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PLANT MICROBE INTERACTIONS

Infection with a Shoot-Specific Fungal Endophyte (*Epichloë*) Alters Tall Fescue Soil Microbial Communities

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Abstract Tall fescue (*Schedonorus arundinaceus*) is a widespread grass that can form a symbiotic relationship with a shoot-specific fungal endophyte (*Epichloë coenophiala*). While the effects of fungal endophyte infection on fescue physiology and ecology have been relatively well studied, less attention has been given to how this relationship may impact the soil microbial community. We used high-throughput DNA sequencing and phospholipid fatty acid analysis to determine the structure and biomass of microbial communities in both bulk and rhizosphere soils from tall fescue stands that were either uninfected with *E. coenophiala* or were infected with the common toxic strain or one of several novel strains of the endophyte. We found that rhizosphere and bulk soils harbored distinct microbial communities. Endophyte presence, regardless of strain, significantly influenced soil fungal communities, but endophyte effects were less pronounced in prokaryotic communities. *E. coenophiala* presence did not change total fungal biomass but caused a shift in soil and rhizosphere fungal community composition, increasing the relative abundance of taxa within the Glomeromycota phylum and decreasing the relative abundance of genera in the Ascomycota phylum, including *Lecanicillium*, *Volutella*, *Lipomyces*, *Pochonia*, and *Rhizoctonia*. Our data suggests that tripartite interactions exist between the shoot endophyte *E. coenophiala*, tall fescue, and soil fungi that may have important implications for the functioning of soils, such as carbon storage, in fescue-dominated grasslands.

Keywords *Epichloë coenophiala* · Mycorrhizae · *Neotyphodium* · Plant-microbe interactions · *Schedonorus arundinaceus* · Soil microbes

Introduction

Grasslands cover 20 % of all land area on Earth [1] and perform valuable ecosystem services, including the provisioning of forage for grazing animals [2]. Many grass species form symbioses with asexual, aboveground fungal endophytes [3], which are thought to be primarily defensive, anti-herbivory mutualisms [4] but have been shown to have other significant ecological effects as well [5, 6]. One of the better studied grass-endophyte systems is that of tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort., a.k.a. *Lolium arundinaceum* and *Festuca arundinacea*), a widespread forage grass and turf species in the USA [7], and its shoot-specific fungal endophyte (*Epichloë coenophiala*, formerly *Neotyphodium coenophialum* [8]). In endophyte-infected tall fescue, *E. coenophiala* grows throughout fescue shoots, consuming nutrients provided by the host plant while producing metabolites that are beneficial to the host. These benefits include improved drought and heat tolerance [9, 10], herbivory

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resistance [4, 11], enhanced photosynthesis [10, 12, 13], and increased nutrient-deficiency tolerance [14].

Ergot and loline alkaloids are key compounds produced by *E. coenophiala* that effectively deter both mammalian and insect herbivores [15]. Ergot alkaloids can be a serious deterrent to grazing livestock and are the primary causative agent of fescue toxicosis, a condition that adversely affects grazing cattle, reduces weight gain and reproduction, and thereby creates significant losses for the livestock industry [16, 17]. Alkaloids are also thought to play a role in creating and maintaining the insect and plant community differences observed between endophyte-infected and endophyte-free fescue stands [5] and may retard rates of fescue litter decomposition [18, 19]. While these aboveground consequences of endophyte-produced compounds have been well documented, to our knowledge, there is only a single paper that has measured alkaloids in soil associated with endophyte-infected tall fescue [20]. However, there is growing evidence that *E. coenophiala* presence aboveground can have a variety of belowground effects [21], including reductions in root nematode herbivory [22], via mechanisms that remain undetermined [23].

Due to obvious adverse effects on valuable livestock, a decade ago, researchers inoculated endophyte-free tall fescue with naturally occurring, recently discovered strains of *E. coenophiala*, so-called novel endophytes that lacked the genetic capacity to produce ergot alkaloids harmful to mammals [24]. Such 'novel' endophytes can still produce lolines and other alkaloids, allowing plants infected with them to retain insect deterrence properties and remain more resistant to stressful abiotic conditions than endophyte-free plants [25, 26]. Over the past decade, several different strains of novel endophytes have been inserted into elite cultivars of tall fescue and made commercially available. Acreage of tall fescue cultivars infected with novel endophytes has been increasing in North America and elsewhere, replacing fescue pastures infected with the so-called common toxic endophyte strain [24].

Changes in the genetics of the tall fescue–fungal endophyte symbiosis represent a potentially important alteration of fescue-dominated ecosystems, yet the effects of these novel endophytes on overall ecosystem ecology remain relatively unknown [27]. In particular, we have a limited understanding of how this aboveground symbiosis impacts soil microbial communities, despite the importance of such microbes to soil fertility and ecosystem-level processes [6]. Previous studies have shown mixed effects of fungal endophyte presence on soil microbial parameters [28]. For example, some studies report that endophyte-infected stands of tall fescue have lower soil microbial activity and utilization of certain substrates [29], and lower abundances of gram-positive bacteria, arbuscular mycorrhizae, and other microbial groups than soils associated with endophyte-free fescue [29, 30]. However, other studies have shown stimulatory effects of endophyte-infected tall fescue exudates on soil microbial activity [31] and no change in the community composition [28]. Alterations to the abundance and composition of soil microbial communities may be associated with changes in soil-to-atmosphere trace gas fluxes and the carbon sequestration potential of these grassland systems [28, 32, 33]. Only one of these studies evaluated whether novel strains of the aboveground endophyte produced similar belowground effects as the common toxic form and found that effects on soil trace gas fluxes were strain-dependent [33]. Given this result and the fact that the acreage of fescue containing novel endophytes is currently increasing worldwide, it is important to determine if there are strain-dependent effects on the soil microbial community.

Here, we investigated the impact of the shoot-specific fungal endophyte, *E. coenophiala*, on the biomass and composition of soil prokaryotic (bacterial and archaeal) and fungal communities. In particular, we wanted to know how soil microbial communities are affected by endophyte presence and strain, whether the effects differ across different fescue cultivars, and whether such effects are evident in both bulk and rhizosphere soils. Based on observed aboveground effects and prior work, we hypothesized that soil microbial community effects would vary across endophyte strains and cultivars and that endophyte/cultivar effects would be most pronounced in rhizosphere soils. To address these questions, we used phospholipid fatty acid (PLFA) analysis to estimate total bacterial and fungal biomass coupled with high-throughput DNA sequencing of rRNA gene regions to characterize the composition of the prokaryotic and fungal communities found in the rhizosphere and bulk soils collected from experimental endophyte-manipulated tall fescue stands.

Materials and Methods

Sample Collection

Soil samples were collected from an experimental tall fescue site in Kentucky, USA, which was established in 2006 for a cattle grazing preference trial (see [34] for more trial details). The site has a uniform soil type (Maury silt loams; fine, mixed, active, mesic Typic Argiudolls) and is on a 2–3 % slope. The grazing preference study included many grass types and cultivars, but we sampled only two experimental tall fescue cultivars (97TF1 and PDF). One cultivar (PDF) has been recently released to the market (Texoma) [35], and the other (97TF1) is planned for release soon (T. Phillips, pers. comm.). Both cultivars originated from fescue selections made at the Samuel Roberts Noble Foundation, and each had populations developed with the following four endophyte infection statuses (for details see [35]): no *E. coenophiala* present (E-), infected with the common toxic strain of the endophyte (CTE+), or infected with one of two different novel endophytes (AR542E+ or AR584E+). Seven replicates of the

cultivar and endophyte combinations were sampled within the randomized complete block experimental trial design for both rhizosphere soil (i.e., soil adhering to plant roots) and bulk soil. In each replicate, multiple clumps of tall fescue were excavated with a shovel. Bulk soil was collected by gently shaking excavated plants. Plant roots were then brushed with a sterilized paintbrush to release rhizosphere soil. Both bulk and rhizosphere soil was sieved to 2 mm to remove any remaining root material. There were a total of 112 samples analyzed (2 cultivars × 4 endophyte statuses × 7 blocks × 2 soil sample types). Samples were taken at the height of the plant-growing season (mid-April 2012), kept cool in transit, and sent to the University of Colorado at Boulder, where they were stored at −20 °C.

PLFA Analyses

To assess soil microbial biomass, we used PLFA analysis, extracting fatty acid methyl esters (FAMEs) utilizing the methodology of Buyer and Sasser [36]. Briefly, FAMEs were extracted from freeze-dried soil samples in Bligh-Dyer extractant, containing an internal 19:0 standard, for 2 h rotating end-over-end. The liquid phase was then collected after centrifugation, 1.0 ml chloroform and water were added, and the lower phase siphoned off and used for lipid separation. Lipids were separated by solid phase extraction (SPE) using a 96-well SPE plate (Phenomenex, Torrance, CA, USA) with chloroform, acetone, and 5:5:1 methanol:chloroform:water. Fatty acids were then transesterified, extracted, and analyzed on an Agilent 7890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with an automatic sampler and flame ionization detector (for more details see [34]). FAMEs were identified and quantified based on MIDI methods (Sherlock Microbial Identification System version 6.2, MIDI Inc., Newark, DE) as described in Buyer and Sasser [36]. Total bacterial biomass was calculated as the sum of iso, anteiso, 10-methyl, cy17:0, cy19:0, and monosaturated FAMEs, and fungal biomass was the sum of 18:2ω6c and 16:1ω5c. The latter of which is considered a biomarker of arbuscular mycorrhizal fungi [37] and was evaluated independently for treatment effects.

Molecular Analyses of Soil Microbial Communities

To characterize the diversity and composition of prokaryotic and fungal communities in each of the 112 soil samples, we used marker gene sequencing approaches. We extracted DNA from each of the soil samples using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). The DNA was then amplified following the approaches described in Crowther et al. [38]. Briefly, we PCR-amplified DNA from two different gene markers to assess both prokaryotic and fungal community composition. To examine prokaryotic communities, the V4 hypervariable region of the bacterial or archaeal 16S rRNA gene was amplified and sequenced using error-correcting 12-bp barcoded primers (515f/806r). For fungal communities, the first internal transcribed spacer (ITS1) region of the rRNA operon was amplified and sequenced using ITS1-F/ITS2 barcoded primers. In both cases, samples were amplified in triplicate. Barcoded primers specific to each sample allowed for multiplexing of samples. PCR products from all samples were quantified using PicoGreen dsDNA assay and pooled together in equimolar concentrations. Amplicons were cleaned and concentrated using the UltraClean PCR Clean-Up Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). Samples were sequenced on an Illumina MiSeq instrument using the paired-end V2 300 cycle MiSeq kit (Illumina, Inc., San Diego, CA, USA) at the University of Colorado Next Generation Sequencing Facility, with separate runs for the 16S rRNA and ITS amplicon pools.

Forward read sequences were used for ITS data, while merged sequences were used for the 16S rRNA data. All sequences were processed using the UPARSE pipeline [39] as in [40], where sequences were quality filtered and clustered de novo into 97 % similar operational taxonomic units (OTUs), and raw reads were mapped to a set of sequences representing these clusters. As an additional quality control measure, OTUs were discarded if they had representative sequences that were not ≥75 % similar to sequences contained in either the Greengenes 13_5 database [41] or UNITE November, 2012 database [42] for 16S and ITS rRNA sequences, respectively. The Ribosomal Database Project (RDP) classifier was used to classify OTUs to taxonomic groups using the aforementioned databases. To compare all samples at equivalent sequencing depths, samples were rarefied to 4000 and 250 sequences per sample for prokaryotes and fungi, respectively. While these sequencing depths are insufficient for capturing the full extent of microbial diversity found within individual samples, they should be sufficient for comparing how the relative abundances of the more abundant taxa change across the collected soils.

Statistical Analyses

To analyze the PLFA results, multifactor ANOVA tests were used to assess the effects of sample type (bulk vs. rhizosphere soil), tall fescue cultivar, endophyte status, and their interactions on total bacterial biomass, total fungal biomass, the ratio of bacterial to fungal biomass, and the abundance of 16:1ω5c. Tests were done using the stats package in R [43], and significance was determined as $P < 0.05$.

To assess differences in microbial community composition, we first calculated Bray-Curtis dissimilarities among samples from square root transformed OTU counts using the 'vegan' package in R [43]. Differences in overall prokaryotic and

fungal community composition across multiple factors were assessed using permutational multivariate ANOVA (PERMANOVA) in PRIMER 6 [44]. The PERMANOVA tests were conducted using tall fescue cultivar, endophyte status, and sample type as fixed factors with block as a random factor. Pairwise PERMANOVA was used to compare community composition within significant fixed factors. Differences in community composition across different endophyte statuses were visualized using constrained ordination as determined using the 'capscale' function in the 'vegan' package in R [43]. This analysis enables the visualization of the relative differences across endophyte statuses while minimizing the influence of other factors. In order to determine which microbial taxa differed across endophyte-infected and -free material, linear mixed effect models were used for each taxon that represented ≥0.01 % of sequences in any sample type. For this analysis, taxa relative abundances were rank transformed prior to model fitting in order to avoid stringency in model assumptions due to the large number of taxa. Soil type, cultivar, and endophyte presence were used as fixed factors, and block was used as a random factor. *P* values were corrected for false discovery rates (FDR) associated with multiple comparisons.

Results

Sample type was the only factor with a significant influence on microbial biomass levels, with rhizosphere soils having higher bacterial and fungal biomass, including amounts of 16:1ω5c, but a lower ratio of bacterial to fungal biomass than bulk soils (Table 1; $P < 0.0001$ for all parameters). There were no significant effects of cultivar, endophyte status, or their interaction on these parameters (Supplementary Table 1). In contrast, the results of the PERMANOVA analyses revealed that all factors significantly influenced the composition of soil prokaryotic and fungal communities (Table 2). For fungal community composition, sample type (rhizosphere vs. bulk soil) had the strongest effect, but all factors had highly significant effects ($P < 0.01$). For prokaryotic communities, sample type was more influential than cultivar or endophyte status (Table 2).

Differences in the bulk and rhizosphere microbial communities were evident even at coarse levels of taxonomic resolution. Although all soils were dominated by the same major bacterial groups, namely Verrucomicrobia (20.6 % on average), Proteobacteria (24.0 %), Bacteroidetes (11.9 %), Actinobacteria (11.4 %), and Acidobacteria (10.3 %), the relative abundances of the archaeal phylum Crenarchaeota and bacterial phyla Verrucomicrobia, Acidobacteria, and Firmicutes were significantly higher in bulk vs. rhizosphere soils ($P < 0.05$ in all cases; Table 3). In contrast, Bacteroidetes, Proteobacteria (Alpha, Beta, Delta, and Gamma), Planctomycetes, and Chloroflexi were significantly more abundant in the rhizosphere soils (Table 3). The fungal communities primarily consisted of members of the phylum, Ascomycota (49.5 % on average), and did not differ in relative abundance between the rhizosphere and bulk soil samples. However, Zygomycota had a significantly higher and Chytridiomycota had a significantly lower relative abundance in bulk soils than in rhizosphere soils (Table 3).

Given the large differences in microbial community composition between the bulk and rhizosphere soils, differences in the relative abundances of individual prokaryotic and fungal taxa between the two sampled cultivars were assessed for each soil fraction separately. In the rhizosphere samples, no prokaryotic or fungal taxa were significantly different in relative abundance between cultivars. However, in bulk soils, PDF had significantly lower relative abundances of bacterial taxa Firmicutes and Alphaproteobacteria than 97TF1 (Table 4). Additionally, the PDF cultivar had a significantly lower relative abundance of Ascomycota and higher abundance of Chytridiomycota compared to 97TF1 in the bulk soil samples (Table 4).

The pairwise PERMANOVA analyses comparing endophyte statuses revealed that there were endophyte-associated significant differences in soil fungal community composition (Table 5). The ordination analysis illustrated strong differences in fungal communities between soils associated with uninfected tall fescue, E−, and each endophyte-infected status, CTE+, AR542E+, and AR584E+ ($P < 0.01$ for each comparison; Fig. 1). However, there was not a significant difference between the endophyte-free samples and any of the endophyte-infected samples for prokaryotic communities (Table 5). Significant, but subtle, differences between the prokaryotic and fungal communities associated with AR542E+ treatment and the other endophyte-infected material were observed (Table 5). Additional pairwise PERMANOVA tests evaluating endophyte-associated differences in soil microbial

Table 1 Mean (±1 sd) total bacterial biomass, total fungal biomass, ratio of bacterial to fungal biomass, and abundance of 16:1ω5c for bulk and rhizosphere soils, averaged across fescue cultivar and endophyte status ($n = 56$ for both sample types)

Sample type	Bacterial biomass (nmol PLFA g soil^{-1})	Fungal biomass (nmol PLFA g soil^{-1})	Bacterial:fungal	16:1ω5c (nmol PLFA g soil^{-1})
Bulk	203.70 ± 13.34b	16.41 ± 3.24b	12.68 ± 1.44a	12.36 ± 0.14b
Rhizosphere	255.81 ± 14.64a	28.11 ± 3.08a	9.17 ± 0.70b	17.54 ± 0.20a

Letters denote statistically significant differences ($P < 0.0001$)

Table 2 PERMANOVA test results for the random (block) and fixed effects of sample type (bulk vs. rhizosphere soil), tall fescue cultivar, and endophyte status (endophyte-free, infected with the common toxic strain, or infected with one of two novel endophyte strains) on soil microbial communities (calculated from Bray-Curtis dissimilarities comparing relative abundances of prokaryotic and fungal OTUs between samples)

	Prokaryotes			Fungi		
	Pseudo-F	*P* value	Component of variation	Pseudo-F	*P* value	Component of variation
Block	2.35	*0.001*	1.2×10^{-2}	2.41	*0.001*	1.9×10^{-2}
Sample type	3.80	*0.001*	7.0×10^{-3}	3.39	*0.001*	9.1×10^{-3}
Cultivar	1.20	0.022	4.9×10^{-4}	1.34	*0.006*	3.4×10^{-3}
Endophyte	1.09	0.041	4.6×10^{-4}	1.45	*0.001*	1.3×10^{-3}

Highly significant effects ($P < 0.01$) are italicized

communities for each of the tall fescue cultivars and for bulk vs. rhizosphere soils separately produced similar results (Supplement Tables 2 and 3), with the endophyte-associated differences being more pronounced for the fungal than the bacterial communities.

Given that the pairwise PERMANOVA and constrained ordination indicated a clear difference in fungal communities between endophyte present and absent soils, we then determined which taxa were driving these differences. At the phylum level, endophyte-infected soils had marginally significant greater relative abundances of Glomeromycota (FDR-corrected $P = 0.089$) and lower relative abundances of Ascomycota than endophyte-free soils ($P = 0.056$; Fig. 2). Additionally, the endophyte effect was examined at a finer scale by comparing the most abundant fungal genera. There were five genera that significantly differed (*Lecanicillium*, *Volutella*, *Lipomyces*, *Pochonia*, *Rhizoctonia*), and of these, most had decreased abundance in endophyte-infected vs. endophyte-free soils (Fig. 3).

Discussion

In contrast to our hypothesis that there would be endophyte strain-specific effects on soil microbial communities, we found that all three fungal endophyte strains tested had similar effects on the belowground communities. *E. coenophiala* presence altered the abundances of several soil fungal groups, including increasing the abundance of the Glomeromycota phylum and reducing the abundance of the phylum Ascomycota and genera *Lecanicillium*, *Volutella*, *Lipomyces*, *Pochonia*, and *Rhizoctonia*. This community shift was not associated with a corresponding change in fungal biomass, indicating that endophyte infection alters the fungal community composition but not the total amount of fungal biomass found in fescue-associated soils. This finding is important because these fungi play diverse roles in soils that can impact other organisms and soil function, such as carbon sequestration.

Table 3 Mean relative abundances of major prokaryotic and fungal taxa that differed between bulk and rhizosphere samples

Taxon		Relative abundance (%)		*P* value	FDR-corrected
		Bulk	Rhizosphere		
Archaea	Crenarchaeota	6.67	4.22	5×10^{-8}	2×10^{-7}
Bacteria	Acidobacteria	10.74	9.83	0.03136	0.03528
	Alphaproteobacteria	7.37	9.32	3×10^{-8}	1×10^{-7}
	Bacteroidetes	10.57	13.41	9×10^{-9}	4×10^{-8}
	Betaproteobacteria	3.96	5.07	2×10^{-10}	1×10^{-9}
	Chloroflexi	1.26	1.53	0.00093	0.00168
	Deltaproteobacteria	6.63	7.34	0.00062	0.00111
	Firmicutes	9.13	7.48	0.02021	0.02598
	Gammaproteobacteria	3.1	4.67	6×10^{-14}	1×10^{-12}
	Planctomycetes	2.42	2.88	0.00001	0.00002
	Verrucomicrobia	22.58	18.37	0.01209	0.01814
Fungi	Chytridiomycota	3.09	5.77	0.00023	0.00137
	Zygomycota	14.45	12.26	0.01387	0.02774

Uncorrected and FDR-corrected *P* values are shown

Table 4 Mean relative abundances of bulk soil bacteria and fungal taxa that differed between PDF and 97TF1 tall fescue cultivars

Taxon		Relative abundance (%)		*P* value	FDR-corrected
		PDF	97TF1		
Bacteria	Alphaproteobacteria	6.68	7.99	0.00218	0.03917
	Firmicutes	7.58	10.51	0.00461	0.04148
Fungi	Ascomycota	43.4	52.96	0.00120	0.00721
	Chytridiomycota	4.25	2.06	0.00971	0.02912

Uncorrected and FDR-corrected *P* values are shown

For example, Glomeromycota are arbuscular mycorrhizal fungi (AMF) and are known to play a role in soil aggregate development and carbon sequestration [45]. AMF are symbiotic with tall fescue roots, as they are with 80 % of terrestrial plants [46]. If endophyte infection of tall fescue stimulates AMF abundance, as our data suggest, this may contribute to the increase in soil carbon storage that has been shown to occur in endophyte-infected vs. endophyte-free stands [28]. A study conducted in concert with this project found increased particulate organic carbon pools in the rhizosphere soil of CTE+ and AR584E+ stands compared to the other endophyte statuses [34], reflecting trends in our endophyte-associated increases in AMF (Fig. 2). However, additional existing studies have shown that presence of *E. coenophiala* in fescue aboveground material decreases mycorrhizal colonization of fescue roots and abundance in soil [29, 47–49]. In perennial ryegrass (*Lolium perenne*), presence of an *Epichloë* endophyte reduced mycorrhizal root colonization; however, the strength of this effect varied depending on phosphorus supply and both ryegrass cultivar and endophyte strain [50]. Our results contrast with these findings in that all three *E. coenophiala* strains produced similar effects, in both tested cultivars, and stimulated (rather than reduced) AMF relative abundance.

Consistent *E. coenophiala* strain effects were also observed in the reduction in relative abundance of the other fungal groups affected by endophyte presence in this study. Although the function of the affected genera is somewhat uncertain, members of *Lecanicillium* and *Pochonia* are known to contain species that are entomopathogenic [51] and parasites of nematodes [52]. Perhaps, their reduced abundance in endophyte-infected soils reflects endophyte effects on their prey, since reductions in nematode and soil-dwelling insect herbivores are well-known ecological consequences of endophyte infection [5, 53]. Such alterations in trophic dynamics can affect nitrogen cycling and acquisition by the plant [54]. *Lipomyces*, *Volutella*, and *Rhizoctonia* are likely saprotrophs, some with potential to become phytopathogens [55–58]. Endophyte infection has been shown to inhibit plant pathogens [59], and reductions in saprotroph abundance could influence carbon cycling, via changes to organic matter decomposition rates and aggregate stabilization [56, 60]. Concomitant endophyte-associated reductions in these fungal groups, increases in AMF, and changes in root biomass production and rhizodeposition are all likely to influence soil carbon pools and may contribute to the enhanced soil carbon sequestration observed in endophyte-infected fescue stands [28].

Table 5 Pairwise PERMANOVA comparisons of different endophyte statuses on soil prokaryotic and fungal communities across sample type (bulk and rhizosphere soil) and cultivar

Endophyte comparisons	Prokaryotes *P* value	Fungi *P* value
E−, CTE+	0.426	*0.001*
E−, AR542E+	0.070	*0.001*
E−, AR584E+	0.294	*0.001*
CTE+, AR542E+	0.042	0.015
CTE+, AR584E+	0.602	0.564
AR542E+, AR584E+	0.035	0.257

Endophyte statuses whose microbial communities strongly differed ($P < 0.01$) from each other are italicized

Our observation of consistent *E. coenophiala* strain effects contrasts with recent work showing varying degrees of endophyte strain effects on perennial ryegrass rhizosphere microbial communities [61]. While some differences were observed between AR542E+ vs. CTE+ and AR584E+ soil microbial communities, strain effects were subtle compared to that of endophyte presence overall (Table 5; Fig. 1). The fact that all three endophyte strains used in our study had similar effects on the soil fungal community suggests that *Epichloë*-produced alkaloids were not responsible for the observed changes in fungal communities [62, 63]. The endophyte strains assessed in this study have different capacities for producing alkaloids [64]. Specifically, neither novel endophyte can produce ergot alkaloids, although the common toxic strain does. Our results suggest that the interaction between endophytes and soil fungi is not related to alkaloid production, which is consistent with the fact that alkaloids are rarely measured in roots [65], but instead, may be due to endophyte-induced changes in tall fescue physiology, possibly altering volatile organic compounds that serve as multifunctional signals [23] or increasing rhizodeposition and root biomass production [66] providing more nutrients to soil symbionts, though it

should be noted we did not observe endophyte-associated differences in measured PLFA in either rhizosphere or bulk soils. However, the latter explanation is consistent with studies conducted on different grasses, *Bromus setifolius*, *Lolium multiflorum*, and *Poa bonariensis*, which demonstrated enhanced mycorrhizal colonization and increased soil fungal activity with *Epichloë* sp. infection [67–69].

The fact that endophyte-associated changes were more pronounced in the soil fungal community than the prokaryotic also contrasts with the limited prior work on the subject. Casas et al. [67] found that soil bacterial community composition was altered by endophyte infection (*Epichloë occultans*) in annual ryegrass (*Lolium multiflorum*) but detected no change in the fungal community. Roberts and Ferraro [62], working on tall fescue, measured increased bacterial diversity in endophyte-infected vs. -free rhizosphere soils, and Wakelin et al. [61] observed endophyte effects on both bacterial and fungal communities inhabiting perennial ryegrass rhizosphere soils.

The degree of environmental stress experienced by tall fescue could explain why our findings differ substantially from prior work. The tall fescue sampled for this study may have been less stressed by grazing, drought, extreme temperatures or nutrient deficiencies, conditions where shoot endophytes may be most beneficial [70]. Malinowsky and Belesky [14] suggested that, under stressed conditions, endophytes may trigger defense mechanisms in tall fescue as if it were infected with pathogenic fungi, releasing reservatrol (an anti-fungal phenol and antioxidant) and chitinase (an enzyme that breaks down fungal cell walls) from the roots. Our field site is located on relatively high phosphorus soils [71] and typically experiences a mesic environment [72]. Perhaps, when conditions are less stressful, alterations to the rhizosphere metabolome are more subtle. This could explain the fact that many of our observed endophyte effects were somewhat limited in magnitude and significance. Microbial communities are dynamic entities, capable of significant variation across time and space, which makes identification of controlling variables challenging in a field context. It is possible that we would have obtained different results if we had sampled at different times of the year. It is also possible that differences in the techniques used to characterize the soil microbial response to endophyte presence (e.g., root AMF colonization rates vs. soil AMF spore counts vs. soil DNA; [73]) and/or the cultivars and strains utilized may explain the discrepancy between our results and those of others.

We found that microbial community composition differed between bulk soil and rhizosphere samples. We measured significantly greater relative abundances of bacteria belonging to

	Phylum	Glomeromycota	Ascomycota
	***P*-value**	0.01278	0.01608
	FDR corrected	0.08943	0.05627
Bulk	E-	6.80	51.03
	CTE+	6.77	46.77
	AR542E+	7.49	49.20
	AR584E+	8.31	46.15
Rhizosphere	E-	6.20	55.83
	CTE+	8.49	50.80
	AR542E+	8.90	48.20
	AR584E+	7.60	47.57

Fig. 2 Mean relative abundances (%) of the fungal phyla that most strongly differed between endophyte treatments. *P* values were generated from linear mixed effect models designed to test for differences between all endophyte-infected (+) and endophyte-free (−) samples for bulk or rhizosphere soils across both cultivars. Higher relative abundances of each phylum are highlighted in *red*, and lower relative abundances are highlighted in *blue* within bulk soil and rhizosphere samples

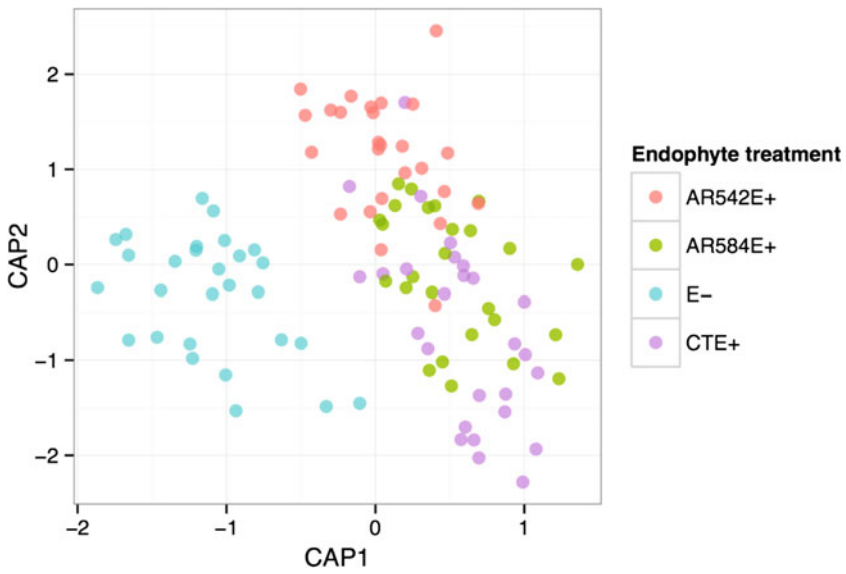

Fig. 1 Principal coordinates analysis ordination of fungal community compositions constrained by endophyte treatment. Both bulk and rhizosphere samples and both cultivars were included in the ordination

	Phylum	Ascomycota												Basidiomycota		Chytridiomycota
	Genera	Lecanicillium	Volutella	Lipomyces	Pochonia	Chalara	Periconia	Cladophialophora	Cercophora	Paraphoma	Bionectria	Articulospora	Alternaria	Erythrobasidium	Rhizoctonia	Powellomyces
	P-value	$5x10^{-12}$	0.00015	0.00125	0.00199	0.00415	0.00916	0.01411	0.01652	0.01749	0.03633	0.03673	0.04325	0.01344	0.00056	0.01749
	FDR corrected	0.00000	0.01179	0.05073	0.06442	0.11206	0.21199	0.25391	0.26761	0.25756	0.45274	0.42497	0.46705	0.27216	0.03036	0.23610
Bulk	E-	0.34	0.40	0.89	0.43	0.20	0.77	0.00	0.00	0.03	0.40	1.00	0.17	0.09	0.06	0.03
	CTE+	0.00	0.71	0.49	0.15	0.00	0.58	0.00	0.00	0.00	0.37	0.25	0.03	0.00	0.00	0.00
	AR542E+	0.00	0.03	0.20	0.20	0.00	0.49	0.00	0.00	0.00	0.26	0.86	0.17	0.00	0.00	0.00
	AR584E+	0.03	0.00	0.62	0.22	0.06	0.43	0.00	0.00	0.00	0.40	0.34	0.06	0.00	0.00	0.00
Rhizosphere	E-	0.97	0.37	0.60	0.26	0.03	0.83	0.11	0.17	0.03	0.63	0.71	0.09	0.00	0.11	0.03
	CTE+	0.00	0.06	0.31	0.23	0.00	0.60	0.00	0.03	0.00	0.31	0.29	0.03	0.00	0.00	0.00
	AR542E+	0.07	0.13	0.20	0.20	0.00	0.63	0.00	0.00	0.00	0.43	0.53	0.00	0.00	0.00	0.00
	AR584E+	0.00	0.00	0.22	0.09	0.06	0.46	0.00	0.00	0.00	0.37	0.25	0.06	0.00	0.00	0.00

Fig. 3 Mean relative abundances (%) of the 15 fungal genera that significantly differed between endophyte treatments. *P* values were generated from linear mixed effect models designed to test for differences between all endophyte-infected (+) and endophyte-free (−) samples for bulk or rhizosphere soils across both cultivars. Higher relative abundances of each phylum are highlighted in *red*, and lower relative abundances are highlighted in *blue* within bulk soil and rhizosphere samples

the phyla Bacteroidetes, Proteobacteria, Planctomycetes, and Chloroflexi in the rhizosphere, while Verrucomicrobia, Acidobacteria, Firmicutes, and the archaeal phylum, Crenarchaeota, were relatively more abundant in the bulk soil (Table 3). These patterns are consistent with previous studies comparing prokaryotic communities in bulk and rhizosphere soil [74–76] and are likely driven, in part, by the nutrient and organic carbon-rich rhizosphere environment favoring copiotrophic microbial taxa [77]. Previous work [76, 78] has shown that members of the β-Proteobacteria and Bacteroidetes phyla are often copiotrophic, with the Acidobacteria phylum containing more oligotrophic taxa. Fungal communities also differed significantly between the two soil sample locations, with greater relative abundance of Chytridiomycota in the rhizosphere and greater Zygomycota in bulk soil, differences that may also be associated with higher resource availability in the rhizosphere. Surprisingly, our hypothesis regarding endophyte and cultivar effects being most pronounced in the nutrient-rich rhizosphere was not supported. Endophyte effects were similarly observed in bulk and rhizosphere soils (Figs. 2 and 3), and cultivar effects were only significant in bulk soil, for two bacterial and fungal phyla (Table 4).

Our study reveals that presence of the *Epichloë* foliar endophyte in tall fescue significantly alters the composition of bulk and rhizosphere soil fungal communities. Specifically, endophyte presence tends to increase phylum Glomeromycota and reduce phylum Ascomycota and other fungal genera. This result suggests complex tripartite interactions exist between tall fescue, *E. coenophiala*, and soil fungal communities. Additional work determining the conditions that influence these interactions and their implications for ecosystem functioning remains to be done. Future studies exploring how biotic and abiotic stressors affect the tripartite relationships, as well as the responses of the fescue root metabolome and belowground production, will improve our understanding of tall fescue—*E. coenophiala* symbiotic effects on soil biogeochemical processes.

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