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# Plant species, nitrogen status and endophytes are drivers of soil microbial communities in grasslands

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## ABSTRACT

**Context.** There is concern that the introduction of 'novel' plant germplasm/traits could outpace our capacity to measure and so assess their impacts on soil microbial communities and function. **Aim.** This study aimed to investigate the effects of plant species/functional traits, nitrogen (N) fertilisation and endophyte infection on grassland soil microbial communities within a short time span of 2 years. **Methods.** Two field experiments with monoculture plots were conducted in a common soil. Experiment 1 compared grasses and legumes, using two cultivars of perennial ryegrass (*Lolium perenne*) that varied in fructan content, along with the legumes white clover (*Trifolium repens*) and bird's-foot trefoil (*Lotus pedunculatus*) that varied in tannin content. Grass treatments received high and low N application levels. Experiment 2 compared the presence/absence of *Epichloë* strains in ryegrass, tall fescue (*Schedonorus phoenix*) and meadow fescue (*Schedonorus pratensis*). Soil microbial communities were analysed by using high-throughput sequencing of DNA isolated from bulk soil cores. **Key results.** Higher abundance of ligninolytic fungi was found in grass soils and pectinolytic fungi in legume soils. Levels of N fertilisation and fructan in ryegrass had only minor effects on soil fungal communities. By contrast, N fertilisation or fixation had a strong effect on bacterial communities, with higher abundance of nitrifiers and denitrifiers in high-N grass soils and in legume soils than in low-N grass soils. *Epichloë* affected soil microbiota by reducing the abundance of certain fungal phytopathogens, increasing mycorrhizal fungi and reducing N-fixing bacteria. **Conclusions.** Chemical composition of plant cell walls, which differs between grasses and legumes, and presence of *Epichloë* in grasses were the main drivers of shifts in soil microbial communities. **Implications.** Impacts of farming practices such as mono- or poly-culture, N fertilisation and presence of *Epichloë* in grasses on soil microbial communities should be considered in pasture management.

**Keywords:** bulk soil, *Epichloë*, legumes, microbiota, nitrogen, pastures, soil microbial community, temperate grasslands.

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## Introduction

The myriad soil microbial species perform a diverse array of metabolic functions and are central to global carbon (C) and nitrogen (N) sequestration and cycling. Interactions and feedback between above- and belowground organisms are increasingly seen to control terrestrial ecosystem processes and services (Wardle *et al.* 2004; De Deyn *et al.* 2008; van der Heijden *et al.* 2008; Zak *et al.* 2011; Tomazelli *et al.* 2023). Sustaining those services, with any degree of certainty, faces increasing challenge.

Managed temperate grasslands occupy 25% of the world's land area, which is 70% of global agricultural land (Ghahramani *et al.* 2019). Those grasslands are a major source or sink for C and N (Oenema *et al.* 2005; McSherry and Ritchie 2013), and their management has significant implications for greenhouse gas emissions (Jones and Donnelly 2004; Reisinger and Ledgard 2013; Parsons *et al.* 2016). In grassland ecosystems, even simple management changes such as N fixation or fertilisation can substantially affect the scale of C and N inputs, as well as the C:N ratio balance, not least because grazing ruminants (as major aboveground heterotrophs) effectively 'uncouple' the C and N cycles (Parsons *et al.* 2013; Soussana and Lemaire 2014). Over the past few decades, pressure for greater

food production has led to substantially increased N fertiliser usage and a decline in reliance on legume N fixation (Brisson *et al.* 2010). There have been increased calls for plants with novel functional traits, such as grasses with high C:N ratios (Parsons *et al.* 2011) and white clover (*Trifolium repens* L.) with high condensed tannins (Hancock *et al.* 2012), or novel forage plant germplasm, for example 'extended phenotypes' of endophyte-infected grasses (Schardl *et al.* 2013), to meet human demands for food. Any introduced desirable traits or extended phenotypes have a substantial impact on some aspect of ecosystem functioning; however, the potential effects of these introductions on soils have received little assessment. Indeed, many of the forage traits targeted by industry directly affect C and N cycling in plants, animals and soils (Parsons *et al.* 2011).

Conventional assessments of the impacts and outcomes on soil C:N states and organic matter, from even simple changes in farm practices, contain a high degree of uncertainty (Kelliher *et al.* 2013) and can require extensive re-measurement across sites and time, for example of some 30+ years (Schipper *et al.* 2010; Mudge *et al.* 2017). Impacts on soil function will be related to, and likely prefaced by, changes in the soil microbial community. Therefore, a first step is to assess whether plant species and their functional traits, N fertilisation and plant extended phenotypes lead to changes that are detectable via a detailed analysis of soil microbial communities, and whether any effects are significant on a short time scale. With the advent of high-throughput next-generation sequencing methods, more detailed studies became possible for analysis of effects of factors such as N fertilisation (Nemergut *et al.* 2008; Nacke *et al.* 2011), global localisation (Prober *et al.* 2015), elevation gradients (Pellissier *et al.* 2014) or endophyte infection (Rojas *et al.* 2016; Mahmud *et al.* 2021; Chen *et al.* 2022) on grassland soil microbial community composition and diversity. Whereas, soil types and their physico-chemical properties, as well as plant species and their associated management, have major impacts on microbial composition (Bezemer *et al.* 2006; Young and Crawford 2004), most studies have been performed across a range of different soils and with limited knowledge about past or present plant cover and management practices.

Changes in the number of species present *per se* might not be the most relevant measure for deducing any change in soil function (van der Wal *et al.* 2013). In addition, studies that attempt to identify and catalogue the wide range of soil organisms present face the difficulty that many of the soil microbial species, fungi in particular, have not been sequenced, let alone functionally classified. In the present study, we attempt to overcome these limitations by using a series of systematically different experimental treatments, analysing only for significant differences between treatments with respect to the microbial communities they foster in what was previously a common soil mineralogy and community. This approach allows us to identify changes in community composition, even when many of the organisms present remain 'unnamed' or where the bulk of the soil community may be unaltered. Specifically, we performed two concurrently run experiments in a common soil, each with a random spatial distribution of treatments and replications, for effects on soil microbial communities. In Experiment 1 (plant species/traits, N), we tested for differential impacts of two major forage legume species (white clover, *Trifolium repens* L.; and bird's-foot trefoil, *Lotus pedunculatus* Cav.) and two cultivars of perennial ryegrass (*Lolium perenne* L.) associated with various N application levels. In Experiment 2 (grass extended phenotype), we tested for differential impacts of grasses containing various strains of *Epichloë* fungal endophytes. Both fungal and bacterial communities were analysed using high-throughput sequencing of DNA isolated from bulk soil cores harvested 2 years after imposing the above treatments. The study focused on implications for C and N cycling, and whether any changes were detectable within a short time span of 2 years.

## Materials and methods

### Experimental design

All experimental field plots were established on Paparua sandy loam soil at the Lincoln University Research Dairy Farm, Lincoln, New Zealand. The whole experimental area had been previously used for cereal cropping and was ploughed prior to sowing forage plants as described below. For a detailed analysis of soil physico-chemical properties, see Cripps *et al.* (2013). Two experiments were set up in April 2010; in both, four experimental replicates (plots) of each treatment were sown in a randomised block design. Fertiliser N in the form of urea was applied to all plots at a rate of 40 kg N $ha^{-1}$ every 4 months, except for the legume and low-N treatments in Experiment 1 (see Table 1), which were fertilised with 10 kg N $ha^{-1}$. The area surrounding the experimental plots was sown with *L. perenne* cv. Alto (containing AR1 endophyte). Individual plot areas were 2.1 m by 6 m in Experiment 1, and 2.1 m by 3 m in Experiment 2. Plots were grazed and mown as described by Cripps *et al.* (2013).

Experiment 1, examining plant species/traits and levels of N, comprised four replicates of six treatments: two legumes, white clover cv. Kopu sown at 4 kg seed $ha^{-1}$ (WCl) and bird's-foot trefoil cv. Barsille sown at 4 kg seed $ha^{-1}$ (LoP); and two *Epichloë*-free perennial ryegrass cultivars, normal sugar cv. Impact (RG) and high sugar cv. AberDart (HSG), both sown at 20 kg seed $ha^{-1}$, and both with a low N (LN, 10 kg N $ha^{-1}$) and a high N (HN, 40 kg N $ha^{-1}$) fertilisation rate treatment. The two legume species differ substantially in their ability to accumulate antimicrobial, protein-binding condensed tannins (Terrill *et al.* 1992), and the two ryegrass cultivars differ significantly in their ability to produce fructans in leaf blades (Parsons *et al.* 2011). A summary of the experiments is shown at Table 1.

**Table 1.** Summary of the design for each experiment.

| Experiment 1: plant species/trait and nitrogen level | | | | |
|---|---|---|---|---|
| **Plant type** | **Species** | **Common name and cultivar** | **Nitrogen applied** | **Treatment abbreviation** |
| Legume | *Trifolium repens* | White clover cv. Kopu | 10 kg N $ha^{-1}$ | WCl |
| | *Lotus pedunculatus* | Bird's-foot trefoil cv. Barsille | 10 kg N $ha^{-1}$ | LoP |
| Grass | *Lolium perenne* | Perennial ryegrass cv. Impact (normal sugar variety) | 10 kg N $ha^{-1}$ | LN RG |
| | | | 40 kg N $ha^{-1}$ | HN RG |
| | | Perennial ryegrass cv. AberDart (high sugar variety) | 10 kg N $ha^{-1}$ | LN HSG |
| | | | 40 kg N $ha^{-1}$ | HN HSG |

| Experiment 2: grass extended phenotype (i.e. endophyte infection) | | | | |
|---|---|---|---|---|
| **Grass common name** | **Species and cultivar** | **Endophyte species** | **Endophyte treatment/strain** | **Treatment abbreviation** |
| Perennial ryegrass | *Lolium perenne* cv. Alto | *Epichloë festucae* var. *lolii* | Uninfected | RG Nil |
| | | | Wild type | RG WT |
| | | | NEA2 | RG NEA2 |
| | | | AR1 | RG AR1 |
| | | *Epichloë* sp. LpTG-3 | AR37 | RG AR37 |
| Tall fescue | *Schedonorus phoenix* cv. Grassland Advance | *Epichloë coenophiala* | Uninfected | TF Nil |
| | | | AR542 | TF AR542 |
| Meadow fescue | *Schedonorus pratensis* | *Epichloë uncinata* | Uninfected | MF Nil |
| | | | U2 | MF U2 |

There were four replicates of each treatment. In Experiment 1, soil bacterial communities were analysed for only three replicates.

Experiment 2, examining grass extended phenotypes, comprised four replicates of nine treatments: five treatments with perennial ryegrass cv. Alto (RG), four of which contained one of three different strains of *Epichloë* spp. (AR1, AR37 and the wild-type strain, WT) or a strain blend of NEA2 and NEA6 (branded 'NEA2', Eady 2021), and one that was endophyte-free (nil); two treatments with tall fescue (*Schedonorus phoenix* (Scop.) Holub) cv. Grasslands Advance (TF), one containing *Epichloë coenophiala* (strain AR542) and one endophyte-free; and two treatments with meadow fescue (*Schedonorus pratensis* (Huds.) P.Beauv.) (MF), one containing *Epichloë uncinata* (strain U2) and one endophyte-free (see Table 1). Each grass treatment was sown at a rate of 15 kg seed $ha^{-1}$. All grass–endophyte combinations were assessed for the presence of endophyte in January 2011 by microscopic examination of leaf sheath tissue stained with lactophenol cotton blue. Endophyte-containing grasses ranged from 85% to 100% infected, and the endophyte-free grasses were 0–5% infected (for further details, see Cripps *et al.* 2013). The commonly used endophytic *Epichloë festucae* var. *lolii* strains (AR1, NEA2 and WT) and *Epichloë* sp. LpTG-3 strain AR37 differ in their ability to produce certain fungal alkaloids.

## Soil sampling and DNA extraction

In April 2012, soil was collected to a depth of 7.5 cm with a metal corer (diameter 3 cm). In Experiment 1 six cores and in Experiment 2 four cores were sampled randomly from each plot. Soil cores from each individual plot were mixed and passed through a 4-mm metal sieve to remove larger stones and plant root parts, bagged, immediately frozen in liquid $N_2$ for transport and stored at −80°C until analysis. The above procedures of collection and treatment for each soil sample were completed within <15 min.

Genomic DNA was extracted from soil by means of the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. DNA concentrations were measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNAs (~25 ng $\mu L^{-1}$) were used for polymerase chain reaction (PCR) as detailed below.

## Analysis of soil fungal community composition: 454 pyrosequencing

Soil fungal community composition was analysed by 454 pyrosequencing on the GS-FLX 454 System (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA) at Waikato University, Hamilton, NZ. DNA samples were amplified using general primers linked to 454 pyrosequencing adapters A and B, and a barcode (multiplex identifier, MID) for each sample, as recommended for the 454 GS-FLX One-Way Read method. The primer pair used in this study, NSI1mod (modified NSI1 primer) and 5.8A2R (Martin and Rygiewicz 2005), was designed to target fungal DNA in the partial 18S SSU and the variable ITS1 region of the non-coding nuclear rDNA ITS region, which is recognised as the universal DNA barcode marker for fungi (Schoch *et al.* 2012). We modified the NSI1 primer, which targets primarily Ascomycota and Basidiomycota, with a degenerate base at the 3′ end in order

to include potential Glomeromycota sequences. Thus, the composite forward primer was NSI1mod 5′-CCATCTCATC CTGCGTGTCTCCGACTCAG xxx *GATTGAATGGCTTAGTG AGK*-3′ and the reverse primer was 5.8A2R 5′-CCTATCC CCTGTGTGCCTTGGCAGTCTCAG*CTGCGTTCTTCATCGAT*-3′, where manufacturer adapters are underlined, barcode is indicated by xxx, and the general primers NSI1mod and 5.8A2R are shown in italics.

For each sample, a PCR mix was prepared containing 1 μL FastStart High Fidelity *Taq* polymerase (Roche Molecular Systems, Pleasanton, CA, USA), 10 μL reaction buffer 10X, 2 μL PCR grade nucleotide mix, and 0.8 μM each primer in a total volume of 100 μL. Three replicate PCRs per DNA sample were performed by adding 1 μL template DNA to 25 μL reaction mix and using the following cycling conditions: 94°C for 4 min; 30 cycles of 30 s at 94°C, 50°C for 1 min and 72°C for 1.5 min; followed by 7 min at 72°C. Replicate PCR products were pooled and separated on 0.8% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer. PCR products were purified from the gel by using the ISOLATE Gel Purification Kit (Bioline, Alexandria, NSW, Australia), and DNA concentrations in the purified products (amplicon libraries) were measured with a QuBit fluorometer (Invitrogen, Eugene, OR, USA). Aliquots (200 pg $\mu L^{-1}$) of each sample were run on a Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) and the number of molecules in each sample was calculated. Amplicon libraries were mixed at equimolar concentrations to make a solution of $1 \times 10^9$ molecules DNA in 200 μL.

A 1-μL sample of the combined library solution was subjected to pyrosequencing and the datasets were processed using mothur v.1.32 (Schloss *et al.* 2009). Reads were subjected to denoising, quality trimming and chimera removal using AmpliconNoise v.1.28 (Quince *et al.* 2011). Sequences shorter than 400 bp after quality trimming were discarded. Sequences were clustered into molecular operational taxonomic units (OTUs) using mothur v.1.32. Distance was calculated using ESPRIT-Tree (pair-wise alignment) (Cai and Sun 2011); of the four stringencies (unique, 1%, 3%, 5% distance) tested with nearest, average and furthest neighbour strategy, we chose the 3% nearest neighbour stringency with a 97% sequence identity threshold. Following OTU clustering, a reference sequence from each OTU was mathematically determined and used for taxonomic identification of the OTU. Representative sequences for each OTU were queried against the non-redundant GenBank database using the BLASTN algorithm.

### Analysis of soil bacterial community composition: MiSeq Illumina Sequencing

Bacterial community composition was investigated via the MiSeq sequencing platform (Illumina, San Diego, CA, USA) at the Massey Genome Service (Massey University, Palmerston North, NZ). Genomic DNA (10 ng $\mu L^{-1}$) was prepared from three replicates of the six treatments from Experiment 1 and from all 36 samples from Experiment 2 according to the specifications of the Massey Genome Service. Amplicon libraries were prepared using a single-step PCR; libraries were purified by the Massey Genome Service. A set of index-tagged PCR primers containing the Illumina complementary adapter sequence for binding to the flowcell, which flanks the 16S fourth hypervariable (V4) region, was used for amplification. The amplicon libraries were pooled at equal molarity. To control for uneven representation of bases at each cycle with 16S libraries, the Illumina PhiX control library was added at ~10% volume before loading onto the Illumina MiSeq instrument.

All bioinformatics analysis steps were performed using mothur (Schloss *et al.* 2009). Sample reads were combined to generate ~250 bp reads (contigs), using FLASH after preliminary quality-control steps. Raw sequence data were filtered by removing any sequences that contained ambiguous call 'N', lengths >275 bp, and low-quality regions. Data were aligned to 16s rRNA reference database (RDP) v.9 and unaligned data were removed. Chimeras were detected using UCHIME (Edgar *et al.* 2011) implemented in mothur. All reads were taxonomically classified using the RDP classifier (Wang *et al.* 2007); reads that were classified as chloroplast, mitochondria, unknown, Archaea and Eukaryota were removed.

### Statistical analyses

Prior to statistical analyses, sequences of very low abundance were removed from the datasets; we used fungal or bacterial sequences of <10 reads on average per treatment as an arbitrary cut-off, i.e. an OTU had to be present at least 60 times in the sample set from Experiment 1 and 90 times in the sample set from Experiment 2. We used partial least square discrimination analysis (PLSDA) to identify microbial genera (i.e. OTUs) contributing the most to a segregation of treatment groups. Those genera with a VIP (variable importance in projection) score >1.0 on each of the relevant PLSDA axes were subsequently selected for analysis of variance (ANOVA). In Experiment 1, one-way ANOVA was used to separate treatment effects (i.e. plant type, plant species, plant cultivar, N applied). In Experiment 2, two-way ANOVA was used to separate effects of endophyte with grass species as factor 1 and endophyte status (endophyte or nil) as factor 2; and one-way ANOVA was used to separate strain dependency within ryegrass group, with nil, WT, AR1, AR37 and NEA2 as treatment groups. All statistical analyses were performed using XLSTAT v.2015.6.01.25106 (Addinsoft, Paris, France). To homogenise the variance, all data were Box–Cox transformed prior to analyses.

## Results

### Experiment 1: impacts of plant species/traits and nitrogen fertilisation level on soil microbial communities

In total, 6648 fungal and 512 bacterial OTUs were identified in soils from Experiment 1 across all treatments, with the most

abundant fungal OTU (*Mycena* sp.) being amplified 12 796 times and the most abundant bacterial OTU (unclassified Actinobacteria_Gp6) being amplified 6691 times. The top 3.8% of fungal OTUs (235 OTUs, total reads 107 406) and 31.25% of bacterial OTUs (160 OTUs, total reads 145 902) were selected for further analysis.

The soil fungal microbiota was dominated by phyla Ascomycota (44%), Basidiomycota (34%), Glomeromycota (6%), Zygomycota (2%), Chytridiomycota (0.4%) and unknown ectomycorrhizal fungi (1%). Approximately 12% of the OTUs aligned to unknown fungi. The abundance was significantly different among treatments for Glomeromycota (arbuscular mycorrhizae; $P = 0.013$) and Chytridiomycota (saprotrophs; $P < 0.001$). Glomeromycota represented by the Glomeraceae family was most abundant in WCl soils and least abundant in grass (both HSG and RG) soils at low N, whereas the Chytridiomycota represented by the Olpidiaceae family was more abundant in RG soils at both N levels than in legume soils. Within the Basidiomycota, the class of Agaricomycetes was more abundant in HSG (both N levels) than WCl soils ($P = 0.004$); and within the Ascomycota, the class Leotiomycetes was more abundant in grass than legume soils ($P < 0.001$). The soil bacterial microbiota was dominated by phyla Proteobacteria (25%), Acidobacteria (12%) and Bacteroidetes (12%). Up to 29% of the OTUs were aligned to unclassified bacteria. The abundance was different among treatments for Armatimonadetes ($P = 0.008$, represented by the Armatimonadaceae family), Bacteroidetes ($P = 0.032$), and Nitrospirae ($P = 0.017$, represented by the Nitrospiraceae family).

With respect to fungal genera, legume soils segregated from grass soils on PLSDA axis 1, whereas WCl soils segregated from LoP soils on axis 2 (Fig. 1*a*), and a weak segregation of HSG vs RG soils and low N vs high N soils was observed on axis 3 (Supplementary Fig. S1a). Regarding bacterial genera, similar to fungi, legume soils segregated from grass soils on PLSDA axis 1 and WCl from LoP soils on axis 2 (Fig. 1*b*), low N segregated from high N soils on axis 3 (Fig. S1b), and there was a weak segregation of HSG from RG soils on axis 4 (Fig. S1b).

In total, 75 fungal OTUs were significantly different (VIP >1.0), with 51 of these being most important for segregation on axis 1, nine on axis 2 and 15 on axis 3 (data not shown). Genera that were significantly affected by treatment are shown in Fig. 2. The Ascomycota *Leohumicola* sp. (root endophyte) and *Chaetomium* sp. (mould) were more abundant in grass soils than in legume soils (Fig. 2*a*). *Crucellisporium* sp. was also more abundant in grass soils but only compared with WCl (not LoP) soil, whereas *Phaeosphaeriopsis* sp. (root endophyte) was more abundant in grass and WCl soils than in LoP soil. The Ascomycota *Epicoccum* sp. (humic acid/melanin forming), *Fusarium* sp. (plant pathogen), *Ilyonectria* sp. (plant pathogen) and *Plectosphaerella* sp. (plant pathogen) were substantially more abundant in legume soils than in grass soils (Fig. 2*a*). The abundance of Xylariales fungi (e.g. *Pestalotiopsis* sp., *Monographella* sp. and *Anthostomella* sp.) appeared to be

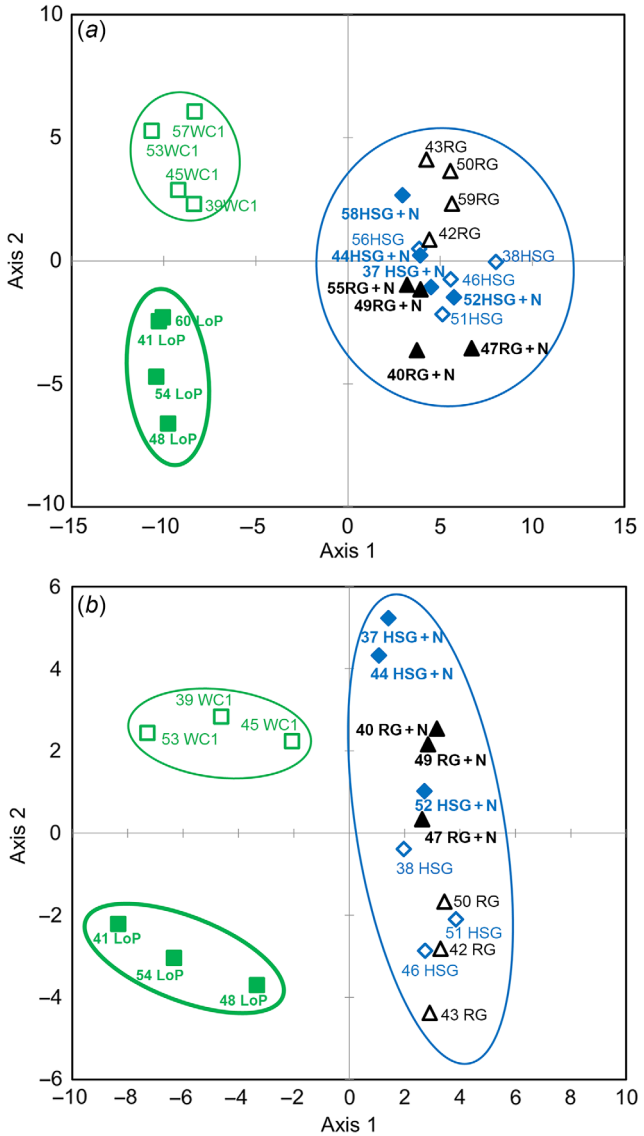


**Fig. 1.** Partial least square discrimination analysis (PLSDA) of impacts of plant species/traits and N fertilisation level on the abundance of (*a*) fungal and (*b*) bacterial genera. Blue open diamonds, HSG (high sugar perennial ryegrass cv. AberDart) (low N); blue closed diamonds, HSG + N (high N); black open triangles, RG (normal sugar perennial ryegrass cv. Impact) (low N); black closed triangles, RG + N (high N); green open squares, WCl (white clover); green closed squares, LoP (bird's-foot trefoil). Abundances were Box–Cox transformed prior to PLSDA. Groups showing major spatial separations are confined within ellipses.

low in legume soils (data not shown). The Basidiomycota *Mycena* sp. (white-rot fungus), *Ceratobasidium* sp. (saprotrophic fungus) and *Sebacina* sp. (mycorrhizal fungus) were more abundant in grass soils than in legume soils, whereas *Holtermannia* sp. (fungal pathogen) was less abundant in grass soils than in legume soils (Fig. 2*b*). The white-rot fungus *Polyporus* sp. was also more abundant in grass soils, except in HSG soil at high N, but only compared with LoP (not WCl) soil.

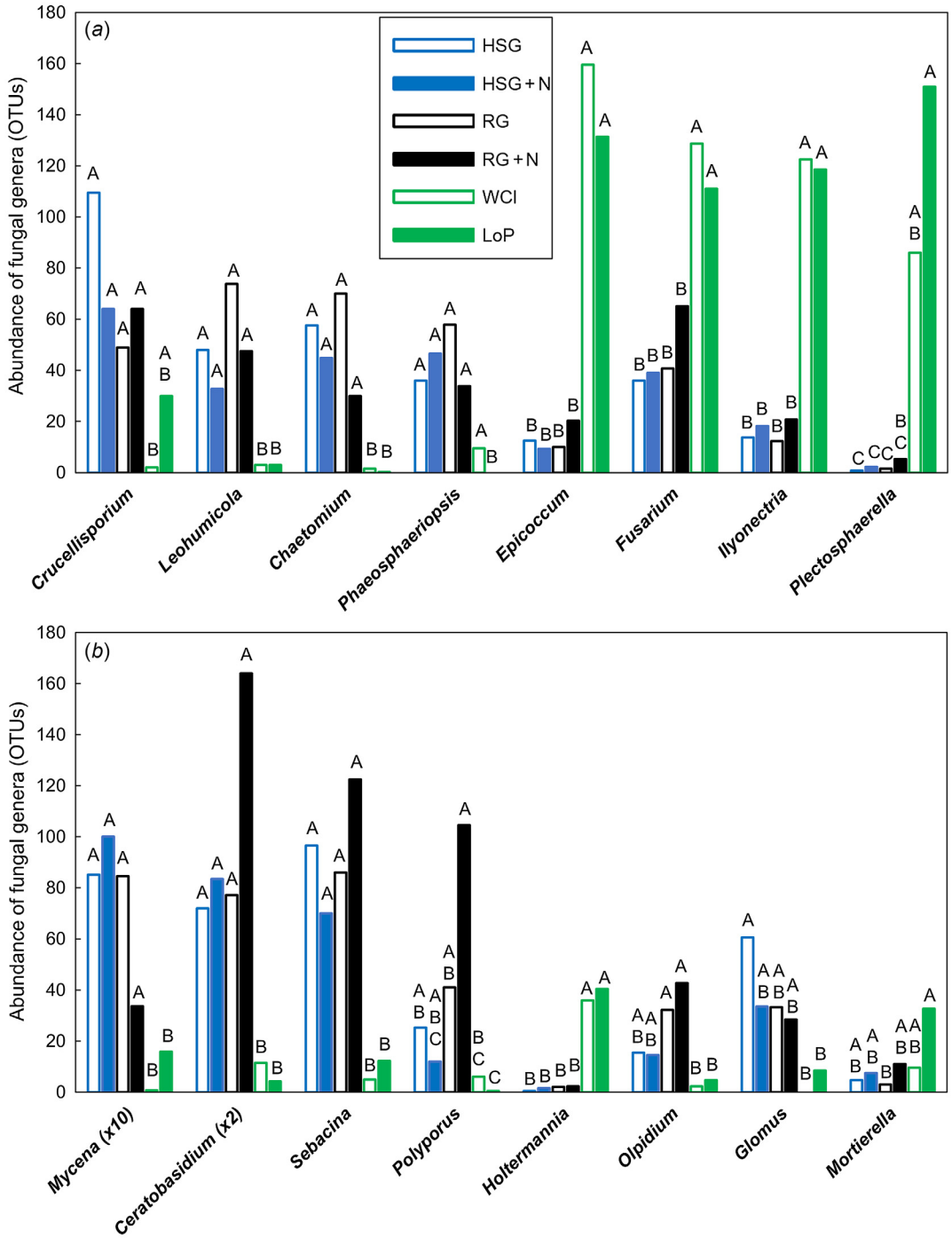


**Fig. 2.** Impacts of plant species/traits and N fertilisation level on the abundance of influential fungal genera within the PLSDA related to Experiment 1. Fungal genera are shown according to the associated phyla within (*a*) Ascomycota and (*b*) Basidiomycota. Only those genera that featured strongly as VIP >1 were analysed further using one-way ANOVA. Untransformed means of statistically significant different genera ($P < 0.05$) are shown. Blue open bars, HSG (high sugar perennial ryegrass cv. AberDart) (low N); blue closed bars, HSG + N (high N); black open bars, RG (normal sugar perennial ryegrass cv. Impact) (low N); black closed bars, RG + N (high N); green open bars, WCl (white clover); green closed bars, LoP (bird's-foot trefoil). Abundances were Box–Cox transformed prior to one-way ANOVA; within each genus, treatment means with the same letter are not significantly different according to Tukey's HSD at $P = 0.05$.

The Chytridiomycota *Olpidium* sp. (root pathogen) was more abundant in RG soils (both N treatments) than in legume soils, and the Glomeromycota *Glomus* sp. (arbuscular mycorrhizal fungus) was more abundant in HSG soil at low N than in legume soils (Fig. 2*b*). By contrast, the Zygomycota *Mortierella* sp. (saprotrophic fungus) was less abundant in RG soil at low N than in LoP soil.

In total, 27 bacterial OTUs were significantly different (VIP >1.0), with 18 of these being most important for segregation on axis 1, two on axis 2, four on axis 3, and three on axis 4 (data not shown). Genera that were significantly affected by treatment are shown in Fig. 3. The nitrifying *Nitrospira* sp. and the N-fixing *Rhizobium* sp. were less abundant in grass soils at low N than in both legume soils (*Nitrospira* sp.), and in LoP soil only (*Rhizobium* sp.) (Fig. 3*a*). The denitrifying unclassified Comamonadaceae were also less abundant in HSG soil at low N and RG soils at both N levels than in LoP soil (Fig. 3*a*). The cellulolytic *Cellvibrio* sp. and the aromatic-degrading *Novosphingobium* sp. were less abundant in all grass soils than in LoP soil (*Cellvibrio* sp.), and in both legume soils (*Novosphingobium* sp.) (Fig. 3*a*). *Peredibacter* sp., a bacterium predating on other bacteria, was less abundant in grass soils (except HSG soil at low N) than in legume soils. The denitrifying *Rhodanobacter* sp. was most abundant in grass soils at low N and least abundant in RG soil at high N and WCl soil (Fig. 3*a*). The cellulolytic *Byssovorax* sp. was more abundant in HSG soil at low N than in all other soils except RG soil at low N (Fig. 3*a*). The carbohydrate-degrading *Flavobacterium* sp., *Adhaeribacter* sp. and *Pedobacter* sp. were highly abundant in legume soils and generally much less abundant in grass soils (Fig. 3*b*). *Flavobacterium* sp. was also affected by N, being less abundant in HSG soil at high N than at low N (Fig. 3*b*). Generally more abundant in legume soils were *Rhodococcus* sp. and *Nocardioides* sp., which are known to be able to degrade phenolics and pesticides. By contrast, the ligninolytic unclassified Micromonosporaceae were highly abundant in all grass soils and, except for the comparison with RG soil at high N, were less abundant in both legume soils (Fig. 3*b*). The chemoheterotrophic *Armatimonas* sp. was more abundant in grass soils than in legume soils, whereas *Luteolibacter* sp. was generally less abundant in grass soils than in legume soils (Fig. 3*b*).

## Experiment 2: impacts of plant extended phenotype on soil microbial communities

In total, 5188 fungal and 569 bacterial OTUs were identified in soils from Experiment 2 across all treatments, with the most abundant fungal OTU (*Mycena* sp.) being amplified 11 941 times, and the most abundant bacterial OTU (Acidobacteria_Gp6) being amplified 15 901 times. The top 3.2% of fungal OTUs (165 OTUs, total reads 120 663) and 19% of bacterial OTUs (108 OTUs, total reads 171 474) were selected for further analysis.

Soil fungal microbiota was dominated by phyla Basidiomycota (34.7%), Ascomycota (34.4%), Glomeromycota (5.4%), Zygomycota (2.1%) and unknown ectomycorrhizal fungi (1%). Up to 22% of the OTUs aligned to unknown fungi. PLSDA for fungal genera showed segregation of MF and TF vs RG soils, and MF_nil vs MF_U2 soils, on axis 1 (Fig. 4*a*); MF vs TF soils on axis 2 (Fig. 4*a*); and a weak segregation between RG soils infected with various strains on axis 3 (Fig. S2a). Bacterial phyla in the soils were dominated by Proteobacteria (24%), Acidobacteria (17%), Verrucomicrobia (12%) and Bacteroidetes (11%). Up to 28% of the OTUs aligned to unclassified bacteria. On the level of phyla abundance, differences were found among treatments for Actinobacteria ($P = 0.013$) and Firmicutes ($P = 0.036$). Abundance of Actinobacteria was higher in RG_AR37 than in RG_nil soil ($P = 0.019$), whereas abundance of Firmicutes was higher in RG_nil and TF_AR542 soils than in RG_NEA2 soil ($P = 0.046$). Bacterial genera in RG_nil and RG_AR37 soils appeared to segregate from the three other endophyte-infected RG groups (WT, AR1 and NEA2), and MF_nil segregated from all other soils on axis 2 (Fig. 4*b*). TF soils segregated from RG soils on axis 3 and from MF soils on axis 4 (Fig. S2b).

In total, 46 fungal OTUs were significantly different (VIP >1.0), with 26 of these being most important for segregation on axis 1, 13 on axis 2 and seven on axis 3 (data not shown). Five fungal OTUs were significantly affected by the interaction between grass species and endophyte infection; these were the endomycorrhizal *Glomus* sp. (Fig. 5*a*), as well as *Boletus* sp. (detected in MF_nil soils only), an unclassified Ascomycete, Zygomycete and an unknown fungus (data not shown). Nine fungal OTUs were significantly affected by the symbiotic endophyte status of the grass. The *Epicoccum* sp. (humic acid producer), plant pathogens *Verticillium* sp. and *Phoma* sp. and turfgrass pathogen *Trechispora* sp. were all less abundant in soils from infected grasses, whereas the ectomycorrhizal *Leohumicola* sp., endophytic *Sclerostagonospora* sp., and arbuscular mycorrhizal *Glomus* sp. were more abundant in these soils (Fig. 5*b*); two OTUs could not be classified. The abundance of 32 fungal genera was affected by grass species. Within these influential genera, the brown-rot fungus *Tapinella* sp., the mycorrhizal fungi *Sebacina* sp. and *Leohumicola* sp., the hemicellulolytic *Xenopolyscytalum* sp., the humic acid producer *Epicoccum* sp., the saprotrophic *Collembolispora* sp. and *Exophiala* sp., the turfgrass pathogen *Leptosphaerulina* sp., and the arbuscular mycorrhizal *Glomus* sp. were all higher in RG soils than in either MF or TF or both fescue soils (Fig. 5*c*). The turfgrass pathogen *Trechispora* sp. and the saprotrophic *Ceratobasidium* sp. and *Nectria* sp. were highest in MF soils, and the pathogenic *Pyrenochaeta* sp. was highest in TF soils (Fig. 5*c*). Five fungal genera were differentially affected by endophytic strains in RG soil. The saprotrophic *Collembolispora* sp. was higher in RG_AR1 and RG_AR37 soils than in RG_WT soil, whereas the mycorrhizal *Leohumicola* sp. was highest in RG_AR37 but lowest in RG_AR1 soil (Fig. 5*d*); three OTUs could not be classified.

In total, 22 bacterial genera were significantly different (VIP >1.0), with seven of these being most important for

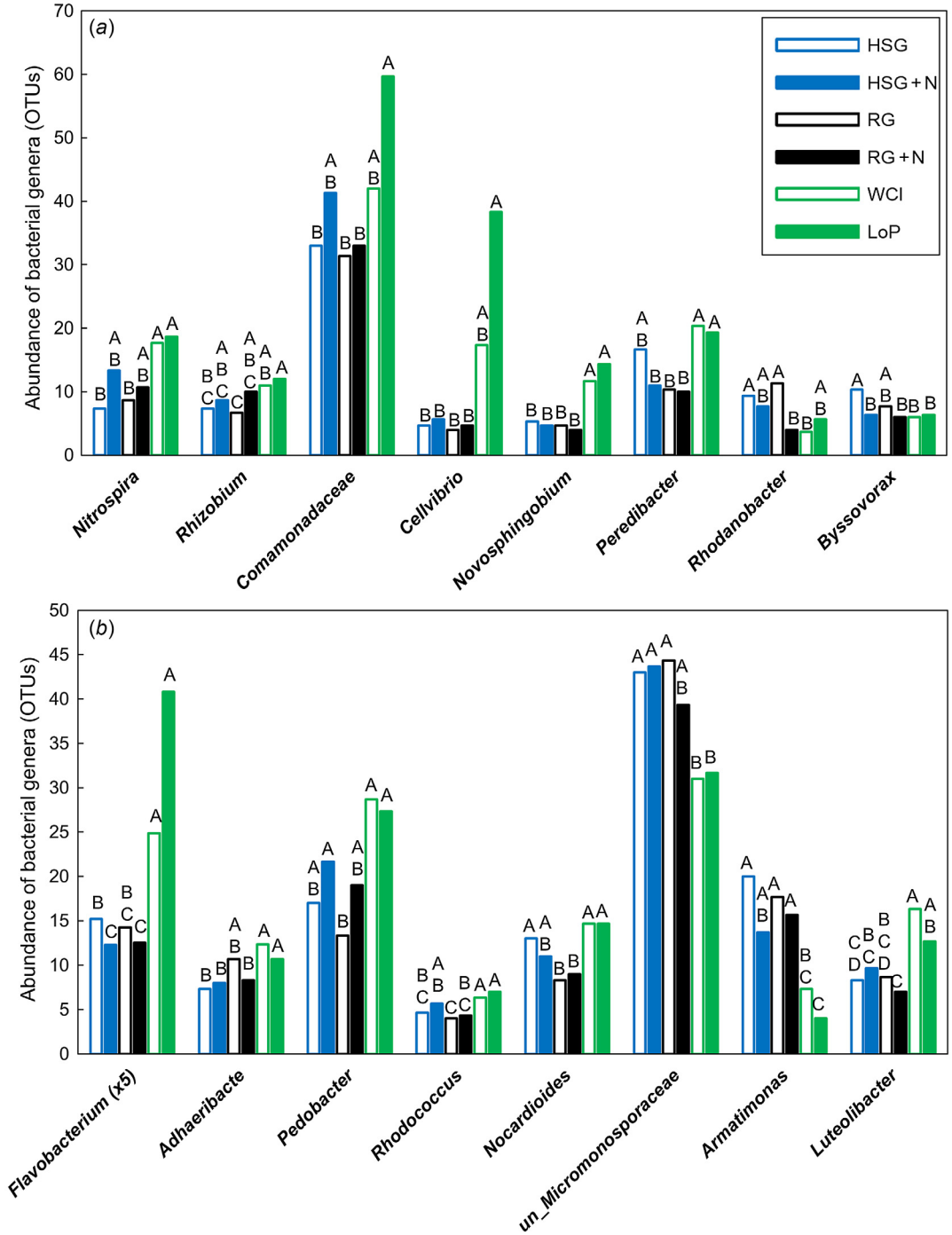


**Fig. 3.** Impacts of plant species/traits and N fertilisation level on the abundance of influential bacterial genera within the PLSDA related to Experiment 1. Bacterial genera were split into (*a*) and (*b*) for illustrative purposes. Only those genera that featured strongly as VIP > 1 were analysed further using one-way ANOVA. Untransformed means of statistically significant different genera ($P < 0.05$) are shown. Blue open bars, HSG (high sugar perennial ryegrass cv. AberDart) (low N); blue closed bars, HSG + N (high N); black open bars, RG (normal sugar perennial ryegrass cv. Impact) (low N); black closed bars, RG + N (high N); green open bars, WCl (white clover); green closed bars, LoP (bird's-foot trefoil). Abundances were Box–Cox transformed prior to one-way ANOVA; within each genus, treatment means with the same letter are not significantly different according to Tukey's HSD at $P = 0.05$.

segregation on axis 1, seven on axis 2, seven on axis 3, and one on axis 4. Six bacterial OTUs were significantly affected by the interaction between grass species and endophyte infection (Fig. 6*a*). *Flavisolibacter* sp. (Bacteroidetes, Chitinophagaceae)

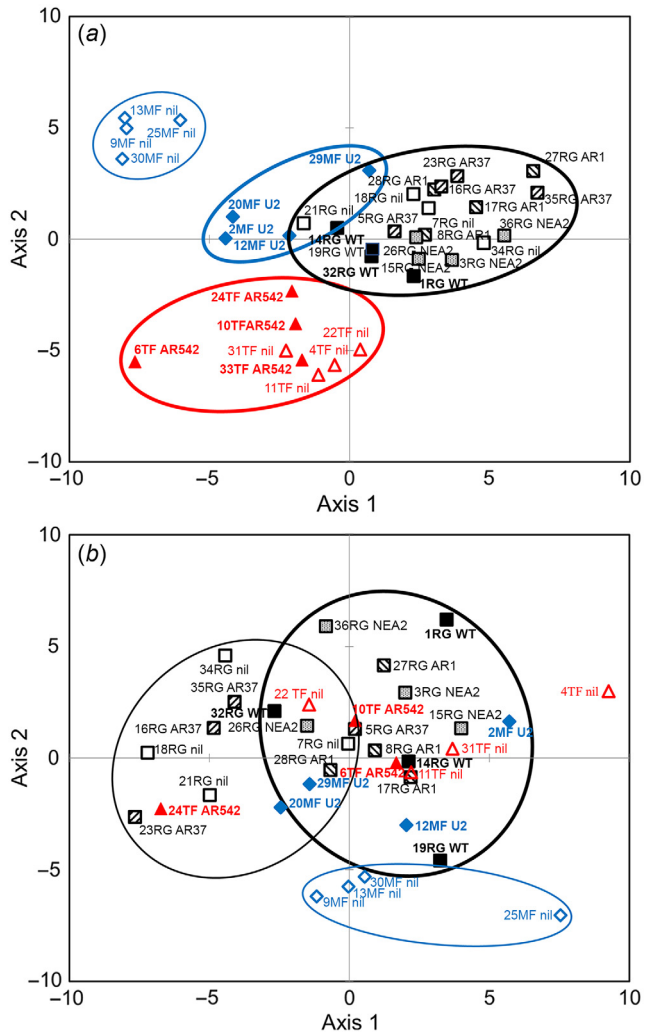


**Fig. 4.** Partial least square discrimination analysis (PLSDA) of impacts of plant extended phenotype (endophyte) and grass species on the abundance of (*a*) fungal and (*b*) bacterial genera. Black open squares, RG_nil (ryegrass without endophyte); black closed squares, RG_WT (ryegrass infected with *Epichloë festucae* var. *lolii* wild-type); black downward-striped squares, RG_AR1 (ryegrass infected with *E. festucae* var. *lolii* AR1); black upward-striped squares, RG_AR37 (ryegrass infected with *Epichloë* sp. LpTG-3 strain AR37); black stippled squares, RG_NEA2 (ryegrass infected with *E. festucae* var. *lolii* NEA2); blue open diamonds, MF_nil (meadow fescue without endophyte); blue closed diamonds, MF_U2 (meadow fescue infected with *Epichloë uncinata* U2); red open triangles, TF_nil (tall fescue without endophyte); red closed triangles, TF_AR542 (tall fescue infected with *Epichloë coenophiala* AR542). Groups showing major spatial separations are confined within ellipses.

and *Opitutus* sp. (Verrucomicrobia) were more abundant in RG_nil soil than in MF_nil and TF_nil soils (*Flavisolibacter* sp.), and in TF_nil soil only (*Opitutus* sp.) (Fig. 6*a*). An unclassified Verrucomicrobiaceae was more abundant in MF_nil than in TF_nil soil, while an unclassified Bacillales and the nitrite-oxidising nitrifier *Nitrospira* sp. were most abundant in TF_EP soil. *Verrucomicrobium* sp. was more abundant in MF_nil soil than in RG and TF_nil soils. Two bacterial OTUs were significantly affected by the endophyte status of the grass species: the N-fixing *Rhizobium* sp. and the Gammaproteobacteria *Legionella* sp., both of which were less abundant in soils from endophyte-infected than uninfected grasses (Fig. 6*b*). Most bacterial genera (14) were affected by grass species and the groups with abundance >15 were further analysed. The Actinobacterium *Conexibacter* sp. and an Acidobacteria_Gp1 were more abundant in RG than in MF and TF soils, whereas the Acidobacteria_Gp3, the Chitinophagaceae *Segetibacter* sp. and an unclassified Myxococcales were more abundant in RG and TF than in MF soils (Fig. 6*c*). The Sphingobacteria *Mucilaginibacter* sp. was most abundant in RG and MF soils. The Bacillales *Mucilaginibacter* sp., an unclassified Polyangiaceae and OD1 were all most abundant in TF soils (Fig. 6*c*). Three bacterial genera were differentially affected by endophytic strain. An unclassified Chitinophagaceae, an unclassified Sphingomonadales and the Actinobacterium *Solirubrobacter* sp. were all highly abundant in RG_nil and RG_AR37 soils (Fig. 6*d*).

## Discussion

There is increasing evidence that aboveground vegetation interacts with belowground microbial populations, with significant outcomes for cycling of both C and N in soils (Wardle *et al.* 2004; van der Heijden *et al.* 2008; Chen *et al.* 2022; Tomazelli *et al.* 2023). In this study, we used a random spatial arrangement of carefully chosen aboveground treatments (plant species/functional traits and N fertilisation level in Experiment 1; grass species and endophyte infection extended phenotype in Experiment 2) across an initially common soil mineralogy and community. Use of a common soil systematically avoids confounding effects of soil biophysical and biochemical characteristics *per se* on microbial communities (Ettema and Wardle 2002; Young and Crawford 2004). Using DNA-based high-throughput sequencing techniques, we are able to focus on those soil organisms that contribute to any significant differences as the soil community responds, while avoiding being overwhelmed by the substantial majority of organisms that might remain common.

### Effects of plant species/traits and N level on soil fungal and bacterial communities

In our study, by far the strongest impacts on soil fungal communities were seen in Experiment 1. Most differences were associated with the distinction between grass and legumes, with relatively minor differences arising between grass cultivars (high vs low sugar), between legume species, or due to N fertilisation level of grasses. Overall, fungal communities were dominated by Asco- and Basidiomycota, with an almost even distribution of these two phyla in all soils except those planted with white clover, in which

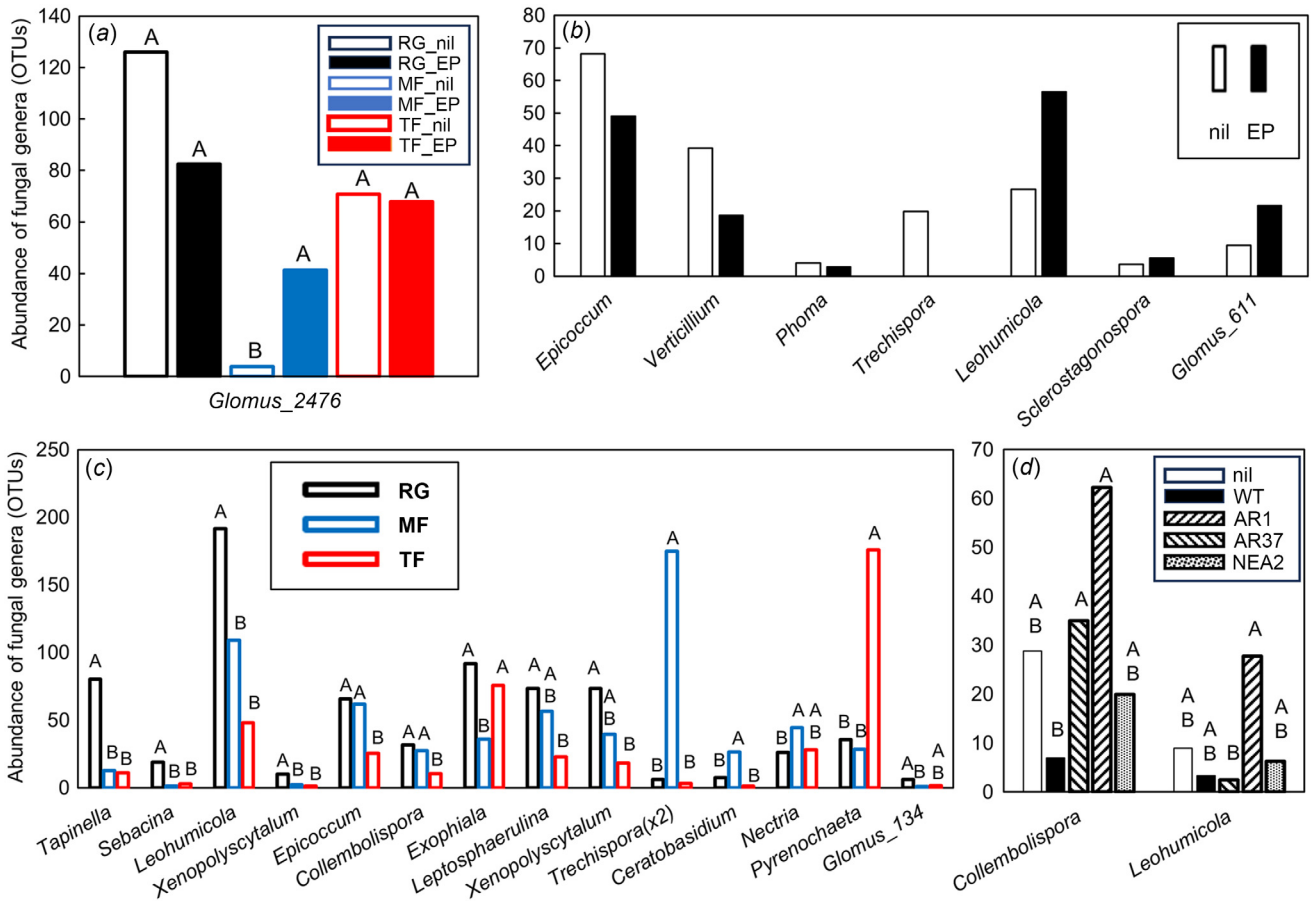


**Fig. 5.** Impacts of plant extended phenotype (endophyte) and grass species on the abundance of influential fungal genera within the PLSDA related to Experiment 2. Only those genera that featured strongly as VIP >1 were analysed further using either (*a–c*) two-way ANOVA or (*d*) one-way ANOVA. Untransformed means of statistically significant different genera ($P < 0.05$) are shown. (*a*) Black open bars, RG_nil (ryegrass without endophyte); black closed bars, RG_EP (ryegrass infected with *Epichloë* sp.); blue open bars, MF_nil (meadow fescue without endophyte); blue closed bars, MF_EP (meadow fescue infected with *Epichloë uncinata* U2,); red open bars, TF_nil (tall fescue without endophyte); red closed bars, TF_EP (tall fescue infected with *Epichloë coenophiala* AR542). (*b*) Open bars, nil (grasses without endophyte); closed bars, grasses infected with *Epichloë* sp.. (*c*) Black bars, RG (ryegrass); blue bars, MF (meadow fescue); red bars, TF (tall fescue). (*d*) Open bars, RG_nil (ryegrass without endophyte); closed bars, RG_WT (ryegrass infected with *E. festucae* var. *lolii* wild-type); downward striped bars, RG_AR1 (ryegrass infected with *E. festucae* var. *lolii* AR1); upward striped bars, RG_AR37 (ryegrass infected with *Epichloë* sp. LpTG-3 strain AR37); stippled bars, RG_NEA2 (ryegrass infected with *E. festucae* var. *lolii* NEA2). Abundances were Box–Cox transformed prior to ANOVA; within each genus, treatment means with the same letter are not significantly different according to Tukey's HSD at $P = 0.05$.

substantially fewer Basidiomycota were detected. The two dominant phyla in grass soils have also been observed in other studies (Borer *et al.* 2014; Prober *et al.* 2015; Yu *et al.* 2021). A sequencing study of the fungal ITS region in soils from grasslands along elevation gradients in the Western Swiss Alps found an even distribution of these two phyla (Pellissier *et al.* 2014). Another sequencing study of the fungal ITS region in soils from 25 temperate grassland sites of the global Nutrient Network experiment (Borer *et al.* 2014) found a strong dominance of Ascomycota in most soils, comprising up to 77% of fungi in tallgrass prairie soils from central USA. Ascomycota, Basidiomycota and Glomeromycota also reportedly dominate fungal communities in the typical steppe soils of northern China (Yu *et al.* 2021). However, unlike our study, none of those studies were performed on monocultures but rather on mixtures of grasses, and notably, where legumes were involved, a mixture of grasses and legumes was used, and so the effects of plant species and N input were confounded.

Within the Ascomycota, we found that *Epicoccum* sp., *Fusarium* sp., *Ilyonectria* sp. (syn. *Cylindrocarpon*) and *Plectosphaerella* sp. were substantially more abundant in legume than grass soils. Species of *Epicoccum* have been shown to form phenolic substances from non-aromatic compounds, which either accumulate as recalcitrant 'humic acid' polymers in soils (Filip *et al.* 1972, 1974) or act as antifungal and

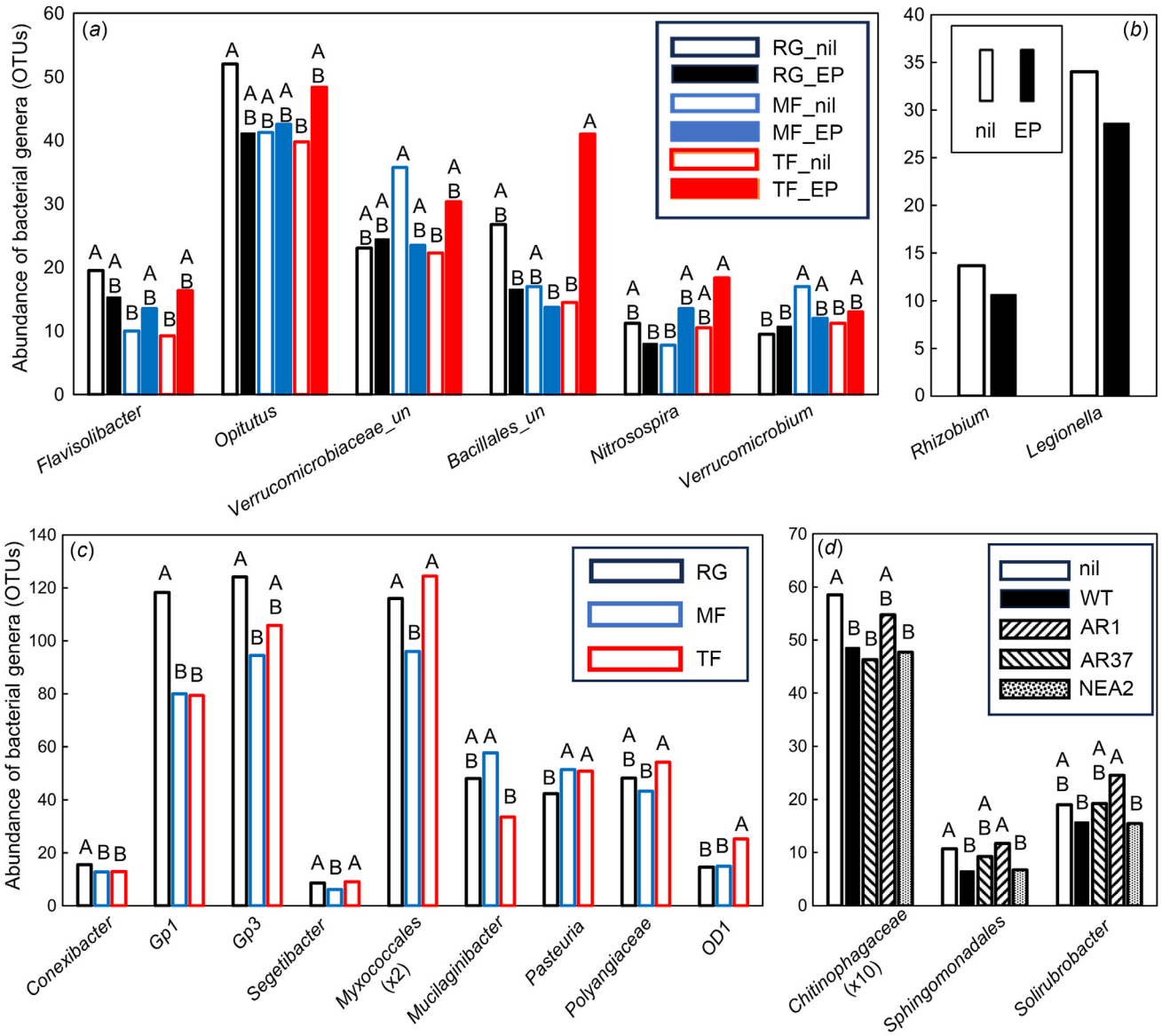


**Fig. 6.** Impacts of plant extended phenotype (endophyte) and grass species on the abundance of influential bacterial genera within the PLSDA related to Experiment 2. Only those genera that featured strongly as VIP >1 were analysed further using either (*a*–*c*) two-way ANOVA or (*d*) one-way ANOVA. Untransformed means of statistically significant different genera ($P < 0.05$) are shown. (*a*) Black open bars, RG_nil (ryegrass without endophyte); black closed bars, RG_EP (ryegrass infected with *Epichloë* sp.); blue open bars, MF_nil (meadow fescue without endophyte); blue closed bars, MF_EP (meadow fescue infected with *Epichloë uncinata* U2); red open bars, TF_nil (tall fescue without endophyte); red closed bars, TF_EP (tall fescue infected with *Epichloë coenophiala* AR542). (*b*) Open bars, nil (grasses without endophyte); closed bars, grasses infected with *Epichloë* sp. endophyte. (*c*) Black bars, RG (ryegrass); blue bars, MF (meadow fescue); red bars, TF (tall fescue). (*d*) Open bars, RG_nil; closed bars, RG_WT (ryegrass infected with *E. festucae* var. *lolii* wild-type); downward striped bars, RG_AR1 (ryegrass infected with *E. festucae* var. *lolii* AR1); upward striped bars, RG_AR37 (ryegrass infected with *Epichloë* sp. LpTG-3 strain AR37); dotted bars, RG_NEA2 (ryegrass infected with *E. festucae* var. *lolii* NEA2). Abundancies were Box–Cox transformed prior to ANOVA; within each genus, treatment means with the same letter are not significantly different according to Tukey's HSD at $P = 0.05$.

antibacterial compounds (Brown *et al.* 1987; Dzoyem *et al.* 2017). *Fusarium* sp. and *Ilyonectria* sp. have been described as plant pathogens (McGee and Kellock 1974) often associated with legumes (Brown *et al.* 1987; Zahid *et al.* 2001; Benitez *et al.* 2016). *Plectosphaerella* sp. has also been described as a plant pathogen (Domsch and Gams 1969). These fungi have been shown to have high pectinase activity (Domsch and Gams 1969) and their high abundance in legume soils might reflect the differences in grass versus dicot cell wall composition, whereby dicot primary cell walls contain up to 7-fold higher levels of pectin (Vogel 2008). In contrast to the above genera, *Crucellisporium* sp., *Leohumicola* sp.,

*Chaetomium* sp. and *Phaeosphaeriopsis* sp. were more abundant in grass soils than legume soils, and all of these have been described as endophytic fungi (Sánchez Márquez *et al.* 2010; Takashima *et al.* 2014), but very little is known about their metabolism. The abundance of *Pestalotiopsis* sp., *Monographella* sp. and *Anthostomella* sp. was also high in grass soils (data not shown); these genera contain members capable of decomposing lignin (Osono 2007).

We also found that the lignin-decomposing fungi in Basidiomycota were more abundant in grass soils. Within the Basidiomycota, several fungal classes (Mycenaceae, Ceratobasidiaceae, Polyporaceae) belonging to the Agaricomycetes were highly represented in soils under grasses compared with those under legumes. Mycenaceae and Polyporaceae are major fungal classes known to contain a large number of ligninolytic white-rot fungal genera (Osono 2007, 2010; Morgenstern *et al.* 2008; Floudas *et al.* 2012; Barrasa *et al.* 2014; Oliveira *et al.* 2020; Atiwesh *et al.* 2022). In this study, the most abundant genus, *Mycena*, was almost absent in soils under white clover and significantly lower in abundance in soils under bird's-foot trefoil, whereas *Polyporus* sp. was most abundant in ryegrass soils under high N fertilisation and least abundant in white clover soils. Both of these genera are white-rot fungi able to degrade lignin, a plant cell-wall phenolic polymer of complex structure that is recalcitrant to decomposition by most organisms, and which forms a protective shield around cellulose (Osono 2007; Dungait *et al.* 2012). These ligninolytic fungi excrete a special set of enzymes: manganese, versatile peroxidases and laccases (Osono 2007; Morgenstern *et al.* 2008; Floudas *et al.* 2012). Most studies on white-rot fungi have focused on wood decay; only little is known about lignin degradation in grasses (Osono 2010; Suhara *et al.* 2012; Nuchdang *et al.* 2015; Shirkavand *et al.* 2016; Oliveira *et al.* 2020; Atiwesh *et al.* 2022). Cell walls of grasses differ significantly from those of dicots (such as legumes) in that they have a higher lignin content and especially high levels of ferulic and *p*-coumaric acid, which crosslink lignin to cellulose, thereby preventing access for microbes to cellulose and other cell wall carbohydrates (Carpita 1996; Grabber *et al.* 2004; Vogel 2008; Oliveira *et al.* 2020; Atiwesh *et al.* 2022). Our results showing a very high abundance of white-rot fungi in grass soils most probably reflect the differences between grasses and legumes, indicating that the chemical composition of plant cell walls that differs between grasses and legumes might be the main driver of shifts in these communities. Qu *et al.* (2016) also found that plant species composition was the key factor influencing the variability of microbial composition within soils.

*Ceratobasidium* sp. (syn. *Rhizoctonia*) and *Sebacina* sp. were also more abundant in grass soils than legume soils. *Ceratobasidium* sp. is a fungal pathogen, known to cause diseases in grasses (Toda *et al.* 1999). Sebacinales are mycorrhizal and endophytic fungi (Weiß *et al.* 2016), some of which (e.g. *Sebacina vermifera*) have been shown to enhance biomass production in switchgrass (*Panicum virgatum* L.) and other plants (Ghimire and Craven 2011; Weiß *et al.* 2016). The only Basidiomycota that was more abundant in legume soils was the *Holtermannia* sp. yeast. Yeasts utilise monomeric and dimeric carbohydrates and amino acids either excreted in root exudates or released during the decomposition of plant cell walls and proteins by other soil microbes (Bisaria and Ghose 1981; Tomme *et al.* 1995); our results might indicate that these types of sugars are more prevalent in soils with legume cover.

Only minor differences in fungal communities were associated with differences in cultivar within the grass species (*L. perenne*), level of N fertilisation or legume species (*T. repens* vs *L. pedunculatus*). *Glomus* sp., an arbuscular mycorrhizal fungus, was more abundant in HSG soil with low N fertilisation than in the legume soils, whereas there were no significant differences among the other grass and legume soils. *Olpidium* sp., a virus-transmitting member of class Chytridiomycetes (Rochon *et al.* 2004), was more abundant in RG soils than legume soils, whereas HSG soils did not differ from any of the soils. The only fungal genus which was affected by legume species was *Phaeosphaeriopsis* sp., which was lower in WCl soils than all other soils including LoP soils.

In this study, soil bacterial communities were dominated by Proteobacteria, Acidobacteria and Bacteroidetes, which is consistent with other studies on grassland soils (Nemergut *et al.* 2008; Fierer *et al.* 2009; Nacke *et al.* 2011; Yu *et al.* 2021). Bacterial communities, particularly the *Rhizobium*, *Nitrospira*, an unclassified Comamonadaceae, *Pedobacter* and *Byssovorax* spp., appeared to be affected by plant species and N levels as well. We found that the N-fixing *Rhizobium* sp. was generally less abundant in grass soils at low N than in legume soils. This was expected because the *Rhizobium* spp. can form symbiotic associations with legumes (Mylona *et al.* 1995). *Rhizobium* spp. can also live non-symbiotically in soils, where they assimilate simple sugars and organic and amino acids, and it has been shown that growth of rhizobia is stimulated by the presence of legumes and non-leguminous dicots, whereas grasses maintain smaller numbers of rhizobia (Tuzimura and Watanabe 1962). In this study, the nitrifying *Nitrospira* sp. and an unclassified genus of the Comamonadaceae family containing denitrifiers (Patureau *et al.* 1996; Khan *et al.* 2002) were also less abundant in grass soils at low N than in legume soils, consistent with a study showing that legume species enriched *Nitrospira* in the soil microbiome (Zhou *et al.* 2017). These findings indicate that N fixed by *Rhizobium* associated with legumes might not only promote plant growth but also provide soil with N-containing substrates that promote propagation of the *Nitrospira* and denitrifiers in the soils. *Nitrospira* are chemolithoautotrophic nitrite-oxidising bacteria, which catalyse the last step in nitrification (Murphy *et al.* 1985; Kowalchuk and Stephen 2001; Leininger *et al.* 2006), and denitrifiers are bacteria capable of performing denitrification as part of the N cycle (Knowles 1982). All of those play key roles in the biogeochemical N cycle and in

the release of nitrous oxide, a potent greenhouse gas, into the atmosphere (Wrage *et al.* 2001). As such, the potential linkage between *Rhizobium, Nitrospira* and denitrifiers in the legume soils may require further study. Compared with the grass soils at low N in this study, a high abundance of *Pedobacter* sp. and a lower abundance of *Byssovorax* sp. were generally found in legume soils. *Pedobacter* sp. has been described as an endophytic bacteria preferentially associating with legumes (Dudeja and Giri 2014; Piechulla *et al.* 2017; Wemheuer *et al.* 2017), and *Byssovorax* sp. was shown to be a cellulose decomposer also able to use nitrate as a source of N (Reichenbach *et al.* 2006). Our results above imply that N fertilisation and biological N fixation have strong effects on bacterial communities, especially on nitrifying and denitrifying genera.

By far the most abundant bacterial genus affected by our treatments was *Flavobacterium*, which was more abundant in legume soils and much less abundant in grass soils at high N. *Flavobacterium* has been described as plant growth-promoting bacterial genus through the production of growth hormones (Hartmann *et al.* 2009; Kolton *et al.* 2016) and phosphate solubilisation from soils (Goldstein 1986). Interestingly, the *Adhaeribacter*, *Luteolibacter* and *Rhodococcus* were also found to be more abundant in legume soils than in grass soils. The *Adhaeribacter* and *Luteolibacter* have been shown to have acid and/or alkaline phosphatases (Yoon *et al.* 2008; Elderiny *et al.* 2017), and these might be able to release phosphorus (P) from insoluble-P sources in the soil and supply legumes, which are often P-limited due to nodulation (Uhde-Stone *et al.* 2003), with additional P. *Rhodococcus* has also been shown to solubilise P by excreting organic acids (Chen *et al.* 2006). These results suggest that plant species and N fertilisation appear to affect bacterial communities associating soil phosphate solubilisation.

In this study, the genera *Flavobacterium, Cellvibrio*, *Novosphingobium* and *Armatimonas*, and an unclassified Micromonosporaceae, were generally affected by plant species as well. Like the impacts on fungi described above, the main impacts of plant species on those genera appear to be associated with the capabilities of bacteria metabolising complex carbohydrates in plants. The genera *Flavobacterium, Cellvibrio* and *Novosphingobium* were all found to be more abundant in legume soils than in grass soils. The xyloglucan production by *Flavobacterium* in dicots is reportedly up to 20-fold higher than that in grass cell walls (Harris and Smith 2006; Vogel 2008). *Cellvibrio* has been shown to produce many enzymes involved in cell wall and storage carbohydrate degradation, especially pectins and galactomannans (DeBoy *et al.* 2008), which are more abundant in legume than in grass cell walls (Vogel 2008). *Novosphingobium* has been shown to produce enzymes that degrade aromatic compounds such as hydroxybenzoate, vanillate and protocatechuic acid (Ohta *et al.* 2015), all of which were detected in relatively high concentrations in legumes (Klejdus *et al.* 2008). By contrast, *Armatimonas* sp. and Micromonosporaceae (both less abundant in legume soils than grass soils) have been shown, respectively, to be oligo-heterotrophic bacteria, preferring nutrient-poor environments and complex carbohydrates over soluble sugars (Tamaki *et al.* 2011; Dunfield *et al.* 2012), or to be degraders of complex cell wall polysaccharides (Yeager *et al.* 2017). These results indicate again that the differences in cell wall composition of grasses vs legumes are major drivers of differences in soil bacteria communities.

### Effects of plant extended phenotype on soil fungal and bacterial communities

Previous experimental reports showed that soils with endophyte-infected grasses had an altered composition of the associated fungal and bacterial communities (Casas *et al.* 2011; Wakelin *et al.* 2015; Rojas *et al.* 2016). In Experiment 2, fungal and bacterial soil communities also differed between soils with endophyte-infected and uninfected grasses. Soils with infected grasses showed low abundance of Ascomycota (here *Epicoccum*, *Verticillium* and *Phoma*) and high abundance of Glomeromycota (e.g. *Glomus*) fungi, findings that agree with previous experimental results (Rojas *et al.* 2016). As discussed, some *Epicoccum* species produce antifungal and antibacterial compounds (e.g. Brown *et al.* 1987; Dzoyem *et al.* 2017), members of *Verticillium* and *Phoma* are plant pathogens (Harris 1986; Klosterman *et al.* 2009), and *Glomus* spp. are beneficial fungi of plants. The reduced abundance of detrimental Ascomycota fungi in soils could be associated with the generally observed *Epichloë*-based inhibition of fungal phytopathogens (and potentially mycopathogens as well) in plants (Card *et al.* 2021; Kou *et al.* 2021). The high abundance of Glomeromycota fungi in soils would be associated with the alteration in the composition of root exudates usually observed in *Epichloë*-symbiotic plants, which promotes the growth and reproduction of members of this phylum (i.e. mycorrhizal fungi) (Vignale *et al.* 2018; Zhong *et al.* 2022). *Epichloë* infection appeared to influence community diversity and abundance of N-cycling bacteria as well (e.g. the ammonia oxidation, nitrite reduction and nitrous oxide reduction bacteria), a pattern that agrees with previous experimental results (Yang *et al.* 2021; Chen *et al.* 2022). Furthermore, soil with *Epichloë*-infected grasses exhibited reduced abundance of N-fixing *Rhizobium*. A similar outcome was documented in soils conditioned by endophyte-associated *Lolium multiflorum* Lam. plants, which reduced the nodulation of N-fixing bacteria in legumes (García-Parisi *et al.* 2017). Additional results in that study showed that despite the reduced abundance of this bacterial group, endophyte-conditioned soils increased plant growth and N acquisition when the availability of *Rhizobium* was low (García-Parisi *et al.* 2017).

## Conclusions

Results associated with Experiment 1 showed that neither N fertilisation level, nor elevated levels of soluble sugars

(in HSG litter) or condensed tannins (in the LoP treatment), had a substantial effect on soil fungal communities. The main driver of shifts in these communities appears to be the chemical composition of plant cell walls, which differs between grasses and legumes. In the case of soil bacterial communities, N fertilisation and biological N fixation affected the abundance of nitrifying and denitrifying genera, while plant species and N fertilisation seemed to affect the bacterial groups associating with the solubilisation of phosphate. Results associated with Experiment 2 showed that *Epichloë* changed the abundance of soil fungal and bacterial groups, including reduction of fungal phyto- and myco-pathogens, an increment of mycorrhizal fungi, and reduction of N-fixing bacteria. The changes in soil microbial communities could be explained in part by the altered root exudate composition exhibited by *Epichloë*-infected plants (Patchett and Newman 2021). Further investigations are warranted to evaluate whether the *Epichloë*-mediated changes in soil microbial communities affect plant responses to the environment and functionality/productivity of plant communities (Bastías *et al.* 2022; Bastías and Gundel 2023).

## Supplementary material

Supplementary material is available online.

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**Data availability.** The data that support this study will be shared upon reasonable request to the corresponding author.

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