



Red and white clover sustain high and stable yields in diversified perennial grasslands under low N input

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ABSTRACT

Context: Increasing species richness and functional diversity in managed grasslands can enhance ecosystem services, particularly when legumes are included. However, it remains unclear whether higher species richness consistently improves productivity and stability in intensively managed grasslands under low nitrogen (N) inputs, or if species identity plays a more dominant role.

Objective: This study evaluated how legume identity, non-legume identity, and species richness influence dry matter (DM) yield, N yield, and biological N₂ fixation (BNF) in grass-legume-forb mixtures managed with low fertilizer inputs.

Methods: In a 3-year field experiment, we tested (i) 2-species mixtures of white clover (*Trifolium repens*, Tr) or red clover (*Trifolium pratense*, Tp) with perennial ryegrass (*Lolium perenne*, Lp), tall fescue (*Festuca arundinacea*, Fa), chicory (*Cichorium intybus*, Ci) or ribwort plantain (*Plantago lanceolata*, Pl); and (ii) multi-species mixtures with 6 and 18 species. Additional 2-species mixtures of ryegrass with six clover genotypes were included. Mixtures received 75 kg N ha⁻¹ yr⁻¹ and were compared with ryegrass monocultures fertilized with 0–450 kg N ha⁻¹ yr⁻¹. BNF was quantified using the ¹⁵N isotope dilution method.

Results: Species identity, particularly the presence of red clover, was the primary driver of productivity, outweighing the effect of species richness. Red clover mixtures produced 14–21 t DM ha⁻¹ yr⁻¹ across three years, matching ryegrass fertilized with 450 kg N ha⁻¹ yr⁻¹, while white clover 2-species mixtures yielded less (8–16 t DM ha⁻¹ yr⁻¹) but gained over time relative to red clover mixtures. N yields in red clover mixtures equaled or exceeded those of highly fertilized ryegrass, whereas white clover mixtures matched ryegrass at 300 kg N ha⁻¹ yr⁻¹. BNF was greatest in red clover mixtures due to higher legume biomass, although the proportion of N derived from the atmosphere (%Nd_{fa}) was similar or higher in white clover mixtures. Increasing species richness beyond two species did not increase yield when red clover was present, but the 18-species mixture maintained more stable legume–non-legume proportions and consistent yields across years.

Conclusions: Red clover drives high productivity in low-input grassland mixtures, whereas higher species richness primarily contributes to temporal yield stability rather than increased yield.

Significance: Red clover-based mixtures can replace high fertilizer N inputs while sustaining yields equivalent to intensively fertilized monocultures, offering substantial gains in N-use efficiency and reduced fertilizer-related emissions. Incorporating higher species richness may enhance long-term stability and resilience of managed grasslands, supporting more sustainable and climate-smart forage production systems.

1. Introduction

Perennial grassland mixtures can enhance productivity and stability by allowing species and functional groups to contribute at different

times and through complementary resource-use strategies, including improved nutrient acquisition and soil fertility buildup (Bardgett et al., 2014; Golińska et al., 2023; Kirwan et al., 2007; Suter et al., 2015). These mechanisms can increase yield stability and resilience (Isbell

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et al., 2017), reduce nitrogen (N) losses to the environment (de Vries and Bardgett, 2016), and support sustainable nutrient management with low fertilizer N inputs (Abalos et al., 2020; Abalos et al., 2019). However, the relative importance of plant species richness versus species identity – particularly the role of legumes – remains difficult to disentangle, as these effects are often confounded in diversity experiments (Abalos et al., 2014; Hooper et al., 2005; Hooper and Vitousek, 1998). Furthermore, most evidence for diversity effects at high species richness comes from natural grassland systems (e.g. Furey and Tilman, 2021), while managed grasslands are understudied and typically examined using fewer and more functionally similar species (Cong et al., 2017; Kirwan et al., 2007; Suter et al., 2015).

Legume-based grassland mixtures reduce greenhouse gas (GHG) emissions related to N fertilization by replacing fertilizer inputs with biologically fixed nitrogen (BNF) through symbiosis with rhizobia (Unkovich et al., 2008), thereby lowering both production- and soil-derived emissions (Chojnacka et al., 2019; Hauggaard-Nielsen et al., 2016; Jensen et al., 2012). The higher N use efficiency improves the economic and environmental sustainability of the grassland system compared to highly fertilized grass monocultures (Isbell et al., 2017; Larsen et al., 2024; Manevski et al., 2017). However, optimizing N management in diversified legume-based grassland systems without compromising productivity remains a key challenge. While dry matter (DM) yield is commonly used to assess agronomic performance, N yield may better reflect production output in legume-based mixtures due to their higher biomass N and protein content (Solati et al., 2017; Suter et al., 2015), which is particularly relevant for feed and biorefining applications (Johansen et al., 2018; Jørgensen et al., 2022). Furthermore, perennial grasslands can increase soil carbon (C) stocks (Bai and Cotrufo, 2022; De Deyn et al., 2011; Ledo et al., 2020), but the magnitude of this effect depends on sward persistence and the time between renewal (Jensen et al., 2022; Smit et al., 2025). Maintaining long sward persistence requires sustained productivity of the mixture, including the continued contribution from legumes. Taken together, grassland performance needs to be evaluated using metrics that integrate N yield and long-term productivity, rather than relying solely on dry matter yield.

Red and white clover differ markedly in their contribution to productivity and persistence in grass-clover mixtures due to contrasting growth and root traits. Red clover (*Trifolium pratense*) often dominates aboveground biomass in mixtures with grasses at low N fertilizer application rates (Hearn et al., 2023; Rasmussen et al., 2019b), but in managed grasslands, red clover remains largely confined to its original sowing spots, as it reproduces only by seed, leading to a gradual decline in plant population over time. In contrast, white clover (*Trifolium repens*) spreads vegetatively via stolons and has a shallow, highly branched root system concentrated in the topsoil (Mortensen et al., 2025b; Pirhofer-Walzl et al., 2012), allowing it to persist and gradually increase its contribution to biomass and N yield as the sward ages (Brophy et al., 2017; Eriksen et al., 2012; Finn et al., 2013). These root and growth characteristics also explain why white clover generally supplies more N to companion grasses than red clover (Pirhofer-Walzl et al., 2012), as its fine roots overlap strongly with grass root zones, whereas red clover develops deep taproots that are more spatially separated from grass roots (Cong et al., 2017; Høgh-Jensen et al., 2006; Mortensen et al., 2025b). In addition, the stolon-based growth strategy of white clover reduces the need for large root N reserves for regrowth after defoliation, further enhancing its contribution to N transfer within the mixture (Pirhofer-Walzl et al., 2012).

In mixtures with legumes, the legume-component facilitates N uptake for the non-legumes through both indirect and direct mechanisms, thereby shaping N dynamics and community balance. Indirect facilitation occurs because legumes rely largely on atmospheric N₂ fixation, increasing the availability of soil N for companion species (a process referred to as “N sparing” by e.g. Temperton et al. 2007), whereas direct or indirect N transfer occur via mycorrhizal networks and rhizodeposition (Chalk et al., 2014; Høgh-Jensen, 2006; Rasmussen et al., 2013;

Tsialtas et al., 2018). Optimizing these processes can enhance complementarity, improve N utilization and productivity while lowering N fertilizer input. However, altered N availability also affects botanical balance, and maintaining a productive sward over time requires that non-legumes are not outcompeted by legumes under low N fertilization (Ergon et al., 2016; Kirwan et al., 2007). This risk is greater in mixtures with red clover, which can dominate under low N inputs but often shows limited persistence, whereas white clover is generally less competitive aboveground but more persistent (Black et al., 2009; Brophy et al., 2017; Brummer and Moore, 2000; Finn et al., 2013; Marshall et al., 2017). Importantly, considerable variation exists among cultivars, offering opportunities to fine-tune N interactions and community balance through the selection of red clover cultivars with improved persistence and reduced dominance, and white clover cultivars with greater yield potential (DSV, 2024; Hoekstra et al., 2017).

In legume-based grassland mixtures, companion species such as grasses and forbs contribute to productivity and stability through complementary above- and belowground traits. Perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*) are highly productive grasses commonly used in intensively managed grassland systems (Cougnon et al., 2014; Cougnon et al., 2017; Zhang et al., 2024) but they differ in resource-use strategies. Perennial ryegrass is a nutrient-acquisitive species and is very effective in nutrient scavenging in the topsoil (Abalos et al., 2019; Schröder et al., 2007). Tall fescue has a larger and deeper root system that enhances drought tolerance and access to subsoil resources (Cougnon et al., 2017; McCormack et al., 2015; Volaire et al., 2014). Combined with legumes, grasses also contribute to seasonal complementarity, as the fast growth of grasses in early spring secures a higher yield in the first cut, while legumes increase productivity in summer and autumn (Kristensen et al., 2022). The addition of forage forbs like chicory (*Cichorium intybus*) and ribwort plantain (*Plantago lanceolata*) can further increase community productivity by extending functional diversity within the mixture (Cong et al., 2017; Dhamala et al., 2016). These forbs exhibit deep rooting and contrasting canopy architectures, enabling complementary resource capture in both soil and light niches and thereby increasing biomass production and resource-use efficiency of the mixture (Cong et al., 2017; Dhamala et al., 2016; Rasmussen et al., 2019a).

Despite evidence from natural and semi-natural grasslands that increasing species richness can enhance productivity and yield stability, it remains unclear whether these positive diversity-productivity relationships translate to intensively managed, agronomically relevant legume-based grassland mixtures under low N input. Large-scale studies have shown that multi-species mixtures with up to six species can outperform fertilized monocultures (O'Malley et al., 2026), yet experimental evidence under practical management conditions and at high levels of species richness in managed grasslands remains limited. To address this gap, we included mixtures on species richness gradient (2, 6, and 18 species), with the 18-species mixture designed to test whether the strong positive relationship between high species richness and productivity observed in natural grasslands (Cardinale et al., 2007; Duffy et al., 2017; Tilman et al., 2001) also applies to intensively managed systems.

The aim of this study was to identify high-yielding legume-based grassland mixtures that sustain stable aboveground biomass and N yield over a 3-year production period. Specifically, we examined how species richness and legume identity influence total aboveground biomass, N yield, and the quantity of BNF. We hypothesized that:

- 1) Mixtures with red clover have a higher total aboveground biomass compared to mixtures with white clover in the 1st year, but not in the 2nd and 3rd years.
- 2) Increasing species richness (1, 2, 6, 18 species) results in a higher aboveground biomass productivity (DM and N yield) over three years.

- 3) Total productivity and yield stability of plant communities varies between red and white clover cultivars.

2. Materials and methods

2.1. Field site and experimental setup

The field experiment, spanning three production years, was initiated in autumn 2021 at AU Viborg, Denmark (56° 30' N, 9° 34' E), in a wet temperate climate zone with a mean annual temperature of 9.13 °C and mean annual precipitation of 833 mm across the three experimental years (Figure S1). The soil type was a loamy sand soil classified as Mollic Luvisol (according to (WRB, 2022)) with 364 g coarse sand kg⁻¹, 303 g fine sand kg⁻¹, 239 g silt kg⁻¹, 70 g clay kg⁻¹, 16 g organic C kg⁻¹, a C:N ratio of 11.6, and a pH of 5.24.

The field had a history of several years of conventional cereal rotation, alternating between winter cereals (rye and wheat) and spring cereals (oat and barley). The preceding crop was winter rye (*Secale cereale*) harvested on the 26th of August 2021, and on 1st of September 2021 plots of 3 × 6 m with mixtures of grasses, forbs and legumes were established. Perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), chicory (*Cichorium intybus*), ribwort plantain (*Plantago lanceolata*), white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) were combined in 2-species mixtures, all including a legume and a non-legume. Further, these six productive species were all combined in a 6-species mixture, as well as added to an 18-species mixture together with another 12 species of grass, legume and forbs (Table 1). Sowing densities were adjusted across mixtures to account for different species richness, with 25 % sown legumes (number of seeds) in all mixtures except for two-species mixtures with red clover where a lower legume proportion of 10 % were applied aiming to reduce competition for the non-legumes. Additional plots were established in the experiment to examine the effect of clover cultivars with different growth patterns. For the two species mixtures of perennial ryegrass and white clover or red clover, three clover cultivars were tested for both white clover (var. *Munida*, *AberCrest*, and *Tr9090011*) and red clover (var. *Larus*, *Semperina*, and *Taifun*) (DSV, 2024). To compare the results of the species mixtures with the yield potential of a perennial ryegrass pure

stand, we established an N response curve for pure stand perennial ryegrass at an increasing N fertilizer rate (0, 75, 150, 300 and 450 kg N ha⁻¹ yr⁻¹) using ammonium nitrate (YaraBela EXTRAN, 27 N). Four replicates were established for all treatments in a completely randomized block design, and a cutting regime of 3 cuts per year was applied for all production years.

All mixtures received 75 kg N ha⁻¹ yr⁻¹ as ammonium nitrate to stimulate non-legume growth, applied as mineral fertilizer split in 2 applications: 2/3 of total N in early spring and 1/3 just after 1st biomass harvest. The pure stand ryegrass plots with more than 75 kg N ha⁻¹ yr⁻¹ also received fertilizer just after the 2nd biomass harvest to sustain autumn productivity with the fertilizer split in three applications: 2/5 in early spring and after 1st biomass harvest, respectively, and 1/5 of total N allocated to the last application. All treatments received the same application of P (24 kg P₂O₅ ha⁻¹ yr⁻¹) and K (303 kg K₂O ha⁻¹ yr⁻¹) divided into 2 applications (early spring and after 2nd biomass harvest) in 2022. In 2023 and 2024, P and K were added in one application per year (early spring). The plots were irrigated during dry summer months, in accordance with common practice in the region.

2.2. Isotopic labelling by ¹⁵N for quantification of N₂ fixation

In each experimental unit (plot), a subplot was established in which soil labelling with ¹⁵N was performed in order to measure the N₂ fixation performed by the legume component in each mixture. The ¹⁵N isotope dilution method (Danso et al., 1993; Unkovich et al., 2008) was used to quantify biological N₂ fixation by the legume component in the grassland mixtures, using the non-legume component as reference crop. Prior to the growing period (2 March), each labelling plot received labelled ammonium sulphate ((NH₄)₂SO₄) (98 atom %) corresponding to 1 kg N ha⁻¹. Thus, the proportion of N derived from the atmosphere (%Nd_{fa}) was estimated as the difference in ¹⁵N enrichment between the legume and the non-legume:

$$\%Nd_{fa} = \left(1 - \frac{\text{atom}\% \text{ } ^{15}\text{N excess in legume crop}}{\text{atom}\% \text{ } ^{15}\text{N excess in reference crop}}\right) * 100 \quad (1)$$

where the atom% ¹⁵N excess is the difference between ¹⁵N enrichment of legume or reference crops in the labelled plots and the natural

Table 1

Overview of all mixtures and pure stand ryegrass treatments used in this study. The proportion of sown legumes (%) in the mixtures are based on number of seeds.

Treatment	Total kg N ha ⁻¹ yr ⁻¹	Sown legume proportion (%)	Species included
Lp0N	0	0	<i>Lolium perenne</i>
Lp75N	75	0	<i>Lolium perenne</i>
Lp150N	150	0	<i>Lolium perenne</i>
Lp300N	300	0	<i>Lolium perenne</i>
Lp450N	450	0	<i>Lolium perenne</i>
Lp+Tr	75	25	<i>Lolium perenne</i> , <i>Trifolium repens</i> (cultivar a)
Fa+Tr	75	25	<i>Festuca arundinacea</i> , <i>Trifolium repens</i> (cultivar a)
Pl+Tr	75	25	<i>Plantago lanceolata</i> , <i>Trifolium repens</i> (cultivar a)
Ci+Tr	75	25	<i>Cichorium intybus</i> , <i>Trifolium repens</i> (cultivar a)
Lp+Tp	75	10	<i>Lolium perenne</i> , <i>Trifolium pratense</i> (cultivar a)
Fa+Tp	75	10	<i>Festuca arundinacea</i> , <i>Trifolium pratense</i> (cultivar a)
Pl+Tp	75	10	<i>Plantago lanceolata</i> , <i>Trifolium pratense</i> (cultivar a)
Ci+Tp	75	10	<i>Cichorium intybus</i> , <i>Trifolium pratense</i> (cultivar a)
MS6	75	25	Lp + Fa + Pl + Ci + Tr + Tp
MS18	75	25	MS6 + additionally 12 species (in total 18)
Lp+Tr(B)	75	25	<i>Lolium perenne</i> , <i>Trifolium repens</i> (cultivar b)
Lp+Tr(C)	75	25	<i>Lolium perenne</i> , <i>Trifolium repens</i> (cultivar c)
Lp+Tp(B)	75	10	<i>Lolium perenne</i> , <i>Trifolium pratense</i> (cultivar b)
Lp+Tp(C)	75	10	<i>Lolium perenne</i> , <i>Trifolium pratense</i> (cultivar c)

Note: MS6 = multi-species mixture with 6 species, MS18 = multi-species mixture with 18 species. MS18 consists of species in MS6 and the following species. Grasses: Timothy (*Phleum pratense*), Meadow fescue (*Festuca pratensis*), Hybrid ryegrass (*Lolium hybridum*) and Cocksfoot (*Dactylis glomerata*). Legumes: Lucerne (*Medicago sativa*), Alsike clover (*Trifolium hybridum*), Bird's-foot trefoil (*Lotus corniculatus*), Black medick (*Medicago lupulina*), Serradella (*Ornithopus sativus*) and Crimson clover (*Trifolium incarnatum*). Forbs: Caraway (*Carum carvi*) and Common yarrow (*Achillea millefolium*). For seeding density, fertilization scheme, and clover cultivars see Table S1 in the supplementary material.

abundance of ^{15}N in the non-labelled legume or reference crops in adjacent subplots. The quantity of N input from biological N_2 fixation (qBNF) (kg N ha^{-1}) was then calculated by multiplying %Ndff by total N in the harvested legume biomass.

2.3. Sampling and botanical composition

Aboveground biomass was harvested from the central 1.5×6 m of the plots for each cut (3 cuts per year), using a plot harvester (Haldrup, Løgstør, Denmark) with 7 cm cutting height. The botanical composition was assessed for each cut, sorting 50×50 cm cut samples into individual species. The samples were dried for 48 h at 60°C for DM determination. Harvest dates were 31 May, 2 August and 11 October in 2022; 30 May, 1 August and 10 October in 2023; and 28 May, 30 July and 8 October in 2024. For total N and ^{15}N determination, 3–4 mg of plant sample were packed in tin capsules and analyzed using a PDZ Europa

ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK). Leaf traits were measured for different clover cultivars, i.e., leaf dry matter content (LDMC; $\text{dry mass fresh mass}^{-1}$), specific leaf area (SLA; $[\text{cm}^2 \text{ mg dry mass}^{-1}]$), one-sided fresh leaf area (LA; $[\text{mm}^2]$), and plant height measured to highest leaf as an average across ten individuals from each plot (Carrascosa et al., 2025; Mueller et al., 2024; Pérez-Harguindeguy et al., 2013). These leaf traits were measured to investigate if any of the most common aboveground plant traits could explain potential effects of different clover cultivars.

2.4. Data analysis and statistics

Data were explored and analyzed in R, version 4.3.2 (R Core Team, 2023). We started by fitting Linear Mixed Models (LMM) for all datasets with package lme4 (Bates et al., 2015), and where the assumptions on

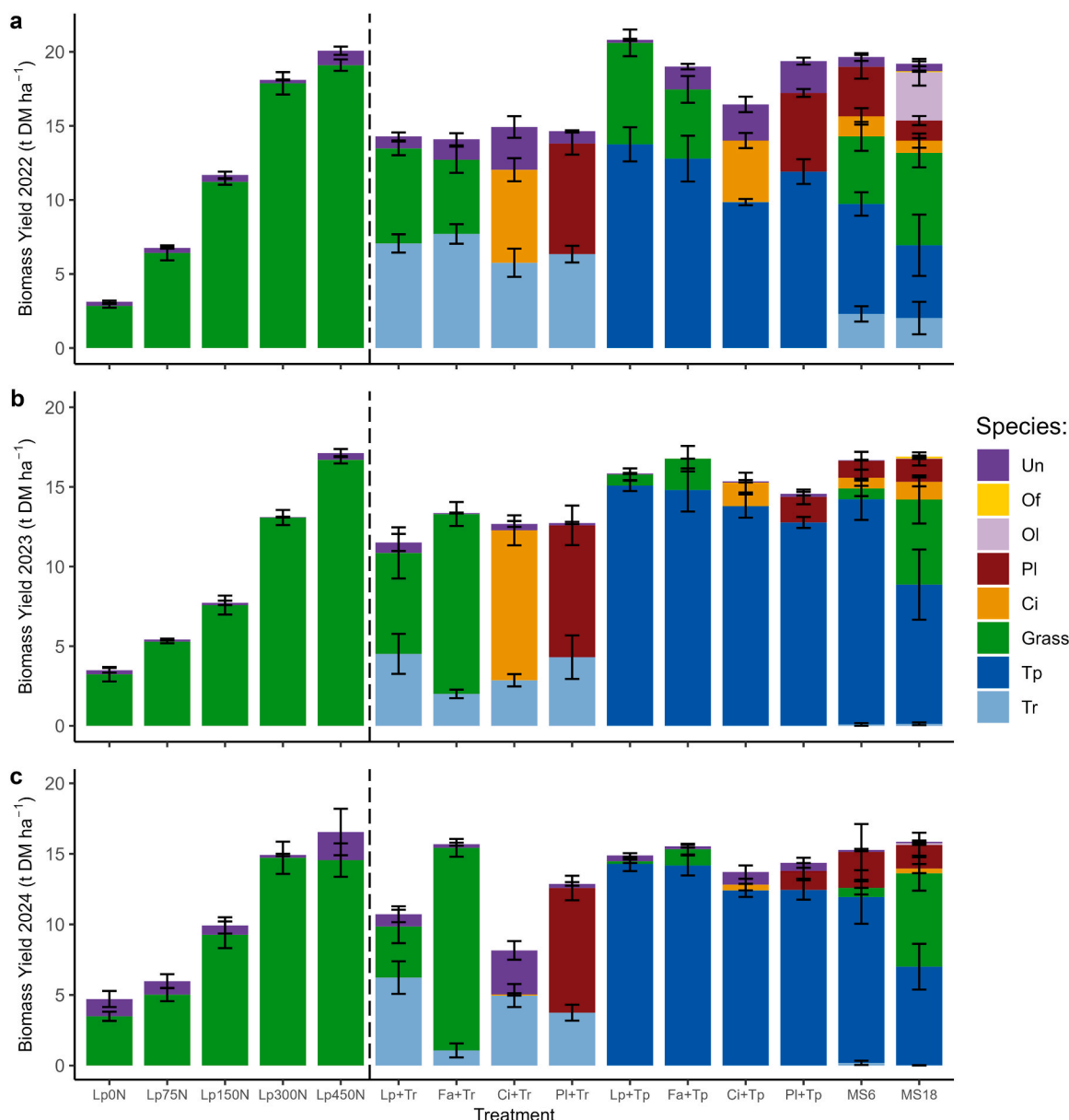


Fig. 1. Biomass dry matter yield (t DM ha^{-1}) in pure stand perennial ryegrass with increasing fertilizer rate (from 0 to 450 kg N ha^{-1}) and in mixtures with legumes, grasses and/or forbs at 75 kg N ha^{-1} . Lp = *Lolium perenne* (perennial ryegrass), Fa = *Festuca arundinacea* (tall fescue), Ci = *Cichorium intybus* (chicory), Pl = *Plantago lanceolata* (ribwort plantain), Tr = *Trifolium repens* (white clover), Tp = *Trifolium pratense* (red clover). MS6 = Multispecies mixture with Lp, Fa, Ci, Pl, Tr and Tp. MS18 = Multispecies mixture incl. MS6 + additionally 12 species (Table 1). Un = unsown species, Ol = other sown legumes, Of = other sown forbs, the latter two referring to the additional species in MS18. Error bars represent standard error ($n = 4$, except $n = 3$ for Pl+Tr in 2022 and Lp450N in 2024).

residual normality and/or homoscedasticity were not met, Generalized Linear Mixed-effects Models (GLMM) were fitted using the package glmmTMB (Brooks et al., 2017). Because yield and N-related response variables were strictly positive, right-skewed, and often exhibited variance increasing with the mean, probability distributions for continuous variables allowing for skew and uneven variances were required. We fitted a GLMM with a gamma probability distribution and a logarithmic link function for continuous, positive, right-skewed data, and a tweedie probability distribution with a log link function for responses showing both skewness and the occurrence of zero values. Model adequacy and assumptions were evaluated visually using residual diagnostic plots. For all fitted models, treatment was considered as a fixed effect and block as a random effect.

For LMM, test of significance of variables was assessed via the *F*-statistics estimated through Satterthwaite's approximation. For GLMM, the significance of variables was assessed using Type II Wald chi-squared

tests implemented through the 'car' package (Fox and Weisberg, 2019). Post-hoc pairwise comparisons between treatments were performed using Tukey's method with the R package emmeans (Lenth, 2023). A significance threshold level of 0.05 was applied to all statistical tests.

3. Results

3.1. Biomass dry matter yield

Biomass yield (t DM ha^{-1}) of perennial ryegrass in pure stand increased linearly with increasing fertilizer rates from 0 to $450 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Fig. 1). In 2022 and 2024, the yield stimulation with increasing fertilizer rates plateaued at the highest N rate. In 2022, 2023, and 2024, the total DM yield was significantly higher for all mixtures including legumes compared to pure stand perennial ryegrass with the same low level of fertilizer (Fig. 1 and Table S2). In all three production

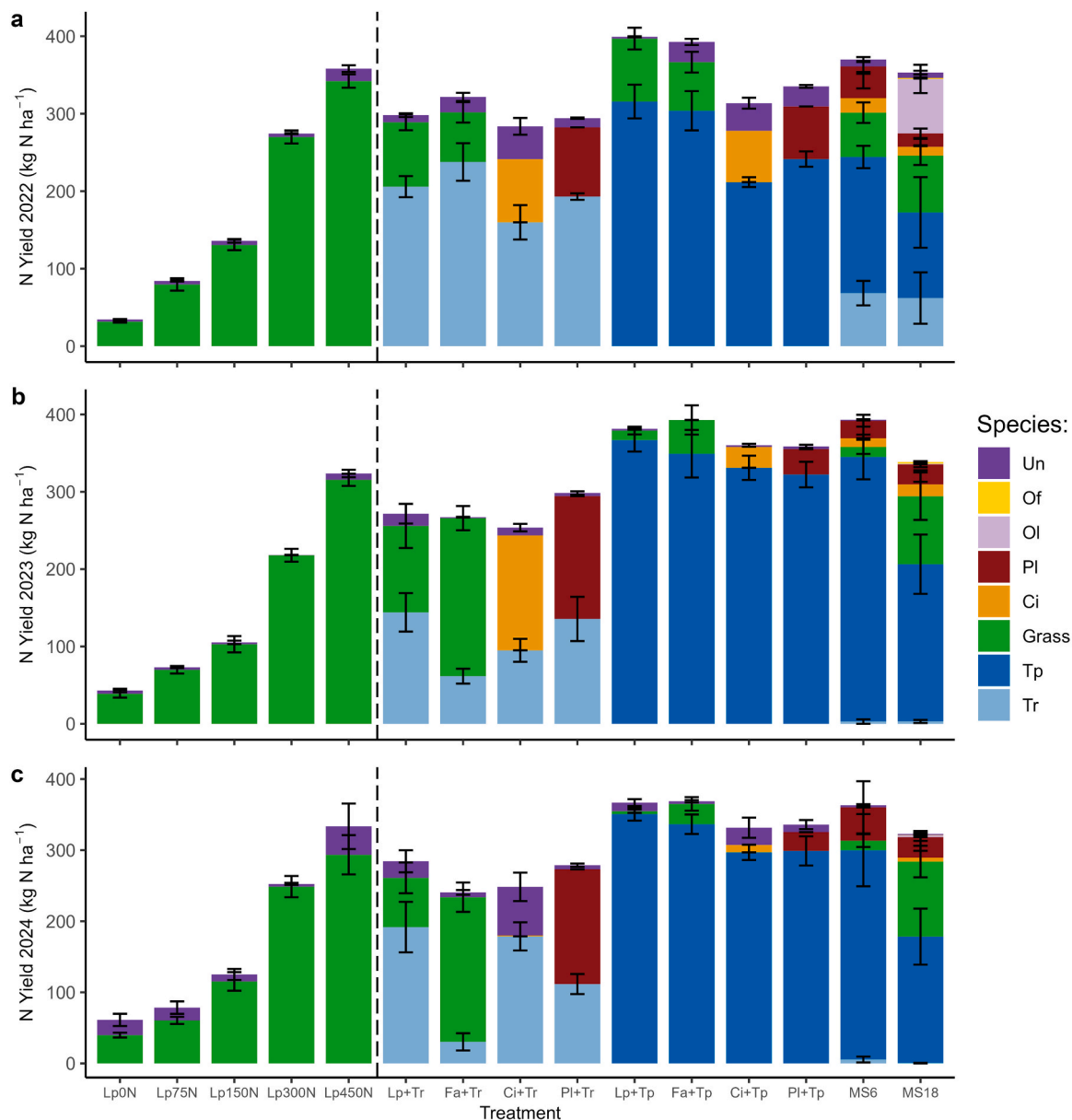


Fig. 2. Nitrogen yield (kg N ha^{-1}) in pure stand perennial ryegrass with increasing fertilizer rate (from 0 to 450 kg N ha^{-1}) and in mixtures with legumes grasses and/or forbs at 75 kg N ha^{-1} . Lp = *Lolium perenne* (perennial ryegrass), Fa = *Festuca arundinacea* (tall fescue), Ci = *Cichorium intybus* (chicory), Pl = *Plantago lanceolata* (ribwort plantain), Tr = *Trifolium repens* (white clover), Tp = *Trifolium pratense* (red clover). MS6 = Multispecies mixture with Lp, Fa, Ci, Pl, Tr and Tp. MS18 = Multispecies mixture incl. MS6 + additionally 12 species (Table 1). Un = unown species, Ol = other sown legumes, Of = other sown forbs, the latter two referring to the additional species in MS18. Error bars represent standard error ($n = 4$, except $n = 3$ for Pl+Tr in 2022 and Lp450N in 2024).

years, the mixtures with red clover showed a DM yield similar to the highest fertilized monoculture grass ($450 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), and generally higher than 2-species mixtures with white clover. In the 1st production year (2022), the 2-species mixtures with white clover in general had a DM yield higher than the monoculture grass receiving $150 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, but lower than that fertilized with $300 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. However, in the 2nd production year (2023), the white clover 2-species mixtures yielded similar to the monoculture grass with $300 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Table S2), reducing the DM yield gap between white clover and red clover 2-species mixtures. In the 3rd production year (2024), 2-species mixtures of white clover with tall fescue or plantain achieved substantially higher total DM yield than mixtures with perennial ryegrass or chicory, due to higher non-legume biomass in the former.

The 2-species mixtures with red clover yielded as high as the multi-species mixtures with 6 and 18 species (MS6 and MS18) over the three years. Thus, increasing species richness above 2 did not increase biomass yield. The contribution from grasses to total harvested biomass was higher in MS18, especially in the 2nd and 3rd production year, while MS6 and all the 2-species mixtures with red clover were dominated by red clover in the 2nd and 3rd year. In contrast, for the 2 species mixtures with white clover, the companion non-legumes were maintained in the sward and dominated the clover component, especially in the 2nd production year.

3.2. Biomass N yield

The N yield (kg N ha^{-1}) in pure stand perennial ryegrass showed a linear increase with higher fertilizer rate from 0 to $450 \text{ kg N ha}^{-1} \text{ yr}^{-1}$

(Fig. 2). In contrast to the biomass DM yield, increasing N rate still followed the linear trend at the highest fertilizer level in all years and did not reach a plateau. All mixtures with legumes showed a higher total N yield than both Lp75N (same fertilizer rate) and Lp150N. The mixtures with the lowest N yield (2-species mixtures with white clover) still had similar or higher N yields than Lp300N in all years. The N yield for all mixtures with red clover was similar to, or higher than, monoculture grass fertilized with $450 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ across all three years. Specifically, in 2023 the N yield was significantly higher for both Fa+Tp and MS6 than for Lp450N (Table S2), and in 2024 the N yield was higher for Lp+Tp compared to Lp450N, although only significantly when using a model restricted to red clover mixtures. Thus, while both the multi-species mixtures and the 2-species grass–red clover mixtures produced about three times the DM yield of Lp75N, their N yield was 4–5 times higher, despite receiving the same low fertilizer input ($75 \text{ kg N ha}^{-1} \text{ yr}^{-1}$).

3.3. Biological N_2 fixation

The quantity of biological N_2 fixation (qBNF) is a product of the percentage of N derived from the atmosphere (%Nd_{fa}) and the N yield in legume biomass. Due to the higher legume yield in mixtures with red clover compared to equivalent mixtures with white clover, qBNF was higher with red clover compared to white clover across companion species in all three years ($p < 0.001$) (Fig. 3, Table S2). The qBNF was not driven by %Nd_{fa}, which was similar between red and white clover in 2022 ($p = 0.569$) and lower in red clover compared to white clover in 2023 ($p < 0.020$) and 2024 ($p < 0.011$) (Table S3). The %Nd_{fa} dropped

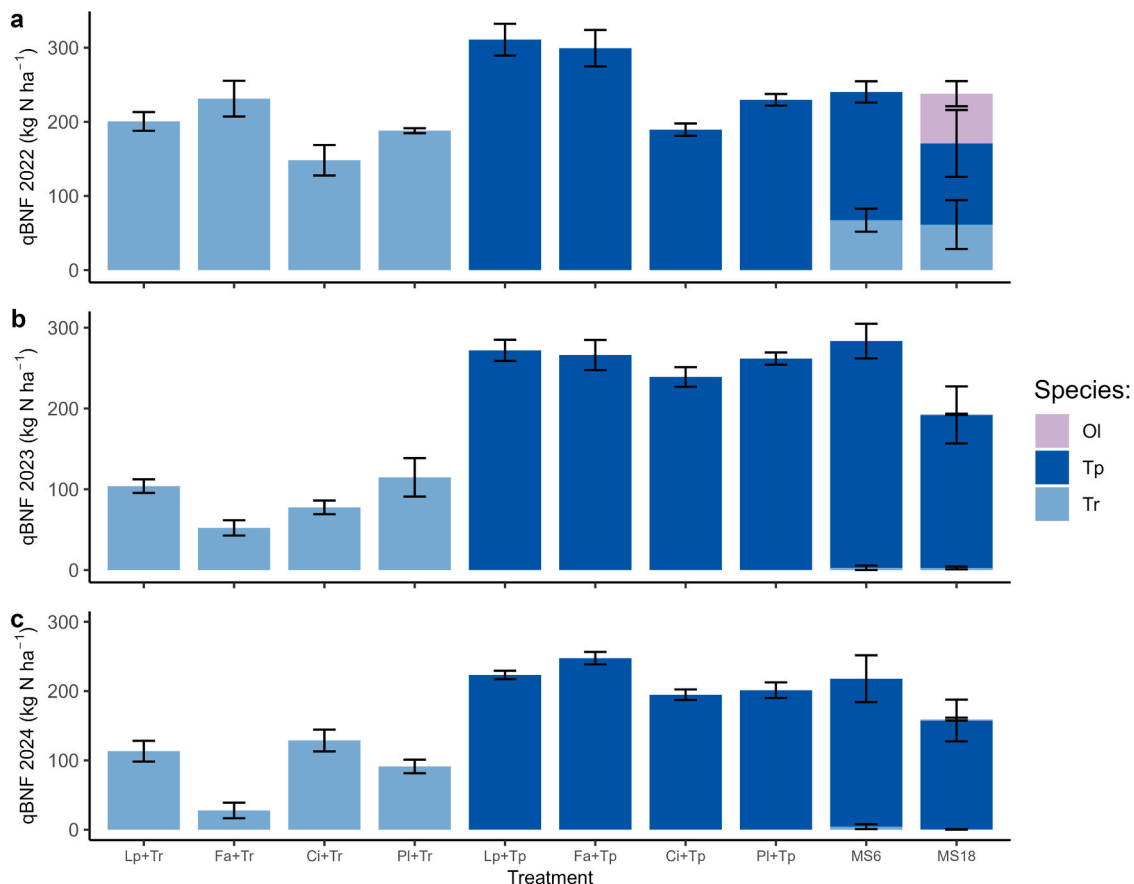


Fig. 3. The quantity of biological nitrogen fixation (qBNF) measured in legume biomass within mixtures with grass or/and forbs. Lp = *Lolium perenne* (perennial ryegrass), Fa = *Festuca arundinacea* (tall fescue), Ci = *Cichorium intybus* (chicory), Pl = *Plantago lanceolata* (ribwort plantain), Tr = *Trifolium repens* (white clover), Tp = *Trifolium pratense* (red clover). MS6 = Multispecies mixture with Lp, Fa, Ci, Pl, Tr and Tp. MS18 = Multispecies mixture incl. MS6 + additionally 12 species (Table 1). Ol = other sown legumes, referring to the additional legumes in MS18. Error bars represent standard error ($n = 4$, except $n = 3$ for Pl+Tr in 2022).

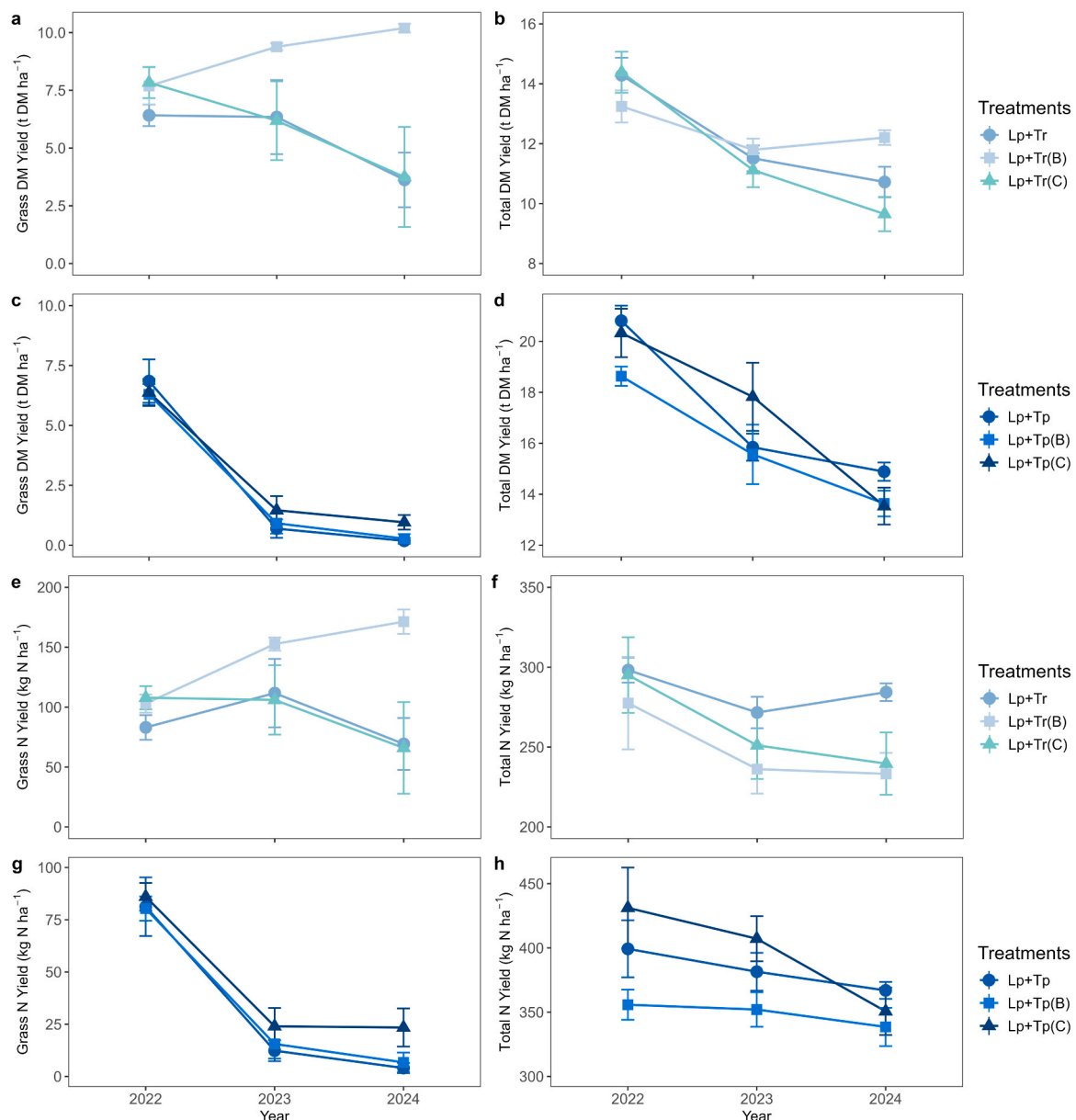


Fig. 4. Grass and total dry matter (DM) and nitrogen (N) yield over 3 production years in 2-species mixtures with perennial ryegrass (*Lolium perenne*: Lp) and different cultivars of white clover (*Trifolium repens*: Tr, panel a+b+e+f) or red clover (*Trifolium pratense*: Tp, panel c+d+g+h). Error bars represent standard error ($n = 4$).

in the 3rd production year for all treatments where legumes were dominant, while in treatments where grasses were still a main part of the botanical composition, e.g. in MS18, Fa+Tr, and Lp+Tr(b) (Figure S2 and Fig. 4a), %Ndffa remained at a high level in 2024 (Table S3).

The higher qBNF in red clover mixtures was particularly pronounced in 2023 and 2024, as the red clover proportion of total biomass increased from the 1st to the 2nd and 3rd production years. This was in contrast to the white clover mixtures, where the non-legume companions increased their relative contribution to the plant community in the 2nd and 3rd year, and white clover yield and qBNF decreased compared to the 1st year (Fig. 3). However, 2-species mixtures with white clover sustained a significantly higher non-legume biomass than the 2-species mixtures with red clover in 2023 and 2024. Specifically, in 2023 all four white clover mixtures sustained a non-legume N yield more than twice the size of the pure stand grass grown with the same low fertilizer rate (Lp75N). While Lp75N had an N yield equal to the input from fertilizer, in 2023 the N yield of the 2-species mixtures with white clover

ranged from 130 to 210 kg N ha⁻¹ yr⁻¹ in non-legume biomass alone (Fig. 2).

Regarding the effect of companion species on %Ndffa, chicory resulted in a lower %Ndffa with both clover species compared to all other companions in 2022 ($p < 0.001$, Table S3). In 2023, there was no general effect of companion on %Ndffa within white clover mixtures but still a lower %Ndffa in red clover with chicory than when combined with plantain ($p < 0.001$). In 2024, the %Ndffa in white clover mixtures sown with chicory as companion was lower than when sown with plantain and tall fescue ($p < 0.001$) (Table S3).

3.4. Effect of clover cultivar selection on community productivity over time

In six additional 2-species mixtures with perennial ryegrass, the effect of clover cultivars was assessed. Significant differences occurred between the three red clover cultivars in terms of DM and N yield in the

1st production year (2022) but not in the 2nd or 3rd year, and between cultivars of white clover in the 3rd production year (2024) but not in 2022 or 2023 (Fig. 4, Table S4).

Among red clover cultivars (*var. Larus*, *var. Semperina*, and *var. Taifun*), the average total yield (DM and N) for mixtures with *Semperina* was lower than with the other two cultivars in 2022 and 2023, although only significantly lower in 2022 compared to *Larus* for DM yield (Fig. 4d, Table S4) and compared to *Taifun* for N yield (Fig. 4h, Table S4), with no yield differences in 2024. *Semperina* had a higher leaf dry matter content (LDMC) than the other two red clover cultivars, and a lower one-sided fresh leaf area (LA) than *Larus*, with no significant differences in specific leaf area (SLA) (Table 2). Plant height was highest for *Larus* and intermediate for *Semperina* when sampled between two cuts, with no plant height differences between red clover cultivars just before harvest. Among white clover cultivars (*var. Munida*, *var. Abercrest* and *var. Tr9090011*), there were no significant differences before the 3rd production year (2024) when the mixture with *Abercrest* showed a higher total average DM yield compared to the other two cultivars (Fig. 4b, Table S4), based on a higher non-legume biomass (perennial ryegrass). However, this difference was only statistically significant compared to *Tr9090011* ($p < 0.001$) with a strong tendency compared to *Munida* ($p = 0.056$). In terms of total N yield, *var. Munida* still outperformed both *Abercrest* and *Tr9090011* in 2024 (Fig. 4f, Table S4), due to a higher legume yield, with legume biomass having higher N content than grass biomass. The cultivars *Munida* and *Tr9090011* were characterized by larger leaf area (LA) and taller plants compared to *Abercrest*, while SLA was higher for *Abercrest* compared to *Tr9090011* with no difference compared to *Munida* (Table 2).

The main cultivar of red clover (*Larus*) and white clover (*Munida*) used in this study showed higher %Ndfa than the other cultivars tested in 2022 ($p < 0.001$), but not in 2023 or 2024 (Table 2). In 2023 and 2024, *Abercrest* had a higher %Ndfa than the other white clover cultivars, while no significant differences occurred for red clover cultivars. For all cultivars except *Abercrest*, the %Ndfa was reduced in the 2nd year and 3rd production year. In mixtures with red clover cultivars, the average qBNF was lowest with *Semperina* compared to *Larus* and *Taifun* in 2022 and 2023, although these differences were not statistically significant (Table S5). No differences in qBNF occurred between mixtures with white clover cultivars before 2024, where *Abercrest* had significantly lower qBNF driven by low legume biomass (Table S5).

3.5. The effect of companion species on community productivity over time

The non-legume companion species investigated in this study (perennial ryegrass, tall fescue, chicory and plantain) showed different patterns in terms of yield contributions over time and synergies with the legume components.

For both grasses, the DM and N yield during the 3 years was higher with white clover than with red clover (Fig. 5a+c+e+g), although the total community yields were higher with red clover (Fig. 5b+f+h), with

a notable exception occurring in the 3rd production year where tall fescue with white clover resulted in a similar (high) total DM yield as with red clover (Fig. 5d). However, the high DM yield of the mixture with white clover and tall fescue in the 3rd production year was based on a high grass proportion and low legume proportion, thus the total N yield was still lower than when tall fescue was combined with red clover (Fig. 5h).

The two forb companions had contrasting behaviors over time. While chicory contributed substantially to the total community biomass yield in the first two years (especially in 2-species mixtures), it disappeared from the mixtures up until the 3rd production year, reducing the total DM yield of the 2-species mixtures in 2024 substantially (Figure S3). In contrast, the yield of plantain was maintained over time, contributing to total community biomass during all three years, in both 2-species mixtures and in the multi-species mixtures with 6 and 18 species (Fig. 6 and Figure S4).

4. Discussion

4.1. Productivity in mixtures with red clover reach the level of high-fertilized pure stand while grass–white clover had more balanced species composition over time

In pure stand perennial ryegrass, DM yield increased linearly with N fertilization up to $450 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ across all three production years (2022–2024), though the response slope declined at the highest fertilizer level in 2022 and 2024. (Fig. 1). The two pure stand reference treatments with the highest N rate (Lp300N and Lp450) represent common practice of high (and very high) fertilized and intensively managed grassland systems in northern Europe (Cougnon et al., 2017; Larsen et al., 2024; Manevski et al., 2017). Importantly, while only fertilized with $75 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, all mixtures with red clover (except Ci+Tp) had a DM yield like the monoculture grass with $450 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ in all production years. In the 2nd and 3rd year, also Ci+Tp had a yield like the highest fertilized monoculture. Thus, low-fertilized mixtures with red clover (including the multi-species mixtures MS6 and MS18) proved to be as productive as high-fertilized monoculture grassland, at least during the first three production years.

In 2023 and 2024, productivity decreased in general for all mixtures and monocultures, with the highest yielding mixtures still being those including red clover, ranging from 13.7 to $16.9 \text{ t DM ha}^{-1} \text{ yr}^{-1}$, compared to 13.1 – 14.9 for Lp300N and 15.9 – $17.2 \text{ t DM ha}^{-1} \text{ yr}^{-1}$ for Lp450N. These values fall within, or slightly exceed, the biomass yields reported in previous studies of highly fertilized pure grass stands. For example, with annual N inputs of $300 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, perennial ryegrass yielded $13.3 \text{ t DM ha}^{-1} \text{ yr}^{-1}$ and tall fescue $18.8 \text{ t DM ha}^{-1} \text{ yr}^{-1}$ (Cougnon et al., 2017). Similarly, Manevski et al. (2017) reported tall fescue yields of $18.5 \text{ t DM ha}^{-1} \text{ yr}^{-1}$ under $500 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. Consequently, our results underline that fertilizer N inputs can be substantially reduced in managed grasslands without biomass yield reductions when including

Table 2

Leaf traits measured for different clover cultivars in 2-species mixtures with perennial ryegrass. Leaf area (measured as one-sided fresh leaf area), specific leaf area (SLA) and leaf dry matter content (LDMC) were measured 4 weeks after 1st cut, and plant height was measured on site at two occasions ("Early" = 4 weeks after 1st cut; "Late" = 8 weeks after 1st cut i.e. just before 2nd cut). The proportion of nitrogen derived from the atmosphere (%Ndfa) was measured for each cut and aggregated for each year.

Clover cultivar	Leaf area (mm^2)	Specific leaf area ($\text{cm}^2 \text{ mg}^{-1}$)	Leaf DM content (dry mass fresh mass $^{-1}$)	Plant Height Early (cm)	Plant Height Late (cm)	%Ndfa 2022	%Ndfa 2023	%Ndfa 2024
Munida	632 ± 69 (b)	0.27 ± 0.01 (ab)	0.229 ± 0.005 (a)	20 ± 1 (b)	32 ± 1 (b)	97.5 ± 0.7 (b)	75.3 ± 6.2 (a)	61.8 ± 6.5 (a)
Abercrest	423 ± 37 (a)	0.32 ± 0.02 (b)	0.211 ± 0.012 (a)	16 ± 1 (a)	28 ± 1 (a)	94.4 ± 1 (ab)	86.9 ± 2.1 (a)	90.9 ± 1.7 (b)
Tr9090011	590 ± 88 (b)	0.25 ± 0.01 (a)	0.223 ± 0.009 (a)	21 ± 1 (b)	30 ± 1 (ab)	93.7 ± 1.2 (a)	75.4 ± 5.5 (a)	65.3 ± 6.7 (a)
Larus	1068 ± 167 (b)	0.21 ± 0.02 (a)	0.254 ± 0.01 (a)	45 ± 1 (c)	69 ± 3 (a)	98.5 ± 0.2 (b)	74.1 ± 1.5 (a)	63.7 ± 1.9 (a)
Semperina	689 ± 46 (a)	0.22 ± 0.01 (a)	0.275 ± 0.005 (b)	36 ± 2 (b)	64 ± 3 (a)	93.7 ± 0.9 (a)	73 ± 4.6 (a)	62.3 ± 3.5 (a)
Taifun	841 ± 95 (ab)	0.24 ± 0.02 (a)	0.248 ± 0.005 (a)	30 ± 1 (a)	67 ± 4 (a)	91.4 ± 1.3 (a)	69.7 ± 1.8 (a)	60.3 ± 1.6 (a)

Note: Standard error (±) is denoted after the average for each value ($n = 4$). Compact letter display indicates statistically significant differences.

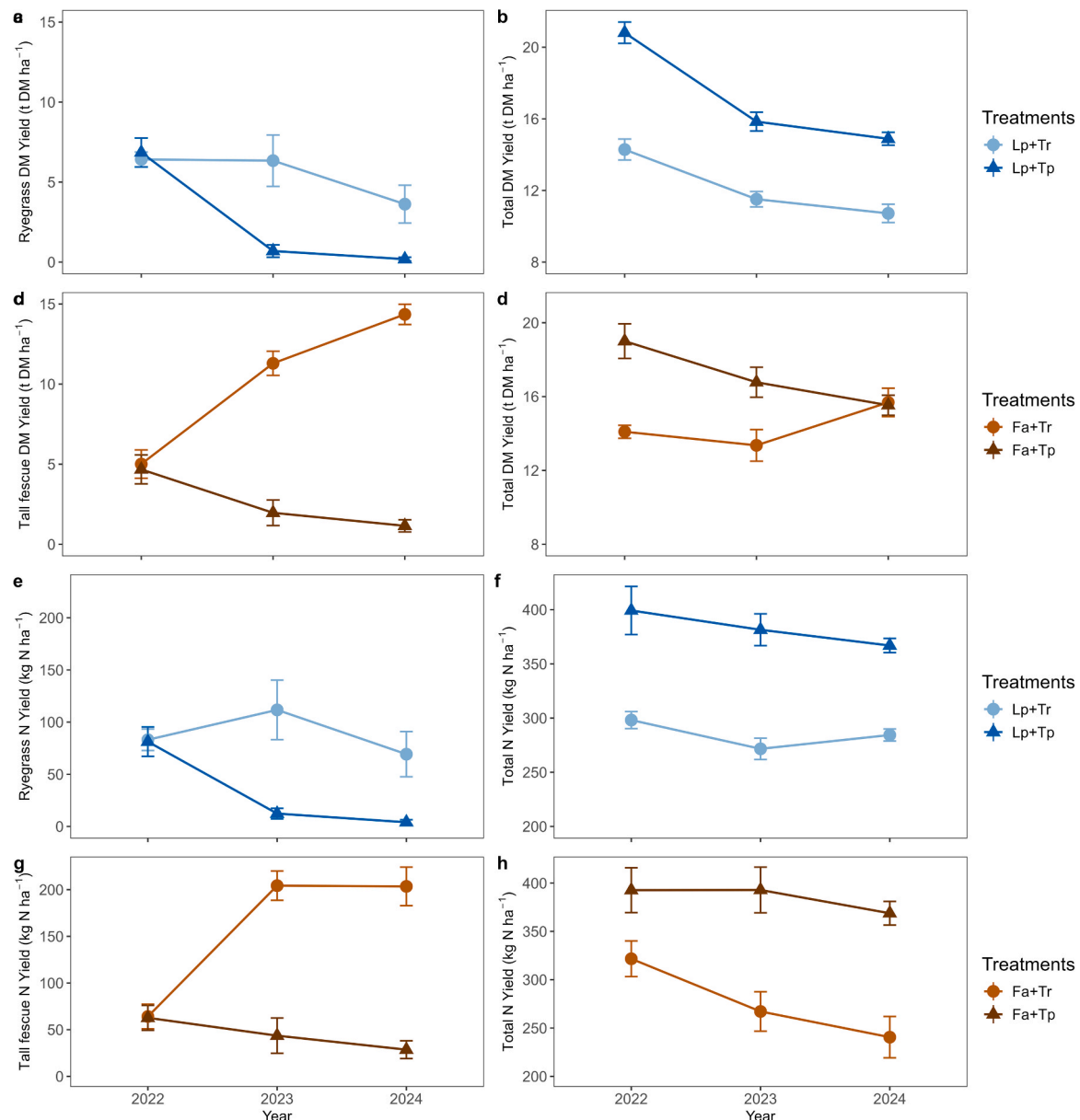


Fig. 5. Grass and total dry matter (DM) and nitrogen (N) yield over 3 production years in 2-species mixtures with perennial ryegrass (*Lolium perenne*: Lp, panel a+b+e+f) or tall fescue (*Festuca arundinacea*: Fa, panel c+d+g+h) with white clover (*Trifolium repens*: Tr) or red clover (*Trifolium pratense*: Tp). Error bars represent standard error (n = 4).

productive forage legumes with benefits for farm economies and with reduced GHG emissions related to fertilizer production and application (Chojnacka et al., 2019; Jensen et al., 2012).

The 2-species mixtures with white clover yielded lower biomass DM than Lp300N and the mixtures including red clover in 2022, which is in line with earlier studies on grass-clover mixtures (Eriksen et al., 2012; Rasmussen et al., 2012). However, in 2023 all 2-species mixtures with white clover had a similar yield as the Lp300N and reduced the yield gap to mixtures with red clover, and in 2024 the mixture with white clover and tall fescue even had a DM yield similar to Lp450N and mixtures with red clover. Hence, the 2-species mixtures with white clover gained productivity over time relative to mixtures with red clover, as expected (hypothesis 1), but only closed the yield gap to red clover mixtures within the first three production years when combined with tall fescue.

We found that the choice of non-legume companion in 2-species mixtures is important for the overall performance of the community in the 2nd and 3rd production year. Tall fescue is recognized as a

competitive and high-yielding grass (Cougnon et al., 2017; Manevski et al., 2017), and indeed it formed a better partnership with the red clover in 2023 and 2024 compared to perennial ryegrass that was to a higher degree outcompeted by red clover in the 2nd and 3rd year. In contrast, perennial ryegrass formed a more equal companionship with white clover in the 2nd and 3rd year than tall fescue, which was dominating white clover in 2023 and 2024, presumably in terms of aboveground resources (i.e. light). However, white clover did not disappear entirely when combined with tall fescue, and even though contributing little to aboveground biomass in 2024, the yield of this mixture was significantly higher than all other 2 species mixtures with white clover this year. The high DM yield of this combination might be explained by the fact that white clover is characterized as a particularly N generous legume (Pirhofer-Walzl et al., 2012) supporting the very productive and nutrient acquisitive tall fescue (Abalos et al., 2020; Abalos et al., 2019). However, a study including more than three production years, is needed to evaluate whether this mixture would

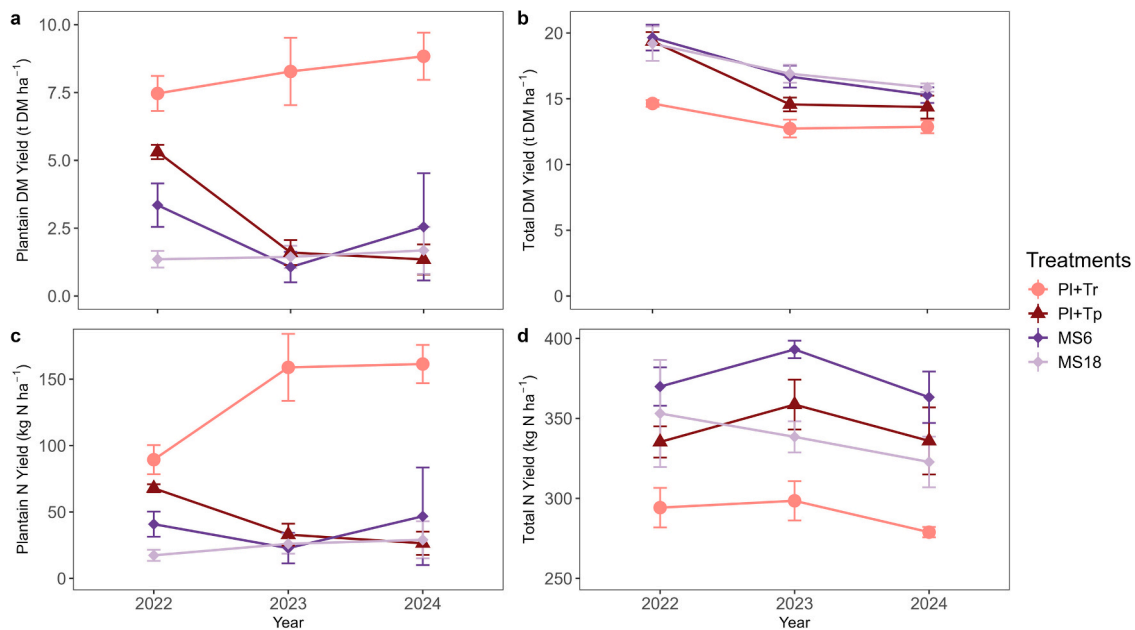


Fig. 6. Plantain and total dry matter (DM) and nitrogen (N) yield over 3 production years in mixtures with white clover (*Trifolium repens*: Tr) or red clover (*Trifolium pratense*: Tp). Error bars represent standard error ($n = 4$, except $n = 3$ for PI+Tr in 2022).

continue to maintain such high yields during the following years, i.e. the 4th and 5th production seasons. Such findings could help extend the typical renewal period for productive grasslands. This would depend on the ability of the white clover to regrow and increase its productivity when soil N and tall fescue productivity are reduced, thus following the oscillating behavior observed in grass-clover swards (Loiseau et al., 2001).

For the forb companions, differences were most prominent after the 2nd production year, when the biannual chicory, as expected, disappeared in all treatments, while plantain increased its yield contribution in the 3rd production year when combined with white clover. In 2-species mixtures with red clover, chicory reduced the total community yield in 2022 but not in 2023 and 2024, where the non-legume companions played a minor role for the total community yield. Thus, while forbs contribute with diversity and inter-specific dynamics that under some conditions can improve total productivity in multi-species mixtures (Cong et al., 2020), also belowground (Cong et al., 2017; Mortensen et al., 2025b), in our study grass companions were generally better than forb companions for aboveground community productivity in 2-species mixtures.

4.2. Mixture composition rather than species richness secure high productivity in managed grasslands

The multi-species mixtures MS6 and MS18 did not increase total aboveground biomass compared to the 2-species mixtures with red clover, only when compared to 2-species mixtures with white clover. Thus, our hypothesis (2) that species richness would increase biomass yield over time was only confirmed up to a species richness level of 2. As red clover was a key determinant of high yield in the mixtures, rather than species richness *per se*, the inclusion of certain productive species proved to be crucial in terms of securing high biomass yields in legume-based mixtures. This is in line with earlier studies on yield comparisons between 2-species grass-legume mixtures and multi-species mixtures (Dhamala et al., 2017; Pirhofer-Walzl et al., 2013), and studies that have observed various ecosystem processes as being dependent on species identity rather than species diversity (Milcu et al., 2006; Mokany et al., 2008; Pérès et al., 2013; Zhang et al., 2017). In contrast, biomass productivity in particular has been found to increase with species richness

in natural grassland systems (Dietrich et al., 2024; Zheng et al., 2024; Zuppinger-Dingley et al., 2014), where management and manipulation of species dynamics is limited.

The mix of productive legumes such as red clover with nutrient acquisitive non-legumes (e.g., perennial ryegrass or tall fescue) is a very strong combination when biomass productivity is the main target, at least when sward age is kept relatively low. Consequently, adding more species (as in our MS6 and MS18 treatments) did not increase total aboveground productivity of the community within three production years, as the addition of these species reduced the abundance of the most productive plant species, as well as presumably increased interspecific competition of above- and belowground resources (Loreau, 2004). This underlines an important difference between natural and managed grassland systems, where the selection effects are fundamental in managed systems, and resource partitioning and niche differentiation is more important in natural and seminatural systems as belowground resources are scarcer (Wang et al., 2023; Zuppinger-Dingley et al., 2014) and thus complementarity effects stronger (Duffy et al., 2017; Loreau and Hector, 2001; Turnbull et al., 2016).

4.3. High species richness promotes long-term yield stability via functional group balance

Species evenness and the sustained presence of both legumes and non-legumes are critical for maintaining high productivity in perennial grass-clover swards over the long term (Ergon et al., 2016; Kirwan et al., 2007). When red clover becomes overly dominant, total aboveground productivity may decline after the first ~3 years, as stationary red clover plants are gradually lost and few companion species remain to compensate (Ergon et al., 2016; Hoekstra et al., 2017; Marshall et al., 2017). While species richness above 2 did not increase total productivity within the first three production years in our study, the only mixture with red clover that maintained a high proportion of non-legume biomass in the latter two production years was MS18 (approximately 50 % in all three years). This was mainly driven by the grasses, which were well maintained during all three production years. In the other mixtures, red clover outcompeted the non-legumes from the end of the 1st production year. Thus, MS18 had a balance between legumes and non-legumes similar to the 2-species mixtures with white clover (e.g.,

Lp+Tr, Figure S2 and Figure S4), whereas red clover dominated aboveground biomass in both MS6 and 2-species mixtures from the end of the 1st production year to the extent that not only chicory but also plantain and grasses almost disappeared from the sward from the beginning of the 2nd production year. The higher temporal stability in species composition observed in the 18-species mixtures suggests that increasing functional diversity may be a viable strategy for maintaining productive swards over multiple years under low N inputs, thereby reducing the need for frequent sward renewal in practical grassland management.

The functional group dynamics observed in MS18 might be explained by the increased competition between species at the highest species richness level, and especially by the addition of competitive but less persistent legumes competing well with red clover in the 1st year. In MS18, the biannual crimson clover (*Trifolium incarnatum*) dominated aboveground biomass in the 1st production year (Figure S4), especially until the first cut. This likely suppressed and delayed the establishment of red clover, allowing the non-legumes, especially grasses, to establish better in the 1st production year and to maintain and contribute to productivity of MS18 in the later years. The crimson clover is also likely to have contributed to elevated C and N rhizodeposition and decay of small roots in the first year (Mortensen et al., 2025b), which would represent an elevated source of soil N for the non-legumes and thus facilitated their growth in the first year and secured them a head start and advantage in the following year(s). This dynamic is supported by a significantly higher %Nd_{fa} in MS18 in 2023 and 2024 compared to the other treatments, indicating a continuous utilization of soil N in MS18. In this way, N inputs from annual or bi-annual legume species in multi-species mixtures may stabilize a mixture composition as an alternative to stimulating the non-legumes with fertilizer N, thereby maintaining higher yield in later production years, which can increase the number of years before sward renewal is needed. Extending the grassland period in a cropping rotation, e.g., from two or three to four or five years, can increase soil C stocks and soil N fertility substantially (Jensen et al., 2022) and reduce the frequency of periods where soil N is vulnerable to be lost during conversion or renewal (Larsen et al., 2024; Thers et al., 2024).

4.4. Stable N yield and high %Nd_{fa} can be obtained by generous legume species in mixtures with N-acquisitive non-legumes

The N yield (kg N ha⁻¹ yr⁻¹) of the monocultures and mixtures followed the same overall pattern as DM yield, however, for N yield the mixtures with legumes performed generally better relative to monoculture perennial ryegrass due to the higher N content in the legume biomass (Johansen et al., 2018). While the high N yield in mixtures with red clover was driven by a high proportion of legume biomass in all three years, interestingly, for white clover mixtures the increased total N yield observed in 2023 was driven by a substantial increase in non-legume biomass and occurred despite a reduction in legume biomass. While non-legume N yield was reduced from 2022 to 2023 in all mixtures including red clover, the N yield increased for the non-legumes in all 2-species mixtures with white clover. These findings show that 1) white clover is less prone to dominate companions in a community than red clover, and that 2) white clover can transfer substantial amounts of fixed N from the atmosphere to its companion non-legumes.

The relatively low qBNF for white clover mixtures in 2023 occurred as a result of increased non-legume N yield, likely explained by the higher N generosity and transfer to companion species from white clover compared to red clover, as demonstrated by Pirhofer-Walzl et al. (2012). This was underlined by the change from 2022 to 2023, where N-yield in non-legume biomass increased 2-fold in white-clover mixtures. This result fits the oscillation of legume vs non-legume productivity from year to year as shown by Eriksen et al. (2015) and Loiseau et al. (2001). Such oscillation occurs when high N inputs from the legume component

to the system one year translate into high biomass of the non-legume and potentially lower %Nd_{fa} in the legume component the following year, which in turn can increase competition for soil N and thus legume biomass and qBNF in the third year, and so forth. However, such oscillation is only possible if both legume and non-legume components remain in the sward. The mixtures with red clover (except MS18) were dominated by this species in the 1st production year to such an extent that an increase of non-legume biomass in the second year was not possible. Instead, %Nd_{fa} in the red clover was reduced significantly (Table 2), likely because the availability of soil N was increased in the 2nd and 3rd year, due to the high N input from legumes in the 1st production year, and a low abundance of nutrient acquisitive non-legumes in year 2 and 3.

The %Nd_{fa} was lower for both white and red clover with chicory as companion, especially in 2022, which is possibly related to differences in root traits (Cong et al., 2017; Louarn et al., 2014; Mortensen et al., 2025a). While the root system of chicory is characterized by a deep taproot, the other companions (i.e. perennial ryegrass, tall fescue and plantain) develop more fine roots in the topsoil, which potentially increase interspecific competition for soil N in the soil layer where most legume roots are located (Mortensen et al., 2025b). Likewise, the significantly higher %Nd_{fa} maintained for red clover in the MS18 mixture in both 2023 and 2024 compared to all other mixtures suggests a higher competition for soil N in this mixture, and potentially higher N transfer from the legumes to the better-established non-legumes in MS18. Similarly, two white clover mixtures stand out with substantially higher average %Nd_{fa} in 2024 than the other 2-species mixtures with white clover. In the mixture with tall fescue (Fa+Tr), the remarkably high non-legume productivity sustained a high N consumption and thus high %Nd_{fa} and total productivity, while in the perennial ryegrass mixture with the small-leaved white clover cultivar (*Abercrist*) the clover maintained a high %Nd_{fa} to continuously sustain the high non-legume biomass in 2024. However, for both these 2-species combinations further studies need to evaluate whether productivity can be maintained after the 3rd production year.

4.5. Cultivars of red clover and white clover can modulate community productivity

Our hypothesis (3) was verified as clover cultivars resulted in significant differences in the productivity of plant communities in 2-species mixtures with perennial ryegrass, although the differences were relatively small and not repeated in all years.

For red clover, yield differences occurred only in the 1st production year, when the cultivar *Semperina* showed lower average legume biomass than the cultivars *Larus* and *TaiFun*, but with similar non-legume biomass, thereby reducing the overall agronomic performance of the community. This difference might be related to the lower leaf area and higher LDMC (Table 2) for this cultivar (Pontes et al., 2007; Smart et al., 2017). No yield differences were found between the red clover cultivars in the 2nd and 3rd production year, and therefore, based on the three cultivars tested in this study, the potential for improving community yield via cultivar selection in red-clover grass swards seems limited. However, Hoekstra et al. (2017) found that differences in yield and persistency of red clover cultivars were most pronounced after the 3rd production year, suggesting that our study was too short to evaluate the effects of different persistency on community yield. Furthermore, most studies on red clover cultivars have focused on improving red clover yield and persistency in monoculture or 2-species mixtures (Herrmann et al., 2008; Hoekstra et al., 2017). If total community productivity in multi-species mixtures is the focus, inclusion of less productive red clover cultivars with high persistence in multi-species mixtures might be a way to reduce the high dominance of red clover that we observed in our MS6 treatment, and maintaining high biomass yield over time. Adjusting the sown proportions of key species is another way of manipulating competition within diverse swards, and further studies

should investigate how targeted changes in legume proportions influence dominance, persistence, and species contributions in perennial multi-species grasslands, particularly for red clover in late-successional swards.

The white clover cultivars did not show yield differences until the 3rd production year. Here, the mixture with the small-leaved *Abercrest* surprisingly resulted in a higher community DM yield compared to the other two cultivars with larger leaves (*Munida* and *Tr9090011*), due to a higher biomass of perennial ryegrass and a sustained high %Ndfa. This observation might be related to both smaller leaves and a lower plant height for *Abercrest* as measured between cutting events, potentially allowing better growth of perennial ryegrass than when this was competing with the large-leaved and taller cultivars. While *Munida* and *Tr9090011* extended their leaves to the top of the canopy already soon after the regrowth, the leaves of the *Abercrest* cultivar were significantly lower when measured in the middle of two biomass harvests (Table 2). However, when measuring the plant height just before harvest, *Abercrest* had reached close to the same height as the other two cultivars. Similarly, for red clover cultivars the difference in plant height disappeared when measured just before harvest. Therefore, in-season growth pattern of clover cultivars should be subject for further studies in relationship grass-clover species dynamics and productivity optimization.

Although cultivar differences were detectable, they were modest compared with the strong and consistent effects of legume species identity (red vs white clover), indicating that species choice remains the primary driver of mixture performance.

5. Significance and Implications

Our study demonstrates that substantial inputs of synthetic N fertilizer can be replaced by biological N₂ fixation without compromising yields in managed grassland systems. In contrast to fertilizer-derived N, which is supplied in pulses and is therefore more vulnerable to losses, legume-fixed N is incorporated directly into plant biomass and released gradually during the growing season. As a result, legume-based grassland systems can maintain high productivity while reducing N losses and greenhouse gas emissions associated with fertilizer production and application. We show that grass–legume–forb mixtures receiving low N fertilizer inputs can produce equal or even higher N yields than highly fertilized monoculture grasslands. Together, these findings highlight the potential of diverse legume-based mixtures to improve N-use efficiency and optimize N cycling, providing simultaneous benefits for environmental sustainability, soil fertility, and farm profitability.

Our results show that both species richness and legume identity play a role in maintaining the balance between legumes and non-legumes in perennial grasslands over time. White clover facilitated a more balanced mixture and better non-legume growth than red clover over 3 production years, which can be important for sustaining high yields in the longer term, as also suggested by previous studies (e.g., Ergon et al., 2016; Kirwan et al., 2007). From the end of the 1st production year, red clover generally outcompeted the non-legumes except in the 18-species mixture. Although all mixtures with red clover still maintained high total yield in the 2nd and 3rd production year, studies over longer periods (e.g., four to five years) are needed to understand the long-term yield dynamics in productive temporary grasslands. Maintaining botanical balance also has important implications for belowground productivity and soil C storage in deeper soil layers (Lange et al., 2023; Mortensen et al., 2025b) as well as the build-up of stable SOC fractions (Mortensen et al., 2025a). Finally, if high yields can be sustained for longer periods, the frequency of sward renewal or conversion of temporary grasslands could be reduced, thereby decreasing soil disturbance events and the associated risks of N and C losses.

The variability observed among clover cultivars in this study suggests potential for further research on multi-species mixture composition, focusing on identifying cultivar combinations that enhance community-level productivity through complementary plant traits,

rather than solely maximizing the performance of individual species. However, from a management perspective, our results indicate that decisions regarding legume species identity and overall mixture diversity have a substantially larger influence on long-term productivity and stability than cultivar choice alone.

CRediT authorship contribution statement

Esben Øster Mortensen: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Diego Abalos:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **De Deyn Gerlinde B.:** Writing – review & editing, Validation, Investigation, Conceptualization. **Jim Rasmussen:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fcr.2026.110426.

Data availability

Data will be made available on request.

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