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The microbiome of Perennial Ryegrass *Lolium perenne L.*

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by

Bernadette Prout

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Abstract

Perennial ryegrass (*Lolium perenne L.*) is the most critical agricultural plant supporting New Zealand's intensive pasture-based dairy industry. A substantial body of research exists on the physiology, genetics, and management of perennial ryegrass; however, the perennial ryegrass microbiome has not yet been investigated in detail. Poor persisting perennial ryegrass pastures are ongoing in some areas of the country. Microbiome research has already targeted one pasture persistence problem by applying *Epichloë* endophytes to combat invertebrate pest herbivory. The success of *Epichloë* encourages future research to explore additional microorganism applications to mitigate other significant contributors to poor pasture persistence, such as drought and water stress.

This study explores the bacterial and fungal communities associated with the perennial ryegrass cultivar/*Epichloë* combination, One50 AR37. In total, 80 samples were collected from plots across four different New Zealand farming regions during April/May 2021 and examined alongside spatial, environmental, and host-plant-related metadata. Cultivation-independent methods, including DNA extraction, 16S and ITS rRNA gene PCR amplicon sequencing analysis (via amplicon sequence variants - ASVs), were used to characterise the perennial ryegrass bacterial and fungal communities.

The below-ground (bulk soil, rhizosphere, and root endosphere) compared to the above-ground (shoot endosphere and phyllosphere) perennial ryegrass microbiome habitats represented significantly different ecological niches, correlating to differences in ASV richness, alpha and beta diversity, and the relative abundance of dominant genera.

This study demonstrated evidence that the perennial ryegrass microbiome is strongly influenced by farming location and management practices. The large proportion of unique, site-specific taxa found at each farming location holds the potential to explain differences in pasture productivity and persistence. Location differences indicate that future microbiome research should compare ryegrass pastures on a regional basis rather than extrapolating the results to all New Zealand farming locations.

A core microbiome was not identified in the current study; however, there is evidence for a wider functional selection of taxa across the individual microbiome habitats. Future studies should incorporate metagenomic sequencing to better understand the functional microbiome trends rather than solely focusing on taxa composition. This study provides the foundation for future perennial ryegrass research as it confirms the general trends and common taxa associated with the five microbiome habitats of this important pasture plant. Future pasture persistence research should compare the microbiome of high producing / persistent ryegrass pastures with low-producing ryegrass pastures whilst controlling as many variables as possible. Bacteria of interest, such as *Bacillus* and *Pseudomonas*, should be explored in greater detail at the species level and isolated and applied in manipulative trials to assess the effects on ryegrass production and persistence.

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1 Literature Review

1.1 Introduction

Perennial ryegrass (*Lolium perenne* L.) is a vital component of pastoral farming in many regions of New Zealand. Compared to other dairy-producing countries, the New Zealand dairy industry is unique in its ability to operate full-time grazing systems. Dairy farmers rely on perennial ryegrass pastures to support the bulk of the nutritional needs of their dairy cow herds. New Zealand's temperate climate is critical to the success of this pasture-based system. A valuable characteristic of perennial ryegrass is its persistence: plants that survive and produce high yields over multiple years. High pasture persistence decreases the need for re-grassing and buying-in supplementary feed and thus lowers the cost of dairy farming. Plant breeding has been essential to produce ryegrass varieties that fit the wide range of climatic conditions and management practices associated with different New Zealand dairy farming locations.

Perennial ryegrass breeding has developed characteristics that are attractive to farmers, such as ease of establishment, high dry matter (DM) yield and insect pest resistance. However, climate change and its flow-on effect on many environmental factors has lowered the 'high persistency' reputation of perennial ryegrass pastures in some regions of New Zealand (Woodward et al., 2020). For years, farmers in the upper North Island have reported that perennial ryegrass pastures have high failure rates, lasting only three to four years post-sowing. Despite continued research efforts, no single factor has been identified to explain the poor persistence of perennial ryegrass. Little work has focussed on the associations between the ryegrass microbiome and productivity. Negative microbial interactions or a poorly structured microbiome could be a key factor contributing to pasture failure. Exploring the scientific gaps in ryegrass persistence research, such as in the microbiome area, can help us support farmers by providing them with new ideas, such as utilising microbial interactions to enhance pasture persistency. Therefore, this project aimed to use 16S rRNA gene and internal transcribed spacer (ITS) PCR amplicon sequencing to examine and compare the microbiome of the perennial ryegrass cultivar 'One50 AR37' across different farming locations in New Zealand.

To achieve this aim, an initial literature review was conducted on perennial ryegrass survival in New Zealand. The review includes an outline of the importance of ryegrass on New Zealand dairy farms; the known factors linked to reduced pasture persistence; an introduction to the role of the perennial ryegrass microbiome; and an outline of the rationale for the molecular techniques and methodology used to achieve the overall research aims.

1.2 Perennial ryegrass in New Zealand farming

1.2.1 Pasture-based dairy farming

The New Zealand dairy industry contributes $7.8 billion (2.8%) to the country's gross domestic product, with dairy products representing the country's largest export sector (DairyNZ, 2021). The industry comprises 11600 milking herds, representing 4.99 million milking cows, and relies heavily on New Zealand's natural environment (DairyNZ, 2021), i.e. a cool temperate climate with fertile soils and abundant water reserves (Edwards & Bryant, 2011). These characteristics make it possible for dairy farmers to efficiently grow large quantities of grass forage all year-round to sustain their livestock. Products derived from the milk produced by dairy cattle are integral to the human diet in many parts of the world (Verkerk, 2003).

Dairy consumers generally regard pasture-based systems as the ideal "natural" farming system (Horan & Roche, 2019). This is due to perceptions of better environmental protection and increased animal welfare compared to intensive indoor supplement-feeding systems. Pastoral farming provides economical and ethical advantages when compared to systems where cows are primarily housed indoors and fed a mixed ration diet, as is common in many other milk-producing countries (Horan et al., 2005).

Pasture-based dairy farming requires balancing the management of pasture growth patterns and cow dietary requirements to achieve sustainable pasture and milk production for an economic profit (Verkerk, 2003)

1.2.2 Ryegrass types

Ryegrass is the most widely sown pasture species in New Zealand. Each ryegrass plant has several tillers ("stems"), and each tiller only maintains three live leaves at one time (Edwards & Bryant, 2011).

Farmers use a mixture of ryegrass types selected to suit their region's land, soil, and climate. Perennial ryegrass is the most persistent ryegrass type, lasting on average, between 5-10 years. Annual or Italian ryegrasses (*L. multiflorum*) are the least persistent, lasting, on average, between 1-2 years. The least persistent ryegrasses are annual or Italian (*L. multiflorum*) – lasting, on average, between 1-2 years (DairyNZ, 2021). Short-rotation hybrid ryegrasses, generally produced by crossing perennial and Italian ryegrass, fall between these two categories, persisting between 2-5 years (Dodd et al., 2018).

Farmers will utilise less persistent, short-grazing rotation ryegrass types (including annual or Italian ryegrass) when they want pastures to establish quickly and produce high volumes of winter early-spring pasture (Charlton, & Stewart, 1999). These grasses are more successful in regions that receive at least some rainfall throughout the warmer summer months (Edwards & Bryant, 2011). Short rotation ryegrasses, characterised by excellent feed quality and good winter growth, are popular in areas where winter conditions limit the growth of other ryegrass types (DairyNZ, 2021).

1.2.3 Perennial ryegrass

Perennial ryegrass is well-adapted to the moist climate and free-draining, fertile soils in New Zealand and other similar regions worldwide (Tozer et al., 2014). It is the dominant temperate grass type used in dairy pastures and is attractive to farmers because of its longer life duration, ease of establishment and management, and high nutritional value. In New Zealand, perennial ryegrass pasture produces average yields of 14 tonnes of DM per hectare a year (t DM/ha/y), with yields over 20 t DM/ha/year achieved under irrigation (Cosgrove, 2011).

In moist summer environments, with good management, perennial ryegrass pastures can last around ten years. However, these favourable, stress-free environments are rare in New Zealand. Perennial ryegrass can face a range of abiotic and biotic stresses in intensively grazed pastures, including high temperatures, soil-moisture deficits, overgrazing, and pest invertebrates (Lucanus et al., 1960). Stresses are typically more severe in summer and early autumn and limit pasture growth, meaning the tiller appearance rate and density are low. In these situations, grazing is more likely to cause tiller death and reduce pasture persistence (Thom, 1991).

Where summer-dry conditions and significant pest pressure prevail, perennial ryegrass persistence may be significantly less than the expected 5-10 years. This is a fundamental drawback of perennial ryegrass (Woodward et al., 2020). Many Northland farmers have switched to annual crop/pasture rotations due to ongoing perennial ryegrass persistence problems with perennial ryegrass (Beukes et al., 2021; Easton et al., 1996). High pasture failure rates increase pasture management costs for farmers. Therefore, increasing perennial ryegrass persistence has become a focus for ryegrass breeding programmes.

Perennial ryegrass was introduced to New Zealand during the nineteenth century (Easton et al., 2001). Active scientific efforts to improve perennial ryegrass began in the 1920s producing the first certified strains (or cultivars) in the 1929-30 season (Easton et al., 2001). These strains were better suited to the New Zealand environment than strains from other countries. Breeding around this time focussed on producing ryegrass that was dense with fine tillers. In the early 1970s, cultivars were released based on the recently discovered open-erect Mangere ecotype, including Nui, Yatsyn and Bronsyn (DairyNZ, 2021; Duder, 1976). Today, there are well over 20 perennial ryegrass cultivars in NZ alongside long-rotation hybrid grasses, all with slightly different agronomic and animal feed strengths.

Plant breeding today attempts to minimise the effects of abiotic and biotic variables, including dry summer conditions and pests, on perennial ryegrass performance and persistence. New perennial ryegrass cultivars are evaluated through a network of trials run by the New Zealand Plant Breeding and Research Association (NZPBRA). The critical breeding goals are focussed on improving persistence and productivity. Perennial ryegrass cultivars today are best classified as a combination of plant strain, *Epichloë* endophyte strain presence, flowering time (heading date) and whether they are chromosomally diploid or tetraploid.

1.2.4 *Epichloë endophyte*

The discovery of *Epichloë* endophytes to combat insect pasture pests, and thus increase pasture persistence, was a substantial breakthrough in the breeding progress of perennial ryegrass. Endophytes are naturally occurring or artificially introduced fungi that reside within plant shoot tissues for at least part of their lifecycle. *Epichloë* is a genus of ascomycete fungi that form an endophytic symbiosis with grasses, including perennial ryegrass (Kauppinen et al., 2016).

The 'endo' component of the word endophyte means 'inside', indicating that the fungus resides within plant tissues. The fungus is not visible on the plant surface and can only be visualised through microscopic examination of plant seeds or leaf sheath tissue after being specifically stained. The fungus obtains nutrients from the plant. In return, the fungus produces chemical deterrents or toxins called alkaloids, protecting the plant from insect and animal consumption.

The perennial ryegrass endophyte (*E. festucae var. lolii*) can significantly improve pasture performance and, thus, animal production by producing these secondary alkaloid compounds (Edwards & Bryant, 2011). The fungal strands, or mycelium, are initially concentrated in the leaf sheath of vegetative tillers at the base of the plant. In spring, the fungal growth expands to the seed heads, infecting the newly formed seeds, thereby transferring to the next ryegrass generation as the seeds germinate (Figure 1.1).

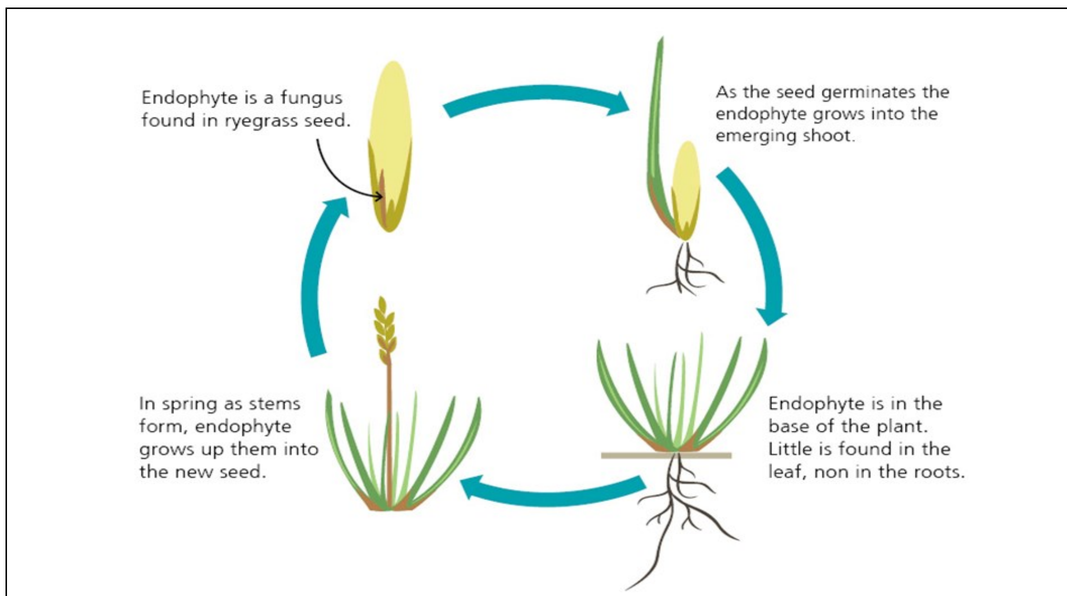


Figure 1.1: The lifecycle of Endophyte in ryegrass plants (sourced from the Dairy NZ website, 2021).

Perennial ryegrass in old dairy pastures is likely infected with the standard (wild type) endophyte. This *Epichloë* endophyte produces multiple alkaloids, some of which provide the plant with protection against several insect pests; however, these have been linked to some animal health problems, including depressed dry matter intake, grass staggers and heat stress (Edwards & Bryant, 2011; Hovermale & Craig, 2001). Due to New Zealand dairy farmers' reliance on perennial ryegrass grazing, developing novel perennial ryegrass/*Epichloë* combinations that provided pest resistance without unfavourable

animal health impacts became crucial. Plant breeders have since developed many endophyte strains, such as AR1, AR37 and NEA2 (Table 1.1). All of these endophyte strains differ slightly in their ability to provide resistance to different species of insect pests.

Epichloë strain AR1 became 'the safer ryegrass endophyte' due to its high level of animal safety and a moderate range of insect protection. It does not produce compounds known to affect animal health, such as lolitrem B or ergovaline (Hovermale & Craig, 2001). It has shown resistance to a range of pest species, including Argentine stem weevil and pasture mealy bug (Edwards & Bryant, 2011); however, it provides limited protection against adult black beetles, which are significant pests in upper North Island pastures.

Table 1.1 The common *Epichloë* endophytes and their indicative insect pest protection ratings. Ratings: no control (-), low-level control (+), moderate control (++), good control (+++), very good control (++++). These ratings are based on diploid perennial ryegrasses and will differ slightly between different cultivars**.**

Endophyte strain	Black beetle	Argentine stem Weevil	Porina	Pasture Mealy bug	Root aphid
Standard	+++	++++	+	++++	++
AR1	+	++++	-	++++	-
AR37	+++	++++	+++	++++	++++
NEA2	+++	+++	Not tested	++++	++

One of the most widely used *Epichloë* around New Zealand is AR37 and is present in ryegrass cultivars such as One50 or Platform. Compared to AR1, AR37 produces a range of janthitrem alkaloids that provide a broad spectrum of pest resistance (Tapper & Lane, 2004). It protects against Argentine stem weevil larvae, Black beetle adults and reduces the survival of porina larvae (Jensen & Popay, 2004). A pot trial showed that porina larvae survival, body weight and head widths, and plant tiller damage were reduced in AR37-infected perennial ryegrass (Jensen & Popay, 2004). Reduced porina survival was a significant breakthrough because before this time, combatting porina with insecticides and grazing management techniques was showing low success (Jensen & Popay, 2004). NEA2 is another endophyte suited to farming systems in all regions of New Zealand. Ryegrass infected with NEA2 is resistant to black beetle and pasture mealy bug, and it provides some protection against Argentine stem weevil (Popay et al., 2003).

Choosing the suitable cultivar and *Epichloë*-endophyte mix has become extremely important for ryegrass pasture success and persistence. In the current study, the specific perennial ryegrass *Epichloë* / cultivar combination analysed was a well-researched standard, One50 AR37. As shown above, different strains of *Epichloë* protect against different pest invertebrates (Table 1.1). Hence, the One50 AR37 cultivar and *Epichloë* combination was controlled to focus the microbiome analysis on the same plant host.

1.3 Factors influencing perennial ryegrass persistence

Re-grassing is a costly process and has become a yearly practice for some farmers to avoid being left with low-producing, low-quality pastures (Drewry et al., 2008).

Understanding the factors that influence pasture species is vital – so that actions can be taken to improve the economic sustainability of pastoral farming. These factors include: summer moisture availability, soil fertility, pest prevalence, endophyte and cultivar type and specific farm management systems (Daly et al., 1999).

1.3.1 Definition of pasture persistence

The term 'persistence' encapsulates expectations of how a sown pasture should perform over time, including plant survival and yield. Poor pasture persistence occurs when the desirable sown species (chiefly ryegrass and clover) reduce and are replaced by undesirable species. Parsons et al. (2011) pointed out that the failure of a new pasture to 'persist' can be categorised in three ways; "(1) a loss of plants from the population established from the seed sown, (2) loss of the specific trait contained in the seed of the cultivar sown and (3) loss of overall yielding ability in the pasture, i.e. plants and specific traits survive, but other yield-related traits are not expressed or are lost altogether". Thus, both physiological (plant growth) and demographic (plant population) factors are considered in pasture persistence (Parsons et al., 2011).

There are published anecdotal reports of farmers who have not renewed perennial ryegrass pastures on their farms in over 18 years (Daly et al., 1999). Despite this, many farmers are experiencing issues since the results from farmer surveys conducted in Northland, Waikato, Taranaki, and Canterbury identified pasture persistence as one of the areas where more research was required (McCahon et al., 2021; Tozer et al., 2011).

1.3.2 Pasture persistence across New Zealand

Pasture productivity (measured in kilograms (kg) or tonnes (t) of dry matter per hectare (DM/ha) is one method of determining perennial ryegrass pasture persistence (DairyNZ, 2022; Dodd et al., 2018). Dairy farmers commonly use assessments of DM when feed budgeting, and DM production is used as a standard measure to compare pasture production across different farming regions. Perennial ryegrass productivity fluctuates across New Zealand depending on the farm location, environmental conditions, and management practices on a given farm. Diploid perennial ryegrass pastures average 14 t DM/ha/year in New Zealand, with yields over 20 t DM/ha/year achieved under irrigation (DairyNZ, 2022). The current dairy farming regions with the highest perennial ryegrass productivity are in the South Island, with irrigated Canterbury sites producing, on average, 15-21 t/DM/ha annually (Vogeler et al., 2019).

Upper North Island farms are unique from the other farming regions, generally experiencing different soil types, weather patterns and higher temperatures (Lane, 2011), which are more challenging for pasture persistence. Nevertheless, it has only been in the last 20 years that farmers in these areas have indicated that their perennial ryegrass pastures show poor persistence (Lane, 2011). Trials investigating dryland North Island sites, including Northland and Waikato, have demonstrated poor perennial ryegrass persistence compared to Southland-Canterbury. The poor persistence at these sites is likely due to increased tiller mortality in response to drought, possibly associated with other effects, such as invasive weeds and pest species (Woodward et al., 2020). Many

other factors have been speculated to cause this poor persistence, including increased stocking rates in the Waikato (2.7 cows/ha in 1996/97 compared to 3.02 cows/ha in 2009) (Environment-Waikato, 2008). More recently, climate change, which is causing increased summer-dry conditions and droughts, has come to the fore as the main factor exacerbating perennial ryegrass persistence in areas like the Waikato (Chapman et al., 2011).

Location seems to be a dominant driver of pasture productivity differences across New Zealand and thus should be tested in any pasture persistence research. Perennial ryegrass in the Waikato (northern North Island) should be compared to other regions, taking into account factors that have yet to be tested and could impact pasture growth, such as the microbiome, soil nutrient profiles and climatic factors.

1.3.3 The effects of climate change on pasture persistence

Climate is the primary determinant of plant growth and, therefore, perennial ryegrass persistence and productivity (Chapman et al., 2015). Many regions of New Zealand experience droughts during the summer months, causing a reduction in pasture and animal production (Pourzand, 2021). Perennial ryegrass pastures thrive in moist, cool-temperate conditions and show moderate to low persistence in summer-dry conditions (low soil moisture) (McCahon et al., 2021). Perennial ryegrass is considered poorly adapted to low moisture availability, attributed to its shallower root systems (Chapman et al., 2011). High summer temperatures are also detrimental to perennial ryegrass pasture growth. The optimum temperature for leaf growth is 18 to 20°C (Chapman et al., 2011). Temperatures close to, or above, 30°C have been reported to cause a decline in growth (Mitchell, 1956).

Water limitations restrict leaf appearance, expansion, and tiller formation, mainly explaining the seasonal variation in plant growth rates. Significant perennial ryegrass tiller losses at Northland and Waikato trial sites have been associated with drought (Lee et al., 2017). Likewise, perennial ryegrass failure in the upper North Island has been correlated to severe droughts (soil moisture deficits of 125 to 150 mm or greater) (Macdonald et al., 2011).

The global surface temperature is expected to increase by 2-4°C by the end of the 21st century due to human-induced climate change (Stocker, 2014). This temperature increase is of great concern to the New Zealand dairy industry, which is reliant on perennial ryegrass species suited to our current cool temperate climate.

Increased frequency and intensity of weather events, including storms and drought, can be attributed to climate change (IPCC, 2010). This has not gone unnoticed in some upper regions of New Zealand, including Northland, Waikato, and Gisborne, which experienced droughts in 2020, severely limiting their perennial ryegrass pastures (Pourzand, 2021). Droughts also facilitate the ingress of annual grass weed species (e.g., yellow bristle grass) due to the death of perennial ryegrass plants. These grasses seem to be better adapted than ryegrass species to warm summer conditions and climate fluctuations and germinate readily from seed set (Campbell et al., 1996).

Due to the poor resilience of perennial ryegrass pastures in dryer regions, some farmers have begun to adopt better-adapted perennial species, including tall fescue (*Festuca arundinacea*) and cocksfoot (*Dactylis glomerata*) (Taylor et al., 2021). Some farmers also use irrigation; however, large areas of the country rely solely on rain-fed farming systems (Rawnsley et al., 2014). Irrigating pastures is unfavourable, as it is economically expensive to farmers and has a negative environmental impact due to utilising vital groundwater reserves.

1.3.4 Biotic factors affecting pasture persistence

The biotic components that make up the dairy farming environment include plants, livestock, invertebrates, and microorganisms/microbiomes. The prevalence of invertebrate pests has an impact on pasture persistence. Climate change has escalated the effects of invertebrate pests, making them a significant threat to perennial ryegrass persistence in New Zealand.

1.3.4.1 Insects

A 2011 New Zealand study of farmers identified insect pests (e.g., grass grub (*Costelytra giveni*) and black beetle (*Heteronychus arator*)) as the primary reason for the death of sown species pasture species (Tozer et al., 2011). Climatic factors (e.g., droughts and waterlogging) have since surpassed this as the most likely primary cause of poor pasture persistence (Beukes et al., 2021). However, pasture pests remain a significant problem for dairy farmers to manage.

Pasture pests vary in importance spatially and seasonally across New Zealand. While some, such as grass grub, are ubiquitous, others, particularly black beetle, have restricted ranges but are still of national importance due to the number of farms within their distribution and their potentially severe impact (Ferguson et al., 2019). In New Zealand, around 45% of dairy herds are situated in areas subject to damage by black beetle. The effects of this pasture pest are extensive economically, costing the dairy industry $223 million annually (Ferguson et al., 2019) and severely impacting the ryegrass persistence. Infestations have the most significant impact in summer and autumn when moisture stress plays a role; therefore, this issue is expected to increase with climate change (Popay & Baltus, 2001).

Grass grub is an endemic species adapted to New Zealand's exotic pastures and can be found throughout the country. The soil-dwelling larvae feed on a wide range of plant roots, with agricultural plants, such as ryegrass, being the most palatable and favourable for their growth (Van Toor & Dodds, 1994). The naturally occurring grass grub disease-causing bacterium *Serratia entomophila* is an effective grass grub biopesticide (Jackson, 2007). This bacterial product is most successful when applied to healthy grass grub populations in the early stages of their development (larval stage) and one or two years after pasture cultivation and sowing (Jackson, 2007). Research has demonstrated a 30-40% increase in winter pasture production and enhanced pasture composition after applying this bacterium (Jackson, 2007). Treatment with *S. entomophila* initiates an epizootic disease that recycles and persists in the grass grub population, reducing their impact.

Utilising microbial species such as *Epichloë* endophytes and bacteria as biopesticides can reduce the impacts of invertebrate pests. This highlights the importance of exploring the perennial ryegrass microbiome to identify other potential microbial options for the control of invertebrate pests.

1.3.4.2 Nematodes

Nematodes are one of the multitudes of organisms inhabiting New Zealand soils and are non-segmented worm-like organisms that obtain nutrients from a wide range of organic sources. Free-living nematodes are abundant in soils and sediments worldwide, utilising bacteria and detritus as food sources (Dropkin, 1964). Nematodes can be plant parasites and cause disease in economically important crops such as white clover and other pasture species. They can constrain pasture production, as was indicated by plant biomass increases of 25–59% after nematicide treatment of soils (Ingham & Detling, 1990). It was also demonstrated that nematodes could alter the rate and direction of nutrient fluxes within grassland ecosystems (R. D. Bardgett et al., 1999), suggesting that competition from nematodes could be a potential factor influencing poor persistence.

Nematodes in New Zealand have been found in high densities in moist environments. Some dominant types of plant-feeding soil nematodes in ryegrass pastures include the genera *Heterodera* (cyst), *Meloidogyne* (root-knot), *Pratylenchus* (lesion), and *Helicotylenchus.* These are plant root parasites, with the first two invading the roots to lay eggs which form cysts or galls, while lesion nematodes feed inside roots but can move freely into and out of roots. Although a single soil core can contain thousands of nematodes, many species do not extensively damage plants when considered alone. However, they become damaging when plants are subject to multiple stresses, including drought and over-grazing (Watson et al., 1986). Therefore, it is crucial to consider the effect of nematodes on ryegrass pastures, as they can alter persistence through parasitic interactions and interfere with nutrient and water uptake from the soil.

1.3.4.3 Gaps in research knowledge (Microbiome area)

Research has identified factors associated with poor perennial ryegrass persistence; however, it remains a significant problem in New Zealand, with many unanswered questions. Farmers have observed that old eco-type-based perennial ryegrass cultivars seem more persistent than new cultivar genotypes.

To address this issue, DairyNZ researchers set up a pasture persistence trial in the autumn of 2011 across three locations: Waikato (Scott Farm), Northland and Canterbury. This trial compared the dairy pasture performance of old versus new ryegrass cultivars. New ryegrass cultivars showed no difference in persistency compared to old cultivars when grown under the same conditions (Lee et al., 2018) (Taylor et al., 2021). It was also found that different ryegrass seed sowing rates had no effect on pasture growth and survival. Location had the most considerable effect on pasture persistence, with the South Island sites (Canterbury) far outperforming the North Island sites (Lee et al., 2018). These results indicate there are unexplained mechanisms causing persistency issues in perennial ryegrass pastures aside from differences in cultivar type, grazing management, stocking rates and sowing rate (Lee et al., 2018).

Microbial interactions associated with ryegrass plants can be to the plant's benefit or detriment (Shi et al., 2021), and there is a chance that some old ryegrass cultivars show sustained persistence due to a well-balanced and stable microbiome network (Lee et al., 2018). However, the role of microorganisms in perennial ryegrass pasture persistence is poorly understood. For this reason, the ryegrass microbiome is of interest to researchers trying to improve ryegrass persistency for New Zealand dairy farmers.

1.4 Plant microbiomes in agriculture

1.4.1 Introduction to microbial interactions

Microorganisms, such as bacteria and archaea, predate plants by several billion years which has allowed complex associations to form (Curtis et al., 2002). Microbes play a part in many plant host functions, including nutrient uptake, defence, and phenology (Ortiz et al., 2015). Individual plant microbiomes represent a vast, largely untapped reservoir which could be used to improve host plant function. The plant microbiome comprises microorganisms from all three primary domains of life - bacteria, archaea and eukarya (C. R. Woese et al., 1990). There are also other domain subgroups to consider, including actinomycetes, fungi, micro-algae, protozoa, and nematodes. Whilst underutilised, the use of beneficial microbial species in modern agriculture dates back to the 1800s. The U.S. Department of Agriculture recommended inoculations of *rhizobium* in legume crops after experiments demonstrated that rhizobium bacteria colonize root nodules and fix nitrogen for the plant (A. Schneider, 1892).

Like all ecological communities, microorganisms interact and play a specialised role in each environment. The increase in abundance or loss of a particular microorganism from an environment can cause an imbalance, potentially resulting in a diseased or infectious state. For example, the overgrowth of the fungus *Tilletia walkeri* causes ryegrass bunt, a disease visible as black-powdered spores on seed that can cause significant reductions in ryegrass and wheat crop yield (Cunfer & Castlebury, 1999). Therefore, it is not only the presence of microbial species that is important; their abundance is also significant in conferring an ecologically stable microbiome.

1.4.2 Factors driving microbial assembly

Several intertwining abiotic and biotic factors can influence microbial community composition. The spatial distribution of microbes worldwide is famously elucidated in the Baas Becking hypothesis as “everything is everywhere, but the environment selects” (Hughes et al., 2006). In other words, microorganisms have a remarkable dispersal potential, but their distribution around the planet is generally shaped by environmental factors rather than geographical distance. Unlike many eukaryotes, bacteria can be found in extreme environments (conditions which are unliveable for most organisms). These bacteria must be well-adapted as specialists (e.g., extremophiles). However, many microbes are more widely adapted (less specialised generalists), allowing them to be more widespread(Logue et al., 2015).

Abiotic factors, such as physical and chemical properties, can affect soil microbial communities. For example, the soil’s pH and nutrient content can explain variation in

community composition (Barnett et al., 2020). Similarly, the water concentration in the rhizosphere (soil immediately surrounding the roots) was found to be the primary driver of bacterial and fungal community structure in wheat root samples (Kavamura et al., 2021).

Other living factors also influence the microbial presence in an environment. Bacterial and fungal species often compete with their neighbours for space and resources. Environmental constraints lead to the selection of bacterial/fungal variants that are best suited to occupy the environment. Microbial species develop many unique mutations due to their rapid growth rates and large population size. Some mutations produce variants better suited to particular niches and are maintained by adverse frequency-dependent selection. This has been illustrated by static cultures of *Pseudomonas fluorescens,* which generated a niche-specialized variant over time, causing the overproduction of extracellular polysaccharides (EPS). This overproduction of EPS enabled the variant to float on the surface of cultures, thus improving its access to oxygen compared to the controls (Shih & Goldenfeld, 2019). Therefore, the term 'survival of the fittest applies to all areas of nature and is a strong driver of microbial diversity and composition.

1.4.3 Plant-associated microbiomes

Over the last few decades, efforts have been continued to inoculate microbial species into crop plants to promote favourable characteristics, including increased growth, nitrogen and phosphorus uptake, and disease resistance (Afzal & Bano, 2008). However, these efforts have focused almost exclusively on individual microbial strains (Afzal & Bano, 2008) and have had varying levels of success, mainly due to the complexity of microbial communities and their interactions within specific environmental settings (Afzal & Bano, 2008).

It is more valuable to explore the idea of microbiomes, rather than individual microbial interactions, due to the range of different microbial species inhabiting specific environments. The term microbiome represents the collective genomes of microorganisms (composed of bacteria, archaea, fungi, protozoa and viruses) that occupy a given area (Berg et al., 2020). Microbiomes exist in all areas of nature and can range in size depending on how specifically they are explored. For example, the human microbiome can be looked at as a whole or split into smaller areas, including the skin surface, eyes, mouth, lungs, gut, and reproductive tract (Turnbaugh et al., 2007). Likewise, natural plant microbiomes can be split into different areas, including the bulk soil, rhizosphere, root endosphere, shoot endosphere and phyllosphere (Turner et al., 2013).

Plant microbiomes can be complicated and include the below-ground root and soil-associated microorganisms and the above-ground leaf and stem-associated microorganisms. The above-ground portion of the plant is subject to harsher environmental and biotic factors, including UV exposure, wind, rainfall and large grazing herbivores (Turner et al., 2013). Seasonal differences such as temperature fluctuations can affect the types of microorganisms that can reside on the exposed aerial parts of plants (Turner et al., 2013).

In contrast, soil environments are more stable, containing the highest abundance of microbial life of any environment on the planet (Delmotte et al., 2009). Soil microorganisms are essential in cycling soil nutrients and decomposing organic matter. Without them, there would be no cycling of elements, meaning the discontinuation of life on Earth (Foster, 1988). The high number and immense diversity of microbial species in the soil mean they must adapt to interspecific competition for resources. Many microorganisms have formed mutualistic relationships with plants to enhance their chances of survival in soils. For example, isotope labelling studies support the concept that plants rely on microbial taxa for nitrogen acquisition because most organic-N is first assimilated by microbial taxa, then subsequently assimilated by plants upon microbial turnover (Richardson et al., 2009).

Exploring all types of plant-microorganism interactions is vital as this can lead to manipulating plant microbiomes (e.g., artificially introduced *Epichloë* endophytes) to support agriculture.

The perennial ryegrass microbiome can be split into five habitats: bulk soil, rhizosphere, root endosphere, shoot endosphere, and phyllosphere (Figure 1.2), each of which is worthy of exploration.

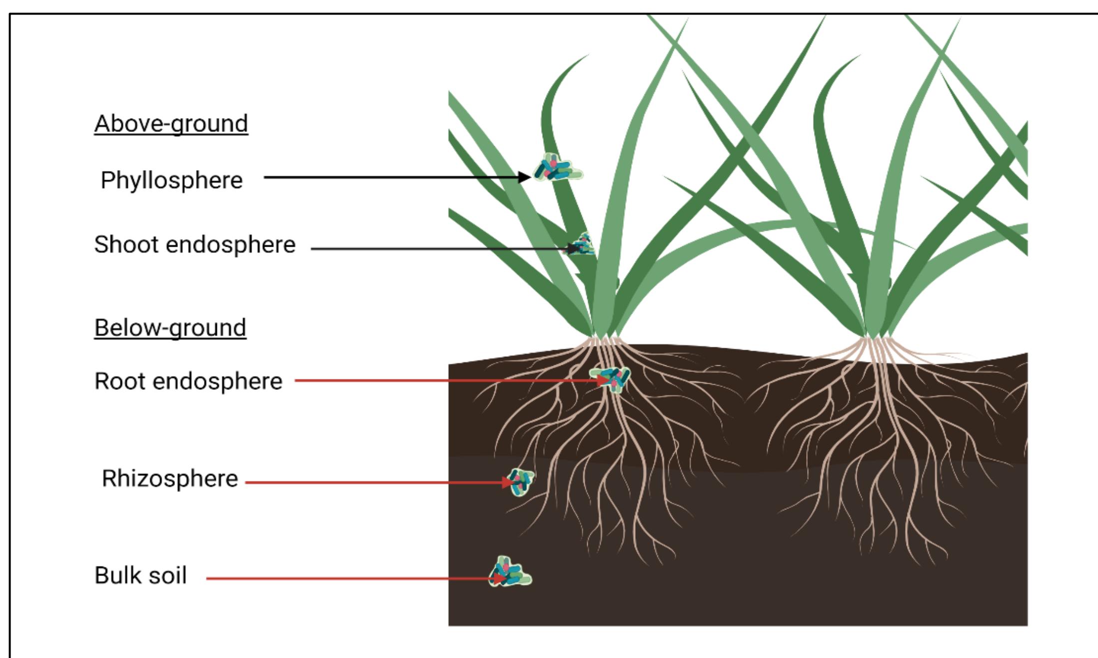


Figure 1.1.2: Illustration of the five microbiome habitats within a perennial ryegrass plant; phyllosphere (the outer surface of the exposed aerial leaves), shoot endosphere (the inner tissues of the ryegrass leaves/shoots), root endosphere (the internal tissues of the ryegrass roots), rhizosphere (the soil in direct contact with the ryegrass roots), and the bulk soil (the soil close/but not directly in connection with the ryegrass roots). Illustration made using BioRender.com.

1.4.3.1 Bulk soil microbiome

The bulk soil microbiome includes any soil not penetrated by living plant roots. Soil microorganisms are essential in maintaining the quality and structure of the soil, with decomposers such as *saprophytic* fungi converting dead organic material into small molecules like organic acids and producing CO_2 that plants can utilise for photosynthesis

(Al-Maliki & Ebreesum, 2020). Some fungal and algae species in soils can act together as cementing agents by binding soil particles. This helps to maintain soil structure and thereby reduce erosion which is incredibly important on all farms (Al-Maliki & Ebreesum, 2020).

Aside from the commonly known nutrient cycling and soil structure benefits, certain soil bacteria can also protect plants from disease-causing pathogens. Plant-microbe interactions can be beneficial or pathogenic to the plant. Soil-borne plant diseases resulting from microbial pathogens that invade their plant host through the soil are an example of a negative interaction (Ajayi ‐ Oyetunde & Bradley, 2018). Common soil-borne diseases include root wilt, vascular wilt and damping-off, which cause plant tissue discolouration, wilting of foliage, root decay and, in worse-case scenarios, death. These microbial soil-borne pathogens can reduce the yields of many crops, contributing to significant losses in agricultural sectors if not managed carefully.

The largest group of microbial pathogens are the fungi and oomycetes; however, plant diseases are also caused by bacteria, protozoa, viruses and nematodes. For example, *Rhizoctonia solani* is a common pathogenic fungus and causes plant rotting and pre- and post-emergence damping-off in soybean plants (Ajayi ‐ Oyetunde & Bradley, 2018). Despite their potential pathogenicity, many disease-causing organisms exist naturally in soil in a non-pathogenic form under normal conditions. Changes in the environment are often the biggest driver of changes to microbial communities. Soil factors, including pH, moisture, temperature and nutrient level, can be critical drivers of disease outbreaks (Lucas, 2006).

Crop researchers are especially interested in beneficial microbial interactions, particularly soil microorganisms that suppress plant diseases and can be primary factors in determining plant health (Schroth & Hancock, 1982). Some well-studied gram-negative *Pseudomona*s species are effective as biocontrol bacteria as they produce several antimicrobial metabolites, including phenazines and hydrogen cyanide (Spence et al., 2014; Thomashow & Weller, 1988). For many years rice farming has been dramatically affected by the blast disease caused by the fungal pathogen *Mangaporthe oryzae*. However, it has been found that the application of a *Pseudomonas* isolate, EA105, effectively inhibits the growth and appressoria formation of *M. oryzae* (Spence et al., 2014). The gram-positive genus *Bacillus* produces antifungal low molecular weight surfactants, and lipopeptides termed ‘kurstakins’, which decrease plant disease (Spence et al., 2014). These examples highlight the potential of beneficial bulk soil microorganisms as biocontrol agents, which could be valuable to current disease protection strategies (Spence et al., 2014).

1.4.3.2 Rhizosphere microbiome

The rhizosphere microbiome represents the soil area in direct contact with plant roots or their secretions (Singh et al., 2007). It is a site of intense interactions between the plant and the rich bank of microbes in the bulk soil. Mutualist microbial species can be involved in biogeochemical soil cycles and nutrient uptake, regulating plant-plant interactions and plant growth (Osorio Vega, 2007; Singh et al., 2007). Resource partitioning is a

mechanism underlying many mutualist relationships, where plants can only access resources through interactions with their associated mutualists. A typical example is symbiotic *rhizobia*, which fix nitrogen for plants (Dardanelli et al., 2010). *Rhizobia* are diazotrophic bacteria that reside within root nodules of legumes, providing plants with a source of acquirable nitrogen. Likewise, nitrogen fixation by rhizobia also mediates the plant species' coexistence of legumes and non-legumes (Kleen et al., 2011). Including legumes (e.g., clover species) in ryegrass pastures creates positive diversity-productivity relationships due to nitrogen fixation (Kleen et al., 2011).

1.4.3.3 Root and shoot endosphere microbiome

Microbial species can colonise the outer surfaces of plants (shoots and roots) as epiphytes and endophytes inside plant tissues for at least part of their lives (Turner et al., 2013). Most endophytic microbes are non-pathogenic on their own, causing no adverse symptoms in plants; however, they can be latent pathogens that cause disease under certain environmental conditions (Hardoim et al., 2015). Plant endosphere environments can be split into the shoot endosphere (microbes within the shoot/leaf tissues) and the root endosphere (microbes within the root tissues).

Endophytic fungi are the dominant colonizers of the plant shoot tissue endosphere (van Overbeek & Saikkonen, 2016). There is likely to be a range of other fungi associated with perennial ryegrass aside from Epichloë fungi, some of which may be equally important but understudied. Arbuscular mycorrhizal fungi are located in the root endosphere and form a symbiotic association with the plant (Bonfante & Anca, 2009). The plant makes organic molecules, such as sugars, by photosynthesis which it supplies to the fungus, and the fungus provides water and mineral nutrients, such as phosphorus, to the plant from the soil (Bonfante & Anca, 2009).

Endosphere bacteria use a variety of means to enter the internal tissues of the host plant. The best evidence indicates that most endosphere microbes enter their host at lateral root junctions or through naturally occurring cracks (H. Liu et al., 2017). Alternatively, many endosphere microbes possess cell-wall degrading enzymes and type three secretion systems (T3SS), allowing microbes to enter or exit the plant tissues (H. Liu et al., 2017). The shoot endosphere generally has lower levels of microbial inhabitants than the root endosphere. Microbes in the shoot endosphere rely on upward movement within their hosts, perhaps through the transpiration stream (Faeth, 2002).

1.4.3.4 Phyllosphere microbiome

The phyllosphere microbiome of a plant comprises the total aerial plant surface (Yadav et al., 2020). In perennial ryegrasses, this habitat represents the surface of the leaves and tillers.

From a microbial perspective, the phyllosphere has been understudied compared to plant soil and root microbiomes. The phyllosphere is the most physically accessible plant microbiome for microbial manipulation. Therefore, insights into the structure of microbial phyllosphere populations are crucial in developing a deeper understanding of plant-microbe interactions, which may lead to applications in promoting plant growth and protection.

The phyllosphere is an extreme habitat for microorganisms due to its exposed environment. For this reason, phyllosphere microorganisms are often more specifically adapted than the microbes in soil habitats, often resulting in a lower diversity of taxa (C.-J. Dong et al., 2019). Microbes present on the leaf surface are subject to temperature, precipitation, and UV exposure fluctuations. However, despite these harsh conditions, plant phyllosphere communities exhibit seasonal spatial and temporal patterns and appear to be consistently dominated by *Gammaproteobacteria* and *Alphaproteobacteria* (Bechtold et al., 2021; Grady et al., 2019). Within *Alphaproteobacteria*, members from the genera *Methylobacterium* and *Sphingomonas* have been found to dominate plant leaf-surface regions (Bechtold et al., 2021). Their dominance has been attributed to the fact that they are generalists, able to survive on many different substrates in low abundance.

The most abundant members of the genus *Methylobacterium* are the pink-coloured facultative methylotrophic (PPFM) bacteria (including *M. oryzae*, *M. mesophilicum* and *M. phyllosphaerae*) ubiquitously found in high abundance in the phyllosphere of several studied plant species (Bechtold et al., 2021; Delmotte et al., 2009). These bacteria are important in seed germination and the growth of crops through the production of cytokinins and auxins and the regulation of the stress hormone ethylene (Chen et al., 2016). They utilise methanol as their primary carbon source, which plants release as a by-product (Delmotte et al., 2009; Yurimoto et al., 2021). PPFM bacteria benefit plants by producing phytohormones and vitamin B12 which promote plant growth and yield. Members of the *Sphingomonas* genus exhibit a high abundance of sugar transporters, demonstrated in phyllosphere metaproteome studies, showing that carbon sugars released by plants provide the critical nutrients for these bacteria (Delmotte et al., 2009).

Previous studies have indicated that many abiotic factors are essential in controlling phyllosphere microbiota populations, including season, weather events (e.g. long-term drought), mineral content, nitrogen fertilisation and pesticide use (Bechtold et al., 2021; Ikeda et al., 2011).

1.4.4 Previous ryegrass microbiome work

After exploring some of the critical microbial interactions across different areas of the plant microbiome, it was evident that plant growth would not be possible without the interplay of microorganisms (Amalric et al., 1999).

Root-associated microbiomes, including the rhizosphere and endosphere, have been previously explored for perennial ryegrass and are dominated by *Proteobacteria* (Chen et al., 2016). *Proteobacteria* are a major phylum of bacteria, including a wide range of pathogens, and include *Escherichia, Salmonella*, *Vibrio*, *Helicobacter*, and many free-living bacteria responsible for nitrogen fixation. Genera, including *Methylobacterium, Rhizobium, Pseudomonas*, *Stenotrophomonas* and *Enterobacter,* are more highly associated with the rhizosphere and endosphere of ryegrass roots compared to the corresponding outer rhizosphere (Chen et al., 2016). Many of these must share symbiotic relationships with perennial ryegrass plants hence their close association (in the root zone) (Chen et al., 2016).

Research on *Epichloë* has demonstrated how fungal species can alter the productivity of a pastoral ecosystem (Attwood et al., 2019). As discussed earlier, this is performed through decreasing insect pest herbivory, and more recently, *Epichloë* infection has also been shown to increase nutrient acquisition. For example, a high pasture white clover percentage is an indicator of a ryegrass failure (Attwood et al., 2019). Perennial ryegrass endophyte strains can respond differently to clover growth in pastures. While white clover improves nitrogen acquisition in perennial ryegrass, extensive white clover growth can cause ryegrass decline due to *rhizobium* symbiosis becoming a dominant process in the community (Attwood et al., 2019). Ryegrass decline in clover-ryegrass pastures has been correlated to infection with *Epichloë* AR1. In comparison, AR37 has been linked to a more stable ryegrass/clover balance (Attwood et al., 2019). This example highlights how the presence of microbes (e.g. *Epichloë* endophyte) can influence feedback loops in pasture environments, resulting in either a legume-dominated pasture with a leaky nitrogen cycle or a ryegrass-dominated pasture that is relatively nitrogen efficient (Attwood et al., 2019).

More recently, soil-borne plant pathogens have been significantly linked to reduced pasture growth in Waikato/upper North Island perennial ryegrass pastures (Dignam et al., 2022). Pasteurising (microwaving) soil is an effective method for exploring the prevalence of soil disease. If the ryegrass shoot/root dry-weight increases when plants are grown in microwaved soils, the original soil likely possessed harmful soil-borne plant pathogens. If plants grown in the soil show decreased shoot/root dry-weight after the soil has been microwaved, the original soil likely contained positive microbial interactions for ryegrass growth. The Waikato region of New Zealand has shown evidence of clover and ryegrass soil and root disease, with pasteurised soils showing an average increase in shoot mass - clover (35%) and ryegrass (19%) - compared to the non-pasteurised controls. Compared to the Waikato region, soils from Canterbury and Southland showed fewer positive effects on ryegrass growth after pasteurisation. Therefore, negative microbial interactions can strongly affect perennial ryegrass growth, particularly in the Waikato region (Dignam et al., 2022). While soil diseases exist across New Zealand farming regions, the microbial species causing these diseases are still unidentified. This highlights the importance of researching the bacterial and fungal species associated with perennial ryegrass, as it could answer some critical questions for pasture persistence research in relation to soil disease.

1.5 Techniques for studying the perennial ryegrass microbiome

1.5.1 Introduction

Gaining an accurate understanding of the extent of microbial diversity in any environment is challenging. When conducting research, the protocols for understanding the structure and composition of a plant microbiome must be carefully considered. The risk of sample contamination by extraneous microbes is a significant issue in microbial research is (Farnsworth et al., 2020). Microbes are everywhere, including in the air, water, surfaces, hands, and breath (Fierer et al., 2008). The prevalence of microbes in all environments is a problem because contamination in a sample adds DNA leading to inaccurate conclusions. The only way to ensure accurate results when conducting microbial research

is through refined fieldwork and laboratory protocols. The current section will review the rationale for the methodology choices associated with this study.

This review identified favourable techniques for the current research project (discussed below). Some were implemented in preliminary trials using ryegrass sourced from the AgResearch Ruakura farm, and those that were successful were integrated into the final methodology of the project.

1.5.2 Microbiome habitats

It was essential to look at all the microbiome habitats separately to accurately portray the microbial communities associated with perennial ryegrass One50 AR37. There are substantial differences between a ryegrass plant's above-ground and below-ground conditions and between the epiphyte and endophyte communities. These differences can significantly impact the microbial species present in each ryegrass microbiome habitat (as detailed previously).

The sampling methodology ensured samples were gathered to represent the five separate microbiome habitats. When retrieving each sample, it was essential to avoid contamination with human DNA and cross-contaminating the microbiome areas (e.g., soil samples contacting ryegrass shoot samples). Strict protocols were followed in all methodology stages to avoid contamination, i.e. when collecting, processing, and extracting DNA samples (refer to Section 2: Methods and Processes).

1.5.3 Sonication for phyllosphere

The phyllosphere is the most complex area of the ryegrass microbiome to analyse. Ryegrass shoot tissues are delicate, and thus there is a high risk of shoot endosphere contamination. Accidentally breaking the outer shoot tissue can contaminate the phyllosphere sample with endosphere microbes and chloroplast DNA. A sonication procedure can separate the phyllosphere microbes while minimising shoot tissue damage. Sonication is the act of applying sound energy vibrations to agitate particles (Suslick, 1990). The use of sonication in an ultrasonic cleaning bath has become a reproducible, conservative, and successful method for isolating phyllosphere microbes from the leaf surface of plants (Müller & Ruppel, 2014). The sonication process often involves submerging the plant leaves in a glass tube containing leaf wash buffer, which is then placed within an ultrasonic cleaning bath. The ultrasonic waves dislodge microbes from the leaf surface and into the wash buffer (Suda et al., 2008). This process was utilised in the current research project because of its gentle manipulation of plant tissue and, thus, minimal contamination from plant organelles.

Although many studies have utilised sonication baths, the exact procedures differ, including the wash buffer constituents, leaf mass (g) to wash buffer volume (mL) ratios, and different ultrasonic frequencies. To determine the adequate sonication time and buffer components for this study, the methodology used by Anya Nobel to explore the mānuka phyllosphere was trialled; 10 mL of wash buffer [PBS, 1% T20] and a 20-minute sonication time at 60 Hz (A. S. Noble et al., 2020). Due to the fragile tissue of ryegrass

shoots, the sonication time was lowered after noticing the post-sonication buffer solution became a faint green colour, indicating chlorophyll and chloroplast contamination.

The ideal Tween 20 concentration of 1% was implemented as part of the final methodology. Glass, instead of plastic flasks, were used for the sonication process as plastic is known to absorb ultrasonic waves (Noble, 2018). Glass tubes successfully transmitted the ultrasonic waves resulting in a suitable quantity of extracted DNA (50-100ng/ml). Lastly, the volume of wash buffer was trialled, ranging from 5 – 30 mL per 5.0 g of ryegrass shoots. Thirty millilitres of leaf wash buffer was sufficient to submerge the shoot tips of 5.0 g ryegrass shoots without reaching the cut ends or overflowing whilst also yielding sufficient DNA concentrations.

The state of the ryegrass shoots was a final factor to consider in the sonication process. The ryegrass samples had cut ends (exposing endosphere) after being harvested in the field with sterile scissors. Therefore, care was taken to ensure the ryegrass shoots were placed into the glass test tube with the cut ends upright with the ryegrass tips facing downward (submerged in the buffer).

1.5.4 DNA extraction and sequencing

Cultivation-independent analysis, using next-generation sequencing methods, has replaced cultivation-dependent analysis due to the high number of unculturable microorganisms associated with plants (Goel et al., 2018). High-throughput DNA sequencing technologies have opened many doors to microbial research. Amplicon sequencing-based research, metagenomics and metatranscriptomics have allowed the thorough analysis of plant microbiome structure and composition (Gupta et al., 2021).

Isolating DNA/RNA from samples is the first step in DNA sequencing. To isolate DNA, cells must be lysed, releasing DNA into the sample, which can be spun down, filtered, and purified. To explore a plant microbiome, total DNA samples must be taken from all the habitats of interest. Choosing an appropriate kit or DNA extraction protocol is critical since all DNA extraction/cell lysis methods contain bias (Feinstein et al., 2009). An unbiased extraction would require that all of the DNA is fully released from all of the cells (i.e., bacteria, fungi, microeukaryotes and unicellular algae). None of the currently available methods would enable a full release of all DNA; therefore, the selected extraction needs to target the groups of interest (bacteria and fungi for this project). It is essential to avoid an excessively long and aggressive cell lysis protocol because this can cause contamination from plant host DNA and DNA shearing.

The perennial ryegrass microbiome analysis includes taking samples from the plant-sub-habitats (the root and shoot endosphere and phyllosphere) and soil sub-habitats (bulk soil and rhizosphere). Soil samples have high microbial diversity, and the bacteria can be hard to extract since some can adhere to soil aggregates or reside within micropores. Plants come with their issues since shoot DNA contains a high amount of chlorophyll DNA, which can mask microbial DNA.

Experiments for the purpose of this study pinpointed commercial DNA extraction kits that provide high DNA yield from plant and soil samples. As part of a preliminary

experiment (not published), AgResearch laboratory scientists tested three DNA extraction kits to determine their suitability for the perennial ryegrass microbiome analysis (Figure 1.3).

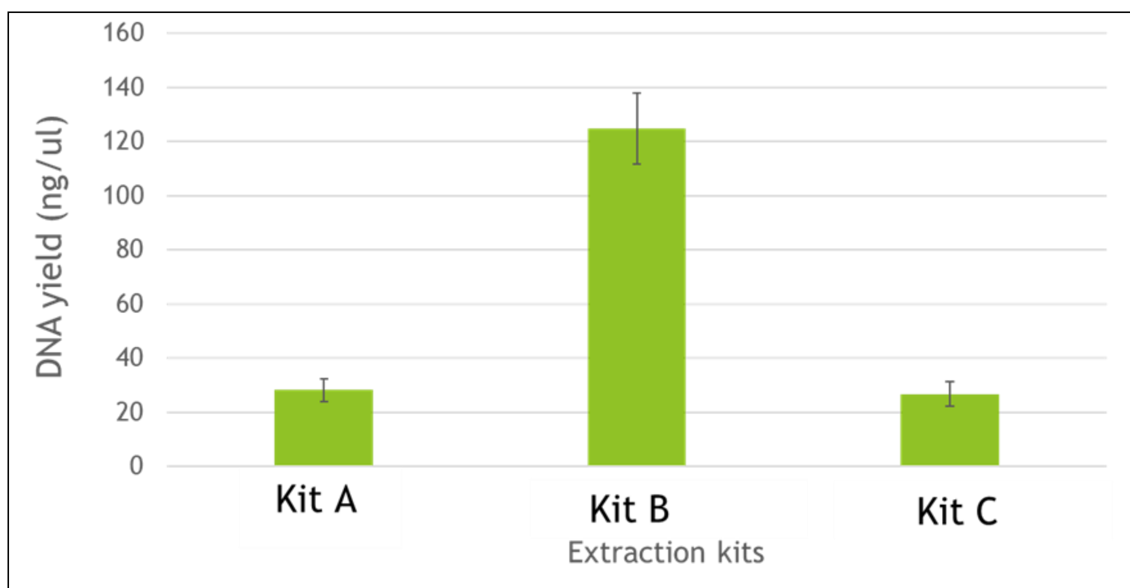


Figure 1.3 Results of an unpublished preliminary trial (produced by Shengjing Shi – AgResearch) showing the average DNA yield of three DNA extraction kits, A= PowerSoil, B= PowerSoil Pro, C= NuclearSpin, for extracting DNA from five perennial ryegrass soil samples (n=5).

The PowerSoil Pro kit (Kit B) showed a significantly higher average DNA yield, and the results were very reproducible compared to the other two kits. As a result, this kit was chosen for the current project. The Qiagen PowerSoil Pro kit is commonly utilised for plant-microbe analysis, including analysis of soil and plant tissue samples (Iturbe-Espinoza et al., 2021). It uses cell lysis chemicals and bead-beating procedures to release the DNA. For the current project, the recommended bead beating length of 10 minutes was reduced to 5 mins to minimise DNA shearing, as this produced high enough DNA yields (20-300 ng/ml). Other minor protocol alterations were made, detailed further in the methods section.

Once DNA of high quality and quantity has been extracted, it can be processed through PCR (polymerase chain reaction). PCR amplifies marker genes of interest (e.g., 16S and 18S rRNA genes) from the DNA present within a sample to focus microbial analysis. Following PCR, sequencing is used to identify the exact sequence of DNA bases (A, C, G and T) present within the samples. In this project, 16S and Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) regions were the amplicon targets used to identify and compare bacterial and fungal microbes in a given sample.

1.5.4.1 16SrRNA and ITS PCR amplicon sequencing

The prokaryotic 16S rRNA gene is approximately 1500 bp long and contains nine variable regions integrated between conserved regions (C. Woese et al., 1990). Every bacterial species has a conserved region, reflecting the phylogenetic relationship among species. While the variable regions reflect differences between species and are frequently used for the phylogenetic classification of genera or species in diverse microbial populations (C. Woese et al., 1990).

The rRNA cistron's internal transcribed spacer 1 (ITS1) region is commonly used as a DNA marker for identifying fungal species in metagenomic samples (Illumina, 2019). Amplification of the ITS1 region provides a culture-independent tool for the identification and characterisation of fungal biota in complex and diverse environmental samples such as soil (A. N. Schneider et al., 2021)

Although culture-independent methods are the standard for assessing a broader spectrum of microbial taxa, they also have drawbacks that can affect the reliability of research data (Janda & Abbott, 2007). For example, they cannot discriminate between live, dormant, or dead microorganisms (Shade & Handelsman, 2012). Any DNA-based sequence analysis detects operational taxonomic units (OTUs) that may be inactive, deceased, or transient (Shade & Handelsman, 2012). Another issue can be caused by differences in the 16S rRNA gene copy number, leading to the overrepresentation of some taxa (Větrovský & Baldrian, 2013). Therefore, DNA-based sequence analysis may not reflect an environment's actual community of interacting species. However, owing to the benefits associated with cultivation-independent techniques, 16S rRNA and ITS1 gene amplicon sequencing were the chosen methods for this study.

1.5.4.2 Amplicon Sequence Variant

Advances in the analysis of amplicon sequence datasets have caused a methodological shift in how microbial diversity should be investigated, moving away from the classification and downstream analyses of traditional operational taxonomic units (OTUs) and towards the usage of amplicon sequence variants (ASVs) (Amir, 2021). Research supports the use of ASVs when analysing alpha-diversity patterns and species-rich environmental samples such as soil samples (Chiarello et al., 2022; Nearing et al., 2018).

The ASV approach differs from OTU clustering by determining the exact sequences and the frequency of reads for each sequence. Utilising the exact sequences allows results to be readily compared between studies using the same target region. Additionally, a given ASV can be compared to a reference database at a much higher resolution than OTUs, allowing for more precise taxonomic identification (Chiarello et al., 2022). Therefore, the ASV taxonomic approach was chosen in the current study.

1.5.5 Metadata analysis

In all microbiome research, it is essential to take note of the environmental parameters in every environment of interest. When exploring the microbiome of perennial ryegrass pastures from multiple locations in New Zealand, each location experiences different environmental conditions, for example, temperature, annual rainfall, soil nutrients and soil type. Collecting and recording environmental data is vital when looking for trends in microbial composition to explore whether the location has a causative effect. For this reason, metadata for each sample site and plot, including soil nutrients, soil type, and climate data, were measured to improve project validity by catering for other factors that could influence ryegrass productivity and the microbiome.

Pasture yield (kg DM/ha) is a measure of pasture productivity and persistence. Pasture yield data was measured during the three-year trial to understand trends across the four

sites. Ryegrass production across New Zealand can fluctuate depending on the season, highlighting the need to capture the seasonal and annual breakdown of the ryegrass dry matter (DM) production. Considering the pasture production data for the four sampling sites suggested which sites could have a more optimised microbiome for pasture growth.

1.6 Project aims and scope

As outlined, perennial ryegrass persistence is a major problem affecting the New Zealand dairy farming industry. Knowledge gaps remain regarding the ryegrass microbiome and the influence of farming location and management practices. An understanding of the types of fungi and bacteria that live in association with perennial ryegrass would provide a valuable stepping-stone in pasture persistence research. Although it is known that pasture persistence varies between farming regions, it remains unknown whether that is also the case for the perennial ryegrass microbiome.

To investigate this, this project aimed to explore the five microbiome habitats of the perennial ryegrass cultivar/*Epichloë* combination, One50 AR37, across four significant farming locations in New Zealand. One50 AR37 is a commercial ryegrass combination used as a standard by the NZ Plant Breeding and Research Association (NZPBRA) as part of the New Zealand National Forage Variety Trials (NFVT). The four farming sites, Cambridge, Havelock North, Palmerston North, and Burnham, have different environmental conditions, management practices and perennial ryegrass pasture productivity. Climate and environmental factors are critical drivers of microbial distribution worldwide, following the famous statement that "everything is everywhere and the environment selects" (Becking, 1934). Therefore, the core hypothesis of this project was that perennial ryegrass would demonstrate site-specific trends, with a subset of unique taxa at each sampling site and significant differences in the bacterial and fungal communities. The observation of site-specific trends and the lack of a core perennial ryegrass microbiome would validate whether the microbiome has the potential to explain regional differences in ryegrass productivity and persistence. Microbiome research aims to guide the future manipulation of these bacterial and fungal communities to promote pasture productivity and persistence for New Zealand dairy farmers.

Unfortunately, details regarding the management of the Burnham sampling site were discovered two months before the submission of this thesis. Unlike the three North Island sites, the Burnham site was mown instead of grazed by livestock during the trial. It is recognised that this management difference reduced the credibility of the project design, which was purposed to examine location-specific trends across the perennial ryegrass microbiome. Mowing pasture instead of grazing would have changed the microbial inputs to the pasture (e.g., grazing livestock introduces microbe-rich faeces). Although there was no time to restart the analysis, sections 3.41 and 3.42 were added to the results chapter to analyse the site-specific trends, excluding the Burnham site. Most of the results still include the Burnham site, with significant consideration of the different management this site received and its probable influence on the associated microbiome trends (discussion sections 4.2.1. and 4.4).

2 Methodology

2.1 Site Selection

Four perennial ryegrass trial sites across New Zealand were chosen for microbiome comparison. These trial sites were chosen to represent the common dairy farming locations across New Zealand and to gain a geographical contrast in soil types and environmental conditions. One South Island, and three North Island sites, were selected due to pasture persistence being a more significant problem for North Island farmers. The trial sites were governed by the NZ Plant Breeding and Research Association (NZPBRA) as a part of the network of National Forage Variety Trials (NFVT). The four trial sites were: Cambridge (Waikato), Havelock North (Hawkes Bay), Palmerston North (Manawatu), and Burnham (Canterbury) (Figure 2.1).

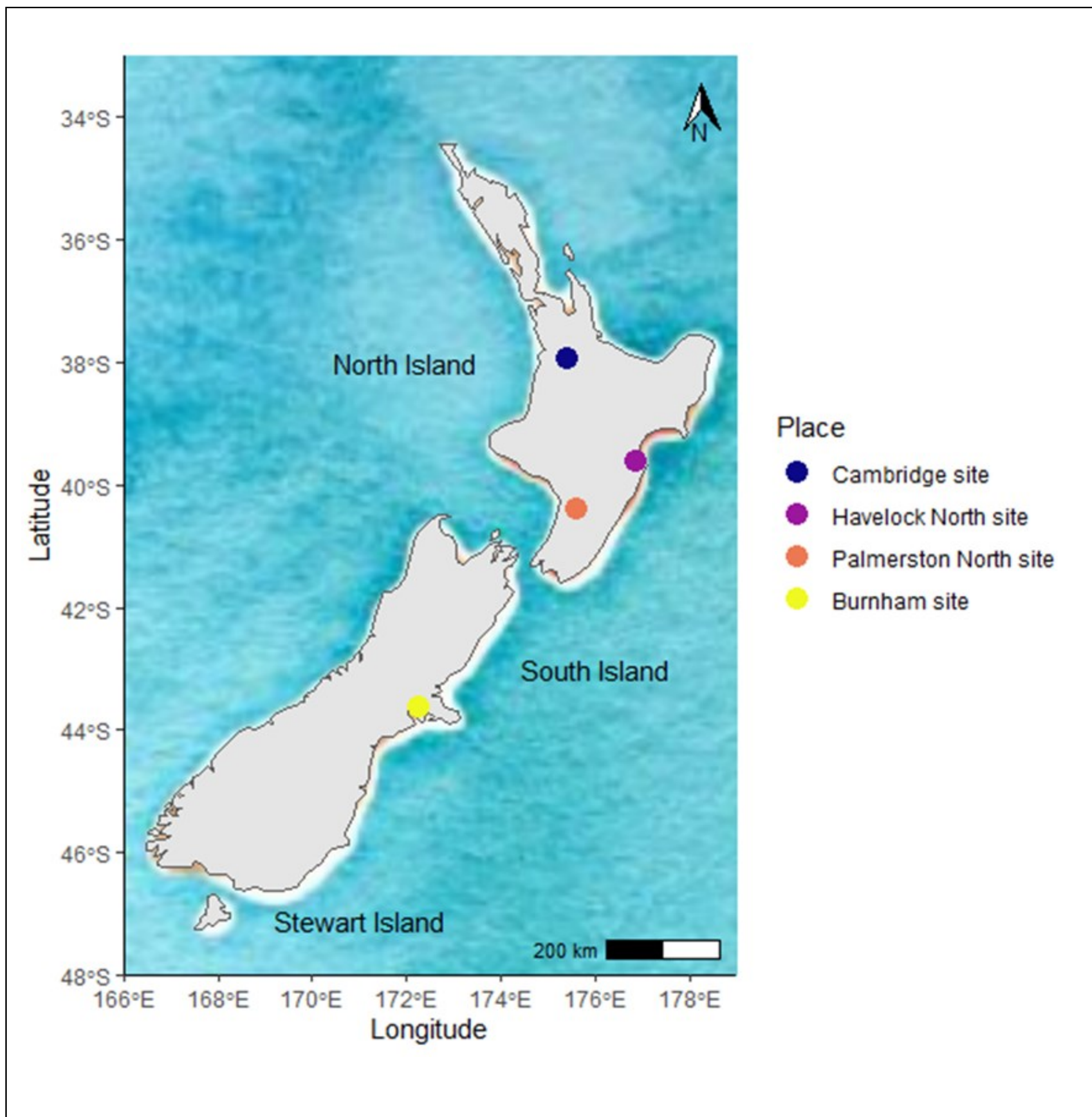


Figure 2.1 Map of New Zealand showing the four NFVT trial locations (Cambridge, Havelock North, Palmerston North, and Burnham) sampled for the current project.

2.2 Experimental design

The NFVT plots were set out in randomised blocks containing 10 different cultivar-*Epichloë* combinations. The current project only sampled the four replicates of the One50 AR37 ryegrass combinations from these plot sites. By randomly allocating the sowing positions of the One50 AR37 ryegrass, it catered for spatial differences in the soil type, pests, elevation, and moisture retention ability along the plot blocks.

2.2.1 Ryegrass plot management

The NZPBRA managed the perennial ryegrass plots at each sampling site. Defoliation of the plots at each site was required after yield sampling, which occurred on average ten times per year (refer to yield sampling details in section 2.8.2). All three North-Island sites were defoliated through livestock grazing: Cambridge and Palmerston North by cows and Havelock North by sheep. The Burnham site ryegrass was manually defoliated with a mower instead of being grazed by livestock throughout the trial. Mowing and grazing achieved the same purpose for defoliating the ryegrass; however, grazing adds nutrient inputs from dung and urine, and livestock treading during grazing can alter the soil structure. The Burnham site was the only site to receive irrigation over the summer months of the three-year trial, replicating the typical Canterbury irrigated dairy system. Both mowing and irrigation became highly important to consider when exploring the microbiome. The plots at all four sites received nitrogen fertilisers during the trial, with the Cambridge site receiving the highest – 446 kgs/ha/year (Table 2.1).

Table 2.1 Defoliation, irrigation, and nitrogen inputs for the four NZPBRA trial sites in the current study.

NZPBRA Trial Site	Summer irrigation	Defoliation	Quantity of Nitrogen applied per year (kgs/ha)	Type of Nitrogen applied
Cambridge	No	Grazed by cows	446	SustaiN
Havelock North	No	Grazed by sheep	154	SustaiN and CropZeal20
Palmerston North	No	Grazed by cows	360	SustaiN
Burnham	Yes	Mown	344	Urea, ammonium sulphate, nitrophoska, cropmaster

2.3 Field sample collection

The field sampling followed the same protocol for each site, with a few minor adjustments described. Each plot replicate was marked with a flag to avoid confusion from neighbouring plots. All equipment, including metal rings, scissors, spades, and soil coring devices, were sprayed and cleaned thoroughly with 70 % ethanol between each plot replicate. Gloves were worn and replaced between each replicate to limit cross-contamination between each plot and human microorganisms.

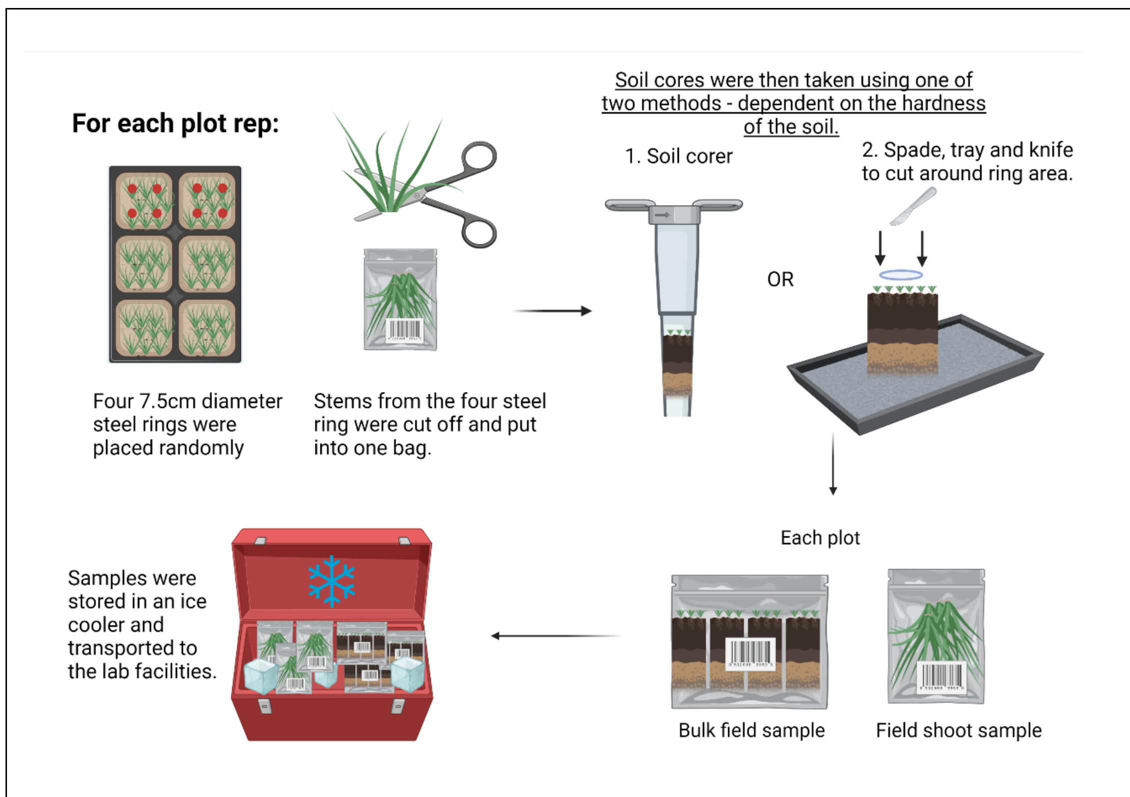


Figure 2.2 Annotation of the field sampling method utilised to obtain the ryegrass stem and soil samples from the four sampling sites across New Zealand. Diagram made using Biorender.com.

For the four One50 AR37 replicates at each sampling site, four sterilised metal rings (7.5 cm diameter) were randomly tossed onto the plot area to limit sampling bias (Figure 2.2). At the location the metal rings landed, the rings were fitted around the base of the ryegrass plant/s, and sterilised scissors were used to carefully cut off the shoots at around 2 cm above the soil to avoid contamination with soil particles. The four ryegrass shoot cut-offs from each plot replicate were pooled and placed into a sterile plastic zip lock bag.

Once shoots were removed from each marked-out core area, cores (7.5 cm diameter x 10 cm deep) were taken from each ring using one of two methods.

A) The first method involved using a soil corer to extract the soil cores. This method was used when the soils at the sites were relatively moist and not compacted. The cores were removed by physically forcing and twisting the corer into the ground to a 10 cm depth (marked by a red line on the corer). Once lodged into the ground, the corer was pulled out, and the core was pushed straight into a sterile plastic Ziplock bag.
B) The second method was the backup method utilised for the Burnham and Havelock North sites when the soil was hard, compacted, or stony. This method involved a spade marked with a 10 cm line to dig out a square of soil around the marked soil core area (Figure 2.3). The soil square was dug out and placed onto a sterile tray. A sterilised knife was used to cut around the metal sore core area to produce a circular core, which was then placed into a sterile Ziplock bag.

Figure 2.3: Photo of myself (Bernadette) and Mark McNeil (AgResearch) demonstrating the spade, tray and knife method (B) for extracting 10cm cores at the NFVT Burnham site.

Once four core samples were extracted from each replicate, the cores were combined as one bulk field sample. The bulk field sample bag was stored in a chilly bin with icepacks, for no longer than half a day, before being relocated into a cool store (4℃).

2.4 Sample processing

All samples were processed within three days of arrival at the laboratory. Samples were split into the five microbiome habitats; bulk soil, rhizosphere, root endosphere, shoot endosphere and phyllosphere (Figure 2.4), taking care to avoid cross-contamination. Once processed, all five ryegrass sub-sample types were stored in a -80 ℃ freezer.

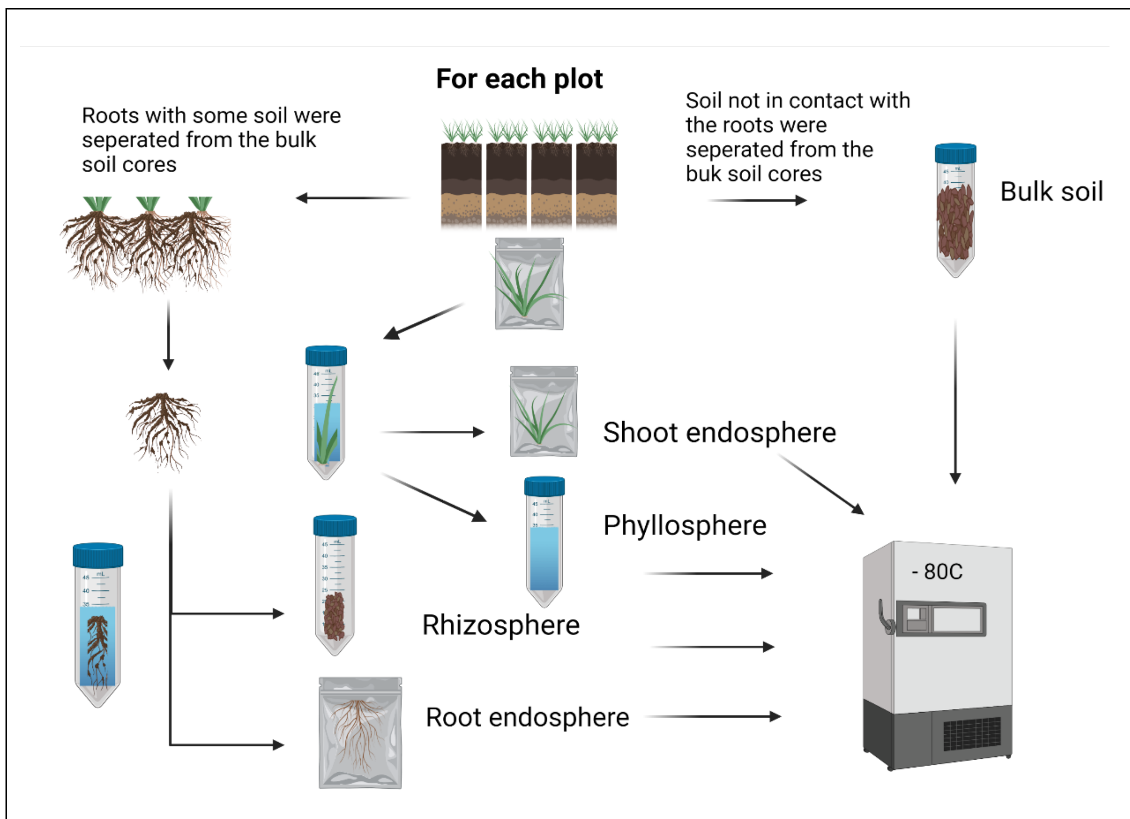


Figure 2.4: Annotated diagram displaying the sample processing overview used in the current project.

2.4.1 Bulk soil

Once arriving at the laboratory, the bulk field samples contained ryegrass cores comprised of shoots, roots, and soil. These bulk samples were taken out from their bags and emptied onto a sterilised tray, where they were processed into the three microbiome habitats: bulk soil, rhizosphere, and root endosphere.

To obtain the bulk soil samples, soil that was not in direct contact with the roots was scooped into a labelled 50 ml Falcon tube until the tube was roughly ¾ full. The Falcon tubes containing the bulk soil were rinsed with 70% ethanol and agitated for 60 seconds before rinsing with distilled water three times, giving the finished ryegrass bulk soil samples.

2.4.2 Root endosphere and rhizosphere

Roots were separated from the bulk field samples to obtain the root endosphere and rhizosphere samples. These root samples (with some soil still attached) were placed into labelled 50 ml Falcon tubes (Tube 1), filling the tubes to just over halfway (loosely packed) (Figure 2.5).

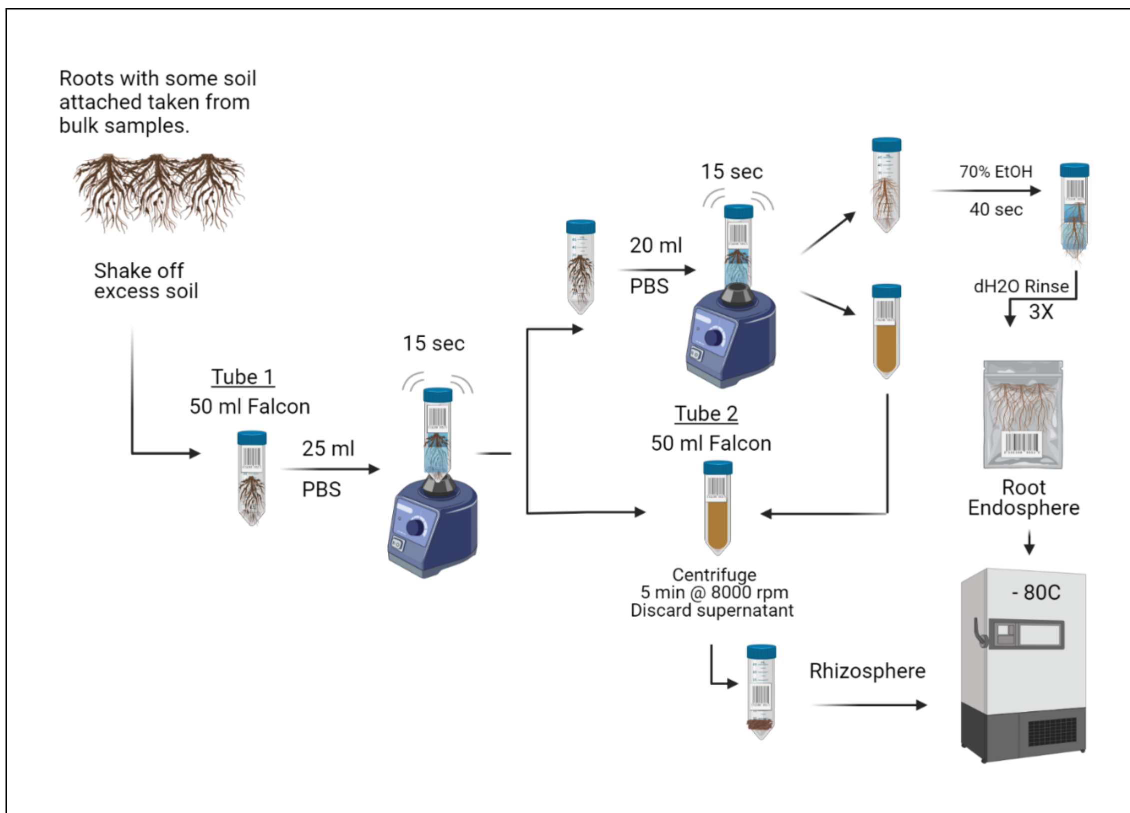


Figure 2.5 Annotated diagram of the sample processing method used to obtain the root endosphere and rhizosphere sample. This process was repeated for the four-bulk sample One50 AR37 reps at each ryegrass plot location. Diagram made using Biorender.com.

Twenty-five mls of phosphate-buffered saline (PBS) was added to the falcon tube (Tube 1) containing the roots and some rhizosphere soil before vortexing for 15 seconds. The liquid supernatant from this tube was poured into another sterile 50 ml Falcon tube (Tube 2). Another 20 mls of PBS was added to Tube 1 before vortexing for another 15 seconds (acting as a second rinse to remove the soil attached to the roots). The liquid supernatant was added to Tube 2, containing the previous supernatant.

Tube 2 containing the supernatant was centrifuged for 5 minutes at 8000 rpm. This produced a soil pellet at the bottom of the tube with liquid floating above. The supernatant liquid could then be poured off carefully, leaving the remaining soil pellet in the Falcon tube. Tube 2, containing the soil pellet, represented the finished ryegrass rhizosphere sample ready for DNA extraction.

The roots remaining in Tube 1 were rinsed using 70% ethanol. This involved adding ethanol to the tube until the roots were fully submerged and then lightly shaking the tube for 60 seconds. The ethanol was then drained off, and the roots were rinsed three times with distilled (sterilised) water. After the rinsing, the processed ryegrass root samples were ready for DNA extraction.

2.4.3 Shoot endosphere and phyllosphere

The ryegrass shoots endosphere, and phyllosphere sub-samples were extracted from the field shoot samples (see section 2.3). The shoot samples were processed in a PC2 laboratory within two days of being collected from the field.

Five-gram sub-samples of ryegrass shoots from the field shoot sample were added to a ziplock bag with 15 mls of PBS and lightly shaken for 1 minute to remove any loosely attached microorganisms (likely contaminants) and soil particles (Figure 2.7). The rinsed shoots were added to 50 ml glass tubes (30 mm x 114 mm) containing 30 mls of PBS. It was essential to ensure the ryegrass shoots were placed into the tube with the cut ends facing upwards and the shoot tips facing downwards to avoid contaminating the PBS medium with endosphere microorganisms (Figure 2.6).

Figure 2.6: An image of the glass tube set up before sonication. Note that the ryegrass cut ends are facing upwards, with the uncut ends of the shoot facing down.

The shoots were transferred to the glass tubes using sterilised metal tweezers. Between each sample, the tweezers were sterilised by dipping the tips into 70 % ethanol and heating under a Bunsen burner blue flame for 5 seconds (Bykowski & Stevenson, 2020). The glass tube samples were secured in a plastic holding rack and placed into a pre-set-up sonicator bath - two-thirds full of room temperature water (Figure 2.7). In each cycle, no more than 20 glass tubes were placed into the sonicator. The sonicator was turned on for 5 minutes. This vibrated the ryegrass tubes and the water, allowing any microorganisms attached to the outer surface of the ryegrass leaves to become dislodged into the liquid PBS medium.

After sonication, the ryegrass shoots were removed from the glass tubes using sterilised metal tweezers and placed into clear plastic bags. The shoots were rinsed with 70 % ethanol, agitated for 60 seconds, and rinsed three times again with distilled water. This resulted in the finished shoot endosphere samples before DNA extraction.

The liquid remaining in the glass tubes was filtered using filter lids into 50ml Falcon tubes. This was to remove any free-floating plant debris or soil. The Falcon tube containing the liquid was centrifuged for 30 mins at 3200 rpm (Figure 2.7). The tube was then centrifuged for a subsequent 30 seconds at 1000 rpm. The supernatant was drained off and discarded, leaving a white pellet on the side of the centrifuge tube. 270 µL of PBS was added, and the Falcon tube was vortexed to resuspend the pellet. 270 µL of the remaining solution was then pipetted into a clean, sterile 1ml tube. This resulted in the finished phyllosphere samples before DNA extraction.

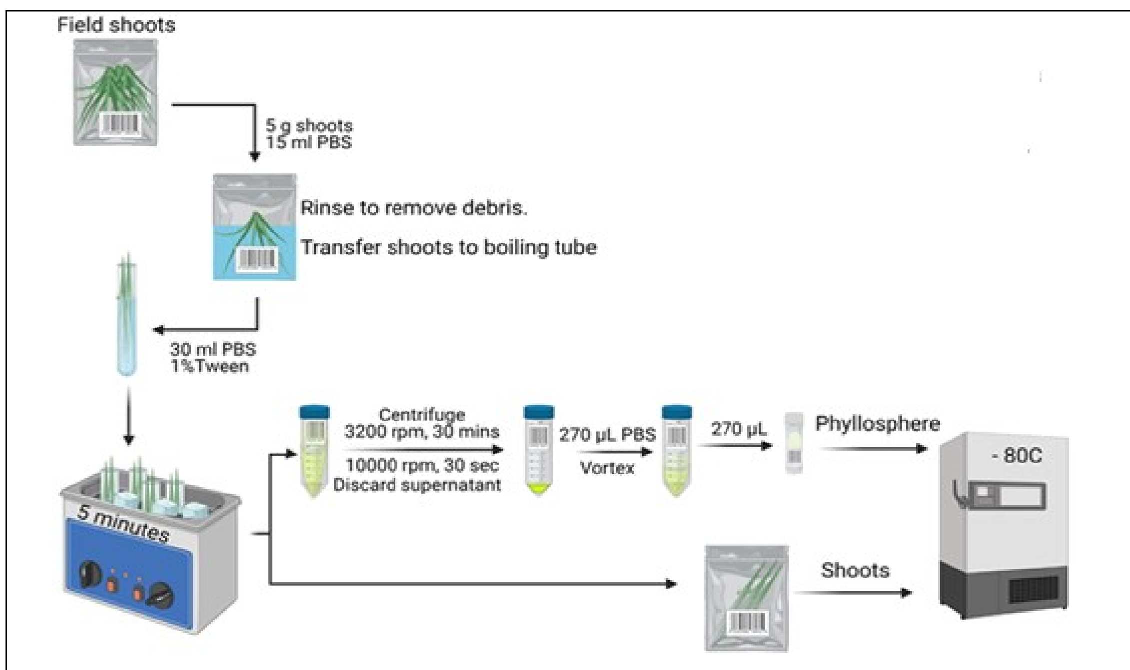


Figure 2.7: Annotated diagram of the sample processing method used to obtain the shoot endosphere and phyllosphere samples. Diagram made using Biorender.com.

2.5 DNA extraction

The prepared shoot and root endosphere sub-samples were freeze-dried, ground for 1 min 30 sec and 1 min respectively at 1,500 rpm (Geno/Grinder SPX 2010-230 with two steel balls sizes 11 mm and 13 mm in each sample) and, a 50 mg subsample taken for DNA extraction. DNA extraction was achieved using the Qiagen DNeasy PowerSoil Pro Kit for all five perennial ryegrass sample types. The DNA was extracted following the standard handbook procedure as of March 2021 (see supplementary material) with minor alterations listed below.

- The weight of the shoot and root material: Compared to the 250 mg of soil recommended for step 1, only 50 mg of ground root and shoot material was used for DNA extraction because the root and shoots were denser and more absorbent than soil.

- Bead-beating: The timeframe was altered from 10 to 5 mins to lessen the chance of DNA shearing.
- Phyllosphere: Before the first step, to maximise DNA concentration, the phyllosphere subsamples were pre-incubated at 60°C for 10 mins before centrifuging for one minute to concentrate the sample as much as possible.
- Phyllosphere: The elusion step used 25 ul of solution C6 (elution solution). This was because the phyllosphere samples had lower DNA concentrations.

DNA extraction was repeated for eight of the ryegrass phyllosphere samples due to low DNA concentrations (>20 ng/µL) in the initial extraction. The highest of the DNA samples from the two extraction attempts was used as the final sample.

The DNA concentration was standardised using dilutions to an end volume of 20 ng/µL with DNA concentrations of 300 ng. Some phyllosphere samples (could not meet this required concentration due to their low DNA concentrations. These samples were concentrated as close to 20 ng/µL as possible.

2.6 PCR amplification and Sequencing

A total of 80 DNA samples were sent to Custom science for 16S and ITS PCR and sequencing. These 80 DNA samples included the five different ryegrass microbiome habitats for the four replicates at each sampling site (Cambridge, Havelock North, Palmerston North and Burnham).

To explore the type of bacteria within the 80 samples, PCR was used to amplify the V5 and V7 regions of the 16S rRNA gene from the extracted DNA using the primer sets 799F (5'-AACMGGATTAGATACCCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3'). To explore the types of fungi present within the 80 samples, PCR was used to amplify the ITS1 region of the ITS rRNA gene from extracted DNA using the primer set ITS5-1737F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2-2043R (5'-GCTGCGTTCTTCATCGATGC-3').

Each PCR reaction contained 5 × PCR buffer (KOD FX Neo), 2 × 2 mM dNTPs (Custom Science, China), 0.3 µM of each 10 µM primer, 1 PCR enzyme (KOD FX Neo - TOYOBO), 0.5 µM MgCl2, 50 ng± 20% of DNA and the reaction was made up to 10 µL with ddH2O. PCR reactions were completed on a Solexa PCR thermocycler under the following thermal cycling conditions: 98°C for 5 min, then 25 cycles of 95°C for 30 sec, 51°C for 30 sec, 72°C for 40 sec, and a final extension of 72°C for 7 min. The PCR product concentrations were checked using ImageJ Software. The samples were pooled according to their concentrations, and the pooled products were purified on an OMEGA DNA column (1.8% Agarose gel - 120V for 40 mins).

DNA sequencing was undertaken at Custom Science. Libraries were sequenced on a paired-end Illumina platform to generate 250 bp paired-end raw reads. No less than 50K pairs of reads were generated per amplicon product.

78 samples for 16S and 78 samples for ITS1 successfully produced >50k reads after sequencing. The two samples that did not generate enough reads were low DNA concentration phyllosphere samples - rep 2 and 4 Burnham (16S), rep 4 Palmerston North

and rep 4 Burnham (ITS). Due to the failed sequencing, these samples were not included in the microbiome analysis.

2.7 Data processing and statistical analysis

2.7.1 16S DADA2 pipeline

The raw reads were trimmed, paired, error-corrected, and associated with representative Amplicon Sequence Variants (ASVs) and chimaera-filtered using the DADA2 pipeline (Callahan et al., 2016) following the pipeline tutorial as of 2022 (https://benjjneb.github.io/dada2/tutorial.html). All ASVs with less than 10 copies were discarded to avoid inflating the number of ASVs by including transient DNA and possible sequencing errors. The taxonomic assignments of the ASVs were obtained using the Qiime "assign_taxonomy" program with standard parameters (Caporaso et al., 2010) and the Silva database version 138 (Quast et al., 2013).

2.7.2 ITS DADA2 pipeline.

The ITS region has enough homology in plant genomes to be amplified using the selected primers, especially in material obtained from leaves or plant shoots. To remove background reads originating from the plant, all reads were mapped to the ryegrass genome using BBMap, and only those without significant homology were kept and further processed.

The DADA2 pipeline was implemented as suggested in DADA2 ITS Pipeline Workflow (1.8) (benjjneb.github.io) as of the 2022 version. ITS amplicons do not have a constant length, so the length variation was considered in the data processing. Long read primers of the other end can be included in the sequence. For this, primer removal was included as read-through can occur in short amplicons. Primer sequences were defined, and the program Cutadapt was used to remove them from the reads before quality control, trimming, assembly, dereplication and chimera removal. Like the 16S, the final ASV table was filtered by keeping only representatives with 10 or more copies.

Initially, the fungi UNITE database was used with Qiime; however, ITS amplicon sequencing is notorious for off-target amplification, causing some contamination in the dataset, such as plant DNA. The problem with the UNITE database was that contaminant plant DNA was occasionally falsely labelled as the closest matched fungal sequence or was not recognised by the library. Therefore, to evaluate not only fungi but also possible sources of contamination, the ASVs were blasted to the NCBI nt database, collecting the top seven matches. To select the taxonomic match, one target was selected first by frequency (appearing most times), sorted by alignment quality and length, and used to associate the query with the target's taxonomic lineage.

2.7.3 Data/statistical analysis of the perennial ryegrass microbiome

Taxonomic assignments were provided at the Kingdom, Phylum, Class, Order, Family, and Genus levels to process the sequencing reads. The species level was analysed for the ITS dataset; however, most of the results section only explored the fungal ASVs to the genus level due to many species being labelled as uncultured genera. The sequencing

data was statistically analysed using R (ver 3.4.3) (R Core Team 2017) and several R packages, including phyloseq (McMurdie & Holmes, 2012), ggplot2 (Wickham & Wickham, 2007), vegan (Oksanen et al., 2013), dplyr (Wickham et al., 2014), reshape2 (Wickham, 2016), tidyverse (Wickham, 2017), microbiomeSeq (Ssekagiri et al., 2017), microbiome(Lahti & Shetty, 2018), micorbiomeutilities(Shetty et al., 2018), fantaxtic (Dixon, 2003), RColorBrewer (Neuwirth & Neuwirth, 2014), phylosmith (Smith, 2019), AICmodavg (Mazerolle & Mazerolle, 2017). Taxonomy data was merged with the ASV.csv and metadata.csv tables to create a phyloseq object in R studio.

Sequence reads were rarefied on a per-sample basis to a minimum library sequencing read depth (12,000 for the 16S bacterial dataset and 7500 for the ITS1 fungal data set) before alpha diversity analyses. In the ITS1 dataset, unclassified fungal ASVs were removed from the dataset at the beginning of the analysis due to low confidence that these ASVs represented true fungal taxa. In the ITS dataset, the low number of reads in the shoot endosphere samples indicated a niche effect of the shoot endosphere habitat with lower richness and diversity. If the shoot endosphere samples were included in the dataset, it would have required a minimum read depth of 1500, which would not have adequately sampled the variation across the soil samples. Therefore, the shoot endosphere samples were not included in the alpha diversity analysis. This allowed rarefying to 7500 reads for the ITS dataset, removing two low-read root endosphere samples, leaving only three reps instead of four for the Palmerston North and Cambridge root endosphere samples. Alpha diversity metrics were calculated to summarize the structure of the bacterial and fungal communities concerning the richness (number of taxonomic groups), evenness (distribution of abundances of the groups), and diversity between samples. The rarefied datasets were only used for the alpha diversity analysis.

The subsequent data analysis was implemented on a normalized dataset by looking at the relative abundance of ASVs. Sub-setting of the data was performed after observing trends in the bacterial and fungal data, including splitting the microbiome habitats into the above-ground and below-ground regions. Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity was chosen for computing dissimilarity between the bacterial and fungal communities (Zorz, 2020). Bray-Curtis dissimilarity accommodates the presence of double zeros (absence) and enables the results of this study to be compared against many other studies (Ricotta & Podani, 2017). Permutational multivariate analysis of variance (PERMANOVA) was conducted using the 'Adonis' R function to explore the trends across the different microbiome habitats and the effect of the sampling site (Kelly et al., 2015). Taxa plots were produced based on the relative abundance of main taxa at the genus level. Alongside the taxa plots, box and whisker graphs were made for taxa of interest and p-values were computed using a Wilcox test by implementing the stat_compare_means (method = 'Wilcox') R function.

Two taxa of interest (*Bacillus* and *Epichloë*) were plotted at the ASV level. An R function 'Envfit' was used to analyse the influence of environmental variables on the perennial ryegrass microbiome by fitting environmental vectors and factors onto an NMDS ordination (Zorz, 2020). Mantel tests using the Pearson product-moment correlation

coefficient were completed to assess the correlation between the microbial communities and environmental distance matrices (Oksanen, 2009).

2.8 Metadata acquisition and analysis

2.8.1 Climate data and annual rainfall

Climate data, including average temperature and annual rainfall, were retrieved from the National Institute of Water and Atmospheric Research (NIWA) for each of the four plot locations. The data was analysed using Microsoft Excel and R studio. The average monthly maximum temperature, minimum temperature and rainfall for each site were calculated for the 25 years before the sowing date of the plots (Feb 1993 - March 2018) and the 3-year NFVT trial period - from the sowing date up until the month of sampling - (April 2018 - May 2021). Seasonal rainfall and temperature trends were calculated using the Forage Value Index (FVI) approach for classifying seasons. This method determined the Cambridge, Havelock North and Palmerston North sites as Upper North Island/Lower North Island (UNI/LNI) sites and the Burnham site as Upper North Island/Lower South Island (USI/LSI), following Chapman et al. (2017). One-way ANOVA models were computed to determine differences in the average rainfall and temperatures between the sites. Tukey tests were used to compare the maximum and minimum winter temperatures and the average early spring rainfall across the four sites.

2.8.2 Perennial ryegrass production data

For the four different sampling sites, ryegrass pasture production cut data (kg DM/ha) was measured and recorded by NZPBRA for the three years of the trial (April 2018 – May 2021), and the harvesting methods described here are theirs. The ryegrass plots were harvested for production cut data when the above-ground herbage mass of the highest-yielding plot reached 2,500 kg DM/ha and before it exceeded 3,000 kg DM/ha. Defoliation at lower than 2500 kg/ha was sometimes necessary if the highest-yielding plot reached canopy closure. During summer (November to January), plots were harvested every 45 days or when the highest yielding plot reached 2,500 – 3,000 kg DM/ha, whichever came first, to minimise the ryegrass plants reseeding. On average, trials were sampled for production cut data ten times a year.

Defoliation was achieved by mowing a single strip in each plot to 5 cm. The location of the mowing strip was changed at each production cut date to avoid altering the natural ryegrass plant growth characteristics. Plots were mown, and a sub-sample was collected for processing in the laboratory for the percentage of dry matter (DM). The dry matter yield (DM kg/ha) was calculated by comparing the dry weight of the perennial ryegrass subsample after oven drying in the oven at (95 °C for 36 hours) with the wet weight measurement and applying this to the mown sample fresh weight.

Annual ryegrass production data was calculated for each trial year as an average of the four reps at each sampling site. A one-way ANOVA was performed on the different production data parameters, including the annual, perennial ryegrass production for each year of the trial across the four different reps at each site. A Tukey test was performed to determine the sites with significantly different DM production.

2.8.3 *Epichloë endophyte testing*

Epichloë endophyte testing was conducted for the shoot samples from the four reps at each sampling site (16 samples). This required weighing 250mg (5 x 50mg) of each pre-ground ryegrass shoot sample (see previous section 2.5) into sterile vials. From these 250 mg ground shoot samples, plant and endophyte whole genomic DNA were isolated using a FastDNA Kit (Q-Biogene Inc.) for plant tissue per the manufacturer's instructions. To type the DNA extracts for their endophyte strains, the methods from (Faville et al., 2020) were followed. This involved utilising the simple sequence repeat (SSR) marker - B11 (Moon et al., 1999) – to discriminate the AR37 endophyte strain from other commercial *Epichloë* endophytes in New Zealand (e.g. SE -standard endophyte), based on the size of the amplicon. Electropherograms were analysed, and the fragments were sized against the GeneMarker v1.75 software (SoftGenetics LLC).

2.8.4 *Soil nutrient analysis*

Bulk soil subsamples for each site were sent to Hill laboratories to undergo nutrient analysis. There were 16 bulk soil samples sent for nutrient sampling, including a sample from each of the four reps at each site. The analysis provided information on many variables, including pH (pH Units), Olsen Phosphorus (mg/L), Sulphate Sulphur (mg/kg), Extractable Organic Sulphur (mg/kg), Potentially Available Nitrogen (15cm Depth) (kg/ha), Anaerobically Mineralisable N (µg/g), Anaerobically Mineralisable N/Total N Ratio (%), Organic Matter (%), C/N Ratio, Ammonium-N (mg/kg), Nitrate-N (mg/kg), Mineral N (sum) (mg/kg), Total Carbon (%), Hot Water Extractable Carbon (mg/kg), Total Nitrogen (%), Dry Matter (%), Moisture (%), Sample Temperature on Arrival (°C), Potassium (me/100g), Calcium (me/100g), Magnesium (me/100g), Sodium(me/100g), CEC (me/100g), Total Base Saturation (%) and Volume Weight (g/ML).

For details on the methods used to analyse each component, refer to the hill laboratory technical note (as of May 2021) for the methodology behind each soil test, Technical Notes | Hill Laboratories - NZ (hill-laboratories.com).

The soil nutrient variables were analysed for variation across the four sampling sites (ANOVA). A Tukey test, and an additional ANOVA model (with the Cambridge site removed), were used to compare the; organic matter, extractable organic sulphur, C/N ratio, total carbon, total nitrogen, and soil moisture at the Cambridge site to the three other sites. The average total nematodes for the four bulk soil reps at each sampling site were compared across all four sampling sites (ANOVA).

2.8.5 *Soil report*

The location of each site was used to match an S-map soil report from Manaaki Whenua Landcare Research, https://smap.landcareresearch.co.nz/. This analysis describes the typical average properties of a specified soil to a depth of 1 metre. This included critical physical properties, including soil classification/family, depth class, texture profile, potential rooting depth, topsoil stoniness, topsoil clay range, drainage class, aeration in the root zone, and permeability profile. It also examined core chemical properties (topsoil P retention) and additional factors to consider, including soil structure integrity.

2.8.6 Nematode analysis

Bulk soil subsamples were analysed for nematodes utilising the tray and beaker method following Bell and Watson (2001) and O'Callaghan et al. (2008). Nematodes were extracted from a 100 g sub-sample of soil from each bulk soil sample. The number of nematodes on each slide was counted to estimate the total nematode abundance. Each slide was examined in transects from left to right across the slide, beginning at a central transect and working towards the top and bottom edges of the slide. This was done to avoid bias towards smaller nematodes which tend to accumulate at the edges of the slide. The first 100 observable nematodes encountered on these transects were identified. Nematodes were classified into genera with up to 100 specimens per slide.

2.8.7 Statistical analysis methods for the metadata

Statistical metadata analysis in Excel was used for initial filtering and averaging the climate, annual rainfall, ryegrass production, soil nutrient, and nematode data. The data was imported into R studio (Version 1.4.1106) and R (Version 4.1.0 "Camp Pontanezen") (R Core Team, 2022) for statistical analysis using R functions: 'aov' and 'TukeyHSD' to compare the site averages statistically as mentioned above.

3 Results

3.1 Site analysis and environmental data

Before exploring the perennial ryegrass microbiome, it was first essential to pinpoint the conditions of the four sampling locations relating to the environmental conditions, pasture productivity, *Epichloë* infection, nutrient analysis, and soil characteristics. Characterising these parameters increased the ability to form accurate conclusions around the bacterial and fungal associations of perennial ryegrass One50 AR37.

3.1.1 Climate analysis

Cambridge had the highest peak summer temperatures (Jan-Feb) among the four sites, reaching average max temperatures above 25°C. All four sites experienced warmer on average temperatures, with at least a 1-3°C increase in the average monthly maximum and minimum temperatures in the 2018-2021 trial period compared to the long-term 25-year temperature trends (Figure 3.1). This temperature increase was distinct at the Burnham site, which showed a 5°C difference in the average monthly minimum temperature. Alongside this, all four sites also received 50 mm less rain in 2018-2021 compared to the 25-year average monthly rainfall (Figure 3.1). This change in average temperature and rainfall demonstrates the increased environmental stress perennial ryegrass plants are under today compared to the last 25 years. The microbiome response to these stresses should be reflected in this study because samples were taken after three years of plant growth.

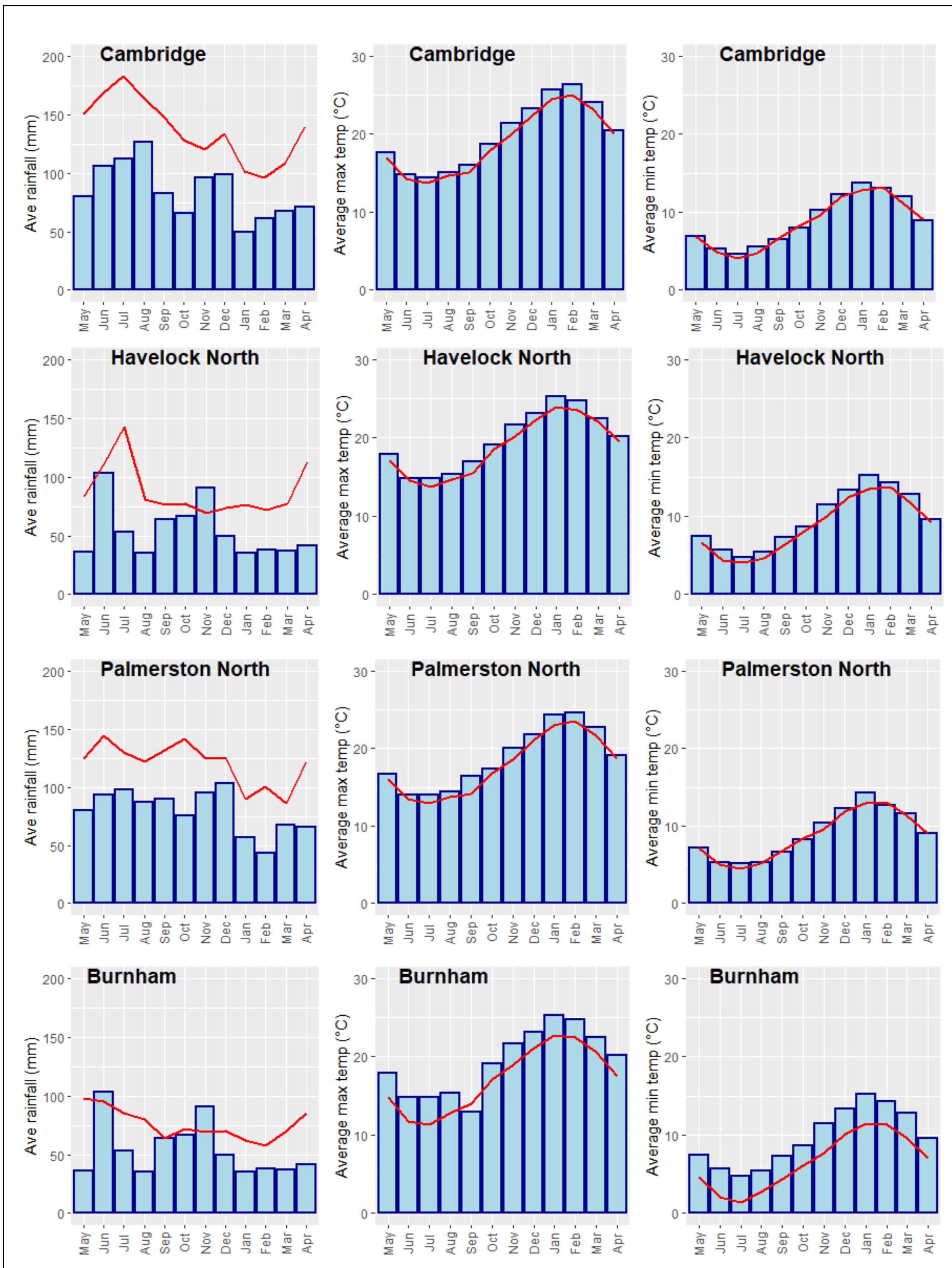


Figure 3.1 Average monthly maximum and minimum temperatures (°C) and average total monthly rainfall (mm) at the Cambridge, Havelock North, Palmerston North, and Burnham sites. The blue bars represent the average data recorded from when the plots were first established (May 2018) until the month of sampling (April 2021), while the red line represents the 25-year monthly average trend for the sites (March 1993 – April 2018).

Overall, the three North Island sites experienced warmer winter conditions during the three-year trial than the South Island Burnham site, with significantly different maximum and minimum winter temperatures (ANOVA p-value < 0.001) (Table 3.1). The results from a Tukey test confirmed that the Burnham site's winter temperatures contributed to this significant difference and that the three North Island sites' winter minimum and maximum temperatures were relatively homogenous (Tukey p-value >0.05). On average, the Cambridge and Palmerston North sites also received higher early spring rainfall than Havelock North and Burnham (Tukey p-value < 0.001). In addition to rainfall, the Burnham site ryegrass plots received summer irrigation (during December, January, and February), while the three other sites relied solely on rainfall throughout the trial period. The irrigation data was not recorded for the current study.

Table 3.1 Climate measurements for the four sites of interest throughout the trial (April 2018-May, 2021). Mean seasonal rainfall (Autumn, Early spring, Late spring, Summer, Winter) was calculated by averaging the total three-year rainfall to get a one-year average. Average temperature measurements were calculated by averaging the total max or min temps over each day of the trial period during the winter and summer seasons. A One-way ANOVA significance P > F codes (‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ NS (>0.005). Values significantly different from the values in each row are underlined (Tucky test output).

Climate measurement	Cambridge	Havelock North	Palmerston North	Burnham	P value significance.
Autumn Rainfall (mm)	200	116	177	165	(NS)
Winter Rainfall (mm)	160	114	150	118	(NS)
Early Spring Rainfall (mm)	**239**	89	**185**	69	**
Late Spring Rainfall (mm)	148	131	165	130	(NS)
Summer Rainfall (mm)	196	141	205	109	(NS)
Summer mean max temp (℃)	24	23	22	22	(NS)
Winter mean max temp (℃)	17	16	15	**12**	***
Summer mean min temp (℃)	12	13	12	11	(NS)
Winter mean min temp (℃)	6	6	6	**2**	***

3.1.2 Productivity of the perennial ryegrass plots

Visually, the perennial ryegrass plots at each site showed variable levels of pasture health and plant survival at sampling (Figure 3.2). The Havelock North site had the poorest visual ryegrass health, and the Palmerston North site was the best. The Burnham site

showed areas of poor plant survival in replicates 1 and 2, which was likely attributed to the high numbers of grass grub observed during sampling.

In preliminary trial notes from NZPBRA, the Cambridge site had low ryegrass plant survival (40% across the plots) in June 2020. Low plant survival is one of the indicators of poor ryegrass persistence.

Unfortunately, it must be reinforced that the Burnham ryegrass plots were under different defoliation management throughout the three-year trial (see Sections 2.2.1 and 3.4.1).

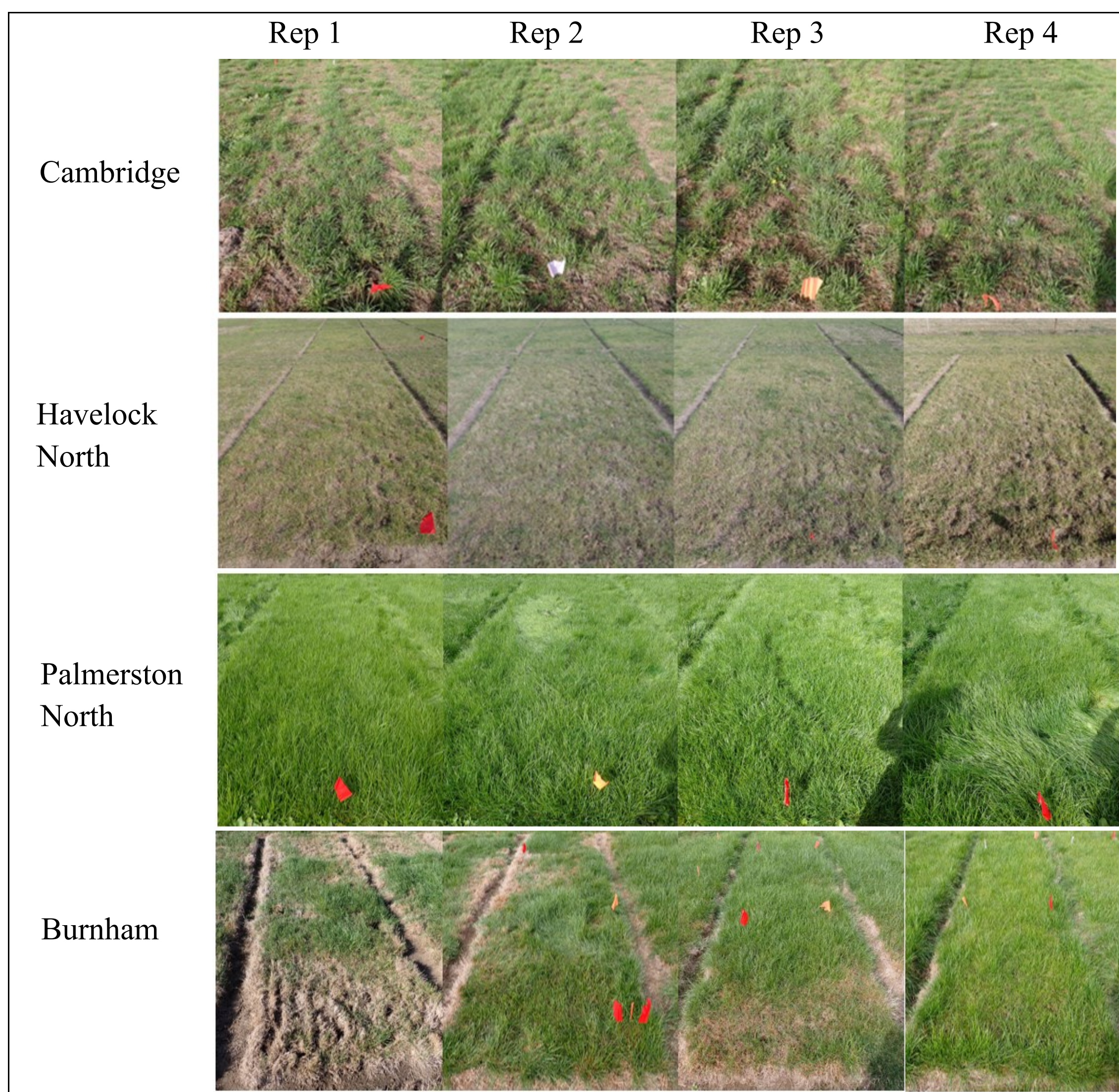


Figure 3.2 Photos taken on the sampling day for each of the four One50 AR37 replicates at the four National Forage Variety Trial sampling sites (Cambridge, Havelock North, Palmerston North, Burnham).

3.1.2.1 Dry matter production of the perennial ryegrass plots

Pasture yields were generally highest for all four sampling sites in late spring/summer, with the lowest yields occurring in winter/autumn (Figure 3.3). Annual yields of DM in the first year were highest at the Havelock North site (Table 3.2), which was attributed to the site's higher summer and autumn production (Figure 3.3). In year 2, ryegrass growth was more balanced across the four sampling locations, with slightly lowered ryegrass growth in early and late spring for the Cambridge site. Pasture production declined in

Autumn year 2 at the Havelock North site. The trial's final year (year 3) demonstrated higher ryegrass production at Burnham compared to the three other sites. Compared to the other sites, Havelock North exhibited lower ryegrass production during winter, early spring, and late spring in year 3 of the trial. At the time of sampling (Autumn - year 3), the Palmerston North site recorded the highest DM yield (Figure 3.3), and the plots had a superior visual appearance compared to the other three sites (Figure 3.2).

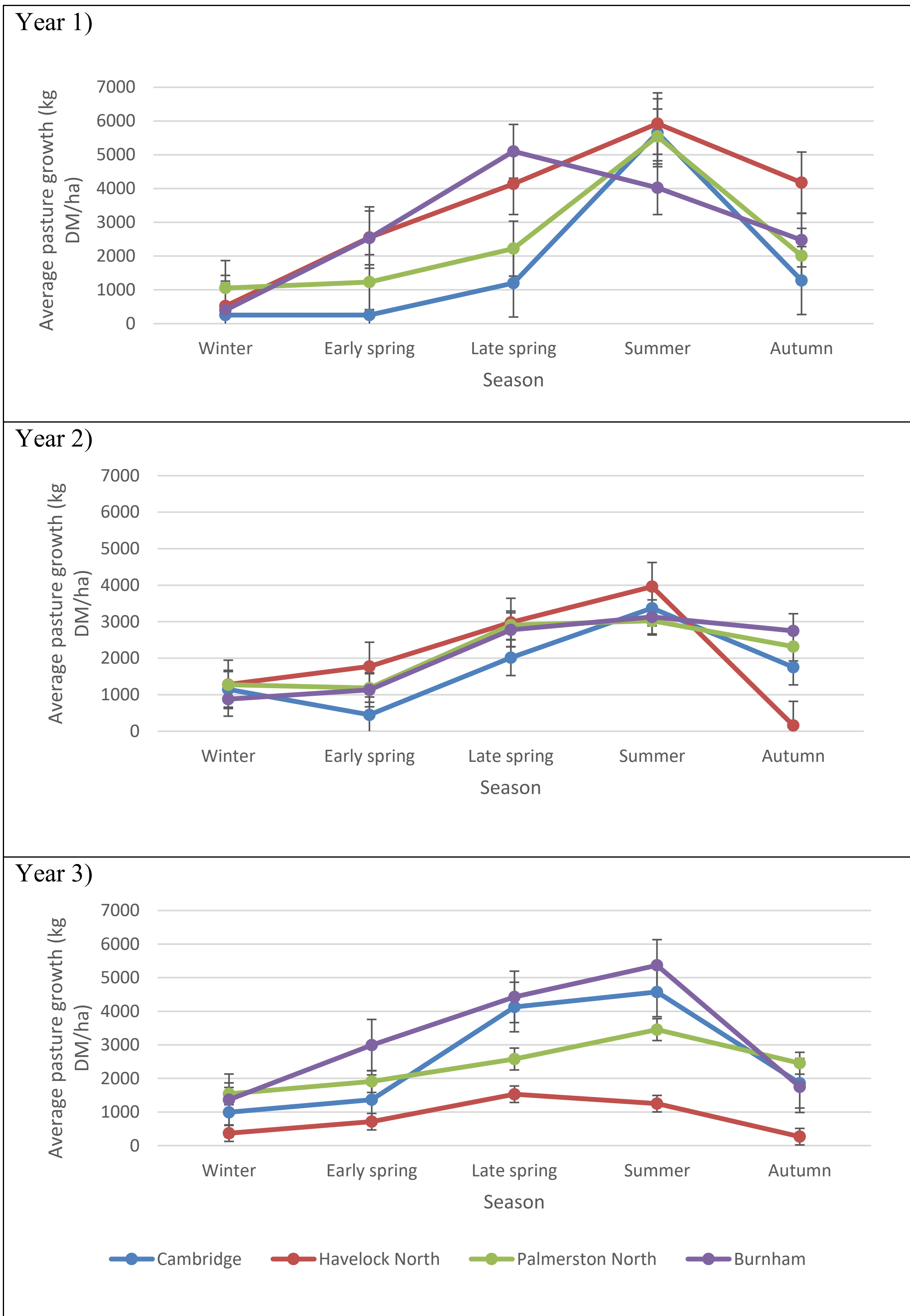


Figure 3.3: The average seasonal pasture production (kg DM/ha) of the four reps at the four sampling sites (Cambridge, Havelock North, Palmerston North, and Burnham) for each of the three years during the period from the plot sowing date up until the sampling date (April 2018-April 2021). Seasonal pasture production was calculated according to the Forage Value Index approach to classifying seasons Chapman et al. (2017).

Aside from the seasonal trends, the average annual ryegrass production was significantly different between the four sampling sites (ANOVA p-value < 0.05) (Table 3.2). The Burnham site had the highest average annual dry matter production of 13641 kg DM/ha, 2077 kgs higher than the Palmerston North site producing 11564 kg DM/ha (Table 3.2). A Tucky test confirmed that Palmerston North and Burnham had a significantly higher mean DM production than Cambridge and Havelock North (P < 0.05).

Table 3.2 The mean annual pasture production (kg DM/ha) for years 1-3 at the Cambridge, Havelock North, Palmerston North, and Burnham sampling sites. The mean production was calculated for the three years of the trial from the plot sowing date up until the sampling date (April 2018 - April 2021).

Year	Cambridge	Havelock North	Palmerston North	Burnham	P value significance
1	8644	17308	12055	14077	0.001 ***
2	8741	10158	10706	10241	0.05 *
3	12919	4129	11933	16606	0.001 ***
Mean	10101	10531	11564	13641	0.01 **

3.1.3 Epichloe endophyte testing

This study aimed to compare the microbiome of one perennial ryegrass cultivar/*Epichloë* combination, One50 AR37. Therefore, it was essential to test whether the perennial ryegrass plants contained the correct 'AR37' *Epichloë* at the end of the trial. It was confirmed that all 16 sampled One50 ryegrass plots contained the expected *Epichloë* 'AR37'; however, there was one plot rep each at the Cambridge and Burnham sites that were also contaminated with Standard *Epichloë* (SE) (Table 3.3). This contamination was important to consider when exploring the associated bacterial and fungal communities for unusual trends.

Table 3.3 The *Epichloë* endophyte results for each shoot DNA sample for the four One50 AR37 reps at the four sampling locations after simple sequence repeat analysis.

Sample	Site	Rep	Expected strain	Strains found
sa61	Cambridge	1	AR37	AR37
sa62	Cambridge	2	AR37	AR37
sa63	Cambridge	3	AR37	AR37
sa64	Cambridge	4	AR37	AR37 + SE
sa57	Havelock North	1	AR37	AR37
sa58	Havelock North	2	AR37	AR37
sa59	Havelock North	3	AR37	AR37
sa60	Havelock North	4	AR37	AR37
sa53	Palmerston North	1	AR37	AR37
sa54	Palmerston North	2	AR37	AR37
sa55	Palmerston North	3	AR37	AR37
sa56	Palmerston North	4	AR37	AR37
sa49	Burnham	1	AR37	AR37
sa50	Burnham	2	AR37	AR37
sa51	Burnham	3	AR37	AR37
sa52	Burnham	4	AR37	AR37 + SE

3.1.4 Soil nutrients

The nutrient content of the soil can contribute to differences in the associated microbiome of plants. There was considerable variation across the four sites regarding the bulk soil nutrient content. An analysis of variance assessment confirmed that 21 variables in the soil nutrient results were considered significantly different (P-value ≤ 0.05) between the four sites (Supplementary Figure 7.1). Only three soil nutrient variables were considered not significantly different between the four sites (p-value > 0.05): pH, nitrate, and mineralisable nitrogen (Supplementary Figure 7.1). Although there was variation across all four sites in the nutrient analysis, the Cambridge site displayed disparate measures of six nutrient variables, including organic matter, extractable organic sulphur, C/N ratio, total carbon, total nitrogen, and soil moisture (Figure 3.4).

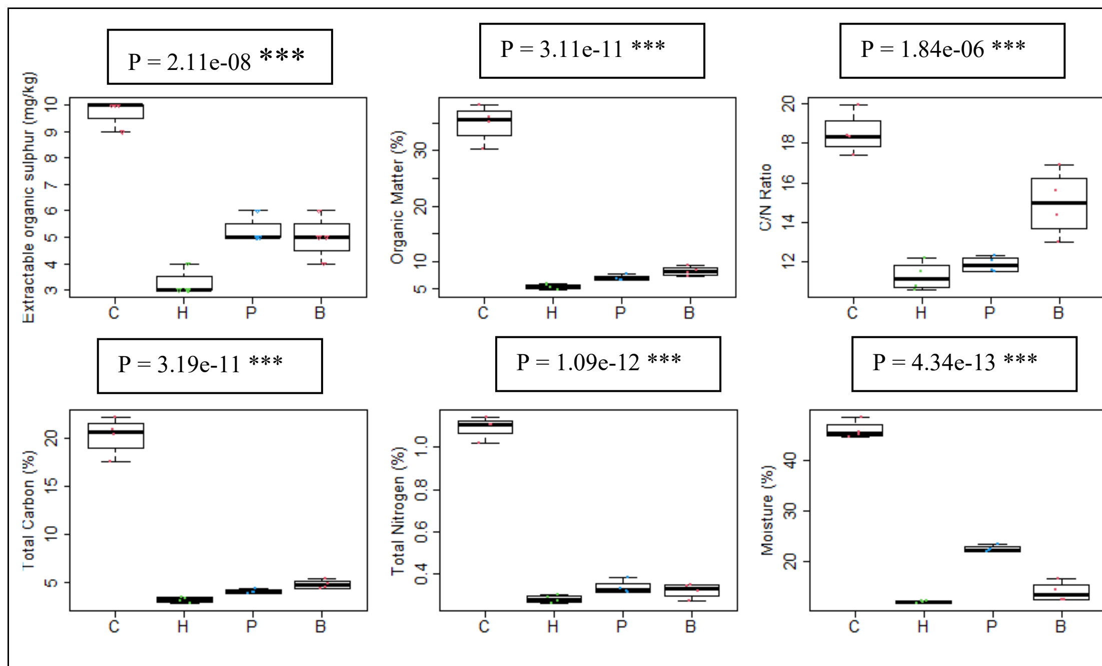


Figure 3.4 The soil nutrient analysis data for the variables; Extractable organic sulphur (mg/kg), Organic matter (%), C/N ratio, Total carbon (%), Total nitrogen (%), and Moisture (%), which were significantly higher (Tucky p-value ⩽ 0.05) for the Cambridge site (C) compared to the other three sites B (Burnham), H (Havelock North) and P (Palmerston North).

The Cambridge site demonstrated the highest variability among the four sites when comparing the above six variables (Figure 3.4). This was determined in two ways. A Tucky test output for all six variables revealed that the Cambridge site was the most unique as it significantly differed from all three other sites (p-value < 0.001). However, the other sites could not be considered entirely homogenous since there were still some significant differences among them (e.g., Havelock N and Burnham showed significantly different extractable organic sulphur, p-value 0.006). The second method used to confirm the heterogeneity of the Cambridge site (in Figure 3.4) was by removing it from each ANOVA model. When it was removed from the ANOVA model, the F-value became smaller, and the p-values became notably less significant for each variable (Table 3.4). The Cambridge site nutrient differences have the potential to influence the associated microbiome since high organic matter content may result in increased microbial heterotrophy.

Table 3.4 One-way ANOVA model of the 6 soil nutrient variables that were higher at the Cambridge site. Two ANOVA models were performed; the first included the Cambridge site samples, and the second did not include the Cambridge site samples.

	1)ANOVA model (including Cambridge samples)		2)ANOVA model (Cambridge samples removed)	
Variable	F value	P (>F)	F value	P (>F)
Extractable Organic sulphur (mg/kg)	86.8	<0.0001 ***	12.2	0.003**
Organic Matter (%)	265.5	<0.0001 ***	19.3	0.005**
C/N Ratio	38.9	<0.0001***	13.7	0.002**
Total Carbon (%)	264.4	<0.0001***	19.5	0.005***
Total Nitrogen (%)	467.7	<0.0001 ***	3.8	0.064 (NS)
Moisture (%)	545.7	<0.0001***	87.8	<0.001 ***

3.1.5 Soil type

Soil type is also a known factor contributing to differences in microbial communities. A soil map report from Manaaki Whenua Landcare Research provided information on the soil type, characteristics, weight, and moisture of the bulk soil samples from the four sites. The Burnham and Palmerston North plots consisted of silt soils, whereas Cambridge consisted of loamy peat soils, and Havelock North had loam over sand soils (Table 3.5). Burnham and Havelock North had well-draining soils, whereas Cambridge and Palmerston North were poorly-draining soils.

Table 3.5 The soil analysis metadata configured from the S-map reports (Landcare Research) for the bulk soil samples from the four site locations.

Site	**Cambridge**	**Havelock North**	**Palmerston North**	**Burnham**
Soil texture	Loamy Peat	Loam over sand	Silt	Silt
Soil type	Mellow Humic Organic	Weathered Fluvial Recent	Argillic-fragic Perch-gley Pallic	Pallic firm Brown
Soil drainage	Poor	Well	Poor	Well
Phosphorous retention (%)	High	Low	Low	Medium

3.1.6 Nematode analysis

Nematodes are common animals in soil environments and can have positive or negative implications for ryegrass growth. Hence, it was valuable to examine the nematode communities associated with perennial ryegrass as they can affect pasture productivity and cause shifts in the associated microbiome. The analysis showed that the Cambridge site had the highest total nematode count (see supplementary Table 7.1). Regarding the plant-feeding identifications, the Palmerston North site contained the least *Paratylenchus*

out of the four sites and showed slightly higher *Pratylenchus*. *Paratylenchus* dominated all three other sites; however, all five of the plant-feeding nematode genera could also be detected at the Cambridge site, including low numbers of *Meloidogyne*, *Heterodera* and *Helicotylenchus* showing up across the plots.

3.1.7 Summary of metadata

In summary, the Burnham site held the highest-producing ryegrass plots, especially in year 3 of the trial, indicating greater One50 AR37 ryegrass persistence. Unfortunately, this may be attributed to the different management this site received, as realised in the final months of the thesis write-up (see section 3.4.1). Of the three north-land sites, Palmerston North maintained consistent One50 AR37 ryegrass production in each year of the trial and had the best visual ryegrass health at the time of sampling, indicative of higher ryegrass persistence compared to Havelock North and Cambridge. Higher ryegrass persistence at the Burnham and Palmerston North sites is of interest in the subsequent microbiome analysis as these sites may show a more optimised microbiome for pasture growth. Although all the reps at each site contained the correct *Epichloë* endophyte (AR37), reps 4 from Cambridge and Burnham did show some contamination by standard endophyte (SE), which may impact other microbial communities. The Cambridge site contributed the highest variation in the soil nutrient results, which could reflect differences across the associated microbiome compared to the other sites. Characterising these parameters early in the analysis increased the ability to form accurate conclusions around the bacterial and fungal associations of perennial ryegrass One50 AR37.

3.2 General characteristics of the perennial ryegrass microbiome

Following the data processing of the 78 successfully sequenced perennial ryegrass samples, the bacterial and fungal genera associated with the perennial ryegrass plant One50 AR37 could be analysed. Firstly, observing the general microbiome trends was necessary to answer the research questions regarding whether there were differences in the fungal or bacterial communities regarding the sampling site and the five microbiome habitats.

3.2.1 Bacterial microbiome

9453 bacterial ASVs were identified from the 78 One50 AR37 ryegrass samples, with an average of 727 bacterial ASVs per sample. After analysing the minimum reads across the 78 samples, each sample was rarefied to an even sequencing depth (12000 reads) for alpha diversity analysis. This sequencing read depth adequately sampled the diversity present as the rarefaction curve had flattened at the minimum sequencing depth of 12000 (Appendices Figure 7.2). This removed 91 OTUs which were no longer present in any sample after random sub-sampling (set.seed1), leaving a total of 9362 bacterial ASVs across the perennial ryegrass samples.

These bacterial ASVs fell into 32 different bacterial phyla dominated by two phyla: *Actinobacteriota* (34.6 %) and *Proteobacteria* (34.2%). The dominant bacterial taxa at the phylum level were similar across all five microbiome habitats. The below-ground microbiome habitats (bulk soil, rhizosphere, root endosphere) were all dominated by

Actinobacteriota, followed by *Proteobacteria.* In contrast, for the above-ground microbiome habitats (shoot endosphere and phyllosphere), *Proteobacteria* took over as the most dominant phylum, followed by *Actinobacteriota* (Table 3.6). For the four sampling sites, Palmerston North had the highest number of ASVs and Havelock North the least. *Proteobacteria* dominated the Cambridge and Palmerston North sites, while the Havelock North and Burnham site had more *Actinobacteriota* (Table 3.7).

Alpha diversity of each microbiome habitat for the four sampling sites was analysed using; Observed (richness), Chao1, Shannon, and InvSimpson indices (Figure 3.5). The rhizosphere generally supported a greater richness of bacterial taxa with samples ranging from (749–1381, n= 16), with locations Palmerston North and Burnham showing the highest richness. The phyllosphere microbiome area demonstrated the lowest richness of bacterial ASVs per sample (ranging from 160–620, n=14). The differences seen between species richness and site (sampling location) were significant for all five microbiome habitats ($P < 0.05$). The Chao1 estimate of richness and Shannon and InvSimpson diversity for the samples were the highest for the below-ground compared to the above-ground, indicating a higher number of bacterial species associated with the soil habitats. The InvSimpson diversity was exceptionally high for the Burnham bulk soil samples compared to the other three sampling locations (Tukey p-value < 0.001).

Table 3.6 The total number of ASVs and the % relative abundance of the two dominant phyla (*Actinobacteriota* and *Proteobacteria*) detected in the five microbiome habitats (Bulk soil, Rhizosphere, Root endosphere, Shoot endosphere and Phyllosphere).

Microbiome Habitat	Number of ASVs	*Actinobacteriota* (% of total ASVs)	*Proteobacteria* (% of total ASVs)
Bulk soil	4260	45.2	25.0
Rhizosphere	5103	42.8	29.6
Root endosphere	4207	36.6	36.6
Shoot endosphere	2374	29.1	44.4
Phyllosphere	1857	22.9	46.1

Table 3.7 The total number of ASVs and the % relative abundance of the two dominant phyla (*Actinobacteriota* and *Proteobacteria*) across the four different sampling locations (Cambridge, Havelock North, Palmerston North, and Burnham).

Sampling site	Number of ASVs	*Actinobacteriota* (% of total ASVs)	*Proteobacteria* (% of total ASVs)
Cambridge	4486	33.3	37.3
Havelock North	3935	39.6	34.5
Palmerston North	4758	33.7	35.2
Burnham	3971	40.2	36.5

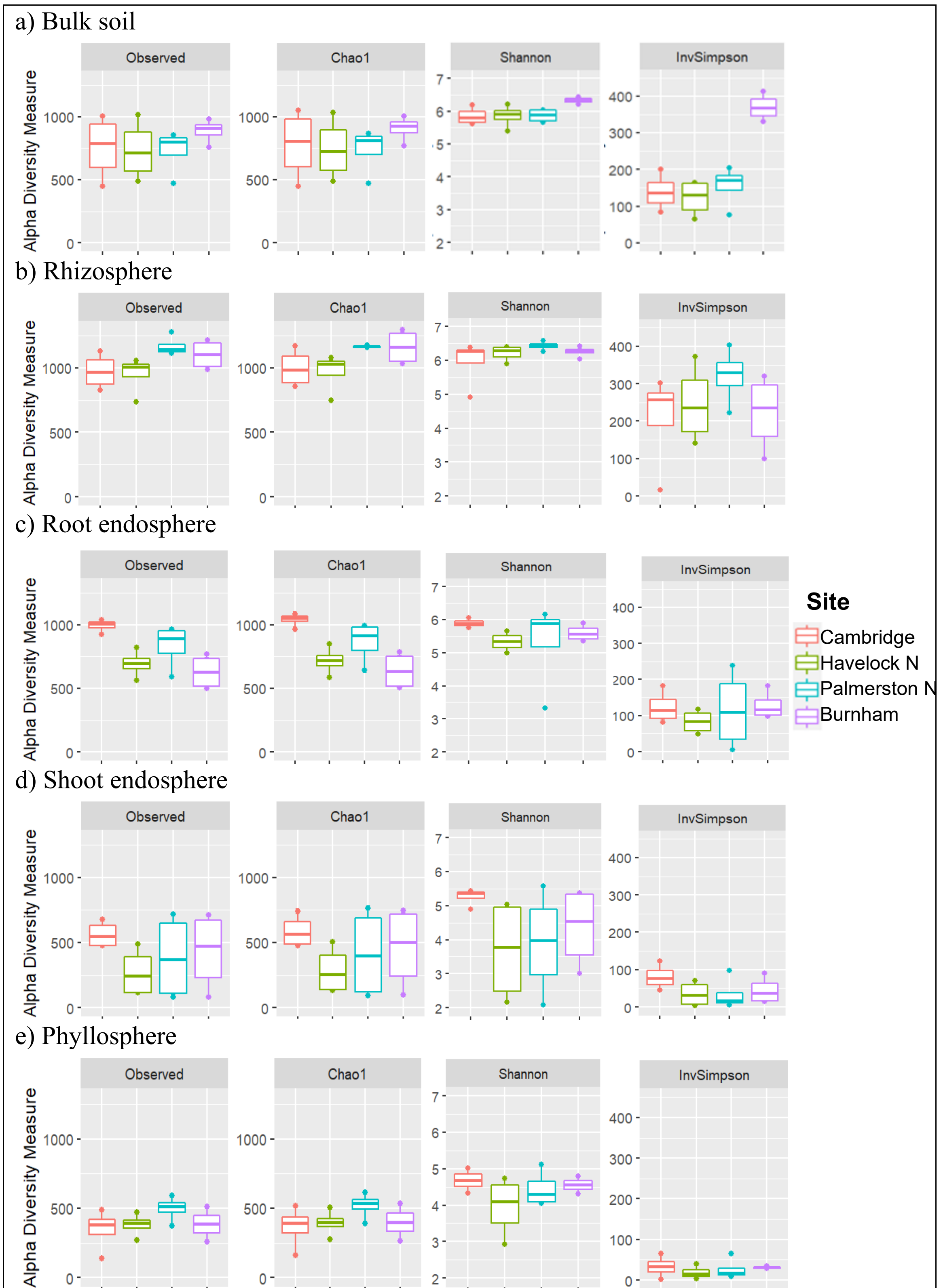


Figure 3.5: Alpha diversity estimation of the One50 AR37 perennial ryegrass bacterial microbiome. Estimations were calculated for a randomized subset of 12000 reads per sample (set. seed(1)` was used to initialize repeatable random subsampling

To gain a more comprehensive picture of the bacterial communities associated with One50 AR37 perennial ryegrass, a non-metric multidimensional scaling (NMDS) ordination was performed based on Bray-Curtis dissimilarity. This analysis revealed a clear separation between the below-ground and above-ground bacterial communities, with an overlap between the bulk soil, rhizosphere, and root endosphere samples and a separate overlap between the shoot endosphere and phyllosphere samples (Figure 3.6). This analysis determined that both the microbiome habitat (sample type) and the sampling location (Site) significantly correlated to variation across the perennial ryegrass bacterial communities (p-value < 0.001) (Figure 3.6).

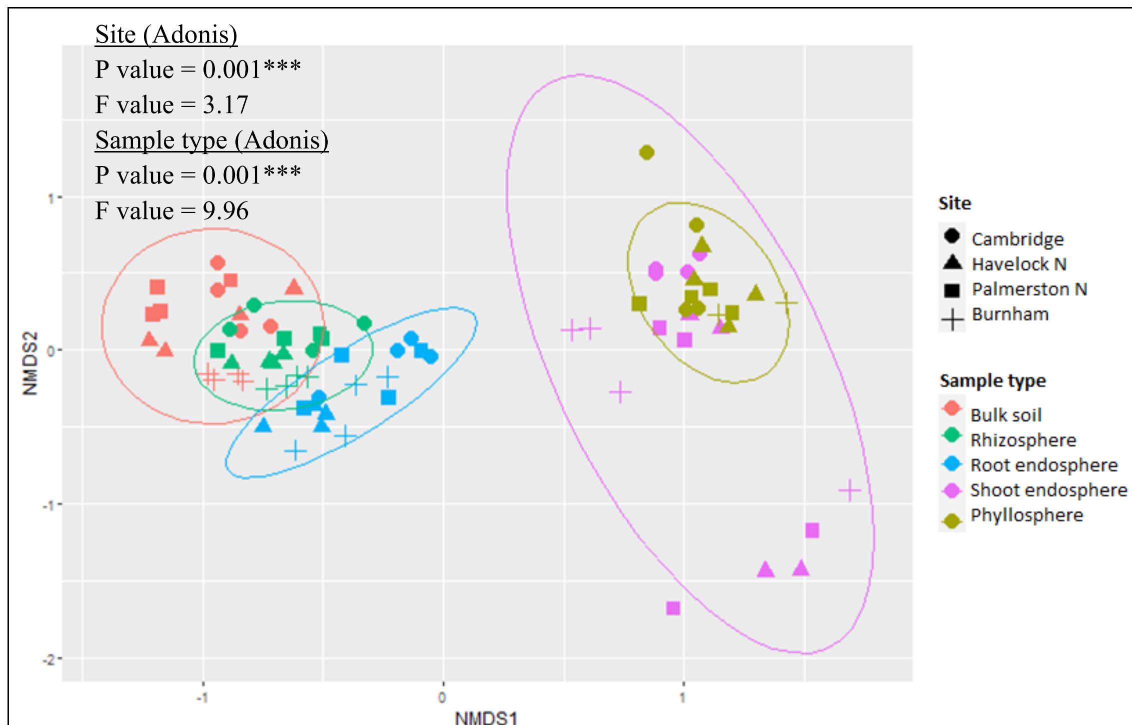


Figure 3.6: Non-metric multidimensional scaling (NMDS) ordination of variation in the bacterial community structure for the five microbiome habitats and the four sampling locations for One50 AR37 perennial ryegrass. Ordination was based on Bray-Curtis dissimilarities among 78 samples. Data points represent individual samples and are coloured/shaped according to microbiome habitat/site.

3.2.2 Fungal microbiome

A total of 3598 ASVs were identified across the 78 One50 AR37 ryegrass samples. Unfortunately, of these fungal ASVs, 1978 could not be classified further than the fungal/eukaryote kingdom. These unclassified fungal ASVs were removed at the beginning of the analysis due to the lack of confidence that these ASVs represented legitimate fungal taxa and the limited information they provided.

It was immediately evident that the shoot endosphere samples had fewer reads than the four other microbiome habitats (Figure 3.7). The lower richness and diversity likely indicates a strong effect of the shoot endosphere niche. The lower reads in the shoot endosphere samples created a problem when trying to rarefy the dataset to a minimum read level before alpha diversity analysis. If the shoot endosphere samples were included in the dataset for alpha diversity analysis, it would have required a minimum read depth

of 1173, which would not have adequately sampled the variation across the soil samples. Therefore, the shoot endosphere samples were included in the subsequent alpha diversity indices analysis; however, a different rarefaction minimum read level was applied (1173) compared to the other four microbiome habitats (7500) (Figure 3.8).

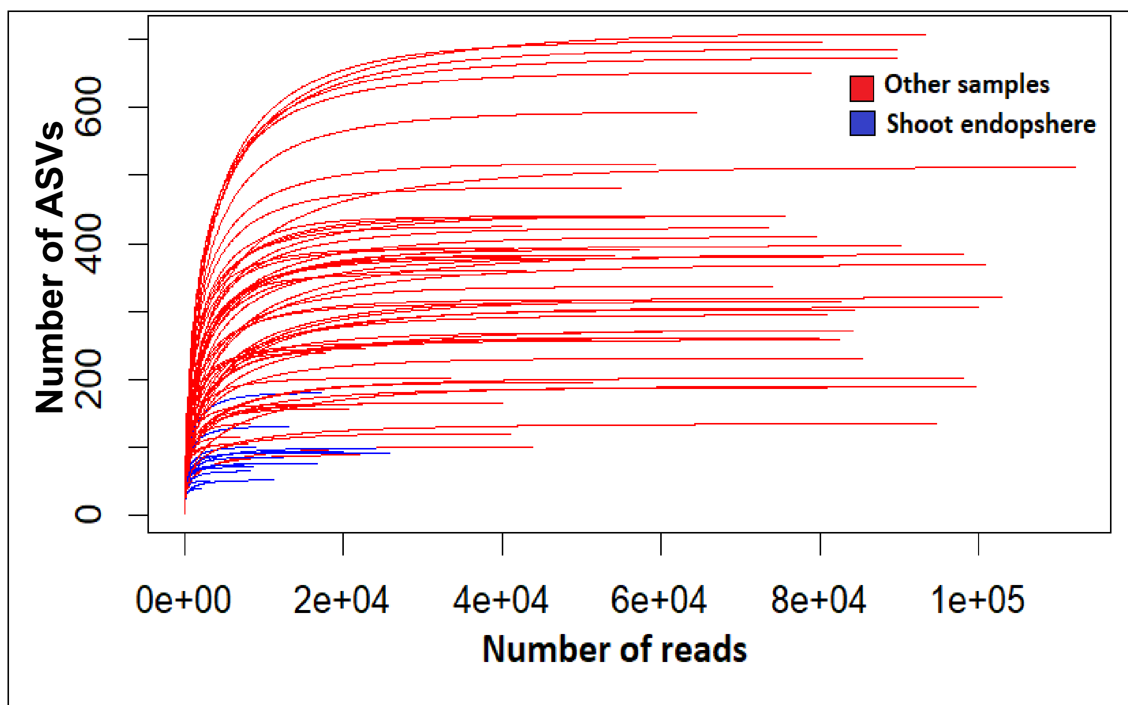


Figure 3.7 Rarefaction curve based on the number of reads vs the number of species (fungal ASVs) for the 78 ryegrass samples, used to analyse the minimum number of reads necessary to adequately sample the variation. The shoot endosphere samples are coloured blue to demonstrate the low reads and ASVs compared to the other sample types.

After rarefying to a minimum sequencing depth of 7500 reads (Appendices Figure 7.3), 1572 fungal ASVs remained across the 59 perennial ryegrass samples. Rarefying to 7500 reads did require removing three low-read root endosphere samples, leaving only two reps for the Cambridge site and three reps for the Palmerston North root endosphere samples. The sequencing read depth chosen adequately sampled the diversity present as the rarefaction curve had flattened for > 90 % of the samples at the minimum sequencing depth of 12000 (Appendices Figure 7.2). Five bulk soil samples contained some un-sampled variation, but this would have included no more than 1-20 fungal ASVs.

The bulk soil generally supported a greater richness of fungal ASVs with samples ranging from 146–320 ASVs, (n= 16) (Figure 3.8). Aside from the shoot endosphere, the root endosphere habitat had the lowest richness of fungal taxa per sample ranging from 48–153 ASVs, (n=13). The differences seen between species richness and shannon diversity for each site' were considered statistically significant (P-value < 0.05) for the rhizosphere and root endosphere microbiome habitats and non-significant for the bulk soil and phyllosphere (P > 0.05). The Chao1 estimate of richness, Shannon and InvSimpson diversity was the highest for the two soil microbiome habitats compared to the root endosphere and phyllosphere, indicating a higher number of fungal species associated with the soil environments.

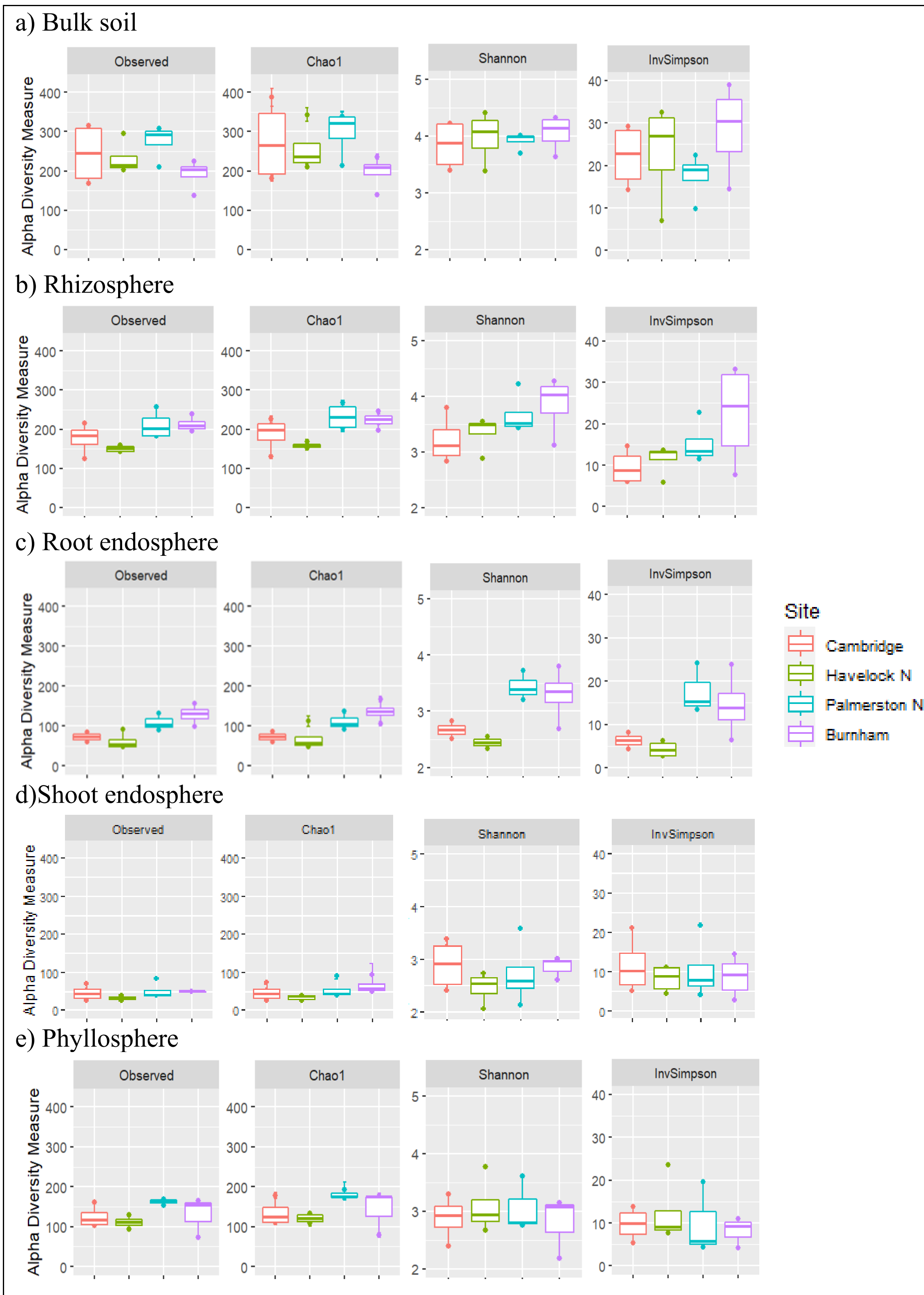


Figure 3.8 Alpha diversity estimation of the One50 AR37 perennial ryegrass fungal microbiome. Estimations were calculated for a randomized subset of 7500 reads per sample for the bulk soil, rhizosphere, root endosphere and phyllosphere and 1173 reads per sample for the shoot endosphere samples. (set. seed(1)` was used to initialize repeatable random subsampling.

The taxa that could be classified into a phylum fell into 19 different phyla, dominated by Ascomycota (54.25%) and Basidiomycota (17.6%). Samples did not differ significantly at the phylum level, with the same phyla, Ascomycota, and Basidiomycota, remaining dominant across all sample types and sampling sites (Table 3.8 and Table 3.9).

Table 3.8 The total number of fungal ASVs detected in the five different microbiome sample types (Bulk soil, Rhizosphere, Root endosphere, Shoot endosphere and Phyllosphere). The table includes the two most abundant phyla in each sample subset (Ascomycota and Basidiomycota), including their relative abundance % of the total ASVs.

Microbiome Habitat	Total Number of ASVs	Ascomycota (% of total ASVs)	Basidiomycota (% of total ASVs)
Bulk soil	1168	56.0	18.2
Rhizosphere	1002	58.8	18.4
Root endosphere	523	63.3	18.3
Shoot endosphere	261	62.4	18.8
Phyllosphere	638	60.5	17.6

Table 3.9 The total number of fungal ASVs detected from the four sampling locations (Cambridge, Havelock North, Palmerston North, and Burnham). The table includes the two most abundant phyla in each sample subset (Ascomycota and Basidiomycota), including their relative abundance % of the total ASVs.

Sampling site	Total number of ASVs	Ascomycota (% of total ASVs)	Basidiomycota (% of total ASVs)
Cambridge	892	58.7	18.2
Havelock North	760	54.1	17.6
Palmerston North	936	59.4	18.3
Burnham	776	58.1	18.1

Similar to the bacterial analysis, an NMDS ordination of the fungal communities revealed a clear separation between the below-ground and above-ground fungal communities with overlap between the bulk soil, rhizosphere, and root endosphere samples; however, the separation was slightly less distinct (Figure 3.9). Interestingly, the shoot endosphere and phyllosphere fungal communities showed no overlap, indicating a distinctly different trend to the bacterial communities in these habitats. There was little overlap between the phyllosphere and the three below-ground microbiome habitats and a slight overlap between the shoot and root endosphere. This analysis determined that both the microbiome habitat (sample type) and the sampling location (Site) correlated to variation across the perennial ryegrass fungal communities (p-value < 0.001, PERMANOVA).

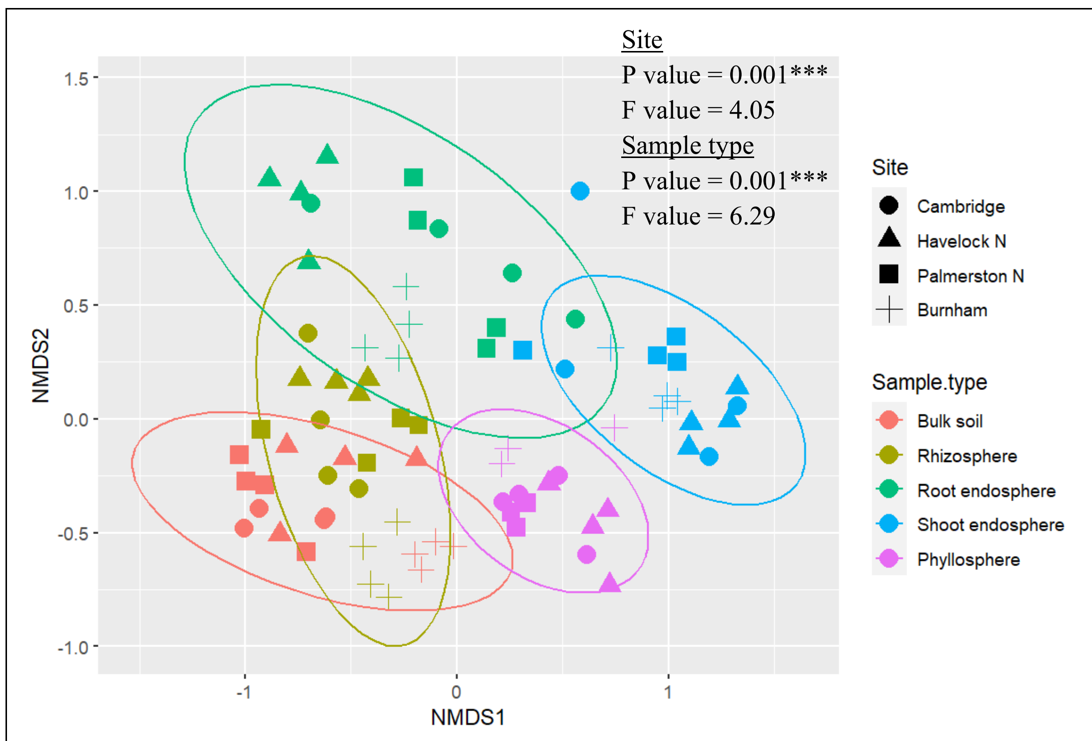


Figure 3.9 Non-metric multidimensional scaling (NMDS) ordination of variation in the fungal community structure for the five microbiome habitats of One50 AR37 perennial ryegrass. Ordination based on Bray-Curtis dissimilarities among 78 samples.

The goal of this section was to observe the overall trends associated with the perennial ryegrass microbiome. The ryegrass microbiome habitats (sample type) and the sampling site significantly correlated to variation across the bacterial and fungal communities. The number of ASVs, alpha, and beta diversity (ordinations), suggested splitting the ryegrass plant habitats into below-ground and above-ground compartments before further analysis.

3.2.3 Does the perennial ryegrass microbiome exhibit site-specific patterns?

The core research hypothesis for this study was that the perennial ryegrass microbiome would exhibit site-specific trends due to environmental heterogeneities across the four sampling sites. Therefore, each of the four sampling locations was expected to display a subset of site-specific taxa that were not present across any of the other three sites. Site effects on the ryegrass bacterial and fungal microbiome were investigated for each different microbiome habitat through an NMDS ordination of variation based on Bray-Curtis dissimilarities among 16 samples (Figure 3.10 and Figure 3.11). The bacterial below-ground microbiome habitats (bulk soil, rhizosphere, and root endosphere) demonstrated strong location effects with prominent clusters between samples from the same sampling site (p-value < 0.001, PERMANOVA). The location effect became less pronounced in the above-ground microbiome habitats (shoot endosphere and phyllosphere), with more substantial overlap between samples from different sites (Figure 3.10). The phyllosphere habitat demonstrated no significant effect of 'Site' (p-value > 0.05, PERMANOVA), with a higher similarity between the bacterial phyllosphere communities at each site and fewer site-specific bacterial ASVs. A similar trend was also evident in the fungal microbiome, with the below-ground samples showing more evident site groupings than the above-ground (Figure 3.11). Unlike the bacterial samples, the site

variable was always significant across the fungal microbiome habitats (p-value < 0.001, PERMANOVA).

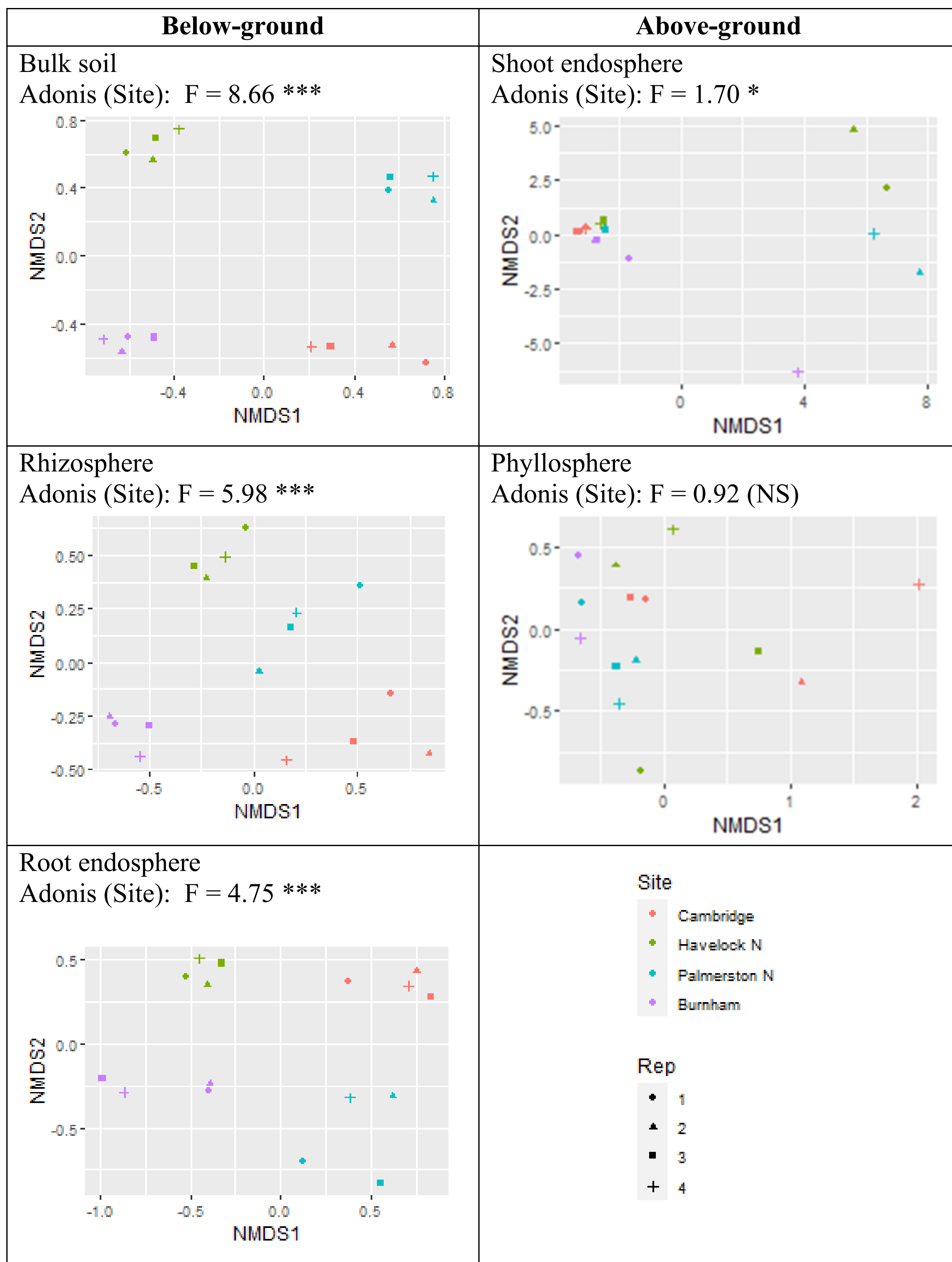


Figure 3.10 Non-metric multidimensional scaling (NMDS) ordination of variation in the bacterial community structure for the four sampling locations regarding the below-ground (a-c) and above-ground (d-e) microbiome habitats of One50 AR37 perennial ryegrass. Significance was judged based on the p-value codes ('***' 0.001 '**' 0.01 '*' 0.05 '.' NS >0.005).

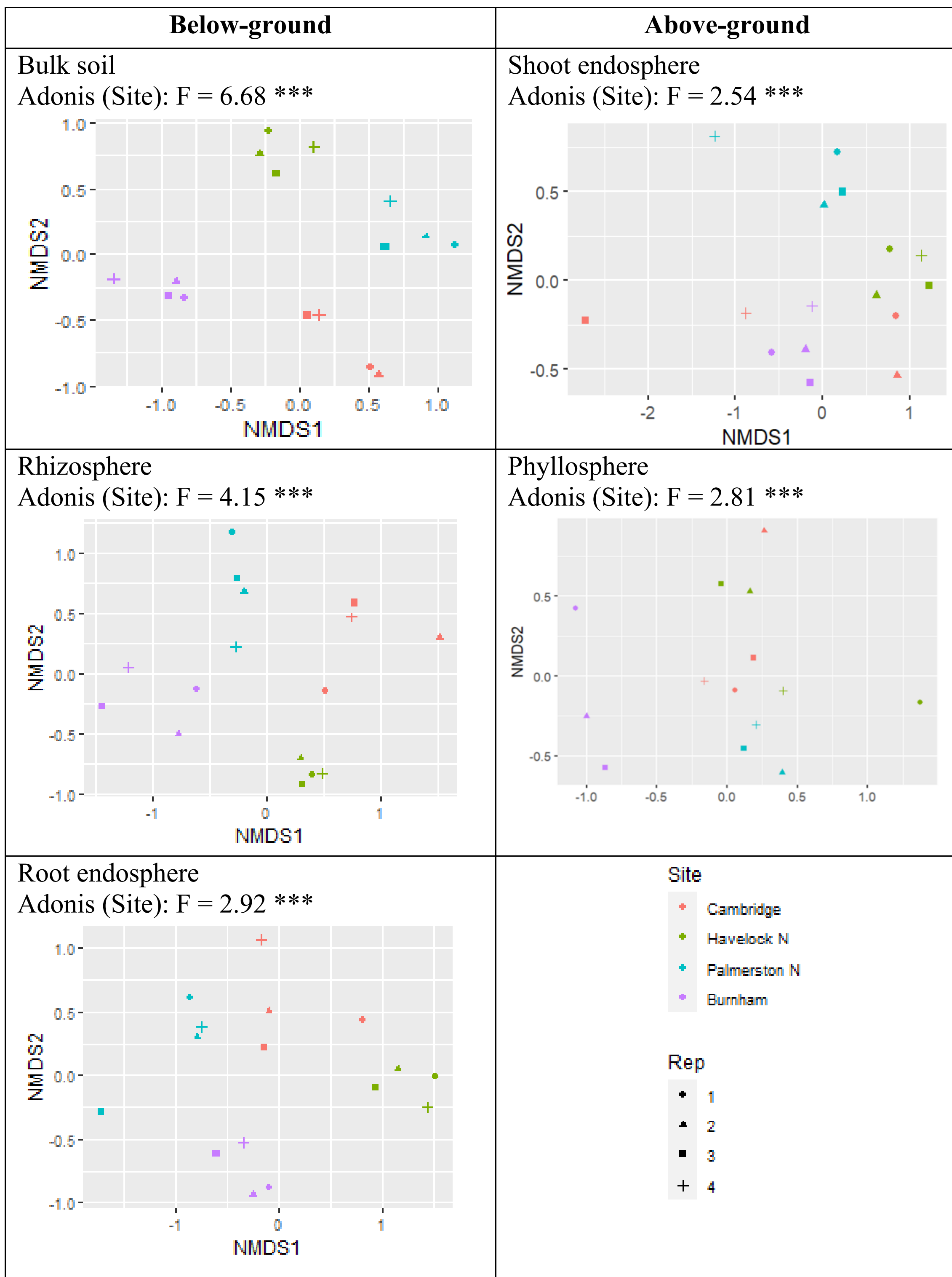


Figure 3.11 Non-metric multidimensional scaling (NMDS) ordination of variation in the fungal community structure for four sites regarding the below-ground microbiome habitats (a-c) and above-ground microbiome habitats (d-e) of One50 AR37 perennial ryegrass. Significance was judged based on the p-value codes ('***' 0.001 '**' 0.01 '*' 0.05 '.' NS (>0.005).

Irrespective of microbiome habitat and relative abundance, each sampling site had specific bacterial and fungal ASVs that were only present at that sampling site and not across the other three sites. Out of the total 9453 bacterial ASVs found across the four sites, the Palmerston North site had the highest number of site-specific ASVs, and Havelock North had the lowest (Table 3.10). Although these taxa were considered site-specific, the taxa of biological interest were those with a high relative abundance greater than 0.001 (0.1%) and 0.01 (1%) in the samples at each site. Of interest, the shoot endosphere microbiome habitat displayed the highest number of site-specific ASVs at a relative abundance of 0.01 or higher for the Burnham, Palmerston North, and Havelock North sites (Table 3.10).

Table 3.10 The number of site-specific bacterial ASVs associated with each site for the five microbiome habitats. ASVs were considered site-specific if they were present at one sampling site only. Site-specific ASVs of interest were those with a high relative abundance (>0.01 or 0.001).

Site + Total ASVs	Total number + (%) of Site-Specific ASVs	Microbiome Habitat	Specific ASVs (Rel abund >0.001)	Specific ASVs (Rel abund >0.01)	Genera of the site-specific ASVs at Rel abun >0.01
Cambridge 4486	1387 30.9 %	Bulk	20	0	
		Rhizo	6	1	*Pseudomonas*
		Root E	14	0	
		Shoot E	17	0	
		Phyllo	0	0	
Havelock North 3935	1029 26.1 %	Bulk	46	0	
		Rhizo	13	0	
		Root E	12	0	
		Shoot E	11	5	*Klenkia, Rubellimicrobium, Turicella, Paracoccus*
		Phyllo	1	0	
Palmerston North 4758	1587 33.4 %	Bulk	40	1	*Pseudomonas*
		Rhizo	13	0	
		Root E	28	1	*Lactobacillus*
		Shoot E	20	7	*Sulfurovum, Streptomyces, Rhodoplanes, Streptomyces, Nocardioides, Bacillus,* and *Kutzneria*
		Phyllo	7	0	
Burnham 3971	1273 32.1 %	Bulk	32	0	
		Rhizo	12	0	
		Root E	13	0	
		Shoot E	22	10	*Actinopolymorpha, Stenotrophomonas,* uncultured-*Ruminococcaceae, Myxosarcina, Flavobacterium, Rubellimicrobium, Microvirga, Marmoricola, Pseudo-Propionibacterium, Flavisolibacter*
		Phyllo	2	0	

Out of the total of 1620 fungal ASVs found across the four sites, the Palmerston North site again showed the highest number of site-specific ASVs, and the Burnham site had the lowest (Table 3.11). There were less apparent trends regarding what microbiome habitat had the highest number of site-specific fungal ASVs at each site. For the Palmerston North and Cambridge sites, the shoot endosphere demonstrated the highest number of site-specific fungal ASVs; for the Burnham site, it was the root endosphere and rhizosphere, and for the Havelock North site, it was the bulk soil and shoot endosphere. Palmerston North had the highest number of site-specific fungal ASVs at a relative abundance of >1 %.

Table 3.11 The number of site-specific fungal ASVs associated with each site for the five microbiome habitats. ASVs were considered site-specific if they were present at one sampling site only. Site-specific ASVs of interest were those with a high relative abundance (>0.01 or 0.001). If known (not uncultured), the associated genus name was given for site-specific ASVs with a relative abundance higher than 0.01.

Site + Total ASVs	Total number + (%) of Site Specific ASVs	Microbiome Habitat	Num of Site Specific ASVs (Rel.abun > 0.001)	Num of Site Specific ASVs (Rel.abun > 0.01)	Genera of the site-specific ASVs at Rel.abun >0.01
Cambridge 892	234 25.1 %	Bulk	19	1	*Metarhizium*
		Rhizo	15	0	
		Root E	18	0	
		Shoot E	41	3	Uncultured *Ascomycota*
		Phyllo	2	0	
Havelock North 760	225 29.6 %	Bulk	17	3	*Penicillium,*
		Rhizo	7	0	
		Root	9	0	
		Shoot	12	3	*Ramularia*
		Phyllo	2	0	
Palmerston North 936	345 36.9 %	Bulk Soil	16	1	*Metarhizium*
		Rhizo	37	3	uncultured Basidiomycota
		Root E	41	6	*Microdochium,* uncultured Basidiomycota
		Shoot E	32	4	*Sporobolomyces*
		Phyllo	10	0	
Burnham 776	175 22.6 %	Bulk	22	1	
		Rhizo	19	4	*Penicillium*
		Root E	27	1	uncultured Trechisporales
		Shoot E	9	1	
		Phyllo	11	0	

The sampling site significantly correlated to variation across the perennial ryegrass microbiome. This was evident in site-specific taxa at each of the four sampling locations. The Palmerston North site contained the highest overall proportion of site-specific bacteria (32 %) and fungi (33 %), likely associated with its high ASV richness. A proportion of site-specific taxa at each site had a high relative abundance (Tables 3.10 and 3.11), likely indicating greater biological significance for the perennial ryegrass microbiome. Although site-specific bacterial and fungal taxa exist across the perennial ryegrass microbiome, the proportion of these taxa ranged between only 20 - 35 % of the total ASVs found at each site. This proportion points to factors other than the farming location, such as host selection, driving variation across the perennial ryegrass microbiome.

3.2.4 Do core taxa exist across the perennial ryegrass microbiome?

Site-specific taxa represent a proportion of the perennial ryegrass microbiome; however, whether a subset of core microbial taxa also existed across sites was of interest. After exploring each microbiome habitat individually, ASVs that were present across all four sampling sites could be identified. An initial, lenient criterion was applied where any ASVs present in at least one sample for each site was identified (second column of Tables 3.12 and 3.13). This gave a broad indication of the total shared ASVs across the perennial ryegrass microbiome. However, like the site-specific taxa analysis (section 3.2.3), core taxa of biological interest were those with a high relative abundance and prevalence across the samples. Some authors suggest bacterial ASVs present at different sites are considered core if they have a relative abundance greater than 0.001 (0.1%) (Neu et al., 2021). Therefore, to explore the highly prevalent and abundant taxa, an ASV was only considered 'core' if it was present in at least 90% of the samples (including reps) in at least one microbiome habitat at a relative abundance threshold greater than 0.1% (Neu et al., 2021).

Based on this criterion, 48 bacterial and 29 fungal ASVs were identified as core taxa across the perennial ryegrass microbiome (Table 3.12 and Table 3.13). Some ASVs were considered core across multiple microbiome habitats, and others were only considered core in one. For example, bacterial ASV-10 belonging to the *Bacillus* genus was considered core across all four sampling locations in three microbiome habitats, bulk soil, rhizosphere, and root endosphere, whereas bacterial ASV-6 belonging to the *Agreia* genus was only considered core in the phyllosphere microbiome habitat (Appendices Table 7.2). No fungal or bacterial ASVs were considered core across all five microbiome habitats and all four sampling sites.

Interestingly, the bacterial shoot endosphere and phyllosphere habitats exhibited the highest percentage of shared bacterial ASVs (13 and 15%) (Table 3.12)). This finding aligns with what was seen when exploring the effects of 'Site' on the microbial taxa (previous Figure 3.10), as both the phyllosphere and shoot endosphere habitats demonstrated less obvious 'Site' groupings across the bacterial communities.

Table 3.12: The number of core bacterial ASVs and the associated genus names occurring in every sample across the four sampling sites for the five microbiome habitats. ASVs were only considered core if they were present in 90% of samples at an abundance threshold of more than 0.001.

Microbiome habitat + Total ASVs across all four sites.	**Total number and % of shared bacterial ASVs (present at least once at all four sites)**	**Number of core bacterial ASVs shared across all four sites (90% of samples > 0.001)**	**Genus names of core ASVs**
Bulk soil 4260 ASVs	223 ASVs = 5.2%	19 =0.45%	*Acidothermus* (1) *Arthrobacter* (1), *Bacillus*(3), *Nocardioides*(1), *Bradyrhizobium*(3), *Catenulispora*(1), IMCC26256 ge (1), *Sphingomonas*(1) Streptomyces(1), *Terrabacter*(1), uncultured(5).
Rhizosphere 5103 ASVs	473 ASVs =9.3%	21 =0.41%	*Acidothermus* (1), *Arthrobacter* (1), *Bacillus* (2), *Bradyrhizobium* (3), *Catenulispora*(1), *Conexibacter*(1), *IMCC26256_ge*(1), *Marmoricola*(1), *Mycobacterium*(2), *Nakamurella*(1), *Nocardioides* (1), *Schumannella*(1), *Solirubrobacter*(1), *Streptomyces* (1), *Terrabacter* (1), *uncultured* (2).
Root endosphere 4207 ASVs	344 =8.18%	17 =0.40%	*Aeromicrobium*(1), *Amycolatopsis*(1), *Bacillus*(1) *Bradyrhizobium*(3), *Nocardioides*(1), *Rhizobacter*(1), *Rhodanobacter*(1), *Schumannella*(1), *Sphingomonas*(1) *Streptomyces*(4), uncultured(1), *Xanthomonas*(1)
Shoot endosphere 2374 ASVs	302 =12.7%	0 =0%	
Phyllosphere 1857 ASVs	269 =14.5%	16 =0.86%	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*(2), *Agreia* (1), *Chryseobacterium*(1), *Clavibacter*(1), *Curtobacterium*(1), *Friedmanniella*(1), *Frigoribacterium*(1), *Microbacterium*(2), *Nocardioides*(1), *Pseudomonas* (2), *Sphingomonas*(1)
Total (Shared ASVs across multiple microbiome habitats counted once)		**48**	

A more focused research question regarding the perennial ryegrass microbiome was whether the presence of the *Epichloë* endophyte 'AR37' would influence a more stable/selective shoot endosphere fungal community compared to the other microbiome habitats. Interestingly, in both the fungal and bacterial ryegrass microbiomes, the shoot endosphere and phyllosphere habitats had the highest total percentage of core taxa before considering the relative abundance (Table 3.12 and Table 3.13). In the phyllosphere habitat, 2.8% of the total fungal taxa were present across 90% of samples at a relative abundance higher than 0.001 (0.1%) (Table 3.13). The shoot endosphere proportion was higher, representing 3.4 % of the total taxa. These taxa included fungal genera such as *Aspergillus*, *Fusarium, Epicoccum*, *Neoascochyta and Pithomyces* (Appendices Table 7.3). Interestingly, across all five microbiome habitats, *five* core fungal ASVs were found at a higher relative abundance of greater than 0.01 (1%), including genera: *Fusarium*, uncultured *Magnaporthaceae*, *Epichloë*, *Neoascochyta* and uncultured *Tremellomycetes*.

Table 3.13: The number of core fungal ASVs and the associated genus names occurring in every sample across the four sampling sites for the five microbiome habitats. ASVs were considered core if they were present in 90% of samples in at least one microbiome habitat at an abundance threshold of more than 0.001.

Microbiome habitat + Total ASVs across all four sites.	**Total number and % of shared fungal ASVs (present at least once at all four sites)**	**Number of core fungal ASVs shared across all four sites (90% of samples > 0.001)**	**Number of core fungal ASVs shared across all four sites (90% of samples > 0.01)**	**Genus names of the core ASVs at a relative abundance of 0.001.** Genus names in bold were also present at a relative abundance of > 1 %
Bulk soil 1168 ASVs	175 =15.0 %	9 =0.22 %	0	*Exophiala* (1), *Fusarium* (3), *Pithomyces* (1), *Mortierella* (1), Uncultured Magnaporthaceae (1), Uncultured Trimorphomycetaceae (1), *Beauveria* (1)
Rhizosphere 1002 ASVs	150 =14.9 %	9 =0.89 %	1 = 0.06 %	***Fusarium*** (3), *Exophiala*(1), *Pithomyces*(1), *Neoascochyta* (1), *Beauveria* (1), uncultured Helotiales (1), uncultured Magnaporthaceae (1)
Root endosphere 523 ASVs	65 =12.4 %	5 =0.95 %	1 = 0.19 %	*Exophiala* (1), *Fusarium* (1), uncultured Helotiales (1), **uncultured Magnaporthaceae** *(1),* uncultured *Ascomycotaa (1)*

Shoot endosphere 261 ASVs	43 =16.5 %	9 =3.4 %	2 = 0.77 %	*Colletotrichum (1),* ***Epichloë*** (1), *Epicoccum*(1), *Fusarium*(1), ***Neoascochyta***(1), Neosetophoma (2) *Pithomyces*(1)
Phyllosphere 638	134 =21.0 %	18 =2.8 %	1 =0.16 %	*Aspergillus*(1), *Colletotrichum*(1), Coniothyrium (1), *Epicoccum*(2), *Fusarium* (1), *Holtermanniella (1), Neoascochyta* (1), *Neosetophoma* (1), *Penicillium* (1), *Pithomyces*(1), *Pyrenochaetopsis*(1), **uncultured Tremellomycetes** *(1)* uncultured(4), *Vishniacozyma* (1)
Total (Core ASVs across multiple microbiome habitats counted once only)		**29**	**5**	

Whilst core ASVs were found across almost all of the perennial ryegrass microbiome habitats, the quantity and relative abundance was not high enough to confirm that perennial ryegrass has a core microbiome (Neu et al., 2021). The total number of these core ASVs was low, with only 48 bacterial ASVs and 29 fungal ASVs at a relative abundance greater than (0.001) 0.1%. Interestingly, five core fungal ASVs were found at a relative abundance higher than 0.01 (1 %); but no bacterial ASVs were found at this threshold. The presence of these singular core bacterial and fungal ASVs holds the potential to be playing important roles in perennial ryegrass growth. Out of the five microbiome habitats, the phyllosphere and shoot endosphere demonstrated the highest total number of shared taxa across the four sites, which could indicate higher selection.

3.2.5 Exploring trends in the below-ground microbiome

Immediately, it was clear that both the perennial ryegrass microbiome habitats and the sampling site could explain variation across the perennial ryegrass microbiome. However, these trends required further investigation by exploring the genera associated with the most abundant ASVs in each microbiome habitat.

An NMDS ordination on the below-ground bacterial and fungal microbiome habitats (Figure 3.12) determined that both the microbiome habitat (sample type) and the sampling location (Site) represented significant variation seen across the below-ground communities (p-value < 0.001, PERMANOVA). For both the bacterial and fungal communities, the bulk soil and rhizosphere demonstrated the most substantial overlap (Figure 3.12). The bacterial root endosphere did not overlap with the two soil microbiome

habitats. A gradient was evident in the fungal below-ground microbiome as the environment transitioned from the soil around the roots (bulk soil) to soil in close contact with the roots (rhizosphere) to the inner tissues of the roots (endosphere) (Figure 3.12). Site separation was apparent, with obvious groupings seen in all three microbiome areas regarding the sampling site.

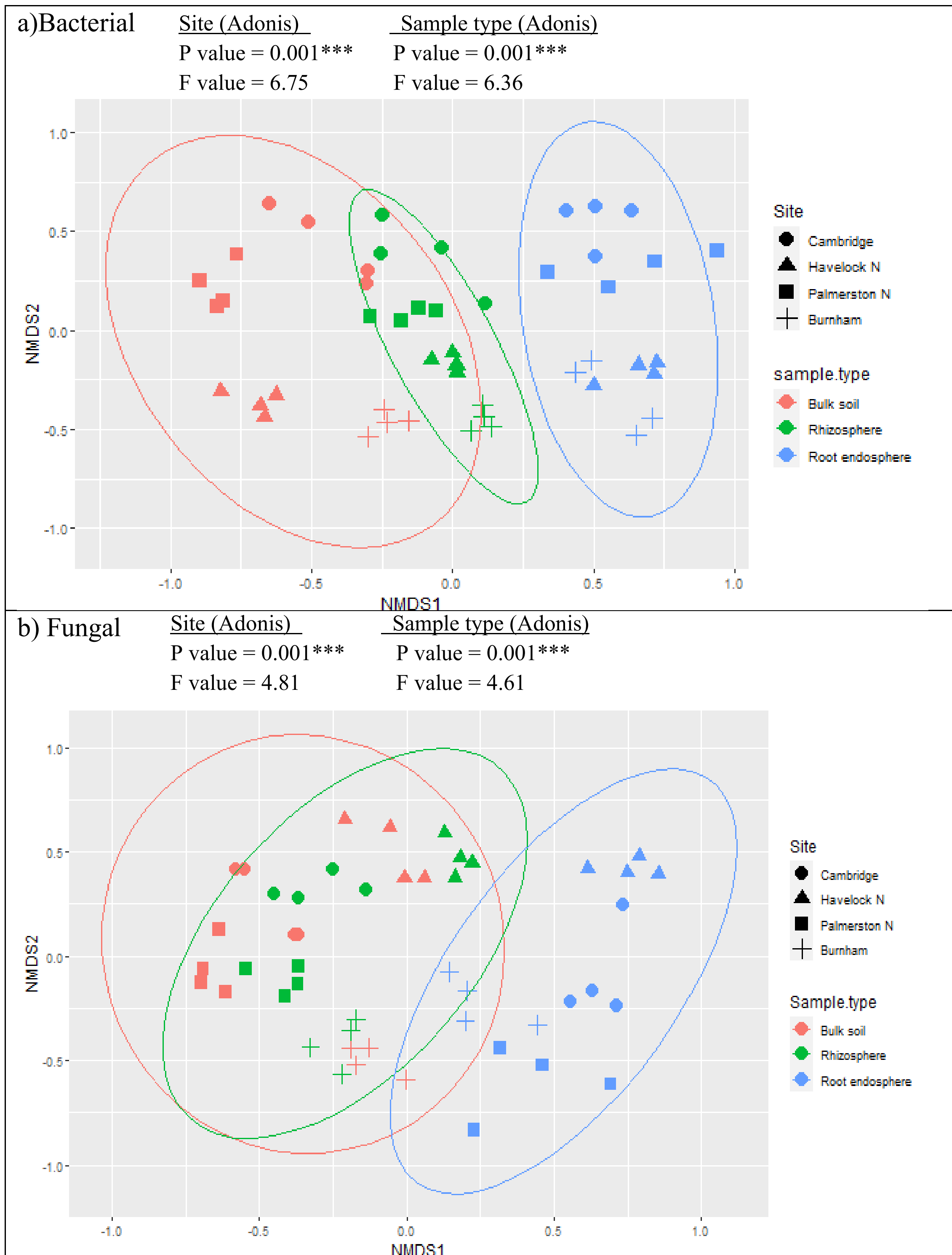
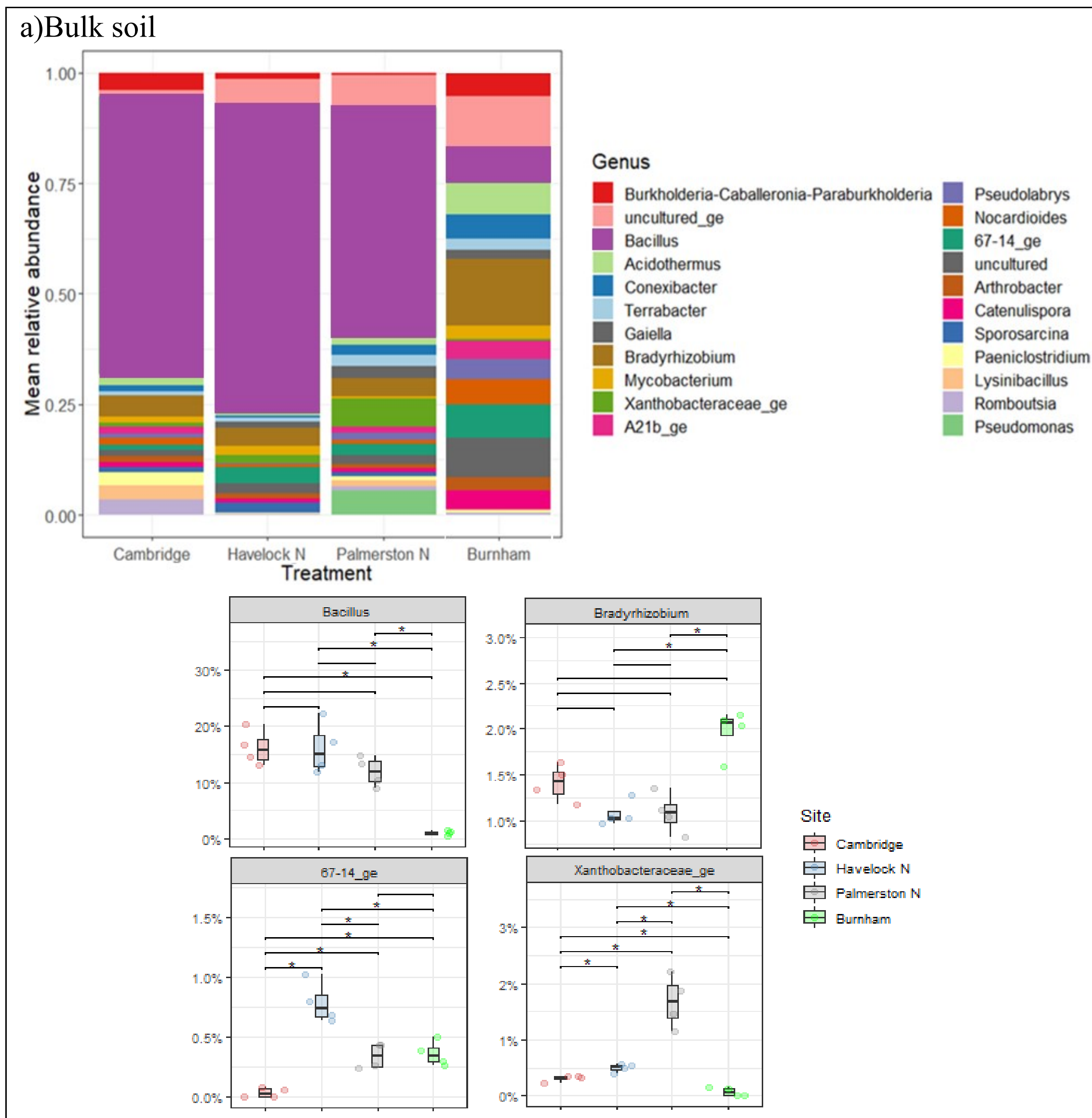


Figure 3.12 NMDS ordinations of variation in the (a) Bacterial and (b) Fungal community structure for the below-ground microbiome habitats of One50 AR37 perennial ryegrass. Each ordination was based on Bray-Curtis dissimilarities among 48 samples.

Interestingly, a signature finding concerning the below-ground perennial ryegrass microbiome was the higher diversity and differential structure of the Burnham bulk soil and rhizosphere bacterial genera compared to the three North Island sites (Figure 3.13). In the bulk soil and rhizosphere, the bacterial communities were dominated heavily by the bacterial genus *Bacillus* for the three North Island sites (Figure 3.13). The Burnham site showed significantly lower *Bacillus* (P-value < 0.05) and a more evenly spread bacterial composition with slight domination by the bacterial genus *Bradyrhizobium* in the bulk soil and a member of the Burkholderiaceae family, *Burkholderia-Cabelleronia-Paraburkholderia* in the rhizosphere. Burnham also showed higher *Mycobacterium* in the rhizosphere than all three North-island sites (P-value < 0.05).



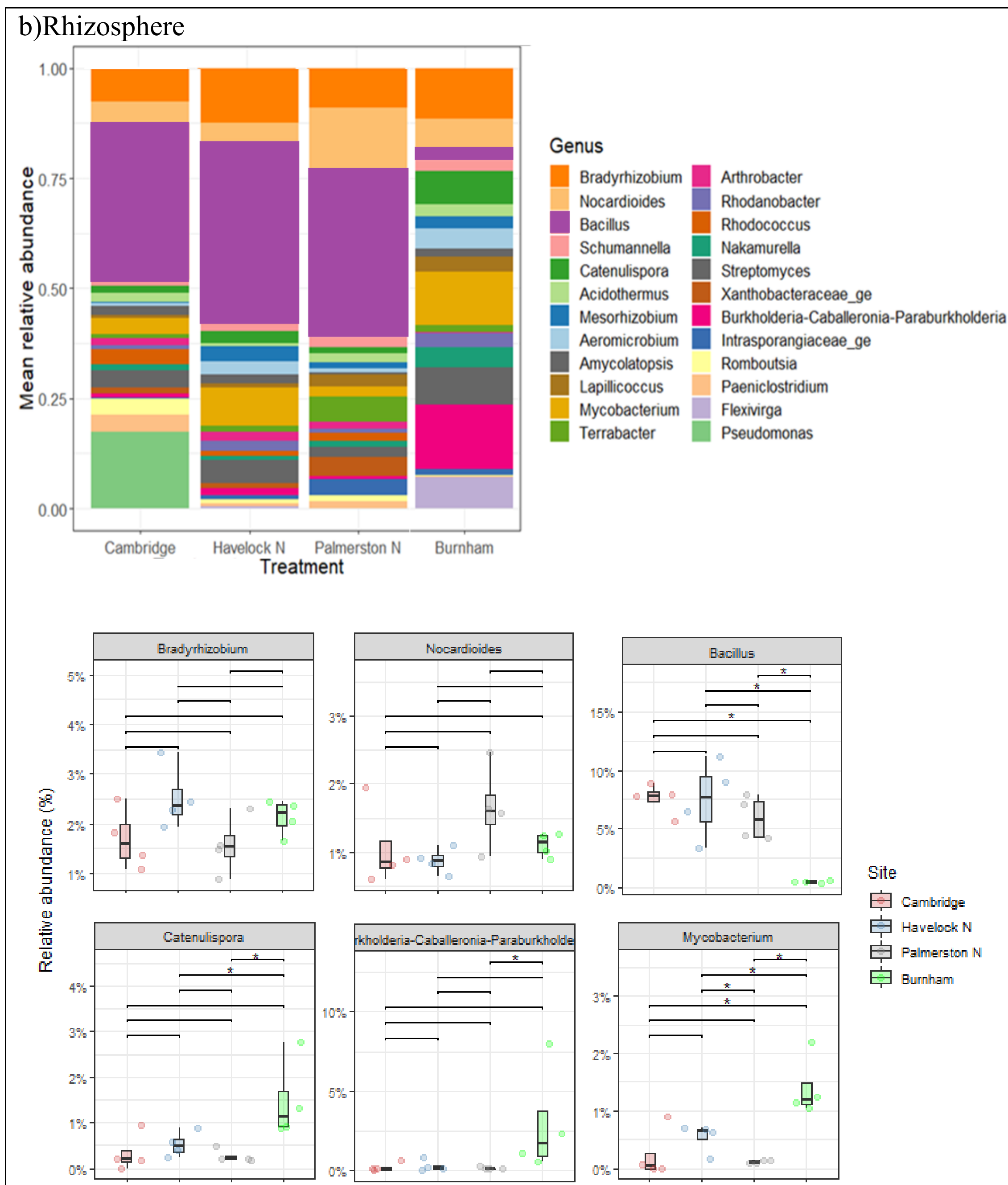


Figure 3.13 The bacterial genera with the highest mean relative abundance across the a) bulk soil and b) rhizosphere microbiome habitats for perennial ryegrass One50 AR37 across the four sites. Note: Due to the differential bacterial genera in the bulk soil compared to the rhizosphere, the colour assignment for each genus differs between graphs a) and b). Bacteria of interest were statistically compared across the sites using Wilcoxon test.

Due to the domination of *Bacillus* across the three North Island sites, it was valuable to explore whether this was due to one or more bacterial ASVs. For the bulk soil and rhizosphere samples together, 170 *Bacillus* ASVs were found. The Burnham site demonstrated a lower absolute abundance of *Bacillus* (Figure 3.14)). There was a significant difference between the absolute abundance of *Bacillus* across the four sampling sites (PERMANOVA, p-value < 0.001, F value: 23.91), however, when Burnham was removed from the model, the difference became less significant (Adonis p value < 0.01, F value: 2.52). The absolute abundance of the top 10 *Bacillus* ASV*s* showed

that ASV-10 was dominant at the Burnham site, whereas the North Island sites showed multiple dominant *Bacillus* ASVs: ASV10, ASV13, ASV24 and ASV29. Interestingly, *Bacillus* ASV10 also represented a core perennial ryegrass ASV (Table 3.12)) across all four sampling sites in the bulk soil, rhizosphere, and root endosphere microbiome habitats.

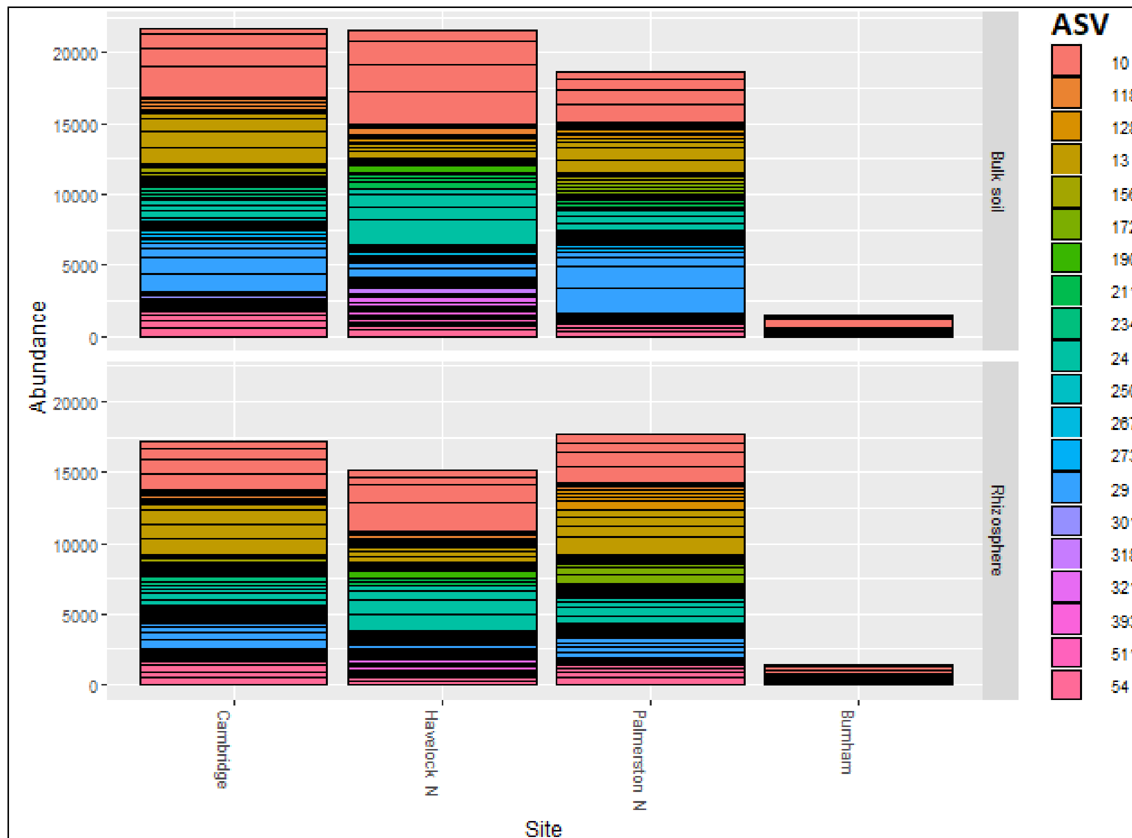


Figure 3.14: The average absolute abundance of the top 20 Bacillus ASVs found across the bulk soil and rhizosphere microbiome regions of perennial ryegrass One50 AR37 for the four sampling sites.

The diversity difference in the bacterial community structure of the Burnham site compared to the three North Island sites was not apparent across the root endosphere samples, with only slight differences in the relative abundances (Figure 3.15). The relative abundance of *Bradyrhizobium* was high across each site, with no significant difference in the mean relative abundance (P-value > 0.05). The bacterial communities were dominated by *Streptomyces* for the Cambridge, Havelock North, and Burnham sites, whereas *Pseudomonas* was the dominant genus across the Palmerston North root endosphere samples (Figure 3.15). However, the relative abundance of *Pseudomonas* at the Palmerston North site was not significantly higher than the other three sites (P-value > 0.05) since only one ryegrass plot rep contributed to this high relative abundance. Palmerston North showed significantly lower Amycolatopsis, and Cambridge and Palmerston North showed significantly lower *Kutzneria* (P-value < 0.05) in the root endosphere samples.

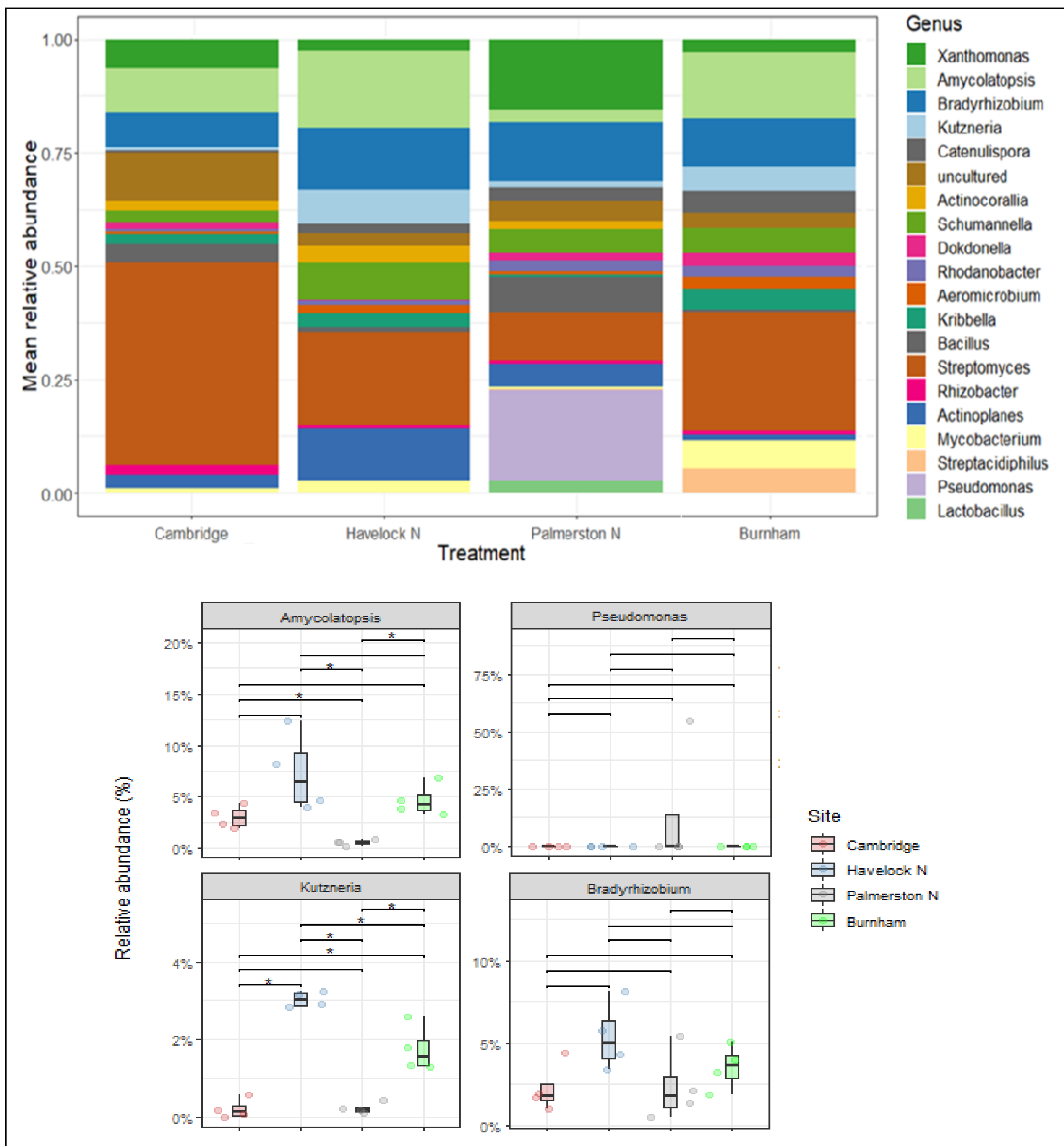
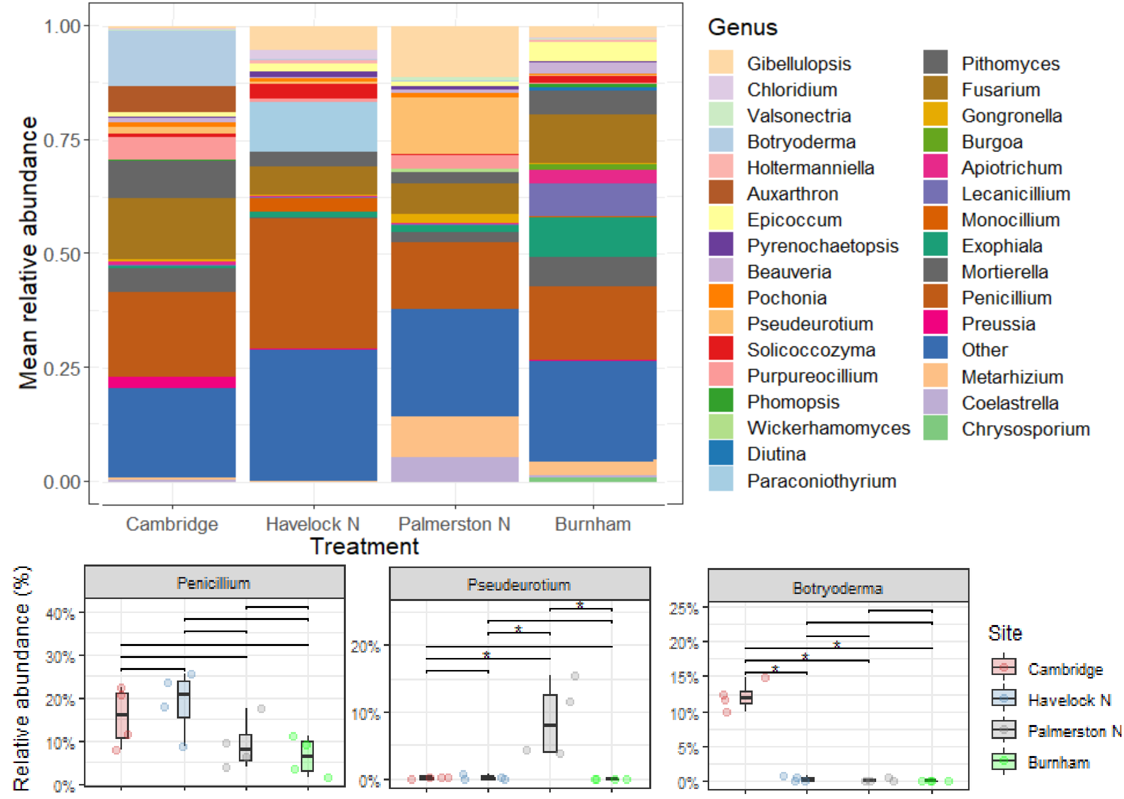
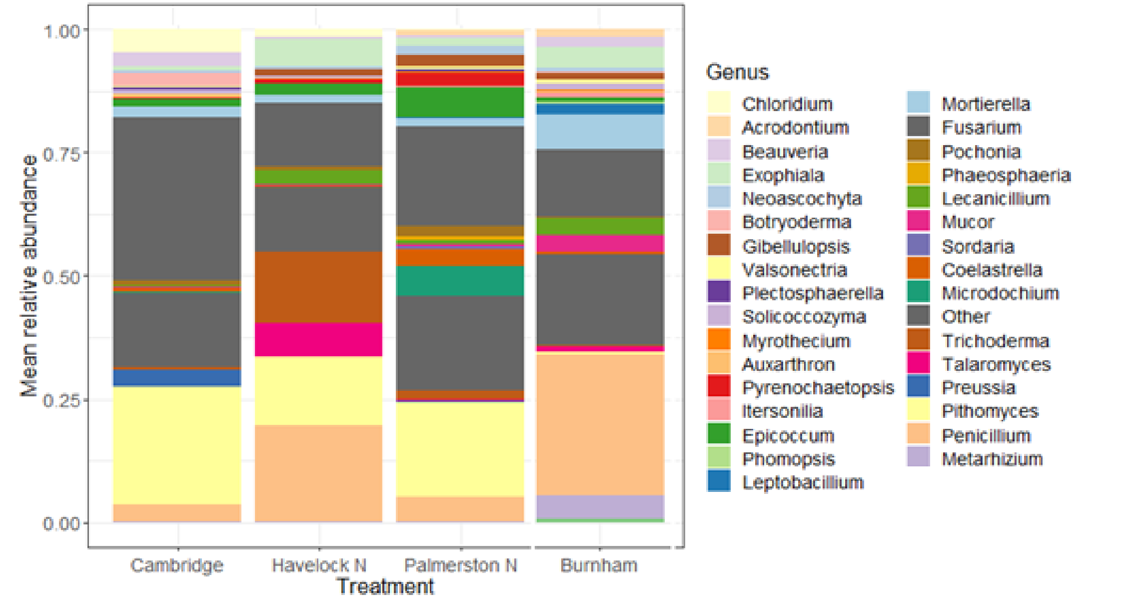


Figure 3.15 The top 20 bacterial genera with the highest mean relative abundance for the root endosphere microbiome habitats for perennial ryegrass One50 AR37 across the four sites. Genera of interest were compared statistically across the sites based on a Wilcox test.

The below-ground fungal perennial ryegrass microbiome habitats showed some overlap between genera (Figure 3.16). The bulk soil and rhizosphere samples shared genera such as *Penicillium*, *Fusarium* and *Pithomyces*. The dominant genus in the bulk soil samples for each site was *Penicillium.*

Intriguingly, in the rhizosphere samples, the three North Island sites were dominated by the fungal genera *Fusarium* and *Pithomyces.* In contrast, Burnham had a significantly lower relative abundance of *Pithomyces* (P-value < 0.05) and instead had a higher number of *Penicillium* (P-value < 0.05). Havelock North also had higher *Trichoderma* in the rhizosphere than the three other sites. (P-value < 0.05) (Figure 3.16).


a)Bulk Soil
Genus
Gibellulopsis
Chloridium
Valsonectria
Botryoderma
Holtermanniella
Auxarthron
Epicoccum
Pyrenochaetopsis
Beauveria
Pochonia
Pseudeurotium
Solicoccozyma
Purpureocillium
Phomopsis
Wickerhamomyces
Diutina
Paraconiothyrium
Pithomyces
Fusarium
Gongronella
Burgoa
Apiotrichum
Lecanicillium
Monocillium
Exophiala
Mortierella
Penicillium
Preussia
Other
Metarhizium
Coelastrella
Chrysosporium
Mean relative abundance
Treatment
Cambridge
Havelock N
Palmerston N
Burnham
Relative abundance (%)
Penicillium
Pseudeurotium
Botryoderma
Site
Cambridge
Havelock N
Palmerston N
Burnham


b) Rhizosphere
Genus
Chloridium
Acrodontium
Beauveria
Exophiala
Neoascochyta
Botryoderma
Gibellulopsis
Valsonectria
Plectosphaerella
Solicoccozyma
Myrothecium
Auxarthron
Pyrenochaetopsis
Itersonilia
Epicoccum
Phomopsis
Leptobacillium
Mortierella
Fusarium
Pochonia
Phaeosphaeria
Lecanicillium
Mucor
Sordaria
Coelastrella
Microdochium
Other
Trichoderma
Talaromyces
Preussia
Pithomyces
Penicillium
Metarhizium
Mean relative abundance
Treatment
Cambridge
Havelock N
Palmerston N
Burnham

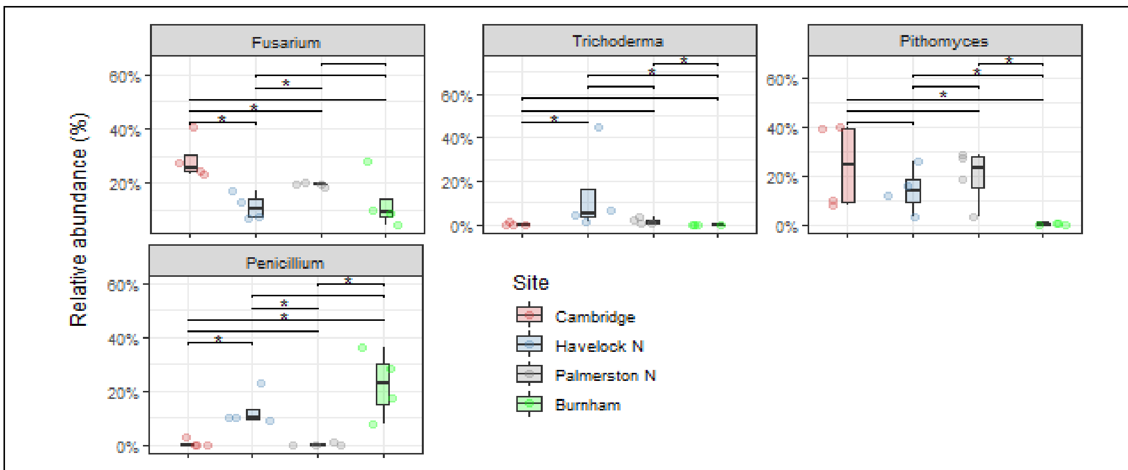


Figure 3.16 The fungal genera with the highest mean relative abundance across the a) bulk soil and b) rhizosphere for perennial ryegrass One50 AR37. Genera of interest were compared statistically across the sites based on a Wilcox test. Note: Due to the differential fungal genera in each microbiome habitat, the colour assignment for each genus differs between graphs a) and b).

The root endosphere samples exhibited high numbers of *Fusarium* across all four sites. Havelock North and Burham also showed slightly higher *Exophiala* than Cambridge and Palmerston North (P-value < 0.05) (Figure 3.17). A notable site-specific difference included the high relative abundance of *Periconia* at the Havelock site compared to the other three sites (P-value < 0.05).

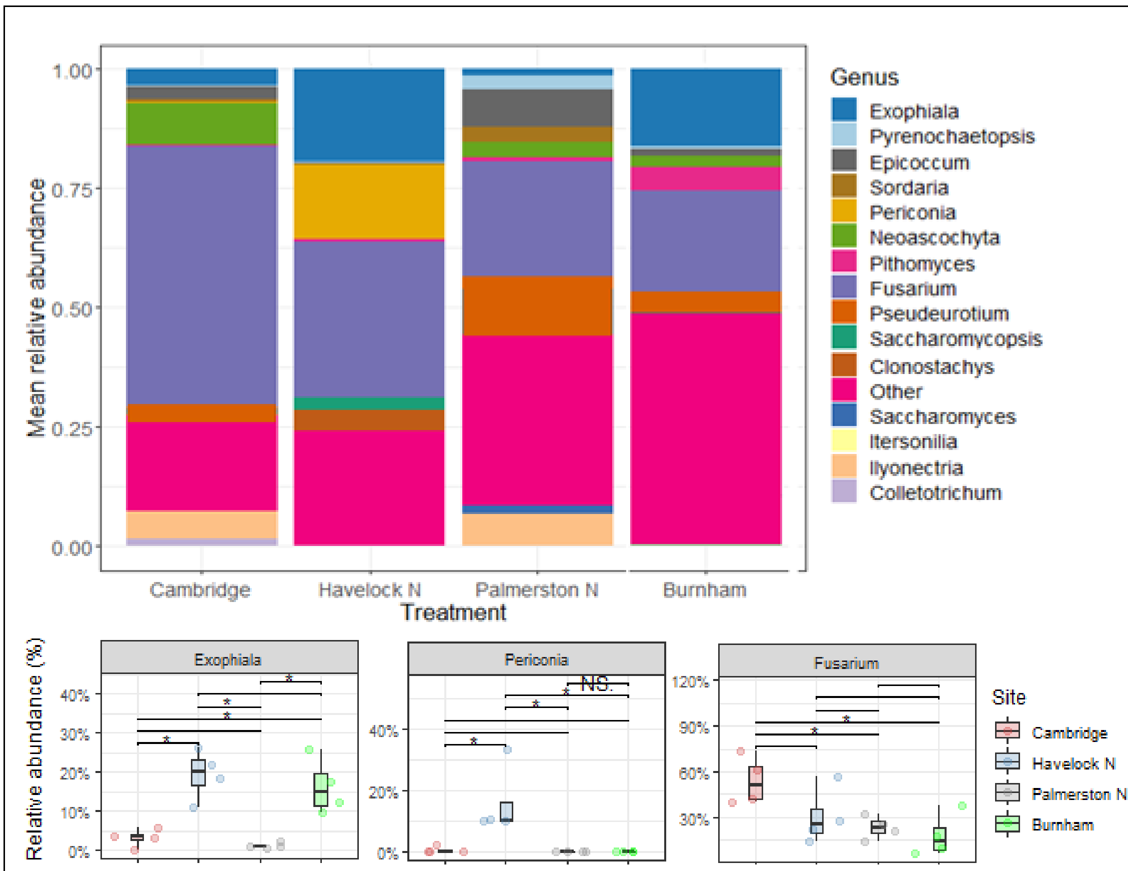


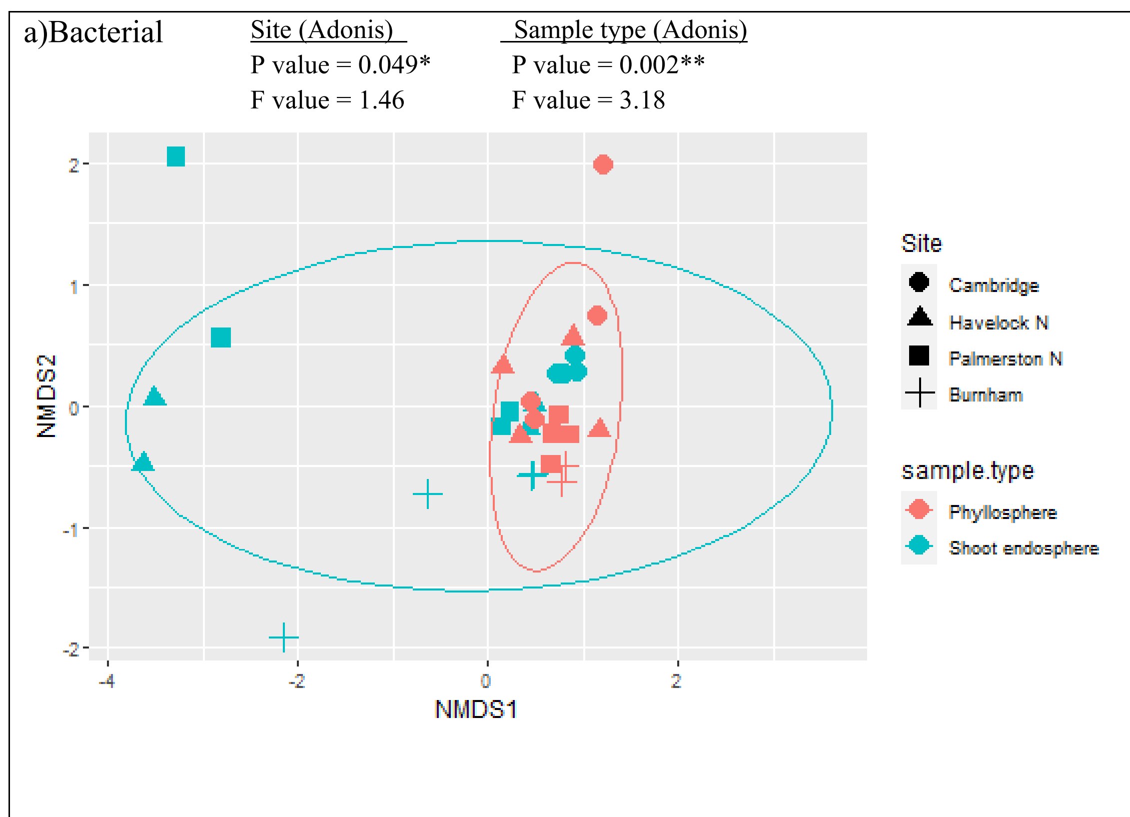
Figure 3.17 The top 15 fungal genera with the highest mean relative abundance across the root endosphere of perennial ryegrass One50 AR37 for the four sites. Genera of interest were compared statistically across the sites based on a Wilcox test.

In summary, an important finding regarding the below-ground perennial ryegrass microbiome was the bacterial community trends of the Burnham site concerning its lack of the *Bacillus* genus in the bulk soil and the *Pithomyces* genus in the rhizosphere. Instead, Burnham had high numbers of *Penicillium*, *Bradyrhizobium*, *Burkholderia-Cabelleronia-Paraburkholderia* (a genus of the Burkholderiaceae family), *Mycobacterium* and *Arthrobacter*.

3.2.6 Exploring trends in the above-ground microbial taxa.

Exploring the high relative abundance bacterial and fungal genera across the above-ground perennial ryegrass microbiome was also important. An NMDS ordination determined that that "sample type' (p-value = 0.002**) could significantly explain more variation compared to the 'site' (p-value = 0.049 *) (Figure 3.18).

The bacterial shoot endosphere and phyllosphere habitats showed substantial overlap - with the phyllosphere bacterial communities fitting well within the range of the endosphere bacterial communities (Figure 3.18). In contrast to the bacteria, there was little to no overlap between the shoot endosphere and phyllosphere fungal communities for the above-ground microbiome habitats (Figure 3.18). The bacterial shoot endosphere samples showed higher variability between the reps, with a wider bacterial community range. One outlier noted in the bacterial phyllosphere samples was Cambridge rep 3 (red circle). For the shoot endosphere samples, there seemed to be at least two reps from each site (aside from Cambridge) that exhibited unique shoot endosphere bacteria, making them seem like outliers.



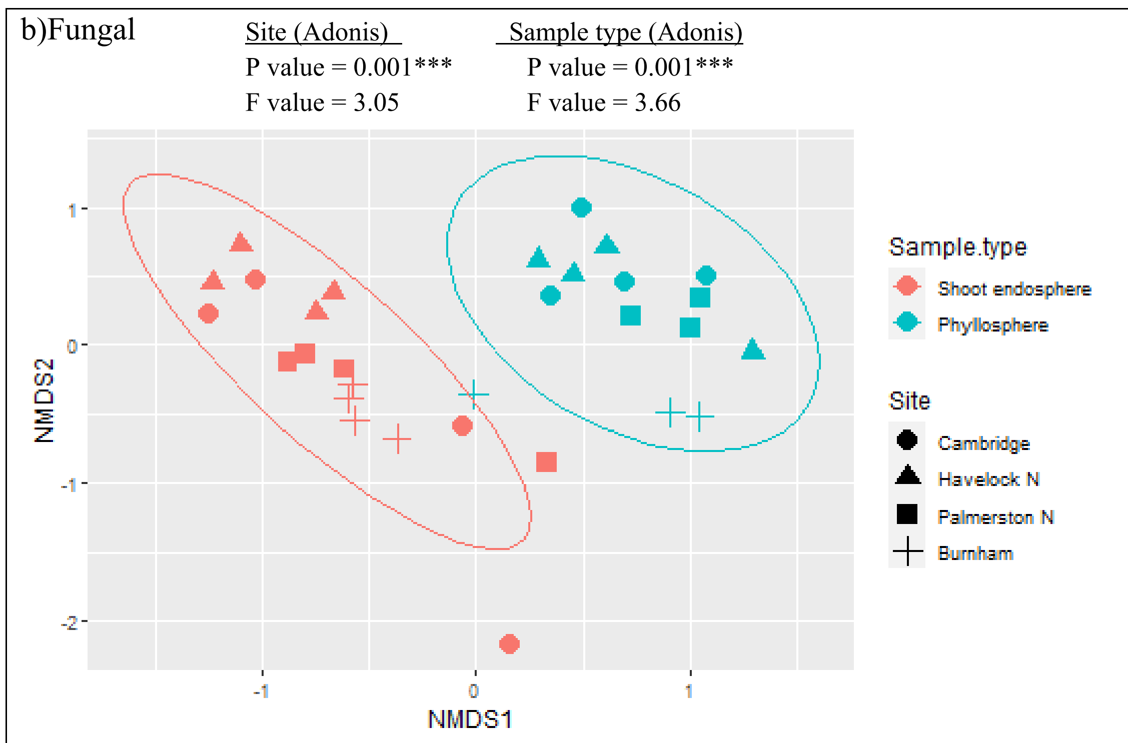


Figure 3.18 Non-metric multidimensional scaling (NMDS) ordination of variation in the a) Bacterial and b) Fungal community structure for the above-ground microbiome habitats of One50 AR37 perennial ryegrass. Each ordination was based on Bray-Curtis dissimilarities among 30 samples.

The shoot endosphere samples showed some bacteria that were consistently dominant across all four sampling sites, including *Curtobacterium*, *Agreia*, and *Xanthomonas* (P-value > 0.05) (Figure 3.19). However, each site was dominated by different Shoot endosphere bacterial genera. The Cambridge site had significantly higher *Paeniclostridium*, *Romboutsia* and *Corynebacterium* (P-value < 0.05). *Serratia* and *Cutibacterium* dominated the Havelock North site, *Staphylococcus* at Palmerston North, and *Cutibacterium* at Burnham. The Cambridge site also lacked specific bacterial genera that were present across the other sites, including *Cutibacterium* and *Staphylococcus*.

The phyllosphere samples also showed some bacterial species present evenly across all four sampling sites, including *Curtobacterium*, *Allorhizobium*, *Sphingomonas* and *Pseudomonas*. *Pseudomonas* dominated the phyllosphere samples at the three North Island sites in contrast to the Burnham site (P-value < 0.05), which instead had higher *Sphingomonas*. *Curtobacterium* was present across the shoot endosphere and phyllosphere samples at all four sampling sites (Figure 3.19).

A)Shoot endosphere

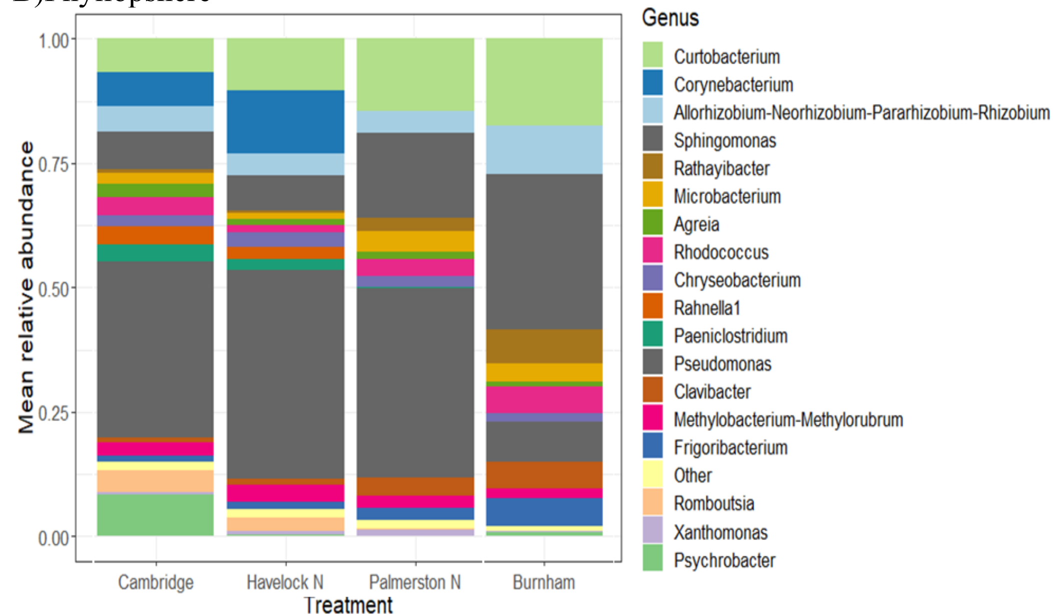
Mean relative abundance
1.00
0.75
0.50
0.25
0.00
Cambridge Havelock N Palmerston N Burnham
Treatment

Genus
Curtobacterium
Agreia
Xanthomonas
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Cutibacterium
Staphylococcus
Microbacterium
Pantoea
Aeromicrobium
Methylobacterium-Methylorubrum
Rhodococcus
Paeniclostridium
Mycobacterium
Mesorhizobium
Other
Serratia
Nocardiopsis
Romboutsia
Brevundimonas
Corynebacterium
Limnohabitans
Stenotrophomonas
Rhodanobacter
Klenkia
Actinopolymorpha
Acinetobacter

Relative abundance (%)
Curtobacterium
Agreia
Xanthomonas
Cutibacterium
Paeniclostridium
Corynebacterium
Romboutsia

Site
Cambridge
Havelock N
Palmerston N
Burnham

B)Phyllopshere



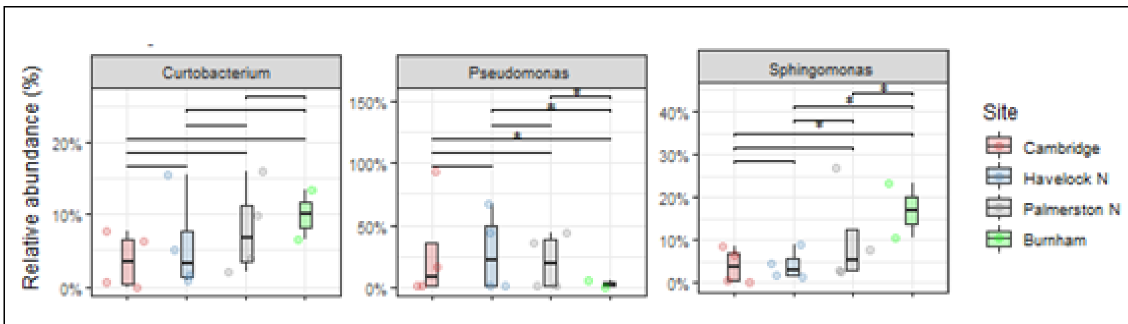


Figure 3.19 The bacterial genera with the highest mean relative abundance for the A) shoot endosphere and B) phyllosphere, microbiome habitats for perennial ryegrass One50 AR37 across the four sites. Genera of interest were compared statistically across the sites based on a Wilcox test. Note: Due to the differential bacterial genera in the shoot endosphere compared to the phyllosphere, the colour assignment for each genus differs between graphs A) and B).

Common speculations around plant microbiomes are whether members of the shoot endosphere and phyllosphere originate from soil communities. Interestingly, dominant members of the above-ground habitats could also be seen at a lower relative abundance in the below-ground soil habitats, including *Curtobacterium* and *Sphingomonas*. When comparing the absolute abundance of these genera, *Curtobacterium* showed a similar number of reads across all five microbiome habitats (Figure 3.20). However, when considering the relative abundance in each sample type, *Curtobacterium* was only of high relative abundance in the shoot endosphere and phyllosphere samples. In comparison, ASV-9 of the *Sphingomonas* genus was dominant in the above-ground microbiome habitat but showed low absolute abundance in the soil habitats (Figure 3.20).

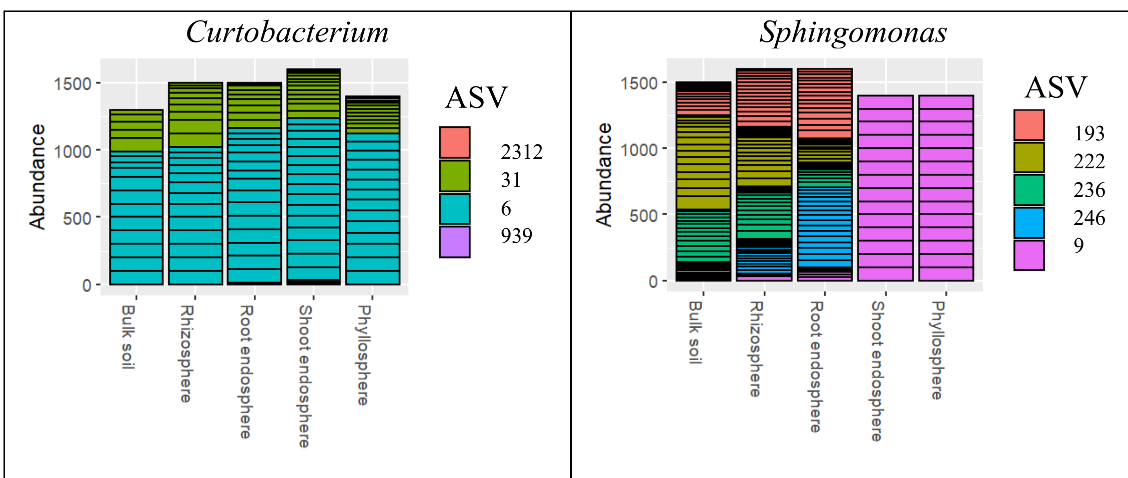


Figure 3.20: The average absolute abundance of the ASVs from the two most dominant above-ground bacterial genera (*Curtobacterium* and *Sphingomonas*) across the five microbiome habitats of perennial ryegrass.

To add credibility to the study, it was essential to compare the amplicon sequencing results to the single sequence repeat *Epichloë* testing results (Section 3.1). All the shoot endosphere samples from the four sites showed *Epichloë* fungal ASVs (Figure 3.21). Two types of *Epichloë* ASVs were found across the One50 AR37 perennial ryegrass shoot endosphere. The Cambridge site contained only ASV 49, whereas the three other sites contained at least some of both *Epichloë* ASVs. The Burnham shoot endosphere samples had high ASV 146; however, this was due to rep 4 containing 3500 reads which biased

the site average (Figure 3.21). Rep 4 at the Burnham site was contaminated with standard endophyte, which likely explains the high number of ASV 146. The amplicon results for *Epichloë* give confidence that a fungus known to be present in the shoot endosphere samples could be detected by this method. Further, this indicates that ASVs may help distinguish amongst some *Epichloë* strains in future studies.

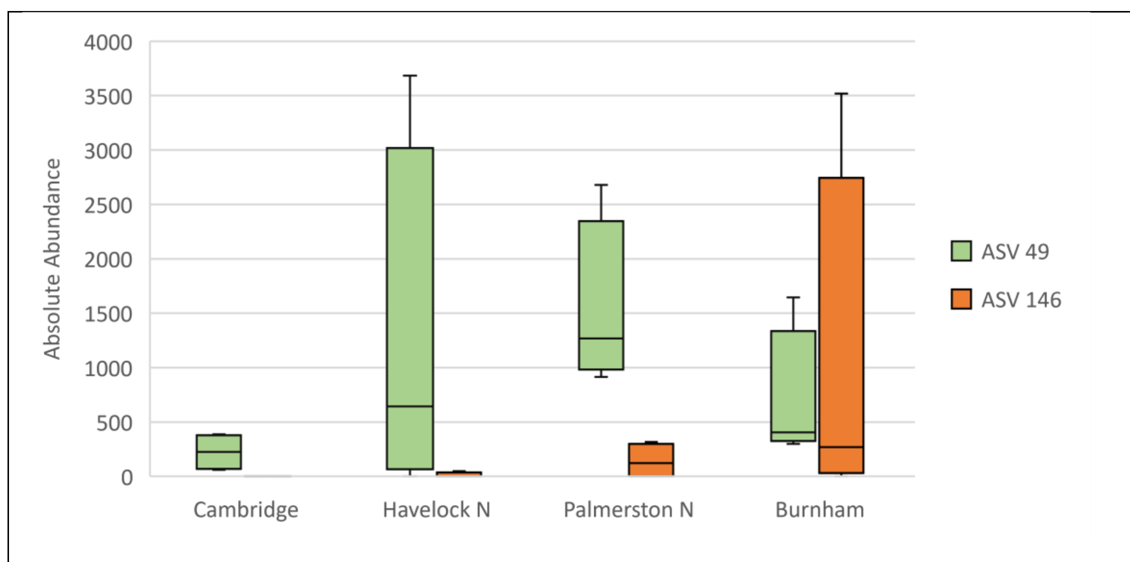
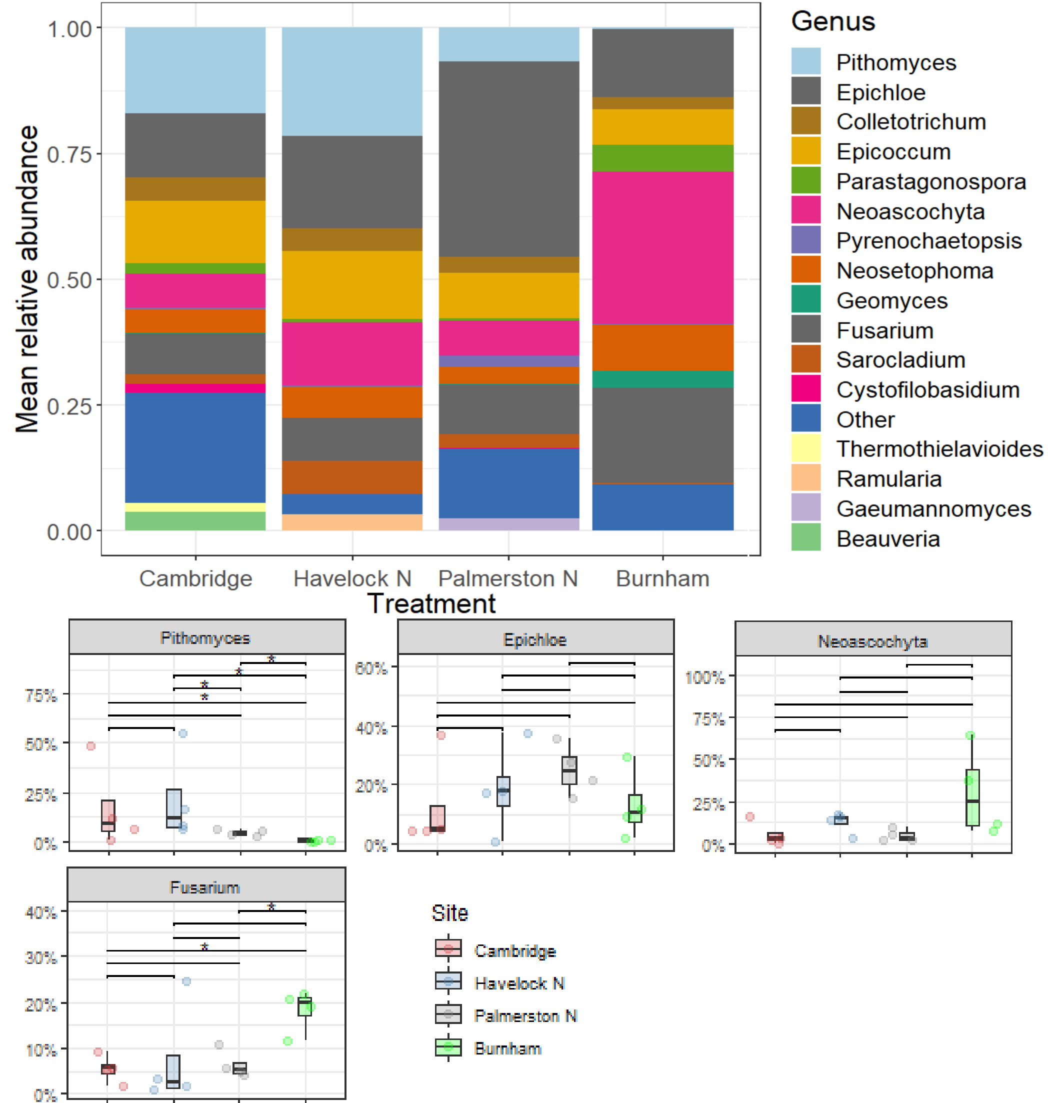
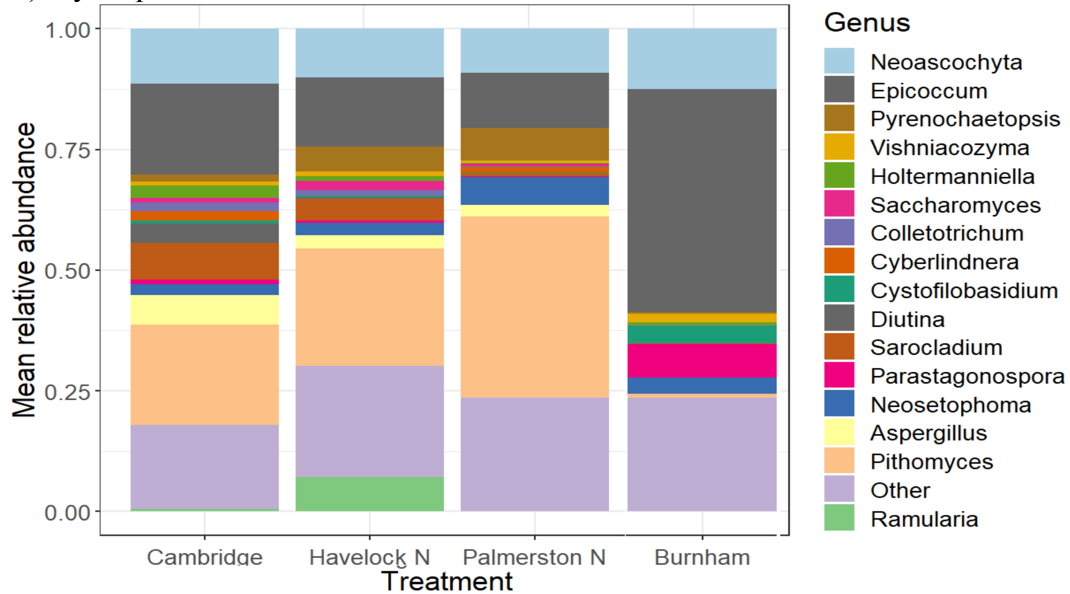


Figure 3.21: The average absolute abundance of the two *Epichloë* ASVs found across the perennial ryegrass shoot endosphere samples for the four sampling sites.

The shoot endosphere and phyllosphere fungal communities across the four sites demonstrated greater similarity than the three below-ground microbiome habitats. Aside from *Epichloë*, the shoot endosphere samples showed other ubiquitous fungal genera over the four sites, including *Pithomyces*, *Neoascochyta*, *Epicoccum* and *Fusarium* (Figure 3.22). Interesting differences between the shoot endosphere genera across the sites included the dominance of *Neoascochyta* and the presence of *Coniothyrium* at the Palmerston North site.

The phyllosphere samples included two main fungal genera at the highest relative abundance in the three North Island sites, including *Pithomyces* and *Epicoccum.* This contrasted with the Burnham site, which had significantly lower Pithomyces and higher *Epicoccum* compared to the three North Island sites (P-value < 0.05) (Figure 3.22). These two fungi could also be found at a high relative abundance in the bulk soil and rhizosphere samples (Figure 3.16), which is important to note for microbial recruitment mechanisms. *Neoascochyta* was also found at a high relative abundance across all four sites (Figure 3.22).


A)Shoot endosphere
Mean relative abundance
1.00
0.75
0.50
0.25
0.00
Cambridge
Havelock N
Palmerston N
Burnham
Treatment
Genus
Pithomyces
Epichloe
Colletotrichum
Epicoccum
Parastagonospora
Neoascochyta
Pyrenochaetopsis
Neosetophoma
Geomyces
Fusarium
Sarocladium
Cystofilobasidium
Other
Thermothielavioides
Ramularia
Gaeumannomyces
Beauveria
Pithomyces
Epichloe
Neoascochyta
Fusarium
Site
Cambridge
Havelock N
Palmerston N
Burnham


B)Phyllosphere
Mean relative abundance
1.00
0.75
0.50
0.25
0.00
Cambridge
Havelock N
Palmerston N
Burnham
Treatment
Genus
Neoascochyta
Epicoccum
Pyrenochaetopsis
Vishniacozyma
Holtermanniella
Saccharomyces
Colletotrichum
Cyberlindnera
Cystofilobasidium
Diutina
Sarocladium
Parastagonospora
Neosetophoma
Aspergillus
Pithomyces
Other
Ramularia

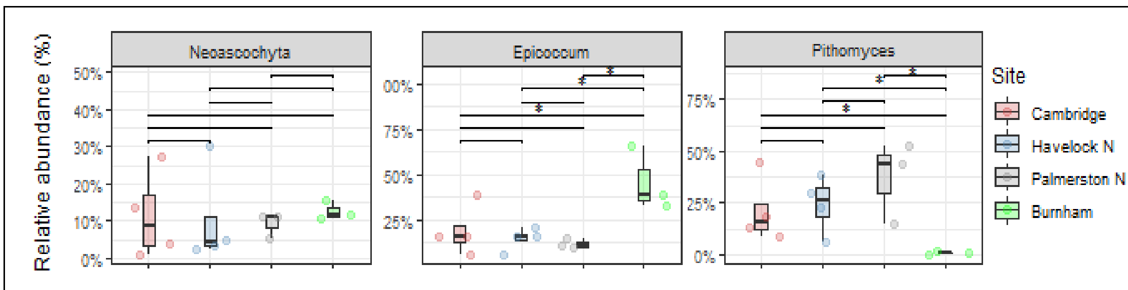


Figure 3.22 The fungal genera with the highest mean relative abundance across the A) shoot endosphere and B) phyllosphere, microbiome habitats for perennial ryegrass One50 AR37 across the four sites. Genera of interest were compared statistically across the sites based on a Wilcox test (P-Value < 0.05 = *). Note: Due to the differential bacterial genera in the shoot endosphere compared to the phyllosphere, the colour assignment for each genus differs between graphs A) and B).

A finding of potential interest to farmers was the higher relative abundance of *Pithomyces* in the shoot endosphere and phyllosphere of the North Island sites compared to the Burnham site (P-value < 0.05). When exploring the species belonging to the *Pithomyces* genus, only one species was found across the perennial ryegrass microbiome, which was *Pithomyces chartarum* (Figure 3.23). The Burnham site showed significantly lower *P. chartarum* than the North Island sites, which is relevant to facial eczema research.

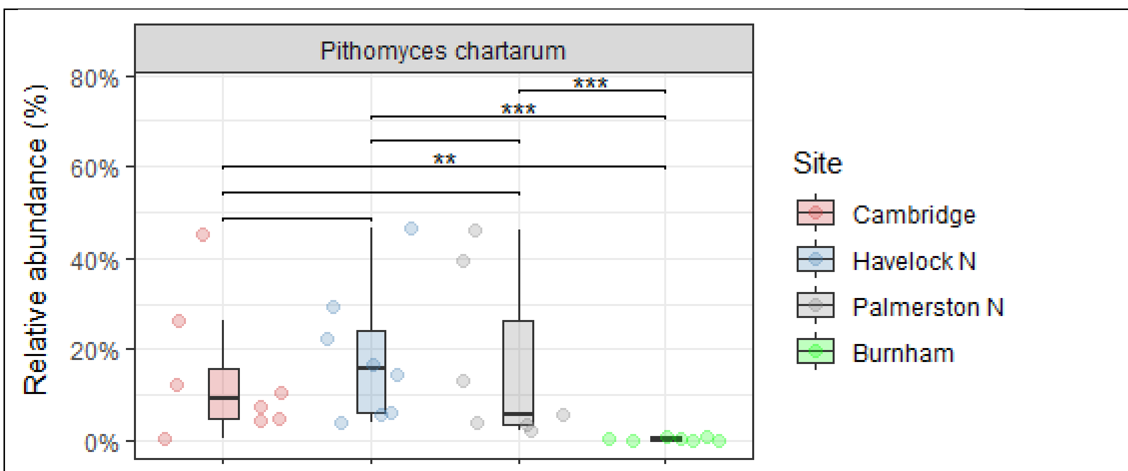


Figure 3.23 The relative abundance of *Pithomyces chartarum* in the shoot endosphere and phyllosphere across the four-sampling sites. The different sites were compared using a Wilcox test. P-value significance codes; '***' 0.001< '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

All four sites show differences in the abundant bacterial and fungal genera, with more considerable differences between the Burnham site and the three North Island sampling sites. The unique characteristics of the Burnham microbiome highlight a clear difference between the Burnham site compared to the three other sites, which could be explained by biogeography, climate, or physical characteristics of the environment. However, the different (unplanned) management at the Burnham site was likely the biggest driver of these microbial differences (see section 3.4.1).

3.3 How did the environment affect the perennial ryegrass microbiome?

There were apparent differences in the highly abundant taxa across the four sampling sites and the five perennial ryegrass microbiome habitats. However, a remaining research question was whether links could be made between the environmental variables and the bacterial and fungal communities at each site.

3.3.1 Soil nutrient metadata at the Cambridge site

From an initial analysis of the soil nutrient data, the Cambridge bulk soil samples stood out compared to the other three sites due to much higher measures of organic matter, extractable organic sulphur, C/N ratio, total carbon, total nitrogen, and soil moisture. This was likely attributed to the Cambridge soil type, a loamy peat soil. The question arose about whether this increase in nutrient availability, such as nitrogen and carbon from the higher organic matter in the soil, would contribute to differences in the associated bacterial and fungal communities.

It first made sense to explore the bulk soil microbiome habitat due to collecting nutrient, soil and nematode metadata based on the bulk soil samples from each sampling site. To explore this, an NMDS ordination was applied to the 16 bulk soil samples across the four different sampling locations (Figure 3.24). All except one of the nutrient variables (Magnesium – Mg) were significant predictors across the bacterial and fungal communities in the bulk soil (EnvFit p-value < 0.05). Weak predictors demonstrated shorter arrows than strong predictors. Notably, anaerobic mineralisable nitrogen, extractable organic sulphur and carbon-nitrogen ratio were all high predictors of the Cambridge microbial communities indicated through the long arrows. The anaerobic mineralisable nitrogen distance matrix had a weak positive relationship with the bulk soil communities Bray-Curtis dissimilarity matrix (Mantel statistic R-value: 0.44, p-value = 0.001). Cambridge reps three and four could be linked to differences in organic matter, cation exchange capacity, nitrogen, sodium levels, Carbon/nitrogen ratio and extractable organic sulphur in the soil. Interestingly, the Havelock North bacterial and fungal associations could also be correlated to soil volume weight and dry matter differences. The Burnham and Palmerston North site could not be linked to any soil nutrient variables, indicating that other mechanisms contribute to differences in the microbiome at these sites.

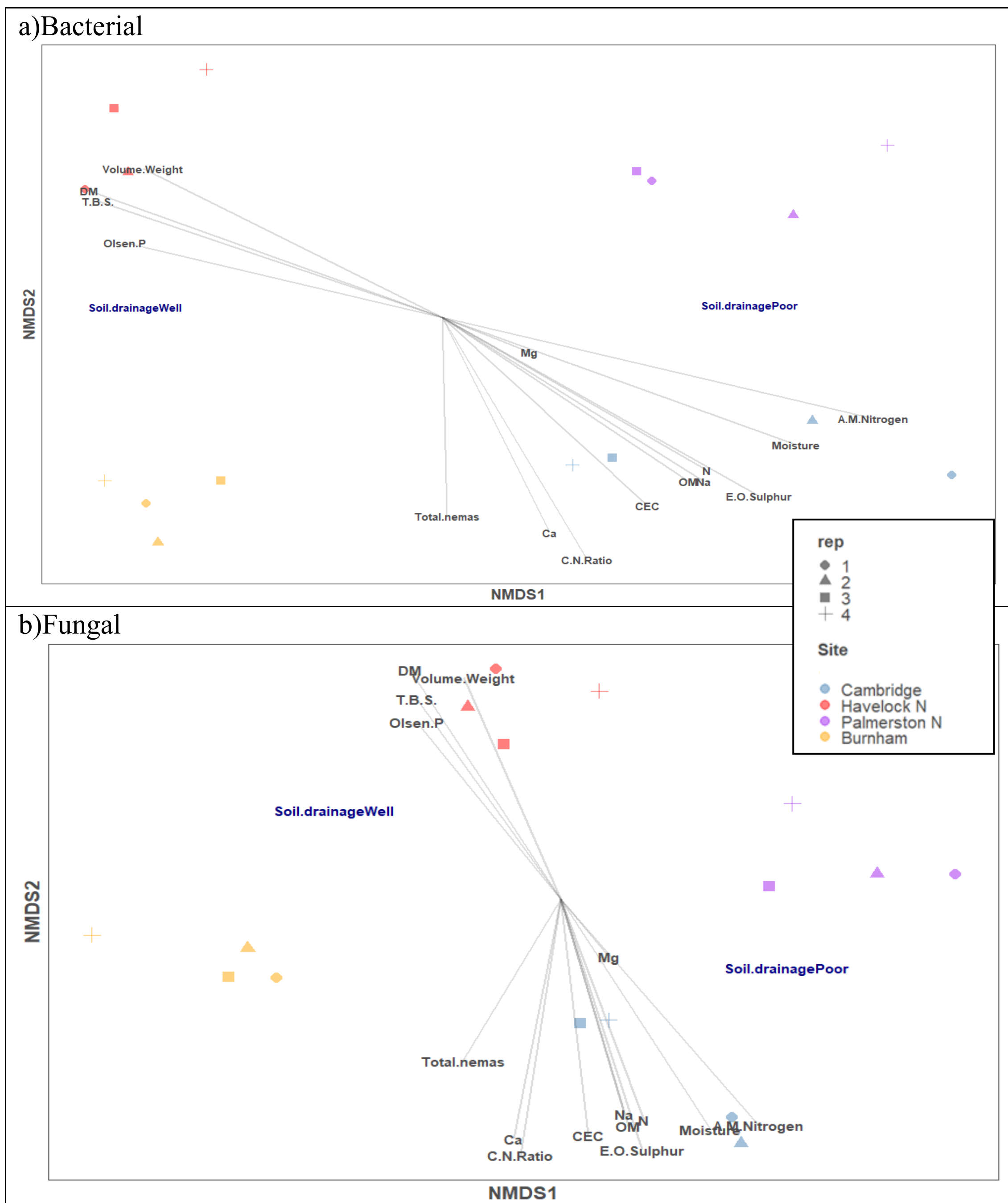


Figure 3.24 Non-metric multidimensional scaling (NMDS) ordination of variation in the a) bacterial and b) fungal community structure overlayed with the soil nutrient variables that were found to be significantly different between the four sites (p-value <0.05). Each ordination was based on Bray-Curtis dissimilarities among 16 samples. Adonis, P< 0.001.

3.3.2 Trends at the Burnham sampling site

Aside from management differences, the Burnham site was the only South Island location sampled in this study and therefore had the largest geographical difference to the next closest site. Burnham also experienced lower average minimum temperatures in the winter season, at least 4°C lower than the three North Island sites (P-value < 0.001). However, in an NMDS ordination, the Burnham site bulk soil bacterial communities could not be linked to differences in the average minimum winter temperature (Figure 3.25). The Palmerston North site did show linkages to higher rainfall in late spring and

summer; however, the difference between the other sites was non-significant (P-value > 0.05 – Section 3.1).

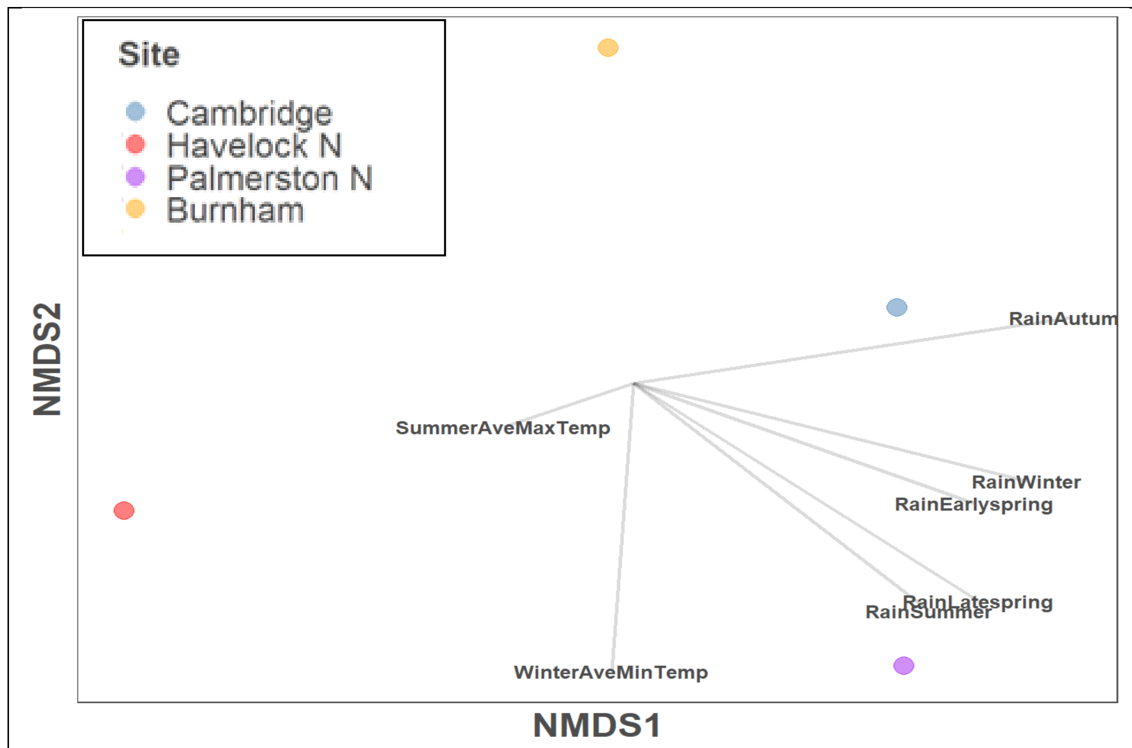


Figure 3.25: NMDS ordination of variation for the bulk soil bacterial community structure for the four sampling locations. The Envfit function was used to overlay the climate data for each site. The ordination was based on Bray-Curtis dissimilarities among four samples. The reps at each site were averaged due to only having climate measurements for each site and not each rep.

The Burnham site had higher ryegrass production than the three North Island sites (ANOVA p-value: 0.002). An NMDS ordination applied to the 16 bulk soil samples bacteria showed that the average annual ryegrass production at the Burnham site could be linked to differences in the bulk soil bacterial communities (Figure 3.26).

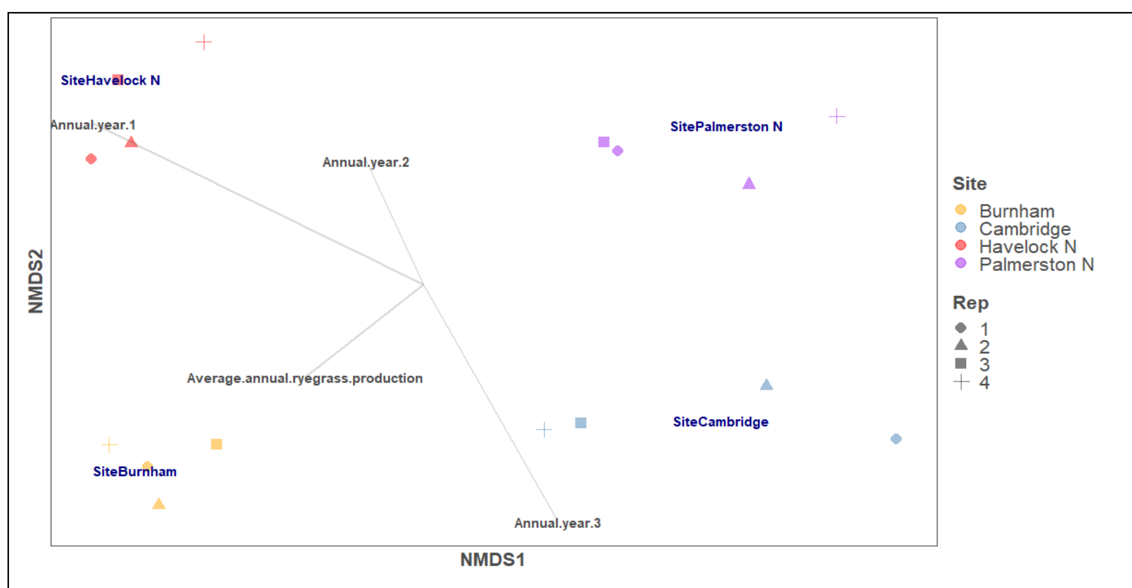


Figure 3.26 NMDS ordination of variation for the bulk soil bacterial community structure for the four sampling locations with consideration of the DM production data using the Envfit function. The ordination was based on Bray-Curtis dissimilarities among 16 samples.

3.4 The perennial ryegrass microbiome and DM production

After observing a moderate correlation between the average annual ryegrass production at the Burnham site and variation in the bulk soil bacterial communities (Figure 3.26), it was valuable to explore whether this was reflected at the ASV level (Figure 3.27).

An NMDS ordination showed that average ryegrass production at each site did not significantly explain trends across the highly abundant bulk soil bacterial ASVs (P-value > 0.05). Three bacterial ASVs were associated with the Burnham site, including *Acidothermus* (ASV 358), an uncultured genus from the *Gaiellales* order (ASV 327) and 67_14_ge from the *Solirubrobacterales* order (ASV 254); however, these associations were not statistically linked to ryegrass production (Figure 3.27). Also, the Cambridge and Havelock North sites seemed to be more correlated to *Bacillus* ASVs.

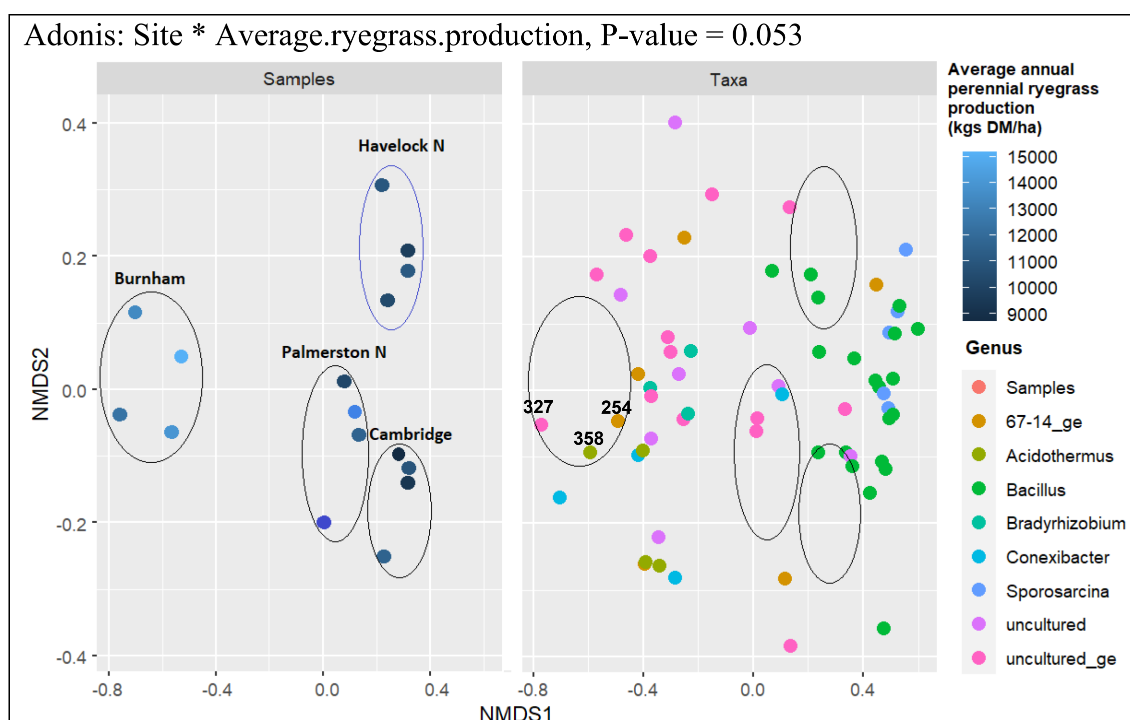


Figure 3.27 NMDS ordination of variation for the high relative abundance bulk soil bacterial ASVs among the four sampling locations. The ordination was based on Bray-Curtis dissimilarities among the four sites with consideration of the annual dry matter production at each site. The ordination output shows the spatial arrangement of the correlated bacterial ASVs and their associated genus.

Interestingly, average ryegrass production across the four sampling sites significantly correlated to trends across the highly abundant bulk soil fungal ASVs (P-value < 0.01). Three fungal ASVs, including *Penicillium* ASVs (28 and 196) and a *Fusarium* ASV (23), were identified in correlation to the Burnham site's higher ryegrass production (Figure 3.28). The lower-producing Cambridge and Havelock North sites also showed associations with different *Penicillium* ASVs.

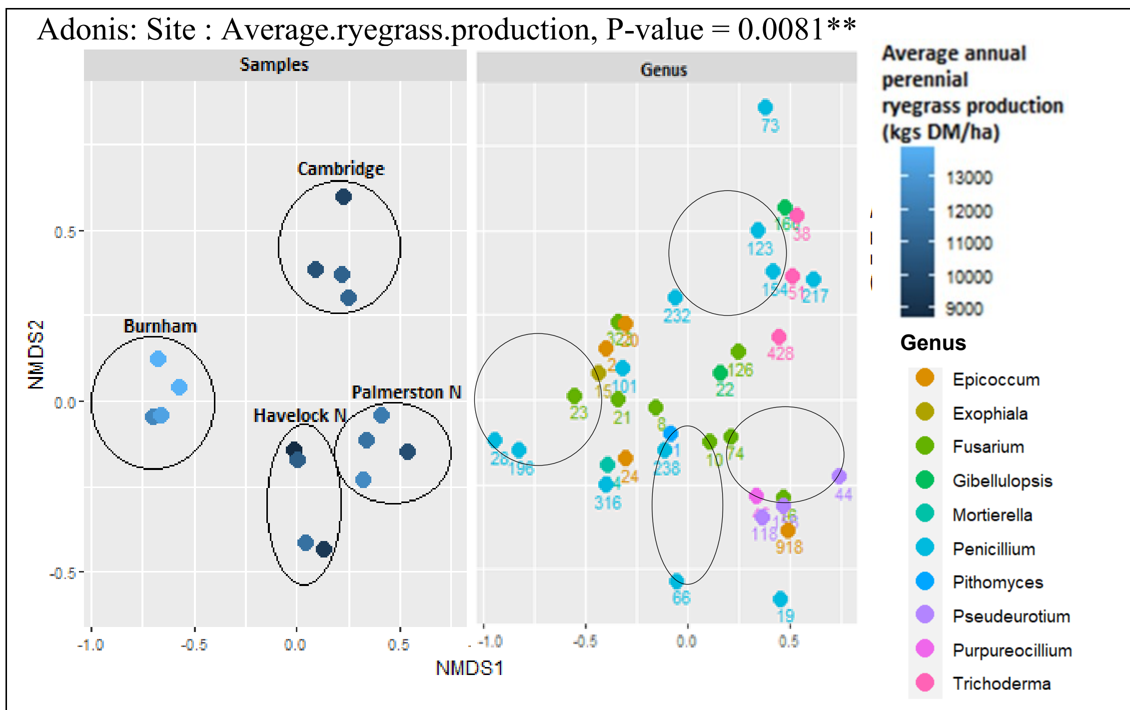


Figure 3.28 NMDS ordination of variation for the top bulk soil fungal ASVs among the four sampling locations. The ordination was based on Bray-Curtis dissimilarities among the four sites with consideration of the annual dry matter production at each site. The ordination output demonstrates the spatial arrangement of the correlated fungal ASVs and their associated genus.

3.4.1 Differences within the Burnham site.

In the final months of the thesis write-up, it was regrettably communicated that the Burnham ryegrass plots were managed differently during the trial period compared to the other three sites. Plot management differences included mowing the pasture rather than grazing by livestock during the trial. Another management difference pre-established for the Burnham site was summer irrigation, replicating the common practice utilised by dairy farmers in the Canterbury region. The summer irrigation did not appear to affect the soil moisture regarding the sampling time (Table 3.4, Table 3.6); however, the irrigation throughout the trial would have reduced moisture stress on the ryegrass plants. Defoliating the Burnham plots through mowing instead of grazing represents a clear difference in management, excluding the addition of animal waste, treading and unbalanced pasture residual during the trial period.

After establishing that the Burnham site was managed differently, it hindered this study's ability to make credible comparisons to the three other sampling sites. Alternatively, the Burnham site was examined individually to explore microbial variation amongst the plot replicates. It must be noted that this analysis is solely qualitative due to inadequate replication.

The annual pasture production was dominant for rep 4 for all three years of the trial (Table 3.14). In year 3 of the trial, reps 1 and 2 produced at least 2000 kgs DM/ha less than reps 3 and 4 (Table 3.14). Rep 1 also showed poor visual pasture health and viable plant loss at the sampling time, attributed to signs of grass grub damage (previous Figure 3.2).

Table 3.14 The annual pasture production (kg DM/ha) for the four perennial ryegrass plot replicates at the Burnham site. The annual production was calculated for the three years of the trial from the plot sowing date up until the sampling date (April 2018 - April 2021).

Year	Rep 1	Rep 2	Rep3	Rep 4
1	15461	12868	14275	15578
2	10053	9437	10613	12603
3	14671	14806	16869	17267
Mean	13396	12370	13919	15149

Although rep 4 at the Burnham site demonstrated a higher DM production, the differences in ryegrass production did not significantly correlate with variation across the below-ground perennial ryegrass bacterial or fungal communities (P-value > 0.05) (Figure 3.29).

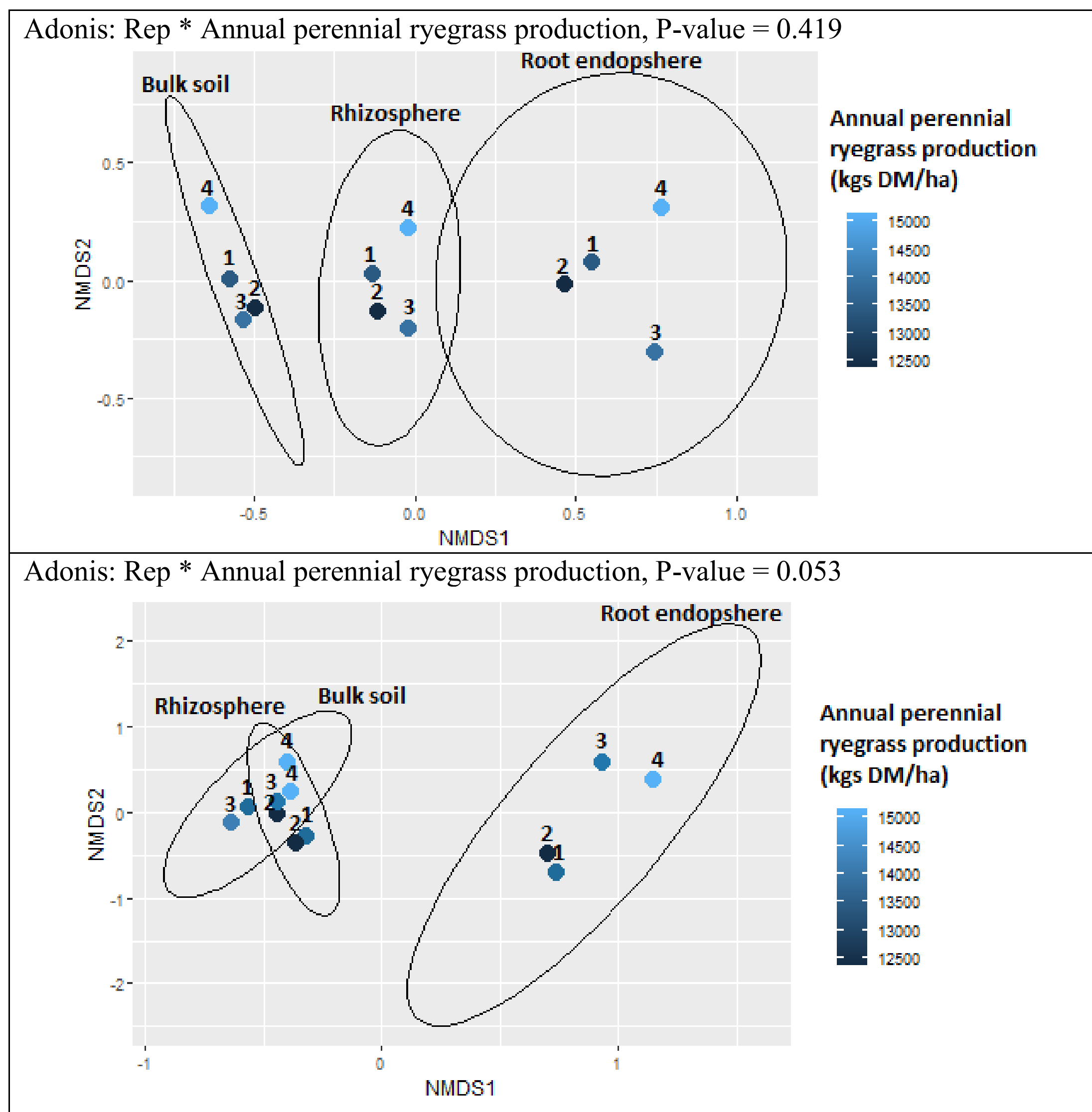


Figure 3.29 NMDS ordination of variation for the below-ground bacterial and fungal ASVs for the four replicates at the Burnham sampling location. The ordination was based on Bray-Curtis dissimilarities among the four reps with consideration of the annual dry matter production.

3.4.2 Focussing on the North Island sites

When removing the Burnham site, there were no noticeable changes to the overall perennial ryegrass microbiome trends. An NMDS ordination of variation confirmed that both the sampling 'Site' and microbiome habitat "Sample type" still significantly correlated to variation across the perennial ryegrass microbiome (P-value <0.001) (Figure 3.30). For this reason, the Burnham site was not removed from any prior sections of the manuscript, as its removal from the dataset did not cause significant changes to the perennial ryegrass microbiome trends.

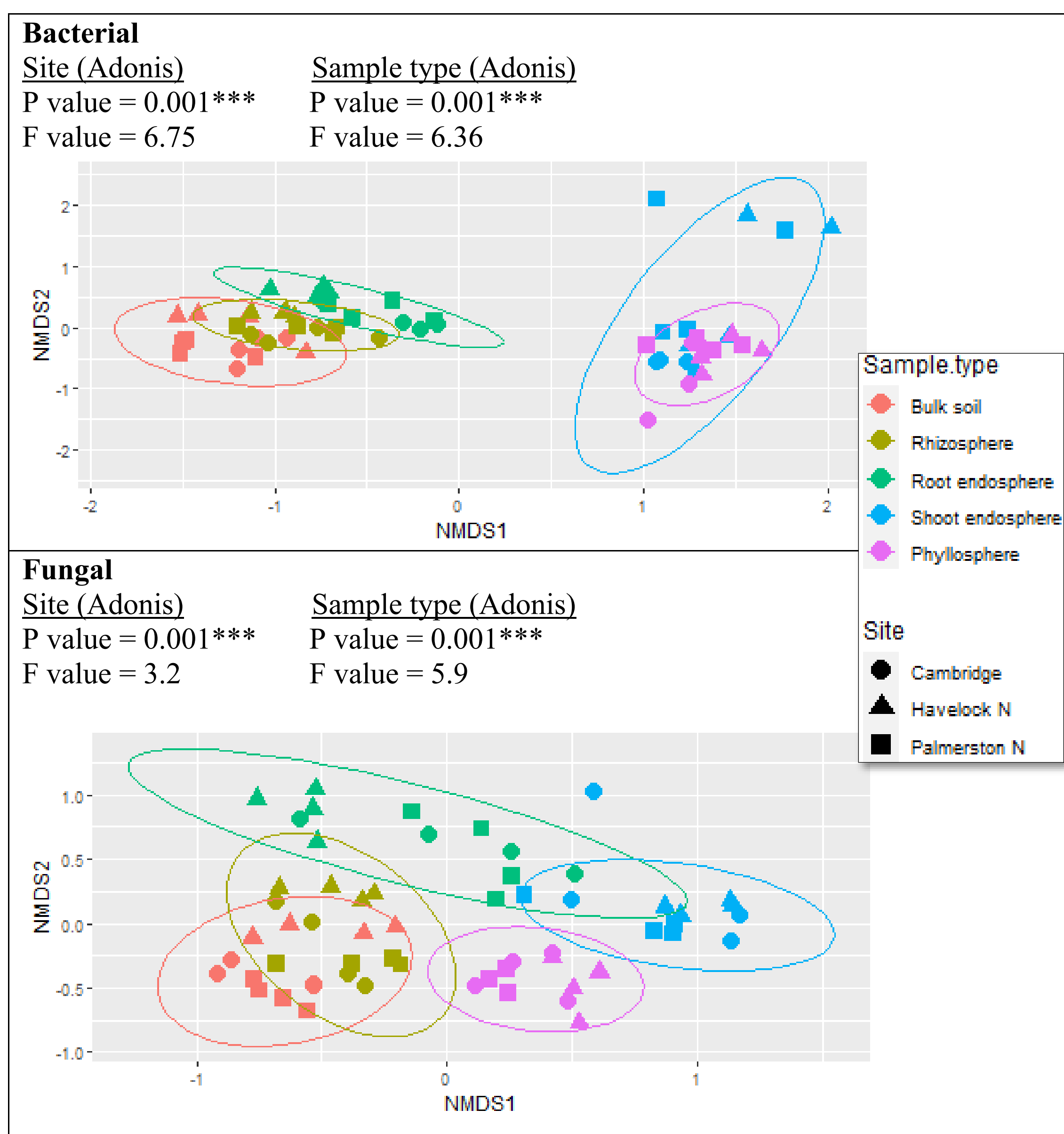


Figure 3.30 Non-metric multidimensional scaling (NMDS) ordination of variation in the a) Bacterial and b) Fungal community structure for the five microbiome habitats of One50 AR37 perennial ryegrass. Each ordination was based on Bray-Curtis dissimilarities among 60 samples from the three North Island sampling sites. F and P values are based on the PERMANOVA model.

There was significant clustering between the bacterial and fungal communities for each microbiome habitat (Figure 3.30). Likewise, the taxonomic analysis showed similarities between the most relatively abundant genera in each microbiome habitat at the three

North Island sites (sections 3.2.5 and 3.2.6). However, some key differences were noted between the three North island sites, including the dominance of *Pseudomonas* in the Palmerston North root endosphere samples (previous Figure 3.15). Therefore, the root endosphere bacterial communities were investigated further, considering the ryegrass productivity at each site.

Palmerston North had the best visual ryegrass health and plant survival at the time of sampling (Autumn 2021) and maintained consistent One50 AR37 ryegrass production in each year of the trial compared to Havelock North and Cambridge, indicative of higher ryegrass persistence. The Havelock North site demonstrated the lowest pasture productivity and visual appearance at the time of sampling. However, the mean annual DM production was not significantly different between Havelock North and Palmerston North (Tukey p value >0.05). Therefore, although the Palmerston North site did not show a higher mean annual DM production, it did demonstrate higher persistence than the other two North-island sites. The Palmerston North site showed increased *Pseudomonas* in the root endosphere compared to the three other sampling sites (Figure 3.15). The root endosphere bacteria were then explored for correlations in average DM production. Although not statistically significant (P-value > 0.05), it was interesting that an uncultured bacterial genus from the *Devosiaceae* family (ASV-735) was associated with the one lower-producing rep at the Palmerston site (Figure 3.31). On the other hand, *Bradyrhizobium* ASV-253 and *Streptomyces* ASV-32 were associated with the three high DM-producing ryegrass plots, possibly providing an advantage for perennial ryegrass growth.

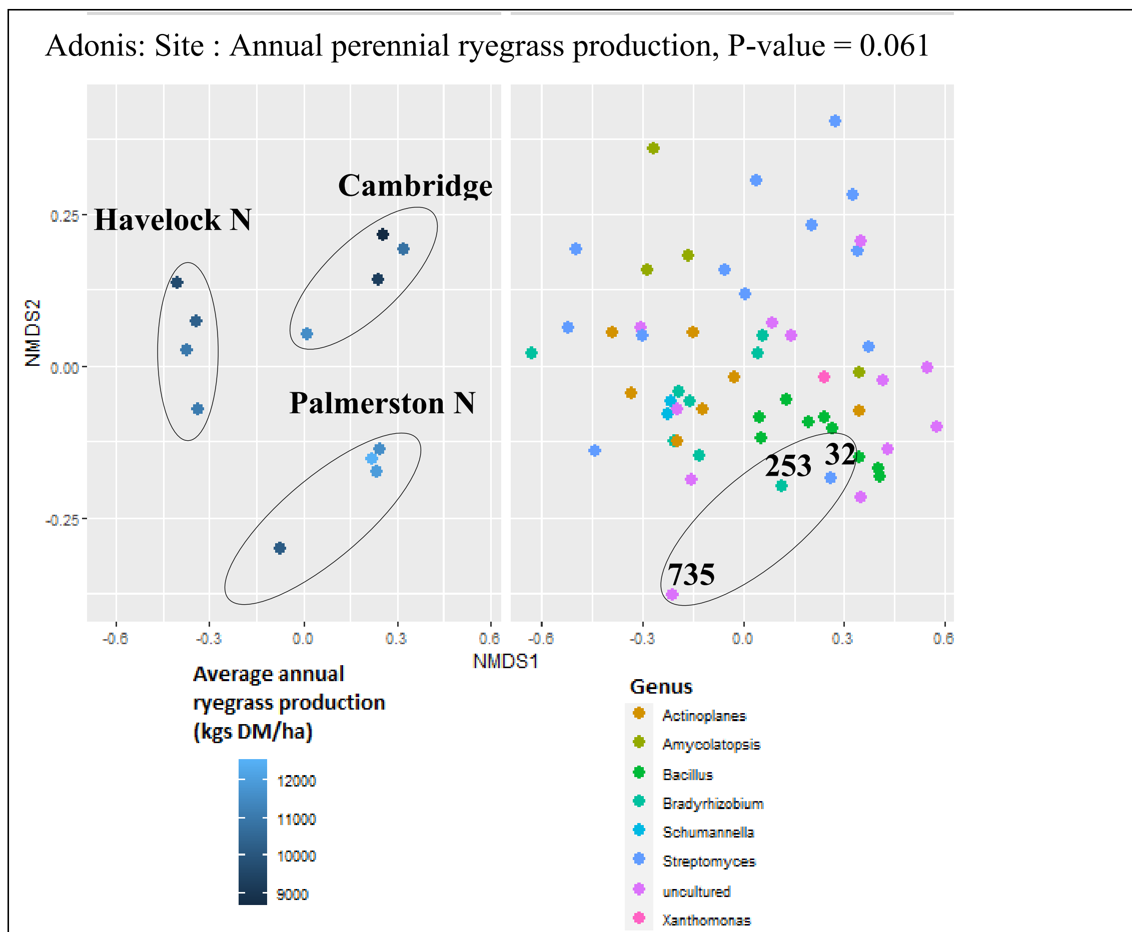


Figure 3.31 NMDS ordination of variation for the root endosphere bacterial community structure across the three North Island sampling locations considering the average annual ryegrass production at each site. The ordination was based on Bray-Curtis dissimilarities among the three sites. The ordination output provides the corresponding spatial arrangement of the most abundant ASVs correlating to the ryegrass production for the four reps at each site. ASVs relating to the Palmerston North ryegrass production are labelled.

In summary, the three North Island sites showed the same microbiome trends as when the Burnham site was included. After excluding the Burnham site from the analysis, there was still strong evidence that the sampling location and microbiome habitats explain variation across the perennial ryegrass microbiome.

4 Discussion

The influence of the perennial ryegrass microbiome on pasture persistence is a relatively understudied area and has gained increasing interest in the New Zealand dairy industry. Plants are shaped by diverse bacterial and fungal communities playing vital functions in plant growth and health enhancement. These microbial assemblages can be influenced by host species and environmental variables (De Wit & Bouvier, 2006; Jones et al., 2019). The composition and driving factors influencing the perennial ryegrass above-ground and below-ground microbiomes remain unexplored.

Over the past decade, investigations have reported that environmental variables, such as nutrient content (Attwood et al., 2019), soil type (Naylor et al., 2017), geographic distance (Gaube et al., 2021), and average temperature and rainfall (Dubey et al., 2019), can correlate to shifts in the microbial community structure of plants (Blaško et al., 2015). Therefore, the presence of a site-specific perennial ryegrass microbiome was likely due to the variability of the environmental factors and management practices across different New Zealand farming locations. This study represents the first detailed exploration of the five microbiome habitats of perennial ryegrass One50 AR37, exploring the effect of farming location. Characterising the trends associated with the perennial ryegrass microbiome lays the research foundation necessary for its future exploitation in improving pasture persistence.

4.1 Trends across the microbiome habitats

Perennial ryegrass provides a diverse environment for bacterial and fungal communities, including the below-ground root and soil-associated portion and the above-ground leaf and stem portion. The five microbiome habitats of perennial ryegrass are fundamentally different in their physiochemical conditions. The above-ground portion of the plant is subject to harsher environmental and biotic factors, including UV exposure, wind, rainfall and large grazing herbivores (Turner et al., 2013). In contrast, soil environments represent more stable, nutrient-rich habitats, usually correlating to higher diversity and abundance of microorganisms (Delmotte et al., 2009). The current study also found this pattern, with a clear separation between the below and above-ground bacterial and fungal communities (Figure 3.6 and Figure 3.9).

The below and above-ground microbiome habitats of the perennial ryegrass differed in terms of the number of bacterial and fungal ASVs, alpha and beta diversity (ordinations) of the communities, the number of core and site-specific taxa, and the composition of highly abundant taxa. However, this study found that the perennial ryegrass microbiome is structured similarly at the phylum level across the different microbiome habitats. Two bacterial phyla; *Actinobacteriota* (34.6 %), *Proteobacteria* (34.2%) and two fungal phyla; Ascomycota (54.25%) and Basidiomycota and (17.6%), dominated the perennial ryegrass microbiome. These phyla were dominant across all five microbiome habitats, except Actinobacteriota was more dominant in the below-ground habitats, and *Proteobacteria* was more dominant in the above-ground habitats. When analysing to the genus level, each

of the ryegrass microbiome habitats had different highly abundant taxa capturing the niche-specificity of the different plant compartments.

The bulk soil and rhizosphere habitats had a higher community richness and diversity than the shoot endosphere and phyllosphere habitats. This trend has been observed in other agricultural plants, including the tomato plant, which exhibited the lowest bacterial diversity in the phyllosphere, the lowest ASV richness in the plant leaf endosphere and the highest diversity and ASV richness in the rhizosphere (C.-J. Dong et al., 2019). Likewise, the wetland grass *Phragmites australis* has previously demonstrated dissimilarity in the composition, structure, and assembly of bacterial communities in the rhizosphere compared to the phyllosphere (Zhou et al., 2019).

4.1.1 Recruitment of microbes from the soil

Although this study found apparent differences in the above- and below-ground perennial ryegrass microbiome, an essential consideration is that the taxa in the shoot endosphere and phyllosphere of plants are often recruited from the soil (Zarraonaindia et al., 2015). The bacterial and fungal taxa found in the shoot endosphere and phyllosphere of perennial ryegrass in this study included Curtobacterium, Sphingomonas, Pithomyces and Epicoccum, could also be found - although in lower abundances - in the bulk soil and rhizosphere samples. Previous studies have demonstrated similar trends, including a study of the grapevine microbiome. Genera commonly found in the soil, such as Curtobacterium and Bacillus, were also found as high relative abundance epiphytic members of the leaves and grapes (Martins et al., 2013). There is evidence that most plant-associated microorganisms originate from soil environments (Zarraonaindia et al., 2015). These microorganisms must migrate to the rhizosphere, rhizoplane, shoot endosphere, and the aerial surfaces of plants before their beneficial effects appear (Zarraonaindia et al., 2015). There are still many unanswered questions regarding the mechanisms of dispersal and the driving factors in selecting certain soil microbes to colonize the above-ground plant habitats.

4.1.2 Relationship between the shoot endosphere and phyllosphere communities

Intriguingly, bacterial communities in the shoot endosphere and phyllosphere habitats showed substantial overlap - with those in the phyllosphere fitting well within the range of the shoot endosphere bacterial communities (Figure 3.18). However, for the fungal communities, there was little to no overlap between the shoot endosphere and phyllosphere (Figure 3.18). This indicates that the perennial ryegrass above-ground bacterial communities are structured differently from the fungal communities, highlighting a difference in selection between these two microbial domains. It is not uncommon for plant species to have significantly different fungal communities in the leaf endosphere and the phyllosphere (Nissinen et al., 2019; Yang et al., 2016). In the current research, the influence of Epichloë endophyte AR37 on the associated fungal communities is a possible driver of this difference. Past researchers have speculated that introduced endophytes can alter the composition of the associated endosphere microbiomes (Roberts & Lindow, 2014). Research to date on the effect of *Epichloë* on fungal communities had varied results, with some studies observing no effect of *Epichloë*

festucae var. lolii and *Epichloë gansuensis* on the fungal microbiome of perennial ryegrass and drunken horse grass (König et al., 2018; B. Liu et al., 2022). Conversely, Nissinen et al. (2019) found an effect of *Epichloë coenophiala* on the tall fescue fungal microbiome, with previous studies also documenting antifungal activity by *E. coenophiala* against fungal pathogens in grasses, including *Limonomyces roseipellis* (pink patch) (Siegel & Latch, 1991). The AR37 *Epichloë* endophyte has already been shown to alter the rhizosphere bacterial and fungal communities in ryegrass with the potential to affect the phyllosphere communities (Wakelin et al., 2015). Although likely, it remains undetermined by this study whether AR37 influenced the segregation between the shoot endosphere and phyllosphere fungal communities. This offers a future research opportunity to compare the microbiome of perennial ryegrass plants with and without *Epichloë* infection.

4.2 Farming location and the perennial ryegrass microbiome

This study shows that farming location significantly correlates to variation across the perennial ryegrass microbiome (Figure 3.6 and 3.9). Even though the Burnham site was managed differently, removing it from the analysis did not change the site-specific trends seen across the other three North Island sites (Figure 3.30).

The evidential driving effect of biogeography (location) has been previously explored across the perennial ryegrass seed microbiome, demonstrating that there were different microbiome clusters in northern vs southern Canterbury farming locations (Tannenbaum et al., 2021). Factors associated with latitudinal geography, including soil types, microclimates, pH, nutrient availability, and management differences, were linked to the microbiome trends (Tannenbaum et al., 2021). The current study noted a similar trend across a broader range of New Zealand, with a higher similarity between the relative abundance of many genera at the three North Island sites compared to the one South Island Burnham site, which often represented an outlier (Figure 3.17). For example, the three North Island sites had significantly higher *Bacillus*, whereas Burnham had significantly higher numbers of *Bradyrhizobium* in the bulk soil, and *Nakamurella*, *Mycobacterium* and a genus of the Burkholderiaceae family: (*Burkholderia-Cabelleronia-Paraburkholderia)*, in the rhizosphere (Figure 3.13). It remains unconfirmed whether a biogeographical difference can explain the uniqueness of the Burnham site or whether it was solely influenced by the different management at this site. Location effects have been explored in other plant species, including the orchid species *Gymnadenia conopsea*, which showed that geographic location was the critical factor in determining the associated bacterial and fungal communities (Lin et al., 2020). Likewise, the extensively studied *Arabidopsis* plant has considerable documentation of geographic location as a significant predictor of variation across the associated microbiome (Talbot et al., 2014).

In the current study, the sampling site was found to significantly explain the variation across all five microbiome habitats, except for the bacterial communities of the phyllosphere (Figure 3.9). The 'site' effect was less evident in the above-ground microbiome habitats compared to the below-ground habitats. The phyllosphere bacterial

communities exhibited no significant effect of sampling location, suggesting that the phyllosphere is under higher selection by the perennial ryegrass plant than the environment.

Each sampling site contained a proportion of site-specific taxa ranging between 22 - 36% of the total taxa. These site-specific taxa were likely selected by the environmental differences at each sampling site rather than selection by the host plant (Lin et al., 2020). Although site-specific taxa were evident at each site, the proportion indicated that there were still between 64 – 78 % of the present taxa that were shared by at least two sites. This higher proportion of non-site-specific taxa indicates that the sampling location does not solely influence the perennial ryegrass microbiome, and other drivers such as host-plant selection and microbe-microbe interactions environmental factors may explain trends (Dastogeer et al., 2020).

4.2.1 Unique attributes of the Burnham sampling site

The Burnham site was unique compared to the other three sampling sites in this study regarding the management practices (mown and irrigated), winter climate, dry matter production and the associated perennial ryegrass microbiome. The Burnham site had a clear biological or physical advantage for ryegrass growth. However, the considerable management differences between it, and the other three North Island sites, compromised the ability to pinpoint whether the microbiome contributed to the higher ryegrass dry matter yield. As aforementioned, perennial ryegrass productivity can be influenced by various factors, including climate, pest prevalence, nutrient availability, and management practices (Daly et al., 1999).

Summer irrigation at the Burnham site would have reduced stress on the ryegrass plants during the trial. It has been well established that low soil moisture reduces perennial ryegrass growth and that droughts are a driver of poor perennial ryegrass persistence (Macdonald et al., 2011). Therefore, the irrigation received by the Burnham site may have influenced the higher DM production compared to the other three sites. Water stress has also been strongly linked to soil rhizosphere bacteria in wheat plants, with irrigated crops demonstrating shifts in the relative abundance of bacteria, including increased *Bacteroidetes* and *Proteobacteria* phyla, and at the class level – *Sphingobacteria* (Mavrodi et al., 2018). Likewise, rice ecotype studies have shown that lowland-irrigated rice ecotypes have enriched bacterial class *Betaproteobacteria* and fungal order Hypocreales compared to upland-rainfed ecotypes. The influence of irrigation on the perennial ryegrass microbiome should be explored in greater depth, treating irrigation as a treatment variable and comparing the associated microbiome to non-irrigated plants. This is important as it would establish whether irrigated, versus rain-fed-only, pastures demonstrate significantly different bacterial and fungal communities.

Mowing pastures prevents the addition of livestock faeces onto the soil. Manure deposition onto pastures of upland soils has been shown to provide an additional substrate for microbial growth and metabolism and alters nutrient availability, including utilisable carbon substrates (Attwood et al., 2019; R. Bardgett et al., 1998). Studies have demonstrated that grazing meadow steppe pastures with livestock can alter the richness

and diversity of soil microbial communities and the availability of soil nutrients (carbon and nitrogen) (Xun et al., 2018). Cow manure has been documented to contain high numbers of the *Alphaproteobacteria, Betaproteobacteria, Actinobacteria*, and *Bacilli* classes, 57 days after it was initially deposited (Wong et al., 2016). This aligns with the trends observed in the current study, with a high relative abundance of *Bacillus* seen across the three grazed sampling sites and a lower relative abundance at the ungrazed Burnham site. Aside from manure deposition, grazing pastures can also alter the soil structure due to treading, and it can be challenging to control the sward grazing residual (Schils et al., 1999).

Although the summer irrigation and mowing defoliation likely influenced the high Burnham perennial ryegrass production, microorganisms can also affect pasture growth. The study did identify two high relative abundance bulk soil fungal ASVs from the *Penicillium* and *Fusarium* genus that correlated to the Burnham site's higher DM production. The *Penicillium* genus has been previously documented to enhance crop productivity and sustainable agriculture (Altaf et al., 2018). Soil inoculation with plant growth providing *Penicillium* species has been documented to significantly promotes the growth, phosphorous uptake, and yield of several important crops, including wheat and sesame (Babu et al., 2015). On the other hand, many *Fusarium* species have been linked to plant diseases, including leaf wilt in tomato plants (Borrero et al., 2004) and ear blight and root rot in barley and malt (Early, 2009). This is not to say that all *Fusarium* have a negative effect on plant growth, as *Fusarium oxysporum* has been documented to produce volatile compounds that improve the shoot and root growth of *Arabidopsis thaliana* and tobacco plants (Bitas et al., 2015). Therefore, there is evidence to suggest that the Penicillium and Fusarium ASVs could contribute to higher ryegrass production. However, this may have been simply an autocorrelation and may not be genuinely linked to the higher DM production. Future studies should control all management variables across the sampling sites so that more confident conclusions can be drawn on whether these ASVs contributed to higher ryegrass DM production. Analysing the ASVs at the species level, and of their functional genes, is necessary to draw more robust conclusions.

4.2.2 Microbiome trends across the three North Island sites

After excluding the Burnham site, it was confirmed that the same microbiome trends remained even when exploring just the three North Island sites (Figure 3.30). Excluding the Burnham site focussed the investigation on some of the apparent differences between the microbiome trends for the three North Island sites. One difference was that Palmerston North had the best visual ryegrass health and plant survival at the time of sampling (autumn 2021) and the highest mean annual DM production compared to the other two North Island sites (Figure 3.3). Therefore, identifying unique attributes of the Palmerston site microbiome became of interest for possible associated pasture persistence benefits. There were significantly higher Pseudomonas in the Palmerston North root endosphere samples (Figure 3.15) compared to Havelock North and Cambridge. *Pseudomonas fluorescens* has been proven to aid agricultural and horticultural crops in coping with biotic stresses by producing antibiotics, siderophores, auxins, cytokines and gibberellin and lowering plant ethylene levels (Sankari Meena et al., 2019). Due to its known success

in other agricultural crops, isolation and subsequent application of *Pseudomonas* species such as *P. fluorescens* to perennial ryegrass plants should be tested to determine whether *Pseudomonas* can directly influence perennial ryegrass growth

4.2.2.1 Higher Pithomyces at the three North Island sites.

The current study found a higher relative abundance of *Pithomyces* in the shoot endosphere and phyllosphere of the North-island sites compared to the Burnham site (P-value < 0.05). The only *Pithomyces* species present across the perennial ryegrass samples in this study was *Pithomyces chartarum.* Although not relevant to pasture persistence research, the high relative abundance of this saprophytic fungus across the North Island sites is a risk for dairy farmers. *P. chartarum* is a known pathogenic fungus due to its production of sporidesmin toxins which is the leading cause of facial eczema (pithomycotoxicosis) in sheep, cattle, goats and deer (Kingsley-Smith, 2021). Facial eczema in dairy herds can strongly affect animal health and milk production by causing liver injury, inflammation, and photosensitisation (Kingsley-Smith, 2021; Little et al., 2011). Cows with facial eczema have shown significantly lower milk production, representing a significant economic loss for dairy farmers. It was unsurprising that *P. chartarum* was more prevalent in the North Island sites as this fungus grows best in humid, warm, and low-altitude climates(Little et al., 2011).

The high prevalence of *P. chartarum* across the North Island site in this study serves as a reminder of the effects climate change may have on pathogenic fungi such as *P. chartarum*. Climate change may exacerbate the impact of *P. chartarum* due to a warmer climate, earlier start to summer, and higher night-time minimum temperatures over summer-autumn (Little et al., 2011).

4.3 Core taxa across the perennial ryegrass microbiome

Many plant species have demonstrated evidence of 'core microbiomes,' i.e., taxa that are common across members of the species despite differences in the environmental conditions (Shade & Stopnisek, 2019) and likely play essential roles in the growth and survival of the host plant. For example, the wheat rhizosphere has previously demonstrated a core microbiome, confirming 177 highly abundant taxa (representing 50% of the total reads) across 12 different soil types and two different agricultural practices. Whilst the concept of a 'core microbiome' has become a hot topic in plant research, when comparing studies, it is important to access the criteria used in the classification of core taxa. Factors to consider in core microbiome analysis include the prevalence or occurrence of taxa across all samples and the relative abundance of these taxa within samples.

Intriguingly, despite the differences in location, climate, soil nutrients, and management practices, the current study identified individual taxa present at all assessed farming locations (Table 3.12 and Table 3.13). A strict criterion was applied, incorporating a high prevalence limit (the ASV was present across 90% of samples) and high relative abundance (the ASV represented a relative abundance of 0.001, 0.1% of the total taxa in the sample). This criterion was based on the idea that a core bacteria or fungi should be consistently seen across almost all ryegrass samples (allowing a non-occurrence of 10%

catering to sampling fall-through), and they should represent at least 0.1% of the total taxa (Neu et al., 2021). This strict criterion serves the statement that core taxa are necessary for plant survival and likely play critical roles in perennial ryegrass growth or survival. Their continued selection by the plant is of interest to pasture persistence research (C. Dong et al., 2021).

A total of 48 bacterial and 26 fungal core ASVs were found across the different microbiome habitats of perennial ryegrass. A higher proportion of core fungal taxa (0.22 – 1.98 %) at a relative abundance higher than 0.001 was found compared to core bacterial taxa (0 – 0.86 %). The proportion of these core taxa across the total taxa in the different microbiome habitats was low (less than 2%). Likewise, no core bacterial ASVs were found at a relative abundance higher than 0.01 (1 %); only once the threshold was decreased to 0.001 (0.1 %) could taxon be found across 90% of samples in at least one of the microbiome habitats. Only five core fungal ASVs could be found at this higher threshold (0.01 – 1 %). These findings indicate insufficient evidence to confirm that perennial ryegrass has a collective core microbiome. The native plant mānuka (*Leptospermum scoparium*) is an exemplary host plant with a specific phyllosphere core microbiome of 10 high relative abundance bacterial OTUs representing 40.1% of the total bacterial reads in the samples (Noble, 2018). The high abundance of these core taxa in the mānuka phyllosphere is indicative of what a clear core microbiome should look like, with the plant selecting highly abundant core bacteria likely playing essential roles in the growth and survival of the host plant.

The lack of evidence for a core microbiome may mean perennial ryegrass does not have a strict dependency on specific taxa at the ASV level. However, a wider functional selection of taxa seems likely due to each microbiome habitat showing distinguishable groupings despite different sampling locations (Figure 3.6 and Figure 3.9). Functional selection of microbial taxa has been explored in the plant rhizosphere of the common weed *Jacobaea vulgaris*, revealing that the plant selected different taxa to the bulk soil with similar functional genes related to transporters, the Embden–Meyerhof–Parnas pathway and hydrogen metabolism (Yan et al., 2017). Also, the genomes of many root-associated microbes have demonstrated functional similarity, such as the ability to encode enzymes to degrade plant-derived carbohydrates (Campos et al., 2016).

The core microbiome analysis found that the *Bacillus* ASV-10 was considered a core ASV across all four sites in all three of the below-ground microbiome habitats. The high prevalence and abundance of this *Bacillus* ASV-10 likely indicates a mutualistic relationship with the bacteria, possibly providing a crucial benefit for perennial ryegrass plant growth and survival of the ryegrass plant. Interestingly, *Bacillus* strains have been previously researched for their drought tolerance benefits for perennial ryegrass where, under severe drought stress (20-day natural drought), plants that were inoculated with the *Bacillus* strains all showed significantly improved fresh shoot weight, dry weight, relative water content (RWC) and chlorophyll compared to the control (Su et al., 2017). This shows the beneficial application that *Bacillus* species could offer for perennial ryegrass drought tolerance – an area of enormous relevance to the pasture persistence issue for specific regions of New Zealand (Su et al., 2017).

4.3.1 Higher specificity of the above-ground perennial ryegrass microbiome habitats

The perennial ryegrass phyllosphere and shoot endosphere habitat demonstrated the least significant effect of sampling location, the highest proportion of core ASVs and a lower proportion of site-specific ASVs. Together these observations suggest that the aboveground microbiome habitats are under higher selection from the perennial ryegrass host plant compared to the below-ground microbiome habitats (Neu et al., 2021).

Higher plant selection of phyllosphere communities makes sense from an ecological perspective since the phyllosphere habitat of plants is considered a hostile environment due to rapid changes in temperature and humidity, limited nutrients, and solar irradiation (Mir et al., 2022). In the 16S dataset, the phyllosphere habitat core ASVs: *Pseudomonas*, *Chryseobacterium*, *Clavibacter*, *Curtobacterium*, *Friedmanniella*, *Frigoribacterium*, *Microbacterim*, *Nocardioides*, and *Sphingomonas*. Previous studies have seen similar bacterial genera occupying the phyllosphere of ryegrass, including *Pseudomonas,* which is documented to possess a wide range of adaption and biocontrol factors allowing its survival in phyllosphere environments (Abraham, 2021). The application of *Pseudomonas* in inhibiting microbial pathogens is an emerging research topic of interest for many agricultural plants (Legein et al., 2020). However, the selection and subsequent application of *Pseudomonas* species must be cautiously approached since certain species are also highly virulent, including *Pseudomonas syringae pv. actinidiae* (Psa), which causes bacterial canker on kiwifruit vines (Kim et al., 2016). In other research, *Sphingomonas* and *Curtobacterium* have also been in the phyllosphere of grasses (Behrendt et al., 2002) and can promote plant growth; however, *Curtobacterium* species are also well-documented plant pathogens (Chase et al., 2016). In the ITS dataset, the shoot endosphere habitat demonstrated the highest core ASVs, including genera: *Aspergillus*, *Epichloë*, *Epicoccum*, *Fusarium*, *Neoascochyta* and *Pithomyces*. As expected, *Epichloë* represented a core member of the shoot endosphere community. Its conservation by the plant during the three-year trial reinforces the biological advantage it carries for the plant in invertebrate pest protection (Tapper & Lane, 2004).

4.4 Limitations of the research and future recommendations

The different management at the Burnham sampling site, and the later notification of this, impeded the ability to draw solid conclusions from this study based on the effect of farming location on the perennial ryegrass microbiome. To effectively explore microbial biogeography, future studies should control all management variables, such as how the plots are defoliated and irrigation regimes. This control would allow more effective conclusions to be drawn about the influence of abiotic and biotic factors associated with biogeography.

In addition, the effects of essential soil nutrients, such as nitrogen, could be explored by applying different quantities of nitrogen to perennial ryegrass plots and observing the associated microbial communities. Nitrogen is a plant growth-limiting resource that is affected heavily by the presence of microbes in the soil (Sankari Meena et al., 2019). Manipulating the potentially available nitrogen, and analysing the associated microbiome, could pinpoint the vital nitrogen-fixing bacteria with essential roles in ryegrass growth.

In a review of the current study, the methodology used in the taxonomic classification of the fungal ASV dataset was not as optimal as the bacterial dataset. The use of the NCBI database for classification was initially thought necessary for two reasons. Firstly, to allow low-level contaminants in the dataset to become apparent by correctly classifying them as bacteria or plants, unlike the fungi version of UNITE, which was sometimes falsely classifying contaminate reads to the closest matched fungi or came up with zero results. The second reason was to seek an improvement in the taxonomic resolution by using the latest fungal annotations available due to fungi being under-researched compared to bacteria. It was expected that the non-redundant database in the NCBI would be more up-to-date for fungal identification compared to UNITE, which lags a bit behind.

A comparison of the sequence match results between the NCBI database used here and the All-Eukaryote version of UNITE should be undertaken in future studies of this design to understand differences in apparent community composition between the two databases (Kõljalg et al., 2019). In addition, it is important to consider that off-target amplification is a common problem regarding the use of ITS primers, not only with the amplification of host DNA, but it can also include mispriming and chimeras. DADA2 has a built-in chimera removal function that caters to most of these occurrences. However, it is essential to remember that just because a sequence matches well to another sequence in GenBank does not mean it is an accurate fungal classification since mispriming is highly reproducible (Breitwieser et al., 2019). Caution must be taken when using a non-curated database such as GenBank, as questions must be asked based on the source of the publication and whether the classification makes biological sense (Breitwieser et al., 2019).

While relying on the NCBI homology for the fungal taxonomic assignment was not optimal, this method was chosen early in the data analysis due to preconceived issues using the curated UNITE database. This highlights a crucial decision for future papers published from the current project, which will reanalyse the fungal data using the UNITE database. Likewise, if given the opportunity again in a similar research design, a curated reference database like RDP, GreenGenes, UNITE, or Silva will be used in the fungal analysis, capturing greater credibility for the conclusions (Breitwieser et al., 2019).

Finally, a study of this exploratory nature can only observe correlations between plant production and microbes and does not hold the statistical power to establish causative occurrences.

4.5 Relevance of findings and future steps for perennial ryegrass pasture persistence research

The goal of this research was - for the first time – to examine and compare the perennial ryegrass microbiome across different New Zealand farming locations to obtain a baseline understanding for use in future pasture persistence research. Whilst it was known that different farming regions have variable climates, soil types, pasture pests, ryegrass production and persistence, it was unknown whether there was also variation across the perennial ryegrass microbiome.

4.5.1 *Farming location influence*

This study did not find evidence that perennial ryegrass has a highly abundant ASV-level core microbiome. The lack of evidence for a core microbiome highlights that the perennial ryegrass bacterial and fungal communities are highly diverse and can be significantly influenced by location-specific differences, likely related to the environment and climatic conditions at specific farming locations. This conclusion was backed by the large proportion of site-specific taxa at each sampling site. As shown in this study, the perennial ryegrass microbiome from one farming region could exhibit a microbiome with 30 % unique taxa compared to a location from another region of New Zealand (E.g., Canterbury vs Waikato). Therefore, future pasture persistence microbiome research should consider perennial plants on a regional basis rather than extrapolating the results from one location to all New Zealand farming locations. This is consistent with other perennial ryegrass farm management and breeding practices which are linked to the conditions experienced at a farming location. The fact that there are site-specific microbiome differences shows the potential for the microbiome to contribute to varying perennial ryegrass pasture persistence and productivity in different farming regions.

4.5.2 *Future core microbiome exploration*

Conversely, although each farming location had a proportion of site-specific taxa, each microbiome habitat saw similar clusters of bacteria and fungi regardless of the sampling location. This seems to indicate a higher-level functional selection of specific bacterial and fungal groups by the different plant niches. Therefore, the future application of 'omic' approaches is suggested to explore the concept of a functional core microbiome rather than an ASV or genus-specific core microbiome for perennial ryegrass. Metagenomic shotgun sequencing would comprehensively sample all genes in all organisms present across the different ryegrass samples. Sequencing in this way is valuable for recovering the functional genes and understanding the physiological roles bacteria and fungi could be playing in the microbiome rather than just the microbiome composition. Research of this nature would expand the concept of the core microbiome beyond taxonomic identification to include community function. This analysis would reveal whether metabolic interdependencies underpin the stability of specific taxa across the perennial ryegrass microbiome and indicate their relevance to perennial ryegrass persistence.

4.5.3 *Narrowing the microbiome habitat focus*

Based on the results from this study, it is recommended that the future manipulation of the perennial ryegrass microbiome should focus on the rhizosphere, shoot endosphere and phyllosphere compartments of the plant. The rhizosphere demonstrated high diversity and lower selection of core taxa, highlighting that it is not under strict selection from the perennial ryegrass plant (Suman et al., 2022). Compared to the bulk soil, bacteria and fungi in the rhizosphere have a higher chance of affecting the perennial ryegrass growth as they are closer to the plant roots and are more likely to form mutualistic relationships with the plant (Logue et al., 2015). This study demonstrates a clear microbial manipulation success story through the presence of *Epichloë* in all the sampled ryegrass shoot endosphere samples three years after sowing. This highlights the ability of the

perennial ryegrass shoot endosphere to conserve a beneficial microbial interaction, even when that interaction is a result of the inoculation of a selected microbe into desirable plant genotypes. The shoot endosphere provides a promising avenue for introducing other microbial species to help improve ryegrass persistence in ways other than insect pest protection.

A core driver of reduced perennial ryegrass persistence is water stress caused by summer-dry conditions and drought. In this study, a likely explanation for the superior Burnham perennial ryegrass DM production compared to the other sites was the reduced soil moisture stress from summer irrigation. Irrigation is becoming a contentious mitigation of water stress on dairy farms due to its economically and sustainably expensive utilisation of groundwater. Identifying soil microbes that support drought tolerance in perennial ryegrass should be a priority. These microbes should be trialled in the rhizosphere compartment of perennial ryegrass under the hypothesis that the root-endosphere will select taxa from the rhizosphere that provide benefits for resource acquisition of water and nutrients. A study on the rhizosphere of great millet plants with trialled strains of *Streptomyces laurentii* and *Penicillium sp*. explored the plant's response to drought stress exposure (Kour et al., 2020). A consequence of drought stress on plants is the reduced bioavailability of phosphorus. The researchers found that the *Streptomyces* and *Penicillium* strains solubilized phosphorus, which resulted in increased plant growth and defence under drought conditions compared to the uninoculated control plants (Kour et al., 2020).

The phyllosphere of perennial ryegrass should also be a focus for future microbiome research. In this study, the phyllosphere had the highest number of core taxa, likely due to higher plant selection and greater specificity linked to the more hostile phyllosphere environment. Inoculating the outside surface of perennial ryegrass shoots offers a simple method of testing the effects of microbial taxa on ryegrass growth compared to the root endosphere, which may be more challenging. Like the rhizosphere, previous plant-microbe experiments have attempted to combat drought through the application of bacterial strains to the phyllosphere of plants. The phyllosphere bacterium *Bacillus megaterium* has been shown to increase the drought tolerance of *Oryza sativa* (rice) with the plants showing greater osmolyte accumulation and plant health (Shaffique et al., 2022).

Farmers commonly apply nitrogen fertiliser on perennial ryegrass pastures to increase forage production. In this study, the Cambridge site had dramatically higher nitrogen application during the trial period, reflected in the high soil nitrogen measure in the soil nutrient analysis. However, the high nitrogen levels were not reflected in high perennial ryegrass production and persistence during the trial period compared to the Palmerston North and Burnham sites. This demonstrates how nitrogen fertiliser may not always be a beneficial option for increasing ryegrass growth in pastures due to confounding variables. Biofertilisers are a recent technology that use approaches such as proprietary formulations of consortia of nitrogen-fixing bacteria, which, in some cases, have shown success in producing higher plant biomass compared to chemical fertiliser treatments (Amy, 2011)

4.5.4 The complexity of the pasture persistence issue

Investigating the perennial ryegrass microbiome is just one approach to tackling the perennial ryegrass persistency issue. The findings of this study present a large number of avenues for future study. This study sought to understand general trends in the perennial ryegrass microbiome for just one perennial ryegrass cultivar/*Epichloë* combination, One50 AR37. It is still unknown whether perennial ryegrass genetics and the type of *Epichloë* endophyte influence the associated microbiome. This highlights the future need for conducting the same investigation across multiple cultivar/*Epichloë* combinations.

In addition, it is well documented that climate and season contribute highly to perennial ryegrass persistence and productivity. However, whether seasonal climatic factors influence the perennial ryegrass microbiome remains unknown. Therefore, it would be of value to sample the same perennial ryegrass plots over different periods of the year to gain a seasonal overview of the perennial ryegrass microbiome. There is a high potential for dry-summer conditions to cause shifts in the associated microbiome, such as the recruitment of plant growth-promoting microbes.

In summary, there are still many more questions than answers remaining that must be explored before an authoritative call can be made on the role of the microbiome in perennial ryegrass persistence. Identifying the bacterial and fungal taxa associated with increased perennial ryegrass production and persistence remains a challenge for future research. This research must incorporate manipulative experiments, such as inoculating plant soils or ryegrass seeds with different bacterial and fungal strains and then measuring the ryegrass DM production and persistence. The current study only observed correlations between plant production and microbes and did not contain the statistical power to establish causative occurrences.

5 Conclusion

The purpose of this project was to explore the unknown area of the perennial ryegrass microbiome for its potential to drive differences in perennial ryegrass persistence and productivity. This research entailed a detailed exploration of the bacterial and fungal communities found across the five microbiome habitats of perennial ryegrass across four significant New Zealand farming locations. This study demonstrates that farming location and management practices strongly influence the perennial ryegrass microbiome. The large proportion of unique, site-specific taxa found at each farming location holds the potential to explain differences in pasture productivity and persistence. Location differences indicate that future microbiome research should compare ryegrass pastures on a regional basis rather than extrapolating the results to all New Zealand farming locations.

Future research should narrow the investigation of the microbiome categories to the rhizosphere, shoot endosphere and phyllosphere since these areas likely have the most considerable relevance for pasture persistence research. A core microbiome was not identified in the current study; however, there is evidence of a wider functional selection of taxa across the individual microbiome habitats. Future studies should incorporate metagenomic sequencing to better understand the functional microbiome trends rather than solely focusing on microbiome composition. The presence of core physiological traits may explain the selection of specific taxa across the different microbiome habitats and their influence on the baseline growth and survival of the perennial ryegrass plants. Bacteria of interest, such as *Bacillus* and *Pseudomonas,* should be explored in greater detail at the species level and applied in manipulative trials to assess the effects on ryegrass production and persistence.

Pasture persistence research needs to identify potential microbial taxa associated with higher-producing ryegrass pastures since they could provide sustainable benefits for aiding pastures with poor ryegrass health and productivity. Microbiome research has already targeted one pasture persistence problem by applying *Epichloë* endophytes to combat invertebrate pest herbivory. This encourages further research to explore other microorganism applications to mitigate other contributors to poor pasture persistence, such as drought and water stress. To achieve this, the primary goal for future studies should be comparing the microbiome of healthy and productive ryegrass pastures with low-producing, declining ryegrass pastures while controlling as many variables as possible (i.e., focusing on one location). In addition, higher-level functional metagenomic sequencing of perennial ryegrass samples would explore not only the microbial taxa present but also the functional genes possessed. Research of this nature would directly extend the concept of core microbial taxa and site-specific taxa by predicting community functions that might be valuable for higher ryegrass productivity.

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7 Appendices

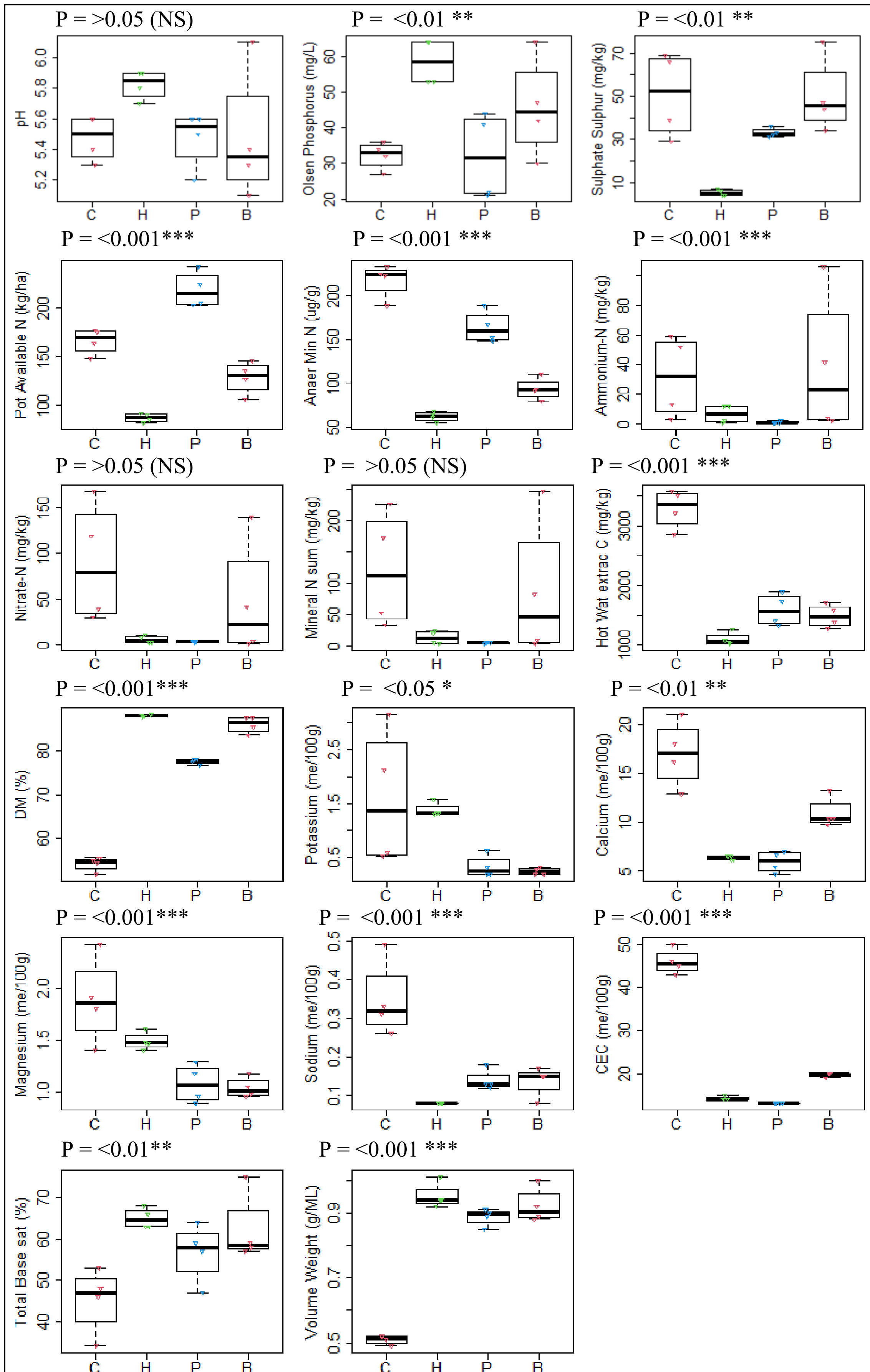

Figure 7.1 The nutrient analysis metadata collected for the 16 bulk soil samples from the four locations; C (Cambridge), H (Havelock North), P (Palmerston North), and B (Burnham). An analysis of variance model was applied to indicate the significantly different soil nutrient variables. Significance was judged based on the p-value codes ('*' 0.001 '**' 0.01 '*' 0.05 '.' NS (>0.005))**

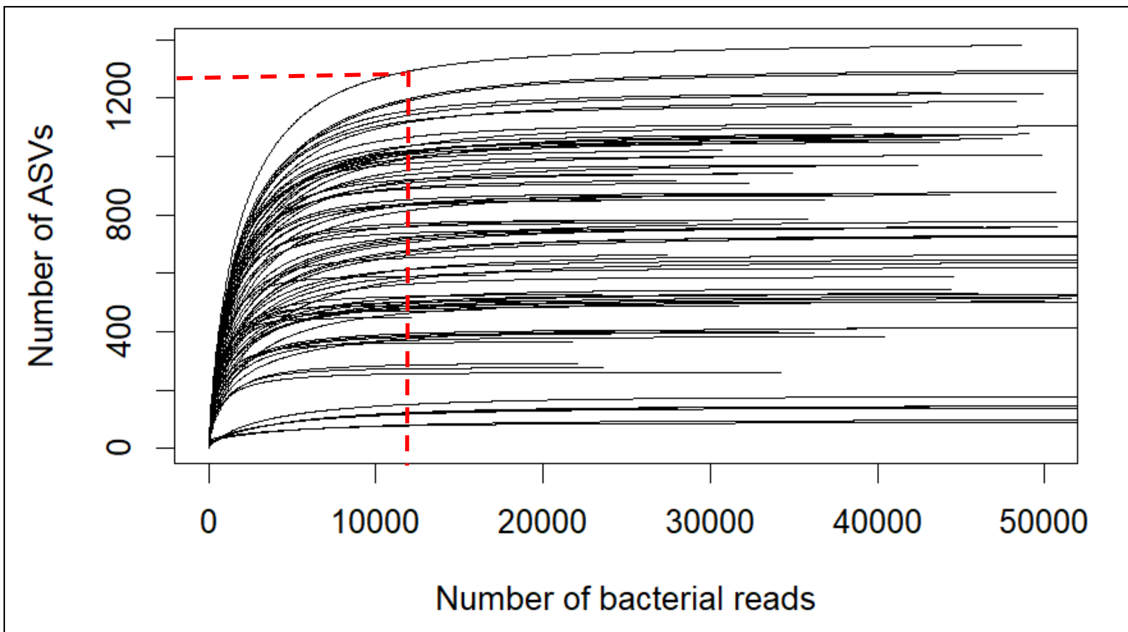

Figure 7.2 Rarefaction curve based on the number of reads vs the number of bacterial ASVs for the 78 ryegrass samples, used to analyse the minimum number of reads necessary to adequately sample the variation. Each sample was rarefied to an even sequencing depth (12000 reads) for alpha diversity analysis.

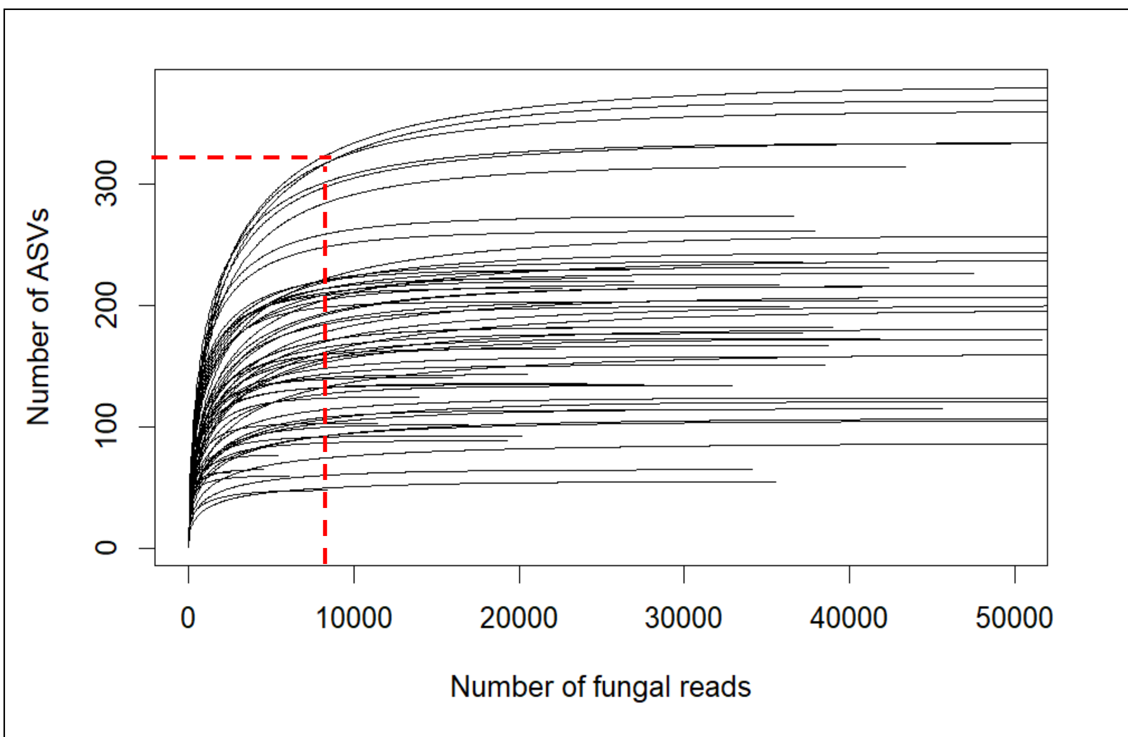

Figure 7.3 Rarefaction curve based on the number of reads vs the number of fungal ASVs for the 62 ryegrass samples, used to analyse the minimum number of reads necessary to adequately sample the variation. Each sample was rarefied to an even sequencing depth (7500 reads) for alpha diversity analysis. The refraction curves show that the top 5 samples may demonstrate a small amount of un-sampled diversity; however, this will include no more than 1-20 fungal ASVs.

Table 7.1 The nematode analysis data for the four ryegrass bulk soil reps from the four site locations, B (Burnham), P (Palmerston North), H (Havelock North) and C (Cambridge).

Site	B	B	B	B	M	M	M	M	H	H	H	H	C	C	C	C
Total nemas (per 100 g dry soil)	4996	7698	6937	4424	1772	1448	684	2352	3906	6281	3284	3244	8018	5859	12328	12998
Meloidogyne (per 100 g dry soil)	0	0	0	0	36	76	0	46	0	0	0	0	0	36	224	17
Heterodera (per 100 g dry soil)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	94	17
Pratylenchus (per 100 g dry soil)	193	582	1392	649	188	439	36	291	206	241	150	469	1024	455	337	244
Paratylenchus (per 100 g dry soil)	3964	5508	3405	2040	16	20	15	0	829	4264	1206	988	389	697	4359	1466
Helicotylenchus (per 100 g dry soil)	0	0	0	0	57	15	10	5	0	0	0	0	0	14	19	0

Table 7.2 The core bacterial ASVs with their associated taxonomical classification for the five microbiome habitats. Bacterial ASVs were considered core if found across at least 90% of samples within a microbiome category at a relative abundance of higher than 0.001. Note: that the shoot endosphere microbiome area did not demonstrate any bacterial ASVs considered as core.

Microbiome Category	ASV	Phylum	Class	Order	Family	Genus
Bulk soil	10	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
	21	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	22	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	24	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
	29	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
	36	*Actinobacteriota*	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
	38	*Actinobacteriota*	*Actinobacteria*	*Catenulisporales*	*Catenulisporaceae*	*Catenulispora*
	45	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	50	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Nocardioides*
	81	*Actinobacteriota*	*Actinobacteria*	*Frankiales*	*Acidothermaceae*	*Acidothermus*
	86	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Intrasporangiaceae*	*Terrabacter*
	145	*Actinobacteriota*	*Thermoleophilia*	*Gaiellales*	*uncultured*	*uncultured_ge*
	147	*Actinobacteriota*	*Thermoleophilia*	*Gaiellales*	*uncultured*	*uncultured_ge*
	164	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*uncultured*
	165	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Arthrobacter*
	222	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*
	276	*Actinobacteriota*	*Thermoleophilia*	*Gaiellales*	*uncultured*	*uncultured_ge*
	304	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*uncultured*
	395	*Actinobacteriota*	*Acidimicrobiia*	*IMCC26256*	*IMCC26256_fa*	*IMCC26256_ge*
	430	*Actinobacteriota*	*Acidimicrobiia*			
Rhizosphere	10	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
	21	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	22	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	24	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
	32	*Actinobacteriota*	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
	33	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Schumannella*

	35	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Nocardioides*
	38	*Actinobacteriota*	*Actinobacteria*	*Catenulisporales*	*Catenulisporaceae*	*Catenulispora*
	45	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	50	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Nocardioides*
	81	*Actinobacteriota*	*Actinobacteria*	*Frankiales*	*Acidothermaceae*	*Acidothermus*
	86	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Intrasporangiaceae*	*Terrabacter*
	90	*Actinobacteriota*	*Actinobacteria*	*Frankiales*	*Nakamurellaceae*	*Nakamurella*
	104	*Actinobacteriota*	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	*Mycobacterium*
	108	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Intrasporangiaceae*	*Lapillicoccus*
	114	*Actinobacteriota*	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	*Mycobacterium*
	140	*Actinobacteriota*	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteracea e*	*Conexibacter*
	147	*Actinobacteriota*	*Thermoleophilia*	*Gaiellales*	*uncultured*	*uncultured_ge*
	159	*Actinobacteriota*	*Acidimicrobiia*	*IMCC26256*	*IMCC26256_fa*	*IMCC26256_ge*
	164	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*uncultured*
	165	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Arthrobacter*
	174	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Marmoricola*
	175	*Actinobacteriota*	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteracea e*	*Solirubrobacter*
Root Endosphere	7	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Xanthomonas*
	10	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*
	14	*Actinobacteriota*	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*Amycolatopsis*
	21	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	22	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	32	*Actinobacteriota*	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
	33	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Schumannella*
	36	*Actinobacteriota*	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
	45	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteraceae*	*Bradyrhizobium*
	50	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Nocardioides*
	66	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Aeromicrobium*
	69	*Actinobacteriota*	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
	80	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*uncultured*

	87	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Rhodanobacteraceae*	*Rhodanobacter*
	144	*Proteobacteria*	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Rhizobacter*
	176	*Actinobacteriota*	*Actinobacteria*	*Streptomycetales*	*Streptomycetaceae*	*Streptomyces*
	193	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*
Phyllosphere	6	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Curtobacterium*
	9	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*
	12	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
	15	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Agreia*
	20	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*
	27	*Actinobacteriota*	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	*Rhodococcus*
	35	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	*Nocardioides*
	57	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*
	61	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
	71	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Clavibacter*
	72	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
	74	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
	88	*Actinobacteriota*	*Actinobacteria*	*Propionibacteriales*	*Propionibacteriaceae*	*Friedmanniella*
	94	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Frigoribacterium*
	117	*Bacteroidota*	*Bacteroidia*	*Flavobacteriales*	*Weeksellaceae*	*Chryseobacterium*
	119	*Actinobacteriota*	*Actinobacteria*			

Table 7.3 The core fungal ASVs with their associated taxonomical classification for the five microbiome habitats. Fungal ASVs were considered core if found across at least 90% of samples (within a microbiome category) at a relative abundance of more than 0.001. Note that the shoot endosphere microbiome area did not demonstrate any bacterial ASVs considered core.

Microbiome Category	ASV	Domain	Phylum	Class	Order	Family	Genus
Bulk soil (6)	1	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Astrosphaeriellaceae	*Pithomyces*
	8	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	10	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	15	Eukaryota	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Exophiala*
	21	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	45	Eukaryota	Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	
Rhizosphere (5)	1	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Astrosphaeriellaceae	*Pithomyces*
	8	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	10	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	15	Eukaryota	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Exophiala*
	21	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
Root endosphere (4)	10	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	11	Eukaryota	Ascomycota	Sordariomycetes	Magnaporthales	Magnaporthaceae	
	15	Eukaryota	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Exophiala*
	88	Eukaryota					
Shoot endosphere (10)	1	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Astrosphaeriellaceae	*Pithomyces*
	2	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*
	3	Eukaryota	Basidiomycota	Tremellomycetes			
	5	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Neoascochyta*
	8	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	13	Eukaryota					
	36	Eukaryota					
	49	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Epichloë*
	68	Eukaryota	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*
	105	Eukaryota	Ascomycota	Dothideomycetes			
Phyllosphere	1	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Astrosphaeriellaceae	*Pithomyces*

(17)	2	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*
	3	Eukaryota	Basidiomycota	Tremellomycetes			
	4	Eukaryota					
	5	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Neoascochyta*
	8	Eukaryota	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
	12	Eukaryota	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	
	17	Eukaryota					
	24	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*
	29	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Pyrenochaetopsidaceae	*Pyrenochaetopsis*
	36	Eukaryota					
	40	Eukaryota	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Neosetophoma*
	42	Eukaryota	Ascomycota	Leotiomycetes	Helotiales		
	66	Eukaryota	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*
	68	Eukaryota	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*
	104	Eukaryota	Ascomycota	Sordariomycetes	Glomerellales	Glomerellaceae	*Colletotrichum*
	114	Eukaryota	Basidiomycota	Agaricomycetes	Trechisporales		