Rhizosphere microbial community dynamics contribute to nitrogen fixation and forage quality in novel perennial intercrops in Rwanda

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Abstract

In low-fertility tropical agroecosystems, intercropping with perennial legumes has the potential to maximize biological nitrogen fixation (BNF) and improve non-legume nitrogen (N) uptake and yields. However, the microbial interactions that facilitate the beneficial effects of intercropping in perennial systems remain largely uncharacterized, particularly in the tropics. In this study, we explored the contribution of root-associated bacteria and fungi to N content and the nutritional characteristics of perennial grasses (*Cenchrus purpureus* and *Brachiaria* cv. Mulato II) and an annual cereal (*Zea mays*) intercropped with a perennial legume (*Desmodium* sp). Sampling approximately every 8 weeks at the time of forage grass anthesis, we collected biomass leaf tissue, bulk soil, and rhizosphere soil. We calculated nitrogen derived from the atmosphere (Ndfa) in *Desmodium* leaf tissue to estimate BNF and found that Ndfa increased in intercropping arrangements relative to single-cropped *Desmodium* by 91.6–147.1% on average in intercropped stands with *Brachiaria* and *C. purpureus*. Intercropping also induced positive changes in non-legume tissue quality in a species- and site-dependent manner. Furthermore, we identified microbial taxa that were significantly enriched in the rhizosphere of intercropped plants relative to monocropped plants and which correlated to multiple forage nutritive quality metrics. Fungal community structure responded more strongly to the presence of a legume intercrop than bacterial communities. We also identified species-specific trends in the degree to which monocropped and intercropped rhizosphere communities differed. Overall, this study suggests that perennial legume intercropping may recruit beneficial rhizosphere microorganisms in rainfed tropical soils to facilitate nutritional benefits in the non-legume companion crop and highlights the complexity of rhizospheric microbial interactions in intercropped systems.

1. Introduction

Mixed crop-livestock systems provide food for millions around the world, yet shortages of high-quality fodder or forage limit their ability to mitigate rural hunger (Herrero et al., 2013; Thornton et al., 2014). Climate-change scenarios in the tropics forecast crop yield declines of 10–20% by 2050, and similar declines are expected to affect crops grown for animal feed, known as forage crops (Thornton et al., 2007). More than 60% of smallholder farmers in Africa cannot afford synthetic fertilizers, and soil conditions such as long drought and nutrient limitation also challenge plant growth (Chianu et al., 2011; Yanggen et al., 1998). In general, previous studies on tropical forage systems have focused on monocropping and singular assessments of forage performance (i.e., yield), with little attention paid to the multidimensional services that are now called for, such as enhanced soil fertility, biodiversity, and improved forage nutritive quality (Nord et al., 2020; Paul et al., 2020).

Intercropping is a diversification strategy in which two or more crops are grown in the same field at the same time, either as a mixture or in spatially distinct arrangements such as rows (Drinkwater et al., 2021). Innovative forage intercrop systems involving the establishment of a permanent, perennial legume that is interspersed among cereal and grass crops are largely unexplored. However, emerging evidence supports the potential of these systems to improve soil structure, tighten nutrient cycles, increase soil microbial diversity, and enhance plant performance relative to monocultures (Duchene et al., 2017; Henneron et al., 2015; Hinsinger et al., 2011). Yield-associated benefits from intercropped systems have been largely attributed to temporal complementary, in which companion crops have different periods of rapid growth or windows for nutrient demand (Li et al., 2020). Alternatively, interspecific differences in root structure and resource acquisition also result in non-competitive use of soil nutrients and thereby improve total biomass productivity (Brooker et al., 2015; Franke et al., 2018). Less-well documented, however, are the plant-microbial mechanisms underpinning belowground facilitative interactions that enhance the growth or performance of co-occurring plant species (Brussaard et al., 2007; Duchene et al., 2017). Elucidating the facilitative microbial interactions in intercropped roots is critical for advancing benefits derived from intercropping, particularly in regions with low nutrient inputs.

Tropical forage legumes can provide yield benefits to companion grass crops by performing biological nitrogen fixation (BNF) and enhancing N use efficiency, yet this remains understudied (Vieira et al., 2010). Nitrogen derived from the atmosphere (Ndfa) from BNF often correlates inversely with soil N availability (Schipanski et al., 2010), making legumes a promising approach to improving soil fertility in N-limited tropical soils. In intercropped systems, legumes are known to fix N at higher rates due to complementary resource use by the non-legume companion plant, which obligately obtains N from the soil (Jensen, 1996; Parsons et al., 1993). Ndfa from legumes can thus serve as a low-cost biological fertilizer in N-limited tropical soils (Abera et al., 2012), with previous work estimating approximately 21 kg of legume-derived N delivered to cereal crops (Wang et al., 2019). Belowground N transfer follows three potential pathways to arrive at the intercropped non-legume: decomposition and mineralization of N-rich biomass, root exudation and diffusion of soluble N-compounds in the rhizosphere, or microbially facilitated N transfer (Thilakarathna et al., 2016). While microorganisms play a direct role in the latter pathway, they are indirectly involved in the first pathway as well, by making biomass-N accessible via decomposition and mineralization.

In general, it is becoming increasingly clear that the presence of a legume intercrop can stimulate microbial processes that enhance plant-available N in the rhizosphere. Past work has demonstrated that legume intercrops can enrich the abundance of bacteria that acquire N from BNF or mineralization (Dang et al., 2020; Schwerdtner and Spohn, 2022), while also limiting the abundance of bacteria that convert N into forms that are unavailable to plants (Sun et al., 2022). For instance, interspecific root exudate signaling may enhance BNF in legumes by stimulating bacterial nodulation and nitrogen fixation genes in both legume and non-legume rhizospheres (Hu et al., 2021). Plant root interactions in intercropped systems can be more effective than inoculation in eliciting these N-acquisitive benefits associated with microbial communities (Cardoso and Kuyper, 2006). Indeed, 28–51% of yield gains in wheat-faba bean and maize-faba bean intercropped systems were attributed to intercrop-driven changes to soil microbial communities that affect nutrient uptake pathways (Qiao et al., 2022; G. Wang et al., 2021). However, these mechanisms have not been explored in tropical intercropped systems that involve perennial legumes.

In addition to favoring N-acquisitive bacteria, legume intercropping may promote fungal nutrient exchange networks that connect interspecific plant roots and facilitate N acquisition (He et al., 2003; Johansen and Jensen, 1996). For instance, arbuscular mycorrhizal fungi (AMF) are known to form a tripartite symbiosis with legumes and rhizobia, an association that can increase tissue N content and nodulation in legumes (Duchene et al., 2017). Legume presence can lead to > 50% increases in root colonization by AMF and biomass yield in subsequent cereal crop rotations (Bagayoko et al., 2000; Lekberg et al., 2008). AMF hyphae may deliver N to non-legumes by colonizing decomposing legume tissue and taking up mineralized N (Cardoso and Kuyper, 2006). In addition to AMF, other types of fungi such as *Basidiomycete* fungi, which cause white rot and degrade lignin, may have an underappreciated role in nutrient cycling and fertility in tropical soils (Lodge et al., 2022). For instance, dissolved aromatic compounds resulting from lignin degradation by white rot aids in the transport of N in acidic soils (Fujii, 2014).

Desmodium sp. are woody perennial legumes commonly used as intercrops and fodder crops in East Africa and, when intercropped, can increase maize yields by 2-3-fold compared to maize monocultures (Midega et al., 2015). When given to livestock as a supplement in addition to other fodder crops (e.g., maize, Brachiaria, and Napier grass), *Desmodium* significantly increases crude protein content and milk production in smallholder dairy cattle (Mutimura et al., 2018). *Desmodium* is also resistant to heat stress and nutrient limitation (Xu et al., 2016), aiding its adoption by more than 150,000 farmers in Kenya, Uganda, Tanzania, and Ethiopia (Murage et al., 2015, 2012). Although it is largely untested as a row crop with perennial grasses, the added belowground N derived from BNF and root exudates (Fustec et al., 2010) may help to sustain crop yields and tissue quality that tend to decline in forage stands after multiple cuttings (Amossé et al., 2014; Boddey et al., 2004). Cadisch et al. (1989) estimated that *D. ovalifolium* derived 44–70% of its N content from BNF, although this is expected to vary dramatically according to soil P content (Zheng et al., 2016),

drought (Fahad et al., 2021), and the presence of compatible rhizobia in the soil (da Silva et al., 2017). Despite the growing relevance of *Desmodium* as a fodder crop across sub-Saharan Africa, little information exists regarding its capacity to fix N, potential benefits to non-legume intercrops, or the root-associated microbiota that may be responsible for facilitating interspecific yield benefits.

Belowground interactions between intercropped roots and soil microorganisms are underexplored, particularly in perennial systems (Brussaard et al., 2007; Duchene et al., 2017) and in the tropics, which are hotspots of uncatalogued global soil biodiversity (Guerra et al., 2022). In this study, we examined plant-microbial dynamics in perennial legume intercropped systems with maize (*Zea mays*), Brachiaria (syn. *Urochloa*), and Napier grass (*Cenchrus purpureus)* under a typical smallholder management regime in Rwanda. We hypothesized that intercropping with the perennial legume *Desmodium intortum* or *D. distortum* would 1) increase N derived from the atmosphere (Ndfa) compared to monocropped stands and improve non-legume forage nutritive quality, 2) drive changes to bacterial and fungal rhizosphere community structure in the legume and non-legume companion crops, and lastly that 3) intercropping-related changes to microbial communities would contribute to forage nutritive quality.

2. Methods

2.1 Site description

This study was conducted at three locations in Rwanda that are situated in major dairy-producing regions of the country: Karama, Rubona, and Burera (Supp Fig. 1). The Karama research center (1°52459’ S, 30°36469’ E) is in the low altitude Eastern Province of Rwanda. This region is the top dairy-producing region in Rwanda, owing partly to larger farm sizes that allow for mixed crop-livestock production. The Rubona research center (2°48330’ S, 29°77554 E) is located in the prominent dairy producing Huye District, Southern Province, which is a mid-altitude tropical central plateau. Lastly, the Rwerere research center (1°49070’ S, 29°88009’ E) is located in the northern Burera district of Rwanda and has a temperate tropical climate, located in the highlands of the western branch of the East African Rift near Lake Kivu. For rainfall measurements, temperature, and soil properties at each site, please refer to Supplemental Materials (Supp Fig. 2, Supp Tables 4, 5).

2.2 Experimental design & forage management

The field trials consisted of randomized complete block designs with four replicates at each of the three research centers. Treatments included single-cropped maize, Napier grass, Brachiaria cv. Mulato II, and *Desmodium* sp. in addition to intercropped treatments that included *Desmodium* planted in alternating rows alongside each of the non-legume species. *Desmodium distortum* was used as the perennial legume intercrop in Karama and Rubona, while *D. intortum* was used in Burera. *D. intortum* tolerates cooler climates (< 27°C) and higher precipitation (Peoples and Baldock, 2001) that are characteristic of northern growing regions in Rwanda. The Rwandan Kigega (ZM607) open pollinated annual maize variety was selected due to its suitability to middle to low altitudes and adaptability to local growing conditions, including drought tolerance (Magorokosho and Tongoona, 2003). Seeding rates and row spacing followed recommendations for perennial forage production (Appendix C Supplemental Methods and Supp Table 1). Inter-row spacing between *Desmodium* and perennial grasses (Napier and Brachiaria) was 50 cm, while spacing between *Desmodium* and annual maize was 25 cm. All forages and annual maize were established at the beginning of the short rainy season in October 2019 (for specific dates, see Supp Table 2).

At the time of establishment in 2019, cattle manure was applied at a rate of 15 kg/plot (approximately 3 kg N/ha) following recommended guidelines for nitrogen application at sowing (Cook et al., 2020). For the remainder of the experiment, no additional amendments were applied to distinguish plant-imposed effects on N-cycling soil

communities. Perennial forages (Brachiaria, *Desmodium*, Napier) were harvested every 60 days by removing all aboveground biomass by hand to a height of 15 cm, preserving the basal portion of the stem defined as stubble (Allen et al., 2011). Annual maize was harvested for grain at approximately 150 days of growth to allow time to reach physiological maturity. *Desmodium* intercropped with maize was harvested on the same schedule as other perennial forages in the study. Supplemental Table 2 contains forage harvest dates and maize growth stages at each date.

2.3 Soil sampling

Bulk soil and rhizosphere soil in all plants (including maize) was sampled approximately every 60 days when the perennial forages reached a harvestable stage at anthesis (Supp Table 2). Sampling occurred between March 2021 - December 2021. Soils in Rubona and Karama were sampled four times, while Burera was sampled three times due to slower plant growth. Rhizosphere soil was collected from one randomly selected plant of each species per plot, sampling one randomly selected plant in monocropped plots, and two in intercropped plots. The entire root system of the plant was gently uprooted and shaken to dislodge loosely bound soil. Rhizosphere soil was defined as the soil that was tightly bound to the root surface and could not be dislodged by shaking. A sterile brush was used to scrape the rhizosphere soil into plastic sample bags. Bulk soil was collected at the plot level by collecting ten soil cores per plot to a depth of 20 cm, then aggregated in sterile bags and passed through a 2mm sieve. In total, we collected 84 bulk soil samples from Burera, 111 bulk soil samples from Rubona, and 112 samples from Karama.

All soil samples were kept cool at 4°C and shipped on ice to the University of Minnesota (UMN) for analysis within one week of sampling. Upon arrival, rhizosphere soils were immediately passed through a 2mm sieve and stored at -20°C, and bulk soil samples tested for mineral N content. Gravimetric water content (GWC) and pH of each of the bulk soil samples were also determined. Soil pH was measured from slurries prepared by mixing 10g air-dried soil and 30 ml deionized H_2O. Gravimetric water content (GWC) was determined by obtaining the mass difference between field-moist and soil air-dried at 95°C.

2.4 Forage biomass sampling

At each sampling period, aboveground forage biomass was sampled from the same plant that was selected for rhizosphere soil collection. The leaf tissue of each sampled plant was collected and dried at 60°C for 48 hours. For the purposes of this study, leaf material included leaflets as well as petioles and stipules; stem material was not collected. Dried plant material was then coarsely ground and shipped to the UMN, where forage leaf tissue was dried at 60°C for an additional 12 hours to remove residual moisture, then ground through a 1mm screen in a Cyclotec (Foss, Hillerød, Denmark). Refer to Supplemental Table 3 for total sample numbers per forage species and location.

2.5 Forage quality

Individual forage samples ground to 1 mm were thoroughly mixed and scanned using near infrared reflectance spectroscopy (NIRS; Model DA 7200; Perten Instruments, Springfield, IL) to estimate forage nutritive value for crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin. Equations for the NIRS procedure were validated by a wet chemistry analysis that was completed at a commercial forage testing facility (DHIA Laboratories, Sauk Centre, MN). We selected 20 forage samples that represented the greatest variability within each of the measurements, with at least four samples for each plant species. The R^2 values (wet chemistry results vs NIRS measurements) for CP, NDF, and ADF were 0.61, 0.85, and 0.63, respectively.

2.6 Forage elemental analysis

Forage leaf material ground to 1 mm was further processed for elemental analysis. We pulverized forage biomass samples with a 2010 Geno/Grinder ball grinder (SPEX SamplePrep, Metuchen, NJ) using a 5 mm diameter stainless

steel ball bearing (Craig Ball Sales, Seaford, DE) for 3–10 min at 1500 rpm. Samples were then homogenized and subsampled into tin capsules (EA Consumables, Pennsauken, NJ) at 20 mg ± 0.005 mg. We measured C, N, and $\delta^{15}N$ isotopic ratios using an Elementar vario PYRO cube EA-IRMS CNS analyzer (Elementar, Langenselbold, Germany).

2.7 ^{15}N Natural abundance and nitrogen derived from the atmosphere

We used $\delta^{15}N$ values to calculate nitrogen derived from the atmosphere (Ndfa) to estimate BNF rates in Desmodium across intercropping treatments. Ndfa was calculated as described previously by Shearer and Kohl (1987):

$$Ndfa\,(\%) = \left(\frac{\delta^{15}N_{ref} - \delta^{15}N_{fix}}{\delta^{15}N_{ref} - B}\right) x100$$

where $\delta^{15}N_{ref}$ is the $\delta^{15}N$ of a non-fixing reference plant, $\delta^{15}N_{fix}$ is the $\delta^{15}N$ of the field-grown *Desmodium*, and *B* is the $\delta^{15}N$% for *Desmodium* grown in an inorganic N-free medium. For *B*, we used the previously published value of -1.55 for *D. intortum* (Boddey et al., 2000; Yoneyama et al., 1986). The $\delta^{15}N$ reference was calculated separately for each location as the average value of $\delta^{15}N$ in monocropped Napier and maize plants. For a complete description of Ndfa calculations, refer to Supplemental Methods.

2.8 Log response ratios

To determine the response of forage biomass characteristics to intercropping, we calculated log-transformed response ratios (LRRs) of N content, $\delta^{15}N$, and forage nutritive quality (protein, lignin, ADF, NDF):

$$LRR = ln\left(\frac{X_{int}}{X_{mono}}\right)$$

where X_{int} and X_{mono} are the response variables of intercropped or monocropped plants, respectively (Rodriguez et al., 2020).

2.9 Soil mineral nitrogen

We measured soil mineral nitrogen (NO_3^--N + NO_2-N and NH_4^+-N) in bulk soil samples to account for the effect of soil N availability on BNF rates. Soil samples were kept at 4°C until N extractions could be performed, typically within 24 hours of arrival to the UMN and no more than 2 weeks from sampling. Mineral nitrogen (minN) was extracted and quantified colorimetrically as described previously (Perrone et al., 2020).

2.10 DNA extraction

Upon arrival at the UMN, a portion of each rhizosphere soil sample was passed through a 2mm sieve, repackaged, and stored at -20°C for DNA extraction. Total genomic DNA was extracted from 0.25g fresh soil using the DNEasy Powersoil Pro DNA Isolation Kit (Qiagen) according to the manufacturer's protocol. The final elution step was modified such that 50ul of elution buffer was used to obtain higher DNA concentrations. DNA quality and concentration were assessed on extracted samples using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA).

2.11 qPCR & amplicon sequencing

Quantitative PCR (qPCR) and amplicon sequencing of the bacterial 16S rRNA gene and the fungal internal transcribed spacer 2 (ITS2) region were performed at the University of Minnesota Genomics Center (UMGC). Briefly, the V4 region of the 16S rRNA gene and the fungal ITS2 region between 5.8S and 23S rRNA gene were amplified by qPCR with the Meta_V4_515F and Meta_V4_806R primer set (Gohl et al., 2016) and the 5.8SR_Nextera and ITS4_Nextera primer set (Castle et al., 2018), respectively. The qPCR mastermix was prepared and the qPCR reactions were carried out as described previously (Gohl et al., 2016; Jang et al., 2022). Amplicon sequencing libraries for the V4 region of the 16S rRNA gene and the fungal ITS2 region were prepared by amplifying the regions as described above. PCR products were subsequently purified and attached to dual index tags and Illumina sequencing adaptors as described previously (Castle et al., 2018; Gohl et al., 2016). Paired-end sequencing was done using a MiSeq platform (Illumina) with V3 chemistry (300-bp read length) at the UMGC.

2.12 Bioinformatics

We used mothur version 1.48.0 to assemble paired-end raw sequence reads and perform all quality-filtering and trimming steps (Schloss et al., 2009). Bacterial sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the OptiClust method in the *cluster.split* command in mothur (Westcott and Schloss, 2017). Bacterial OTUs were assigned taxonomic classifications using the Silva 138.1 database (Quast et al., 2013). Following Tedersoo et al. (2022), we used an OTU-based approach for ITS2 amplicons. Fungal ITS2 sequences were clustered into OTUs at 95% sequence similarity using the abundance-based greedy clustering method in the cluster command in mothur (*method = agc*). Fungal OTUs were assigned taxonomic classifications using the UNITE version 9.0 reference dataset (Abarenkov et al., 2022).

The number of high-quality sequences per sample ranged from 33 to 34,313 and from 227 to 37,198 for the bacterial 16S rRNA gene and fungal ITS2 regions, respectively. For all subsequent analyses, we rarefied to 2,097 and 1,923 sequences per sample, respectively, for 16S and ITS2. Good's coverage at this rarefaction depth was 70.6% and 92.4% on average for bacteria and fungi, respectively. Following rarefaction, the number of rhizosphere samples retained at each study site for 16S and ITS2, respectively, were 114 and 115 in Burera, 157 and 158 in Karama, and 157 and 159 in Rubona. We assigned trophic modes and functional guilds to fungal OTUs using FunGUILD (Nguyen et al., 2016). For a full discussion of this process, refer to Supplemental Methods. Only classifications with a confidence ranking of 'Highly Probable' or 'Probable' were used (44.8% of reads) for statistical analysis. Bacteria such as *Bradyrhizobium* that are known N-fixing endophytes were also classified based on genus-level identification (Supplemental Methods).

2.13 Statistical analysis

We analyzed differences in the log response ratios (LRRs) of forage nutritive quality among non-legume companion plants using mixed effects models with *lmer* in the lme4 package (Bates et al., 2015). Plant species was used as the fixed effect, while block and sampling time were set as random intercepts for each location separately. In cases where the plant species term was significant, we performed means separation with Tukey's Honest Significant Difference (HSD) test with the emmeans package in R (Lenth et al., 2019). To acquire overall treatment effects across locations, we followed the same procedure and included site as an additional random factor. We also used Spearman's correlations (*cor.test*) in the stats package (R Core Team, 2020) to investigate relationships among plant tissue measurements, soil minN, Ndfa, soil gravimetric water content (GWC), and the abundance of intercrop-responsive microbial taxa.

Indicator species analysis was used to identify putative fungal and bacterial OTUs that responded to the presence of an adjacent allospecific crop in intercropped systems. Hereafter, we will refer to these intercrop-responsive OTUs as 'indicator OTUs'. We used the *multipatt* function from the R package indicspecies (Cáceres and Legendre, 2009) with 1,000 permutations to perform this analysis. Indicator species that were positively associated with intercropping and

with an adjusted P-value of < 0.01 were retained for further analyses. We then validated these indicator OTUs using Wilcoxon Rank Sum tests (*wilcox.test*) in the stats package. Wilcoxon p-values were adjusted using the Benjamani-Hochberg correction with *p.adjust(method= "BH")* from the stats package.

We tested the response of microbial functional guilds to intercropping with Wilcoxon Rank Sum tests as previously described. Data were separated according to sampling time, location, and plant species before testing for differences between monocropped and intercropped rhizospheres. We were particularly interested in whether the abundance of arbuscular mycorrhizal fungi (AMF), other plant-associated non-AMF symbiotrophs, and white rot fungi increased in intercropped rhizospheres. We also investigated intercropping effects on bacterial OTUs identified at the genus level that are known to be endosymbiotic rhizobia (Supplemental Methods).

To compare microbial community responses to intercropping across plant species, we generated rarefied Bray-Curtis distance matrices generated with *vegdist* in vegan (Oksanen et al., 2022). From the distance matrices, we used tidyverse functions in R (Wickham et al., 2019) to filter and select for distances between monocropped and intercropped rhizospheres within the same plant species, block, timepoint, and location. With Bray-Curtis distance as the response variable, we used mixed effects models (fixed = plant species, random = location/block/timepoint) to test for differences in ecological distance across plant species. We then performed means separation with Tukey's HSD test as described previously.

We visualized the contribution of intercropping, plant species, location, and sampling time to microbial community structure using NMDS (*metaMDS* in vegan) ordinations of Bray-Curtis distances. To formally test the significance of each of these factors, we used PERMANOVA tests (*adonis2* in vegan) constrained by block. We also used Mantel tests with Spearman's correlations (*mantel* in vegan) to examine linkages between rhizosphere microbial community composition and biomass quality (%N, δ^{15}N, protein, lignin, ADF, NDF). For the Mantel tests, we used *vegdist* (vegan) to generate Euclidean distance matrices on scaled biomass data and Bray-Curtis distances for microbial community data.

Finally, we tested the relationships between intercropping, microbial communities, and forage nutritive quality. To do so, we performed log-ratio analysis (LRA) weighted on column means (LRA; Greenacre and Lewi, 2009) using *pcwOrd* in ordinator (Ewing, 2020). Weighting relative abundance by mean relative abundance across samples accounts for certainty in relative abundance and reduces the influence of rare or poorly sequenced samples in LRA solutions (Benitez et al., 2021; Greenacre and Lewi, 2009). We replaced all zeros in the community matrices to allow for logarithmic transformation and transformed relative abundances to centered log ratios with *CLR* from *easyCODA* (Greenacre, 2019). We performed LRA independently for each site and extracted the first three axes from the ordination for use in mixed effects models.

We used *Anova* from the car package to test the hypothesis that intercropping explains variation in microbial community composition represented by individual LRA axes after accounting for plant species:

$$H_1 Axis1/2/3 \sim intercrop + species + location + 1|block/timepoint/location$$

$$H_0 Axis1/2/3 \sim species + location + 1|block/timepoint/location$$

We used the same approach to test the hypothesis that microbial community composition (represented by each LRA axis individually) mediates biomass characteristics independently of plant species:

$$H_2$$
$$\%N/\delta^{15}N/CP/lignin/ADF/NDF \sim Axis1/2/3 + species + location + 1|block/timepoint/location$$

$H_0 \%N/\delta^{15}N/CP/lignin/ADF/NDF \sim species + location + 1|block/timepoint/location$

2.14 Nucleotide sequence accession numbers and data availability

The 16S rRNA gene and fungal ITS2 amplicon sequences were deposited to the GenBank database under the BioProject numbers PRJNA970362 and PRJNA970694, respectively. The datasets and R code used for all analyses are available on https://github.com/schaedem/Forage_Rhizosphere_Microbiome_Paper.

3. Results

3.1 Intercropping alters Ndfa in legumes and biomass characteristics in non-legumes

On average, nitrogen derived from the atmosphere (Ndfa) increased in intercropped *Desmodium* regardless of location or sampling timepoint ($P < 0.01$). Furthermore, Ndfa in *Desmodium* depended on the species identity of the intercropped non-legume ($P < 0.01$). *Desmodium* intercropped with Napier had the highest Ndfa overall (82.3%, $P = 0.04$), followed by *Desmodium* intercropped with Brachiaria (63.8%). Intercropping with Brachiaria and Napier led to overall increases in Ndfa relative to single-cropped *Desmodium* (33.3%) by 91.6% and 147.1%, respectively. However, Ndfa in *Desmodium* intercropped with maize did not differ from monocropped *Desmodium* ($P = 0.92$) in any location (Fig. 1). On average, Ndfa was greatest in Rubona (69.6%, $P < 0.01$) compared to Burera (42.6%) or Karama (48.7%). In addition to species effects, Ndfa was also affected by soil conditions that differed between sites (Fig. 1B, C). Ndfa was negatively correlated with both soil minN ($\rho = -0.40$, $P < 0.001$) and GWC ($\rho = -0.21$, $P = 0.02$) across all sites.

While some forage nutritive quality metrics (CP, NDF, and %N) were more strongly related to Ndfa, others (ADF and lignin) were more strongly related to soil minN levels (Supp Figs. 24 & 25). Within *Desmodium* plants, Ndfa was positively correlated with CP ($\rho = 0.24$, $P < 0.01$) and negatively correlated with NDF ($\rho = -0.24$, $P < 0.01$) overall. Conversely, CP and ADF were only marginally associated with soil minN ($\rho = -0.16$, $P = 0.06$ and $\rho = -0.22$, $P = 0.01$, respectively). Leaf %N was negatively correlated with Ndfa ($\rho = -0.32$, $P < 0.01$) but not related to soil minN ($\rho = -0.03$, $P = 0.72$) in *Desmodium*. Conversely, ADF and lignin content were not correlated to Ndfa in *Desmodium* but both were negatively correlated with soil minN ($\rho = -0.19$, $P = 0.03$, $\rho = -0.23$, $P = 0.01$, respectively).

Intercropping was also associated with differences in %N in the non-legume companion crops. Across all sampling times and locations, maize demonstrated a positive log response ratio (LRR) for %N, indicating that legume intercropping increased tissue N content ($P < 0.05$). Within location, however, maize had a significantly positive LRR in Karama only ($P < 0.01$) irrespective of sampling time. Maize had a marginally significant LRR for %N in Rubona ($P = 0.06$), but not Burera ($P = 0.36$). In addition, %N in intercropped Brachiaria increased relative to single-cropped Brachiaria, but only in Karama at the second sampling time ($P = 0.03$).

Intercropping altered CP in non-legume companion crops in Rubona ($P < 0.01$) but not in Karama or Burera. The contribution of intercropping to CP in Rubona arose from a positive LRR for protein in maize, which differed significantly from other species and amounted to a marginal increase of 1.13% compared to monocropped maize ($P = 0.07$; Fig. 2B). Napier had a negative LRR for lignin content ($P = 0.09$) in Rubona, as did Brachiaria in Burera ($P = 0.01$; Fig. 2C). ADF in nonlegumes did not respond to intercropping in any study location (Fig. 2D). Finally, intercropped maize had a marginally negative LRR for NDF ($P = 0.08$) in Karama (Fig. 2E).

3.2 Intercropping selectively enriches microbial taxa but does not alter diversity

To assess intercrop-driven changes to rhizosphere community dynamics, we first tested whether community diversity was dependent on a binary intercropping term across plant species and locations. Alpha diversity (Shannon-Weaver) of bacterial OTUs did not differ between intercropped and monocropped rhizospheres across study sites (Supp Fig. 9). Bacterial beta-diversity (Bray-Curtis) also did not differ overall between intercropped and monocropped rhizospheres, nor was a plant species x intercrop interaction term significant in explaining variation in 16S beta-diversity across sites and sampling times (Supp Figs. 11, 13). Rhizosphere bacterial community structure was most strongly influenced by plant species (PERMANOVA, P = 0.001) and sampling time (PERMANOVA, P = 0.001) across all three study locations (Supp Figs. 12, 14).

Fungal alpha diversity was similarly unaffected by intercropping (Supp Fig. 10), although fungal community structure was responsive to intercropping effects when aggregating across locations and sampling times. Bray-Curtis dissimilarity in fungal communities was marginally related to intercropping in Burera (PERMANOVA, P = 0.08) and Karama (PERMANOVA, P = 0.06), but not Rubona (Supp Fig. 15). Likewise, rhizosphere fungal beta-diversity in both Burera (PERMANOVA, P = 0.001) and Karama (PERMANOVA, P = 0.03) was significantly related to a plant species *x* intercrop interaction term (Supp Fig. 17). Lastly, rhizosphere fungal beta-diversity was strongly related to sampling time (PERMANOVA, P = 0.001) and plant species (PERMANOVA, P = 0.001) across all study locations (Supp Figs. 16, 18).

Although a binary intercropping term did not detect differences between intercropped and monocropped rhizospheres, the Bray-Curtis distances between intercropped and monocropped communities within each plant species and location revealed species-specific trends. The Bray-Curtis distance between monocropped and intercropped rhizospheres depended significantly on species ($P < 0.001$) in each location for bacterial communities (Fig. 3). Maize exhibited the greatest distances between intercropped and monocropped rhizobacterial communities in both Burera and Karama. In Rubona, *Desmodium* and Napier rhizospheres had the greatest differences in bacterial community structure between monocropped and intercropped plants. Fungal community responses to intercropping depended on plant species in Karama and Burera ($P < 0.001$), but not Rubona ($P = 0.90$; Fig. 4). In Karama, fungal community responses to intercropping were the greatest in maize rhizospheres, followed by *Desmodium* and Napier grass. In Burera, Brachiaria and maize rhizospheres exhibited the greatest fungal community responses to intercropping (Fig. 4).

We used an indicator species analysis to identify specifically enriched microbial taxa in intercropped rhizospheres relative to monocropped rhizospheres. Overall, we identified more bacterial indicator OTUs (24) than fungal indicator OTUs (20) that responded to the presence of an adjacent allospecific row crop in intercropped systems. However, the relative abundance of fungal indicator OTUs was more than 4 times greater (0.3%) than the relative abundance of bacterial indicator OTUs (0.07%) on average. Brachiaria and Napier grass had the highest number of bacterial OTUs that were enriched in intercropped rhizospheres. Only one bacterial OTU identified as *Hyphomicrobium* was preferentially enriched in intercropped *Desmodium* rhizospheres in only one location (Burera). Indicator 16S OTUs in maize were largely unidentified beyond the family in kingdom levels (Fig. 5), although cross-referencing with the RDP Bayesian classifier (Appendix C Supplemental Methods) suggested designations of candidate division WPS-1 (OTU 571) and *Gaiellaceae* (OTU 381; Supp Table 6). Contrary to our expectations, there were no known rhizobial endosymbionts among the indicator bacterial OTUs. Similarly, endophytic bacterial N-fixers were not enriched in intercropped rhizospheres, nor did we find evidence that plant species was significant in driving their relative abundance.

Brachiaria had the smallest number of intercrop-responsive fungal indicator OTUs (2) compared to Napier (7), *Desmodium* (6), or maize (5; Fig. 6). Of the twenty total fungal indicator OTUs we identified across plant species, only three had known functional guilds or trophic modes according to the FUNGuild database. Of these, only one was

identified as an AMF. This OTU (OTU 828), belonging to the *Dentiscutata* genus, was enriched in intercropped Napier grass rhizospheres in Rubona. Another OTU (OTU 43) belonging to the *Acrocalymma* genus was enriched in both maize and Napier grass intercropped rhizospheres and was identified as saprotrophic. Lastly, OTU 499 belonging to the family *Phaeosphaeriaceae*, was enriched in intercropped Napier grass rhizospheres and was identified as a plant-associated necrotroph or saprotroph.

In addition to indicator OTUs, intercropped rhizospheres were also enriched in certain fungal functional guilds in a site and time-dependent manner. We investigated whether the relative abundance of AMF, plant-associated non-AMF symbiotrophs, and white rot fungi differed between intercropped and monocropped rhizospheres. Of these three functional guilds, AMF had the greatest number of significant comparisons between monocropped and intercropped rhizospheres across time and study location (Supp Fig. 20). AMF relative abundance was also dependent on plant species (P = 0.02) across all locations and timepoints (Supp Fig. 21). The relative abundance of non-AMF symbiotrophs (Supp Fig. 20b) and white rot fungi (Supp Fig. 20c) was also greater in intercropped rhizospheres across several plant species and timepoints

3.3 Aboveground biomass characteristics are related to rhizosphere community dynamics

Belowground microbial community dynamics were related to forage biomass characteristics (%N, δ^{15}N, CP, ADF, NDF, lignin). Notably, we found that fungal communities were more strongly related to forage biomass characteristics than were bacteria. Fungal community composition was consistently linked to biomass characteristics in all locations (Table 1). However, bacterial community composition was significantly related to biomass characteristics only in one location (Table 1). This location, Burera, demonstrated the strongest correlations between community composition and biomass characteristics for both fungi and bacteria. Likewise, Ndfa was strongly correlated with the relative abundance of several fungal indicator OTUs associated with *Desmodium* (P < 0.001; Supp Fig. 19), but not the bacterial indicator OTU 472.

Table 1
Mantel test results indicating correlations between microbial community structure and leaf tissue quality measures. Significant Mantel correlations are shown in bold for bacteria and fungi within each location and across all sites combined.

	Fungi		***Bacteria***	
	R_{Mantel}	*P*	R_{Mantel}	*P*
All locations	0.08	**0.001**	0.03	**0.03**
Burera	0.22	**0.001**	0.11	**0.02**
Karama	0.19	**0.001**	0	0.57
Rubona	0.14	**0.001**	0.02	0.31

We also found that the relative abundance of intercrop-responsive indicator OTUs were related to multiple forage biomass characteristics. Both bacterial (Fig. 7) and fungal (Fig. 8) indicator OTUs maintained a low abundance in the rhizosphere that did not respond linearly to sampling time. While every fungal indicator OTU had a significant correlation to at least one biomass characteristic, several bacterial indicator OTUs were not correlated to any of the

measured biomass properties. These included Napier-associated bacterial OTUs such as Unclassified *Actinomycetales*, Unclassified *Planctomycetaceae, Gemmata*, and others (Fig. 7).

There were strong species-specific trends in the correlations between the relative abundance of indicator OTUs and forage nutritive quality metrics. For instance, *Desmodium* was higher in %N, lignin and CP, and tended to be lower in NDF and δ^{15}N than all other forage species (Supp Fig. 26). All *Desmodium*-associated fungal OTUs were likewise positively correlated to %N, lignin, and CP and negatively correlated to δ^{15}N and NDF (Fig. 8). A similar pattern held true for the *Desmodium*-associated bacteria *Hyphomicrobium*, whose abundance increased with %N and CP but decreased with NDF (Fig. 7). Likewise, bacterial and fungal indicator OTUS associated with *Brachiaria* demonstrated consistent associations with *Brachiaria*-associated nutritive characteristics. Napier and maize associated fungal OTUs had a greater number of correlations to biomass characteristics than bacterial indicator OTUs. In most cases, bacterial and fungal indicator OTU correlations with nutritive quality mirrored each other. Interestingly however, the Napier-associated bacterial indicator OTUs identified as *Gemmatimonas* and *Phenylobacterium* were positively correlated to %N (Fig. 7) while the fungal OTUs identified as *Microascus, Penicillifer*, and *Acrocalymma* were negatively correlated to %N (Fig. 8).

Next, we tested whether the top three centered log ratio analysis (LRA) axes in each sampling location were explained by intercropping after accounting for forage species. Each of the LRA axes accounted for less than 20% of the variation in microbial community structure for both bacteria and fungi (Tables 2, 3). The intercropping term did not explain LRA axis coordinates derived from bacterial or fungal community composition in any sampling location. However, intercropping was marginally significant (P = 0.08) in explaining fungal community composition along LRA axis 3 in Karama.

Finally, we used the LRA axes as fixed effects in mixed models to determine the contribution of microbial community structure to individual forage nutritive characteristics (Tables 2, 3). Bacterial and fungal communities contributed to divergent forage biomass properties that varied by location. In Burera, bacterial community structure explained %N, δ^{15}N, CP, and lignin while fungal community structure explained ADF and NDF. In Rubona, bacterial communities contributed to δ^{15}N and ADF, while fungal communities explained %N and CP. Fungal community structure in Karama exhibited no relationships to forage nutritive quality, while bacterial community structure only explained δ^{15}N.

Table 2

Summary of mixed model results using the top three axes from a centered log-ratio analysis. We tested the hypotheses that intercropping (presence/absence of a *Desmodium* intercrop) explains variation in bacterial community composition (H_1) and that bacterial community composition mediates biomass characteristics independently of plant species or intercropping (H_2). LRA axes were tested individually for each biomass characteristic for H_2.

Bacteria			
	Axis 1	Axis 2	Axis 3
Burera			
Variance	10.10%	7.26%	4.73%
H_1: axis ~ species + *intercrop*	n.s.	n.s.	n.s.
H_2: forage quality ~ species + *axis*			
%N	n.s.	**P = 0.04**	P = 0.09
δ^{15}N	**P = 0.03**	P = 0.08	n.s.
CP	n.s.	**P = 0.01**	n.s.
Lignin	**P = 0.02**	n.s.	n.s.
ADF	P = 0.09	n.s.	n.s.
NDF	n.s.	n.s.	n.s.
Karama			
Variance	13.00%	5.06%	3.92%
H_1: axis ~ species + *intercrop*	n.s.	n.s.	n.s.
H_2: forage quality ~ species + *axis*			
%N	n.s.	n.s.	n.s.
δ^{15}N	n.s.	n.s.	**P = 0.03**
CP	n.s.	n.s.	n.s.
Lignin	n.s.	n.s.	P = 0.06
ADF	n.s.	n.s.	n.s.
NDF	n.s.	n.s.	n.s.
Rubona			
Variance	10.30%	5.61%	3.87%
H_1: axis ~ species + *intercrop*	n.s.	n.s.	n.s.
H_2: forage quality ~ species + *axis*			

Bacteria			
%N	n.s.	n.s.	n.s.
δ^{15}N	n.s.	n.s.	**P = 0.05**
CP	n.s.	n.s.	n.s.
Lignin	P = 0.07	n.s.	n.s.
ADF	**P = 0.03**	P = 0.08	n.s.
NDF	n.s.	n.s.	n.s.

Table 3

Summary of mixed model results using the top three axes from a centered log-ratio analysis. We tested the hypotheses that intercropping (presence/absence of a *Desmodium* intercrop) explains variation in fungal community composition (H_1) and that fungal community composition mediates biomass characteristics independently of plant species or intercropping (H_2). LRA axes were tested individually for each biomass characteristic for H_2.

Fungi			
	Axis 1	Axis 2	Axis 3
Burera			
Variance	15.10%	11.40%	10.4%%
H_1: axis ~ species + *intercrop*	n.s.	n.s.	n.s.
H_2: forage quality ~ species + *axis*			
%N	n.s.	n.s.	n.s.
δ^{15}N	n.s.	n.s.	n.s.
CP	n.s.	n.s.	n.s.
Lignin	n.s.	n.s.	n.s.
ADF	n.s.	**P < 0.01**	n.s.
NDF	n.s.	**P = 0.02**	n.s.
Karama			
Variance	16.70%	10.10%	8.10%
H_1: axis ~ species + *intercrop*	n.s.	n.s.	P = 0.08
H_2: forage quality ~ species + *axis*			
%N	n.s.	n.s.	n.s.
δ^{15}N	n.s.	n.s.	n.s.
CP	n.s.	n.s.	n.s.
Lignin	n.s.	n.s.	n.s.
ADF	n.s.	n.s.	n.s.
NDF	n.s.	n.s.	n.s.
Rubona			
Variance	19.00%	9.99%	8.43%
H_1: axis ~ species + *intercrop*	n.s.	n.s.	n.s.
H_2: forage quality ~ species + *axis*			
%N	P = 0.10	n.s.	**P = 0.03**

Fungi			
$\delta^{15}N$	n.s.	n.s.	n.s.
CP	P = 0.06	n.s.	**P = 0.01**
Lignin	n.s.	n.s.	n.s.
ADF	n.s.	n.s.	n.s.
NDF	n.s.	n.s.	n.s.

4. Discussion

We hypothesized that intercropping the tropical perennial legume *Desmodium* with a perennial forage grass (Brachiaria or Napier) or an annual grain (maize) crop would enrich the abundance of facilitative microbial taxa and lead to rhizosphere community-dependent changes in Ndfa and forage biomass characteristics. Our results partially support our hypotheses because intercropping selectively enriched certain microbial taxa and functional groups within the rhizosphere but did not alter overall community diversity. Our expectation that BNF would increase in intercropped *Desmodium* systems was confirmed because Ndfa significantly increased relative to monocropped stands regardless of location or sampling time. Furthermore, intercropping also improved non-legume intercrop N content and quality, although this was dependent on site and species. Given that intercrop-responsive bacterial and fungal taxa correlated to multiple biomass characteristics, our study best supports a model of indirect microbial facilitation for productivity benefits.

4.1 Intercropping has implications for N fixation and nutritive quality

The biomass quality measures we used (ADF, NDF, CP, and lignin) have been previously linked to N supply. Past studies have identified positive correlations between fertilizer N application rates and CP in forage grasses such as timothy grass (Cherney and Cherney, 1997) and eastern gamagrass (Moyer and Sweeney, 2016). While CP tends to demonstrate the most consistent response to fertilizer N application among forage nutritive quality (Coblentz et al., 2017) there is also evidence for moderate negative correlations between N supply and ADF, NDF, and lignin content in grass (Coblentz et al., 2010) and grain crops (Li et al., 2010).

We used an unfertilized perennial pasture system in our study design, in which the only N inputs were derived from legume Ndfa. Therefore, the negative response to legume intercropping that we identified in lignin (Napier, Brachiaria) and NDF (maize) and the positive responses of CP and %N (maize) must derive from increased N supply in the rhizosphere. CP was weakly negatively correlated with bulk soil minN (Supp Fig. 25a), while %N was not correlated to minN. Together, this implies that the rhizosphere may be a more important source of minN than bulk soil for intercropping-driven changes to forage nutritive quality.

In this study, we showed that legume Ndfa from BNF increased in all intercropped systems relative to single-cropped stands with the exception of maize intercropping. Across all locations, Ndfa in *Desmodium* + Napier intercropped plots was higher than other intercropped treatments. Bulk soil minN also tended to be lower in *Desmodium* + Napier plots than other treatments irrespective of location (Supp Fig. 22). Our results imply that interspecific root interaction and differences in access to N pools within the soil profile drive BNF, with maximal BNF occurring in intercropped systems with Napier. This effect has been referred to as ‘N sparing’, in which increasing interaction intensity from the non-

legume results in decreased N concentration in the legume rhizosphere, enhancing Ndfa (Danso et al., 1987; Xiao et al., 2004). Our findings agree with those of Fan et al. (2006), who found that competition for minN drove increased BNF in faba bean (*Vicia faba* L.) intercropped with perennial wheat grass (*Triticum aestivum*), but not with maize. Perennials have 2–17 times more root biomass than annual maize (Black et al., 2017), and Napier grass has a deep root structure that penetrates down to at least one meter (Cardoso et al., 2015). *Desmodium intortum* also has a deep taproot and extensive secondary roots that compete for soil nutrients with allospecific crops in intercropping systems (Kifuko-Koech et al., 2012). Therefore, niche overlap explains the significant increases in Ndfa we observed in *Desmodium* intercropped with Napier or Brachiaria. Maize, however, has a shallower rooting depth and is less likely to interact directly with *Desmodium* roots.

4.2 Intercropping drives changes in rhizosphere community membership

Intercropping was associated with increased abundance of select microbial functional groups and taxa, rather than significant shifts in rhizosphere community diversity more broadly. However, we found evidence for greater differences in fungal community structure than bacterial community structure between intercropped and single-cropped rhizospheres when aggregating across all plant species. This finding contradicts previous studies that have shown that bacteria, more than fungi, respond to intercropped arrangements (Gong et al., 2019; Zhang et al., 2022). Many soils in sub-Saharan Africa experience phosphorus (P) deficiency, which also limits BNF in legumes (Nziguheba, 2007). Compared to Rubona and Karama, Burera soils were more acidic and P limited (Supp Table 5). Previous work has demonstrated the role of P availability in driving fungal microbial dynamics in intercropped systems with wheat and faba bean (Tang et al., 2016). Nutrient limitation and pH stress could explain the strong effect of the plant species x intercrop interaction in explaining fungal rhizosphere community structure in Burera (Supp Fig. 17) if intercropping enhanced P access or increased P demand in the rhizosphere.

We also observed that the Bray-Curtis distances between monocropped and intercropped rhizosphere communities were dependent on plant species. This finding provides evidence for plant species-specific effects on microbial community responses to intercropping in all locations for both bacteria and fungi. The modulators of species-specific responses to intercropping remain unresolved, particularly in field conditions. Non-legume plant species have also demonstrated differential responses to intercropping in terms of rhizospheric microbial enzyme activity and nutrient cycling potential (Kumar et al., 2022). Uncovering the mechanisms of plant-microbial responses in intercropped rhizospheres will help us to better understand the utility of this effect in predicting facilitative intercropping arrangements.

The selective enrichment of fungal and bacterial OTUs that we observed in intercropped rhizospheres of all plant species corroborate previous work by Hu et al. (2021), who similarly found that certain rhizobacteria respond strongly (2.7-10.5-fold increases in relative abundance) to intercropping arrangements. The greater number of selectively enriched fungal and bacterial taxa in intercropped Napier rhizospheres compared to other companion crops in this study is interesting to note and may relate to the higher Ndfa we observed in these systems. Follow-up studies could investigate linkages between selective microbial enrichment in the rhizosphere, nutrient mobilization, and BNF rates under abiotic stress. Contrary to our expectations, intracellular symbionts seemed to play a small role in intercropping related changes to rhizosphere community structure and forage nutritive quality.

Nevertheless, many of the bacterial intercrop-responsive indicator OTUs we identified at the genus level have documented resistance to a number of abiotic stressors and have been linked to putative plant growth promoting functions. For instance, members of *Gemmatimonas* (OTU 1953) have been implicated in salt stress responses in the artichoke microbiome (Yue et al., 2020). *Gemmata* (OTU 516) and *Phenylobacterium* (OTU 1452), also enriched in

intercropped Napier rhizospheres, contain several species that are acidophilic (Kulichevskaya et al., 2017), drought resistant (Khan et al., 2017), or demonstrate resistance to heavy metal toxicity in soil (Li et al., 2019; Singh et al., 2020). Intercrop-responsive bacterial genera in Brachiaria rhizospheres have similarly been implicated in abiotic stress resistance and nutrient solubilization. Bacterial genera *Aciditerrimonas* (OTU 1256) and *Singulisphaera* (OTU 3700) are temperature-tolerant (Itoh et al., 2011; Kulichevskaya et al., 2017) and associated with nutrient cycling (J. Wang et al., 2021)d solubilization (Zhou et al., 2020). Members of *Paenibacillus* (OTU 1574) are widely studied soil-associated microorganisms with functions as diverse as promoting soil aggregation and porosity (Bezzate et al., 2000), enhancing BNF and P solubilization, increasing nutrient cycling and hydrolytic enzyme activity, and suppressing pathogenic organisms through antibiotic production (Lal and Tabacchioni, 2009; McSpadden Gardener, 2007).

Cross-referencing the FUNGuild database yielded only three matches with intercrop-responsive fungal OTUs that have confirmed trophic modes or functions. Of these, *Acrocalymma* (OTU 43) was enriched in both maize and Napier intercropped rhizospheres. *Acrocalymma* species are saprotrophic and potentially endophytic as well, linked to biotic and abiotic stress resistance (Jin et al., 2018). However, some members of *Acrocalymma* are also plant pathogens (Das et al., 2020). Similarly, OTU 499, belonging to the *Phaeosphaeriaceae* family, was enriched in intercropped Napier rhizospheres and is a monocotyledon-associated plant saprotroph or pathogen (Cannon and Kirk, 2007; Phookamsak et al., 2014). Lastly, *Dentiscutata* (OTU 828) in Napier is a known beneficial AMF genus that has been identified in acidic tropical agricultural soils, including India (Khade, 2010) and Brazil (Krüger et al., 2014).

In addition to fungal indicator OTUs, we also found that the relative abundance of OTUs classified by FUNGuild as AMF, symbiotrophs, and white rot fungi significantly increased in intercropped rhizospheres. White rot fungi are responsible for degrading lignin (Hatakka, 1994) and may therefore be relevant to the turnover of root-derived SOM in cropping systems that include woody perennials such as *Desmodium* sp. Future work could investigate this mechanism further, as increased SOM turnover could result in higher rates of N mineralization in the rhizosphere, thereby improving forage grass yield and nutritive quality. The enhanced AMF abundance we observed in intercropped rhizospheres concurs with past studies that note the role of legumes in promoting AMF abundance in low-input agricultural soils (Pivato et al., 2017; Scheublin et al., 2004). Overall, there is evidence that intercropping with perennial legumes enhances the abundance of fungal guilds and specific taxa that may be involved in critical roles that support plant growth, such as nutrient mobilization and stress resistance.

4.3 Indirect facilitation explains intercropping effects

Our results best support a model of indirect facilitation, in which intercropping-driven changes to microbial abundance in the rhizosphere enhance nutrient availability and uptake in the companion crop. This resulted in species-specific improvements to forage nutritive quality that occurred alongside increased rates of BNF and selective enrichment of microbial taxa in the rhizosphere. Enhanced Ndfa in intercropped legumes and access to nitrogen in the rhizosphere has implications for aboveground plant tissue characteristics including %N, δ^{15}N and CP. Emerging evidence from previous studies suggests that annual legume intercropping improves non-legume yield and nutritional quality by recruiting beneficial rhizosphere microorganisms that aid in improving N uptake and limiting N losses (Sun et al., 2022; Wang et al., 2022).

In this study, we showed that similar benefits could be achieved with a perennial legume intercrop. Nearly all of the intercrop-responsive OTUs that were identified to the genus level have been previously linked to plant growth promotion. Zhou et al. (2019) found that changes in plant communities can shift the functional potential of soil microbial communities, which could explain the benefits we observed in non-legume tissue quality. Our study does not exclude the potential role of direct facilitation in this system, which relates to the transfer of nutrients from one plant to

another via a microbial interface (Duchene et al., 2017). To confirm a direct microbially mediated transfer of nutrients, other techniques such as isotope labeling are required.

Intercrop-responsive fungal and bacterial OTUs were associated with multiple biomass quality metrics. However, while there is a clear relationship between the abundance of certain microbial taxa and forage nutritive quality, causality is difficult to establish. For instance, rhizosphere community structure may contribute to forage quality such as CP or %N; conversely, these same metrics may also correlate to rhizosphere community structure if aboveground traits determine belowground root inputs, for instance rhizodeposition (Fustec et al., 2010). Benitez et al. (2021) note a similar challenge in their discussion of rhizosphere community responses to diverse crop rotations and biomass yield. However, Hirte et al. (2018) found that aboveground yield indicators were independent of root carbon inputs to soil. This finding lends support for bottom-up, rather than top-down, interactions between microbial communities and plant biomass.

We also tested the contribution of intercropping in explaining microbial community structure (H_1) and the contribution of microbial community structure in explaining biomass quality (H_2). Our approach with integrating LRA coordinates into mixed effects models rejected the hypothesis that a binary intercropping term explains fungal or bacterial community composition. This concurs with PERMANOVA results that did not find differences in fungal or bacterial beta-diversity between intercropped and monocropped rhizospheres when aggregating across plant species (Supp Figs. 11, 15). However, it is important to note that our approach with LRA encapsulated a relatively small proportion of community variation (~ 22% for bacteria and ~ 38% for fungi). Therefore, the large amount of variation in microbial community composition left out of the analysis could contribute to our negative results. However, LRA was the most successful ordination method that we identified for maximizing the amount of variation accounted for in our dataset.

Using the same approach with LRA coordinates and mixed models, we confirmed the hypothesis that fungal and bacterial communities mediated the effect of plant species on forage nutritive quality (H_2), with the exception of fungal communities in Karama (Table 4.2). The contribution of fungal communities to forage nutritive quality was dependent on location, as rhizosphere fungi in Rubona contributed mostly to %N and CP, while fungi in Burera contributed to cellulosic properties (ADF, NDF). Location-based distinctions were less clear for bacteria, which related to δ^{15}N in all locations. Bacterial community structure in Burera also predicted %N, CP, and lignin while bacteria in Rubona predicted ADF (Table 4.1). These results reinforce the context dependency of benefits derived from plant-microbial interactions, as the three sites differed markedly in precipitation and soil properties (Supp Tables 4, 5).

The balance between facilitation (productivity gains) and competition (productivity declines) in intercropped systems is delicate (Hauggaard-Nielsen and Jensen, 2005). Our results support past work that has found facilitation in intercropped systems to be species-specific (Bedoussac and Justes, 2010; Corre-Hellou et al., 2006), highlighting the importance of crop selection. Plant-plant facilitation by microbial mechanisms is also context-dependent and sensitive to soil type and disturbance such as drought stress (Callaway and Howard, 2007). Leveraging biotic interactions may be critical to maximizing BNF and alleviating nutrient deficiency (Grönemeyer and Reinhold-Hurek, 2018; Smithson and Giller, 2002), although evidence from perennial tropical cropping systems is lacking. Overall, our results suggest that intercropping grain or grass crops with a perennial legume selectively enriches low-abundance taxa that have implications for non-legume tissue quality and Ndfa. More work is needed to design successful intercropped systems at local and regional scales in rainfed tropical agroecosystems, taking into account the many variables (row spacing, species selection, soil type, and environmental conditions) that are expected to impact the outcome.

5. Conclusion

We have shown that perennial legume intercropping is associated with improvements in biomass characteristics of the companion plant in a tropical forage system. Intercropping also resulted in the selective enrichment of specific microbial taxa in the rhizosphere that have been previously linked to abiotic stress resistance and nutrient mobilization. Indirect microbial facilitation of nutrients and enhanced nitrogen fixation is a reasonable explanation for intercropping-derived benefits. However, more work lies ahead in understanding the context-dependent mediating factors that underlie facilitative microbial interactions in the rhizosphere and species-specific responses to intercropping. Overall, this research advances the goal of innovating new perennial cropping systems that deliver multifunctional benefits for food security, income generation, and soil improvement by leveraging biotic interactions.

Declarations

Competing Interests

The authors declare no competing interests.

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Figures

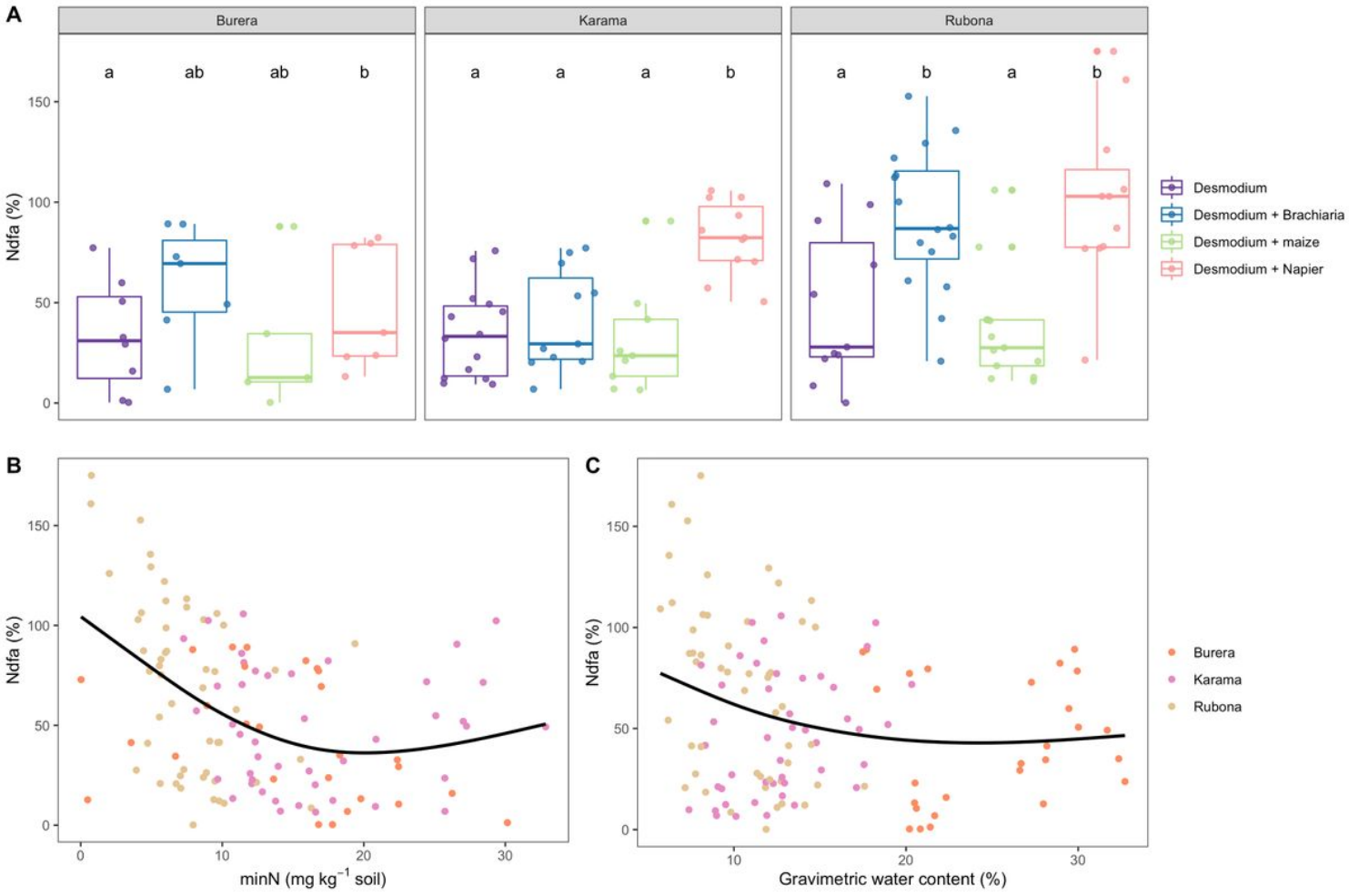

Figure 1

Nitrogen derived from the atmosphere (Ndfa) in *Desmodium* intercrops in each study location (A) and relationships between Ndfa and soil mineral N (B) and gravimetric water content (C).

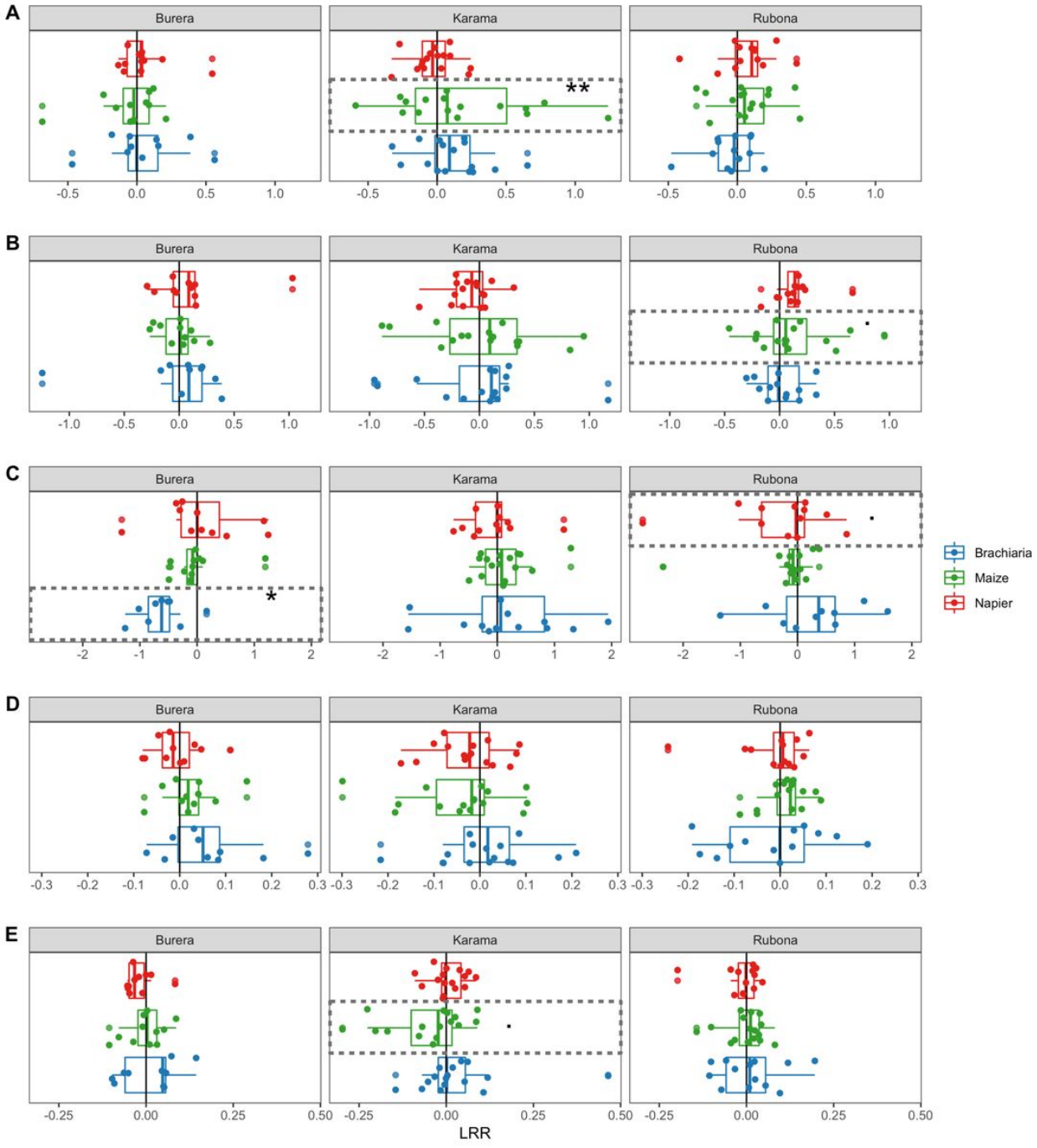

Figure 2

Log-response ratios (LRRs) of companion crop responses to legume intercropping by %N (A), crude protein (B), lignin (C), ADF (D), and NDF (E) across study locations. An LRR of zero, indicating no difference between monocropped and intercropped plants, is shown by a solid vertical line. Positive LRR values to the right of the vertical line suggest increases in forage nutrive metrics in intercropped plants relative to monocropped plants. Negative LRR values to the

left of the vertical line suggest reduced forage nutrive metrics in intercropped plants relative to monocropped plants. Dotted lines highlight differences from zero at the level of P <0.01 (**), P <0.05 (*), and P <0.10 (.).

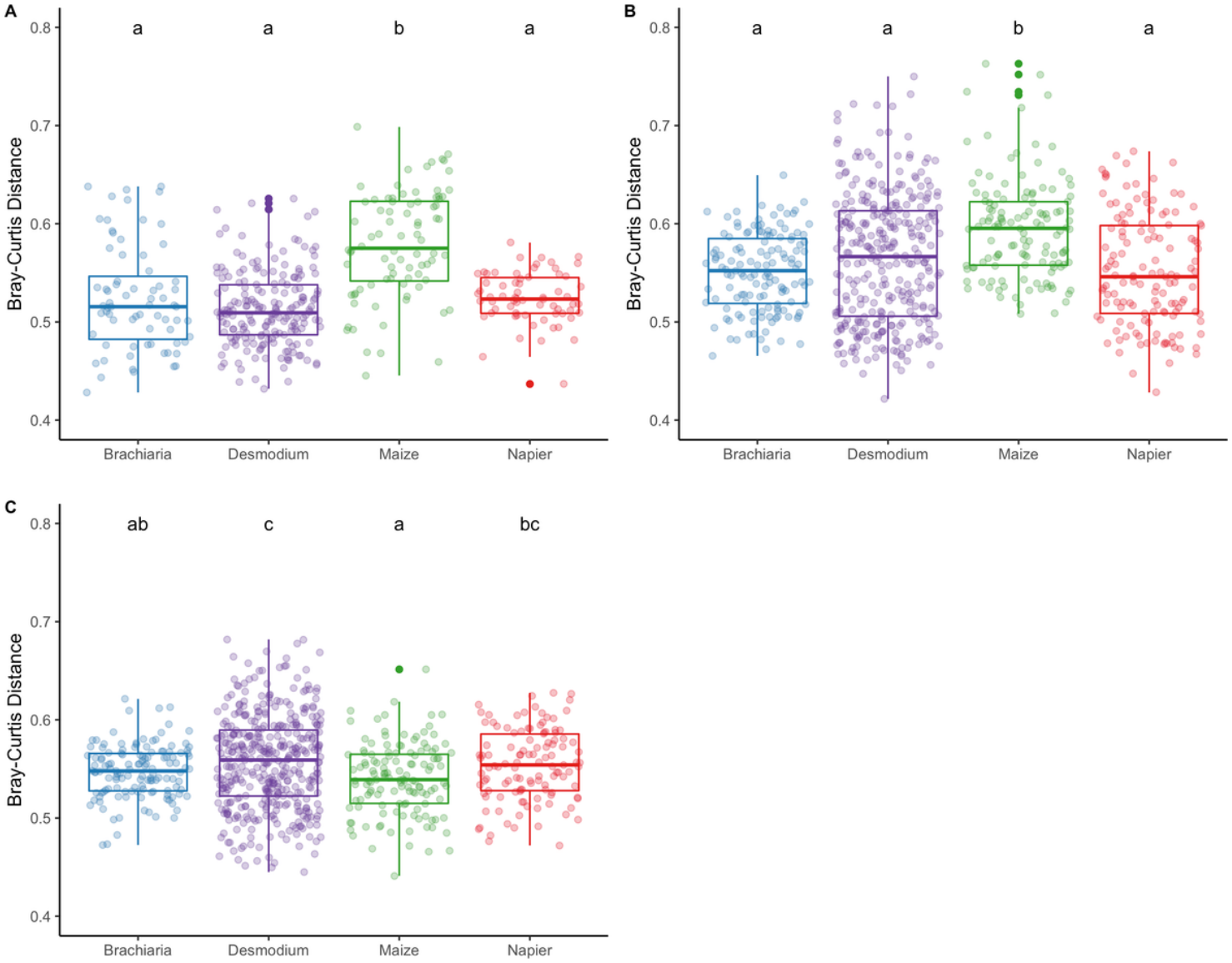

Figure 3

Bray-Curtis distances for 16S rhizosphere bacterial communities between monocropped and intercropped rhizospheres in Burera (A), Karama (B), and Rubona (C). Distances between monocropped and intercropped rhizosphere communities were extracted within forage species and location. Significance groupings refer to species differences at the level of P < 0.05 according to mixed-effects models (random = timepoint, block) and post-hoc Tukey tests.

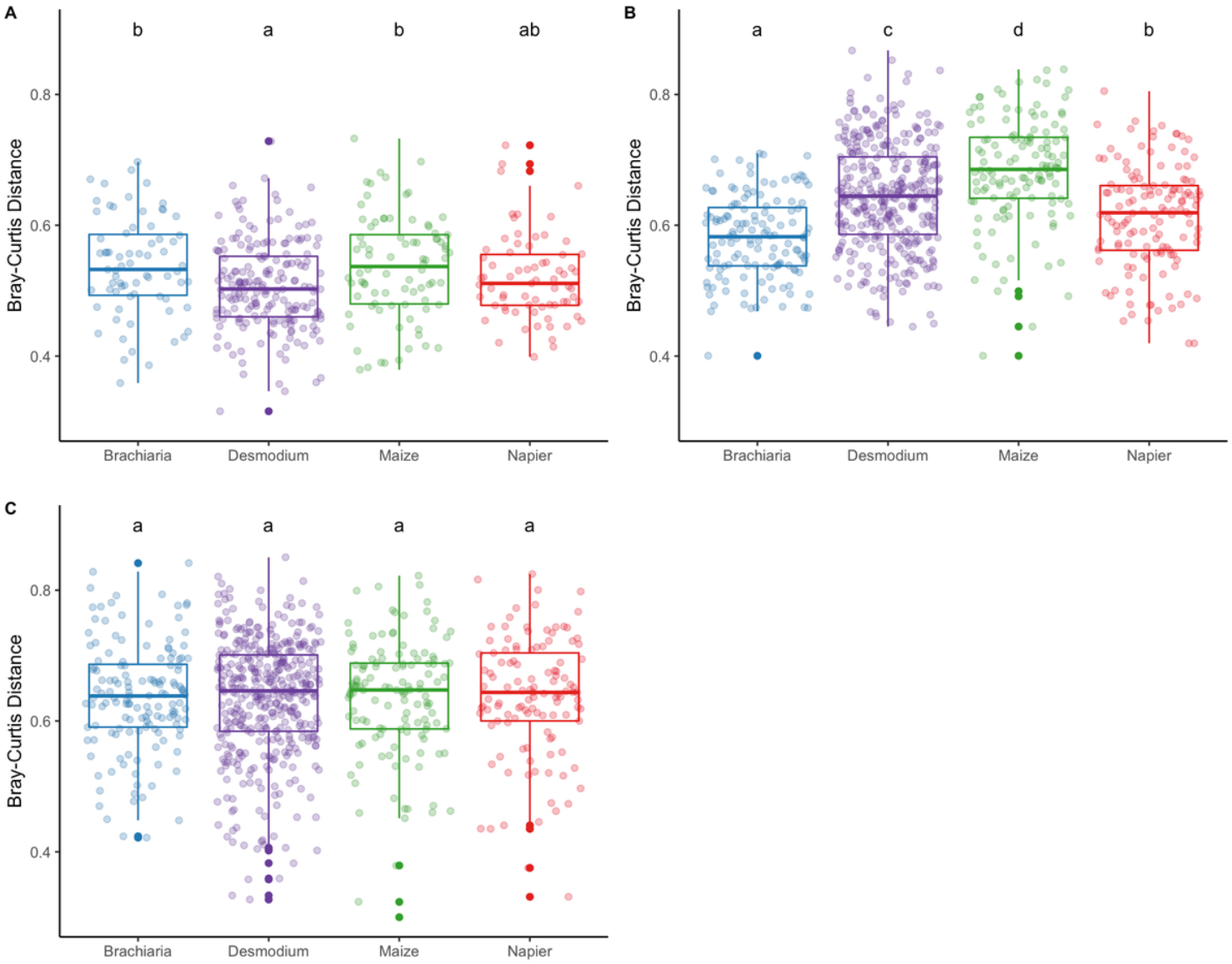

Figure 4

Bray-Curtis distances for ITS2 rhizosphere fungal communities between monocropped and intercropped rhizospheres in Burera (A), Karama (B), and Rubona (C). Distances between monocropped and intercropped rhizosphere communities were extracted within forage species and location. Significance groupings refer to species differences at the level of $P < 0.05$ according to mixed-effects models (random = timepoint, block) and post-hoc Tukey tests.

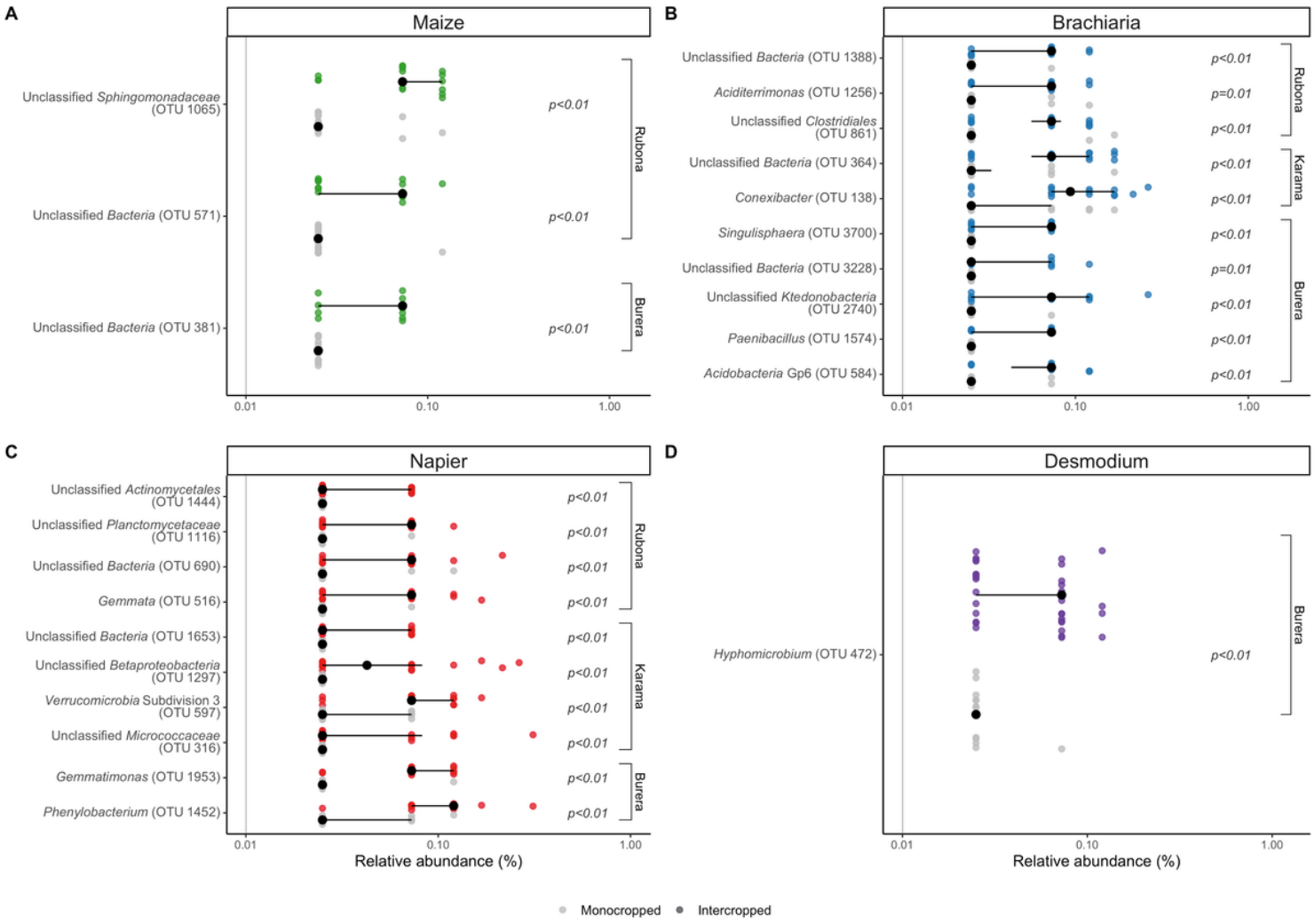

Figure 5

Significantly enriched 16S indicator OTUs in intercropped rhizospheres. Indicator OTUs are shown for each forage species, with location in which the indicator OTU appeared indicated by the brackets. The p-values shown are adjusted for multiple comparisons and derive from Wilcoxon Rank Sum tests comparing the relative abundance of each indicator OTU in intercropped relative to monocropped rhizospheres.

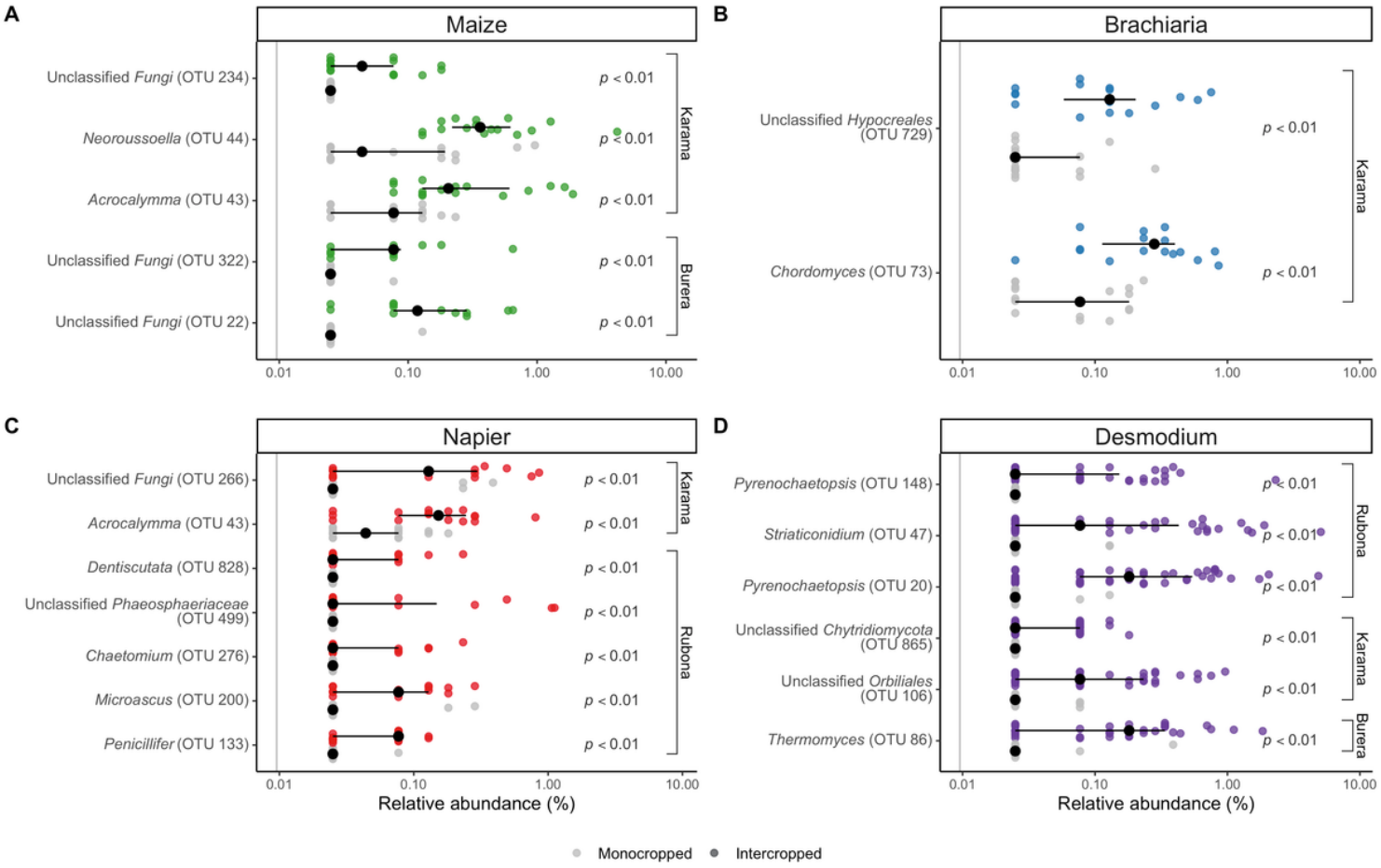

Figure 6

Significantly enriched ITS2 indicator OTUs in intercropped rhizospheres. Indicator OTUs are shown for each forage species, with location in which the indicator OTU appeared indicated by the brackets. The p-values shown are adjusted for multiple comparisons and derive from Wilcoxon Rank Sum tests comparing the relative abundance of each indicator OTU in intercropped relative to monocropped rhizospheres.

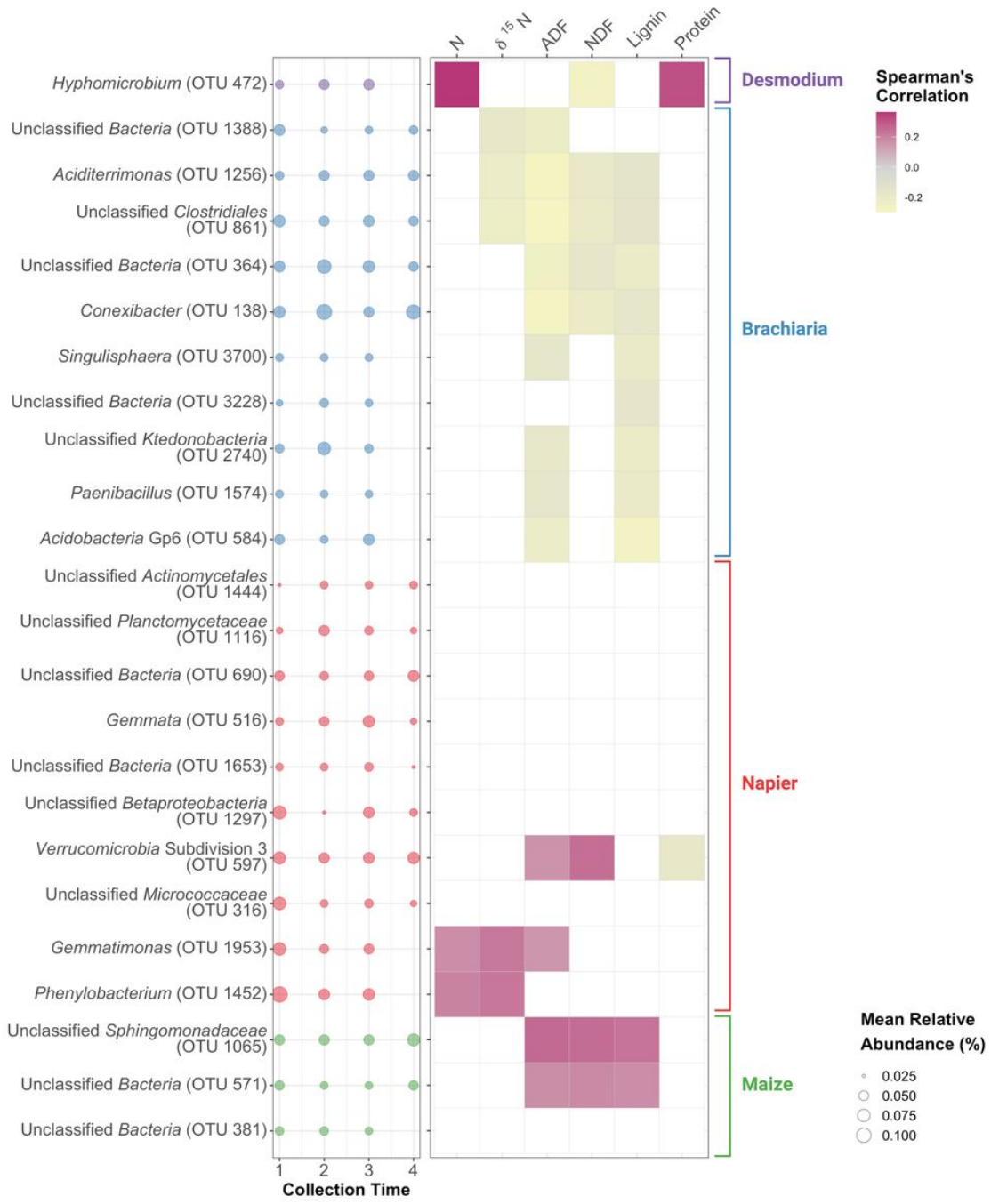

Figure 7

Bacterial indicator OTU relative abundance according to collection time and correlation to forage leaf biomass characteristics. Indicator OTUs are grouped vertically according to forage host species. Spearman's correlations between indicator OTU relative abundance and individual biomass characteristics were calculated, with p-values adjusted for multiple comparisons. Only significant correlations ($P < 0.05$) are shown, with white tiles indicating non-significance.

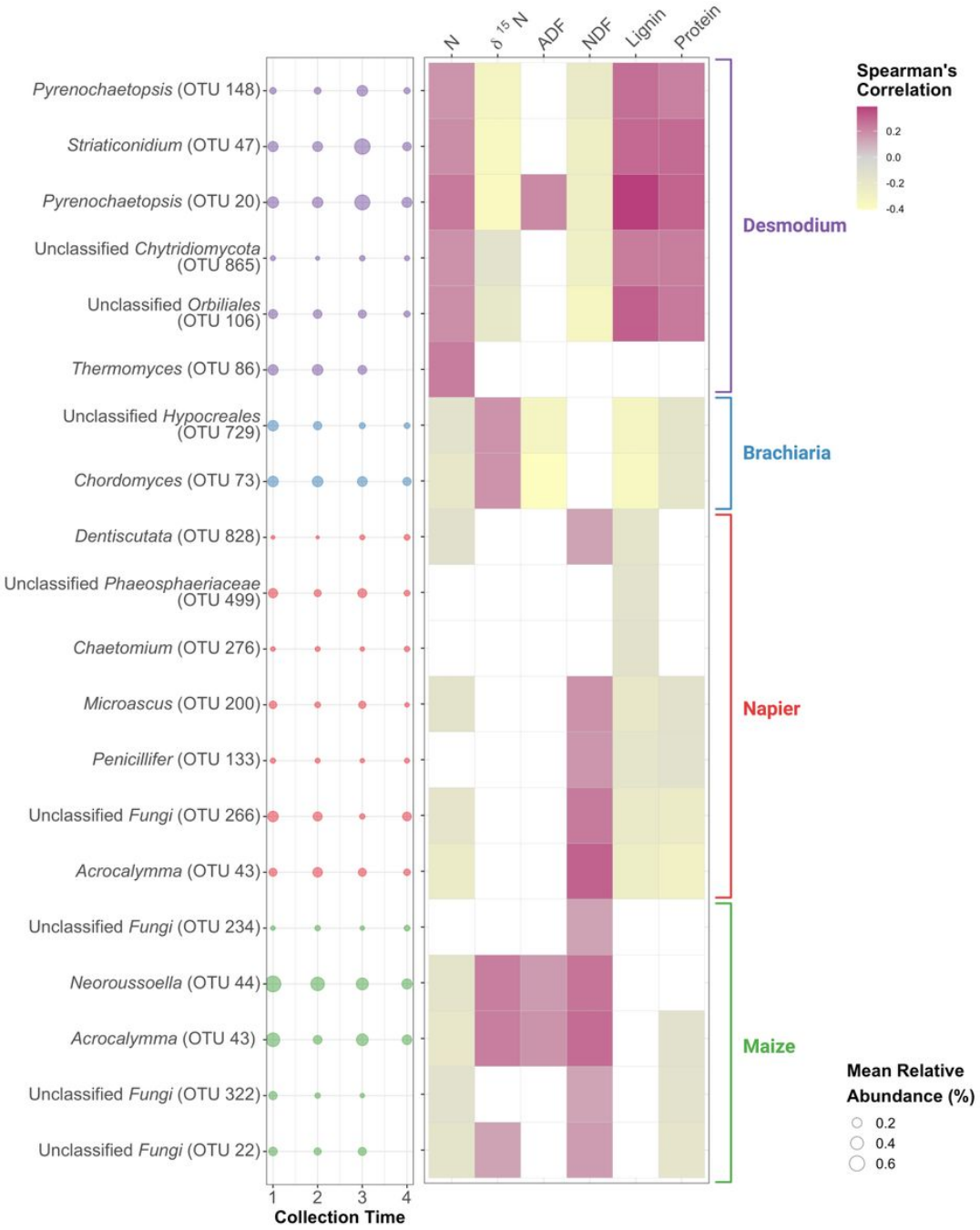

Figure 8

Fungal indicator OTU relative abundance according to collection time and correlation to forage leaf biomass characteristics. Indicator OTUs are grouped vertically according to forage host species. Spearman's correlations between indicator OTU relative abundance and individual biomass characteristics were calculated, with p-values adjusted for multiple comparisons. Only significant correlations ($P < 0.05$) are shown, with white tiles indicating non-significance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalMaterials.pdf