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Forage Species Identity Shapes Soil Biota in a Temperate Agroecosystem

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Abstract: Increasing plant diversity in the perennial phase of pasture-crop rotations is predicted to positively affect belowground productivity and microbial communities and, in turn, augment belowground agroecosystem services including soil health and carbon storage. Using two grass and one legume forage species grown as monocultures and combined in four intercropped combinations, we evaluated how species identity and richness influence belowground productivity, soil microbial communities, and soil C pools. Though grass-legume intercrops demonstrated higher aboveground productivity than component species grown in monoculture, higher species richness was not associated with increased productivity belowground. Root biomass was greatest in tall fescue (*Festuca arundinacea* Schreb.) monoculture, and intercrops including this species. Species identity was similarly associated with soil microbial community attributes. Orchardgrass (*Dactylis glomerata* L.) monoculture exhibited lower total microbial abundance and lower bacterial abundance than grass-legume intercrops. Bacterial abundance was also lower in orchardgrass compared to white clover (*Trifolium repens* L.) monoculture. A common indictor of soil function, the fungal:bacterial ratio, was higher in grass-only than clover-only stands. The prevalence of species-specific impacts on roots and microbial communities in this study suggests that species identity may have a stronger influence than species richness on belowground agroecosystem services from perennial forages in temperate regions.

Keywords: ecosystem services; soil carbon; soil microbial communities; pasture-crop rotation; intercropping; soil health; sustainable agriculture

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1. Introduction

Increasing the capacity of agricultural systems to provide multiple agroecosystem services (i.e., the benefits humans derive from agriculture such as food production; soil, water, and air quality regulation; and support for nutrient cycling) is critical to meeting the needs of the global population while also mitigating negative environmental impacts of agriculture [1]. A potential strategy to enhance agricultural sustainability and increase service provision from row crops is to increase plant diversity [2]. For instance, pasture-crop rotations that incorporate multiple years of a perennial forage within row crop production can enhance agronomic crop yield while improving water and soil quality [3]. Agroecosystem services expected from perennial forages in a pasture-crop rotation, such as soil carbon (C) sequestration and soil health, are mediated by plant roots and soil microbes [4], yet few studies on the short-term effects of crop selection practices on soil biota have been conducted. In this paper, we examine the impacts of species identity and richness on belowground biota and soil C dynamics using perennial forage species common to the temperate northeastern United States.

Rotating perennial forages with annual crops (a pasture-crop rotation) can increase soil organic carbon (SOC) compared to annual cropping systems, promoting mitigation of C emissions and enhancing soil health [3,5,6]. One driver of SOC accrual in rotations that include perennials is increased root biomass [5], as plant roots are the primary source of C

inputs to soil [7–9]. Root biomass production is particularly consequential in rotations that manage perennial forages for hay due to the removal of aboveground biomass. Management strategies to promote belowground productivity from forage crops are expected to increase SOC stocks and support soil C-derived agroecosystem services [10–14].

Decades of research on multi-species forage stands has demonstrated that intercropping (the planting of two or more species in a single field) increases aboveground productivity and also reduces weed establishment, boosts forage quality, and improves fertility management [15–18]. Increased productivity belowground from forage intercrops is also expected based on ecological diversity theory and evidence from experimental grasslands [19,20]. Several studies comparing root biomass production in grass-legume bicultures to stands with greater species richness (intercrops containing three to eleven species) have reported increased belowground productivity associated with higher species richness [21–24]. Comparisons of belowground productivity between monocultures and intercrops are, however, limited (but see [25]). Such comparisons are essential to identifying approaches to crop selection (i.e., the identity and number of species to include in a forage stand) that optimize root biomass production.

One mechanism through which intercrops lead to increased productivity is complementarity, which occurs among species that exhibit differences in resource use that allow for greater overall resource capture [26]. Grass-legume bicultures, for example, leverage the complementary nitrogen (N) acquisition strategies of component species (i.e., grasses that utilize soil N resources and legumes that fix atmospheric N_2) to support internal N cycling [27]. This facilitation has been cited as the mechanism leading to increased aboveground primary production [28,29]. Belowground productivity is also responsive to N availability [30]; thus a grass-legume intercrop may exhibit overyielding, producing more root biomass than the average of its component species grown in monoculture, or transgressive overyielding, producing more biomass than the highest performing component monoculture [31]. Grasses and legumes also exhibit complementary root architecture. Pairing a monocot such as a grass, which has a fibrous root system, with a tap rooted dicot such as white clover (*Trifolium repens* L.), could expand spatial exploitation of soil resources and result in increased root biomass [32,33].

Plant functional identity (i.e., grass versus legume) also mediates the quantity and composition of resources available to the soil microbial communities that drive agroecosystem services, influencing their size, structure, and function [34–37]. Current C stabilization paradigms suggest that agricultural practices that increase microbial abundance and shift composition to dominance by fungi (as indicated by the ratio of fungal to bacterial biomass) will promote SOC accrual [38,39]. Increasing diversity through intercropping is expected to augment microbial abundance and activity in perennial forage stands through enhanced belowground productivity and provision of heterogenous C inputs [40–42]. Shifts in the composition of the microbial community may, however, be driven by plant identity [25]. Field-based studies have reported divergent findings regarding grass-legume biculture effects on microbial communities. A study by van Eekeren et al. [43] found that bacterial and fungal abundance were each higher in a grass monoculture (ryegrass, *Lolium perenne* L.) compared to a legume (white clover). Dhakal and Islam [44] reported the opposite, with higher total microbial, bacterial, and fungal abundances in alfalfa (*Medicago sativa* L.) and alfalfa-grass bicultures compared to grass monocultures. These studies also found different responses of fungal:bacterial biomass ratio (F:B ratio) to species identity and richness; van Eekeren et al. [43] reported no differences among grasses, legumes, and grass-legume mixes, while Dhakal and Islam [44] reported a higher F:B ratio in grass monocultures compared to a legume monoculture and grass-legume mixes. Whether there are generalizable impacts of legumes and grasses—grown singly and in intercrops—on microbial community composition remains an open question.

To address current gaps in our knowledge of the impacts of plant species identity and richness on belowground biota and, consequently, C cycling, in perennial forages, we measured root and microbial characteristics in seven forage treatments. Species were

drawn from those commonly used for forage in the northeastern United States and included two perennial grasses, tall fescue (*Festuca arundinacea* Schreb.) and orchardgrass (*Dactylis glomerata* L.), and the perennial legume white clover. Phospholipid fatty acid analysis (PLFA) was used to determine the size and structure of microbial communities associated with each treatment, and C dynamics were assessed through measurement of microbial respiration, labile C, and total organic C (TOC). We assessed roots, microbial communities, and C pools in the second and third growing seasons following forage planting to capture the short-term impacts of species identity and richness on these attributes. Though detectable changes in soil C may not manifest in this short timeframe, the species-driven influences on roots and microbial communities we measured are indicative of the effectiveness of crop selection as a tool to create conditions favoring SOC accrual. In addition, two to three years represents a realistic timeframe for the pasture phase of a pasture-crop rotation. We hypothesized that productivity belowground would be higher in intercrops compared to monocultures and reflect patterns observed in aboveground productivity. Intercrops were also expected to support more microbial biomass than monocultures, while abundances of bacteria and fungi and the F:B ratio were expected to differ among grass and legume monocultures.

2. Materials and Methods

2.1. Site Description

This field experiment was established at the Whittaker Environmental Research Station in Trappe, Pennsylvania, USA (40°11′ N 75°28′ W, 82 m elevation) in April 2017. This location receives approximately 1175 mm of precipitation annually, and mean monthly temperatures range from −1.6 °C (January) to 23.2 °C (July) (Northeast Regional Climate Center, nrcc.cornell.edu). Climate data for the experimental period were collected from NOAA weather station USW00054782 (Table 1; Midwest Regional Climate Center, mrcc.illinois.edu). The soil type in experimental plots was Penn silt loam (fine-loamy, mixed, superactive, mesic Ultic Hapludalf) with pH of 7.0 ± 0.05 and soil organic matter content of 2.5% ± 0.06% (n = 16) when the experiment was initiated.

Table 1. Annual precipitation, air temperature, and growing degree days (base 10 °C) in Trappe, Pennsylvania, USA for the years 2017 to 2019.

Parameter	2017	2018	2019
Annual precipitation, mm	1066.8	1828.5	1269.0
Average annual temperature, °C	11.8	11.5	11.4
Average annual maximum temperature, °C	17.2	16.3	16.8
Average annual minimum temperature, °C	6.4	6.5	6.0
Total growing degree days	3357	3496	3435

2.2. Experimental Design

This study employed a randomized complete block design with four replicates of seven treatments. Experimental plots measuring 7.3 × 12.2 m were planted on 19 April 2017 in a field planted to no-till corn the previous year. Treatments included a monoculture of each species (tall fescue [TF], orchardgrass [OG], and white clover [WC]), three bicultures representing all possible species combinations (TF + OG, TF + WC, OG + WC), and a mixture combining the three focal species plus alfalfa. The alfalfa failed to establish in this mix and comprised less than 2% of standing aboveground biomass (Figure S1); therefore, this treatment is considered and referred to as a triculture (TF + OG + WC). All monoculture treatments were sown at a rate of 1000 pure live seed (PLS) m^{-2}. The seeding rate of individual species in mixtures (two or more species) was determined by dividing the monoculture seeding rate by the number of species in the intercrop [45]. Following disking, treatments were planted using an Esch no-drill fitted with a cone-seeder. Legume seed was mixed with *Rhizobium* ssp. inoculant prior to seeding. The field received a uniform fertility application of 39 kg N ha^{-1}, 27 kg P_2O_5 ha^{-1} and 118 kg K_2O ha^{-1} prior to planting. An additional 140 kg N ha^{-1} and 200 kg K_2O ha^{-1} were applied to all plots in June 2019,

following soil sampling (see data collection below). Fertility applications were withheld between spring 2017 and summer 2019 to avoid potentially confounding effects of fertilizer application on root and microbial dynamics [30,43,46]. Fertilizer application rates were based on soil fertility analysis and recommendations provided by Penn State University. Experimental treatments were managed as hay, with aboveground biomass cut, baled, and removed from the field three times each season.

2.3. Data Collection

Aboveground biomass production was measured prior to each hay harvest in 2018 and 2019. At each sampling event, aboveground biomass was destructively harvested in two 0.25 m^2 quadrats per plot. Samples were dried at 60 °C and weighed. Dry matter yield at each harvest was summed within year to estimate annual dry matter yield. At each spring harvest (prior to the first hay harvest of the season), forage and weed biomass were segregated and forage biomass further sorted by species prior to drying to determine botanical composition.

Standing root biomass measurements were taken in spring 2018 (two years after planting) and spring 2019 (three years after planting) to compare belowground productivity among treatments [46]. Soil cores for root biomass determination were collected within six hours of hay cutting in year 2 (1 June 2018) or prior to hay cutting in year 3 (22 May 2019). At each root sampling event, four soil cores (5.1 cm i.d.) were collected from each plot to a depth of 30 cm, with two cores taken within a plant row and two cores taken between plant rows. Soil cores were extracted from areas with low weed biomass to the extent possible. Soil cores were separated into two depths (0–15 cm and 15–30 cm). Samples from each depth were then homogenized within plot and stored at 4 °C until root extraction. To prepare for root extraction, soil samples were shaken in 500 mL 2.5% hexametaphosphate for 60 min. Root material was washed on a stacked sieve system (2 mm round sieve on top of a 250 µm sieve) to segregate coarse (>2 mm) and fine roots (>250 µm and <2 mm) [24]. Root material was dried at 60 °C for a minimum of 48 h, weighed, and ground for elemental analysis. The C and N concentration of coarse roots was determined by combustion analysis (Elementar Vario MICRO CHN Analyzer, Ronkonkoma, NY, USA) [47].

Soil samples for microbial analyses and carbon pool quantification were collected annually in June. At each sampling event, eight cores (15 cm depth by 2.5 cm i.d.) were collected from random locations in each plot and homogenized. Samples were placed in coolers for transport to the lab and stored at −20 °C (2018) or 4 °C (2019) prior to wet sieving to 2 mm. Representative subsamples were collected from each plot sample and stored at −20 °C for phospholipid fatty acid analysis or air-dried for carbon analysis. An additional 10 g subsample was dried at 105 °C for at least 48 h to determine gravimetric water content. Soil sampling and laboratory processing were conducted in an aseptic manner to the extent possible.

Phospholipid fatty acid (PLFA) analysis was used to determine the size and structure of the soil microbial community on soils that had been stored at −20 °C and lyophilized. The PLFA biomarkers were extracted and esterified into fatty acid methyl esters using the modified Bligh-Dyer extraction as described by Buyer and Sasser [48]. Samples were analyzed on an Agilent 6890 (Agilent Technologies, Wilmington, DE, USA) gas chromatograph outfitted with an autosampler, split-splitless injector, and flame ionization detector. The internal standard for the analysis was 19:0 phosphatidylcholine. MIDI Sherlock® software (MIDI, Inc., Newark, DE, USA) was used in conjunction with an Agilent Chemstation to control the system for the analysis. Fatty acids were separated on an Agilent Ultra 2 column (25 m × 200 µm I.D. × 0.33 µm film thickness) using hydrogen gas as the carrier (1.2 mL min^{-1} flow rate) and identified using the MIDI PLFAD1 software package. The following fatty acids were summed to determine bacterial abundance: iso and anteiso branched fatty acids (Gram-positive bacteria) [49]; monounsaturated fatty acids, cyclopropyl 17:0, and cyclopropyl 19:0 (Gram-negative bacteria) [50]; and 10-methyl 16:0 and 10-methyl 18:0 (actinomycetes) [49]. Fungal abundance was the sum of 16:1 ω5 cis (ar-

buscular mycorrhizal fungi) [51] and 18:2 ω6 cis (saprophytic fungi) [52]. The ratio of fungal to bacterial biomarkers (F:B ratio) was calculated as the sum of fungal biomarker concentrations divided by the sum bacterial biomarker concentrations [34]. Results are reported in nanomoles g^{-1} soil based on biomarker molecular weight.

Microbial basal respiration was measured as an indicator of soil biological activity in 2019. At initiation, 20 g of fresh, sieved soil was placed in a 120 mL Wheaton serum bottle and pre-incubated at 22 °C for seven days. Following this period, deionized water was added to adjust each vial to 50% water holding capacity. Each vial was capped with a butyl stopper, and an aluminum ring was used to create an air-tight seal. Vial headspace was sampled with a syringe after a six-hour incubation period, and carbon dioxide concentration in the headspace gas was quantified immediately with an infrared gas analyzer (LI-8100A, LiCor, Inc., Lincoln, NE, USA). Results are presented as the hourly respiration rate in grams of dry soil (μg CO_2-C g^{-1} soil h^{-1} ± 1 standard error of the mean).

Permanganate oxidizable carbon (POX-C), often referred to as 'active C,' is an indicator of microbially-available C. It is also considered a processed labile C pool that serves as an early indicator of soil C stabilization [53,54]. Quantification of POX-C was based on the procedure of Weil et al. [55]. Briefly, 2.5 g of 2 mm sieved, air-dried soil was shaken with 18 mL deionized water and 2 mL 0.02 M KMnO4 for exactly 2 min at 240 rpm. After a settling period of exactly 10 min, 0.5 mL of supernatant was transferred to a tube containing 49.5 mL deionized water. The sample absorbance at 550 nm was determined on a SpectraMax spectrophotometer (Molecular Devices, San Jose, CA, USA). Results are reported in mg kg^{-1} dry soil. Total soil carbon was quantified on an air-dried, pulverized (8000M Mixer/Mill, Spex Sample Prep, Metuchen, NJ, USA) subsample by combustion analysis (Elementar Vario MICRO CHN Analyzer, Ronkonkoma, NY, USA). All C quantified through combustion analysis was considered organic because the field had not recently received limestone and pH was below 7.1 [56].

2.4. Data Analysis

Analysis of variance (ANOVA) was used to test for differences in above and below-ground biomass at the first hay harvest of each season across treatments using a mixed model with treatment, sampling year, and their interaction as fixed effects and block as a random effect. For this analysis, coarse and fine root biomass from 0 to 30 cm were summed to quantify standing root biomass. Coarse root biomass, coarse root C:N, and fine root biomass were subject to ANOVA using the same mixed model, with separate ANOVA performed for each depth (0–15 cm and 15–30 cm). Microbial parameters and carbon pool sizes measured in surface soils (0–15 cm) were compared across treatments using the same mixed model approach. When a significant treatment by year interaction was identified, separate analyses were conducted for each year. Data were square root transformed as needed to meet assumptions of normality, apart from root C:N and F:B ratios, which were log transformed. Where significant treatment differences were detected, Tukey's adjustment was used to separate means with $p < 0.05$ indicating statistical significance. All analyses were performed in R statistical software [57].

3. Results

3.1. Above- and Below-Ground Biomass

Dry matter yield at the first hay harvest of each season differed across crop treatments (Figure 1A; $F_{6,45} = 13.72$, $p < 0.0001$) and was higher in 2018 (3099 ± 232 kg ha^{-1}) compared to 2019 (2556 ± 263 kg ha^{-1}; $F_{1,45} = 6.94$, $p = 0.015$). The grass-legume bicultures (TF + WC, OG + WC) exhibited transgressive overyielding aboveground, producing more biomass per unit area than the most productive component species grown in monoculture (Figure 1A). The triculture performed similarly to grass-legume bicultures (Figure 1A) but did not produce more biomass than the highest performing component species (TF, $p = 0.238$). The triculture did, however, produce more biomass per unit area than the average yield of the component species grown as monocultures, an indicator of overyielding (Table 2).

On average, bicultures also produced more biomass than monocultures (Table 2). Among intercrops, the grass biculture produced less biomass than the TF + WC and the triculture (Figure 1A), and there was a trend toward lower production in TF + OG compared to OG + WC, but this difference was not statistically significant. Annual dry matter yield reflected similar trends, though the overyielding response was dampened by the summer fertility application in 2019 that followed spring biomass sampling (Table S1).

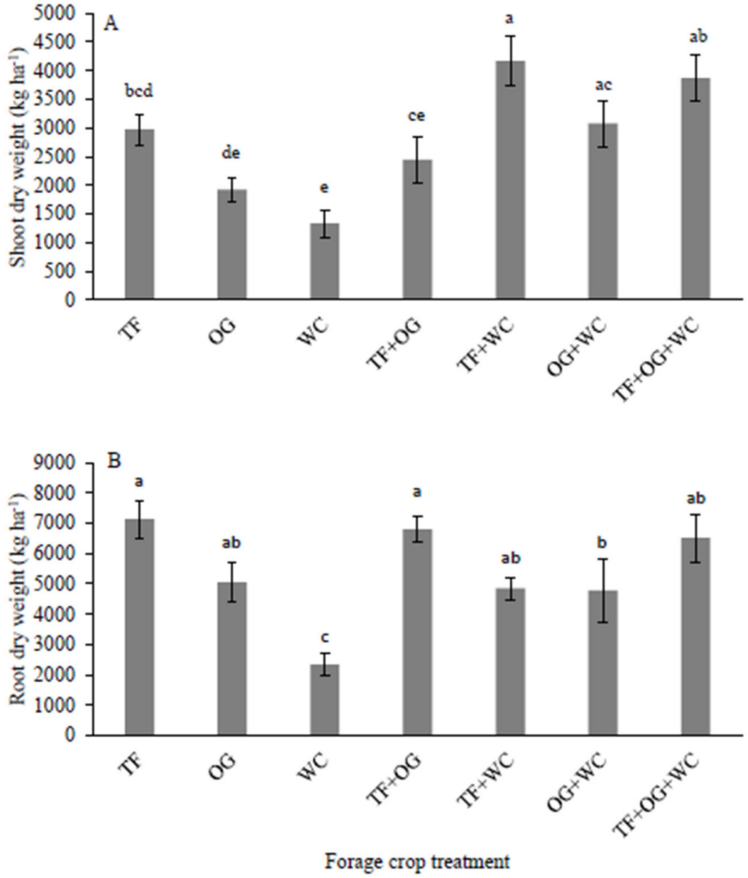


Figure 1. Primary productivity (**A**) aboveground and (**B**) belowground to 30 cm in forage crops as measured at the time of spring cutting two and three years after establishment in southeast Pennsylvania, USA. TF, tall fescue; OG, orchardgrass; WC, white clover. Lowercase letters indicate treatment differences based on mean separation using Tukey's honestly significant difference (HSD) at $\alpha = 0.05$. Error bars are 1 standard error of the mean.

Table 2. Above and belowground productivity of forage monocultures and intercrops measured as standing biomass at the time of first cutting two and three years after establishment in southeast Pennsylvania, USA. Root biomass was collected to a depth of 30 cm. Values are the mean dry matter (kg ha^{-1}) of all observations collected from each diversity level at two time points (monoculture: n = 24; bicultures: n = 24; triculture: n = 8) ± 1 standard error.

Treatment Category	Aboveground Biomass (kg ha^{-1})	Belowground Biomass (kg ha^{-1})
Monocultures	2080 ± 948 b †	4740 ± 512
Bicultures	3229 ± 273 a	5408 ± 396
Triculture	3866 ± 410 a	6510 ± 790

† Lowercase letters indicate differences among values within each column based on mean separation using Tukey's honestly significant difference (HSD) at $\alpha = 0.05$.

Belowground, root biomass to a depth of 30 cm differed across crop treatments (Figure 1B; $F_{6,43}$ = 14.08, $p < 0.0001$) and was higher in 2018 (6296.6 ± 391.9 kg ha^{-1}) compared to 2019 (4277.1 ± 383.3 kg ha^{-1}; $F_{1,43}$ = 29.67, $p < 0.0001$). Root biomass was lowest in WC, consistent with aboveground results (Figure 1A). Tall fescue monoculture and intercrops containing tall fescue tended to exhibit the highest root biomass (Figure 1B), though only WC and OG + WC produced significantly less biomass belowground than TF and TF + OG. There was no evidence of transgressive overyielding belowground by any of the intercrops (Figure 1B), and there were no differences in average root biomass within each level of diversity (Table 2). Root biomass in the top 0–15 cm ranged from 6230.8 ± 595.7 kg ha^{-1} to 2286.5 ± 416.0 kg ha^{-1}, and 84–90% of all roots recovered were in this soil layer (Table 2). Root biomass differed across treatments in the top soil layer (0–15 cm, $F_{6,41}$ = 8.05, $p < 0.0001$) and differences between treatments were similar to those observed for overall root biomass. In the 15–30 cm layer, root biomass also varied across treatments ($F_{6,43}$ = 7.48, $p < 0.0001$) because WC exhibited lower biomass than all other treatments (Table 3). Fine root biomass was not different across treatments in either soil layer (0–15 cm: $F_{6,42}$ = 1.61, p = 0.168; 15–30 cm $F_{6,43}$ = 2.28, p = 0.053). The C:N ratio of coarse roots tended to be lower in surface layer than the deeper soil layer.

Table 3. Root characteristics at two soil depths (0–15 cm and 15–30 cm) from seven forage crop treatments in southeast Pennsylvania, USA. Data are means ± 1 standard error of observations collected at the first hay cutting two and three years after establishment.

0–15 cm Soil Depth				
Treatment	**Total Root Dry Weight (kg ha^{-1})**	**% of Total Root Biomass in This Layer**	**Fine Root Dry Weight ‡ (kg ha^{-1})**	**Root C:N ***
Tall fescue (TF)	6230.8 ± 595.7 a †	87	2153.2 ± 321.5	44.2 ± 1.6 a
Orchardgrass (OG)	4460.1 ± 571.2 ab	88	1512.5 ± 242.4	38.8 ± 2.6 ab
White clover (WC)	2286.5 ± 416.0 c	90	1315.3 ± 392.4	20.4 ± 1.9 c
TF + OG	5873.7 ± 415.2 ab	86	1944.8 ± 331.9	45.6 ± 1.9 a
TF + WC	4048.8 ± 320.1 bc	84	1452.1 ± 221.3	40.5 ± 2.5 ab
OG + WC	4244.4 ± 933.4 abc	89	1334.2 ± 354.4	35.7 ± 2.8 b
TF + OG + WC	5774.7 ± 686.3 ab	89	1557.5 ± 246.8	40.0 ± 3.7 ab
15–30 cm Soil Depth				
Treatment	**Total Root Dry Weight (kg ha^{-1})**	**% of Total Root Biomass in This Layer**	**Fine Root Dry Weight (kg ha^{-1})**	**Root C:N**
Tall fescue (TF)	865.8 ± 84.0 a	13	310.4 ± 36.2	60.5 ± 5.0
Orchardgrass (OG)	588.7 ± 90.8 a	12	345.5 ± 56.9	52.6 ± 6.3
White clover (WC)	188.6 ± 51.7 b	10	152.7 ± 50.4	21.3 ± 1.1
TF + OG	930.0 ± 95.8 a	14	378.8 ± 44.1	60.2 ± 5.3
TF + WC	798.2 ± 111.2 a	16	352.9 ± 50.7	50.7 ± 5.1
OG + WC	638.4 ± 152.1 a	11	335.0 ± 99.3	50.9 ± 1.4
TF + OG + WC	735.2 ± 125.8 a	11	248.0 ± 63.4	48.2 ± 5.6

† Lowercase letters indicate differences among values within each column based on mean separation using Tukey's honestly significant difference (HSD) at α = 0.05. ‡ Root size > 250 µm and < 2 mm. * As determined by combustion analysis of coarse roots (>2 mm).

3.2. Microbial Community Size, Structure, and Activity

Microbial community parameters were measured in the top 0–15 cm of the soil profile. The size of the soil microbial community, measured as the abundance of PLFA biomarkers, differed across crop treatments (Table 4; $F_{6,45}$ = 3.22, p = 0.010) and was higher in 2019 (111.0 ± 2.9 nmol g^{-1}) compared to 2018 (86.4 ± 1.9 nmol g^{-1}; $F_{1,45}$ = 72.82, $p < 0.0001$). Microbial abundance was lowest in OG and tended to be highest in two and three species grass-legume intercrops (Table 4). Bacterial abundance also differed by treatment ($F_{6,45}$ = 3.40, p = 0.008) and was lower in OG compared to WC and the three grass-legume intercrops. There was no effect of treatment on fungal abundance ($F_{6,45}$ = 1.59, p = 0.171), but F:B ratios differed among treatments ($F_{6,45}$ = 3.18, p = 0.011). Both TF and OG exhibited a higher F:B

ratio than WC (Table 4). Basal respiration, a measure of microbial activity, did not differ across treatments (Table 4).

Table 4. Microbial characteristics in surface soils collected from seven forage crop treatments in southeast Pennsylvania, USA. Data are means ± 1 standard error from bulk soil samples collected two and three years after crop planting.

Treatment	Total PLFAs (nmol g^{-1})	Bacterial Biomarkers (nmol g^{-1})	Fungal Biomarkers (nmol g^{-1})	F:B Ratio	Basal Respiration (μg CO_2-C g^{-1} soil h^{-1})
Tall fescue (TF)	100.3 ± 5.8 ab †	74.1 ± 4.0 ab	7.7 ± 0.7	0.102 ± 0.007 a	11.5 ± 0.5
Orchardgrass (OG)	86.6 ± 5.4 b	64.2 ± 3.9 b	6.4 ± 0.5	0.099 ± 0.004 a	12.7 ± 2.0
White clover (WC)	100.6 ± 7.5 ab	76.5 ± 6.0 a	6.9 ± 0.9	0.089 ± 0.005 b	11.0 ± 1.0
TF + OG	91.9 ± 6.6 ab	68.5 ± 4.6 ab	6.7 ± 0.8	0.095 ± 0.007 ab	11.7 ± 1.3
TF + WC	103.6 ± 5.3 a	77.6 ± 3.8 a	7.2 ± 0.6	0.092 ± 0.004 ab	12.4 ± 1.4
OG + WC	105.3 ± 6.1 a	78.6 ± 4.4 a	7.7 ± 0.7	0.096 ± 0.005 ab	14.2 ± 2.2
TF + OG + WC	102.7 ± 6.6 a	76.4 ± 4.6 a	7.4 ± 0.8	0.095 ± 0.006 ab	13.0 ± 0.9

† Lowercase letters indicate differences among values within each column based on mean separation using Tukey's honestly significant difference (HSD) at $\alpha = 0.05$. PLFA = phospholipid fatty acid; F:B = fungal:bacterial.

3.3. Carbon Pools

Carbon pools were quantified in the top 0–15 cm of the soil profile. In this layer, POX-C concentrations ranged from 402.8 mg kg^{-1} to 471.6 mg kg^{-1}, though no difference among treatments was detected (Table 5). Differences in TOC, which ranged from 14.1 g kg^{-1} to 15.8 g kg^{-1}, were not identified, though TOC tended to be lowest in orchardgrass (Table 5).

Table 5. The mean concentration of permanganate oxidizable carbon (POX-C, mg C kg^{-1} soil ± 1 standard error) and total organic carbon (TOC, g C kg^{-1} soil) in soils collected from seven forage treatments in southeast Pennsylvania, USA. Bulk soil samples were collected in June 2018 and 2019, two and three years, respectively, after forage planting.

	POX-C (mg C kg^{-1} Soil)	TOC (g C kg^{-1} Soil)
Tall fescue (TF)	454.1 ± 12.1	15.4 ± 0.2
Orchardgrass (OG)	402.8 ± 22.1	14.1 ± 0.3
White clover (WC)	414.7 ± 16.8	15.5 ± 0.3
TF + OG	412.3 ± 22.2	15.6 ± 0.5
TF + WC	454.8 ± 27.5	15.8 ± 0.5
OG + WC	471.6 ± 26.4	15.8 ± 0.6
TF + OG + WC	466.2 ± 44.0	15.0 ± 0.7

4. Discussion

Pasture-crop rotations can provide multiple agroecosystem services, including those mediated by belowground biota such as C emission mitigation and soil health [3]. The objective of this study was to determine how crop selection (i.e., the identity and number of species) for the pasture phase of a rotational system influences root productivity and quality; microbial community size, structure, and function; and soil carbon pools. Our results indicate that despite overyielding aboveground, intercropped forages did not increase root productivity compared to monocultures. Forage species identity did, however, influence microbial abundance and community structure within three years of establishment.

4.1. Intercropping Effects on Productivity

Consistent with numerous studies documenting a positive relationship between increasing diversity and productivity, our results demonstrated the aboveground production benefit frequently observed from forage intercrops [15–18]. Given that grass-legume intercrops resulted in overyielding—and in some cases transgressive overyielding—but the grass-grass intercrop did not, our results suggest that one mechanism driving this response

was complementary N acquisition traits exhibited by white clover and the grasses [28,58,59]. Previous research has demonstrated that legumes, including white clover, can transfer N to associated grasses in perennial systems [27]. While indicative of an intercropping benefit to aboveground productivity, these results cannot be interpreted as evidence of a generalizable diversity-productivity relationship for forages given the limited number of species included in the experiment. Three species is, however, a realistic species richness for an agricultural intercrop due to management challenges such as maintaining diversity over multiple seasons [22]. When species richness is limited, the effects of diversity, per se, may also be limited, but selection of crops with specific, and potentially complementary, traits can leverage diversity as a means to increase targeted agroecosystem functions [45,60].

We predicted that any aboveground productivity benefits associated with higher species richness would be mirrored belowground, a hypothesis that was not supported. We expected that productivity benefits associated with intercrops would extend belowground, given evidence of this outcome from experimental grasslands [20,61] and because mechanisms such as complementarity that drive enhanced productivity aboveground would, theoretically, operate similarly belowground [26]. In our study, root biomass in the top 30 cm of soil was higher in grass monocultures than in white clover. Van Eekeren et al. [43] similarly observed higher root biomass in surface (0–10 cm) soils under unfertilized ryegrass (*Lolium perenne* L.) compared to white clover. There were, however, few differences in root biomass between grass monocultures and the intercropped stands. One possible explanation for the lack of response to increased diversity is that high performing grasses were present in all intercrops, commonly referred to as a 'selection effect' [58,62]. Previous research has similarly cited a selection effect associated with tall fescue as a possible driver of root biomass increases in high diversity forage mixtures [22]. Though roots were not sorted by species in our study, aboveground species composition at the time of root sampling (Figure S1) indicates that a greater proportion of biomass in intercrops was contributed by grasses than white clover, which supports this interpretation.

Previous field studies of forage intercrops have cited greater root depth distribution as a driver of root biomass increases in higher diversity intercrops relative to bicultures [21,22,24]. A higher proportion of roots below 30 cm was also identified as a driver of a positive relationship between root biomass and plant species richness in the Cedar Creek experimental grassland [20]. Wardle and Peltzer [63] suggested competition near the soil surface, promoting deeper rooting as a mechanism for this response. Differences in root biomass across treatments were similar in surface (0–15 cm) and deeper (15–30 cm) soils in our experiment, indicating no intercropping effect on root depth distribution. One limitation of our study is that roots were sampled only to 30 cm, and differences in root depth distribution may manifest below this depth, as was the case at Cedar Creek [20] and some of the previous studies of forage intercrops [21,22]. With recent evidence that processes governing SOC accrual can vary between surface and deeper soils, continued studies of intercrop impacts on root depth distribution and root chemistry are essential to improving predictive models for C sequestration in agroecosystems [14,64]. Our observations that both root biomass and root quality (measured as root C:N) decreased with depth indicate that rates of processes such as decomposition and microbial respiration will differ through the soil profile. These findings highlight that direct measurements of biota and C pools and fluxes in subsurface soils will be informative in future studies and caution against extrapolation of C dynamics observed in surface soils to greater soil depths.

Grass-legume intercrops also exhibit complementary N acquisition traits that we suggest as a mechanism for increased productivity aboveground. This dimension of complementarity did not provide the same benefit belowground, potentially due to different responses to N availability in roots versus shoots. We measured root biomass two and three years after a pre-planting N addition, making it likely that grass monocultures experienced greater N deficiency than grass-legume intercrops. Soil inorganic N concentrations at the time of sampling, however, were not different among intercrop treatments (Table S2). In a greenhouse study, Skinner and Comas [30] found that N stress reduced shoot biomass by

an average of 50% in 10 forage grasses. Root biomass in orchardgrass and two cultivars of tall fescue, however, were not reduced under these low N conditions, and low N had little impact on resource allocation to roots across the grass species examined [30]. Thus, while grass productivity might be expected to benefit from intercropping with a legume, the limited response of root biomass to N variability could contribute to similar belowground productivity when unfertilized grasses are grown with or without legumes. Because roots were not sorted by species, this theory cannot be tested in the current experiment, but further studies of biomass allocation under varying N conditions for forage species grown singly and as intercrops will further our understanding of the interactions among soil C and N dynamics and root production.

4.2. Crop Selection Effects on Microbial Communities and Carbon Pools

Crop selection influenced both the size and structure of soil microbial communities. Microbial abundance tended to be highest in grass-legume intercrops, though only the OG monoculture had a significantly lower microbial biomass than these intercrops. This response provides weak support for our hypothesis that intercrops would support greater microbial abundance than monocultures. In a grassland diversity experiment, Lange et al. [41] found that, in addition to environmental factors, plant species richness positively influenced microbial biomass (also measured as total PLFA concentration). It is plausible that the differences in microbial abundance we detected after three years will strengthen over time, as has been observed in other perennial systems [65]. Groffman et al. [35] also posed the argument that variation in microbial biomass carbon, which reflects abundance, within a single location should be driven by variation in labile C inputs. That we detected no differences in POX-C among treatments may indicate that crop diversity had limited influence on labile C inputs and, thus, microbial abundance.

Differences in microbial biomass appear to have been driven by variation in bacterial abundance, as fungal abundance did not differ among treatments. Bacterial abundance tended to be higher in WC and intercrops containing WC compared to OG and TF monocultures and the grass-only biculture. This association likely reflects a response to the low C:N ratio of white clover roots. Increased bacterial abundance on high quality residues (those with a low C:N ratio) has been observed in lab-based studies [66] and, similar to our findings, observed in field-grown alfalfa and alfalfa-grass bicultures [44]. Van Eekeren et al. [43], however, reported lower bacterial abundance in white clover relative to perennial ryegrass. They attributed this outcome to a greater overall volume of microbe-enriched rhizosphere soil in the grass, as Mawdsley and Bardgett [67] had previously shown that when root biomass was equal, white clover supported higher microbial abundance than ryegrass. Our findings, combined with previous observations, offer evidence that leguminous crops likely support increased bacterial abundance in agricultural soils relative to grasses. Bacterial abundance may contribute to higher C and N mineralization rates that have been observed in leguminous forages [43,44], though the effects of microbial functional identity and substrate quality on mineralization are difficult to disentangle [11]. While increased mineralization can support internal nutrient cycling, higher rates of mineralization may also reduce the potential for C stabilization in bacteria-dominated soils [68].

Though crop selection effects on fungal abundance were not detected in our study, variability in total microbial and bacterial abundance across treatments led to higher F:B ratios in TF and OG monocultures compared to WC. There are several factors that may have led to this increase in grasses versus the legume. First, increasing N fertility has been observed to lower F:B ratio in grasslands [69]; therefore, the presence of white clover could lower the F:B ratio in the WC monoculture as well as intercrops with WC [41]. This response is thought to be driven by suppression of fungi in high N environments [69] but may also be due to increased bacterial abundance as we observed in the present study. Second, a corollary to the association between bacterial abundance and high quality substrates is that fungal biomass tends to be higher on low quality substrates [66], thus higher F:B ratios would be expected with a high root C:N ratio. Both TF and OG exhibited higher root C:N

than WC in our study, consistent with this reasoning. Factors determining F:B ratio in intercropped systems are less clear. The intermediate F:B ratio in grass-legume intercrops may be due to bacterial abundance associated with white clover, as noted above. The contrast of TF and OG to WC would have been expected to lead to a higher F:B ratio in the grass-only biculture compared to WC, but it did not.

The F:B ratio is commonly regarded as an indicator of functionality of the soil microbial community [70,71]. Of particular interest in managed agroecosystems is an association between increasing fungal dominance and enhanced C accrual [38,39]. The results of this study demonstrate that crop selection influences F:B ratio, and, in the context of a reduced tillage forage rotation, grass monocultures can induce changes to microbial community structure in the near term (two to three years after planting) that may support C accrual. It is essential to note that additional features of the microbial community such as growth efficiency also mediate SOC formation and stabilization; thus, changes to structure alone cannot predict C outcomes [72,73]. Despite differences in the quantity of C inputs indicated by root biomass and variation in microbial abundance and structure across treatments, we did not detect differences in microbial activity, measured as basal respiration. Further, we observed no changes in labile or total C, which was not unexpected, given the short time scale of this experiment [74] and may be explained, in part, by the lack of difference in microbial activity levels [75]. Recent research has also identified the potential for limited C gains in perennial systems when increases in decomposition rates, which are mediated by a combination of climate, substrate quality, and soil biota, offset any increases in root C inputs [14]. Continued observation in our study system over a longer timescale will provide further insight into the effects of individual species and species combinations on root characteristics and microbial communities to inform crop selection that supports soil C accrual in perennial forage systems.

4.3. Implications for Agroecosystem Services

Increased yield and yield stability are key motivators for the use of intercropping [76]. Here, we have shown that though intercropping forage species benefits productivity aboveground, productivity belowground does not respond similarly. One intercrop, WC + OG, exhibited overyielding aboveground but not below, and it produced less root biomass than TF and TF + OG. On the other hand, the combination of two grasses (TF + OG) did not exhibit overyielding aboveground but exhibited high productivity belowground. These cases suggest the potential for a trade-off between above and belowground productivity for some forage combinations. This trade-off could be of consequence for farmers intercropping forages not only to provide feed but also to build soil C stocks as part of a pasture-crop rotation. Building on current findings with continued research on root traits and trait expression in intercropped stands will help identify linkages between roots and soil-derived agroecosystem services and assist farmers in selecting crops to address goals they have defined for their fields [45,60]. The crop identity influences on microbial and other soil food web communities that this and other studies have identified [25,43,44] indicate that in order to achieve an enhanced mechanistic understanding of how crop selection can be leveraged to promote soil-derived services, continued research integrating multiple aspects of soil biology is needed.

5. Conclusions

Both research and practice have demonstrated the capacity of increasing crop diversity in time and/or space to support a range of agroecosystem services. With heightened emphasis on engineering agroecosystems to provide services such as enhanced soil health and C storage, an improved understanding of how the identity and number of species present in an agroecosystem influences belowground biota is critical to helping farmers fine-tune crop selection for expanded service provision. Our results showed that mechanisms driving increased productivity aboveground from intercropped forage species did not promote increased root production belowground in a temperate agroecosystem. Thus,

simply adding species to a forage stand is not likely to be a consistently effective means to augment soil C inputs in this context. We also found that increases in bacterial abundance were associated with the legume, white clover, while a higher F:B ratio was observed in association with grass species. This outcome suggests that the microbially-mediated processing of soil C inputs and soil health in general could be more strongly influenced by the identity of plant species present than by the number of species present when species richness is low. Taken together, our findings show that among forage species commonly used in the northeastern US, the identity of species selected for a pasture-crop rotation will shape the belowground biota, namely roots and microbial communities, that mediate agroecosystem services. Selecting species with root traits and microbial associations that promote soil health and C storage is expected to enhance the provision of these desired services from rotational systems and promote agricultural sustainability.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su13105689/s1, Table S1: Annual forage yield, Figure S1: Species composition; Table S2: Soil inorganic nitrogen.

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