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Comparative Analysis of Rhizosphere and Endosphere Microbiome of Different Blueberry Species (Vaccinium sp.)

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COMPARATIVE ANALYSIS OF RHIZOSPHERE AND ENDOSPHERE MICROBIOME OF DIFFERENT BLUEBERRY SPECIES (*VACCINIUM* SP.)

by

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ABSTRACT

Blueberries are an important agricultural commodity in all over the United States. Due to its health benefits, there is a huge demand globally, thus expanding the industry. Breeding programs are essential to maintain such industries. Challenges that play a role in contemporary breeding programs are various biotic and abiotic stress factors. Studies have shown that microorganisms are recruited by plants to alleviate them during stressful conditions. Though blueberries have been cultivated for about 100 years, how the microbiome has been affected due to this is poorly understood. We hypothesized that interspecific crosses and artificial selection have significantly changed the microbiome of the blueberry and it has affected the overall community. We tested the hypothesis by comparing the microbiome and metagenome of five different species of blueberry comprising both wild and cultivated species. The results showed that statistically significant differences were seen between the wild and cultivated species of blueberry with respect to the microbial composition of the rhizosphere and root endosphere. The metagenome analysis showed the presence of various metabolic pathways associated with the interaction. The physiological profiling showed the utilization of different carbon sources by the microbes associated with the rhizosphere of the plants. All this information can be used in traditional plant breeding programs. Microbiome-supported breeding should be performed instead of using excessive fertilizers and pesticides and it can be complemented by looking at the interactions involved with key beneficial or pathogenic plant-microbe interactions. This approach can be employed in blueberries to improve their resistance to various stress factors.

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DEDICATION

To my friends and family.

To Rabindranath Tagore, whose creations motivate me and keep me moving forward.

“Where the mind is without fear and the head is held high.”

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LIST OF ABBREVIATIONS

AM	Arbuscular Mycorrhiza
ASV	Amplicon Sequence Variants
AUC	Area Under Curve
AWCD	Average Well Color Development
EM	Ericoid Mycorrhizae
EPS	Exopolysaccharide
FDR	False Discovery Rate
ITS	Internal Transcribed Spacer
LDA	Linear Discriminant Analysis
MAGs	Metagenome Assembled Genomes
NCBI	National Center for Biotechnology Information
NIGMS	National Institute of General Medical Sciences
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
QIIME	Quantitative Insights Into Microbial Ecology
TCA	Tricarboxylic Acid
USDA-ARS	United States Department of Agriculture – The Agricultural Research Service
VOC	Volatile Organic Compounds

CHAPTER I - INTRODUCTION

1.1 Plant microbiome and its importance

Microbial communities that live on and within eukaryotic organisms contribute to many key aspects of the host biology. All multicellular eukaryotes harbor diverse microbial communities that are recruited and supported by a steady supply of carbon and nitrogen. In return, the microbiome expands the genetic potential of plants and animals by shaping their physiology, and developmental and reproductive phenotype (Fitzpatrick et al., 2018; Hacquard et al., 2015). For example, the human gut microbiota produces essential vitamins and amino acids and aids digestion by breaking down various dietary substrates. It positively affects human health by outcompeting pathogens, producing antimicrobial metabolites, and modulating toxin levels (Berendsen et al., 2012; Derrien et al., 2010; Fagundes et al., 2012). The establishment of the gut microflora in early life is critical for the proper development of the innate immune system and its ability to distinguish pathogens from symbionts and commensal microbes (Bron et al., 2012; Chinen & Rudensky, 2012; Lathrop et al., 2011).

Plants also harbor numerous parasitic, commensal, and symbiotic microbes that form complex ecological communities or the “phytobiome” (a term describing a plant, the environment in which it is residing and all the organisms) (Figure 1.1.1). The association between plants and microorganisms is ancient and thought to have originated over 400 million years ago (Santoyo, 2021). The plant-associated microbial communities reside within plant tissues (endosphere), on aerial surfaces (phyllosphere) and around plant roots (rhizosphere). The term “rhizosphere” was first coined by Lorenz Hiltner to describe the dynamic interface between plant roots and soil influenced by root exudates

(Hartmann et al., 2008). This unique environment houses the bulk of the phytobiome and is characterized by a high level of microbial activity (Morrissey et al., 2004). Similar to the human gut microbiome, root-associated microorganisms positively influence plant nutrient status (Barea et al., 2005; Bowen & Rovira, 1999).

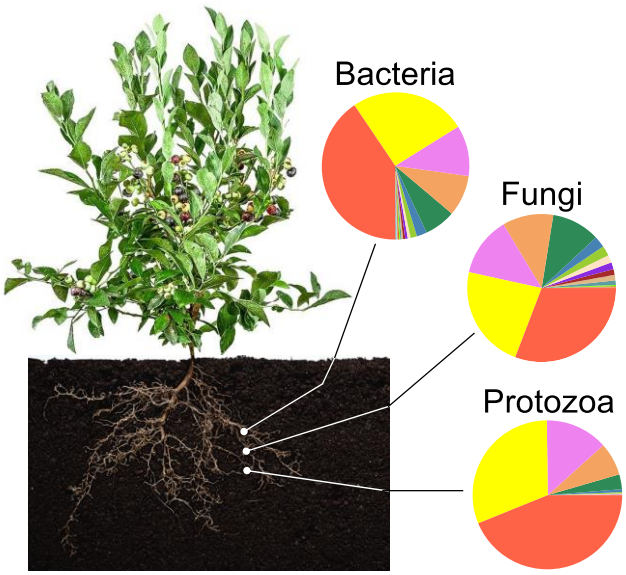

Figure 1.1 *The plant microbiome (phytobiome)*

Alpha- or Betaproteobacteria known as rhizobia can infect roots of certain leguminous plants and form nodules, in which the bacteria supply their host with nitrogen by converting N_2 to NH_3 (Udvardi & Poole, 2013). This interaction is mutualistic in nature, and plant supports its root nodule bacteria with the source of carbon and energy. Another significant supply of macronutrients is provided by mycorrhizal fungi that form symbioses with an estimated 70–90% of plant species (Parniske, 2008). The arbuscular mycorrhizal (AM) fungi are members of the Glomeromycota that form extensive hyphal networks capable of mining phosphorus (P_i) and inorganic nitrogen (NH^{4+} and NO^{3-}) from the soil and delivering them to the plant via intraradical mycelium (Sikes et al., 2010) (Figure 1.1.2). In return plants support mycorrhizae by allocating up to 20% of

photosynthetically fixed carbon to the fungus. The beneficial effects are best observed in poor soils where mycorrhizal plants thrive due to more efficient absorption of nutrients through the fungal mycelium (Hawkins et al., 2000). Another well-studied fungal mutualist is *Serendipita indica*, an endophyte of the order Sebacinales that, in contrast to obligately biotrophic AM fungi, can be cultured *in vitro* (Weiß et al., 2016). *Serendipita indica* colonizes many different plant species and triggers induced systemic resistance, modulates phytohormone levels and mobilizes nutrients thus boosting plant's tolerance to biotic and abiotic stresses (Gupta & Pandey, 2019).

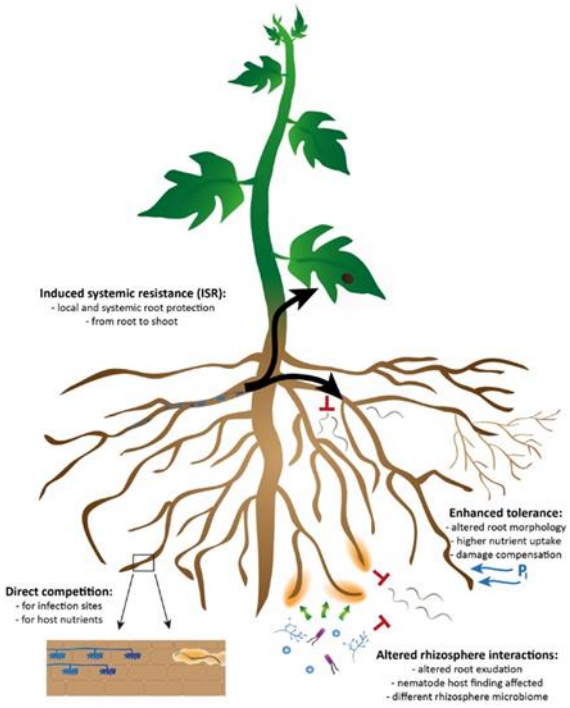

Figure 1.2 *Beneficial effects of arbuscular mycorrhizal (AM) fungi.*

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The stimulation of nutrient uptake and organ development also has been demonstrated in numerous non-rhizobial bacteria, commonly referred to as plant growth-promoting rhizobacteria (PGPR). The promotion of plant growth by microorganisms is a

multifactorial phenotype and subject of ongoing active research (Y. C. Kim et al., 2011). Phosphorus (P) is one of the essential macronutrients required for plant growth. Despite the widespread presence of P in soil, most of it is present in the form of highly insoluble mineral and organic phosphates that are not suitable for assimilation by plants. Beneficial rhizobacteria can improve the availability of P available to plants by solubilizing the soil phosphates via production of organic acids (gluconic and citric), acid phosphatases, redox-active secondary metabolites and the release of protons (Alori et al., 2017; McRose & Newman, 2021; Richardson et al., 2009). In addition to macronutrients, plants require several microelements for proper growth and development. One of these, iron, is abundant in the environment, but mostly in the insoluble oxidized form, which is biologically inaccessible. To cope with the limited supply of iron, microorganisms scavenge it by producing siderophores, low-molecular weight metabolites with high affinity for Fe^{3+} (Soares, 2022). Siderophores are secreted in the environment, where they bind iron, and the resultant Fe-siderophore complexes are taken up by dedicated membrane receptors. Siderophores produced by rhizobacteria can efficiently supply iron to plants and improve their growth under conditions of heavy metal pollution (C. Dimkpa et al., 2008; C. O. Dimkpa et al., 2009).

Many rhizosphere microorganisms actively modulate host phytohormone levels, which stimulates germination of seed and tubers, promotes stem and root growth, and alleviates the negative effects of various abiotic stresses. Treatment of plants with strains producing auxins, cytokinins, and/or gibberellins commonly results in the better formation of root hairs, increased root growth and branching, and, as a result, in improved mineral and nutrient uptake (Glick, 2012). Certain groups of rhizobacteria also

produce an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves the immediate precursor of the plant hormone ethylene (Ravanbakhsh et al., 2018). Lower ethylene levels result in longer roots and less inhibition of ethylene-sensitive plant growth following environmental-induced stress. Rhizobacteria also produce volatile organic compounds (VOCs), a class of small molecules that diffuse via a gaseous phase and modulate interactions between plants and root-associated microbial communities (Garbeva & Weisskopf, 2020). Microbial VOCs like 2,3-butanediol positively affect plant growth and improve tolerance to salinity, drought, and other abiotic stresses (Cho et al., 2008).

Recent studies of drought-stressed phytobiome revealed additional ways in which PGPR exert beneficial effects on their plant hosts. Drought is a major abiotic stress condition that adversely affects agriculture on a global scale. Over 30% of the earth is classified as arid and most general circulation models predict increases in the length and severity of droughts (IPCC, 2022). Plants respond and adapt to the deprivation of water by employing morpho-physiological adaptations that result in the stomatal closure, increased water uptake due to changes in the root (A. Gupta et al., 2020) architecture, and tissue-specific modulation of hormonal signaling to adjust osmotic processes. Soil moisture deficit also shapes the belowground plant microbiome, and the drought-adapted rhizosphere communities confer water stress tolerance (Jochum et al., 2019; Lau & Lennon, 2012; Naylor et al., 2017; Santos-Medellín et al., 2017). Treatment of drought-stressed plants with beneficial rhizobacteria coincides with the accumulation of compatible solutes such as choline and glycine betaine and a significant decrease in the

concentration of reactive oxygen species due to the activation of different antioxidant enzymes (Vurukonda et al., 2016; H. Zhang et al., 2010).

Microorganisms associated with plant roots form biofilms embedded in complex mixtures of polysaccharides, DNA, and proteins. This exopolysaccharide (EPS) matrix has high water content and protects microorganisms from desiccation in dry soils (Chang et al., 2007; Schmid et al., 2015). The presence of hydrating microbial EPS strongly influences soil moisture by promoting the formation of soil aggregates and inhibiting evaporation by changing soil water repellency (Guo et al., 2018). It is thought that these changes may benefit drought- and salt-stressed plants by supporting the flow of water, nutrients, and ions to the root system. For example, the inoculation of sunflower seedlings with the EPS-producing strain *Pseudomonas* sp. GAP-P45 significantly increased root-adhering soil, leading to better uptake of water and nutrients and survival under drought stress (Sandhya et al., 2009). The treatment of maize seeds with EPS-producing strains of *Proteus*, *Pseudomonas*, and *Alcaligenes* (along with their exopolysaccharides) promoted plant growth and improved the content of protein, sugar, proline, and antioxidant enzymes (Naseem & Bano, 2014b). Other examples include the inoculation of chickpea with salt-tolerant strains of *Halomonas variabilis* and *Planococcus rifietoensis,* which promoted plant growth and improved the aggregation of rhizosphere soil under high salinity (Qurashi & Sabri, 2012). Similarly, the inoculation of quinoa seeds with halotolerant strains of *Enterobacter* and *Bacillus* improved plant growth as well as osmotic and ionic stress under saline irrigation (Yang et al., 2016).

1.2 The control of soilborne pathogens by rhizosphere microbiome

Soilborne pathogens that cause root and crown rots, seed and seedling damping-off and wilts are major yield constraints in crop production. They can reduce attainable yields by 10-20% resulting in billions of dollars in monetary losses annually (Pascale et al., 2020). Most plants lack genetic resistance to soilborne diseases and have instead developed a defense strategy against soilborne pathogens that involves the stimulation and support of populations of antagonistic rhizosphere microorganisms (Cook et al., 1995). Disease-suppressive soils represent the best example of indigenous microorganisms protecting plants against pathogens (Weller et al., 2002). These are "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (Baker & Cook, 1974). In contrast, conducive (non-suppressive) soils are soils in which disease readily occurs. Suppressive soils are known for many pathogens, including *Gaeumannomyces graminis* var. *tritici* (take-all of wheat), *Fusarium oxysporum* (Fusarium wilt of melons and strawberries), *Rhizoctonia solani* (bare patch and root rot of wheat, damping-off of sugar beet), *Thielaviopsis basicola* (black root rot of tobacco), and *Streptomyces scabies* (potato scab) (Schlatter et al., 2017; Weller et al., 2002).

The disease decline in suppressive soils is often initiated and sustained by crop monoculture, which results in the proliferation of select groups of antagonistic microorganisms. For example, the suppressiveness to take-all disease is associated with the proliferation of *Pseudomonas* bacteria that protect wheat plants against *G. graminis* var. *tritici* by producing the antifungal compound 2,4-diacetyl phloroglucinol (DAPG)

(Berendsen et al., 2012). A different group of antagonistic pseudomonads was identified in the Dutch soil suppressive to damping-off of sugar beet (Mendes et al., 2011). These bacteria proliferated in the plant rhizosphere and secreted a complex of chlorinated lipopeptides that inhibited the causative agent of the disease, *R. solani* AG2-2. The microbiome profiling of Korean soil suppressive to the Fusarium wilt of strawberry identified a complex of beneficial root-colonizing *Streptomyces* (Cha et al., 2016). The follow-up analysis of one of these strains, *Streptomyces* sp. S4-7, attributed its ability to inhibit fungal pathogens to the production of a novel lantipeptide antibiotic (D.-R. Kim et al., 2019). Finally, a different mode of disease suppression was identified in the Fusarium wilt suppressive soil from the Châteaurenard region of France (Alabouvette, 1999). In this case, the disease decline was partially attributed to the presence of non-pathogenic strains of *F. oxysporum* that induced plant resistance and displaced pathogen from the rhizosphere through competition for nutrients and iron (Alabouvette et al., 2009). Similarly, the natural suppression of potato scab in Minnesota soils was associated with both the antibiosis and competition between the beneficial nonpathogenic *Streptomyces* and the causative agent, *S. scabies* (Neeno-Eckwall et al., 2001). It should be noted that the suppressive soil analysis often focused on antagonistic microorganisms that can be cultured and genetically manipulated *in vitro*. However, recent next-generation sequencing studies revealed the far more complex nature of suppressiveness, which is accompanied by shifts in the abundance of multiple and diverse groups of rhizosphere and endophytic bacteria and fungi (Schlatter et al., 2017).

1.3 Factors influencing the assembly and function of the plant microbiome

Rhizosphere microorganisms are derived mainly from the local soil, and geography is a critical factor that determines the structure of the phytobiome (Edwards et al., 2015). The seed bank of soil microbes is affected by the soil's physical and chemical properties and the legacy of plants previously grown on the same site (Hannula et al., 2021). It has been suggested that the plant microbiome is assembled in two steps: i) recruitment to the vicinity of the root and ii) the invasion of root tissues controlled by species-specific genetic factors (Bulgarelli et al., 2013). Plants recruit microorganisms from the bulk soil into rhizobacterial communities by releasing photosynthates as rhizodeposits or root exudates (Bais et al., 2006). The secretions released by plant roots contain complex mixtures of low and high-molecular-weight compounds that serve as nutrient sources for microbes (Barea et al., 2005; Toal et al., 2000). Root exudates contain diverse carbohydrates, amino acids, organic acids, proteins, and mucilages that attract soil microorganisms by providing abundant energy and carbon source (Kuzyakov & Xu, 2013). As a result, the rhizosphere microbiota is dominated by heterotrophic copiotrophs like Bacteroidetes, Proteobacteria, and Actinobacteria (Ling et al., 2022). In contrast, bulk soil communities contain mostly oligotrophs like Chloroflexi, Acidobacteria, Gemmatimonadetes, and Nitrospirae.

The patterns of root exudation vary depending on the plant's species, age, and physiological state. It is thought that the changes in the amount and composition of rhizodeposits exert selective pressure on microbes and determine which taxa are recruited into rhizobacterial assemblages (Fitzpatrick et al., 2018; Li et al., 2020). For example, the establishment of legume-rhizobia symbiosis is triggered by flavonoids exuded by the

roots of nitrogen-starved plants. These exometabolites attract nitrogen-fixing symbiotic rhizobia and induce the expression of nodulation or *nod* genes (Abdel-Lateif et al., 2012). The production of Nod factors (lipochitooligosaccharides) initiates the invasion of plant roots by bacteria and the formation of nitrogen-fixing nodules. The variable structure of the bacterial Nod factors determines the host specificity observed between rhizobia and legume plants (Santoyo, 2021). The elevated levels of osmoprotectants in exudates of water-stressed plants may contribute to the enrichment of beneficial rhizosphere *Pseudomonas* spp. in microbiomes of wheat grown in low precipitation parts of the Inland Pacific Northwest, USA (Bhattacharyya et al., 2021).

The process of plant domestication involves the adoption of wild species followed by artificial selection for desirable traits, such as altered morphology, seed size, seed dormancy, seed dispersal mechanisms, photoperiod sensitivity, and root architecture. Compared to wild ancestors, domesticated crops often exhibit a moderate reduction in genetic diversity across the genome and severely reduced diversity for genes targeted by artificial selection (Flint-Garcia, 2013). Several studies reported pronounced differences in the diversity and composition of rhizosphere microbiomes associated with the domestication of different plant species (Bulgarelli et al., 2015; Cardinale et al., 2015; Coleman-Derr et al., 2016; Li et al., 2020; Szoboszlay et al., 2015; Zachow et al., 2014) (Martínez-Romero et al., 2020). The meta-analysis of the effects of domestication on the composition of rhizosphere microbiomes of barley, lettuce, common bean, bittercress, and *Arabidopsis* revealed consistent shifts in the abundance of Bacteroidetes, Actinobacteria, and some Proteobacteria (Pérez-Jaramillo et al., 2018). Another microbiome study reported an abundance of Actinobacteria and Proteobacteria on roots

of modern bean accessions, while Bacteroidetes and Verrucomicrobia were enriched in the rhizosphere of wild relatives (Pérez-Jaramillo et al., 2017). Similar trends were discovered in the rhizobacterial communities of wild and domesticated accessions of rice (Kim et al., 2020). Finally, a moderate but significant effect of domestication on the structure of rhizosphere bacterial and fungal communities was identified in wild and domesticated wheat (Spor et al., 2020). The cultivated species had decreased relative abundance of some Sordariomycetes, including known plant pathogens *Ophiostoma, Raffaela,* and *Togniniella*. In contrast, the cultivated emmer had a higher abundance of Glomeromycetes, including arbuscular mycorrhizal fungi of the Glomerales group. The wild and cultivated emmer wheat also harbored more Gemmatimonadetes and members of the Flavobacteriaceae and Comamonadaceae, all of which exemplify groups of bacteria exhibiting a significant rhizosphere effect. Interestingly, a subset of wheat genotypes representing key steps in the domestication of tetraploid wheat also varied significantly in the composition of their root exudates (Iannucci et al., 2017). It is plausible that such changes in the rhizodeposition select for bacteria and fungi with varying abilities to utilize metabolites secreted by wild and domesticated plants.

1.4 Blueberry rhizosphere microbiome

Blueberries belong to the *Ericaceae* family of flowering plants, which are prominent in forests, alpine and arctic tundras, and bogs of the northern hemisphere (Schwery et al., 2015). Many ericaceous plants, including wild blueberries, grow in acidic soils with low NO_3, low Ca, high organic matter, and elevated levels of toxic metals and aromatic compounds. To cope with these stressful habitats, ericaceous plants form symbiotic associations with ericoid mycorrhizae (EM) (Meharg & Cairney, 1999;

Smith & Read, 2008). The dominant EM fungal species are ascomycetes of the class Leotiomycetes, especially members of *Hyaloscypha, Pezoloma, Oidiodendron,* and *Cairneyella* (Perotto et al., 2018). Other fungi known to form ericoid mycorrhizae are certain basidiomycetes, such as *Sebacina* and *Kurtia*. Typical EM fungi penetrate hair roots and form dense hyphal coils that facilitate the exchange of material between the host and fungal symbiont (Cairney & Ashford, 2002). In addition to typical ericoid mycorrhizae, ericaceous plants also form associations with dark septate endophytic fungi, which colonize roots with melanized hyphae arranged in loops instead of EM coils (Daghino et al., 2022).

Recent studies suggest that interactions between blueberries and mycorrhizae may have some level of host-fungus specificity (Li et al., 2020). Ericoid mycorrhizae supply their hosts with phosphorus, nitrogen (in the form of NH^{4+}, NO^{3-} or amino acids and peptides) and iron (Retamales & Hancock, 2018). The mycorrhizal plants also benefit from the ability of EM fungi to detoxify phenolic compounds and modulate resistance to toxic metals (Caspersen et al., 2016). In blueberries, ericoid mycorrhizae increase root and shoot dry weight, nutrient uptake, the efficiency of fertilizer and water use, and tolerance to aluminum toxicity (Yang et al., 2002). Although EM are most prevalent in natural habitats, they also are important in commercial blueberry production (Scagel & Yang, 2005).

Apart from the ericoid mycorrhiza, the roots of ericaceous plants harbor diverse and dynamic bacterial and protozoan communities. The core rhizosphere bacteriome of blueberries is dominated by Proteobacteria, Actinobacteria, and Acidobacteria, with members of *Bradyrhizobium* and Pedosphaerales acting as hub taxa (Li et al., 2020;

Yurgel et al., 2018; Zhang et al., 2021). The rhizosphere communities also harbored diazotrophs like *Bradyrhizobiaceae, Methylocystaceae, Burkholderiaceae* and *Beijerinckiaceae*. Blueberry species also seem to differ in the richness and evenness of their rhizosphere communities and abundance of certain Alpha- and Betaprotebacteria, Actinobacteria, and Firmicutes, many of which have been implicated in plant growth promotion and/or suppression of soilborne pathogens (Li et al., 2020; Yurgel et al., 2018; Zhang et al., 2021).

1.5 Wild and cultivated species of blueberry

Blueberries are perennial flowering plants native to Northern America. They include several wild and cultivated species of the genus *Vaccinium* that are grown worldwide for their fruits rich in minerals and antioxidants (Massarotto et al., 2016; Retamales & Hancock, 2018). The commercial production includes the lowbush blueberry, *V. angustifolium* harvested from managed wild patches across Canada and the northeastern United States. The Northern highbush blueberry, *V. corymbosum*, is the most widely cultivated type of blueberry in the U.S. and grown throughout the Northeast, the Atlantic Coastal Plain, and the Pacific Northwest. In contrast, species that grow well throughout the southeastern United States include the rabbiteye blueberry, *V. virgatum*, and Southern highbush blueberry (SHB), an interspecific hybrid of *V. corymbosum* and *V. darrowii* (Retamales & Hancock, 2018) (Figure 1.5).

Blueberries have excellent nutritional value, flavor, and health properties, and their production is expanding worldwide. The industry is also rapidly expanding into new parts of the globe, such as the Asia-Pacific region and Africa, and new environments and global climate change present an obstacle for blueberry growers. Breeding programs are

responding to these challenges by focusing on cultivars with better cold hardiness, reduced chilling hours, and tolerance to higher pH, radiative and temperature stress (Lobos & Hancock, 2015). Recent studies suggest that stressed plants recruit specific microbes that alleviate the suffering from biotic and abiotic stresses. Hence, there has been a surge of interest in the incorporation of microbiome into breeding programs as a source for genes that may help to improve plant growth and survival in response to pathogens and climate change (Ke et al., 2021). However, the success of these strategies depends on the understanding of microbial traits involved in the colonization of specific plant species and the plant genes that shape the associated microbiome.

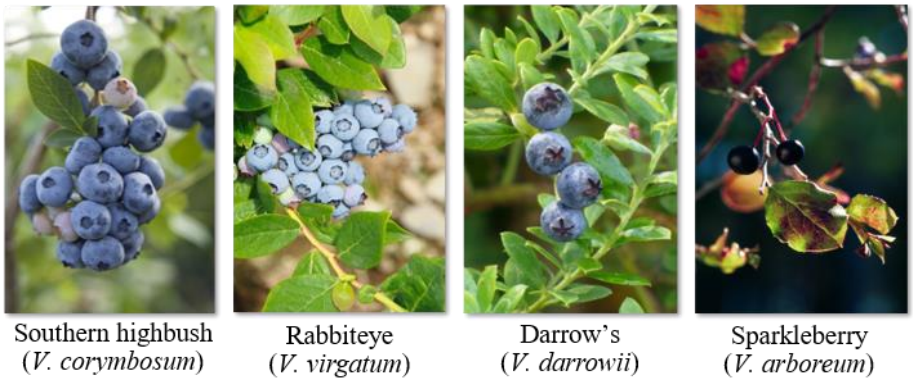

Figure 1.3 *Blueberry species studied in this project*

1.6 Aims of this study

Blueberries are an important agriculture commodity in the southern states. Active breeding programs resulted in the development of numerous accessions and varieties that differ in the yield and fruit quality and their tolerance to diseases and abiotic stress. The effects of these extensive manipulations on the blueberry microbiome are poorly understood. My research project aimed to understand how decades of interspecific crossing and artificial selection have significantly changed the blueberry microbiome and

affected both pathogens and microorganisms known to control plant diseases and alleviate abiotic stress. The project seeks to compare rhizosphere microbiomes and metagenomes of five different species of blueberries, including *V. virgatum*, *V. darrowii*, *V. corymbosum*, and *V. arboreum* (Fig. 3). Two of these, *V. arboreum* and *V. darrowii*, are wild species whereas *V. virgatum* and *V. corymbosum* are cultivated species. The research will also include the Robeson variety, a pentaploid interspecific hybrid of *V. virgatum* and *V. corymbosum*. The specific aims of my project are:

1) Comparative profiling of bacterial and fungal communities associated with wild and domesticated blueberries.
2) Metagenomic and physiological profiling of rhizosphere microbiomes of different blueberry species.

CHAPTER II - MATERIALS AND METHODS

2.1 Plant material used in the study

This study involved comparative microbiome and metagenome analyses of rabbiteye blueberry (*Vaccinium virgatum* Aiton, 2n = 6*x* = 72, 'Premier' and MS 1389), highbush blueberry (*V. corymbosum* Linnaeus, 2n = 4*x* = 48, 'Pearl' and 'Star'), Darrow's blueberry (*V. darrowii* Camp, 2n = 2*x* = 24, clone 2163 and 'Native Blue'), *V. arboreum* Marsh, 2n = 2*x* = 24, clones 59 and 63, and 'Robeson', a pentaploid hybrid of *V. corymbosum* and *V. virgatum* (2n = 5*x* = 60). Plant material was sampled from a field plot located at the USDA-ARS Thad Cochran Southern Horticultural Laboratory in Poplarville, Mississippi (N 30° 50' 18.406", W 89° 32'41.625"). Samples of roots and bulk soil (*n* = 12 per genotype) were collected in the fall of 2020, placed into coolers, and moved to USM for processing and analysis.

2.2 Rhizosphere and root endosphere DNA extraction

To extract rhizosphere DNA, plant roots were cut, shaken to remove excess soil, and transferred into 50-ml centrifuge tubes containing 40 mL of 10% glycerol. The tubes were vortexed for 1 min followed by treatment for another minute in an ultrasonic water bath. The resultant root wash was then centrifuged at 3,000 × g for 15 min, the supernatant was removed, and 0.25-g aliquots of soil pellet were transferred into PowerBead tubes (part of the DNeasy PowerSoil Pro Kit manufactured by - Qiagen, Germantown, MD). For bulk soil DNA, 0.25 g of soil was weighed directly into PowerBead tubes. The rhizosphere and bulk soil DNA were extracted following the DNeasy PowerSoil Pro kit protocol, quantified with NanoDrop OneC (ThermoFisher Scientific, Waltham, MA), and stored at -20°C until analysis.

For endophyte analysis, roots processed by sonication were rinsed 3-5 times with distilled water and surface sterilized by sequential treatment for 5 min each in 70% ethanol, 3% H_2O_2 and 5% bleach. The sterilized roots were then rinsed with sterile distilled water, dried in a biosafety cabinet, cut aseptically, lyophilized and stored at -80°C until needed. To extract DNA, protocol for purification of total DNA from plant tissue (mini protocol) was used. The lyophilized root material was homogenized by bead beating in TissueLyser LT (Qiagen, Germany) for around 15 mins at 50 Hz. After extraction DNA was quantified with NanoDrop, and stored at -20°C. All DNA samples were shipped for high throughput amplicon and metagenome sequencing to the Integrated Microbiome Resource (IMR) laboratory at Dalhousie University (Nova Scotia, Canada).

2.3 Microbiome profiling

The rhizosphere communities of different blueberry species were analyzed by high-throughput sequencing of 16S rDNA and ITS2 amplicons, processing reads, and classifying them into operational taxonomic units (OTUs) that serve as "species tags." Barcoded amplicons were generated from the purified microbial DNA by PCR with primers B969F and BA1406R targeting the V6-V8 region of the 16S rRNA gene. The ITS86F and ITS4R primer set was used to amplify the ITS2 sequences of the fungal community. Twelve biological replicates of 16S and ITS2 amplicons were generated per blueberry cultivar/species. The amplicons were purified, quantified, and used to prepare libraries that were next sequenced on a MiSeq instrument (Illumina, San Diego, CA) using 300-bp paired-end V3 chemistry. MiSeq forward and reverse reads were processed with the Microbiome Helper amplicon analysis pipeline (Comeau et al., 2017). Briefly, the reads were trimmed, merged with VSEARCH (Rognes et al., 2016), and denoised

with Deblur in QIIME2 (Caporaso et al., 2010) to generate a table of amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using a Naive-Bayes approach implemented in the scikit learn Python library and the SILVA or UNITE databases (Pruesse et al., 2007). Any sequences that accounted for less than 1% of the total OTUs and samples with low read counts were removed. Estimators of alpha diversity, including richness and evenness, and beta diversity analyses were performed with the R packages Phyloseq (McMurdie & Holmes, 2013) and by exporting ASV tables, taxonomy, and metadata into MicrobiomeAnalyst (Chong et al., 2020). Differential abundance analysis of bacterial and fungal communities of different *Vaccinium* species was also performed at the family and genus level by calculating LEfSe (Linear discriminant analysis Effect Size) scores that indicate the degree of consistent difference in relative abundance between treatments (Segata et al., 2011).

2.4 Metagenome profiling

For metagenome profiling, samples of DNA extracted for the rhizosphere of *V. arboreum*, *V. darrowii*, *V. corymbosum*, and *V. virgatum* were shotgun sequenced on Illumina NextSeq platform (2 ×150-bp paired-end mode, $n = 6$ per plant species). The raw sequence data were quality filtered with Trimmomatic (Bolger et al., 2014) and FastQC followed by analysis with the KBase metagenomic pipeline (Henry et al., 2016). The taxonomic assignment of reads was achieved by comparing them to a reference database using Kaiju (Menzel et al., 2016) and GOTTCHA2 (Freitas et al., 2015). The metagenome assembly was performed with metaSPAdes (Nurk et al., 2017), MEGAHIT (D. Li et al., 2015), IDBA-UD (Peng et al., 2012) and the results were compared for length and size distribution because longer contigs are more desirable. The number of

contigs, N50/75, L50/75, GC content, number of uncalled bases (Ns), and predicted genes were evaluated with QUAST (Gurevich et al., 2013). Metagenomic contigs from the best assemblies were clustered with CONCOCT (Alneberg et al., 2014) into "bins" corresponding to putative genomes, annotated with Prokka (Seemann, 2014) and processed with GTDB-Tk, a software toolkit for assigning taxonomic classifications to bacterial and archaeal genomes (Chaumeil et al., 2020). The phylogenetic relationship of the source genomes was visualized using the KBase Build Species Tree app and metabolic annotations of assemblies was performed with DRAM (Shaffer et al., 2020). Draft metabolic models representing the overall physiology of the assembled metagenomes (i.e., a matrix of metabolic reactions and their associated biochemical compounds) were constructed with the KBase Build Metagenome Metabolic Model app (Figure 2.4).

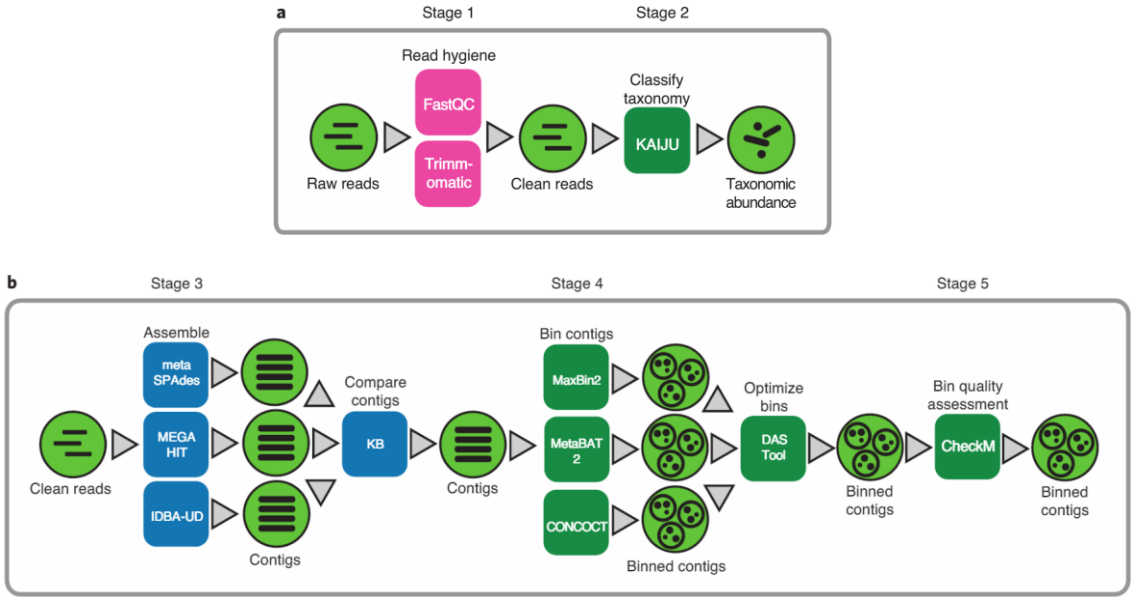

Figure 2.1 *Overview of MAG extraction data and analysis workflow using KBase apps (Stages 1-5.)*

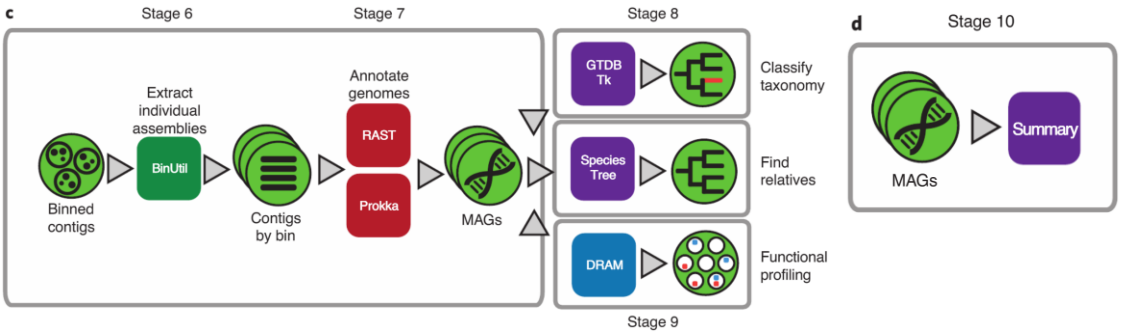

Figure 2.2 *Overview of MAG extraction data and analysis workflow using KBase apps (Stages 6-10).*

Circles represent data objects in KBase, and square icons identify analysis steps. (a) Stages 1 and 2: Quality control of read libraries and taxonomic profiling of shotgun reads. (b) Stages 3–5: metagenome assembly, genome binning, bin refinement, and bin quality filtering. (c) Stages 6–9: contig extraction for each bin, genome annotation into MAGs, taxonomic classification, phylogenetic placement, and functional profiling of MAGs. (d) Stage 10: Summary of MAGs, including bioelement active genes, taxonomy, MAG quality scores, genome statistics, and ribosomal RNA genes.

The figure is taken from Arkin et al., 2018. Nat. Protoc. 36: 566–569.

We also used the OmicsBox metagenomics pipeline (BioBam Bioinformatics, Cambridge, MA) to generate functional annotations and explore ortholog relationships and gene evolutionary histories. Briefly, sequence data were processed with Trimmomatic and assembled with MEGAHIT followed by the analysis with FragGeneScan (Rho et al., 2010), a tool used for the prediction of prokaryotic genes in incomplete assemblies or complete genomes. Functional annotation was performed using PfamScan (Finn et al., 2014) and EggNOG Mapper (Cantalapiedra et al., 2021) and the resultant data were subjected to differential abundance testing with EdgeR (Robinson et al., 2009).

2.5 Physiological profiling

For the physiological profiling of culturable rhizobacteria, six plants per genotype were randomly selected and their roots were excised, shaken to remove loose soil, weighed, and placed in 50 ml conical centrifuge tubes. The tubes were filled with nine

parts (w/w) of phosphate-buffered saline (pH 7.0), vortexed for 5-10 sec, and then sonicated for 1 min. The root wash then was serially diluted ten-fold (3×) and 100-µl aliquots of the last dilution was dispensed into EcoPlates (Biolog, Hayward, CA). The inoculated plates were covered with foil and incubated statically at room temperature. The bacterial growth and color development was monitored by measuring absorbance at two different wavelengths (590 nm and 750 nm) with a BioTek Synergy HTX multi-mode reader (Agilent, Santa Clara, CA). The first absorbance reading was recorded at 15 h, followed by readings taken every 24 h for a total of 10 days. The community-level physiological profiles were assessed for key characteristics such as pattern stability (similarity), rate of color change in each well (activity), and richness of well response (diversity). Data were analyzed using R package rstatix (Kassambara, 2022). Differences among treatments were tested by One-Way Analysis of Variance (ANOVA) followed by mean comparisons by Fisher's protected least significant difference (LSD) test (P=0.05) or by Kruskal Wallis test (P=0.05).

CHAPTER III - RESULTS

3.1 Diversity of microbial communities from bulk soil, rhizosphere and endosphere

The blueberry phytobiome was characterized via high-throughput sequencing of 16S and ITS amplicons generated from samples of rhizosphere, endosphere, and bulk soil DNA. To profile the bacterial communities, the 2,612,371 high-quality 16S reads were processed with the Microbiome Helper amplicon analysis pipeline (Comeau et al., 2017) and binned by assigning taxonomy to the resultant amplified sequence variants (ASVs) using the SILVA database (Glöckner et al., 2017). The ASVs were rarefied to a uniform depth per sample and analyzed with MicrobiomeAnalyst (Chong et al., 2020). Results revealed the presence of species representing 14 different bacterial phyla, of which Proteobacteria, Actinobacteriota, Acidobacteriota and Chloroflexi were most abundant in the bulk soil and rhizosphere communities (Figure 3.1A). In contrast, Actinobacteriota, Proteobacteria and Bacteroidota dominated the endosphere microbiome.

There were differences in the alpha diversity, with significantly lower (Kruskal-Wallis, $p < 0.0001$) bacterial richness and diversity in the endosphere compared to the rhizosphere and bulk soil (Figure 3.1B). The bacterial communities also clustered distinctively in the ordination space (NMDS stress 0.09; ANOSIM (Analysis Of Similarities) $R=0.78$, $p < 0.001$) indicating differences in the microbiome composition between the three sources (Figure 3.1C).

The analysis of fungal community was performed based on a dataset of 2,170,768 high quality reads that were processed as described above and rarefied to an even count depth of 1,026 per sample. The analysis revealed members of six different phyla, including Ascomycota, Basidiomycota, Chytridiomycota, Mortierellomycota,

Kickxellomycota and Mucoromycota. At the order level, Mortierellales, Auriculariales, and Cantharellales were most abundant in bulk soil, while different Agaricales, Mortierellales, and Mucorales dominated the blueberry rhizosphere (Figure 3.1D). The endosphere microbiome was largely composed of Agaricales and had significantly lower alpha diversity than the rhizosphere and bulk soil (Figure 3.1E). Fungal communities inhabiting the three niches also differed significantly in their overall structure (NMDS stress 0.16; ANOSIM $R=0.26$, $p < 0.001$) (Figure 3.1F).

3.2 Differences between bacterial communities of wild and cultivated blueberries

The central hypothesis was tested by contrasting bacterial communities of wild and cultivated blueberries. The alpha diversity estimates revealed significantly higher (Kruskal-Wallis, $p < 0.001$) richness (Chao1) and evenness (Shannon) of bacteria in the rhizosphere and endosphere of cultivated species (Figure 3.2A).

The differences in the composition were further supported by the ordination analysis, which showed pronounced differences in the structure of rhizobacterial communities (NMDS stress 0.15; ANOSIM $R=0.29$, $p < 0.001$) (Figure 3.2B). In contrast, the endosphere microbiome of wild and domesticated plants had similar overall structures (Figure 3.2E). The bacterial communities were also subjected to biomarker analysis by calculating LEfSe scores, which indicate the degree of consistent differences based on relative abundances of predominant phyla.

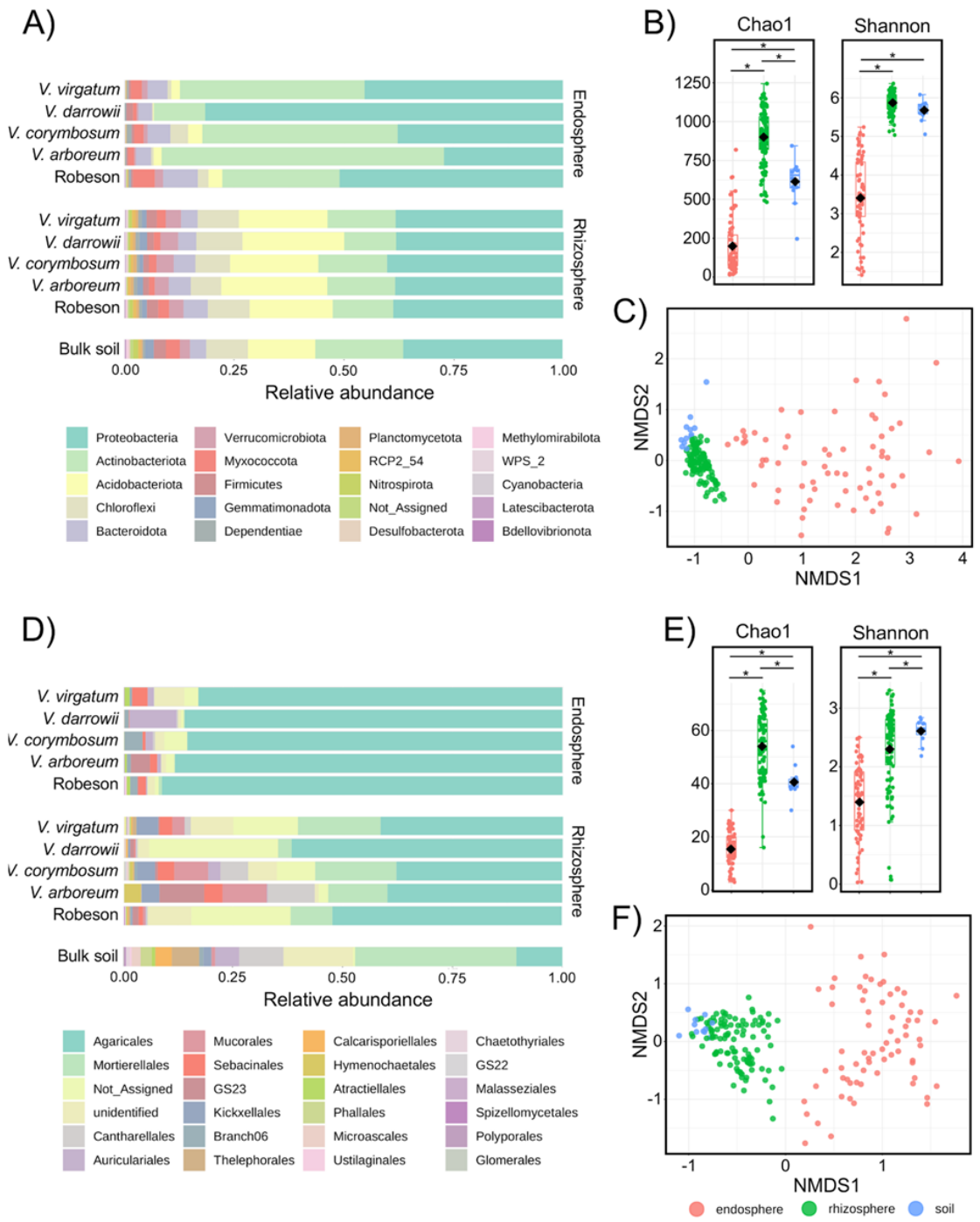

Figure 3.1 *The diversity and structure of microbial communities residing in the blueberry endosphere, rhizosphere and bulk soil.*

Panels A and D show relative abundance of bacterial (phylum level) and fungal (order level) lineages. Panels B and E show alpha diversity (Chao1 and Shannon indices), while C and F represent the results of NMDS ordination analysis (Bray-Curtis distances) of the bacterial and fungal communities.

The LEfSe analysis of the rhizosphere revealed an abundance of Acidobacteriota and Gemmatimonadota in wild blueberries whereas Actinobacteriota, Firmicutes, Myxococcota, Nitrospirota, and Latescibacterota were associated with domesticated species. Genus level differences showed enrichment of *Pseudolabrys*, *Gaiella*, *Bacillus*, and *Streptomyces* in domesticated species, while *Actinospica*, *Dinghuibacter*, *Dyella*, *Gemmatimonas*, and *Chitinophaga* were among biomarkers specifically associated with the wild species (Figure 3.2C). Similar alpha and beta diversity trends were observed in the blueberry endosphere (Figures 3.2D and 3.2E). The LEfSe analysis revealed an abundance of *Mycobacterium* and *Methylobacterium* in the endosphere of wild species, while the roots of cultivated species were enriched in *Streptomyces*, *Actinoplanes*, *Phenylobacterium*, *Plantactinospora*, and a few other taxa (Figure 3.2F).

3.3 Differences between fungal communities of wild and cultivated blueberries

The comparison of rhizosphere fungal communities revealed no differences in the richness and evenness between the wild and domesticated blueberries (data not shown). The community structure was similar with some differences (NMDS stress 0.2; ANOSIM $R=0.23$, $p < 0.001$) (Figure 3.3A). However, the LEfSe analysis revealed multiple biomarkers that were differentially distributed between the wild and cultivated plant hosts (Figure 3.3B).

Among notable taxa associated with wild blueberries were the ectomycorrhizal *Rhizopogon* and *Hyphodontia* (syn. *Kurtia*), which forms sheathed ericoid mycorrhizae. Two other mycorrhizal fungi, *Mycena* and *Calvatia*, were more abundant in the rhizosphere of cultivated blueberries. Similar trends were observed with endosphere fungi, which had similar alpha diversity (data not shown) and significant overlap in the

community composition (NMDS stress 0.09; ANOSIM R=0.14, $p < 0.001$) (Figure 3.3C). The differential abundance analysis revealed the presence of *Gymnopus* and *Auricularia* in wild species of *Vaccinium*, while ericoid mycorrhizal symbionts *Clavaria* and Serendipitaceae were more abundant in domesticated species (Figure 3.3D).

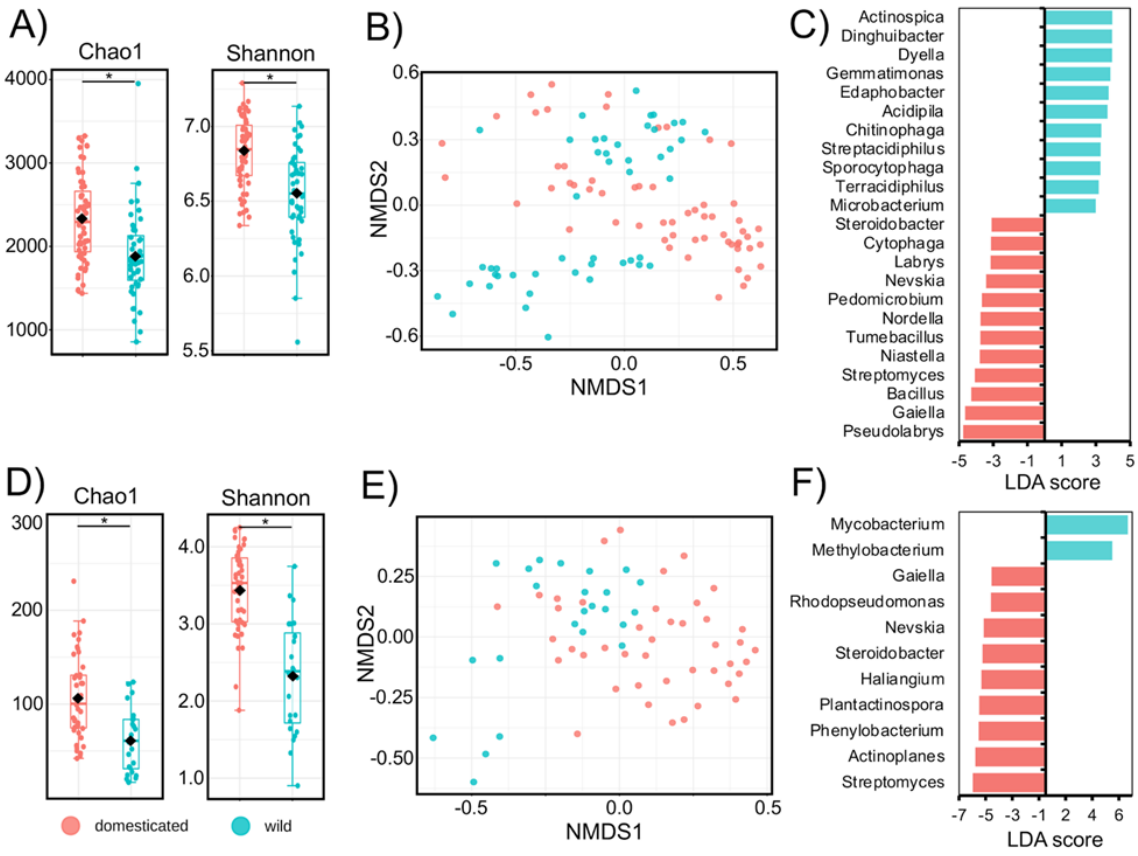

Figure 3.2 *Structure of bacterial communities associated with domesticated and wild species of blueberries*

Alpha diversity estimates and NMDS ordination analysis (Bray-Curtis distances) for the rhizosphere (A, B) and endosphere (D, E) microbiomes are depicted. Also shown are bacterial taxa that were differentially distributed between the rhizosphere (C) and endosphere (F) of domesticated and wild blueberries.

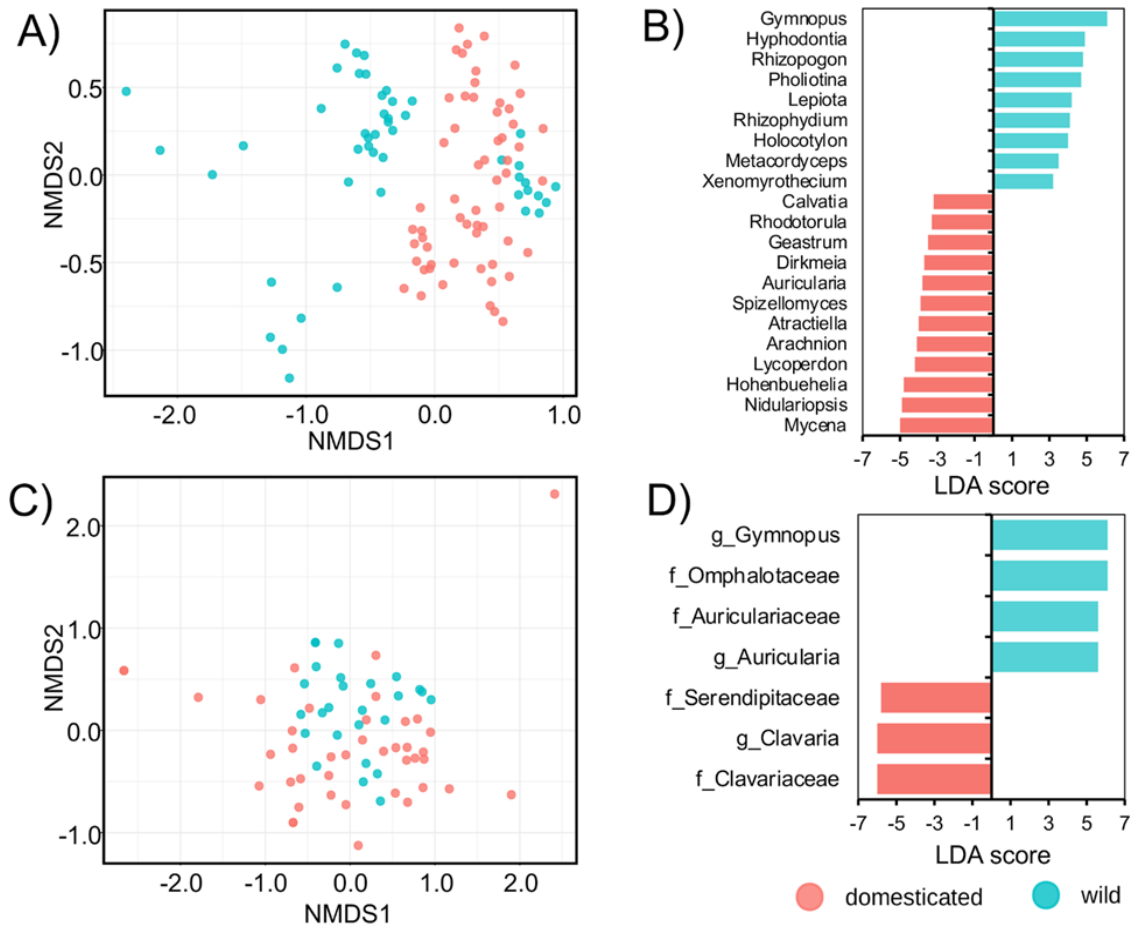

Figure 3.3 *Structure of fungal communities associated with domesticated and wild blueberries.*

The left panels depict the NMDS ordination analysis (Bray-Curtis distances) of the rhizosphere (A) and endosphere (C) microbiomes. The right panels show the LEfSe analysis of taxa differentially distributed between the rhizosphere (B) and endosphere (D) of domesticated and wild blueberries.

3.4 Plant species-level differences in the bacterial community composition of blueberry microbiome

The comparison of bacterial rhizosphere communities revealed significant differences in the richness and evenness indices (Kruskal-Wallis, $p < 0.001$) and overall community structure (NMDS stress 0.15; ANOSIM $R=0.5$, $p < 0.001$) (Figure 3.4A, B). These differences extended into plant endosphere, which harbored bacterial communities

that also varied in their diversity (Kruskal-Wallis, $p < 0.001$) and had close but distinct composition (NMDS stress 0.2; ANOSIM $R=0.45$, $p < 0.001$) (Figure 3.4D, E).

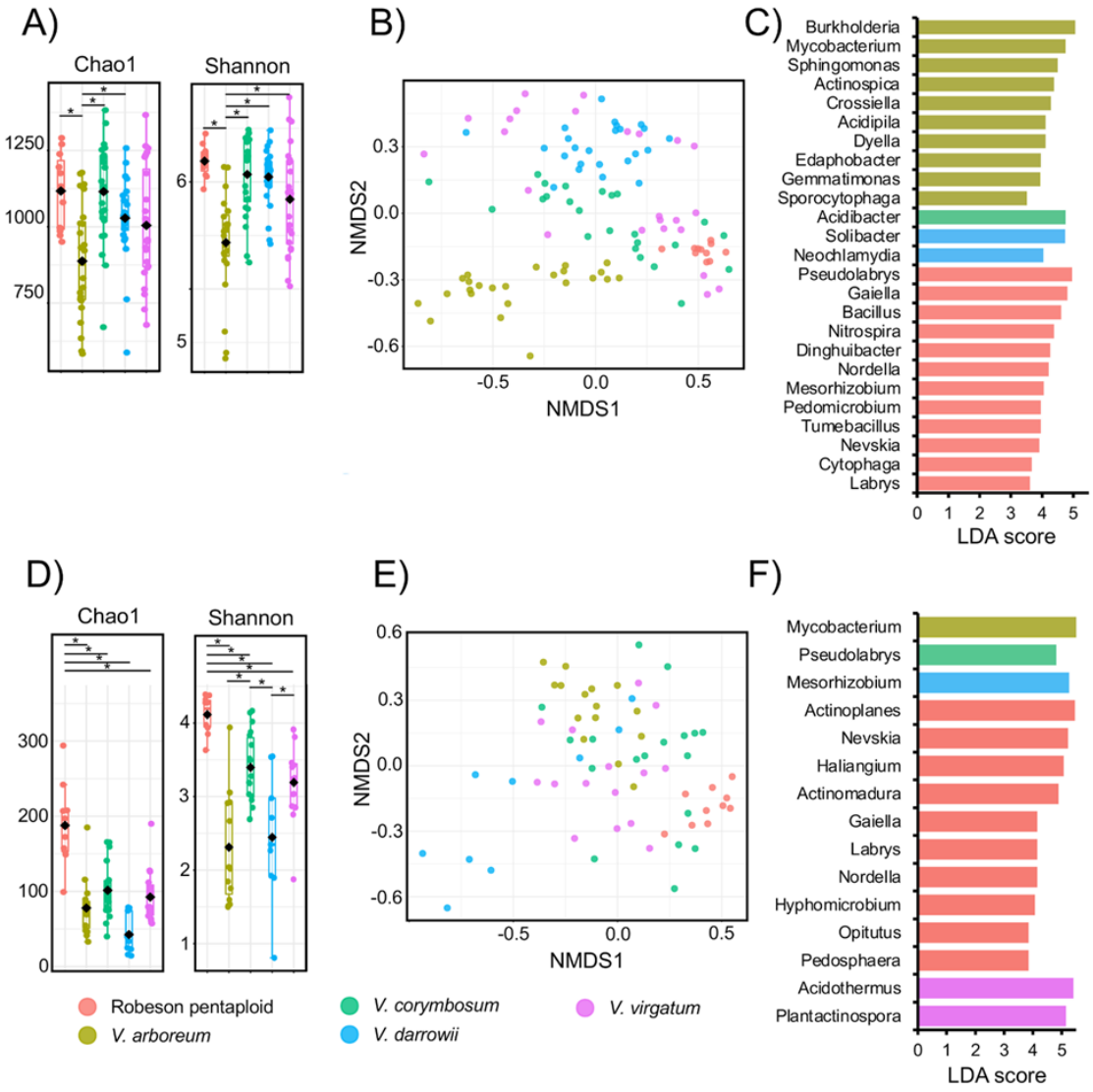

Figure 3.4 *Bacterial communities from the rhizosphere (A, B, C) and endosphere (D, E, F) of different blueberry species.*

Shown are the results of alpha diversity analysis (Chao1 and Shannon indices) (panels A and D), ordination by NMDS scaling based on Bray-Curtis dissimilarity matrices (B and E), and LEfSe analysis of differentially abundant taxa (LDA threshold > 3) (C and F).

The differential abundance analysis revealed multiple genera that were specifically associated with the rhizosphere and endosphere of the studied blueberry species, especially *V. arboretum* and the Robeson pentaploid variety (Figure 3.4C, F).

These differences in the microbiome composition correlated with changes in the relative abundance of 28 fungal biomarkers identified using an LDA threshold score of >2.0 (Figure 3.5C).

3.5 Plant species-level differences in the fungal community composition of blueberry microbiome

There were also significant differences (Kruskal-Wallis, $p < 0.003$) in the richness and diversity of rhizosphere fungi, with higher Chao1 and Shannon indices in Robeson pentaploid, *V. arboreum* and *V. virgatum* compared to *V. darrowii* (Figure 3.5A). The beta diversity analysis revealed significant differentiation (NMDS stress 0.2; ANOSIM $R=0.45$, $p < 0.001$) of the fungal community by *Vaccinium* species (Figure 3.5B).

Some of these phylotypes matched beneficial mycorrhizal and endophytic taxa including at least four different ericoid mycorrhizal fungi (*Hyphodontia*, *Serendipita*, *Clavaria*, *Spizellomyces*). The diversity of endosphere fungi was similar with some minor differences in the community structure (NMDS stress 0.09; ANOSIM $R=0.2$, $p < 0.01$) (Figure 3.5E). A handful of differentially distributed taxa included *Gymnopus* from *V. arboreum*, members of the Serendipitaceae from *V. corymbosum*, and *Clavaria*, which was enriched in the roots of Robeson pentaploid (Figure 3.5F).

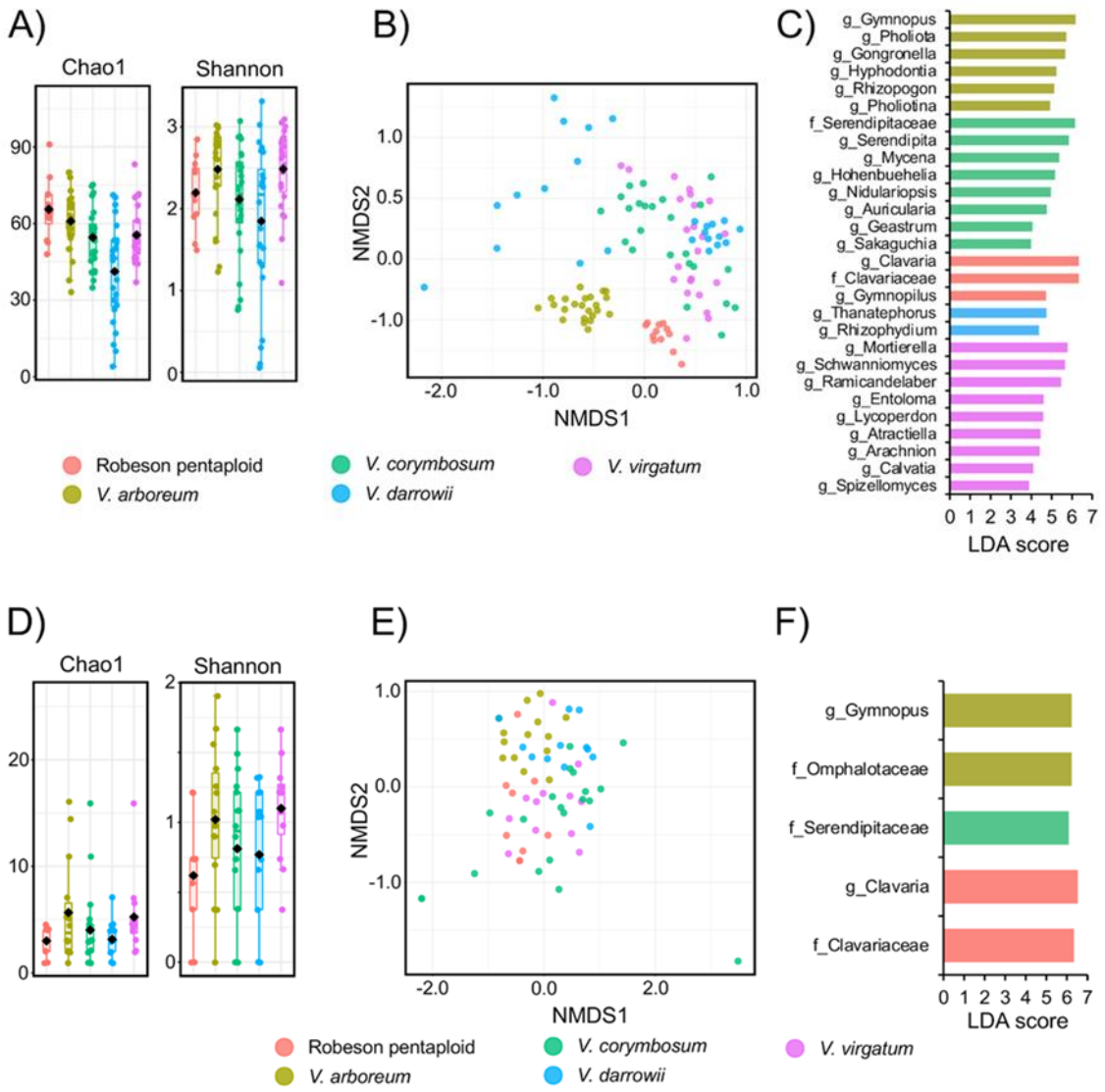

Figure 3.5 *Fungal communities from the rhizosphere and endosphere of different blueberry species.*

Shown are the results of alpha diversity analysis (A, D), ordination by NMDS scaling based on Bray-Curtis dissimilarity matrices (B,E), and LEfSe analysis of differentially abundant taxa (LDA threshold > 2) (C, F).

3.6 Physiological profiling of culturable rhizobacteria

The capacity of culturable rhizobacteria to utilize C substrates from Biolog EcoPlates was analyzed by comparing the OD590 absorbance values after 10 days of incubation at room temperature. The analysis revealed that microbes catabolized carbohydrates, amino acids, carboxylic acids, polymers, amines and aromatics, with the

most active growth observed in the presence of several carbohydrates (D-mannitol, N-acetyl-D-glucosamine, D-galactonic acid gamma-lactone), some amino acids (L-arginine, L-asparagine), carboxylic compounds (pyruvic acid methyl ester, D-galacturonic acid, γ-amino butyric acid), Tween 80 and Tween 40. In contrast, almost no growth was observed in the microplate wells containing 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, L-threonine and glycyl-L-glutamic acid (Figure 3.6.1). For each sample, the OD_{590} absorbance values recorded after 120 h of incubation were used to calculate Richness (R), Shannon-Wiener diversity (H'), Pielou evenness (J') and Simpson diversity (D) indices using the method of Ge et al. (2018) (Table 3.6). The capacity of culturable microorganisms to catabolize different carbon sources was expressed by calculating and plotting average well-color development (AWCD) values and then converting them by trapezoidal method into Area Under AWCD curve (AUC) values to capture the dynamics of substrate utilization by the compared rhizosphere communities. The analysis revealed that EcoPlates inoculated with the rhizosphere soil suspensions of *V.darrowii* clone 59 had overall lower AWCD values compared to those inoculated by samples from other blueberry species, whereas the highest readings were observed in *V.corymbosum* Pearl, *V. arboreum* clone 59 and *V. virgatum* clone MS 1389 (Figure 3.6.2). However, the observed differences in the microbial diversity estimated and AWCD area under curve values were not statistically significant (data not shown).

Chemicals	Compound	Cor 1	Cor 2	Vir 1	Vir 2	Pent	Arb 1	Arb 2	Dar 1	Dar 2	Soil
D-Cellobiose	Carbohydrate	0.74	0.78	0.25	1.06	0.62	0.59	0.75	0.07	0.10	0.89
Alpha-D-Lactose	Carbohydrate	0.22	0.23	0.01	0.07	0.14	0.01	0.29	0.01	0.00	0.11
Beta-Methyl-D-Glucoside	Carbohydrate	0.8	0.23	0.69	0.89	0.79	0.78	1.11	0.32	0.34	0.30
D-Xylose	Carbohydrate	0.39	0.61	0.34	0.72	0.54	0.35	0.55	0.04	0.21	0.02
i-Erythritol	Carbohydrate	0.4	0.21	0.04	0.76	0.65	0.12	0.36	0.28	0.04	0.08
D-Mannitol	Carbohydrate	1.18	1.37	0.94	1.14	0.51	1.79	1.32	0.51	1.11	1.09
N-Acetyl-D-Glucosamine	Carbohydrate	0.95	1.06	1.14	1.21	0.99	1.65	1.23	1.34	0.68	1.01
Glucose-1-Phosphate	Carbohydrate	0.49	0.36	0.14	0.45	0.45	0.40	0.63	0.22	0.33	0.15
D,L-Alpha-Glycerol Phosphate	Carbohydrate	0.26	0.21	0.14	0.23	0.44	0.27	0.18	0.17	0.20	0.16
D-Galactonic Acid Gamma-Lactone	Carbohydrate	0.9	0.64	0.71	0.37	0.60	0.94	0.96	0.66	0.49	0.57
L-Arginine	Amino Acid	0.23	0.65	0.31	0.24	0.65	0.55	0.61	0.22	0.30	0.69
L-Asparagine	Amino Acid	0.86	1.07	0.70	1.23	1.19	1.55	1.12	0.49	0.77	1.28
L-Phenylalanine	Amino Acid	0.42	0.40	0.37	0.42	0.25	0.35	0.39	0.19	0.22	0.36
L-Serine	Amino Acid	0.66	0.25	0.08	0.53	0.33	1.16	0.29	0.34	0.21	0.74
L-Threonine	Amino Acid	0.11	0.04	0.11	0.11	0.03	0.14	0.09	0.03	0.03	0.03
Glycyl-L-Glutamic Acid	Amino Acid	0.25	0.05	0.05	0.31	0.06	0.41	0.03	0.10	0.01	0.08
Pyruvic Acid Methyl Ester	Carboxylic Acid	0.57	0.67	0.65	0.65	0.62	0.73	0.64	0.51	0.44	0.65
D-Glucosaminic Acid	Carboxylic Acid	0.34	0.33	0.38	0.45	0.19	0.50	0.34	0.46	0.29	0.42
D-Galacturonic Acid	Carboxylic Acid	1.1	1.13	0.89	1.49	1.08	1.08	1.17	0.96	1.06	1.04
Gamma-Amino Butyric Acid	Carboxylic Acid	0.77	0.70	0.75	0.70	0.75	1.42	0.86	0.56	0.79	1.00
Itaconic Acid	Carboxylic Acid	0.13	0.01	0.01	0.24	0.01	0.24	0.02	0.08	0.03	0.28
Alpha-Keto Butyric Acid	Carboxylic Acid	0.04	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.00
D-Malic Acid	Carboxylic Acid	0.13	0.02	0.52	0.10	0.09	0.30	0.40	0.06	0.22	0.35
Tween 40	Polymer	0.9	0.83	0.73	0.74	0.76	0.79	0.78	0.65	0.82	0.65
Tween 80	Polymer	0.55	1.08	0.58	0.75	0.76	1.04	0.58	0.42	0.56	0.80
Alpha-Cyclodextrin	Polymer	0.17	0.04	0.01	0.32	0.16	0.26	0.13	0.01	0.04	0.05
Glycogen	Polymer	0.14	0.03	0.22	0.70	0.17	0.56	0.33	0.18	0.06	0.31
Phenylethyl-amine	Amines	0.22	0.13	0.22	0.42	0.18	0.12	0.12	0.16	0.05	0.52
Putrescine	Amines	0.75	0.47	0.25	0.70	0.73	0.31	0.69	0.20	0.25	0.32
2-Hydroxy Benzoic Acid	Aromatics	0	0.01	0.00	0.01	0.00	0.01	0.04	0.00	0.01	0.00
4-Hydroxy Benzoic Acid	Aromatics	0.15	0.22	0.37	0.08	0.18	0.25	0.22	0.26	0.26	0.30

Figure 3.6 *Heatmap summarizing efficacy in the oxidation of 31 carbon sources by rhizosphere microbial communities of different blueberry species after 10 days of incubation at room temperature*

Table 3.1 *Estimates (means ± standard deviation) of microbial diversity in the blueberry rhizosphere by the community-level physiological profiling with Biolog EcoPlates.*

Plant species	Accession	Richness index	Shannon diversity	Pielou evenness	Simpson diversity	McIntosh evenness
V. corymbosum	Pearl	15.7 ± 6.9	2.8 ± 0.3	1.1 ± 0.1	0.9 ± 0.0	1.0 ± 0.1
V. corymbosum	Star	15.0 ± 2.8	2.7 ± 0.2	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
V. arboreum	clone 59	18.3 ± 3.7	2.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
V. arboreum	clone 63	17.2 ± 4.2	2.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.0
Pentaploid	Robeson	14.3 ± 6.0	2.7 ± 0.3	1.1 ± 0.1	0.9 ± 0.0	1.0 ± 0.0
V. virgatum	Premier	15.8 ± 0.8	2.8 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
V. virgatum	MS 1389	14.2 ± 7.0	2.7 ± 0.3	1.1 ± 0.1	0.9 ± 0.0	1.0 ± 0.0
V. darrowii	clone 2163	12.3 ± 6.1	2.5 ± 0.3	1.1 ± 0.1	0.9 ± 0.0	1.0 ± 0.0
V. darrowii	Native Blue	13.2 ± 2.5	2.6 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.0
Bulk soil	N/A	16.2 ± 5.9	2.7 ± 0.3	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0

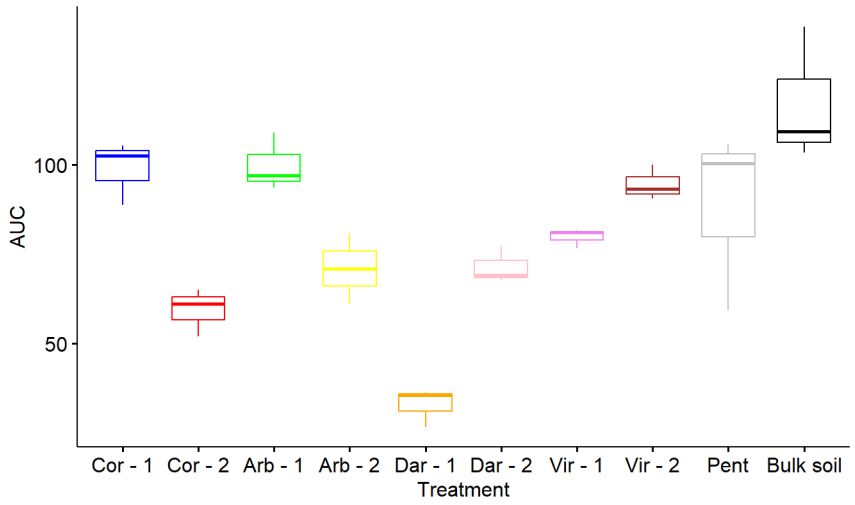

Figure 3.7 *Area Under AWCD Curve (AUC) values generated by the community-level physiological profiling of the blueberry rhizobiome with Biolog EcoPlates.*

The treatments are abbreviated as follows: Cor-1, cultivar Pearl; Cor-2, Star; Arb-1, clone 59; Arb-2, clone 63; Dar-1, clone 2163; Dar-2, Native Blue; Vir-1, Premier; Vir-2, MS 1389; Pent, Robeson pentaploid.

3.7 Metagenomic profiling

The processing of metagenome sequence data resulted in a pooled dataset of 301.2M reads totaling 38Gbp that were subjected to taxonomic profiling with Kaiju using the microbial subset of the NCBI BLAST non-redundant protein database. The results of this taxonomic classification revealed that the bulk of metagenome reads matched eubacterial phyla Proteobacteria, Actinobacteria, Acidobacteria, Planctomyces, Firmicutes and Bacteroidetes (Figure 3.7.1). At the class level, most reads mapped to Actinobacteria, Acidobacteria, Alpha-, Beta- and Gammaproteobacteria. The reads were further assembled into 46,082 contigs, 3,308 of which separated into bins, three of which ranged in completeness between 79.6 and 70.6% with ≤12 % of contamination (the contamination was calculated based on the fraction of marker genes that occur as duplicates, while the completeness was assessed by estimating the proportion of missing markers relative to the total number of markers used) (Table 3.7). The taxonomic classification analysis identified these higher-quality bins as *Gemmatimonas* sp. (Bin.002), *Candidatus Koribacter* (Bin.003) and *Pseudolabrys* sp. (Bin.004) (Figure 3.7.2).

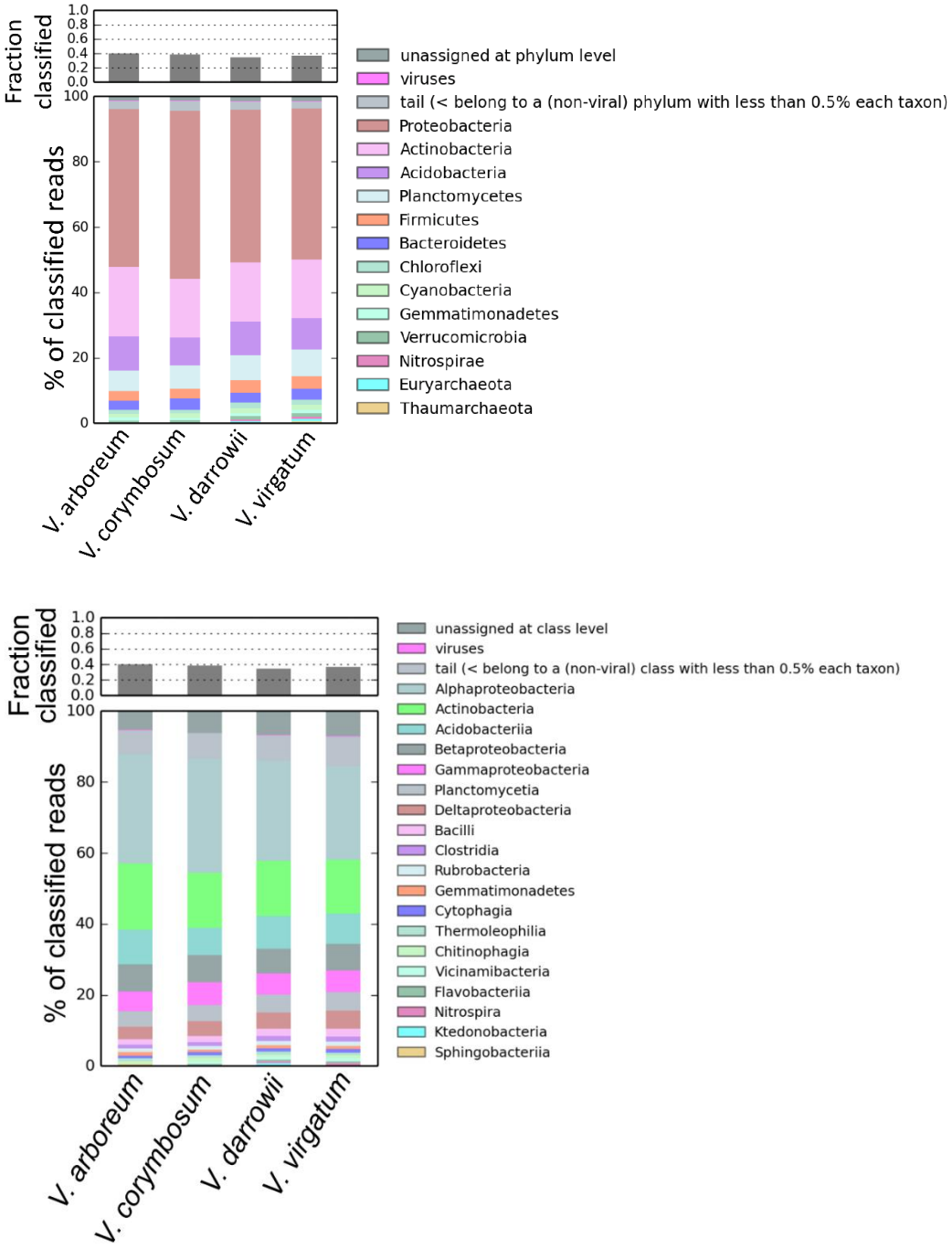

Figure 3.8 *Taxonomic diversity and relative abundance of bacterial phyla (top) and classes (bottom) present within rhizosphere metagenomes of V. arboreum, V. corymbosum, V. darrowii and V. virgatum.*

Categories with relative abundance below 0.5% were merged and shown as “tail.

Table 3.2 *Detailed information (taxonomic classification, completeness, contamination, GC content, genome size, number of contigs, number of features, number of 5S and 16S rRNA genes, number of tRNAs and tRNAs for different amino acids) of the selected metagenome-assembled genomes (MAGs).*

MAG	Classification	Completeness (%)	Contamination (%)	GC (%)	Length (bp)	Contigs	Features	5S rRNAs	16S rRNAs	tRNAs	tRNAs for different amino acids
Bin.002	d__Bacteria p__Gemmatimonadota c__Gemmatimonadetes o__Gemmatimonadales f__Gemmatimonadaceae	70.56	2.85	66.98	2,876,889	518	2,888	1	0	15	11
Bin.003	d__Bacteria p__Acidobacteriota c__Acidobacteriae o__Acidobacteriales f__Koribacteraceae	71.42	6.41	60.38	4,301,506	819	4,328	0	0	34	12
Bin.004	d__Bacteria p__Proteobacteria c__Alphaproteobacteria o__Rhizobiales f__Xanthobacteraceae g__Pseudolabrys	79.6	12.48	58.49	6,549,732	785	7,493	1	0	75	20

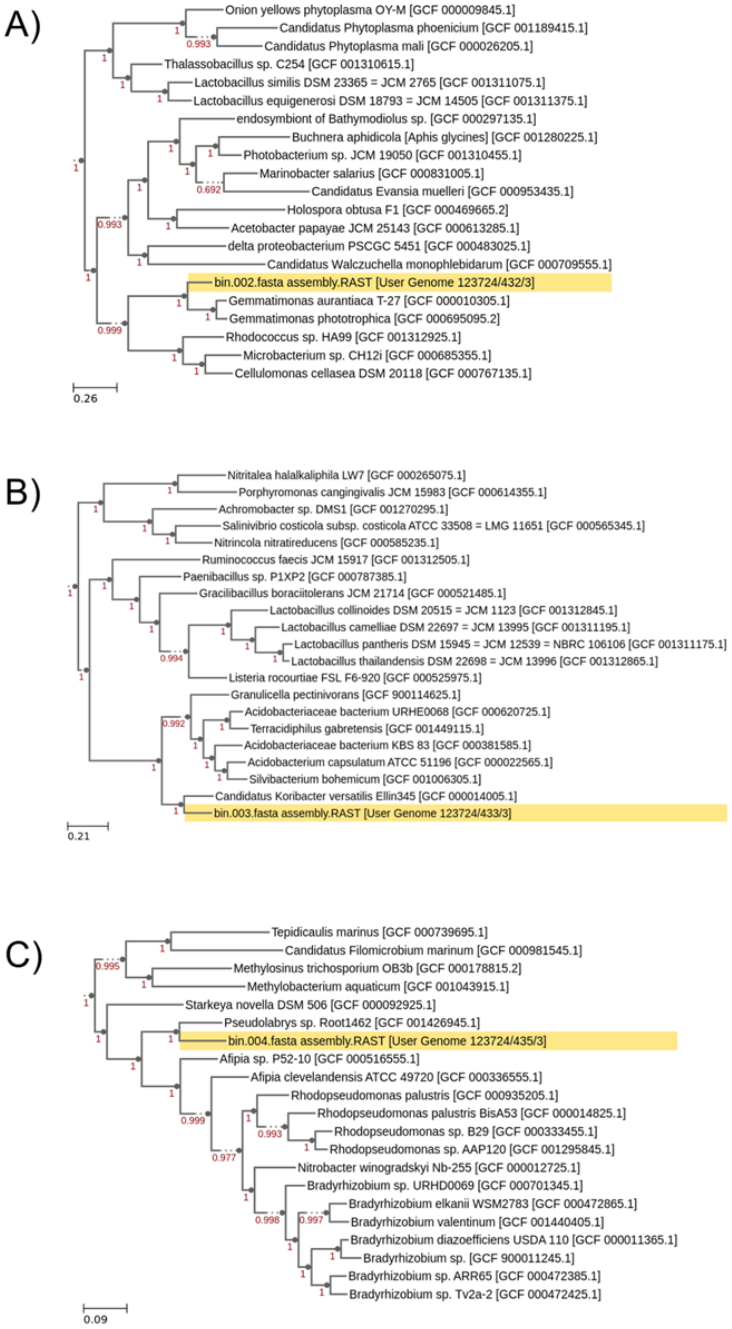

Figure 3.9 *Taxonomic status of the selected metagenome-assembled genomes (MAGs)*

The analysis was performed with the KBase "Insert Genome Into SpeciesTree" app (v2.2.0) using a set of shared 49 core genes defined by COG (Clusters of Orthologous Groups) gene families. The assembled MAGs were compared to sets of closely related genomes which are selected from the KBase database.

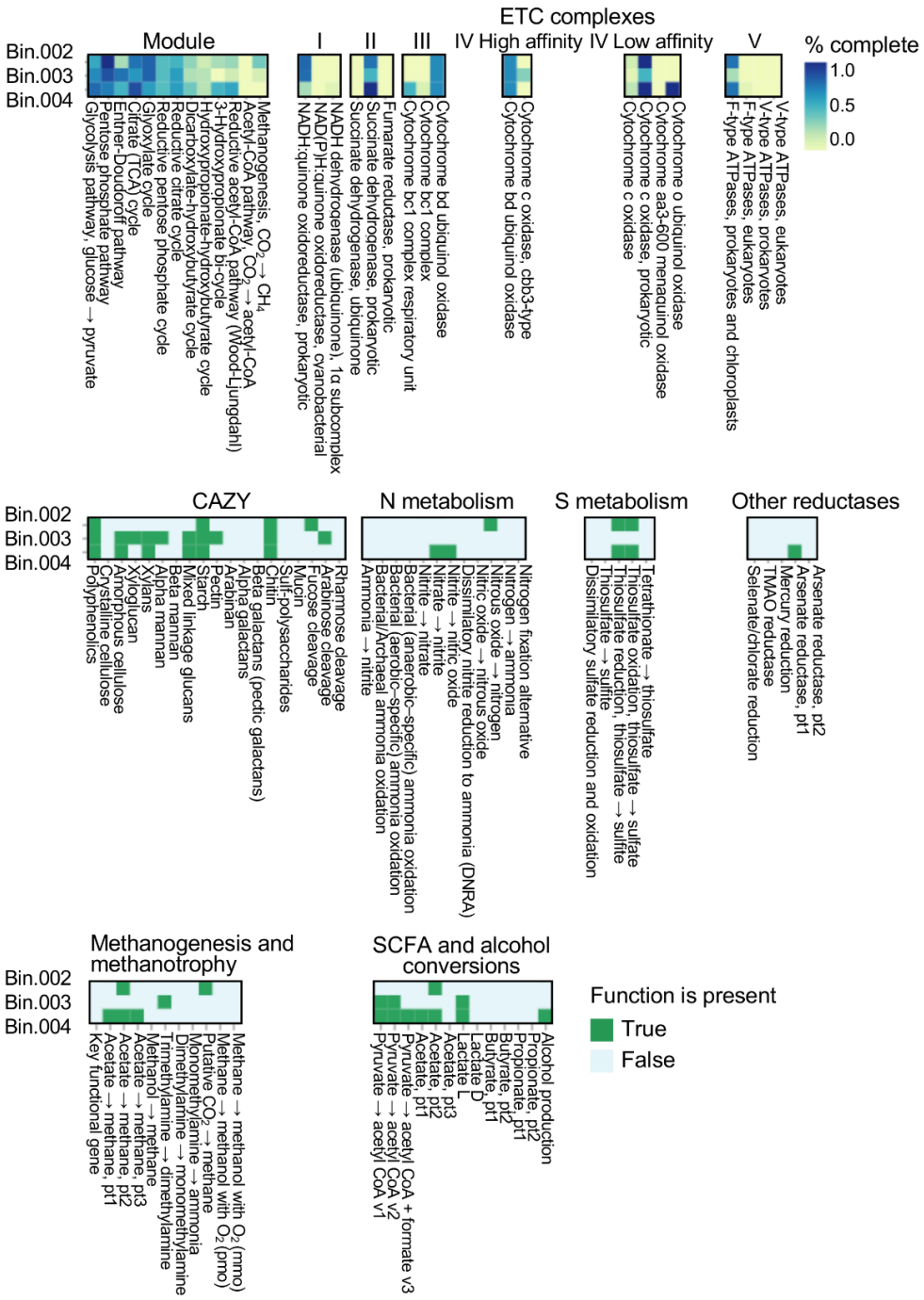

Figure 3.10 *DRAM metabolic annotations of the blueberry rhizosphere metagenome-assembled genomes (MAGs).*

Figure 3.11 *The differential abundance analysis of ortholog group descriptions (COG/KOG/NOG) with the OmicsBox pipeline.*

In the top panel *V. corymbosum* and *V. darrowii* were set as a reference group and *V. arboreum* and *V. virgatum* as a contrast group. In the bottom panel, *V. arboreum* and *V. darrowii* as a reference and *V. corymbosum* and *V. virgatum* as a contrast. Differentially abundant features were plotted against their logFC values that signify the effect size. The dot colors indicate the significance (FDR), while the dot size represents the number of genes in the global dataset annotated with this feature.

Metabolic annotations of the metagenome assembled genomes (MAGs) revealed genes with central metabolism, breakdown of complex carbohydrates, cleavage of polyphenolics, nitrogen-transforming processes and conversion of short-chain fatty acids and alcohols (Figure 3.7.3). Finally, the processing of metagenome reads with the OmicsBox pipeline (BioBam Bioinformatics, Cambridge, MA) revealed several groups of orthologs with differential abundance among the studied blueberry species. The

analysis using *V. corymbosum* and *V. darrowii* as a reference group and *V. virgatum* and *V. arboreum* as a contrast group revealed an overrepresentation of features associated with protein kinase c (logFC = 25.5126, FDR = 4.282e-05) and sodium-potassium-transporting ATPase (logFC = 25.2385, FDR = 2.796e-03) (Figure 3.7.4). In contrast, the use of *V. arboreum* and *V. darrowii* as a reference group and *V. corymbosum* and *V. virgatum* as a contrast revealed an overrepresentation of proteins containing zinc finger domains (logFC = 24.6292, FDR = 4.072e-02).

CHAPTER IV - DISCUSSION

In this work, we employed both amplicon and metagenomic sequencing methods to compare the rhizobiomes of nine different blueberry genotypes representing four different *Vaccinium* species and one interspecific hybrid. The comparison of the studied varieties revealed overall similar bacterial communities with a significant overlap between the rhizosphere and bulk soil compartments both in the composition and alpha diversity. In contrast, the endosphere was colonized by a distinct prokaryotic community with significantly reduced species richness relative to the rhizosphere and bulk soil. Similar trends were observed during the analysis of soil and root-associated fungal microbiomes. These results agree with earlier studies (Li et al., 2020; Yurgel et al., 2017; Zhang et al., 2021) and suggest that different blueberry species harbor conserved core communities of rhizosphere and endosphere microorganisms. These findings also corroborate the notion that plant roots support active and specialized microbiomes that are recruited from the surrounding soil and fueled by carbon sources present in root exudates (Reinhold-Hurek et al., 2015)

Despite the overall similarity in the composition of bacterial and fungal communities, all studied blueberry species had distinct microbiome signatures. We observed multiple taxa that were differentially associated with roots of *V. arboreum* and Robeson pentaploid, including *Bacillus, Burkholderia*, and *Sphingomonas*. These genera encompass numerous strains capable of promoting plant growth and/or suppressing plant pathogens by outcompeting them, secreting antimicrobials or inducing systemic resistance (Elshafie & Camele, 2021; Fira et al., 2018; Legein et al., 2020). A recent study by Rodriguez-Mena et al. (Rodriguez-Mena et al., 2022) used the 16S and ITS

metabarcoding approach to compare rhizosphere fungal and bacterial communities of healthy blueberry plants and plants infected by the pathogenic fungus *Macrophomina phaseolina.* Results of that study revealed that the rhizosphere of healthy plants had a significantly higher proportion of Proteobacteria and Actinobacteria, including the actinomycete genus *Actinospica*. Studies in other crops, revealed positive association between the abundance of Gaiellaceae and Streptomycetaceae and soil suppressiveness to soilborne fungal pathogens (Xue et al., 2015; Zhao et al., 2019). Interestingly, our results revealed that *Actinospica* along with *Crossiella* were overrepresented in the rhizosphere of *V. arboreum,* while other actinomycetes were enriched on the roots of Robeson pentaploid (*Actinoplanes, Actinomadura, Gaiella*) or *V. virgatum* (*Plantactinospora*). The differential association with certain blueberry species was also observed for diazotrophs *Burkholderia* and *Mesorhizobium* and other taxa (*Gemmatimonas*, *Nitrospira*) involved in soil nitrogen cycle (Chee-Sanford et al., 2019; Kalam et al., 2020; Mujakić et al., 2022). Wild blueberries are adapted to acidic soils with slow rates of litter decomposition that result in a high C: N ratio and low phosphorus availability (Vega et al., 2009). The low pH often leads to the accumulation of trace metals and phenolic acids that are toxic to plants. Blueberries cope with these harsh conditions by forming mutualistic associations with ericoid mycorrhizal (EM) fungi that form hyphal networks around thin Ericaceae roots and penetrate their epidermal layer forming characteristic mycelial coils (Smith & Read, 2008). Ericoid mycorrhizae improve nitrogen nutrition of the host by taking up both inorganic (NH^{4+}, NO^{3-}) and organic (amino acids, peptides) N compounds. They also increase the uptake of phosphorus and iron and provide protection

against elevated levels of aluminum, copper, and zinc often observed in soils with low pH (Mitchell & Gibson, 2006; Scagel & Yang, 2005).

Typical ericoid mycorrhizae are formed by several ascomycete genera that belong to the order Helotiales of the class Leotiomycetes and have been observed in the roots of multiple blueberry species (Daghino et al., 2022; Wei et al., 2022; Zhang et al., 2016). Previous research by our group revealed the differential enrichment of the EM fungus *Hyaloscypha* and dark septate endophytes *Meliniomyces* and *Phialocephala* in the rhizosphere of *V. virgatum* (Li et al., 2020). In contrast, *V. darrowii* had higher levels of *Pezoloma* and *Oidiodendron*, whereas the EM basidiomycete *Clavaria* was enriched in the rhizosphere of *V. corymbosum.* This project further highlighted the diversity of fungi capable of symbiosis with *Vaccinium* roots and the differential nature of interactions between different blueberry species and mycorrhizae. Interestingly, in contrast to Li et al. (Li et al., 2020), the roots of plants analyzed in this study did not carry members of the Helotiales but instead harbored basidiomycetes *Clavaria*, *Calvatia*, *Rhizopogon,* and *Hyphodontia*, which form sheathed ericoid mycorrhizae (Vohník, 2020; Vohník et al., 2012). We also detected the basidiomycete *Mycena*, which in *V. corymbosum* has been shown to have a growth promotion effect comparable to that of the classical EM ascomycete *Pezoloma ericae* (Grelet et al., 2017).

Several factors may explain discrepancies in the diversity of mycorrhizal fungi between this work and the study by Li et al. (Li et al., 2020), which was also conducted at the USDA-ARS Thad Cochran Southern Horticultural Laboratory in Poplarville, Mississippi. First, that study was based on young blueberry plants growing in a mixture of pine bark mulch and sand under greenhouse conditions, whereas we used in our

project mature field-grown plants. Second, all plants used in our study came from a plot that was conventionally managed, and the colonization of blueberries by mycorrhizae depends on the amount and type of organic matter present in the soil and the rate of fertilization. Generally, higher amounts of fertilizers correlate with a decrease in mycorrhizal colonization (Hanson, 2006). Finally, due to COVID-19-related restrictions our field sampling was conducted later in the fall of 2020, which may also have impacted the diversity and population levels of EM fungi. Similar seasonal variations in the ratio of Basidiomycota to Ascomycota were detected in ericoid mycorrhizal communities by other studies and attributed to changes in monthly mean temperature and soil moisture (Zhang et al., 2016).

Blueberries were first domesticated at the end of the 19th century with the first breeding of *V. corymbosum* varieties dating back to 1908 (Retamales & Hancock, 2018). Over the years, blueberries were rebranded as a "superfood" and became an international crop that is grown in North America, South America, Europe, China and around the Pacific Rim. Although wild *Vaccinium* spp. has been harvested by humans for thousands of years, the tremendous growth in blueberry production fueled active breeding efforts and resulted in the selection of numerous cultivars with early ripening, improved plant vigor and disease resistance, later flowering, higher yields, better flavor, and suitability for mechanical harvesting. The effect of these genetic manipulations on the blueberry microbiome remains poorly understood. Recent studies demonstrated significant differences in the composition of microbial communities associated with the roots of modern crops and their wild ancestor. The effect of domestication on specific bacterial and fungal taxa was observed in the rhizosphere microbiomes of sugar beet, barley,

wheat, and lettuce (Bulgarelli et al., 2015; Cardinale et al., 2015; Spor et al., 2020; Zachow et al., 2014). A meta-analysis of published microbiome data revealed an abundance of Bacteroidetes on roots of wild plants, which differed from the rhizosphere of domesticated species enriched in Actinobacteria and Proteobacteria (Pérez-Jaramillo et al., 2018). In contrast, our study revealed enrichment of Actinobacteria and Firmicutes in the rhizosphere of domesticated blueberries, whereas Acidobacteria and Gemmatimonadetes were more abundant in the wild *V. darrowii* and *V. arboreum*. An opposite trend was observed in the endosphere, where Actinobacteria and Firmicutes were abundant in the wild species, while Acidobacteria and Bacteroidetes were associated with cultivated species. At a finer taxonomic level, we observed differential distribution of multiple prokaryotic and fungal taxa, including species with known plant growth-promoting, biocontrol, and mycorrhizal properties.

Although amplicon sequencing-based microbiome analysis is a powerful technique, it provides insight only into the taxonomic makeup of the microbial community. Computational methods such as PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2020) can be used to infer biological functions based on particular 16S sequences. However, the accuracy of such predictions depends on how well a studied community is represented by microbial genomes available in sequence databases, making this approach of limited value for studying novel or highly diverged microorganisms. Shotgun metagenomics avoids these limitations by sequencing an entire pool of DNA extracted from environmental sample of interest. The resulting reads are analyzed for the presence and distribution of specific taxa and/or assembled to provide insight into biological functions encoded in the sequenced

microbial genomes. Although shotgun metagenomics is increasingly used to characterize rhizosphere communities, we are aware of only one study that employed this approach to analyze the blueberry rhizobiome. In that work, Yurgel et al. (Yurgel et al., 2019). characterized the functional potential of rhizosphere microbiomes from managed and wild lowbush blueberries (*V. angustifolium*). By sorting functional pathways into co-occurrence networks, the authors determined that central metabolism pathways (e.g., glycolysis/gluconeogenesis, pentose phosphate pathway, TCA cycle, metabolism of cofactors and vitamins) were underrepresented in the rhizosphere. In contrast, the rhizosphere had higher relative abundance of carbohydrate and putrescine transport genes and pathways involved in the biodegradation of complex organic compounds and metabolism of terpenoids and polyketides.

Our metagenomic survey of the blueberry rhizobiome assigned most of the classified reads to Proteobacteria, Actinobacteria, Acidobacteria, Planctomycetes, Firmicutes, Bacteroidetes, and Chloroflexi. This community composition was close but not identical to the results of 16S amplicon sequencing, which predicted a rhizobiome comprised of Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Bacteroidetes, Verrucomicrobiota, and Myxococcota. In addition to the analysis of short reads for broad functional summaries of samples, we also generated genome-level insights from the metagenome sequence data. The high level of complexity of the analyzed communities and modest coverage depth forced us to pool sequence data generated from different blueberry species. A total of three metagenome-assembled genomes (MAGs) were selected from the merged dataset for further analysis based on their completeness and contamination scores. The selected MAGs belonged to species of Gemmatimonadetes

(Bin.002 - *Gemmatimonas* sp.), Acidobacteria (Bin.003 - *Candidatus Koribacter*) and Alphaproteobacteria (Bin.004 - *Pseudolabrys* sp.). The selected MAGs featured components of glycolysis, pentose phosphate, Entner-Doudoroff, citrate and glyoxylate cycle pathways, as well as electron transport chain complexes associated with aerobic metabolism. Also were present multiple carbohydrate-active enzymes (CAZymes) associated with the cleavage of polyphenolics and complex carbohydrates, nitrogen, and sulfur metabolism, and conversions of short-chain fatty acids and alcohols.

Although studies targeting taxa represented by MAGs are limited, *Gemmatimonas* and *Pseudolabrys* seem to be frequently associated with plants and the rhizosphere where they play a role in nitrogen by fixing nitrogen, oxidizing ammonia to nitrite or reducing nitrous oxide (Oshiki et al., 2022; Yu et al., 2022). *Candidatus Koribacter* is a ubiquitous slow-growing acidobacterium that accounts for up to 14% of some soil microbial communities (https://genome.jgi.doe.gov/portal/aciel/aciel.home.html). The wide distribution in soils and broad metabolic potential suggest that this organism is a significant player in the terrestrial carbon cycle (Ward et al., 2009). However, the exact role of these taxa in the rhizosphere communities of blueberries requires further investigation.

In conclusion, our results showed an extensive diversity of pro- and eukaryotic organisms inhabiting the rhizosphere and root endosphere of the different blueberry species. Our results also revealed that wild and domesticated *Vaccinium* species vary in the abundance of multiple microorganisms, including diverse beneficial rhizobacteria and ericoid mycorrhizal (EM) fungi. Like other members of the *Ericaceae* family, wild blueberries rely on EM to thrive in acidic soils rich in organic matter but low in NO_2 and

calcium. The mycorrhizal association also improves the ability of wild plants to use water and tolerate toxic metals that accumulate in low-pH soils. Therefore, our study suggests that breeding may indirectly impact blueberry health by affecting the abundance of beneficial bacteria and EM fungi that play a vital role in the ability of these plants to cope with biotic and abiotic stress. Our findings warrant further investigation of this phenomenon with the ultimate aim of amending breeding efforts with a microbiome-supported approach to improve the resistance of blueberry cultivars against diseases, tolerance against heat and drought, and ability to thrive in a broader range of soil conditions.

APPENDIX A - Oligonucleotide Primers Used in This Study

Table A.1 - Oligonucleotide primers used in this study

Primer	Sequence	Reference
B969F	5’-ACGCGHNRAACCTTACC-3’	Comeau et al., 2011
BA1406R	5’-ACGGGCRGTGWGTRCAA-3’	Comeau et al., 2011
ITS86(F)	5’-GTGAATCATCGAATCTTTGAA-3’	Op De Beeck et al., 2014
ITS4(R)	5’-TCCTCCGCTTATTGATATGC-3’	Op De Beeck et al., 2014

APPENDIX B - Bar Plots Showing Utilization of Biolog Ecoplate C Sources by Culturable Bacteria Associated With Different Blueberry Species.

The treatments are abbreviated as follows: Cor-1, cultivar Pearl; Cor-2, Star; Arb-1, clone 59; Arb-2, clone 63; Dar-1, clone 2163; Dar-2, Native Blue; Vir-1, Premier; Vir-2, MS 1389; Pent, Robeson pentaploid.

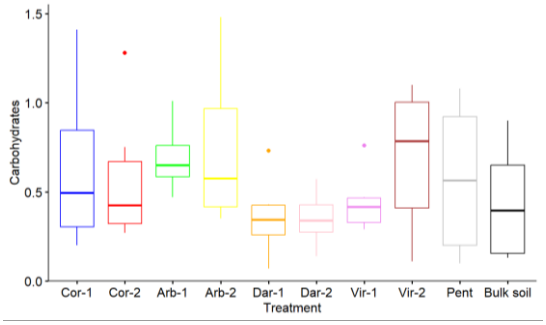

Figure B.1 - Catabolism of carbohydrates.

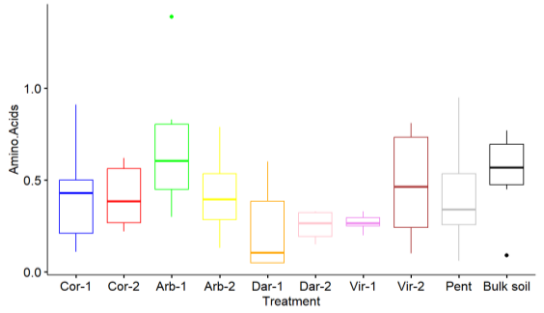

Figure B.2 - Catabolism of amino acids.

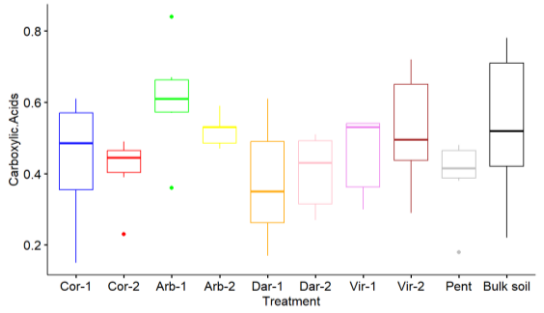

Figure B.3 - Catabolism of carboxylic acids.

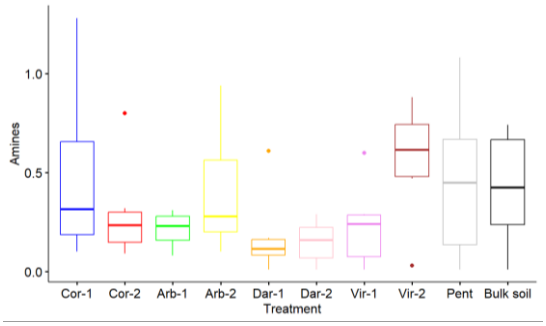

Figure B.4 - Catabolism of amines.

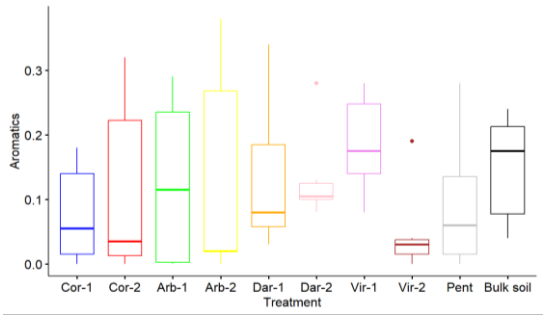

Figure B.5 - Catabolism of aromatic compounds.

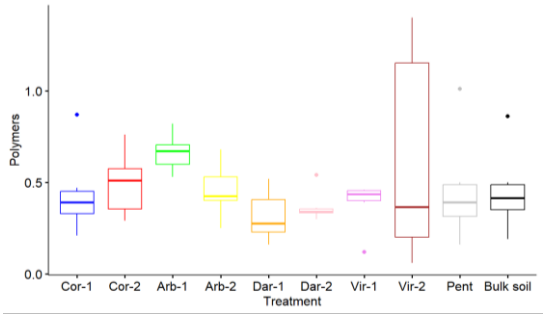

Figure B.6 - Breakdown of biological polymers.

APPENDIX C - The Diversity of Culturable Rhizosphere Bacteria Inferred from the Patterns of Utilization of Biolog Ecoplate C Sources.

The treatments are abbreviated as follows: Cor-1, cultivar Pearl; Cor-2, Star; Arb-1, clone 59; Arb-2, clone 63; Dar-1, clone 2163; Dar-2, Native Blue; Vir-1, Premier; Vir-2, MS 1389; Pent, Robeson pentaploid.

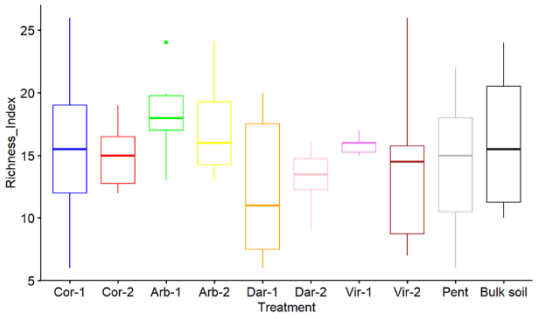

Figure C.1 - Richness index.

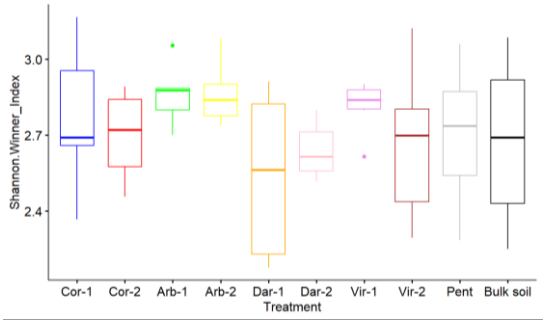

Figure C.2 - Shannon-Winner index.

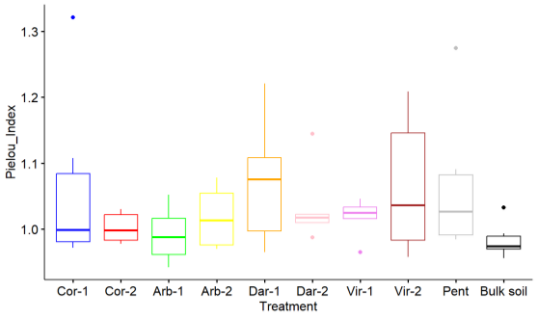

Figure C.3 - Pielou index.

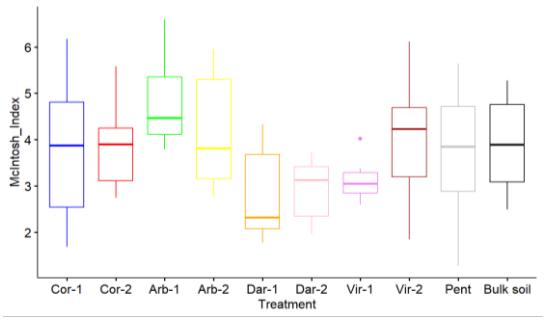

Figure C.4 - McIntosh index.

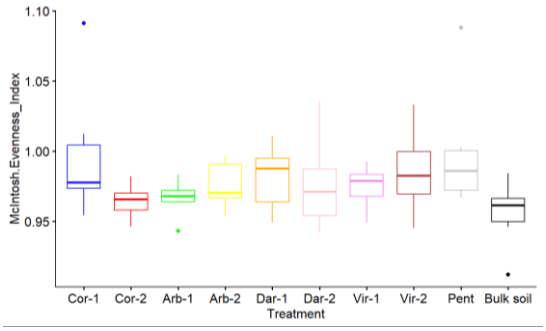

Figure C.5 - McIntosh Evenness index.

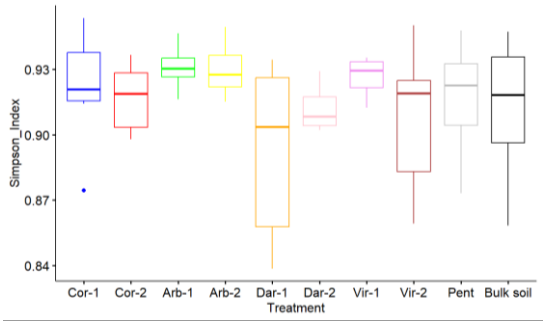

Figure C.6 - Simpson index.

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