Effects of *Epichloë festucae* var. *lolii* fungal endophyte strains associated with *Lolium perenne* on root exudate chemistry and microbial communities in the rhizosphere

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ABSTRACT

EFFECTS OF *EPICHLOË FESTUCAE* VAR. *LOLII* FUNGAL ENDOPHYTE STRAINS ASSOCIATED WITH *LOLIUM PERENNE* ON ROOT EXUDATE CHEMISTRY AND MICROBIAL COMMUNITIES IN THE RHIZOSPHERE

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Identifying key metabolites that are changing in the *Lolium perenne* - *Epichloë festucae* var. *lolii* metabolome will contribute to our understanding of their role in the belowground microfood-web and subsequently in ecosystem level processes. I designed two glasshouse studies to investigate the role of a plant-fungal endophyte symbiosis in shaping the grass-fungal metabolome and the microbial community in the rhizosphere. I tested the influence of several strains of *E. festucae* var. *lolii* on *L. perenne* growth and the composition of root exudate metabolites in hydroponic and soil mediums, and on the composition of microorganisms in a soil medium using metabolomic and next-generation sequencing techniques. I found that plant and endophyte growth, and the metabolomic profiles of root exudate and rhizosphere soils saw significant endophyte strain specific effects. While, compositions of the microbial communities in the rhizosphere had only subtle, and mostly non-significant changes as a result of endophyte soil conditioning treatments. The significant results of the metabolomics analyses provide a strong rational for more targeted investigations of the root exudate grass-fungal endophyte metabolome.

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CHAPTER 1: INTRODUCTION

This thesis focused on a plant-fungal symbiosis between *Lolium perenne*, a globally distributed and economically important forage and turf grass, and the fungal endophyte *Epichloë festucae* var. *lolii*. This thesis was conducted within the frame of the research program in plant-fungal ecology, whose special interest is the metabolomics of a plant-fungal symbiosis and its influence on microorganisms in the rhizosphere. It is necessary to identify the endophytic fungi and plant metabolites to elucidate this complex field of plant-fungal interactions; which metabolites are exiting the plants via the roots, which are unique to specific plant-fungal strain combinations, and which are important in rhizosphere interactions. The bulk of previous work has focused on fungal derived metabolites because of their economic importance in the agricultural and turf grass industries. However, with improvements in molecular techniques, new technologies, and more sensitive instruments, the contributions of the plant-fungal metabolome as a whole has begun to be investigated. This has led to the identification and characterization of several previously unknown metabolites as well as the identification of many more unknown metabolites that may be of importance to the success of the plant-fungal endophyte symbiosis and its impact on other organisms. Many of the molecular studies on plant-fungal endophyte symbiosis have utilized aboveground shoot tissue, but little is known about the make-up of the metabolites leaving the plants via the roots. Thus the aim of this thesis was to identify key changes in the plant-fungal metabolome and any influences these changes may have on microorganisms in the rhizosphere utilizing metabolomics to analyse root exudate and soil, and next generation sequencing of the soil derived from plant-fungal associations with several fungal strains. In this study I focused on perennial ryegrass (*Lolium perenne*), because of its global distribution and economic importance as a pasture and turf grass.

An introduction to grass-endophyte mutualisms

Lolium perenne (perennial ryegrass) is a globally distributed cool season perennial grass, originating in Europe, Asia, and North Africa (Balfourier *et al*., 2000). This grass has significant economic importance in both the agricultural industry, as feed for ruminants, and the turf industry as the ground cover for golf courses and sports fields. This grass is commonly host to fungal endophytes, microbial symbionts that live within the tissues of host plants, that may provide their hosts with fitness benefits such as improved yield, drought tolerance, nutrient acquisition, and deterrence or toxicity to herbivorous pests (Schardl *et al*., 2004; Saikkonen *et al*., 2004; Gond *et al*., 2010). The mechanisms responsible for these fitness benefits are not fully understood, however anti-herbivory properties have been attributed to changes in the host plant's chemistry, specifically to the presence of secondary metabolites synthesized by the fungal endophyte (Aasen *et al*., 1969). The contribution of fungal endophytes to the host plant's chemistry is not limited to secondary metabolism however, they can also influence the host plant's primary metabolism (Rasmussen *et al*., 2008b; Cao *et al*., 2008; Dupont *et al*., 2015). These changes in the host plant's chemistry have broader reaching ecological consequences both above and belowground, including influencing plant community composition, herbivore health, and non-target organisms. The majority of research investigating the interactions, influences, and consequences of plant-fungal endophyte relationships has focused on aboveground effects, while research investigating the belowground impacts of these relationships is still limited. Belowground ecosystem processes, such as decomposition and nutrient cycling, are made possible by the interaction of plants and soil organisms. These processes are essential in the transferring of resources throughout the foodweb. The bulk of these interactions are occurring in the rhizosphere, the interface of the root surface and the soil, and are heavily influenced by the

quality and quantity of plant outputs via the roots (i.e. root exudation). It is therefore important to improve our understanding of the belowground component of plant-fungal endophyte relationships so that we may determine their role in grassland ecosystems. More specifically, to determine the effects of plant-fungal metabolism on the quality and quantity of root exudates, and the subsequent response of the microbial community to these inputs, in terms of structure and function.

I have organized this review into three main sections: 1) the plant-fungal endophyte relationship; 2) the plant-fungal endophyte metabolome; and 3) the plant-fungal endophyte rhizosphere microbiome. In the first section I briefly cover how the endophyte is distributed within the plant and its resource needs, the different strains of endophytes, and how the presence of a fungal endophyte affects the host plant and associated organisms above and belowground. In the next section I discuss what is known about the plant-fungal metabolome, highlighting known changes in both primary and secondary metabolisms. In the last section I discuss the rhizosphere ecosystem, how plant-fungal metabolites enter the soil, their fate in soil, and their relationship with soil bacteria and fungi.

THE PLANT-FUNGAL ENDOPHYTE RELATIONSHIP

Many cool season grasses form associations with the fungal endophytes in the family Clavicipitaceae. The family Clavicipitaceae is made up of a diverse group of fungi with more than 40 genera and 300 species. Of these genera, seven include fungal endophytes (Clay 1988; White 1994; White and Reddy, 1998), one of which is *Epichloë*. The naming and categorization of the *Epichloë* genus, and the species within it have undergone several revisions (see Leuchtmann *et al*., 2014 for a review). *Epichloë* endophytes infect a variety of grasses from the subfamily Pooideae (White 1988), including *L. perenne* L. (Glenn *et al*., 1996). *Epichloë* fungi are systemic within their host, and their relationship with the host can range from parasitic to mutualistic (Saikkonen *et al*., 2006). Regardless of the relationship, fungi may be transmitted asexually through the host seed (Becker *et al*., 2016). Parasitic relationships are most often linked with a sexual reproductive strategy in which sexual forms of the fungal endophyte may lead to the inhibition of plant reproduction (Clay 1986). In parasitic relationships, infection becomes visible during the reproductive stage as a result of stromata forming over the inflorescence of the grass (Leuchtmann and Schardl 1998). In contrast, mutualistic relationships are always associated with asexual forms of *Epichloë* fungi which rarely become visible because they are transferred from one plant generation to the next only via transmission through the plant host seed (Schardl 1996; and see Panaccione *et al*., 2014, and Tadych *et al*., 2014 for detailed reviews of sexual and asexual fungal endophytes). When the seed germinates, the fungal endophyte's mycelia spread intercellularly through most of the organs of the plant (Musgrave, 1984; Christensen *et al*., 2008), though they are rarely found in roots (Azevedo and Welty 1995).

One of the most common, naturally occurring, fungal endophytes in *L. perenne* is *Epichloë festucae* var. *lolii*, formerly *Acremonium lolii* and later *Neotyphodium lolii*

(Leuchtmann *et al*., 2014). This symbiotic grass-fungal endophyte relationship is considered mutualistic in nature: the endophyte benefits from the nutrients and the protection which the host provides, while the grass receives several fitness benefits both above and belowground. Although endophytes such as *E. festucae* var. *lolii* live within their host and obtain resources from them, they are equipped with their own metabolism and are known to synthesize secondary metabolites that differ from those that the host can synthesize. Additionally, these endophytes can influence the synthesis and uptake of compounds by the host grass which can alter plant nutrition (Malinowski *et al*., 2004, Rasmussen *et al*., 2007, Soto-Barajas *et al*., 2015). Some of the fitness benefits associated with these changes in plant chemistry supplied by the endophyte to its plant host are welcomed within both the agricultural and turf industries such as improved yield, drought tolerance, nutrient acquisition, and toxicity to insect pests. However, some of the secondary metabolites synthesized by the endophyte are also toxic to vertebrates. While toxicity to ruminants is generally not of concern to turf grass managers, it is of great concern to farmers managing pastoral grasses. For example, 'ryegrass staggers,' a neurotoxic condition in sheep, has been linked to *L. perenne* infected with *Epichloë festucae* var. *lolii* (Fletcher and Harvey 1981).

The link between ryegrass staggers and fungal endophytes directed research toward forage improvement. The primary goal was to maintain the beneficial properties of the association while reducing the animal toxicity (see review Johnson *et al*., 2013). A useful discovery was the existence of naturally occurring strains ('novel') of the endophyte lacking the secondary metabolites associated with ruminant toxicity. Researchers began to cultivate these novel endophytes by isolating them from the host plant tissue, culturing them on growth media in the lab, and then inoculating uninfected ryegrass seed with the cultured fungal endophyte (West *et al*., 1998; Fletcher 1999). These novel strains possess secondary metabolite profiles that differ

from the common toxic strain (E+). *L. perenne* seeds inoculated with these novel endophytes have been made commercially available, and are now found in agricultural fields, and other grassland ecosystems. However, experimental research on these novel endophytes, particularly with respect to their below-ground impacts, is still limited. There are industry specific economic interests that select for particular secondary metabolite profiles. The criteria for the marketing of 'safe' novel endophytes do not consider the potential effect(s) on non-target soil organisms, rather the research and screening process of novel grass-fungal endophyte associations focusses on their field performance, persistence, and animal toxicity (specifically for insect pests and ruminants) (Fletcher 2005).

The symbiosis between asexual species of *Epichloë* fungi and their plant host is often considered a mutualism, where both parties are benefiting from the relationship, though the nature of this relationship is debated (Clay 1988, Cheplick *et al*., 1989; Saikkonen *et al*., 2010; Cheplick and Faeth 2009; Brosi *et al*., 2012). Determining the mechanisms involved in this relationship has been a long standing challenge for scientists, however advances in molecular and cellular techniques have progressed our understanding of intra and inter species signalling, metabolic pathways, and gene regulation. In recent years the fungal endophyte *Epichloë festucae* and the grass *Lolium perenne* have been used as the model system to understand the mechanisms involved within the relationship (see review by Tanaka *et al*., 2012). The genome sequencing for two strains of *E. festucae* (Schardl *et al*., 2013) as well as the generation of a draft genome of *L. perenne* (Byrne *et al*., 2015) has been instrumental in advancing our understanding.

Novel *Epichloë* fungi strains

Natural variants of *Epichloë* fungi exist which differ in their alkaloid profiles (Christensen *et al*., 1991). These variants are often referred to as 'novel' or 'selected'

endophytes. The goal of cultivating novel endophytes is to maintain their beneficial attributes for the grass host while eliminating the fungal metabolites toxic to ruminants (Fletcher and Easton, 1997). These novel endophytes play an important role in agriculture and are thoroughly evaluated for their impact on insect pests and ruminants, as well as their overall performance and persistence in the field. The majority of novel endophyte work is conducted in New Zealand by Grasslanz Technology and AgResearch (Johnson *et al*., 2013). They identify and patent many novel endophytes. The first novel endophyte strain to be marketed as a ruminant friendly strain was called Endosafe (this strain 187BB isolated from *Acremonium typhinum* (Christensen *et al*., 1991)). This particular strain did not synthesize the alkaloid lolitrem B, a known neurotoxin. It was a short-lived product as it was found to synthesize more ergovaline, another toxic alkaloid produced in *Epichloë,* than the common toxic strain. Thus, sheep feeding on *L. perenne* infected with Endosafe had reduced ryegrass staggers symptoms, but their live weight gains were similar to those of sheep feeding on *L. perenne* infected with the common toxic strain.

Several *E. festucae* var. *lolii* novel strains exist today such as AR1, AR37, and NEA2. AR1, released for sale in 2000, produces peramine and terpendoles (Young *et al*., 2009); AR37, released in 2007, produces epoxy-janthitrem alkaloids; and NEA2, released in 2005, produces very low levels of peramine, ergovaline and lolitrem B (Rasmussen *et al*., 2007). What is known about the effects of these endophyte strains on animals, the grass-fungal metabolome, and on soil microorganisms will be discussed throughout this review.

Impact of *Epichloë* fungi on the plant host

Changes to host morphology and physiology are common when infected with *Epichloë* endophytes. However, the direction and nature of these changes are dependent on the host genotype, host cultivar, endophyte strain, resource availability, and environmental conditions

(Belesky and Fedders 1996; Amalric *et al*., 1999; Cheplick and Cho, 2003; Faeth and Sullivan 2003; Rasmussen *et al*., 2008; Tian *et al*., 2013; Card *et al*., 2014). Beneficial effects on plant growth parameters, and for plant vigour under biotic and abiotic stresses have been observed (Fletcher and Harvey 1981; Latch *et al*., 1985; Pedersen *et al*., 1988; Breen 1994; Ravel *et al*., 1997). However, these benefits are not always seen and the presence of the endophyte can have neutral effects or detrimental effects to plant growth and vigour (Eerens *et al*., 1998; Cheplick *et al*., 2000; Hunt *et al*., 2005; Cheplick 2007). It is suggested that metabolic costs to the plant host are responsible for the negative impacts of fungal endophytes (Cheplick *et al*., 2000; Hesse *et al*., 2004). Indeed, recent molecular studies provide strong evidence for a metabolic cost to the plant host, wherein many genes associated with secondary metabolism are increasing in expression, at a cost to primary metabolism (Ambrose and Belanger, 2012; Dupont *et al*., 2015). A decline in the expression of genes associated with primary metabolism may explain a decrease in plant growth, while an increase in the expression of genes associated with secondary metabolism may, in part, explain certain abiotic tolerances. Under ideal growing conditions, with the appropriate level of light, moisture, and nutrients, the effect of the metabolic cost of hosting a fungal endophyte may be less prominent.

In this section I have shown that fungal endophytes have a highly regulated relationship with their grass hosts, and that the presence of these fungal endophytes can alter host morphology and physiology which, directly and indirectly, lead to impacts on other organisms. Increased biomass, changes in root architecture, and altered plant chemistry can all have impacts on the belowground ecosystem. In the next section I focus on what is known about the grass-fungal metabolome.

GRASS-FUNGAL METABOLOME

Plant metabolism can be broadly separated into primary and secondary components, where primary can then be subdivided into three main classes: carbohydrate, amino acid, and lipid metabolism. Within kingdoms primary metabolism is relatively conserved, while secondary metabolism has a large degree of variability. Within an organism, we also find metabolomic variability. This variability is due to changes in biotic and abiotic factors, tissue types, and differences in the extraction methods and analytical instruments used.

The study of plant metabolism is important in several areas of research including agriculture. Understanding plant metabolism aids in selecting for advantageous traits related to abiotic and biotic tolerances which improve overall plant fitness, and plant quality for ruminant feed. It is also important to understand plant metabolism in an ecological context. Changes in plant metabolism may lead to changes in the quality and quantity of plant outputs, which can influence microbial communities, nutrient cycling, plant-soil feedbacks, and ultimately ecosystem functions. The metabolic pathways of primary and secondary metabolism are complex and numerous and a thorough explanation of these processes goes beyond the scope of this review. Therefore, in this section I will briefly highlight common primary and secondary metabolites that have been observed to change in grass-fungal endophyte associations.

Metabolome of the grass-fungal symbiosis

The plant-fungal metabolome as a whole system has not been thoroughly investigated within grass-*Epichloë* associations. This is, in part, due to a focus on the fungal derived compounds in the association, but it is also because of the incomplete picture of both organisms' genomes. For example, only a draft genome exists for *L. perenne* and although the genomes for two strains of *E. festucae* have been sequenced, 44% of its genes still require functional

characterization (Eaton *et al*., 2015). The majority of research has focused on secondary metabolites associated with pest deterrence and toxicity. This is understandable due to the economic importance of cultivating high yielding and persistent pasture grasses. These anti-herbivory properties have been attributed to secondary metabolites produced by the fungal endophyte: phenols and alkaloids (Malinowski *et al*. 1998), which will be outlined in more detail later in this section.

There are a number of factors contributing to the production and concentration of metabolites in the plant-fungal metabolome including environmental variables, such as: temperature, precipitation, light, nutrient management, and soil type; and biological variables, such as: tissue type, plant genotype, and endophyte strain. The focus will be on biological variables.

Primary metabolism

Primary metabolism is an understudied area in plant-fungal endophyte relationships. In this subsection I review differences between the plant metabolome and the plant-fungal metabolome, that have been observed in the *E. festucae* var. *lolii – L. perenne* system.

Carbohydrates

The presence of fungal endophytes can alter the composition of carbohydrates and carbohydrate alcohols in grasses (Cheplick and Cho, 2003; Hunt *et al*., 2005; Rasmussen *et al*., 2007). Non-structural carbohydrates which include water soluble carbohydrates (WSCs), sugar alcohols, and storage carbohydrates have been shown to increase in infected plants (Cheplick and Cho 2003; Hunt *et al*., 2005; Rasmussen *et al*., 2008; Dupont *et al*., 2015). In rhizodeposit of endophyte infected *S. arundinaceus*, Van Hecke *et al*. (2005) observed an increase in total

soluble carbon and carbohydrates, which they suggest could be a result of increased photosynthetic rates. An upregulation of genes associated with photosynthesis have been observed in *Festuca rubra* infected with *E. festucae* (Ambrose & Belanger, 2012); However, other studies have shown a decrease in photosynthetic rates of grasses infected with *E. festucae* (Dupont *et al*., 2015), and *E. festucae* var. *lolii* (Spiering *et al*., 2006). WSCs were also increased in the sheath and root tissue of endophyte infected *L. perenne* exposed to high nitrogen fertilization (Ren *et al*., 2009). As were the sugar alcohols arabitol, threitol, and mannitol in the apoplastic fluids of infected *L. perenne* plants (Dupont *et al*., 2015). Mannitol was also increased in leaf tissue (Cao *et al*., 2008; Rasmussen *et al*., 2008). Mannitol is produced by both the plant and the fungal endophyte, and it has been shown that there is a positive correlation with endophyte concentration and the amount of mannitol present in the host grass (Rasmussen *et al*., 2008).

The influence of endophyte infection on fructan storage is dependent on the genotype of the host. Cheplick and cho (2003) found that it was the grass host genotype that played a significant role in *L. perenne* growth and storage of fructans. Total non-structural carbohydrates were not significantly effected by endophyte infection alone, but were significantly effected by plant genotype, and by a plant genotype x endophyte infection interaction (Cheplick and Cho, 2003). Interestingly, although we see increases in water soluble carbohydrates in plant tissues, the expression of the genes associated with major carbohydrate synthesis are shown to be downregulated in endophyte infected *L. perenne* (Dupont *et al*., 2015). This counter-intuitive result demonstrates the complexity of biological systems.

Structural carbohydrates can also be effected by endophyte presence. For example, the expression of cell wall-associated genes, like those responsible for hemicellulose synthesis, are

upregulated in endophyte infected *L. perenne* (Dupont *et al*., 2015). Using transmission electron microscopy Dupont *et al*. (2015) observed the cell walls of infected and uninfected *L. perenne* and found that the cell walls of infected plants were significantly thinner than uninfected plants, accept where hyphae were present. Endophyte nutrition may have a requirement for sugars contained in the cell wall (Ryan *et al*., 2015). This is supported by the identification of fungal sugar transporters that demonstrate a higher affinity for cell wall bound sugars (Rasmussen *et al*., 2012b).

Water soluble carbohydrates (WSCs) serve many roles in grasses including as chemical energy, structural components, and signal molecules. Storage carbohydrates aid as energy reserves for plants during growth in the spring, as well as regrowth after cutting or herbivory, and during abiotic stress (Prud'homme *et al*., 1992; Abeynayaka *et al*., 2015). The alteration in carbohydrate metabolism by the presence of fungal endophytes can be detrimental to plant performance under certain conditions. But, this may be offset by the upregulation in secondary metabolism, caused by the presence of fungal endophytes, which may aid in plant defense against insect herbivory, and infection by fungal pathogens (Pańka *et al*., 2013; Ambrose and Belanger 2012; Dupont *et al*., 2015).

Lipids

Lipids provide energy, protection, and structural material for plant tissues. There are several categories of lipids including triglycerides, oils, and phospholipids. Triglycerides and oils are for energy storage, and are composed of three fatty acids (FAs) and one glycerol. Oils are most abundant in grass seeds, an important resource for the developing seedling. Common fatty acids are palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) (Bauchart *et al*., 1984). C18:3 makes up at least half of the total FA content in fresh *L. perenne*

followed by C16:0, C18:2, C18:1, and C18:0; similar results are seen in dried grass (Bauchart *et al*., 1984; Elgersma *et al*., 2003; Mir *et al*., 2006).

In the presence of fungal endophyte some lipids may increase in aboveground tissues of *L. perenne* (Rasmussen *et al*., 2008). Rasmussen *et al*. (2008) found that three fatty acids (C16:0, C16:1, and C18:3) were increased under high nitrogen treatments, but all decreased under low nitrogen treatments. While two fatty acids (C17:0 and C18:0) were reduced in endophyte infected plants. However, the expression of genes associated with lipid synthesis were downregulated in the Dupont *et al*. (2015) study. This may demonstrate that, as regularly seen, the plant cultivar and genotype combined with endophyte strain may result in different physiological and chemical plant responses.

L. perenne root exudates can contain lauric (C12:0), myristic (C14:0), pentadecanoic, palmitoleic (C16:1), C16:0, C18:1 and C18:0 which have been found to have allelopathic affects (Takahashi *et al*., 1993). The residence time of lipids in soil can be decades making them a very stable class of metabolites (Wiesenburg *et al*., 2004). Although plant root exudates are a major contributor of lipids and FAs to the soil lipid pool, inputs over short time scales (i.e. day to week) are harder to measure (Wiesenburg *et al*. 2010). Changes in lipid content based on endophyte infection and endophyte strain have been found in plant root exudates. Wakelin *et al*. (2015) were interested in the belowground effects of the plant-fungal endophyte association. Using *L. perenne* infected with fungal endophyte strains AR1 and AR37, they conducted untargeted metabolomics on the rhizosphere soil (Wakelin *et al*., 2015). Using GC-MS, they were able to detect 50 compounds (25 of which they were able to identify) in the soil rhizosphere. A suite of alkane hydrocarbon derivatives was responsible for the greatest amount of differences in metabolomic profiles between endophyte treatments. Metabolomic profiles

clearly separated between endophyte free (E-) and infected (strains AR1 and AR37), and further separation between the two novel endophyte strains was apparent, however due to low statistical power they were unable to report this with any confidence (Wakelin *et al*., 2015). This is the only study conducted on endophyte infected *L. perenne*, and the results of this study warrant further investigation of the influences of this relationship on the quality of root exudates.

Nitrogen and Amino Acids

Amino acids are the building blocks of proteins and are involved with many functions within plants including plant growth and development, signaling, and precursors for secondary metabolite synthesis (Atilio and Causin, 1996; Tegeder 2012; Hildebrandt *et al*., 2015). Amino acid composition varies from organ to organ and is sensitive to the source and level of nitrogen available. Amino acids exist in plant tissue as either free or protein-bound. Free amino acids pools are smaller than protein-bound amino acid pools (Hildebrandt *et al*., 2015). The amino acids present in *L. perenne* in the highest amounts are glutamine, glutamate, aspartate, and serine. Those present in smaller amounts are glycine, threonine, asparagine, alanine, and valine. And those in trace amounts are arginine, phenylalanine, isoleucine, and leucine. Nitrogen fertilization has a greater impact on major amino acids than minor amino acids (Rasmussen *et al*., 2008a).

When nitrogen levels are low in the soil, endophyte infected plants can have an advantage over uninfected plants (Ravel *et al*., 1997; Lewis 2004). However, Ren *et al*., (2009) found that endophyte infection only improved plant growth at high N levels. Though this may not be the result of the fungal endophyte as Rasmussen *et al*. (2007) found endophyte concentration to be significantly reduced at high N levels. Plant nitrogen metabolism can be altered by the presence of a fungal endophyte (Lyons 1985; Lyons *et al*., 1990). For example,

nitrogenous compounds in plant tissue can be reduced in the presence of endophyte (Hunt *et al*., 2005; Rasmussen *et al*., 2008; Ren *et al*., 2009). Rasmussen *et al*. (2008a) saw a 50% reduction in nitrate levels in *L. perenne* blade tissue when infected with strains of *E. festucae* var. *lolii*. The reduction of nitrogenous compounds can effect the biosynthesis of certain amino acids and Rasmussen *et al*. (2008) found that the majority of amino acids were reduced in the presence of a fungal endophyte with asparagine being the most effected (Rasmussen *et al*., 2008a). There may be indirect implications of reduced nitrogen in the plant-fungal system in terms of nutrient quality for herbivores and the levels of secondary metabolites present.

Organic Acids

The presence of fungal endophytes can increase organic acids depending on its identify. For example, citrate and succinate have been observed to decrease, while malate increase in endophyte infected blades (Rasmussen *et al*., 2008a). In general, the roots of endophyte infected *L. perenne* can contain a higher quantity of organic acids than endophyte free roots (Ren *et al*., 2007). Organic acids are an important component in plant metabolism interacting with carbohydrates, lipids, and amino acids (Burris 1953). They are also linked with some of the adaptive qualities in plants to abiotic stress, and with plant-microbe interactions and nutrient uptake in the rhizosphere (Ohkama-Ohtsu and Wasaki, 2010). Organic acids increase under drought stress (Foito *et al*., 2009). Organic acids like citrate and malate can be secreted from plant roots to chelate metal cations (Ohkama-Ohtsu and Wasaki, 2010). Malate helps to prevent pH changes. Nitrogen fertilization can increase malate, succinate, and citrate (Rasmussen *et al*., 2008a). Malinowski *et al*. (2004) observed that *L. perenne* root exudates had increased Cu^{2+} -binding activity when in a phosphorous poor environment.

Secondary Metabolism

Alkaloids and phenols are the dominant secondary metabolites produced by both common toxic and novel endophyte strains. Since the early 1980s the majority of fungal endophyte secondary metabolite research has focused on alkaloids and phenols (Gallagher *et al*., 1981; Lyons *et al*., 1986; Rowan and Gaynor 1986; Rowen *et al*., 1986). Qualitative and quantitative evaluation of alkaloids and phenols have been conducted for the aboveground tissues, guttation fluid, and seeds of *L. perenne* infected with *Epichloë* endophytes (Ball *et al*., 1997; Fletcher 1999; Thom *et al*., 1999; Panaccione 2005; Koulman *et al*., 2007a; Koulman *et al*., 2007b; Cao *et al*., 2008; Rasmussen *et al*., 2008; Cripps and Edwards, 2013), but minimal studies have considered evaluating the roots and root exudates of *L. perenne* (Lewis *et al*., 1996; Ball *et al*., 1997b; Zhou *et al*., 2003; Ren *et al*., 2007, 2009; Wakelin *et al*., 2015).

Phenols

Phenols make up the largest group of secondary plant metabolites. They have been connected with antimicrobial, allelopathic, antioxidant, and pest deterrent activities. Although plants produce phenols independently, the presence of fungal endophytes often can influence the quantity and quality of phenols found in plant tissue (Ju *et al*., 1998; Malinowski and Belesky 1999; Rasmussen *et al*., 2008; Ponce *et al*., 2009; Vazquez-De-Aldana *et al*., 2011; Qawasmeh *et al*., 2012; Pańka *et al*., 2013). In general, the presence of endophyte increases the amount of total phenolic compounds in plant host tissue (Pańka *et al*., 2013), which may help protect plant cells against oxidative stress (Grace 2005). The influences of a fungal endophyte on plant phenols can be endophyte strain specific. Qawasmeh *et al*. (2012a) generated phenolic profiles of *L. perenne* (cv. Samson) blade tissue either uninfected (E-), or infected with different endophyte strains (E+, AR1, and AR37). They found that the phenol content of blade tissue differed in quantity between

endophyte treatments with AR1 having the highest phenol content and E+ the lowest. They found the same pattern when they measured antioxidant activity. Qawasmeh *et al*. (2012b) also found an endophyte strain specific effect on the antioxidative capacity of grasses. Torres *et al*. (2012), suggest that increases in the production of phenolic compounds and in antioxidant activity in endophyte infected plant tissue may be due to the production of reactive oxygen species (ROS) by the fungal endophyte. In high enough concentrations, ROSs can cause cell damage or death.

Alkaloids

Alkaloids are a large group of chemicals that are produced by many plants, pathogens, and symbionts, which have defensive properties against other competing species in their environment. The majority of research on *Epichloë* has focused on the alkaloids it produces. As previously discussed, *E. festucae* var. *lolii* naturally infects *L. perenne*. The common toxic strain (E+) of *E. festucae* var. *lolii* produces three classes of alkaloids: pyrrolopyrazines (peramine), associated with resisting insect herbivory (Gaynor *et al*., 1983; Tanaka *et al*., 2005); indole diterpenes (lolitrem B) (Fletcher and Harvey, 1981), associated with animal toxicity; and lysergyls (ergovaline) (Rowan and Shaw 1987; Filipov *et al*., 1998), associated with both anti-herbivory properties and animal toxicity. The biosynthetic pathways for these alkaloids are now known and the specific genes and gene clusters have been identified in the genome (Schardl *et al*., 2013). The synthesis of the alkaloid peramine is unique as it only requires a single nonribosomal peptide synthetase *perA* (Tanka *et al*., 2005). For the indole diterpene alkaloids (*IDT/LTM*), there are seven genes associated with paxilline, and ten genes associated with lolitrem B (Fletcher *et al*., 1993; Young *et al*., 2005, 2006, 2009). Many of these genes are the same as in other indole diterpenes producing fungi, but *ltmE, ltmF, ltmJ* and *ltmK* are genes

unique to epichloae (Saikia *et al*., 2012). There are 11 genes associated with the synthesis of ergot alkaloids (*EAS*) (Panaccione *et al*., 2001; Fleetwood *et al*., 2007). The ergot alkaloid pathway starts with the primary metabolites L-tryptophan and dimethylallylpyrophosphate. The NRPS gene *lpsB* is responsible for the synthesis of ergovaline, a main alkaloid produced by *E. festucae* var. *lolii* (Fleetwood *et al*., 2007). In the common toxic strain, the *perA* gene, and the *EAS* and *LTM* clusters are complete and functional. However, in the novel endophytes AR1 and AR37 some genes are present but non-functional, or missing, resulting in different alkaloid profiles. For example, in AR1 the *perA* gene is complete and functional, but the *EAS* cluster is present but non-functional. Furthermore, two genes from the *LTM* cluster are missing resulting in the production of terpendole, an early pathway metabolite, but not lolitrem B. In AR37, the *perA* gene is present but it is non-functional due to a missing reductase domain, therefore no peramine is produced. Also in AR37, two *LTM* genes are absent but the early pathway still functions and it has additional *IDT/LTM* gene(s) for an extra prenylation step. These genes synthesize epoxy-janthitrems (Tapper and Lane 2004), an alkaloid not found in other *E. festucae* var. *lolii* strains. The gene cluster for epoxy-janthitrem synthesis has been identified but not yet published. Lastly, in AR37, the *EAS* cluster is missing, therefore this strain is unable to produce ergot alkaloids. The importance of identifying the genes involved in the production of fungal alkaloids is primarily in plant breeding. Plant-fungal endophyte associations can be screened for beneficial and detrimental metabolites based on the genes present. However, the presence of a gene does not confirm its expression *in planta*. Further analyses such as the use of transcriptomics and proteomics would confirm the expression of the gene and the presence of the gene product.

In addition to alkaloid profiles changing based on endophyte strain, alkaloid concentrations can also vary based on endophyte concentration, alkaloid class, plant tissue type,

plant age, nutrient levels and environmental conditions. **Table 1.1.** provides a breakdown of alkaloid concentrations based on alkaloid class and plant tissue type, and where possible, plant age, and season are included. Belesky *et al*. (1988) found that the level of ergopeptine was higher in tall fescue leaf tissue that had high endophyte infection. This pattern has also been found with *L. perenne*, with peramine, ergovaline, and lolitrem B (di Menna *et al*., 1992; Ball *et al*., 1995a; Easton *et al*., 2002; Spiering *et al*., 2005) suggesting a linear relationship between endophyte concentrations and alkaloid concentrations (Rasmussen *et al*., 2008; Reed *et al*., 2011b).

Table 1. 1. Summary of alkaloid concentrations (ppm) (Mean ± SD) in Lolium perenne plants infected with Epichloë festucae var. lolii. The alkaloids are separated by class and divided into four categories: plant tissue type, cultivar, plant age, and season. This summary is based on data from 34 papers in the literature. This is not the entirety of the data available; some papers were excluded due to measurement techniques that could not be converted to ppm units of measure. Where a 0.00 value is present for the standard deviation, this indicates that there was only one data point, and therefore no deviation from the mean. Below the table is a list of the literature in short form; a full citation can be found in the reference section.

	Epoxy-Janthitrems	**Ergovaline**	**Lolitrem B**	**Peramine**	**Literature**
Average Concentration (ppm)	11.96 ± 16.90	1.80 ± 3.56	2.14 ± 2.27	17.20 ± 11.97	
Tissue Type					
Blade	3.55 ± 6.55	0.55 ± 0.46	1.46 ± 1.21	24.38 ± 12.53	15, 16, 18, 25, 27, 30
Pseudostem	46.38 ± 38.27	5.04 ± 5.49	4.25 ± 3.43	17.22 ± 11.14	8, 15, 16, 25, 26, 34
Stem	75.82 ± 0.00	0.42 ± 0.39	3.16 ± 1.45	17.86 ± 7.24	3, 6, 10, 25
Whole Tiller	13.08 ± 11.55	0.52 ± 0.38	1.53 ± 1.32	11.29 ± 8.09	1, 2, 4-7, 9-13, 17, 19, 21, 22, 29, 31-33
Root				0.93 ± 1.85	3, 10
Seed			3.50 ± 0.00		6
Faecal matter (ewes)	18.00 ± 0.00	0.15 ± 0.00	1.90 ± 0.00	2.15 ± 0.21	5
Cultivar					
Aberdart	0.90 ± 0.00	0.63 ± 0.15	2.70 ± 0.33	27.43 ± 3.21	24, 30
Aberdove	0.55 ± 0.00	0.84 ± 0.16		27.00 ± 0.00	30
Alto	26.73 ± 28.69	0.17 ± 0.18	2.35 ± 2.21	17.74 ± 12.46	25
Avalon		0.47 ± 0.14	1.00 ± 0.55		29
Bronsyn	0.30 ± 0.00	0.55 ± 0.32	0.54 ± 0.60	8.43 ± 7.68	29, 30, 34
Commando	13.03 ± 11.73	0.47 ± 0.33	2.28 ± 1.53		32, 33
Ellett			3.98 ± 1.99		6
Extreme		0.73 ± 0.27			1
Fennema	1.22 ± 0.00	0.52 ± 0.09	2.15 ± 1.11	35.57 ± 4.69	24, 30
Impact	0.80 ± 0.00	0.96 ± 0.28	0.41 ± 0.00	12.83 ± 10.61	22, 30
Mixed	0.80 ± 1.00	0.72 ± 0.48	1.65 ± 2.54	29.03 ± 11.83	7, 21, 23, 24, 29, 30

	Epoxy-Janthitrems	Ergovaline	Lolitrem B	Peramine	Literature
Cultivar cont.					
Northrup King				14.10 ± 12.80	10
Nui		3.58 ± 4.96	1.58 ± 1.33	11.55 ± 6.71	8, 9, 11, 15, 22, 29
Pacific			0.16 ± 0.00	6.20 ± 0.14	22
PG113	0.40 ± 0.00	0.37 ± 0.03		17.00 ± 0.00	30
Repel				34.85 ± 7.00	10
Samson	10.60 ± 21.31	0.24 ± 0.13	1.78 ± 1.63	10.69 ± 8.75	5, 12, 16, 18, 19, 22
Unknown		0.49 ± 0.41	3.71 ± 5.46	18.56 ± 7.17	4, 13, 14, 17, 20, 27, 28
Victorian		0.48 ± 0.44	0.83 ± 0.49		29
Wild origin		0.53 ± 0.43	1.40 ± 1.17	20.12 ± 14.88	2, 3, 26, 29
Yatsyn 1		1.20 ± 0.36	1.78 ± 1.21		31
Plant Age (Months)					
1-2	1.42 ± 1.27	0.26 ± 0.14	4.05 ± 1.48	14.80 ± 11.27	18, 19
3-6	30.64 ± 37.75	0.85 ± 0.89	1.81 ± 1.34	26.59 ± 12.41	15, 16, 24, 34
7-12	0.73 ± 0.57	6.44 ± 5.67	0.52 ± 0.81	13.82 ± 9.00	8, 12, 22, 30
13+		0.28 ± 0.06	1.31 ± 0.46	7.83 ± 1.28	12, 13
Unknown	15.25 ± 15.47	0.49 ± 0.38	2.31 ± 2.51	16.99 ± 12.17	1-7, 9-11, 14, 17, 20, 21, 23, 25-29, 31-33
Experiment Type					
Field	15.25 ± 15.47	0.46 ± 0.37	2.09 ± 1.87	15.40 ± 11.94	1, 3, 5-7, 9, 11-13, 17, 19, 20, 22, 25, 26, 29, 31-33
Glasshouse	6.23 ± 18.07	3.79 ± 5.01	1.84 ± 1.40	20.43 ± 11.81	8, 10, 15, 16, 18, 24, 30, 34
Unknown		1.02 ± 0.16	6.44 ± 8.65	13.42 ± 6.84	2, 4, 14, 21, 23, 27, 28
Season					
Spring	3.72 ± 2.74	0.30 ± 0.24	1.11 ± 1.38	13.57 ± 13.21	2, 3, 6, 9, 11, 16, 19, 29, 32-34
Summer	14.94 ± 19.98	0.62 ± 0.41	2.31 ± 1.84	14.46 ± 11.75	1-3, 6, 7, 9, 11-13, 16-18, 22, 29, 31-33
Autumn	17.58 ± 6.53	0.51 ± 0.32	2.43 ± 2.05	18.11 ± 7.45	2, 5-7, 9, 11, 20, 29, 31, 33

	Epoxy-Janthitrems	Ergovaline	Lolitrem B	Peramine	Literature
Season cont.					
Winter		0.60 ± 0.57	0.33 ± 0.21	4.93 ± 6.99	2, 11, 31
Unknown	11.13 ± 21.63	3.40 ± 4.86	2.44 ± 3.07	20.42 ± 11.75	4, 6, 8, 10, 14, 15, 21, 23-28, 30

*Special note that N-formyl loline (340 ppm) and N-methyl loline (63 ppm) were found in roots (Rostas *et al*., 2015).

List of papers included in the above Table 1

1. Aiken *et al* 2011
2. Ball *et al*., 1995
3. Ball *et al*., 1997b
4. Bush *et al*., 1997
5. Cripps and Edwards 2013
6. di Menna *et al* 1992
7. Easton *et al*., 1996
8. Easton *et al*., 2002
9. Eerens *et al*., 1998
10. Fannin *et al*., 1990
11. Fletcher 1999
12. Fuchs *et al*., 2017a
13. Fuchs *et al*., 2017b
14. Gallagher *et al*., 1987
15. Hahn *et al*. 2008
16. Hennessy *et al*., 2016
17. Hovermale and Craig 2001
18. Hume *et al*., 2007
19. Hunt *et al*., 2005
20. Keogh *et al*., 1996
21. Krauss *et al* 2007a
22. Krauss *et al* 2007b
23. Latch and Fletcher
24. Liu *et al*., 2011
25. McKenzie 2014
26. Oliveira *et al* 1997
27. Panaccione 2005
28. Prestidge and Galliger 1988
29. Reed *et al*. 2011
30. Ryan *et al*., 2015
31. Thom *et al*. 1999
32. Thom *et al*., 2010
33. Thom *et al*., 2014
34. Tian *et al*., 2013

The distribution of alkaloids within the plant host varies depending on the alkaloid class and chemical behaviour (Spiering *et al*., 2005). Peramine is water-soluble, highly polar, with a high molecular weight. It is found throughout all organs of the plant with the greatest concentrations in sheath tissue (Spiering *et al*., 2002), but sometimes peramine concentrations are higher in blade tissue (Ball *et al*., 1995; Spiering *et al*., 2002). Very low amounts of peramine (~0.5 μg/g) can also be found in the root tissues (Ball *et al*.,

1997b). Lolitrem B is lipophilic, concentration is highest in the leaf sheath and inflorescence (Ball *et al*., 1995; Keogh *et al*., 1996; Repussard *et al*., 2014), but is also found in the leaf blade (Ball *et al*., 1997), and in the roots (Ball *et al*., 1993; Azevedo *et al*., 1993). Lolitrem B also accumulates in older and senescent plant tissues (Keogh *et al*., 1996). Ergovaline has low water-solubility, and is found in the lowest concentrations, relative to other alkaloids. Ergovaline is not mobilized in *L. perenne* (Koulman *et al*., 2007a). It tends to accumulate in the stem and mature sheath tissue, and is not found in guttation fluid or apoplastic fluid (Spiering *et al*., 2002; Spiering *et al*., 2005; Koulman *et al*., 2007a), but is also found in the inflorescence and seeds (Lane *et al*., 1997) and has been found in roots (Ball *et al*., 1993; Azevedo *et al*., 1993). Epoxy-janthitrems are lipophilic and are not thought to translocate through the plant. Epoxy-janthitrems are found in the pseudostems and leaves, but are in higher concentrations in the pseudostems (Hennessy *et al*., 2016). There are no published studies that have tested for the presence of epoxy-janthitrems in the roots. However, Popay and Gerrard (2007) comment on unpublished data where they found low concentrations of ergovaline and epoxy-janthitrems in the root tissue of infected plants.

There is also seasonal variation in alkaloid concentrations (Ball *et al*., 1995; Ball *et al*., 1997b; Reed *et al*., 2011a). Reed *et al*. (2011a) found that as temperature and solar radiation increased ergovaline and lolitrem B concentrations decreased. However, Hennessy *et al*. (2016) found that high temperature actually significantly increased the concentration of epoxy-janthitrems in the leaves and pseudostems of grass infected with the endophyte strain AR37, while low temperature conditions (7°C) had the opposite effect (Hennessy *et al*., 2016). It should be noted that in the Reed *et al*. (2011a) study their highest temperatures exceeded 25°C, though the trend in decreasing alkaloid concentrations with increasing temperatures could

already been seen by 20°C. Also, the Reed *et al*. (2011a) study was conducted in the field while the Hennessy *et al*. (2016) study was conducted in a glasshouse. A field study conducted by Thom *et al*. (2014) showed a consistent trend, over a four-year period, of peak alkaloid concentrations in all endophyte treatments occurring in mid to late summer through to early to mid autumn. Seasonal variation in alkaloid production can also correlate with endophyte concentrations. Endophyte concentration, peramine, ergovaline and lolitrem B are higher in summer and fall than winter and spring (Ball *et al*., 1995; Thom *et al*., 1999; Thom *et al*., 2010; Fuchs *et al*., 2017b), mean ergovaline concentrations was not found to change throughout the seasons (Ball *et al*., 1995b).

Alkaloids independently synthesized by grasses can also be influenced by fungal endophytes. The grass alkaloid perloline, was reduced in blade and sheath tissue in the presence of fungal endophyte (Cao *et al*., 2008). Little it known about the biosynthesis of perloline, or the mechanism which decreases its production in the presence of endophyte. The grass steroidal alkaloids trihexoside, tetrahexoside, and pentahexoside were increased in sheath tissue of endophyte infected *L. perenne* (Cao *et al*., 2008).

Although the fungal alkaloids mentioned above play a significant role in the grass-fungal endophyte relationship, many recent studies provide evidence for a broader range of alkaloids present not yet identified, characterized, or have their biological role identified (Rasmussen; Cao *et al*., 2008; Koulman *et al*., 2007b). Observations of yet unknown alkaloids, an area well-studied for more than three decades, suggests that there are many metabolites from other compound classes still to be discovered.

Other metabolites

Advances in molecular techniques like mass spectrometry, PCR and genome sequencing have demonstrated that there is other endophyte derived metabolites contributing to the plant-fungal endophyte association (Johnson *et al*., 2007; Koulman *et al*., 2007b; Cao *et al*., 2008; Schardl *et al*., 2013). These metabolites include siderophores, cyclic modified oligopeptides, VOCs, and flavonoids. Using MS and NMR, Koulman *et al*. (2012) found extracellular siderophores (epichloënin A, epichloënin B, and epichloëamide) in the guttation fluid of infected plants as well as in fungal culture. The fungal endophyte secretes siderophores to obtain iron, an essential nutrient required by fungi, from the plant host (Johnson *et al*., 2013). Using degenerate PCR Johnson *et al*. (2007) identified new secondary metabolism genes. The most abundantly expressed gene is called *gigA*, previously known as Nc25. The *gigA* gene encodes a new class of *Epichloë* secondary metabolites called epichloëcyclins, they are secreted cyclic modified oligopeptides. Bioactivity has not been determined yet (Johnson *et al*., 2015).

Volatile organic compounds are also produced by both plant and endophyte. The presence of endophytes alone increases the production of phenolics and VOCs in the plant, in the presence of stress, such as invasion by a pathogen, these levels increase even more (Pańka *et al*., 2013). Fungal endophyte infection effects the quantity of VOCs produced, but not the quality. Compounds emitted by infected plants in the highest amounts were β-caryophyllene, (Z)-3-hxen-1-yl acetate, (Z)-3-hexenal, and linalool (Pańka *et al*., 2013). While, (Z)-ocimene, benzyl acetate, and indole were only emitted in low amounts and did not differ between infected and uninfected grasses (Pańka *et al*., 2013). Qawasmeh *et al*. (2014) looked at volatile oil profiles in *L. perenne* infected with E+, AR1, or AR37 fungal endophyte and found that volatile oil profiles were differentially affected based on endophyte strain. In E+ plants the volatile oils 2-ethyl-1-hexanol

acetate, (Z)-2-octen-1-ol, and butylated hydroxyl were all found in greater amounts than in E-. While bytyl hexanoate and hexanal were found in the same amounts, and the 13 other volatile oils were at least 10% less than in E-. Increases in volatile organic compounds have been associated with enhanced grass fitness, due to enhanced resistance to biotic stresses (Pańka *et al*., 2013), but it is also suggested that changes in volatile compounds may play a role in mediating the grass-fungal relationship (Qawasmeh *et al*., 2014).

Flavonoids are another group of secondary metabolites which are produced both by plants and fungi. Flavonoids tend to be increased in endophyte infected tissues and it has been suggested that they are responding as antioxidants to endophyte-produced ROS (White and Torres 2010).

Plant roots are an understudied organ in grass-fungal endophyte associations. Although some alkaloids have been captured in the roots (Azevedo *et al*., 1993; Ball *et al*., 1993; Bush *et al*., 1993; Rostas *et al*., 2015), suggesting that they have been translocated via the vascular tissues of the plant (Koulman *et al*., 2007a), for many years this finding was not extended to investigate the presence of alkaloids in root exudates. As well, broader investigation of other metabolites present in root tissue and root exudate is needed. Guo *et al*. (2015, 2016), and Wakelin *et al*. (2015) are the first studies to examine the root exudate of endophyte infected plants from the rhizosphere or isolated in hydroponic studies. To date, no extensive root exudate study has been conducted for *L. perenne* infected with *E. festucae* var. *lolii*. Without this information, it is difficult to make more complex connections between aboveground grass-fungal metabolism and potential belowground impacts on the soil-dwelling organisms. Van Hecke *et al*. (2005) did consider the effect of fungal endophyte on root exudate composition when looking at tall fescue infected with the fungal endophyte *Epichloë coenophiala*, however alkaloid content

was not measured. Alkaloids and phenols are not the complete story however (Siegrist *et al*., 2010; Soto-Barajas *et al*., 2015); it is possible that other metabolites may be responsible for some of the positive aspects of the grass-endophyte relationship for the host grass. This is potentially important when we consider engineering novel strains of the endophytes. If the focus has been on eliminating the toxic mycotoxins while maintaining other beneficial properties of the endophytes, we need to know what compounds, if any, are contributing to plant fitness. Two proposed mechanisms for changes to plant exudate composition are the presences of secondary metabolites of fungal origin and enhancements in plant photosynthetic rates which also increase the quantity of root exudates (Van Hecke *et al*., 2005). Changes in the quality and quantity of root exudates may influence the microbial community in the rhizosphere.

Guo *et al*. (2016), expanded upon their previous root exudate work (Guo *et al*., 2015) to include compounds in the soil rhizosphere. The compounds identified were grouped into sugars, polyols, growth factors and vitamins, lipids, amines, phenolics, carboxylic acids, nucleosides and others (Guo *et al*, 2016). The two *S. arundinaceus* cultivars chosen (PDF and 97TF1) produced distinct metabolic profiles, and within the cultivars the inclusion of endophyte treatments (E-, CTE+, AR584E+, and AR542E+) introduced another level of variability in the metabolites present. Guo *et al*., (2016) found that the cultivar-endophyte interaction significantly impacted 26 compounds including arabinose, dihydroxyacetone, palmitic acid, caffeic acid, syringic acid, and terephtalic acid.

In this section I have shown that host plant primary and secondary metabolism is significantly altered by endophyte infection, which can be observed in both above- and belowground tissues. In the next section I explore the effects of endophyte infection on the belowground soil community.

THE RHIZOSPHERE

In the previous section (the Plant-Fungal Metabolome) I highlighted some of the ways in which the presence of a fungal endophyte alters plant host chemistry. In this section I will first review the potential routes of plant-fungal metabolites into the soil ecosystem with an emphasis on plant root exudates; then I will consider the effect, if any, of plant-fungal metabolites on microbial communities in the soil; and lastly, I will highlight potential changes in soil feedbacks as a result of changes in microbial communities.

The soil is often separated into the rhizosphere and bulk soil. These two environments often differ in their pH, chemistry, and the microbial communities that they host. The rhizosphere, the region of the soil under the influence of plant roots, first described by Hiltner in 1904 (Hartmann *et al*., 2008), is a dynamic interface between plant and microbial processes. Plants not only acquire water, nutrients, and trace elements through their roots, they also exude hundreds of biochemical compounds into the rhizosphere. These compounds include sugars, amino acids, organic acids, phenolics, and secondary metabolites (considered low molecular weight (LMW)); and mucilage and proteins (considered high molecular weight (HMW)) (Walker *et al*., 2003). Collectively, these compounds are referred to as root exudates and they serve many functions within the rhizosphere including transport, by acting as metal chelators to increase the availability of important micronutrients (i.e. iron (Fe)); and in the mediation of biological interactions in the rhizosphere (i.e., plant-plant and plant-microbe) (Bais *et al*., 2006). Root exudates represent up to 20% of the plants photosynthetically derived carbon, a considerable but necessary cost to the plant (Walker *et al*., 2003). Plants shape the structure of the microbial community in the rhizosphere and a plants long-term survival depends on the microbial community present (see review Segura *et al*., 2009; Bandyopadhyay *et al*., 2016). Plants can

affect the microbial community composition of the rhizosphere through their root exudates, which can both attract beneficial organisms and deter harmful organisms like pathogens (Marschner *et al*., 2001).

The rhizosphere is composed of many organisms including a consortium of microorganisms representing diverse enzymatic systems. Soil microorganisms are connected with ecosystem level functions such as decomposition, nutrient cycling (e.g. carbon and nitrogen mineralization, nitrification, and denitrification), and degradation of contaminants (Doran and Zeiss, 2000; Kaneda and Kaneko, 2008). Important areas of research include communications between plants and microorganisms, how microorganisms respond to root exudates and how they interact with root surfaces (Segura *et al*., 2009). For example, Kosuta *et al*. (2003), provided evidence for arbuscular mycorrhizal fungi (AMF) using a signalling molecule that activates plant genes which aid in symbiosis. Also, a study by Meharg and Killham (1995) using ^{14}C labelled CO_2 found that certain microbial species in the rhizosphere increased root exudation anywhere from 3 to 34%. Many abiotic processes can influence biotic processes belowground such as plant-microbe interactions, and the efficiency of degradation and sorption by plants and microbes. These abiotic processes include the temperature, pH, aeration, O_2 tension, electron acceptors, and organic content of the soil (de Lorenzo, 2008).

As previously discussed, the presence of fungal endophytes can alter the morphology and physiology of the grass host which can influence associated organisms aboveground, but there is also evidence that there are effects belowground in the roots and in the rhizosphere. Belowground effects such as an altered root morphology, chemical changes in the roots, root surfaces, and soil, and changes to microbial community composition have been studied more frequently in tall fescue infected with *Epichloë coenophiala* (Malinowski *et al*., 1998;

Franzluebbers *et al*., 1999; Franzluebbers and Stuedemann 2002; Franzluebbers and Stuedmann 2005; Van Hecke *et al*., 2005; Franzluebbers 2006; Iqbal *et al*., 2012; Hosseini *et al*., 2015; Hosseini *et al*., 2016), but there is also evidence of belowground effects in endophyte infected *L. perenne* (Bell *et al*., 2009; Popay and Thom 2009; Bowatte *et al*., 2011; Cripps and Edwards 2013). **Table 1.2.** provides a list of studies that investigated belowground responses to *L. perenne* infected with *Epichloë festucae* var. *lolii*. However, the influence of foliar fungal endophytes on the belowground ecosystems is an understudied area and still little is known about how the plant-fungal metabolome affects belowground communities and biogeochemical processes. In the following four sub-sections, I will discuss how plant-fungal metabolites enter the soil, their fate in soil, their impact on soil microorganisms, and the potential effect, if any, on soil feedbacks and on larger ecosystem processes.

Table 1. 2 Belowground effects of *Lolium perenne* infected with *Epichloë festucae* var. *lolii*. The endophyte effect could be (+) increase, (-) decrease, (0) no change, or (1) a change occurred but direction of change not known.

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Growth of neighbouring plants					
	-	Percival and Duder 1983	E-, E+	Nui, Ruanui, Ellett	*Trifolium repens*
	-	Sutherland and Hoglund 1989	E-, E+	Ariki, Nui	*Trifolium repens* (Field) Wheat seedlings grown in E+ conditioned soil
	-	Stevens and Hickey 1990	E-, E+	Unknown	*Trifolium repens*
	-	Sutherland and Hoglund 1990	E-, E+	Unknown	*Trifolium repens*
	+	Watson 1990	E-, E+	Ellett	*Trifolium repens* nematodes
	-/0	Lewis 1992	E-, E+	Nui	*Trifolium repens*
	-	Thom *et al*., 1999	E-, E+	Yatsyn 1	*Trifolium repens*
	-	Thom 2008	E-, AR1, AR37	Unknown	*Trifolium repens* reduced by AR37
	-/+/0	Bryant *et al*., 2009	E-, AR1, AR37	Aberdart, Samson, Quartet	*Trifolium repens*
	+	Cripps *et al*., 2013	E-, E+, AR1, AR37, NEA2	Alto	*Trifolium repens* Soil conditioning all responses showed positive growth but endophyte treatments were significantly different from each other
	-	Thom *et al*., 2014	E-, E+, AR1, AR37	Commando	*Trifolium repens* reduced by AR37

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Root exudation and nutrient uptake					
	0	Lewis *et al*., 1996	E-, E+	Unknown	Nitrogen uptake, Hydroponic
	0	Malinowski *et al*., 2004	E-, AR1	Aries, Quartet	Cu^{2+} binding activity
	-/+	Ren *et al*., 2007	E-, E+	SR4000	At low P, did not increase P uptake rate but improved P use efficiency
	+	Soto-Barajas *et al*., 2015	E-, E+	Naturalised	Field. Indirect. Changes in nutrients in the plant tissue
	1	Wakelin *et al*., 2015	E-, AR1, AR37	Samson	alkanes
Nematodes					
	0	Yeates and Prestidge 1986	E-, E+	Ellett, Ruanui	Many genera investigated
	0	Watson 1990	E-, E+	Ellett	Many ectoparasitic (*Helicotylenchus, Tylenchus,* and *Paratylenchus*) and endoparasitic (*Heterodera, Meloidogyne,* and *Pratylenchus*) genera present in soil
	0, +	Cook *et al*., 1991	E-, E+	Unknown	*Meloidogyne naasi*
	-	Stewart *et al*., 1993	E-, E+	Unknown	*Meloidogyne naasi*
	+/-/0	Watson *et al*., 1995	E-, E+	Pacific	*Pratylenchus* sp. ↑ *H. pseudorobustus* and *Tylenchus* ↓ but not significant

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Nematodes					
	-	Ball *et al*., 1997a	E-, AR17, AR19, AR20, AR21, AR23, AR24	Nui	*Meloidogyne marylandi* All endophyte strains hosted lower nematode numbers, but AR19 was significantly different than E-
	-	Eerens *et al*., 1998	E-, E+, AR6	Nui, Ruanui, and Pacific	Negative correlation between *Paratylenchus* and the presence of endophyte, but confounded by plant biomass
	-	Panaccione 2005	NA	NA	*Pratylenchus scribneri* Ergot alkaloids – synthesized Ergotamine
	-	Panaccione *et al*., 2006	E-, E+, *lpsA* knockout, *dmaW* knockout	Rosalin	*Pratylenchus scribneri*
	+/-/0	Bell *et al*., 2009	E-, E+, AR1, AR37	Samson	Cephalobidae nematodes increased with E+ and AR37 Rhabditidae nematodes increased with E- and AR1
Soil microbial activity and decomposition rates					
	+	Bowatte *et al*., 2011	E-, E+, AR1, AR6, AR37	Samson	Soil nitrification
	-	Cripps and Edwards 2013	E-, E+, AR1, AR37	Alto	All endophyte treatments resulted in a slower faecal decay rate

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Compounds found in roots					
	+	Ball *et al*., 1997b	E+	Unknown	Peramine – very low concentrations ~0.5 ppm
	+	Zhou *et al*., 2003	E-, E+	Unknown	Phenolic content increased at low phosphorus levels
	-	Ren *et al*., 2007	E-, E+	SR4000	At low P, total phenolics and organic acids content (but not concentration) was greater
	+	Ren *et al*., 2009	E-, E+	SR4000	At high N, sugar and mineral elements (B, Mn and Mg) increased in the roots
Root growth					
	+	Latch *et al*., 1985	E-, E+	Nui	Drought stress
	0	Eerens *et al*., 1988	E-, E+, AR6	Ruanui, Nui, Pacific	Root weight
	+	Hesse *et al*., 2003	E-, E+	Naturalised	Root dry weight and root:shoot ratio increased
	+	Zhou *et al*., 2003	E-, E+	Unknown	Phosphorous deficiency, root length increased
	+/-	Cheplick 2004	E-, E+	Yorktown III	Drought stress
	+	Crush *et al*., 2004	E-, E+, AR1, AR37	Samson	Root angle
	+	Hesse *et al*., 2003	E-, E+	Unknown	Drought stress
	+	Hesse *et al*., 2005	E-, E+	Unknown	Drought stress
	-	Cheplick 2007	E-, E+	Naturalized	Nutrient stress
	+	Ren *et al*., 2007	E-, E+	SR4000	At low P, roots longer and heavier
	+	Ren *et al*., 2009	E-, E+	SR4000	At high N level

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Other invertebrates					
	-	Ball and Prestidge 1992	E-, E+	Ellett	Black beetle larvae feed on roots
	-	Prestidge and Ball 1993	E-, E+	Unknown	New Zealand grass grubs
	-	Popay and Wyatt 1995	E-, E+, AR37	Nui, Ruanui	Argentine stem weevil larvae
	+/-	Easton *et al*., 2000	AR1	Unknown	Black beetle (*Heteronychus arator*)
	-/+/0	Popay *et al*., 2004	E-, E+, AR1, AR37	Samson	Root aphid AR1 ↑ AR37 ↓ No difference between E+ and E-
	-	Hume *et al*., 2007	E+, AR1, AR37	Samson	African black beetle Root aphid AR37 performed better than E+ and AR1
	-	Popay and Gerard 2007	E-, E+, AR1, AR6, AR12, AR22, AR23, AR37	Nui, Samson, Impact	Root aphid AR37 and AR6 suppressed root aphid numbers
	-	Bryant *et al*., 2009	E-, AR1, AR37	Aberdart, Samson, Quartet	Root aphid deterred by AR37
	0/-	Popay and Thom 2009	E-, E+, AR1, AR37	Commando	Black beetle, effect of endophyte changed from year-to-year, but AR37 had the strongest effect
	0/-	Popay and Thom 2009	E-, E+, AR1, AR37	Commando	*Costelytra zealandica* AR37 had the strongest effect

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Other invertebrates					
	-	Popay and Thom 2009	E-, E+, AR1, AR37	Commando	*A. lentisci* (root aphid) AR37 had the strongest effect
	0	Popay and Thom 2009	E-, E+, AR1, AR37	Commando	*Sitona Lepidus* (clover root weevil)
	-	Moate *et al*., 2012	E+, AR1, AR37	Commando	Root aphids, mealy bugs, pasture tunnel moths AR37 had the strongest effect
	-	Thom *et al*., 2014	E-, E+, AR1, AR37	Commando	African back beetle larvae and root aphids, E+ and AR37 decreased Grass grubs lower in AR37
Root symbioses (Mycorrhizae)					
	-	Müller, 2003	E-, E+	9155, Lacerta	*Sclerocystis* sp.
	-	Liu *et al*., 2011	E-, E+, AR1	AberDart, Fennema	AM fungi: *G. mosseae* and *G. intraradices*
Soil microbial community					
	+/-/0	Bell *et al*., 2009	E-, E+, AR1, AR37	Samson	AR1 – *Pseudomonas* and Actinobacteria communities AMF communities were significantly different in structure
	+	Wakelin *et al*., 2015	E-, AR1, AR37	Samson	Changes in bacterial and fungal community

Belowground Parameter	Endophyte Effect	Supporting Work	Endophyte Strain	Grass Cultivar	Additional information
Compounds in soil					
	+	Sutherland and Hoglund 1989	E-, E+	Ariki, Nui	Suggest the possibility of allelopathic toxins, but this was not measured
	0	Sutherland and Hoglund 1990	E-, E+	Unknown	Tested leachates from shoots and roots. Shoot leachate was allelopathic, root leachate was not. Root leachate came from ground up roots not exudates.
	-	Quigley *et al*., 1990	E-, E+	Unknown	Tested leachates, but not from root exudate. Did not identify or measure any compounds in the leachate.
	-	Sutherland *et al*., 1999	E-, E+, AR4	Nui, Pacific	Did not test the soil for alkaloids. Did not use the roots to make their allelopathic leachate, they used pseudostems
	1	Cripps *et al*., 2013	E-, E+, AR1, AR37, NEA2	Alto	Did not identify or measure any compounds in the soil but suggested they had an influence on plant growth
	0	Mace *et al*., 2016	E-, AR95	Colosseum	Did not test the soil for alkaloids, tested crop plants grown in soil that endophyte infected perennial ryegrass had grown in
	+	Wakelin *et al*., 2015	E-, AR1, AR37	Samson	Alkane hydrocarbon derivatives

Routes of plant-fungal metabolites into the soil ecosystem

Three potential routes for plant-fungal metabolites entering the soil are: faecal matter and urine from herbivores feeding on infected grasses (Cripps and Edwards, 2013); litter fall (Hume *et al*., 2007); and exudation from roots of infected grass (Panaccione *et al*., 2014). In contrast to animal inputs and litter fall, where there would be intermittent inputs, plant root exudates provide a steady flux of resources into the rhizosphere and contribute to a dynamic relationship with soil organisms.

Animal inputs

Ruminants feeding on grass infected with fungal endophytes are not able to completely metabolize mycotoxins produced by the fungal endophyte (Hill *et al*., 1994). Therefore, un-metabolized and partially metabolized secondary metabolites can exit the ruminant in its waste products (Westendorf *et al*., 1993). Secondary metabolites, such as the fungal alkaloid loline, have been found in the feces and urine of sheep feeding on endophyte infected *Schedonorus pratensis* seed (Gooneratne *et al*., 2012), and the fungal alkaloids peramine, lolitrem B, and epoxy-janthitrems have been found in the feces of sheep feeding on *L. perenne* infected with *E. festucae* var. *lolii* strains E+, AR1, and AR37 (Cripps and Edwards, 2013). Franzluebbers *et al*. (1999), hypothesized that alkaloids could be transferred from infected plant tissue (in the form of leaf litter) and animal waste (in the form of feces and urine) to the soil matrix and that their presence in the soil may influence the microbial community and subsequently the production of soil organic matter. Franzluebbers and Hill (2005) confirmed the presence of ergot alkaloids in soils where endophyte infected *S. arundinaceus* plants had grown over a long-term, suggesting that at least one of the alkaloids synthesized by fungal endophytes can persist in soil. As there

were cattle, leaf litter, and exudation at this site it was unclear to the authors the origin of the alkaloids (Franzluebbers and Hill 2005).

Litter fall

Some fungal derived metabolites can persist in dead plant tissue. For example, ergovaline degrades quickly when exposed to the environment but can persist in silage when processed immediately after cutting and stored appropriately (Fletcher 2005). Ergovaline persists in hay at much lower levels than fresh grass and in less than a year the concentration of ergovaline is almost negligible (Fletcher 2005). Lolitrem B persists at relatively similar concentrations regardless of being silage or hay (Fletcher 2005). Dead and senescence leaves have little to no peramine and ergovaline content, however lolitrem B and janthitrems are still present (Hume *et al*., 2007). Hume *et al*. (2007) were interested in the fate of peramine, ergovaline, lolitrem B, and epoxy-janthitrems alkaloids in above-ground *L. perenne* tissue after clipping and left in the field. Although they did not measure leaching into the soil, they did observe that alkaloids persisted in plant tissue to varying degrees, suggesting that there is potential for alkaloids to end up in the soil matrix as the plant tissue decomposes. Antunes *et al*. (2008), demonstrated that dead shoot tissue from tall fescue infected with *N. coenophialum* left on the soil surface could limit plant colonization by AMF, suggesting that fungal metabolites are being leached from litter fall. Quigley *et al*. (1990) used endophyte infected *L. perenne* shoot tissue of to prepare aqueous extracts that were applied to legume species. They found that the presence of endophyte reduced the root length of the legume species by 10%, on average.

Plant root exudates

The first mention of the potential of fungal endophyte produced alkaloids to be translocated via the roots into the soil was by West *et al*. (1988). It is rare to find studies that measure alkaloids in grass roots. Loline alkaloids can be found in the roots of endophyte-infected *S. arundinaceus*, *S. pratensis*, and *S. pratensis* x *L. perenne* (Bush *et al*., 1993; Patchett *et al*., 2011; Rostas *et al*., 2015), and peramine can be found in the roots of *L. perenne* (Fannin *et al*., 1990). However, there are unpublished accounts that report alkaloids not being detected in root exudates.

The number of studies where the quantity and quality of root exudates of endophyte infected grasses are investigated are so few. For endophyte infected *L. perenne* there are two (Malinowski *et al*., 2004; Wakelin *et al*., 2015). Malinowski *et al*., (2004) were interested in the copper binding activity of extracellular root exudates of endophyte infected tall fescue and *L. perenne*. They found that endophyte had no effect for *L. perenne* copper accumulation, but did have an effect for *S. arundinaceus*. Several *S. arundinaceus* studies have demonstrated that endophyte infection can influence the quality and quantity of metabolites exiting the plant in the form of root exudates, hence providing evidence of root exudates as a method of fungal derived or influenced metabolites entering into the soil (Malinowski *et al*., 1998a, 1998b; Guo *et al*., 2015).

Fate of metabolites in the soil

Decomposition of animals and plants is essential in the formation of soil organic matter and the cycling of nutrients. Soil organic matter can be formed by both the recalcitrant and labile components of plant litter (Cotrufo *et al*., 2015). Early in decomposition it is the non-structural plant residues that are lost from plant litter. Root exudates, composed of non-structural

metabolites, are considered a labile form of plant residues, and are quickly utilized by soil organisms,

Carbon compounds entering the soil can be re-taken up by plant roots, immobilized as soil organic matter (e.g. humus, or assimilated by living microorganisms), and mineralized into inorganic matter. Using isotope labeling studies, it is clear that when a solution of ^{12}C-glucose is directly added to soil, microbial organisms in the rhizosphere immediately utilize and convert it to $^{14}CO_2$ (Cheng *et al*., 1993). However, when it is ^{14}C-labeled photosynthates there can be a time lag between when it is deposited through root exudates and when it is taken up and utilized by microorganisms. The microbial assimilation efficiency of carbon can range from approximately 30 to 60% (Elliott *et al*., 1983; Johansson 1992). The nitrogen fixation process requires a lot of energy, and microbes that fix N_2 can obtain this energy from sugars exuded by plant roots. Root exudates prime the soil for faunal grazers (e.g. bacterial feeding nematodes), by increasing microbial biomass.

Impact of plant-fungal symbiosis on organisms in the rhizosphere

Plants provide the primary source of carbon, in the form of root exudates, to the rhizosphere food web. The quantity and quality of root exudates influence changes in the rhizosphere community activity and structure. Regardless of the importance of plant root exudation to the soil ecosystem, very few studies have addressed the role of foliar endophyte infection on root exudates quantity and quality and its effect on the microbial community. A recent meta-analysis by Omacini *et al* (2012) addressed this gap by synthesizing the available knowledge on the below-ground impacts of several plant-fungal endophyte associations. The authors considered only literature that had both experimentally manipulated endophyte infection (i.e. E+ and E-), and provided statistical information. There were six response variables of

interest: root biomass, arbuscular mycorrhizal fungi, soil fauna, root exudates, microbial respiration and litter decomposition. There were only 27 papers (all within the last two decades) that met their criteria. Although their aim was to understand general trends in grass fungal relationships with respect to the above noted six response variables, very limited data were contributed by research related to *L. perenne* infected with *E. festucae* var. *lolii* and no data collected considered novel endophytes (although some papers did include novel endophytes in their research). From these papers Omacini *et al*., (2012) were able to abstract 133 data pairs, and only 16 of these data pairs were from *L. perenne* research. These 16 data pairs represented studies on root biomass, mycorrhiza and rhizobium soil fauna, and did not touch on root exudates, litter decomposition, or soil respiration. Across all 133 data pairs representing all grass endophyte relationships they found that only root biomass, root exudates, and mycorrhizal colonization showed significant endophyte effects, with significant reductions in root biomass and mycorrhizal colonization, and a significant stimulating effect on the root exudates of infected vs uninfected grasses. Additionally, of the literature Omacini *et al*. (2012) reviewed, there was no apparent endophyte effect on soil fauna regardless of whether all organisms were grouped together or subdivided into microfauna, mesofauna, and macrofauna. Host-specific responses were not measured due to low literature replication (Omacini *et al*., 2012). Since the Omacini *et al*. (2012) meta-analysis, three important papers have been published which address metabolomic and genomic features of the below-ground ecosystem (Guo *et al*., 2015, 2016, Wakelin *et al*., 2015); of these, only one uses *E. festucae* var. *lolii* infected L. perenne (Wakelin *et al*., 2015).

The presence of fungal endophytes can have an effect on soil organisms such as nematodes, fungi, and bacteria (Panaccione 2005; Panaccione *et al*., 2006; Antunes *et al*., 2008;

Bacetty *et al*., 2009a,b; Rojas *et al*., 2016). While some studies have concluded that the presence of fungal endophytes would have very little short-term impact on all three of these communities (Bell *et al*., 2009), others have suggested that even subtle changes may have wider reaching ecological impacts (Guo *et al*., 2014, 2015; Wakelin *et al*., 2015). In depth investigation into belowground responses to the presence of fungal endophytes in grass hosts is still limited.

Fungal communities (AM and other)

In addition to forming symbiotic relationships with fungal endophytes from the genus *Epichloë*, grasses can also form symbiotic relationships with mycorrhizal fungi. These mycorrhizal fungi colonize the roots of the grass and can provide similar benefits to the grass as *Epichloë*, including improvements in nutrient status (i.e. phosphorous), tolerance to abiotic stresses, and improvements in yield (Smith and Read 1997). It is possible for a grass to be infected by both fungi simultaneously and rather than doubling the benefits for the plant host, they often compete with one another (Müller 2003; Liu *et al*., 2011). Müller (2003) used *L. perenne* infected with either *E. typhina* or *E. festucae* var. *lolii* fungal endophytes and the mycorrhizal fungi *Sclerocystis* sp. to determine the effect of fungal endophyte presence on both mycorrhiza and the plant host. They found that mycorrhizal colonisation of host plant roots was significantly less in fungal endophyte infected plant hosts than uninfected. This was also found by the Liu *et al*. (2011) study which focused on the competitive interaction between *E. festucae* var. *lolii* (E+ and AR1) and the mycorrhizal fungi *Glomus mosseae* and *G. intraradices* in two cultivars of *L. perenne* (AberDart which is a high sugar grass, and Fennema, a conventional grass) under phosphorus limited conditions. The presence of mycorrhizal fungi resulted in a decrease in both fungal endophyte concentrations and fungal derived alkaloids (peramine and lolitrem B). While fungal endophyte presence resulted in significant reductions in mycorrhizal

colonization. When colonization was successful, the effect of fungal endophyte presence on mycorrhizal concentrations was species specific, significantly reducing *G. intraradices* concentrations, but not *G. mosseae*. There are several possible mechanisms by which foliar fungal endophytes limit mycorrhizal colonization success including access and utilization of resource supply, timing of colonization (i.e. fungal endophytes subside in the grass seed and grow with the plant, whereas AMF establish associations with the grass roots via the soil), and the production of allelopathic compounds by the grass-fungal metabolome (Müller 2003; Liu *et al*., 2011).

There are other fungal species in the soil rhizosphere, that may be beneficial or pathogenic to grasses. *In vitro* studies demonstrate *E. festucae* var. *lolii* can inhibit the growth of some pathogenic fungi like *Fusarium culmorum*, *F. equiseti*, and *Dreschlera dictyoides* (Holzmann-Wirth *et al*., 2001; Pańka 2008). Though this effect is often lower when *in planta* (Pańka 2008). The compounds responsible for the growth inhibition were not isolated or identified in either of these studies.

Fungal communities can differ between E+ and E- soils. This has been observed in fungal endophyte infected *Festuca arundinacea*, *L. multiflorum*, and *L. perenne* (Wakelin *et al*., 2015; Rojas *et al*., 2016). Rojas *et al*., (2016) used the fungal strains E+, AR542, and AR584, and found a significant difference in the fungal communities of E+ and E- soils, but did not find that there were endophyte strain-specific effects. Endophyte infection altered fungal community composition but not overall fungal biomass. The phylum Glomeromycota (AMF) had greater relative abundances and the phylum Ascomycota had lower relative abundances when compared to E- soils. At the genera level *Lecanicillium*, *Volutella*, *Lipomyces*, *Pochonia*, and *Rhizoctonia* were significantly different between endophyte treatments with all but *Volutella* having lower

relative abundances when compared to E- soils (Rojas *et al*., 2016). Similarly, Using *L. perenne* infected with several strains of *E. festucae* var. *lolii* Wakelin *et al*. (2015) also found differences in fungal community composition. There was clear grouping by endophyte strain, but the within endophyte strain variation was high. In contrast, Casas *et al*. (2011) found that *L. multiflorum* infected with *E. occultans* increased soil fungal activity but did not effect the fungal community composition.

Bacterial communities

The presence of fungal endophytes can alter the structure and function of the bacterial community in the soil, often with endophyte strain specific effects (Guo *et al*., 2015; Roberts and Ferraro 2015). McNear and McCulley (2012) isolated the exudates from whole *S. arundinaceus* root infected with *E. coenophiala* strains, and applied them, at three different concentrations, to bacterial cultures of *Sinorhizobium meliloti*, a key symbiotic soil microorganism. They found that the exudates from uninfected and endophyte infected (E+ and AR542) plants differed in their inhibition of the bacteria. Root exudates from E+ infected plants had the highest inhibition of the bacteria at 20 and 30% concentrations, while E- had the highest at 10%. Root exudates from AR542 inflected plants had the lowest inhibition of bacterial growth. Bell *et al*. (2009) explored the effect of *L. perenne*, cv. Samson, infected with E+, E-, AR1 or AR37, directly grown in soil, on microorganisms. They found that there were endophyte strain dependent effects on bacterial community structure. Actinobacteria and *Pseudomonas* abundances were significantly different between E+ conditioned soils and E-, AR1 and AR37 conditioned soils. Betaproteobacteria abundances were significantly different between E+ conditioned soils and E- and AR1 conditioned soils. While Alphaproteobacteria abundances were significantly different between AR1 and E- conditioned soils. Wakelin *et al*. (2015) also found differences in bacterial

community structure, that were strain specific. They found that the bacterial community structure of AR1 conditioned soils had the least similarity to the other treatments. Unlike in Bell *et al*. (2009), Wakelin *et al*. (2015) did not find significant differences in *Pseudomonas* between endophyte soil conditioning treatments.

Potential Changes to Soil Feedbacks

The rhizosphere food web is fueled by nutrient and carbon fluxes influenced by plant roots, bacteria, fungi, and their consumers. These fluxes are essential to the stability of the food web and consequently to greater ecosystem functions. Fluxes translate as positive and negative feedbacks throughout an ecosystem at different spatial and temporal scales. In plant-soil feedbacks it is the plants which effect soil properties and soil resource availability which, in turn, can affect the soil microorganisms.

Endophyte infected grasses can influence plant community composition by competing with other plant species, potentially reducing community diversity; or by inhibiting or promoting the growth of other plant species through soil conditioning (Rudgers and Clay, 2007; Cripps *et al*., 2013). Guo *et al*. (2015) identified several metabolites in the root exudate of endophyte infected grasses that could potential inhibit the germination and growth of other plant species (e.g., syringic acid, and myristic acid). Changes in the identity of plant species may have an effect on the properties of the soil (Sutherland and Hoglund, 1989; Matthews and Clay 2001). For example, endophyte presence may result in greater productivity of the soil with increased carbon and nitrogen content (Franzluebbers 2006). Root exudates stimulate microbial activity which is responsible for the mineralisation of nutrients (Casas *et al*., 2011) which leads to growth of plants. However, if E+ plants do better than E- plants and are able to more efficiently utilise resources they could potentially consume more soil nutrients making nutrient poor soil

(Handayani *et al*., 2011; Cripps *et al*., 2013). There have been several studies that do not indicate that endophyte presence results in nutrient poor soils (Franzluebbers *et al*., 1999; Franzluebbers 2006; Iqbal *et al*., 2012).

The presence of fungal endophytes can potential alter the quantity and quality of plant litter and root exudates being inputted into the soil. These changes have been correlated with shifts in microbial community structure and function (Van Hecke *et al*., 2005; Casas *et al*., 2011; Wakelin *et al*., 2015). These shifts may have consequences for key biogeochemical processes. Casas *et al*. (2011) found that the capacity of soil to metabolize substrates differed between E+ and E-. In soils grown with E+ plants, respiration rates were higher for glucose, starch and E+ litter substrates when compared to E- plants. However, the whole endophyte infected plant tissue may represent a poorer quality resource for organisms and result in a reduction in the rate of organic matter decomposition (Rudgers and Clay 2007; Walela *et al*., 2014). Consequently, this could slow down the cycling of carbon, nitrogen, and other key nutrients. Carbon and nitrogen storage can be higher in soils conditioned with endophyte infected plants (Franzluebbers, 2006; Iqubal *et al*., 2012; Guo *et al*., 2016). Contrasting with this, greater CO_2 and N_2O fluxes have been observed (Iqbal *et al*., 2013).

CONCLUSIONS

The effect of foliar fungal endophytes on microbial communities in the soil are highly variable in the limited literature available. This is most likely due to differences in analytical techniques, plant age, plant genotype, endophyte strain, and environmental variables. Using advanced molecular techniques there is some evidence for fungal endophyte presence resulting in changes in the metabolome of above and belowground plant tissues, and root exudates (Rasmussen *et al*., 2007, 2008, 2009; Wakelin *et al*., 2015). Additionally, these changes in the plant-fungal metabolome can also be confirmed through transcriptomics work where is has been found that a significant proportion of the *L. perenne* transcriptome is influenced by the presence of *E. festucae* (Dupont *et al*., 2015). As so few studies have been conducted using *L. perenne* infected with strains of *E. festucae* var. *lolii*, it is therefore important to identify the chemical composition of the root exudates entering the soil that contribute to the chemical signalling in the rhizosphere, and to determine if the quality and/or quantity of these root exudates influence the microfood-web.

Studying plant-endophyte associations at a molecular level has relevance in agriculture, turf, and in bioremediation. The improvements in forage cultivars bred for growth under environmental constrains, the welfare of livestock, which have environmental benefits, in turf improvements in tolerance to varying environments can greatly decrease the need for fertilization inputs, and in bioremediation the more we understand the plant microbiome, can aid in the synthesis of functional microbiomes that can be helpful in the agricultural, turf, and bioremediation industries. Although the agronomic argument for developing novel strains of endophytic fungi has clear benefits, how these strains will affect grassland systems and ecosystem processes is less understood. When common toxic and novel strains are introduced,

they represent changes in the grass-endophyte chemistry. The aboveground effects of this association have been extensively investigated; however, the belowground effects of the grass-endophyte association remain an understudied area.

The purpose of this thesis was to improve our understanding of the indirect effects of grass-fungal mutualisms on belowground soil organisms. This thesis will help to answer the following research question; How do changes in the plant-fungal metabolome effect the belowground microbial community? In order to answer this, we need to extend our understanding of metabolomic changes which occur in grass-fungal mutualisms to the root exudates. My three main objectives were to (1) isolate plant-fungal metabolites being exuded from the plant roots and determine if there are endophyte strain specific changes in the root exudate metabolomic profile; (2) isolate rhizosphere metabolites and determine if there are endophyte strain specific changes in the rhizosphere metabolomic profile; and (3) determine if endophyte presence changes the structure of the soil microbial community.

In Chapter 2 I will conduct untargeted metabolomics on plant root exudates using liquid chromatography mass spectrometry (LC-MS), to investigate changes in metabolites in the *L. perenne- E. festucae* var. *lolii* fungal metabolome. In chapter 3 metabolomics is further discussed used in more complex soil environment, and coupled with next generation sequencing (NGS) to explore the effects of plant-fungal relationships on microbial community. In this chapter I will also outline my soil priming experiment again utilizing *L. perenne* plants infected with several *E. festucae* var. *lolii* strains.

OBJECTIVES

In this thesis my objectives were to 1) establish how fungal endophyte strains of *Epichloë festucae* var. *lolii* influence the chemical composition of *L. perenne* root exudates; 2) determine if changes in plant root exudate chemistry effect bacterial, and fungal diversity in the soil rhizosphere; and 3) discern the effect of endophyte infection concentration on root exudate chemistry and soil microbial communities. To address my first objective, I used a hydroponic set-up to collect plant root exudates. To measure the metabolomic profiles of the plant-endophyte treatments the plant root exudates were subjected to liquid chromatography mass spectrometry (LC-MS). I hypothesize that the presence of *E. festucae* var. *lolii* alters plant host chemistry, and that each fungal endophyte strain will present a unique root exudate chemical profile. To address my second objective, I extracted the metabolites from rhizosphere soil and again used a non-target metabolomics approach with LC-MS. I also conducted next-generation sequencing on the rhizosphere soil to determine fungal and bacterial structure. I hypothesize that changes in plant host chemistry can indirectly effect soil organisms in the rhizosphere, via changes in the quality of root exudates, which will result in shifts in community composition unique to each endophyte strain that can first be seen when comparing soil conditioning treatments to bare soil, and second when comparing endophyte free conditioned soils with endophyte infected conditioned soil. For my third objective, to measure endophyte concentrations in the plant host, I conducted quantitative polymerase chain reaction (qPCR) using primers specifically designed for a fungal gene in our endophyte. I hypothesize that fungal endophyte strains differ in their level of plant host colonization (i.e. endophyte concentration), and that endophyte concentration will be correlated with changes in plant morphology and physiology, as well as changes in soil microbial community structure.

CHAPTER 2: COMPARISON OF PLANT METABOLITES IN ROOT EXUDATES OF LOLIUM PERENNE INFECTED WITH DIFFERENT STRAINS OF THE FUNGAL ENDOPHYTE EPICHLOË FESTUCAE VAR. LOLII USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

ABSTRACT

Lolium perenne infected with the fungal endophyte *Epichloë festucae* var. *lolii* have specific, endophyte strain dependent, chemical phenotypes in their aboveground tissues. Differences in these chemical phenotypes have been largely associated with classes of fungal derived alkaloids which protect the plant against many insect pests. However, the utilization of new methodologies, such as various omic techniques, have demonstrated that many other chemical changes, in both primary and secondary metabolites, occur. Few studies have investigated changes in plant metabolites exiting the plant in the form of root exudates. As root exudates play an essential role in the acquisition of nutrients, microbial associations, and defence in the belowground environment, it is of interest to understand how plant root exudate chemistry is being influenced by the presence of strains of a fungal endophyte. This experiment tested the influence of four strains of *E. festucae* var. *lolii* (E+, AR1, AR37, NEA2) on *L. perenne* growth and the composition of root exudate metabolites. Plants, germinated and grown in rockwool for nine weeks, were transferred to a hydroponic set-up and grown for four weeks before the plant biomass and root exudates were harvested. The extent of endophyte infection was assessed by qPCR from a subsample of sheath tissue. Root exudates present in the hydroponic water were assessed by untargeted metabolomics using Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC-MS. There was a significant effect of endophyte treatment on plant biomass ($F_{4,80}$ = 7.8, $p<0.001$), endophyte concentration ($F_{3,63}$ = 4.3715, p = 0.007363), and root exudate metabolites. There were

a total of 73 metabolites that were differentially expressed in at least one of the endophyte treatments when compared to E- plants. There were several compounds that were strongly associated with one endophyte treatment, like in AR37 (m/z 135.0546 RT 1.17), and E+ (m/z 517.1987 RT 9.26). These results provide evidence for significant changes in *L. perenne* physiology in the presence of several fungal endophyte strains. Further research should aim to connect changes in root exudate chemical composition with soil ecosystem processes.

Keywords:, *Lolium perenne*, *Epichloë festucae* var. *lolii*, plant-fungal endophyte associations, fungal endophyte strains, fungal endophyte concentration, root exudate metabolites, qPCR, LC-MS

INTRODUCTION

Fungal endophytes of the genus *Epichloë* can be found in many cool season grasses (Schardl, 2010). *Lolium perenne* is one such grass that commonly forms an association with *Epichloë festucae* var. *lolii* (Leuchtmann *et al*., 2014). This symbiotic grass-fungal endophyte relationship is considered mutualistic in nature: the endophyte benefits from the nutrients and the protection which the host provides, while the grass receives several fitness benefits such as improved growth in terms of biomass production, drought tolerance, nutrient acquisition, and deterrence or toxicity to herbivorous pests (Saikkonen *et al*., 2004; Schardl *et al*., 2004; Gond *et al*., 2010). Due to the nature of the plant-fungal endophyte relationship their metabolomes are inextricably linked, therefore it is only possible to consider the plant-fungal metabolome as a whole. Many changes in the plant-endophyte metabolome may contribute to the fitness of the grass host (Cao *et al*., 2008; Rasmussen *et al*., 2008b; Dupont *et al*., 2015). Both transcriptomic and metabolomic analyses have demonstrated that *L. perenne* infection by *E. festucae* results in significant changes in primary

and secondary metabolism, as well as stress-related gene expression (Dupont *et al*. 2015). These changes in the host plant's chemistry have ecological consequences both above and belowground.

Metabolomics is an emerging technique in the toolbox of plant ecologists, several of whom have begun to utilize it for analyses of shoot tissue, guttation fluid, apoplastic fluid (Rasmussen *et al*., 2007, 2008, 2009; Cao *et al*., 2008; Cao *et al*., 2012; Dupont *et al*., 2015), and to a lesser extent root tissue and root exudate of infected hosts (Guo *et al*., 2015, 2016; Wakelin *et al*., 2015). These metabolomic techniques have not only led to the discovery of new metabolites, such as alkaloids, extracellular siderophores, and cyclic oligopeptides, they have also provided insight into changes in primary and secondary metabolism in the plant-fungal endophyte metabolome (Cao *et al*., 2008; Rasmussen *et al*., 2008a; Koulman *et al*., 2007b,2012).

Key changes observed in primary metabolism include increases in non-structural carbohydrates (water soluble carbohydrates (WSCs), sugar alcohols, and storage carbohydrates) (Cheplick and Cho 2003; Hunt *et al*., 2005; Rasmussen *et al*., 2007,2008; Ren *et al*., 2009; Dupont *et al*., 2015) and decreases in nitrogenous compounds (Hunt *et al*., 2005; Rasmussen *et al*., 2008; Ren *et al*., 2009). In perennial ryegrass (cv. Samson), the leaf and pseudostems of endophyte-infected plants have higher concentrations of fructans, a primary storage carbohydrate, and glucose, fructose, and sucrose, involved in carbohydrate metabolism, and a lower concentration of starch and soluble protein (Hunt *et al*. 2005). Non-structural carbohydrates and nitrogen are important to both plant and endophyte for growth, development, and signaling (Prud'homme *et al*., 1992; Rasmussen *et al*., 2012; Abeynayaka *et al*., 2015; Hildebrandt *et al*., 2015). Changes in the C:N ratio in infected plants may be a result of a metabolic cost associated with hosting the endophyte, wherein the fungal endophyte has a higher nitrogen demand (Cheplick *et al*., 1989; Rasmussen *et al*., 2007; Ren *et al*., 2009). Utilizing the plant host's resources, these fungal

endophytes can synthesize various nitrogen rich bioactive secondary metabolites, such as alkaloids. The concentration of alkaloids present in plant tissue is linearly related to the concentration of fungal endophyte in plant tissue (di Menna *et al*., 1992; Ball *et al*., 1995a; Easton *et al*., 2002; Spiering *et al*., 2005; Rasmussen *et al*., 2008; Reed *et al*., 2011b). The most studied alkaloids in grass-fungal endophyte associations include pyrrolopyrazines (peramine) (Gaynor *et al*., 1983), indole diterpenes (lolitrem B) (Fletcher and Harvey, 1981), and lysergyls (ergovaline) (Rowan and Shaw 1987), all produced by the 'common toxic' (E+) strain of the endophyte. Natural variants of *Epichloë* fungi exist which differ in their alkaloid profiles (Christensen *et al*., 1991). These variants are often referred to as 'novel' or 'selected' endophytes — terms used in the grass breeding industry. The novel endophyte strain AR37 produces a unique group of compounds called epoxy-janthitrems (Tapper and Lane 2004). These fungal-derived alkaloids play a major role in plant host resistance to invertebrate herbivores (Prestidge *et al*., 1982; Saikkonen *et al*., 2010). Many other secondary metabolites that play a role in plant host resistance are phenolic and volatile organic compounds that can change in composition and quality in fungal endophyte-infected host tissue (Qawasmeh *et al*., 2012a,b; Pańka *et al*., 2014; Wiewióra *et al*., 2015).

The majority of research investigating the interactions, influences, and consequences of plant-fungal endophyte relationships has focused on aboveground effects, while research investigating the belowground impacts of these relationships is still limited. Belowground ecosystem processes, such as decomposition and nutrient cycling, are made possible by the interaction of plants and soil organisms. These processes are essential to the transfer of resources throughout the foodweb. The bulk of these interactions occur in the rhizosphere, the interface of the root surface and the soil, and are heavily influenced by the quality and quantity of plant outputs via the roots (i.e. root exudation). It is therefore important to improve our understanding of the belowground component

of plant-fungal endophyte relationships so that we may determine their role in grassland ecosystems.

The majority of plant-fungal endophyte metabolome research has focussed on aboveground tissues, with less being known about root and root exudate chemistry. Those studies that investigate root and root exudate chemistry have predominantly focused on a similar grass-fungal endophyte association between *Schedonorus arundinaceus* (tall fescue) and *Epichloë coenophiala*. As in aboveground tissues, fungal endophytes can induce significant changes in root exudate composition and quantity (Van Hecke *et al*., 2005; Guo *et al*., 2015). There are increases in soluble organic carbon and carbohydrates (Van Hecke *et al*., 2005). Guo *et al*. (2015) found that E+ infected plants had significantly more total carbon and phenolic compounds in their root exudates than plants infected with novel endophytes, which were also significantly different from each other. They also found that amines, growth factors and vitamins were significantly affected by endophyte status. In *L. perenne*, Ren *et al*. (2009) found that soluble sugars increased in the roots of endophyte-infected plants, but this was only observed at high nitrogen levels. Wakelin *et al*. (2015) extracted metabolites from the rhizosphere of *L. perenne* plants infected with selected strains of *E. festucae* var. *lolii* and found that metabolite profiles between E+ and E- plants differed most in the alkane hydrocarbon derivatives (i.e. lipids).

There is very limited information available on root exudate composition of *L. perenne* infected with different strains of *E. festucae* var. *lolii*. Therefore, the main purpose of this research was to improve our understanding of specific compounds that constitute the root exudates in perennial ryegrass with and without endophyte infection as well as how different strains of the endophyte alter root exudate composition. A hydroponic growth system, while limited in ecological relevance, was chosen to facilitate detection and quantification of the changes between the plant

and plant-fungal endophyte metabolome. Plant metabolites are diverse and complex; no one analytical method will capture the whole plant-fungal metabolome. In this study, I conducted untargeted metabolomics using Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC-MS to detect polar compounds. I hypothesized that endophyte presence, and endophyte concentration would have significant strain specific effects on plant growth, and root exudate composition.

Methods

To isolate the effects of endophyte infection on perennial ryegrass plant metabolites in root exudates, I designed a hydroponic experiment to collect root exudate for identification of individual metabolites and to capture its metabolic profile. The concentration of endophyte infection was also estimated in order to determine if it was a contributing factor in the metabolic profile. Additional measurements were taken to determine the effect of endophytes on plant growth.

Experiment design

The experiment was performed in a glasshouse from January to April 2016. The temperature was maintained at ~23°C and supplementary lighting was provided on a 16:8 hour light:dark cycle. The hydroponic system comprised 90 pots set up as a randomized block design (9 blocks x 5 endophyte treatments with 2 replicates of each endophyte treatment per block).

Sample preparation

Lolium perenne seeds *(*cultivar Alto), either un-infected (E-) or infected with one of four endophyte strains (AR1, AR37, NEA2, E+), were provided by Courtney Inch (Agriseeds Ltd, New Zealand) and stored at -20˚C. Endophyte status of seeds was previously confirmed using an immunoblot assay (Phytoscreen seed endophyte detection kit; Agrinostics Ltd. Co., Watkinsville, GA, USA). It was assumed that the genetic variation among seeds was randomly distributed

among the experimental groups; therefore, genotypic homogeneity was not considered. Individual seeds were sown into Rockwool and grown for nine weeks in the glasshouse (starting January 25, 2016) to allow for vegetative propagation and establishment of roots in the absence of soil. Plants in Rockwool were then transferred to the hydroponic system by inserting each into the centre hole of a 4.5 cm diameter disk made of Styrofoam. These disks were then floated in a 1 L silver clay pot (ceramic, inert), filled to the top with fertilizer water (Plant Products®, 20-8-20 All Purpose High Nitrate, 1.25 g per litre, 250 parts nitrogen, pH adjusted to 6.0). Fertilizer water was replenished every other day. Pots were aerated through a plastic tube that pumped in a steady flow. Plants were allowed to acclimate and grow for an additional four weeks (starting March 23, 2016). This allowed for further root development in order to produce a sufficient amount of root exudate, and allowed for the growth of the endophyte. At the end of this growth period the fertilizer water in the pots was discarded and replaced with deionized water in order to sample root exudate.

Sample collection

After 24 hours in deionized water, all plants and root exudate were harvested (April 20, 2016). Two individual tillers (consisting of stem, sheath, and blade tissue), and a sample of the root tissue from each plant were removed and preserved in liquid nitrogen. The tiller and root tissue were then freeze-dried, and stored at -20˚C for further molecular analyses. From one tiller, the stem and sheath tissue were separated from the blade for use in qPCR. All freeze-dried samples of plant tissue (tiller and root) were weighed and then ground using a 2010 Geno/Grinder® (SPEX® SamplePrep, USA) tissue homogenizer. Ground plant tissue was stored at -20˚C for immediate use, and at -80˚C for future metabolomic analyses.

All remaining plant tissue (roots and shoots) was harvested by cutting the shoot and root portions as closely as possible to the Rockwool medium. The tissue was placed in a drying oven at 65˚C for at least three days, and then weighed to determine overall plant biomass. The weights of the samples of plant tissue were included in the overall plant biomass calculation. Lastly, 50 mL of the deionized water from the hydroponic pots was collected and placed in falcon tubes (Fisherbrand), vacuum filtered through nylon 0.45 μm filters (Merck Millipore Ltd., Ireland), and stored in 10 mL Falcon tubes at -80˚C.

Endophyte concentration

To estimate endophyte concentration in plant tissue, I utilized quantitative polymerase chain reaction (qPCR), following methods as described in Ryan *et al*. (2014, 2015). Briefly, genomic DNA (gDNA) was extracted from 20 mg of ground stem and sheath tissue, using DNeasy Plant Mini Kit (Qiagen Inc., Toronto, Canada) in conjunction with the QIAcube® (Qiagen Inc., Toronto, Canada), for automated sample prep, as per manufacturer's instructions. For each sample, total gDNA (plant and fungal) was measured by placing 2 μL of sample on a NanoDrop® 2000, run in triplicate. The gDNA was then diluted to a working concentration of 0.5 ng total gDNA/μL using Millipore water. PCR plates were set up with each well receiving 9 μL PCR mix (forward primer and reverse primer (0.75 μL each, 0.5 μmol concentration), LightCycler® 480 SYBR Green I Master (7.5 uL, 2x concentration)), and 6 μL gDNA (0.5 ng/μl concentration). Sample wells were tested in triplicate. Both dilutions and plating were carried out by an automated PCR set-up instrument, QIAgility® (Qiagen Inc., Toronto, Canada). PCR reactions were performed on a LightCycler® 480 Instrument II (Roche, Canada). The PCR thermocycling conditions were as follows: initial denaturation for one cycle at 95 °C for 5 min, followed by amplification for 45 cycles of 95 °C for 10 s, 64 °C for 10 s, and 72 °C for 10 s. The

formation of a single PCR product in the PCR reactions was confirmed by melting analysis where the PCR products were raised to 95°C for 5 s, and then lowered to 65 °C and raised back to 97 °C over 1 min with continuous fluorescence data acquisition. The T_m of the PCR product was 83.75 ± 0.25. One alteration to the methodology was needed when samples were extrapolated from the curve. In this case, the sample was re-run using 9 ng of gDNA instead of 3 ng. If endophyte infection was still below the detection limit, the sample was considered to have no endophyte infection.

Metabolomic profile of root exudate

Frozen root exudate samples were thawed, and 200 µl of each were transferred into 350 µL glass vials (Thermo Scientific™ National MS Certified, MSCERT5000-37LVW). No solvent extraction step or concentration step was conducted.

To determine the root exudate composition, liquid chromatography–mass spectrometry (LC-MS) analyses were performed on an Agilent 1200 HPLC liquid chromatograph interfaced with an Agilent UHD 6530 Q-Tof mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. A C18 column (Agilent Poroshell 120, EC-C18 50 mm x 3.0 mm 2.7 µm) was used for chromatographic separation with the following solvents: water with 0.1% formic acid (A) and acetonitrile with 0.1 formic acid (B). The mobile phase gradient was as follows: initial conditions were 10% B hold for 1 min then increasing to 100% B in 29 min followed by column wash at 100% B for 5 min and 20 min re-equilibration. The flow rate was maintained at 0.4 mL/min. The mass spectrometer electrospray capillary voltage was maintained at 4.0 kV and the drying gas temperature at 250° C with a flow rate of 8 L/min. Nebulizer pressure was 30 psi and the fragmentor was set to 160. Nitrogen was used as both nebulizing and drying gas. The mass-to-charge ratio was scanned across the m/z range of 50-1500

m/z in 4GHz (extended dynamic range) positive and negative ion modes. The acquisition rate was set at 2 spectra/s. The instrument was externally calibrated with the ESI TuneMix (Agilent). The sample injection volume was 10 µL.

Metabolomic Data Analysis

The mass spectrometry data were further processed using Agilent Mass Hunter Work-station software (MassHunter Profinder B.08.00). Recursive molecular feature extraction (rMFE) was used for binning and alignment of molecular features. The rMFE is an algorithm which groups related co-eluting ions (i.e. isotopes, adducts, and dimers) into a single compound, and then creates compound chromatograms. The rMFE step also filters out noise and reduces false positives. Molecular features were aligned based on a retention time window of 0.40 min and a mass window of 40.00 ppm + 2.00 mDa, and an absolute height of at least 3000 counts. Aligned features that were found in at least six replicates in one treatment group (n = 18) were retained. Molecular features were extracted as compound exchange format (cef) files and imported into Agilent's Mass Profiler Professional (MPP) software version B14.5. We used Mass Profiler Professional (MPP) for statistics visualization, and annotation and identification of compounds.

In MPP, compounds that were statistically significant and/or unique to certain treatments were noted. To aid in improving compound identification, a subset of samples that had the highest intensities of statistically significant compounds were re-run using MS/MS. Compounds were searched against an in-lab annotated METLIN Personal Metabolite Database (Agilent Technologies) and against KNApSAck: Species-Metabolite Relationship Database (Nakamura *et al*., 2014).

Statistics

The following statistical analyses were performed in R 3.3.1 (R Core Team 2016). Potential differences in endophyte concentrations based on endophyte strain were tested with one-way analysis of variance (ANOVA). Only endophyte infected samples were included for this test. One-way analysis of covariance (ANCOVA's) were conducted to determine a statistically significant difference between endophyte treatments on total, root, and shoot biomass controlling for endophyte concentration. One sample in the NEA2 treatment group had an extreme outlier. I ran the model with and without the outlier and found that the results were not qualitatively different. The results presented below include the outlier. To determine the effects of endophyte concentration on individual metabolites, permutational multivariate analysis of variation (PERMANOVA) were performed using the 'vegan package.'

Metabolite data, collected in positive and negative ion mode, were subjected to statistical and visual differential analysis in MPP separately. ANOVA was conducted to determine metabolites showing statistical differences across endophyte treatments. The Benjamini Hochberg correction (Benjamini and Hochberg, 1995) was applied to control for the number of false positives, resulting from multiple testing of p-values ($p < 0.05$). An additional fold change filter ≥ 2.0 was applied to statistically significant metabolites. Visualisation of data included principal component analysis (PCA) and hierarchical cluster analysis (HCA). Hierarchical clustering was used to group significant compounds (as determined by an ANOVA and fold change) in clusters by metabolite and by endophyte treatment using a Euclidean distance metric (Taylor *et al*., 2002; Anderson 2006) and Ward's Linkage rule (Ward 1963).

RESULTS

Endophyte Infection

The endophyte strains differed in their concentrations in plant sheath tissue ($F_{3,63}$ = 4.3715, p = 0.007). Differences were evident between NEA2 and E+, and NEA2 and AR37, with the E+ and AR37 endophyte concentrations being significantly higher than NEA2 (Tukey multiple comparisons test, $p < 0.05$) (Fig. 2.1.). The average concentration of endophyte differed between endophyte strains with E+ having the highest number of gene copies ng^{-1} gDNA and NEA2 the lowest (Table 2.1.). An ANCOVA failed to reject the null hypothesis that endophyte concentration had no significant effect on plant growth, however there was a significant endophyte strain x endophyte concentration interaction for NEA2 shoot biomass (Table 2.2.). There was also a non-significant upward trend of increasing total biomass with increasing endophyte concentration for E+, AR1, and AR37. Whereas, NEA2 total biomass decreased as endophyte concentration increased (data not shown).

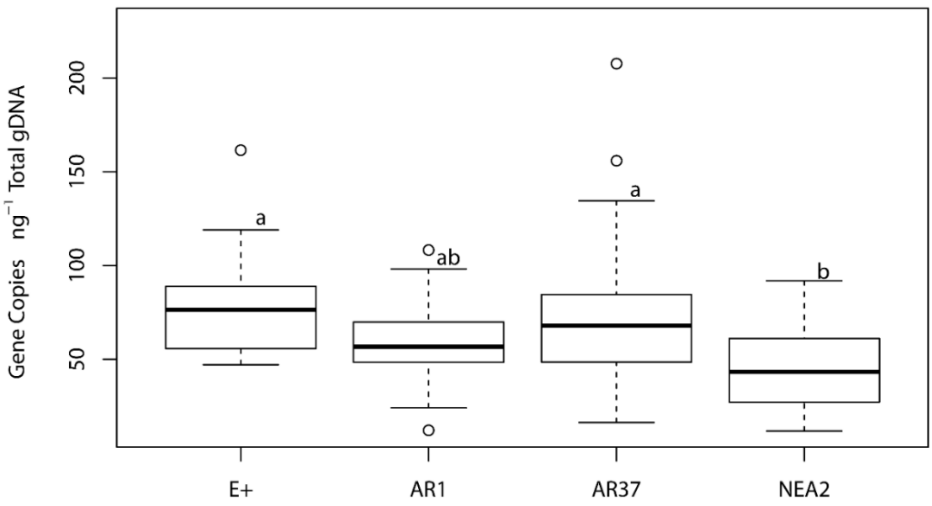

Figure 2. 1. Fungal endophyte concentrations for *Lolium perenne* plants infected with different strains of *Epichloë festucae* var. *lolii* endophyte. Sample sizes differed between the treatments

(E+, n = 18; AR1, n = 17; AR37, n = 17; NEA2, n = 15). Means followed by the same letter do not differ significantly (Tukey test, $p > 0.05$). See Table 1 for treatment means.

Table 2. 1. The effects of *Epichloë festucae* var. *lolii* on *Lolium perenne* biomass. Means ± SD are shown. See treatment medians for Gene copies (Fig 2.1) and Total Biomass (Fig 2.2).

Endophyte	Shoot Biomass (g)	Root Biomass (g)	Total Biomass (g)	Root:Shoot	Gene copies (ng^{-1} total genomic DNA)
E-	5.60 ± 3.04	1.16 ± 0.42	6.76 ± 3.23	0.25 ± 0.09	3.44 ± 2.11*
E+	6.71 ± 2.45	1.40 ± 0.47	8.11 ± 2.70	0.23 ± 0.08	79.11 ± 29.02
AR1	5.58 ± 3.45	1.25 ± 0.61	6.84 ± 3.96	0.25 ± 0.08	58.99 ± 25.36
AR37	8.06 ± 3.27	1.53 ± 0.53	9.59 ± 3.56	0.22 ± 0.09	78.59 ± 48.78
NEA2	9.59 ± 3.07	2.22 ± 1.24	11.81 ± 2.72	0.42 ± 0.93	46.42 ± 23.86

*Sample size differed for Gene copies ng^{-1} total genomic DNA for each treatment (AR1 (n=17); AR37 (n=17); NEA2 (n=15); E+ (n = 18); and E- (n=3). E- samples are only a small subset as it is assumed that there is little to no colonization by the fungal endophyte.

Table 2. 2. ANCOVA summary of *Epichloë festucae* var. *lolii* effects on growth of *Lolium perenne*.

Source	Total DW	Root DW	Shoot DW	Root:Shoot DW
Endophyte	*** $F_{4,80} = 7.8$	* $F_{4,80} = 3.2$	*** $F_{4,80} = 6.4$	NS
Gene Copies	NS	NS	NS	NS
Endophyte:GeneCopies	NS	NS	* $F_{4,80} = 2.5$	NS

Significant at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); not significant (NS); dry weight (DW)

Biomass

We measured shoot and root plant dry mass at harvest. Overall, total biomass was greatest for NEA2 followed by AR37, E+, AR1, and then E- (Table 2.1). A one-way ANCOVA was conducted to compare the effect of endophyte on plant growth while controlling for endophyte concentration (Table 2.2). There was a statistically significant effect of endophyte strain on shoot biomass ($F_{4,80} = 6.4143$, $p < 0.0002$; Fig. 2.2). This was a result of the NEA2

endophyte infected plants having more biomass than E- ($p < 0.001$), and AR1 ($p < 0.001$) infected plants, but there was no significant difference between NEA2 and E+ ($p = 0.0508$) or NEA2 and AR37 ($p = 0.1877$) infected plants shoot biomass. NEA2 infected plants had the highest amount of shoot biomass on average 9.59 g ± 3.07 (SD), followed by AR37 8.06 g ± 3.27 (SD) (Table 2.1). There was a statistically significant effect of endophyte on root biomass ($F_{4,80} = 3.2077$, $p = 0.017$). This result was due to the NEA2 endophyte infected plants having significantly more root biomass than E- ($p = 0.0287$), and AR1 ($p = 0.0155$). The root:shoot ratio was not significantly effected by endophyte treatment ($F_{4,80} = 0.0794$, $p = 0.98841$).

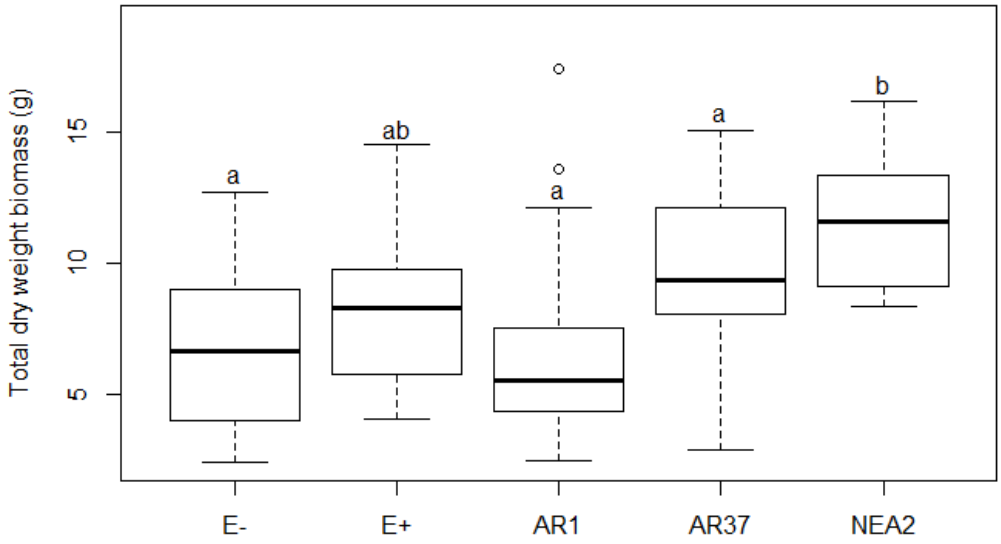

Figure 2. 2. Total dry weight biomass (shoots and roots) of *Lolium perenne* either uninfected (E-), or infected with different strains of *Epichloë festucae* var. *lolii* endophyte (common toxic strain, E+; and novel strains AR1, AR37, and NEA2). Each treatment n = 18. See Table 2.1. for treatment means.

Metabolites in Root Exudate

To determine changes in the plant-fungal metabolome with different endophyte strains, an accurate-mass Q-TOF LC/MS-based analysis of metabolites in root exudates was performed.

The goal was to obtain a general overview of metabolomic similarities and differences in plant root exudates infected with a common toxic strain (E+) or one of three novel strains (AR1, AR37, NEA2) of the fungal endophyte *E. festucae* var. *lolii* compared to uninfected hosts.

Feature extraction from raw data found 62 features from the positive ion data, and 115 features from the negative ion data.

Positive ion mode

An ANOVA showed that 41 of the 62 entities were significantly different at a corrected p-value (Benjamini Hochberg FDR) cut-off of 0.05. A subsequent Tukey HSD showed where the differences lay (Table 2.3). A fold change filter was applied to the 41 compounds with significant differences. Table 2.4 lists the 23 metabolites found to be differentially-expressed in at least one of the endophyte treatments, with $p<0.05$ and fold-change ≥ 2.0. Perhaps not surprisingly, the main differences were found in the up and downregulation of the compounds, rather than the presence or absence of compounds. Overall, 12.5% of all metabolites were significantly upregulated in all endophyte treatments when compared to E- treatments. There were no metabolites that were significantly downregulated in all endophyte treatments.

Table 2. 3. Number of metabolites detected in positive ionization mode that are differentially expressed (blue) or not differentialy expressed (green) between treatment groups based on a TukeyHSD Post Hoc test ($p < 0.05$).

Treatment	E-	E+	AR1	AR37	NEA2
E-	41	12	8	15	9
E+	29	41	20	19	26
AR1	33	21	41	5	10
AR37	26	22	36	41	4
NEA2	32	15	31	37	41

Table 2. 4. Differentially expressed root exudate metabolites detected in positive ionization mode from *Lolium perenne* plants infected with strains of *Epichloë festucae* var. *lolii* (E+, AR1, AR37, and NEA2; each treatment with n = 18). Fold-change data is based on the abundance difference between uninfected (E-) plants and individual treatment groups. Log_2 fold-change data that is $\geq \pm 1$ represents significantly downregulated (red) or upregulated (blue) metabolites relative to endophyte free plants. Values in black show either downregulated (negative value) or upregulated (positive value) metabolites that are not significantly different than endophyte free plants.

								Fold-Change (Log_2)			
Tentative Compound Name	**MF [M + H]$^+$**	**Mass**	**m/z**	**RT**	**Fragments**	**P(1)**	**p value**	**E+**	**AR1**	**AR37**	**NEA2**
Alkaloids											
N-Methyltyramine	C9 H13 N O	151.0984	152.0967	1.74	65.1095, 67.0532, 68.9968, 92.0562, 95.0479	0.043	9.13E-03	1.36	-1.26	1.68	0.52
Valeroidine	C13 H23 N O3	241.1657	242.1716	7.47	79.0523, 99.0434, 133.1000, 147.1141, 207.1337	0.181	6.56E-08	1.36	-2.49	-3.94	-2.07
(-)-N-(2-Oxopyrrolidinomethyl) cytisine	C16 H21 N3 O2	287.1607	288.1695	3.34	58.0649, 125.1066, 147.0436, 168.1123, 288.1684	-0.376	1.21E-13	7.60	8.06	6.93	-0.91
Benzanoids											
Eupatoriochromene	C13 H14 O3	218.0929	219.0988	7.15	91.0534, 117.0314, 121.0629, 145.0618, 187.0733	0.118	9.13E-03	3.87	1.63	5.24	5.24
Precocene II	C13 H16 O3	220.1085	221.1179	7.02	95.0503, 162.8361, 177.1276	0.128	1.62E-03	6.19	1.58	5.43	7.18
1-Methoxy-1-(2,4,5-trimethoxyphenyl)-2-propanol	C13 H20 O5	256.1311	257.1365	5.70	105.0683, 119.0838, 123.0781, 165.0887, 175.1106	0.146	2.98E-07	2.31	-0.93	-4.97	-4.87
Flavonoids											
Purpuritenin A	C19 H16 O3	292.1076	293.1167	4.01	219.1086, 234.1209, 245.1129, 293.1163	-0.424	4.62E-16	7.40	7.84	5.62	-4.28
Dextrorphan O-glucuronide	C23 H31 N O7	433.205	434.2121	5.06	124.1104, 288.1565, 434.2117	0.089	1.68E-03	-3.53	-2.39	0.92	2.23
Peptides											
Pro Arg	C11 H21 N5 O3	271.1669	272.1741	4.16	97.0751, 125.1066, 131.0482, 168.1123	-0.379	1.61E-11	5.95	6.85	5.53	-2.84
Ala Trp	C14 H17 N3 O3	275.1267	276.1330	9.27	57.0700, 149.0207, 159.1144, 171.1357, 177.1268	0.149	2.17E-13	1.77	-2.92	-2.74	-1.94
Thr Gly Thr	C10 H19 N3 O6	277.124		9.26		0.263	1.97E-08	1.79	-5.37	-4.79	-2.31
Pro Asn Cys	C12 H20 N4 O5 S	332.1135	333.1155	5.24	289.0938, 317.0889, 333.1201	0.052	4.84E-02	0.34	-1.38	0.46	-0.35

Tentative Compound Name	MF [M + H]$^+$	Mass	m/z	RT	Fragments	P(1)	p value	Fold-Change (Log_2) E+	AR1	AR37	NEA2
Peptides (Cont.)											
Leu Ala Arg	C15 H30 N6 O4	358.2297	359.2363	10.83	99.0435, 129.0881, 359.2350	0.019	1.80E-03	-2.40	0.24	-0.85	0.21
Gln Pro Gln	C15 H25 N5 O6	371.1752	372.1870	4.49	45.0339, 59.0498, 89.0607, 103.0395, 147.0663	0.088	3.79E-08	4.07	1.45	8.36	8.28
Terpenoids, Lipids & Lipid Derivatives											
Dehydrovomifoliol	C13 H18 O3	222.1232	223.1296	6.97	79.0533, 91.0534, 93.0692, 149.0943, 150.1018	0.185	1.55E-05	2.04	-1.97	-3.01	-1.34
Methyl Jasmonate	C13 H20 O3	224.1392	225.1462	7.40	79.0528, 91.0534, 105.0685, 119.0836, 133.0993	0.361	2.29E-06	2.06	-5.25	-5.38	-1.74
9Z,11E-Hexadecadienal	C16 H28 O	236.212	259.2001	4.21	55.0537, 59.0120, 83.0843, 100.1112, 115.0749	-0.039	1.97E-03	-1.37	0.93	1.16	1.49
N-linoleoyl taurine	C20 H37 N O4 S	387.2441	388.2516	4.54	45.0334, 57.0699, 89.0595, 133.0855, 149.0223	0.029	5.89E-04	0.48	0.37	1.48	1.74
a-Tocotrienol	C29 H44 O2	424.3398		5.36		-0.075	2.44E-02	0.41	3.27	2.52	3.35
beta-Citraurol	C30 H42 O2	434.3239	435.3262	5.47	57.0680, 109.1018, 118.0844, 151.0729, 176.1059, 207.0601, 211.1391, 298.0424, 421.1824	-0.069	4.84E-02	-0.33	2.36	1.74	2.55
Unknown											
	C7 H6 N2 O	134.0474	135.0546	1.17	53.0380, 56.9632, 80.0493, 107.0589, 135.0548	-0.220	7.50E-19	-0.09	-1.29	11.40	-1.56
	C15 H37 N4 O5	353.2758	354.2816	10.72	45.0329, 57.0692, 59.0483, 87.0431, 103.0738	0.013	4.08E-04	-2.28	0.17	-0.81	0.15
	C16 H35 N11	381.3048		10.82		0.004	2.29E-06	-5.46	0.17	-0.67	0.35

Molecular formula positive ion mode (MF [M + H]$^+$); retention time (RT); first principal component score (P(1)). Statistically significant (p-value < 0.05).

Principal component analysis (PCA) provides a three-dimensional visualization of the 23 entities recognized in 75% of samples from at least one endophyte treatment group. PCA findings did not show a full separation between treatment groups, however there is clustering by treatment groups with moderate overlap between groups (Fig. 2.3). Principal component 1 (PC1) explained 23.99% of the variation, PC2 explained 20.97%, and PC3 explained 15.58%.

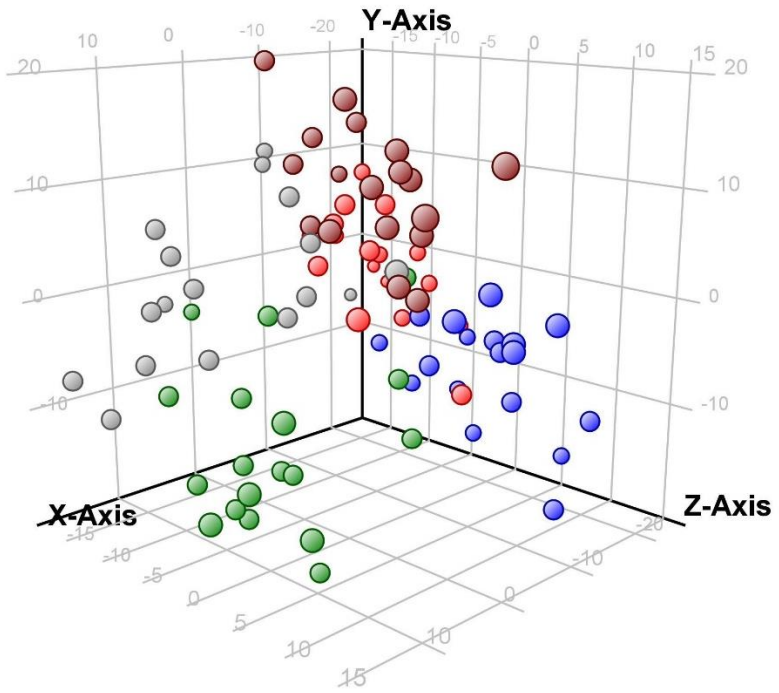

Figure 2. 3. Principal component analysis (PCA) performed on the positive ion mode metabolomic profiles of *Lolium perenne* root exudates infected with strains of *Epichloë festucae* var. *lolii* endophyte (E- (grey), E+ (maroon), AR1 (red), AR37 (blue), and NEA2 (green)). This PCA is based on 23 metabolites found to be differentially-expressed in at least one of the endophyte treatments, with $p<0.05$ and fold-change >2.0. Each treatment n = 18.

Hierarchical clustering of the data showed that endophyte treatments are separating into two groups with E-, and NEA2 forming one group and E+, AR1, and AR37 forming the other group (Fig. 2.4). Within the second grouping, AR1 and AR37 are more similar to each other than to E+. The majority of compounds are similar between endophyte treatment groups, however there are trends worth noting. Within E- and NEA2 there are three compounds (Purpuritenin A, m/z 293.1167 RT 4.0071 (Fig. 2.5A); Pro Arg, m/z 272.1741 RT 4.1623 (Fig. 2.5B); and (-)-N-(2-Oxopyrrolidinomethyl)cytisine, m/z 288.1695 RT 3.3391 (Fig. 2.5C)) at low intensity clustering together,

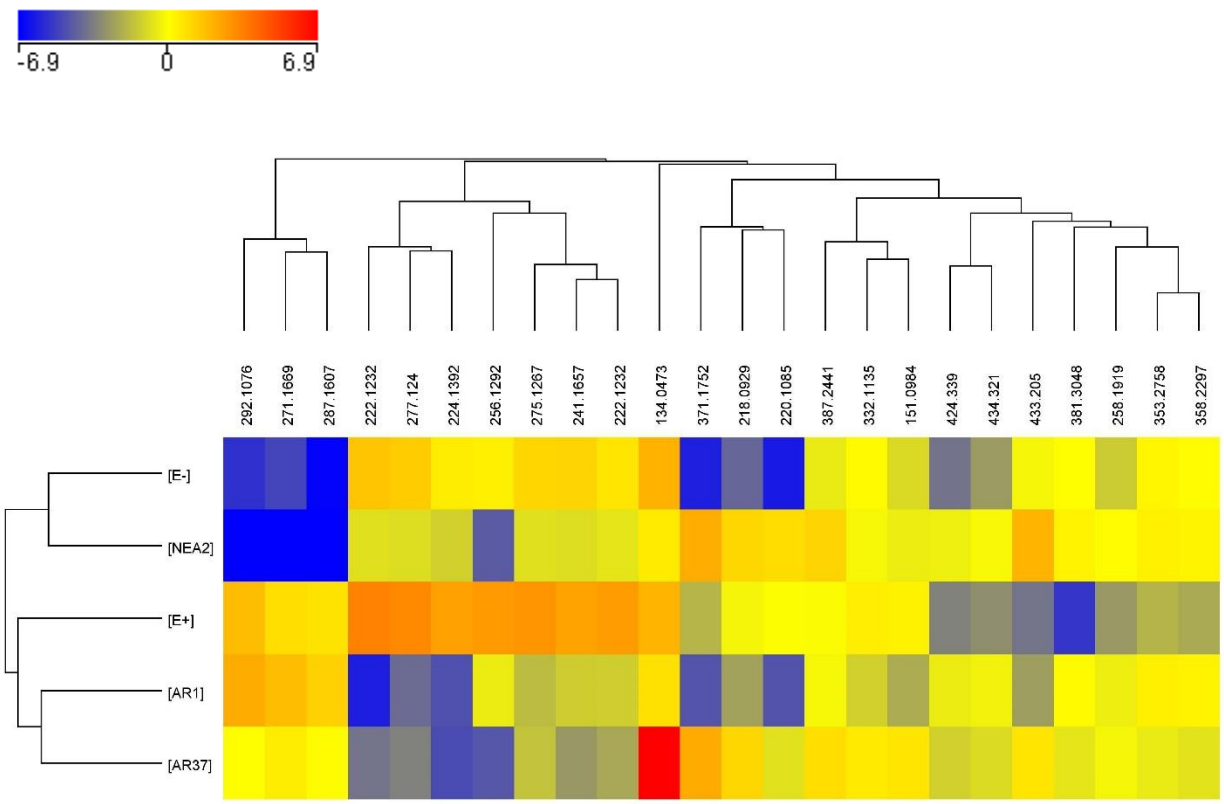

Figure 2. 4. Unsupervised hierarchical clustering of *Lolium perenne* root exudate metabolites, obtained by LC-MS in the positive ion mode. These are the 23 metabolites found to be differentially-expressed in at least one of the endophyte treatments, with $p<0.05$ and fold-change >2.0. Metabolites are grouped by fungal endophyte treatments based on similar peak intensity profiles. Colours represent normalized intensity values (yellow is the centre, blue represents high intensity, red represents low intensity). Each treatment n = 18.

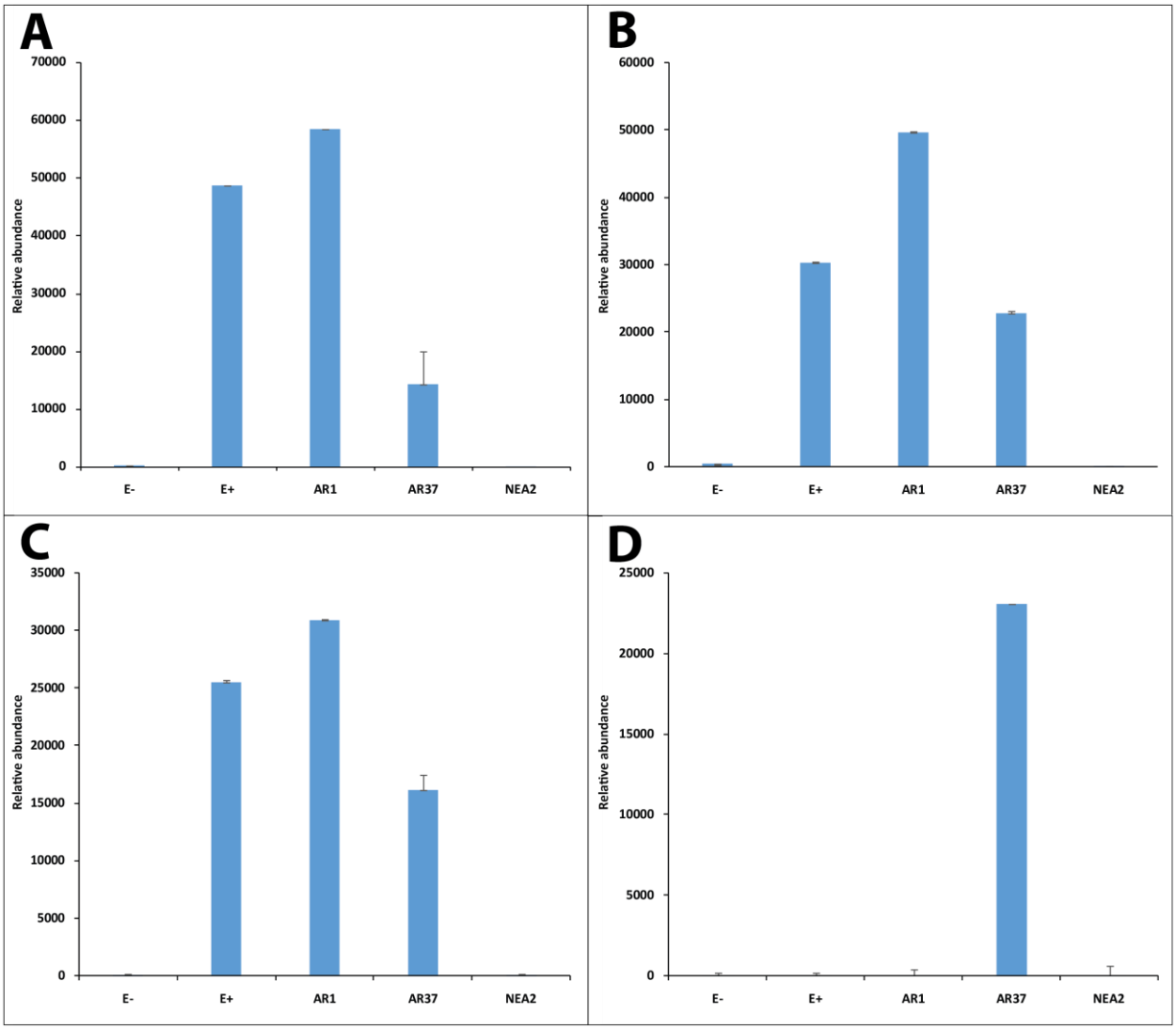

Figure 2. 5. *Epichloë festucae* var. *lolii* endophyte infected *Lolium perenne* root exudate metabolites captured in positive ion mode. Bar graphs showing significant variation in the relative intensity of metabolites: A) m/z 293.1167 RT 4.00; B) m/z 272.1741 RT 4.16; C) m/z 288.1695 RT 3.34; and D) m/z 135.0546 RT 1.17.

while in the other three endophyte treatments these compounds had higher intensities. AR37 had one compound (C7H6N2O, m/z 135.0546 RT 1.1744 (Fig. 2.5D)) with a much higher intensity, than in other endophyte and non-endophyte treatment groups.

Negative ion mode

An ANOVA found that 102 of 115 compounds were significantly different at a corrected p-value (Benjamini Hochberg FDR) cut-off of 0.05. A subsequent Tukey HSD showed where the differences lay (Table 2.5). A fold change was applied to the 102 compounds with significant differences. There were 60 entities that passed a fold change cut-off of ≥ 2.0 (Table 2.6). Overall, 60% of metablites were significantly upregulated in E+, 49% in NEA2, 46% in AR37, and 26% in AR1 relative to E- plants. Interestingly, 38.5% of metabolites were upregulated in E+ while being downregulated in the novel endophytes. There were no metabolites that were significantly upregulated concurrently across all endophyte treatments relative to E- treatments.

Table 2. 5. Number of metabolites detected in negative ionization mode that are differentially expressed (blue) or not differentialy expressed (green) between treatment groups based on a TukeyHSD Post Hoc test ($p < 0.05$).

Treatment	E-	E+	AR1	AR37	NEA2
E-	102	41	27	35	34
E+	61	102	78	73	76
AR1	75	24	102	8	20
AR37	67	29	94	102	5
NEA2	68	26	82	97	102

Table 2. 6. Differentially expressed root exudate metabolites detected in negative ionization mode from *Lolium perenne* plants infected with strains of *Epichloë festucae* var. *lolii* (E+, AR1, AR37, and NEA2; each treatment with n = 18). Fold-change data is based on the abundance difference between uninfected (E-) plants and individual treatment groups. Log_2 fold-change data that is $\geq \pm 1$ represents significantly downregulated (red) or upregulated (blue) metabolites relative to endophyte free plants. Values in black show either downregulated (negative value) or upregulated (positive value) metabolites that are not significantly different than endophyte free plants.

Tentative Compound Name	MF [M - H]$^-$	Mass	m/z	RT	Fragments	P(1)	p value	Fold-Change (Log_2) E+	AR1	AR37	NEA2
Alkaloids											
(1xi,3S)-1,2,3,4-Tetrahydro-1-methyl-beta-carboline-1,3-dicarboxylic acid	C14 H14 N2 O4	274.0932	273.0923	7.52	79.9571, 120.9018, 239.0403, 275.0176	0.185	1.46E-07	2.50	-4.28	-3.74	-1.61
Malasseziazole A	C20 H12 N2 O3	328.0847		8.54		0.190	5.53E-11	1.33	-5.75	-5.87	-4.26
Amino Acids, Peptides & Derivatives											
N2-Succinyl-L-ornithine	C9 H16 N2 O5	232.1037		5.72		0.193	5.91E-08	2.24	-2.78	-5.46	-2.42
Gly-Ser-OH	C10 H10 N2 O7	270.0511	269.1059	5.25	55.0200, 135.0830, 163.1151, 181.1243, 208.1078	0.179	4.01E-10	1.46	-5.54	-4.04	-2.60
Asp-His	C10 H14 N4 O5	270.0982		7.09		0.189	8.04E-10	1.29	-5.70	-4.67	-2.81
N-Succinyl-L-2,6-diaminopimelate	C11 H18 N2 O7	290.1091		7.00		0.194	1.10E-08	1.16	-4.75	-5.71	-3.03
(2S,4S)-Monatin	C14 H16 N2 O5	292.1065	291.1030	4.00	55.0297, 231.0995, 257.1145, 291.1018	0.004	6.63E-15	7.23	7.98	5.57	-1.22
Glu-Leu-Ser	C14 H25 N3 O7	347.1699	346.1657	8.55	61.9881, 73.8238, 959.0072, 1097.0344	0.013	5.18E-05	2.94	0.15	4.08	7.17
Gln-Thr-Asp	C13 H22 N4 O8	362.1455	361.1418	4.21	90.7250, 160.7155, 174.0564, 269.1017	0.008	2.03E-05	-0.34	1.72	0.50	-2.33
Z-Gly-Pro-Leu-Gly-Pro	C28 H39 N5 O8	573.2839		5.36		-0.074	1.70E-02	-0.90	1.32	1.44	1.11
Glabrin C	C41 H64 N8 O9	812.4801		5.99		-0.043	4.50E-03	-1.46	0.50	0.16	-0.04
Benzanoids											
Ginkgotoxin	C9 H13 N O3	183.0886	182.0839	5.06	61.9898, 112.0311, 128.2595	0.019	2.69E-02	1.60	0.64	0.84	1.23
Hordatine A	C28 H38 N8 O4	550.3005	549.2983	5.39	78.9593, 96.9619, 98.9566, 549.3004	-0.064	4.15E-03	-0.38	1.18	1.34	1.41
Thonningianin B	C35 H30 O17	722.1509		3.92		-0.087	1.98E-07	2.69	0.25	6.54	7.71

Tentative Compound Name	MF [M - H]$^-$	Mass	m/z	RT	Fragments	P(1)	p value	Fold-Change (Log_2) E+	AR1	AR37	NEA2
Carbohydrates & Derivatives											
Cycasin	C8 H16 N2 O7	252.0971	251.0942	5.86	57.0348, 105.0353, 190.0653, 221.0824	-0.005	1.20E-02	2.13	0.11	3.34	3.19
(Z)-3-Hexenyl beta-D-glucopyranoside	C12 H22 O6	262.1412	261.1404	3.64	197.9015, 351.5887	-0.064	3.65E-03	-0.76	0.81	1.03	1.23
5'-Oxoinosine	C10 H10 N4 O5	266.0647		9.26		0.092	3.98E-15	1.25	-1.09	-2.65	-2.44
N-Glycosyl-L-asparagine	C10 H18 N2 O8	294.1074	293.1051	6.77	106.0435, 165.0555, 172.0913, 221.0823, 231.1034	0.075	2.67E-05	1.41	-1.97	-0.64	0.40
Flavonoids											
Pongachalcone II	C21 H20 O5	352.133	351.1321	3.59	57.0366, 59.0154, 99.0455, 101.0254, 194.5610	-0.044	2.73E-02	1.16	0.16	3.66	4.95
Denticulaflavonol	C35 H42 O6	558.2953		5.36		-0.087	3.59E-02	0.38	2.14	1.86	2.51
Lipids & lipid like											
2-O-(beta-D-galactopyranosyl-(1->6)-beta-D-galactopyranosyl) 2S-hydroxytridecanoic acid	C25 H46 O13	554.2965		5.36		-0.070	1.58E-02	-0.82	0.93	1.33	1.49
Cyclopassifloside VII	C37 H62 O13	714.4162		6.00		-0.045	4.51E-03	0.55	1.77	1.50	1.60
PI(P-16:0/20:5(5Z,8Z,11Z,14Z,17Z))	C45 H77 O12 P	840.5097		5.99		-0.039	4.49E-03	0.75	0.79	2.12	2.28
Secondary Metabolites: bacteria, fungi, and other organisms											
Decarbamoylsaxitoxin	C9 H16 N6 O3	256.1277	255.1250	5.75	125.0986, 255.1237, 281.2153	0.224	9.81E-09	1.39	-4.23	-7.36	-2.70
5-amino-1-[3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl)imidazole-4-carboxamide	C9 H14 N4 O5	258.0982	257.0962	8.49	145.3180, 178.1321, 221.1178	0.118	9.81E-09	2.46	-6.03	-6.49	-5.03
	C12 H12 N5 O2	258.0992		9.25		0.118	2.14E-15	1.46	-2.50	-2.76	-2.24
Decarbamoylneosaxitoxin	C9 H16 N6 O4	272.1221	271.1209	5.10	165.1305, 207.1042, 209.1204, 227.1302, 271.1204	0.175	9.44E-07	1.79	-4.32	-3.81	-3.20
Pinolidoxin	C18 H26 O6	338.1705	337.1666	7.89	106.0433, 150.0336, 165.0558, 337.1660, 338.1663	0.022	2.22E-03	2.69	0.63	2.57	4.07

Tentative Compound Name	MF [M - H]⁻	Mass	m/z	RT	Fragments	P(1)	p value	Fold-Change (Log_2) E+	AR1	AR37	NEA2
Terpenoids											
Pulchellamine C	C24 H33 N O9	479.2113	478.2061	5.10	44.9980, 115.0385, 286.1415, 315.4149, 401.1563	-0.067	1.32E-03	-4.26	-3.06	-0.07	2.51
Gossypol	C30 H30 O8	518.1968		9.26		0.240	7.05E-15	6.50	-3.40	-3.60	-5.34
Taxinine M	C35 H44 O14	688.2765	687.2796	5.27	281.0919, 581.2150, 687.2786, 688.2822	-0.005	3.79E-05	-5.95	-2.52	-0.91	-4.44
Hovenidulcioside B1	C44 H70 O16	854.4642		5.99		-0.074	2.22E-03	-1.76	1.29	1.05	0.89
UnKnown											
	C7 H6 N6 O S	222.0325	221.0305	5.75	79.9590, 157.0675, 221.0303	0.030	1.89E-04	1.99	-1.23	3.33	3.31
	C9 H14 N6 O	222.1225	221.1179	7.02	95.05037, 162.83616, 177.12765, 284.07957	0.158	2.97E-07	1.32	-3.01	-4.16	-2.31
	C9 H16 N6 O	224.1381	223.1362	7.01	61.9898, 178.1371, 208.9768, 223.1332	0.105	2.14E-15	1.24	-2.53	-2.38	-2.50
	C8 H18 N8	226.1655	225.1462	7.40	79.0528, 91.0534, 105.0685, 119.0836, 133.0993	-0.100	1.45E-02	0.50	2.44	2.23	2.94
	C9 H12 N6 O2	236.1025	235.0976	7.15	105.03408, 158.07458, 177.05570, 235.09480	0.046	1.39E-03	2.73	-0.75	3.54	3.32
	C9 H16 N6 O2	240.1329	239.1303	6.24	57.0356, 62.9934, 107.0507, 123.0823, 239.1305	0.233	1.47E-03	2.74	-2.50	2.46	2.21
	C12 H10 N5 O2	256.0832	255.0256	7.05	79.9573, 83.26375, 129.09133, 143.05869	0.077	2.52E-14	1.45	0.44	-2.33	-2.37
		260.0921		9.25		0.120	4.39E-15	1.48	-2.55	-2.84	-2.22
		270.1065	269.1059	5.25	55.0200, 135.0830, 163.1151, 181.1243, 208.1078	0.228	8.04E-10	1.45	-3.63	-7.85	-4.29
	C11 H22 N5 O3	272.1726	271.1663	3.61	44.9984, 83.0498, 186.21285	-0.065	1.92E-03	-0.85	0.98	0.91	1.01
	C15 H22 O5	282.1427	281.1423	6.33	44.9993, 125.9432, 237.1522, 238.1544, 281.1406	0.175	1.12E-07	1.82	-3.75	-5.46	-3.17
		296.0722		9.25		0.177	1.30E-07	2.14	-3.69	-3.43	-3.20
	C12 H14 N8 O2	302.1238	301.1220	4.97	117.0584, 160.0413, 161.0501, 283.1124	0.077	3.59E-02	0.20	-1.30	1.17	1.74
		318.1008	317.1014	7.12	61.9893, 121.0290, 278.2160, 299.5229	0.189	7.25E-08	1.41	-5.30	-4.10	-3.45
	C11 H13 N5 O8	343.0757		9.25		0.177	2.84E-09	1.82	-4.62	-3.68	-2.55
	C15 H N O14	418.9405	417.9399	9.26	160.8439, 162.8406, 417.9403, 419.9374	0.164	9.81E-09	1.30	-3.70	-4.30	-2.76

Tentative Compound Name	MF [M - H]$^-$	Mass	m/z	RT	Fragments	P(1)	p value	Fold-Change (Log_2) E+	AR1	AR37	NEA2
Unknown (Cont.)											
	C12 H5 O15 S	420.9358	419.9326	8.57	189.9559, 333.1031, 346.0412	0.152	4.57E-09	1.70	-3.49	-3.88	-2.22
	C14 H N O15	422.933		9.25		0.170	7.74E-09	2.31	-4.30	-3.04	-3.88
	C21 H46 N3 O7	452.3336		5.36		-0.075	1.10E-02	-1.04	1.24	1.05	1.22
	C32 H36 N2 O3	496.2736		5.36		-0.071	7.49E-03	0.17	1.68	1.83	1.90
		537.3072		5.36		-0.059	1.01E-03	-0.67	1.01	0.90	1.05
	C18 H31 N20 O3	575.2889		5.36		-0.142	6.77E-03	-1.98	1.93	2.00	2.51
	C21 H37 N19 O4 S	651.3005		5.36		-0.132	7.07E-03	-0.18	3.19	2.48	3.59
	C33 H33 N16 S	685.2794		5.36		-0.105	1.49E-04	0.69	3.58	3.39	3.51
		999.2011	998.2025	3.90	96.9584, 815.2975, 998.2022	-0.099	4.22E-06	0.97	-1.07	7.10	6.11
		1403.0048		5.99		-0.137	3.36E-02	-0.99	1.36	2.50	3.08

Molecular formula positive ion mode (MF [M - H]$^-$); retention time (RT); first principal component score (P(1)). Statistically significant (p-value < 0.05).

Principal component analysis (PCA) provides a three-dimensional visualization of the 60 entities recognized in 75% of samples from at least one endophyte treatment group. PCA findings did not show a full separation between treatment groups, however there is clustering by treatment groups with extensive overlap between groups (**Fig. 2.6**). Principal component 1 (PC1) explained 37.15% of the variation, PC2 explained 14.92%, and PC3 explained 7.34%.

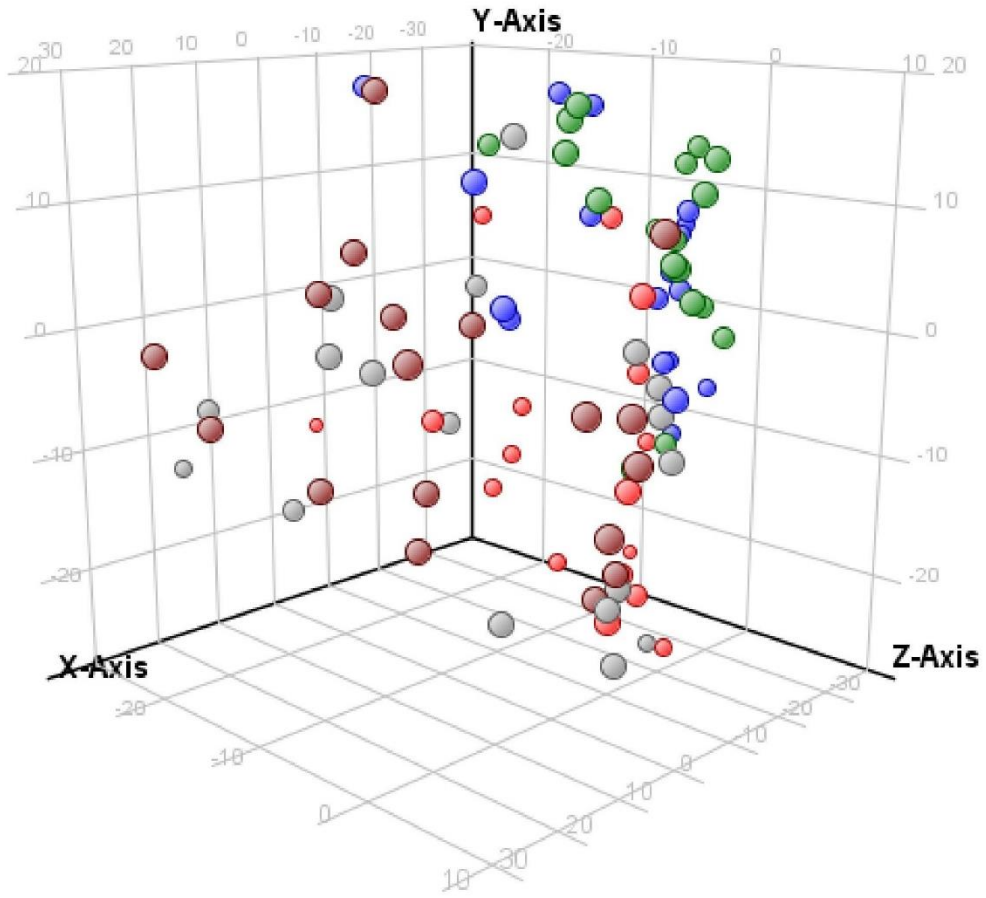

Figure 2. 6. Principal component analysis (PCA) performed on the negative ion mode metabolomic profiles of *Lolium perenne* root exudates infected with strains of *Epichloë festucae* var. *lolii* endophyte (E- (grey), E+ (maroon), AR1 (red), AR37 (blue), and NEA2 (green)). This PCA is based on 60 metabolites found to be differentially-expressed in at least one of the endophyte treatments, with $p<0.05$ and fold-change >2.0. Each treatment n = 18.

Hierarchical clustering of the data shows that endophyte treatments are separating into two groups with E- and E+ forming the first group and the novel endophytes (AR1, AR37, and NEA2) forming the second group (Fig. 2.7). Within the second grouping, AR37 and NEA2 are more similar to each other than to AR1. For the first 26 compounds in the hierarcical clustering map there is a general trend of higher intensities for the E+ and E- treatments and lower intensities for the novel endophyte treatments. However, the E+ treatment consistently had higher intensities than the E- treatment. Compounds with highest intensity within a treatment were: E+ and E- (Gossypol, m/z 517.1987 RT 9.26); AR1((2S,4S)-Monatin, m/z 291.1030 RT 4.00); AR37 and NEA2 (Glu Leu Ser, m/z 346.1657 RT 8.55).

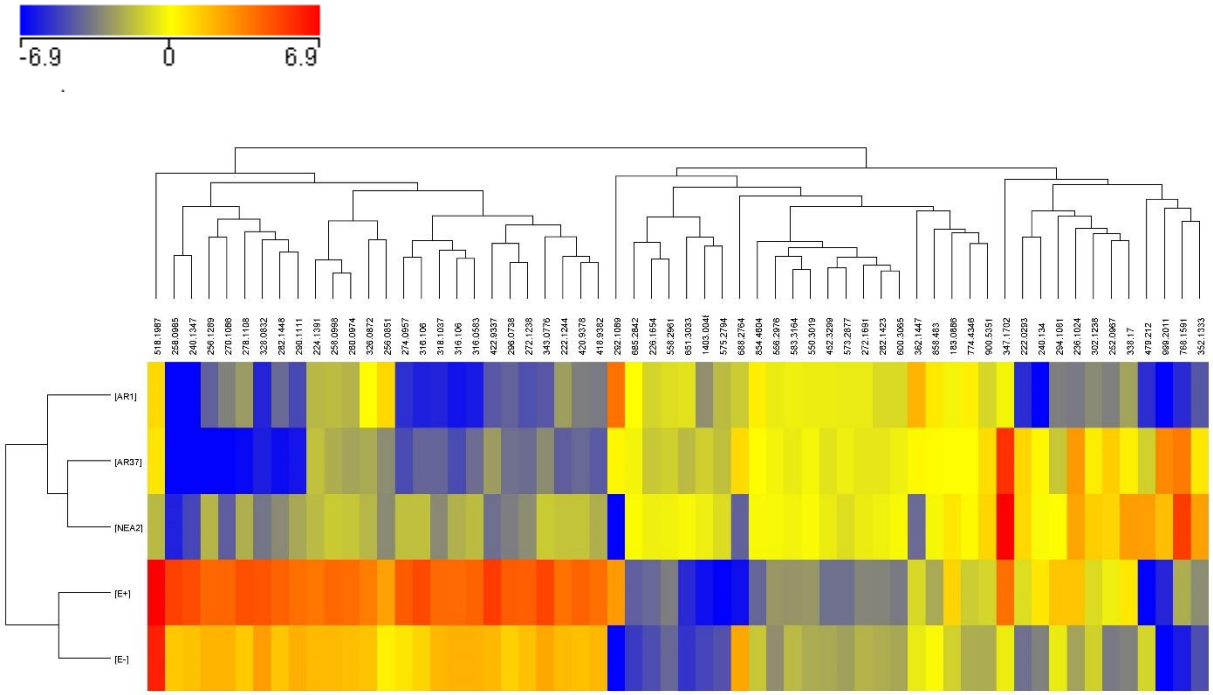

Figure 2. 7. Unsupervised hierarchical clustering of *Lolium perenne* root exudate metabolites, obtained by LC-MS in the negative ion mode. These are the 60 metabolites found to be differentially-expressed ($p<0.05$ and fold-change >2.0). Metabolites are grouped by fungal endophyte treatments based on similar intensity profiles. Colours represent normalized intensity values (yellow is the centre, blue represents high intensity, red represents low intensity). Each treatment n = 18.

DISCUSSION

The presence of *E. festucae* var. *lolii* in *L. perenne* alters grass physiology, including morphology and phytochemistry, which can have multitrophic implications in the aboveground ecosystem. How changes in *L. perenne* morphology and phytochemistry influence the belowground ecosystem remains unclear. Identification of changes in root exudate chemistry of fungal endophyte infected grasses will facilitate our understanding of plant-fungal endophyte contributions to the soil ecosystem, and their potential ecological outcomes.

In this study I measured the influence of endophyte strain and concentration on plant growth (root and shoot), and plant root exudate metabolites to determine if the level of endophyte concentration, in addition to endophyte identity, contributed to plant growth, or the regulation of root exudate metabolites. I found that *E. festucae* var. *lolii* did have significant strain specific effects on plant growth and root exudates.

Endophyte Infection

Fungal endophytes provide a sizeable input to the plant-fungal metabolome. The estimated genome size of *L. perenne* is 2,068 Mb, encoding for between approximately 11,000 and 28,000 genes (Byrne *et al*., 2015). Whereas, *E. festucae*'s genome is 35 Mb and the number of protein encoding genes is still being investigated. However, it is believed to be similar to that of *Neurospora crassa* which is estimated to encode for 11,000 gene products (Mannhaupt *et al*., 2003). Although *E. festucae*'s genome is 1 to 2% the size of *L. perenne*'s genome, it encodes for comparable numbers of genes. Therefore, it is suggested that the inclusion of endophyte concentration measurements in plant-fungal endophyte studies, and not merely presence absence confirmation, is important when interpreting the results of plant-fungal metabolic interactions (Rasmussen *et al*., 2007). We found that the level of endophyte infection (copies ng^{-1} gDNA) of

plant sheath tissue was dependent on endophyte strain. In five of 67 sheath tissue samples, endophyte concentration was below the detection limit (AR1 = 1, AR37 = 1, and NEA2 = 3). E+ and AR37 had the highest concentrations (E+ 79.11 ± 29.02, AR37 78.59 ± 48.78), not significantly different from one another. E+ and AR37 had 34% higher concentrations than AR1 and 69% higher concentrations than NEA2. AR1 had 27% higher concentration than NEA2. Different levels of endophyte concentration between strains is consistent with the literature, however, rather than being of equal concentrations as in this current study, others have found the E+ strain to have significantly higher concentrations than AR37 (Rasmussen *et al*., 2007; Ryan *et al*., 2015). While Tian *et al*. (2013) found E+, AR37, and NEA2 to have similar endophyte concentrations, which were all significantly higher than the AR1 strain. In contrast to this study where NEA2 had the lowest levels of endophyte infection. Different cultivars of *L. perenne* were used in each of these studies. Rasmussen *et al*. (2007) used two cultivars, one high sugar cultivar 'AberDove', and one normal sugar cultivar 'Fennema'. Tian *et al*. (2013) studied the normal sugar cultivar 'Bronsyn'. While Ryan *et al*. (2015), included 'AberDove', 'Fennema', and 'Bronsyn', along with the high sugar cultivars 'AberDart', 'PG113', and the normal sugar cultivar 'Impact'. Interestingly, Ryan *et al*. (2015) found that cultivar explained less than 2% of the variability in endophyte concentration, while host plant genotype explained nearly half of the variability, and endophyte strain almost a third. Additionally, the average number of gene copies between studies also differed widely. The average number of gene copies found in this study most closely reflected those found in Rasmussen *et al*. (2007). Tian *et al*. (2013) reported values up to 5x lower than in the current study, and although they used clonal replicates there was a large amount of variability within endophyte treatments, with the exception of AR1. In contrast, the number of gene copies in Ryan *et al*. (2015) were 2x to upwards of an order of magnitude

higher than my results for E+ plants, while on par with my results for AR37 plants, depending on cultivar and light and temperature regimes. There are most likely several factors contributing to variability in endophyte infection levels, including within plant colonization success from one tiller to another; environmental factors such as light and temperature; and host-endophyte genetics (Tian *et al*., 2013; Faville *et al*., 2015; Ryan *et al*., 2015). Several studies have found both fungal endophyte and plant genes involved in the regulation of hyphal growth (Tanaka *et al*., 2006, 2008; Takemoto *et al*., 2006; Scott *et al*., 2007; Eaton *et al*. 2010; Tanaka *et al*. 2013; Faville *et al*., 2015; Voisey *et al*., 2016). While the majority of those studies identified fungal genes, Faville *et al*. (2015), using quantitative trait loci (QTL) analysis, were able to identify discrete regions of the *L. perenne* genome controlling a portion of fungal growth.

An assessment of the relationship between endophyte concentration and plant biomass revealed a significant endophyte strain x endophyte concentration interaction for NEA2 shoot biomass only ($F_{4,80} = 2.4913$, $p = 0.0496$). A scatterplot showed a negative linear relationship between NEA2 endophyte concentration and total biomass, as endophyte concentration increased total biomass decreased. This is possibly the result of a dilution effect, wherein NEA2 biomass growth increased faster than fungal growth, although Rasmussen *et al*. 2007 discounts this in their study because they did not find differences in regrowth rates between treatments. Furthermore, we know that hyphal growth is synchronized with that of its host and when the plant's leaves cease growing so do the fungal hyphae (Tan *et al*., 2001; Tanaka *et al*., 2006; Christensen *et al*., 2008). This unique growth strategy of the fungal endophyte suggests, that I dilution effect would not be likely. In a companion study (Chapter 3), there was an increase in endophyte concentration, and an increase in plant growth over time. Therefore, in this particular NEA2 endophyte-host interaction there may be regulatory genes on the part of the endophyte

and/or host minimizing hyphal growth (Tanaka *et al*., 2012). In this study, a positive linear trend of endophyte concentration and total biomass was observed for E+, AR1, and AR37, though not statistically significant. Ryan *et al*. (2015) found a significant positive linear relationship between endophyte concentration and plant growth for E+ infected plants. They were able to provide evidence in their study based off of endophyte concentration and endophyte inoculation success into host plant, that suggested endophyte strain E+ was more compatible with its host than AR37.

Biomass

The fungal endophyte strain NEA2 outperformed all other endophyte strains, producing more root and shoot biomass on average. This is similar to Tian *et al*. (2013) who found that the root and leaf blade dry weight for NEA2 was significantly more than E+ and AR37, however they also found NEA6 and E- plants to have significantly more biomass than E+ and AR37, which was not the case for E- in my current study. Several field studies have demonstrated that perennial ryegrass infected with novel endophyte strains provide improvements in pasture productivity and persistence, when compared to E- plants (Popay *et al*. 1999; and Popay and Hume 2011). Additionally, field studies have found that novel endophyte strains can provide comparable improvements to each other in terms of yield (Moate *et al*., 2012). However, some studies have found that novel endophyte strains can outcompete each other. For example, AR37 resulted in higher yields than E+ and AR1 (Hume *et al*., 2007), and another where AR37 was higher than AR1 (Thom *et al*., 2013). In a glasshouse study, Ryan *et al*. (2015) found a significant cultivar x endophyte interaction on blade biomass attributed to one AR37 cultivar having significantly higher shoot biomass than some, but not all, of the other AR37 and E+ cultivars. In contrast E+ plants produced significantly less shoot biomass than E-, AR1, and

AR37 (Bell *et al*., 2009). Both Bell *et al*. (2009) and the current study considered only one grass cultivar, but still observed strain specific effects in plant growth. Further research focussing on the genetic components of these grass-fungal endophyte strain specific differences would perhaps elucidate the mechanisms involved.

Metabolites in Root Exudate

The degree to which fungal endophytes benefit host plant fitness can depend on several factors including endophyte strain and plant host genotype. Endophyte strain and plant host genotype have been found to significantly influence the synthesis of fungal derived alkaloids (Liu *et al*., 2011; Tian *et al*., 2013). For example, AR37 is one novel strain that synthesizes epoxy-janthitrems which is an alkaloid not so far found in other endophyte strains. It stands to reason that if alkaloid quality and quantity vary between strains, then other metabolites may be changing between strains.

Through liquid chromatography mass spectrometry-based assays, I identified metabolites as being significantly differentially regulated between the strains of endophyte infected plants when compared to uninfected plants. Recursive molecular feature extraction resulted in a total of 177 molecular features (positive and negative ion mode totals combined). Only compounds significantly different between at least one treatment group, and further passed a fold-change filter, were considered. This reduced the number of total compounds to 73 (pos = 23; neg = 60). In the positive ionization mode, the metabolomic profiles for E- and NEA2 treatments were more similar to each other, and E+, AR1, and AR37 treatments were more similar to each other. In the negative ionization mode, E- and E+ were more similar to each other, and AR1, AR37, and NEA2 were more similar to each other. This is in contrast to Wakelin *et al*. (2015) who found that endophyte infected samples separated out from uninfected samples in *L. perenne* (cv. Samson). They also

found that root exudate chemistry differed between the two novel endophyte strains AR1 and AR37. Although in my study the metabolomic profiles did not separate out the E- from the endophyte infected, there was a substantial amount of individual metabolites that varied between the endophyte treatments, and often significantly different than E- plants. Endophyte treatment groups have also been found to have distinct metabolite patterns in aboveground tissues (Koulman *et al*., 2007b, 2009; Cao *et al*., 2008). In the Wakelin *et al*. (2015) they found alkane-type (lipid) compounds to be the most variable between the endophyte treatments. In the current study, almost every compound class in both positive and negative ion mode had high degree of variability between endophyte treatments when compared to E-. Although the Wakelin *et al*. (2015) study utilizes *L. perenne* infected with AR1 or AR37 fungal endophyte strains, or left uninfected (E-), their study differs in plant cultivar, growth and extraction medium (soil), and analytical technique (GC-MS). There is an overlap in the chemical classes that can be captured with GC-MS and LC-MS, but there are also certain chemical classes that are most compatible with one technique or the other. Guo *et al*. (2015) was the first comprehensive root exudate study using *S. arundinaceus* and *E. coenophiala*. They also utilized GC-MS and found that the composition of root exudates was influenced by both endophyte strain and plant cultivar. Most notably were the differences in some lipids, phenolics, amines, and sugars between the endophyte strains. Although in the current study I did not capture sugars commonly observed in root exudates, the pattern of endophyte strain specific effects on root exudate composition found in Guo *et al*. (2015) were similar to this study.

Field experiments are often desired for studying ecological systems, but they are not always ideal when trying to isolate metabolites from a biologically complex, and inherently dynamic system. Therefore, I used individual plants in this experiment, grown in a hydroponic set-up to simplify the system. In a natural soil medium, and in the presence of other plants, the

plant metabolites excreted by roots will change significantly in response to the environment (Badri *et al*., 2012). It is important to pair experiments conducted in simplified systems with those conducted in more complex systems, to gain more biologically meaningful results.

The study of root exudate metabolites in grass-fungal endophyte associations is rare. It was therefore difficult to make clear connections with the compounds I observed with those of other studies. Additionally, the compounds I was able to tentatively identify were not the common metabolites talked about in the broader literature on plant metabolites. This made it difficult to draw conclusions about why we may be seeing endophyte strain specific changes in metabolite regulation. Improvements in the root exudation collection, and preparation for LC-MS could be made. Root exudates were measured directly from deionized water, with no concentration of the samples or use of an extraction solvent. Sampling of the root exudates directly from water most likely had a strong dilution effect, resulting in lower peak intensities and potentially the loss of metabolites during recursive molecular feature extraction. The use of multiple extractions solvents to separate polar and non-polar fractions, as well as protein fractions of the root exudate would allow for multiple different LC-MS analyses to capture a broader range of metabolites.

Conclusion

In this study I was interested in identifying changes in the plant metabolome during fungal endophyte infection, the “plant-fungal metabolome”. I focussed specifically on changes in root exudate composition as very few studies have been conducted on root exudate chemistry in plant-fungal endophyte associations. I found that the presence of fungal endophytes in *Lolium perenne* significantly effected plant growth, and root exudate chemical composition, and that these effects were significantly related to the fungal strain. The isolation of an unknown

metabolite (m/z 135.0546 RT 1.17) unique to the AR37 endophyte strain suggests that there are more compounds yet to discover in these associations that may provide mechanistic explanations for fungal endophyte's fitness benefits to their plant hosts. This study demonstrates the complexity of the plant-fungal association, and the role of the fungal endophyte in altering plant host chemistry beyond alkaloids. My results provide justification for further research in this area, and warrant more targeted metabolomic approaches, paired with other omics techniques, and NMR. Such targeted methods will aid in compound identification and mapping of metabolomic pathways to connect the chemistry with the biology.

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CHAPTER 3: EFFECTS OF *EPICHLOË FESTUCAE* VAR. *LOLII* 'SELECTED' STRAINS ON COMMUNITY STRUCTURE OF BACTERIA AND FUNGI INHABITING THE *LOLIUM PERENNE* RHIZOSPHERE

ABSTRACT

Lolium perenne is a cool season grass which is host to a foliar fungal endophyte *Epichloë festucae* var. *lolii*. The presence of this endophyte can alter aspects of the grass's morphology and physiology, including changes in root growth and structure, and shifts in the production of primary and secondary metabolites. Such changes may influence the activity, structure, and function of soil organisms, which could have ecological consequences belowground impacting the decomposition of organic matter and the cycling of carbon, nitrogen, and other nutrients. I conducted a soil conditioning experiment using endophyte free (E-) and endophyte infected (common toxic (E+), and novel strains (AR1, AR37, NEA) *L. perenne* seeds which were germinated and grown in a potting soil:unsterilized field soil mix (1:2), for four, eight, and twelve weeks in a controlled glasshouse environment. My objectives were to measure plant and fungal endophyte growth over time, identify changes in plant-fungal endophyte metabolomic profiles via rhizosphere soil, and to identify changes in the structure of the microbial community in rhizosphere soil. Plant growth did significantly increase over time with endophyte infected plants performing better than uninfected plants. Using qPCR, endophyte concentration was significantly increased over time, and varied between the endophyte strains with AR37 having the highest endophyte concentration by week 12. Using LC-MS, the rhizosphere metabolome showed significant differences in profiles between soil conditioning treatments relative to bare soil, and relative to uninfected plants. E+ had the greatest percentage of metabolites at high intensities relative to E- plants. Only five metabolites appeared to be directly contributed by the

presence of plants, while the majority of the metabolites were found in the bare soil. This suggests that they were contributed by the microbial community, but were differentially effected between uninfected plants and the different strains of fungal endophyte infected plants. Using NGS, I found significant differences in bacterial community composition at the phylum level between control soils and both AR1 and NEA2 conditioned soil, and strong differences between control soils and E+ and AR37 conditioned soil. Differences in fungal community composition between soil conditioning treatments were clearer at the class level, and some significance was seen with finer classification. AR37 conditioned soils had the lowest average bacterial abundances at the phylum level, followed by E+. While control soils had the highest average abundances for 65% of the phyla. E+ conditioned soils had the lowest average fungal abundances at the phylum level, while E- had the highest average abundances for Ascomycota and Zygomycota; AR37 for Basidiomycota; AR1 for Chytridiomycota; and NEA2 for Glomeromycota. Furthermore, tiller number, root biomass, and soil moisture significantly influenced bacterial community composition. My results show that the presence of fungal endophytes can significantly influence aspects of its grass host's morphology and physiology which can subsequently impact the belowground microbial community. The significant changes in metabolic profiles, and subtle shifts in the microbial communities between endophyte strains warrant more targeted studies in the future.

Keywords: *Lolium perenne*, *Epichloë festucae* var. *lolii*, plant-fungal endophyte associations, fungal endophyte strains, rhizosphere metabolites, qPCR, LC-MS, Next-generation sequencing (NGS), microbial community structure

INTRODUCTION

Foliar fungal endophytes from the genus *Epichloë* have co-evolved with grasses in the *Poaceae* family (Saikkonen et al., 2016) and provide plant benefits of economic importance in both the agricultural and turf industries. The most notable feature of grass-fungal endophyte associations is the plants improved resistance to herbivory through the endophytes' production of bioactive alkaloids which can act as deterrents and/or toxins to some animals (Fletcher and Harvey, 1981; Pedersen *et al*., 1988; Breen, 1994; Malinowski *et al*. 1998). The contribution of fungal endophytes to the host plant's chemistry is not limited to alkaloids however, they can also influence other aspects of secondary metabolism as well as the host plant's primary metabolism (Hunt et al., 2005; Rasmussen et al., 2008b; Cao et al., 2008; Ryan et al., 2014; Dupont et al., 2015). These changes in the plant-fungal metabolome may also contribute to the improvements seen in plant host fitness, but the influence of these physiological changes on many aspects of the phytobiome are still unclear. In particular, the influence of foliar endophytes on the belowground foodweb and biogeochemical processes have been little studied.

Lolium perenne (perennial ryegrass) is one such species of grass that is commonly host to the fungal endophyte *Epichloë festucae* var. *lolii*. In addition to changes in host morphology, there is growing evidence that the presence of fungal endophytes in grasses can dramatically alter the host metabolome and these changes can have subsequent effects on soil organisms and biogeochemical processes. Furthermore, there are many naturally occurring strains of *E. festucae* var. *lolii* which may have unique metabolomic profiles, which each may affect belowground processes differently. Hypothesized mechanisms driving belowground changes include: indirect effects like the leaching of allelochemicals, including alkaloids, into the soil, which can have a negative impact on soil organisms; shifts in plant community composition due to improved

competitiveness, as a result of improved tolerance to biotic and abiotic stress; improvements to plant production, mainly due to resistance to herbivory (Popay and Hume, 2011); and direct effects on root biomass, root structure, and quantity and quality of root exudates (Malinowski et al. 1998; Van Hecke et al., 2005; McNear and McCulley, 2012).

The use of molecular techniques, such as metabolomics, in more recent years has allowed for more in-depth investigations of the plant-fungal metabolome. These investigations have predominantly utilized aboveground tissues (Koulman et al., 2007a; Rasmussen et al., 2008; Dupont et al., 2015), however the limited studies on roots and root exudates have demonstrated that the quantity and quality of root exudates are also affected by grass-endophyte associations (Malinowski et al., 1998; Omacini et al., 2012; Guo et al., 2015; Wakelin et al., 2015). For example, increases in the amount of carbon and nitrogen in root exudate have been observed in a similar grass-fungal association between *Schedonorus arundinaceus* and *Epichloë coenophiala* (Guo et al., 2015). Metabolites, such as phenolics, involved in plant defence, and nutrient acquisition, also can increase in the root exudates of endophyte infected plants (McNear and McCulley, 2012; Guo et al., 2015). Root exudate composition can also change in the presence of fungal endophytes. Guo et al. (2015) found 43 metabolites to be differentially expressed in the root exudates of *S. arundinaceus* infected with various strains of *E. coenophiala*. Composition of root exudates from the common toxic and uninfected plants was more similar and differed from the similar composition associated with plants infected with the two novel endophyte strains. The metabolite classes significantly influenced by endophyte presence were sugars, growth factors and amines. Several of the affected compounds were associated with nutrient acquisition and allelopathy, and are known to alter microbial community composition in the soil (Guo et al., 2015). Wakelin et al. (2015) also found that the rhizosphere metabolome of *L. perenne* plants

infected with novel endophytes (AR1 and AR37), was clearly different than E- plants. They also found that the metabolomic profiles differed between the two endophyte strains. There were seven alkane hydrocarbon derivatives, and two unidentified metabolites that were responsible for the greatest amount of difference in the metabolomic profiles. Alkane hydrocarbon derivatives are prevalent in rhizosphere soil and may play a role in carbon cycling in the rhizosphere (Wakeline et al., 2015). Metabolomics has also revealed many unidentified compounds, demonstrating that the identity and role of root exudate metabolites is far from being clarified.

Soil organisms play a pivotal role in soil processes such as the generation of soil organic material through decomposition of plants and animals, and the cycling of carbon and nutrients (Badri 2009). In order to understand how these soil processes may be impacted by grass-fungal endophyte associations, we must look to the soil organisms. The presence of fungal endophytes can alter the activity, structure, and function of soil organisms. Soil microbial responses to endophyte presence are highly variable being influenced by many factors including plant cultivar, plant genotype, endophyte strain, temporal and edaphic factors, or some combination of these variables. In the presence of fungal endophytes, it is possible to see changes in activity in microorganisms but no changes in composition, and vice versa (Van Hecke et al., 2005). It is also possible that these differences are only seen in one microbial community (i.e. bacterial, or fungal) and not the other. For example, Casas et al. (2011) found that in the presence of endophytes, fungal activity increased while bacterial activity was not affected, and they also found that bacterial community composition in endophyte infected soils was different than uninfected soils, while fungal community composition was not changed. Using endophyte infected *S. arundinaceus*, Rojas et al. (2016) found that, while there was no significant endophyte effect on bacteria communities, soil fungal community composition, but not total

fungal biomass, were influenced by endophyte infection regardless of endophyte strain. The composition associated with each endophyte strain significantly differed from the composition associated with E- plants. In *L. perenne* endophyte strain specific effects have been found for certain classes of bacteria and fungi (Bell et al., 2009). Wakelin et al. (2015) also found significant, endophyte strain dependent, differences between the bacterial communities and fungal communities of endophyte infected vs. uninfected soils.

There can be a stimulatory effect on microbial activity, without changing population density (Van Hecke et al., 2005). Though often increases in soil carbon and nitrogen are observed (Franzluebbers 2006; Iqbal et al., 2012), suggesting that changes in composition may be important with respect to differing roles of these species in the soil. For example, they found that there was a stimulatory affect on arbuscular mycorrhiza fungi (AMF) which could lead to an increase in carbon sequestration in the soil. Indeed, Guo et al. (2016) did observed an increase in particulate organic carbon pools in the rhizosphere soil in some endophyte treatments. However, there are studies which show that the presence of an endophyte in a grass host can lead to a reduction in mycorrhiza colonization (Liu et al., 2011). Changes in soil processes, such as increases in soil organic C and N (Franzluebbers 2006; Iqbal et al., 2012; Guo et al., 2016), increases in nitrification (Bowatte et al., 2011), and decreases in litter decomposition (Omacini et al., 2004; Lemons et al., 2005), suggest changes in the soil microbial community structure and function.

Few studies have investigated the influence of endophytes in the rhizosphere both with respect to changes in plant inputs, in the form of root exudates, and to changes in soil community composition. The objective of this study was to investigate the microbial communities in the rhizospheres of *L. perenne* plants infected with different strains of the fungal endophyte *E. festucae* var. *lolii*. I examined how plant biomass, endophyte concentration, root exudate metabolites, and

soil microbial community composition differed between endophyte strains. For plant biomass and endophyte concentration, time, and time x endophyte interaction were also considered. I was also interested in the relationship between endophyte concentration and plant growth, and endophyte concentration and relative intensity of root exudate metabolites. I hypothesized that the physiology of *L. perenne* would differ in both plant growth and root exudate chemistry in the presence of *E. festucae* var. *lolii*, and that these differences would be endophyte strain specific. The presence of *E. festucae* var. *lolii* would also alter the community composition of microorganisms in the rhizosphere: 1) the community composition of the bare soil should be distinctly different from plant conditioned soils, regardless of endophyte status; 2) among plant conditioned soils, there should be significant differences between endophyte free plants and endophyte infected plants; 3) among endophyte infected plants, there should be significant differences between endophyte strains; and 4) the level of endophyte concentration will influence the community composition in the rhizosphere.

MATERIALS AND METHODS

To understand the effects of fungal endophyte strains of *Epichloë* var. *lolii* associated with *Lolium perenne* (perennial ryegrass) on microbial communities in the rhizosphere, I designed a soil conditioning experiment. I measured total root and shoot biomass, endophyte concentration in stem and sheath tissue, and conducted metabolomics and metagenomic sequencing on rhizosphere soil.

Experimental design

The experiment was performed in a glasshouse during the months of September to December 2015. The temperature was maintained at ~23°C and supplementary lighting was provided on a 16:8 hour light:dark cycle. Pots, constructed of 15” PVC tubes with fiberglass mesh bottoms,

were placed in 6 x 6 cell PVC frames in a glasshouse (Figure 3.1 A-D). Pots were arranged in blocks. Each block contained 144 randomly distributed pots that were left out for either 4, 8 or 12 weeks. There were 3 blocks placed out at three different dates to account for variation in environmental conditions over time. There were 8 replicates/pot treatment/time treatment. (8 replicates x 6 pot treatments (Control, E-, E+, AR1, AR37, and NEA2) x 3 time treatments (4, 8, and 12 weeks) x 3 blocks (n = 24) for a total of 432 pots.

Figure 3. 1. Experimental set-up of 6 x 6 frames for holding experimental pots in glasshouse after initial placement (A), week 4 (B), week 8 (C), and week 12 (D).

Sample preparation

Seed and endophyte status

Individual seeds of *Lolium perenne* (perennial ryegrass), cultivar Alto, either infected with one of four strains of the fungal endophyte *Epichloë festucae* var. *lolii* (common toxic strain (E+), and 3 novel strains (AR1, AR37 and NEA2)) or uninfected (E-) were used. These Alto seed lines were received from Courtney Inch (Agriseeds Ltd, New Zealand) and stored in the freezer at -20˚C, to preserve endophyte prior to planting.

Prior to planting, endophyte status and frequency within the *L. perenne* seeds was determined by using an immunoblot assay (Agrinostics Ltd. Co., Watkinsville, GA, US). Seeds were then germinated and allowed to grow for six weeks. Further endophyte testing was conducted on sheath tissue utilizing quantitative Polymerase Chain Reaction (qPCR). The qPCR protocol was established for *S. arundinaceus* association with *Neotyphodium coenophialum* by Ryan *et al*. (2014). A search in the National Center for Biotechnology Information (NCBI) gene bank confirmed that the tef-A gene present in *N. coenophialum*, and utilized for the production of qPCR primers, was also present in *E. festucae* var. *lolii*, therefore new primers were not designed.

Soil

Soil was prepared by mixing one-part sterilized potting soil Sunshine Mix #4 (Sun Gro Horticulture), and two parts unsterilized soil (orthic grey brown luvisol). The unsterilized soil, from the surface to a depth of 30 cm, was collected from our field site at the Guelph Turfgrass Institute and Research Centre (GTI) located in Guelph, Ontario (43.549414, -80.213130). Soil characterization was conducted by Laboratory Services, University of Guelph: Soil parameters,

expressed as soil dry weight: Soil organic carbon (2.25 %), inorganic carbon (0.70 %), total carbon (2.96 %), total nitrogen content (0.24 %), phosphorous (18.4 ppm), potassium (111 ppm), magnesium (302 ppm), sodium (15 ppm), calcium (2440 ppm), electrical conductivity (0.103 mS/cm), pH (7.5). The soil was sieved through a 4.75 mm screen to remove roots, rocks, and other large particles, and then homogenized.

Pots were made of PVC piping cut to 39 cm length tubes with a 7.62 cm inner diameter and an 8.89 cm outer diameter. The bottom of the piping was covered with window screen to allow for adequate soil drainage during watering. Soil (Volume ~1687.34 cm^3) was then added to each pot leaving 2 cm of space at the top. Each pot then received 250 ml of deionized water prior to adding seeds to ensure that seeds would not get dislodged from the soil. Five seeds of each endophyte treatment (E-, E+, AR1, AR37, and NEA2) were sown in pots at a depth of 0.2 cm. Control pots received no seeds. Pots were then arranged in the glasshouse as described in the *experimental design*. Once seeds germinated, seedlings were pruned back so that only one plant remained per pot. Pots were watered with deionized water three times a week, however the control pots (i.e. no plants present) received a smaller volume as they remained wet. All pots were given an application of nutrients (fertilizer water: Plant Products®, 20-8-20 All Purpose High Nitrate, 1.25 g per litre, 250 parts nitrogen, pH adjusted to 6.0) applied at weeks 3, 6, and 9. Nutrient supplementation was not an experimental treatment; our field soil is nutrient poor so nutrients were added to ensure adequate plant development in the timeframe required for this study.

Sample collection

Pots were destructively harvested at each time point (4, 8, and 12 weeks). Aboveground growth was clipped at the soil surface. Two individual tillers were flash frozen in liquid nitrogen

for molecular analyses (estimation of endophyte concentration and plant metabolomics). The remaining aboveground growth was placed in a drying oven (65ºC) and dry-weight biomass was measured. Then the soil with intact plant roots was removed from pots. Rhizosphere soil, considered the soil adhering to and within 5 mm of the root surface, was collected, homogenized, and separated into four subsamples. Two of the four subsamples of rhizosphere soil were frozen at - 20°C. The subsamples were then freeze-dried, sieved (500 μm sieve), and then ground using a 2010 Geno/Grinder® (SPEX® SamplePrep, USA) tissue homogenizer. These samples were used to measure metabolites as well as microbial communities. The other two subsamples were kept fresh, one was used to assess nematode diversity and abundance (not reported here), and the other was used for the determination of soil moisture (gravimetric) and pH. Roots were washed and oven-dried to obtain dry-weight.

Estimation of endophyte concentration

The plant tissue flash frozen in liquid nitrogen was then lyophilized (FreeZone 4.5 Liter Console Freeze Dry System, Labconco, USA). Samples were transferred into 5 mL tubes with a 9.5 mm ball bearing and ground using a 2010 Geno/Grinder® (SPEX® SamplePrep, USA) tissue homogenizer. Genomic DNA was extracted from 20 mg of ground sheath tissue using DNeasy®Plant Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer's protocol, in conjunction with the QIAcube® (Qiagen Inc., Toronto, Canada), for automated sample prep, as per manufacturer's instructions. For each sample, total gDNA (plant and fungal) was measured by placing 2 μL of sample on a NanoDrop® 2000, run in triplicate. The gDNA was then diluted to a working concentration of 0.5 ng total gDNA/μL using Millipore water. The qPCR reactions were set up with a total volume of 15 μL. Each reaction contained 9 μL PCR mix (forward primer and reverse primer (0.75 μL each, 0.5 μmol concentration), LightCycler® 480 SYBR

Green I Master (7.5 µL, 2x concentration)), and 3 ng of gDNA (6 µL gDNA, 0.5 ng/µL concentration). Sample wells were tested in triplicate. Both dilutions and plating were carried out by an automated PCR set-up instrument, QIAgility® (Qiagen Inc., Toronto, Canada). PCR reactions were performed on a LightCycler® 480 Instrument II (Roche, Canada). The PCR thermocycling conditions were as follows: initial denaturation for one cycle at 95 °C for 5 min, followed by amplification for 45 cycles of 95 °C for 10 s, 64 °C for 10 s, and 72 °C for 10 s. The formation of a single PCR product in the PCR reactions was confirmed by melting analysis where the PCR products were raised to 95°C for 5 s, and then lowered to 65 °C and raised back to 97 °C over 1 min with continuous fluorescence data acquisition. The T_m of the PCR product was 83.75 ± 0.25. One alteration to the methodology was needed when samples were extrapolated from the curve. In this case, the sample was re-run using 9 ng of gDNA instead of 3 ng. If endophyte infection was still below the detection limit, the sample was considered to have no endophyte infection.

Metabolites in rhizosphere soil

A preliminary assessment of the metabolites present in rhizosphere soil was conducted on soils from the *L. perenne* plants infected with *E. festucae* var. *lolii* grown for 12 weeks. There were three replicates per treatment (bare soil (control); plant without endophyte infection (E-); plant infected with common toxic strain (E+); plant infected with one of three novel endophytes (AR1, AR37, or NEA2)). Briefly, 2 g of ground lyophilized rhizosphere soil were measured out into 50 mL conical flasks and 8 mL of ice cold extraction solution (isopropanol/methanol/water; 3:3:2 v/v/v) was added. Flasks were placed on an orbital shaker for 1 hour at 200 rpm at 4◦C. The soil solution was then centrifuged at 8500 rpm for 15 minutes. Supernatant was collected

and passed through a 0.45 um vacuum pumped filter and transferred to a MS certified (MSCERT5000-37LVW) 350 µL glass vial (Thermo Scientific™) for further LCMS analysis.

To determine the composition of metabolites, present in rhizosphere soil, liquid chromatography–mass spectrometry (LC-MS) analyses were performed on an Agilent 1200 HPLC liquid chromatograph interfaced with an Agilent UHD 6530 Q-Tof mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. A C18 column (Agilent Poroshell 120, EC-C18 50 mm x 3.0 mm 2.7 µm) was used for chromatographic separation with the following solvents: water with 0.1% formic acid (A) and acetonitrile with 0.1 formic acid (B). The mobile phase gradient was as follows: initial conditions were 10% B hold for 1 min then increasing to 100% B in 29 min followed by column wash at 100% B for 5 min and 20 min re-equilibration. The flow rate was maintained at 0.4 mL/min. The mass spectrometer electrospray capillary voltage was maintained at 4.0 kV and the drying gas temperature at 250° C with a flow rate of 8 L/min. Nebulizer pressure was 30 psi and the fragmentor was set to 160. Nitrogen was used as both nebulizing and drying gas. The mass-to-charge ratio was scanned across the m/z range of 50-1500 m/z in 4GHz (extended dynamic range) positive and negative ion modes. The acquisition rate was set at 2 spectra/s. The instrument was externally calibrated with the ESI TuneMix (Agilent). The sample injection volume was 10 µL.

Metabolomic Data Analysis

The mass spectrometry data were further processed using Agilent Mass Hunter Work-station software (MassHunter Profinder B.08.00). Recursive molecular feature extraction (rMFE) was used for binning and alignment of molecular features. The rMFE is an algorithm which groups related co-eluting ions (i.e. isotopes, adducts, and dimers) into a single compound, and then creates compound chromatograms. The rMFE step also filters out noise and reduces false positives.

Molecular features were aligned based on a retention time window of 0.40 min and a mass window of 40.00 ppm + 2.00 mDa, and an absolute height of at least 3000 counts. Aligned features that were found in at least one replicate in one treatment group (n = 3) were retained. Molecular features were extracted as compound exchange format (cef) files and imported into Agilent's Mass Profiler Professional (MPP) software version B14.5. I used Mass Profiler Professional (MPP) for statistics visualization, and annotation and identification of compounds.

In MPP, compounds that were statistically significant and/or unique to certain treatments were noted and tentative identification was performed. Compounds were searched against an in-lab annotated METLIN Personal Metabolite Database (Agilent Technologies) and against KNApSAck: Species-Metabolite Relationship Database (Nakamura et al., 2014).

DNA extraction of rhizosphere soil and library preparation

Rhizosphere microbial communities were assessed using Illumina sequencing analysis of 16S rDNA and ITS amplicons of a total of 72 samples from five treatments (E+, E-, AR1, AR37, NEA2), and a soil control (12 replicates per treatment). Only results from soil samples from the 12 weeks of plant growth are reported here. DNA was extracted from two 0.5 g subsets of each freeze-dried soil sample using PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), according to the manufacturer's protocol. Two subsets of each soil sample were extracted to improve yield of total DNA. Absorbance ratios of 260/280 nm and 260/230 nm were obtained using a NanoDrop® 2000 spectrophotometer (Thermo Scientific, USA) to evaluate DNA quality and concentration. Soil is known to have poor DNA yield, therefore 40 ng/ul was set as the goal concentration to allow for sufficient amplification of target DNA. Samples were concentrated when necessary. Following the Illumina MiSeq protocol, two-step PCR was applied for 16S rDNA V3 and V4 region and ITS 2 region amplification using KAPA

HiFI HotStart ReadyMix PCR kit. PCR 1 amplification was performed in a total volume of 25 µL containing 2.5 µL microbial genomic DNA (40 ng/ul in 10 mM Tris pH 8.5), 5 ul of each primer (1 uM), and 12.5 ul 2x KAPA HiFi HotStart Ready Mix. The 16S bacterial primer pair S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Klindworth et al., 2012), and the ITS2 fungal primer pair fITS7 and ITS4 (Ihrmark et al. 2012) were used in the first step (Gene-specific sequences, including Illumina adapter overhang nucleotide sequences, can be found in Table 3.1). This bacterial primer pair tends to have higher bacterial diversity and broader taxonomic coverage when compared to other commonly used 16S primers (Thijs et al., 2017). The products of PCR1 were purified with AMPure XP Beads (Beckman Coulter, Inc.).

Table 3. 1. Full length primer sequences used for library preparation (green represents the Illumina adapter overhang nucleotide sequences that were added to the gene-specific sequences).

Target	**Region**	**Primer Direction**	**Primer Name**	**Primer Sequence**	**Reference**
Bacteria	16S V3 and V4	Forward	S-D-Bact-0341-b-S-17	5’- CCTACGGGNGGCWGCAG-3’	Klindworth et al. 2012
				5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’	
		Reverse	S-D-Bact-0785-a-A-21	5’- GACTACHVGGGTATCTAATCC-3’	
				5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’	
Fungi	ITS2	Forward	fITS7	5’-GTGARTCATCGAATCTTTG-3’	Ihrmark et al. 2012
				5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCGAATCTTTG-3’	
		Reverse	ITS4	5’-TCCTCCGCTTATTGATATGC-3’	
				5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3’	

The products of the PCR reaction were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.2 ug/ml) prior to the second PCR step. The purified samples were then used for PCR 2. In this second PCR step the Nextera XT Index Kit was used to attach Illumina sequencing adapters. Libraries were sequenced at the Advanced Analysis Center, University of Guelph, Canada, using Illumina MiSeq.

Next Generation Sequence data analysis

The raw data reads were paired using SeqPrep (John 2011); I set a minimum quality of 20, and an overlap of at least 35. The paired reads were then trimmed of the primer sequences and subjected to a sequence quality check with cutadapt (Martin 2011). I set the minimum length of my sequences to be 300bp and the maximum to be 400bp. I allowed for three ambiguities with the primer. The qualified reads were de-replicated, chimeras identified and removed, and the remaining reads were sorted, and clustered to generate operational taxonomic unit (OTUs) at the 97 similarity level using VSEARCH (Rognes et al., 2016). I assigned OTUs to the genus level (cut off of 0.80) using the Ribosomal Database Project (RDP) database by the RDP classifier (Wang et al., 2007).

Statistical analysis

The effect of endophyte treatment on endophyte concentration, tiller number, total, root, and shoot biomass, and root:shoot ratio was determined using a general mixed effects model in JMP version 14.0 (SAS Institute, Cary, NC) testing first for the effect of time (4, 8, or 12 weeks of growth) and secondly for the effects of endophyte strain, and time by endophyte interactions. A similar test was also conducted to see if plant growth was correlated with endophyte concentration. Least squares means of untransformed data and standard errors are reported. Data not normally distributed were Box-Cox transformed and reanalysed to obtain F-statistics and p-

values. Tukey Honest Significant Difference tests (Tukey HSD) were used to determine the identity of significant treatment effects.

Metabolite data, collected in positive and negative ion mode, were subjected to statistical and visual differential analysis in MPP separately. ANOVA was conducted to determine metabolites showing statistical differences across endophyte treatments. The Benjamini Hochberg correction (Benjamini and Hochberg, 1995) was applied to control for the number of false positives, resulting from multiple testing of p-values ($p < 0.05$). An additional fold change filter ≥ 2.0 was applied to statistically significant metabolites. Visualisation of data included principal component analysis (PCA) and hierarchical cluster analysis (HCA). Hierarchical clustering was used to group significant compounds (as determined by an ANOVA and fold change) in clusters by metabolite and by endophyte treatment using a Euclidean distance metric (Taylor et al., 2002; Anderson 2006) and Ward's Linkage rule (Ward 1963).

To evaluate how endophyte strain influenced microbial community composition, the OTU counts were transformed and Bray-Curtis dissimilarity matrices were calculated. These distance matrices were then fit to linear models including permutational multivariate analysis of variance (PERMANOVA) tests (Anderson, 2001), with Adonis () function, used to assess the effects of endophyte status, endophyte concentration, plant growth parameters, and soil moisture on fungal and bacterial community structure in the rhizosphere. To test whether microbial communities within a soil conditioning treatment are homogenized compared to communities between soil conditioning treatments, I compared the species composition matrix between all treatment groups using tests of homogeneity of multivariate dispersion (PERMDISP) (Anderson and Walsh, 2013). To determine which phylum were contributing towards differences between treatment groups percent similarity percentages were calculated used the simper function

(Oksanen et al., 2017). Non-parametric multidimensional scaling (NMDS) was used to visualize the composition of organisms between treatment groups. Additionally, a constrained correspondence analysis (CCA) was plotted, displaying the best set of environmental variables that describe the community structure. All statistical tests were performed using the 'vegan' package (Oksanen et al., 2017) in R v.3.3.1 (R Development Core Team, 2016), and significance was determined as $P<0.05$. NMDS and CCA plots, as well as the stacked bar graph of bacterial community relative abundances by phylum were generated utilizing code creating by Torondel et al. (2016).

One additional test was performed in R to look at microbial composition, called analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015). This test was performed on fungal data only, as it was a much smaller data-set and the analysis is computationally intensive.

RESULTS

Endophyte Concentration

Analysis of endophyte concentration in plant sheath tissue using qPCR revealed significant differences between endophyte strains ($F_{3,237} = 3.81$, $P = 0.0108$). E+, and AR37 had equally higher gene copies than NEA2 (100 vs 128 copies ng^{-1} gDNA). There was also an increase in the average (mean ± SE) endophyte concentration over time (Wk 4 = 33.45 ± 9.99, Wk 8 = 104.19 ± 9.15, Wk 12 131.19 ± 8.66 copies ng^{-1} gDNA), with week 4 being significantly different than weeks 8 and 12 ($F_{2,237} = 33.42$, $P<0.0001$) (Fig 3.2). The effect of endophyte strain on endophyte concentration was not dependent on time.

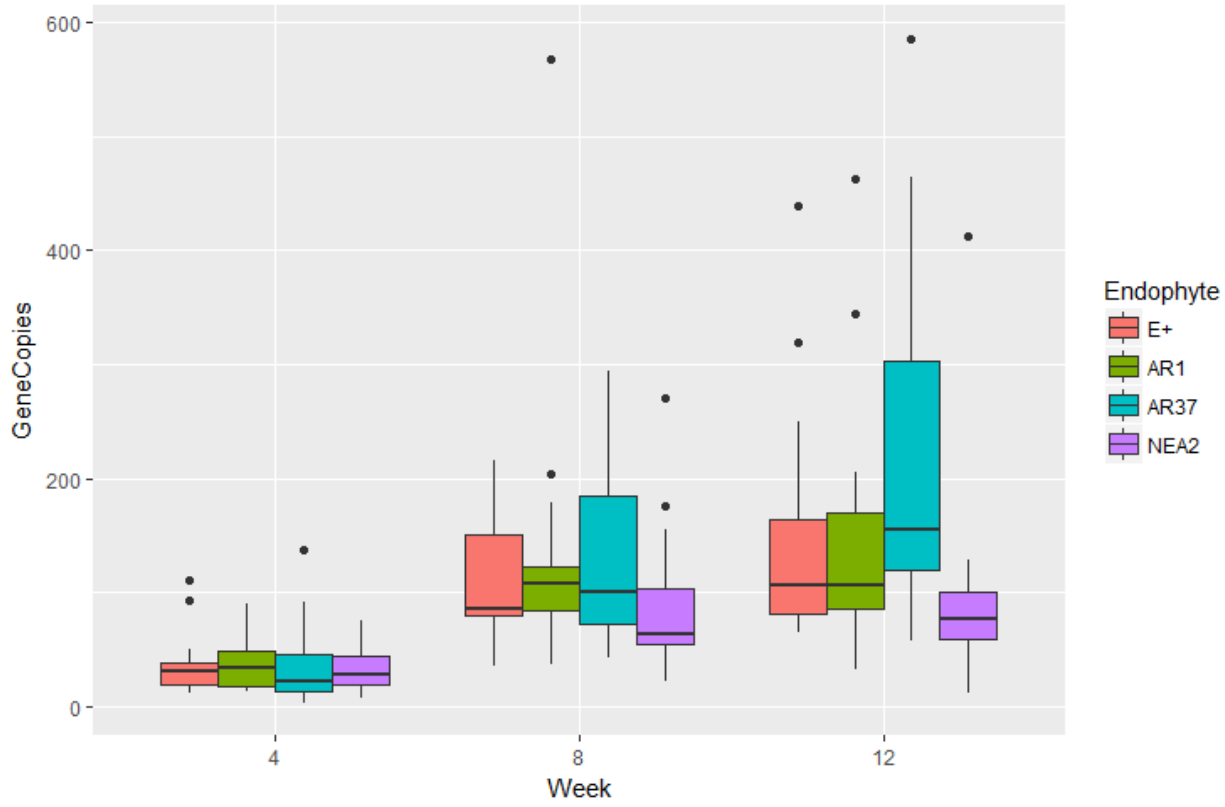


Figure 3. 2. Fungal endophyte concentrations at each sampling week for *Lolium perenne* plants infected with different strains of *Epichloë festucae* var. *lolii* endophyte. Sample sizes differed between the treatments (E+, n = 61; AR1, n = 63; AR37, n = 61; NEA2, n = 66).

I ran generalized linear mixed effects models with endophyte concentration to examine the relationship of endophyte concentration on plant growth. The regressions were significant for all plant growth parameters except root:shoot ratio; plant growth increased with endophyte concentration (Tillers: $F_{1,250} = 5.32$, $P = 0.0219$, $R^2_{adj} = 0.88$; Total Biomass: $F_{1,250} = 5.84$, $P = 0.0164$, $R^2_{adj} = 0.87$; Total Shoot Biomass: $F_{1,250} = 4.72$, $P = 0.0308$, $R^2_{adj} = 0.88$; Total Root Biomass: $F_{1,250} = 8.07$, $P = 0.0049$, $R^2_{adj} = 0.82$) (Table 3.2). As NEA2 treatments had the highest amount of biomass, but the lowest amount of endophyte concentration on average, I ran a separate linear regression to confirm a linear relationship of increasing growth with increasing endophyte concentration. All plant growth measurements for NEA2 were significantly correlated

with endophyte infection (Tillers: $F_{1,69} = 7.936$, $P = 0.1032$, $R^2 = 0.1032$; Total Biomass: $F_{1,69} = 14.32$, $P = 0.0003$, $R^2 = 0.1719$; Total Shoot Biomass: $F_{1,69} = 13.77$, $P = 0.0004$, $R^2 = 0.1664$; Total Root Biomass: $F_{1,69} = 14.31$, $P = 0.0003$, $R^2 = 0.1597$). Tiller number increased by 0.14, and total, shoot, and root biomass increased by 0.30 g, 0.29 g, and 0.31 g respectively, for every one-unit increase in gene copy.

Table 3. 2. Mixed Effects Mode summary of *Epichloë festucae* var. *lolii* concentration effects on growth of *Lolium perenne*.

Source	Tillers	Total DW	Root DW	Shoot DW	Root:Shoot DW
GeneCopies	***	***	***	***	***
	$F_{1,250} = 307.8$	$F_{1,250} = 329.9$	$F_{1,250} = 239.0$	$F_{1,250} = 337.8$	$F_{1,251} = 13.6$
Week	***	***	***	***	***
	$F_{2,250} = 717.0$	$F_{2,250} = 692.0$	$F_{2,250} = 442.3$	$F_{2,250} = 753.5$	$F_{2,251} = 8.7$
Endophyte	NS	NS	NS	NS	NS
Week:Endophyte	NS	NS	NS	NS	NS
Block	***	***	NS	***	NS
	$F_{2,250} = 66.9$	$F_{2,250} = 11.2$		$F_{2,250} = 16.2$	

Significant at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); not significant (NS); dry weight (DW)

Biomass

Endophyte presence and time significantly increased *L. perenne* biomass (Fig 3.3, Table 3.3, 3.4). Overall, plants infected with NEA2 had the highest total, root, and shoot biomass on average, while uninfected plants (E-) had the lowest. Not surprisingly, plant biomass increased significantly over time. NEA2 and E+ infected plants had significantly higher total, and shoot

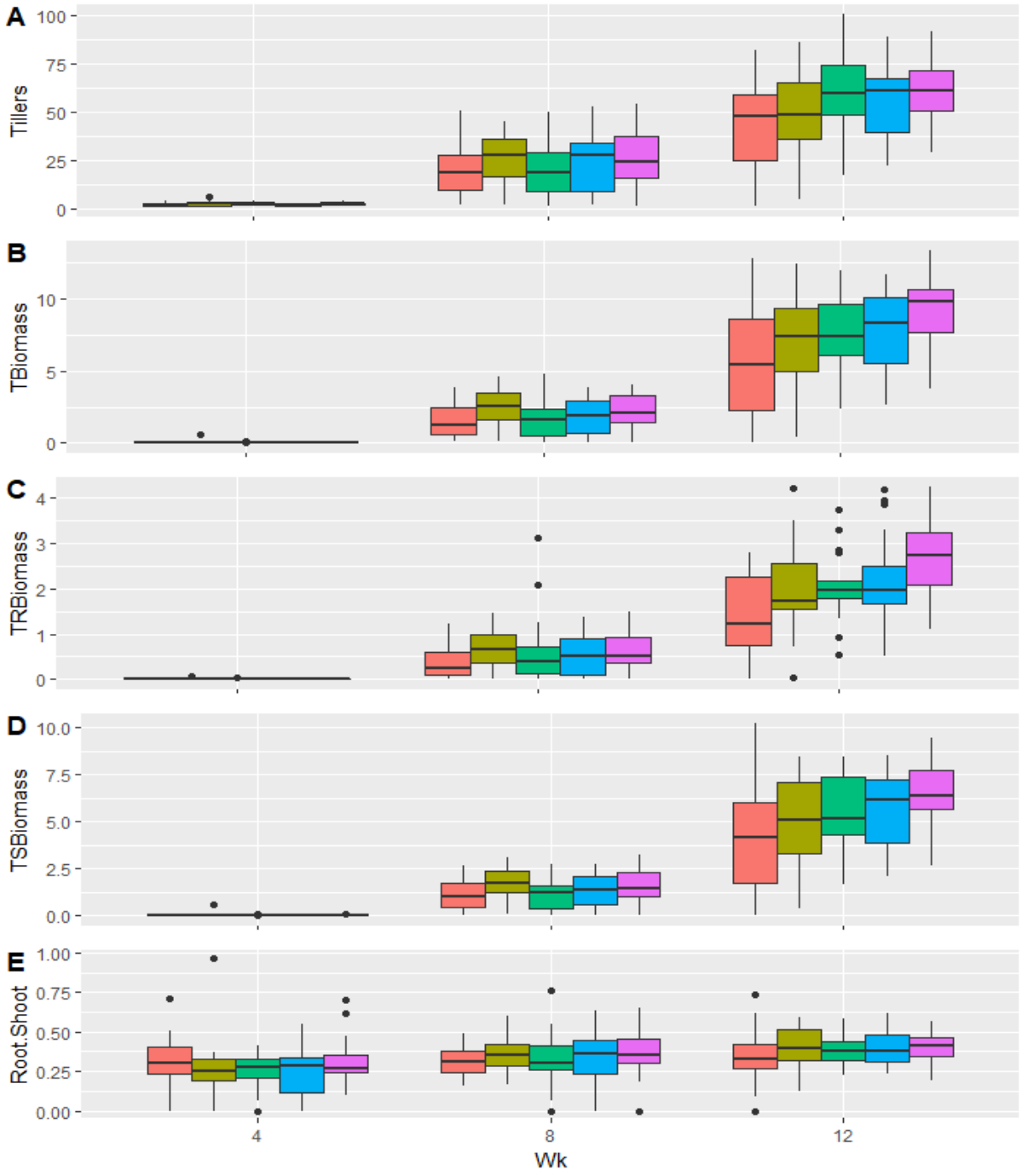


Figure 3. 3. Plant growth measurements at each sampling week for *Lolium perenne* either uninfected (E- (pink)), or infected with different strains of *Epichloë festucae* var. *lolii* endophyte (common toxic strain (E+ (olive); and novel strains (AR1 (green), AR37 (blue), and NEA2 (purple)). Growth measurements were taken for total number of tillers (A: E- n = 68, E+ n = 69, AR1 n = 69, AR37 n = 67, NEA2 n = 71), total dry weight biomass (g) (B: E- n = 67, E+ n = 68, AR1 n = 70, AR37 n = 67, NEA2 n = 70), total dry weight root biomass (g) (C), total dry weight shoot biomass (g) (D), and total dry weight root:shoot ratio (E). At week 12, E- is significantly different from AR1, AR37, and NEA2 for all biomass measurements except for root:shoot ratio.

Table 3. 3. Mixed Effects Model summary of *Epichloë festucae* var. *lolii* effects on growth of *Lolium perenne*.

Source	Tillers	Total DW	Root DW	Shoot DW	Root:Shoot DW	Gene Copies
Week	***	***	***	***	***	***
	$F_{2,342} = 874.9$	$F_{2,326} = 876.2$	$F_{2,324} = 639.5$	$F_{2,326} = 919.9$	$F_{2,326} = 15.7$	$F_{2,237} = 33.4$
Endophyte	**	***	***	***		*
	$F_{4,342} = 3.7$	$F_{4,326} = 5.1$	$F_{4,324} = 5.1$	$F_{4,326} = 5.0$	NS	$F_{3,237} = 3.8$
Week:Endophyte	*	*	*	*		
	$F_{8,342} = 2.1$	$F_{8,326} = 2.1$	$F_{8,324} = 2.0$	$F_{8,326} = 2.3$	NS	NS
Block	***	***	*	***		*
	$F_{2,342} = 66.1$	$F_{2,326} = 12.3$	$F_{2,324} = 4.3$	$F_{2,326} = 16.6$	NS	$F_{2,237} = 4.6$

Significant at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); not significant (NS); dry weight (DW)

Table 3. 4. The effects of *Epichloë festucae* var. *lolii* strains on *Lolium perenne* biomass. Least Squares Means ± SE for untransformed data. **Note**: NA = not-applicable, as a subset of E- samples indicated negligible endophyte infection.

	GeneCopies (ng^{-1} total genomic DNA)	Tiller #	Total Biomass (g)	Root Biomass (g)	Shoot Biomass (g)	Root:Shoot
Week						
4	35.76 ± 9.99	2.07 ± 1.13	1.05 ± 0.17	1.01 ± 0.06	1.04 ± 0.12	0.29 ± 0.03
8	106.23 ± 9.15	23.44 ± 1.13	2.96 ± 0.17	1.57 ± 0.05	2.38 ± 0.12	0.39 ± 0.03
12	129.98 ± 8.66	54.07 ± 1.10	8.47 ± 0.16	3.06 ± 0.05	6.41 ± 0.12	0.40 ± 0.02
Endophyte						
E-	NA	21.59 ± 1.46	3.40 ± 0.21	1.62 ± 0.07	2.78 ± 0.16	0.33 ± 0.03
E+	93.77 ± 10.99	25.95 ± 1.45	4.16 ± 0.21	1.89 ± 0.07	3.27 ± 0.16	0.35 ± 0.03
AR1	94.58 ± 10.69	28.09 ± 1.45	4.14 ± 0.21	1.89 ± 0.07	3.25 ± 0.16	0.39 ± 0.03
AR37	111.56 ± 10.83	27.66 ± 1.48	4.31 ± 0.22	1.93 ± 0.07	3.37 ± 0.16	0.34 ± 0.03
NEA2	62.73 ± 10.34	29.34 ± 1.42	4.80 ± 0.21	2.08 ± 0.07	3.71 ± 0.15	0.37 ± 0.03

biomass than E- plants. Whereas, NEA2, E+, and AR37 infected plants had significantly higher root biomass than E- plants. There was a significant week x endophyte interaction for all biomass measurements for week 12 samples. NEA2, AR37, and AR1 plants had significantly higher biomass measurements than E- plants at week 12. Although plant biomass was not significantly different between endophyte treatments between weeks 4 and 8, E+ and NEA2 consistently had the highest biomass, while E-, AR37, and AR1 treatments fluctuated. At week 4, total biomass and root biomass was E+>NEA2>E->AR37>AR1, and shoot biomass was E+>NEA2>AR37>E->AR1. At week 8 it was E+>NEA2>AR37>AR1>E- for all biomass measurements.

Metabolites in rhizosphere soil

To determine changes in the plant-fungal metabolome with different endophyte strains, an accurate-mass Q-TOF LC/MS-based analysis of metabolites in rhizosphere soil was performed. The goal was to obtain a general overview of metabolomic similarities and differences in the plant root exudates of 12 week old plants uninfected (E-) or infected with a common toxic strain (E+) or one of three novel strains (AR1, AR37, NEA2) of the fungal endophyte *E. festucae* var. *lolii* compared to controls (bare soil).

Feature extraction from raw data found 457 features from the positive ion data, and 537 features from the negative ion data.

Positive ion mode

An ANOVA showed that 34 of the 457 entities were significantly different at a corrected p-value (Benjamini Hochberg FDR) cut-off of 0.05 (Table 3.5). A fold change was applied to the 34 metabolites with significant differences. Table 4 lists the 34 metabolites found to be differentially-expressed in at least one of the endophyte treatments relative to the Control, with

Table 3. 5. Differentially expressed rhizosphere soil metabolites detected in positive ionization mode from *Lolium perenne* plants infected with strains of *Epichloë festucae* var. *lolii* (E+, AR1, AR37, and NEA2; each treatment with n = 3). The two columns of fold-change data are based on the abundance difference between 1) soils with no plants grown (Control) and individual treatment groups; and 2) uninfected (E-) plants and individual treatment groups. Log_2 fold-change data that is ≥ ± 1 represents significantly downregulated (red) or upregulated (blue) metabolites relative to endophyte free plants. Values in black show either downregulated (negative value) or upregulated (positive value) metabolites that are not significantly different than endophyte free plants.

						Fold-Change (Log_2) Relative to Control Soil					Fold-Change (Log_2) Relative to E-			
Tentative Compound Name & Classification	**MF [M + H]⁺**	**MW**	**RT**	**P(1)**	**p-value**	**E-**	**E+**	**AR1**	**AR37**	**NEA2**	**E+**	**AR1**	**AR37**	**NEA2**
Alkaloids														
Cassine	C18 H35 N O2	297.2636	10.57	-0.1824	3.37E-05	-4.54	4.49	-10.44	0.15	-4.64	9.03	-5.90	4.69	-0.10
Atalanine	C34 H30 N2 O9	610.1947	16.37	-0.2080	1.06E-05	5.83	5.72	-11.33	0.67	0.17	-0.11	-17.17	-5.16	-5.66
Carbohydrates & Derivatives														
Lychnose	C24 H42 O21	666.2218	0.63	0.0275	1.57E-02	1.58	-0.14	1.59	-3.14	1.92	-1.72	0.01	-4.72	0.35
Carboxylic Acids & Derivatives														
p-Hydroxytiaprofenic acid	C14 H12 O4 S	276.045	0.59	0.0349	8.48E-04	6.37	12.54	8.68	4.17	-3.15	6.17	2.31	-2.20	-9.52
Dopaxanthin	C18 H18 N2 O8	390.1059	12.68	-0.2151	1.11E-04	0.46	6.23	-11.81	0.87	0.25	5.77	-12.27	0.42	-0.21
Panose	C18 H32 O16	504.167	0.63	0.0825	1.74E-02	-9.90	0.48	1.54	-3.73	-4.11	10.38	11.43	6.17	5.79
Pteroyltriglutamic acid	C29 H33 N9 O12	703.2099	17.17	-0.2338	1.19E-05	6.44	6.29	-12.62	0.74	-6.00	-0.15	-19.06	-5.70	-12.44
Lipids & Lipid-like														
3-Hydroxy-3-methyl-glutaric acid	C6 H10 O5	144.0419	0.64	0.0575	2.34E-02	-4.55	-0.88	1.14	-4.21	1.37	3.67	5.69	0.35	5.92

Tentative Compound Name & Classification	MF [M + H]$^+$	MW	RT	P(1)	p-value	Fold-Change (Log_2) Relative to Control Soil					Fold-Change (Log_2) Relative to E-			
						E-	E+	AR1	AR37	NEA2	E+	AR1	AR37	NEA2
Lipids & Lipid-like (Cont.)														
(6β,7α,12β,13β)-7-Hydroxy-11,16-dioxo-8,14-apianadien-22,6-olide	C23 H28 O5	384.1968	11.84	-0.1837	1.47E-07	-4.98	4.73	-10.68	0.48	0.36	9.71	-5.70	5.46	5.34
MG(0:0/22:1(13Z)/0:0)	C25 H48 O4	412.3582	14.97	-0.2009	3.82E-07	0.16	4.95	-11.29	0.54	-5.34	4.79	-11.44	0.38	-5.50
4-O-Methylmelleolide	C24 H30 O6	414.2035	9.39	-0.0983	1.64E-06	-4.84	-5.99	-16.73	-10.74	-16.44	-1.15	-11.89	-5.90	-11.60
Longispinogenin	C30 H50 O3	458.3791	16.05	-0.0007	7.05E-06	17.32	15.82	16.36	16.85	16.91	-1.50	-0.97	-0.48	-0.41
Madlongiside D	C41 H66 O14	764.4405	15.82	-0.1706	1.65E-05	-5.26	3.73	-10.10	0.91	0.58	8.99	-4.84	6.17	5.84
Phenylpropanoids & Polyketides														
Cinnamic Acid	C9 H8 O2	148.052	10.56	0.0733	5.00E-02	-5.82	-1.94	-0.41	-5.65	-5.19	3.88	5.41	0.17	0.63
Farnesiferol A	C24 H30 O4	382.2151	15.50	-0.1903	3.82E-07	4.43	4.06	-10.77	0.67	0.52	-0.38	-15.20	-3.76	-3.91
Secondary Metabolites Produced by Other Organisms														
(22E, 24x)-Ergosta-4,6,8,22-tetraen-3-one	C28 H40 O	392.3095	16.43	-0.1895	2.12E-06	5.04	3.71	-11.08	-0.44	-0.50	-1.33	-16.12	-5.48	-5.54
Epothilone A	C26 H39 N O6 S	493.2462	10.28	-0.0021	4.81E-02	0.14	-1.12	0.06	0.50	0.26	-1.26	-0.09	0.36	0.12
Unknown														
	C4 H4 N3 O2	126.0317	0.64	-0.0143	3.50E-05	10.91	9.78	11.27	5.54	17.07	-1.13	0.36	-5.37	6.15
	C16 H10 N O2 S	280.0456	16.37	-0.1755	4.18E-05	-4.31	4.90	-9.86	0.59	-4.63	9.21	-5.54	4.90	-0.31
	C18 H12 N8 S	372.0946	16.37	-0.1886	1.65E-05	5.20	5.27	-10.17	0.72	0.12	0.07	-15.36	-4.48	-5.08
	C21 H32 O6	384.1968	12.92	-0.1865	1.47E-07	-5.18	4.68	-10.83	0.33	-5.11	9.86	-5.65	5.50	0.06

Tentative Compound Name & Classification	MF $[M + H]^+$	MW	RT	P(1)	p-value	Fold-Change (Log_2) Relative to Control Soil					Fold-Change (Log_2) Relative to E-			
						E-	E+	AR1	AR37	NEA2	E+	AR1	AR37	NEA2
Unknown (Cont.)														
	C22 H47 N5 O8	509.3521	11.94	-0.1579	1.91E-05	-0.11	4.37	-10.66	0.50	0.21	4.49	-10.54	0.61	0.32
	C24 H51 N11 O	509.4325	16.15	-0.1873	1.06E-05	5.54	5.89	-10.05	0.47	0.59	0.35	-15.60	-5.07	-4.96
	C37 H16 N3 O	518.1355	15.41	-0.2117	3.28E-04	1.36	6.71	-11.36	0.93	1.10	5.35	-12.72	-0.44	-0.26
	C43 H64 O	596.5051	16.42	-0.2027	2.08E-05	5.68	3.61	-11.71	-0.07	-0.06	-2.07	-17.39	-5.75	-5.73
	C27 H17 N17 O2	611.1801	17.25	-0.2299	2.33E-05	7.68	6.84	-12.12	1.38	0.61	-0.84	-19.80	-6.29	-7.07
	C31 H62 N10 O6	670.4844	13.45	-0.0890	1.20E-04	11.34	18.43	5.98	6.02	6.01	7.10	-5.36	-5.31	-5.32
	C31 H29 N19 S2	731.2408	17.17	-0.2065	1.00E-05	5.69	5.52	-11.09	0.66	-5.20	-0.17	-16.78	-5.02	-10.89
		801.3995	14.70	-0.1956	9.25E-05	1.65	5.30	-10.55	0.96	0.05	3.65	-12.20	-0.69	-1.60
		806.3538	14.70	-0.1946	1.18E-04	6.55	4.74	-10.25	0.85	-4.74	-1.81	-16.80	-5.71	-11.30
		878.28	17.82	-0.2143	7.63E-06	6.80	5.62	-11.67	0.43	-5.73	-1.18	-18.47	-6.37	-12.53
		917.5765	14.91	-0.2178	4.69E-06	6.78	5.57	-11.92	0.34	0.74	-1.21	-18.70	-6.44	-6.05
		1066.7449	16.15	-0.2330	1.12E-05	6.85	6.37	-12.43	1.08	-5.73	-0.48	-19.27	-5.77	-12.57
		1519.1152	16.52	-0.2137	2.33E-05	6.52	5.92	-11.84	0.39	-0.23	-0.60	-18.36	-6.13	-6.75

Molecular formula positive ion mode (MF $[M + H]^+$); retention time (RT); first principal component score (P(1)). Statistically significant (p-value < 0.05).

$p<0.05$ and fold-change ≥ 2.0. While the main differences were found in the up and downregulation of the metabolites, there were metabolites absent in treatment groups worth noting. Soils conditioned with AR1 infected plants lacked 73.5% of the metabolites identified. While, there were three metabolites absent from control soils (Longispinogenin, m/z 440.3702 RT 16.05; C31H62N10O6, m/z 670.4844 RT 13.45; C4H4N3O2, m/z 126.0317 RT 0.64), and there were two metabolites absent from NEA2 conditioned soils (p-Hydroxytiaprotenic acid, m/z 276.0450 RT 0.59; 4-O-Methylmelleolide, m/z 414.2035 RT 9.39). Whereas, all metabolites were present in E-, E+, and AR37 conditioned soils. Overall, 9% of metabolites were significantly upregulated when compared to controls, and only 2.9% when compared to E- conditioned soils. While 3% and were significantly downregulated in all soil conditioning treatments when compared to Controls, and 17.6% when compared to E- conditioned soils. Soils conditioned with E+ infected plants had the highest percentage of upregulated metabolites, 82% relative to Controls and 44% relative to E- conditioned soils. While NEA2 had the highest percentage of downregulated metabolites, 38% relative to Controls and 58.8% relative to E- conditioned soils. AR1 was technically higher, but this was due to the majority of metabolites being absent.

Principal component analysis (PCA) provides a visualization of the 34 entities recognized in 75% of samples from at least one endophyte treatment group. PCA findings did not show a full separation between treatment groups, however there is clustering by treatment groups with moderate overlap between groups (Fig. 3.4). Principal component 1 (PC1) explained 71.05% of the variation, PC2 explained 7.06%, and PC3 explained 5.61%. There were ten compounds that were responsible for the maximum variation in component 1 (Fig. 3.5).

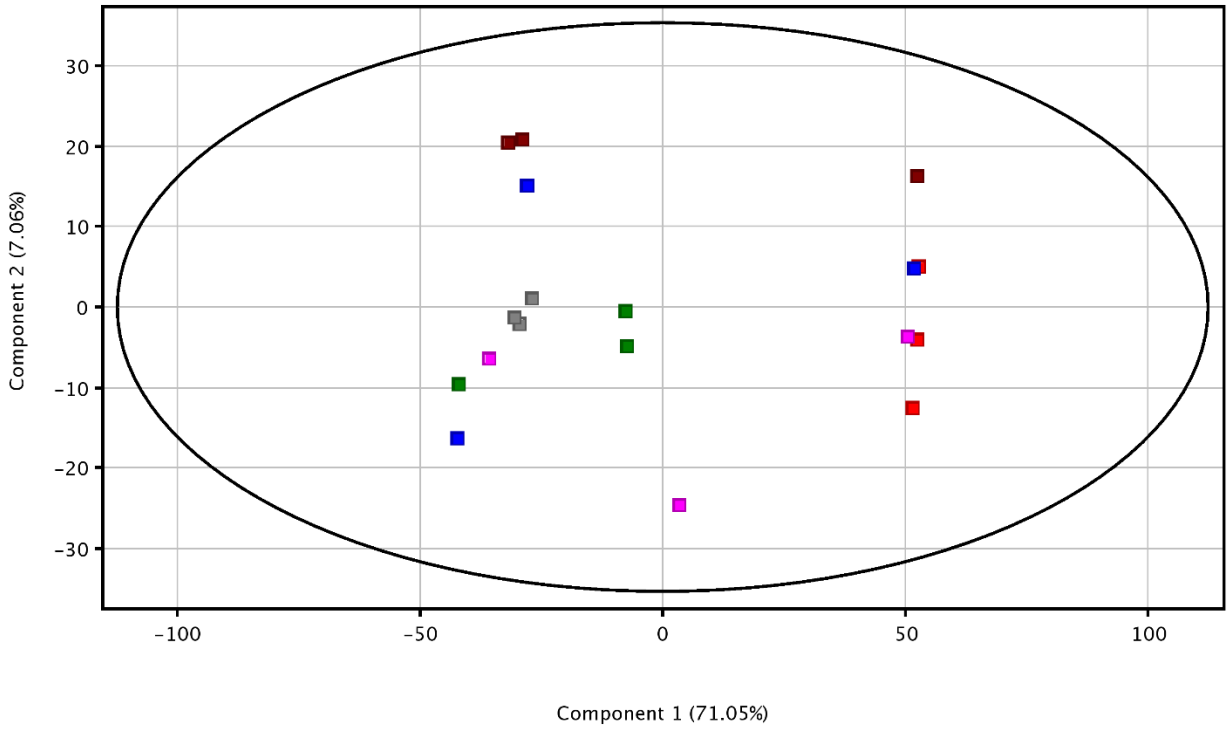
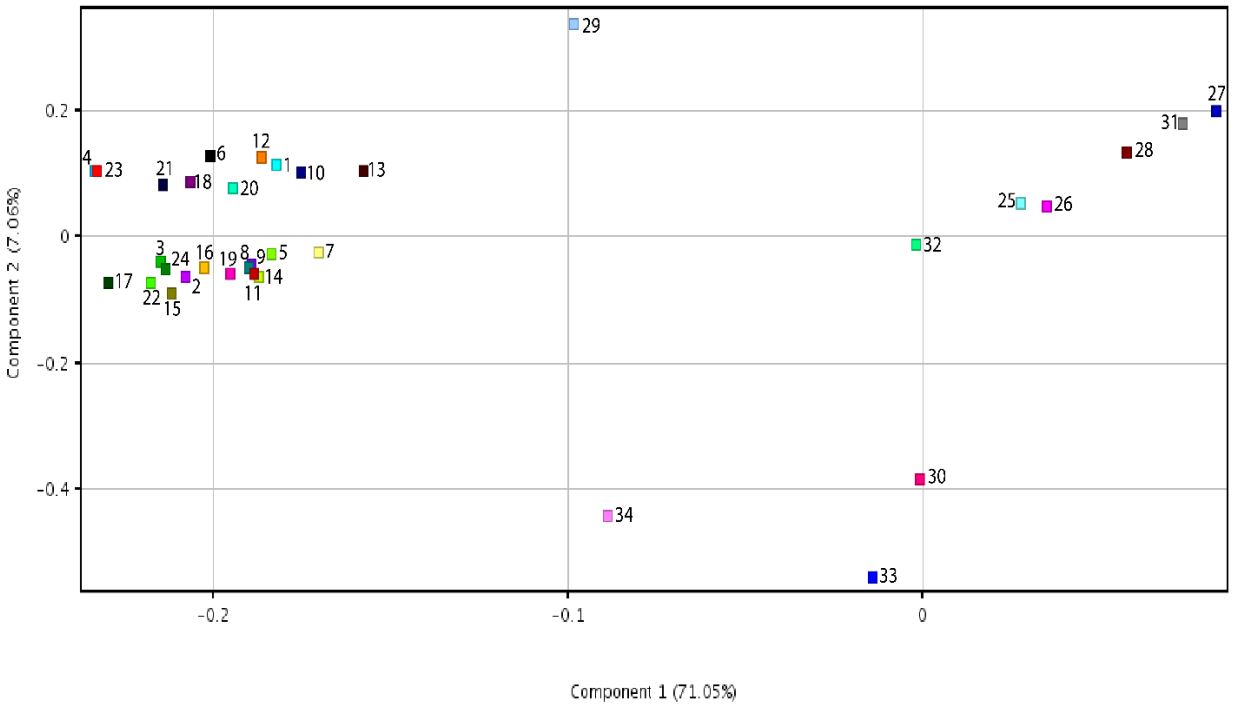


Figure 3. 4. Principal component analysis (PCA) performed on the positive ion mode metabolomic profiles of rhizosphere soil conditioned with *Lolium perenne* plants infected with strains of *Epichloë festucae* var. *lolii* endophyte (E+ (grey), AR1 (red), AR37 (blue), and NEA2 (pink)), *L. perenne* plants without infection (E- (green), and no plants present (Control (maroon)). This PCA is based on 34 metabolites found to be differentially-expressed in at least one of the endophyte treatments, with $p<0.05$ and fold-change >2.0. Each treatment n = 3.



Legend for loadings

1. Cassine	10. C16H10NO2S	19. m/z 801.3995	**28. 3-Hydroxy-3-methyl-glutaric acid**
2. Atalanine	11. C18H12N8S	20. m/z 806.3538	**29. 4-O-Methylmelleolide**
3. Dopaxanthin	12. C21H32O6	21. m/z 878.28	**30. Longispinogenin**
4. Pteroyltriglutamic acid	13. C22H47N5O8	22. m/z 917.5765	**31. Cinnamic acid**
5. (6β,7α,12β,13β)-7-Hydroxy-11,16-dioxo-8,14-apianadien-22,6-olide	14. C24H51N11O	23. m/z 1066.7449	**32. Epothilone A**
6. MG(0:0/22:1(13Z)/0:0)	15. C37H16N3O	24. m/z 1519.1152	**33. C4H4N3O2**
7. Madlongiside D	16. C43H64O	**25. Lychnose**	**34. C31H62N10O6**
8. Farnesiferol A	17. C27H17N17O2	**26. p-Hydroxytiaprofenic acid**	
9. (22E, 24x)-Ergosta-4,6,8,22-tetraen-3-one	18. C31H29N19S2	**27. Panose**	

Figure 3. 5. Positive ion mode principal component analysis loadings for component 1 (P(1)) and 2. Compounds in the legend that are bolded represent those responsible for the largest amount of variability in metabolites between treatment groups. The details for these compounds can be found in Table 5.

Hierarchical clustering of the data showed that endophyte treatments are separating into two groups with AR1 separating off from all other soil conditioning treatments. All other treatments separated into two groups with E-, and E+ forming one group and Control, AR37, and

NEA2 forming the other group (Fig. 3.6). Within the second grouping, Control and AR37 are more similar to each other than to NEA2. The majority of metabolites are similar between endophyte treatment groups, however there are a few high intensity metabolites. The metabolite 4-O-Methylmelleolide (m/z 414.2035 RT 9.39) had at least a 1.54 order of magnitude higher relative intensity in control soils than E-, E+, and AR37 conditioned soils, and is not present in AR1 or NEA2 conditioned soils (Fig. 3.7A). The metabolite C31H62N10O6 (m/z 670.4844 RT 13.448) has at least a 2.41 order of magnitude higher relative intensity in E+ than the other plant conditioned soils, and is not present in control soils (Fig. 3.7B). There are another three metabolites within E+ conditioned soils ((6β,7α,12β,13β)-7-Hydroxy-11,16-dioxo-8,14-apianadien-22,6-olide, m/z 384.1968 RT 11.835 (Fig. 3.8A); Cassine, m/z 279.2577 RT 13.845 (Fig. 3.8B); and C16H10NO2S, m/z 280.0456 RT 16.366 (Fig. 3.8C) at high intensity clustering together, while in the control, E-, AR37, and NEA2 these metabolites had low intensities, and in AR1 conditioned soils these metabolites were not present.

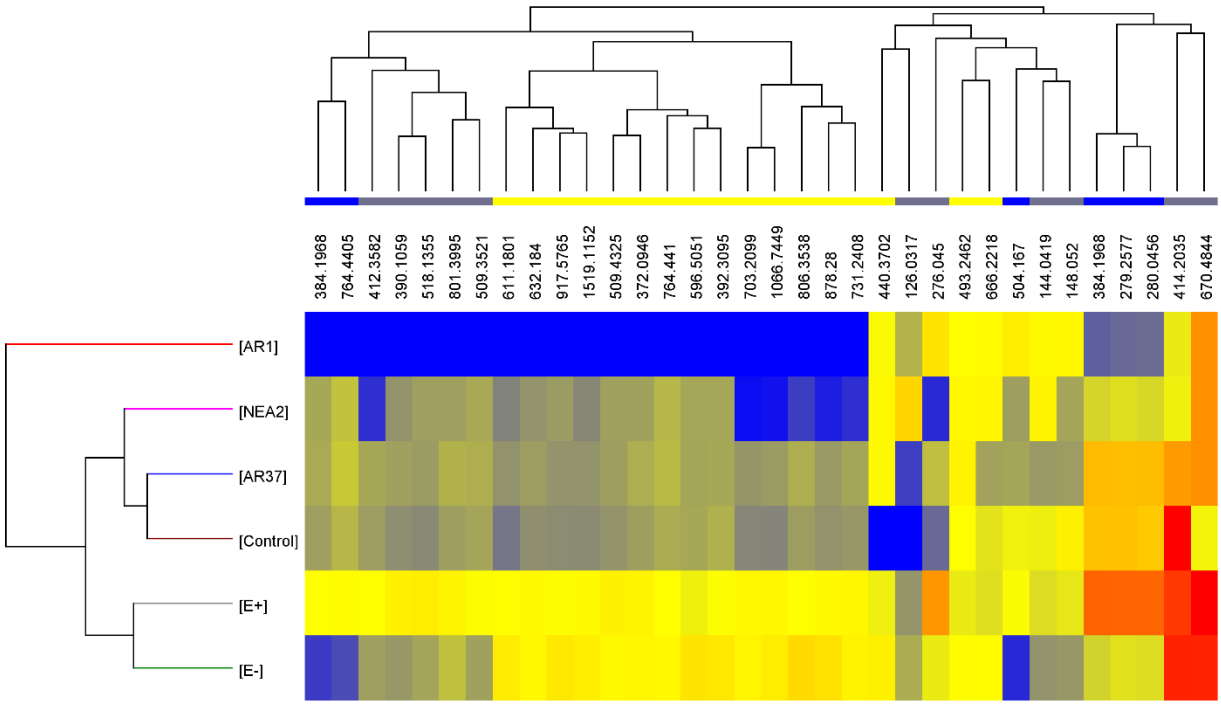


Figure 3. 6. Unsupervised hierarchical clustering of rhizosphere soil conditioned by *Lolium perenne* plants, obtained by LC-MS in the positive ion mode. These are the 34 metabolites found to be differentially-expressed ($p<0.05$ and fold-change >2.0). Metabolites are grouped by fungal endophyte treatments based on similar intensity profiles. Colours represent normalized intensity values (yellow is the centre, blue represents low intensity, red represents high intensity). Each treatment n = 3.

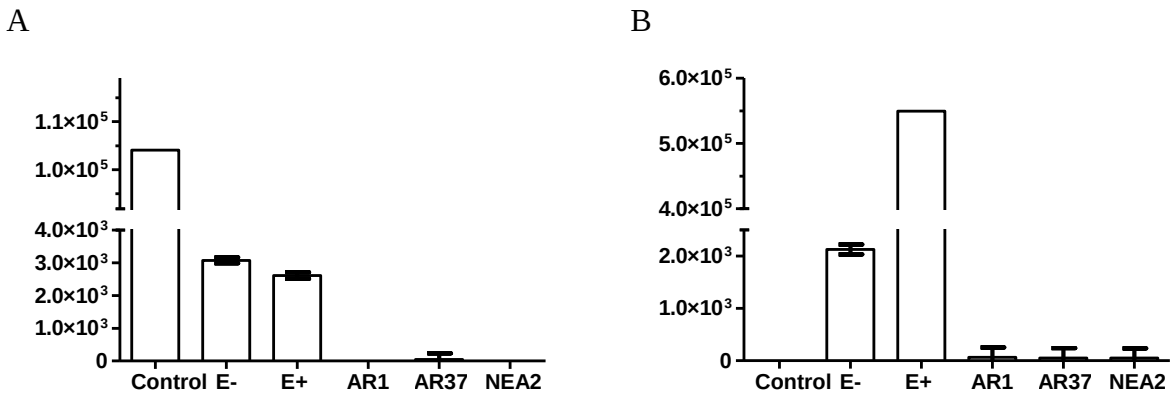


Figure 3. 7. Metabolites in positive ion mode clustering together in the hierarchical clustering map 4-O-Methylmelleolide (A); and C31H62N10O6 (B).

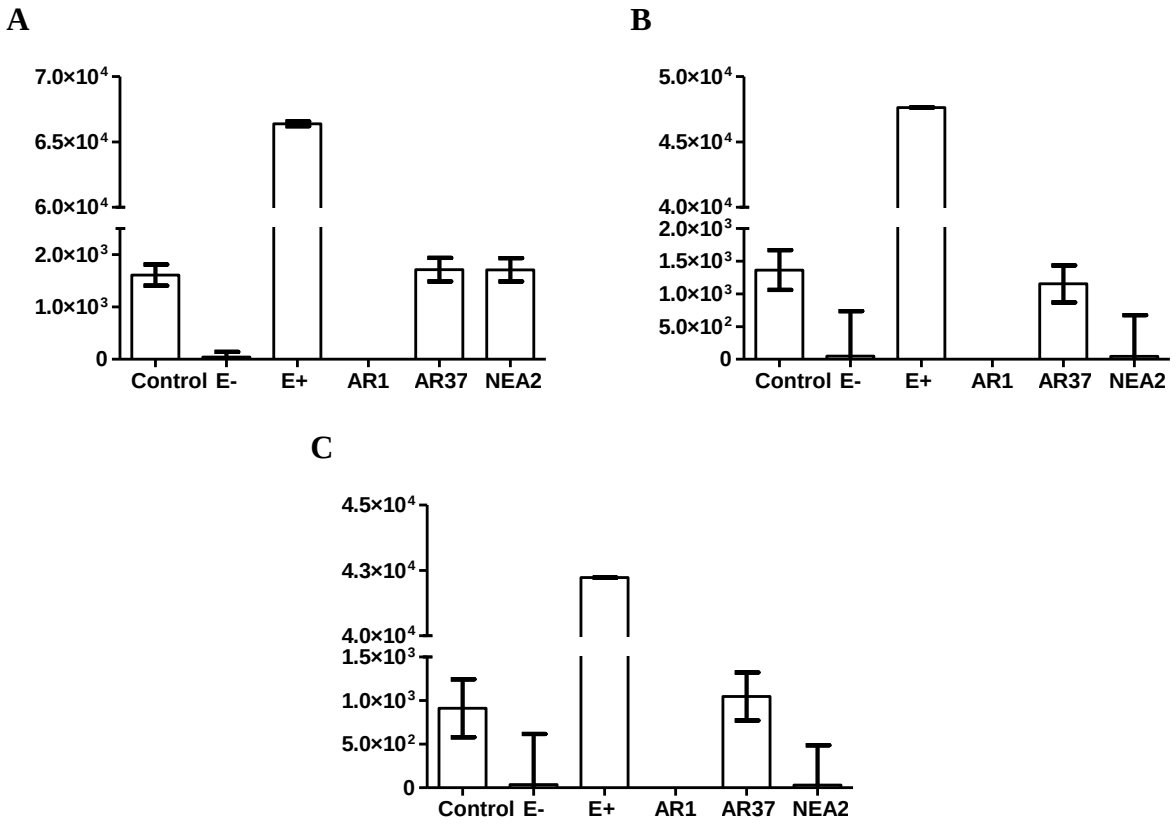


Figure 3. 8. E+ metabolites in positive ion mode with high relative intensities clustering together in the hierarchical clustering map: (6β,7α,12β,13β)-7-Hydroxy-11,16-dioxo-8,14-apianadien-22,6-olide, m/z 384.1968 RT 11.835 (A); Cassine, m/z 279.2577 RT 13.845 (B); and C16H10NO2S, m/z 280.0456 RT 16.366 (C).

Negative ion mode

An ANOVA with unequal variance found that 28 of 537 metabolites were significantly different at a corrected p-value (Benjamini Hochberg FDR) cut-off of 0.05. A fold change was applied to the 28 metabolites with significant differences. There were 21 entities that passed a fold change cut-off of ≥ 2.0 (Table 3.6). Overall, 42.9% of metablites were significantly upregulated in E-, 38% in E+, 19% in AR1, 23.8% in AR37, and 14.3% in NEA2, relative to the control soils. While, 23.8% of metabolites were significantly upregulated in E+, 9.5% in AR1,

28.6% in AR37, and 23.8% in NEA2 relative to E- conditioned soils. Across all soil conditioning treatments 9.5% of metabolites were significantly upregulated and 23.8% were significantly downregulated relative to control soils. While, only one metabolite was significantly upregulated concurrently across all soil conditioning treatments relative to E- treatments, and none were significantly downregulated.

Table 3. 6. Differentially expressed rhizosphere soil metabolites detected in negative ionization mode from *Lolium perenne* plants infected with strains of *Epichloë festucae* var. *lolii* (E+, AR1, AR37, and NEA2; each treatment with n = 3). The two columns of fold-change data are based on the abundance difference between 1) soils with no plants grown (Control) and individual treatment groups; and 2) uninfected (E-) plants and individual treatment groups. Log_2 fold-change data that is ≥ ± 1 represents significantly downregulated (red) or upregulated (blue) metabolites relative to endophyte free plants. Values in black show either downregulated (negative value) or upregulated (positive value) metabolites that are not significantly different than endophyte free plants.

Tentative Compound Name & Classification	MF [M - H]$^-$	MW	RT	P(1)	p-value	Fold-Change (Log_2) Relative to Control Soil					Fold-Change (Log_2) Relative to E-			
						E-	E+	AR1	AR37	NEA2	E+	AR1	AR37	NEA2
Amino Acids, Peptides, & Proteins														
Glycyl-Tyrosine	C11 H14 N2 O4	238.0967	0.61	-0.3075	2.00E-02	7.01	9.86	4.93	2.72	-3.16	2.85	-2.08	-4.29	-10.17
Carboxylic Acids & Derivatives														
Phaseolic acid	C13 H12 O8	296.0552	0.61	-0.4418	1.73E-03	13.63	16.47	8.60	6.84	3.57	2.84	-5.03	-6.79	-10.06
Fumonisin B3	C34 H59 N O14	751.3925	15.06	-0.0345	8.84E-03	1.27	1.00	0.65	0.01	0.31	-0.27	-0.63	-1.27	-0.96
Secondary Metabolites Produced by Other Organisms														
CE-108	C37 H57 N O14	785.3814	15.49	0.0422	4.93E-02	-1.01	-0.65	-0.71	0.34	0.38	0.36	0.29	1.35	1.39
Terpenes														
Betavulgaroside IV	C41 H62 O15	794.4078	14.87	-0.0325	4.23E-02	1.17	0.87	0.18	0.06	0.09	-0.30	-0.99	-1.11	-1.08
Shiromodiol diacetate	C19 H30 O5	338.2507	9.07	-0.0333	3.19E-02	0.34	2.47	-1.17	-0.34	0.11	2.13	-1.51	-0.67	-0.23
Unknown														
	C8 H15 Cl3 N2 O3 S4	419.8992	0.56	0.0181	1.85E-02	-1.03	-1.58	-1.12	-1.42	-1.21	-0.55	-0.09	-0.39	-0.18

Tentative Compound Name & Classification	MF [M - H]$^-$	MW	RT	P(1)	p-value	Fold-Change (Log_2) Relative to Control Soil					Fold-Change (Log_2) Relative to E-			
						E-	E+	AR1	AR37	NEA2	E+	AR1	AR37	NEA2
Unknown (Cont.)														
	C38 H37 O2	525.2816	11.74	-0.0537	3.84E-02	1.52	1.70	0.52	-0.38	0.14	0.18	-1.01	-1.90	-1.38
	C20 H5 Cl N O9 S3	533.8785	0.56	0.0203	2.00E-02	-1.18	-1.80	-1.34	-1.55	-1.40	-0.62	-0.16	-0.37	-0.22
		535.8776	0.56	0.0209	3.02E-02	-1.09	-1.79	-1.18	-1.35	-1.30	-0.69	-0.09	-0.25	-0.21
	C16 H36 N8 O13 S	580.2107	0.63	-0.3905	6.69E-05	17.37	19.35	15.46	14.00	8.99	1.98	-1.91	-3.36	-8.38
		663.8351	0.56	0.0234	2.00E-02	-1.43	-2.12	-1.43	-1.83	-1.61	-0.69	0.00	-0.39	-0.18
	C54 H51 N O	729.3955	14.90	0.0203	1.04E-02	-0.16	0.35	0.11	1.22	0.52	0.51	0.27	1.38	0.68
		751.3785	15.51	0.4844	2.59E-05	-5.13	-5.26	0.24	10.48	10.66	-0.13	5.37	15.61	15.79
		753.3923	15.06	-0.0413	1.54E-02	1.42	1.18	0.67	-0.10	0.30	-0.24	-0.75	-1.52	-1.12
		777.4179	14.88	-0.0251	1.15E-02	1.09	0.95	0.22	0.23	0.33	-0.14	-0.88	-0.87	-0.76
		777.8178	0.56	0.0229	5.66E-04	-1.41	-1.94	-1.49	-1.89	-1.50	-0.54	-0.08	-0.48	-0.10
		783.3825	15.50	0.0394	3.02E-02	-1.02	-0.52	-0.71	0.42	0.32	0.51	0.31	1.44	1.34
		806.3896	15.50	0.0454	4.17E-02	-1.17	-0.67	-0.74	0.28	0.39	0.50	0.43	1.45	1.56
		833.3483	15.07	-0.0622	2.69E-02	2.14	1.80	1.25	-0.02	0.52	-0.34	-0.89	-2.16	-1.62
		865.3356	15.50	0.5501	5.53E-06	-16.71	-11.42	-6.00	0.38	0.27	5.29	10.71	17.08	16.98

Molecular formula negative ion mode (MF [M - H]$^-$); retention time (RT); first principal component score (P(1)). Statistically significant (p-value < 0.05).

Principal component analysis (PCA) provides a visualization of the 21 entities recognized in 75% of samples from at least one endophyte treatment group. PCA findings showed separation between treatment groups, however there is clustering by treatment groups with some overlap between groups (Fig. 3.9). Principal component 1 (PC1) explained 65.09% of the variation, PC2 explained 17.61%, and PC3 explained 7.89%.

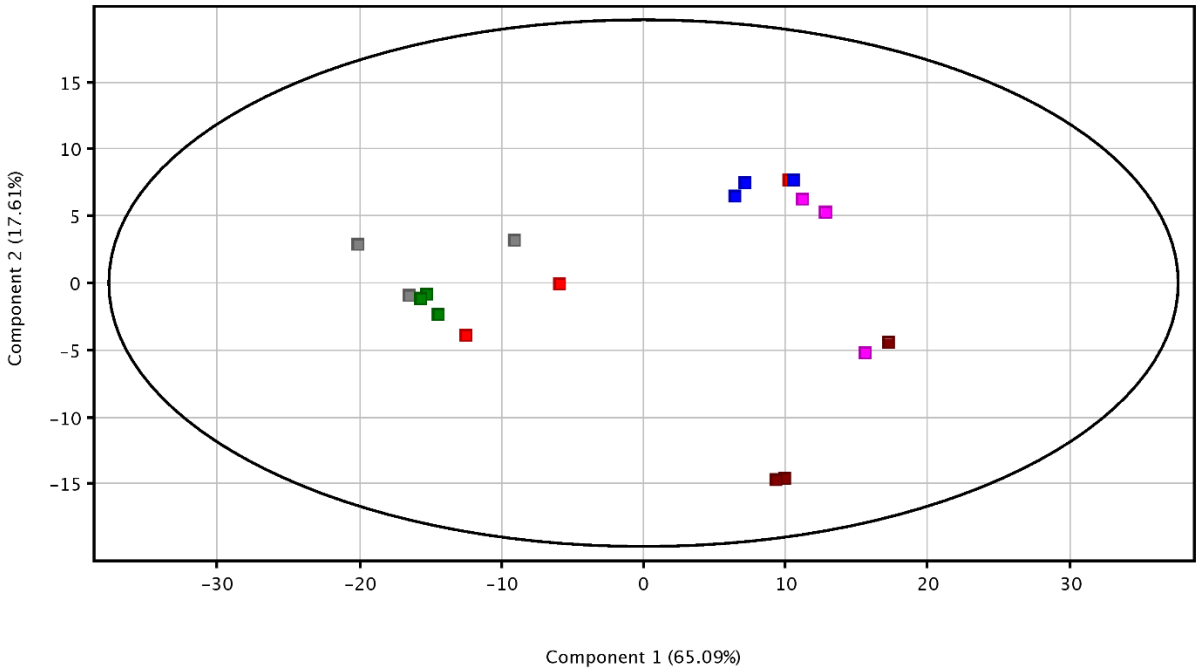


Figure 3. 9. Principal component analysis (PCA) performed on the negative ion mode metabolomic profiles of rhizosphere soil conditioned with *Lolium perenne* plants infected with strains of *Epichloë festucae* var. *lolii* endophyte (E+ (grey), AR1 (red), AR37 (blue), and NEA2 (pink)), *L. perenne* plants without infection (E- (green), and no plants present (Control (maroon)). This PCA is based on 21 metabolites found to be differentially-expressed in at least one of the endophyte treatments, with $p<0.05$ and fold-change >2.0. Each treatment n = 3.

Hierarchical clustering of the data showed that endophyte treatments separated into two groups with E- , E+, and AR1 forming the first group and Control, AR37, and NEA2 forming the second group (Fig. 3.10). Within the first grouping, E- and E+ were more similar to each other

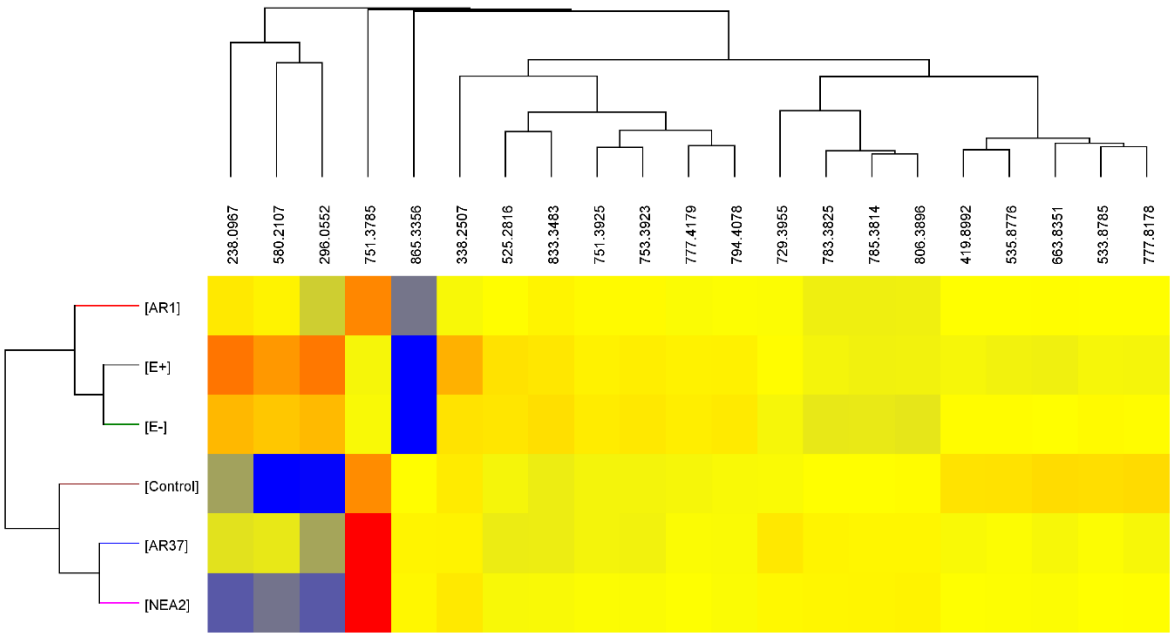


Figure 3. 10. Unsupervised hierarchical clustering of rhizosphere soil conditioned by *Lolium perenne* plants, obtained by LC-MS in the negative ion mode. These are the 21 metabolites found to be differentially-expressed ($p<0.05$ and fold-change >2.0). Metabolites are grouped by fungal endophyte treatments based on similar intensity profiles. Colours represent normalized intensity values (yellow is the centre, blue represents low intensity, red represents high intensity). Each treatment n = 3.

than to AR1. Within the second grouping, AR37 and NEA2 were more similar to each other than to Control. Clear differences between treatment groups can be seen for five metabolites. Three of the metabolites clustered together and had the highest relative intensities in the E+ conditioned soils (Glycyl-Tyrosine, m/z 238.0967 RT 0.605 (Fig. 3.11A); C16H36N8O13S, m/z 580.2107 RT 0.63 (Fig. 3.11B); and Phaseolic acid, m/z 296.0552 RT 0.606 (Fig. 3.11C)). The other two metabolites separated out independently. The first metabolite (Unidentified, m/z 751.3785 RT 15.512 (Fig. 3.12A)) had the highest relative instensities in the NEA2 and AR37 condition soils, with low relative intensities found in the control soils and the AR1 conditioned soils, but was not

present in E- or E+ conditioned soils. The second metabolite (Unidentified, m/z 865.3358 RT 15.498 (Fig. 3.12B)) had the highest relative intensities in the AR37 and NEA2 conditioned soils, with low relative intensities in E+ and AR1 conditioned soils, but was not present in E- conditioned soils.

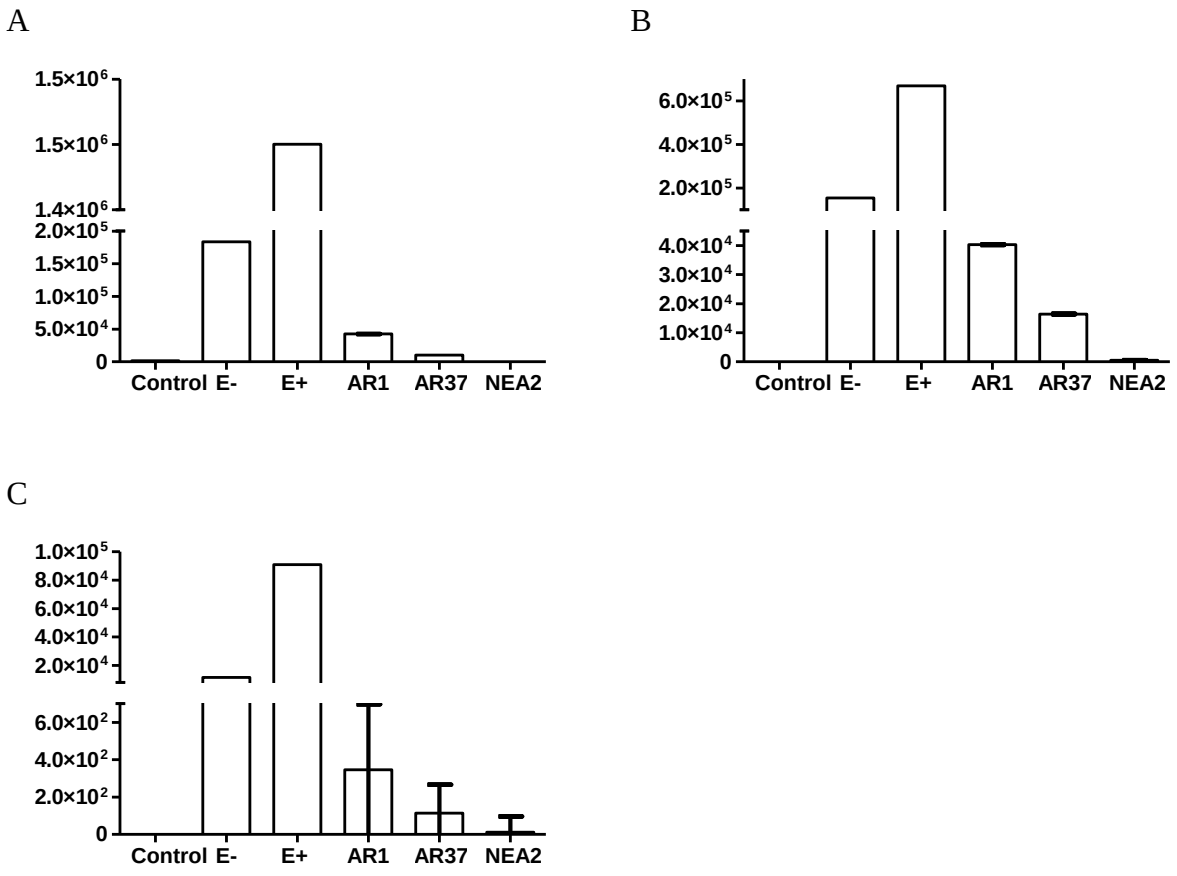
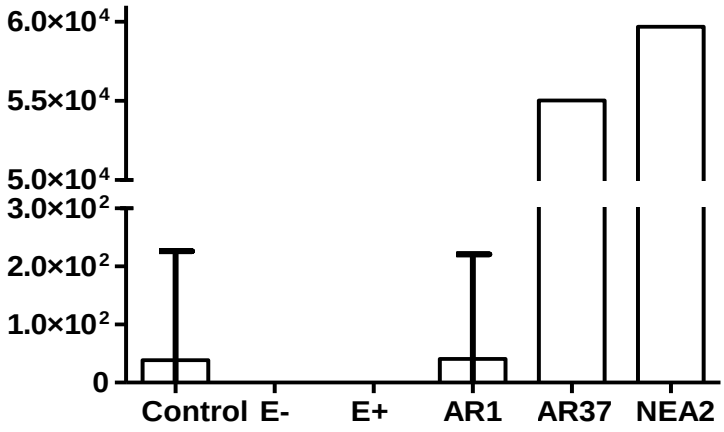


Figure 3. 11. E+ metabolites in negative ion mode with high relative intensities clustering together in the hierarchical clustering map: Glycyl-Tyrosine, m/z 238.0967 RT 0.605 (A); C16H36N8O13S, m/z 580.2107 RT 0.63 (B); and Phaseolic acid, m/z 296.0552 RT 0.606 (C).

A



B

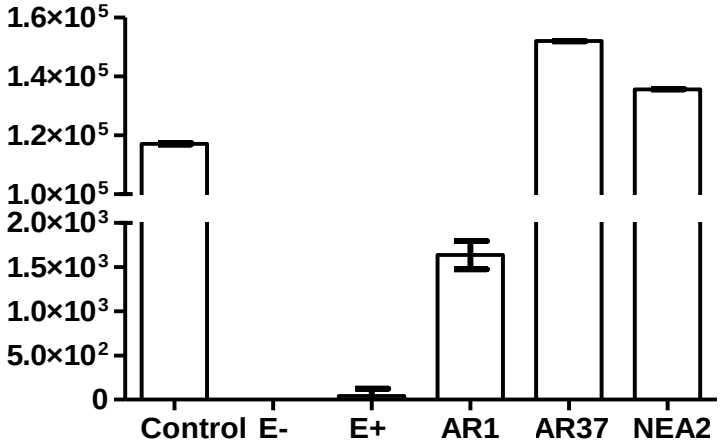


Figure 3. 12. Negative metabolites: Unidentified, m/z 751.3785 RT 15.512 (A); and Unidentified, m/z 865.3358 RT 15.498 (B).

Microbial community in the rhizosphere

After sequence processing; 2,949,321 and 746,296 sequences remained which were binned into 13,159 and 3,901 different OTUs at the 0.03 dissimilarity level for 16S and ITS respectively.

Bacterial community

A total of 27 phylum made up the 16S OTUs. One third of the phyla accounted for 70% of the total OTUs, the other 2/3rd of the phyla accounted for 5%, while 25% of the OTUs remained unclassified to phylum (Fig. 3.13). The phylum with the largest abundance contributions were Proteobacteria (26.85%), Planctomycetes (11.17%), Actinobacteria (6.63%), Bacteroides (6.45%), Acidobacteria (5.12%), Verrucomicrobia (3.72%), Chlamydiae (3.48%), Parcubacteria (3.42%), and Firmicutes (2.31%). The most influential phyla contributing to the differences between treatment groups were Actinobacteria, Proteobacteria, Acidobacteria, Planctomycetes, and unclassified.

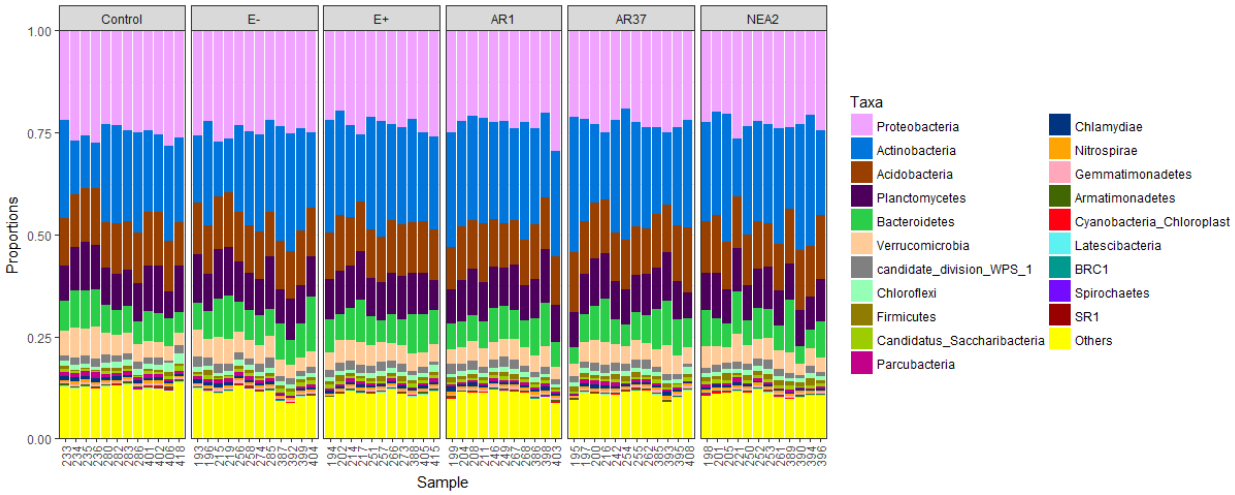


Figure 3. 13. Bacterial community composition based on relative abundance for each soil conditioning treatment by phylum. All replications included: Control, n = 12; E-, n = 12; E+, n = 11; AR1, n = 11; AR37, n = 12; NEA2, n = 12.

A PERMANOVA found that soil conditioning treatments significantly effected bacterial community abundance ($p < 0.005$) (Table 3.7). Pairwise PERMANOVA analyses showed that control samples were significantly different than AR1 and NEA2 soil conditioning treatments, and had strong differences from E+ and AR37 ($p = 0.06$), but no endophyte conditioned soils were significantly different from each other (Table 3.8). In both AR1 and NEA2, Actinobacteria had higher abundances than Control soils. Pairwise comparisons show that there were also other differences in average abundances for the other soil treatment groups, though these were not significant. E- and E+ were most similar to AR37, and AR1, AR37, NEA2, and Control were most similar to E-. AR1 consistently had the highest dissimilarity to all soil conditioning

Table 3. 7. Results of PERMANOVA analysis of Bray-Curtis dissimilarities in bacterial and fungal community structure in relation to soil conditioning treatment, plant biomass, soil moisture, and endophyte concentration, Pseudo-F = F value by permutation, bold font indicates statistical significance at corrected $p<0.05$, p-values based on 999 permutations.

	Bacteria			Fungi		
	Pseudo-F	R2	p-value	Pseudo-F	R2	p-value
Endophyte	2.7639	0.13368	**0.005**	1.4039	0.07723	**0.036**
TRBiomass	16.7025	0.16157	**0.001**	1.5037	0.01654	0.338
PC	2.9262	0.05661	**0.043**	1.5481	0.03406	0.327
Block	5.9603	0.05766	**0.002**	17.2725	0.19003	0.215

Table 3. 8. Pairwise PERMANOVA comparisons of different endophyte statuses on the composition of bacterial and fungal communities. Corrected p-value (Benjamini Hochberg FDR) cut-off of 0.05.

Endophyte Comparisons	Bacteria P value	Fungi P value
E-, E+	0.5100	0.7961
E-, AR37	0.4328	0.4050
E- vs NEA2	0.2200	0.6232
E- vs AR1	0.2200	0.6232
E-, Control	0.4444	0.6232
E+, AR37	0.9820	0.4650
E+, NEA2	0.9210	0.6232
E+, AR1	0.9820	0.6232
E+, Control	0.0600	0.6232
AR37, NEA2	0.9820	0.8500
AR37, AR1	0.9820	0.7961
AR37, Control	0.0600	0.2700
NEA2, AR1	0.9820	0.8500
NEA2, Control	**0.0300**	0.6232
AR1, Control	**0.0300**	0.6232

treatments. E- conditioned soil had higher abundances for all phyla than E+ and AR37, except for the phylum Firmicutes. Overall, E- conditioned soil had lower abundances of the phylum Firmicutes than all other soil conditioning treatments. NEA2 conditioned soil had higher abundances, on average, than E- for 70% of phylum. Between E- and AR1, 43% of phyla had higher abundances in E- and 57% of phyla had higher abundances in AR1. The majority of high abundance taxa were higher in AR1 than in E-. Control samples had higher abundances than E- for all phyla except Bacteroidetes and Candidatus Saccaribacteria.

A PERMANOVA also found bacterial abundance to be significantly effected by root biomass (Table 3.7). Correlation tests using the Pearson coefficient found that the direction of the relationship differed depended on the phylum. Actinobacteria and Acidobacteria abundance increased as root biomass increased (r = 0.5137, 0.229). While, Proteobacteria, Planctomycetes,

and Unidentified species decreased in abundance as root biomass increased (r = -0.5199, -0.1691, -0.5800).

The NMDS ordination for bare soil (control) and soil conditioning treatments (E-, E+, AR1, AR37, NEA2) produced a 2 dimensional solution with a final stress of 0.12 after 45 iterations (Fig. 3.14). The ordination analysis showed no clear differences in bacterial communities between treatment groups, except for AR1 whose 95% ellipses was out of line with the other ellipses. A PERMANOVA was also run, without including control samples, to determine the effect of plant growth parameters and soil moisture on bacterial community abundance. Tiller number, total root biomass, and soil moisture all significantly effected bacterial community abundance (Table 3.9). A CCA plot was generated to visualize the relationship between significant environmental variables and the soil conditioning treatment groups without the control groups present (Fig. 3.15).

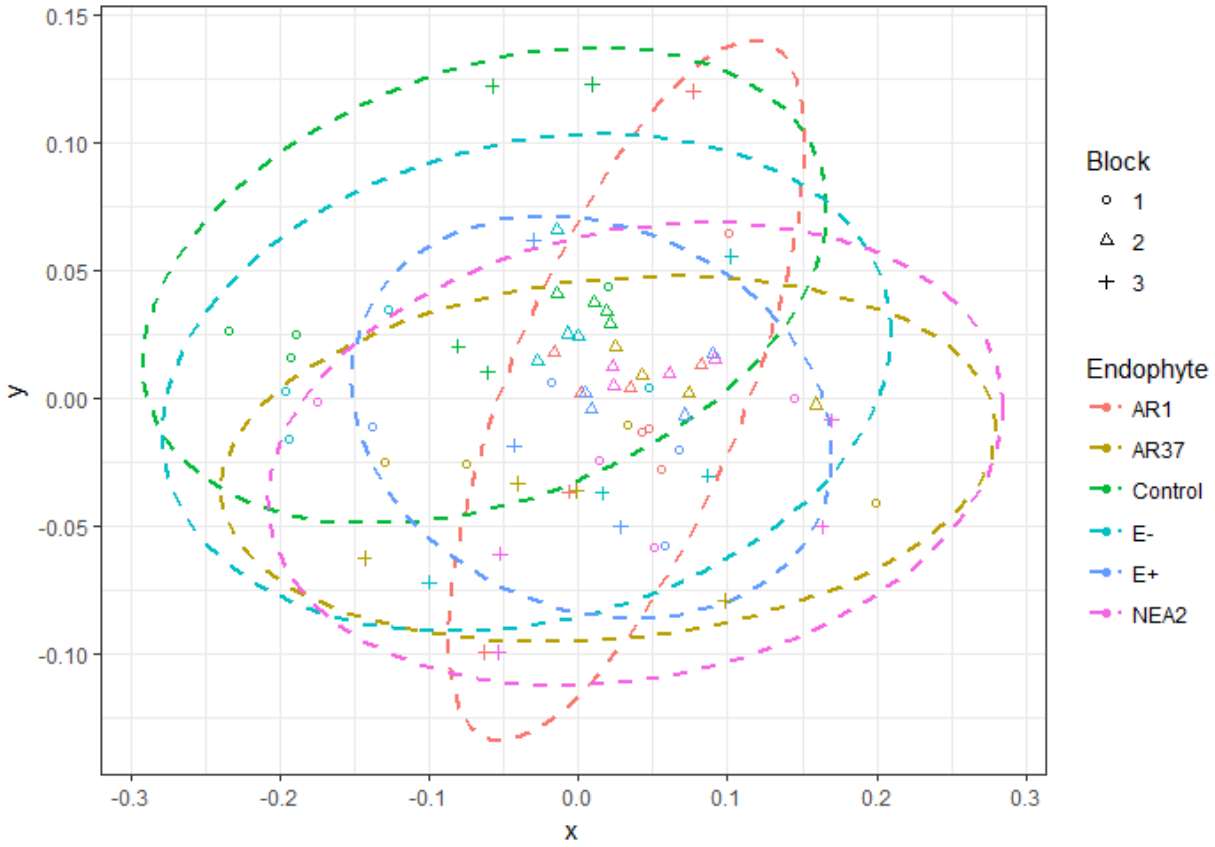


Figure 3. 14. Non-metric multidimensional scaling (NMDS) of bacterial community composition for all soil conditioning treatments based on Bray-Curtis distance matrix of all OTUs at the 0.03 dissimilarity level. Ellipses based on 95% confidence. Dimensions = 2, Stress = 0.12.

Table 3. 9. Summary of PERMANOVA analysis of Bray-Curtis dissimilarities in bacterial community structure in relation to soil conditioning treatment, plant biomass, soil moisture, and endophyte concentration, Pseudo-F = F value by permutation, bold font indicates statistical significance at corrected $p<0.05$, p-values based on 999 permutations.

	Pseudo-F	R2	p-value
Endophyte	1.2431	0.07323	0.245
Tillers	3.8325	0.05644	**0.019**
Total root biomass	8.2384	0.12133	**0.002**
Total shoot biomass	0.5650	0.00832	0.634
Root:Shoot	0.7356	0.01083	0.523
Soil moisture	3.1206	0.04596	**0.032**
Gene copies	0.4368	0.00643	0.726

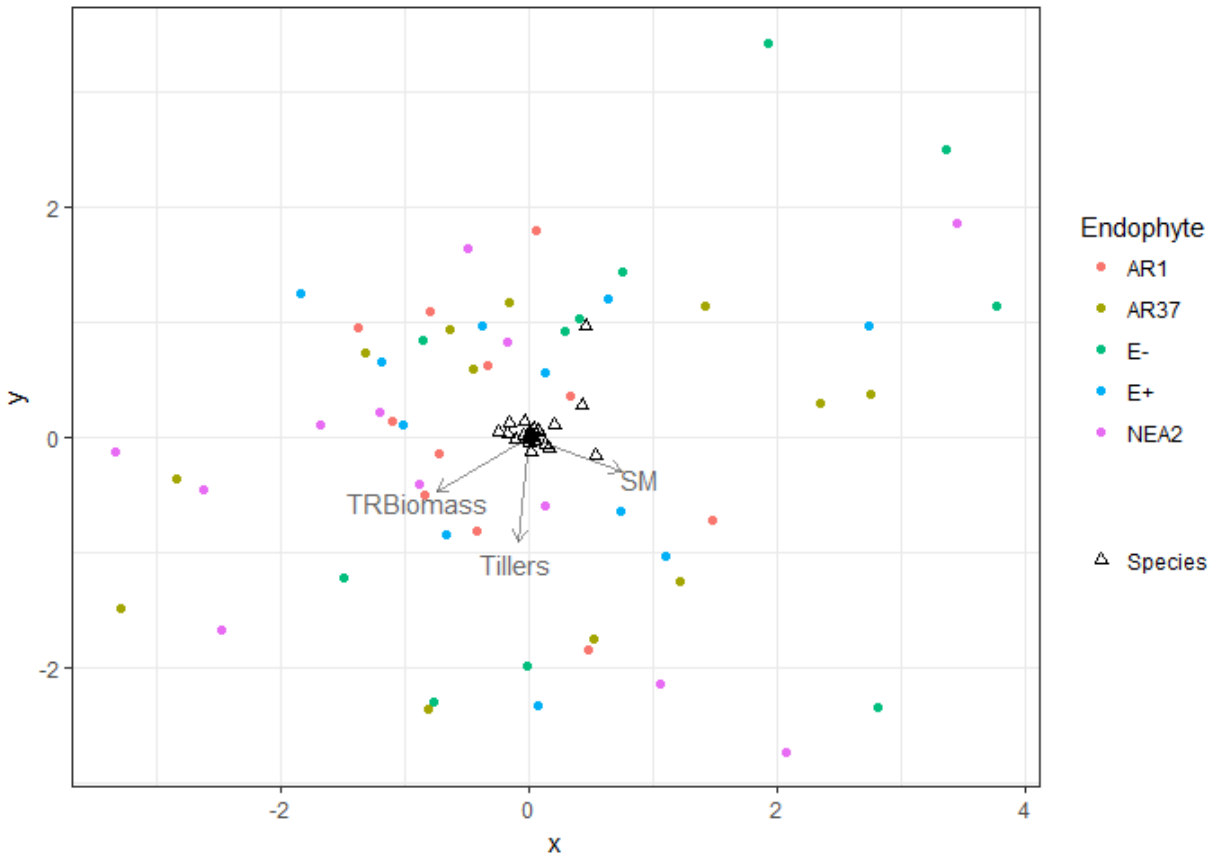


Figure 3. 15. Constrained correspondence analysis (CCA) plot indicating significant environmental variables that best describe the community structure of bacterial communities for all soil conditioning treatments based on Bray-Curtis distance matrix of all OTUs at the 0.03 dissimilarity level. Significant environmental variables are indicated by vectors with arrow direction indicating increasing gradients and length indicating strength of association.

Presence/absence differences between soil conditioning treatments were not observed at the phylum or class level, however they could be seen at the order level (Table 3.10). The absence of taxa at the order level did not follow any consistent patterns within or between treatment groups.

Table 3. 10. Summary of bacterial taxa, classified to order, absent from at least one soil conditioning treatment category. The "X" represents where no sequence reads were found in these orders for a particular treatment.

	Control	E-	E+	AR1	AR37	NEA2
Armatimonadetes gp2	X	X		X		X
Deinococcales	X		X	X	X	X
Enterobacteriales			X	X	X	
Gammaproteobacteria incertae sedis		X		X	X	
Halanaerobiales	X		X	X		
Haloplasmatales		X	X		X	
Ktedonobacterales					X	
Lactobacillales		X	X	X	X	X
Thermoanaerobacterales					X	
Thiotrichales	X	X		X		X
Unclassified Cyanobacteria/Chloroplast		X				
WPS-2 genera incertae sedis	X		X	X		X
Xiphinematobacter	X		X			
ZB3 genera incertae sedis		X	X	X	X	X

Fungal community

The fungal communities consisted of members of five phyla, in ascending order of abundance, including Ascomycota (51.82%), Zygomycota (18.07%), Basidiomycota (11.10%), Chytridiomycota (4.35%), and Glomeromycota (1.68%). Unclassified taxa made up 12.98% of sequence abundance. There was very little difference in abundance between treatment groups (Fig. 3.16). However, the highest abundance within a phylum varied between treatment groups, with E+ conditioned soils having the lowest abundances for all phylum except the unclassified taxa. Clearer differences were observed at the class level (Fig. 3.17), though also not significant.

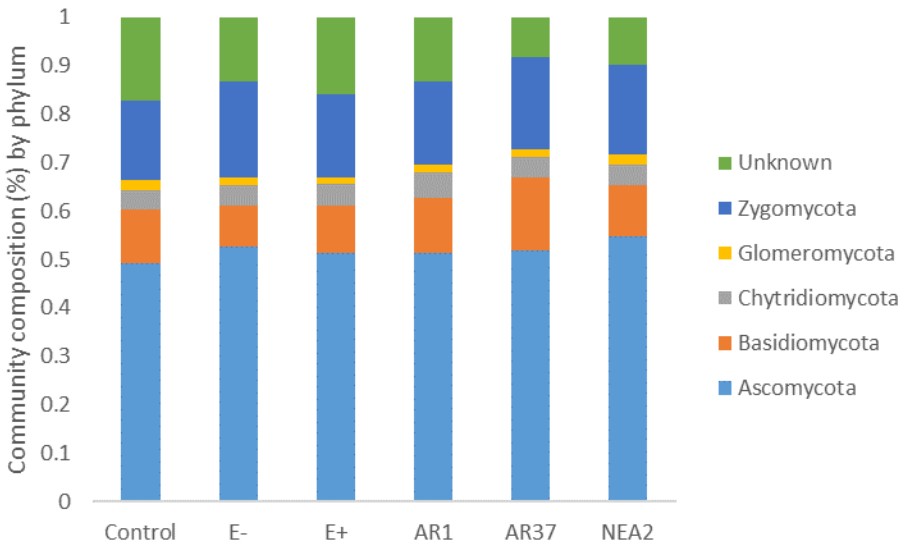


Figure 3. 16. Fungal community composition based on relative abundance for each soil conditioning treatment by phylum.

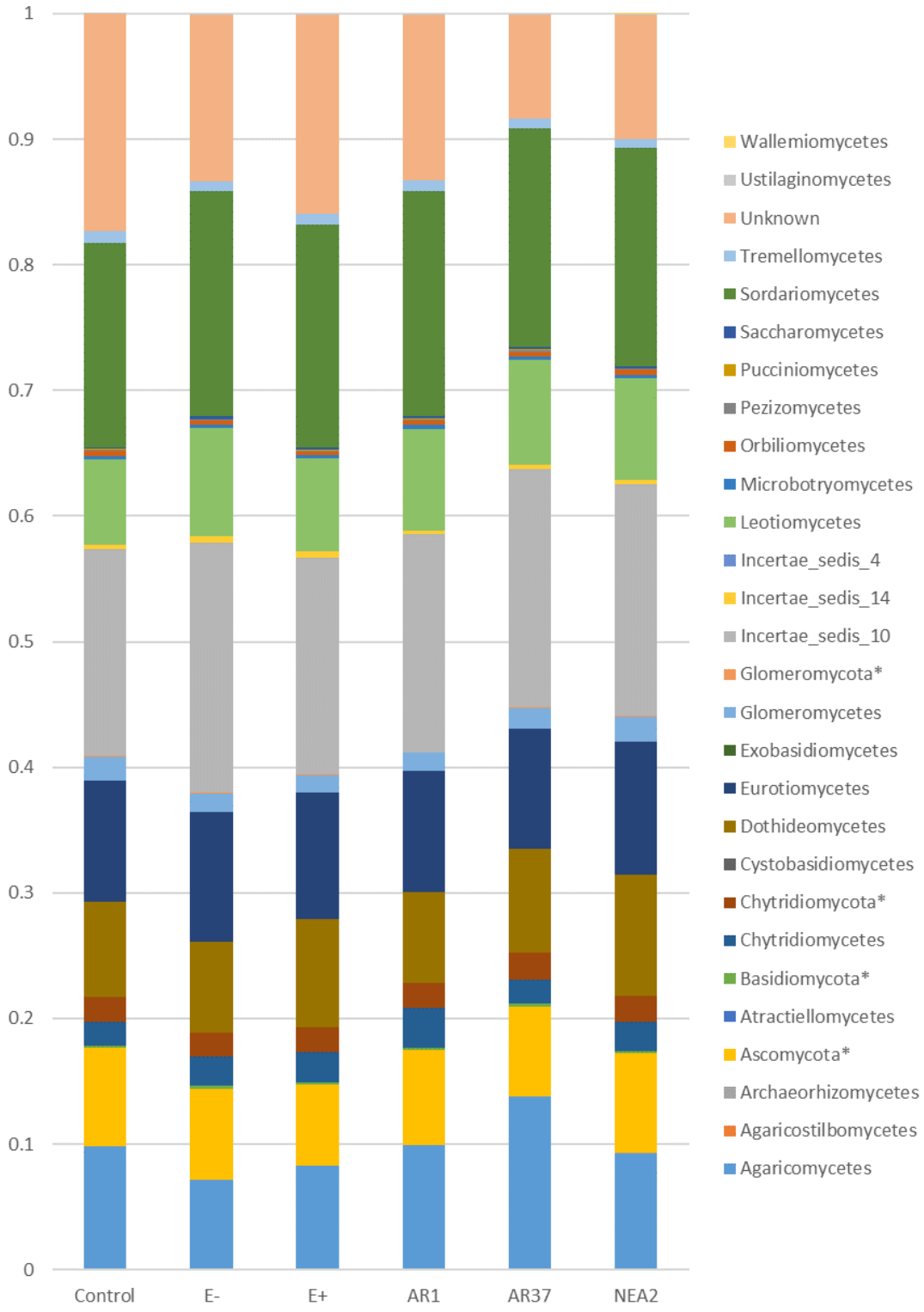


Figure 3. 17. Fungal community composition relative abundance for each soil conditioning treatment by class. *indicates the group was given an "unclassified" status at the class level but retained a designation at the phylum level.

Using analysis of composition of microbiomes (ANCOM), differences in microbial mean taxa abundance were detected for two OTUs (Fig. 3.18). When referenced against the taxonomy table generated through the UNITE database, I was able to identify the genus and species (at 0.80 cut-off) of both OTUs, *Davidiela tassiana* (OTU 61) and *Ilyonectria robusta* (OTU 67).

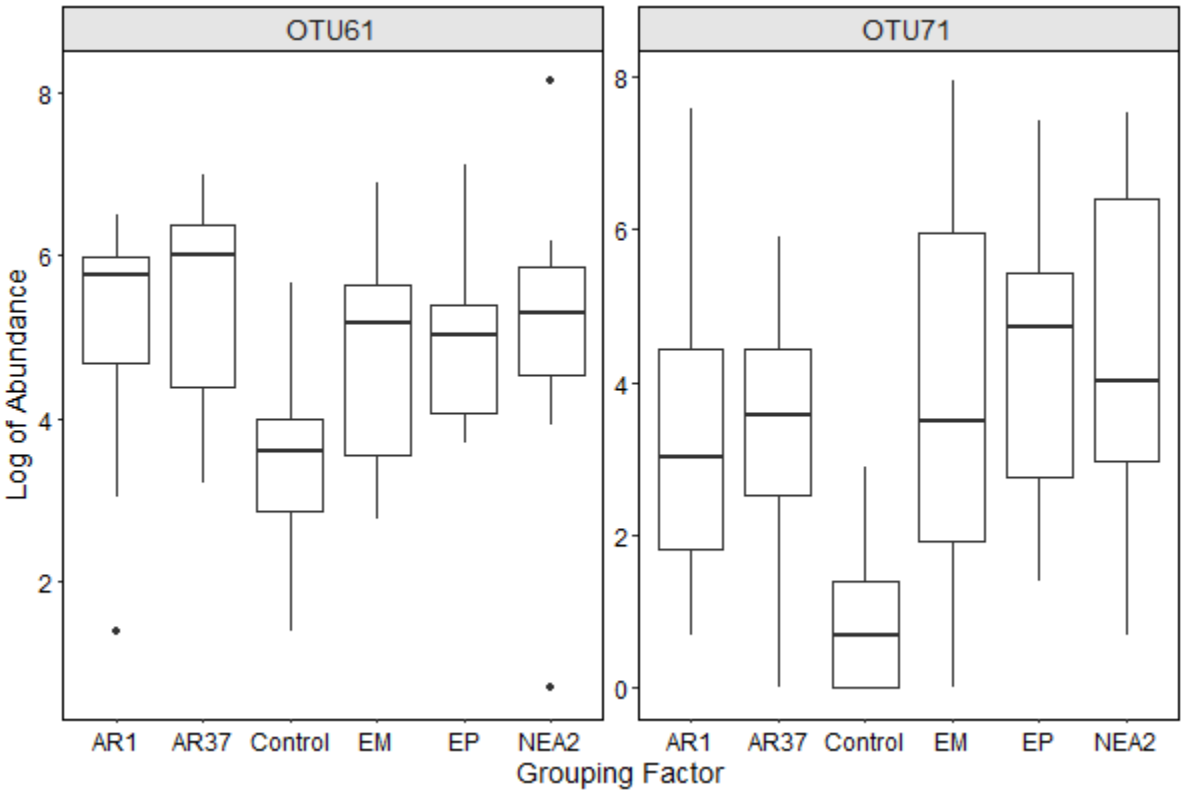


Figure 3. 18. The detection of two differentially abundant OTUs using Analysis of composition of microbiomes (ANCOM) for fungal data obtained from ITS2 rDNA genes.

A PERMANOVA found that soil conditioning treatments significantly affected fungal community abundance ($p = 0.036$) (Table 3.7). Pairwise PERMANOVA analyses showed that, with an adjusted p-value, these differences were no longer significant (Table 3.8). The NMDS ordination for bare soil (control) and soil conditioning treatments (E-, E+, AR1, AR37, NEA2) produced a 2 dimensional solution with a final stress of 0.15 after 84 iterations (Fig. 3.19). The

ordination analysis showed no differences in fungal communities between treatment groups, this included control soils where no plants were grown.

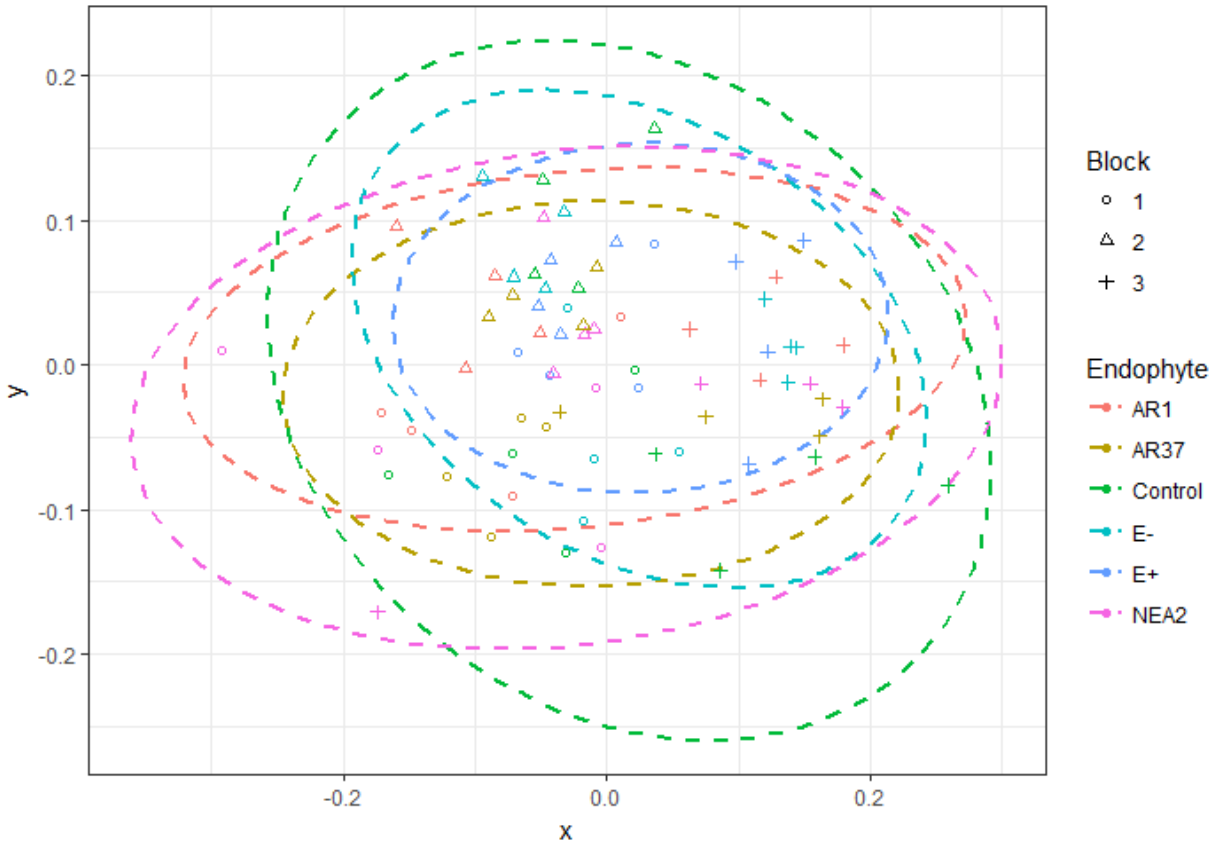


Figure 3. 19. Non-metric multidimensional scaling (NMDS) of fungal community composition for all soil conditioning treatments based on Bray-Curtis distance matrix of all OTUs at the 0.03 dissimilarity level. Ellipses based on 95% confidence. Dimensions = 2, Stress = 0.15.

The average similarity in fungal communities between soil conditioning treatments was 56.6%. This was 10% lower, than the average similarity in bacterial communities. Soils conditioned with E- plants were most similar to control soils (64.5%), and least similar to AR1 conditioned soils (53.1%). E+ conditioned soils were most similar to AR37 conditioned soils (60.1%), and only 51.6% similar to both AR1 and NEA2. AR1 conditioned soils were the least similar to NEA2 (47.8%). AR37 conditioned soils were most similar to control soils (63.9%),

and least similar to AR1 conditioned soils (53.9%). NEA2 was most similar to control soils (56.5%).

Presence/absence differences between soil conditioning treatments were not observed at the phylum or class level, however they could be seen at the order level (Table 3.11). At the order level there were no consistent patterns.

Table 3. 11. Summary of fungal taxa, classified to order, absent from at least one soil conditioning treatment category. The "X" represents where no sequence reads were found in these orders for a particular treatment.

	Control	E-	E+	AR1	AR37	NEA2
Basidiobolales	X	X	X			X
Boliniales			X		X	X
Chytridiales	X	X			X	X
Corticiales			X			
Cystobiasidiales			X	X	X	X
Diaporthales		X		X	X	X
Endogonales				X		
Erysiphales	X		X	X		X
Erythrobasidiales		X			X	X
Exobasidiomycetes unidentified	X	X		X		
Geminibasidiales	X	X	X	X	X	
Incertae sedis	X	X	X			
Incertae sedis 18	X	X		X		X
Incertae sedis 51	X	X	X	X		X
Incertae sedis 58			X			
Pezizomyceles unidentified	X	X	X	X		X
Phyllachorales		X			X	X
Tilletiales	X		X	X	X	
Verrucariales	X	X	X	X		X

DISCUSSION

Grass-fungal endophyte associations are common in unmanaged and managed grasslands. The presence of endophytes fundamentally changes plant host chemistry which can have broad effects on plant interactions both above- and belowground, through improvements in plant fitness, growth, competitive ability, and tolerances to abiotic and biotic stresses. Aboveground interactions are relatively well understood, while the belowground contributions of grass-fungal endophyte associations are less known. My objective was to determine if the presence of different strains of fungal endophytes can influence the microbial community in the rhizosphere via changes in the chemistry of root exudates.

Endophyte concentration

It is common in endophyte studies to confirm the endophyte status of the plant without measuring the actual level of infection. Studies have shown however, that endophyte concentration can be correlated with the production of alkaloids and other metabolites, plant performance, and microbial processes (Rasmussen et al., 2007; Casas et al., 2011). The results of these studies suggest that determining the concentration of endophyte in plant tissues may help interpret the strength of an endophyte effect on any given parameter. In this experiment, I found that endophyte concentration varied between endophyte strains, and that endophyte concentration increased over time. The highest concentration of endophyte was found in AR37 plants. This was similar to my previous hydroponic experiment (Chapter 2) however E+ and AR37 did not significantly differ in their concentrations. The plants in these two experiments were of similar age for the week 12 measurements, however the hydroponic experiment yielded lower endophyte concentrations (78.59 ± SE 11.83, n = 18), than the current experiment (220.42 ± SE 35.17, n = 24). In Eerens (1999) it was determined that a hydroponic set-up has little effect

on endophyte infection levels. However, endophyte infection levels were determined using microscopy rather than molecularly. It is possible that using a more sensitive technique, like qPCR, has demonstrated that the growth medium may effect the levels of endophyte infection in plant tissue. Variation in endophyte concentration between endophyte strains has also been observed in other studies (Rasmussen et al., 2007; Tian et al., 2013; Faville et al., 2015; Ryan et al., 2015). Increases in endophyte concentration over time is not particularly surprising due to the unique nature of the in-plant distribution of these asexual fungal endophytes via tip growth and intercalary division and extension[1] (Christensen et al., 2008; Voisey 2010). This growth strategy allows the fungal endophyte to extend its hyphae with the growth of the plant, the oldest hyphae cells being found higher up the leaf blade, and the newest cells closest to the base, just like as for the plants cells (Christensen et al., 2008; and see Voisey 2010 for full review of intercalary growth).

Plant Growth

Endophyte status had a significant effect on plant growth, which were most prominent after 12 weeks of growth. Overall, endophyte free plants performed poorer for all plant growth parameters when compared to endophyte infected plants. Although not always observed, improvements in plant performance in the presence of endophyte is often reported in the

[1] Host cell division occurs in the meristematic zone. Fungal hyphae colonize the meristematic zone and attach to host cell walls, growing by apical extension (Tan et al., 2001). In this region the hyphae are heavily branched. As plant cells divide, older cells are pushed upwards into an area called the leaf expansion zone. In this zone cells stop dividing and start to increase in size via intercalary extension. This extension continues until the cell has reached its maximum growth. Concurrently with plant cell extension, attached hyphae are extending via intercalary growth (increasing intercalary compartments) (Christensen et al., 2008).

literature (Guo et al., 2015). Enhanced root growth is of particular interest because of the potential influences on the soil ecosystem (Malinowski et al., 1999; Hesse et al. 2003; Ren et al., 2007).

There was a significant correlation between endophyte concentration and plant growth parameters. Interestingly, NEA2 had the highest biomass but the lowest concentration of endophyte infection among the endophyte strains. While E-, with no endophyte present, had the lowest biomass of all treatments. This suggests that there may be something specific about the NEA2 endophyte strain's relationship with its host (i.e. compatibility). Unfortunately, no manipulation of endophyte concentration was conducted in the current experiment. Therefore, it is not possible to say if low endophyte levels result in better plant productivity than high endophyte levels or endophyte free plants, or if there is something special about the plant-NEA2 relationship. Spiering et al. (2006) did manipulate endophyte concentration and found that, although endophyte had an effect on plant growth, it was independent of endophyte concentration.

Root Exudates

In the previous hydroponic study (Chapter 2) I found that root exudate composition was significantly influenced by endophyte presence and endophyte strain. As root exudates are an important carbon source for soil microorganisms, and endophytes can alter the quantity and quality of root exudates, I was curious if an endophyte effect could be seen in the metabolic composition of rhizosphere soil.

There were significant differences in soil metabolomic profiles between all endophyte treatment groups, and the bare soil control group. These results were due to the absence of metabolites in some treatment groups, as well as the up- and downregulation of metabolites.

Endophyte free and endophyte infected grasses can differ in terms of the quantity and quality of root exudates (Malinowski et al., 2998; Van Hecke et al., 2005), but in this study there was no consistent pattern of endophyte related responses within metabolite classes. Although, E+ conditioned soils often, but not always, had the highest intensity of metabolites when compared to the other treatment groups. In AR1 conditioned soils nearly 75% of metabolites were missing in the positive ion mode, however all metabolites were present in negative ion mode. There were no likely candidate steps in the experimental design, the sample processing, or the sample analysis, that could explain these results. At each step, randomization was used to prevent the incursion of systematic bias or error. Nevertheless, there is equally no obvious biological explanation for these results either. Surprisingly, there were only five compounds that were absent from the bare soil controls. Two of the compounds were tentatively identified as phaseolic acid and longispionogenin, the other three compounds were not identified. Phaseolic acid (caffeoyl-L-malic acid) is a phenolic compound, more specifically a fatty acid derivative. Phaseolic acid has been isolated in pulses, and lettuce (Mai and Glomb, 2013). It is synthesized through the phenylpropanoid metabolic pathway in response to wounding, but it is also known to be involved in the regulation of some aspects of plant development (Redmann et al., 1968), and is also involved in signalling between plants and microorganisms (Farmer 1994). Phaseolic acid was found in the highest intensities in E+ conditioned soils. The metabolite longispinogenin is a triterpenoid which are a group of defensive compounds. Longispinogenin has been isolated from *Calendula officinalis* (Zitterl-Eglseer et al., 1997), but there is no literature on its presence in other plants or its specific biological role within plants. Longispinogenin had the highest intensities in E- conditioned soils. Having the majority of compounds present in bare soil suggests that most of the metabolites identified in the rhizosphere soil were either 1) produced by plants and highly stable in soil,

explaining why they are present in bare soil; 2) produced by algae that was found on surface soils; or 3) produced by soil microorganisms. In the first case, many of the metabolites remained unidentified, and for those that were identified it was a tentative identification based off molecular weight, retention time, and how well it matched current metabolites in databases. Therefore, the nature of many of these metabolites in soil would be difficult to determine. Lipids are one class of compounds that are well known for their stability in soils (Wiesenberg et al., 2010; Jandl et al., 2013), but I was unable to find a consistent pattern among metabolites placed in this class. In the second case, during the processing of the soil, care was taken not to include surface soil. Although not impossible, it is unlikely that the source of metabolites came from algae. In the third case, if these metabolites are originating from microbes, the results could be interpreted based on the suppressive or stimulatory effect of plant presence and/or effect of endophyte infected plants on soil microbial communities. There were three metabolites tentatively identified as being produced by other organisms.

The similarity in metabolomic profiles between soil conditioning treatment groups differed between positive and negative ion mode. In positive ion mode AR1 separated out from all other treatments, this was not surprising considering the majority of metabolites were absent. I had predicted that all plant treatments should be more similar to each other than to bare soil, and that endophyte infected treatments should be more similar to each other than endophyte free. In both ion modes E+ and E- were more similar to each other, and control treatments did not separate out from AR37 and NEA2. This pattern differed from the previous study in Chapter 2 where root exudate was collected from a hydroponic set-up and not from a soil medium. In that experiment E- and NEA2 clustered together separate from E+, AR1, and AR37 which clustered together, in positive ion mode, and AR1, AR37, and NEA2 clustered together, while E+ and E- like in the

current experiment clustered together in negative ion mode. In other studies, endophyte free metabolomic profiles did clearly separate from endophyte infected profiles (Guo et al., 2015; Wakelin et al., 2015).

It should be noted that this metabolomics investigation of the rhizosphere soil had low replication (n = 3). Due to high variability in the quantity of plant metabolites both biologically and technically, an n of three is considered quite low for metabolomics studies. Additionally, no chemical standards were included during the LC-MS runs therefore making compound identification challenging.

Microbial community

Despite significant changes in the rhizosphere metabolome of endophyte infected grasses, there were few significant changes in the rhizosphere's microbial community. Bacterial and fungal communities responded differently to the soil conditioning treatments, and to environmental changes. There were no significant differences between plant conditioned soils (i.e. soils with uninfected or infected plants present), but there were significant differences between AR1 and NEA2 conditioned soils when compared to the bare soil controls. These differences were only observed for the bacterial communities, where higher abundances of Actinobacteria were observed in AR1 and NEA2 conditioned soils when compared to control soils. This is in contrast to Singh et al. (2007) who found that non-plant soils were dominated by Firmicules and Actinobacteria. Such minimal differences between soils with and without plants present is surprising as we know that biotic interactions shape microbial composition and function in the soil (Crowther et al., 2015). It has been found that the presence of plants tends to modify soil chemical and biological processes primarily through the release of root exudates and consequent stimulation of the microbial community (Wardle et al., 2004; Bais et al., 2006). For

example, rhizosphere soils can have more carbon and nitrogen fractions, and higher enzymatic activity (Guo et al., 2016). Some endophyte studies have compared the microbial communities of rhizosphere soil versus bulk soil, and have found that there are clear differences between these soil fractions for several bacterial and fungal taxonomic classes (Jenkins et al., 2006; Guo et al., 2016).

Perhaps the length of time allotted for this soil condition experiment was not enough to see significant treatment effects. Jenkins et al. (2006) saw increases in eubacteria in the rhizosphere occurring at weeks 20 and 36, with the first sampling of the soil occurring at week 8. In the current study the soil was tested at week 12. However, Wakelin et al. (2015) was able to see changes after four weeks of growth. In both of these studies the volume of soil and the number of plants placed in one container may have resulted in more plant biomass contributing to the environment, both with respect to habitat and food resources. Their study took place in 50 cm^3 of soil with five seedlings growing per tube. While the Jenkins et al. (2006) study was conducted in the field, plants were grown in 176 cm^3 pots with five tillers per plot. In the current study it was one plant per pot growing in approximately 1687 cm^3 pots, which is more than 30 times larger in volume from the Wakelin et al. (2015) study, and nearly 10 times larger in volume than Jenkins et al. (2006). By week 12 plant roots had grown the length of the pot (39 cm), which suggested sufficient exposure of the soil to root influence. However, only allowing for the growth of one plant per pot may have diluted the effect of changes to the plant-fungal endophyte metabolome, in a field setting it would not be uncommon for individual grass plants growing in close proximity to one another.

The shifts in bacterial community structure correlated with tiller number, root biomass, and soil moisture. No environmental variables explained the fungal community structure.

Together, this suggests that the bacterial community is more sensitive to changes in the soil environment. This is a similar result to Casas et al. (2011) who found changes in the structure of the bacterial community, but not for the fungal community. They did however find that bacterial and fungal activity were significantly influenced by endophyte presence. Activity measurements were not considered for the current experiment, however future microbial community studies should include both structure and function measurements as a standard practice. My results are contrary to Wakelin et al. (2015), where they found endophyte related effects on fungal community structure. However, the magnitude of the effect was minor due to high within treatment variability. I also found high within treatment variability, which may be due to inherent biological variation, but also may be the result of technical error throughout the next generation sequencing workflow. However, without rarefaction of the data, E+ and AR37 samples had lower abundances of total sequences for both 16S and ITS compared to the other treatment groups. This may indicate suppression of microbial organisms in soils conditioned with E+ and AR37 endophyte strains. McNear and McCulley (2012) evaluated the bioactivity of unfiltered root exudates, from uninfected or endophyte infected plants (both common toxic and novel strains), on cultures of a single species of bacteria. They found that the exudates from E+ plants had the most deleterious effect on bacterial growth. Interestingly, although the novel endophyte had higher total phenolics than E-, it was the least inhibitory on bacterial growth. Using chromatography, they found three peaks of interest, one that was unique to E+, one that was shared by E+ and NE, and another that was present in all three treatments (E+, NE, and E-), but found in E+ and NE in larger amounts (McNear and McCulley, 2012).

There were observable trends in the soil conditioning treatments that were specific to the soil conditioning treatment. In bacterial communities, the majority of phylum had the highest

abundances in Control samples, on average. NEA2 hosted the highest abundances of Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Candidatus Saccharibacteria, and Cyanobacteria. This is in contrast to Singh et al. (2007) who found that non-plant soils were dominated by Firmicules and Actinobacteria, and rhizosphere soils were dominated by Proteobacteria and Acidobacteria. AR1 hosted the highest abundances of Bacteroidetes and Candidate Division WPS 1. In the bacterial community composition, AR1 consistently had the highest level of dissimilarity to all other treatments. A similar result was also found in Wakelin et al. (2015), where bacterial community composition had the strongest differences in AR1. Additionally, Bell et al. (2009) found significant differences between E+ and AR1 for Actinobacteria, Betaproteobacteria (phylum: Proteobacteria), and the genus *Pseudomonas* (phylum: Proteobacteria), and between AR1 and E- for Alphaproteobacteria (phylum: Proteobacteria). In my study, the control samples hosted the highest abundance of Proteobacteria, with AR1 conditioned soils having only slightly lower abundances. Bell et al. (2009) also found that E- and AR1 hosted higher abundances of bacterial feeding nematodes, suggesting that resource availability for bacteria was greater in these two endophyte conditions than in E+ and AR37. They suggested root exudates as the source of increased resource availability (Bell et al., 2009), however this was not tested.

In the fungal communities there were fewer phylum and the highest abundances were distributed across the soil conditioning treatments. On average, E- had the highest abundances of Ascomycota and Zygomycota, Control had the highest abundances of unclassified phyla, AR1 had the highest abundances for Chytridomycota, AR37 had the highest abundances of Basidomycota, and NEA2 had the highest abundances of Glomeromycota. Soils conditioned with E+ plants supported the lowest abundances of Glymeromycota, on average. The

Glymeromycota phylum are made up of arbuscular mycorrhizal fungi (AMF), and are among the most commonly studied organisms in the rhizosphere of grass-fungal endophyte associations. Reductions in AMF root colonization and abundance in soil are often observed in endophyte infected grasses (Chu-Chou et al., 1992; Guo et al., 1992; Mack and Rudgers 2008; Buyer et al., 2011; Omacini et al., 2012), however a stimulatory effect has also been seen (Rojas et al., 2016). Bell et al. (2009) found only significant differences between AR1 and E- for general fungi, but found almost all treatment combinations to be significant for AMF. In the current study, abundances of Glymeromycota were different across treatment groups. Due to the symbiotic nature of some genus within this phylum, with *L. perenne*, and the competitive nature of some genus within this phylum, with *E. festucae* it may be a phylum worth focussing on in future studies.

CONCLUSION

In this study I examined how time, *Epichloë festucae* var. *lolii* strain, and their interaction effected plant biomass development, and endophyte concentration. I also looked at the effect of endophyte strain on root exudate metabolite composition, and soil microbial community composition of 12 week old plants grown in soil in a controlled glasshouse environment. I found that there was a clear endophyte effect on plant growth, endophyte concentration, and the quality of metabolites in rhizosphere soil. There was also a significant difference between bare soil and some endophyte treatments with respect to bacterial community abundance, but not fungal community abundance. Most environmental parameters did not significantly contribute to microbial community abundance, however there were linear relationships between root abundance and bacterial abundance. The direction of these relationships was dependent on phylum.

Although a great deal has been studied on the ecological consequences of *Epichloë* fungal endophytes, particularly with respect to the aboveground ecosystem, the belowground ecosystem continues to be an understudied area. How these fungal endophytes contribute to soil biogeochemical processes, and the structure and function of microbial communities as well as higher trophic levels, is of great importance particularly for managed grasslands where these grass-fungal endophyte associations may be the predominant species. Glasshouse based research can control for environmental variables identifying key features of the plant-fungal endophyte relationship, however they are limited in their external validity. Evidence suggests that changes in plant growth parameters and chemistry can influence soil rhizosphere soil and the organisms that inhabit it. Future studies should place emphasis on field trials including temporal variation.

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CHAPTER 4: CONCLUSIONS

Investigations of plant-soil interactions, in general, have doubled in the last decade, revealing a complex system of signalling and feedback loops between organisms both above- and belowground. These interactions regulate ecosystem level processes such as organic matter decomposition, and carbon and nitrogen cycling, and contribute to water quality, soil aggregate stability, and overall plant and soil health. Despite more than a century of research in grass-fungal endophyte associations, the role of foliar fungal endophytes in grass-soil interactions is little understood. This gap exists because our understanding of above- and belowground ecosystems have been, for the most part, established independently of one another (Wardle, 2002). The knowledge gap also exists because grass-fungal endophyte research has been predominantly driven by an agricultural perspective, focused on ruminant animal health, insect pest deterrence, and overall productivity and persistence of agricultural fields. Fungal derived alkaloids (a class of secondary metabolites) were thought to be responsible for much of the phenomenon observed in these grass-fungal endophyte associations, and while they are unequivocally involved in ruminant animal health (Fletcher and Harvey 1981) and insect pest deterrence (Mortimer et al., 1982; Prestidge et al., 1982), mechanisms explaining other phenomenon are still unclear. Additionally, *Epichloë* fungal endophytes infect aboveground tissues (Christensen et al., 2008), and fungal hyphae and fungal derived alkaloids are rarely found in root tissues (Azevedo and Welty 1995). Due to this, grass-fungal endophyte effects on the belowground ecosystem were rarely investigated. More recent research has found that significant changes are occurring in the plant-fungal metabolome (Koulman et al., 2007b; Rasmussen et al., 2008; Ambrose and Belanger, 2012; Koulman, 2012; Dupont et al., 2015; Guo et al., 2015), confirming that alkaloids alone do not present the full picture. The isolation of metabolites from the roots (Malinowski et al., 1998; Bacetty et al., 2009a,b) and from the root exudates (Creek and Wade

1985; McNear and McCulley 2012) of *S. arundinaceus* infected with *E. coenophiala* revealed the presence of non-alkaloid compounds, such as phenols, that may impact root feedings insects, nematodes, and microbial communities in soil. The extraction of metabolites from rhizosphere soil of endophyte infected plants has recently been undertaken (Wakelin et al., 2015), but the isolation of root exudate metabolites has not been conducted in the *L. perenne-E. festucae* var *lolii* relationship. The research described in this thesis addresses these gaps by conducting preliminary investigations on the effects of the fungal endophyte *Epichloë festucae* var. *lolii* (Latch, Christensen & Samuels) Glenn, Bacon & Hanlin, (Clavicipitaceae: Poëideae), an important symbiont of *Lolium perenne* in temperate grasslands. The goals of this research were: 1) to examine the influence of each *E. festucae* var. *lolii* strain on root exudate chemical composition in a hydroponic system; and 2) to examine the effect of each *E. festucae* var. *lolii* strain on the rhizosphere soil metabolome, and on microbial community structure in a glasshouse setting using potted field soil.

Endophyte strain dependent changes in plant growth

The effect of fungal endophytes on plant growth is by no means a resolved area of the grass-endophyte mutualism, and is a common measurement included in studies. In both the hydroponic and soil conditioning experiments I found that endophyte infected plants consistently had higher total, root, and shoot biomass than endophyte free plants. Furthermore, NEA2 endophyte infected grasses, on average, outperformed the other endophyte treatment groups in both studies. E- plants had the lowest production of biomass, on average, in both studies. In the hydroponic study (Chapter 2), NEA2 infected plants had significantly higher biomass than E-, AR1, and AR37 plants, but not E+. In the soil conditioning study (Chapter 3), week 12 E- plants produced significantly less biomass than AR1, AR37, and NEA2. E+ infected plants were not

significantly different than any of the other treatment groups. NEA2 and E+ infected plants had significantly higher total, and shoot biomass than E- plants, while NEA2, E+ and AR37 had significantly higher root biomass than E- plants.

Endophyte strain specific differences in plant growth are not uncommon (Popay et al., 1999; Hume et al., 2007; Bell et al., 2009; Popay and Hume 2011; Moate et al., 2012; Tian et al., 2013; Ryan et al., 2015). These differences are hypothesized to be the result of: 1) insect resistance as a consequence of endophyte and alkaloid content (Popay et al., 1999; Hume et al., 2009; Popay and Hume 2011; Moate et al., 2012); 2) a cultivar effect (Rasmussen et al., 2008; Hume et al., 2009); 3) host-endophyte specific genetic effects (Rasmussen et al., 2008; Tian et al., 2013), and 4) other yet unknown factors. With respect to my two studies, using a glasshouse setting eliminated issues with invertebrate pests, at least ones seen with the naked eye. I also only considered one cultivar, therefore the cultivar effect hypothesis does not explain the biomass differences. The most likely hypothesis for endophyte strain specific differences in plant growth would be one of host-endophyte genetic effects. Identifying the mechanisms involved in genetic related changes is an area of active research, and therefore no conclusive answers are available. In recent years *E. festucae* and *L. perenne* have been used as the model system to understand the mechanisms involved within the relationship (Tanaka et al., 2012). The genome sequencing for two strains of *E. festucae* (Schardl et al., 2013) as well as the generation of a draft genome of *L. perenne* (Byrne et al., 2015) has been instrumental in advancing our understanding. However, the picture of both organisms' genomes is incomplete. For example, only a draft genome exists for *L. perenne* and although the genomes for two strains of *E. festucae* have been sequenced, 44% of its genes still require functional characterization (Eaton et al., 2015). Combined studies utilizing transcriptomics and metabolomics techniques will aid progress in gene functional

characterization. As this genome based picture comes into focus, the hope is that new mechanistic explanations of the grass-fungal endophyte association will emerge.

Endophyte strain dependent changes in the metabolomes of root exudate and rhizosphere soils

Many of the molecular studies on plant-fungal endophyte symbiosis have utilized aboveground shoot tissue, but little is known about the make-up of the metabolites leaving the plants via the roots. Thus the aim of this thesis was to identify key changes in the plant-fungal metabolome based on root exudates. My research has demonstrated that there are significant endophyte and endophyte strain specific effects on the metabolomic profiles of root exudates and rhizosphere soils.

One of the primary goals of coupling a hydroponic study with a soil study was to isolate root exudate metabolites that were different between endophyte treatments. The thought being that identifying metabolites in a simplified system would aid in the identification and understanding of the metabolites present in a substantially more complex soil system. Ultimately, I found no overlap in the compounds collected from root exudates from a hydroponic system (Chapter 2) with the metabolites collected from the soil rhizosphere (Chapter 3). LC-MS is an extremely sensitive process requiring a large amount of biological replication and very precise sample preparation techniques. Additionally, plant metabolites are diverse and complex and no one analytical method will capture the whole plant-fungal metabolome. I used two different extraction methods to collect the metabolites, which potentially captured two different aspects of the same metabolome resulting in a lack of similarity in metabolite identities and whole profiles between the two experiments. Furthermore, in the hydroponic experiment (Chapter 2) there were 18 biological replicates within each treatment group, while in the soil conditioning experiment there were only three. Regardless of differences in individual metabolites, it should be reasonable to expect similar responses within metabolite classes. However, in both studies, as

many as half of the metabolites were left unidentified and, particularly in the case of the soil conditioning experiment, low replication and the absence of known standards warrants caution when interpreting the tentative identification of compounds.

In the hydroponic study (Chapter 2) there were a total of 73 metabolites (positive and negative ion modes combined) that were differentially expressed in at least one of the endophyte treatments when compared to E- plants. There were several compounds that were strongly associated with one endophyte treatment, like in AR37 (m/z 135.0546 RT 1.17), and E+ (m/z 517.1987 RT 9.26). These results provide evidence for significant changes in *L. perenne* physiology in the presence of several fungal endophyte strains. Further research should aim to connect changes in root exudate chemical composition with soil ecosystem processes. Relative to the hydroponic study, the soil conditioning study (Chapter 3) yielded far fewer significantly differentially expressed metabolites (i.e. positive and negative ion modes combined resulted in a total of 55 metabolites). Of these metabolites, three were specific to plants and absent from control soils (Longispinogenin, m/z 440.3702 RT 16.05; C31H62N10O6, m/z 670.4844 RT 13.45; C4H4N3O2, m/z 126.0317 RT 0.64). The remaining 52 metabolites may have been contributed by soil organisms. If this is the case, there is further interpretation to be done on the endophyte strain specific suppression or stimulation of organisms producing these metabolites. Future studies involving the rhizosphere metabolome should adopt metabolite extraction techniques that discriminate endogenous soil metabolites from those derived from organisms in the soil (Swenson et al., 2015). Such an addition would significantly aid in the interpretation of these results.

The metabolomics conducted in the two experiments were based on un-targeted approaches, therefore no focus on specific metabolites, metabolite classes, or metabolic pathways was considered in the research designs. The strong evidence provided in these two

experiments warrants more targeted metabolomic approaches using known standards to aid in identification and quantification of metabolites of interest. Furthermore, research by Rasmussen et al. (2008), Ambrose and Belanger (2012), Dupont et a. (2015), and Wakelin et al. (2015) also provide strong justification for approaches targeting specific primary (e.g. carbohydrate and lipid metabolism), and secondary (e.g. phenylpropanoid pathway) metabolic pathways in future research.

Endophyte strain dependent changes in microbial community composition in the rhizosphere

Despite significant changes in the root exudate and rhizosphere soil metabolomes, neither endophyte presence nor endophyte strain were found to significantly effect the structure of bacterial and fungal communities at the phylum level. Furthermore, even the absence of plants in the soil provided little differences in microbial composition. We know that biotic interactions shape microbial composition and function in the soil (Crowther et al., 2015), which makes the minimal response of the microbial community composition in the soil conditioning experiment so surprising. Other studies have found variability in microbial community responses (Sayer et al., 2004; Bell et al., 2009; Rojas et al., 2016), but differences between the microbial composition of bare soil versus plant inhabited soil are generally apparent (Singh et al. 2007), as are differences in soil fractions (i.e. bulk vs. rhizosphere) (Jenkins et al., 2006; Guo et al., 2016). There were some significant differences between community composition of control soils and soil conditioning treatments, but this was not consistent for all soil conditioning treatments.

At course taxonomic resolution there were no significant differences in microbial community structure between soil conditioning treatments. However, the percent similarity in composition between soil conditioning treatments was lower than expected. For example,

bacterial community composition in E- and AR37 conditioned soils had 75.7% similarity, at the high end, while E+ and AR1 conditioned soils had 60.8% similarity at the low end. Fungal community composition in E- and AR37 had 63.6% similarity, at the high end, while NEA2 and AR1 had 47.8% similarity at the low end. Viewing the data at a finer taxonomic resolution revealed shifts in composition. For example, abundance differences for species from the Actinobacteria and Agaricomycetes classes were observed. Future work should consider including primers specific to taxonomic class in addition to universal primers, for use in next generation sequencing. This would allow for a more detailed picture of potential classes of interest.

In this thesis I set out to isolate the root exudates produced by *Lolium perenne* either uninfected (E-) or infected by the common toxic strain (E+) of the fungal endophyte *Epichloë festucae* var. *lolii* or one of three novel strains (AR1, AR37, NEA2) of the endophyte and determine their metabolomic profiles. I was successful in tentatively identifying several metabolites of interest that are differentially expressed between endophyte strains. I was also able to highlight two unidentified compounds, one specific to the E+ endophyte strain, and the other specific to the AR37 endophyte strain. As endophyte derived alkaloids have been ruled out as a mechanism for deterring root feeding insects, or altering soil microbial composition, these two metabolites may warrant further investigation as potential influencers of belowground community dynamics. This research confirmed the results of previous metabolomic work with seeds and aboveground tissues of *L. perenne* infected with the fungal endophyte *E. festucae* var. *lolii*, which found that not only does endophyte presence dramatically alter the plant's metabolome, these effects can also differ from one endophyte strain to another. This research also provided insight into the complexity of the grass-fungal endophyte association, and strain dependent effects on root exudate chemistry and demonstrates that the metabolomics of endophyte infected plant root exudate and rhizosphere soil is a rich area for future research.

In this thesis I also set out to investigate the effects of fungal endophyte strain on the belowground microbial community, focussing on bacterial and fungal communities utilizing next-generation sequencing. I did find shifts in microbial community composition between the soil conditioning treatments, particularly at the finer taxonomic resolution. But, these shifts were generally not significant. Subtle shifts in community composition may have more significant effects over longer temporal scales, and also effect broader soil processes. I did not measure changes in soil activity, which would have been helpful in predicting greater ecosystem impacts. Although there are several studies which have found little to no effect of endophytes on the soil microbial community, there are also as many studies that have found significant endophyte effects. This variability in results, combined with the fact that only few studies have even been conducted on fungal endophyte effects on the belowground environment, and even fewer that have been conducted on the same grass species, warrants further research in this area. This research should consider larger scale experiments over time and space, encompassing more than one trophic level.

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