used to test for differences among plant type groups (clones and cultivar, $p<0.05$).

To estimate the fungi normalized abundance in a compositional form across all the clones studied, the ASVs were filtered at a prevalence of 20%, and a relative abundance of 0.01% data at species level was plotted through a heatmap.

Algorithms designed to determine features that are differentially abundant between two or more groups were used to evaluate differential abundance. Between the control (unbagged) and (bagged) treatment, DESeq2 (Li & Andrade, 2017) was used to normalize the bacterial ASVs and to detect significantly differential relative abundance between the two groups. To identify consistent trends observed across all samples, the differential expression (DE) analysis filtering criteria was based on p-value < 0.05, and estimated fold change <1/4, taxa units were considered significantly abundant between 2 of the examined classes. A multi-factor analysis with interaction between genotype and treatment was also applied using the same parameters.

Differential abundance for fungi and bacteria between bagged and unbagged plants was tested with edgeR (Li & Andrade, 2017), using Benjamini and Hochberg false-discovery rate (FDR) adjusted *p*-value (<0.05). For bacterial and fungal comparisons between genotypes, the LEfSe analysis tool was used after normalization and filtering the ASVs from MicrobiomeAnalyst (Chong et al., 2020). LEfSe is an algorithm for high-dimensional biomarker discovery and explanation that identifies and characterize the differences between two or more groups. It first applies a non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance with respect to the variable of interest. Biological significance is subsequently investigated using a set or pairwise tests among subclasses using (unpaired) Wilcoxon rank-sum test. The last step is Linear Discriminant Analysis to estimate the effect size of each differentially abundant feature and to perform dimension reduction.

For nectar DHA and sugar content, comparisons between genotypes and treatment were completed using SPSS (IBM) using a general linear model with genotypes and treatment as factors. Sugars were analysed with a Kruskal-Wallis non-parametric test.

2.3. RESULTS

2.3.1. Sequencing analysis

For all plant genotypes high quality sequences were obtained, and after denoising and filtering, 370382 ASVs were obtained for bacteria (TableS2.8.1). For Fungi, 133620 ASVs were obtained after denoising and filtering (TableS2.8.2). All samples rarefied with the sampling effort done suggesting the availability of enough reads to represent each microbiome from the community (Fig.S2.9.1, Fig.S2.9.2).

2.3.2. Microorganism diversity

The microbial taxonomic composition of mānuka nectar encompasses representatives in Eubacteria, Archaea, and Fungi. The majority of Bacteria and Archaea were represented by just a few orders including Pseudomonadales 43%, Rhizobiales 20%, Sphingomonadales 7%, Enterobacteriales 7%, Xanthomonadales 4%, Baccillales 4%, Corynebacteriales 4%. Fungal community was mostly comprised of classes of Microbotryomycetes 37.3%, Dothideomycetes 4.30%, Malasseziomycetes 17.49%, Sordariomycetes 10.80%,

Ustilaginomycetes 7.41%, Tremellomycetes 3.46%, Agaricomycetes 1.90%, and Cystobasidiomycetes 1.0%.

A core microbiome composition refers to the set of taxa that are detected at a certain abundance threshold in a population. To produce a core microbiome, the count data was transformed to compositional (relative) abundances and a heatmap produced that represents the overall community composition at a certain taxonomic level. A core microbiome at genus level is shown in Figure 2.1.

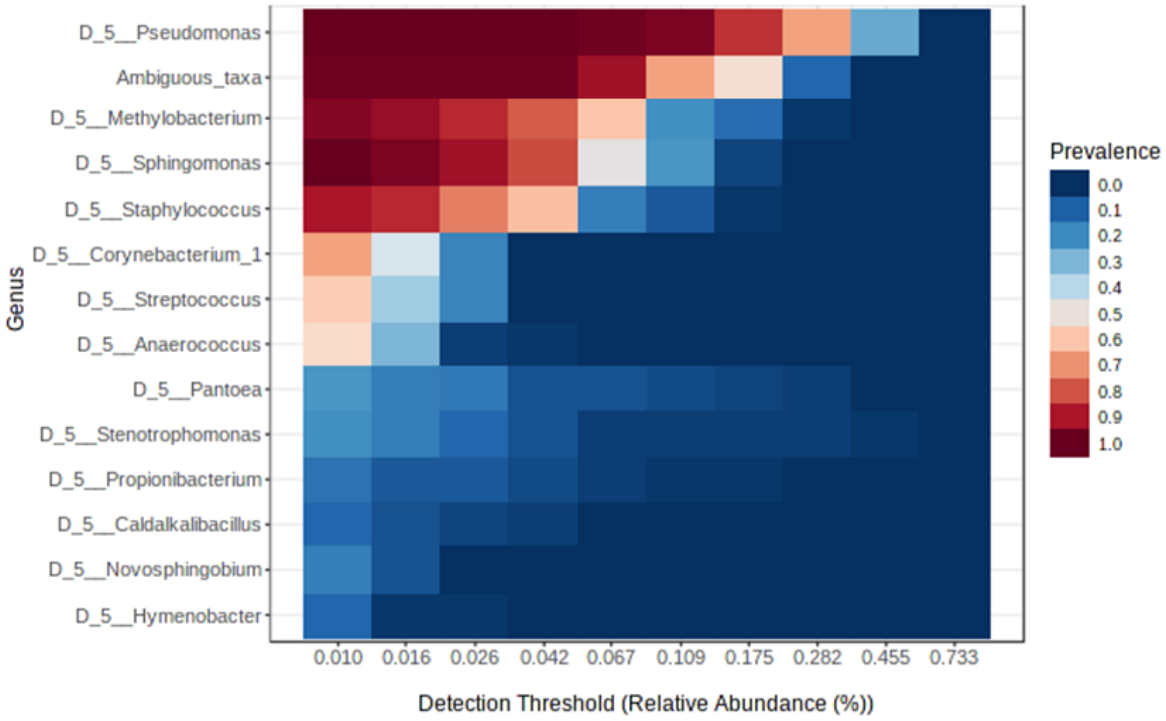


Figure 2.1. Heatmap of the core mānuka nectar microbiome at a genus level. The count data is transformed to compositional (relative) abundances, sample prevalence at a threshold of 10 and relative abundance 0.01%.

2.3.3. Bacterial Community composition

Mānuka nectar bacterial communities were dominated by Pseudomonadales, followed by Rhizobiales, Sphingomonadales, Corynebacteriales, Baccillales, Enterobacteriales, Xanthomonadales, Clostridiales, Lactobacillales and other taxa in smaller proportions (Fig. 2.2). Bacteria community composition per sample and genotype are shown in supplementary figures (Fig.S2.10.1- S2.10.5)

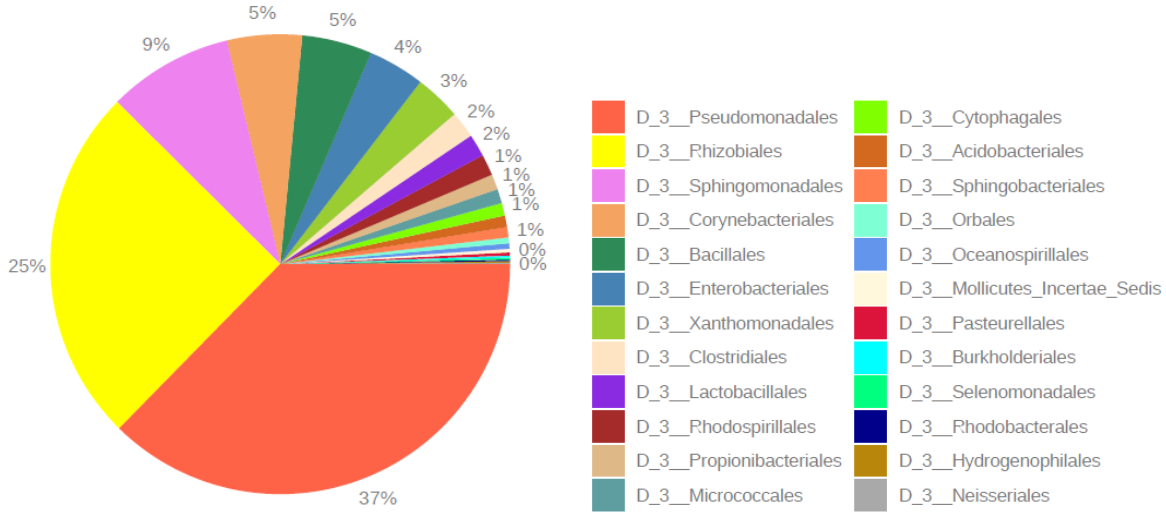
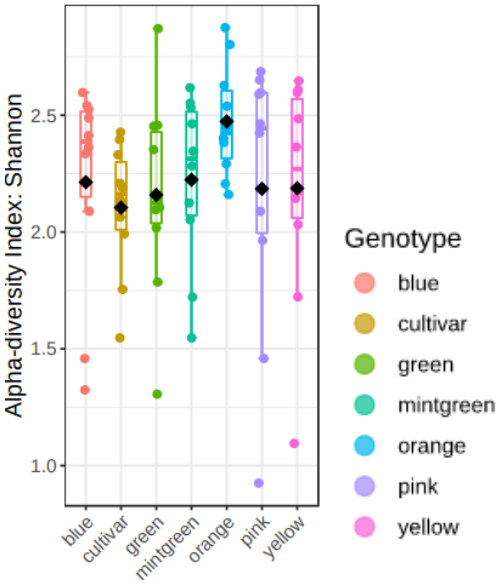
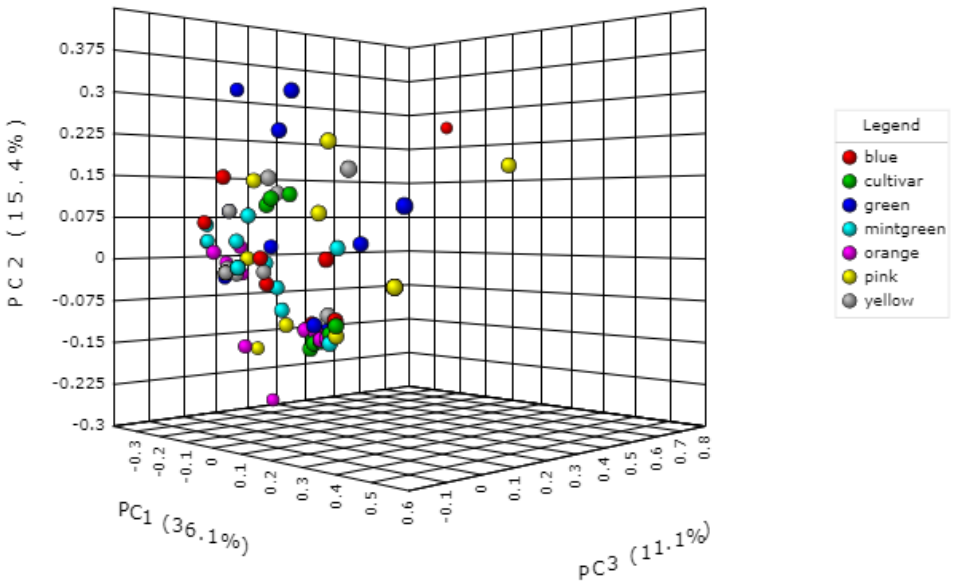


Figure 2.2. Composition of mānuka nectar bacterial community at order level across all samples.

Bacterial α diversity (species richness and evenness) did not differ among the genotypes studied (Mann-Whitney/Kruskal-Wallis, Shannon, P=0.3) (Fig. 2.3., Panel A) or between treatments (bagged and unbagged) (Mann-Whitney/Kruskal-Wallis, Shannon, P=0.6). However, β diversity (differences between community composition) was significantly different (dissimilarity distances between samples) (PERMANOVA, Bray-Curtis dissimilarity index, P =0.01) (Fig. 2.3. panel B).



Panel A.



Panel B.

Figure 2.3. Panel A: Plot for α diversity per plant genotype, Shannon diversity index compared at class taxonomy level. **Panel B** Principal Coordinates Analysis (PCoA) ordination plot of bacterial β diversity across all genotypes studied, PERMANOVA, Bray-Curtis distances, species level, $p \leq 0.05$).

The analysis with PCoA with Bray-Curtis shows clustering of genotypes indicated by spatial clustering under the PC1(36.1%), PC2 (15.4%) and PC3 (11.1%) axis. Bacterial genera with

the most counts across genotypes expressed in relative abundance were: *Pseudomonas*, *Methylobacterium*, *Sphingomonas*, *Staphylococcus* and *Pantoea* (Fig. S2.10.1-S2.10.5).

2.3.4. Genotype effect on bacteria biomarkers

Linear discriminant analysis effect size (LEfSe) analysis was performed to identify specific taxa that varied in abundance consistently among plant genotypes and thus could be used as biomarkers. In total, five bacterial and seven fungal biomarkers with LDA scores > 3.5 were determined (Fig. 2.4.).

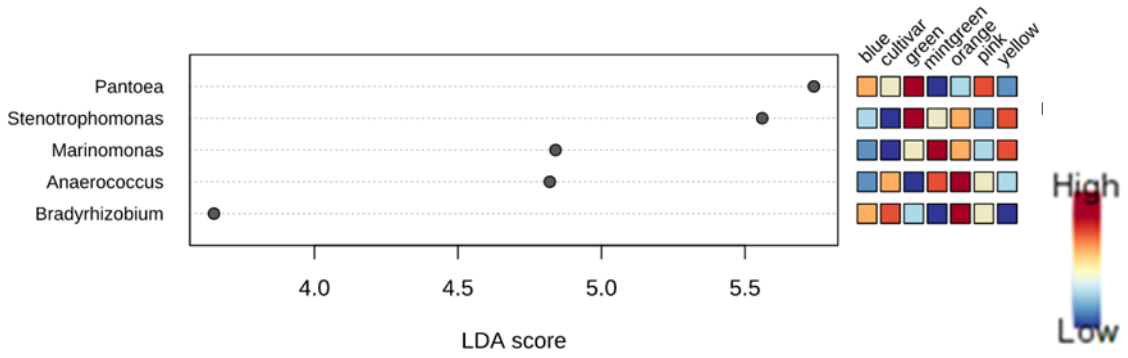
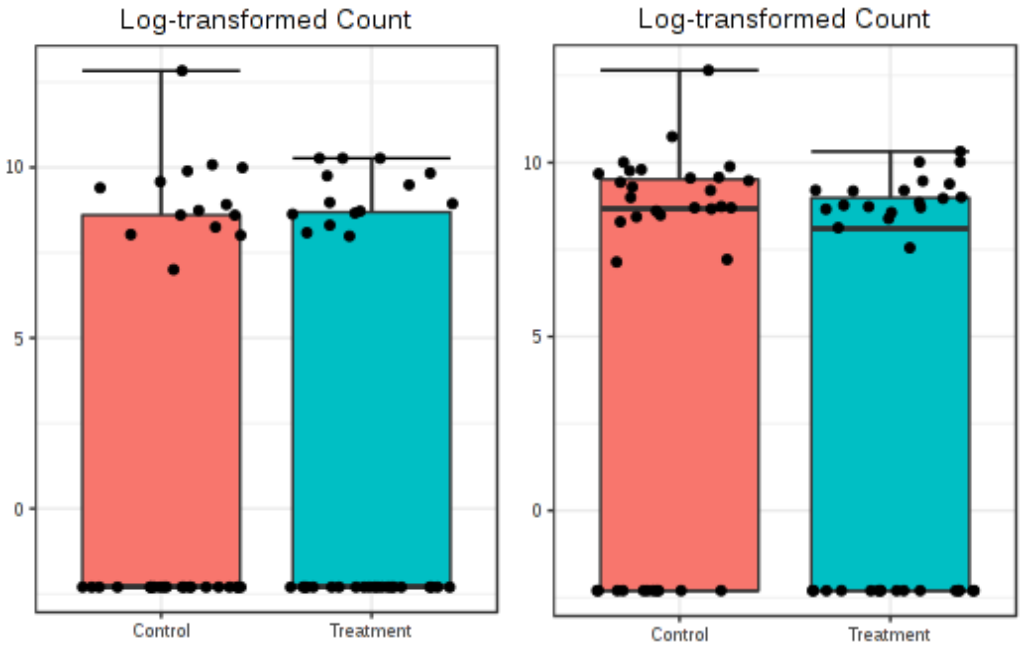


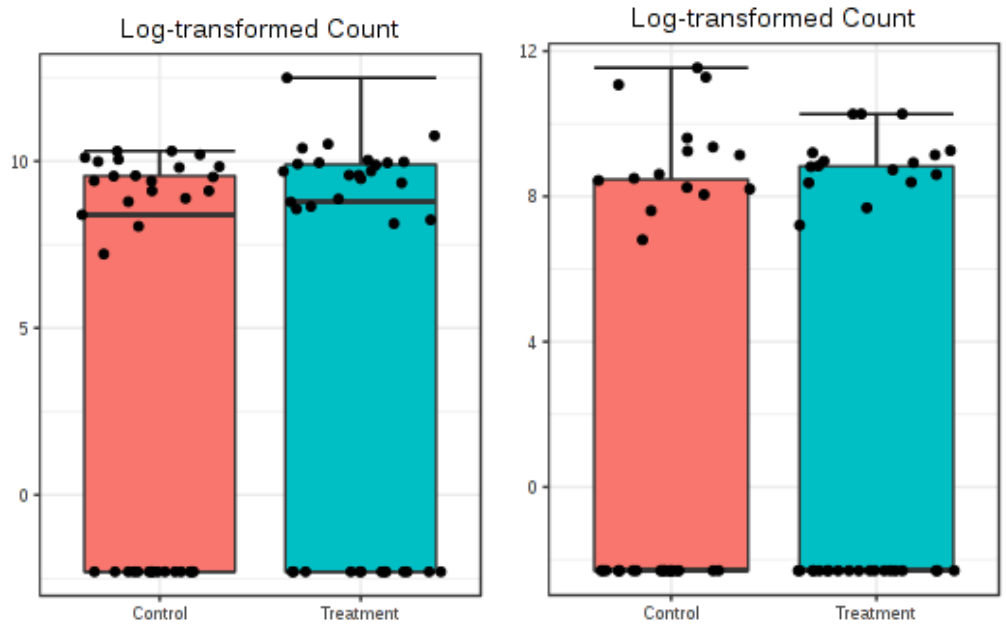
Figure 2.4. LEfSe analysis showing the highest LDA (Lineal Discriminant Analysis) ranks scores at bacteria genera level between all the genotypes with P value cut-off < 0.05 and Log LDA score of 2.0 as threshold.

2.3.5. Effect of the treatment on the flowers on bacteria diversity

Bagged and unbagged flowers, showed no differences in community composition, α diversity (Kruskal-Wallis test, Shannon diversity P=0.43) or β diversity in the PERMANOVA, Bray-Curtis, P=0.20). Nevertheless, analysing the community at genus level and with proportional statistical methods, differences between treatments of bacteria genus arise. Differences in the abundance for *Acinetobacter*, *Curtobacterium*, *Paraburkholderia spp.* and *Deinococcus* for control (unbagged) and treatment plants were found using edgeR (Fig. 2.5.).



A) *Acinetobacter* B) *Curtobacterium*



C) *Paraburkholderia sp.* D) *Deinococcus*

Figure 2.5. Plots of bacteria for control and treatment edgeR, A) *Acinetobacter* (P=1.120 E-5 , False Discovery Rate (FDR) = 4.48 E-4), B) *Curtobacterium* (P=1.5703E-4 , FDR = 0.0030471), C) *Paraburkholderia spp.* (P=8.8312e-4, FDR=0.009052), D) *Deinococcus* (P=8.8312E-4, FDR = 0.009052).

2.3.6. Interaction effect of the bagging treatment on the plant genotypes on bacteria abundance.

A Multi-Factor edgeR with FDR adjusted-P value shows that bacteria from *Orbaceae* family are significantly more abundant in bagged flowers than in unbagged flowers for the pink clone (P=0.000411; FDR=0.0195). For the same clone there was a significant difference in the abundance of two bacteria families. A higher abundance of the families *Propionibacteriaceae* (P=9.0213e-05; FDR=0.00857) and *Xanthomonadaceae* (P=0.0007702; FDR=0.02439) was found in unbagged flowers. The cultivar has bacteria significantly more abundant in bagged flowers from the family *Acidobacteriaceae* (P=0.000239; FDR=0.01238) and the family *Acetobacteraceae* (P=0.00026; FDR=0.01238).

2.3.7. Fungal community composition

The most abundant fungi species were *Curvibasidium cygneicollum, Cladosporium delicatulum, Malassezia restricta, Farysia acheniorum, Malassezia arunalokei, Schizophyllum commune, Malassezia globosa, Botrytis caroliniana, Sporobolomyces roseus, and Vishniacozyma psychrotolerans.* A core microbiome analysis at species level shows the most abundant fungi across all samples (Fig. 2.6.). Fungal community composition per sample and genotype are shown in supplementary materials (Fig.S2.11).

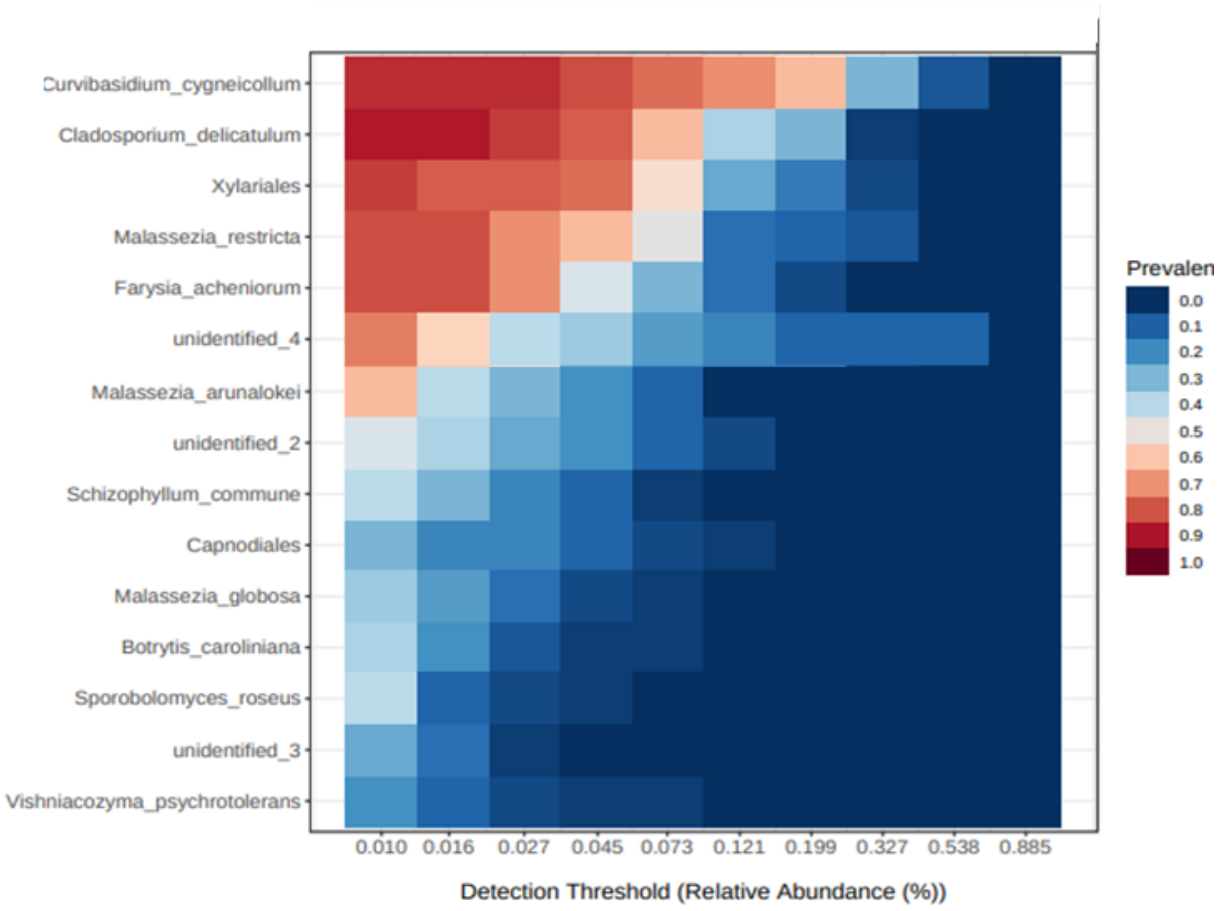


Figure 2.6. Heatmap of core fungi at species level. The count data is transformed to compositional (relative) abundances, sample prevalence 10% and relative abundance 0.01%.

Fungal diversity differs in α diversity between treatments (bagged and unbagged) (Mann-Whitney/Kruskal-Wallis, observed ASVs and Chao1, $P<0.05$), and among genotypes (Mann-Whitney/Kruskal-Wallis, observed ASVs and Chao1, $P<0.05$) (Fig. 2.7.). Significant differences were also found in the β diversity (Fig. 2.8.).

Fungi in both bagged and unbagged flowers were less abundant than bacteria and were difficult to amplify in bagged flowers. As a result, fungal DNA was amplified only in eight bagged plants. The statistical analysis was based on total sum normalization. This analysis revealed differences in β diversity both for observed ASVs and Chao1 indices (Kruskal-Wallis test, $P<0.05$) (Fig. 2.8.).

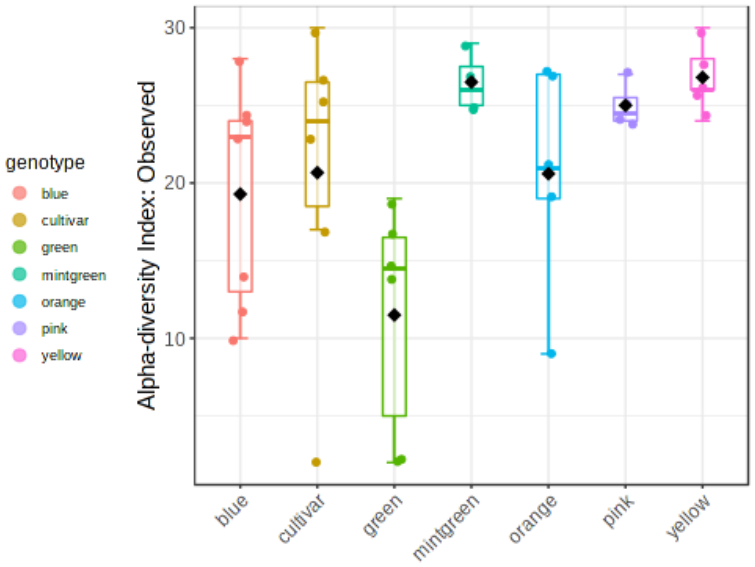


Figure 2.7. Plot for fungal α diversity per genotype, diversity Index Observed, normalization TTS.

For β diversity there were differences amongst all the samples for two different distance statistical methods (PERMANOVA, and analysis of group similarities (ANOSIM), P<0.05) (Fig. 2.8.).

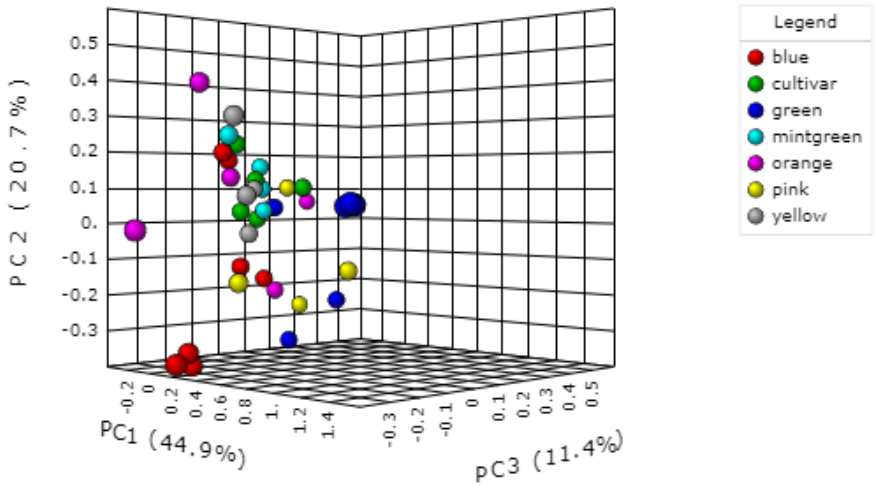


Figure 2.8. Principal Coordinates Analysis (PCoA) ordination plot of fungal β diversity across all genotypes studied, PERMANOVA, Bray-Curtis distance, species level, P<0.05.

Analysis with PCoA with Bray-Curtis shows clustering of genotypes indicated by spatial clustering under the PC1(44.9%), PC2 (20.7%) and PC3 (11.4%) axis.

2.3.8. Genotype effect on fungal biomarkers

LEfSe analysis was performed with the pooled data to find significant differences in fungi across mānuka plant genotypes (Fig. 2.9.). The fungi species found to be biomarkers for each clone were *Farysia acheniorum*, *Schizophyllum commune*, *Vishniacozyma victoriae*, and *Sporobolomyces*. Their lineal discriminant analysis scores can be seen at Figure 2.9.

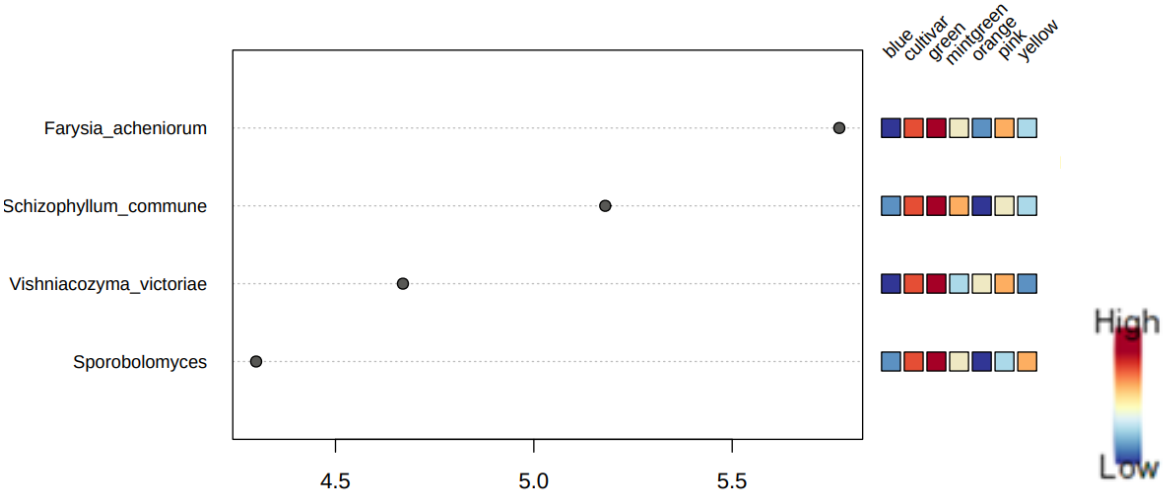
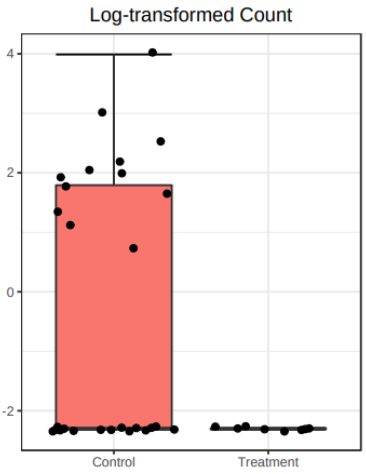


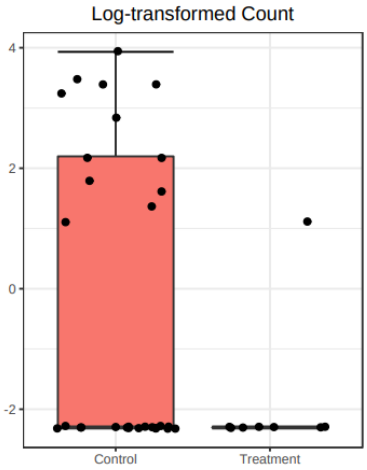
Figure 2.9. LEfSe analysis showing the highest LDA (Lineal Discriminant Analysis) ranks scores at fungal genera level between all the genotypes with P value cut-off < 0.05 and Log LDA score of 2.0 as thresholds.

2.3.9. Effect of pollinator visitation on Fungal communities

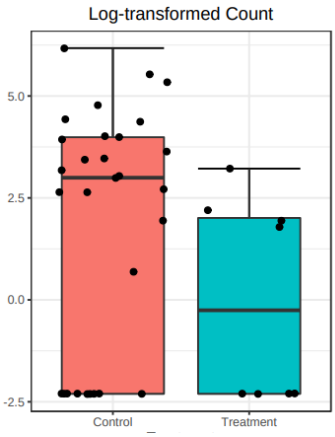
Differential expression analysis with DESeq2 revealed three fungi with significantly lower abundance in the bagged (treatment flowers) than in the unbagged flowers (control). Significantly different fungal species based on p-value ($p<0.05$) and FDR are shown on Figure 2.10.



A) *Vishniacozyma victoriae*



B) Genolevuria amylolytica



B) *Capnodiales sp.*

Figure 2.10. Plots of bacteria for Control and Treatment *Vishniacozyma victoriae* (P= 2.1135E-4; FDR = 0.0067535), *Genolevuria amylolytica* (P= 0.0156; FDR = 0.1064), and *Capnodiales sp.* (P= 0.0014793; FDR = 0.023668). Computational calculation, a Negative Binomial Wald Test using standard maximum likelihood estimates for coefficients assuming a zero-mean normal prior distribution, implemented in the binomial Wald Test with MicrobiomeAnalyst.

2.3.10. Quantification of DHA and total sugars by HPLC

Normalized DHA comparisons between genotypes

I found variation in the levels of normalised DHA among clones and cultivars. With green and pink clones showing a higher DHA production, followed by mint-green and orange, while the blue and yellow clone, and the wild cultivar had the lowest DHA production for the

season sampled in October 2017 (GLM, P<0.05). However, there were no significant differences between the treatment (bagged flowers) and control flowers, for each genotype (GLM, P<0.05) (Fig 2.11. and supplementary materials Table S2.12).

Total sugars (TS) that accounts for fructose, glucose and sucrose were not significant between treatments or genotypes (Kruskal-Wallis, P<0.05).

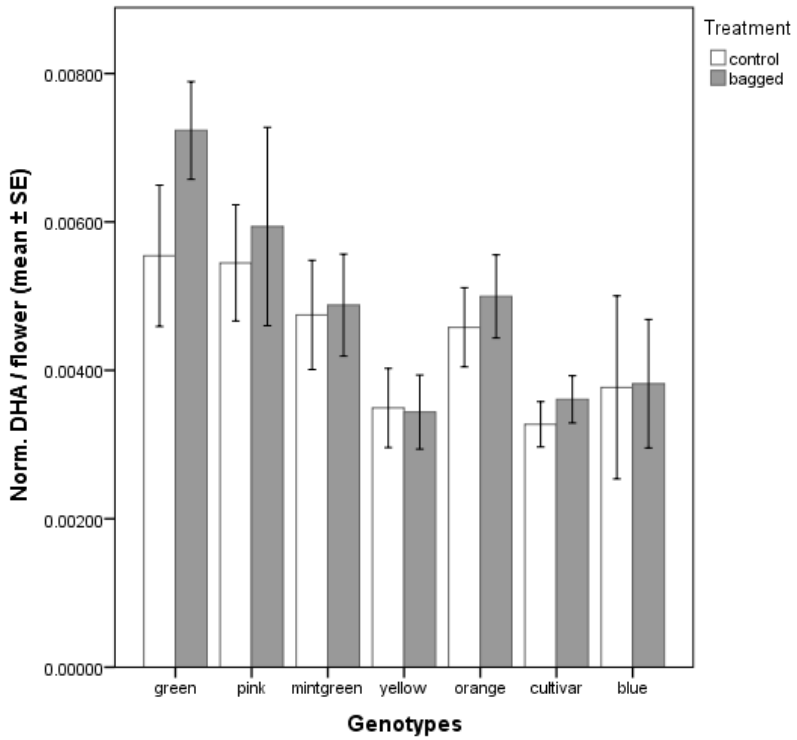


Figure 2.11. Mean nectar normalized DHA values for mānuka plants. Five plants per treatment and genotype, 20 flowers for control and treatment. Colour code clones each one refers to different genotype and a cultivar originated from Northland (NZ): from left to right: green, pink, mint green, yellow, orange, cultivar and blue.

2.4. DISCUSSION

Bacterial diversity

The most abundant bacteria in the nectar microbiome were *Pseudomonas*. These are known to play a major role in plants, with implications in the secretion of secondary metabolites for defence or glycerol uptake (Mithani, Hein, & Preston, 2010). Interconnections between glycerol metabolism and biofilm production may be a key factor that contributes to *Pseudomonas* having persistence in different environments by promoting adaptation and colonization to the environment as described for *P. aeruginosa* (Scoffield, Duan, Zhu, & Wu, 2017). Furthermore, *Pseudomonas fluorescens* are able to utilize fructose by glycolysis with thermodynamic and kinetics constraints (Lien, Niedenführ, Sletta, Nöh, & Bruheim, 2015).

Rhizobiaceae was the second most abundant bacteria. It has nitrogen-fixing capabilities that are encoded in a symbiotic plasmid that is the main genetic determinant of nitrogen-fixing symbiosis, is mobilisable by horizontal gene transfer (Yang et al., 2020), and plays an interesting role in the biochemical process of adaptation to the plant (Brencic et al., 2005). *Methylobacterium* was found in high abundance and may stimulate plant development through phytohormone production (Kutschera, 2007). The enrichment in taxa belonging to the Rhizobiales order, such as *Bradyrhizobium spp.* could also result in antibiotic production and nitrogen fixation (Naamala, Jaiswal, & Dakora, 2016).

Enterobacteriaceae is the third important group; important plant bacteria belong to this family. They are able to colonize the plant through the roots or glandular trichomes (Kim, 2019). Interactions between plant metabolites and enterobacteria have been shown in the plant genus *Arugula*, which has health properties (Cernava et al., 2019).

Acetobacteraceae were in high relative abundance. They are also described as diazotrophic, and this capability could be of ecological relevance in conjunction with other bacteria belonging to the order *Rhizobiales* and genus *Bradyrhizobium* (Yoneyama, Terakado-Tonooka, Bao, & Minamisawa, 2019). I found differences for bacteria in beta diversity, suggesting that the genotype might cause differences in the type of bacteria adapted to the nectar by random or selection process, as seen in the microbiomes of *Brassica napus* seeds (Rochefort et al., 2019).

Fungal diversity

Regarding fungal diversity, I found a characteristic community of fungi species in the mānuka nectar. Some species have been found in extreme environments like *Vishniacozyma* (Buzzini et al., 2018) that could have biochemical properties that enhance their adaptation to the nectar environment in mānuka plants.

Vishniacozyma is detected in relative high abundance in all the clones studied and was also reported in the nectar of *Gossypium hirsutum* (*Malvaceae*) in Mexico (Canto et al., 2017). Besides this, there are differences in its abundance on each type of clone; *Curvibasidium* and *Cladosporium* were also detected in high relative abundance and they can be transmitted by air and pollen;- the capability of these fungi spores to become airborne (Pavan & Manjunath, 2014) could explain their presence in the nectar of bagged and unbagged flowers. From the differential abundance analysis between bagged and unbagged flowers the fungi *Vishniacozyma victoriae*, *Genolevuria amylolytica, and Capnodiales* were found in bagged flowers in significantly higher abundance. *Vishniacozyma victoriae* is an Ascomycetes yeast that has been found to be endophytic in leaf galls (Glushakova & Kachalkin, 2017), could be in a close relationship with aerial pollinators and also could have other ways to colonise to mānuka nectaries. *Genolevuria amylolytica* is a Basidiomycetous yeast described from leaves that can be in a close relationship with mānuka plants. Members of this group of fungi have the capability to assimilate ethylamine gas (Landell, Inácio, Fonseca, Vainstein, & Valente, 2009), and, in epiphytic leaves, D-glucuronic acid (Inácio, Portugal, Spencer-Martins, & Fonseca, 2005), that could be generated from glucose in the nectar (Seo et al., 2013), and a substrate for *Genolevuria amylolytica*. Fungi rely on dispersal agents like pollinators as opposed to bacteria that have several ways to reach the nectar (Vannette et al., 2020). *Capnodiales* belongs to the Dothideomycetes and are epiphytes fungus associated with honeydew of insects including ants (Crous et al., 2009).

Mānuka flowers form an enclosed tube adnate to the corolla but not an enclosed tube; this is another important anatomical factor that shapes fungal communities in the *Leptospermum* genus. Plants in which *Saccharomyces* are found in high abundance typically possess a corolla that provides protection for this type of fungi (Klaps et al., 2020). In contrast, mānuka plants' flowers are devoid of an enclosed tube adnate to the corolla and as a result their nectar

is totally exposed, and it might be one of the reasons for finding *Saccharomyces* yeast in low abundance.

The effect of pollinator visitation on nectar microbial composition

The differences observed in fungal (but not bacterial) composition between unbagged and bagged plants suggests that pollinators are important fungal vectors, but that other factors may contribute to the diversity of bacterial communities in nectar. This means that fungi may be linked to a certain type of pollinator, and thus appear in different numbers across different types of plants due to an unequal number of visits from pollinators, or a different adaptation process of the fungi to the plant. The basis of these differences might be due to different shapes and colours of flowers that may lead to different relationships with pollinators (Chalcoff, Aizen, & Galetto, 2006). Other studies also suggest that pollinators act as microbial vectors (Sugiura & Masuya, 2010), and fungi related to a social bee that is in a symbiosis with *Scaptotrigona depilis* (Menezes et al., 2015).

Bacteria in nectar can be also acquired through pollinators, but other processes such as airborne fixation or mobility through the phloem flow from internal plant circulation are also common, as described in other plants (Bové & Garnier, 2003; Eichmeier et al., 2019), Bacteria can also be carried on very small insects (thrips) (Fig.S2.7.3 and Fig.S2.7.4) that can pass a breathable mesh and were also found on mānuka flowers. In this study, few individual bacteria differed between treatments, suggesting a relationship with pollinator visitation, for example *Deinococcus*, *Acinetobacter* and *Curtobacterium. Deinococcus* is a bacterium with free radical protection systems (Jin et al., 2019) that should be an advantage in the extreme nectar chemistry environment. *Acinetobacter* is also differentially abundant between control and treatment plants and is a pollinator-related bacterium (Álvarez-Pérez, Lievens, Jacquemyn, & Herrera, 2013). *Curtobacterium* is also in greater proportions in control and treatment plants and have also been reported as a nectar-related bacterium with functions in substrate degradation and nitrogen fixation (Ruiz-González et al., 2019). *Curtobacterium spp.* have also been detected in the nectar of orchid flowers exposed to pollinators (Jacquemyn et al., 2013).

Microorganisms and DHA production

Bacteria revealed by LEfSe analysis as potential biomarkers of each genotype may have biochemical capabilities to modify DHA content in the nectar. *Pantoea* was the highest LDA rank (Fig. 2.4.) and was found in high abundance in high DHA producing clones (green and pink). This bacterium has been used industrially to metabolize glycerol or other sugars (Zhou et al., 2013), and it is reported as an endophyte in plants (Marag & Suman, 2018). I found no evidence of marker fungi being related to elevated DHA production, suggesting that fungi do not impact DHA production.

The results of normalized DHA show that clones (pink and green) with the highest values also showed high LDA scores of the dominant bacteria (*Pantoea-Enterobacteriales*), that might be relevant in DHA production in the nectaries. These clones also have been reported to be more visited than other varieties (Bohórquez Rodríguez de Medina, 2018), although, there are no statistical differences in the normalized DHA between bagged and control flowers, which means pollinators should not be the only source of bacteria involved in DHA production. The importance of DHA in the nectar of these plants could be a key aspect for the stages of seed development. Bacteria-plant interactions could play a role in DHA production in nectar along plant development stages.

2.5. CONCLUSION

Mānuka plants possess an interesting diversity of fungi and bacteria where fungi are related to pollinator activity while the bacterial community structure is quite independent of pollinators. DHA content is a characteristic of *Leptospermum scoparium* independent of pollinator interaction and it varies with plant genotype. Bacteria and fungi with different metabolic

capabilities also change their abundance among plant genotypes. However, bacterial taxa with versatile metabolic capabilities, such as *Pseudomonas* and *Pantoea,* could determine DHA production through their own metabolic pathways or by enzymatic action on secondary metabolites. As predicted, I found a varied community of microorganism in the nectar of mānuka plants. However, I only partially confirmed our hypothesis regarding the effect of pollinators in the nectar microbial community and chemistry. The effect of pollinators is evident in the fungal community, while the bacteria community seems to be present independently of pollinator presence, possibly due to alternative pathways for bacteria to reach the nectar. Restricting pollinators did not have a significant effect on the nectar chemical parameters measured, but the DHA content (but not sugars) differed among plant clones.

These results highlight the importance of plant phenotypic characteristics in determining nectar microbial diversity, and potentially open the question whether bacteria could change the chemical phenotype of plants through biochemical reactions. These multiple possibilities have implications for tri-trophic interactions (plant-pollinators-microorganisms) and their ecosystem services.

2.6. REFERENCES

Adams, C. J., Manley-Harris, M., & Molan, P. C. (2009). The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. *Carbohydrate Research, 344*(8), 1050-1053. https://doi.org/10.1016/j.carres.2009.03.020

Alm, J., Ohnmeiss, T.E., Lanza, J. *et al.* Preference of cabbage white butterflies and honey bees for nectar that contains amino acids. *Oecologia* **84**, 53–57 (1990). https://doi.org/10.1007/BF00665594

Álvarez-Pérez, S., Lievens, B., Jacquemyn, H., & Herrera, C. M. (2013). Acinetobacter nectaris sp. nov. and Acinetobacter boissieri sp. nov., isolated from floral nectar of wild Mediterranean insect-pollinated plants. *International Journal of Systematic and Evolutionary Microbiology, 63*(4), 1532-1539. https://doi.org/10.1099/ijs.0.043489-0

Brencic, A., & Winans, S. C. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiology and molecular*

biology reviews : MMBR, *69*(1), 155–194. https://doi.org/10.1128/MMBR.69.1.155-194.2005

Bohórquez Rodríguez de Medina, J. (2018). *Companion biota associated with Leptospermum scoparium (mānuka; Myrtaceae) : a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Ecology at Massey University, Manawatū, New Zealand.* (Doctor of Philosophy (PhD) Doctoral), Massey University, Retrieved from http://hdl.handle.net/10179/14300

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology, 37(8), 852-857.

Bové, J., & Garnier, M. (2003). *Phloem-and xylem-restricted plant pathogenic bacteria* (Vol. 164). 10.1016/S0168-9452(03)00033-5

Buzzini, P, Turchetti, B, Yurkov, A. Extremophilic yeasts: the toughest yeasts around? *Yeast*. 2018; 35: 487– 497. https://doi.org/10.1002/yea.3314

Canto, A., Herrera, C. M., & Rodriguez, R. (2017). Nectar-living yeasts of a tropical host plant community: diversity and effects on community-wide floral nectar traits. *PeerJ*, *5*, e3517. 10.7717/peerj.3517

Carter, C., Healy, R., O'Tool, N. M., Naqvi, S. M. S., Ren, G., Park, S., . . . Thornburg, R. W. (2007). Tobacco Nectaries Express a Novel NADPH Oxidase Implicated in the Defense of Floral Reproductive Tissues against Microorganisms. *Plant Physiology*, *143*(1), 389-399. 10.1104/pp.106.089326

Cernava, T., Erlacher, A., Soh, J., Sensen, C. W., Grube, M., & Berg, G. (2019). Enterobacteriaceae dominate the core microbiome and contribute to the resistome of arugula (Eruca sativa Mill.). *Microbiome*, *7*(1), 1-12.

Chalcoff, V. R., Aizen, M. A., & Galetto, L. (2006). Nectar Concentration and Composition of 26 Species from the Temperate Forest of South America. *Annals of Botany*, *97*(3), 413-421. 10.1093/aob/mcj043

Chen, M., & Thelen, J. J. (2010). The essential role of plastidial triose phosphate isomerase in the integration of seed reserve mobilization and seedling establishment. *Plant Signaling & Behavior*, *5*(5), 583-585. 10.4161/psb.11496

Chong, J., Liu, P., Zhou, G., & Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nature protocols, 15(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1

Clearwater, M. J., Revell, M., Noe, S., & Manley-Harris, M. (2018). Influence of genotype, floral stage, and water stress on floral nectar yield and composition of mānuka (Leptospermum scoparium). *Annals of Botany, 121*(3), 501-512. 10.1093/aob/mcx183

Cox, M. P., Peterson, D. A., & Biggs, P. J. (2010). SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics, 11*(1), 485. 10.1186/1471-2105-11-485

Crous, P., Braun, U., Wingfield, M., Wood, A., Shin, H., Summerell, B., Alfenas, A., Cumagun, Christian J., & Groenewald, J.Z.. (2009). Phylogeny and taxonomy of obscure genera of microfungi. Persoonia. 22. 139-61. 10.3767/003158509X461701.

Del Giudice, L., Massardo, D.R., Pontieri, P., Bertea, C.M., Mombello, D., Carata, E., Tredici, S.M., Talà, A., Mucciarelli, M., Groudeva, V.I., De Stefano, M., Vigliotta, G., Maffei, M.E. and Alifano, P. (2008), The microbial community of Vetiver root and its involvement into essential oil biogenesis. Environmental Microbiology, 10: 2824-2841. https://doi.org/10.1111/j.1462-2920.2008.01703.x

Dyer, A., Whitney, H., Arnold, S. *et al.* Bees associate warmth with floral colour. *Nature* **442**, 525 (2006). https://doi.org/10.1038/442525a

Effmert, U., Kalderás, J., Warnke, R., & Piechulla, B. (2012). Volatile Mediated Interactions Between Bacteria and Fungi in the Soil. *Journal of Chemical Ecology, 38*(6), 665-703. 10.1007/s10886-012-0135-5

Eichmeier, A., Kiss, T., Necas, T., Penazova, E., Tekielska, D., Bohunicka, M., . . . Baldrian, P. (2019). High-Throughput Sequencing Analysis of the Bacterial Community in Stone Fruit Phloem Tissues Infected by "Candidatus Phytoplasma prunorum". *Microbial Ecology, 77*(3), 664-675. 10.1007/s00248-018-1250-9

Glushakova, A. M., & Kachalkin, A. V. (2017). Endophytic yeasts in leaf galls. *Microbiology, 86*(2), 250-256. 10.1134/s0026261717020096

Heil M. (2011). Nectar: generation, regulation and ecological functions. *Trends in plant science*, *16*(4), 191–200. https://doi.org/10.1016/j.tplants.2011.01.003

Herrera, C. M., García, I. M., & Pérez, R. (2008). Invisible Floral Larcenies: Microbial Communities Degrade Floral Nectar of Bumble Bee-Pollinated Plants. *Ecology, 89*(9), 2369-2376. doi: 10.1890/08-0241.1

Inácio, J., Portugal, L., Spencer-Martins, I., & Fonseca, Á. (2005). Phylloplane yeasts from Portugal: Seven novel anamorphic species in the Tremellales lineage of the Hymenomycetes (Basidiomycota) producing orange-coloured colonies. *FEMS Yeast Research, 5*(12), 1167-1183. 10.1016/j.femsyr.2005.05.007

Jacquemyn, H., Lenaerts, M., Tyteca, D., & Lievens, B. (2013). Microbial diversity in the floral nectar of seven Epipactis (Orchidaceae) species. *MicrobiologyOpen*, *2*(4), 644-658. 10.1002/mbo3.103

Jin, M., Xiao, A., Zhu, L., Zhang, Z., Huang, H., & Jiang, L. (2019). The diversity and commonalities of the radiation-resistance mechanisms of Deinococcus and its up-to-date applications. *AMB Express, 9*(1), 138. 10.1186/s13568-019-0862-x

Junker, R. R., & Keller, A. (2015). Microhabitat heterogeneity across leaves and flower organs promotes bacterial diversity. *FEMS Microbiology Ecology, 91*(9), fiv097-fiv097. 10.1093/femsec/fiv097

Kevan, P. G., Eisikowitsch, D., Fowle, S., and Thomas, K. (1988). Yeast-Contaminated Nectar and its Effects on Bee Foraging. J. Apic. Res. 27, 26–29

Kevan, P.G., 1999. Pollinators as bioindicators of the state of the environment: species, activity and diversity. Agric. Ecosyst. Environ. 74, 373e393. https:/doi.org/10.1016/b978-0-444-50019-9.50021-2.

Kevan, P. G., & Viana, B. F. (2003). The global decline of pollination services. *Biodiversity*, *4*(4), 3-8. 10.1080/14888386.2003.9712703

Kim, W., Gilet, T., & Bush, J. W. M. (2011). Optimal concentrations in nectar feeding. *Proceedings of the National Academy of Sciences, 108*(40), 16618-16621. 10.1073/pnas.1108642108

Kim, K.W. Plant trichomes as microbial habitats and infection sites. Eur J Plant Pathol 154, 157–169 (2019). https://doi.org/10.1007/s10658-018-01656-0

Klaps, J., Lievens, B. & Álvarez-Pérez, S. Towards a better understanding of the role of nectar-inhabiting yeasts in plant–animal interactions. *Fungal Biol Biotechnol* **7**, 1 (2020). https://doi.org/10.1186/s40694-019-0091-8

Kutschera, U. (2007). Plant-associated methylobacteria as co-evolved phytosymbionts: a hypothesis. *Plant Signaling & Behavior, 2*(2), 74-78. 10.4161/psb.2.2.4073

Landell, M. F., Inácio, J., Fonseca, Á., Vainstein, M. H., & Valente, P. (2009). Cryptococcus bromeliarum sp. nov., an orange-coloured basidiomycetous yeast isolated from bromeliads in Brazil. *International Journal of Systematic and Evolutionary Microbiology*, *59*(4), 910-913. https://doi.org/10.1099/ijs.0.005652-0

Li, Y., & Andrade, J. (2017). DEApp: an interactive web interface for differential expression analysis of next generation sequence data. *Source code for biology and medicine*, *12*, 2-2. 10.1186/s13029-017-0063-4

Lien, S. K., Niedenführ, S., Sletta, H., Nöh, K., & Bruheim, P. (2015). Fluxome study of Pseudomonas fluorescens reveals major reorganisation of carbon flux through central metabolic pathways in response to inactivation of the anti-sigma factor MucA. *BMC Systems Biology*, *9*(1), 6. 10.1186/s12918-015-0148-0

Lozupone, C., & Knight, R. (2005). UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Applied And Environmental Microbiology*, *71*(12), 8228-8235. 10.1128/aem.71.12.8228-8235.2005

Marag, P. S., & Suman, A. (2018). Growth stage and tissue specific colonization of endophytic bacteria having plant growth promoting traits in hybrid and composite maize (Zea mays L.). *Microbiological Research*, *214*, 101-113. https://doi.org/10.1016/j.micres.2018.05.016

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., . . . Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, *110*(9), 3229-3236. 10.1073/pnas.1218525110

Menezes, C., Vollet-Neto, A., Marsaioli, A. J., Zampieri, D., Fontoura, I. C., Luchessi, A. D., & Imperatriz-Fonseca, V. L. (2015). A Brazilian Social Bee Must Cultivate Fungus to Survive. *Current Biology*, *25*(21), 2851-2855. https://doi.org/10.1016/j.cub.2015.09.028

Mithani, A., Hein, J., & Preston, G. M. (2010). Comparative Analysis of Metabolic Networks Provides Insight into the Evolution of Plant Pathogenic and Nonpathogenic Lifestyles in Pseudomonas. *Molecular Biology and Evolution*, *28*(1), 483-499. 10.1093/molbev/msq213

Naamala, J., Jaiswal, S. K., & Dakora, F. D. (2016). Antibiotics Resistance in Rhizobium: Type, Process, Mechanism and Benefit for Agriculture. *Current Microbiology*, *72*(6), 804-816. 10.1007/s00284-016-1005-0

Nilsson, R. H., Larsson, K.-H., Taylor, A. F S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., . . . Abarenkov, K. (2018). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research*, *47*(D1), D259-D264. 10.1093/nar/gky1022

Pavan, R., & Manjunath K. (2014). Qualitative Analysis of Indoor and Outdoor Airborne Fungi in Cowshed. *Journal of Mycology*, *2014*, 8. https://doi.org/10.1155/2014/985921

Peay KG, Schubert MG, Nguyen NH, Bruns TD (2012) Measuring ectomycorrhizal fungal dispersal: macroecological patterns driven by microscopic propagules. Mol Ecol 21:4122–4136. https://doi.org/10.1111/j.1365-294X.2012.05666.x

Pozo, M. I., Herrera, C. M., & Bazaga, P. (2011). Species richness of yeast communities in floral nectar of southern Spanish plants. *Microb Ecol, 61*(1), 82-91. 10.1007/s00248-010-9682-x

Rochefort, A., Briand, M., Marais, C., Wagner, M. H., Laperche, A., Vallée, P., ... & Sarniguet, A. (2019). Influence of environment and host plant genotype on the structure and diversity of the Brassica napus seed microbiota. Phytobiomes Journal, 3(4), 326-336.

Ruiz-González, X. M., Leroy, C., Dejean, A., Gryta, H., Jargeat, P., Armijos Carrión, D. A., & Orivel, J. (2019). Do Host Plant and Associated Ant Species Affect Microbial Communities in Myrmecophytes? *Insects, 10*(11) 10.3390/insects10110391

Schaeffer, R. N., Vannette, R. L., & Irwin, R. E. (2015). Nectar yeasts in Delphinium nuttallianum (Ranunculaceae) and their effects on nectar quality. *Fungal Ecology, 18*, 100-106. https://doi.org/10.1016/j.funeco.2015.09.010

Scoffield, J. A., Duan, D., Zhu, F., & Wu, H. (2017). A commensal streptococcus hijacks a Pseudomonas aeruginosa exopolysaccharide to promote biofilm formation. *PLoS pathogens, 13*(4), e1006300-e1006300. 10.1371/journal.ppat.1006300

Seo, P. J., Wielsch, N., Kessler, D., Svatos, A., Park, C.-M., Baldwin, I. T., & Kim, S.-G. (2013). Natural variation in floral nectar proteins of two Nicotiana attenuata accessions. *BMC Plant Biology, 13*(1), 101. 10.1186/1471-2229-13-101

Sugiura, S., & Masuya, H. (2010). Leaf-mining beetles carry plant pathogenic fungi amongst hosts. *Journal of Natural History, 44*(35-36), 2179-2186. 10.1080/00222933.2010.487161

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. *PLOS ONE, 7*(7), e40863. 10.1371/journal.pone.0040863

Ushio, M., Yamasaki, E., Takasu, H., Nagano, A. J., Fujinaga, S., Honjo, M. N., . . . Kudoh, H. (2015). Microbial communities on flower surfaces act as signatures of pollinator visitation. *Scientific Reports, 5*, 8695. 10.1038/srep08695

Vannette, R. L. The floral microbiome: plant, pollinator, and microbial perspectives. *Annu. Rev. Ecol. Evol. Syst.* **51**, 363–386 (2020)

Wang, W., Meng, D., Li, Q., Li, Z., & You, C. (2019). Characterization of a hyperthermophilic phosphatase from Archaeoglobus fulgidus and its application in in vitro synthetic enzymatic biosystem. *Bioresources and Bioprocessing, 6*(1), 22. 10.1186/s40643-019-0257-5

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., . . . Stevenson, P. C. (2013). Caffeine in Floral Nectar Enhances a Pollinator's Memory of Reward. *Science, 339*(6124), 1202-1204. 10.1126/science.1228806

Yang, L. L., Jiang, Z., Li, Y., Wang, E. T., & Zhi, X. Y. (2020). Plasmids Related to the Symbiotic Nitrogen Fixation Are Not Only Cooperated Functionally but Also May Have Evolved over a Time Span in Family Rhizobiaceae. *Genome biology and evolution*, *12*(11), 2002–2014. https://doi.org/10.1093/gbe/evaa152

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., . . . Glöckner, F. O. (2013). The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Research, 42*(D1), D643-D648. 10.1093/nar/gkt1209

Yoneyama, T., Terakado-Tonooka, J., Bao, Z., & Minamisawa, K. (2019). Molecular Analyses of the Distribution and Function of Diazotrophic Rhizobia and Methanotrophs in the Tissues and Rhizosphere of Non-Leguminous Plants. *Plants, 8*(10), 408.

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., . . . Gilbert, J. A. (2015). The Soil Microbiome Influences Grapevine-Associated Microbiota. *mBio, 6*(2), e02527-02514. 10.1128/mBio.02527-14

Zheng, Z., Luo, M., Yu, J., Wang, J., & Ji, J. (2012). Novel Process for 1,3-Dihydroxyacetone Production from Glycerol. 1. Technological Feasibility Study and Process Design. *Industrial & Engineering Chemistry Research, 51*(9), 3715-3721. 10.1021/ie201710h

Zhou, Y. J., Yang, W., Wang, L., Zhu, Z., Zhang, S., & Zhao, Z. K. (2013). Engineering NAD+ availability for Escherichia coli whole-cell biocatalysis: a case study for dihydroxyacetone production. *Microbial Cell Factories, 12*(1), 103. 10.1186/1475-2859-12-103

2.7. SUPPLEMENTARY MATERIALS - MĀNUKA PLANT MATERIAL

Fig.S2.7.1. Mānuka genotypes planted in lines of six plants corresponding to each genotype, and in blocks, Palmerston North

Fig.S2.7.2a. Mānuka genotypes in the field at the end of the flowering season. Flowers and capsules are present in each plant. From left to right: blue, pink and yellow

Fig.S2.7.2b. Mānuka genotypes at the end of the flowering season. Flowers and capsules, from left to right orange, green and mint-green.

Fig.S2.7.3. Mānuka flower showing all the components and insects (thrips) on top left, next to the pollen grains orange genotype. Stereo microscope Olympus SZX7, camera Olympus SC100, magnification 1X**.**

Fig. S2.7.4. Pollen grain in the mānuka flower showing thrips next to the pollen grains, reference bar of the scale is shown at the bottom. Stereo microscope Olympus SZX7, camera Olympus SC100, scale is at the left bottom of the picture

2.8. SUPPLEMENTARY MATERIALS - TABLES

Table S2.8.1 Sequencing and bioinformatic filtering with denoising for bacterial samples Plant genotypes: b (blue), Cv (cultivar), O (orange), P (pink), G (green), Mg (mint-green), Y (yellow); the letter c stands for a covered plant.

Sample	After Denoising	Initial Counts	Sample	After Denoising	Initial Counts
b11	26664	87549	g1	11090	50650
b11c	23672	92667	g10	10000	51020
b12	26883	75482	g10c	38911	91148
b12c	35021	136644	g1c	6424	37628
b2	9259	30744	g2	39556	164173
b2c	172	36595	g2c	41338	141476
b4	19019	90535	g6	10058	47689
b4c	21124	80041	g6c	12671	43153
b5	37815	169371	g8	15664	59107
b5c	22604	113408	g8c	41400	151431
Cv1	14733	75311	mg1	11471	45535
Cv10	14939	94825	mg11	19765	84942
Cv10c	172	16645	mg11c	21876	79309
Cv1c	31297	150247	mg12	15691	47679
Cv2	8420	61451	mg12c	14370	65915
Cv2c	45691	147496	mg1c	13611	51980
Cv8	41047	117723	mg5	9412	50322
Cv8c	21711	72153	mg5c	13367	60706
Cv9	37282	127710	mg7	17271	84942
Cv9c	25135	85372	mg7c	10736	30312

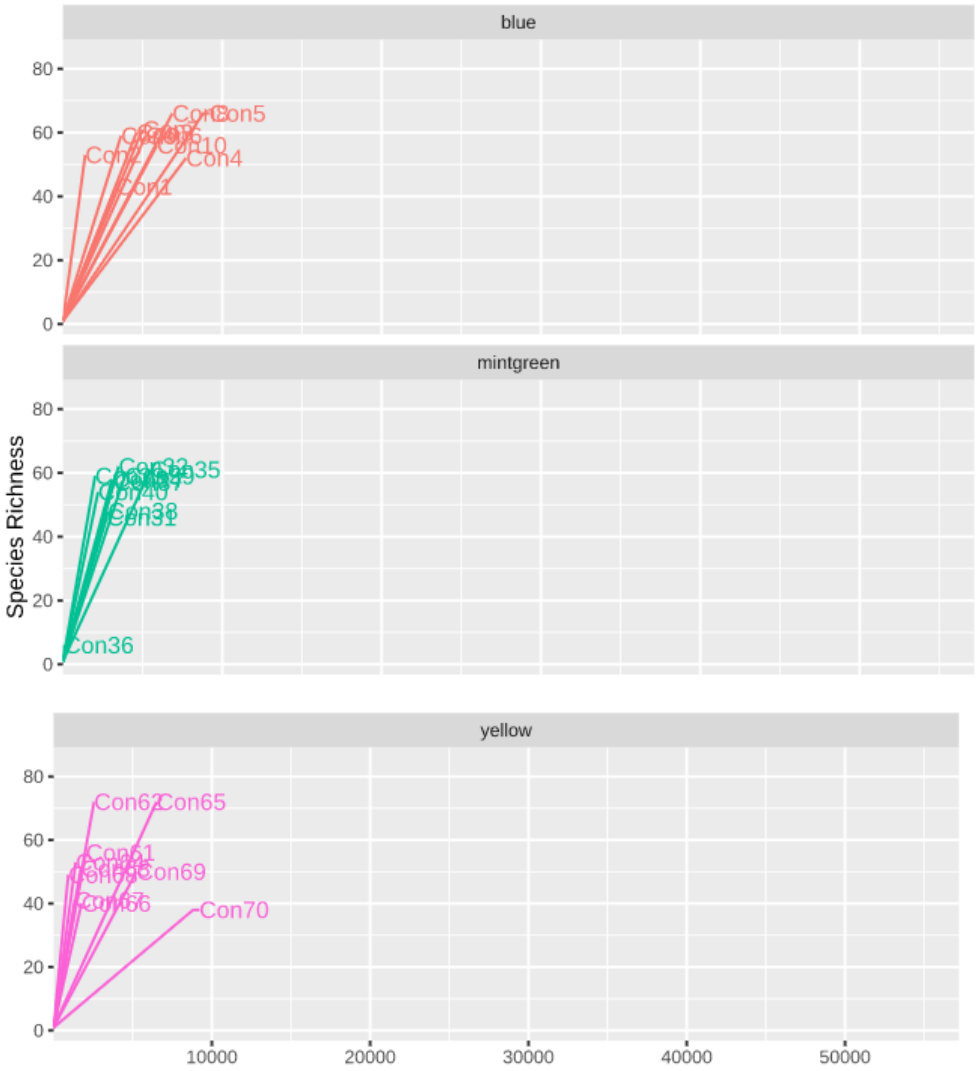
Sample	After Denoising	Initial Counts	Sample	After Denoising	Initial Counts
O2	3515	49465	Y10	10647	38840
O2c	18143	83734	Y10c	11282	51949
O3	15499	46910	Y11	14068	58257
O3c	20975	81672	Y11c	8702	47758
O5	16735	49311	Y3	33177	130719
O5c	14074	54843	Y3c	11690	58184
O6	10046	60706	Y6	6424	37628
O6c	15123	54959	Y6c	6424	35520
O9	11550	63777	Y7	74546	217797
O9c	8603	39400	Y7c	19390	63038
P1	17889	86934			
P11	10000	61688			
P11c	10780	52942			
P12	83775	357420			
P12c	25707	114182			
P1c	11496	62977			
P4	46582	134963			
P4c	24798	84644			
P6	9079	51976			
P6c	17707	83787			

TableS2.8.2 Sequencing and bioinformatic filtering with denoising for fungal samples

Table: Indicates fungal samples ASVs counts after denoising algorithm applied. Sample-b is for blue, Sample-bc is for blue covered, Sample-cv is for cultivar, Sample-g: green, Sample-gc: green cover, Sample-mg: mint-green, Sample-mgc: mint-green covered, Sample-o: orange, Sample-oc: orange covered, Sample-p: pink, Sample-y: yellow.

Sample	After Denoising	Initial counts
Blue	208	39732
Blue	2149	53751
Blue	3194	80213
Blue	3053	172641
Blue	479	50016
Blue covered	202	60154
Sample-bc	6188	142601
Sample-cv	995	56848
Sample-cv	3713	90606
Sample-cv	8380	167024
Sample-cv	941	80112
Sample-cvc	3859	43787
Sample-cvc	1932	70708
Sample-g	3695	160493
Sample-g	445	36420
Sample-g	1352	86168
Sample-gc	1438	9294
Sample-gc	23323	173884
Sample-mg	11513	95332
Sample-mg	2788	61966
Sample-mg	4505	128576
Sample-mg	3269	118358
Sample-mgc	1804	59046
Sample-o	2486	135663
Sample-o	1177	57300
Sample-o	2759	49560
Sample-o	2483	53950
Sample-oc	3097	50763
Sample-p	3225	68318
Sample-p	4224	192519
Sample-p	1588	48059
Sample-p	1839	63078
Sample-y	5040	187730
Sample-y	8806	127504
Sample-y	2268	81780
Sample-y	2743	63140
Sample-y	2574	56032

2.9.1. Rarefaction curves. Bacterial amplicon sequence variants grouped by genotype.



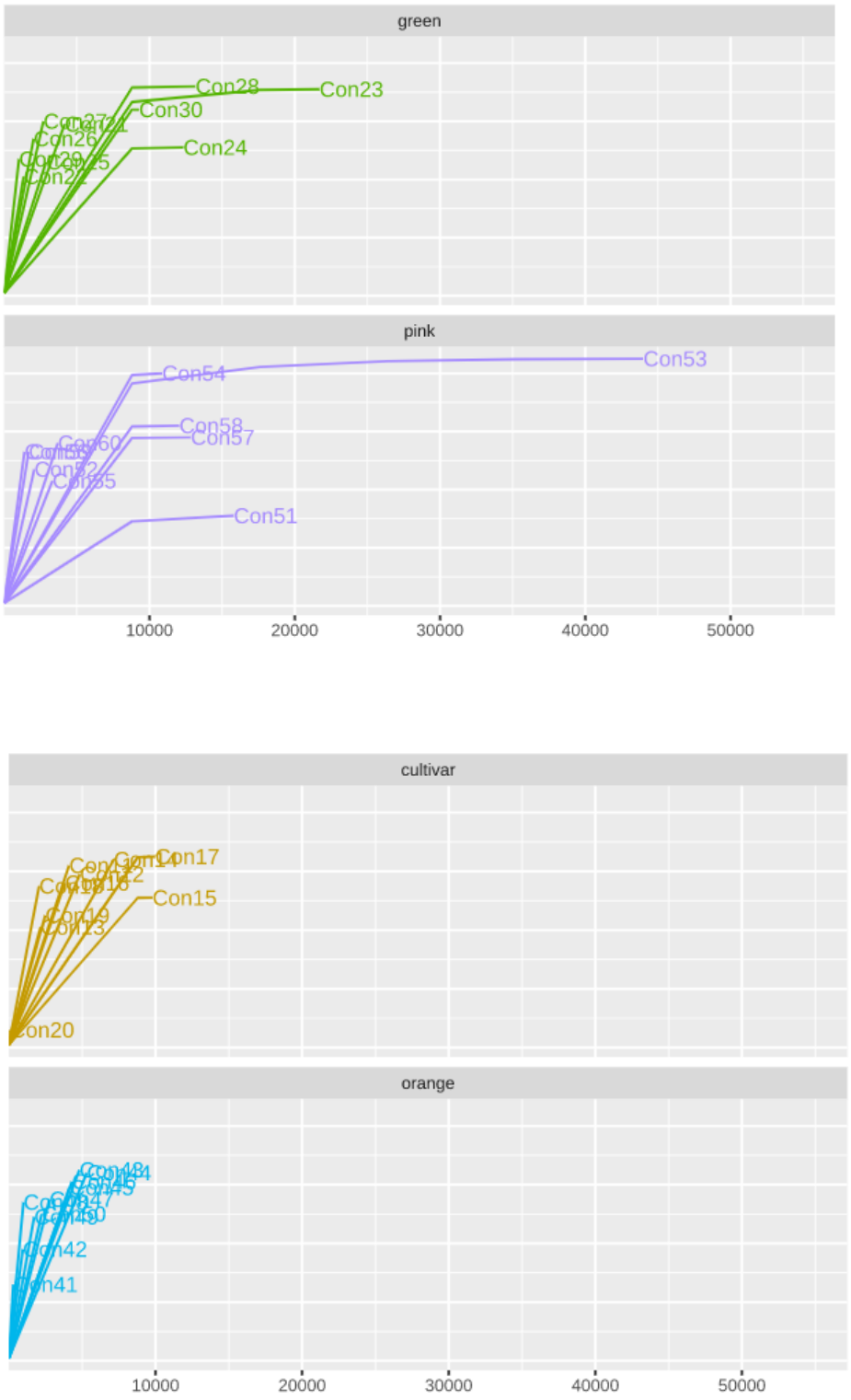
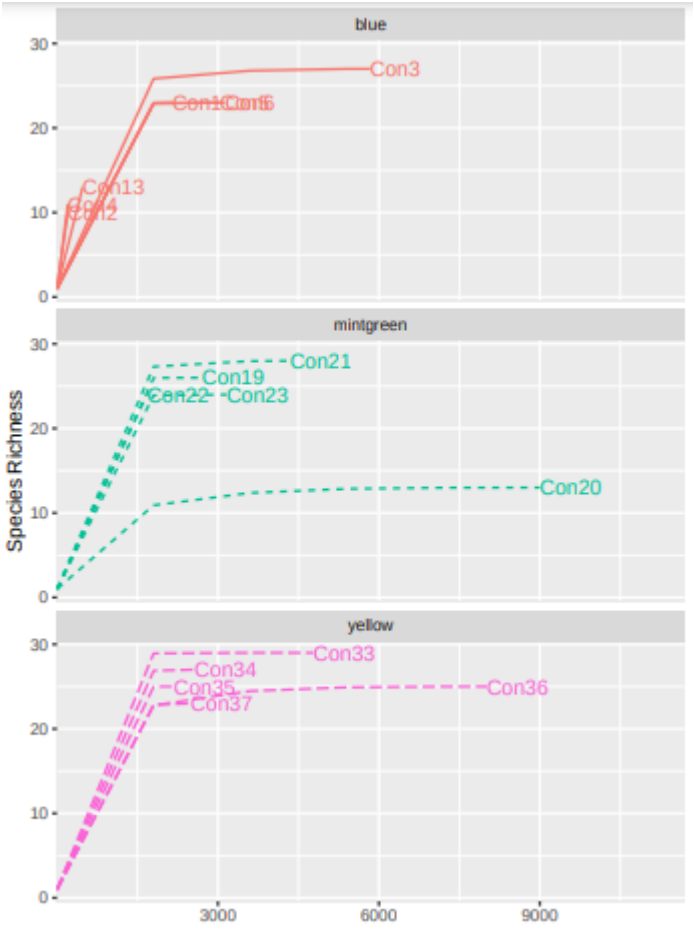


Fig.S.2.9.1. Rarefaction curves for bacterial samples separated per sample and genotype as blue, mint-green, yellow, green, pink, cultivar and orange. At the y axis is indicated species richness and at the x axis is indicated sequence sample size

2.9.2 Rarefaction curves for fungal amplicon sequence variants grouped by genotype.



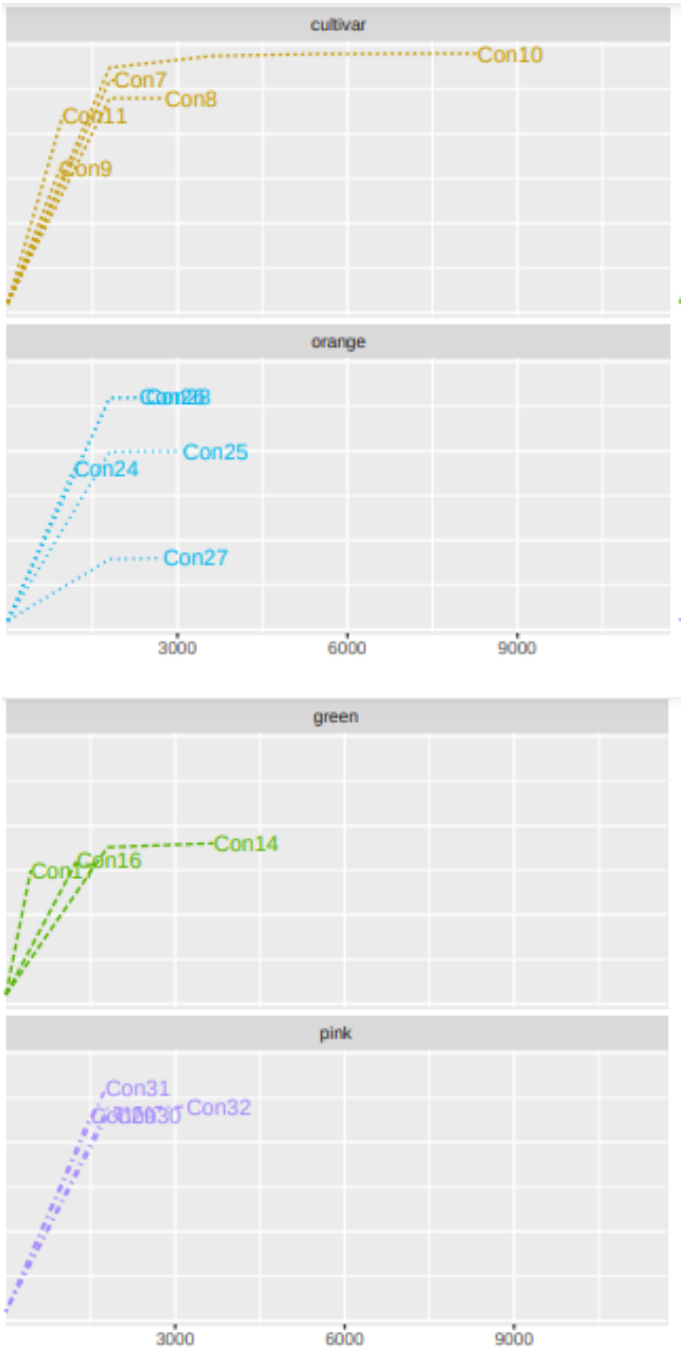


Fig S2.9.2 Rarefaction curves describing the observed fungal richness for amplicon sequence variants. Sequence sample size is on the x axis, and on the y axis the species richness**.**

2.10. SUPPLEMENTARY MATERIALS BAR PLOTS FOR BACTERIA PER SAMPLE AND GENOTYPE

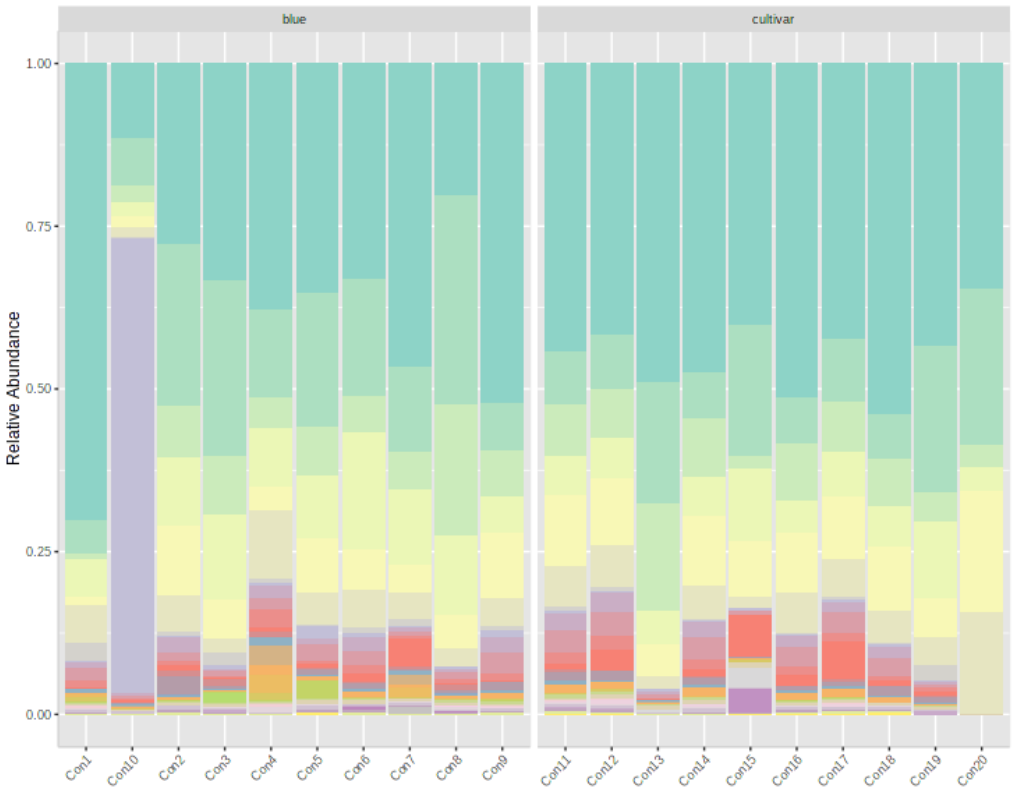


Fig. S2.10.1. Relative abundance for samples grouped by blue genotype on the left and cultivar on the right

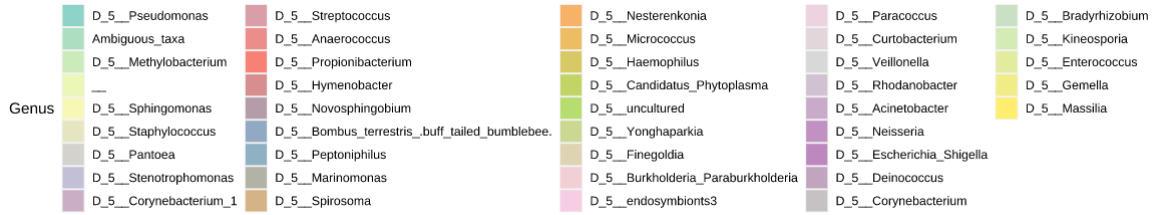


Fig. S2.10.5. Reference heatmap colours for genus taxonomic resolution for each sample

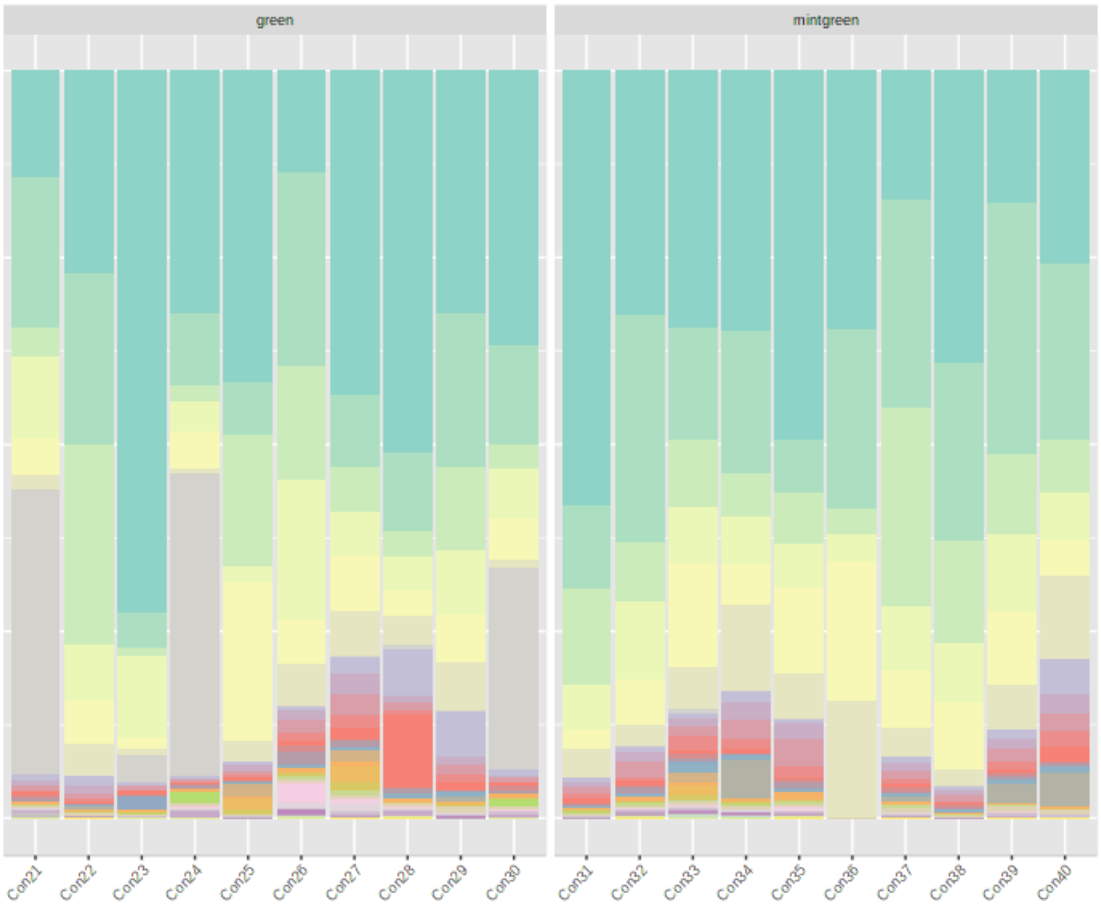


Fig. S2.10.2. Relative abundance for samples grouped by green on the left and mint-green on the right, individual samples ordered for each genotype

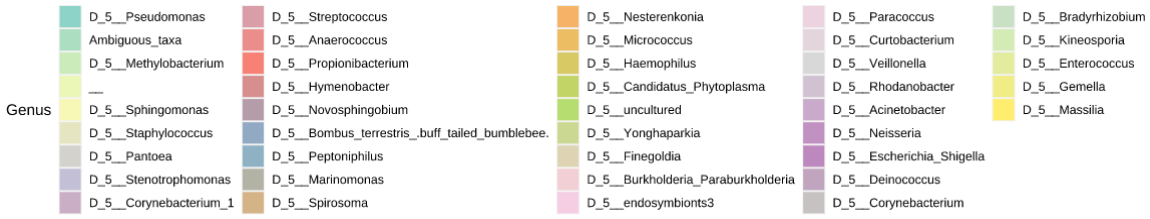


Fig. S2.10.5. Reference heatmap colours for genus taxonomic resolution for each sample

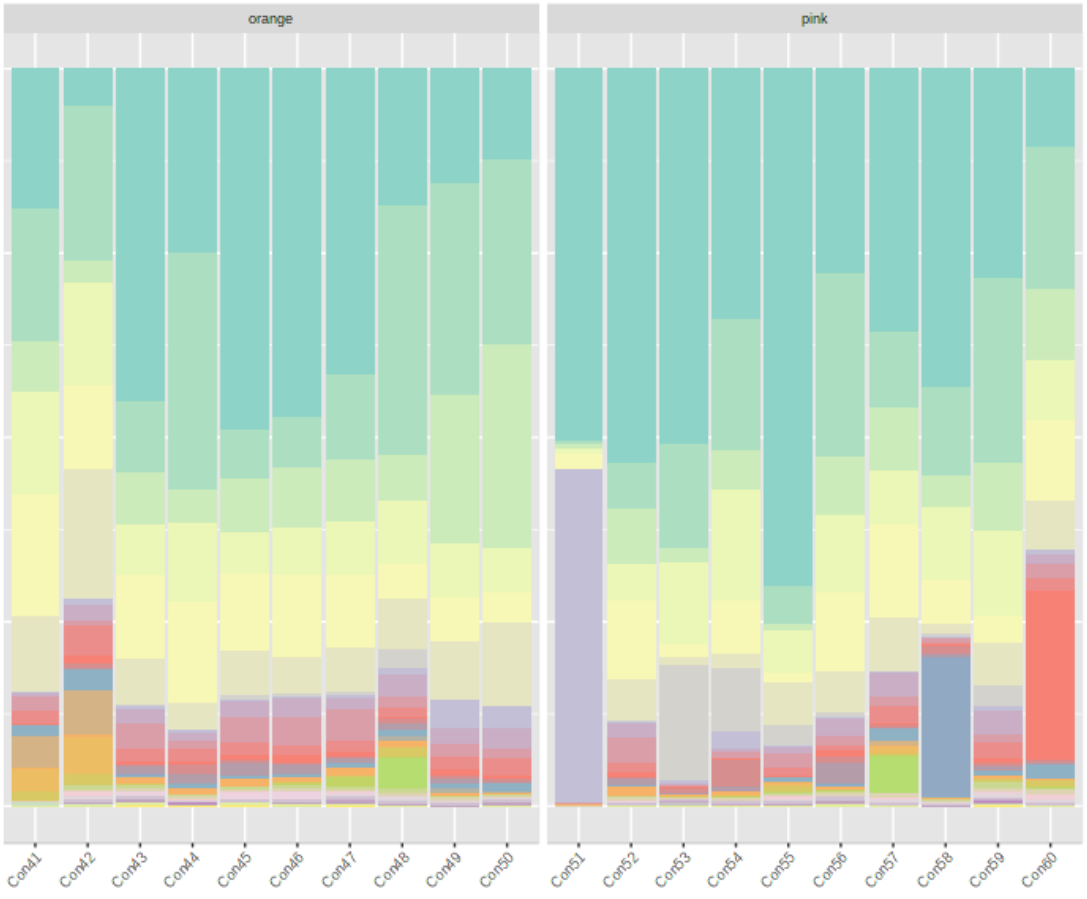


Fig. S2.10.3. Relative abundance for orange genotype on the left and pink genotype on the right, individual samples ordered for each genotype

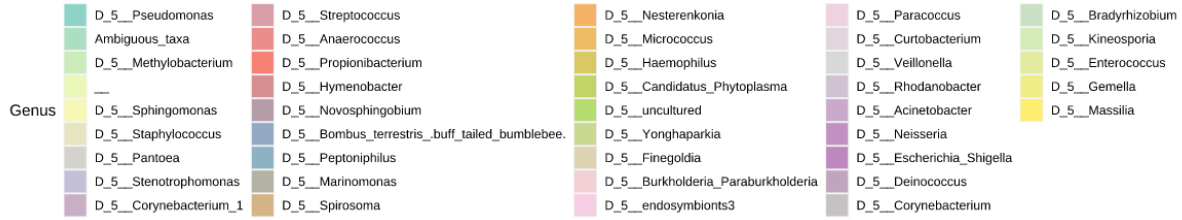


Fig. S2.10.5. Reference heatmap colours for genus taxonomic resolution for each sample

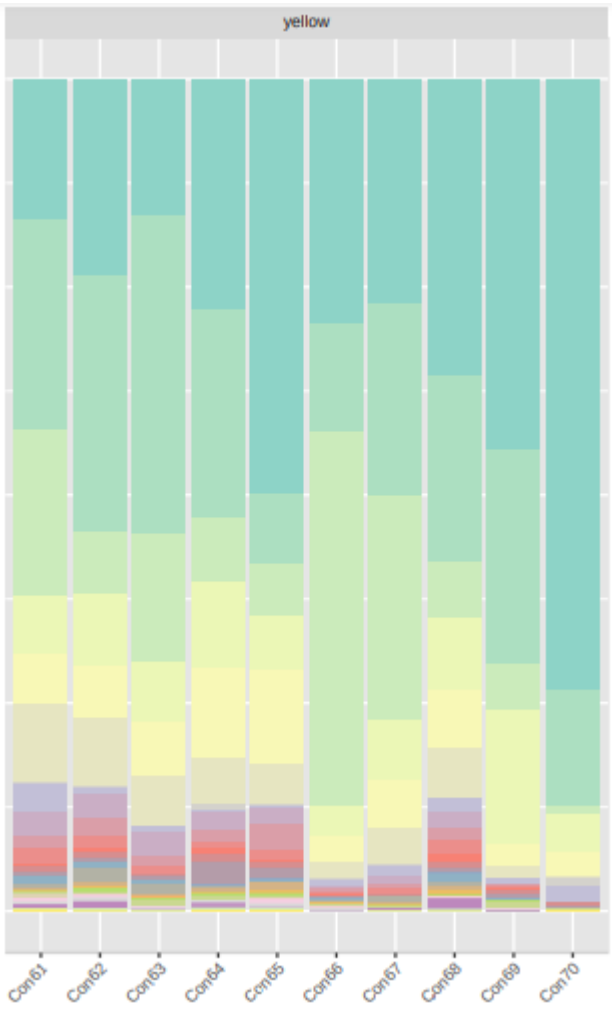


Fig. S2.10.4. Relative abundance for yellow genotype (y axis), individual samples ordered on the x, axis.

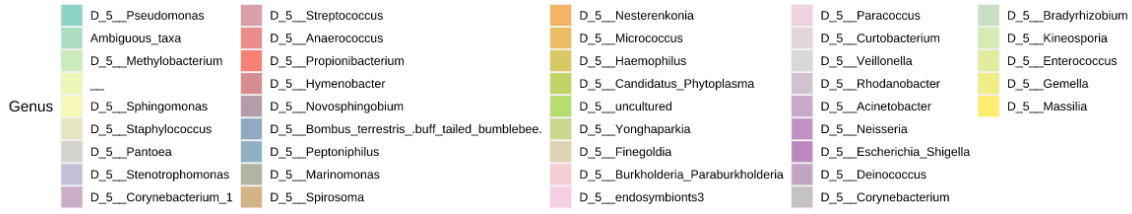
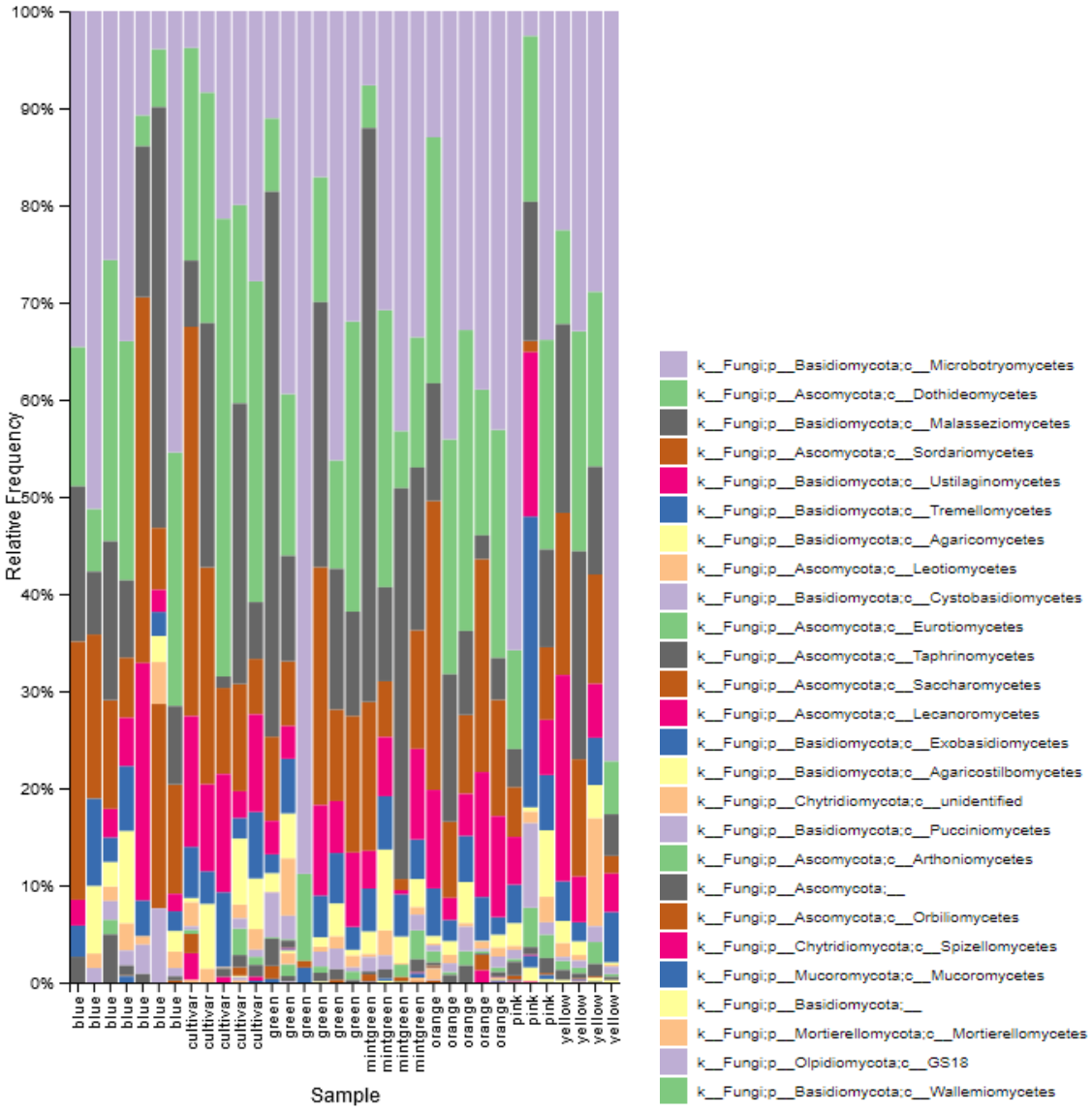


Fig. S2.10.5. Reference heatmap colours for genus taxonomic resolution for each sample

2.11. SUPPLEMENTARY MATERIALS BAR PLOTS FOR FUNGI PER SAMPLE AND GENOTYPE



PANEL A) PANEL B)

Fig. S2.11. Panel A. Bar plot showing the fungal relative frequency for each sample, and grouped by type of genotype: blue, cultivar, green, mint-green, orange, pink, yellow. Panel B. Reference colours for the bar plot with corresponding taxonomy at class level

2.12. SUPPLEMENTARY MATERIALS - Total Sugars and DHA per genotype and treatment

Table S2.12 Concentration of TSugars (mg/ml), TSugars mg/flower, DHA (mg/ml) and Normalized DHA (DHA/TS mg/flower), per clone genotype and treatment concentrated and per flower

Clone-treatm	TSugars mg/ml	TS mg/flower (concentrated)	DHA mg/ml	Norm DHA/TS mg/flower
Green	0.0253	1.2903	0.003	0.002325041
Green	0.0279	1.4229	0.01	0.007027901
Green	0.0291	1.4841	0.0112	0.007546661
Green	0.0487	2.4837	0.0156	0.006280952
Green	0.0813	4.1463	0.0188	0.004534163
green cov	0.0291	1.4841	0.0083	0.005592615
green cov	0.0329	1.6779	0.0116	0.006913404
green cov	0.0345	1.7595	0.0125	0.007104291
green cov	0.0419	2.1369	0.0148	0.006925921
green cov	0.0513	2.6163	0.0252	0.009631923
Pink	0.0159	0.8109	0.0055	0.006782587
Pink	0.0267	1.3617	0.0056	0.004112506
Pink	0.0402	2.0502	0.0104	0.005072676
Pink	0.0587	2.9937	0.0107	0.003574172
Pink	0.0675	3.4425	0.0265	0.007697894
pink cov	0.0345	1.7595	0.008	0.004546746
pink cov	0.0428	2.1828	0.013	0.005955653
pink cov	0.0644	3.2844	0.014	0.004262575
pink cov	0.1013	5.1663	0.0198	0.00383253
pink cov	0.1324	6.7524	0.0749	0.011092352
mint green	0.0145	0.7395	0.0045	0.006085193
mint green	0.0309	1.5759	0.0046	0.002918967
mint green	0.0341	1.7391	0.0056	0.003220056
mint green	0.0453	2.3103	0.0114	0.004934424
mint green	0.0534	2.7234	0.0179	0.006572667
mint green cov	0.0254	1.2954	0.0067	0.005172148
mint green cov	0.042	2.142	0.0073	0.00340803
mint green cov	0.0534	2.7234	0.0088	0.003231255
mint green cov	0.0544	2.7744	0.0161	0.005803057
mint green cov	0.0552	2.8152	0.0191	0.006784598
Yellow	0.019	0.969	0.0042	0.004334365
Yellow	0.0504	2.5704	0.0058	0.002256458
Yellow	0.0515	2.6265	0.0091	0.003464687
Yellow	0.0545	2.7795	0.0139	0.005000899
Yellow	0.1156	5.8956	0.0142	0.002408576

yellow cov	0.0235	1.1985	0.0048	0.004005006
yellow cov	0.0449	2.2899	0.0053	0.002314512
yellow cov	0.0497	2.5347	0.0055	0.002169882
yellow cov	0.0498	2.5398	0.0117	0.004606662
yellow cov	0.0772	3.9372	0.0161	0.0040892
Orange	0.0401	2.0451	0.0065	0.003178329
Orange	0.0435	2.2185	0.0088	0.003966644
orange	0.046	2.346	0.01	0.004262575
orange	0.0499	2.5449	0.0133	0.005226139
orange	0.1374	7.0074	0.0439	0.006264806
orange cov	0.0493	2.5143	0.0087	0.003460208
orange cov	0.0497	2.5347	0.0119	0.004694836
orange cov	0.0558	2.8458	0.0193	0.006781924
orange cov	0.0765	3.9015	0.0218	0.005587595
orange cov	0.1024	5.2224	0.0233	0.00446155
cultivar	0.0248	1.2648	0.0038	0.003004428
cultivar	0.0405	2.0655	0.0063	0.003050109
cultivar	0.0534	2.7234	0.0065	0.002386722
cultivar	0.0644	3.2844	0.0127	0.003866764
cultivar	0.0716	3.6516	0.0148	0.004053018
cultivar cov	0.0676	3.4476	0.0096	0.002784546
cultivar cov	0.0716	3.6516	0.0125	0.003423157
cultivar cov	0.083	4.233	0.0161	0.003803449
cultivar cov	0.0805	4.1055	0.0193	0.004701011
cultivar cov	0.1324	6.7524	0.0225	0.003332149
blue	0.0337	1.7187	0.0138	0.008029324
blue	0.0466	2.3766	0.0079	0.003324076
blue	0.0581	2.9631	0.0129	0.004353549
blue	0.0615	3.1365	0.0081	0.002582496
blue	0.0977	4.9827	0.0028	0.000561944
blue cov	0.0402	2.0502	0.0034	0.001658375
blue cov	0.0407	2.0757	0.004	0.001927061
blue cov	0.0553	2.8203	0.0142	0.005034925
blue cov	0.0729	3.7179	0.0224	0.006024907
blue cov	0.1413	7.2063	0.0321	0.004454436

CHAPTER 3.

The role of bacteria in dihydroxyacetone production in mānuka plants

ABSTRACT

In mānuka plants the production of dihydroxyacetone (DHA) in nectar is related to methylglyoxal (MGO), the compound commonly associated with the antioxidant and antibacterial properties of mānuka honey. The nectar is inhabited by bacterial communities that can impact metabolite production; however, little is known about these communities and whether they influence DHA production. High throughput sequencing of microbial communities is a commonly used method to identify interactions between community members followed by co-occurrence network analysis. In this chapter, the bacterial networks for six mānuka clones and one cultivar were analysed to identify highly connected 'hub' bacteria that might have functional importance in the bacterial network and DHA production. Network topology and connectivity revealed the *Pantoea* genus as one of the important connector nodes. To analyse DHA production from the *Pantoea spp*. *in vitro* and *in vivo* conditions, *Pantoea agglomerans* was cultivated on natural nectar and selective media and inoculated on mānuka flowers in the field. Flowers inoculated with *P. agglomerans* were found to have elevated DHA compared with the control flowers (no inoculation). The network central distances between bacteria at the genus level reveals similar co-occurrence values and with positive values between both *Phytoplasma* and *Corynebacterium* with *Pantoea,* suggesting that species of these genera may occur together in the nectar. *Corynebacterium* spp. is known to produce DHA from dihydroxyacetone phosphate (DHAP) or glycerol. In the nectar, an acidic environment facilitates these conversions by enabling optimal enzyme function, and, also, chemical stability of newly formed DHA. Together, these results highlight the importance of the nectar bacterial community in DHA production in mānuka plants and could explain the observed variability in DHA content among plant genotypes and between New Zealand regions.

3.1. INTRODUCTION

Mānuka plants, native to New Zealand, are valued for their honey. Dihydroxyacetone (DHA) in the nectar of these plants is converted to methylglyoxal (MO) in honey, which is one of the quality markers for mānuka honey (Adams et al., 2009). Methylglyoxal has an antimicrobial effect and therefore increases the value of mānuka honey (Lu et al., 2014). In addition, other molecules such as glycosides are under study due to their antimicrobial properties (Alvarez-Suarez et al. 2014).

Plants can produce DHA as an intermediate in carbohydrate metabolism (Córdova et al., 2002). Metabolic pathways that give plants capabilities to produce DHA are found across different plant taxa (Gee et al. 1988) and are not unique to *Leptospermum*. Interestingly, some bacteria and fungi can produce DHA via fermentation or organic synthesis routes. For example, under aerobic conditions *Corynebacterium glutamicum* converts sugars into DHA by dephosphorylation of DHAP (Dihydroxyacetone phosphate) to DHA using dihydroxyacetone phosphate dephosphorylase (*hdpA*) (Jojima et al., 2015). The dephosphorylase enzyme from *C. glutamicum* has been used in *Escherichia coli* expression systems to produce DHA (Li et al., 2010). In fungal systems, the enzyme glycerol dehydrogenase (GDH) can produce DHA from glycerol as a substrate as shown in experiments using recombinant *Saccharomyces cerevisiae* (Nguyen and Nevoigt 2009).

Other precursors of DHA could be carbon dioxide and methane. Bacteria can participate in this process and also in the conversion of DHA into other molecules such as ethanol and succinate (Wang et al., 2018). A possible role of the DHA molecule could be as an antimicrobial, since after its conversion into methylglyoxal it turns into a toxic molecule (Borysiuk et al., 2018). DHA is also toxic by itself as it can affect cells through its connection with protein deterioration linked to glycation and end-product protein formation (Molin et al., 2007). In microorganisms, the contribution of fructose to cell stability though its conversion into DHAP could be a way to avoid toxic effects of DHA inside the cells, as has been shown for *S. cerevisiae* (Nomura et al., 2018).

There is no information on mānuka plants about the metabolites produced in different parts of the plant, but according to what we know from model plants such as *Arabidopsis*, leaf metabolism might be important in the contribution to metabolites of the aerial phloem circulation (Carrera et al., 2021).

Due to the metabolic pathways that can produce DHA from different substrates, it has been hypothesised that the microorganisms present in nectar might play a role in DHA production in mānuka plants as bacteria in the nectar have an environment with reactives to complete the reaction to obtain DHA. One evidence in support of this hypothesis is the elevated production of DHA in regions with higher temperatures (Williams et al., 2014), which overlaps with those in which bacteria have higher metabolic rates (Schulte, 2015).

Given the complexity of bacterial communities, co-occurrence networks produced from high throughput sequencing data are frequently used to identify interactions between community members and select "hub" or highly interconnected bacteria, for further exploration. Network analysis has been widely used by scientists from several disciplines to explore interactions between entities (Bascompte et al., 2006). Entities are defined in the study system and could be species in a food web (Krause et al., 2003) including bacteria across environmental gradients (Farrer et al., 2019), or fungal species across soils depths (Schlatter et al., 2018).

To analyse the network and its topological properties relevant for community roles, the following functions are evaluated in microbial ecology (Faust and Raes 2012): (i) mean degree: the number of edges of each node averaged over all the nodes in the network; (ii) degree distribution: the frequency of nodes vs. their (increasing) degree; (iii) average shortest path length: the shortest path between any two nodes is the single path with fewest edges between them (used as a measure of the efficiency of mass transport on a network); iv) betweenness centrality: the number of shortest paths between any two nodes in the graph passing through that node. The mean is calculated from all nodes in the network.

Members of an ecological community can be divided between generalists and specialists in terms of their distribution in the environment (Lindström and Langenheder 2012). Further studies under this classification have demonstrated that generalist bacteria are shaped by local environmental conditions (Lindh et al., 2016). Members of microbial communities studied by network analysis are classified as generalists when they bridge several different nodes within their own modules or among different modules, whereas specialists link to few nodes (Barberán et al. 2012). Therefore, generalists are key organisms for community function (Lu

et al., 2013). The main exchange of materials and information (traduced as signal exchange) among microbial species occurs amongst generalists. In contrast, specialists' nodes are more peripheral, having less connections with other nodes, reflecting less interactions with other bacteria as a result of less adaptations to multiple hosts or environments.

Highly interacting bacterial taxa, denominated "microbial hubs", mediate between abiotic factors and host genotyping signatures, for instance, they can mediate microbe-microbe interactions and the process of microbial colonization (Agler et al., 2016). Taxa that have the hub role stabilize populations of specific microbes on individual plants and connect modules or groups of highly connected microbes (Poudel et al., 2016). Some of the microbial hub species are also keystone species (bacteria that are essential to ecosystem structure and function, and without them the ecosystem would be very different or would not exist), and they could be identified through network analysis by their topological features as path, transitivity (measure of the tendency of the nodes to cluster together), mean degree and centrality; trends for keystone taxa are to have high transitivity, high mean degree and low betweenness centrality (Berry and Widder 2014). This concept, when applied to a microbiome enables us to detect bacteria that have multiple biotic interactions of importance to ecosystem function out of proportion with their abundance (Müller et al., 2018; Power et al., 1996).

Pantoea belongs to the order Enterobacteriales, which includes bacteria with a high diversity of the Haloacid dehalogenase (HAD) enzyme family, as an e.g. phosphoserine phosphatase SerB from *Methanococcus jannaschii*, (Wang et al., 2001 and β-phosphoglucomutase from *Lactococcus lactis* (Lahiri et al., 2002). Hydrolases have the ability to dephosphorylase DHAP (Kuznetsova et al. 2006), which is one of the substrates in the nectaries and a potential precursor of DHA. The microbial capability of conduct hydrolyse DHAP in the nectaries could contribute to the DHA presence nectar.

Pantoea agglomerans has been experimentally proven to possess phytate (inositol polyphosphate)-degrading enzyme(s) with optimal activity at pH 4.5 (Greiner et al., 2004); which shows its potential activity on phosphorous compounds. Another substrate used by *P. agglomerans* for DHA production is glycerol, thanks to the activity of the enzyme glyceradehyde-3-phosphate dehydrogenase (GAP-DH) (Barbirato et al. 1997). The *Pantoea sp.* Genome, which possesses genes encoding for phosphatases, also includes a putative regulatory activity for GAP-DH regulation and function (Suleimanova et al. 2015). *P. agglomerans* also has the ability to produce indole-3-acetic acid (IAA), solubilize phosphate,

and inhibit plant pathogens (Luziatelli et al. 2020). In summary, this means that several biochemical capabilities could give *P. agglomerans* a key role in microbial communities. *P. agglomerans* seems to have the capabilities to adapt to the nectar environment and to use plant metabolites with its multiple metabolic pathways.

This study aimed to analyse the bacterial network interactions in the mānuka nectar and explore the capability of a selected hub bacterium (*Pantoea agglomerans*) to produce DHA under laboratory conditions with natural nectar and under field conditions in mānuka flowers. Based on previous literature, I hypothesize that nectar microbes such as *P. agglomerans* are capable of modifying nectar chemistry, mainly DHA production.

3.2. METHODS

3.2.1. Study areas and systems

The study was conducted in a population of mānuka clones (six different colour code genotypes) at Moginie block of the Pasture & Crop Research Unit (PCRU), Massey University (Palmerston North – New Zealand). The mānuka plants were planted in 2011 and propagated by cuttings as six genotype lines. Sampling was conducted in three different seasons: March 2017, October 2017, and October 2019. Nectar drops were recovered from five plants at the end of the flowering season and from mature flowers (bagged the previous day with a breathable mesh) by pipetting flower nectar with 10 µl sterile water and pooling it together. Samples were transported to the laboratory the same day on ice.

3.2.2. Sampling and bacteria identification

From the pooled nectar sample, 20 µl was streaked on Tryptone Soy Agar (TSA, Difco, Le Point de Caix, France) plates. Plates were incubated at 25 °C for 72 h. Yellow-coloured bacterial colonies from bacteria were picked for colony PCR identification using the universal prokaryote phylogenetic marker, 16S rRNA-encoding gene. The PCR amplicons were generated with bacterial universal primers (27F and 1492R), which generated an almost full length of gene encoding for 16S rRNA (Lane, 1991). Reactions were prepared to a total of 50 µL with nuclease-free water as follows: For 16S, reactions contained 200 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.8X Platinum Taq HiFi buffer (Invitrogen, MA, USA), 1 U Platinum Taq HiFi polymerase (Invitrogen, MA, USA), and 10 ng of template. Thermal cycling was carried out using a Mastercycler (Roche, USA), using the following conditions: For 16S, an initial 95 ºC for 2 min, followed by 26 cycles of 94 ºC for 45 s, 52 ºC for 30 sec and 72 ºC for 1 min followed by a final extension at 72 ºC for 10 min.

The fragments from individual bacteria were Sanger-sequenced, and the obtained sequences were trimmed with chromas and assembled with Geneious (Kearse et al. 2012) and identified by closest match with Blast using GenBank as the reference database.

3.2.3. Network analysis

Once metabarcoding data was denoised, filtered, normalized and amplicon sequenced variants assigned to taxa (Chapter 2), co-occurrence patterns were explored using network inference based on significant correlations using the algorithm sparCC, developed for the compositional nature of the data for microbiomes based on its relative abundance (Friedman & Alm, 2012).

A phylogenetic molecular ecological network was constructed with Molecular Ecological Network Analyses, MENA (Deng et al., 2012). First, a standardization of high-throughput data is applied, then a pair-wise similarity of abundances and a determination of adjacency matrix by the RMT approach. A Pearson correlation coefficient (*r*-value) was calculated between each pair of OTUs, and a symmetric similarity matrix was formed after all *r*-values were calculated. The generated matrix was then resubmitted to MENA for further calculations of the networks. The threshold for defining a network is determined by

calculating the transition from Gaussian orthogonal ensemble to Poisson distribution of the nearest-neighbour eigenvalues; and hence the network is automatically defined based on the data structure itself. Afterwards, modules inside the network were detected by greedy modularity optimization. The network graph was generated using different OTUs (nodes) with positive or negative interactions (edges). Nodes are organised into modules, which are comprised of microbial species that strongly interact with each other but rarely with nodes in other modules. Therefore, modularity (M) measures the degree of organization of a network into delimited modules. Networks were generated with MicrobiomeAnalyst (Dhariwal et al. 2017; Chong et al. 2020) for Person and sparCC algorithms; related co-occurrence plots were defined for both distances' algorithms. sparCC uses a log ratio transformation and performs multiple iterations to identify taxa pairs that are outliers to background correlations.

Bacteria were analysed for their connectivity, node degree and network position to be selected for inoculation experiments.

3.2.4. Nectar sampling for *in vitro* experiments

Twenty flowers per treatment and per plant genotype were taken to the laboratory and stored at -20 °C. Once thawed the nectar was taken by pipette. During this process, it is inevitable (due to the change in turgidity of the cell walls) that fluids from the inside of the nectaries are mixed with the nectar. The resulting liquid was stored at -80 °C.

3.2.5. Bacterial selection for DHA production

Selection of bacteria for inoculation experiments, both *in vivo* and *in vitro,* was based on the following criteria:

1) Isolation of bacteria with relatively high abundance on the culture plates.
2) LEfSe (Lineal Discriminant Analysis Effect Size) from metabarcoding data (see Chapter 2, Section 3.2.2.).
3) Identification of functional significance in the microbial metacommunity network modelling used to analyse the topology and connectivity of hub bacteria.

3.2.6. Bacterial preparation for nectar inoculation

A pure culture of *Pantoea agglomerans,* inoculated from a single colony on a 3-day old TSA (Tryptone soy agar) plate, was grown in LB (Luria-Bertani) broth media, up to the start of exponential phase (25 °C, 6 hrs, aerobic, light conditions with agitation on a Rocket 25 platform at 60 RPM) immediately prior to inoculation of the nectar.

3.2.7. Nectar inoculation *in vitro*

The nectar was thawed and divided into 10 × 30 µl aliquots. Five nectar replicates were inoculated with bacteria and five with LB broth only as a control. To mimic *in vivo* conditions, the sample tube was placed in a light-proof container (to simulate the absence of light in the interface of hypanthium-nectaries) with the extracted nectar solution and cotton wool was placed on top to allow aeration. The samples were placed on the platform of a Rocker 25 (Labnet) at a speed of 60 RPM at 25 °C for 24 hrs and afterwards stored at -80 °C.

3.2.8. Nectar inoculation *in vivo*

Prior to inoculation, *P. agglomerans* was grown in LB from a pure culture up to exponential phase (2.0 X 10^8 bacteria/ml). The fresh liquid culture was taken on ice to the field and 10 flowers per plant in a total of 8 plants of the blue clone were inoculated with 10 µl of the *P. agglomerans* solution estimated to contain 2 x 10^6 bacteria cells, Control flowers were inoculated with 10 µl of the growth medium (LB) on the same day. Flowers were already mature and at the end of the flowering season. After inoculation all the flowers were covered with breathable mesh. After three days the flowers were recovered and taken to the laboratory on ice in one bag per plant for each treatment. Flowers were stored at -20 °C.

3.2.9. HPLC quantification of DHA

To quantify DHA from *in vivo* and *in vitro* samples. High-Performance Liquid Chromatography (HPLC) was used. HPLC requires two phases: phase A (a solution of nanopure water combined with acetonitrile (ACN) at ratio of 70:30 (v/v) and phase B (100% ACN). HPLC reaction solution contained hidroxyacetone (HA) (3.01 mg/ml) with O-(2,3,4,5,6-pentaflurobenzyl) hydroxylamine (PFBHA) as a derivatising reagent (19.8 mg/ml), which was diluted in 0.1 M citrate buffer adjusted to pH 4 with sodium hydroxide (4M NaOH). Six DHA standards (100, 75, 60, 50, 25, 0 µl) aliquots were taken from a stock solution of DHA (3.88 mg/ml) made up to 10 ml. For HPLC analysis, 20 µl of the standard or the nectar sample and 25 µl of HA were mixed and shaken on a rotator for an hour. A derivatizing reagent (PFBHA) in volumes of 40 µl was added and samples were stored for a further hour. Finally, 1.5 ml ACN and 0.5 µl deionised Milli-Q water were added to the samples and filtered (0.22 µm) before being measured by HPLC with a UV Diode array detector (λ=263 nm, UVD340U) using a Synergy Fusion column (size 4.6 X 7.5 mm, 4 µm particle size). The column was kept at 30 °C.

3.2.10. Determination of nectar pH

The pH of nectar was measured as the acidity influenced the chemical reactions and the microorganisms in the given environment. The pH on the plants was measured by adding drops of nectar onto pH strips and then compared with manufacturer's standard reference colours (Macherey-Nagel, Germany). Flowers were stored at -20 °C then the nectar was taken with 10 µl of water and the resulting fluid was a mixture composed of hypanthium fluids and nectar. The pH was measured in the field, and again in the laboratory before and after reaction with the bacteria.

3.2.11. Statistical analysis

Obtained DHA values were tested for normality with a Shapiro-Wilk test and homogeneity of variances (Shapiro and Wilk 1965). Afterwards a t-test in R (R Core Team, 2017) was used for comparing DHA levels in inoculated nectar (bacteria and control) under *in vitro* and *in vivo* conditions.

3.3. RESULTS

3.3.1. Network analysis

Network analysis revealed that among the total bacterial community, *Staphylococcus, Pantoea, Marinomonas, Escherichia, Peptoniphilus, Micrococcus, Methylobacterium, and Yonghaparkia* (Fig.1, Tab S3.8.1) were the bacteria with higher degree and centrality measures. They were found to be well connected and had a high level of betweenness centrality when high throughput illumina data was analysed with a co-occurrence network. All these bacteria are good connectors and could therefore be considered “hub” bacteria in the nectar network of mānuka plants. Nodes corresponding to bacteria are represented in a graphical network in Figure 1.

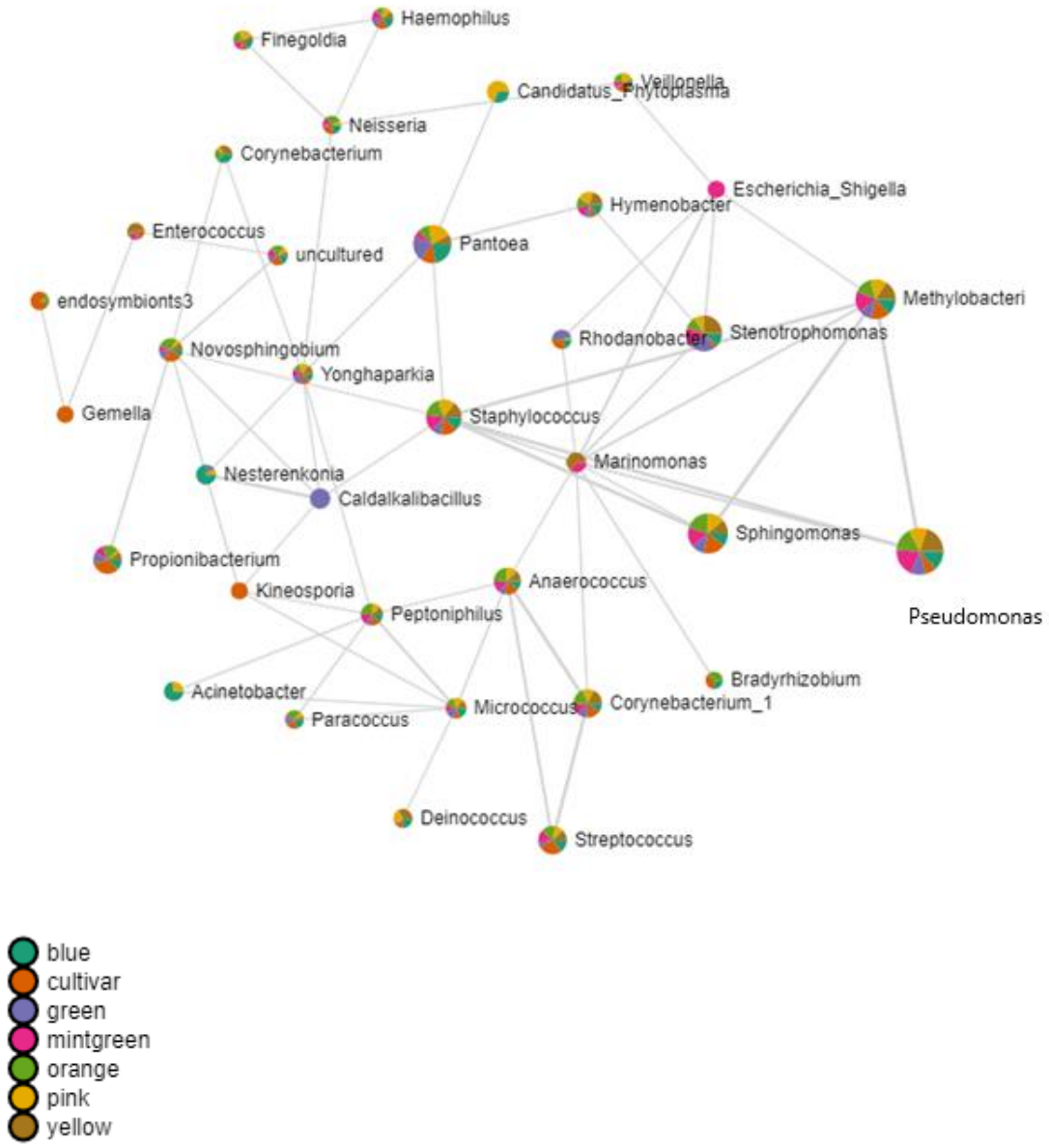


Figure 1. Network projection constructed with sparCC (i.e., assumes a sparse network and uses log-ratio transformation and then performs iterations to identify taxa pairs that are outliers to background correlations). Parameters: 180 permutations, data filtered with Inter-quantile range 10%, low count features remove at a prevalence in samples of 10%, P-value < 0.05, correlation threshold 0.3, coloured by median, genus level, experimental factor: genotype. The size of the circle represents the total abundance.

3.3.2. Network description

The resulting nectar microbial network (Figure 1) consisted of 37 nodes (average degree or node connectivity: 6.45 and average for betweenness centrality: 40.96). *Marinomonas* were the bacteria with highest node degree (20), followed by *Staphylococcus* (14), *Novosphingobium* (12), *Yonghaparkia* (12), *Peptoniphilus* (12), *Micrococcus* (12), *Anaerococcus* (10), *Caldakalibacillus* (10), *Escherichia-Shigella* (10), *Methylobacterium* (10), *Neisseria* (8), *Pantoea* (8), *Kineosporia* (8), *Nesterenkonia* (8), Stenotrophomonas (6), *Corynebacterium* (6), and *Sphingomonas* (6) (Table ST.8.1).

To analyse all the possible bacterial interactions and to corroborate the network topology, a different algorithm (MENA) was applied. A correlation matrix was constructed using Pearson distances with a minimum threshold cut-off (0.310). The total nodes obtained was 53 and the total links 569, with an average connectivity of 21.47 and average length of 1,592 (Table TS3.8.3).

3.3.3. Bacteria selection for inoculation

Based on the network analysis data, ease of cultivation, and potential for DHA production (as reported in the literature), *Pantoea* was selected for further inoculation experiments (I conducted additional phylogenetic analysis to establish the relationship between the cultivated bacteria used in this study and other publish sequences and found it to be *Pantoea agglomerans* – Figure S3.2) .

Pantoea was found to be abundant in all the plant genotypes, its presence was consistent across different seasons (Chapter 2), and it was easy to cultivate. Network analysis and LEfSe analysis show that *Pantoea* has at the same time a central position in the network and a high degree and connectivity, suggesting it is a hub/keystone species (Table S3.8.1). Its presence was positively correlated with other bacteria such as *Phytoplasma, Hymenobacter*, and *Yonghaparkia* (although a negative correlation was found with Staphylococcus).

Furthermore, the results of the LEfSe analysis and the Lineal Discriminant Analysis (LDA) (section 3.2.2.) show that *Pantoea* has a high LDA value (6.5, $P \leq 0.05$, log LDA score cut-

off ≤ 2). This means that there are significant differences in relative abundance for *Pantoea* genus among the mānuka genotypes from LEfSe analysis.

3.3.4. Hydrogen concentration of nectar

Nectar pH measured in the flowers in the field was near neutral (pH 6.5). Nectar was recovered from 10 frozen flowers and samples were pooled together. The pH was measured again and was pH 5.5. Then pooled nectar samples were aliquoted and inoculated with *P. agglomerans*. After aerobic incubation with *P. agglomerans*, the pH of the sample was pH 4.5. The turbidity of the nectary solution decreased after incubation with *P. agglomerans*. These values are summarized in Table 1.

TABLE 1. Measurements of pH and temperature for the nectar in the plant, the nectary fluids and after bacterial reaction.

Environmental/solutions	**pH [+/- 0.2]**	**Temperature °C**
pH nectar on the plant	6.5	20
pH nectar (pre-reaction)	5.5	20
pH nectar after incubation with *P. agglomerans*	4.5	20

3.3.5. DHA production

P. agglomerans was selected for nectar inoculation trials both *in vivo* and *in vitro*, because of the degree value in the topology and good level of connectivity of the *Pantoea* genus in the network, and also because of its ease of *in vitro* culture. In this section, I report its effects on DHA production, but in the supplementary material I include a separate assay where I assessed the effect of *Pantoea* on floral sugars Figure S3.3.).

Inoculation of natural nectar under laboratory conditions with *P. agglomerans* resulted in a significant increase in DHA (T-test, $p<0.05$, The *t*-value is -2.83589) (Figure 2.). Values for DHA production in natural nectar under laboratory conditions are shown in Table TS3.8.5.

Under field conditions, the results show a trend towards increased DHA production, but the standard deviations were wider, and the results were not significantly different for a Kruskal-Wallis test (P=0.0587) (Figure 3 and Table TS3.8.6).

3.3.6. Dihydroxyacetone statistical analysis

3.3.6.1. Laboratory production of DHA

The DHA mean for laboratory conditions (*in vitro)*: For the control group was 8.180E-03 ± 1.777E-03 [g/l], and for the group inoculated with *P. agglomerans* the DHA mean was 2.770E-02 ± 2.770E-02 [g/l]. The data is normally distributed Shapiro-Wilk (W = 0.86302, p-value = 0.2393), and the variances are homogeneity distributed F-test (F = 3.0503, p-value = 0.1644). There were significant differences between the control and the inoculated group (T-test, P= 0.022), with higher DHA production in the inoculated treatment (Figure 3.4.1).

3.3.6.2. Field production of DHA

The DHA mean for the field conditions (*in vivo*). For the control group was 1.282E-02 ± 1.276E-02 [g/l], and for the group inoculated with *P. agglomerans the* DHA mean was 3.118E-02 ± 2.229E-02 [g/l]. The data is not normally distributed (Shapiro-Wilk, 0.868, P=0.026), so a non-parametric test was used (Kruskal-Wallis), which showed no significant differences between the control and the inoculated group (Kruskal-Wallis test (P= 0.0587). Although there was a trend towards higher DHA production in the inoculated treatment (Figure 3.4.2).

3.4. Graphical representation of DHA production

3.4.1) Dihydroxyacetone laboratory production after inoculation with *Pantoea agglomerans* (Figure 2).

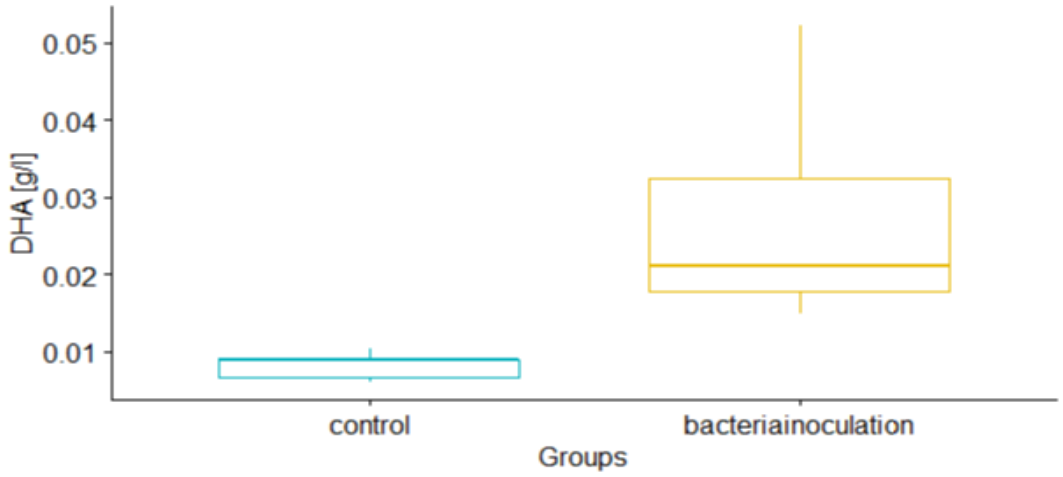


Figure 2.) DHA production in laboratory conditions (aerobic conditions and agitation, T=20 °C, time course reaction 20 hours). The control is mānuka nectar and LB medium, the treatment is nectar with bacteria inoculation in the same medium (10 µl).

3.4.2.) Dihydroxyacetone field production after inoculation with *Pantoea agglomerans* (Figure 3).

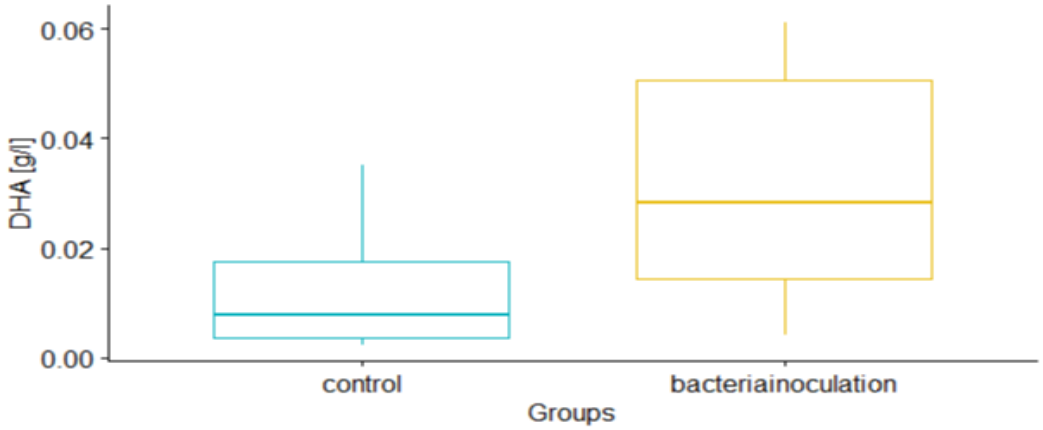


Figure 3.) DHA production under field conditions (October 2019, Palmerston North, mānuka blue clone, 10 flowers inoculation 10 µl, 72 hrs bagged flowers). Control: mānuka flowers nectar inoculated with LB medium and Treatment: mānuka flowers nectar with bacteria inoculation (10 µl).

3.5. DISCUSSION

This study characterised the cultivable bacterial network in mānuka nectar, suggesting several hub bacteria. Inoculation of a selected hub bacteria, *Pantoea agglomerans,* was shown to increase dihydroxyacetone content in nectar under *in vitro* conditions, supporting the important role of microbes in DHA production. The results for *in vivo* conditions also showed a trend increase in the DHA concentration, but this was not significant, probably due to the introduction of the variability of the overall system, (plant physiology and nectar secretion) and more challenging conditions for working in the field introduced more variability in the manipulation of the flowers.

Pantoea agglomerans was present in the plants studied in three different seasons and in abundance both in cultures and in the metabarcoding (Chapter 2). Network analysis for all clones and cultivar for this bacterium shows high connectivity and node values, which suggests it is a hub bacterium that connects functions with others (Agler et al., 2016) with generalist ecological functions in the nectar network and with potential to be a keystone bacterium in the nectar network due to its centrality measures (Martín González et al., 2010).

In addition, in the sparCC network analysis *P. agglomerans* showed a negative correlation coefficient with *Staphylococcus* (TS3.8.1), meaning that they may be competitive rather than cooperative in the nectar microbial ecology. By contrast, a positive correlation coefficient was found with *Bacillus* and *Phytoplasma* bacteria, meaning that they may have a similar function or cooperative interactions.

Other positive correlations are found with *Yonghaparkia,* belonging to the phylum *Actinobacteria,* and *Hymenobacter* from the phylum *Bacteroidetes*. Biological networks are characterized for presenting local assortative (nodes tend to be connected with other nodes with similar degree values) (Piraveenan et al., 2013), the similarities in nodes degree can be defined as similarity in co-occurrence or mutual exclusion (Newman, 2006).

Interestingly, this co-occurrence pattern with bacteria from different phyla differs from what has been found in soils with positive interactions between members of the same phyla (Barberán et al., 2012). Competition scenarios in nectar through complex chemical conditions and low availability of nutrients might switch these correlations to different phyla in order to assess metabolic complementation under the same niche. The positive correlation found between *Phytoplasma* and *Pantoea* reveals a strong tendency for co-occurrence between both bacteria. Phytoplasma life environment or ecological niche is usually the phloem of plants (Hogenhout et al., 2009). The positive correlation that has with *Pantoea* could mean a similar environmental context for both bacteria that could be related with a similar ecological niche for *Phytoplasma* and *Pantoea,* suggesting that *Pantoea* is also related to the plant phloem in terms of their living environment or its physiological role. The occurrence of *Pantoea* and *Phytoplasma* could also mean a selection advantage for better performance for the host through interactions with a restricted endosymbiotic bacterium and *Pantoea* (Pacifico et al. 2019).

In terms of carbon metabolism, there is also a positive correlation between *Pantoea* and an important bacteria *Streptococcus*, species from this genus are known for their capabilities to

fix CO_2 because of its carboxylase activity (Arioli et al., 2009), and also as important fermenters where plants isolates have better performance at syneresis (liquids phase separation) (Saito et al., 2020) and with capabilities to work at low pH and to acidify while is growing (Linares et al., 2016). This may indicate that a shared niche between both bacteria could reflect similar functions in terms of mechanisms for carbon fixation, acidification, and formation of resistant structures for phase physical separation.

The leaf and nectaries environment could be a niche where bacteria could contribute to carbon fixation through different ways in complementation with the natural activity of the plant i.e. (Calvin cycle Figure S3.1.). Interconnections between bacteria with carbon fixation capabilities and the plant could be the main metabolic network for the overall lateral reactions in the upper organs of the plant that could be involved in dihydroxyacetone production. Dihydroxyacetone is a compound that can be generated and degraded by bacteria: known bacteria that degrade it include *Halomonas* (Bardavid & Oren, 2008), although it was not present in the metabarcoding which suggests the majority of the DHA produced in the nectar could end in the seed and afterwards start a conversion into MGO.

The capability of mobility that *Pantoea* has between the interior of cells and the nectar may explain a metabolic role in DHA formation and a relationship with pollinators in the nectar out of the nectary cavity or cells, as seen in other plants (Farré-armengol & Junker, 2019). Other known seed endophytes bacteria such as *Anaerococcus* have a positive correlation with *Corynebacterium* which possesses dephosphorylations capabilities, meaning that an endosymbiotic bacterium in the nectaries could have the capability of interacting with bacteria related to DHA production.

P. agglomerans has capabilities to decrease the pH and as a consequence to change the microbial community in the soil, it can also increase salt tolerance in maize through the increase of photosynthetic pigments (Gond et al., 2015). These important changes could also happen in the nectar and the position in the network backs this function.

The pH in the natural mānuka nectar is 6.5 (Table 1), which is suitable for forager's choice and plants attractiveness (Hendriksma et al., 2014), and provides optimal conditions for stability of dihydroxyacetone (Ciriminna et al., 2018). A pH close to neutral ensures less water oxidation and therefore less peroxide formation (Liu et al., 2019). A neutral pH means fewer free radical compounds and lends stability to the dihydroxyacetone molecule. In nectar, a pH of 5.5 is suitable for the transformation of DHAP into DHA (van Herk et al., 2009),

since this pH value is in the optimum range for the enzyme *hdpA*. With the availability of DHAP provided in an acidic environment, the hypothesis of the overflow metabolism, according to which DHA is formed by the action of *hdpA* only when excess DHAP accumulates in cells (Jojima et al., 2015), seems to explain DHA formation in the nectar. This also can explain the higher production of DHA in the experiment *in vivo* in comparison to *in vitro* conditions. This is the first work that investigates bacteria nectar inoculation to study DHA production. The DHA values observed here are in the range recorded in another mānuka study in the North Island (Noe et al., 2019). The increase in DHA production was higher in the field (Fig 2.) than in the laboratory conditions (Fig 3.) possibly because of the availability of more DHAP under natural plant conditions, or other positive feedbacks between *P. agglomerans* and resident bacteria that increase the activation of pathways that could enhance enzyme bacteria activity. Interactions such as these have been shown to affect the DHA production by *Escherichia coli* under laboratory conditions in an artificial medium (Peiro et al., 2019). The differences were significant in the *in vitro* inoculation, while in the field they did not reach a significant threshold.

Nectar and its metabolites have been shown elsewhere to be reabsorbed by mānuka plants (Clearwater et al. 2018). The *in vivo* inoculation of the flowers could have affected DHA reabsorption, which makes it more difficult to measure the real amount of DHA produced by the bacteria as the molecule can move into the nectaries. The movement of fluids with molecules will affect the DHA measures and the translocations of DHA to other organs with a direct effect on the DHA concentration in the nectar. By contrast, the tube where the reaction took place for the in vitro experiment was not affected by the reabsorption dynamics of the nectaries in the flowers, and all the DHA produced remained in the tube under controlled conditions.

Nectaries are an important component of mānuka plants and their distribution, surface and number in plant branches facilitate plant-bacteria interactions, by increasing the number of possible interactions between bacteria and nectaries components. Their chemical composition with an aqueous component in an acidic environment seems to be a natural reactor for DHA production where bacteria could be playing a major role. A water-based composition favours the catalytic activity of *dihydroxyacetone phosphate dephosphorylase* to complete reaction where DHA and phosphate could be the final end or even intermediate products that could be substrates for further reactions, either for plants or bacteria.

The correlation between *Pantoea* and *Corynebacterium* was 0.423 (table S3.8.3) suggests a mutualistic interaction between them. In nature, the bacterium *Corynebacterium glutamicum* converts sugars to DHA under aerobic conditions as it possesses the enzyme *dihydroxyacetone phosphate dephosphorylase*, and the possibility for the bacteria to produce DHA in only one step from DHAP (Jain et al., 2016). *Pantoea* species have several mechanisms of dephosphorylation (Brady, 2010), and some of these pathways are regulated through quorum sensing mechanisms (Xavier & Bassler, 2005). The positive correlation coefficient found in the network correlation analysis could mean that *Corynebacterium* and *Pantoea* possess similar functions. Therefore, the formation of DHA in mānuka nectar might be due to the interaction between these two bacteria and the plant as a source of DHAP for bacteria metabolisms. *Pantoea* also possesses glycerol dehydrogenase (Barbirato et al., 1997), and more recently it was determined that this enzyme catalyses the production of higher amounts of DHA than other bacteria without this enzyme (Li et al., 2010). Furthermore, *Pantoea* possesses capabilities to solubilise phosphates particularly on acid media (Son et al., 2006) via the secretion of organic acids and siderophores (Castagno et al., 2011).

Pantoea was found in all the genotypes and in the network, topology has a high level of degree and betweenness that shows connectivity reflecting interactions with other bacteria. Because of its central position and connectivity in the network *Pantoea* can be considered a hub bacterium. The position that *Pantoea* has in the network also matches with the generalist concept, and the versatile metabolism that presents also contributes to the generalist's ecological role (Lang et al., 2017). Though *Pantoea* is not the bacteria with the highest levels of connectivity and abundance, meaning that it could have other relationships for e.g., the plant (host), and also keeps interactions with the rest of the nectar microbial community. Bacteria that present dephosphorylation capabilities that are in the nectar e.g., *Pantoea* and *Corynebacterium*; can dephosphorylate DHAP and transform it into DHA. The availability of more than one species of bacteria in the nectar with capabilities of DHA production will keep levels of DHA in the nectar. Furthermore, the acidification of the media facilitates phosphate dissolution and activates other metabolic pathways that could incorporate phosphorous in other plant metabolism or bacteria functions. Further research introducing isotope labelling will be necessary to determine dihydroxyacetone and phosphate movements in the metabolic pathways. Gaining knowledge of metabolites origins will be important to clarify plant-bacteria interactions and enhance plant performance.

3.6. CONCLUSION

Mānuka plants possess high levels of DHA in the nectar, a trait that has not been recorded in other plants. Bacteria are known to be capable of producing DHA through different metabolic pathways. Interactions between bacteria and metabolites provided by mānuka plants may explain their high DHA levels. In this study, I found that inoculation of *Pantoea agglomerans* increased dihydroxyacetone content in nectar both *in vivo* and *in vitro*, though only the *in vitro* results were statistically significant, without significantly affecting sugar contents. The literature suggests that bacteria can use, versatile plant metabolic molecules like DHAP or glycerol and convert them into DHA in mānuka nectar, which serves several biological purposes such as UV protection, removal of free radicals, and protection against pathogens.

There also seems to be an interconnection between dihydroxyacetone production and the bias towards high fructose levels in the mānuka nectar. Fructose metabolism increases DHAP levels, and this pathway would favour DHA production, therefore, high levels of fructose may be correlated with high DHA levels in mānuka plants, but this requires further exploration.

The presence of bacteria with capabilities of producing DHA and also to consume DHA is a perfect scenario to regulate DHA concentrations in the cells of nectar parenchyma. Mānuka plants seem to select bacteria that are able to produce DHA rather than consume it, and to regulate fructose levels in the nectar with the production of DHAP favouring plant-bacteria interactions, and DHA accumulation in the nectar.

This work confirms our hypothesis regarding the ability of nectar microorganisms to influence DHA nectar production, as there was an increase in the DHA content after the reaction with the bacteria *P. agglomerans* and natural nectar under laboratory conditions. While a similar trend was observed in the field the results were not significant, multiple environmental factors and challenges related to inoculation and sample collection could have contributed to this outcome. I recommend repeating these measurements to confirm this observation and to explore the effect of environmental factors on microbe-mediated DHA production.

3.7. REFERENCES

Alvarez-Suarez, José M, Massimiliano Gasparrini, Tamara Y Forbes-Hernández, Luca Mazzoni, and Francesca Giampieri. 2014. “The Composition and Biological Activity of Honey: A Focus on Mānuka Honey.” *Foods* . https://doi.org/10.3390/foods3030420.

Adams, C. J., Manley-Harris, M., & Molan, P. C. (2009). The origin of methylglyoxal in New Zealand mānuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, *344*(8), 1050–1053. https://doi.org/10.1016/j.carres.2009.03.020

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S.-T., Weigel, D., & Kemen, E. M. (2016). Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLOS Biology*, *14*(1), e1002352. https://doi.org/10.1371/journal.pbio.1002352

Arioli, S., Roncada, P., Salzano, A. M., Deriu, F., Corona, S., Guglielmetti, S., Bonizzi, L., Scaloni, A., & Mora, D. (2009). The relevance of carbon dioxide metabolism in *Streptococcus thermophilus*. *Microbiology (Reading, England)*, *155*(Pt 6), 1953–1965. https://doi.org/10.1099/mic.0.024737-0

Barberán, A., Bates, S. T., Casamayor, E. O., & Fierer, N. (2012). Using network analysis to explore co-occurrence patterns in soil microbial communities. *The ISME Journal*, *6*(2), 343–351. https://doi.org/10.1038/ismej.2011.119

Barbirato, F., Chedaille, D., & Bories, A. (1997). Propionic acid fermentation from glycerol: comparison with conventional substrates. *Applied Microbiology and Biotechnology*, *47*(4), 441–446. https://doi.org/10.1007/s002530050953

Bascompte, J., Jordano, P., & Olesen, J. M. (2006). Asymmetric coevolutionary networks facilitate biodiversity maintenance. *Science*, *312*(5772), 431 LP – 433. https://doi.org/10.1126/science.1123412

Berry, D., & Widder, S. (2014). Deciphering microbial interactions and detecting keystone species with co-occurrence networks. *Frontiers in microbiology*, *5*, 219. https://doi.org/10.3389/fmicb.2014.00219

Borysiuk, K., Ostaszewska-Bugajska, M., Vaultier, M. N., Hasenfratz-Sauder, M. P., & Szal,

B. (2018). Enhanced Formation of Methylglyoxal-Derived Advanced Glycation End Products in *Arabidopsis* Under Ammonium Nutrition. *Frontiers in plant science*, *9*, 667. https://doi.org/10.3389/fpls.2018.00667

Bardavid, E. R., & Oren, A. (2008). Dihydroxyacetone metabolism in *Salinibacter ruber* and in *Haloquadratum walsbyi*. Extremophiles : life under extreme conditions, 12(1), 125–131. https://doi.org/10.1007/s00792-007-0114-x

Brady, M. J. (2010). Allosteric trumps covalent in the control of glycogen synthesis. *Cell Metabolism*, *12*(5), 428–430. https://doi.org/https://doi.org/10.1016/j.cmet.2010.10.008.

Carrera, D. Á., George, G. M., Fischer-Stettler, M., Galbier, F., Eicke, S., Truernit, E., Streb, S., & Zeeman, S. C. (2021). Distinct plastid fructose bisphosphate aldolases function in photosynthetic and non-photosynthetic metabolism in Arabidopsis. Journal of experimental botany, 72(10), 3739–3755. https://doi.org/10.1093/jxb/erab099

Castagno, L. N., Estrella, M. J., Sannazzaro, A. I., Grassano, A. E., & Ruiz, O. A. (2011). Phosphate-solubilization mechanism and in vitro plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado River Basin (Argentina). *Journal of Applied Microbiology*, *110*(5), 1151–1165. https://doi.org/10.1111/j.1365-2672.2011.04968.x

Chong, J., Liu, P., Zhou, G., & Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature Protocols*, *15*(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1

Clearwater, M.J.; Revell, M.; Noe, S.; Manley-Harris, M. Influence of genotype, floral stage, and water stress on floral nectar yield and composition of mānuka (*Leptospermum scoparium*). *Ann. Bot.* 2018, *121*, 501–512

Ciriminna, R., Fidalgo, A., Ilharco, L. M., & Pagliaro, M. (2018). Dihydroxyacetone: An updated insight into an important bioproduct. *ChemistryOpen*, *7*(3), 233–236. https://doi.org/10.1002/open.201700201

Córdova, A., Notz, W., & Barbas III, C. F. (2002). Direct organocatalytic aldol reactions in buffered aqueous media. *Chemical Communications*, *24*, 3024–3025. https://doi.org/10.1039/B207664K

Dhariwal, Achal, Jasmine Chong, Salam Habib, Irah L King, Luis B Agellon, and Jianguo

Xia. 2017. “MicrobiomeAnalyst: A Web-Based Tool for Comprehensive Statistical, Visual and Meta-Analysis of Microbiome Data.” *Nucleic Acids Research* 45 (W1): W180–88. https://doi.org/10.1093/nar/gkx295.

Deng, Y., Jiang, Y.-H., Yang, Y., He, Z., Luo, F., & Zhou, J. (2012). Molecular ecological network analyses. *BMC Bioinformatics*, *13*(1), 113. https://doi.org/10.1186/1471-2105-13-113

Edgar R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Elevi Bardavid, R., Oren, A. Dihydroxyacetone metabolism in *Salinibacter ruber* and in *Haloquadratum walsbyi* . *Extremophiles* **12**, 125–131 (2008). https://doi.org/10.1007/s00792-007-0114-x

Farré-armengol, G., & Junker, R. R. (2019). Bacteria associated with flowers affect the reproductive success of *Brassica napus* L . via scent-mediated interactions with pollinators. *BioRxiv*, 1–34. https://doi.org/10.1101/685453

Farrer, E. C., Porazinska, D. L., Spasojevic, M. J., King, A. J., Bueno de Mesquita, C. P., Sartwell, S. A., Smith, J. G., White, C. T., Schmidt, S. K., & Suding, K. N. (2019). Soil microbial networks shift across a high-elevation successional gradient . In *Frontiers in Microbiology*(Vol.10,p.2887). https://www.frontiersin.org/article/10.3389/fmicb.2019.02887

Faust, Karoline, and Jeroen Raes. 2012. “Microbial Interactions: From Networks to Models.” *Nature Reviews Microbiology* 10 (8): 538–50. https://doi.org/10.1038/nrmicro2832.

Friedman, J., & Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLOSComputationalBiology*,*8*(9),e1002687. https://doi.org/10.1371/journal.pcbi.1002687

Gee, R W, R U Byerrum, D W Gerber, and N E Tolbert. 1988. “Dihydroxyacetone Phosphate Reductase in Plants.” *Plant Physiology* 86 (1): 98–103. https://doi.org/10.1104/pp.86.1.98.

Greiner, R. Purification and Properties of a Phytate-degrading Enzyme from *Pantoea agglomerans*. *Protein J* 23, 567–576 (2004). https://doi.org/10.1007/s10930-004-7883-1

Gond, S K, M S Torres, M S Bergen, Z Helsel, and J F White Jr. 2015. "Induction of Salt Tolerance and Up-Regulation of Aquaporin Genes in Tropical Corn by *rhizobacterium Pantoea Agglomerans*." *Letters in Applied Microbiology* 60 (4): 392–99. https://doi.org/https://doi.org/10.1111/lam.12385.

Hendriksma, H. P., Oxman, K. L., & Shafir, S. (2014). Amino acid and carbohydrate tradeoffs by honey bee nectar foragers and their implications for plant–pollinator interactions. *Journal of Insect Physiology*, *69*, 56–64. https://doi.org/https://doi.org/10.1016/j.jinsphys.2014.05.025

Hogenhout, S. A., Oshima, K., Ammar, e., Kakizawa, S., Kingdom, H. N., & Namba, S. (2008). Phytoplasmas: bacteria that manipulate plants and insects. Molecular plant pathology, 9(4), 403–423. https://doi.org/10.1111/j.1364-3703.2008.00472.x.

Jain, V. K., Tear, C. J. Y., & Lim, C. Y. (2016). Dihydroxyacetone production in an engineered *Escherichia coli* through expression of *Corynebacterium glutamicum* dihydroxyacetone phosphate dephosphorylase. *Enzyme and Microbial Technology*, *86*, 39–44. https://doi.org/https://doi.org/10.1016/j.enzmictec.2016.01.015

Jojima, T., Noburyu, R., Sasaki, M., Tajima, T., Suda, M., Yukawa, H., & Inui, M. (2015). Metabolic engineering for improved production of ethanol by *Corynebacterium glutamicum*. *Applied Microbiology and Biotechnology*, *99*(3), 1165–1172. https://doi.org/10.1007/s00253-014-6223-4

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)*, *28*(12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Krause, A. E., Frank, K. A., Mason, D. M., Ulanowicz, R. E., & Taylor, W. W. (2003). Compartments revealed in food-web structure. *Nature*, *426*(6964), 282–285. https://doi.org/10.1038/nature02115

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular biology and evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kuznetsova E., Proudfoot M., Gonzalez C.F., Brown G., Omelchenko M.V., Borozan I.,

Carmel L., Wolf Y.I, Mori H., Savchenko A.V., Arrowsmith C.H., Koonin E.V., Edwards A.M., Yakunin A.F. Genome-wide analysis of substrate specificities of the *Escherichia coli* haloacid dehalogenase-like phosphatase family. *J Biol Chem*. 2006 Nov 24;281(47):36149-61. doi: 10.1074/jbc.M605449200. Epub 2006 Sep 21. PMID: 16990279.

Lahiri, S. D., Zhang, G., Dunaway-Mariano, D., & Allen, K. N. (2002). Caught in the act: The structure of phosphorylated β-phosphoglucomutase from Lactococcus lactis. *Biochemistry*, *41*(26), 8351-8359.

Lane, D. J. (1991). 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

Lang, J., Vigouroux, A., El Sahili, A., Kwasiborski, A., Aumont-Nicaise, M., Dessaux, Y., Shykoff, J. A., Moréra, S., & Faure, D. (2017). Fitness costs restrict niche expansion by generalist niche-constructing pathogens. *The ISME Journal*, *11*(2), 374–385. https://doi.org/10.1038/ismej.2016.137

Li, M., Wu, J., Liu, X., Lin, J., Wei, D., & Chen, H. (2010). Enhanced production of dihydroxyacetone from glycerol by overexpression of glycerol dehydrogenase in an alcohol dehydrogenase-deficient mutant of *Gluconobacter oxydans*. *Bioresource Technology*, *101*(21), 8294–8299. https://doi.org/10.1016/j.biortech.2010.05.065

Lindh, M. V, Sjöstedt, J., Casini, M., Andersson, A., Legrand, C., & Pinhassi, J. (2016). Local environmental conditions shape generalist but not specialist components of microbial metacommunities in the Baltic Sea . In *Frontiers in Microbiology* (Vol. 7, p. 2078). https://www.frontiersin.org/article/10.3389/fmicb.2016.02078

Linares, D. M., O'Callaghan, T. F., O'Connor, P. M., Ross, R. P., & Stanton, C. (2016). *Streptococcus thermophilus* APC151 Strain Is Suitable for the Manufacture of Naturally GABA-Enriched Bioactive Yogurt. Frontiers in microbiology, 7, 1876. https://doi.org/10.3389/fmicb.2016.01876

Lindström, Eva S, and Silke Langenheder. 2012. "Local and Regional Factors Influencing Bacterial Community Assembly." *Environmental Microbiology Reports* 4 (1): 1–9. https://doi.org/10.1111/j.1758-2229.2011.00257.x.

Liu, Y., Han, Y., Zhang, Z., Zhang, W., Lai, W., Wang, Y., & Cao, R. (2019). Low

overpotential water oxidation at neutral pH catalyzed by a copper(ii) porphyrin. *Chemical Science*, *10*(9), 2613–2622. https://doi.org/10.1039/C8SC04529A

Lu, J., Turnbull, L., Burke, C. M., Liu, M., Carter, D. A., Schlothauer, R. C., Whitchurch, C. B., & Harry, E. J. (2014). Mānuka-type honeys can eradicate biofilms produced by *Staphylococcus aureus* strains with different biofilm-forming abilities. *PeerJ*, *2*, e326–e326. https://doi.org/10.7717/peerj.326

Lu, L., Yin, S., Liu, X., Zhang, W., Gu, T., Shen, Q., & Qiu, H. (2013). Fungal networks in yield-invigorating and -debilitating soils induced by prolonged potato monoculture. *Soil Biology and Biochemistry*, *65*, 186–194. https://doi.org/https://doi.org/10.1016/j.soilbio.2013.05.025

Luziatelli, F., Ficca, A. G., Cardarelli, M., Melini, F., Cavalieri, A., & Ruzzi, M. (2020). Genome sequencing of *Pantoea agglomerans C1* provides insights into molecular and genetic mechanisms of plant growth-promotion and tolerance to heavy metals. *Microorganisms*, *8*(2), 153. https://doi.org/10.3390/microorganisms8020153

Martín González, A., Dalsgaard, B., & Olesen, J. (2010). Centrality measures and the importance of generalist species in pollination networks. *Ecological Complexity*, *7*. https://doi.org/10.1016/j.ecocom.2009.03.008

Molin, Mikael & Pilon, Marc & Blomberg, Anders. (2007). Dihydroxyacetone-induced death is accompanied by advanced glycation endproduct formation in selected proteins of *Saccharomyces cerevisiae* and *Caenorhabditis elegans.* Proteomics. 7. 3764-74. 10.1002/pmic.200700165.

Müller, J., Noss, R. F., Thorn, S., Bässler, C., Leverkus, A. B., & Lindenmayer, D. (2018). Increasing disturbance demands new policies to conserve intact forest. *Conservation Letters*, e12449. https://doi.org/10.1111/conl.12449

Newman, M. E. J. (2006). Modularity and community structure in networks. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 8577–8582. https://doi.org/10.1073/pnas.0601602103

Nguyen, H T T, and E Nevoigt. 2009. "Engineering of *Saccharomyces cerevisiae* for the Production of Dihydroxyacetone (DHA) from Sugars: A Proof of Concept." *Metabolic Engineering* 11 (6): 335–46. https://doi.org/10.1016/j.ymben.2009.07.005.

Noe, S., Manley-Harris, M., & Clearwater, M. J. (2019). Floral nectar of wild mānuka (*Leptospermum scoparium)* varies more among plants than among sites. *New Zealand Journal of Crop and Horticultural Science*, *47*(4), 282–296. https://doi.org/10.1080/01140671.2019.1670681

Nomura, S., Satoh, M., Fujita, T. et al. Cardiomyocyte gene programs encoding morphological and functional signatures in cardiac hypertrophy and failure. *Nat Commun* 9, 4435 (2018). https://doi.org/10.1038/s41467-018-06639-7

Pacifico, D., Squartini, A., Crucitti, D., Barizza, E., Lo Schiavo, F., Muresu, R., Carimi, F., & Zottini, M. (2019). The role of the endophytic microbiome in the grapevine response to environmental triggers. In *Frontiers in Plant Science* (Vol. 10, p. 1256). https://www.frontiersin.org/article/10.3389/fpls.2019.01256

Peiro, C., Millard, P., de Simone, A., Cahoreau, E., Peyriga, L., Enjalbert, B., & Heux, S. (2019). Chemical and metabolic controls on dihydroxyacetone metabolism lead to suboptimal growth on *Escherichia coli*. *Applied and Environmental Microbiology*, *85*(15), e00768-19. https://doi.org/10.1128/AEM.00768-19

Piraveenan, M., Prokopenko, M., & Hossain, L. (2013). Percolation centrality: quantifying graph-theoretic impact of nodes during percolation in networks. *Plos One*, *8*(1), e53095. https://doi.org/10.1371/journal.pone.0053095

Poudel, R., Jumpponen, A., Schlatter, D. C., Paulitz, T. C., Gardener, B. B. M., Kinkel, L. L., & Garrett, K. A. (2016). Microbiome networks: A systems framework for identifying candidate microbial assemblages for disease management. *Phytopathology*, *106*(10), 1083–1096. https://doi.org/10.1094/PHYTO-02-16-0058-FI

Power, M.E., Tilman, D., Estes, J.A., Menge, B.A., Bond, W.J., Mills, L.S., Daily, G., Castilla, J.C., Lubchenco, J., Paine, R.T., (1996). Challenges in the quest for keystones. Bioscience 46 (8), 609–620.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Saito, Y., Tonouchi, A., Harada Y., Ogino, Ryouhei & Toba, Takahiro. (2020). Isolation of *Streptococcus thermophilus* strains from plants in Japan and their application to milk fermentation. Food Science and Technology Research. 26. 1-8. 10.3136/fstr.26.1.

Schlatter, D. C., Kahl, K., Carlson, B., Huggins, D. R., & Paulitz, T. (2018). Fungal community composition and diversity vary with soil depth and landscape position in a no-till wheat-based cropping system. *FEMS Microbiology Ecology*, *94*(7). https://doi.org/10.1093/femsec/fiy098

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *The Journal of Experimental Biology*, *218*(12), 1856 LP – 1866. https://doi.org/10.1242/jeb.118851

Shapiro, S S, & M B Wilk. 1965. "An Analysis of Variance Test for Normality (Complete Samples)†." *Biometrika* 52 (3–4): 591–611. https://doi.org/10.1093/biomet/52.3-4.591

Son, H.J., Park, G.T., Cha, M.S., & Heo, M.S. (2006). Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans R-42* isolated from soybean rhizosphere. *Bioresource Technology*, *97*(2), 204–210. https://doi.org/10.1016/j.biortech.2005.02.021

Suleimanova, A. D., Beinhauer, A., Valeeva, L. R., Chastukhina, I. B., Balaban, N. P., Shakirov, E. V., Greiner, R., & Sharipova, M. R. (2015). Novel Glucose-1-Phosphatase with High Phytase Activity and Unusual Metal Ion Activation from Soil Bacterium *Pantoea sp. strain 3.5.1*. *Applied and environmental microbiology*, *81*(19), 6790–6799. https://doi.org/10.1128/AEM.01384-15

van Herk, T., Hartog, A. F., Babich, L., Schoemaker, H. E., & Wever, R. (2009). Improvement of an acid phosphatase/DHAP-dependent aldolase cascade reaction by using directed evolution. *ChemBioChem*, *10*(13), 2230–2235. https://doi.org/10.1002/cbic.200900102

Wang, W., Kim, R., Jancarik, J., Yokota, H., & Kim, S. H. (2001). Crystal structure of phosphoserine phosphatase from Methanococcus jannaschii, a hyperthermophile, at 1.8 Å resolution. *Structure*, *9*(1), 65-71.

Wang, L., Chauliac, D., Rhee, M. S., Panneerselvam, A., Ingram, L. O., & Shanmugam, K. T. (2018). Fermentation of dihydroxyacetone by engineered *Escherichia coli* and *Klebsiella variicola* to products. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(17), 4381–4386. https://doi.org/10.1073/pnas.1801002115

Williams, S., King, J., Revell, M., Manley-Harris, M., Balks, M., Janusch, F., Kiefer, M.,

Clearwater, M., Brooks, P., & Dawson, M. (2014). Regional, annual, and individual variations in the dihydroxyacetone content of the nectar of mānuka (*Leptospermum scoparium*) in New Zealand. *Journal of Agricultural and Food Chemistry*, *62*(42), 10332–10340. https://doi.org/10.1021/jf5045958

Xavier, K. B., & Bassler, B. L. (2005). Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. *Journal of Bacteriology*, *187*(1), 238 LP – 248. https://doi.org/10.1128/JB.187.1.238-248.2005

3.8. SUPPLEMENTARY MATERIALS

Table S3.8.1. sparCC degree and betweenness centrality values for each taxon

Taxon	Label	Degree	Betweenness
D_5__Marinomonas	*D_5__Marinomonas*	20	192
D_5__Staphylococcus	*D_5__Staphylococcus*	14	130
D_5__Novosphingobium	*D_5__Novosphingobium*	12	165
D_5__Yonghaparkia	*D_5__Yonghaparkia*	12	133
D_5__Peptoniphilus	*D_5__Peptoniphilus*	12	94
D_5__Micrococcus	*D_5__Micrococcus*	12	46
D_5__Anaerococcus	*D_5__Anaerococcus*	10	93
D_5__Caldalkalibacillus	*D_5__Caldalkalibacillus*	10	46

D_5__Escherichia_Shigella	*D_5__Escherichia_Shigella*	10	44
D_5__Methylobacterium	*D_5__Methylobacterium*	10	0
D_5__Neisseria	*D_5__Neisseria*	8	84
D_5__Pantoea	*D_5__Pantoea*	8	54
D_5__Kineosporia	*D_5__Kineosporia*	8	32
D_5__Nesterenkonia	*D_5__Nesterenkonia*	8	0
D_5__Stenotrophomonas	*D_5__Stenotrophomonas*	6	15
Ambiguous_taxa	*Ambiguous_taxa*	6	0
D_5__Corynebacterium_1	*D_5__Corynebacterium_1*	6	0
D_5__Sphingomonas	*D_5__Sphingomonas*	6	0
D_5__uncultured	*D_5__uncultured*	4	93
D_5__Enterococcus	*D_5__Enterococcus*	4	64
D_5__Corynebacterium	*D_5__Corynebacterium*	4	42
D_5__Gemella	*D_5__Gemella*	4	33
D_5__Veillonella	*D_5__Veillonella*	4	31
D_5__Hymenobacter	*D_5__Hymenobacter*	4	7
D_5__Acinetobacter	*D_5__Acinetobacter*	4	0
D_5__Finegoldia	*D_5__Finegoldia*	4	0
D_5__Haemophilus	*D_5__Haemophilus*	4	0
D_5__Paracoccus	*D_5__Paracoccus*	4	0
D_5__Rhodanobacter	*D_5__Rhodanobacter*	4	0
D_5__Streptococcus	*D_5__Streptococcus*	4	0
D_5__Bradyrhizobium	*D_5__Bradyrhizobium*	2	0
D_5__Candidatus_Phytoplasma	*D_5__Candidatus_Phytoplasma*	2	0
D_5__Deinococcus	*D_5__Deinococcus*	2	0
D_5__endosymbionts3	*D_5__endosymbionts3*	2	0
D_5__Propionibacterium	*D_5__Propionibacterium*	2	0

Table S3.8.2. sparCC correlations values

Taxon1	Taxon2	Correlation	P. value
Ambiguous taxa	D_5__Marinomonas	0.543	0.011
Ambiguous taxa	D_5__Methylobacterium	0.9027	0.0276
Ambiguous taxa	D_5__Staphylococcus	0.8623	0.0276
D_5__Acinetobacter	D_5__Micrococcus	-0.3322	0.0055
D_5__Acinetobacter	D_5__Peptoniphilus	-0.3921	0.0055
D_5__Anaerococcus	D_5__Corynebacterium_1	0.8587	0.0055
D_5__Anaerococcus	D_5__Marinomonas	0.3361	0.011
D_5__Anaerococcus	D_5__Micrococcus	0.4153	0.0055
D_5__Anaerococcus	D_5__Peptoniphilus	0.3296	0.0166
D_5__Anaerococcus	D_5__Streptococcus	0.6382	0.0055
D_5__Bradyrhizobium	D_5__Marinomonas	-0.4058	0.0055
D_5__Caldalkalibacillus	D_5__Kineosporia	-0.3119	0.011
D_5__Caldalkalibacillus	D_5__Nesterenkonia	0.8358	0.0055
D_5__Caldalkalibacillus	D_5__Novosphingobium	-0.3596	0.0055
D_5__Caldalkalibacillus	D_5__Staphylococcus	0.3398	0.0166
D_5__Caldalkalibacillus	D_5__Yonghaparkia	-0.343	0.0166
D_5__Candidatus_Phytoplasma	D_5__Pantoea	0.3618	0.0221
D_5__Corynebacterium	D_5__Novosphingobium	-0.3136	0.0055

D_5__Corynebacterium	D_5__Yonghaparkia	-0.3178	0.0055
D_5__Corynebacterium_1	D_5__Anaerococcus	0.8587	0.0055
D_5__Corynebacterium_1	D_5__Marinomonas	0.3627	0.011
D_5__Corynebacterium_1	D_5__Streptococcus	0.8212	0.0055
D_5__Deinococcus	D_5__Micrococcus	-0.3149	0.0055
D_5__endosymbionts3	D_5__Gemella	0.3126	0.0055
D_5__Enterococcus	D_5__Gemella	0.3736	0.0055
D_5__Enterococcus	D_5__uncultured	-0.3069	0.011
D_5__Escherichia_Shigella	D_5__Marinomonas	0.5866	0.0055
D_5__Escherichia_Shigella	D_5__Methylobacterium	0.3446	0.0221
D_5__Escherichia_Shigella	D_5__Rhodanobacter	-0.3243	0.0055
D_5__Escherichia_Shigella	D_5__Stenotrophomonas	0.4068	0.0055
D_5__Escherichia_Shigella	D_5__Veillonella	-0.342	0.0055
D_5__Finegoldia	D_5__Haemophilus	0.3371	0.0055
D_5__Finegoldia	D_5__Neisseria	0.3836	0.011
D_5__Gemella	D_5__endosymbionts3	0.3126	0.0055
D_5__Gemella	D_5__Enterococcus	0.3736	0.0055
D_5__Haemophilus	D_5__Finegoldia	0.3371	0.0055
D_5__Haemophilus	D_5__Neisseria	0.314	0.011
D_5__Hymenobacter	D_5__Pantoea	0.4696	0.0055
D_5__Hymenobacter	D_5__Stenotrophomonas	0.3045	0.011
D_5__Kineosporia	D_5__Caldalkalibacillus	-0.3119	0.011
D_5__Kineosporia	D_5__Micrococcus	-0.3535	0.011
D_5__Kineosporia	D_5__Nesterenkonia	-0.3949	0.0055
D_5__Kineosporia	D_5__Peptoniphilus	-0.3425	0.0055
D_5__Marinomonas	Ambiguous taxa	0.543	0.011
D_5__Marinomonas	D_5__Anaerococcus	0.3361	0.011
D_5__Marinomonas	D_5__Bradyrhizobium	-0.4058	0.0055
D_5__Marinomonas	D_5__Corynebacterium_1	0.3627	0.011
D_5__Marinomonas	D_5__Escherichia_Shigella	0.5866	0.0055
D_5__Marinomonas	D_5__Methylobacterium	0.5666	0.0055

D_5__Marinomonas	D_5__Rhodanobacter	-0.3356	0.0166
D_5__Marinomonas	D_5__Sphingomonas	0.339	0.0331
D_5__Marinomonas	D_5__Staphylococcus	0.4429	0.011
D_5__Marinomonas	D_5__Stenotrophomonas	0.4737	0.0055
D_5__Methylobacterium	Ambiguous taxa	0.9027	0.0276
D_5__Methylobacterium	D_5__Escherichia_Shigella	0.3446	0.0221
D_5__Methylobacterium	D_5__Marinomonas	0.5666	0.0055
D_5__Methylobacterium	D_5__Sphingomonas	0.8901	0.0221
D_5__Methylobacterium	D_5__Staphylococcus	0.8257	0.0055
D_5__Micrococcus	D_5__Acinetobacter	-0.3322	0.0055
D_5__Micrococcus	D_5__Anaerococcus	0.4153	0.0055
D_5__Micrococcus	D_5__Deinococcus	-0.3149	0.0055
D_5__Micrococcus	D_5__Kineosporia	-0.3535	0.011
D_5__Micrococcus	D_5__Paracoccus	0.335	0.0055
D_5__Micrococcus	D_5__Peptoniphilus	0.602	0.0055
D_5__Neisseria	D_5__Finegoldia	0.3836	0.011
D_5__Neisseria	D_5__Haemophilus	0.314	0.011
D_5__Neisseria	D_5__Veillonella	0.3847	0.0055
D_5__Neisseria	D_5__Yonghaparkia	-0.3965	0.0055
D_5__Nesterenkonia	D_5__Caldalkalibacillus	0.8358	0.0055
D_5__Nesterenkonia	D_5__Kineosporia	-0.3949	0.0055
D_5__Nesterenkonia	D_5__Novosphingobium	-0.3731	0.0055
D_5__Nesterenkonia	D_5__Yonghaparkia	-0.3033	0.011
D_5__Novosphingobium	D_5__Caldalkalibacillus	-0.3596	0.0055
D_5__Novosphingobium	D_5__Corynebacterium	-0.3136	0.0055
D_5__Novosphingobium	D_5__Nesterenkonia	-0.3731	0.0055
D_5__Novosphingobium	D_5__Propionibacterium	0.4988	0.0055
D_5__Novosphingobium	D_5__Staphylococcus	-0.3112	0.0387
D_5__Novosphingobium	D_5__uncultured	0.4232	0.0055
D_5__Pantoea	D_5__Candidatus_Phytoplasma	0.3618	0.0221
D_5__Pantoea	D_5__Hymenobacter	0.4696	0.0055

D_5__Pantoea	D_5__Staphylococcus	-0.3365	0.0276
D_5__Pantoea	D_5__Yonghaparkia	0.4003	0.0055
D_5__Paracoccus	D_5__Micrococcus	0.335	0.0055
D_5__Paracoccus	D_5__Peptoniphilus	0.3666	0.0055
D_5__Peptoniphilus	D_5__Acinetobacter	-0.3921	0.0055
D_5__Peptoniphilus	D_5__Anaerococcus	0.3296	0.0166
D_5__Peptoniphilus	D_5__Kineosporia	-0.3425	0.0055
D_5__Peptoniphilus	D_5__Micrococcus	0.602	0.0055
D_5__Peptoniphilus	D_5__Paracoccus	0.3666	0.0055
D_5__Peptoniphilus	D_5__Yonghaparkia	-0.3378	0.0055
D_5__Propionibacterium	D_5__Novosphingobium	0.4988	0.0055
D_5__Rhodanobacter	D_5__Escherichia_Shigella	-0.3243	0.0055
D_5__Rhodanobacter	D_5__Marinomonas	-0.3356	0.0166
D_5__Sphingomonas	D_5__Marinomonas	0.339	0.0331
D_5__Sphingomonas	D_5__Methylobacterium	0.8901	0.0221
D_5__Sphingomonas	D_5__Staphylococcus	0.8705	0.0166
D_5__Staphylococcus	Ambiguous taxa	0.8623	0.0276
D_5__Staphylococcus	D_5__Caldalkalibacillus	0.3398	0.0166
D_5__Staphylococcus	D_5__Marinomonas	0.4429	0.011
D_5__Staphylococcus	D_5__Methylobacterium	0.8257	0.0055
D_5__Staphylococcus	D_5__Novosphingobium	-0.3112	0.0387
D_5__Staphylococcus	D_5__Pantoea	-0.3365	0.0276
D_5__Staphylococcus	D_5__Sphingomonas	0.8705	0.0166
D_5__Stenotrophomonas	D_5__Escherichia_Shigella	0.4068	0.0055
D_5__Stenotrophomonas	D_5__Hymenobacter	0.3045	0.011
D_5__Stenotrophomonas	D_5__Marinomonas	0.4737	0.0055
D_5__Streptococcus	D_5__Anaerococcus	0.6382	0.0055
D_5__Streptococcus	D_5__Corynebacterium_1	0.8212	0.0055
D_5__uncultured	D_5__Enterococcus	-0.3069	0.011
D_5__uncultured	D_5__Novosphingobium	0.4232	0.0055
D_5__Veillonella	D_5__Escherichia_Shigella	-0.342	0.0055

D_5__Veillonella	D_5__Neisseria	0.3847	0.0055
D_5__Yonghaparkia	D_5__Caldalkalibacillus	-0.343	0.0166
D_5__Yonghaparkia	D_5__Corynebacterium	-0.3178	0.0055
D_5__Yonghaparkia	D_5__Neisseria	-0.3965	0.0055
D_5__Yonghaparkia	D_5__Nesterenkonia	-0.3033	0.011
D_5__Yonghaparkia	D_5__Pantoea	0.4003	0.0055
D_5__Yonghaparkia	D_5__Peptoniphilus	-0.3378	0.0055

Table S3.8.3. Pearson co-occurrence correlation values

Taxon1	**Taxon2**	**Correlation**	***P*-value**
D_5__Anaerococcus	*D_5__Burkholderia_Paraburkholderia*	-0.3123	0.0442
D_5__Anaerococcus	*D_5__Corynebacterium_1*	0.9552	0.011
D_5__Anaerococcus	*D_5__Hymenobacter*	0.4467	0.0331
D_5__Anaerococcus	*D_5__Micrococcus*	0.4181	0.0497
D_5__Anaerococcus	*D_5__Pantoea*	0.4945	0.0387
D_5__Anaerococcus	*D_5__Streptococcus*	0.9244	0.0055
D_5__Burkholderia_Paraburkholderia	*D_5__Anaerococcus*	-0.3123	0.0442
D_5__Burkholderia_Paraburkholderia	*D_5__Corynebacterium_1*	-0.3113	0.0387
D_5__Burkholderia_Paraburkholderia	*D_5__endosymbionts3*	0.3298	0.0387
D_5__Burkholderia_Paraburkholderia	*D_5__Marinomonas*	-0.3643	0.0331
D_5__Burkholderia_Paraburkholderia	*D_5__Veillonella*	0.4331	0.0055
D_5__Candidatus_Phytoplasma	*D_5__Haemophilus*	-0.3608	0.0221

D_5__Candidatus_Phytoplasma	*D_5__Pantoea*	0.3856	0.011
D_5__Candidatus_Phytoplasma	*D_5__Streptococcus*	-0.3002	0.0442
D_5__Corynebacterium_1	*D_5__Anaerococcus*	0.9552	0.011
D_5__Corynebacterium_1	*D_5__Burkholderia_Paraburkholderia*	-0.3113	0.0387
D_5__Corynebacterium_1	*D_5__Marinomonas*	0.3138	0.0331
D_5__Corynebacterium_1	*D_5__Pantoea*	0.4247	0.0276
D_5__Corynebacterium_1	*D_5__Streptococcus*	0.9452	0.0055
D_5__endosymbionts3	*D_5__Burkholderia_Paraburkholderia*	0.3298	0.0387
D_5__endosymbionts3	*D_5__Pantoea*	-0.3969	0.0055
D_5__Finegoldia	*D_5__Neisseria*	0.3301	0.0331
D_5__Finegoldia	*D_5__Stenotrophomonas*	-0.3234	0.0331
D_5__Haemophilus	*D_5__Candidatus_Phytoplasma*	-0.3608	0.0221
D_5__Haemophilus	*D_5__Neisseria*	0.6017	0.0055
D_5__Haemophilus	*D_5__Spirosoma*	0.4078	0.011
D_5__Hymenobacter	*D_5__Anaerococcus*	0.4467	0.0331
D_5__Hymenobacter	*D_5__Micrococcus*	0.5791	0.0055
D_5__Hymenobacter	*D_5__Pantoea*	0.3879	0.0497
D_5__Hymenobacter	*D_5__Propionibacterium*	0.4096	0.0331
D_5__Hymenobacter	*D_5__Veillonella*	-0.356	0.0276
D_5__Marinomonas	*D_5__Burkholderia_Paraburkholderia*	-0.3643	0.0331
D_5__Marinomonas	*D_5__Corynebacterium_1*	0.3138	0.0331
D_5__Marinomonas	*D_5__Methylobacterium*	0.5524	0.0331
D_5__Marinomonas	*D_5__Rhodanobacter*	-0.3653	0.0221
D_5__Marinomonas	*D_5__Stenotrophomonas*	0.4471	0.0055
D_5__Methylobacterium	*D_5__Marinomonas*	0.5524	0.0331
D_5__Micrococcus	*D_5__Anaerococcus*	0.4181	0.0497
D_5__Micrococcus	*D_5__Hymenobacter*	0.5791	0.0055
D_5__Micrococcus	*D_5__Peptoniphilus*	0.4924	0.0166
D_5__Micrococcus	*D_5__Veillonella*	-0.4569	0.0166

D_5__Neisseria	*D_5__Finegoldia*	0.3301	0.0331
D_5__Neisseria	*D_5__Haemophilus*	0.6017	0.0055
D_5__Neisseria	*D_5__Pantoea*	-0.3235	0.0497
D_5__Neisseria	*D_5__Veillonella*	0.3521	0.0276
D_5__Neisseria	*D_5__Yonghaparkia*	-0.4125	0.011
D_5__Novosphingobium	*D_5__Propionibacterium*	0.5619	0.011
D_5__Novosphingobium	*D_5__uncultured*	0.5357	0.011
D_5__Pantoea	*D_5__Anaerococcus*	0.4945	0.0387
D_5__Pantoea	*D_5__Candidatus_Phytoplasma*	0.3856	0.011
D_5__Pantoea	*D_5__Corynebacterium_1*	0.4247	0.0276
D_5__Pantoea	*D_5__endosymbionts3*	-0.3969	0.0055
D_5__Pantoea	*D_5__Hymenobacter*	0.3879	0.0497
D_5__Pantoea	*D_5__Neisseria*	-0.3235	0.0497
D_5__Pantoea	*D_5__Streptococcus*	0.4464	0.0442
D_5__Paracoccus	*D_5__Peptoniphilus*	0.501	0.0055
D_5__Paracoccus	*D_5__uncultured*	0.3108	0.0497
D_5__Peptoniphilus	*D_5__Micrococcus*	0.4924	0.0166
D_5__Peptoniphilus	*D_5__Paracoccus*	0.501	0.0055
D_5__Peptoniphilus	*D_5__uncultured*	0.5727	0.0055
D_5__Propionibacterium	*D_5__Hymenobacter*	0.4096	0.0331
D_5__Propionibacterium	*D_5__Novosphingobium*	0.5619	0.011
D_5__Rhodanobacter	*D_5__Marinomonas*	-0.3653	0.0221
D_5__Spirosoma	*D_5__Haemophilus*	0.4078	0.011
D_5__Spirosoma	*D_5__Veillonella*	0.4256	0.0055
D_5__Spirosoma	*D_5__Yonghaparkia*	0.4009	0.0331
D_5__Stenotrophomonas	*D_5__Finegoldia*	-0.3234	0.0331
D_5__Stenotrophomonas	*D_5__Marinomonas*	0.4471	0.0055
D_5__Streptococcus	*D_5__Anaerococcus*	0.9244	0.0055
D_5__Streptococcus	*D_5__Candidatus_Phytoplasma*	-0.3002	0.0442
D_5__Streptococcus	*D_5__Corynebacterium_1*	0.9452	0.0055
D_5__Streptococcus	*D_5__Pantoea*	0.4464	0.0442

D_5__uncultured	*D_5__Novosphingobium*	0.5357	0.011
D_5__uncultured	*D_5__Paracoccus*	0.3108	0.0497
D_5__uncultured	*D_5__Peptoniphilus*	0.5727	0.0055
D_5__Veillonella	*D_5__Burkholderia_Paraburkholderia*	0.4331	0.0055
D_5__Veillonella	*D_5__Hymenobacter*	-0.356	0.0276
D_5__Veillonella	*D_5__Micrococcus*	-0.4569	0.0166
D_5__Veillonella	*D_5__Neisseria*	0.3521	0.0276
D_5__Veillonella	*D_5__Spirosoma*	0.4256	0.0055
D_5__Yonghaparkia	*D_5__Neisseria*	-0.4125	0.011
D_5__Yonghaparkia	*D_5__Spirosoma*	0.4009	0.0331

Table S3.8.4. Network Global Properties – (MENA -Pearson)

Network Indexes	**bactMENA (0.310)**
Total nodes	53
Total links	569
R square of power-law	7
Average degree (avgK)	21472
Average connectivity	21.47
Average clustering coefficient (avgCC)	671
Average path distance (GD)	1592
Geodesic efficiency (E)	706
Harmonic geodesic distance (HD)	1417
Maximal degree	49
Centralization of degree (CD)	550

Maximal betweenness	149736
Centralization of betweenness (CB)	103
Maximal stress centrality	755
Centralization of stress centrality (CS)	481
Maximal eigenvector centrality	240
Centralization of eigenvector centrality (CE)	116
Density (D)	413
Reciprocity	1
Transitivity (Trans)	576
Connectedness (Con)	1
Efficiency	598
Hierarchy	0
Lubness	1

Table S3.8.5. DHA measurements of the control and bacteria-inoculated natural nectar in the laboratory.

Control **DHA [g/l]**	Bacterial inoculation **DHA [g/l]**
0.0089	0.0149
0.0103	0.021
0.009	0.0178
0.006	0.0325
0.0067	0.0523

Table S3.8.6. DHA measurements of *P. agglomerans* – inoculated nectar in the field.

Control **DHA [g/l]**	Bacterial inoculation **DHA [g/l]**
0.0352	0.0496
0.0109	0.0373
0.0042	0.017
0.0046	0.0066
0.0023	0.0541
0.0293	0.0612
0.0023	0.0041
0.0138	0.0195

Table S3.8.7. DHA measurements of *P. agglomerans* – inoculated nectar in the field (log transformation).

Control **DHA [g/l]**	Bacterial inoculation **DHA [g/l]**
-1.45346	-1.30452
-1.96257	-1.42829
-2.33724	-1.76955
-2.33724	-2.18046
-2.63827	-1.2668
-1.53313	-1.21325
-2.63827	-2.38722

-1.86012	-1.70997

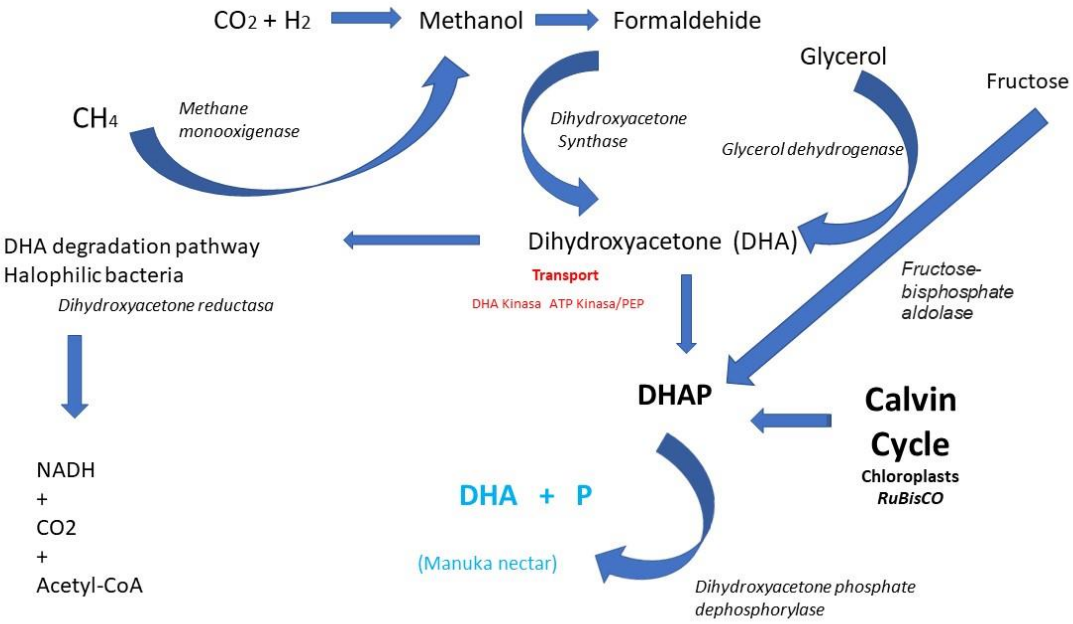


Figure S3.1.) Metabolic pathways and interconnections for the production of dihydroxyacetone from fructose, methane, glycerol or carboxy dioxide, main enzymes, reactives and products for the main conversions that end in the production of dihydroxyacetone in mānuka nectar are indicated by arrows.

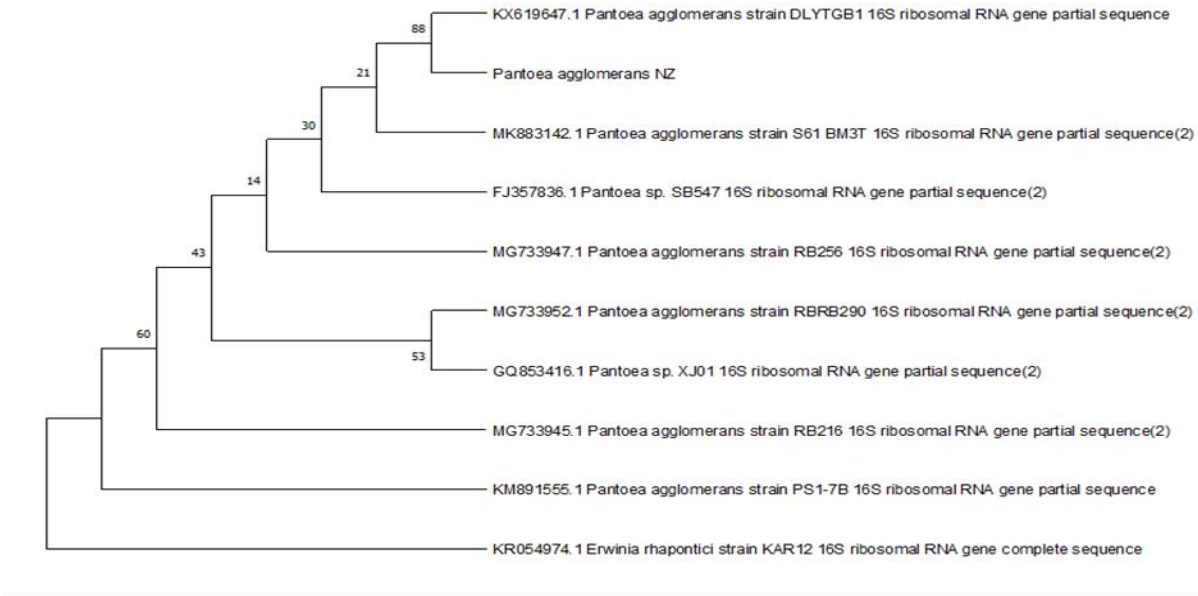


Figure S3.2.) Maximum-likelihood tree based on 16S rRNA gene *Pantoea agglomerans* sequences. *Erwinia rhapontici strain KAR12 16S* sequence was used as an outgroup. The strain isolated from New Zealand - Palmerston North, is indicated as *Pantoea agglomerans* NZ.

Brief methodology: The isolated culture of *P. agglomerans* was analysed phylogenetically by 16S rRNA sequence. The closest match as in Genbank (NCBI) were downloaded as 16S rRNA FASTA files, and for the sister group *Erwinia*, the alignment was performed with the algorithm MUSCLE (Edgar, 2004), and the phylogeny determined with maximum likelihood algorithm under 1000 repetition bootstrap, with the software MEGA X (Kumar et al. 2018).

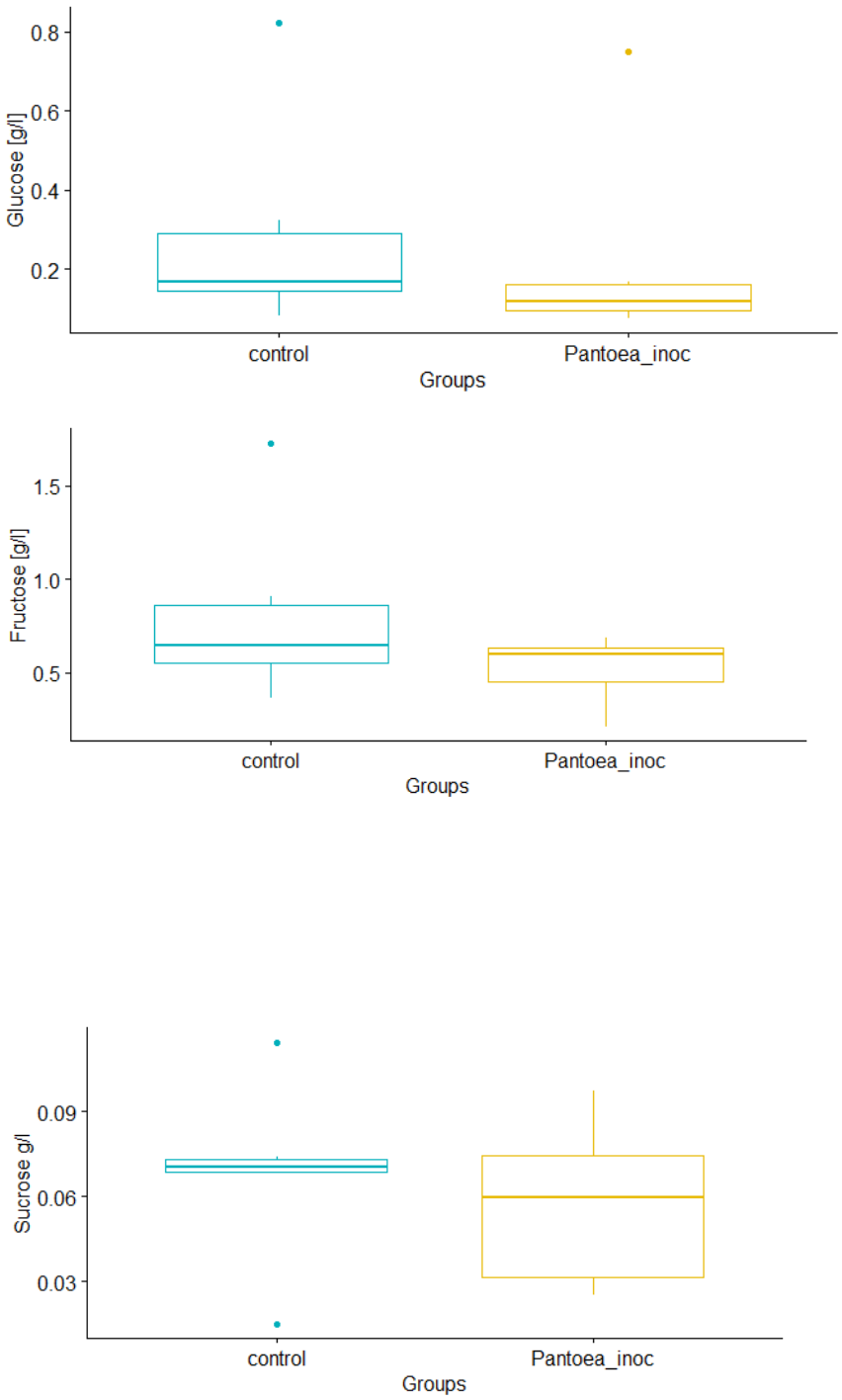


Figure S3.3.) Effect of *P. agglomerans* inoculation on glucose, fructose, and sucrose concentration under field conditions (October 2019, Palmerston North, mānuka blue clone, 10 flowers inoculation 10 µl, 72 hrs bagged flowers). Control: mānuka flower nectar inoculated with LB medium and Treatment: mānuka flowers nectar with bacteria inoculation (10 µl).

Brief methodology: Prior to inoculation; *P. agglomerans* was grown to exponential phase (2.0 X 108 bacteria/ml). The fresh liquid culture was taken on ice to the field and 10 flowers in 6 plants of the blue genotype were inoculated. Control flowers were inoculated with the growth medium Luria-Bertani (LB) on the same day and considered the same number of flowers (10). After inoculation all the flowers were covered with a breathable mesh. After three days flowers were recovered and taken to the laboratory on ice in one bag per plant for each treatment. At the laboratory flowers were stored at minus 20 °C. To analyse the sugars, sucrose, glucose, and fructose, were separated by HPLC (Perkin Elmer series 200); a column Sugar-Pak I (Waters, size 6.5x300mm) was used. This column was kept at 75 °C. The single mobile phase solution was prepared by dissolving ethylenediaminetetraacetic acid (50mg/L) in deionised milli-Q water and run using an isocratic elution system at a constant flow rate of 0.6 ml per minute. Samples were run at a volume of 20 µl using a RI detector (Shodex RI-101). For each sugar, a 1% stock solution was made, and five standards were prepared at different concentrations. The standards ranged in concentration from 0.0025% to 0.5%.

CHAPTER 4.

Cultivable bacteria, osmotolerance and catalase activity, and the chemical environment in the nectary/nectar interphase

ABSTRACT

Mānuka flowers have photosynthetically active nectaries having a dynamic mechanism for nectar production. In addition, nectaries can selectively release and reabsorb nectar controlling the concentration of sugar other metabolites and tend to have lower pH. The nectar environment has a high concentration of hexoses, antibiotics, and free radicals. Bacteria that inhabit nectar must be adapted to tolerate pH fluctuations, high ultraviolet light, and low moisture content. Osmotolerance and enzymes with capabilities to avoid free radicals (e.g., catalase) are key traits that enable bacteria to survive in these environments. In this work I aimed to 1) Identify the diversity of nectar cultivable bacteria based on morphology and in the sequence of the 16S rRNA gene, 2) Explore the osmotolerance and catalase activity of some cultivable bacteria, and 3) Determine the pH of mānuka nectar outside and inside nectaries. I found a diversity of cultivable bacteria (corresponding to six different families). Most bacteria had high osmotolerance (*P. agglomerans, Erwinaceae*, *Arthrobacter*) and catalase activity. The pH inside the nectaries was acidic while in the nectar outside of them was close to neutrality, the differences in the pH may contribute to attract pollinators and favour the production of key metabolites. Altogether, this work contributes to our understanding of nectar bacteria and the chemical environment they encounter in the nectar/nectaries interface.

4.0. INTRODUCTION

Microbial habitats that contain an excess of carbohydrates include plant tissues, phloem, fruits and nectar (Lievens et al., 2015). In nectar, carbohydrate compounds generate high osmotic pressure, and there is limited protein content (Gonzalez-Teuber et al., 2009). Local adaptation and phenotypic plasticity will determine the microbial species that can be found in the nectar, as they might be on a natural competition process with other bacteria and interacting with the plant metabolism to be adapted to this environment (Chappell & Fukami, 2018). Osmotolerance (the ability to grow in an environment with high osmotic pressure), and catalase activity (the ability to break oxygen peroxide into water and oxygen, preventing oxidative damage in aerobic environments) are essential attributes for bacteria to survive in the nectar environment (Schmitt, 2014).

The nectar in mānuka plants is produced in structures called nectaries, which are photosynthetically active in mānuka. Nectar production involves a process of secretion, accumulation and reabsorption, with nectar accumulating on the inner surface of the hypanthium (cuplike or tubular enlargement of the receptacle of a flower) and on the upper surface of the gynoecium (the innermost whorl of a flower) (O'Brien et al., 1996). The process of reabsorption occurs concurrently with nectar secretion and may contribute to nectar homeostasis (Nepi & Stpiczyńska, 2007). Evolutionary aspects related to the attraction of a broad range of pollinators have been attributed to nectar reabsorption (Nepi, Grasso, and Mancuso 2018). In mānuka plants, when reabsorption occurred it appeared to be partially selective to sugars diffusion, with a gradual but significant drift to higher levels of fructose in relation to glucose, particularly in older flowers of high nectar-producing genotypes (Clearwater et al., 2018).

Nectar accumulates in the inner and outer base of the hypanthium (Clearwater et al., 2018). These two parts have a different chemistry, differing in their pH, which may influence the type of microorganisms found in one or the other. The accumulation of nectar under two different chemical contexts might result in different concentrations of DHA, as this compound

is very dependent on the pH and is photochemically reactive under UV light (Lebedeva et al., 2010).

The dynamic of hexoses (simple sugars) in phloem and within nectar is also a complex process that varies depending on whether nectaries photosynthesise. Given that hexoses are typically not components of phloem sap (Lohaus & Schwerdtfeger, 2014), the proportion of hexoses in nectar depends on the presence and activity of sucrose-cleaving enzymes. Sucrose cleavage in plants can be catalysed by at least two types of enzymes: reversible sucrose cleavage is catalysed by sucrose synthase (SuS; EC 2.4.1.13), a glycosyltransferase, and irreversible sucrose cleavage is catalysed by invertases (β-fructofuranosidases; EC 3.2.1.26).

Given the importance of hexoses (sugars) in nectar and the role of nectaries on nectar production. This chapter aims to identify some cultivable bacteria recovered from mānuka nectar, their growth under different hexose concentrations, their catalase chemical activity and the varying pH conditions inside and outside mānuka nectaries that may influence metabolite production and bacterial activity. This will be done by 1) Identifying the diversity of nectar cultivable bacteria based on morphology and the 16S rRNA gene 2) Characterising bacterial growth under different concentrations of carbon sources and their catalase activity and 3) Determining the pH of mānuka nectar inside and outside the nectary.

Pursuing these objectives will allow me to determine if it is possible to find cultivable bacteria with biological significance in the nectar, and if these bacteria have metabolic capabilities in the nectar environment. The data from this chapter will provide additional information to assess the biological diversity of bacteria in the nectar (Chapter 2) and allow me to gain a better understanding of their metabolic capabilities (Chapter 3), and the conditions they may experience in the nectar/nectary environments.

4.1. METHODS

4.1.1. Study areas and systems

The study was conducted in a population of mānuka clones and cultivars at Moginie block of the Pasture & Crop Research Unit (PCRU), Massey University (Palmerston North – New Zealand). Sampling was done in two different ways in October 2019. For the pink genotype, nectar was recovered from five plants in the field and on the tree with 10 µl sterile diluted water and pooled per plant in a tube and transported to the laboratory the same day on ice. Bacteria were cultivated the same day. In the second sampling, whole flowers were collected from five plants from six genotypes (including pink) and transported to the laboratory to be frozen at -20 °C. Flowers were later thawed, and nectar harvested by dilution with 10 µl of pure distilled water. From this, nectar aliquots were taken to cultivate bacteria per genotype.

4.1.2. Sampling and bacteria identification

From the pooled of nectar, a volume of 20 µl was taken by pipette and afterwards stroked in TSA (Tryptone soy agar, Difco) culture plates. Plates were incubated at 25 °C for three days. A diversity of bacterial colony morphologies was obtained in the culture plates, from these, yellow colonies were picked and purified by further culture on TSA plates. The DNA content of each colony was extracted in sterile ultrapure water by thermic shock and the 16S rRNA gene was amplified by PCR and sequenced with primers 27F and 1492 R (Lane, 1991).

Reactions were prepared to a total of 50 µL with nuclease-free water as follows: For 16S, reactions contained 200 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.8X Platinum Taq HiFi buffer (Invitrogen, MA, USA), 1 U Platinum Taq HiFi polymerase (Invitrogen, MA, USA), and 10 ng of template. Thermal cycling was carried out using a Mastercycler (Roche, USA), using the following conditions: For 16S, an initial 95 ºC for 2

min, followed by 26 cycles of 94 ºC for 45 s, 52 ºC for 30 sec and 72 ºC for 1 min followed by a final extension at 72 ºC for 10 min.

Amplicons were sequenced by Sanger method and the obtained sequences were trimmed with Chromas (v.2.6.2 Technelysium Pty Ltd., Brisbane, Australia) and assembled with Geneious (Kearse et al. 2012) and identified by closest match with BLAST using GenBank as a reference database.

4.1.3. Osmotolerance and catalase activity

Glucose, Fructose and Sucrose tolerance was assessed by culturing isolates at 20 °C, aerobic, light conditions with agitation on a Rocket 25 platform at 60 RPM for up to 7 days in transparent tubes containing LB (Luria-Bertani, Difco) broth supplemented with 0% (positive control), 10%, 20% and 30% glucose, fructose, and sucrose (w/v, Sigma-Aldrich), in triplicate. The appearance of turbidity in the tubes with respect to negative controls (i.e., tubes containing no inoculated media) was recorded as a positive result.

For detection of catalase activity, a bacterial colony was taken from an axenic culture on TSA with a microbiology loop and was suspended in 3% hydrogen peroxide. The appearance of bubbles was recorded as a positive result (Aslanzadeh 2006).

4.1.4. Determination of pH in the nectar and nectary fluids

The pH (H^+ proton concentration) of nectar was measured since the acidity of an environment determines the chemical reactions and the microorganisms that can have metabolic functions in that environment. The pH of the nectar was measured by adding drops of nectar on pH strips and then compared with manufacturer standard reference colours (Macherey-Nagel, Germany). Flowers were stored at -20 °C then the nectar was taken with 10 µl of water; the resulting fluid was a mix of hypanthium fluids and nectar. Fresh flowers were taken from the tree and immediately taken to the laboratory in a thermic container with ice, In the laboratory,

in a sterile chamber, a transversal cut in the ovaries was performed (Figure 4.1) to measure the pH with drops of the solution that is interconnecting through inside the nectaries and ovaries without diluting the fluids with water. The pH accuracy for the strip kit is +/- 0.2 pH.

A) Blue genotype **B) Yellow genotype**

Figure 4.1) Photograph of a transversal section of a mānuka ovaries and conducts that communicates with the nectary, showing pigments, fluids, and penta-lobular structure A) blue genotype, B) yellow genotype. Stereo microscope Olympus SZX7, camera Olympus SC100, magnification 1X.

4.2. RESULTS

4.2.1. Nectar bacteria

A total of 100 bacterial colonies were isolated and identified through sequencing of the 16S rRNA gene for selected morphotypes. Sampling was done from the nectar corresponding to flowers on the tree and the nectaries fluid corresponding to frozen flowers.

An initial taxonomical classification of different morphotypes was assessed and the top hits from the BLAST with GenBank are shown in Table 4.0.

Table 4.0.) Taxonomical affiliation of cultivable manuka nectar bacteria

Name Classification	Per. Ident (*) (%)	Description	Accession num.
Pantoea agglomerans	98.24	*Pantoea agglomerans* DLYTGB1	KX619647.1
Arthrobacter sp.	98.68	*Arthrobacter sp. St GBRS11*	MT373551.1
Brevundimonas sp.	97.60	*Brevundimonas sp. FW305-C-20-1 16S*	MT160402.1
Psychrobacter (genus)	96.78	*Psychrobacter alimentarius st. JM48*	MN758808.1
Enterobacteriales (order)	91.44	*Pantoea sp.* St. ICMP 20883	MG786394.1
Erwinaceae (class)	82.47	*Pantoea agglomerans* st. S61_BM3T	MK883142.1
Erwinaceae (class)	87.33	*Pantoea sp.* st. ORTB2 16S rRNA	MN685248.1
Erwinaceae (class)	85.7	*Pantoea agglomerans* DLYTGB1	KX619647
Burkholderiales (order)	90.16	*Delftia acidovorans*	MN330317
Gammaproteobacteria	78.26	*Mesorhizobium sp.* Amf-4	AF509923.1
Pseudomonas (family)	93.85	*Pseudomonas syringae st.* PDD-58b-28	KR922139.1
Pseudomonas (class)	83.38	*Pseudomonas sp.* B AS 33	JF901705.1
Pseudomonas (class)	80.56	Pseudomonas sp. I-111-27 16S	FJ786058.1
Pseudomonas (genus)	96.7	*Pseudomonas lurida strain P 513/18*	NR042199
Sphingobacteriia (class)	85.67	*Sphingobacterium faecium*	LN995717.1
Actinomycetales (order)	90.31	*Janibacter melonis* st. CM2104	NR_025805
Flavobacteria (class)	86.83	*Elizabethkingia miricola* sp. ST3C	GU458290
Bacillales (class)	86.34	*Paenibacillus amylolyticus st.* KK 9a	KP858920.1

(*) Per. Identity is the ratio of the number of matching nucleobases to the total length of the alignment within the obtained sequence and the references sequences at the Genbank database

4.2.1.1. Nectar cultivable bacteria

Nectar sampling of the flowers was carried out to cultivate and isolate bacteria from flowers without interfering with the natural nectar dynamics. As a result, the following bacteria were identified: *Pantoea agglomerans, Arthrobacter sp.* and *Brevundimonas sp*.

Pantoea agglomerans was initially identified because of its yellow colonies, gram-negative rods and its growth in culture plates from different genotypes. *Brevundimonas sp*. was identified as rod-shaped aerobic cells, *Arthrobacter* were identified as rod-shaped cells.

4.2.1.2. Nectary fluids cultivable bacteria

Bacterial sampling was made from the nectary fluids from frozen samples.

After an initial identification of the isolates, they were assigned based on sequence analysis to the orders: Pseudomonadales, Enterobacterales, Actinomycetales, Baccillales, and Sphingobacteriales, corresponding to the families Pseudomonaceae, Erwinaceae, Sphingobacteriaceae, Bacillaceae, Moraxellaceae, and Micrococcaceae.

Pseudomonadales and Enterobacterales presented several isolates across the different culture plates. The isolated bacteria belong to families already described from the metabarcoding in high abundance (Chapter 2). The different taxonomic affiliations that could be identified with an initial sampling are shown with their percentage of identity with previous isolates on Genbank in Table 4.0.

4.2.1.3. Bacterial chemical traits: osmotolerance and catalase reaction

All tested bacteria showed a catalase positive reaction meaning that they are aerobic or facultative aerobic bacteria. They also have flexibility to grow in only one carbon source; the results after growing them in three different carbon sources are shown in Table 4.1.

Table 4.1.) Growth of bacteria inoculated in sugar solutions with different concentrations of glucose (Glc), fructose (Fru) and sucrose (Suc) (10%, 20% and 30%).

Bacteria (isolates)	**Glc 10**	**Glc 20**	**Glc 30**	**Fru 10**	**Fru 20**	**Fru 30**	**Suc 10**	**Suc 20**	**Suc 30**
Erwinaceae	+	+	+	+	+	+	+	+	+
Pantoea agglomerans	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	+	+	+	+	+	+	+	-	-
Sphingobacteria	+	+	+	+	+	+	+	-	-
Alphaproteobacteria-Rhizobiales	+	+	+	-	-	-	+	+	+
Actinomycetales	+	+	-	+	+	-	+	+	-
Psychrobacter	+	+	-	+	+	-	+	+	-
Burkholderiales	+	+	+	+	+	-	+	+	-
Pseudomonas	+	+	+	+	+	+	+	+	-

Bacteria at different sugar concentrations: glucose 10% (Glu 10), glucose 20% (Glu 20), glucose 30% (Glu 30), fructose 10% (Fru10), fructose 20% (Fru20), fructose 30% (Fru30), sucrose 10% (Suc10), sucrose 20% (Suc20), sucrose 30%(Suc30). The symbol (+) or (–) represents appearance of turbidity relative to a negative (uninoculated) control for each sugar solution.

Pantoea agglomerans and *Erwinaceae* were the most osmotolerant bacteria, followed by isolates from the Pseudomonaceae family. Most of the isolates show adaptability to fructose and glucose, which are found in high concentration in the nectar of mānuka plants.

4.2.2. NECTARY HYDROGEN ION ACTIVITY

4.2.2.1. pH for nectar and nectary fluids

The pH of the nectary was measured by doing a transverse cut on the nectary; then drops located inside the nectaries were measured for their pH. The fluids secreted by the nectary and nectar on the hypanthium surface were also measured. The nectar on fresh flowers was also measured. Results are presented in Table 4.2.

TABLE 4.2) Measurements of pH and temperature for the nectar from fresh flowers (on the plant), nectary fluids (from frozen flowers), and nectary fluids (after a transversal cut to the nectary).

Environmental/solutions	pH [+/- 0.2]	Temperature °C
pH nectar fresh flowers	6.5	20
pH nectary fluids frozen flowers	5.5	20
pH nectary fluids (drops inside nectary lobule)	5.0	20

The nectar pH measured from fresh flowers is near neutrality (pH 6.5), while the pH inside the nectaries is acidic. This indicates a pH gradient from phloem proximity to pollinator interaction that varies from acidic pH (5.0) to close to neutral pH (6.5).

4.3. DISCUSSION

The cultivable bacteria in nectar belong to the families Pseudomonaceae, Erwinaceae, Sphingobacteriaceae, Bacillaceae, Moraxellaceae, and Micrococcaceae. *Pseudomonas* have been described as an important clade in flowers because of their genetic diversity seen through their homologous recombination (Álvarez-Pérez, de Vega, and Herrera 2013), and because of pathogenic members found in kiwifruit (Purahong et al. 2018). Erwinaceae members are important for nutrient availability of the seeds as they can increase growing abilities on exudates release during germination and are acquired through flower stigma (Torres-Cortés et al. 2018). *Sphingomonas* can have an important role as they can control pathogens e.g. *P. syringae* (Innerebner, Knief, and Vorholt 2011). Bacillaceae members produce odorants that are attractive towards insects (Peach et al. 2020). Moraxellaceae, and Micrococcaceae members were also found in flowers, but their roles are less understood (Morris et al. 2020).

The bacteria isolated showed high osmotolerance and the capacity to grow in only one carbon source. They also presented catalase activity. Both characteristics enable these bacteria to survive in changing environments, and with potential high exposure to free radicals. Sphingobacteriaceae members possess several compounds and lipids that act against free radicals (Asker et al. 2012) and provide special resistance in these environments. Actinomycetales bacterial species show adaptation to extreme conditions (Trenozhnikova and Azizan 2018), and high GC genome content which provides adaptation to difficult environments through higher stabilization of the nucleic acid due to stronger chemical bonds (Mann and Chen 2010), and some of their bacteria members, for example: *Janibacter sp*. were reported as a symbiotic bacterium from termites (Dueholm et al. 2019), a symbiotic relationship with an insect of mānuka plants could be a possibility for the presence of *Janibacter* in mānuka nectar. Adaptation to the nectaries acidic conditions is also found in Burkholderiales e.g. *Delftia acidovorans* that also promotes several responses in plants such as root development, seed emergence and increase nodule number (Perry et al. 2017).

From the different morphotypes studied, we can observe bacteria from the same class and families that were obtained in the meta-barcode from the same group of plants with two-years

difference between both studies, meaning that the isolated bacteria belong to taxonomic groups stably associated with mānuka nectar, and this is consistent across the period studied.

Enterobacteriales and Pseudomonadales were found together in the nectar from fresh and frozen flowers, while *Arthrobacter sp.* was found only in the nectar sampled on the plant. In other regions *Arthrobacter* species were found in the nectar of *Asphodelus aestivus;* in an extensive study conducted in Israel, the species *Arthrobacter oryzae* was found at two remote locations (Samuni-Blank et al., 2014). Their presence in the mānuka nectar could be important as they could produce alone or in combination with other bacteria, the antibiotic compound methylglyoxal, one of the metabolites that contributes to mānuka honey quality.

Neutral pH favours *Arthrobacter* growth (Levering et al. 1981). The pH of the nectar on the tree was (pH 6.5), which is near the optimal working pH for *Arthrobacter* bacteria. This chemical context could favour the thermodynamic and kinetics of microbial enzymes (Jin and Kirk 2018), towards methylglyoxal production should nectar abundance be suitably high. Methylglyoxal can be formed from carbonyl compounds (carbohydrates e.g., glucose, fructose, aldehyde, ketones) by enolization and this series of reactions occurs during the storage of mānuka honey and can be enhance by amino acids such as alanine (non-enzymatic reactions) (Grainger et al. 2016), and microorganisms can produce methylglyoxal from DHA or trioses (Subedi et al. 2008).

Microorganisms with both aerobic and anaerobic metabolism can produce methylglyoxal. Bacteria such as *Arthrobacter, Lactobacillus, Fusobacterium* and *Proteus spp.* possess methylglyoxal synthase and have been demonstrated to produce this compound (Baskaran et al., 1989). *Arthrobacter* possess the metabolic pathways to produce methylglyoxal from carbohydrates (Chakraborty et al., 2014). They also have the capability to produce methylglyoxal from DHAP (Chakraborty et al., 2014). This could explain the findings of methylglyoxal in mānuka nectar and related species such as kānuka (*Kunzea ericoides*), which do not have DHA in their nectar (McDonald et al., 2018).

The mānuka plant genotypes studied, possess different phenotypes, differing in flower size, colour and size of their hypanthium. They also differ in the colour of their nectar; when the nectaries fluids are taken the difference is more contrasting and the nectaries fluids vary from red to white. These different colours are due to different pigments. Anthocyanins and anthoxanthins can produce the full spectrum of colours from pale yellow to blue purple (Zhao & Tao, 2015). Internal changes inside the nectaries could initiate different types of reactions

resulting in different DHA concentrations. When red in colour, anthocyanins maintain an acidic pH, while when colourless or neutral the pH, is correspondingly almost neutral. The mānuka genotypes have different colours in their nectary fluids, from white to pink, meaning they might have different pigments (Figure 4.1).

Further studies could focus on internal measurements inside the nectaries, although with the methodology used, harvesting diluted nectar and measuring with a normal scale, these differences were not detected but, for example, differences in colour of the solution from pink to white were found between the pink clone and yellow clone (Figure 4.1) and seem to be related to differences in acidity inside the nectaries but this was not detectable with the standard method used.

Differences in the pH could be one of the factors that modify the dihydroxyacetone content (Pantini et al. 2007). As also seen in the soil, microbial metabolism and community structure are usually strongly determined by pH (Rousk et al., 2010). In aquatic communities even small pH changes determine important diversity variations in the bacterial community (Krause et al., 2012). Further studies should investigate the effect of pH on dihydroxyacetone production.

Nectar reabsorption and the availability of several carbon compounds in the nectar (Smallfield, Joyce, and van Klink 2018), may contribute to the adaptation of some bacteria to the nectar environment (Pusey, 1999). In addition, mānuka nectaries have enzymes, that, when their functions collaborate with the sugars, result in chemical equilibrium in the nectar. The origin and maintenance of sugar concentrations in the nectar can be explained by the action of the invertase enzyme that cleaves sucrose into glucose and fructose while the nectar is under the dynamic nectar reabsorption process that keeps the sugar content outside the hypanthium stable (Clearwater et al. 2018).

Mānuka plants have a high rate of glucose reabsorption and with this mechanism they can retain higher levels of glucose inside the nectaries (Noe et al., 2019). The continuous reabsorption of nectar could retain sugars in flowers that are exposed to pollinators and several environmental factors during the full day. The very low concentrations of sucrose in the nectar indicates that differences between the phloem and the nectar in sucrose concentration (Smallfield, Joyce, and van Klink 2018), is due to nectary activities. The acquisition of the invertase enzyme is likely to have been a key evolutionary process in the

Leptospermum genus that maintains glucose and fructose levels in the nectar, as also has been recorded in other plant species (Bocock et al. 2008).

Reabsorption of the nectar could also help to control the amount of other important metabolites, such as dihydroxyacetone, in the nectar. Well-adapted bacteria such as *P. agglomerans* can form conglomerates with high metabolic activity (Tecon and Leveau 2016), while not interfering with the plants' metabolic pathways. Plant performance retains glucose, fructose and sucrose levels while other important metabolites increase their concentration as found with DHA in Chapter 3. The high levels of fructose could also be a means of maintaining high levels of DHAP, as they are metabolically interconnected through the conversion of fructose-1-P into DHAP (Yang et al. 2015).

The presence of bacteria in the nectar and nectaries could also have important effects on other aspects of plant-bacterial interactions as they have been determined to increase amino acid concentrations in other plants (Vannette & Fukami, 2018). Bacteria also can induce an increase in the amino acid proline, and this can affect plant reabsorption of floral nectar or plant stress response (Fabro et al., 2004). The full day open flowers that this plant possesses could facilitate the acquirements of bacteria at day and night. The flow of nectar can give more possibilities to bacteria for entering flowers and into the internal plant circulation through the phloem contributing to the complex microbial communities in nectar.

4.4. CONCLUSION

The cultivable fraction of bacteria in mānuka nectar is adapted to withstand extreme conditions in the nectar including the low osmotic potential, presence of antimicrobial compounds and free radicals. The constantly changing conditions caused by efflux and reabsorption of the nectar for a one-day period, could lessen the adaptability of some bacteria. However, some species seem well adapted to these changes and have the capacity to enhance plant functions while keeping sugars levels stable without affecting plant pollinator interactions. Being in a photosynthetic environment increases the possibility for the bacteria

to encounter carbon compounds with changing concentrations, which creates a versatile set of substrates for bacteria metabolism.

Nectaries in mānuka plants seem to be the perfect filter to regulate pH, metabolites, and bacterial activity, to facilitate several plant physiological functions. The regulation of nectary metabolites by nectar reabsorption could determine that relevant metabolites for mānuka honey production such as dihydroxyacetone can be increased through different routes, for instance, by providing to the flower's additional amounts of DHA-producing bacteria through the phloem as seen in chapter 3.

This chapter supports the hypothesis presented in chapter 2 regarding the diversity of bacteria in the nectar. I can confirm that the bacteria from the mānuka microbiome are part of a varied and stable community, and part of their members can be culture from the nectar. They are adapted through biochemical capabilities to the nectar environment, showing metabolic capacity in the nectar environment. Comparing these cultures results with the bioinformatic analysis, we can observe the main bacteria groups present in the metabarcoding that are also present in the cultures, suggesting that the key taxa of the nectar microbial communities can be well preserved and studied through cultivation.

4.5. REFERENCES

Álvarez-Pérez, Sergio, Clara de Vega, and Carlos M Herrera. 2013. "Multilocus Sequence Analysis of Nectar *Pseudomonads* Reveals High Genetic Diversity and Contrasting Recombination Patterns." *PLOS ONE* 8 (10): e75797.

Asker, Dalal, Tarek S Awad, Teruhiko Beppu, and Kenji Ueda. 2012. "Novel Zeaxanthin-Producing Bacteria Isolated from a Radioactive Hot Spring Water BT - Microbial Carotenoids from Bacteria and Microalgae: Methods and Protocols." In , edited by José-Luis Barredo, 99–131. Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-61779-879-5_5.

Aslanzadeh, Jaber. 2006. "Biochemical Profile-Based Microbial Identification Systems BT - Advanced Techniques in Diagnostic Microbiology." In , edited by Yi-Wei Tang and Charles W Stratton, 84–116. Boston, MA: Springer US. https://doi.org/10.1007/0-387-32892-0_6.

Baskaran, S., Rajan, D. P., & Balasubramanian, K. A. (1989). Formation of methylglyoxal by bacteria isolated from human faeces. Journal of medical microbiology, 28(3), 211-215.

Bocock, Philip, Alison Morse, Chris Dervinis, and John Davis. 2008. "Evolution and Diversity of Invertase Genes in *Populus Trichocarpa*." *Planta* 227 (March): 565–76. https://doi.org/10.1007/s00425-007-0639-3.

Chakraborty, Sangeeta, Kapudeep Karmakar, and Dipshikha Chakravortty. 2014. "Cells Producing Their Own Nemesis: Understanding Methylglyoxal Metabolism." *IUBMB Life* 66 (10): 667–78. https://doi.org/10.1002/iub.1324.

Clearwater, Michael J, Maria Revell, Stevie Noe, and Merilyn Manley-Harris. 2018. "Influence of Genotype, Floral Stage, and Water Stress on Floral Nectar Yield and Composition of Mānuka (*Leptospermum scoparium*)." *Annals of Botany* 121 (3): 501–12. https://doi.org/10.1093/aob/mcx183.

Chappell, C. R., & Fukami, T. (2018). Nectar yeasts: a natural microcosm for ecology. *Yeast*, *35*(6), 417-423.

Dueholm, Morten Simonsen, Kasper Skytte Andersen, Francesca Petriglieri, Simon Jon McIlroy, Marta Nierychlo, Jette Fisher Petersen, Jannie Munk Kristensen, et al. 2019. "Comprehensive Ecosystem-Specific 16S rRNA Gene Databases with Automated Taxonomy Assignment (AutoTax) Provide Species-Level Resolution in Microbial Ecology." *BioRxiv*, January, 672873. https://doi.org/10.1101/672873.

Fabro, G., Kovács, I., Pavet, V., Szabados, L., & Alvarez, M. E. (2004). Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. Molecular Plant-Microbe Interactions, 17(4), 343-350.

González-Teuber, M., & Heil, M. (2009). Nectar chemistry is tailored for both attraction of mutualists and protection from exploiters. *Plant signaling & behavior*, *4*(9), 809-813.

Grainger, Megan N C, Merilyn Manley-Harris, Joseph R Lane, and Richard J Field. 2016. "Kinetics of Conversion of Dihydroxyacetone to Methylglyoxal in New Zealand Mānuka Honey: Part I – Honey Systems." *Food Chemistry* 202: 484–91. https://doi.org/https://doi.org/10.1016/j.foodchem.2016.02.029.

Innerebner, Gerd, Claudia Knief, and Julia A Vorholt. 2011. "Protection of *Arabidopsis thaliana* against Leaf-Pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a Controlled Model System." *Applied and Environmental Microbiology* 77 (10): 3202–10. https://doi.org/10.1128/AEM.00133-11.

Jin, Qusheng, and Matthew F Kirk. 2018. "pH as a Primary Control in Environmental Microbiology: 1. Thermodynamic Perspective." *Frontiers in Environmental Science. 6:21. doi: 10.3389/fenvs.2018.00021*

Kearse, Matthew, Richard Moir, Amy Wilson, Steven Stones-Havas, Matthew Cheung, Shane Sturrock, Simon Buxton, et al. 2012. "Geneious Basic: An Integrated and Extendable Desktop Software Platform for the Organization and Analysis of Sequence Data." *Bioinformatics (Oxford, England)* 28 (April): 1647–49. https://doi.org/10.1093/bioinformatics/bts199.

Krause, E., Wichels, A., Giménez, L., Lunau, M., Schilhabel, M. B., & Gerdts, G. (2012). Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach.

Lane. (1991). 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt, E., and Goodfellow, M., eds., John Wiley and Sons, New York,, NY, 115 - 175

Lebedeva, Natalia V, Thomas K Chen, and Malcolm D E Forbes. 2010. "Solvent- and Wavelength-Dependent Photochemistry of 1,3-Dihydroxyacetone Studied by TREPR: Implications for Commercial Sunless Tanning Lotions." *Applied Magnetic Resonance* 38 (2): 155–66. https://doi.org/10.1007/s00723-009-0105-4.

Levering, P. R., J. P. van Dijken, M. Veenhuis, and W. Harder. 1981. "*Arthrobacter P 1*, a Fast Growing Versatile Methylotroph with Amine Oxidase as a Key Enzyme in the Metabolism of Methylated Amines." *Archives of Microbiology* 129 (1): 72–80. https://doi.org/10.1007/BF00417184.

Lievens, B., Hallsworth, J. E., Pozo, M. I., Belgacem, Z. B., Stevenson, A., Willems, K. A., & Jacquemyn, H. (2015). Microbiology of sugar-rich environments: diversity, ecology and system constraints. Environmental Microbiology, 17(2), 278-298.

Lohaus, G., & Schwerdtfeger, M. (2014). Comparison of sugars, iridoid glycosides and amino acids in nectar and phloem sap of Maurandya barclayana, Lophospermum erubescens, and Brassica napus. *PloS one*, *9*(1), e87689. https://doi.org/10.1371/journal.pone.0087689

Mann, Scott, and Yi-Ping Phoebe Chen. 2010. "Bacterial Genomic G+C Composition-Eliciting Environmental Adaptation." *Genomics* 95 (1): 7–15. https://doi.org/https://doi.org/10.1016/j.ygeno.2009.09.002.

McDonald, Claire M, Suzanne E Keeling, Mark J Brewer, and Steve C Hathaway. 2018. "Using Chemical and DNA Marker Analysis to Authenticate a High-Value Food, Mānuka Honey." *Npj Science of Food* 2 (1): 9. https://doi.org/10.1038/s41538-018-0016-6.

Morris, Megan M, Natalie J Frixione, Alexander C Burkert, Elizabeth A Dinsdale, and Rachel L Vannette. 2020. "Microbial Abundance, Composition, and Function in Nectar Are Shaped by Flower Visitor Identity." *FEMS Microbiology Ecology* 96 (3). https://doi.org/10.1093/femsec/fiaa003.

Nepi, M., & Stpiczyńska, M. (2007). Nectar resorption and translocation in Cucurbita pepo L. and Platanthera chlorantha Custer (Rchb.). Plant biology (Stuttgart, Germany), 9(1), 93–100. https://doi.org/10.1055/s-2006-924287

Nepi, Massimo, Donato A Grasso, and Stefano Mancuso. 2018. "Nectar in Plant–Insect Mutualistic Relationships: From Food Reward to Partner Manipulation." *Frontiers in Plant Science* ,9, 1063.

Noe, Stevie, Merilyn Manley-Harris, and Michael J Clearwater. 2019. "Floral Nectar of Wild Mānuka (*Leptospermum scoparium*) Varies More among Plants than among Sites." *New Zealand Journal of Crop and Horticultural Science* 47 (4): 282–96. https://doi.org/10.1080/01140671.2019.1670681.

O'Brien, S. P., B. R. Loveys, and W. J. R. Grant. 1996. Ultrastructure and function of floral nectaries of Chamelaucium uncinatum (Myrtaceae). Annals of Botany 78: 189–196.

Pantini, Giovanni, R Ingoglia, F Brunetta, and A Brunetta. 2007. "Sunless Tanning Products Containing Dihydroxyacetone in Combination with a Perfluoropolyether Phosphate." *International Journal of Cosmetic Science* 29 (July): 201–9. https://doi.org/10.1111/j.1467-2494.2007.00381.x.

Peach, D A H, C Carroll, S Meraj, S Gomes, E Galloway, A Balcita, H Coatsworth, et al. 2020. "Nectar-Dwelling Microbes of Common Tansy Are Attractive to Its Mosquito Pollinator, *Culex Pipiens L*." *BioRxiv*, January, 2020.04.03.024380. https://doi.org/10.1101/2020.04.03.024380.

Perry, Benjamin J, Jordyn Bergsveinson, Dinah D Tambalo, Christopher K Yost, Nurul H Khan, and Mike Whiting. 2017. "Complete Genome Sequence of *Delftia Acidovorans RAY209*, a Plant Growth-Promoting *Rhizobacterium* for Canola and Soybean." *Genome Announcements* 5 (44): e01224-17. https://doi.org/10.1128/genomeA.01224-17.

Purahong, Witoon, Luigi Orrù, Irene Donati, Giorgia Perpetuini, Antonio Cellini, Antonella Lamontanara, Vania Michelotti, Gianni Tacconi, and Francesco Spinelli. 2018. "Plant Microbiome and Its Link to Plant Health: Host Species, Organs and *Pseudomonas syringae Pv. Actinidiae* Infection Shaping Bacterial Phyllosphere Communities of Kiwifruit Plants." *Frontiers in Plant Science*.7; 9:1963.

Pusey, P. L. (1999). Effect of nectar on microbial antagonists evaluated for use in control of fire blight of pome fruits. Phytopathology, 89(1), 39-46.

Rousk, J., Brookes, P. C., & Bååth, E. (2010). Investigating the mechanisms for the opposing pH relationships of fungal and bacterial growth in soil. Soil Biology and Biochemistry, 42(6), 926-934.

Samuni-Blank, M., Izhaki, I., Laviad, S., Bar-Massada, A., Gerchman, Y., & Halpern, M. (2014). The Role of Abiotic Environmental Conditions and Herbivory in Shaping Bacterial Community Composition in Floral Nectar. PLOS ONE, 9(6), e99107. doi: 10.1371/journal.pone.0099107

Smallfield, Bruce Murray, Nigel Ian Joyce, and John William van Klink. 2018. "Developmental and Compositional Changes in *Leptospermum scoparium* Nectar and Their Relevance to Mānuka Honey Bioactives and Markers." *New Zealand Journal of Botany* 56 (2): 183–97. https://doi.org/10.1080/0028825X.2018.1446450.

Subedi, Krishna P, Insook Kim, Junho Kim, Bumchan Min, and Chankyu Park. 2008. "Role of GldA in Dihydroxyacetone and Methylglyoxal Metabolism of *Escherichia coli K12*." *FEMS Microbiology Letters* 279 (2): 180–87. https://doi.org/10.1111/j.1574-6968.2007.01032.x.

Tecon, Robin, and Johan H J Leveau. 2016. "Symplasmata Are a Clonal, Conditional, and Reversible Type of Bacterial Multicellularity." *Scientific Reports* 6 (1): 31914. https://doi.org/10.1038/srep31914.

Torres-Cortés, Gloria, Sophie Bonneau, Olivier Bouchez, Clémence Genthon, Martial Briand, Marie-Agnès Jacques, and Matthieu Barret. 2018. "Functional Microbial Features Driving Community Assembly During Seed Germination and Emergence." *Frontiers in Plant Science*, 9:902.

Trenozhnikova, Lyudmila, and Azliyati Azizan. 2018. "Discovery of Actinomycetes from Extreme Environments with Potential to Produce Novel Antibiotics." *Central Asian Journal of Global Health* 7 (1): 337. https://doi.org/10.5195/cajgh.2018.337.

Vannette, Rachel L, and Tadashi Fukami. 2018. "Contrasting Effects of Yeasts and Bacteria on Floral Nectar Traits." *Annals of Botany* 121 (7): 1343–49. https://doi.org/10.1093/aob/mcy032.

Yang, Jiangang, Jitao Li, Yan Men, Yueming Zhu, Ying Zhang, Yuanxia Sun, and Yanhe Ma. 2015. "Biosynthesis of L-Sorbose and L-Psicose Based on C—C Bond Formation Catalyzed by Aldolases in an Engineered Corynebacterium glutamicum strain." Edited

by V Müller. *Applied and Environmental Microbiology* 81 (13): 4284 LP – 4294. https://doi.org/10.1128/AEM.00208-15.

Zhao, D., Tang, W., Hao, Z., & Tao, J. (2015). Identification of flavonoids and expression of flavonoid biosynthetic genes in two coloured tree peony flowers. Biochemical and biophysical research communications, 459(3), 450-456.

CHAPTER 5.

SYNTHESIS

1) Microbial diversity in nectar

Plants have developed multiple associations with microorganisms that colonize their exterior (epiphytes) or their interior (endophytes). Both types are known to interact with the host plant influencing metabolic or defence functions (Turner et al., 2013). In reproductive organs of plants like flowers, microorganisms play important biological and ecological roles. For instance, they can change flower smell and nectar properties (Aleklett et al., 2014). There are several bacteria and fungi that modify nectar metabolite compositions (Vannette & Fukami, 2016; Vannette et al., 2018).

The diversity of bacteria in the nectar of plants shows the adaptation of several bacteria families to the nectar environment. In mānuka there is a high diversity of bacteria groups with similarities to other studies with isolates from the nectar of plants in the Southern hemisphere. The similar findings have been found for 48 other plant species belonging to 16 angiosperm families in South Africa, where the most identified phyla were Proteobacteria (including α-, β- and γ-Proteobacteria), Actinobacteria and Firmicutes. The most frequently isolated genera were *Pantoea, Asaia, Pseudomonas* and *Enterobacter*. Interestingly from 5 plants (*Albuca nelsonii*, *Aloe dominella*, *Cyrtanthus contractus*, *Dipcadi viride* and *Tulbaghia natalensis*), they could not isolate any type of bacteria or fungi, while from the *Haemanthus humilis, Silene bellidioides, and Kniphofia sp. plants,* they isolated a very high abundance of *Pantoea* (de Vega et al., 2021). This means that there is a selection process in the establishment of the bacterial community for each type of plant, and that there are special interactions between certain plant species and some types of bacteria.

The bacteria found in this study with culturing technics (Chapter 4, Table 4.0.) are similar to the ones found by metabarcoding analysis on the same population with two years difference (Chapter 2). The similarity found via cultivable and non-cultivable methods shows a well-established microbiota for mānuka plants that is adapted to the particular physicochemical characteristics of these flowers. The methodology used was enough to be able to culture bacteria from mānuka nectar. Several bacteria strain isolations were made, though due to time constrains not all were sequenced. From the strains that were sequenced, we can see well adapted bacteria in the nectar of mānuka plants as supported by biochemical experiments on their osmotolerance and catalase activity (Chapter 4, Table 4.1.). These results suggest stable evolutionary interactions between the plant and certain microbial groups, which may influence the nectar chemistry. Interestingly, the isolated bacteria *Arthrobacter sp.* could be involved in the production of methylglyoxal in closely plant species, such as Kānuka, that are not reported to have DHA in their nectar, further studies will be necessary to analyse the presence of *Arthrobacter sp*. in kānuka plants and the similarities and differences between mānuka and kānuka plants in terms of bacterial composition.

Comparisons with the meta-barcode data did not yield differences between pollinator restricted (bagged) and pollinator accessible (unbagged) flowers, however, molecular essays were performed on the limits of the polymerase enzyme to initiate the amplification reaction. The nectar DNA extraction methodology employed and the use of frozen flowers, made it more difficult to differentiate between the bacterial community in the nectar and the one that is in the nectaries. Ideally, the DNA extraction should be done from flowers that are not frozen so that the differences between bugged and unbagged flowers can be detected more accurately.

Due to the limitations of meta-barcoding technics, low phylogeny definitions in meta-barcodes and low identification at species are common. Particularly the Enterobacteriaceae family is difficult to identify at species level (McLean et al., 2019). Mānuka flowers are small and with very low nectar volume, with only few drops of nectar. In order to isolate a minimal amount of DNA, the nectar was pooled from a group of flowers which caused more errors because of the handling of higher amounts of volume. Besides these practical circumstances the PCR products were obtained at a level enough for sequencing and were representative and as close possible as to the real microbial community in the nectar. All the available bioinformatic filters were applied to eliminate chimeras and non-desirable molecular products during reactions. The most accurate possible results that could be obtained by the

available methods were the identification and representativeness of the bacteria present in the nectar, which was as close as it could get in real life conditions. The statistical methods were applied considering the compositional nature of the bioinformatic data and the results could be compared with the obtained cultures. I hope that the advancement of metagenomics and other techniques will allow a more accurate species identification in the future.

2) Network analysis and key functional bacteria in the nectar

Interactions within bacteria members determine the carbon flow on a certain ecosystem. The meta-barcode and the analysis with network theory has provided the possibility to determine interactions within bacteria in the nectar.

Competition scenarios in nectar through complex chemical conditions and low availability of nutrients might switch correlations within bacteria from different phyla in order to assess metabolic complementation under the same niche. The nectar microbial ecology network will have the members and interconnections of bacteria to assure the cycle of carbon and nitrogen in the nectar.

Pantoea agglomerans was identified as a keystone/hub species in nectar microbial communities which has positive and negative corelations with other bacteria. There is an increasing number of studies showing several applications of this species in agriculture, as a consequence of its interaction capabilities for substrate competition and antibacterial metabolite production (Smits et al., 2011). It has a versatile metabolism with capabilities of pathogen inhibition (Walterson, Smith, and Stavrinides, 2014) and also shows positive interactions with several bacteria, including the promotion of traits of rhizobacteria in the nitrogen and phosphorous fixation (Silini-Chérif et al., 2012), In another important crop: rice (*Oryza sativa*), *P. agglomerans* also interacts with the host with phytohormones to regulate the photosynthate and stimulate plant growth (Feng, Shen, and Song 2006).

The interactions that *P. agglomerans* have developed with other bacteria and plants could be other examples and useful models to study the interactions that are present in mānuka plants and to use as a baseline to elucidate its role in the carbon, nitrogen and overall energetic metabolism in the nectar. Through network analysis, we have a first indication of positive correlations between *Pantoea* and other bacteria that may indicate an endosymbiotic role with

capabilities to interfere with photosynthesis and mineral metabolisms e.g., correlation with Phytoplasma, and with bacteria with carbon fixation capabilities (*Synechococcus*) (Chapter 3, ST.3.8.3). There is an indication that it is a hub bacterium, and it has interconnections with important roles in the microbial ecology of the nectar with implications in the carbon metabolism of the plant that end in the secretion of the compound dihydroxyacetone in the nectar of mānuka plants.

This work reports on the first data on nectar microbial biodiversity and abundance, although other efforts to determine which and how many types of bacteria could be transported along the phloem and xylem of these flowers will be very important.

3) Microorganisms in the nectar and the pollinators' effect

I compared bacterial and fungal communities between flowers exposed to pollinators and bagged with breathable mesh in order to restrict their access. Fungal communities, but not bacteria, where influenced by pollinator visitation. This is likely due to bacteria having multiple ways to enter nectar (aside of pollinators). However, I also want to acknowledge some limitations in our approach. Limitations in the amount of DNA needed for the first amplification of nucleic acids is certainly the first step that make difficult comparisons within studies and also linked to bioinformatic analysis of molecular products gives some bias of the natural community structure that is present in the nectar of the flowers. The step of DNA extraction and the afterward amplification with phylogenetic assessment of the community could end in overrepresentation of bacteria phyla based on better taxonomy resolution this can end in a large amount of sequences belonging to high study groups e.g. *Pseudomonas*, that counts with several members with 16S rRNA sequences submitted to the bioinformatic databases, that make possible their assignment to taxa, while new bacteria belonging to genus that are not well studied and with a large proportion of non-culturable members will be underestimated. In addition, the number and volumes of PCR reaction that each author uses can end in different molecular rearrangements and subsequent different purification process of the sequences that could be very different withing studies what makes difficult compare results from studies of different authors. Overall meta-barcoding studies are representing the finding with culturing studies techniques in the different geographic regions. They have been adding rare bacteria of the nectar and quantitative relationship within and between bacteria

genera and families per study, that makes possible the use of advance approaches to find and explain microbial ecology scenarios with a functional meaning based on biochemistry knowledge of each bacteria group.

The structure of the bacterial community was very similar between control and treatment flowers, and I did not find microbiome structural differences between control and treatment flowers, although differences in particular bacteria genus with proportional statistics methods were found what means pollinators effectively contribute to bacteria composition (Chapter 2, Figure 2.5.). These bacteria seem to be adapted to the nectar environment besides the several antibiotic compounds present in the nectar. As several types of pollinators regularly visits these flowers it could be interesting to determine if evolutionary adaptation to honeybees has been developed as the original pollinators to visit these flowers were flies, moth, geckos and birds (Whitaker, 1987).

Bacteria can also move by themselves, and several species can do swarming which gives them an independent way to access to the nectar. This could also be an important source and movement of bacteria along flower's components as they may have different types of nutrients that change during flower's development.

Ecological adaptation processes as priority effects could be important in terms of bacteria movements and intermediate dispersion mechanisms (Fukami 2015). Selection processes could determine the niche of bacteria in the nectar, and competition between phylogenetically related bacteria is more intense in the nectar environment because of higher ecological similarity requirements for closer phylogenetic bacteria (Peay, Belisle, and Fukami 2012). The nature of the competitions and bacteria assembly will affect the community composition and jointly with priority effects will determine the timing of inoculation in agricultural practices with microorganisms (Verbruggen et al. 2013).

Fungi are also other components of mānuka nectar, although they are in smaller concentration, and they were linked to pollinator visitation. In comparison with other studies there are fewer *Saccharomyces* yeast probably because of the high UV radiation in New Zealand as *Saccharomyces cerevisiae* are sensitive to low doses of UV radiation (Guerrero-Beltren & Barbosa-Cenovas, 2006), An additional explanation is that these flowers have no depth in the corolla and *Saccharomyces* yeasts are sensitive to physical and chemical agents. Other types of very small yeast such as Capnodiales and Pucciniales were found in mānuka nectar, that might be important pathogenic actors, but bacteria could play an important role in

their biocontrol, as bacteria are always found in the nectar from both bagged and exposed flowers, with inhibition capabilities against fungi.

4) Bacteria cultures, metabarcoding and plant genotypes

The bacteria found in the cultures from different morphotypes (Chapter 4, table 4.0.) belong to the same families as the important families found in the metabarcoding (Chapter 2, Figure 2.1). This indicates that these families belong to the natural community of mānuka nectar and represent the core microbiome of these flowers. It is important to note that these components can be cultured. This gives possibilities to manipulate the nectar microbiome towards the production of metabolites of interest for a variety of ecosystem services as high-quality mānuka honey, biocontrol of pathogens in surroundings plantations of agricultural plants, and therefore high-quality fruits production and better reproduction performance.

Differences in mānuka genotypes for beta diversity for bacteria reveal a genotype effect on bacteria assemblage in the nectar of the different genotypes. Exploring the genotypes with LEfSe analysis (Chapter 2, Figure 2.4.) shows specific taxa that vary in abundance consistently among the different genotypes. *Pantoea* was the genus with the highest differences in LDA score (Chapter 2, Figure 2.4. first position) meaning that its contribution could make a difference in the metabolites considering the mānuka genotypes. The activity of *Pantoea* could show an effect on the DHA values for the different genotypes. Nectar inoculation experiments with *P. agglomerans* show an effect of this bacterium increasing DHA production. Furthermore, network analysis reveals high connectivity for *Pantoea* genus in the overall network for all the genotypes (Chapter 3, Figure 1.). An important ecological meaning is that the function of these bacteria in the community can affect the overall community structure and important roles as a keystone bacterium could be explored based on the network topology and LDA scores for *Pantoea* genus.

Pollinators seem to play a role in the incorporation of bacteria in the nectar (Rebolleda-Gómez et al. 2019), and in other plants were suggested to contribute to the evolution of the flowers (Gómez, Perfectti, and Klingenberg 2014). In mānuka, they do not modify the structure of the nectar community, although they could be driving the adaptation and evolution of mānuka flowers to different environmental changes such as higher UV radiation, metabolism, and adaptation to water stress.

A multifactorial edgeR analysis (Chapter 2, 2.3.6) shows an effect of the treatment (bagged flowers) on two genotypes and the cultivar, which means that pollinators could be differentially visiting these genotypes. *Pantoea* did not appear in this analysis, meaning that its presence is independent of the treatment. Further research in the quantification of bacterial abundance with pollinator visitation could give information about the contribution of the pollinators to specific types of bacteria and the overall microbial community in the nectar.

Further studies are also needed to explore the contribution of different pollinators to the structure of mānuka flowers over time and during the different development stages of the flowers, as some metabolites like DHA are known to change during the different flower development stages (Smallfield, Joyce, and van Klink 2018). Exploring the contribution of other factors such as wind and water in terms of bacteria structure will give knowledge for understanding of microbial nectar dynamics and the input and evolutionary drivers of these complex plant systems. Interactions with other plants will also give a further understanding for managing ecosystems with different agricultural plants and evaluating the best practices to enhance the productivity of each plant, nectar, and fruit. The better flow of bacteria between ecosystem components will determine ecosystem services with higher individual plant productivity.

Based on the discovery of the interaction between *Pantoea agglomerans* and mānuka plants, the importance of *P. agglomerans* for the production of high-quality nectar, and as a consequence, high-quality honey, has been revealed. The identification of bacteria involved in the production of key nectar metabolites will be an important field in ecology and agriculture microbiology as would determine the success of agricultural practices and the level and quality of the overall ecosystem services.

5.1. REFERENCES

Aleklett, K., Hart, M., & Shade, A. (2014). The microbial ecology of flowers: an emerging frontier in phyllosphere research. Botany, 92(4), 253-266. doi: 10.1139/cjb-2013-0166

de Vega, C., Álvarez-Pérez, S., Albaladejo, R. G., Steenhuisen, S.-L., Lachance, M.-A., Johnson, S. D., & Herrera, C. M. (2021). The role of plant–pollinator interactions in structuring nectar microbial communities. Journal of Ecology, 109, 3379– 3395. https://doi.org/10.1111/1365-2745.13726

Feng, Y, D Shen, and W Song. 2006. "Rice Endophyte Pantoea Agglomerans YS19 Promotes Host Plant Growth and Affects Allocations of Host Photosynthates." *Journal of Applied Microbiology* 100 (5): 938–45. https://doi.org/https://doi.org/10.1111/j.1365-2672.2006.02843.x.

Fukami, Tadashi. 2015. "Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects." *Annual Review of Ecology, Evolution, and Systematics* 46 (1): 1–23. https://doi.org/10.1146/annurev-ecolsys-110411-160340.

Gómez, José María, Francisco Perfectti, and Christian Peter Klingenberg. 2014. "The Role of Pollinator Diversity in the Evolution of Corolla-Shape Integration in a Pollination-Generalist Plant Clade." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 369 (1649): 20130257. https://doi.org/10.1098/rstb.2013.0257.

Guerrero-Beltren, J. A, and Barbosa-Cenovas G. 2006. "Inactivation of Saccharomyces Cerevisiae and Polyphenoloxidase in Mango Nectar Treated with UV Light." *Journal of Food Protection* 69 (2): 362–68. https://doi.org/10.4315/0362-028X-69.2.362.

McLean, K., Rosenthal, C. A., Sengupta, D., Owens, J., Cookson, B. T., Hoffman, N. G., & Salipante, S. J. 2019. Improved Species-Level Clinical Identification of Enterobacteriaceae through Broad-Range dnaJ PCR and Sequencing. Journal of clinical microbiology, 57(11), e00986-19. https://doi.org/10.1128/JCM.00986-19

Peay, Kabir G, Melinda Belisle, and Tadashi Fukami. 2012. “Phylogenetic Relatedness Predicts Priority Effects in Nectar Yeast Communities.” *Proceedings of the Royal Society B: Biological Sciences* 279 (1729): 749–58. https://doi.org/10.1098/rspb.2011.1230.

Rebolleda-Gómez, María, Nicole J Forrester, Avery L Russell, Na Wei, Andrea M Fetters, Jessica D Stephens, and Tia-Lynn Ashman. 2019. “Gazing into the Anthosphere: Considering How Microbes Influence Floral Evolution.” *New Phytologist* 224 (3): 1012–20. https://doi.org/https://doi.org/10.1111/nph.16137.

Silini-Chérif, H, A Silini, M Ghoul, and S Yadav. 2012. “Isolation and Characterization of Plant Growth Promoting Traits of a Rhizobacteria: Pantoea Agglomerans Lma2.” *Pakistan Journal of Biological Sciences : PJBS* 15 (6): 267–76. https://doi.org/10.3923/pjbs.2012.267.276.

Smallfield, Bruce Murray, Nigel Ian Joyce, and John William van Klink. 2018. “Developmental and Compositional Changes in Leptospermum Scoparium Nectar and Their Relevance to Mānuka Honey Bioactives and Markers.” *New Zealand Journal of Botany* 56 (2): 183–97. https://doi.org/10.1080/0028825X.2018.1446450.

Smits THM, Rezzonico F, Kamber T, Blom J, Goesmann A, Ishimaru CA, et al. (2011) Metabolic Versatility and Antibacterial Metabolite Biosynthesis Are Distinguishing Genomic Features of the Fire Blight Antagonist Pantoea vagans C9-1. PLoS ONE 6(7): e22247. https://doi.org/10.1371/journal.pone.0022247

Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. Genome biology, 14(6), 1-10.

Vannette, Rachel L, Marie-Pierre L Gauthier, and Tadashi Fukami. 2013. “Nectar Bacteria, but Not Yeast, Weaken a Plant–Pollinator Mutualism.” Proceedings of the Royal Society B: Biological Sciences 280 (1752): 20122601. https://doi.org/10.1098/rspb.2012.2601.

Vannette, Rachel L, and Tadashi Fukami. 2018. “Contrasting Effects of Yeasts and Bacteria on Floral Nectar Traits.” *Annals of Botany* 121 (7): 1343–49. https://doi.org/10.1093/aob/mcy032.

Verbruggen, Erik, Marcel G A van der Heijden, Matthias C Rillig, and E Toby Kiers. 2013. “Mycorrhizal Fungal Establishment in Agricultural Soils: Factors Determining

Inoculation Success." *New Phytologist* 197 (4): 1104–9. https://doi.org/https://doi.org/10.1111/j.1469-8137.2012.04348.x.

Walterson, Alyssa M, Derek D N Smith, and John Stavrinides. 2014. "Identification of a Pantoea Biosynthetic Cluster That Directs the Synthesis of an Antimicrobial Natural Product." *PLOS ONE* 9 (5): e96208. https://doi.org/10.1371/journal.pone.0096208.

Whitaker, A. H. (1987). The roles of lizards in New Zealand plant reproductive strategies. New Zealand journal of botany, 25(2), 315-328.